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**Contribution des phytoglobines et des nitrate réductases
à la régulation de l'oxyde nitrique et de la fixation de
l'azote dans la symbiose *Medicago truncatula* /
*Sinorhizobium meliloti***

Antoine Berger

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THÈSE DE DOCTORAT

Contribution des phytooglobines et des
nitrate réductases à la régulation de
l'oxyde nitrique et de la fixation de l'azote
dans la symbiose *Medicago truncatula* /
Sinorhizobium meliloti

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Contribution des phytoglobines et des nitrate réductases à la régulation de l'oxyde nitrique et de la fixation de l'azote dans la symbiose *Medicago truncatula* / *Sinorhizobium meliloti*

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Contribution des phytoglobines et des nitrate réductases à la régulation de l'oxyde nitrique et de la fixation de l'azote dans la symbiose *Medicago truncatula* / *Sinorhizobium meliloti*

Résumé :

La symbiose fixatrice d'azote entre les légumineuses et les bactéries du sol de type *Rhizobium* permet de réduire l'azote atmosphérique (N_2) en ammoniacque (NH_3) grâce à la présence de la nitrogénase bactérienne au sein d'un organe racinaire appelé nodosité.

Dans le modèle symbiotique *Medicago truncatula/Sinorhizobium meliloti*, l'oxyde nitrique (NO) est produit tout au long du processus symbiotique, du début de l'interaction entre la plante et les bactéries jusqu'à la sénescence de la nodosité. Les effets toxiques, de signal ou de métabolite du NO dépendent principalement de sa concentration à son site d'action. Sa concentration au sein des cellules des nodosités doit être régulée afin de limiter ses effets toxiques et lui permettre de remplir ses fonctions de signalisation et de métabolite. Chez les plantes, les principales sources de NO identifiées sont la nitrate réductase (NR) et la chaîne de transfert d'électrons mitochondriale (ETC). Par ailleurs, les phytoglobines (Phytogb) sont connues pour être impliquées dans le catabolisme du NO. D'après leur homologie de séquence et leur affinité pour l'oxygène, trois classes de Phytogb ont été décrites chez les légumineuses : les Phytogb non-symbiotiques (Phytogb1), les leghémoglobines (Lb) -spécifiques des légumineuses- et les Phytogb tronquées (Phytogb3).

Les principaux objectifs de cette thèse ont été, d'une part, de caractériser et étudier le rôle des NR et des Phytogb dans la régulation du NO lors de la symbiose entre *M. truncatula* et *S. meliloti* et, d'autre part, d'analyser le rôle du NO dans le développement et le fonctionnement du processus symbiotique.

Chez *M. truncatula*, 3 gènes codent pour des NR et 17 pour des Phytogb. L'étude phylogénétique des séquences de Phytogb de *M. truncatula* a permis d'identifier 12 Lb, 3 Phytogb1 et 2 Phytogb3. L'analyse de l'expression des gènes de NR et de Phytogb, ainsi que la mesure de l'activité totale des NR et la production du NO a permis de suivre le niveau de NO durant la symbiose fixatrice d'azote, ainsi que le rôle respectif des différentes NR et Phytogb dans sa régulation. Au cours du processus symbiotique, quatre pics de production de NO ont été observés, correspondant à quatre étapes du processus symbiotique, pendant (1) l'établissement de l'interaction la plante et la bactérie, (2) le début de l'organogénèse de la nodosité, (3) le fonctionnement de la nodosité mature et (4) lors de l'entrée en sénescence des nodosités. Lors de ces différentes étapes, la production de NO a pu être particulièrement corrélée à l'expression des gènes des NR1 et NR2, d'une Phytogb1 (*Phytogb1.1*) et d'une Phytogb3 (*Phytogb3.1*). L'utilisation de divers inhibiteurs des voies de synthèse du NO a montré que la production de NO dépend principalement de l'activité NR et de la chaîne de transfert d'électrons mitochondriale. L'utilisation de donneurs de NO a permis de montrer que, lors du développement nodulaire, le NO induit l'expression des Phytogb1 et de plusieurs gènes de défense, mais réprime celle des Lb et Phytogb3. Une analyse fonctionnelle de *Phytogb1.1* pendant l'établissement, le fonctionnement et la sénescence de la nodosité, a été initiée via la production de plants de *M. truncatula* sur-exprimant ou sous-exprimant ce gène. La surexpression et la sous-expression de ce gène ont abouti respectivement à une diminution et une augmentation du niveau de NO dans les nodosités, mais s'est traduit dans les deux cas par une diminution du nombre de nodosités par plante. L'analyse de l'expression d'un certain nombre de gènes marqueurs de l'interaction symbiotique, des réponses de défense, du métabolisme azoté et de l'hypoxie, et les mesures de fixation de l'azote dans les nodosités matures ont mis en évidence le rôle particulier de *Phytogb1.1* dans la régulation du NO au cours du développement nodulaire et lors de l'entrée en sénescence des nodosités.

Contribution of phytoglobins and nitrate reductases to the nitric oxide regulation and nitrogen fixation in *Medicago truncatula* / *Sinorhizobium meliloti* symbiosis

Abstract:

The nitrogen-fixing symbiosis between legumes and soil bacteria of Rhizobia type reduces atmospheric nitrogen (N_2) to ammonia (NH_3) through the presence of bacterial nitrogenase in a root organ, called nodule.

In the symbiotic model *Medicago truncatula* / *Sinorhizobium meliloti*, nitric oxide (NO) is produced throughout the symbiotic process, from the beginning of the interaction between the plant and the bacteria until the senescence of the nodule. The toxic, signal or metabolite effects of NO depend mainly on its concentration at the action site. Its concentration within the nodule cells must be regulated in order to limit its toxic effects and leads its signaling and metabolite functions. In plants, the main sources of NO identified are nitrate reductase (NR) and the mitochondrial electron transfer chain (ETC). In addition, phytoglobins (Phytogb) are known to be involved in the catabolism of NO. According to their sequences homology and affinity for oxygen, three classes of Phytogb have been described in legumes: non-symbiotic Phytogb (Phytogb1), legume-specific leghemoglobin (Lb) and truncated Phytogb (Pgb3).

The main objectives of this PhD were, on the one hand, to characterize and study the role of NR and Phytogb in the NO regulation, during the symbiosis between *M. truncatula* and *S. meliloti* and, on the other hand, to analyse the role of NO in the development and in mature nodule during the symbiotic process.

In *M. truncatula*, 3 genes code for NR and 17 for Phytogb. The phylogenetic study of *M. truncatula* Phytogb identified 12 Lb, 3 Phytogb1 and 2 Phytogb3. Analysis of NR and Phytogb gene expression, as well as measurement of total NR activity and NO production, allowed to monitor the level of NO during the N_2 -fixing symbiosis, and to determine the respective roles of the different NR and Phytogb in the NO regulation. During the symbiotic process, four peaks of NO production were observed, corresponding to four periods of the symbiotic process, during (1) the establishment of the interaction between the plant and the bacteria, (2) at the setup of the nodule organogenesis (3) inside the functioning and mature nodule and (4) at the onset of nodule senescence. During these different periods, the production of NO is particularly correlated with the expression of the NR1 and 2, one Phytogb1 (Phytogb1.1) and one Phytogb3 (Phytogb3.1) genes. The use of various inhibitors of NO synthesis pathways has shown that NO production depends mainly on NR activity and the mitochondrial electron transfer chain. The use of NO donors has shown that, during nodular development, NO induces the expression of Phytogb1 and several defence genes but represses Lb and Phytogb3 genes. Functional analysis of Phytogb1.1, during nodule establishment, functioning and senescence, was initiated via the production of *M. truncatula* plants overexpressing or silencing this gene. Overexpression and under-expression of this gene resulted, respectively, in a decrease and an increase in the NO level in the nodules, but in both cases resulted in a decrease in the number of nodules per plant. Analysis of the expression of several markers genes of the symbiotic interaction, defence responses, nitrogen metabolism and hypoxia, and analysis of nitrogen fixation in mature nodules highlight the particular role of Phytogb1.1 in the NO regulation during nodule development and during the senescence process.

*Si vous voulez faire une tarte aux pommes à partir de rien,
il vous faudra d'abord créer l'univers.*
Carl Sagan

*Oh Mec !! Regarde ce caillou là-bas !! Absolument incroyable !!
Un moment épique de ma vie !!*
**Harrison Schmitt, géologue lunaire lors de
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Table 3.1: Number and size of nodules in control plant and *Phytogb1.1* transformed plant at 14 dpi.

Table 3.2: Nitrogenase activity in control plant and *Phytogb1.1* transformed plant at 3 wpi.

Table 3.3: Nitrogenase activity in control plant and *Phytogb1.1* transformed plant at 3 wpi after 2 days of 10 mM nitrate treatment

Table 3.4: Analyse du phénotype de nodulation des racines transformées pour *Phytogb3.1* et le contrôle

Abréviations

ADH : Alcool déshydrogénase	MoCo : Cofacteur au molybdène
ADN : Acide désoxyribonucléique	MPT : Modification post-traductionnelle
ADP : Adénosine di-phosphate	N₂ : Diazote
AOX : Alternative oxydase	NAD(P)H : Nicotinamide adénine dinucléotide (phosphate) réduit
APX : Ascorbate peroxydase	NH₄⁺ : Ammonium
ARA : Activité réductrice d'acétylène	NiR : Nitrite reductase
ATP : Adénosine tri-phosphate	NO : Monoxyde d'azote
BH₄ : Tétrahydrobioptérine	NO[•] : NO radicalaire
CaM : Calmoduline	NO⁻ : Anion nitroxyde
CAT : Catalase	NO⁺ : Cation nitrosonium
CO : Monoxyde de carbone	NO₂⁻ : Nitrite
CO₂ : Dioxyde de carbone	NO₃⁻ : Nitrate
CP : Cystéine protéase	NOFNIR : Nitric oxide forming nitrite reductase
cPTIO : carboxyphényl-tétraméthylimidazoline-oxyloxide	NOR : NO réductase
CS : Chalcone synthase	NOS : NO synthase
DAF : Diaminofluoresceine	NR : Nitrate reductase
DEA : Diéthylamine	O₂ : Dioxygène
DEA-NO ; Diéthylamine NONOate	O₂^{-•} : Anion superoxyde
Dpi/jpi : jours post-inoculation	PDC : Pyruvate décarboxylase
EDRF : Endothelium derived relaxing factor	Phytogb : Phytoglobine
ETC/CTE : chaîne de transfert d'électrons mitochondriale	PGPR : Plant growth promoting rhizobacteria
FAD : Flavine adénine dinucléotide	Phytogb1 : hémoglobine non-symbiotique 1
Fe²⁺ : Fer ferreux	Phytogb2 : hémoglobine non-symbiotique 2
Fe³⁺ : Fer ferrique	Phytogb3 : hémoglobine tronquée
FMN : Flavine mononucléotide	pO₂ : Pression partielle en oxygène
GOGAT : Glutamine oxoglutarate aminotransférase	RNS : Espèces réactive de l'azote
Gpx : Glutathion peroxydase	ROS : Espèces réactive de l'oxygène
GSH : Glutathion	SNO : S-nitrosothiol
GSNO : Glutathion S-nitrosylé	SNP : Nitroprussiate de sodium
GSNOR : GSNO réductase	SOD : Superoxyde dismutase
GST : Glutathion S-transférase	Spi/wpi : Semaines post-inoculation
GS1a : glutaminie synthetase 1a	
H₂S : Sulfure d'hydrogène	
Hb : Hémoglobine	
HDAC : Histone désacétylase	
Hmp : Flavohémoglobine bactérienne	
HNO : Nitrosyle	
Hpi : Heures post-inoculation	
Lb : Légghémoglobine	
LCO : Lipo-chito-oligosaccharides	
L-NAME : L-N ^G -Nitro-arginine methyl ester	
L-NMMA : L-N ^G -monoéthyl-arginine citrate	

Introduction générale

La symbiose entre les légumineuses et les bactéries fixatrices d'azote (rhizobium) représente un enjeu majeur pour la réduction des intrants azotés en agriculture. La réduction de l'azote moléculaire (N_2) en ammonium (NH_4^+) par la nitrogénase bactérienne, puis son assimilation par la cellule végétale constituent les fonctions centrales des nodosités symbiotiques. Plusieurs études ont démontré l'importance de la production du monoxyde d'azote (NO) par la plante et la bactérie lors de la symbiose Légumineuse-Rhizobium, notamment au cours des premières étapes de l'interaction dans le fonctionnement de la nodosité et dans le processus de sénescence. Pour comprendre le rôle du NO, il est nécessaire de comprendre, d'une part, de quelle façon il est produit, puis métabolisé et, d'autre part, comment il régule l'activité des protéines et l'expression des gènes au cours du processus symbiotique.

Lorsque je suis arrivé en thèse, l'équipe Symbiose avait montré que, dans des nodosités fixatrices d'azote, la nitrate réductase végétale et la chaîne de transfert d'électron mitochondriale sont impliquées dans la synthèse du NO. D'autre part, des travaux de l'équipe avaient montré que les leghémoglobines sont capables d'oxyder le NO en nitrate. Par ailleurs, le rôle du NO dans l'interaction symbiotique était, et reste, controversé. Certaines études montraient que le NO inhibe la nodulation, tandis que d'autres montraient qu'il est nécessaire à la nodulation et au développement des nodosités.

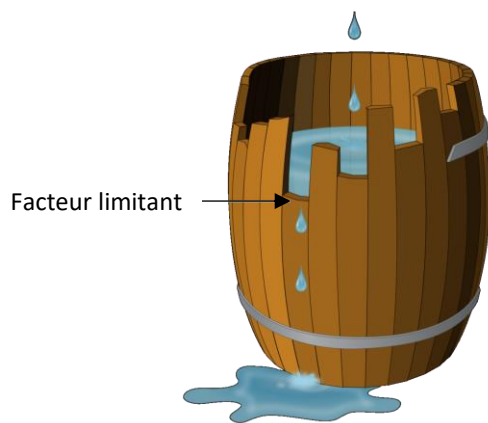
Plusieurs questions se posaient donc, à savoir : 1) quel est le rôle des nitrate réductases et des hémoglobines (phytoglobines) dans la régulation du niveau de NO au cours de l'ensemble du processus symbiotique ; 2) de quelle manière le NO régule-t-il le processus symbiotique ?.

Les principaux objectifs de cette thèse ont été, d'une part, de caractériser et étudier le rôle des nitrate réductases et des phytoglobines dans la régulation du NO lors de la symbiose entre *M. truncatula* et *S. meliloti* et, d'autre part, d'analyser le rôle du NO dans le développement et le fonctionnement du processus symbiotique.

Ce manuscrit présente, d'une part, un état de l'art bibliographique sur la symbiose : le NO, les nitrate réductases, et les hémoglobines. D'autre part, après un chapitre consacré aux matériels et méthodes, les résultats obtenus au cours de ce travail sont présentés en trois chapitres. Le premier chapitre est consacré à la caractérisation des nitrate réductases de *M. truncatula* et à leur rôle dans la synthèse du NO lors du processus symbiotique. Le second chapitre est consacré à la caractérisation des phytoglobines de *M. truncatula* et à leur rôle dans la régulation de l'homéostasie du NO tout au long du processus symbiotique. Le dernier chapitre est consacré à l'étude d'une phytoglobine, PhytoGb1.1, lors des premières étapes de la symbiose, ainsi que dans la nodosité fixatrice d'azote. Le manuscrit se termine par une conclusion générale et par l'identification de quelques pistes pour poursuivre ce travail.

Introduction Bibliographique

(A)



(B)

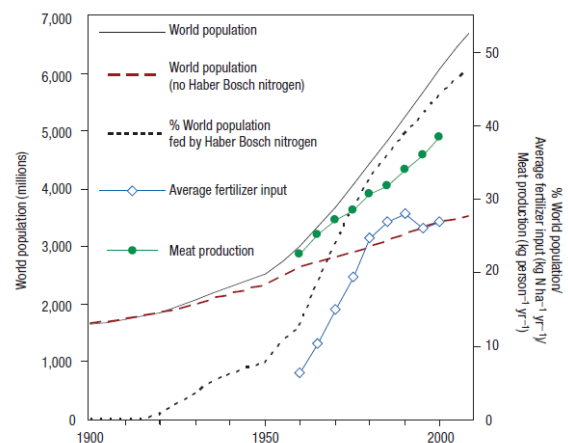


Figure 1 : Rôle de l'azote sur la croissance des plantes et impact des engrais azotés

(A) Représentation schématique de la loi de Liebig ou loi du minimum. En 1840, Justus von Liebig formule cette loi selon laquelle la croissance d'une plante est limitée par l'élément assimilable dont la concentration dans le milieu est la plus faible (facteur limitant).

(B) Évolution de la population mondiale et de l'utilisation des engrais azotés au cours du XX^{ème} siècle. A partir de la population mondiale totale (trait continu), une estimation d'une population qui n'utiliserait pas le procédé Haber-Bosch est réalisée (trait rouge pointillé). En trait noir pointillé est représentée la population mondiale nourrie grâce à ce procédé. L'augmentation de l'utilisation moyenne d'engrais par hectare de terre agricole est symbolisée par la courbe bleue. L'augmentation de la production de viande par habitant (courbe verte) est également indiquée (Erisman *et al.*, 2008).

1. La symbiose fixatrice d'azote

1.1. Symbiose et généralités

L'azote est un élément important pour la croissance et le développement des végétaux (constituant moléculaire des protéines, des acides nucléiques, des polyamines, cofacteur enzymatique ...). Il peut, tout comme l'eau, devenir un facteur limitant de la production végétale puisque les plantes n'assimilent que les formes minérales de l'azote telles que le nitrate (NO_3^-) et l'ammonium (NH_4^+) qui sont tous deux présents en quantité limitée dans le sol (Fig. 1A).

Cependant, certaines bactéries, dites fixatrices d'azote, peuvent réduire l'azote atmosphérique (N_2) en ammoniac grâce à la présence d'une enzyme spécifique, la nitrogénase. Trois catégories de bactéries fixatrices d'azote peuvent être distinguées : (1) les cyanobactéries responsables de 40 à 50% de la fixation biologique de l'azote, (2) des microorganismes du sol qui sont capables de vivre sous forme libre ou (3) en symbiose avec un végétal. La symbiose correspond à une situation où deux, voire plusieurs, organismes appartenant à différentes espèces vivent en étroite interaction pendant une période de temps prolongée (De Bary, 1879). Deux types de symbioses plantes/microorganismes peuvent être distinguées. Tout d'abord, les symbioses dites associatives dont la plus connue fait intervenir des bactéries rhizosphériques stimulatrices de la croissance des plantes ou Plant Growth-Promoting Rhizobacteria (PGPR) (Dobbelaere *et al.*, 2001; Franche *et al.*, 2009). D'autres part, les symbioses dites mutualistes qui aboutissent à une différenciation morphologique chez l'un ou les deux partenaires (Bertrand *et al.*, 2011). Les symbioses mutualistes plantes/microorganismes les plus étudiées sont les symbioses mycorhiziennes entre les plantes et les champignons (Hebeloma, Glomus, etc.), ainsi que les symbioses fixatrices d'azote impliquant des légumineuses (avec des Rhizobium) ou des plantes actinorhiziennes (avec Frankia) (Marmeisse *et al.*, 2004; Normand *et al.*, 2007; Franche *et al.*, 2009).

L'interaction symbiotique qui réunit les légumineuses et les rhizobia permet à la plante d'utiliser les 78% de l'azote (N_2) présent dans l'atmosphère, en utilisant la capacité de fixation du N_2 de la bactérie.

Malgré la diversité des interactions plantes/micro-organismes, peu d'espèces sont capables de mettre en place cette symbiose fixatrice d'azote. C'est pourquoi, la découverte de la synthèse de l'ammoniac à partir du N_2 au début des années 1900 par Fritz Haber¹ et Carl

¹ A la fin du 19^{ème} siècle, Fritz Haber décide de travailler à la synthèse du nitrate pour permettre à l'Allemagne de disposer d'autant d'explosifs qu'elle veut. En 1909, il réussit à fixer l'azote contenu dans l'air pour fabriquer de l'ammoniac. En 1913, avec Carl Bosch, ils mettent au point le procédé de synthèse de l'ammoniac. Il se trouve que ce procédé permet, du coup, de fabriquer aussi des engrais sauvant ainsi l'humanité d'une famine mondiale annoncée. Haber aura d'ailleurs le Prix Nobel de chimie en 1919, au titre de l'avancée de

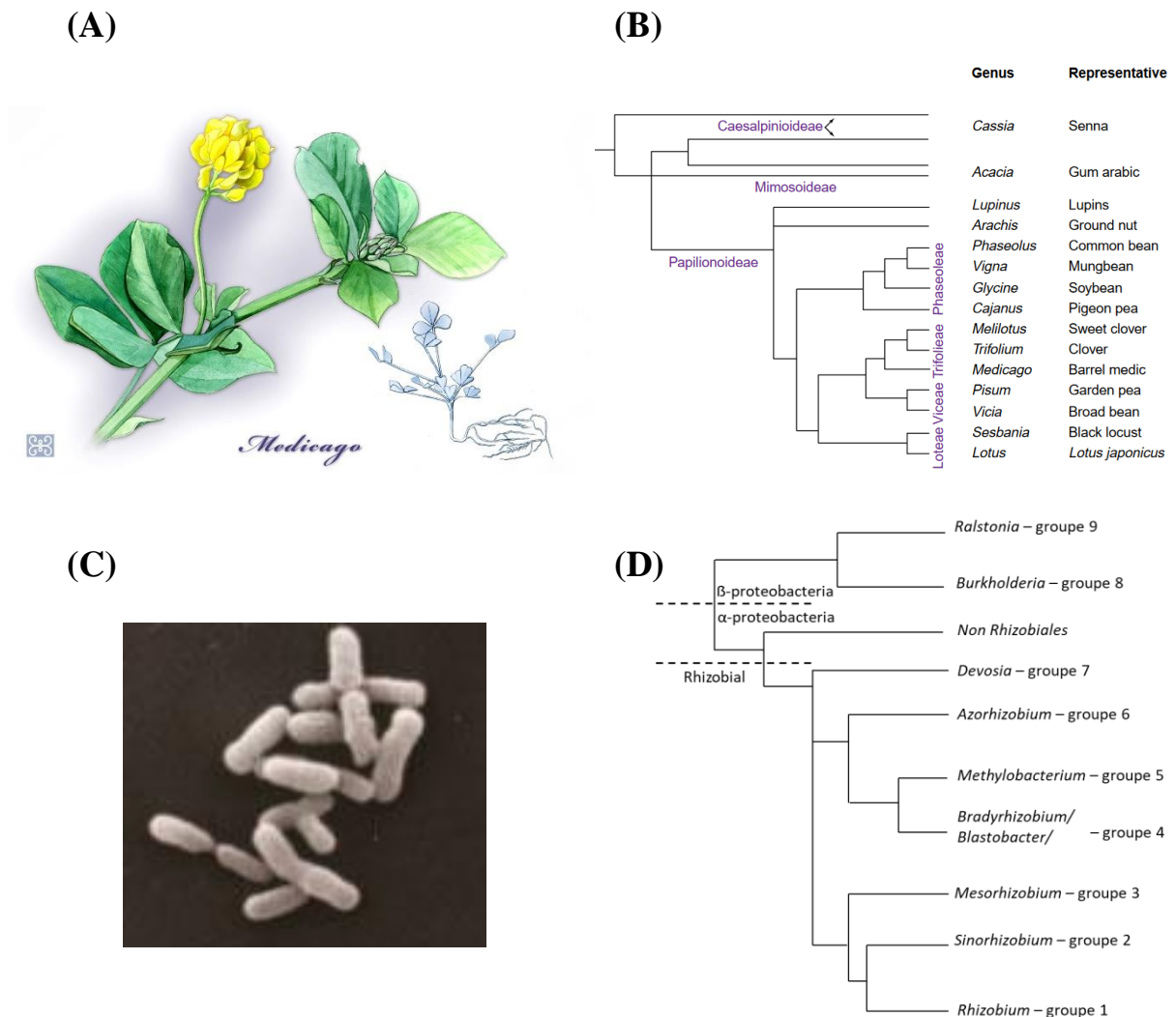


Figure 2: Classification du genre Légumineuse et du genre *Rhizobium*.

(A) Feuille, fleur et racines du genre *Medicago* (Zina Deretsky, NSF)

(B) Représentation phylogénétique du genre des légumineuses. La figure montre les trois sous-familles principales ainsi que certaines espèces cultivées dans les *Papilionoidea* (adapté de Udvardi *et al.* (2005)).

(C) Cellules de *S. meliloti* analysées en microscopie électronique à balayage (photo par William Margolin)

(D) Représentation phylogénétique simplifiée du genre *Rhizobium*. La figure montre les 9 groupes monophylétiques (adapté de Sawada *et al.* (2003))

ses travaux dans "le bien-être de l'humanité". Cette attribution est hautement controversée, de par sa participation à la conception des gaz de combat employés dans les tranchées et notamment le gaz moutarde. Par la suite, avec l'un de ses anciens collaborateurs, (Hans Heerdt) ils mettent au point un pesticide (Zyklon A), très efficace et qui est suffisamment odorant pour alerter n'importe quel humain autour qu'il y a danger. Les nazis, quelques années plus tard, se débarrasseront de cette odeur et augmenteront un petit peu la concentration du produit. Ils mettent ainsi au point un gaz (le Zyklon B) qui sera responsable de la mort de plus de six millions d'hommes, de femmes et d'enfants y compris pas mal de membres de la famille de Fritz Haber.

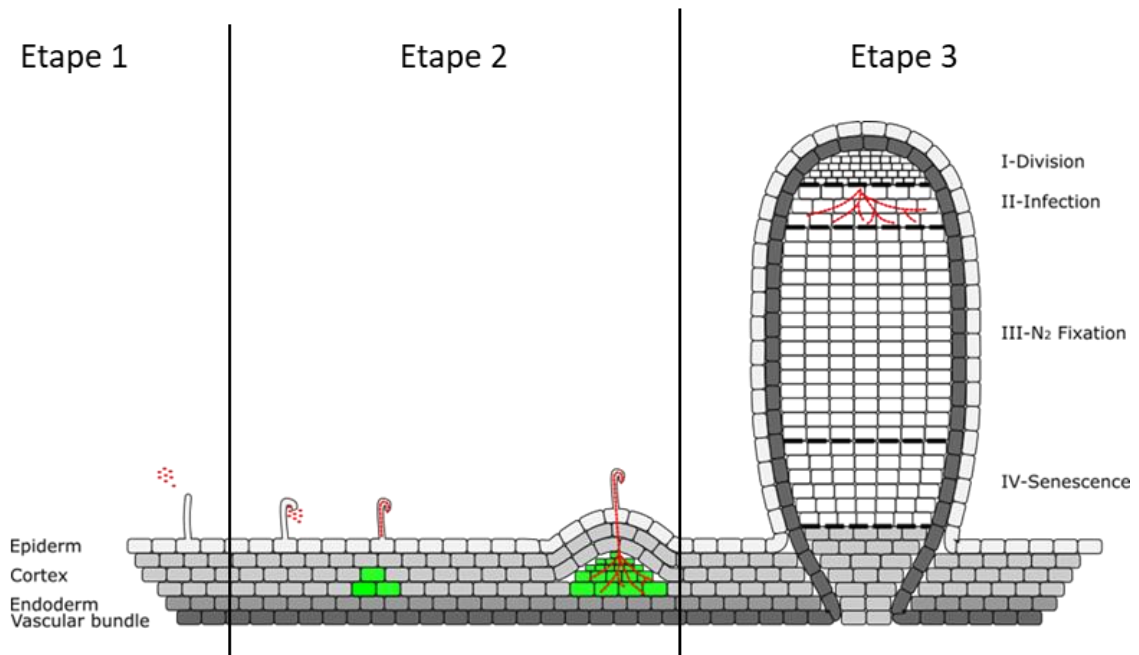
Bosh, fut une avancée majeure pour la production industrielle d'engrais azoté. Erisman *et al.* (2008) estiment que 40% de la population mondiale est aujourd'hui dépendante des engrais azotés pour produire sa nourriture (Fig. 1B). Cependant la production et l'utilisation de ces engrais ne sont pas sans impact sur l'environnement (prolifération d'algues vertes, phénomène d'eutrophisation, pollution de l'air et des nappes phréatiques) et la vie humaine (intoxication) (Sutton *et al.*, 2011). Il est donc nécessaire de trouver d'autres stratégies permettant de minimiser l'utilisation des engrais azotés tout en gardant un fort rendement de production. L'une de ces stratégies consiste en la culture d'espèces végétales capables de fixer et de remobiliser l'azote efficacement, comme les légumineuses, et qui permettent de diminuer en partie l'apport en intrants azotés (Foyer *et al.*, 2016).

1.1.1. Les Légumineuses

Les Légumineuses (Fabacées) sont des dicotylédones appartenant à l'ordre des Fabales. Les Fabacées comptent environ 18 000 espèces regroupées en 650 genres (Sprent, 2001), réparties en 3 sous-familles : les Césalpiniées (arbre de judée), les Mimosacées (acacia, mimosa) et les Papilionacées (luzerne, soja, pois, trèfle, haricot...) (Doyle & Luckow, 2003) (Fig. 2B). Les Légumineuses possèdent l'aptitude de former des symbioses fixatrices d'azote avec des bactéries du sol (De Faria *et al.*, 1989). Cependant, toutes les Légumineuses ne peuvent pas établir cette interaction, en effet bien que 90% des Papilionacées et des Mimosacées peuvent rentrer en symbiose, seul 25% des Césalpiniées en sont capables (Hirsch *et al.*, 2001).

Sur un plan scientifique, la Légumineuse modèle *Medicago truncatula* (Fig. 2A), est l'une des plus utilisées. D'un point de vue phylogénétique, *M. truncatula* se rapproche des principales Légumineuses cultivées dont *Medicago sativa* et *Pisum sativum* (Zhu *et al.*, 2005). C'est une espèce diploïde ($2n=16$) ce qui facilite les études génétiques et moléculaires. Son génome, 4 fois plus grand que celui d'*Arabidopsis thaliana* (500 Mbp), est complètement séquencé depuis 2011 (Young *et al.*, 2011). De nombreux outils génétiques comme génomiques sont disponibles, ainsi qu'une banque de mutants par insertion de transposon (<https://medicago-mutant.noble.org/mutant/>). *M. truncatula* présente aussi l'avantage d'être facilement transformable par *Agrobacterium rhizogenes/tumefaciens* selon les méthodes de Boisson-Dernier *et al.* (2001) et Vieweg *et al.* (2005).

(A)



(B)

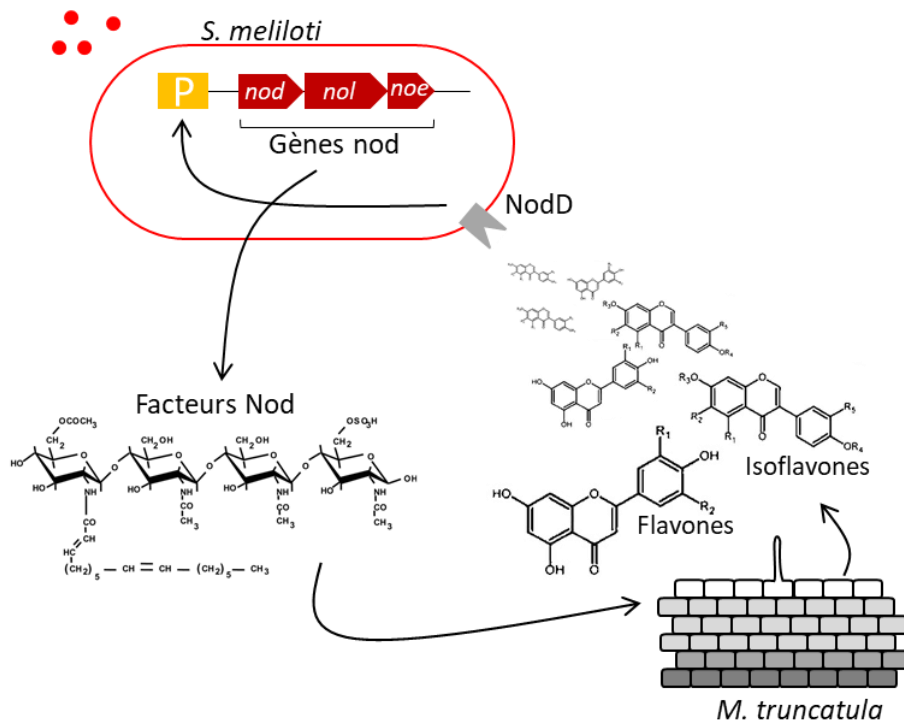


Figure 3 : Les premières étapes de la symbiose

(A) Représentation schématique de la mise en place de la nodosité. Étape 1 : l'étape de reconnaissance entre la plante et la bactérie, Étape 2 : l'infection et le développement, Étape 3 : la formation de la nodosité mature.

(B) Représentation du dialogue moléculaire entre *M. truncatula* et *S. meliloti*. *M. truncatula* sécrète des flavonoïdes qui vont être perçus par *S. meliloti* via son récepteur NodD. NodD va alors induire, via l'expression des gènes nod, la sécrétion des facteurs Nod qui vont à leur tour être perçus par la plante hôte (Oldroyd & Downie, 2004).

1.1.1. Les rhizobiacées

Les bactéries du genre *Rhizobium*, sont de type gram négative et appartiennent au phylum des α -protéobactéries qui peut être divisé en 9 groupes monophylétiques différents (Sawada *et al.*, 2003) (Fig. 2D). Parmi les rhizobiacées, *Sinorhizobium meliloti* (appartenant au groupe 2) a été retenu comme un organisme modèle pour les bactéries symbiotiques fixatrices d'azote. *S. meliloti* (Fig. 2C), bactérie du sol aérobie, est capable de vivre sous forme libre, mais aussi en association avec les légumineuses du genre *Medicago*, *Melilotus* ou *Trigonella*. Son génome de 6,7 mégabases est séquencé depuis 2001 (Galibert *et al.*, 2001) et ré-annoté par Becker *et al.* (2009) Le génome de *S. meliloti* est composé d'un chromosome de 3,65 Mb et deux mégaplasmides, pSymA et pSymB, respectivement de 1,36 Mb et 1,68 Mb. La plupart des gènes impliqués dans la symbiose sont présents sur le plasmide pSymA, tandis que des gènes codant pour des fonctions de prélèvement de nutriments et d'invasion de la plante sont présents sur le plasmide pSymB (Barloy-Hubler *et al.*, 2000).

1.2. Les différentes étapes de la formation de la nodosité

La mise en place de la symbiose Légumineuse-Rhizobium se caractérise par 3 étapes principales (Fig. 3A) : (1) une étape de reconnaissance entre la plante et la bactérie par l'intermédiaire d'un dialogue moléculaire, (2) l'infection de la racine par la bactérie et le développement du primordium nodulaire, et (3) la formation d'un nouvel organe racinaire, la nodosité au sein duquel se déroule la réduction et la fixation de l'azote atmosphérique.

1.2.1. Dialogue moléculaire et reconnaissance

Le processus de reconnaissance entre les Rhizobia et une Légumineuse est une étape essentielle dans l'établissement de cette symbiose. La plante hôte sécrète dans le sol des composés de diverses natures tels que des glucides, des acides organiques, et différents dérivés phénoliques qui peuvent induire des changements sur le microbiote² du sol. Parmi les composés sécrétés, des flavonoïdes (flavone, chalcone, isoflavonoïdes ...) peuvent être perçus par les rhizobia au niveau d'un récepteur de type LysR appelé NodD.

² L'ensemble des microorganismes présents dans un environnement défini. Le terme microbiote a été défini pour la première fois par Lederberg & McCray, (2001), qui ont souligné l'importance des micro-organismes présents dans le corps humain pour la santé et la maladie (Marchesi & Ravel, 2015).

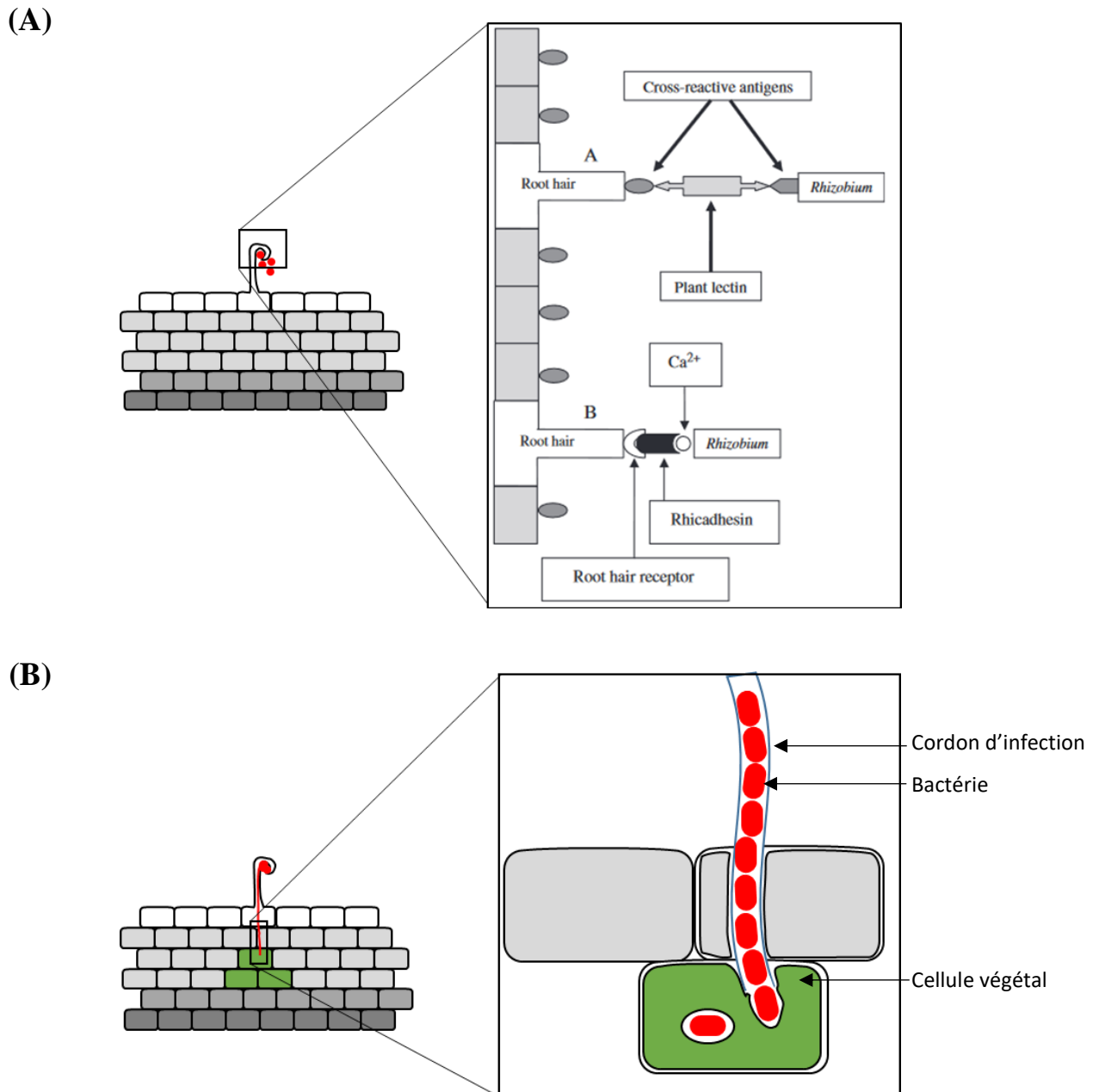


Figure 4: Etape d'adhésion et d'infection des rhizobia

(A) Etape d'adhésion des *rhizobia* aux poils absorbants. Deux modèles d'attachement sont représentés, l'un médié par la présence de lectines et qui reconnaît les polysaccharides bactériens. L'autre, par la présence de rhiCADHÉSINES au niveau bactérien qui reconnaît un récepteur végétal (Rodríguez-Navarro *et al.*, 2007).

(B) Développement du cordon d'infection et formation du symbiosome par invagination de la membrane. Les cordons d'infection sont principalement composés de bactéries issues d'une même souche et leur croissance au sein du cordon d'infection participe à son extension. Les rhizobia quittent les cordons d'infection via une structure dite de goutte d'infection. Ces gouttes sont en fait des invaginations de la membrane plasmique du cordon d'infection qui enferme un Rhizobium. Par ce processus similaire à l'endocytose, les rhizobia sont internalisées dans la cellule végétale sans être libres dans le cytoplasme.

Les rhizobia sont alors attirés par chimiotactisme vers les racines (Perret *et al.*, 2000) (Fig. 3B). Il existe un spectre d'hôte pour chaque rhizobium conditionné par la nature de l'exsudat racinaire sécrété par la plante (Pueppke & Broughton, 1999). Une espèce de rhizobium ne peut réaliser de symbiose qu'avec un nombre limité d'espèces de légumineuses et réciproquement (Pueppke & Broughton, 1999).

Une fois les flavonoïdes de la plante reconnus par le récepteur NodD, celui-ci va induire l'expression des opérons de nodulation (*nod*, *nol*, et *noe*) (Perret *et al.*, 2000) (Fig. 3B). Ces gènes codent pour des enzymes impliquées dans la synthèse, la maturation et le transport de composés lipo-chito-oligosaccharidiques (LCO) appelés facteurs Nod. Ces composés sont constitués d'une succession de 4 à 5 β_{1-4} N-acétyl-D-glucosamines auxquelles est liée une chaîne d'acides gras insaturée (Oldroyd & Downie, 2004). Selon les espèces de rhizobium, le squelette de LCO est décoré avec différents groupements méthyle, acétyle, sulfuryle, carbonyle, fucosyle ou encore arabinosyle (Oldroyd & Downie, 2008). Ces modifications du squelette du LCO, spécifiques selon l'espèce de rhizobium, participent à la fois au spectre d'hôte, mais aussi à la stabilité des facteurs Nod (Oldroyd & Downie, 2008).

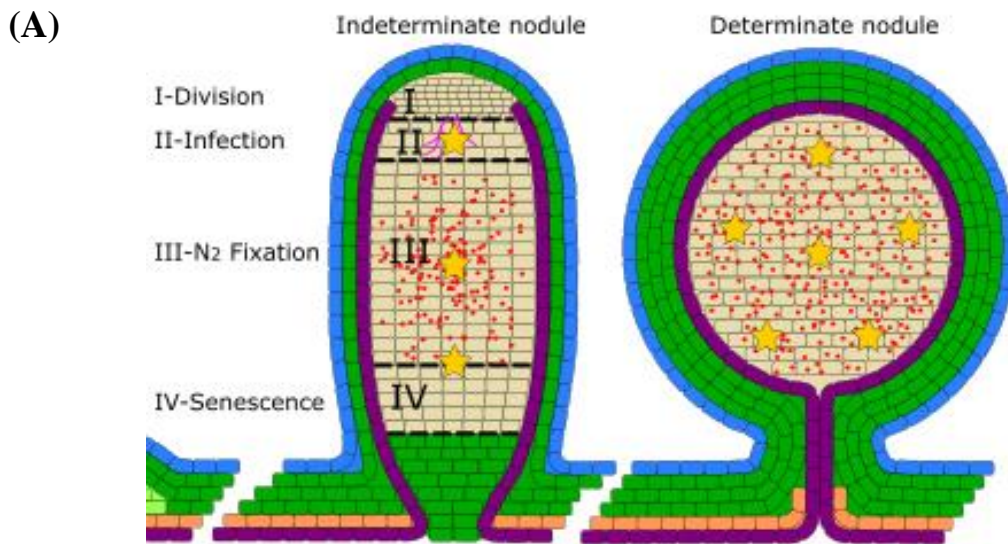
Le dialogue moléculaire entre la légumineuse et le rhizobium est essentiel et permet par la suite la bonne initiation du développement du processus symbiotique (Cooper, 2007; Gibson *et al.*, 2008).

1.2.2. Infection et développement de la nodosité

Les facteurs Nod sont perçus par la plante jusqu'à de très faibles concentrations (10^{-4} nM) grâce à la présence de récepteurs membranaires LysM-RKs (Gough & Cullimore, 2011). Ces récepteurs sont composés d'un domaine LysM extra-cellulaire (pour la liaison au facteur Nod) et d'un domaine kinase cytosolique (Arrighi *et al.*, 2006). La perception des facteurs Nod induit chez la plante de multiples réponses, et permet simultanément l'invasion bactérienne et le développement des divisions cellulaires, ce qui aboutit à la formation du primordium nodulaire.

1.2.2.1. Invasion bactérienne

La pointe des poils absorbants est la principale cible d'infection par le rhizobium, ce qui est en partie expliqué par leurs parois cellulaires plus minces et moins réticulées. L'adhésion des bactéries aux poils absorbants est possible grâce à la présence de rhicadhésines (adhésine



	Nodosité indéterminée	Nodosité déterminée
Site des 1 ^{er} évènement de division cellulaire	Cortex racinaire interne	Cortex racinaire externe
Type du méristème	Méristème persistant	Pas de méristème persistant
Forme normal de la nodosité	Cylindrique	Sphérique
Cordon d'infection	Large	Étroit
Cellules infectées	Hautement vacuolé	Peu vacuolé
Forme du bactéroïde	Large, ramifié faible viabilité, un par symbiosome	Normal, haute viabilité, plusieurs par symbiosome
Région géographique de la plante	Région tempérée	Région tropicale/subtropicale
Exemples	Medicago, Trèfles, Pois.....	Soja, Fève, Lotier

Figure 5: Caractéristiques des nodosités déterminées et indéterminées

(A) Caractéristiques morphologiques des deux types de nodosités, déterminée et indéterminée.

(B) Différences majeures entre les nodosités déterminées et indéterminées (adapté de Fergusson *et al.*, 2010)

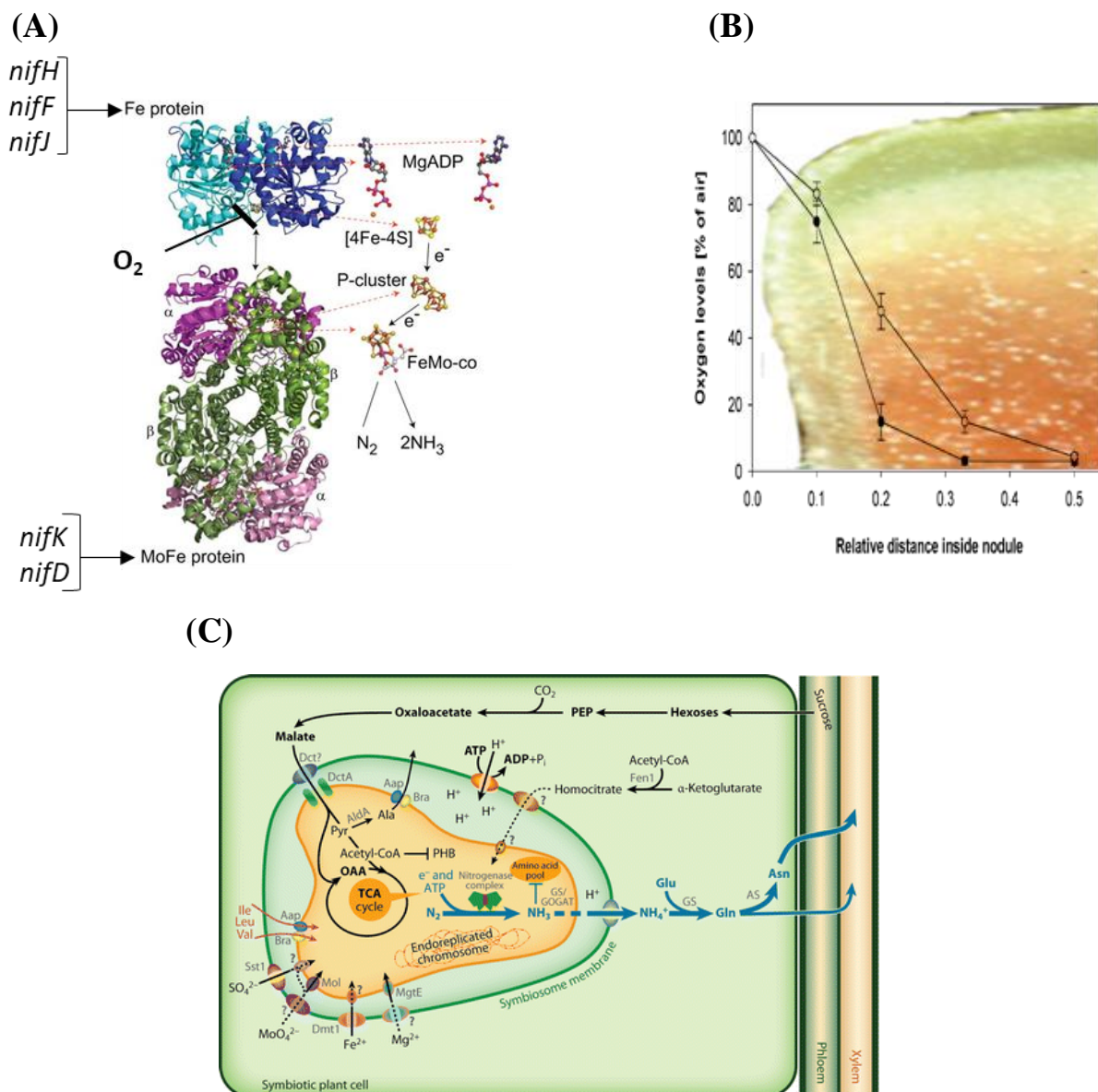
calcium dépendante) à la surface des bactéries et par la présence à la surface des poils racinaires de lectines qui peuvent lier les polysaccharides présents sur la surface des bactéries (Laus *et al.*, 2006) (Fig. 4A). La fixation des rhizobia aux poils absorbants et l'influence des facteurs Nod provoquent, en 6 à 8 heures, la déformation des poils absorbants en une structure dite de « crosse de berger » (Yao & Vincent, 1969; Bhuvaneshwari *et al.*, 1981; Gage, 2004).

Encapsuler à l'intérieur de cette structure, les bactéries qui se divisent pour infecter la plante par l'intermédiaire d'un cordon d'infection (Fig. 4B). Le cordon d'infection est une structure tubulaire extérieure à la cellule végétale comportant des composants identiques à ceux de la paroi végétale (Gage, 2004). Chez le soja, l'infection des poils racinaires à travers la formation du cordon d'infection se met en place 12 h après le contact avec la bactérie (Turgeon & Bauer, 1982, 1985). C'est à l'intérieur de cette structure que les bactéries se divisent et ainsi progressent en direction du cortex racinaire (Gage, 2002) vers les cellules du futur primordium nodositaire (Fig. 4B). Les bactéries pénètrent ensuite au sein des cellules végétales grâce à une invagination de la membrane plasmique *via* un phénomène d'endocytose (Fig. 4B). Ainsi les bactéries sont séparées du cytoplasme de la cellule végétale par une membrane nommée membrane peribactéroïdienne, qui forme le symbiosome à l'intérieur duquel la bactérie se différencie en bactéroïde (Leborgne-Castel *et al.*, 2010).

1.2.2.2. Formation du primordium nodulaire

Parallèlement au processus d'infection par les bactéries symbiotiques, le processus de formation de la nodosité s'initie. La perception des facteurs Nod induit, par l'intermédiaire d'un signal calcique, l'activation de facteurs de transcription qui ciblent des gènes (nodulines) impliqués dans la différenciation du primordium nodositaire (Geurts *et al.*, 2005). Les premières cellules à rentrer en division active sous l'effet des facteurs Nod sont les cellules du pericycle (16 – 18 h post-inoculation), puis celles du cortex. Elles forment le primordium nodositaire (18-24 h post inoculation). Ce sont ces cellules qui seront infectées par les bactéries libérées par les cordons d'infections (48 h post-inoculation). Puis, entre 60 et 72 h après infection, le méristème nodositaire se forme à partir des cellules primordiales des cortex médian et externe qui ne sont pas infectés par les cordons d'infections (Timmers *et al.*, 1999; Xiao *et al.*, 2014).

Il existe deux types de nodosités : déterminé et indéterminé (Fig. 5A). Les nodosités déterminées sont caractérisées par une forme ronde et l'absence d'un méristème persistant. Les légumineuses qui forment des nodosités déterminées sont principalement des espèces tropicales ou subtropicales comme le soja (*Glycine max*), pongamia (*Pongamia pinnata*), mais peut aussi



Udvardi M, Poole PS. 2013. Annu. Rev. Plant Biol. 64:781–805

Figure 6: Les éléments caractéristiques de la nodosité mature

(A) Structure de la nitrogénase. La nitrogénase est formée de la sous unité Fe codée par les gènes *nifH*, *nifF* et *nifJ*, et la sous unité MoFe codée par les gènes *nifK* et *nifD*. Le site d'inhibition par l'oxygène est indiqué par une flèche plate (adapté de Seefeldt *et al.* (2009)).

(B) Variation de la concentration en oxygène au travers du cortex jusqu'au centre de la nodosité. L'image illustre le gradient de concentration en légghémoglobine dans la nodosité de l'extérieur vers l'intérieur (Downie, 2005; Ott *et al.*, 2005).

(C) Schéma simplifié du métabolisme symbiotique au sein des cellules de la nodosité fixatrice (Udvardi & Poole, 2013). L'activité nitrogénase étant très endergonique la plante hôte fournit aux bactéroïdes des glucides issus de la photosynthèse. Ces glucides sont assimilés sous forme de malate qui alimente directement ou indirectement le cycle de Krebs permettant avec la chaîne respiratoire la synthèse d'ATP nécessaire à l'activité nitrogénase. La nitrogénase réduit ainsi le diazote atmosphérique en ammoniacque qui sous forme d'ammonium sera assimilé par la plante sous forme d'asparagine (Asn) et de glutamine (Gln)

inclure des espèces plus tempérées telles que le lotier (*Lotus japonicus*). Chez le soja, le méristème cesse de se développer 10 jours après l'infection (Newcomb *et al.*, 1979). Les premiers événements de divisions cellulaires d'une nodosité déterminée se produisent généralement sous l'épiderme dans le cortex externe. Des exceptions existent, telles que les nodosités de *Lotus japonicus*, qui ne présentent pas les divisions cellulaires initiales sous-épidermiques (Wopereis *et al.*, 2000).

Les nodosités indéterminées ont un méristème plus persistant, ce qui donne des nodosités de forme cylindrique, comme en témoignent les nodosités de luzerne (*Medicago sativa*), de trèfle (*Trifolium repens*), de pois (*Pisum sativum*) et de *Medicago truncatula* (Bond, 1948; Libbenga & Harkes, 1973; Newcomb, 1976; Newcomb *et al.*, 1979). Les premiers événements de divisions cellulaires d'une nodosité déterminée se produisent dans le cortex interne (Xiao *et al.*, 2014).

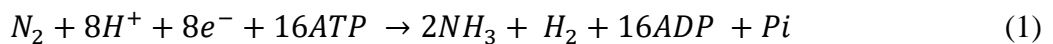
Quatre zones sont distinguées sur une nodosité indéterminée mature et fonctionnelle (Fig. 5A) :

- La zone 1, dite zone méristématique, est une zone de taille constante qui assure la croissance de la nodosité pendant plusieurs semaines. Cette zone, où les cellules végétales se divisent en permanence, ne renferme jamais de bactérie.
- La zone 2, dite zone d'infection, correspond au site de développement terminale des cordons d'infection. Dans cette zone, les rhizobia sont libérés dans les cellules végétales qui ont terminé leur division cellulaire. Les bactéries commencent à se différencier en bactéroïdes (Vasse *et al.*, 1990).
- La zone 3, dite zone de fixation, où les bactéries sont complètement différenciés en bactéroïdes et capable de fixer le N₂ grâce à la nitrogénase.
- La zone 4, dite zone de sénescence, se met en place après 4-6 semaines post-inoculation (Puppo *et al.*, 2005a; Van de Velde *et al.*, 2006). Cette zone ne joue aucun rôle dans le processus de fixation de l'azote, mais correspond à la dégradation progressive des bactéroïdes, puis des cellules hôtes végétales.

Le type de nodosité est déterminé par la plante hôte et les principales différences concernent la présence d'un méristème persistant et la forme de la nodosité mature (Ferguson *et al.*, 2010) (Fig. 5B).

1.3. Le fonctionnement de la nodosité

Un des changements clefs de la différenciation des bactéries en bactéroïdes est l'induction des gènes codant les différentes sous-unités de la nitrogénase, l'enzyme responsable de la fixation de l'azote atmosphérique. Le complexe nitrogénase est composé de 2 métalloprotéines : la protéine MoFe (codée par les gènes *NifK* et *NifD*) qui est la nitrogénase au sens strict et une protéine à fer (codée par les gènes *NifH*, *NifF* et *NifJ*) appelée « diazote réductase » (Fig. 6A) (Seefeldt *et al.*, 2009). La réduction d'une molécule d'azote en deux molécules d'ammoniaque par cette enzyme consomme 16 ATP (1).



L'énergie nécessaire au maintien de l'activité nitrogénase est assurée par la plante qui fournit aux bactéroïdes des composés carbonés issus de la photosynthèse, majoritairement sous forme de malate, et secondairement de succinate et fumarate (Zirilli *et al.*, 1988). En contrepartie, l'ammoniaque produit par le bactéroïde est assimilé dans le cytoplasme de la cellule végétale par la voie « glutamine synthétase (GS) – glutamate synthase (GOGAT) » (Fig 6C).

Comme la nitrogénase est inhibée par l'O₂, la nodosité possède un environnement microaérobie avec une pO₂ de moins de 1 µM au sein des cellules fixatrices, (Minchin, 1997) (Fig. 6B). Deux éléments, en particulier, participent à la mise en place de cet environnement. D'une part, la présence d'une barrière à l'O₂, présente dans le cortex, qui limite la diffusion des gaz extérieurs comme l'O₂ à l'intérieur de la nodosité. Cette barrière de diffusion à l'O₂ est faite d'un épithélium avec une forte densité cellulaire et dont les espaces intercellulaires sont obstrués par des sécrétions de glycoprotéines (Batut & Boistard, 1994). D'autre part, la présence en grande quantité (jusqu'à 40% de la fraction protéique soluble totale d'une nodosité mature) d'une protéine végétale de la famille des hémoglobines, la leghémoglobine (Nash & Schulman, 1976). Cette protéine possède une forte affinité pour l'O₂ et permet de faciliter la diffusion de l'oxygène vers les mitochondries pour alimenter la chaîne de transfert d'électrons (Ott *et al.*, 2005).

1.4. La sénescence du processus symbiotique

La fixation de l'azote est optimale pendant les premières semaines post-infection. Au-delà de cette période, l'efficacité de fixation diminue et conduit à la mise en place progressive

d'un processus de sénescence (Puppo *et al.*, 2005). Visuellement, la sénescence nodositaire est caractérisé par un changement de couleur de la zone de fixation qui passe du rose au vert. Ce changement est en partie provoqué par la dégradation de la leghémoglobine.

Les premiers signes de la sénescence apparaissent dans des cellules au centre de la zone de fixation, puis la zone de sénescence s'étend progressivement dans les couches cellulaires périphériques pour lui donner une forme conique (Perez Guerra *et al.*, 2010). Le développement de la sénescence peut être divisée en deux étapes. Une première étape, au cours de laquelle les bactéroïdes sont dégradés et les symbiosomes fusionnent. Cette fusion forme des compartiments de lyse qui permettent la remobilisation des nutriments (Vasse *et al.*, 1990; Limpens *et al.*, 2009). La deuxième étape correspond à la dégradation des cellules végétales hôtes (Vasse *et al.*, 1990; Van de Velde *et al.*, 2006). Le phénomène de dégradation cellulaire, s'accompagne de la mise en place d'une forte activité protéolytique *via* l'activation de gènes codant pour des protéases telles que les cystéine protéases (CP), des nucléases, des lipases et différents types d'hydrolases (Van de Velde *et al.*, 2006).

La leghémoglobine fait partie de ces protéines dégradées. Sa dégradation provoque la libération de fer qui participe à la production d'espèces réactives de l'oxygène (ROS) *via* la réaction de Fenton (Puppo *et al.*, 2005). Cette augmentation des ROS est responsable de la mise en place d'un stress oxydatif au sein des nodules sénescents (Puppo *et al.*, 2005a; Loscos *et al.*, 2008). Par conséquent, la diminution de l'expression des gènes codants pour les leghémoglobines, ainsi que la diminution de la teneur en Lb, sont corrélées à la mise en place du processus de sénescence (Puppo *et al.*, 2005).

Il est possible de déclencher artificiellement la sénescence nodositaire en appliquant soit un stress carboné en privant les plantes de lumière, soit en provoquant un stress nitrate en augmentant les quantités de nitrate dans le milieu (Matamoros *et al.*, 1999). Des différences physiologiques et moléculaires existent entre des nodosités qui ont subi une sénescence développementale ou une sénescence induite. Notamment, lors d'une sénescence induite par l'obscurité, les bactéroïdes perdent leur contenu sans dégradation des membranes pér bactéroides, contrairement à une sénescence développementale (Perez Guerra *et al.*, 2010). De plus, sur un échantillon de 58 gènes induits dans les nodules de *M. truncatula* au cours d'une sénescence naturelle, seuls 50% sont régulés au cours d'une sénescence induite par l'obscurité (Perez Guerra *et al.*, 2010). Il semble donc que la sénescence induite et la sénescence naturelle sont régulées selon différents mécanismes.

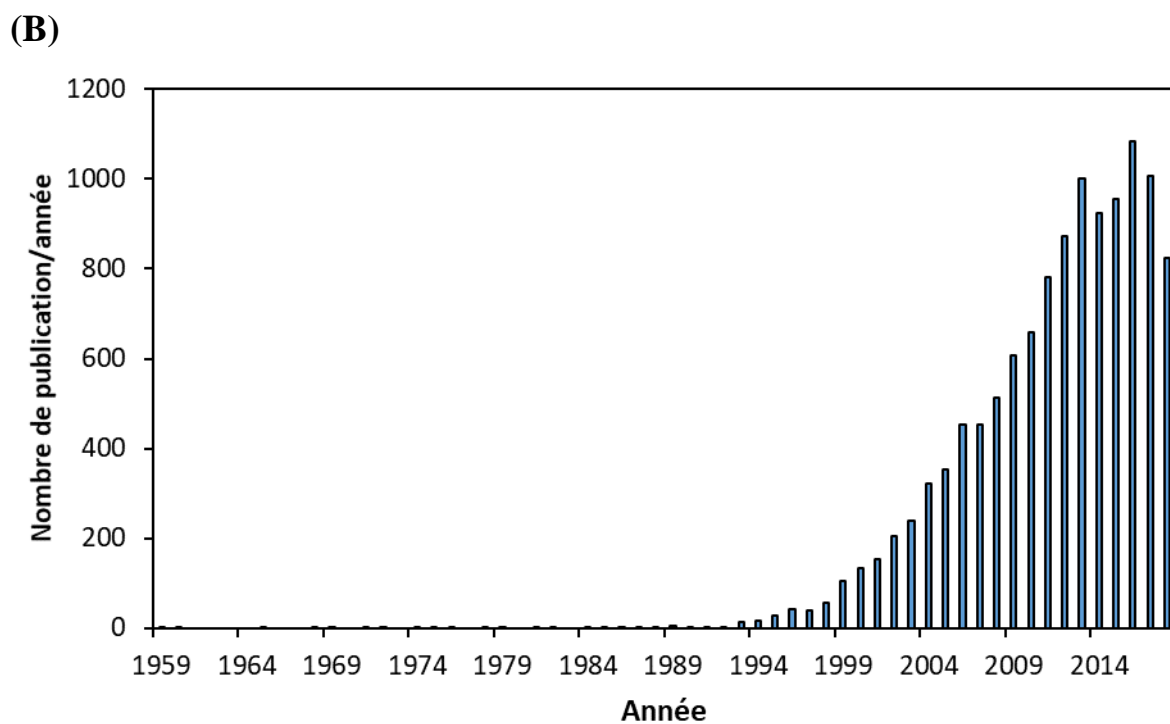
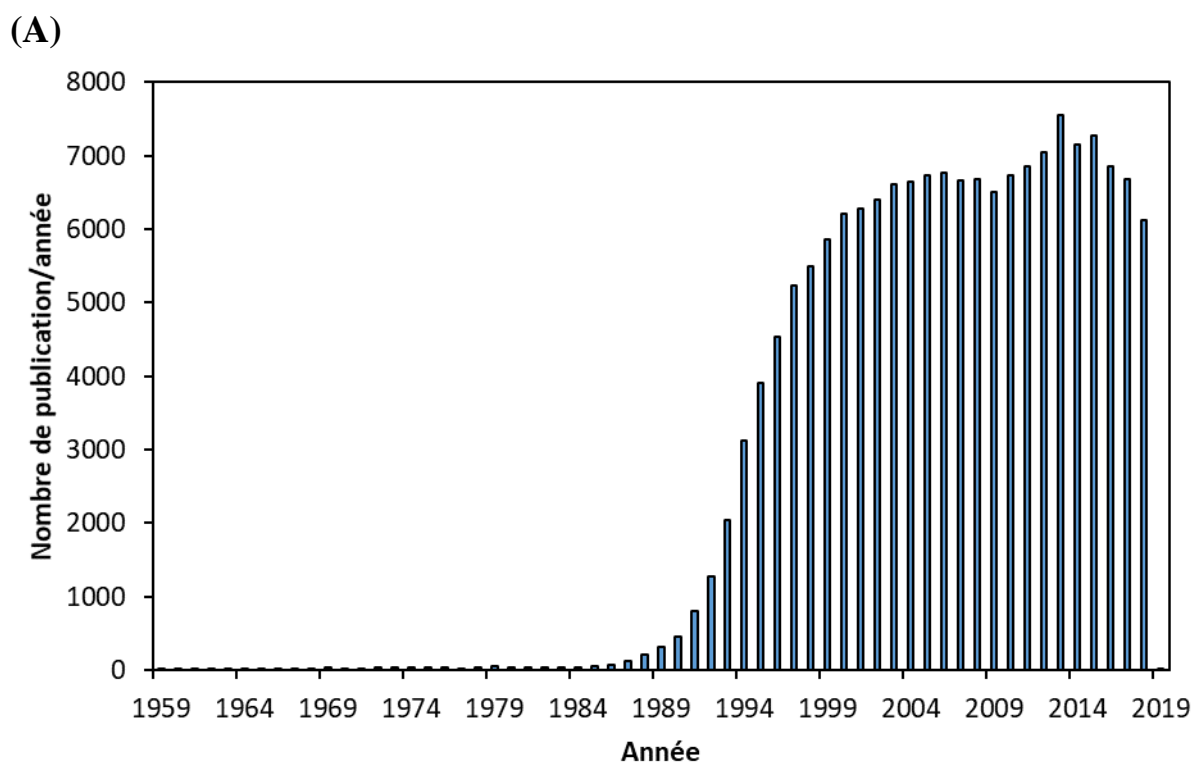


Figure 7 : Evolution du nombre de publications référencées sur la plateforme NCBI.

Sujet de recherche (A) « Nitric oxide » et (B) « Nitric oxide plant » de 1959 à 2019.

2. Le monoxyde d'azote

Le monoxyde d'azote (NO) est un gaz diatomique, radicalaire et hydrophobe impliqué dans de nombreux processus biologiques (Torreilles, 2001). Il a été découvert en 1772 par Joseph Priestley qui le décrit comme un gaz incolore avec une durée de vie *in vivo* de 6 à 10 secondes (Priestley, 1772). Les propriétés chimiques du NO sont étudiées depuis plus de 200 ans (Gow, 2006), mais c'est à la fin des années 1970 que ses propriétés biologiques ont été analysées. En 1977, Murad montre que des composés libérant du NO tel que la nitroglycerine ou le nitroprussiate de sodium, provoquent une vasodilatation des muscles lisses et stimulent la guanylate cyclase, augmentant ainsi les niveaux de cGMP dans les tissus (Katsuki *et al.*, 1977b,a). Au cours de l'année 1980, Furchgott découvre que les cellules endothéliales produisent un composé appelé EDRF (endothelium-derived relaxing factor) qui agit comme un produit de relaxation des muscles lisses vasculaires (Furchgott *et al.*, 1980). En 1987, Ignarro *et al.*, identifient l'EDRF comme étant le NO. En 1992, le magazine Science déclare le NO comme « Molecule of the Year »³ et 7 ans plus tard le prix Nobel de Physiologie et de Médecine est attribué à Furchgott, Ignarro et Murad pour leur découverte sur l'importance du NO.

A la fin des années 1970, il est démontré que le NO est produit par les plantes après un traitement avec différents herbicides (Klepper, 1979). Par la suite, de très nombreuses publications scientifiques ont été réalisées (Fig. 7A, 7B). Les premiers travaux sur les effets physiologiques du NO chez les plantes (Leshem & Haramaty, 1996; Noritake *et al.*, 1996) ont démontré que le NO est impliqué dans la croissance des plantes, mais également dans l'adaptation au stress et le contrôle de la sénescence. Par la suite, des recherches ont permis d'identifier les rôles du NO dans de nombreux processus moléculaires et signalétiques chez les plantes (Gouvêa *et al.*, 1997; Besson-Bard *et al.*, 2008; Wilson *et al.*, 2008).

2.1. La chimie du NO

Il est courant de parler du NO comme s'il s'agissait d'une molécule unique. Cependant, ses propriétés chimiques font qu'il existe 3 formes différentes de ce même gaz. Le NO est une espèce réactive de l'azote mais aussi de l'oxygène. Sous sa forme radicalaire (NO[•]), le NO possède un électron sur son orbital non liant $2p-\pi$ (Stamler *et al.*, 1992) (Fig. 8). Sous cette forme le NO possède un temps de demi-vie de quelques secondes.

³ Parmi les « finalistes » pour le titre de molécule de l'année de 1992, on peut notamment retrouver la nitrogénase, et la découverte de sa structure 3D.

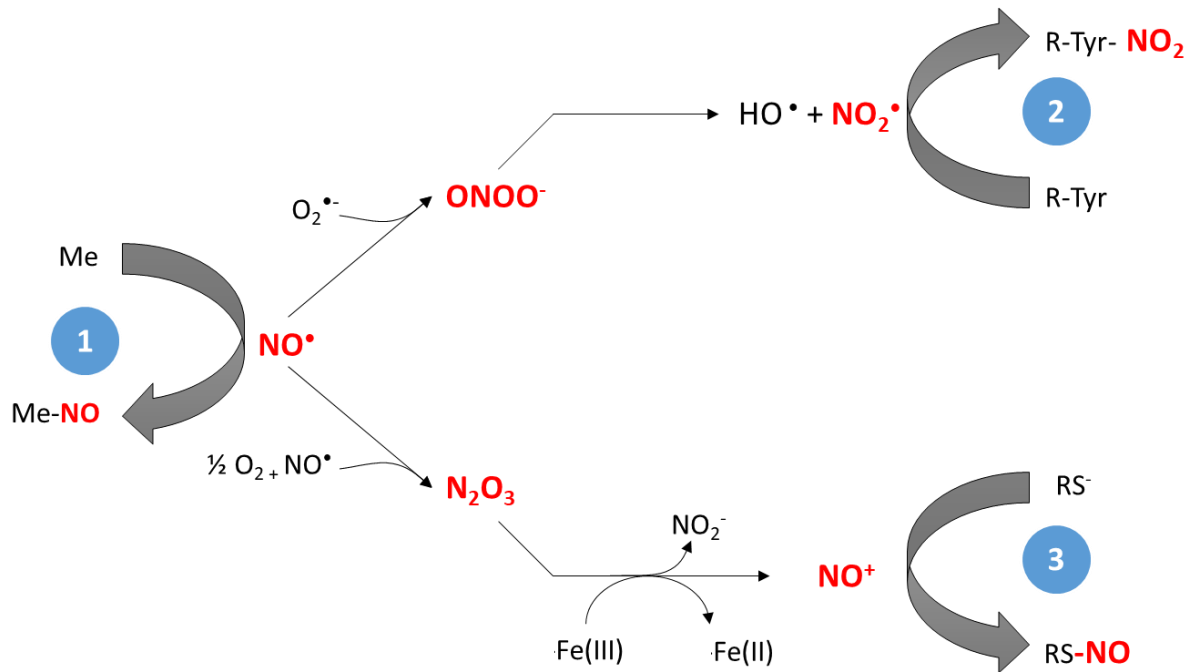


Figure 9 : Principales modifications post-traductionnelles liées au NO et ses dérivés

1) Métal-nitrosylation : Le NO radicalaire se lie par liaison dative au métal de transition d'une métalloprotéine. 2) Tyrosine nitration : en présence de l'anion superoxyde ($\text{O}_2^{\bullet-}$) le NO forme du peroxynitrite (ONOO^-) qui se décompose en radical hydroxyle et en dioxyde d'azote radicalaire. Ce dernier réagit avec le cycle aromatique de la tyrosine pour former un résidu tyrosine nitrée. 3) S-nitrosylation : le NO interagit avec l'oxygène moléculaire pour former du trioxyde d'azote (N_2O_3) qui agit sur des métalloprotéines et libère un cation nitrosonium qui interagit avec les groupes thiol des résidus cystéines et forme un groupement nitrosothiol (SNO)

Lorsque le NO[•] perd son électron non apparié cela engendre un cation nitrosonium (NO⁺) d'une demie vie de 3.10⁻¹⁰s, alors qu'inversement lors du gain d'un électron il se forme un anion nitroxyle (NO⁻) d'une demie vie de 2 à 3 min sous forme protonée (Fig. 8). Ces différents composés (réunis sous le terme NO) présentent des propriétés et réactivités propres comme une forte réactivité avec l'oxygène, les dérivés azotés et les métaux de transition (Lamattina *et al.*, 2003).

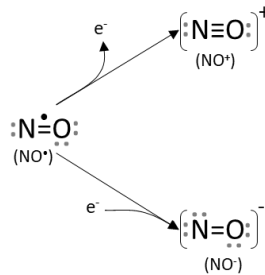


Figure 8: Différentes formes chimiques du NO (adapté de Lamattina *et al.*, 2003)

2.2. Modes d'action du NO

Le NO peut exercer ses effets principalement *via* des modifications post-traductionnelles (MPT) des protéines ou des acides gras, et la modification transcriptionnelle des gènes.

2.2.1. Modifications post-traductionnelles

Le NO module l'activité de certaines protéines par l'intermédiaire de 3 types de MPT qui sont : la S-nitrosylation/S-nitrosation, la métal-nitrosylation et la tyrosine nitration (Hess *et al.*, 2005; Besson-Bard *et al.*, 2008) (Fig. 9).

Les réactions de S-nitrosylation et de métal nitrosylation sont des modifications réversibles. La S-nitrosylation consiste en l'addition de NO sur le groupement sulfhydrile d'un résidu cystéine pour former un S-nitrosothiol (SNO) (Fig. 9). De nombreuses études ont permis de caractériser à l'échelle structurale et fonctionnelle, des protéines modifiées par S-nitrosylation (Romero-Puertas *et al.*, 2008; Lindermayr & Durner, 2009). Parmi ces protéines, on peut citer la glutathione peroxydase (Gpx) dont l'activité est inhibée réversiblement par S-nitrosylation (Castella *et al.*, 2017). De plus, au sein des cellules végétales, le NO réagit par S-nitrosylation avec le glutathion (GSH) présent à des concentrations de l'ordre du millimolaire

(Folkes & Wardman, 2004) pour former le S-nitrosoglutathion (GSNO) considéré comme un réservoir stable du NO (Liu *et al.*, 2001; Sakamoto *et al.*, 2002). Par trans-nitrosylation, le GSNO peut ensuite transférer le NO sur des résidus cystéines présents dans un environnement basique au sein de protéines cibles. Un mutant d'*Arabidopsis thaliana* dépourvu de GSNO réductase (GSNOR), l'enzyme qui catabolise le GSNO, montre une augmentation globale de protéines S-nitrosylées. Cette augmentation s'accompagne d'un défaut de développement et d'une sensibilité accrue aux stress abiotiques et biotiques. Ces observations mettent en évidence les diverses fonctions des modifications post-traductionnelles telles que la S-nitrosylation chez les plantes (Feechan *et al.*, 2005; Lee *et al.*, 2008; Malik *et al.*, 2011).

La métal-nitrosylation fait référence à la liaison dative de NO sur un métal de transition d'une métalloprotéine tel que le Fe (Fig. 9). Le NO agit en tant que donneur d'électrons (acide de Lewis), tandis que l'ion métallique est l'accepteur d'électrons (base de Lewis). Ainsi, chez les mammifères, le NO produit par les protéines NOS peut se lier à l'atome de fer du groupement hème de la guanylate cyclase, ce qui provoque un changement conformationnel de la protéine. Cette action du NO sur la guanylate cyclase entraîne la production de GMPc (Russwurm & Koesling, 2004). Chez les plantes, relativement peu de données sont disponibles sur la métal-nitrosylation. La metal-nitrosylation des hémoglobines a été décrite comme un mécanisme de protection contre un stress oxydatif lié à une forte production de NO (Astier & Lindermayr, 2012).

La tyrosine nitration, quant à elle, touche les résidus tyrosine des protéines par addition irréversible d'un groupe nitro (-NO₂) en position ortho du cycle aromatique de la tyrosine (Stamler *et al.*, 2001) (Fig. 9). Chez plusieurs espèces végétales, en situation de stress, une augmentation du nombre et de la quantité des protéines Tyr-nitrées a été observée (Daniela Cecconi *et al.*, 2009; Leitner *et al.*, 2009; Corpas *et al.*, 2009a). Dans tous les exemples rapportés jusqu'à présent, la nitration des protéines inhibe exclusivement l'activité des protéines. Certaines superoxyde dismutases, qui sont des antioxydants importants au cours de la réponse de défense, sont inhibées par le peroxy-nitrite, qui bloquent l'accès du substrat au site actif (Holzmeister *et al.*, 2015). La Gpx peut aussi être modifiée par tyrosine nitration ce qui inhibe de façon irréversible son activité (Castella *et al.*, 2017).

2.2.2. Modifications de la transcription des gènes

Les premières données de l'implication du NO dans la régulation des gènes ont été obtenues au sein des cellules animales. Il a été montré que le NO peut moduler l'activité de facteurs de transcription tels que NF- κ B (Lander *et al.*, 1993), influencer la stabilité des ARNm et la traduction (Wang *et al.*, 2008). Cependant, il n'y a aucune preuve à ce jour de l'existence d'une séquence régulatrice au sein des promoteurs eucaryotes pouvant réagir ou lier directement le NO.

Au sein des cellules végétales, la capacité du NO à moduler l'expression d'un grand nombre de gènes a été démontrée par l'intermédiaire d'études transcriptomiques (revue par Grün *et al.* (2006)). Chez *A. thaliana*, l'expression de 342 gènes est modulée après un traitement par fumigation où l'adjonction d'un donneur de NO (Huang *et al.*, 2002; Polverari *et al.*, 2003; Parani *et al.*, 2004; Ahlfors *et al.*, 2009). Chez le tabac, divers gènes sont aussi régulés par le NO (Zago *et al.*, 2006). Chez *M. truncatula*, l'amplification des ADNc par AFLP a permis de comparer l'expression de gènes induits par le NO entre des racines de *M. truncatula* inoculées avec *S. meliloti* et des feuilles inoculées avec un champignon pathogène *Colletotrichum trifolii* (Ferrarini *et al.*, 2008). Cette étude a permis d'identifier 47 gènes différentiellement exprimés entre les 2 conditions, dont certains impliqués dans la mise en place de réponse de défense dans les feuilles inoculées avec le champignon (Ferrarini *et al.*, 2008). Plus récemment, Boscari *et al.* (2013a) ont montré que le cPTIO (piégeur de NO) affecte le transcriptome de *M. truncatula* au cours des premières étapes de la symbiose. Les analyses de RNA-seq montrent qu'après un traitement de 8 h par un piégeur de NO (cPTIO) sur des racines inoculées avec *S. meliloti*, 2030 gènes sont exprimés de manière différente en comparaison avec des plantes non traitées.

Ces études ont montré que la majorité des gènes régulés par le NO chez les végétaux sont reliés à des mécanismes de défense, de réponse au stress oxydatif, au métabolisme et au développement (Grün *et al.*, 2006). Cependant, contrairement aux modèles animaux, très peu de données sont disponibles sur le mécanisme par lequel le NO régule la transcription chez les végétaux.

Une étude récente chez *A. thaliana* a montré que l'activité biologique du NO peut être transférée par des modifications redox-dépendantes telle que la S-nitrosylation (Yu *et al.*, 2014), comme par exemple sur le facteur de transcription NPR1 lié à la régulation de la synthèse de l'acide salicylique (Tada *et al.*, 2008).

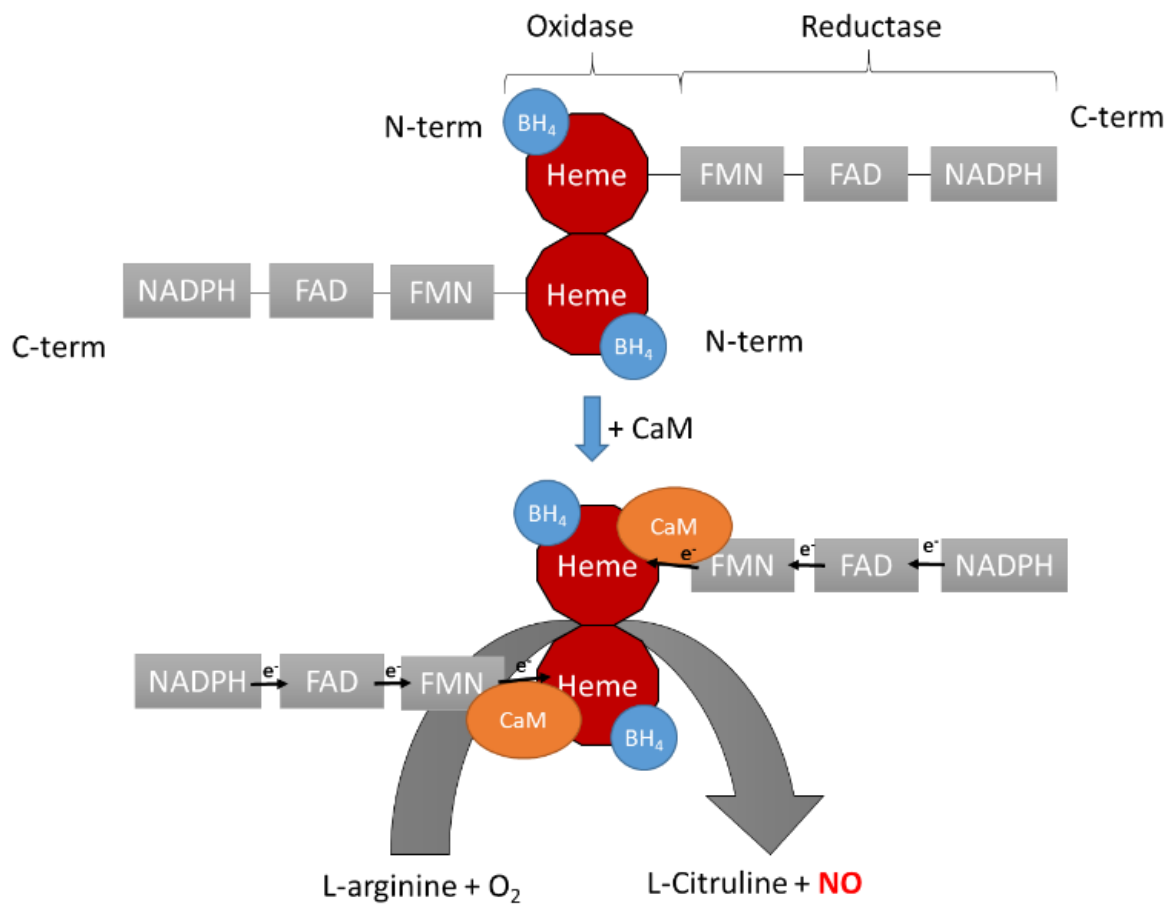


Figure 10 : Structure de la NOS et biosynthèse du NO
 (adapté de Wendehenne *et al.*, 2003)

2.3. La synthèse du NO

2.3.1. La synthèse du NO chez les animaux

Chez les mammifères, dont l'homme, la synthèse du NO est principalement catalysée à partir de la L-arginine et de l'oxygène (O₂) par l'oxyde nitrique synthase (NOS) (Fig. 10). Il existe 3 isoformes de la NOS chez les mammifères ; une isoforme neuronale (nNOS), une endothéliale (eNOS) et une isoforme inductible (iNOS) présente dans les macrophages (Mayer & Hemmens, 1997). La structure enzymatique des NOS consiste en un domaine C-terminal réductase et un domaine N-terminal oxygénase. Ces deux domaines sont liés par un domaine de liaison à la calmoduline (CaM). D'autres cofacteurs sont nécessaires pour assurer le fonctionnement des NOS tels que la flavine mononucléotide (FMN), la flavine adénine dinucléotide (FAD, la tétrahydrobioptérine (BH₄) et un groupement hème (Mayer & Hemmens, 1987) (Fig. 10). L'activité des NOS constitutives (nNOS et eNOS) est fortement dépendante de la fixation de Ca₂⁺ à la calmoduline. Ces deux NOS permettent une libération de NO en de faibles quantités et de courte durée pour une fonction de signalisation (Mayer & Hemmens, 1997). La iNOS est capable de lier la calmoduline en absence de calcium, ce qui induit une production de NO plus importante et de manière prolongée. Cette activité importante est souvent liée à des mécanismes de réponse immunitaire ou le NO joue un rôle toxique (Beck *et al.*, 1999).

D'autres sources de production de NO existent au niveau animal : par oxydation de l'hydroxylamine ou encore, dans certaines conditions, de façon non-enzymatique par réduction chimique du nitrite dans un environnement acide (Weitzberg & Lundberg, 1998; Zweier *et al.*, 1999).

2.3.2. Biosynthèse du NO chez les bactéries

Chez les bactéries libres, la principale voie de production de NO est la voie de dénitrification du nitrate (Zumft, 1997a). Le NO a été identifié comme un intermédiaire de la dénitrification anaérobie chez la bactérie marine *Pseudomonas perfectomarinus* (Barbaree & Payne, 1967). La capacité de dénitrification, est une propriété commune des rhizobia. Chez ces bactéries, la voie de dénitrification dépend des gènes *napEDABC*, *nirKV*, *norCBQD* et *nosRZDYFLX* qui codent respectivement pour la nitrate réductase (NR), la nitrite réductase (NiR), la NO réductase (Nor) et la N₂O réductase (Bedmar *et al.*, 2005).

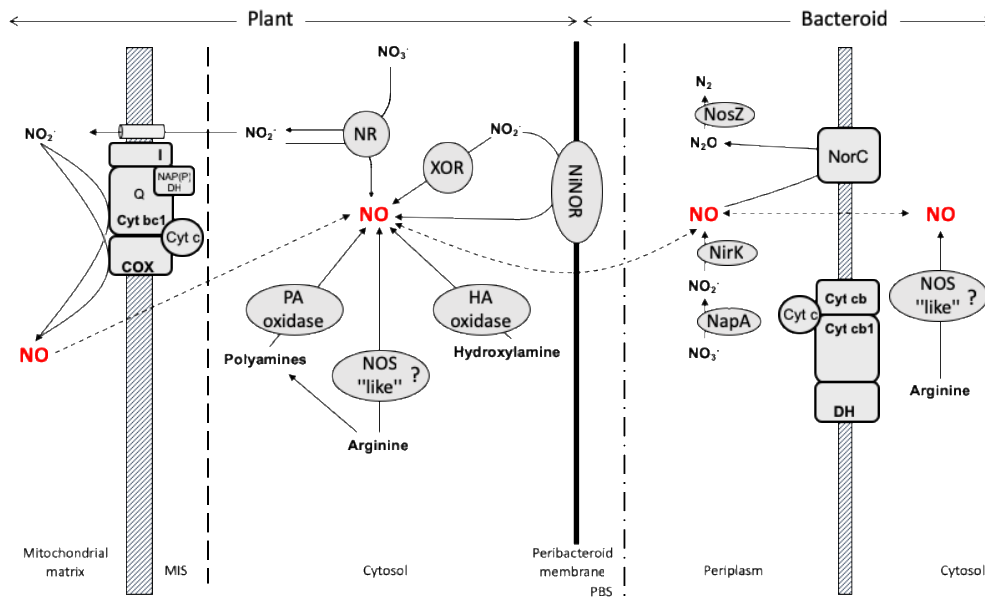


Figure 11 : Différentes voies de synthèse du NO lors de la symbiose. Du côté plante, le NO peut être produit via la réduction du nitrite (NO₂⁻) par la nitrate réductase (NR), la nitrite NO réductase (NiNOR), xanthine oxido-réductase (XOR) et la chaîne respiratoire mitochondriale. Les voies oxydatives de production du NO impliquent la polyamine oxidase (PA oxidase), la voie NOS like, et l'hydroxylamine oxydase (HA oxydase). Côté bactérie, les voies de production du NO impliquent des enzymes de la voie de dénitrification comme la nitrate réductase (NapA) et la nitrite réductase (NirK)

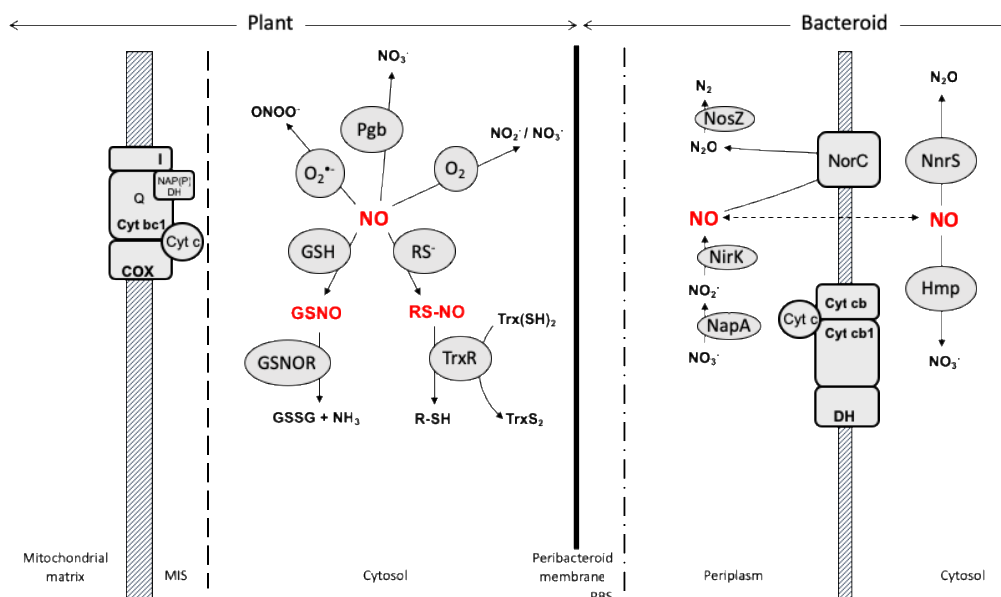


Figure 12 : Différentes voies de métabolisation du NO lors de la symbiose. Le NO peut interagir avec l'O₂ pour former du peroxy-nitrite (ONOO⁻), encore l'anion superoxyde (O₂^{•-}). Le NO peut aussi interagir avec le glutathion (GSH) ou les thiols de certaines protéines et forment respectivement le glutathion S-nitrosylé (GSNO) ou une protéine S-nitrosylée (RS-NO). Après, le GSNO formé est ensuite réduit par la GSNO réductase (GSNOR) en glutathion disulfide (GSSG). Les hémoglobines (Hb) sont aussi capables de cataboliser le NO pour former du nitrate. Du côté bactérien la dégradation du NO fait intervenir la NO réductase (norC) et la flavohémoglobine (Hmp)

Une activité de dénitrification a été mise en évidence chez les bactéroïdes de *S. meliloti* (O'Hara *et al.*, 1983), et dans des nodosités fonctionnelles de soja, l'expression des gènes de dénitrification *nirK*, *norC* et *nosZ* a été rapportée (Mesa *et al.*, 2004). Également dans les nodosités de soja, l'utilisation de mutants déficients sur *napA* et *nirK*, a montré que les enzymes bactéroïdiennes NR et NiR contribuent pour plus de 90% de la production de NO, dans des conditions hypoxiques (Meakin *et al.*, 2007; Sánchez *et al.*, 2010).

Une autre manière potentielle de produire du NO dans les bactéries fixant le N₂ implique les enzymes de type NOS. Les NOS procaryotes ont été identifiées principalement chez les bactéries à Gram positif et les Archaea (Wang & Ruby, 2011) et sont dépourvues du domaine réductase. Néanmoins, elles utilisent des réductases proches comme donneurs d'électrons pour produire du NO *in vivo* (Gusarov *et al.*, 2008). Chez des bactéries libres de *S. meliloti*, une production de NO-dépendante de la L-arginine a été découverte (Pii *et al.*, 2007). À ce jour, bien qu'aucun gène analogue à la NOS n'a été identifié dans le génome, l'implication d'une telle enzyme ne peut être exclue.

2.3.3. La synthèse du NO chez les plantes

Chez les végétaux, plusieurs voies de production de NO ont été mises en évidence (Fig. 11). Deux voies majeures sont distinguées les voies réductrices et les voies oxydatives (Gupta *et al.*, 2011a).

2.3.3.1. Synthèse oxydative du NO

Parmi les voies de production oxydatives, il existe une production de NO à partir de l'arginine qui fait intervenir une enzyme de type « NOS-like ». Cette dénomination NOS-like a été adoptée en raison des similarités avec le fonctionnement des NOS animales, l'utilisation du même substrat mais aussi la sensibilité à des inhibiteurs de NOS animales tels que le L-N^G-monomethyl Arginine citrate (L-NMMA) (Corpas *et al.*, 2009b). Ces inhibiteurs (L-NMMA et L-NAME) sont capables d'inhiber la production de NO dans différents contextes physiologiques tels que le développement (Corpas *et al.*, 2006) ou la réponse immunitaire face à un pathogène (Delledonne *et al.*, 1998; Asai & Yoshioka, 2009). Cependant, malgré la détection d'activité de type NOS, aucun gène codant pour un orthologue de NOS animales n'a été identifié à ce jour dans les génomes des plantes terrestres séquencées (Jeandroz *et al.*, 2016). Néanmoins, 15 séquences complètes qui possèdent une similarité suffisante pour être identifié

comme NOS ont été retrouvées dans un total de 265 espèces d'algues dont *Ostreococcus tauri* (Jeandroz *et al.*, 2016; Santolini *et al.*, 2017).

D'autres voies de production oxydative du NO existent chez les plantes qui font intervenir les polyamines oxydases (Yamasaki & Cohen, 2006). Il a été montré chez *A. thaliana* qu'un traitement par la spermine et/ou la spermidine provoque une production de NO importante (Tun *et al.*, 2006). Chez le tabac d'autres travaux ont mis en évidence une production de NO par l'intermédiaire de l'hydroxylamine (Rümer *et al.*, 2009b,a).

2.3.3.2. Synthèse réductrice du NO

Les principales voies réductrices font intervenir le nitrite (NO_2^-) comme substrat pour produire du NO (Fig. 11). Il a été montré, chez le tournesol et la canne à sucre que l'augmentation de la concentration en nitrate dans le milieu nutritif augmente la production de NO durant les nuits après le traitement (Wildt *et al.*, 1997). D'autres expériences ont montré que chez des mutants de soja qui possèdent une faible ou pas d'activité nitrate réductase (NR), aucune émission de NO n'était mesurable (Nelson *et al.*, 1983; Klepper, 1990). La capacité de la NR de catalyser la réduction du NO_2^- en NO, en utilisant le NADH comme pouvoir réducteur, a été confirmée *in vitro* (Yamasaki & Sakihama, 2000) et *in vivo* (Rockel *et al.*, 2002) en condition hypoxique et avec une concentration en nitrite élevée.

Chez *Chlamydomonas reinhardtii*, il a été montré que la NR peut interagir avec une protéine NOFNIR (nitric oxide-forming nitrite reductase) pour produire du NO à partir du nitrite en condition normoxique (Chamizo-Ampudia *et al.*, 2017). Stöhr *et al.* (2001) ont également mis en évidence la présence d'une activité nitrite-NO réductase sur des membranes plasmiques de racines de tabac.

D'autres enzymes sont aussi capables de produire du NO à partir du nitrite comme la xanthine oxydase (Wang *et al.*, 2010b) ou encore par l'intermédiaire de la chaîne respiratoire d'électrons en condition d'hypoxie (Gupta & Igamberdiev, 2011). Cette dernière voie de synthèse sera détaillée dans le paragraphe (§. 2.6.2).

2.4. Catabolisme du NO chez les plantes

A forte concentration, le NO peut devenir toxique, ainsi au-delà de 0,1 mM, le NO inhibe l'activité de la nitrogénase (Kato *et al.*, 2010). Le contrôle de la concentration de NO dans la cellule dépend donc de sa synthèse, mais aussi de son catabolisme. La dégradation du

NO passe par trois voies principales faisant intervenir les hémoglobines, l'anion superoxyde et le couple GSNO/GSNOR (Groß *et al.*, 2013) (Fig. 12).

2.4.1. Les hémoglobines

Les hémoglobines végétales sont capables grâce à leur activité NO dioxygénase de transformer le NO en nitrate et sont la principale voie de dégradation du NO chez les plantes. Le rôle des hémoglobines est détaillé dans le §4 de l'introduction bibliographique.

2.4.2. GSNO et GSNOR réductase

Une autre voie de métabolisation fait intervenir la GSNOR (Fig. 12). La GSNOR est capable de réduire le GSNO en glutathion sulfinamide en utilisant un pouvoir réducteur fourni par le NADH. Cette enzyme est localisée principalement dans le cytosol, mais des localisations nucléaires et péroxisomales ont également été rapportées (Fernández *et al.*, 2003; Reumann *et al.*, 2007). La GSNOR joue un rôle important dans les réponses aux stress biotiques et abiotiques ainsi que dans la résistance aux maladies (Sakamoto *et al.*, 2002; Díaz *et al.*, 2003; Achkor *et al.*, 2003; Lee *et al.*, 2008). Il a été suggéré que la GSNOR réduit la S-nitrosylation des protéines en métabolisant le GSNO et en réduisant le niveau de NO stocké. Par conséquent, une diminution de l'activité du GSNOR entraîne une accumulation de SNOs (Feechan *et al.*, 2005).

2.4.3. L'anion superoxyde

Le NO peut réagir avec l'anion superoxyde ($O_2^{\cdot-}$) pour former du peroxydinitrite qui est considéré comme un acteur de l'homéostasie du NO (Vandelle & Delledonne, 2011). La formation du peroxydinitrite permet à la fois de réguler la concentration en NO mais aussi celle en H_2O_2 . En effet, la vitesse de réaction entre le NO et l'anion superoxyde est plus rapide que la réaction de transformation de l'anion superoxyde avec la SOD (Salvemini *et al.*, 2006). Cependant, le peroxydinitrite est aussi considéré comme une forme réactive de l'azote et peut provoquer la peroxydation des lipides, la tyrosine nitration ou l'oxydation des protéines et des dommages oxydatifs de l'ADN. Le peroxydinitrite est donc considéré comme un composé cytotoxique (Vandelle & Delledonne, 2011).

2.5. Catabolisme du NO chez les bactéries

Du côté bactérien, plusieurs protéines sont impliquées dans le catabolisme du NO dont les principales sont une flavohémoglobine Hmp et la NO réductase Nor de la voie de dénitrification (Cam *et al.*, 2012; Meilhoc *et al.*, 2013) (Fig. 12). Cam *et al.* (2012) ont montré que Hmp est essentiel pour maintenir un niveau de NO compatible avec le processus symbiotique tandis que, l'utilisation de mutants *norC*, montre une augmentation significative du niveau de NO (Sánchez *et al.*, 2010; Gómez-Hernández *et al.*, 2011; Meilhoc *et al.*, 2013). Plus récemment, il a été proposé que deux autres gènes, *nnrS1* et *nnrS2*, seraient impliqués dans le catabolisme du NO (Blanquet *et al.*, 2015). Chez les bactéries pathogènes *Vibrio cholerae* et *Neisseria meningitidis*, NnrS est impliqué dans la résistance face au NO (Stern *et al.*, 2012; Arai *et al.*, 2013), et un rôle similaire a été suggéré dans les nodules symbiotiques (Blanquet *et al.*, 2015). Par conséquent, les bactéries telles que *S. meliloti* possèdent au moins quatre systèmes de détoxification du NO (Hmp, Nor, NnrS1 et NnrS2). Les raisons d'une telle redondance du système de régulation du NO dans les rhizobia ne sont pas encore claires, mais cela souligne l'importance de la régulation dans le temps et dans l'espace du NO (Torres *et al.*, 2016).

2.6. Principales fonctions physiologiques du NO chez les plantes

Chez les animaux, le NO est impliqué dans de nombreuses fonctions cellulaires comme la régulation de la pression sanguine, la réponse immunitaire, la neurotransmission et la différenciation cellulaire (Hirst & Robson, 2011). Chez les plantes, le NO est impliqué dans la régulation de différents processus développementaux, de la réponse aux stress abiotiques et dans les interactions biologiques pathogènes ou symbiotiques (Yu *et al.*, 2014; Corpas *et al.*, 2017).

2.6.1. Rôles du NO au cours de la croissance et du développement

Le NO est impliqué dans la levée de dormance des graines chez *A. thaliana* où un traitement cPTIO maintient la dormance (Bethke *et al.*, 2004, 2006; Libourel *et al.*, 2006). (Liu *et al.*, 2009) ont montré une augmentation de la production de NO dans l'endosperme des graines d'*A. thaliana* après l'imbibition durant la germination. Cette production de NO est

inhibée après un traitement par le L-NAME ce qui indique une synthèse potentielle de NO par la voie NOS-like au cours de la germination (Lombardi *et al.*, 2012).

Le NO joue aussi un rôle dans le développement racinaire ou le gravi-tropisme en interférant avec des auxines (Hu *et al.*, 2005; Lanteri *et al.*, 2006a). Chez le concombre il a été montré que le NO induit les réponses à l'auxine lors de la formation de racine adventive (Pagnussat *et al.*, 2002). Chez des mutants d'*A. thaliana* (*nia1/nia2*), une faible production de NO réduit l'élongation des racines primaires et provoque des divisions anormales du méristème racinaire (Sanz *et al.*, 2014). La baisse du niveau de NO perturbe la biosynthèse de l'auxine, le transport et la signalisation (Sanz *et al.*, 2014). Le NO agit comme un régulateur du développement *via* l'inhibition des réponses médiées par l'auxine (Fernández-Marcos *et al.*, 2011, 2012).

Le NO joue aussi un rôle important dans le développement des fleurs et la floraison chez *A. thaliana*, par l'intermédiaire de la S-nitrosylation d'un récepteur de phytohormone, TIR1 (Kwon *et al.*, 2012; Terrile *et al.*, 2012). Le NO est aussi impliqué dans la maturation des fruits en la ralentissant (Corpas & Palma, 2018). Il a été montré que la quantité de NO diminue durant la maturation du fruit chez le poivron (*Capsicum annuum*), alors que les MPT telles que Tyronitration et S-nitrosylation augmentent (Chaki *et al.*, 2015; Rodríguez-Ruiz *et al.*, 2017).

2.6.2. Rôles du NO dans la réponse aux stress abiotiques

Les plantes sont continuellement soumises à de nombreux stress environnementaux, tels que la salinité, la présence de métaux lourds ou les inondations (qui provoquent un stress hypoxique). De plus en plus de données indiquent que le NO est impliqué dans les processus d'acclimatation de la plante à ces différents stress (Arasimowicz-Jelonek & Floryszak-Wieczorek, 2014).

Le rôle du NO en réponse à un stress salin a été mis en évidence chez de nombreuses espèces (Zhang *et al.*, 2007; Zhao *et al.*, 2007). Une approche pharmacologique, par traitement avec un donneur de NO (SNP), montre que le NO joue un rôle protecteur contre le stress salin en induisant la croissance de la plante d'une part, et la défense contre le stress oxydatif d'autre part (Uchida *et al.*, 2002; Zhang *et al.*, 2007). Chez le blé, le maïs et la tomate, le NO augmente la tolérance au stress froid (Neill *et al.*, 2003).

Dans le cas d'un stress métallique, une production forte et rapide de NO est observée (Arasimowicz-Jelonek *et al.*, 2012). Le NO appliqué de manière exogène offre une protection

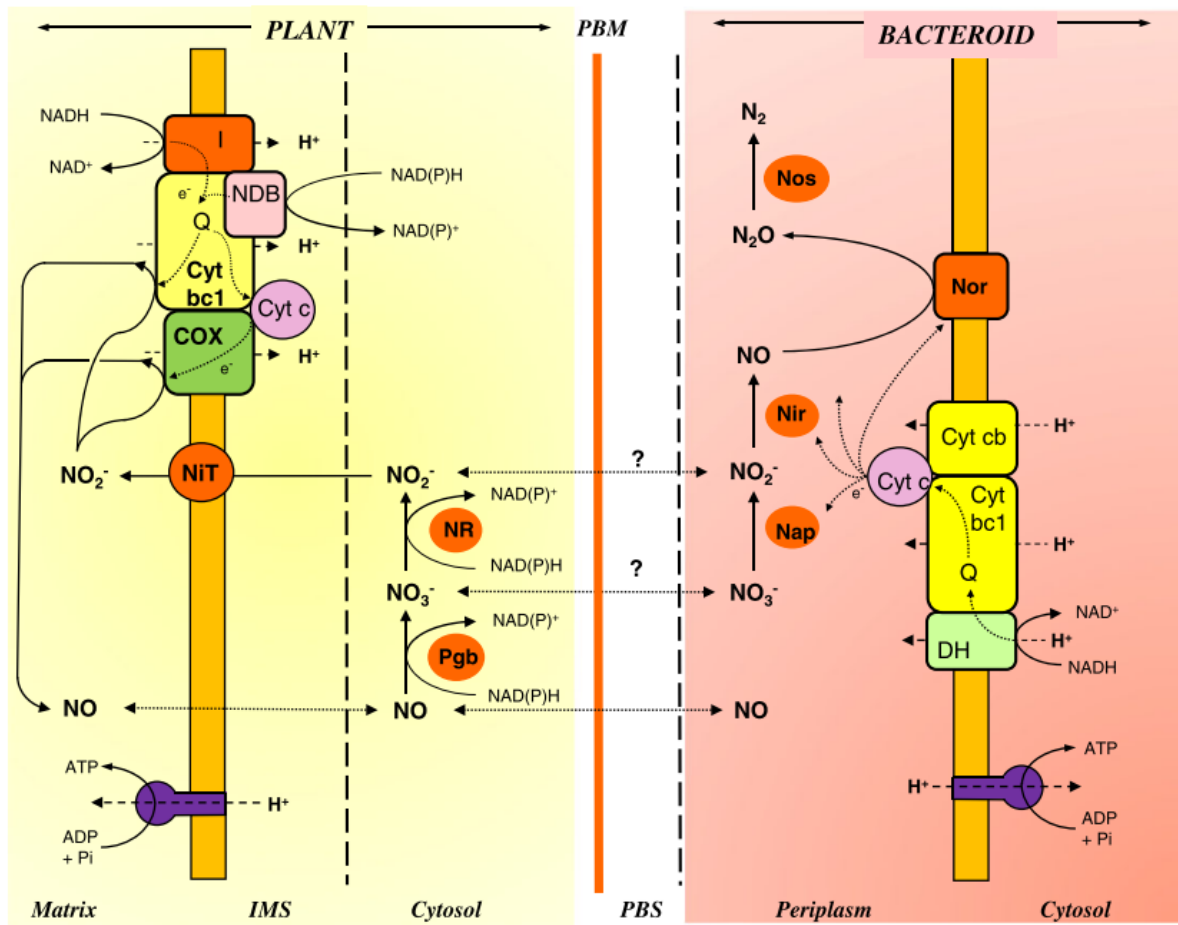


Figure 13 : Représentation schématique de la respiration « Phytogb-NO » et de la voie de dénitrification des bactéroïdes dans des nodosités hypoxiques.

Du côté de la plante, le complexe mitochondrial I, (I) et les déshydrogénases externes (NDB) oxydent respectivement la NADH et le NADPH cytosoliques. Pour simplifier, les NADH et NADPH déshydrogénases ne sont représentées que par un seul complexe. Les électrons sont transférés successivement en passant de l'ubiquinone (Q), par le cytochrome bc1 du complexe III (Cyt bc1), le cytochrome c (Cyt c) pour arriver jusqu'à la cytochrome oxydase (COX). Les nitrites (NO_2^-) sont réduits en NO sur les sites Cyt bc1 et COX. Le NO diffuse dans le cytosol où il est oxydé en nitrate (NO_3^-) par les phytoglobines (Phytogb). La nitrate réductase (NR) réduit le NO_3^- en NO_2^- qui est transporté dans les mitochondries par un transporteur de nitrites (NiT). Du côté des bactéroïdes, le pouvoir réducteur, issu de l'oxydation du NADH par la NADH-quinol oxydoréductase (DH), est fourni à chaque étape de dénitrification *via* le Cyt c. Le NO_3^- est réduit successivement en NO_2^- , NO et N_2 par la nitrate réductase (Nap), la nitrite réductase (Nir), la NO réductase (Nor) et la NO synthase (Nos). Les mécanismes d'échange de NO et de NO_2^- entre la matrice, le cytosol et le périplasm ne sont toujours pas identifiés. L'ATP est synthétisée par le gradient électrochimique transmembranaire généré par le pompage du proton (H^+) sur les différents sites des chaînes de transfert d'électrons, tant chez les plantes que chez les bactéroïdes partenaires. IMS = espace intermembranaire mitochondrial; PBM = membrane péri bactéroïde; PBS = espace péri bactérien

contre la toxicité des métaux lourds, tels que l'aluminium ou le cadmium au sein de différentes espèces végétales (revue par He *et al.* (2012) et Gill *et al.* (2013)). Le NO agit en réduisant (ou en compartimentant) l'accumulation des métaux ou en atténuant le stress oxydatif qui en résulte (Cerana & Malerba, 2015). Par exemple, un traitement par le SNO protège les racines de riz contre la toxicité de l'arsenic en éliminant les ROS, réduisant ainsi les dommages oxydatifs (Singh *et al.*, 2009). Par ailleurs, le SNP neutralise l'effet inhibiteur des métaux lourds sur la croissance racinaire du lupin par stimulation de l'activité de la SOD et/ou en éliminant directement l'anion superoxyde (Kopyra & Gwózdź, 2003). De nombreux travaux décrivent l'effet protecteur du NO chez plusieurs espèces de plantes exposées au cadmium en modulant les activités des enzymes antioxydantes, telles que CAT, SOD et APX (revue par Gill *et al.* (2013)).

Lors d'un stress hypoxique, une accumulation de NO est observée au sein des plantes stressées. Au cours des 24 premières heures d'un traitement hypoxique, une quantité importante de NO est observé dans des cellules de maïs et dans des cultures de racines de luzerne (Dordas *et al.*, 2003a). De plus, les auteurs observent qu'en l'absence de phytoglobines (qui sont capables de se complexer avec le NO), le niveau d'ATP diminue lors de l'hypoxie. En se basant sur ces observations, l'hypothèse que le NO et les phytoglobines serait impliqués dans la réponse des plantes à l'hypoxie a été posée. Cette hypothèse a été renforcée par différents travaux : d'une part, il a été montré qu'en hypoxie les phytoglobines forment du nitrate à partir du NO via une activité NO dioxygénase (Dordas *et al.*, 2004), d'autre part des analyses avec du $^{15}\text{NO}_3^-$ montrent que le NO produit en hypoxie provient du nitrate (Dordas *et al.*, 2004). Par la suite, Stoimenova *et al.* (2007) ont démontré qu'en condition hypoxique et en présence de pouvoir réducteur (NAD(P)H), les mitochondries purifiées d'orge et de riz sont capables d'utiliser le nitrite comme accepteur final de la chaîne de transfert d'électrons pour le réduire en NO. Cette production de NO induit l'alternative oxydase (Benamar *et al.*, 2008; Gupta *et al.*, 2012) et est impliqué dans la production d'ATP lors de ce stress (Stoimenova *et al.*, 2007).

Ces différentes observations ont conduit à un modèle appelé «respiration PhytoGb-NO» (Igamberdiev & Hill, 2004; Igamberdiev *et al.*, 2005; Gupta & Igamberdiev, 2011; Gupta *et al.*, 2017) qui fonctionne selon un cycle constitué de 4 étapes (Fig. 13) :

- 1) Le nitrate présent dans le cytosol est réduit par la NR pour donner du nitrite. En situation normale, le nitrite est pris en charge par la nitrite réductase pour former de l'ammoniaque. Cependant en situation d'hypoxie, la réduction du nitrite est inhibée et celui-ci s'accumule dans les tissus (Botrel & Kaiser, 1996).

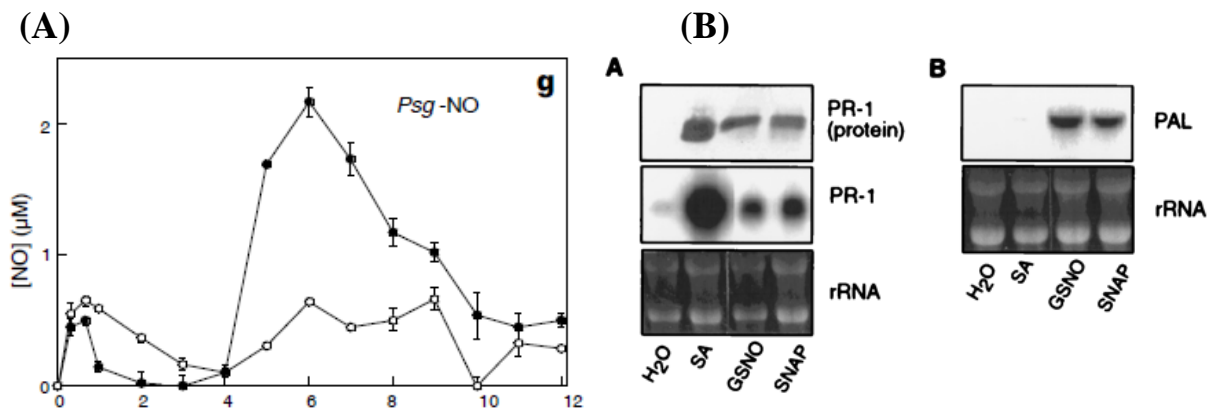


Figure 14 : Le NO dans le cadre des interactions pathogènes

(A) Accumulation du NO dans des cellules en suspension de soja traitées par une souche virulente de *P. syringae* pv. *Glycinea* (rond plein) ou avirulente (rond vide) au cours du temps (en heure) (Delledonne *et al.*, 1998).

(B) Induction des gènes de défense PR-1 et Pal dans des cellules en suspension de tabac après traitement avec de l'acide salicylique SA (100 μM), du glutathion S-nitrosylé GSNO (200 μM), ou du s-nitroso-n-acetyl penicillamine SNAP (100 μM) (Durner *et al.*, 1998)

2) Le Nitrite est ensuite transporté du cytosol dans la mitochondrie par l'intermédiaire d'un transporteur mitochondrial qui n'a pas encore été caractérisé (Gupta & Kaiser, 2010).

Récemment, un transporteur a été identifié à partir de mitochondrie racinaire purifié de *M. truncatula* (Berger *et al.*, 2018b).

3) Une fois dans la mitochondrie, le nitrite est réduit en NO au niveau de l'alternative oxydase et du cytochrome c oxydase par les électrons de la chaîne respiratoire mitochondriale.

4) Le NO formé diffuse ensuite librement à travers la membrane mitochondriale vers le cytosol où il est oxydé en nitrate par les phytoglobines (Igamberdiev & Hill, 2004) grâce à leur activité NO dioxygénase.

2.6.3. Rôles du NO dans les interactions avec les micro-organismes

2.6.3.1. Interactions plante/pathogène

Le rôle du NO dans les interactions biotiques, a été identifié pour la première fois chez la pomme de terre qui, en réponse à un donneur de NO (NOC-18), accumule de la rishitine, une phytoalexine antimicrobienne (Noritake *et al.*, 1996). Différents travaux ont observé une production de NO lors de différentes interactions plantes/pathogènes *A. thaliana*/*P. syringae* (Modolo *et al.*, 2005), *Pelargonium peltatum*/*Botrytis cinerea* (Floryszak-Wieczorek *et al.*, 2007) ou encore *A. thaliana*/*A. euteiches* (Thalineau *et al.*, 2016). Par ailleurs, il a été montré que le NO (en synergie avec les ROS) est capable de déclencher la réponse hypersensible et activer l'expression de gène de défense *PAL* et *PRI* chez le tabac et *A. thaliana* (Fig. 14) (Durner *et al.*, 1998 ; Delledonne *et al.*, 1998). En effet, des plantes d'*A. thaliana* qui possèdent une activité NR très réduite (mutant *nialnia2*) ou traitées par des inhibiteurs de la synthèse de NO montrent une réponse altérée de la réponse hypersensible au cours de l'infection par *P. syringae* (Delledonne *et al.*, 1998 ; Modolo *et al.*, 2005). La cryptogéine est un éliciteur protéique d'une dizaine de kDa synthétisé par l'oomycète *Phytophthora cryptogera* (Ricci *et al.*, 1989). Cet activateur de la réponse immunitaire induit la réponse hypersensible ainsi que la production de NO chez le tabac (Besson-Bard *et al.*, 2008).

Par une approche de génétique inverse, Feechan *et al.*, (2005) ont mis en évidence des SNO (S-nitrosothiol) dans la réponse de défense des plantes. Chez un mutant GSNOR d'*A. thaliana*, l'accumulation de SNO s'accompagne d'une susceptibilité accrue face à *Pseudomonas syringae*. (Feechan *et al.*, 2005). Il a également été montré que les modifications de la concentration de SNO cellulaire, régule à la fois l'accumulation d'un activateur de la défense, l'acide salicylique (Loake & Grant, 2007) et l'expression de gènes dépendant de l'acide salicylique (Feechan *et al.*, 2005).

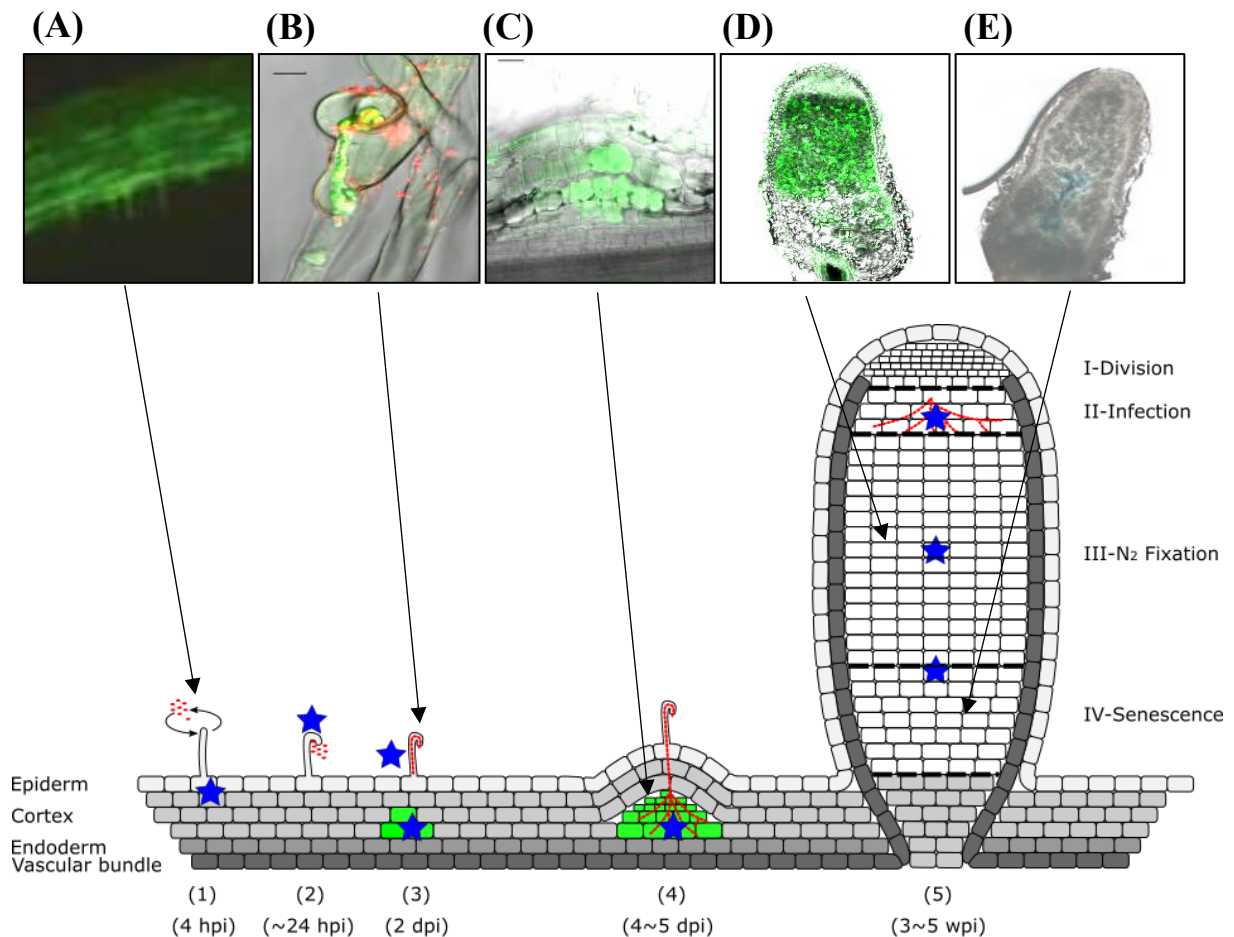


Figure 15: Détection du NO au cours du processus symbiotique. Le NO a été détecté grâce à l'utilisation de la sonde fluorescente DAF-2DA (a) dans la racine à 4 hpi (Nagata *et al.*, 2008); (b) dans le cordon d'infection; (c) dans le primodium nodulaire à 4 dpi (del Guidice *et al.*, 2011); (d) dans la nodosité mature à 3 wpi (Baudoin *et al.*, 2006) et par détection de l'activité β -galactosidase (e) dans la nodosité mature (Cam *et al.*, 2012).

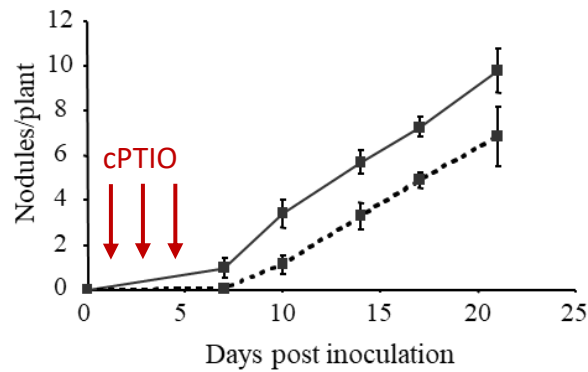
Ces données suggèrent que le NO *via* la S-nitrosylation est un régulateur clé des réponses de défense dépendante de l'acide salicylique mais qu'à forte concentration le SNO rend la plante moins résistante aux maladies. Ces données ont ensuite été confirmées en analysant la S-nitrosylation du co-activateur transcriptionnel NPR1, un régulateur majeur de la voie de signalisation du SA en réponse à un agent pathogène (Tada *et al.*, 2008). Cette protéine se trouve sous forme d'homo-oligomère inactifs dans le cytosol. Lors de la stimulation des réponses de défense, NPR1 est dissocié en monomères et le NO dans un premier temps, va faciliter la migration de la protéine dans le noyau pour activer le facteur de transcription TGA1 qui régule positivement les gènes de défense (Lindermayr *et al.*, 2010). Dans un second temps, le NO exerce un rétrocontrôle négatif en provoquant la S-nitrosylation de NPR1 ce qui le rend inactif (Tada *et al.*, 2008).

Récemment d'autres protéines importantes impliquées dans la réponse immunitaire ont été identifiées comme CDC48, HDACs et CAM qui peuvent être S-nitrosylés (Rosnoblet *et al.*, 2016).

2.6.3.2. Interaction plante/symbiote

Durant les 20 dernières années, plusieurs travaux ont démontré l'implication du NO dans les interactions symbiotiques. Le NO a été décrit dans les interactions mycorhiziennes et les lichens (Hichri *et al.*, 2016). Ainsi, une production de NO a été observée aussi bien au début qu'à la fin de l'interaction avec les mycorhizes à arbuscule (Calcagno *et al.*, 2012; Zhang *et al.*, 2013; Espinosa *et al.*, 2014). Une production de NO a aussi été observée dans les premières étapes de l'interaction entre la plante et *Azospirillum* (Molina-Favero *et al.*, 2008) ainsi que dans la mise en place des lichens lors de la réhydratation de *Ramalina* (Weissman *et al.*, 2005; Catalá *et al.*, 2010). Dans les premières étapes de l'interaction mycorhizienne, le NO semble agir dans la voie de signalisation qui stimule la formation de racines latérales (Oláh *et al.*, 2005; Mukherjee & Ané, 2010). Cette observation s'appuie notamment par la présence de NO dans les méristèmes racinaires et sur les sites d'apparition des racines latérales (Correa-Aragunde *et al.*, 2004, 2006). Il a également été démontré que les variations du niveau de NO influent sur la synthèse de la cellulose dans les racines (Correa-Aragunde *et al.*, 2008). L'augmentation des teneurs d'une cellulose synthase présumée au cours des toutes premières étapes de l'interaction mycorhizienne (Siciliano *et al.*, 2007), laisse entrevoir un rôle possible dans le remodelage de la paroi cellulaire au cours du processus d'hébergement du champignon dans les cellules épidermiques. Toutes ces observations montrent que le NO est produit lors de plusieurs interactions symbiotiques et tout au long du processus. Cela soulève la question de la fonction

(A)



(B)

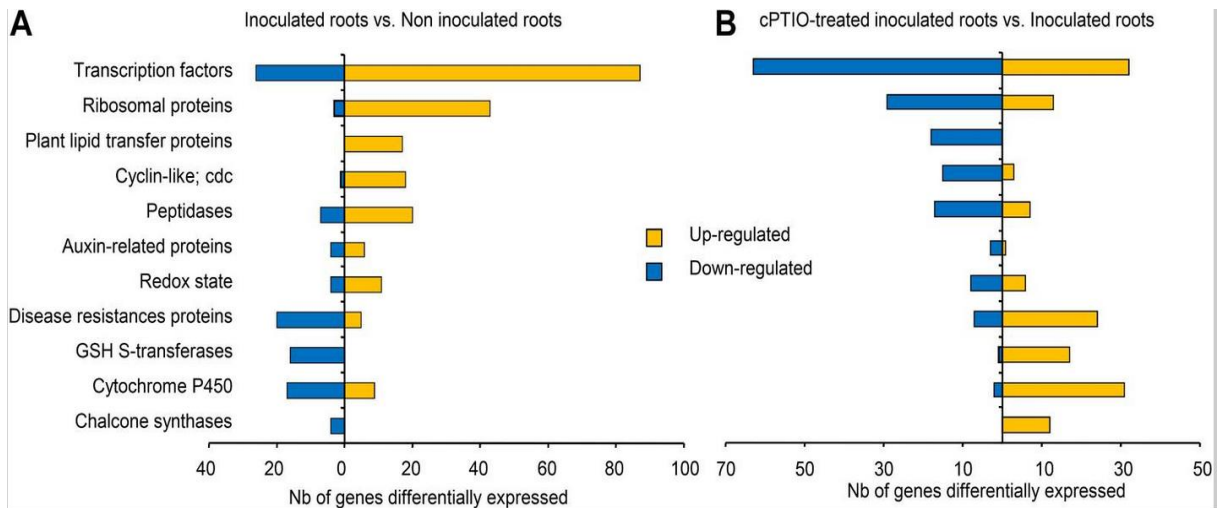


Figure 16 : Le NO dans l'établissement de l'interaction symbiotique

(A) Impact d'un traitement cPTIO (ligne pointillée) sur le phénotype de nodulation chez *M. truncatula*. (del Giudice *et al.*, 2011)

(B) Comparaison des régulations transcriptionnelles de famille de gènes sur des racines de *M. truncatula* inoculées par *S. meliloti* traitées ou non avec du cPTIO. (Boscari *et al.*, 2013a)

du NO lors des premières étapes de l'établissement de la symbiose, mais aussi dans la régulation de son fonctionnement et de sa rupture. Le prochain chapitre a pour but de donner un aperçu des rôles du NO lors des différents étapes de la symbiose fixatrice d'azote

2.6.3.2.1. Principales fonctions du NO lors de la symbiose fixatrice d'azote

2.6.3.2.1.1. Implication du NO dans l'établissement de l'interaction plante/symbiote

Au cours de la symbiose fixatrice d'azote entre les légumineuses et les bactéries de type rhizobium, une augmentation de la production de NO est observée dès les premières heures de l'interaction. Chez le lotier (*Lotus japonicus*) et la luzerne (*M. sativa*), Nagata *et al.*, (2008) ont montré que 4 heures après inoculation avec leur symbiote respectif (*B. japonicus* et *S. meliloti*) une production transitoire de NO est observée à la surface des racines de *Lotus japonicus* et *M. sativa* (Fig. 15A). De plus, cette production de NO au cours des premières heures de l'interaction n'est pas observée lorsque *M. truncatula* ou *L. japonicus* sont inoculées avec des rhizobiums non compatibles ce qui indique la spécificité de ce signal (Nagata *et al.*, 2008). Chez *M. truncatula*, del Giudice *et al.* (2011) ont observé une production de NO plus tardivement à 4 jpi au niveau de la poche d'infection et du cordon d'infection (Fig. 15B). Ces auteurs ont aussi testé l'importance de cette production de NO sur la nodulation en utilisant soit un piègeur de NO, le cPTIO soit en surexprimant la flavohémoglobine *Hmp* de *S. meliloti* sous un promoteur spécifique de la nodulation (Enod20). Dans les deux cas, la diminution du contenu en NO chez *M. truncatula* provoque un retard de nodulation (Fig. 16A) et une inhibition de l'expression des gènes impliqués dans le développement de la nodosité tel que *MtCRE1* et *MtCCS52A* (del Giudice *et al.*, 2011). Cependant, chez des plantes de *L. japonicus* mutées pour le gène *LjGlb1.1* (codant pour une phytoglobine non symbiotique – Phyto**g**lb1), une réduction du nombre de nodosités a été observée (Fukudome *et al.*, 2016). Inversement, lors d'une surexpression de *Phyto**g**lb1* chez le lotier ou *Alnus firma*, une augmentation du nombre de nodosités a été observée (Shimoda *et al.*, 2009). Ces observations semblent être en opposition apparente concernant le rôle positif ou négatif de NO dans le processus de nodulation, et la question s'est posée de savoir si cette contradiction était liée au modèle symbiotique, aux stades de la symbiose analysée ou à la durée des traitements (Hichri *et al.*, 2015).

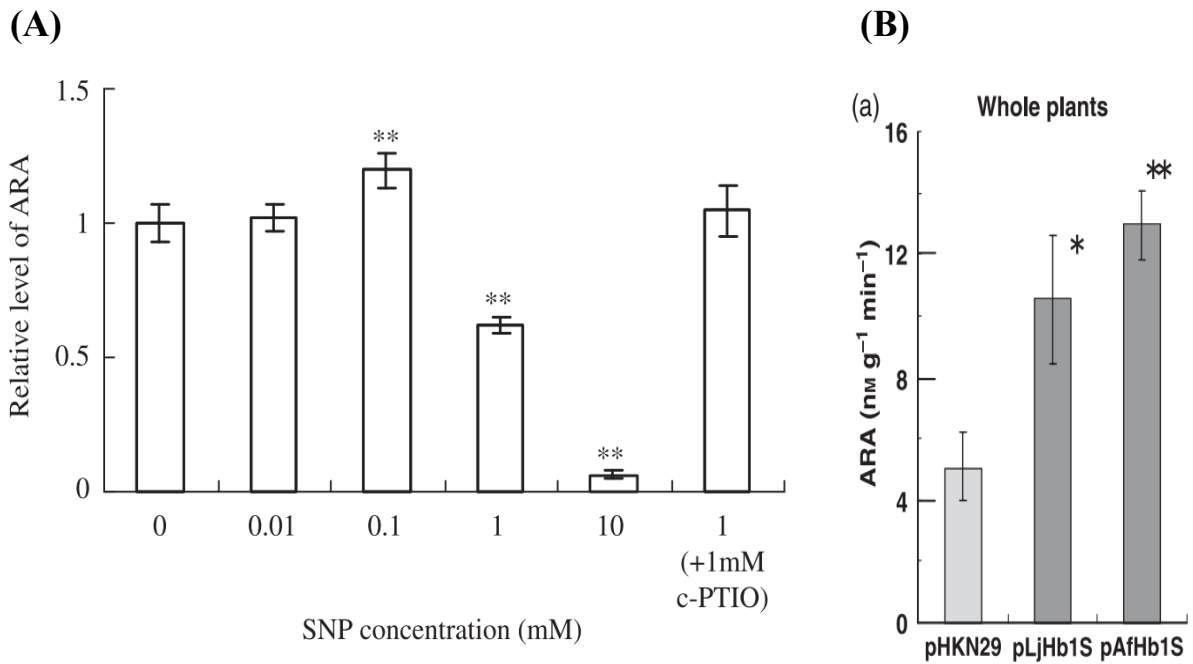


Figure 17 : Influence du NO sur le fonctionnement de la nodosité

(A) Influence du NO par traitement avec un donneur de NO (SNP) sur l'activité fixatrice d'azote (Kato *et al.*, 2010).

(B) Analyse de la fixation de l'azote sur des plantes transformées surexprimant un gène codant pour une phytohémoglobine non symbiotique chez le lotier et *Alnus firma* (Shimoda *et al.*, 2008).

2.6.3.2.1.2. Implication du NO dans le développement et l'organogénèse de la nodosité

Lors de l'interaction entre *M. truncatula* et *S. meliloti*, une production de NO est observée à 4 jours post inoculation au sein des cellules corticales du primordium nodulaire (del Giudice *et al.*, 2011) (Fig. 15C). Une analyse transcriptomique sur des racines inoculées de *M. truncatula* traitées ou non avec du cPTIO (pendant 8h) a permis d'identifier les gènes potentiellement régulés par le NO au cours du développement du primordium nodulaire (Boscari *et al.*, 2013a). Au total plus de 2000 gènes sont retrouvés exprimés différemment entre les racines traitées avec du cPTIO et les racines contrôles. Parmi ces cibles moléculaires du NO, un grand nombre codent pour des facteurs de transcription et des protéines impliquées dans le développement cellulaire, telles que des cyclines, des peptidases ou des protéines ribosomiques, ce qui suggère une implication du NO dans la dédifférenciation des cellules corticales et l'induction de la division cellulaire au cours de la formation des nodosités. L'autre fait marquant de cette étude réside dans l'observation de l'induction, lors du traitement au cPTIO, de la plupart des gènes de défense normalement réprimés au cours de la mise en place de l'interaction symbiotique. Ainsi, un grand nombre de gènes impliqués dans la synthèse de métabolites secondaires (terpène, flavonoïdes, phénylpropanoïdes,...) et des gènes codant pour des protéines de type PR sont induits par le traitement cPTIO. Ces observations indiquent que le NO pourrait être impliqué dans la répression des réactions de défense des plantes, favorisant la mise en place de l'interaction entre la plante et son microsymbionte (Boscari *et al.*, 2013a). Un rôle similaire du NO a été proposé lors de la symbiose mycorhizienne (Espinosa *et al.*, 2014). Cependant son rôle dans la symbiose diffère de son rôle lors des interactions pathogènes, dans lesquelles le NO induit notamment la réponse hypersensible (Delledonne *et al.*, 1998). Cela signifie qu'en fonction du moment de la symbiose, NO régule différemment la réponse de défense de la plante : en l'induisant pendant l'établissement de l'interaction et en la réprimant au début du développement des nodules.

2.6.3.2.1.3. Implication du NO dans la régulation de la fixation de l'azote au sein de la nodosité mature

Le NO est fortement présent dans la zone de fixation de N₂ des nodosités de *L. japonicus* et de *M. truncatula* et principalement dans les cellules contenant des bactéroïdes, (Baudouin *et al.*, 2006) (Fig. 15D).

Il a été signalé, grâce à des mesures *in vitro* que le NO est un puissant inhibiteur de l'activité de la nitrogénase (Trinchant & Rigaud, 1982). Dans des nodosités de soja soumises à un stress de submersion, l'augmentation de la production de NO entraîne à une réduction de trois fois de l'expression des gènes codant pour la nitrogénase, *nifH* et *nifD*, inhibition partiellement annulée par l'application de cPTIO (Sánchez *et al.*, 2010). Une activité nitrogénase plus élevée a également été mesurée sur des nodosités détachées de *L. japonicus*, surexprimant le gène *LjHb1*, et présentant un niveau de NO réduit par rapport aux nodules témoins (Fig. 17B) (Shimoda *et al.*, 2009). Cependant, les résultats de Kato *et al.* (2010) montrent qu'un traitement avec 0,1 mM de SNP, la fixation de l'azote est plus importante qu'avec des concentrations une plus faible (0,01 mM) ou plus forte (10 mM) de ce donneur de NO (Fig. 17A). Ces données indiquent que le niveau de NO dans la nodosité est contrôlé pour permettre le bon fonctionnement de la fixation de l'azote (Hichri *et al.*, 2016). La régulation de l'homéostasie du NO dans la nodosité est notamment effectuée par les Lbs (Sánchez *et al.*, 2010), mais aussi les PhytoGb1 (Shimoda *et al.*, 2009) ainsi que par des protéines bactériennes qui agissent comme capteur de NO (hmp) (Herold & Puppo, 2005; Sánchez *et al.*, 2010). Les nodosités fonctionnelles sont caractérisées par un environnement micro-oxique, ce qui pose la question de l'approvisionnement en énergie au sein de cet organe. De nombreuses évidences suggèrent que la signification biologique de la présence du NO dans les nodosités matures est en lien avec le statut microoxique du nodule (Hichri *et al.*, 2015 ;2016). Il a été montré qu'au sein des nodosités, se produit une respiration alternative dite « Pgb-NO » dans laquelle les NR et l'ETC sont impliqués (Horchani *et al.*, 2011) (pour plus de détail voir le §.2.6.2). Cette respiration utilise le nitrite comme accepteur d'électron final, au lieu de l'O₂, pour être réduit en NO, ce qui permet de conserver le statut énergétique des cellules (Gupta & Igamberdiev, 2011; Berger *et al.*, 2018b).

Le NO apparaît également comme un régulateur clé du métabolisme du carbone et de l'azote au sein du nodule. En effet, la fixation symbiotique de l'azote génère de l'ammonium (NH₄⁺), qui est libéré dans le cytosol et peut empoisonner les cellules végétales à forte concentration (Li *et al.*, 2014). La glutamine synthétase cytosolique (GS1) est une enzyme clé du piégeage du NH₄⁺ et du métabolisme de l'azote, car elle permet la synthèse de la glutamine (Gln) suite à la condensation de NH₄⁺ avec le glutamate (Glu) (Silva & Carvalho, 2013). Chez *M. truncatula*, GS1a est responsable de 90% de l'activité totale des GS au sein des nodosités (Carvalho *et al.*, 2000) et peut être inactivée par le NO via Tyr-nitration (Melo *et al.*, 2011). L'inactivation de la GS par le NO semble réguler également la réponse antioxydante. En effet, le glutamate est un substrat nécessaire pour la GS, mais également un précurseur de la

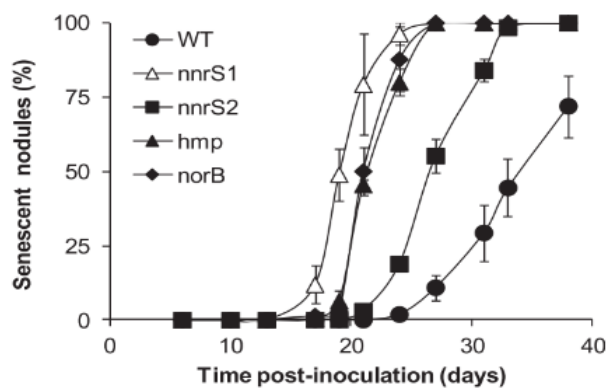


Figure 18 : Cinétique d'apparition de la sénescence sur des nodosités inoculées avec des mutants de *S. meliloti* pour des gènes impliqués dans le catabolisme du NO. (Blanquet *et al.*, 2015)

biosynthèse du glutathion (GSH), un composé antioxydant essentiel, ainsi que le précurseur du GSNO (Melo *et al.*, 2011). En plus de la GS, environ 80 protéines S-nitrosylées ont été identifiées, à la fois chez le partenaire plante et bactérien, notamment des enzymes de la glycolyse (aldolase, glycéraldéhyde déshydrogénase, émolase, pyruvate kinase), cycle du TCA (citrate synthase, succinate déshydrogénase, malate déshydrogénase), la saccharose synthase, les ATPases ou l'asparagine synthétase (Puppo *et al.*, 2013). L'activité de la plupart de ces enzymes s'est avérée inhibée par les donneurs de NO (Boscari *et al.*, 2013a; Igamberdiev *et al.*, 2014). Ces données suggèrent que le NO pourrait agir en tant que régulateur négatif du métabolisme primaire.

2.6.3.2.1.4. Implication du NO dans la sénescence de la nodosité

Au cours de la sénescence nodulaire, un niveau de NO plus important a été mis évidence dans la partie la plus proximale de la nodosité (Fig 15E). Cette localisation a été déterminé grâce à l'utilisation d'une souche de *S. meliloti* bio-sensor du NO (Cam *et al.*, 2012). Chez *M. truncatula*, une augmentation de la production de NO observée soit dans des nodosités issues d'un mutant *hmp* de *S. meliloti* soit suite à un traitement avec du DEA-NO est corrélée à une sénescence plus précoce (Cam *et al.*, 2012 ; Blanquet *et al.*, 2015 ; Meilhoc *et al.*, 2013). Inversement, l'utilisation de souches de *S. meliloti* qui sur-expriment *hmp* produit des nodosités avec un niveau de NO plus faible et une sénescence retardée (Fig 18) (Cam *et al.*, 2012). Il a donc été émis l'hypothèse que le NO contrôle le processus de sénescence des nodosités de *M. truncatula* (Cam *et al.*, 2012). Cependant aucune donnée directe ne permet de conclure si le NO est un signal inducteur de la sénescence ou s'il est juste un produit intermédiaire du processus de sénescence.

2.7. Interaction entre le NO et les ROS dans la nodulation

Plusieurs espèces réactives de l'oxygène (ROS), distincte du NO telles que l'anion superoxyde ou H₂O₂, sont produites à différents moments du processus symbiotique. Il a été montré que parallèlement au NO, les ROS régulent aussi la réponse immunitaire et l'expression des gènes au cours de la symbiose (Andrio *et al.*, 2013 ; del Giudice *et al.*, 2011; Damiani *et al.*, 2016). H₂O₂ et NO sont tous deux capables de réagir avec les protéines cibles et de moduler leurs activités (Fig. 19). Des cibles protéiques S-sulfénylées et S-nitrosylées communes ont été

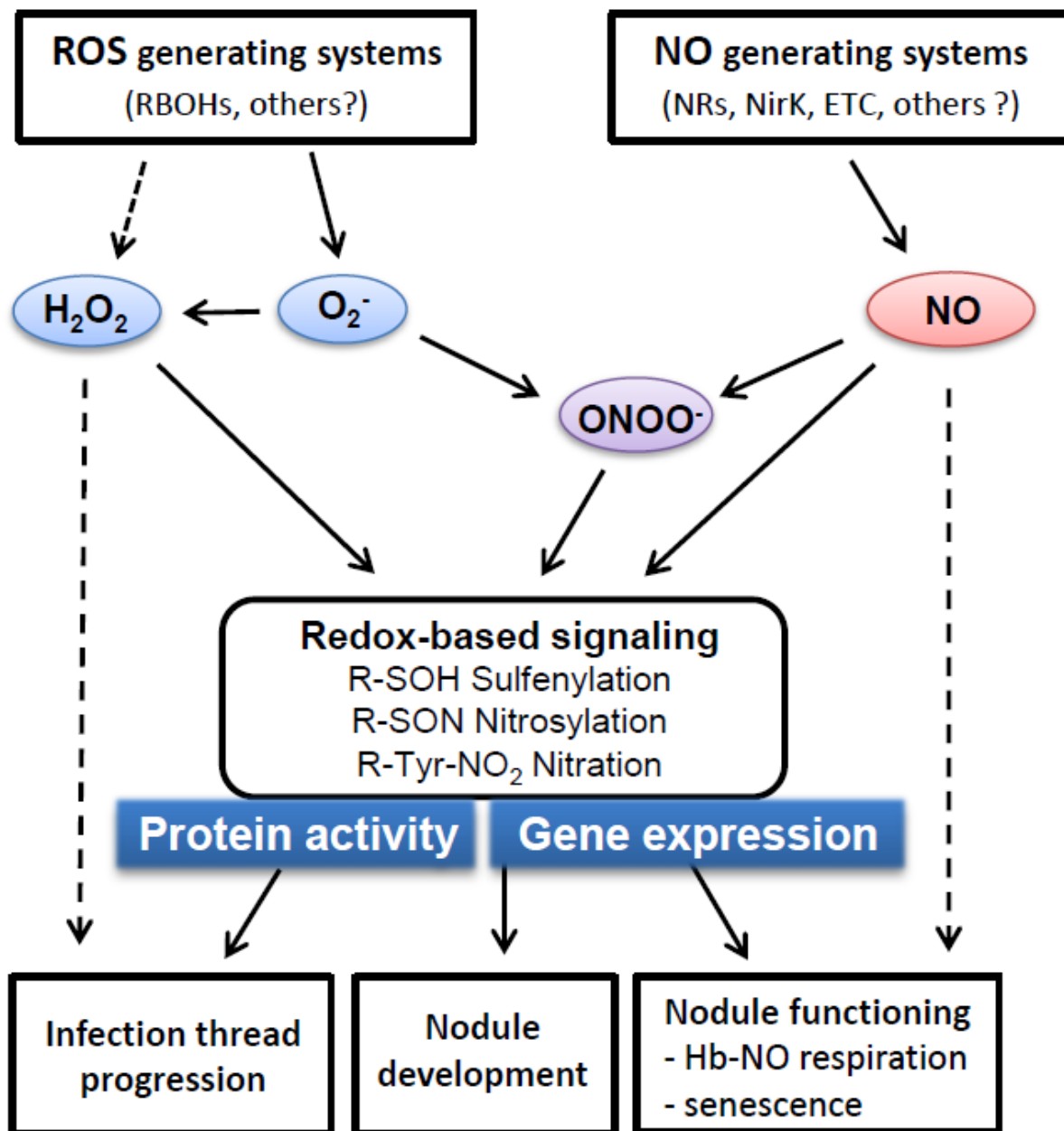


Figure 19 : Schéma des sources de NO et d'espèces réactives de l'oxygène (ROS), des voies de signalisation et des diaphonies modulant les étapes essentielles du processus symbiotique. La régulation du processus symbiotique basée sur l'oxydo-réduction est en partie induite par les modifications post - traductionnelles dépendantes du NO et des ROS (sulfabénylation, nitrosylation et tyrnation) qui régulent l'activité des protéines et l'expression des gènes. NRs = nitrate réductase

identifiées dans les nodosités racinaires (Andrio *et al.*, 2013). En outre, des analyses transcriptomiques sur des racines inoculées de *M. truncatula* suite à un traitement soit avec un inhibiteur de NO (cPTIO), soit avec un inhibiteur de la NADPH oxydase (diphénylèneiodonium), montrent qu'il existe un chevauchement entre les voies de signalisation impliquant le NO et celles impliquant la NADPH oxydase (Andrio *et al.*, 2013; Boscaro *et al.*, 2013a). Parmi les plus de 300 gènes régulés négativement, on trouve des gènes impliqués dans les processus de formation et de développement de la paroi cellulaire, alors que des gènes impliqués dans la défense de la plante et le métabolisme secondaire sont induits (Andrio *et al.*, 2013).

Certaines de ces protéines sont impliquées dans le métabolisme primaire du carbone et de l'azote et peuvent réguler le métabolisme cellulaire énergétique général des deux symbiotes. L'exemple le plus caractéristique de *M. truncatula* est, la GS1a, qui est soumis à une inactivation médiée par NO via Tyr-nitration (Melo *et al.*, 2011) mais qui est également soumise à la sulfoxydation de la méthionine au cours de la sénescence des nodosités (Matamoros *et al.*, 2013). Cette double modification post traductionnelle montre que cette enzyme clé est régulée à la fois par le ROS et le RNS. Le rôle régulateur potentiel du NO sur la NADPH oxydase (Yun *et al.*, 2011) pourrait également être impliqué dans la diaphonie entre les ROS et les RNS dans les nodosités racinaires en modifiant la production de ROS.

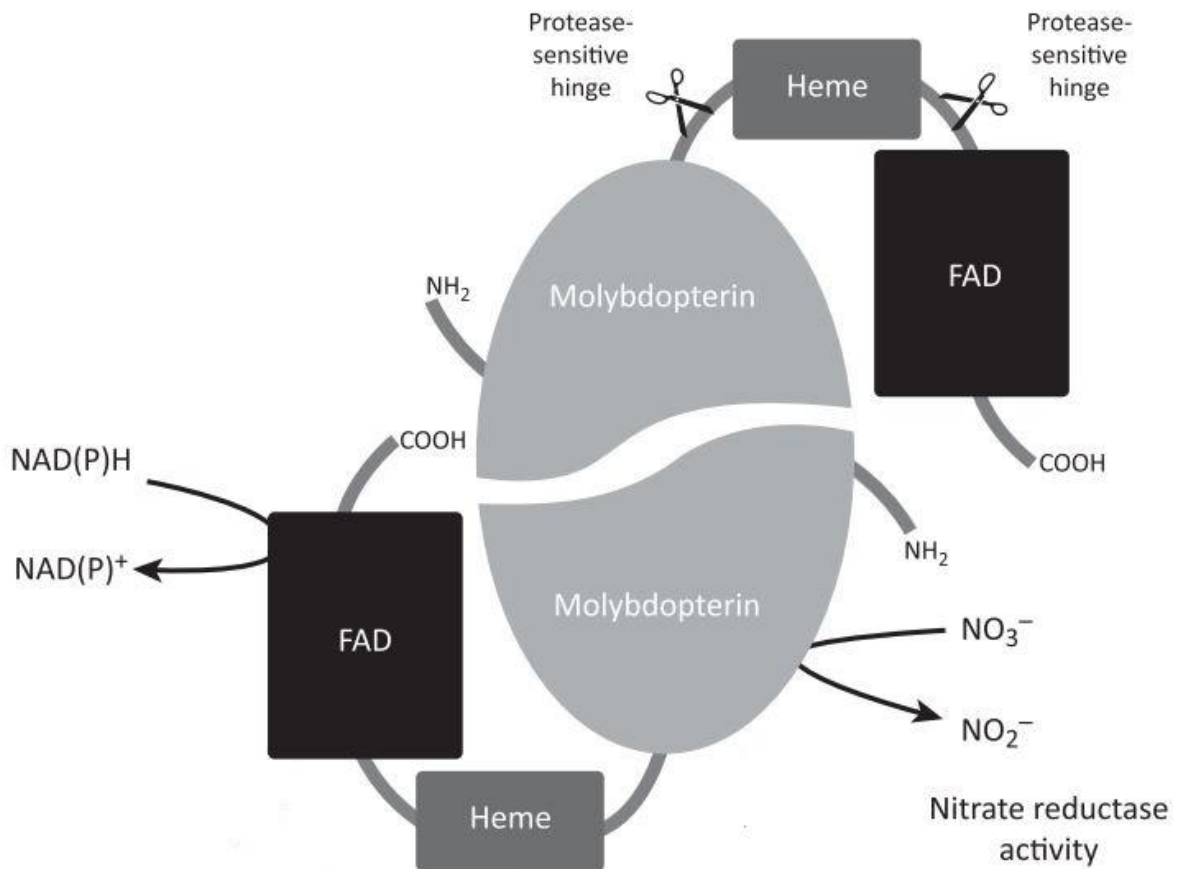
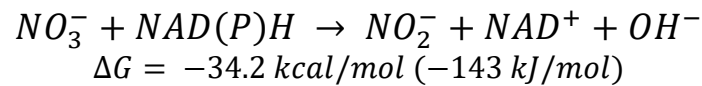


Figure 20 : Représentation schématique de la structure de la NR. Les deux sous-unités constituant la protéine active sont formées, en partant de l'extrémité N-ter, du domaine Molybdopterin (Moco) du domaine hème et du domaine FAD. (Adapté de Chamizo-Ampudia *et al.*, 2017).

3. Les Nitrate Réductases (NR) végétales

La nitrate réductase (NR; EC 1.6.6.1-3) catalyse la réduction NAD(P)H-dépendante du nitrate en nitrite selon l'équation suivante :



Cette réaction est irréversible, en condition normale, à cause de la faible énergie libre nécessaire pour réduire le nitrate en nitrite (Campbell, 1999). Trois formes de NR existent : la forme spécifique du NADH présente chez les plantes supérieures et les algues, la forme qui utilise le NADH et/ou le NADPH présente chez les plantes, les algues et les champignons, et enfin, la forme NADPH spécifique présente uniquement chez les champignons (Campbell, 1999). Cette enzyme catalyse la première étape de l'assimilation du nitrate chez tous ces organismes. Le nitrate étant la source d'azote la plus importante chez les plantes cultivées (Bloom, 2015), la compréhension du rôle des NR chez les plantes supérieures revêt une importance économique, notamment pour son rôle central dans le métabolisme azoté.

3.1. Structure, fonction et localisation des NR

La NR n'est active que sous forme homo-dimère avec deux sous-unités d'environ 100 kDa (Campbell, 2001). Chacune de ces sous-unités est composée de 3 groupements prosthétiques : un co-facteur FAD, un hème et un cofacteur au molybdène (MoCo) (Fig. 20). L'interaction entre les deux sous-unités se fait au niveau du domaine MoCo et les trois domaines FAD-Hème-Moco, dans l'ordre, permettent le transfert des deux électrons provenant du NAD(P)H au nitrate (Fig 20). Entre les domaines FAD et hème ainsi qu'entre les domaines hème et MoCo, se trouvent deux domaines « charnières » (Hinge) qui sont flexibles et qui contiennent des sites sensibles à la protéolyse (Campbell, 1996, 1999).

En plus de son activité de diaphorase⁴ du nitrate par le transfert de deux électrons, la NR catalyse également les activités de diaphorase ne nécessitant le transfert que d'un seul électron. Ainsi, la NR peut utiliser l'oxygène moléculaire comme accepteur d'électrons pour produire l'anion superoxyde (Ruoff & Lillo, 1990). De la même façon, le nitrite peut être réduit en NO et comme cette réaction est en compétition avec la réduction du nitrate (Rockel *et al.*, 2002), celle-ci a lieu au centre du domaine MoCo, comme pour la réduction du nitrate.

⁴ La diaphorase est une flavoprotéine capable d'oxyder le NADH

Tableau 1: Exemple de stimuli qui influencent la transcription et l'activité de la NR (Buchanan *et al.*, 2015)

Stimuli	Effet sur la NR
Glutamine	Inhibition de la transcription
Stress par privation d'azote	Inhibition de la transcription
Rythme circadien	Modulation de la transcription en fonction du jour ou de la nuit
Nitrate	Augmentation de la transcription
Cytokinin	Augmentation de la transcription
Sucrose	Augmentation de la transcription
Lumière	Augmentation de la transcription et de l'activité
Obscurité	Inhibition de la transcription et de l'activité
Oxygène	Diminution de l'activité
Anoxie	Augmentation de l'activité

La nitrate réductase et la réduction des nitrates sont localisées dans le cytosol des cellules dans les organes végétatifs de la plante (Meyer & Stitt, 2001). L'existence de certains analogues liés à la membrane plasmique a également été rapportée (Stöhr & Ullrich, 1997). Dans la plupart des espèces de plantes, la NR se trouve dans les parties aériennes et les racines, sa répartition entre les différents organes dépende des conditions environnementales (Andrews, 1986). Cependant, chez certaines espèces (canneberge, trèfle blanc et chicorée) les NR sont presque toutes localisées dans les racines, tandis que chez d'autres (lampourde⁵) les NR sont presque exclusivement exprimées dans les feuilles (Buchanan *et al.*, 2015). Au sein d'un même organe, la NR montre une localisation spécifique à certain type de cellule. Dans les racines, et en présence d'une faible concentration externe de nitrate (0,2 mM), la NR se trouve principalement dans les cellules épidermiques et les cellules corticales proches de la surface des racines (Rufty *et al.*, 1986). En revanche, à des concentrations externes élevées de nitrates (20 mM), la NR est détectée dans les cellules du cortex, mais aussi les cellules du système vasculaire (Rufty *et al.*, 1986).

3.2.Mécanisme de régulation des NR

3.2.1. Modifications post-transcriptionnelles

La concentration en nitrate est le facteur majeur qui contrôle la transcription des NR (Kaiser *et al.*, 2010). En présence de faible quantité de nitrate ou en présence d'ammonium la NR est faiblement exprimée. A contrario, en présence de grande quantité de nitrate, les quantités de transcrits et de protéine sont élevées (Hoff *et al.*, 1994; Scheible *et al.*, 1997). D'autres facteurs induisent la transcription des NR (Tab. 1), comme les niveaux en sucre et/ou la photosynthèse (Vincentz *et al.*, 1993; Huber *et al.*, 2002; Lillo *et al.*, 2004). La régulation simultanée des NR par ces deux facteurs, permet de synthétiser les enzymes seulement si le nitrate et les composés carbonés nécessaires à la synthèse des acides aminés sont disponibles (Kaiser *et al.*, 2010). La transcription de la plupart des gènes de NR déjà caractérisé est également régulée selon le rythme circadien. Ainsi, la transcription des NR est élevée vers la fin de la nuit, reste élevée ou augmente légèrement pendant les deux premières heures du jour puis décline pendant la photopériode restante et re-augmente progressivement durant la nuit (Tucker *et al.*, 2004).

⁵ La lampourde, *Xanthium strumarium* est une plante herbacée annuelle de la famille des Astéracées.

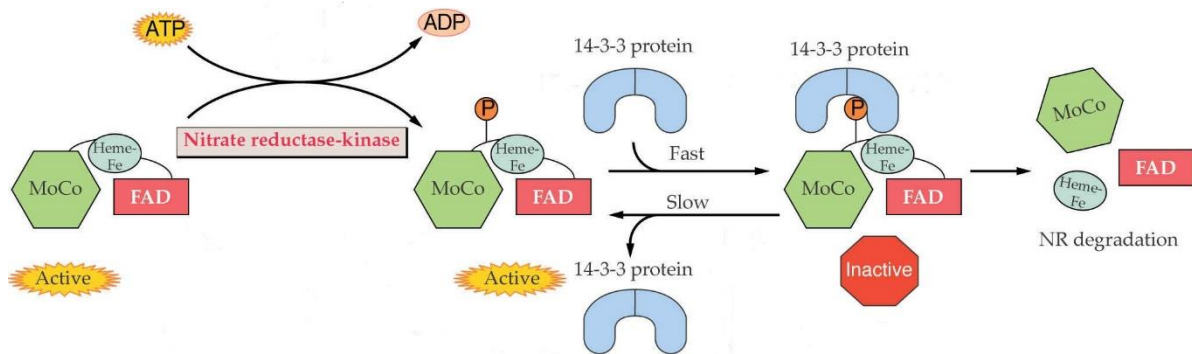


Figure 21 : Modèle proposé pour la régulation de l'activité nitrate réductase par phosphorylation et liaison réversible avec les protéines 14-3-3. La NR peut-être phosphorylée par des protéines kinases sur une sérine. La NR phosphorylée reste active mais c'est la fixation par les protéines 14-3-3 qui la rend inactive (adapté Buchanan *et al.*, 2015).

3.2.2. Modifications post-traductionnelles

Les régulations post-traductionnelles modulent de manière réversible l'activité de la NR, en quelques minutes, et permettent une réponse rapide aux changements de l'environnement ou du métabolisme cellulaire (Wang *et al.*, 2012). Des régulations par phosphorylation, ont été identifiées, sur la région « charnière » entre le domaine MoCo et hème, au niveau du résidu sérine chez *A. thaliana* (S534), l'épinard (S543) ou le tabac (S521) (Bachmann *et al.*, 1996; Su *et al.*, 1996; Lillo *et al.*, 2003). Cette régulation par phosphorylation inhibe rapidement la NR en réponse à différents signaux comme la diminution du niveau de CO₂, ou l'augmentation du pH cytosolique (Campbell, 1999 ; Su *et al.*, 1996). La NR sous forme phosphorylée peut se complexer avec des protéines de type 14-3-3, et sous cette forme complexée, la NR est plus facilement dégradée (Kaiser & Huber., 2002) (Fig. 21).

Cependant la phosphorylation au niveau de la région « charnière » entre les domaines hème et FAD chez *A. thaliana* (S627), provoque une augmentation de l'activité NR (Wang *et al.*, 2010a). De plus, l'accumulation de ROS notamment lors de la transition jour-nuit, provoque une rapide activation de la NR par phosphorylation au niveau de ce domaine (op den Camp *et al.*, 2003; Cookson *et al.*, 2005).

De récentes études montrent que la NR peut être modifiée par S-nitrosylation (Hu *et al.*, 2015; Thalineau *et al.*, 2016; Fu *et al.*, 2018). La S-nitrosylation peut entraver l'assemblage de l'oligomère protéique ou le repliement de la chaîne peptidique naissante, et inhibe donc l'activité enzymatique (Lindermayr *et al.*, 2005; Zhang *et al.*, 2017). Il est donc possible que le NO généré par la NR puisse désactiver la NR elle-même, ce qui aboutit à une boucle de régulation négative. Cette régulation peut être importante pour l'équilibre entre les signaux de croissance médiés par le nitrate et les signaux de réponse au stress médiés par le NO. Un autre effet inhibiteur du NO sur la NR a également été décrit : Sanz-Luque *et al.* (2015) ont montré qu'une hémoglobine tronquée, trHb1, est impliqué dans l'inhibition de la NR par le NO. Cette hémoglobine possède une activité NO dioxygénase (voir §. 4.3.1) et utilise l'électron du groupe FAD de la NR. En présence de NO, trHb1 peut catalyser la conversion de NO en NO₃⁻, ce qui diminue l'activité de la NR en diminuant le pouvoir réducteur disponible pour l'enzyme (Sanz-Luque *et al.*, 2015).

3.3. Rôles des NRs

L'activité NR est considérée comme un des facteurs limitants de la croissance, du développement et de la production de protéines chez les végétaux (Solomonson & Barber, 1990).

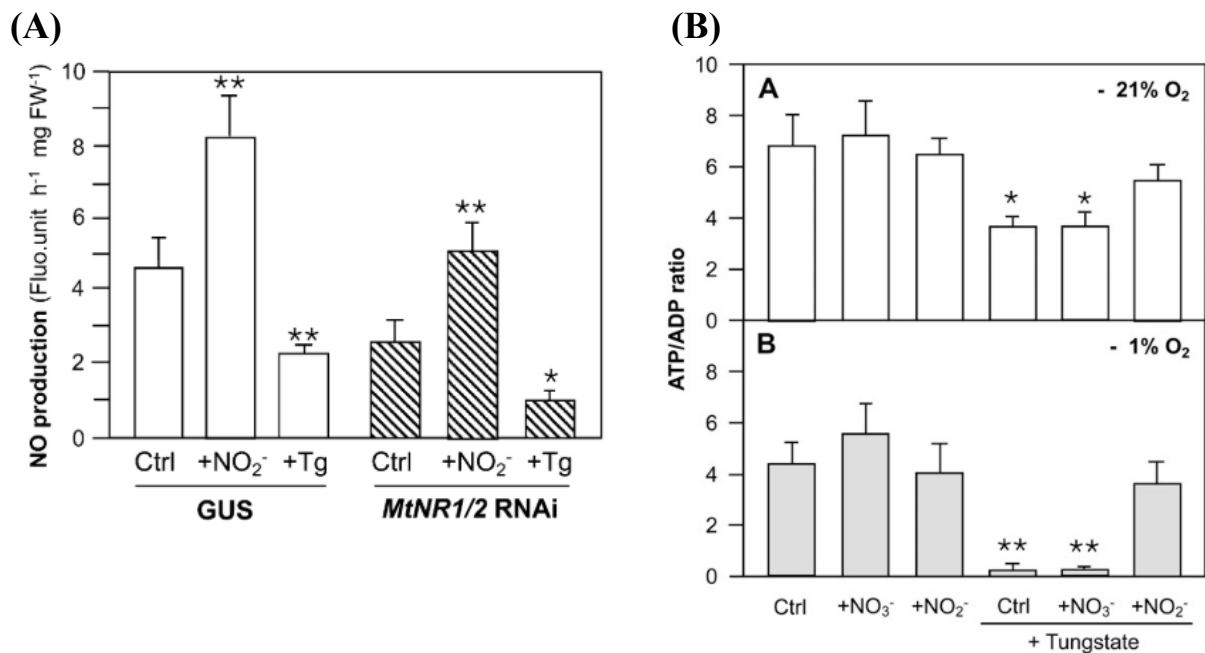


Figure 22 : Impact de la NR sur la production de NO et le statut énergétique dans la nodosité mature

(A) Production de NO par des nodosités de *M. truncatula* contrôle (GUS) et sous-exprimant deux nitrate réductases (NR1/2). Les nodosités sont traitées avec du nitrite (1mM) ou un inhibiteur de la NR, le tungstate (Tg ; 1mM) (Horchani et al., 2011).

(B) Effet des effecteurs de la NR sur l'état énergétique des nodosités de *M. truncatula*. Les ratios ATP/ADP sont mesurés en présence de 21% et 1% d'oxygène. Les concentrations des effecteurs sont : 10 mM NaNO₃ (NO₃⁻), 1 mM NaNO₂ (NO₂⁻), et 1 mM NaTg.

D'autre part, l'importance de cette enzyme dans le métabolisme du NO et sa signalisation ((Magalhaes *et al.*, 2000; García-Mata & Lamattina, 2003) suggère que la NR est impliquée dans les processus régulés par le NO (Hao *et al.*, 2010; Mur *et al.*, 2013a; Lu *et al.*, 2014; Yuan *et al.*, 2016). Il est donc difficile de décrire le rôle de la NR sans la mettre en relation avec l'assimilation du nitrate et la signalisation du NO.

Un des premiers exemples est que la NR est impliquée dans le développement des plantes et dans les réponses au stress en modulant le niveau de NO, mais également le niveau de nitrite (Campbell, 2001). Une étude récente a aussi démontré que l'activité de la NR affecte le statut nutritionnel en potassium (Chen *et al.*, 2016). L'activité de la NR est impliquée dans la régulation des flux de carbone et provoque la régulation de gènes codant pour la biosynthèse et le métabolisme de la chlorophylle ainsi que la fixation et le métabolisme du carbone chez *Phaeodactylum tricorutum* (McCarthy *et al.*, 2017). La NR est également impliquée dans l'acclimatation des plantes aux stress nutritionnels. Une autre étude a montré que la NR est impliquée dans la protection des plantules d'orge contre un stress au cuivre en renforçant les activités des enzymes antioxydantes via la production de NO (Hu *et al.*, 2015).

Dans le contexte de l'interaction plantes/pathogènes, l'assimilation du nitrate est impliquée dans les réponses de défense de différentes manières. Chez plusieurs écotypes de *M. truncatula*, l'augmentation de la quantité en nitrate dans le milieu, peut augmenter ou réduire la résistance de la plante face à *A. euteiches* (Thalineau *et al.*, 2018). De plus, il a été suggéré que le nitrate augmente l'activité des NR, ce qui peut aboutir à une augmentation de la production de NO (Gupta *et al.*, 2005; Planchet *et al.*, 2005). Yamamoto-Katou *et al.* (2006) ont indiqué que, chez le tabac la sous expression de la NR entraîne une diminution de la teneur en NO après un traitement à l'élicite qui mime l'attaque par un pathogène.

Peu de données sont disponibles concernant l'implication des NR végétales lors de l'interaction symbiotique. Une activité NR importante a tout de même été identifiée dans le cytosol des nodosités de soja. (Streeter, 1982, 1985; Stephens & Neyra, 1983).

Par la suite, il a été montré que la NR est indirectement impliquée dans la production de NO, via la respiration Pgb-NO et dans la régénération de l'ATP, lors de la symbiose en *M. truncatula* et *S. meliloti* (Horchani *et al.*, 2011). Les auteurs montrent que suite à la sous expression des NR de *M. truncatula*, la production de NO était diminuée (Fig. 22A) ainsi que le statut énergétique de la nodosité (Fig. 22B) (Horchani *et al.*, 2011).

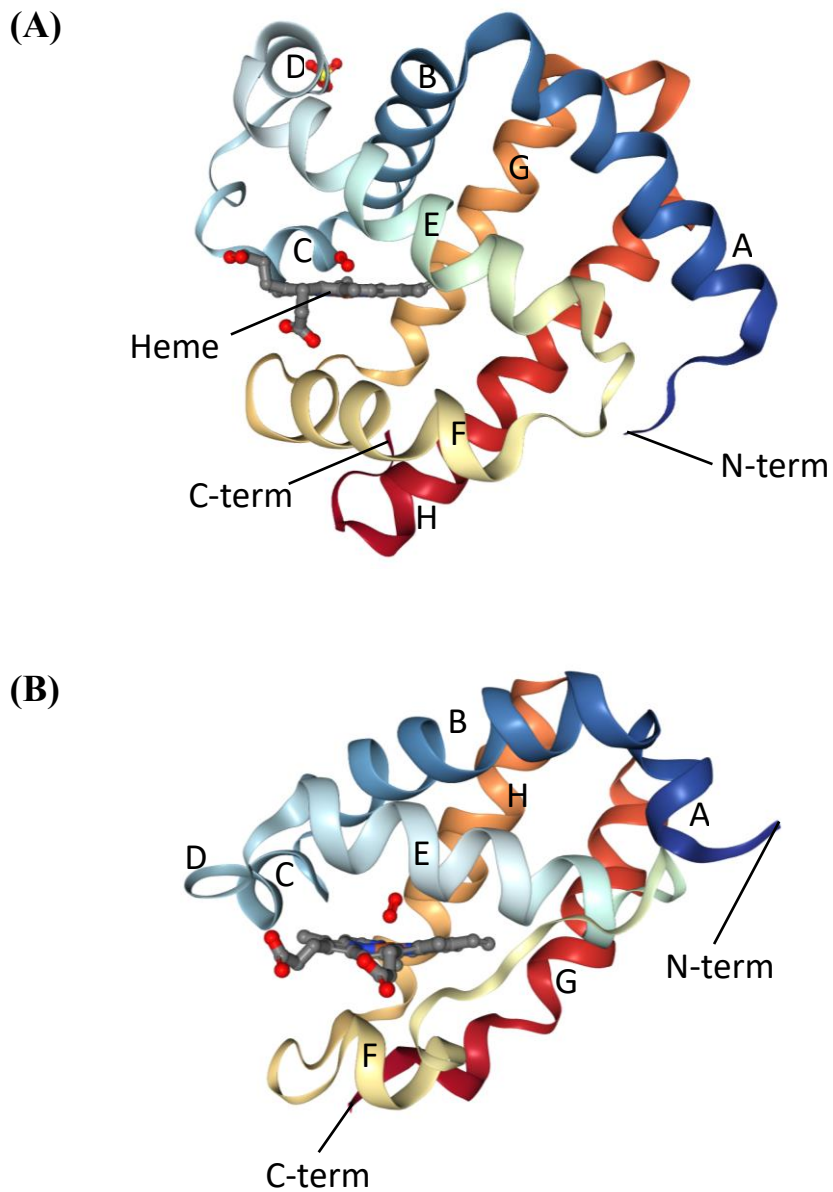


Figure 23 : Caractéristiques de la structure tertiaire des hémoglobines. La figure représente (A) la structure tertiaire de la myoglobine (code PDB : 2mgm ;<https://www.rcsb.org/>) qui correspond à un repliement 3 on 3 (B) la structure tertiaire d'une hémoglobine tronquée (code PDB 3aq5) qui correspond au repliement 2 on 2. Les hélices α conservées entre les deux structures sont nommées par leur lettre correspondante (A-H).

4. Les hémoglobines

4.1. Généralités sur les hémoglobines

L'hémoglobine (Hb) est principalement connue pour être une protéine abondante du sang⁶, essentielle au transport de l'O₂ chez les mammifères. Elle est présente dans les trois domaines du vivant : les bactéries, les archées et les eucaryotes. La découverte et la dénomination « hémoglobine », par Felix Hoppe-Seyler⁷, date de 1862.

Hoppe-Seyler avait émis l'idée que la couleur rouge du sang était expliquée par l'absorption de la lumière à des longueurs d'ondes spécifiques. Pour tester son idée, il construisit un spectroscope basé sur la lumière du soleil. Son appareil fonctionnait comme suit : la lumière du soleil, qui provient d'un héliostat⁸, est orientée vers une fente située au centre d'un objectif achromatique, dirigée vers un prisme en verre. Derrière le prisme se trouvait une cuvette en verre de 1 cm d'épaisseur avec une solution de sang diluée. Le spectre qui était obtenu pouvait être visualisé au travers d'un télescope ou projeté sur un écran de papier blanc. Grâce à cet appareil, Hoppe-Seyler découvrit la bande d'absorption de l'oxyhémoglobine et montra que l'hémoglobine de différents mammifères, oiseaux et poissons, possédait le même spectre d'absorption (Perutz, 1995). Deux ans plus tard, George Gabriel Stokes, plus connu pour son implication en physique des fluides, répéta les expériences de Hoppe-Seyler, et découvrit le spectre d'absorption de la déoxyhémoglobine et démontra spectroscopiquement la réaction réversible de fixation de l'O₂ à l'hémoglobine (Stokes, 1864). En 1960, Perutz et Kendrew identifièrent la structure tridimensionnelle de l'hémoglobine et de la myoglobine par diffraction des rayons X (Perutz *et al.*, 1960) et seront récompensés par le prix Nobel de chimie en 1962.

4.1.1. Structure et fonctions

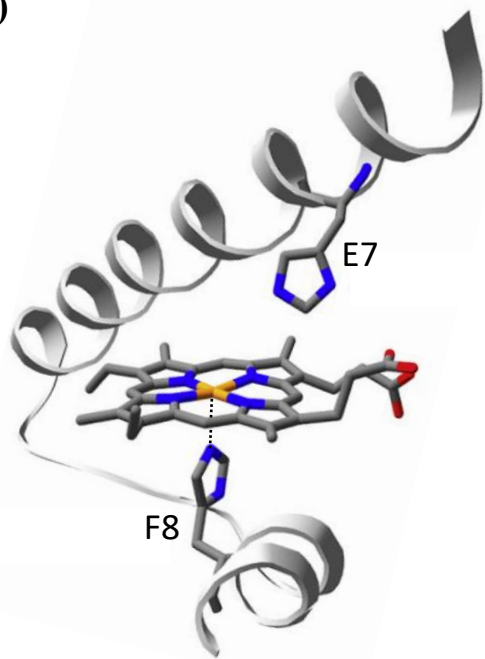
Les hémoglobines sont toutes construites avec une structure de base conservée, qui comprend 6 à 8 hélices α (identifiées de A à H) (Fig. 23). Cette structure, appelé globine, fut déterminée pour la première fois au sein de la myoglobine du muscle du cachalot (Kendrew *et al.*, 1958, 1960). L'étude structurale de la myoglobine fournit la structure de référence à laquelle toutes les autres séquences et structures d'hémoglobines sont comparées (Lesk & Chothia, 1980).

⁶ La concentration en hémoglobine dans le sang humain est de 15g/100ml

⁷ Hoppe Seyler est aussi considéré comme un des fondateurs de la biochimie

⁸ L'héliostat est un miroir qui suit le soleil et permet de rediriger ses rayons

(A)



(B)

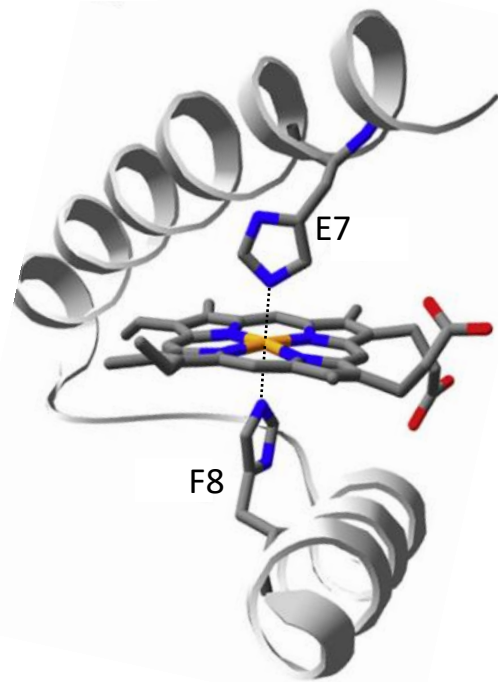


Figure 24: Hémoglobines pentacoordonnées ou hexacoordonnées. A) La structure 3D montre la poche distale ouverte des hémoglobines pentacoordonnées, tandis que B) montre la coordination de l'histidine distale (E7) avec le sixième site de liaison du fer hémique. Les hélices E et F, les histidines distales et proximales et l'hème sont représentés. (Adapté de Hoy & Hargrove, (2008))

Par exemple, la position F8 désigne le résidu d'acide aminé structurellement équivalent au huitième résidu de l'hélice F de la myoglobine du cachalot et qui correspond à une histidine. Cette position correspond au seul résidu conservé à 100% dans toutes les hémoglobines (Kapp *et al.*, 1995; Freitas *et al.*, 2004).

La partie globine est repliée autour d'un groupement prosthétique, l'hème qui est une protoporphyrine IX. L'hème est une molécule amphiphile avec deux groupes de propionates qui peuvent interagir avec les chaînes latérales des acides aminés polaires à la surface de la globine. Le reste du groupement est en grande partie de nature hydrophobe et se lie à l'intérieur hydrophobe de la globine. L'hème possède un atome de fer central coordonné par quatre liaisons équatoriales avec les atomes d'azote des pyroles de l'hème et un lien axial formé avec l'histidine en position F8 du côté proximal de l'hème (Milani *et al.*, 2001). L'atome de fer a une géométrie de coordination octaédrique et peut donc établir une 6^{ème} liaison covalente avec des ligands gazeux diatomiques tels que O₂, CO, NO, HNO, NO₂⁻ et H₂S (Voets & Voets, 2011). L'environnement qui entoure l'hème, formé par le repli de la globine, module les cinétiques d'association et de dissociation entre l'atome de fer et le ligand.

Une première classification structurale permet de différencier les deux principaux repliements tridimensionnels de la globine. Le premier, le plus représenté, correspond à un repliement dit 3-sur-3 (3-on-3) (Fig. 23A). Ce repliement fait référence au « sandwich » formé d'un côté par les hélices A-G-H et de l'autre B-E-F qui entourent le groupement hémique (Holm & Sander, 1993). Les hélices C et D correspondent à des structures de soutien et ne sont pas toujours présentes. Le second repliement, correspond à une classe particulière d'hémoglobines, les hémoglobines tronquées, dont le repliement est dit 2-sur-2 (2-on-2) (Fig. 23B). Dans ce repliement, les hélices A, C, D et F sont très réduites voir absentes et le « sandwich » qui entoure le groupe hémique est composé d'un côté des hélices B et E et de l'autre des hélices G et H (Pesce *et al.*, 2000).

4.1.2. Classes structurales des hémoglobines

Une autre subdivision en deux classes existe, basée sur l'état de coordination de l'atome de fer. La première classe, comprend les hémoglobines dites pentacoordinées (Fig. 24A), et correspond à la structure de l'hémoglobine ou de la myoglobine. La deuxième classe structurale correspond aux hémoglobines qui sont hexacoordinées (Fig. 24B) en l'absence de ligands exogènes, c'est-à-dire que le fer est coordonné aux six positions et notamment avec une histidine (E7) distale.

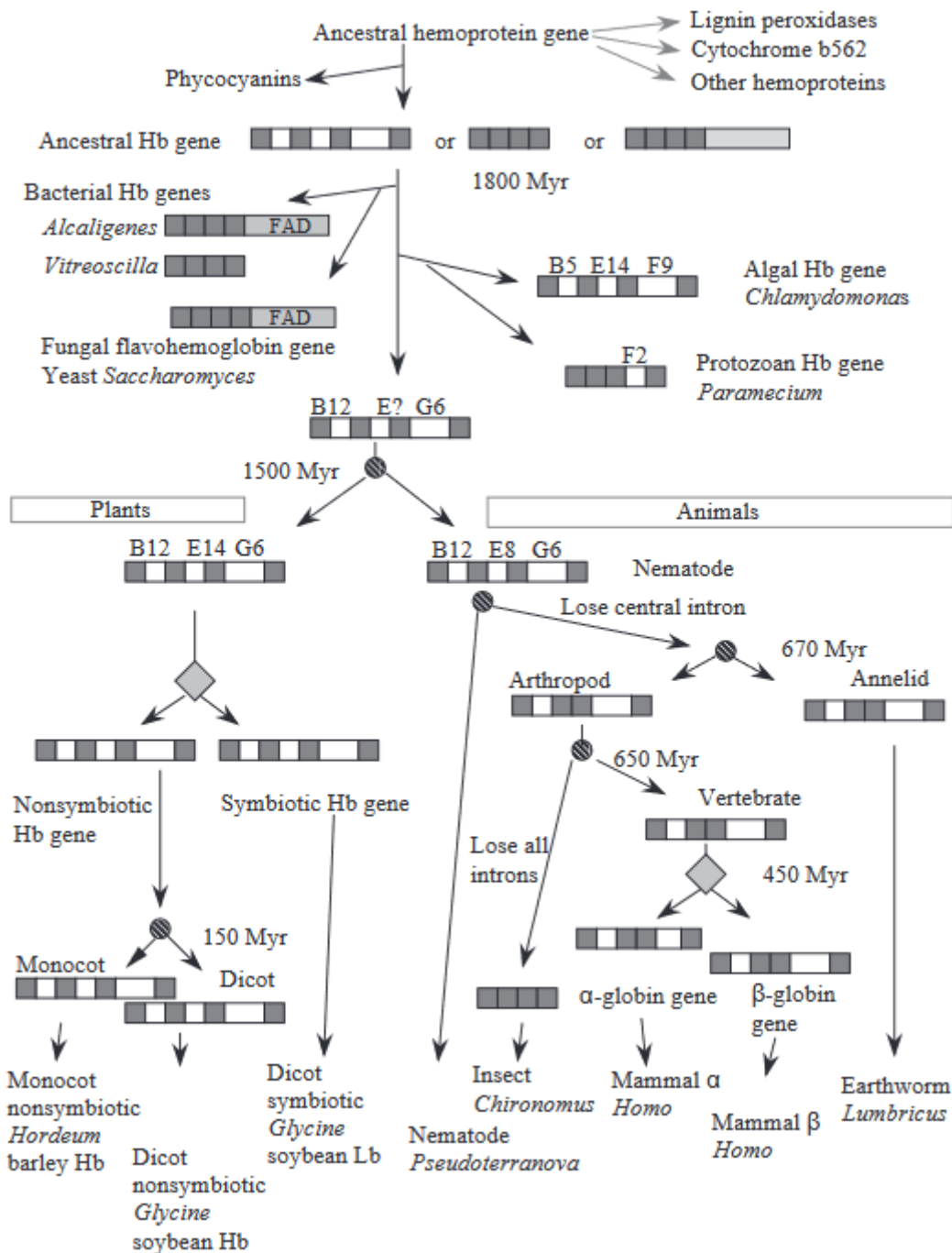


Figure 25: Evolution schématique du gène de l'hémoglobine. Les exons et les gènes codant la globine sont représentés par des cases sombres et les introns par des cases ouvertes dans les gènes. Les événements de spéciation sont représentés par des cercles rayés en diagonale et les duplications de gènes par des losanges gris. Myr, millions d'années; Monocot, Monocotylédone; Dicot, dicotylédone.

La coordination réversible du fer hémique par l'histidine distale rend la liaison du ligand très complexe. Néanmoins, par rapport aux hémoglobines pentacoordinées, elles sont toujours capables de lier des ligands avec une très grande affinité (Hargrove, 2000; Trent & Hargrove, 2002).

4.2. Les hémoglobines dans le règne du vivant

Les analyses génomiques de nombreuses espèces ont permis de faire remonter l'apparition d'un gène d'hémoglobine ancestral à 1,8 milliard d'années (Fig. 25). A cette époque l'O₂ commençait à s'accumuler dans l'atmosphère notamment grâce au développement de la photosynthèse (Lyons *et al.*, 2014). Cette corrélation entre l'apparition de l'O₂ *via* la photosynthèse, et l'apparition et l'utilisation des hémoglobines par les organismes primitifs, notamment les archaebactéries (Freitas *et al.*, 2004), permet de poser certaines hypothèses. L'O₂ est, et reste, une espèce réactive capable de réagir et d'endommager divers composants cellulaires (ADN, protéines, lipides...). L'apparition des hémoglobines qui lient l'O₂ aurait permis, de protéger les cellules de cette espèce réactive et d'autres existantes telles que le NO (Thannickal, 2009). Par la suite, l'O₂ est devenu un accepteur d'électron couramment utilisé au sein des chaînes respiratoires, et les hémoglobines liées à l'O₂ pouvaient servir d'accepteurs d'électrons terminaux. Ensuite, d'autres modifications, par duplication ou divergence de gènes, ont permis d'obtenir d'autres fonctions telles que la capacité de catalyser d'autres réactions redox, ou la capacité de transporter ou piéger l'O₂ (Hardison, 1998).

4.2.1. Evolution du micro-organisme à l'humain en passant par les plantes

C'est du gène de l'hémoglobine ancestral que vont dériver les gènes d'hémoglobines présents chez les procaryotes, les champignons, les plantes et les animaux. L'analyse du nombre, de la position des introns et des exons des gènes contemporains permet de faire le lien avec le gène d'hémoglobine ancestral (Goodman *et al.*, 1988) (Fig. 25). Chez les micro-organismes, il existe une grande diversité sur le nombre et la disposition des introns. Aucun intron n'est retrouvé chez les bactéries, les champignons et les levures, un seul chez les protozoaires et 3 chez les algues. La structure génique composée de 4 exons séparés par 3 introns est considérée comme la structure du gène à l'origine des hémoglobines animales et

Tableau 2: Caractéristiques physiologiques et biochimiques des phytoglobines. Les valeurs des K_{mO_2} ont été mesurées pour les Phytogb1 et Phytogb2 d'*A. thaliana* (Trevaski *et al.*, 1997)¹, pour une Lb du soja (Gibson *et al.*, 1989)², et pour la Phytogb3 d'*A. thaliana* (Watts *et al.*, 2001)³. La valeur du K_{mNO} a été mesurée pour la Lb du soja (Harutyunyan *et al.*, 1995)⁴

	Plante	Etat de coordination	Constante affinitée (Km)		Localisation expression
			O ₂	NO	
Phytogb1	Monocot / Dicot	Penta / Hexacoordiné (60%)	1.6 nM (1)		tissus vasculaire, graines, racines tissus souches,
Phytogb2	Dicot	Hexacoordiné	130 nM (1)		tissus en cours de développement, végétatif
Lb	legumineuse	Penta coordiné	48 nM (2)	7.10 ⁻⁴ nM (4)	tissus nodositaire
Phytogb3	Monocot / Dicot	Penta/ Hexacoordiné	1500 nM (3)		tissus nodositaire, tige, racine

végétales, il y a 1,5 milliard d'années, lors de la séparation entre les règnes animal et végétal (Goodman *et al.*, 1988). Au sein du règne animal, le 3^{ème} intron a été perdu, alors qu'au sein du règne végétal, les gènes des hémoglobines ont conservé la structure ancestrale comportant trois introns et quatre exons (Fig. 24).

L'analyse phylogénétique des séquences d'hémoglobines végétales (Anderson *et al.*, 1996) distingue deux branches, l'une avec les hémoglobines dites « symbiotiques » et l'autre avec les hémoglobines dites « non symbiotiques » (la classification des hémoglobines végétales sera détaillée dans le chapitre suivant). Ces dernières sont présentes chez un grand nombre d'espèces végétales des bryophytes jusqu'aux angiospermes.

4.3. Les hémoglobines dans le règne végétal

Au sein des plantes terrestres, trois principales classes d'hémoglobines ont été identifiées (Tab. 2), les hémoglobines symbiotiques, les hémoglobines non symbiotiques et les hémoglobines tronquées (Arredondo-Peter *et al.*, 1998; Garrocho-Villegas *et al.*, 2007). Toutes les plantes n'ont pas forcément un membre de chaque classe, mais possèdent au minimum un membre des hémoglobines non symbiotiques et un membre des hémoglobines tronquées (Hunt *et al.*, 2001; Garrocho-Villegas *et al.*, 2007). En 2016, une nouvelle nomenclature a été proposée lors de la XVIIth Conference on Oxygen-Binding and Sensing Proteins, (Hill *et al.*, 2016). Elle permet d'étendre et d'homogénéiser la classification des hémoglobines végétales qui ont été renommées « phytoglobines ».

Dans le cadre des interactions symbiotiques, 2 types de phytoglobines symbiotiques ont été identifiés. Les premières, les **symPhytogb** correspondent à des phytoglobines symbiotiques présentes au sein de plantes qui réalisent une symbiose fixatrice d'azote, mais qui ne sont pas des légumineuses. Ce cas particulier concerne des plantes tels que *Parasponia* (Appleby *et al.*, 1983), *Casuarina* (Jacobsen-Lyon *et al.*, 1995) et *Chamaecrista* (Gopalasubramaniam *et al.*, 2008). La deuxième classe de phytoglobines symbiotiques correspond aux **legHémoglobines** (Lb) présentes spécifiquement chez les espèces légumineuses fixatrices d'azote.

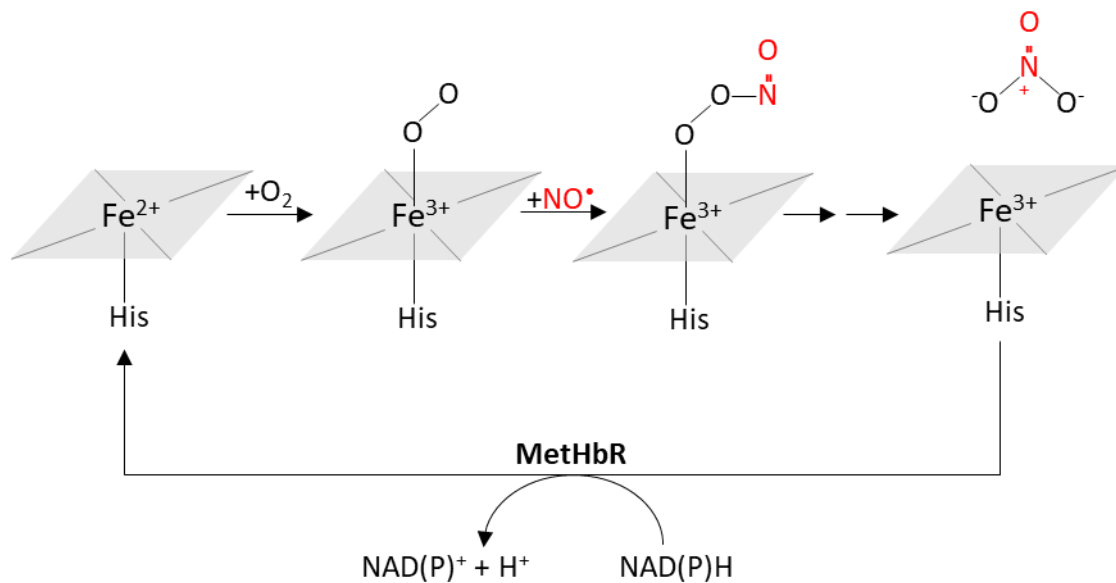


Figure 26: Mécanisme réactionnel de l'activité NO dioxygénase. Durant cette réaction, l'oxygène peut se lier au fer sous forme ferreux (Fe^{2+}) qui passe alors sous forme oxygéné ferrique (Fe^{3+}). Le NO peut réagir avec l' O_2 fixé et par réaction d'isomérisation donner du nitrate qui est relargué. L'hémoglobine sous forme ferrique appelé MetHb est ensuite réduite par la réductase correspondante (MetHbR) pour redonner du fer ferreux et être à nouveau oxygéné.

Au sein de la classe des phytoglobines non symbiotiques, 3 subdivisions sont faites : Les **Phytogb0** correspondent aux phytoglobines non symbiotiques présentes chez les algues, les bryophytes et les gymnospermes. Les **Phytogb1** et **Phytogb2** subdivisent les phytoglobines non symbiotiques, présentes chez les angiospermes, selon leur affinité avec l'O₂ et de leur similarité de séquence (Trevaskis *et al.*, 1997; Smaghe *et al.*, 2009). Dans cette nouvelle nomenclature les **Phytogb3** correspondent à la nouvelle dénomination des phytoglobines tronquées.

4.3.1. Les phytoglobines non symbiotiques, Phytogb1

Les Phytogb1 sont présentes à la fois chez les monocotylédones et les dicotylédones. Elles sont principalement exprimées dans les graines, les racines et les tiges (Arredondo-Peter *et al.*, 1998; Hill, 1998). Les Phytogb1 existent à la fois sous forme penta- et hexacoordinées (40%/60%) (Bruno *et al.*, 2007). Elles possèdent une très forte affinité pour l'O₂ (K_m ~ 1-2 nM) ainsi que le NO (Duff *et al.*, 1997). Cette caractéristique suggère que les Phytogb1 ne peuvent pas jouer le rôle de transporteurs ou capteurs d'O₂ (Smaghe *et al.*, 2009). Cependant, plusieurs travaux indiquent que les Phytogb1 sont impliquées dans la régulation des niveaux intracellulaires de NO (Igamberdiev & Hill, 2004; Hill, 2012; Hebelstrup *et al.*, 2013). Cette fonction est due à leur activité NO dioxygénase (Igamberdiev & Hill, 2004; Perazzolli *et al.*, 2004). En présence d'O₂, le NO est converti en nitrate via l'oxygénation du fer ferreux (Fe²⁺) en fer ferrique (Fe³⁺). Cette étape aboutit à la formation de méthémoglobine qui est réduite par l'intermédiaire de la méthémoglobine réductase (MetHbR) pour redonner une phytoglobine avec du Fe²⁺ (Fig. 26) (Gardner, 2012).

4.3.2. Les phytoglobines non symbiotiques, Phytogb2

Les Phytogb2 sont présentes majoritairement chez les dicotylédones et la plupart des monocotylédones en sont dépourvues (Smaghe *et al.*, 2009), bien qu'une Phytogb2 ait été caractérisée chez le maïs (Garrocho-Villegas *et al.*, 2007). Les Phytogb2 sont exprimées dans les tissus en cours du développement ainsi que dans les tissus végétatifs et reproductifs (Wang *et al.*, 2003; Ross *et al.*, 2004; Smaghe *et al.*, 2009). Les Phytogb2 se trouvent exclusivement sous forme hexacoordinée et possèdent une forte affinité pour l'O₂ et le NO (K_{mO₂} ~100-200 nM ; (Gupta *et al.*, 2011b)). Une activité NO dioxygénase est possible pour les Phytogb2, bien que celle-ci soit moins favorable (Nienhaus *et al.*, 2010). Par conséquent, le rôle supposé

des Phytogb2 correspond à une fonction de senseur d'O₂, de stockage et de diffusion (Kakar *et al.*, 2010). Dans le cas des légumes ou autres dicotylédones capables de nodulation, les Phytogb2 ont évolué en phytoglobines symbiotiques aussi appelées leghémoglobines (Hunt *et al.*, 2001 ; Garrocho-Villegas *et al.*, 2007 ; Smagghe *et al.*, 2009).

4.3.3. Les Leghémoglobines, Lbs

Les leghémoglobines (Lb) ont été décrites pour la première fois par Kubo, (1939). Elles ont une structure tertiaire similaire à la myoglobine animale. Elles sont pentacoordonnées et possèdent une forte affinité pour l'O₂ et le NO (Hoy *et al.*, 2008). Les Lbs sont présentes, en concentration importante (0.7 mM), exclusivement dans les nodosités racinaires. Dans les nodosités racinaires, 20% des Lb sont sous forme oxygénées (oxyLb), et 80% sont désoxygénées (désoxyLb) (Mathieu *et al.*, 1998). Il a été proposé que, la forme désoxyLb puisse moduler les niveaux de NO en liant le NO avec une haute affinité (Herold & Puppo., 2005). L'identification du complexe nitrosyl-leghémoglobine (Lb-NO) a permis de démontrer cette capacité de piégeage du NO (Harutyunyan *et al.*, 1995) et du peroxy-nitrite (Sanchez *et al.*, 2010).

4.3.4. Les phytogb3

Les Phytogb3 se distinguent par leur structure proche des hémoglobines bactériennes dites 2-sur-2 et sont plus petites de 75-80% par rapport aux hémoglobines 3-sur-3 (Wittenberg *et al.*, 2002). Les deux états de coordination sont retrouvés chez les phytogb3, mais celle-ci possède une faible affinité (1500 nM) pour l'O₂ et le NO (Watts *et al.*, 2001). Ces hémoglobines n'ont été découvertes que très récemment au sein des plantes (Watts *et al.*, 2001) du fait de leur présence en faible concentration (de l'ordre du nM). Chez les bactéries, une sous-division en 3 groupes existe, I, II et III ou, selon l'ancienne nomenclature, trHbN, trHbO et trHbP (Vinogradov *et al.*, 2006). D'un point de vue phylogénétique, il apparaît que les gènes du groupe II correspondent aux gènes originaux, tandis que les gènes du groupe I et III ont été obtenus par duplication et transfert (Vuletich & Lecomte, 2006). Les fonctions des trHb sont diverses. Par exemple, une trHb du bacille tuberculeux *Mycobacterium tuberculosis* (trHbN) est impliquée dans la défense contre le NO généré en réponse à une infection (Nathan & Shiloh, 2000; Ouellet *et al.*, 2002). En revanche, il a été proposé que la trHb de *M. tuberculosis*, trHbO, joue le rôle de facilitateur de la diffusion de l'O₂ pour les oxydases terminales en conditions

hypoxiques (Pathania *et al.*, 2002). Les phyto**gb**3 sont phylogéniquement plus proches du groupe II (trHbN) des trHb (Wittenberg *et al.*, 2002).

4.4. Rôles des phytoglob**ines**

Plusieurs rôles biochimiques et physiologiques importants ont été identifiés pour les phyto**glob**ines tels qu'un rôle de transporteur, de stockage et de capteurs d'O₂, mais aussi un rôle de transfert d'électrons (Arredondo-Peter *et al.*, 1998). L'analyse des mutants de phyto**glob**ines d'*A. thaliana* a mis en évidence le rôle vital des phyto**glob**ines lors du développement des plantes et montre que la présence d'au moins un gène fonctionnel de phyto**gb** est essentiel à la survie des jeunes plantes (Hebelstrup *et al.*, 2007).

Les Phytogb**1**

Les *Phyto**gb**1* sont exprimées lors de la germination des monocotylédones et des dicotylédones dans la couche d'aleurone, la radicule et le coléoptile (Hebelstrup *et al.*, 2007). Chez le blé, les *Phyto**gb**1* sont exprimées 2 heures après l'imbibition (Guy *et al.*, 2002), puis les niveaux d'expression augmentent pour des périodes allant de 2 à 6 jours après l'imbibition (Zhao *et al.*, 2008) comme chez le riz (Ross *et al.*, 2001). L'expression des *Phyto**gb**1* a aussi été identifiée lors de l'embryogénèse *in vitro* de cellules somatiques chez la chicorée (Smaghe *et al.*, 2009), et une étude montre que l'addition d'hémoglobine commerciale stimule l'embryogénèse chez *Arachis hypogaea* L. (Jayabalan *et al.*, 2004). Inversement, chez le maïs la suppression des *Phyto**gb**1*, normalement exprimées dans les cellules de l'embryon, provoque l'avortement de ce dernier (Huang *et al.*, 2014). Chez *A. thaliana*, la surexpression de *Phyto**gb**1* provoque l'induction de la croissance de la racine primaire par élongation de la zone de différenciation cellulaire (Hunt *et al.*, 2002; Zhao *et al.*, 2008), mais aussi stimule la floraison (Hebelstrup *et al.*, 2007; Hebelstrup & Jensen, 2008).

Une des fonctions principales des *Phyto**gb**1* est associée à la modulation des niveaux de NO (Hebelstrup *et al.*, 2013). L'interaction entre le NO et les phyto**glob**ines a principalement été étudiée par traitement des plantes avec des donneurs de NO, tels que le SNP, ou dans des conditions de croissance connues pour générer du NO. L'une de ces conditions est le stress hypoxique, connu pour augmenter les émissions de NO au sein des cellules végétales (Dordas *et al.*, 2003). Sowa *et al.* (1998) ont été les premiers à constater que les cellules de maïs surexprimant une *Phyto**gb**1* d'orge maintenaient les taux d'ATP dans des conditions hypoxiques plus efficacement que les cellules de type sauvages. Par la suite, il a été montré que la

concentration en NO dans les cellules de maïs avait une relation inverse avec le niveau d'expression des *Phytogb1* (Dordas *et al.*, 2004), ce qui suggère que les *Phytogb1* sont capables de métaboliser le NO.

Chez le coton, des traitements par l'éthylène, l'acide jasmonique ou encore l'infection par un pathogène fongique, induisent l'expression de *Phytogb1* (Qu *et al.*, 2006). Cette induction est associée à une résistance de la plante face au champignon *Verticillium wilt*. Les gènes de *Phytogb1* sont également surexprimés lors de différents stress tels que l'hypoxie et les stress osmotiques et salins (Trevaskis *et al.*, 1997; Lira-Ruan *et al.*, 2001; Zhao *et al.*, 2008). Les *Phytogb1* sont aussi surexprimées suite à différents traitements avec du nitrate, nitrite, NO, de l'acide salicylique ou jasmonique, de l'éthylène et de l'H₂O₂ (Wang *et al.*, 2000a; Sakamoto *et al.*, 2004; Ohwaki *et al.*, 2005; Qu *et al.*, 2006; Sasakura *et al.*, 2006).

Ces dernières années, il est apparu que les *Phytogb1* sont devenues des acteurs importants de la symbiose fixatrice d'azote. Des données obtenues suite à l'application de donneur de NO [S-nitroso-N-acétyl-D, L-pénicillamine (SNAP)] et de piègeur de NO (c-PTIO) sur les racines de *L. japonicus*, ont montré respectivement l'induction et la répression de *LjPgb1* (Shimoda *et al.*, 2005; Nagata *et al.*, 2008). Ces observations ont permis aux auteurs d'émettre l'hypothèse que pendant les premières étapes de l'interaction symbiotique une diminution de l'expression de *Phytogb1* entraîne une augmentation des niveaux de NO, qui revient par la suite à son niveau physiologique de base *via* la surexpression de *Phytogb1* (Murakami *et al.*, 2011).

Par ailleurs, la surexpression de *Phytogb1* induit une augmentation du nombre de nodosités par plante et améliore la fixation symbiotique de l'azote (Shimoda *et al.*, 2009). Ces différentes observations montrent le lien étroit qu'il existe entre le NO, les phytoglobines et la symbiose fixatrice d'azote.

Les Phytogb2

Chez *A. thaliana*, l'expression de *Phytogb2* est induite durant la maturation de la graine et durant l'embryogénèse (Hunt *et al.*, 2001). Les gènes de *Phytogb2* ne sont pas régulés de la même manière que ceux de *Phytogb1*. Ils sont inductibles par le froid ou les cytokines, mais ne sont pas modifiés en réponse à l'hypoxie, le stress osmotique ou le stress salin, ni en réponse à la plupart des traitements qui modifient l'expression des *Phytogb1* (Trevaskis *et al.*, 1997 ; Hunt *et al.*, 2001).

Les leghémoglobines

Les leghémoglobines (Lbs) constituent les hémoglobines les plus abondantes présentes dans les nodules des légumineuses et jouent un rôle important dans la symbiose fixatrice d'azote (Gupta *et al.*, 2011b), avec 2 fonctions principales : i) la réduction du taux d'O₂ libre pour obtenir une concentration de l'ordre du nanomolaire et ii) le transport de l'O₂ vers les mitochondries et les bactéroïdes (Singh & Varma, 2017). Les Lbs jouent un rôle dans le maintien d'un environnement microaérobie approprié pour permettre la respiration et la production d'énergie par l'O₂ sans inactivation de la nitrogénase (Appleby, 1984; Ott *et al.*, 2005). La fonction de ces protéines dans le métabolisme du NO est limitée dans des conditions de faible teneur en O₂ en raison de la différence significative entre l'affinité vis-à-vis de l'O₂ et du NO par rapport aux PhytoGb1 (Berger *et al.*, 2018). Cependant, les Lb peuvent tout de même contribuer à la récupération du NO sous forme d'un complexe LbNO (Mathieu *et al.*, 1998). Le fonctionnement des PhytoGb1 et des Lb au sein de la nodosité permet un ajustement précis des concentrations d'O₂ et de NO dans ce tissu (Berger *et al.*, 2018).

Les phytoGb3

Les PhytoGb3 n'ont été découvertes que récemment chez les plantes (Watts *et al.*, 2001) et le nombre d'études analysant leur rôle fonctionnel est très limité. Il a été démontré que les gènes de *PhytoGb3* ne sont pas induits en réponse à l'hypoxie ou suite à un traitement avec des phytohormones (Watts *et al.*, 2001). Chez *M.truncatula*, les deux gènes de *PhytoGb3* n'ont pas le même profil d'expression et de localisation. *PhytoGb3.1* est exprimé dans les cellules infectées de la zone de fixation d'azote, alors que *PhytoGb3.2* est principalement exprimé dans le tissu vasculaire nodulaire (Vieweg *et al.*, 2005). De plus, une induction des gènes de *PhytoGb3* chez *M. truncatula* a été observée lors de l'interaction symbiotique dans les nodules racinaires et dans les racines colonisées par des champignons mycorhiziens (Vieweg *et al.*, 2005). Il a par ailleurs été constaté que l'induction des gènes de *PhytoGb3* ne se limite pas qu'aux endosymbiotes, mais qu'une induction transitoire (5 hpi et 2 dpi) est observée après interaction avec les champignons ectomycorhiziens *Leccinum populinum* et *Xerocomus subtomentosus* (Jokipii *et al.*, 2008).

*Maman disait toujours, « la vie, c'est comme une boîte de chocolats :
on ne sait jamais sur quoi on va tomber. »*
Forrest Gump

Matériels & Méthodes

1. Matériels biologiques et conditions de culture

1.1. Matériel végétal

Les graines de luzerne tronquée (*M. truncatula cv Jemalong A17*) ont été utilisées pour toutes les expériences selon deux types de cultures :

- des cultures *in vitro* sur milieu gélosé, utilisées principalement pour l'analyse des premières étapes de la nodulation (de l'inoculation jusqu'à 12 jours post inoculation, jpi).
- des cultures *in vivo* en pot avec un mélange perlite/vermiculite comme support, utilisées dans les expériences nécessitant d'aller jusqu'à 8 semaines post-inoculation (spi).

1.1.1. Stérilisation et germination

Les graines sont scarifiées dans l'acide sulfurique 12 M pendant 4 min. Elles sont ensuite rincées à l'eau distillée et stérilisées dans une solution de NaOCl à 6 % de chlore actif pendant 3 min. Les graines sont ensuite rincées, puis imbibées pendant 3 h dans de l'eau, et finalement déposées sur boîte de Pétri (100 graines/boîte) contenant de l'agar 0,4 % (p/v), et gardées à l'abri de la lumière. Après 2 jours à 4 °C, les graines sont transférées pendant 2 jours à 16 °C.

1.1.2. Milieu et conditions de culture *in vitro*

Après germination, les plantules sont transférées sur boîte de Pétri carrée (10 plantes/boîte) contenant du milieu Fahræus (Tableau. M1) gélosé (agar 1.3% p/v). Le tiers supérieur de la boîte est libre de milieu, ce qui permet la bonne croissance des parties aériennes, tandis que la partie inférieure est protégée de la lumière avec du papier aluminium afin que le système racinaire se développe à l'obscurité.

Tableau M.1 : Composition du milieu Fahræus

	Concentration
MACROELEMENTS	
magnésium sulfate (MgSO ₄)	0,5 mM
Citrate de fer	20 µM
Phosphate de potassium (KH ₂ PO ₄)	0,7 mM
Hydrogénophosphate de sodium (Na ₂ HPO ₄)	0,8 mM
Chlorure de calcium (CaCl ₂)	0,5 mM
Nitrate de potassium (KNO ₃)	0 / 0,2 / 2 mM
MICROELEMENTS	
Sulfate de manganèse (MnSO ₄)	100 µg/l
Sulfate de zinc (ZnSO ₄ , 7 H ₂ O)	100 µg/l
Acide borique (H ₃ BO ₃)	100 µg/l
Sulfate de cuivre (CuSO ₄ , 5 H ₂ O)	100 µg/l
Molybdate de sodium (Na ₂ MoO ₄ , 2 H ₂ O)	100 µg/l

Les boîtes sont placées dans une chambre de culture climatisée dont l'éclairage est assuré par des tubes fluorescents assurant une énergie lumineuse de 180 - 240 $\mu\text{mol.m}^2.\text{s}^{-1}$. La photopériode est de 16 h. La température est de 23 °C pendant l'héméroperiode et de 20 °C pendant la nyctipériode. L'humidité relative est de 60 %.

1.1.3. Milieu et conditions de culture *in vivo*

Pour permettre la culture des plantes pendant 8 à 10 semaines, les plantules germées sont transférées dans des pots de 2 litres (\varnothing :14,8 cm ; H :17,4 cm) contenant un mélange vermiculite et perlite (2:1, v/v). Les pots sont placés dans une chambre de culture aux caractéristiques identiques à celles décrites précédemment. Le milieu de culture est arrosé avant la germination avec du milieu nutritif (Tableau M.2) et les pots sont recouvert d'un film plastique transparent pendant 5 jours afin d'éviter la dessiccation. Par la suite, pour maintenir un bon niveau d'hydratation les pots sont arrosées tous les 2 – 3 jours avec une alternance (1/2) de milieu nutritif et d'eau distillée complémentée avec 0,2 mM de KNO_3 .

Tableau M.2 : Composition du milieu nutritif

	Concentration
MACROELEMENTS	
magnésium sulfate (MgSO_4)	200 mg/l
EDTA-Fe-Na	25 mg/l
potassium dihydrogen phosphate	200 mg/l
potassium chloride (KCl)	200 mg/l
calcium sulfate(CaSO_4)	125 mg/l
potassium nitrate (KNO_3)	0,2 mM
MICROELEMENTS	
manganese sulfate (MnSO_4)	100 $\mu\text{g/l}$
zinc sulfate ($\text{ZnSO}_4, 7 \text{H}_2\text{O}$)	1 mg/l
boric acid (H_3BO_3)	1 mg/l
copper sulfate ($\text{CuSO}_4, 5 \text{H}_2\text{O}$)	30 $\mu\text{g/l}$
sodium molybdate ($\text{Na}_2\text{MoO}_4, 2 \text{H}_2\text{O}$)	5 mg/l
aluminium chloride ($\text{AlCl}_3, 6 \text{H}_2\text{O}$)	54 $\mu\text{g/l}$
nickel chloride ($\text{NiCl}_2, 6 \text{H}_2\text{O}$)	30 $\mu\text{g/l}$
potassium iodure (KI)	10 $\mu\text{g/l}$

1.2. Souches bactériennes et plasmides

Les caractéristiques des souches et plasmides utilisés sont décrites dans le tableau ci-dessous :

Tableau M.3 : Souches et plasmides utilisés au cours de ce travail

Souches/plasmide	Caractéristiques	Références
<i>S. meliloti</i>		
Sm 2011	Sm ^R ,	Rosenberg <i>et al.</i> , 1981
<i>E. coli</i>		
DH5α	F-supE44ΔlacU169 (Ø80lacZΔM15) HsdR17 (rK-mK+)recA1	Sambrook <i>et al.</i> 1989
<i>A. rhizogenes</i>		
Arqua1	Sm ^R , dérivé de la souche R1000	Quandt <i>et al.</i> , 1993
Plasmides		
pDONR207	Gen ^R , vecteur donneur Gateway	Invitrogen
pK7WG2D	Sm ^R ,Sp ^R , vecteur d'expression sous promoteur 35s et exprimant la GFP	Invitrogen
pK7GWIW	Sm ^R ,Sp ^R , vecteur d'expression sous promoteur 35s pour construction RNAi et exprimant la GFP	Karimi <i>et al.</i> , 2002
pK43rollDGFP	Sp ^R , vecteur de destination pour les clonages à 3 entrées	Karimi <i>et al.</i> , 2002
pENTR-T35S	Km ^R , vecteur comportant le terminal 35S	Karimi <i>et al.</i> , 2002
pDONR P4-P1r-NCR001	Km ^R , vecteur comportant le promoteur NCR001	Horchani <i>et al.</i> , 2011
pK7WG2D-nshb1/trhb1	Sp ^R , vecteur sur-exprimant nshb1 ou trhb1 sous promoteur 35s	Ce travail
pK7GWIWG2D-nshb1/trhb1	Sp ^R , vecteur sous-exprimant nshb1 ou trhb1	Ce travail
pk43NCR001-nshb1/trhb1	Sp ^R , vecteur sur-exprimant nshb1 ou trhb1 sous promoteur NCR001	Ce travail

1.2.1. Milieux bactériens et antibiotiques

La souche d'*E. coli* DH5α est cultivée, à 37 °C, en milieu riche Luria-Bertani, LB (Miller, 1972). Lorsque cela est nécessaire, les antibiotiques sont ajoutés aux concentrations suivantes : kanamycine 30 µg/ml, streptomycine 100 µg/ml, ou spectinomycine 50 µg/ml.

La souche de *S. meliloti* Sm 2011 est cultivée pendant 2 jours à 30°C sur un milieu LB supplémenté avec 2,5 mM CaCl₂ et MgSO₄.

La culture de la souche d'*A. rhizogenes* se fait sur un milieu riche Tryptone-Yeast extract, TY (5 g/l de bacto-tryptone, 3 g/l yeast-extract supplémenté de 6 mM de CaCl₂) pendant 48 h à 28 °C.

2. Techniques de transformations pour l'obtention de racines transgéniques

2.1. Transformation des racines par la méthode de Boisson-Dernier *et al.*, 2001

Les racines de *M. truncatula* sont transformées par *A. rhizogenes* (Arqua1) selon le protocole décrit par Boisson-Dernier *et al.* (2001). Cette technique nécessite 25 jours pour obtenir des plantes « composites » (seule les racines sont transformées) prêtes à être inoculées.

Après germination, les racines de 1,5 – 2 cm sont coupées à 3 mm environ de l'apex. La surface sectionnée de la plantule est alors recouverte d'*A. rhizogenes* provenant d'une culture fraîche de 48 heures. Les plantules sont ensuite transférées sur boîte de Pétri (10 plantes/boîte) contenant du milieu Fahræus gélosé (agar 1,3 % p/v) supplémenté avec 1 mM de nitrate d'ammonium (NH₄NO₃) et 20 µg/ml de kanamycine pour permettre une première sélection. Les boîtes sont placées en chambre climatique à 20 °C avec une photopériode de 16 heures pendant 7 jours, puis à 25 °C dans les chambres climatiques décrites précédemment. Cette croissance en 2 étapes permet dans un premier temps d'optimiser l'agro-infection des plantes, puis dans un deuxième temps d'assurer la bonne croissance de la plante.

Après les 7 jours de croissance à 25°C, les plantules sont sélectionnées à l'aide de la détection de la GFP sous microscope (Leiza MZ FL Fluo), les racines non transformées sont coupées au scalpel pour ne garder que celles fluorescentes. Les plantes transformées sont alors transférées sur boîtes de Pétri (6 plantes/boîte) contenant du milieu Fahræus gélosé (agar 1,3 % p/v) avec 0,2 mM de KNO₃ et placées en chambre climatique à 25 °C.

2.2. Transformation des racines par pique

Cette méthode de transformation permet d'étudier les nodules matures de plantes composites. La transformation des racines se fait selon le protocole décrit par Vieweg *et al.*, (2004) et permet d'obtenir des plantes transformées et prêtes à être inoculées au bout de 46 jours.

Les graines sont scarifiées et mises à germer 24 h, à 24 °C, puis placées 3 jours à 4 °C pour permettre un épaissement de l'hypocotyle. L'inoculum d'*A. rhizogenes* est préparé à partir d'une culture sur milieu solide de 2 jours. Les colonies sont récupérées et diluées dans 1 ml de tampon peptone/sucrose, PS (sucrose 10 g/l, bactopectone 10 g/l, pH 7,4). Les racines de *M. truncatula* sont inoculées par trois injections de l'inoculum au niveau de l'hypocotyle. Les plantes sont mises en culture dans des pots (l : 8/ L : 8/ H : 8 cm) contenant un mélange sable B5 et perlite (2:1, v/v) et couvert d'un film plastique pendant 7 jours pour éviter la dessiccation. Les pots sont placés en chambre de culture à 20 °C avec une photopériode de 16 h. Après 7 jours, les pots sont transférés à 25 °C et au bout de 3 semaines les plantes transformées

sont sélectionnées par détection de la GFP et transplantées dans un pot plus grand (1 : 9/ L : 9/ H 9,5 cm) contenant le même milieu de culture que précédemment.

3. Inoculation avec *S. meliloti*

Les plantules sont inoculées avec une suspension de *S. meliloti*. L'inoculum est préparé à partir d'une boîte de *S. meliloti* dont les colonies sont récupérées, re-suspendues et lavées dans 50 ml d'eau stérile, puis centrifugées (2200 g, 5 min). Le culot de bactéries est ensuite re-suspendu dans 30 ml d'eau stérile. A partir de cette solution bactérienne, un inoculum est préparé à une DO_{600nm} de 0,01. Les plantes cultivées en condition *in vitro* sont inoculées en déposant 200 µl de l'inoculum sur chaque racine, et en condition *in vivo* chaque pot arrosé avec 20ml d'inoculum.

4. Techniques de physiologie et de biochimie

4.1. Analyses phénotypiques

Les nodosités sont dénombrées à 14 jpi, puis déposées sur un milieu contenant de l'agar 1% (p/v) pour être photographiées. La surface et la taille des nodosités sont estimées avec le logiciel ImageJ (<https://imagej.nih.gov>).

4.2. Mesure de la production de NO

La détection du NO est effectuée grâce à une sonde fluorescente, la 4,5-diaminofluoresceine (DAF-2 $\lambda_{ex} = 485 \text{ nm}$; $\lambda_{em} = 520 \text{ nm}$), selon le protocole décrit par Horchani *et al.*, (2011). Le matériel végétal (racines, nodosités, racines + nodosités) est pesé et incubé dans un tube Eppendorf de 2 ml contenant 1 ml de milieu de détection (10 mM Tris-KCL, pH 7.4 et 10 µM de DAF-2 ajoutée extemporanément). La production de NO est mesurée à l'aide d'un spectrofluorimètre (Xenius, SAFAS, Monaco) toutes les heures pendant 4 h après addition de la sonde. La production de NO est ensuite normalisée avec le poids de l'échantillon.

4.2.1. Utilisation d'effecteurs

4.2.1.1. Inhibiteurs de la production de NO

Les inhibiteurs de la production de NO sont utilisés dans les conditions décrites par Horchani *et al.*, (2011). Les inhibiteurs utilisés sont : le tungstate (Tg) comme inhibiteur des nitrate réductase, à 1mM, l'allopurinol comme inhibiteur de la xanthine oxydo-réductase, à 300 µM, le propyl gallate comme inhibiteur de l'alternative oxydase, à 300 µM, et le cyanure de potassium (KCN) comme contrôle négatif, à 300µM.

4.2.1.2. Donneurs de NO

Pour les analyses à 4 jours post-inoculation, les donneurs de NO ou leurs contrôles respectifs sont ajoutés toutes les 24 h sur les racines (200µl/racines) des plantes cultivées en boîte de Pétri.

Tableau M.4 : Donneurs de NO utilisés

Donneur	Contrôle	Temps de ½ vie (T _{1/2}) du donneur	Forme du NO libérée	T _{1/2} de la forme libérée	Concentration final
Sodium nitroprussiate (SNP)	Ferricyanure	1-4 min	NO ⁺	3.10 ⁻¹⁰ s	0,1 mM
S-nitrosogluthation (GSNO)	Glutathion (GSH)	101 min (25°C)	NO ⁺	3.10 ⁻¹⁰ s	0,5 mM
Diethylamine nonoate (DEA-NONOate)	Diethylamine	16 min (22/25°C)	NO•	~ s	0,5 mM
Spermine nonoate (SP-NONOate)	Spermine	230 min (22/25°C)	NO•	~ s	0,5 mM

4.3. Mesure de l'activité Nitrate Réductase

Les échantillons tissulaires sont broyés au mortier et au pilon dans l'azote liquide. Les protéines totales sont extraites à partir de 100 mg de poudre en utilisant le tampon d'extraction suivant : Tris HCl pH 8,5 25 mM, EDTA 1 mM, FAD 20 µM, Triton 0,04 % (v/v), NaMO₄ 10 µM, DTT 1 mM, E64 20 µM, PMSF 2 mM. Les extraits sont centrifugés (15000 g, 15 min) et le surnageant est récupéré pour mesurer l'activité enzymatique. L'extrait enzymatique contient 0,2 M d'HEPES pH 7, 15 mM KNO₃ et 250 µM de NADH (Miranda *et al.*, 2001). Un extrait enzymatique bouilli est utilisé comme contrôle négatif. La réaction est stoppée après 30 min en bouillant les échantillons 3 min à 100°C. Le nitrite produit est mesuré en utilisant le réactif de Griess (1% (p/v) sulphanilamide dans 1 M HCl et 0,01% (p/v) NEDD [N-1-naphtylethylenediamine dihydrochloride] dans l'eau) et mesuré à 540 nm. Les protéines des échantillons sont dosées selon la méthode de Bradford (1976). L'activité NR est mesurée en quantifiant le NO₂⁻ produit à partir du NO₃⁻ apporté au sein du mélange réactionnel.

4.4. Mesure de réduction de l'acétylène

L'analyse de réduction de l'acétylène (C₂H₂) en éthylène (C₂H₄) permet une mesure indirecte de la capacité de fixation de l'azote de la nitrogénase (Halliday & Pate, 1976).

L'activité réductrice d'acétylène (ARA) est donc estimée en mesurant la proportion de ces deux gaz.

L'ARA est mesurée à l'aide de pilulier hermétique contenant 3 systèmes racinaires de plantes. Dix pourcents de l'air présent dans les piluliers sont remplacés par 10 % d'acétylène pure et les piluliers sont incubés 30 min à 28 °C. Un prélèvement de 0,2 ml de gaz présent dans le pilulier est réalisé grâce à une seringue hypodermique. L'analyse des gaz se fait par chromatographie gazeuse (Agilent Technologie, 6890N) équipé d'une colonne GS-Alumina avec N₂ comme gaz vecteur et une détection par ionisation de flamme.

Les mesures d'ARA sont ensuite normalisées soit par le nombre de nodosités, soit par la masse fraîche.

5. Techniques de biologie moléculaire et génétique

5.1. Analyses bio-informatique

5.1.1. Analyses de séquences

Les comparaisons de séquences sont réalisées avec le programme BLAST de la plateforme NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), et les alignements multiples de séquences sont réalisés à l'aide du programme MUSCLE de la plateforme European Bioinformatic Institute (<https://www.ebi.ac.uk/Tools/msa/muscle/>). La recherche de motifs consensus au sein de plusieurs séquences est réalisée avec la suite MEME (<http://meme-suite.org/>).

5.1.2. Analyses phylogénétiques

Les données de phylogénie ont été obtenues en utilisant le mode « one click » du site <http://www.phylogeny.fr> (Dereeper *et al.*, 2008). Il inclut un alignement de séquences avec les programmes MUSCLE et Gblocks qui permettent de retirer les portions de séquences mal alignées et très peu conservées. La reconstruction phylogénétique se fait grâce au programme PhyML en utilisant la méthode du maximum de vraisemblance. Les séquences de *M. truncatula* utilisées ainsi que celles de *L. japonicus*, *A. thaliana* et *G. max* sont listées en annexe (A1). Les nœuds possédant une robustesse inférieure à 80 % sont regroupés dans un même sous-groupe phylogénétique.

5.2. Technique d'extraction, de purification et d'analyses d'ARN

Les ARNs présents dans le matériel végétal (racines, nodosités et racines + nodosités) sont extraits en utilisant le RNazol®RT (Euromedex) selon les recommandations du fabricant.

Le matériel végétal est broyé à l'azote liquide avec pilon et mortier préalablement nettoyés avec une solution de 0,1 N NaOH et 0,1 % (p/v) SDS et rincés à l'eau milliQ. Une quantité de 100 mg de poudre est prélevée et mise en contact avec 1 ml de RNAzol®RT et 400 µl d'eau DEPC (diethylpyrocarbonate). L'ensemble est mélangé 15 s, incubé à température ambiante pendant 15 min puis centrifugé (12000 g, 15 min, 4 °C) pour éliminer les débris. Le surnageant est récupéré, puis traité pendant 10 min avec 500 µl d'isopropanol (précipitation des ARNs) avant centrifugation (12000 g, 10 min, 4 °C). Le surnageant est prélevé et le culot d'ARNs est lavé 2 fois avec 500 µl (centrifugation à 12000 g, 5 min, 4 °C) et 400 µl (centrifugation à 12000 g, 10 min, 4 °C) d'éthanol à 70%. L'éthanol est ensuite éliminé par évaporation sous hotte pendant 10 min. Le culot d'ARNs est solubilisé dans 30 µl d'eau stérile exempte de nucléase (« nuclease free »).

La concentration des ARNs est analysée par spectrophotométrie à l'aide du Nanodrop (Thermo Scientific). L'intégralité des ARNs est vérifiée par électrophorèse sur un gel d'agarose à 1,2%.

5.3. Production d'ADN complémentaire par transcription inverse

Les ADNc sont synthétisés à partir de 1 µg d'ARN. Un traitement DNase est préalablement réalisé avec la RQ1 RNase-Free DNase (Promega) dans un volume final de 10 µl. La réaction est réalisée à 37 °C, pendant 30 min, puis stoppée avec l'ajout de 1 µl de solution RQ1 DNase STOP et une incubation à 75 °C pendant 10 min.

La synthèse des ADNc se fait avec le système GoScript™ Reverse Transcription (Promega). Les ARNs sont incubés avec les amorces dégénérées et les oligodt à 70 °C pendant 5 min puis à 4 °C pendant 5 min. Le mélange réactionnel contenant la transcriptase inverse est ajouté à la solution d'ARNs dans un volume final de 20 µl. La réaction se déroule en 40 cycles composés de 3 étapes : l'hybridation à 25 °C pendant 5 min, l'élongation à 42 °C pendant 1 h, et l'inactivation à 70 °C pendant 15 min.

Tableau M.5 : Composition du milieu réactionnel pour la réaction de Transcription inverse

Composants	Concentration finale
GoScript™ 5x reaction buffer	1 X
PCR nucleotide mix	0.5 mM each dNTP
Recombinant RNAsin®ribonuclease inhibitor	20 u
GoScript™ reverse transcriptase	1 µl
Nuclease free water	qsp 8 µl

L'absence de contamination par de l'ADN génomique au sein des ADNc est évaluée par PCR en utilisant des amorces entourant un intron du gène codant pour la glutathion synthétase (GSHS) (Frendo *et al.*, 2001). La contamination par l'ADNg est alors visible avec l'apparition d'une bande à 500 bp en plus de la bande à 300 bp qui correspond à l'ADNc.

5.4. Technique d'analyse de l'accumulation des transcrits par PCR quantitative en temps réel

Les PCR quantitatives (qPCR) sont réalisées à l'aide du kit GoTaq® qPCR Master Mix (Promega) et du thermocycleur AriaMx Real-Time PCR System (Agilent). La réaction est réalisée dans un volume de 15 µl selon la composition suivante :

Tableau M.6 : Composition du milieu réactionnel pour la réaction de qPCR

Composants	Concentration finale
GoTaq® qPCR Master Mix, 2X	1 X
CXR	0,15 µL
Amorces sens et anti-sens (10 µM)	0,3 µM
ADNc (1/40°)	5 µl
Nuclease free water	qsp 10 µl

L'amplification PCR se fait selon le cycle suivant : une première étape de dénaturation (95°C, 3 min), 40 cycles composés d'une étape de dénaturation (95 °C, 3 sec) et d'une étape d'hybridation/élongation (60 °C, 30 sec). La courbe de fusion est déterminée en mesurant la fluorescence tous les 0,5 °C de 65 °C à 95 °C et permet de vérifier la bonne qualité des amplifications. Toutes les expériences sont réalisées sur plaque 96 puits en triplicatas techniques et biologiques. Chaque plaque comporte 2 gènes de références (Mtc27 et Mta38) qui permettent de normaliser l'expression des gènes cibles étudiés. Les données sont recueillies à l'aide du logiciel AriaMX (Agilent) et sont analysées statistiquement avec le logiciel R et le package RqPCRBase fournie par Hiliou & Tran, (2006).

5.5. PCR et techniques de clonage

5.5.1. Amplification par PCR à partir des ADNc

Les séquences d'intérêt sont amplifiées à partir des ADNc de *M. truncatula* avec les amorces appropriées en utilisant une ADN polymérase haute-fidélité à correction d'épreuve (Phusion, NEB). La réaction est réalisée selon les recommandations du fabricant. Le produit de PCR final est purifié avec le système Wizard® SV Gel and PCR clean (Promega) selon les recommandations du fabricant.

Tableau M.7 : Composition du milieu réactionnel pour la réaction de PCR

Composants	Concentration finale
5X Phusion HF Buffer	1 X
10 mM dNTP	200 μ M each
Amorces sens et anti-sens (10 μ M)	0,5 μ M
ADNc (1/10 ⁹)	5 μ l
Phusion DNA polymerase	0.02 u/ μ l
Nuclease free water	qsp 50 μ l

5.5.2. Technique de clonage Gateway et transformation

La technologie Gateway est une technique de clonage universelle qui utilise les propriétés de recombinaison site-spécifique du bactériophage lambda (Landy, 1989). Ceci permet de déplacer et insérer rapidement avec une haute efficacité une séquence d'ADN dans de multiples vecteurs d'expression.

5.5.2.1. Réaction de recombinaison de type « BP »

La première étape nécessite d'ajouter par PCR les 2 sites de recombinaisons spécifiques *attB1* en amont et *attB2* en aval de la séquence d'intérêt. Les amplicons de PCR, flanqués des sites *attB*, sont alors clonés dans le vecteur donneur pDONR207 (Invitrogen) qui possède les sites *attP1* et *attP2* par réaction de BP clonase. Le mélange réactionnel (2 μ l de pDONR207 à 50 ng/ μ l, 1,5 μ l d'insert, 0,5 μ l de TE buffer, 1 μ l de BP clonase) est incubé toute une nuit à 25 °C, puis la réaction est stoppée avec l'ajout de 1 μ l de protéinase K, à 37 °C, pendant 10 min. Il se forme alors par cette réaction un *vecteur d'entrée*. Celui-ci possède le gène de résistance à la kanamycine (Km^R), les sites de recombinaison homologue *attL1* et *attL2*, et le gène d'intérêt.

5.5.2.2. Transformation d'*E. coli* par électroporation

Une fois la réaction de recombinaison de type « BP » terminée, 1 μ l du produit de recombinaison est ajouté à 75 μ l de bactéries compétentes DH5 α . Le mélange est placé dans une cuve d'électroporation de 0,2 cm et un choc électrique de 2520 V pendant 2,10 ms est délivré (Micropulser, Biorad). Le mélange est ensuite transféré dans 925 μ l de milieu de culture LB supplémenté avec 6 mM de CaCl₂. Après 1 h d'incubation à 37 °C, la culture est étalée sur boîte de Pétri contenant du milieu LB avec l'antibiotique approprié.

5.5.2.3. Purification des plasmides

Les colonies bactériennes transformées possédant la résistance à l'antibiotique sont récupérées et mis en culture liquide de 5 ml de LB contenant l'antibiotique approprié à 37°C. Après une

nuit de culture, les plasmides sont extraits et purifiés à l'aide du système Isolate II Plasmide Mini Kit (Bioline) selon les recommandations du fabricant.

5.5.2.4. Réaction de recombinaison de type « LR »

La réaction de recombinaison de type « LR » permet de transférer la séquence intérêt du vecteur d'entrée vers le vecteur final d'expression. Dans le cas des sur-expression et sous-expression sous contrôle du promoteur 35s

La réaction est dite « simple » quand elle consiste au transfert d'une séquence unique du vecteur d'entrée au plasmide de destination ;et « multiple » si le mélange réactionnel comporte trois vecteurs d'entrées pour le transfert de trois séquences (promoteur NCR001-séquence d'intérêt-terminateur 35S) vers un vecteur de destination. Le mélange réactionnel (Tab M.8) est incubé de la même manière que la réaction de type « BP » avec l'enzyme LR clonase II ou LR clonase II plus selon que la réaction recombinaison soit « simple » ou « multiple » respectivement.

Tableau M.8 : Composition des mélanges réactionnels pour les réactions de LR clonase

Réaction LR simple		Réaction LR multiple	
Composants	Concentration finale	Composants	Concentration finale
Vecteur d'entrée	5 fmol/μl	Vecteur d'entrée L4-L1	5 fmol/μl
Vecteur de destination	10 fmol/μl	Vecteur d'entrée L1-L2	5 fmol/μl
LR clonase	1 μl	Vecteur d'entrée L2-L3	5 fmol/μl
		Vecteur de destination	10 fmol/μl
		lr clonase II plus	1 μl

Des bactéries DH5α sont ensuite transformées par électroporation avec les produits de recombinaison, puis les plasmides récupérés sont extraits et purifiés comme décrit précédemment.

La vérification des plasmides est réalisée par PCR avec les amorces appropriées et par séquençage (Genewiz corporation, Takeley, United Kingdom)

5.6. Transformation d'Agrobactéries par la méthode de congélation/décongélation

La transformation d'*A. rhizogenes* est réalisée selon le protocole établi par Wise *et al.*, 2006. Une préculture d'*A. rhizogenes* (Arqua1) est réalisée dans 5 ml de milieu TY contenant de la streptomycine, incubée tout une nuit sous agitation à 200 rpm et à 28 °C. Une fois que la densité optique à 600 nm (DO600) de la culture est comprise entre 0,5 et 1, celle-ci est refroidie

sur glace (15 s), puis centrifugée (1200 g, 6 min, 4 °C). Le culot de bactérie est resuspendu dans 1 ml de CaCl₂ (20 mM) à 4°C et réparti par aliquote de 100 µl dans des tubes Eppendorf. Un tube permet de réaliser une transformation. La transformation est réalisée en ajoutant 1 µg de plasmide, et le mélange bactérie/plasmide est ensuite congelé dans l'azote liquide, puis décongelé 4 min à 37 °C. Les bactéries sont ensuite transférées dans 900 µl de milieu de culture TY et incubées 4 h sous agitation à 28 °C, puis étalées sur boîte de culture TY contenant l'antibiotique approprié et incubées 48 h à 28 °C.

6. Détection histochimique de l'activité β-glucuronidase (GUS)

Une fois collectées, les tissus (racines ou nodosités) sont fixés pendant 1 h dans l'acétone à 90 % (v/v) à -20°C. Puis, une fois rincés avec un tampon phosphate (100 mM, pH 7), les tissus sont mis en présence d'une solution de coloration composée de 0,5 mg/ml de X-gluc ; 0,5 mM de ferricyanide de potassium ; 0,5mM de ferrocyanide de potassium) préparée dans du tampon phosphate 1 mM à pH 7,4. Les échantillons sont alors mis à incuber à 37°C à l'obscurité sur un temps plus ou moins important en fonction de la force du promoteur d'intérêt. Après arrêt de la réaction par rinçage avec un tampon phosphate (100 mM, pH 7), les échantillons sont préparés pour être observés au microscope optique Axio Plan II (Zeiss).

7. Analyses statistiques

Les analyses statistiques ont été réalisées en utilisant les logiciels XLSTAT et R.

8. Liste des amorces utilisées

La page suivante contient les tableaux des amorces utilisées en qPCR et lors des clonages.

Tableau M.9 : Liste des amorces utilisées en qPCR

Genes	Code gene	Description	Amorces sequences 5'-3'	Efficacité	Références
Mtc27	Medtr2g436620	housekeeping gene	F : GTGGGAGGTTGAGGGAAAAGT; R : TTGAAGGTCCTTGAGCTTGC	97%	del Guidice et al., 2011
a38	Medtr4g109650	housekeeping gene	F : TCGTGGTGGTGGTTATCAAA ; R : TTCAGACCTTCCCATGACA	92.50%	del Guidice et al., 2011
ENOD20	Medtr8g145270	early noduline	F : TCAACTCCAATTCCTCATCC; R : AATCTGAAGGTGACGGTG	95%	del Guidice et al., 2011
CRE1	Medtr8g106150	cytokinin receptor histidine kinase	F : CTCTGCCATCCTTGTTCAA; R : GTGCATAGGCCACTCCACTAA	91%	del Guidice et al., 2011
phytogb1.2	Medtr4g068870	no symbiotic hemoglobin	F : GGACAATGCCAATTTGATAAGCAG ; R : CTGGTGGAGCAATCTCAAGG	90%	Ce travail
phytogb1.3	Medtr0026s0210	no symbiotic hemoglobin	F : TTCTCATGACATGTGAATCAGC; R : GTGACCACATTTCAAGTAATGC	102%	Ce travail
lb 3	Medtr1g090810	leghemoglobine	F : GGACAATGCCAATTTGATAAGCAG ; R : CTGGTGGAGCAATCTCAAGG	80%	Ce travail
lb 4	Medtr1g011540	leghemoglobine	F : GAGCGAAGAATTGAGCACTGCT ; R : TGCCTTCTTAATTGCAGTTGCC	88.50%	Ce travail
phytogb3.1	Medtr3g109420	truncated hemoglobin	F : GCTTCATCACACACACATAC ; R : AATCATGATCTATATCTGAAATGTT	83%	Ce travail
phytogb3.2	Medtr1g008700	truncated hemoglobin	F : AACTTTATAAGTTTTCTTTGTTTG ; R : GATAGACATATAGACGTTCAATCTT	84%	Ce travail
GS1	Medtr3g065250	glutamine synthetase isoforme 1	F : CTTGACCTCTCCGAAACCA; R : CTTGGGAAGCTGTGAAGGG	93%	Ce travail
CP6	Medtr4g079800	cysteine protease	F : CCTGCTGCTACTATTGCTGGATATG; R : CACTCGCATCAATGGCTACGG	97%	Pierre et al., 2014
nr1	Medtr3g073180	Nitrate reductase isoforme 1	F : GTTCAGTTTGAGTAAAGCC; R : ATACATACAGCGTCTACTC	91%	Horchani et al., 2011
nr2	Medtr5g089820	Nitrate reductase isoforme 2	F : CCACCTATGATTCATTTGCTG ; R : TCTATTACTTGCCTAGAACAC	97%	Horchani et al., 2011
nr3	Medtr3g073150	Nitrate reductase isoforme 3	F : GCATGGGATCTGGCTAATAACAC ; R : TTATTCTTAGGTCTGGGTCAGAG	93%	Boscari et al., 2013
nir	Medtr4g086020	Nitrite Reductase	F : AAATGGTAAGGCTACTGAAGG ; R : CTACAATAGGCACCAAGTCC	96%	Boscari et al., 2013
GST	Medtr7g065600	glutathion S-transferase	F : TTTGTTCACTAGTGAGAAATTTCC; R : GAAGACTTTCATAACGAGCTTTAA	115%	Boscari et al., 2013
CS	Medtr1g124600	chalcone synthase	F : AAAGA5TAAATCCACCAGAG; R : AAACACCAAACCTCAAGTCTC	120%	Boscari et al., 2013
ADH	Medtr3g089940	alcool déshydrogénase	F : GGGACTATGTTCTCAATCTGG; R : TAGGTACCAAATGTCACAGTCTC	100%	Rovere et al., 2019
PDC	Medtr2g015560	pyruvate decarboxylase	F : GCCCCGCTTAAGATCAAC; R : CCAAGTATTCCACTGCCT	98%	Rovere et al., 2019
B2.2	Medtr1g087920	ethylene response factor	F : TGCCACTAATAATGTTCAAGGA; R : TTCACAGAAGAAACCGAAGCA	107%	Rovere et al., 2019

Tableau M.10 : Liste des amorces utilisées pour les clonages

Nom	Description	Amorces sequences 5'-3'
attb-adaptateur	Complète les séquence attb1 et 2 qui flanque la séquence d'intérêt	F : GGGGACAAGTTTGTACAAAAAAGCAGGCT; R : GGACAATGATCCATGCTG
phytogb1.1-seq	amplifie la séquence pleine taille de phytoGb1.1	F : AAAAAAGCAGGCTTCCACATGGGCATTTGGATACAAAAG; R : AGAAAGCTGGGTGTTAAGAGGAAGGTTTCATTTTCAGAT
phytogb3.1-seq	amplifie la séquence pleine taille de phytoGb3.1	F : GTACAAAAAAGCAGGCTACCATGCGAATCTGCAAGAGAAAGGC; R : ACAAGAAAGCTGGGTCTAAAAGTTTTTACATGGATGCCTT
RNAi-phytoGb1.1	Amplifie 100 pg de phytoGb1.1 pour la construction RNAi	F : AAAAAAGCAGGCTTCCATGGGCATTTGGATACAAAAG; R : ACAAGAAAGCTGGGTTTAGTCTCAACTCAGCAGAA
RNAi-phytoGb3.1	Amplifie 100 pg de phytoGb3.1 pour la construction RNAi	F : GTACAAAAAAGCAGGCTTGGTGTGAGACAAATCATG; R : ACAAGAAAGCTGGGTGAAACCACTCCTCATGTC

Chapitre 1 :
Caractérisation des Nitrate Réductases de
M. truncatula, et rôle potentiel dans la
régulation du NO au cours de la symbiose

1. Contexte et objectif du travail : Characterization of *Medicago truncatula* Nitrate Reductase in relation with nitric oxide production during the nitrogen-fixing symbiosis

Dans le cadre de la symbiose entre *Medicago truncatula* et *Sinorhizobium meliloti*, une production spécifique de NO a été observée lors des premières étapes de la mise en place de l'interaction symbiotique, dans les premières cellules en division du cortex interne de la racine à l'origine de la nodosité (del Giudice *et al.*, 2011), ainsi que dans la nodosité mature. Des travaux du laboratoire, sur des racines transgéniques affectées sur le niveau de transcrits des deux principales NRs de la nodosité par la technique d'ARN interférence (ARNi *MtNR1/2*) indiquent un rôle des NRs dans la synthèse de NO via le nitrate dans les nodosités (Horchani *et al.*, 2011). De plus, les auteurs montrent que les NRs sont impliquées dans la régénération de l'énergie dans un environnement hypoxique comme c'est le cas dans la nodosité mature (Horchani *et al.*, 2011).

L'objectif de ce chapitre est donc de d'identifier les NRs présentes chez *Medicago truncatula* et d'étudier leur implication au cours de l'interaction symbiotique, notamment dans la synthèse de NO lors des premières étapes. Pour cela nous avons analysé l'expression de chaque NR ainsi que leur activité globale tout au long du processus symbiotique. Par la suite, l'implication des NR dans la régénération du statut énergétique de la nodosité a été étudiée par des approches de RMN et biochimique. Enfin, l'implication respective de NR1 et NR2 sur la production de NO dans la nodosité mature a été analysée par une approche génétique permettant la sous-expression soit de *NR1* soit de *NR2* ou des deux gènes.

2. Publication N°1

Characterization of *Medicago truncatula* Nitrate Reductase in relation with nitric oxide production during the nitrogen-fixing symbiosis

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Supporting information: .. Figures, .. Table.

Summary

- The nitrogen-fixing symbiosis between legumes and rhizobia leads to the formation of a new root organ called nodule. In legumes nodules, NO is produced throughout the whole symbiotic process as well as by both, the plant partner and the bacterial partner. In N₂-fixing symbiosis, plant nitrate reductases (NR) are the best-characterised source of NO production and are involved in “Phytob-NO” respiration. In this study, we surveyed the three NR genes present in *Medicago truncatula* genome, and address their expression, activity and involvement in NO production during the symbiosis between *M. truncatula* and *S. meliloti*. Then, we investigated the relationship between NR and hypoxia in mature nodule.
- By using pharmacological and genetic approaches, we determined gene expression, NR activity and NO production during the whole symbiotic process and specially during the first step of symbiotic interaction. Using a NMR spectrometric approach, we investigated the implication of NR on hypoxia state in mature nodule
- Our results reveal that *NR1* and *NR2* gene expression and activity are correlated with NO production during the symbiotic process. NR are involved together with the electron transfer chain of mitochondria in NO production during nodule development
- In mature nodule, NR was found to participate in the regeneration of energy state during hypoxia stress and NR1 is specially implicated in NO production in mature nodule.

Keywords: hypoxia Legumes, *Medicago truncatula*, nitric oxide, nitrogen-fixing symbiosis, nitrate reductase, nodules.

Introduction

Nitric oxide (NO) is a diatomic, radical and hydrophobic gas involved in biological processes (Torreilles, 2001). In animals, NO is known to be involved in many cellular functions such as regulation of blood pressure, immune response, neurotransmission, and cell differentiation (Hirst & Robson, 2011). In plants, many studies have reported the importance of NO as a key molecule in the regulation of developmental processes and in the response to either abiotic or biotic stress (Yu *et al.*, 2014; Corpas *et al.*, 2017). NO is known to be an important regulatory molecule in the growth and development process (Bethke *et al.*, 2004, 2006; Libourel *et al.*, 2006). It plays a role in root development or gravi-tropism by interfering with auxins (Hu *et al.*, 2005; Lanteri *et al.*, 2006b), and it was found to be involved in plant acclimation processes to salt stress, cold stress (Neill *et al.*, 2003) or heavy metals stress (Arasimowicz-Jelonek & Floryszak-Wieczorek, 2014). The role of NO in the response to biotic interactions was first identified in potato (Noritake *et al.*, 1996). It has also been shown that NO is capable of triggering the hypersensitive response and activating defense gene expression (Delledonne *et al.*, 1998; Durner *et al.*, 1998). NO production was then observed in different plant/pathogen interactions such as *A. thaliana*/*P. syringy* (Modolo *et al.*, 2005), *Pelargonium peltatum*/*Botrytis cinerea* (Floryszak-Wieczorek *et al.*, 2007) or *A. thaliana*/*A. euteiches* (Thalineau *et al.*, 2016). There is also evidence that NO plays a role in symbiotic interactions. Many studies have described the production of NO during the nitrogen-fixing symbiosis and emphasized its importance for the different stages of the interaction (Meilhoc *et al.*, 2011; Hichri *et al.*, 2015, 2016).

During the first hours after inoculation with the symbiotic partner, NO was observed in the roots of *L. japonicus* and *M. sativa* (Nagata *et al.*, 2008). NO production was also observed later during the infection process, all along the infection thread and in the dividing cells of the *M. truncatula* nodule primordium (del Giudice *et al.*, 2011). Similar results in *M. truncatula* showed the importance of NO production in the nodulation events (Pii *et al.*, 2007). Furthermore, transcriptomic analyses revealed that the expression of many genes was differentially regulated during the various stages of the interaction and that NO plays a role in this regulation (Ferrarini *et al.*, 2008; Boscari *et al.*, 2013a). In mature *M. truncatula* nodules, Baudouin *et al.* (2006) showed that NO accumulates particularly in the N₂-fixing zone. Both the plant and the bacteria partner are involved in NO production in the mature nodules (Meakin *et al.*, 2007; Sánchez *et al.*, 2010; Horchani *et al.*, 2011). In the bacteroid, the denitrification pathway is a main source of NO (Bedmar *et al.*, 2005). In *B. japonicum*, the denitrification

pathway depends on the *napEDABC* (Delgado *et al.*, 2003), *nirK* (Velasco *et al.*, 2001) *norCBQD* (Mesa *et al.*, 2002) and *nosRZDYFLX* (Velasco *et al.*, 2004) genes, encoding nitrate-, nitrite-, nitric oxide- and nitrous oxide reductase enzymes, respectively. In mature nodules, the denitrification pathway was shown to contribute for 30% (Horchani *et al.*, 2011) to over 90% (Meakin *et al.*, 2007; Sánchez *et al.*, 2010) of NO production in nodules. On the plant side, seven NO sources have been described (Gupta *et al.*, 2011a; Mur *et al.*, 2013b; Yu *et al.*, 2014). Evidence for the existence of both a reductive and an oxidative pathway for NO synthesis in plants was obtained (Gupta *et al.*, 2011a). The better characterized NO synthesis pathway in the plant partner during the nitrogen-fixing symbiosis is the pathway that implements the NR and the mitochondrial electron transport chain (ETC) (Hichri *et al.*, 2016). Plant NRs are located in the cytosol and in the plasma membrane, and catalyse reduction of nitrate to nitrite the first and rate-limiting step in nitrate assimilation (Campbell, 1999). Plant NRs have been shown to produce NO from nitrite using NADH as reducing power (Yamasaki & Sakihama, 2000; Rockel *et al.*, 2002). In the symbiotic association between legumes and rhizobia, the activity of NR has been observed in nodules of soybean (Streeter, 1982, 1985; Hunter, 1983; Heckmann & Drevon, 1987), in yellow lupin (Polcyn & Luciński, 2001) and in *M. truncatula* (Horchani *et al.*, 2011).

Using both pharmacological and genetic approaches, Horchani *et al.* (2011) addressed the role of NR in NO production in *M. truncatula*–*S. meliloti* mature nodules. NO production was thus found to be inhibited by tungstate (Tg), a NR inhibitor. In addition, nodules obtained with plant RNAi double knockdown on *NRI/2* exhibited reduced NR activities and NO production levels. The reduction of NO production was reversed by the addition of nitrite, both in the Tg-treated nodules and in *NRI/2* RNAi nodules, indicating that NO synthesis depends on NR activity, but that NR does not produce NO directly. The inhibition of NO production by ETC inhibitors indicated that mitochondrial ETC was the site of nitrite reduction into NO (Horchani *et al.*, 2011). Thus, in *M. truncatula* nodules, nitrate may be reduced into NO in a two-step mechanism involving successively NR and ETC. Moreover the energy status of the nodules depends either partly, or almost entirely, on NR functioning under normoxic, or hypoxic conditions, respectively (Horchani *et al.*, 2011). Indeed, NR is involved in the respiratory cycle named Phyto gb -NO respiration allowing the regeneration of ATP in nodule. The Phyto gb -NO respiration is divided into 4 steps including (1) NO_3^- reduction to NO_2^- by cytosolic NR, (2) NO_2^- transport from the cytosol to the mitochondrial matrix, (3) NO_2^- reduction to NO by the ETC, and (4) NO diffusion to the cytosol and oxidation to NO_3^- by Phyto gbs (Igamberdiev & Hill, 2004; Hichri *et al.*, 2016). Although many data are available on

the involvement of bacterial NR, few studies have been conducted on the involvement of plant NR during the symbiotic process.

In this work, we first identified the three different NR genes present in the *M. truncatula* genome. Then we addressed NR expression and activity from the first hours of symbiotic interaction up to eight weeks post-inoculation, at the onset of nodule senescence. Last, we investigated the impact of NR on NO production during the first step of the symbiotic interaction and the relationship between NR and hypoxia in mature nodule. Based on our data, we discussed the potential roles of NR on NO homeostasis during the symbiotic process.

Materials and methods

Plants growth and inoculation conditions

Medicago truncatula (cv Jemalong A17) were scarified, sterilized and germinated as in del Giudice *et al.*, (2011). Seedlings were cultivated and inoculated with *Sinorhizobium meliloti* 2011 strain either in Petri dishes as described in del Giudice *et al.*, (2011), or in planters as described in Horchani *et al.*, (2011). Cultures in Petri dishes were used for short-term experiments, between 0 and 14 days post-inoculation (dpi), while those in planters were used for long-term experiments between 0 and 8 weeks post-inoculation (wpi). Roots and/or nodules were harvested at various times of the kinetics. For short-term experiments, gene expression and NO production were analyzed in the 2 cm-long root segments around the inoculation zone, while for long-term experiments they were analyzed only in the nodules.

Construction of RNAi vector

Using *M. truncatula* cDNA as template, 432-bp and 441-bp fragments of NR1 (TC137636; Mtr.10604.1.S1_at) and NR2 (TC130773; Mtr.42446.1.S1_at) genes, respectively were obtained via polymerase chain reaction (PCR) with specific primers. PCR products were independently ligated into pGEM-T easy vector (Promega) and subsequently subcloned into pENTR4 vectors in BamHI - KpnI restriction sites for NR1 and EcoRI and KpnI restriction sites for NR2. The pENTR4 vector carrying the NR1 or the NR2 fragment was recombined with pK7GWIWG2R vector using the LR clonase enzyme mix (CatNo.11791-019, Invitrogen) to create the RNA interference expression vectors. Constructs were checked by sequencing, introduced by electroporation into *Agrobacterium rhizogenes* strain ARqua1, and used for *M. truncatula* root transformation as described by Boisson-Dernier *et al.* (2001).

Measurement of NO production

Detection of NO was performed using the 4,5-diaminofluorescein probe (DAF-2) (Horchani *et al.*, 2011). Segments of 2 cm roots or nodules were incubated in 1 ml of detection buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl) in the presence of 10 μ M DAF-2. The production of NO was measured with a spectrofluorimeter (Xenius, SAFAS, Monaco) every 30 min for 4 hours after addition of the probe. The production of NO was then normalized with the weight of the sample. Inhibitors are used under the conditions described by Horchani *et al.* (2011). The inhibitors are added to the reaction medium for the determination of NO at the concentration of; 1 mM for tungstate (Tg), allopurinol and propyl gallate and 300 μ M for potassium cyanide (KCN). The measurement of NO production is done after one hour of incubation.

Measurement of Nitrate Reductase Activity

Tissue samples are ground with mortar and pestle in liquid nitrogen. The total proteins are extracted from 100 mg of powder using the following extraction buffer: 25 mM Tris HCl pH 8.5, 1 mM EDTA, 20 μ M FAD, 0.04% Triton, 10 μ M NaMO₄, 1 mM DTT, E64 μ M, 2 mM PMSF. The extracts are centrifuged (15000 g, 15 min). The NR activity is measured by quantifying the NO₂ - produced in the reaction mixture containing: the 0.2 M enzyme extract of HEPES pH7, 15 mM KNO₃ and 250 μ M of NADH (Miranda *et al.*, 2001). The reaction is stopped after 30 min by boiling the samples for 3 min at 100 ° C. The nitrite produced is measured using Griess reagent (1% sulphanilamide in 1 M HCl and 0.01% NEDD [N-1-naphthylethylenediamine dihydrochloride] in water) and measured at 540 nm. Soluble proteins are assayed according to the method of Bradford, (1976).

RNA isolation, reverse transcription and genes expressions

RNAs were isolated from 100 mg of frozen material ground in liquid N₂ using the RNAzol following the manufacturer's recommendations (Sigma-Aldrich). RNA quality was checked and DNase treatment was carried out prior to the synthesis by GoScript reverse transcriptase (Promega) of the cDNAs. The RT-qPCR and data analysis was made as described in Berger *et al.* (2018). RT-qPCR analyses were carried out in triplicate, using the primers reported in Table 1. The reference value "1" was attributed to the first time when the Ct of the analysed gene was significantly detectable.

Phosphorus NMR

For each experiment, 0.9 to 1.1 g fresh weight of 3 wpi-old nodules (around 1400 to 1700 nodules) were harvested and incubated at ambient temperature in an aerated perfusion medium containing 1 mM KH_2PO_4 , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 mM K_2SO_4 , 0.5 mM CaCl_2 , 10 mM MES/KOH, pH 6.0, and 25 mM glucose. At the end of the preparation period, of approximately 3 h, the nodules were placed between two filters into a 10-mm tightly closed NMR tube, part of a homebuilt perfusion system. The latter, evolved from experimental device described previously (Roby *et al.*, 1987), allows circulation of the perfusion medium controlled in solute composition, temperature and pH, through the living nodule sample. The partial oxygen pressure in the perfusion medium was established by bubbling mixtures of oxygen and nitrogen into the medium reservoir. At various time, effectors were added into the perfusion medium.

^{31}P NMR spectra were acquired at 202.47 MHz using a 500.16 MHz NMR spectrometer (Avance III, Bruker). For in vivo experiments, ^{31}P NMR spectra were recorded for 36 min, using a 10 mm ATMA broadband observe probe. A solution of 500 mM Hexamethylphosphoramide (HMPA, ref H3380, Sigma) contained in a concentric capillary provide the chemical shifts and intensity references for ^{31}P NMR spectra. 3072 scans of 16 K data points were acquired with a 60° pulse angle, a spectral width of 14,204 Hz, acquisition time of 0.58 s and recycle delay of 0.1 s. Preliminary data processing was carried out with TOPSPIN 3.0 software (Bruker Biospin, Karlsruhe, Germany). Each Free Induction Decay (FID) was Fourier transformed (10 Hz line broadening), manually phased and baseline corrected. The resulting spectra were aligned by setting the HMPA signal to 30.73 ppm. The resonance assignments were based on chemical shifts. ^{31}P chemical shift were determined according to (Rolin *et al.*, 1989). Subcellular pH was estimated by use of a standard reference curve of pH as a function of chemical shift, which was obtained according to the method of Roberts *et al.* (1980).

Enzymatic and HPLC metabolite analyses

Nodule metabolites (amino acids, organic acids, soluble sugars) were extracted by the alcoholic extraction method and resuspended in water as described in Brouquisse *et al.* (1991). Sucrose was determined enzymatically (Velterop & Vos, 2001) at 340 nm using a microplate spectrophotometer. Succinate and malate were determined by anion exchange HPLC (Dionex) with conductivity detection (Moing *et al.*, 1998). Free amino-acids were analysed by HPLC using the AccQ.Tag method from Waters (Milford, MA) with fluorescence detection (Moing *et al.*, 1998).

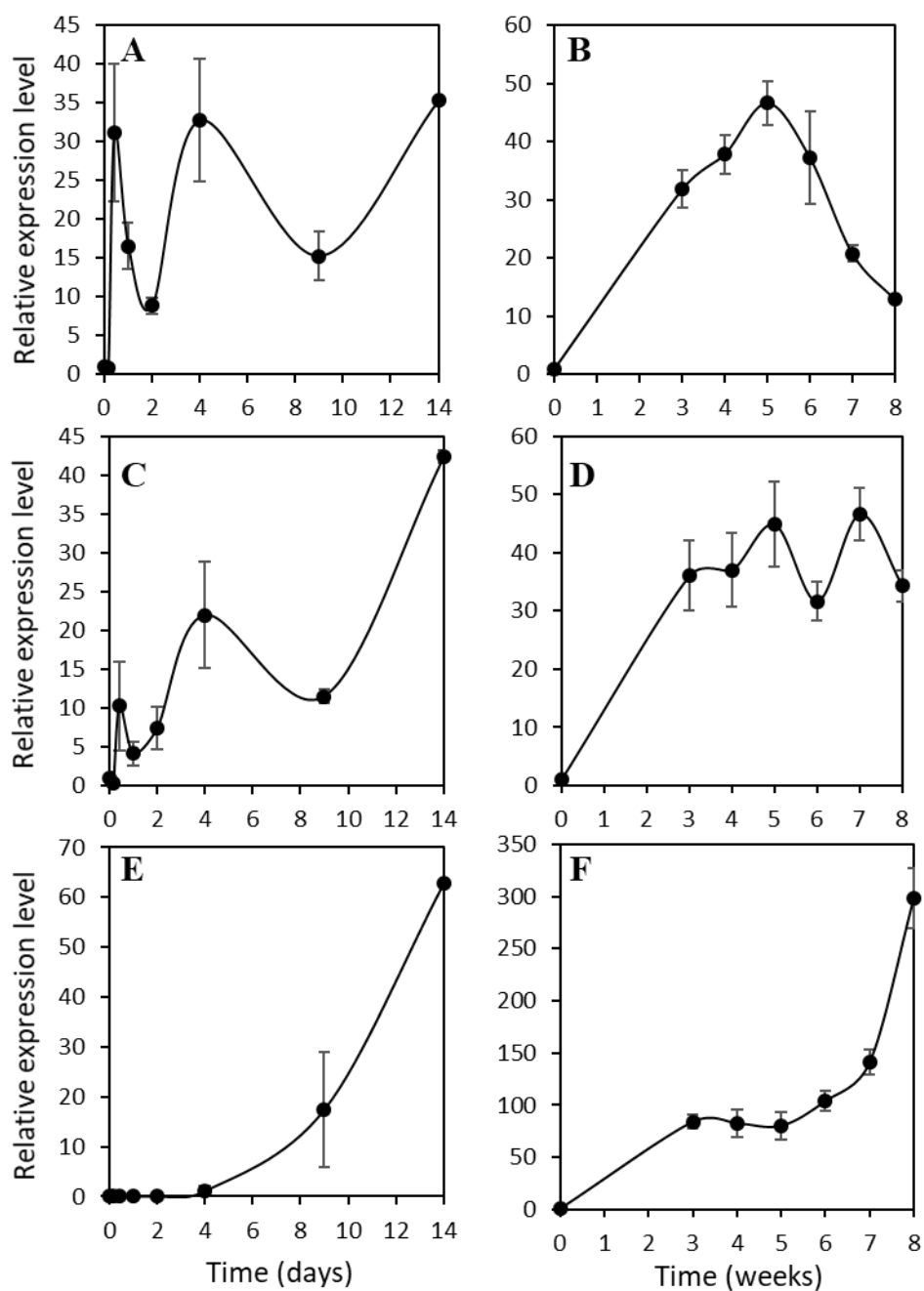


Figure. 1.1: Expression of NR genes during the symbiotic process

Expression analysis of the NR genes in roots and nodules during the symbiotic process. Short term kinetic 14 dpi (a, c, e), long term kinetic 8 wpi (b, d, f). Expression of NR1 (a, b), NR2 (c,d) and NR3 (e,f). Value are means \pm SE of three biological replicates, each with three technical replicates.

β -glucuronidase detection in planta

For β -glucuronidase detection, 4 dpi roots and 14 dpi nodules transformed with pKGFWS7-pNR1/2/3-GUS constructions were incubated for 1 h in acetone 90% (diluted with a phosphate buffer: Na₂HPO₄/ NaH₂PO₄ 0.1 M, pH 7.4) at -20°C. Roots were then washed twice with the phosphate buffer and incubated at room temperature, in the dark, in phosphate buffer containing potassium ferricyanide (0.5 mM) and X-gluc (0;5 ng.ml⁻¹) for 16 h. the roots were observed by optical microscopy (Axioplan II, Zeiss).

Results

Nitrate reductase genes expression during the symbiosis process

In *Medicago truncatula* genome databases (Mt4.0), 3 sequences encoding nitrate reductase, with the same gene structure composed by 4 exons and 3 introns, have been identified (Fig. S1B). The sequences, named *NR1*, *NR2* and *NR3* encode respectively proteins of 902, 884 and 876 amino acids. Both *NR1* and *NR3* are closely present in the chromosome 3, while *NR2* is present in chromosome 5 (Fig. S1A). The three genes exhibit strong homologies between them (of the order of 71%), but also with the *A. thaliana* orthologs (homology of 68 to 72%). In previous reports, NR expression and activity have been investigated in the N₂-fixing nodules (Streeter, 1985; Arrese-Igor *et al.*, 1990; Silveira *et al.*, 2001; Kato *et al.*, 2003; Horchani *et al.*, 2011), but not throughout the whole process, from the inoculation to the nodule senescence. To this end, we used two types of *M. truncatula* cultures: a short-term culture from 0 to 14 dpi, and a long-term culture from 0 to 8 wpi.

Both *NR1* and *NR2* were expressed at significant level in non-inoculated roots (Fig. 1.1; Table S1). Following inoculation *NR1* expression significantly increased (between 30-45 folds) and exhibited three overexpression peaks at 10 hpi, 4 dpi and 5 wpi (Fig. 1.1A and B). Similarly, *NR2* expression increased, although to a lesser extent than *NR1*, with two peaks at 10 hpi and 4 dpi, and then increased to reach a plateau between 3 and 8 wpi (Fig. 1.1C and D). As reported in Affimetrix data (Fig. S2), *NR3* is specifically expressed in nodules. During the symbiotic process, *NR3* expression was detected only from 4 dpi, increased to reach a plateau a between 3 and 5 wpi, when the N₂-fixation activity of *M. truncatula* nodules is maximal. After this period, the expression of *NR3* strongly increased (300 times) when the nodule senescence process is engaged (Fig. 1.1E,F).

Considered globally, four periods of time may be distinguished from this kinetics: 1) 10 hpi, during the first hours of the interaction between the plant and the symbiont, 2) 4 dpi,

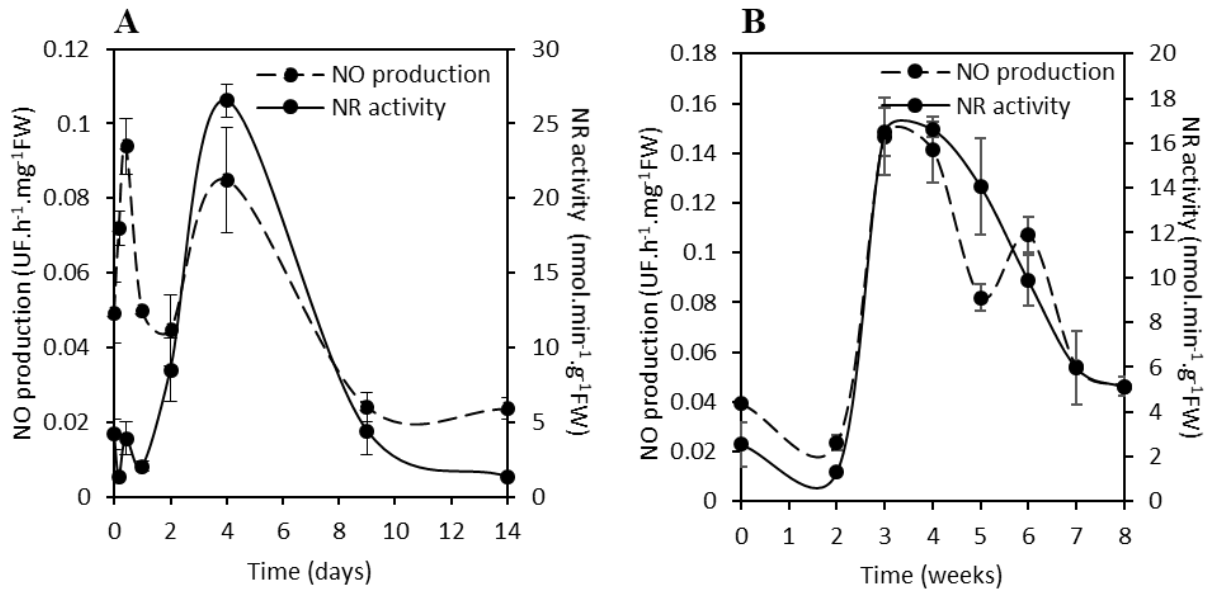


Figure. 1.2: NO production and NR activity during the symbiotic process

Short term kinetic 14 dpi (a), long term kinetic 8 wpi (b, d, f). The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe. The nitrate reductase activity is expressed in nmol per min per g of fresh weight. Value are means \pm SE of three biological replicates, each with three technical replicates.

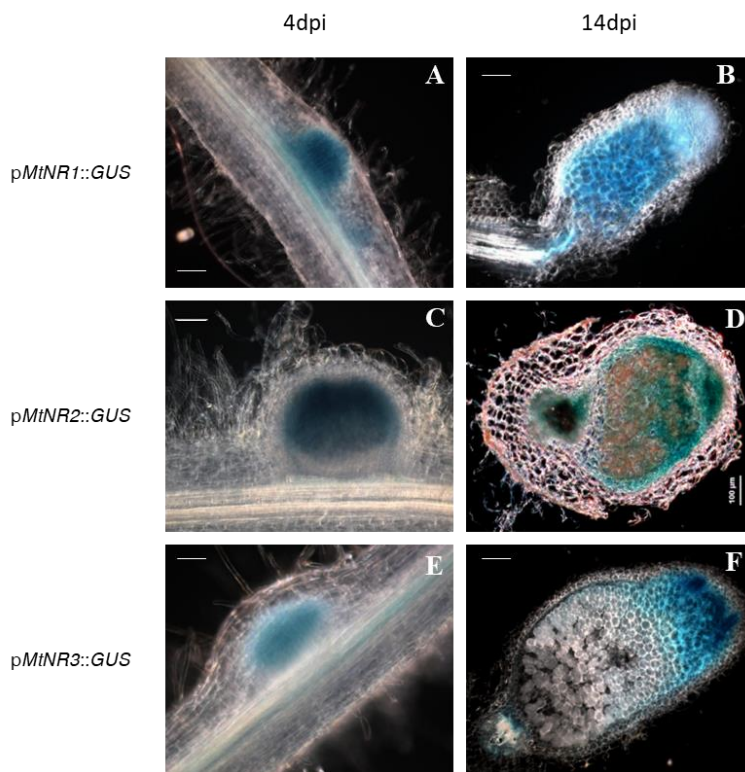


Figure. 1.3: Histochemical localization of GUS activity

Localization of GUS activity in transgenic roots of *M. truncatula* expression pNR1 (A, B) pNR2 (C, D) and pNR3 (E, F). 4 dpi whole root segment with *S. meliloti* (A, C, E) and 2 wpi old nodules (B, D, F) were stained for 16h with X-gluc for the GUS activity.

at the setup of nodule organogenesis (these two periods are characterized by expression peaks of *NR1* and *NR2*); 3) between 3 and 6 wpi, when nodule reaches maturity, marked by a strong expression of the three *NRs*, and 4) beyond 6 wpi, at the onset of nodule senescence, a period accompanied by a strong increase of *NR3* expression, while that of *NR1* genes decrease.

Nitrate reductase activity and NO level during the symbiosis process

The involvement of NR activity in NO production has already been evidenced in several plant organs and tissues (Dean & Harper, 1988; Rockel *et al.*, 2002; Gupta *et al.*, 2005; Planchet *et al.*, 2005). Thus, to assess the contribution of NR in NO production, the total activity of NR was analysed and compared with the NO level during the nodulation process. As compared to non-inoculated roots, NO level increased during the symbiotic process and exhibited 4 transient production peaks, at 10 hpi, 4 dpi, 3-4wpi and 6 wpi (Fig 1.2), corresponding to the four periods characterized with NR gene expressions. Total NR activity in non-inoculated roots was close to 2.5-4.0 nmol.min⁻¹.gFW⁻¹ (Fig. 1.2). Following inoculation, NR activity exhibited a significant and reproducible 60-70% decrease within 4 hpi, before returning close to its initial value at 10 hpi. After a further decrease at 24 hpi, NR activity strongly increased to 25-27 nmol.min⁻¹.gFW⁻¹ at 4 dpi, fell again, and exhibited a third peak around 3-5 wpi to fall back to a level close to that in non-inoculated roots. As a whole, the pattern of NR activity showed a parallel with the production of NO (Fig. 1.2). It fitted with the expression of *NR1* and *NR2* during the two first weeks of the symbiosis, and with that of *NR1* between 3 and 8 wpi.

Histochemical expression of NRs in *M. truncatula* nodules

Histochemical detection of GUS activity under the control of NR promoters was investigated to analyse the spatial expression of the 3 *NR* genes at two stages of the symbiosis, i.e. at 4 dpi at the onset of nodule development, and at 2 wpi when N₂-fixation starts to occur. In 4 dpi transgenic roots, the 3 *NR* genes were found to be expressed in the controlled area (Xiao *et al.*, 2014) of nodule primordium (Fig. 1.3A, C and E). In 14 dpi-old nodules, *NR1* was expressed in the whole nodule, but mainly in interzone 2-3 and zone 3 (Fig. 1.3B), as confirmed by Symbimix data (Table S1). In nodule, *NR2* expression seems to be localised in zone 2 and partially in zone 3 as Symbimix data show (Fig. 1.3D, Tab S1). *NR2* was mainly expressed at the periphery of the nodule, as well as in zones 1, and 2, in interzone 2-3, and unequally in zone 3 (Fig. 1.3D), which is consistent with the data Symbimix (Table S1).

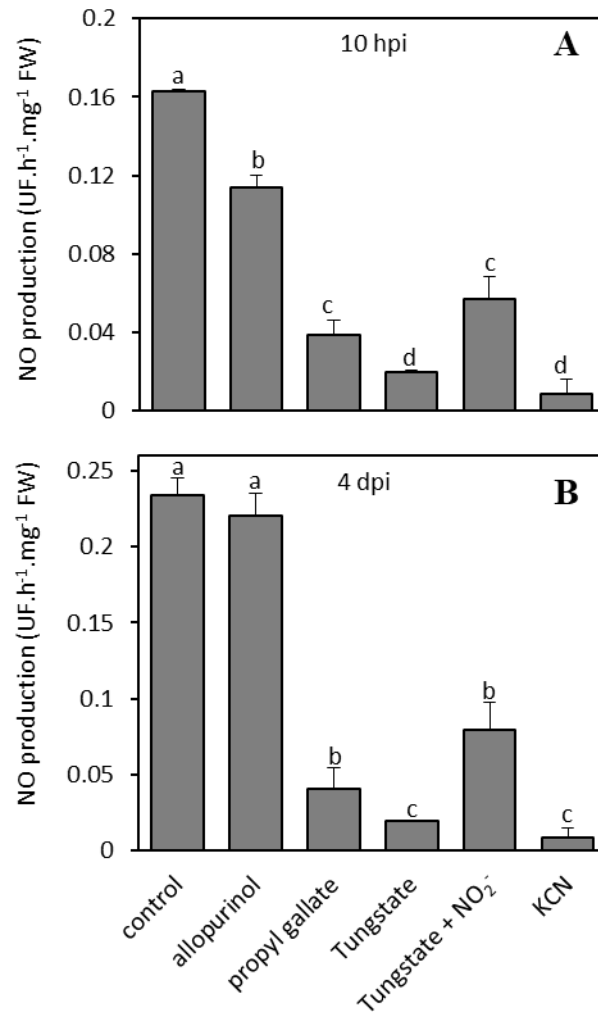


Figure. 1.4: Effects of different inhibitors on NO production

NO production was measured on roots inoculated at 10 hpi (A) and 4 dpi (B) in the presence of 1 mM tungstate, allopurinol and propyl gallate and 300 μ M KCN. NO production is expressed in units of fluorescence per mg of fresh weight. The data correspond to the average of 3 independent biological experiments performed in triplicate. The error bars correspond to the standard error (n = 3).

In case of *NR3* promoter, staining appear clearly at the center of nodule primordia and more precisely in nodule at the level of zone I and II (Fig 1.3E,F). As for *NR3*, which is very weakly expressed in the nodules (Table S1), it was expressed mainly in zone 1 and less in zone 2 (Fig. 1.3F).

Involvement of NR activity in NO production during nodule development

In previous work, Horchani *et al.* (2011) showed that, in mature *M. truncatula* nodule, NR is involved in NO production in relation with the mitochondrial electron transport chain (ETC). Using an inhibitor approach, NO production was analysed in 10 hpi and 4 dpi roots. As reported in Fig. 1.4, it was inhibited over 95% by KCN used as a negative control. Allopurinol, an inhibitor of xanthine oxido-reductase, moderately inhibited NO production (by 28%) in 10 hpi roots, whereas it was without effect in 4 dpi roots. In both 10 hpi and 4 dpi roots, propylgallate (inhibitor of the mitochondrial alternative oxidase) inhibited NO production by 70-90%, indicating that mitochondria are involved in this reaction. Interestingly, tungstate (Tg), which is a NR inhibitor, was found to inhibit NO production by 88% and 92% in 10 hpi and 4 dpi roots, respectively, and this inhibition was partially relieved by the addition of nitrite, the product of NR reaction, in the medium (Fig. 1.4). Considered together, these results indicate that, similarly to what was observed in mature nodules (Horchani *et al.*, 2011), both the NR and the respiratory chain are involved in the production of NO, probably via the reduction of nitrate to nitrite by NR, and the subsequent reduction of nitrite to NO at the ETC level.

Involvement of nitrate reductase in energy, carbon and nitrogen metabolism in mature nodules

It has been shown that in hypoxic plant roots in hypoxia, NR activity is necessary to maintain the energy state of N₂-fixing nodules, via the functioning of a cyclic respiration, called "Phytogb-NO respiration", in which the nitrite resulting from the nitrate reduction by the NR, is reduced to NO by the mitochondrial ETC, and then NO is reoxidized in nitrate by the phytooglobins (Dordas *et al.*, 2003b, 2004; Stoimenova *et al.*, 2007). The operation of this respiration, in which nitrite substitutes for O₂ as the ETC electron terminal acceptor, makes it possible to support the regeneration of ATP when the O₂ content falls in the cells (Igamberdiev & Hill, 2009). In a previous work, we showed that in *M. truncatula* nodules, NR activity supports the production of NO and allows the maintenance of a high ATP/ADP ratio despite the microoxic environment that prevails in nodules (Horchani *et al.*, 2011). In the present work, we aimed at investigating more precisely the role of NR activity in the metabolism of

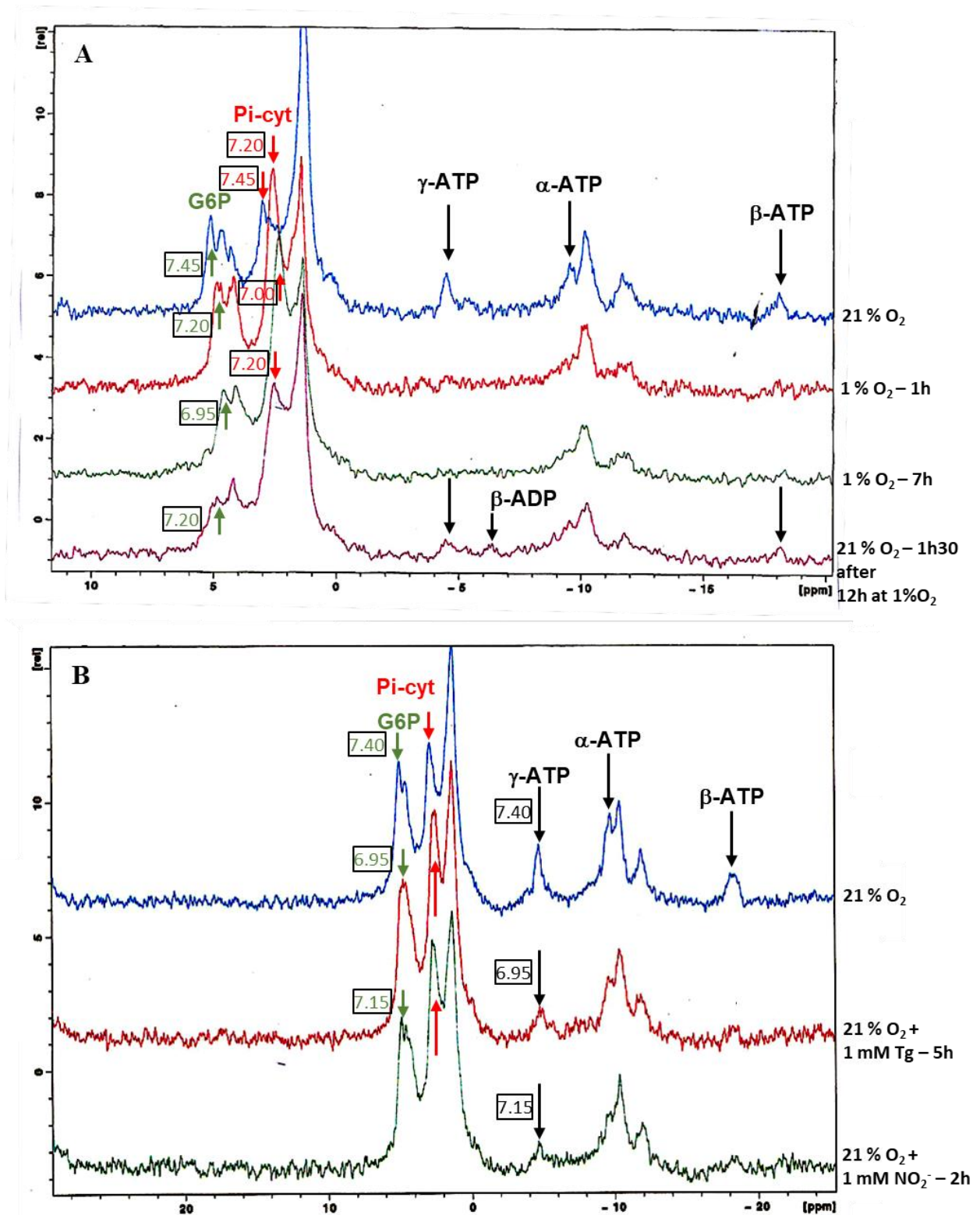


Figure 1.5: *In vivo* ^{31}P -NMR study of metabolic transitions induced in nodule.

(A) Proton-decoupled ^{31}P -NMR spectra of nodules in normoxia at 21°C, perfused with a nutritive medium at pH 6.0 with successively 21% O_2 (control), 1% O_2 and 21% O_2 . For each line, duration of the treatment is indicated up to the end of the spectrum. (B) Proton-decoupled ^{31}P -NMR spectra of nodules in normoxia at 21°C, perfused with a nutritive medium at pH 6.0 containing successively 21% O_2 (control), 1 mM Tg, and 1 mM NO_2^- . For each line, duration of the treatment is indicated up to the end of the spectrum. Exponential apodization and zero filling. Abbreviations: Pi, inorganic phosphate; G6P, Glc-6-P; Pi-cyt, cytoplasmic

M. truncatula N₂-fixing nodules. To this end, we first followed the *in vivo* effects of a "normoxia-hypoxia-normoxia" transition on the energy metabolism of 3 wpi nodules. When using a perfusion system adapted to NMR spectrometer (Roby *et al.*, 1987), *in vivo* NMR spectroscopy is a well-established technique to follow the concentration of abundant mobile metabolites within a sample as a function of time. Figure 1.5A displays typical ³¹P spectra obtained with living *M. truncatula* nodules during normoxia-hypoxia-normoxia transition experiments. In the present experiments, ³¹P-NMR was used to analyze the most abundant phosphorylated compounds, such as glucose-6-phosphate (G6P), cytosolic and vacuolar inorganic phosphate (Pi-cyt and Pi-vac, respectively), and ATP (Roby *et al.*, 1987). Transition from 21% O₂ (normoxia) to 1% O₂ (hypoxia) led to an important reduction in ATP content and in a significant acidification of cytosolic pH, from 7.45 to 6.95 after 7 h of hypoxia, as measured by the shift of G6P and Pi-cyt resonances (Fig. 1.5A). Transition from 1% O₂ back to 21% O₂ was accompanied by an increase in ATP and a progressive return of cytosolic pH to more alkaline values (7.2). In a second series of experiments, nodules were incubated at 21% O₂ in the presence of 1 mM Tg (Fig. 1.5B). After 5 h of Tg treatment, ATP level decreased by a factor 2.5, and cytosolic pH decreased from 7.4 to 6.95, indicating a decrease in energy state and an acidification of the nodules. The addition of 1 mM NO₂⁻ failed to restore ATP level, but triggered an increase in cytosolic pH to 7.15, indicating a progressive recovery of the cell metabolism (Fig. 1.5B). Thus, the inhibition of NR by Tg and its subsequent relieve by NO₂⁻ partially mimicks "normoxia-hypoxia-normoxia" transitions and support the involvement of NR in the energy metabolism of nodules.

In a second set of experiments, 3 wpi-old *M. truncatula* nodules were incubated for 4h in either the presence, or absence, of 1 mM Tg, 10 mM nitrate and 1 mM nitrite. As reported in Fig. 1.6, the nitrogenase activity, as measured by its acetylene reducing activity (ARA), was inhibited in the presence of either nitrate or Tg (or both), but was unaffected by the presence of nitrite. Nitrate was well known to be an inhibitor of ARA (Streeter & Wong, 1988; Fujikake *et al.*, 2002). ARA inhibition by Tg and its relief by nitrite support a key role of NR in the N₂-fixing metabolism. The variations of various metabolites related to the carbon and nitrogen metabolism of nodules were consistent with this hypothesis. Thus, following NR inhibition by either Tg or Tg + nitrate, sucrose content increased, indicating a slowdown of its consumption by nodules (Fig 1.6B). As a consequence, first, succinate and malate contents decreased (Fig. 6 C and D), indicating a lower supply of carbon nutrients to bacteroids, and second, asparagine content also decreased (Fig. 1.6E) as a result of the shortage of carbon substrate supply and

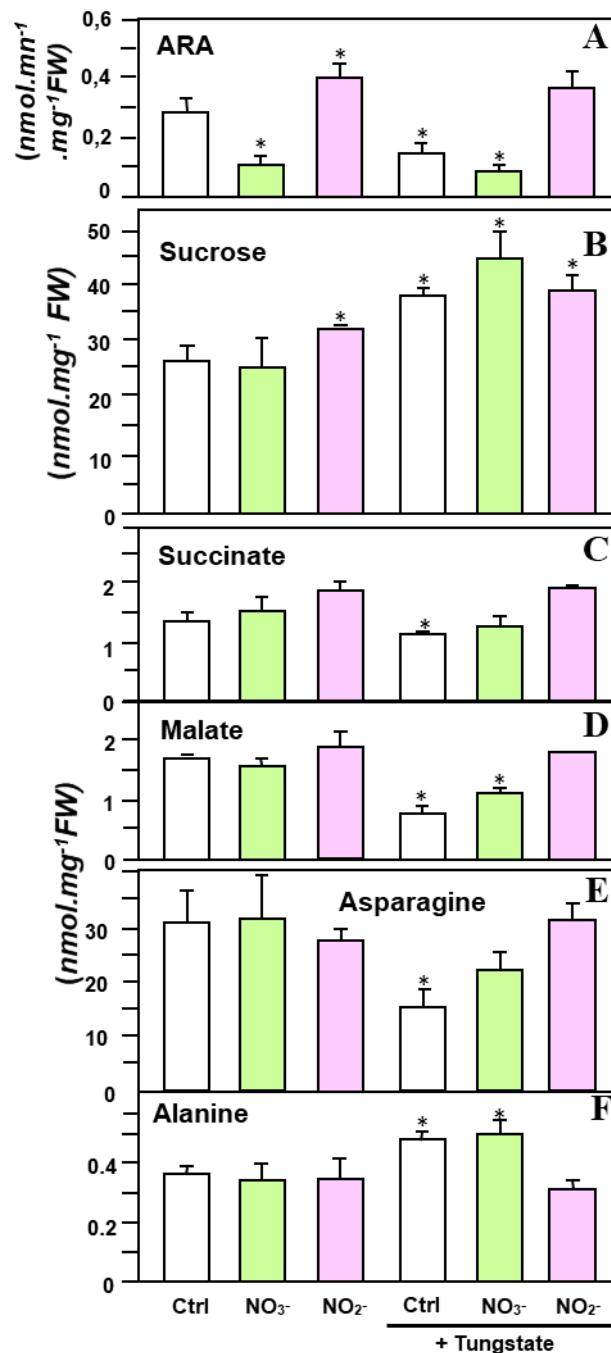


Figure. 1.6: Effects of NR effectors on nitrogenase activity, Sucrose, succinate, malate and amino-acids contents.

(A) Nitrogenase activity (estimated as ARA) was measured at 3 wpi and normalized per nodule fresh weight. (B) Sucrose concentration was calculated from enzymatic analysis (C) Succinate, (D) Malate, (E) Asparagine, (F) Alanine concentrations were calculated after HPLC analysis. Effector concentrations were 10 mM NaNO₃⁻ (NO₃⁻), 1 mM NaNO₂⁻ (NO₂⁻) and 1 mM NaTg. Asterisks indicate significant difference (P<0,05) when compared with the control (Ctrl) according to Student's t test.

ARA inhibition. Interestingly, alanine, which is a marker of hypoxia in plant tissues (Gibbs & Greenway, 2003), was found to increase in the presence of Tg (Fig. 1.6F), indicating that NR inhibition mimics hypoxia probably *via* the inhibition of the Phyto**g**b-NO respiration.

NR1 is involved in NO production in mature nodules

In a last step, we aimed at identifying the NR isoform involved in the production of NO within the mature nodule. As *NR3* is very weakly expressed, we focused our study on *NR1* and *NR2*. To study the involvement of NRs in the production of NO at the nodule level, without affecting the nitrogen metabolism of the plant, an RNAi strategy was used. *M. truncatula* RNAi on the *NR1*, *NR2* and *NR1-2* genes were constructed under the control of a zone 3 specific promoter, the MtNCR001 promoter (Mergaert *et al.*, 2003a; Horchani *et al.*, 2011). Nodules of 3 wpi were collected and analyzed for NR activity and NO production. As shown in Fig. 1.7A, the total NR activity was decreased by 47 and 56%, respectively, in the RNAi::*NR1* and *NR2* nodules. On the other hand, the double RNAi did not make it possible to further reduce the total NR activity. The NO production measurements show that NO production dropped by 45% in RNAi::*NR1* nodules, but only by 18% in RNAi::*NR2* nodules (Fig. 1.7B). The decrease in NO production in the nodules of double RNAi::*NR1-2* was of the same order of magnitude as that observed in RNAi::*NR1* nodules. These results clearly show that the decrease in NR activity is accompanied by a fall in NO production, and therefore that NRs are involved in the production of NO in nodules. Moreover, since the fall in NO production correlates with the decrease in NR activity in RNAi::*NR1* nodules, but not in RNAi::*NR2*, it can be assumed that NO production is particularly related to NR1 activity, rather than NR2.

Discussion

The analysis of NO production during the symbiotic process shows that NO is produced continuously during symbiosis, but with level variations (Fig. 1.2). Distinct periods can be considered: i) at 10 hrs, during the first hours of the interaction between the plant and the rhizobia; ii) at 4 dpi, corresponding to early development of the nodule ((Xiao *et al.*, 2014)); iii) at 3-4 wpi, when the N₂-fixation zone ceases to grow and the nodule reaches the status of "mature N₂-fixing nodule" (Ferguson *et al.*, 2010), and iv) after 6 wpi, corresponding to the global onset of nodule senescence (Puppo *et al.*, 2005b). In plants, NR has been shown to be involved in the production of NO in intact mature nodules of *M. truncatula* (Horchani *et al.*, 2011). Starting with nodule extracts or sections, several studies have shown that NO can also

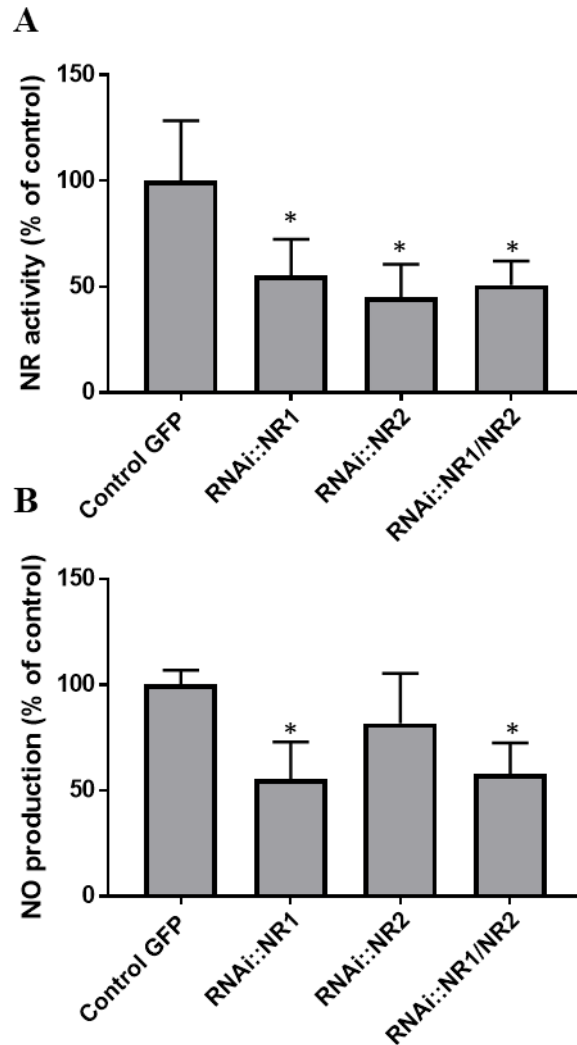


Figure. 1.7: NR activity and NO production in NR RNAi transformed nodules

The nitrate reductase activity is expressed in nmol per min per g of fresh weight (a), The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe (b). Value are means \pm SE of three biological replicates, each with three technical replicates. Asterisks indicate significant difference ($P < 0,05$) when compared with the control according to Student's t test.

be produced by NO synthase-like activities (Cueto *et al.*, 1996; Baudouin *et al.*, 2006; Leach *et al.*, 2010), but these approaches were biased because they were performed with non-intact and non-microoxic material. Three isoforms coding for NR were identified in *M. truncatula*. NR1 and NR2 correspond respectively to inducible and constitutive forms which have been found in other higher plants such as *A. thaliana*, *G. max*, or *L. japonicus* (Santucci *et al.*, 1995). Although there are 4 isoforms of NR in rice (Li *et al.*, 2009), most of the higher plants described in the literature have only two. The third isoform of NR found in *M. truncatula* is specifically expressed in N₂-fixing nodules (Puppo *et al.*, 2013). However, the reason for the presence of this isoform in *M. truncatula*, and its absence in other legumes, remain unanswered today due to the low number of fully sequenced legume genomes.

The peaks of expression of *NR1* and *NR2* at 10 hpi and 4 dpi (Fig. 1), and the location of their expression in the nodular primordium (Fig. 3), suggest that NR activity is involved in NO production during the first stages of symbiosis. Measurements of NO production in the presence of inhibitors (Fig. 4) definitely confirm this hypothesis. However, 1) partial inhibition reversal of NO production by Tg in the presence of nitrite, and 2) inhibition of NO production by mitochondrial ETC inhibitors (Fig. 4), clearly indicate that NR activity is indirectly involved in NO production via reduction of nitrate to nitrite, and nitrite is reduced to NO by mitochondrial ETC. These results are similar to those obtained in mature nodules (Horchani *et al.*, 2011) and indicate that from the beginning of the symbiotic process, a large part of NO is produced from nitrite via a reducing pathway (Gupta *et al.*, 2011a). This result was unexpected since the roots of *M. truncatula* are not particularly microoxic. However, the analysis of NO production data with pharmacological inhibitors (Fig. 4) indicates that at 10 hpi. XOR could also participate for one part to this production. XOR is a peroxisomal enzyme capable of producing NO[•] and superoxide anion (O₂^{•-}) that can complex together to give peroxynitrite, ONOO⁻ (del Río *et al.*, 2004). ONOO⁻ plays a key role in the induction of defense responses, particularly via tyrosine nitration of proteins (Saito *et al.*, 2006), which is consistent with the involvement of this NO production in defense responses to 10 hpi.

Up to 1 dpi, the measured NR activity was low. Such a low activity could be explained by a post-transcriptional regulation of NR. Indeed, it has recently been shown in *A. thaliana* that NR can be phosphorylated by a mitogen activated protein kinase, MAPK6, which results in the increase of its reducing activity (from NO₃⁻ to NO₂⁻ and NO₂ in NO) (Qi *et al.*, 2019). In addition, in *M. truncatula* roots it has been shown that, in response to *S. meliloti* inoculation, the induction of MAPK6 gene is lower than its induction in response to inoculation by the PstDC3000 pathogen (Chen *et al.*, 2017). The inoculation of tobacco cells with PstDC300

induces an increase in NO production (Delledonne *et al.*, 1998), which subsequently results in the induction of defense gene expression (Durner *et al.*, 1998). These observations suggest that inoculation with *S. meliloti* may reduce MAPK6-dependent signaling, thereby reducing activation of NR activity and NO production. This regulation would reduce the NO-dependent defense responses and allow the symbiont to enter the root. Therefore, it would be interesting to test the hypothesis that the induction of the expression of NR1 and NR2 at 10 hpi is related to the establishment of the immune response during the first hours of the symbiotic interaction. Indeed, it has been previously shown that the expression of NR increases in response to PAMP in potato, which implies that NR plays a role in the immune response (Delledonne, 2005). In addition, it has been shown that, in *G. max* roots, a large number of plant immunity related genes were induced within 12 hours post-inoculation by *B. japonicum*, but their expression levels gradually decreased to background levels within 24h of inoculation (Libault *et al.*, 2010). These observations suggest the existence of two parallel signaling pathways: 1) one involving the recognition of *S. meliloti* as a pathogen and the induction of genes related to the immune response via NO production, and 2) another passing through the MAPK6 signaling pathway to decrease the production of NO and consequently the immune response of the plant, thus allowing the symbiotic bacteria to enter the cells of the root.

The third peak of NO production at 3-4 wpi (Fig. 1.2) corresponds to the optimum of N₂-fixing activity in mature nodules. The NR activity and the NR1 expression kinetics are correlated with NO production, while NR2 and NR3 are steadily expressed at 3 to 5 wpi (Fig. 1.1 and 1.2). This suggests that NR1 is the major cause of the total measured NR activity and potentially of NO production. The location of NR1 expression in zone 3 (Fig. 3), corresponding to the NO accumulation site in mature nodules (Baudouin *et al.*, 2006), reinforces this hypothesis and the analysis of NO production in RNAi nodules (Fig. 7) confirms that NR1, rather than NR2, is primarily responsible for NO production in the mature nodule.

Mature nodules are characterized by a microoxic environment, raising the issue of energy supply within this organ. Accumulated data support the existence of a Phyto**g**b-NO respiration in legumes nodules exposed to hypoxia, in which both mitochondrial ETC and NR are involved in ATP regeneration (Meakin *et al.*, 2007; Sánchez *et al.*, 2010; Horchani *et al.*, 2011). In this study, the role of NR activity in energy, carbon and nitrogen metabolism was investigated by using the NR inhibitor Tg. *In vivo* 31P-NMR experiments (Fig. 5A) showed that hypoxia leads to a fall in ATP and to the acidification of cytosolic pH inside the nodules. These data are consistent with earlier observations with maize root tips (Roberts *et al.*, 1984) and sycamore maple cells (Gout *et al.*, 2001). In the presence of Tg, the fall in ATP and the cytosol

acidification resulting from the inhibition of NR activity and their partial reversion by the addition of nitrite (Fig. 1.5B) confirm that NR activity is fully involved in the energy metabolism of nodules via "Phytoglobin-NO" respiration, as already shown by Horchani *et al.* (2011). The inhibition of energy metabolism triggers an inhibition of the carbon and nitrogen metabolism in nodules, with an accumulation of sucrose, a subsequent decrease in the supply of carbon substrates to bacteroids (succinate, malate), and ultimately, a decrease in nitrogen fixation (ARA) and assimilation (asparagine) activities (Figure 6). It is interesting to note that, with the accumulation of alanine upon Tg treatment, the inhibition of NR activity mimics a situation of hypoxia (Fig 6), which is an additional argument of the role played by NR in the energy metabolism of N₂-fixing nodules.

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Supporting information

Tab S1: Access code and symbionics expression of *M. truncatula* NR genes

Genes	code affymetrix	code gene Mt4.0	Mt20120830-LIPM	DESEQ MEAN				
				FI	FIID	FIIP	IZ	ZIII
NR1	Mtr.42446.1.S1_at	Medtr3g073180	Mt0006_00730	20.7	89.1	21.3	193.7	693.5
NR2	Mtr.10604.1.S1_at	Medtr5g059820	Mt0008_10301	156.6	166.9	266.7	712.9	843
NR3	Mtr.31448.1.S1_at	Medtr3g073150	Mt0006_00731	1.3	0.5	1.3	0	1.4 ± 1.4

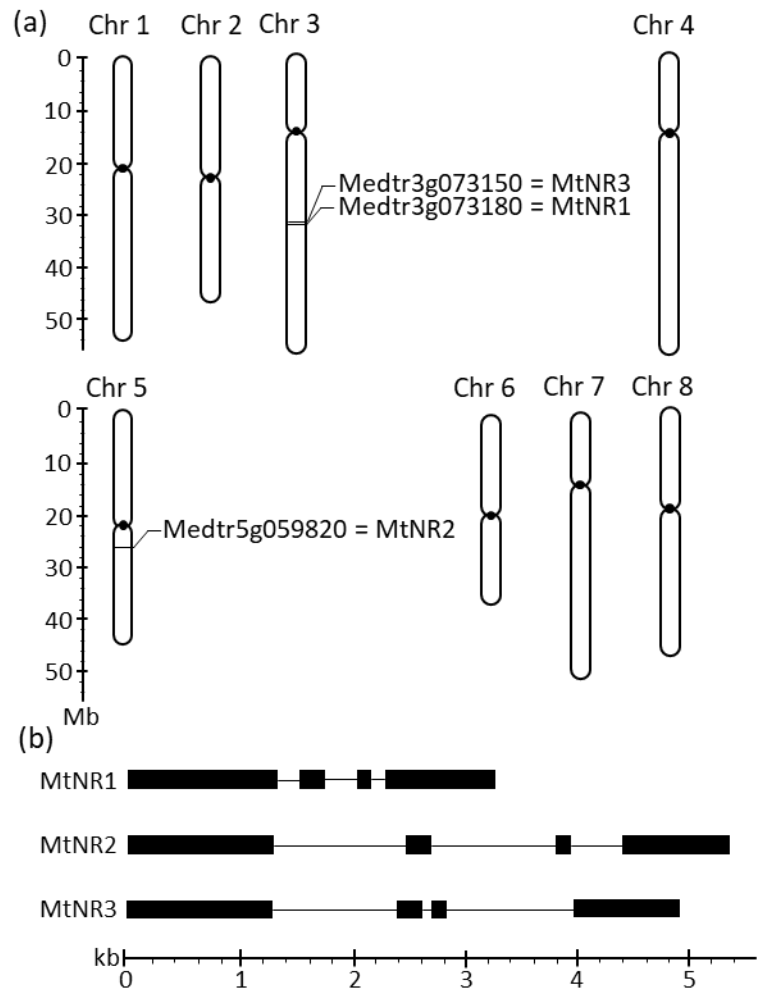


Figure S1: NR genes localisation and Exon-Intron structure

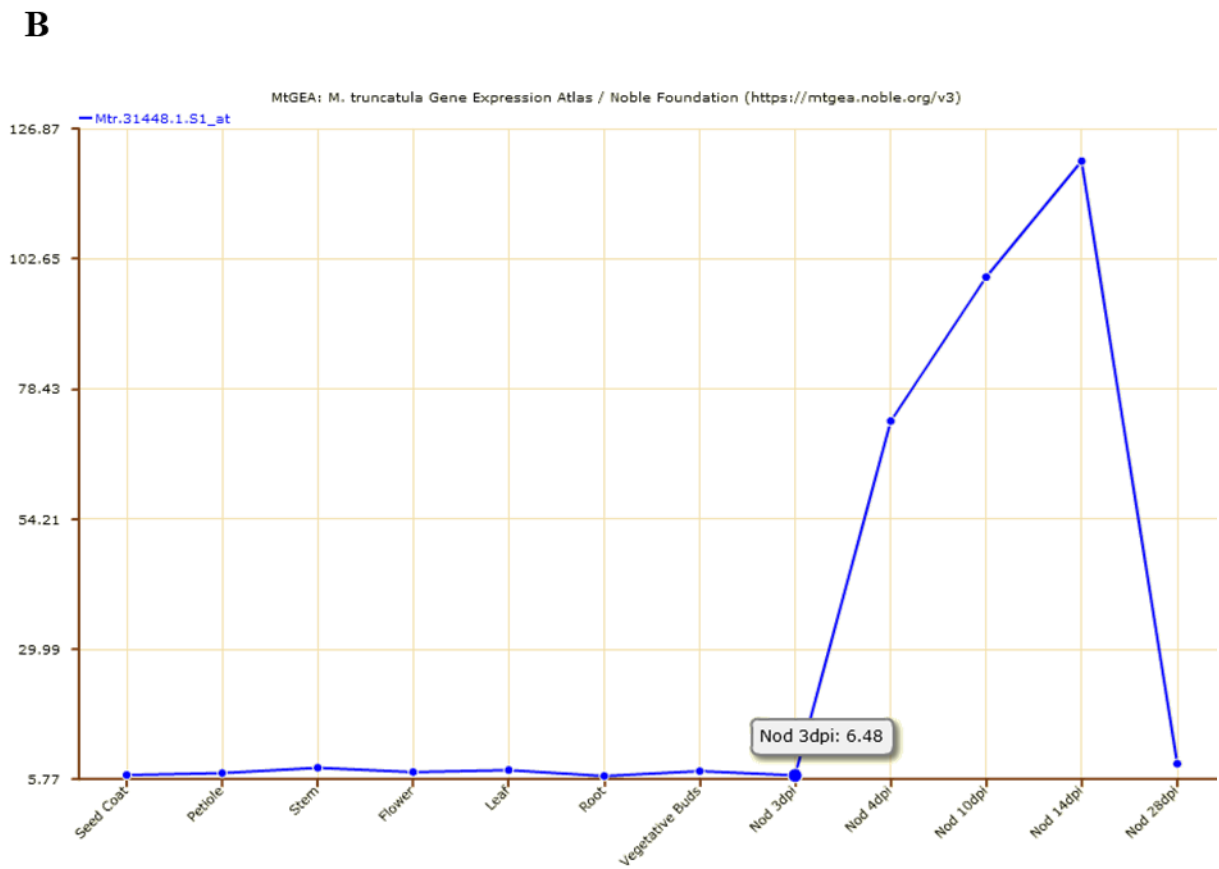
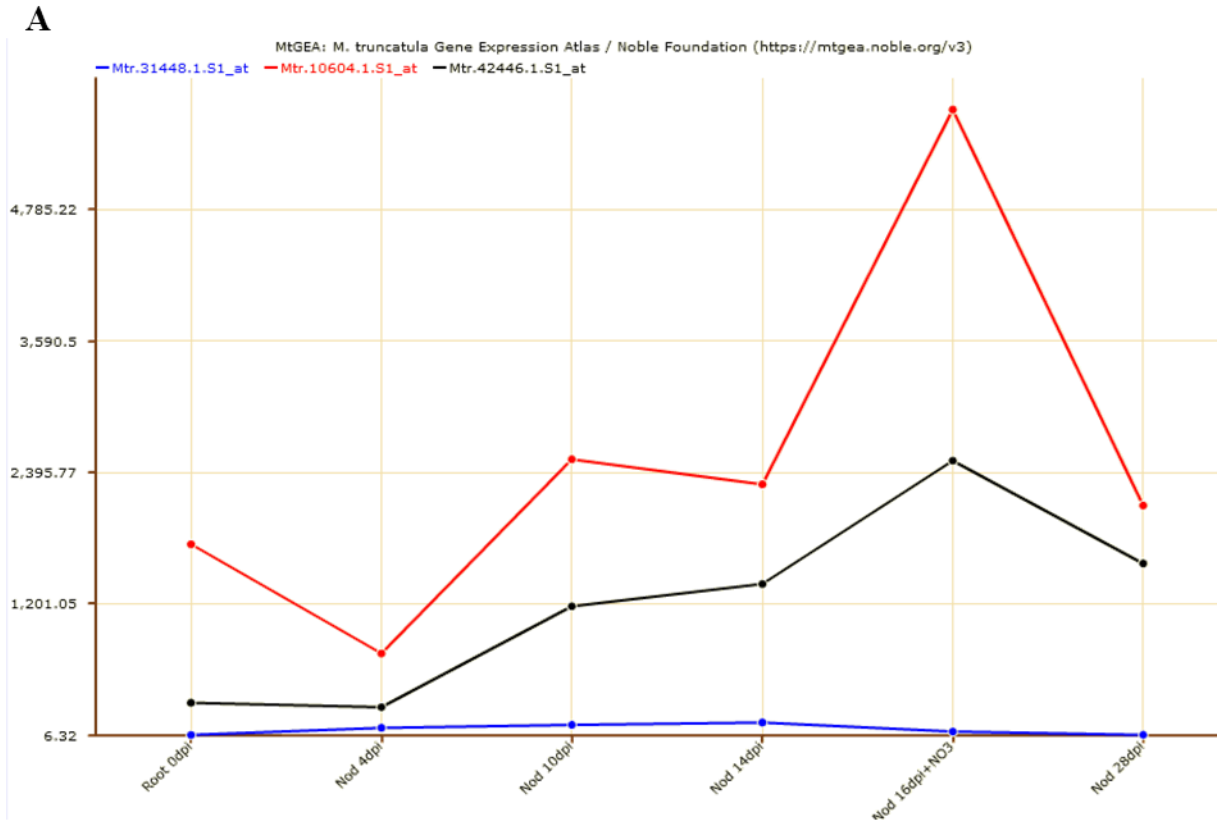


Figure S2: Microarray data of *Medicago truncatula* NR genes

Chapitre 2 :
Caractérisation des Phytoglobines de
M. truncatula, et rôle potentiel dans la
régulation du NO au cours de la symbiose

1. Contexte et objectifs du travail: Regulation of *Medicago truncatula* phytoleghemoglobin genes expression in relation with nitric oxide production throughout the nitrogen-fixing symbiosis

Pendant de nombreuses années, les leghémoglobines ont été les seules hémoglobines connues chez les plantes (Kubo, 1939). Par la suite, d'autres classes de phytoleghémoglobines ont été identifiées, qui possèdent chacune des caractéristiques biochimiques différentes. Plusieurs études phylogénétiques ont identifié certaines phytoleghémoglobines présentes chez *M. truncatula* (Vinogradov *et al.*, 2007 ; Garrocho-Villegas *et al.*, 2006 ; Bustos-Sanmamed *et al.*, 2010), cependant aucune ne les a dénombrées précisément. De plus, les études réalisées sur les phytoleghémoglobines et le NO dans la symbiose fixatrice d'azote ont été effectuées la plupart du temps à un moment précis du processus symbiotique (Shimoda *et al.*, 2009 ; Fukudome *et al.*, 2016). (Nagata *et al.*, 2008 ; del Guidice *et al.*, 2011 ; Pii *et al.*, 2007 ; Cam *et al.*, 2006), mais aucune n'a envisagé la totalité du processus.

Le premier objectif de notre étude a été d'identifier et classer toutes les phytoleghémoglobines de *M. truncatula*. En se basant sur la nouvelle nomenclature des phytoleghémoglobines végétales proposée par Hill *et al.*, (2016), nous avons homogénéisé la nomenclature pour les phytoleghémoglobines de *M. truncatula*. Dans un second temps, nous avons analysé l'expression des phytoleghémoglobines tout au long du processus symbiotique, de l'inoculation (0 dpi) jusqu'à la période où la sénescence de la nodosité est initiée (8 wpi). Au cours de la même cinétique, la production de NO a été analysée. Pour finir, nous avons voulu étudier plus précisément la relation entre le NO et l'expression des phytoleghémoglobines à deux moments physiologiques de la symbiose, l'établissement de la symbiose et lors du processus de sénescence.

Les résultats obtenus ont abouti à l'identification de 17 phytoleghémoglobines chez *M. truncatula*, réparties en 12 Lb, 3 Phytoleghb1 et 2 Phytoleghb3. Quatre pics de production de NO ont été observés, correspondant à quatre étapes du processus symbiotique, pendant : (1) l'établissement de l'interaction la plante et la bactérie, (2) le début de l'organogénèse de la nodosité, (3) le fonctionnement de la nodosité mature et (4) lors de l'entrée en sénescence des nodosités. Lors de ces différentes étapes, la production de NO a pu être corrélée à l'expression des gènes d'une *Phytoleghb1* (*Phytoleghb1.1*) et d'une *Phytoleghb3* (*Phytoleghb3.1*). L'utilisation de donneurs de NO a permis de montrer que, lors du développement nodulaire, le NO induit l'expression des *Phytoleghb1* et de plusieurs gènes de défense (*GST*, *CS*), mais réprime celle des *Lb* et *Phytoleghb3*.

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Regulation of *Medicago truncatula* phytohemoglobin genes expression in relation with nitric oxide production throughout the nitrogen-fixing symbiosis

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Regulation of *Medicago truncatula* phytoleglobin genes expression in relation with nitric oxide production throughout the nitrogen-fixing symbiosis

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Supporting information: 3 Figures, 1 Table.

Summary

- The nitrogen-fixing symbiosis between legumes and rhizobia leads to the formation of a new root organ called nodule. In legumes nodules, three types of phytohemoglobins, were characterized: phytohemoglobin1, leghemoglobin and phytohemoglobin3. They are known to buffer oxygen and to scavenge nitric oxide (NO). NO is a signalling/metabolic molecule involved in developmental processes and in plant-microorganism interactions, including symbiosis. In this study, we survey the phytohemoglobin genes present in the *Medicago truncatula* genome, address their expression and NO production during the symbiosis between *M. truncatula* and *Sinorhizobium meliloti*, and investigate the relationship between NO and Phytohgb.
- Gene expression and NO production were determined by Q-PCR during the whole symbiotic process, from inoculation up to the onset of senescence. At two time points of the symbiosis, plants were exposed to either NO-donor, or dark and nitrate treatments.
- Phytohemoglobin and NO showed four expression and production peaks, respectively, at 10 hours, 4 days, 3-5 weeks, and 6-8 weeks post-inoculation. NO was found to upregulate phytohemoglobins1, but down-regulate leghemoglobins and phytohemoglobins3.
- The update of the nomenclature and the precision of kinetics highlight the importance of phytohemoglobins in NO homeostasis, and brings key to understand the role of NO during symbiosis.

Keywords: Legumes, *Medicago truncatula*, nitric oxide, nitrogen-fixing symbiosis, nodules, phytohemoglobins.

Introduction

The symbiotic interaction between legumes and Rhizobium bacteria results in the formation of a new root organ, the nodule, whose main function is the reduction and fixation of atmospheric nitrogen (N_2). The process starts with the mutual recognition of both the plant and the bacterial partners involving plant flavonoids and bacterial lipochito-oligosaccharides, called "nodulation factors". Bacteria enter the root hairs via a specific structure, the infection thread, while some cell's root cortex divide to form the nodule (Long, 2001). Inside the infection thread that progresses and reaches the cortical cells, bacteria divide and are released into the host cells of the developing nodule. Bacteria then differentiate into bacteroids that reduce N_2 via nitrogenase activity (Oldroyd & Downie, 2008). Nodules may be of either determinate or indeterminate type (Hirsch, 1992). Determinate nodules, such as those of soybean, cowpea, or bean, are round shaped, lack a persistent meristem, and grow through cell expansion rather than cell division. A central area hosts infected cells where N_2 fixation occurs (Franssen *et al.*, 1992). Indeterminate nodules such as those of alfalfa, clover or pea are cylinder shaped, possess a persistent meristem and comprise four distinct zones: zone I, the meristematic cells, zone II, where the bacteria enter the host cells and differentiate into bacteroids, zone III, where bacteroids reduce N_2 to ammonia (NH_3), and zone IV, characterized by the breakdown of the symbiosis and the set-up of senescence (Timmers *et al.*, 2000). As nitrogenase is irreversibly inhibited by traces of oxygen (O_2), N_2 fixation requires the microaerophilic conditions found in nodules (Appleby, 1992).

Nitric Oxide (NO) is a bioreactive gaseous molecule found in all living organisms. In plants, it participates in the regulation of the main stages of development, from germination to senescence, through several physiological processes (Corpas & Barroso, 2015; Domingos *et al.*, 2015). NO also participates in the response of plants to many abiotic stresses including hypoxia (Simontacchi *et al.*, 2015). It is involved as well in the plant-microbe interactions where it acts as a signal and regulatory molecule in the induction of cell death, defense gene expression, or interaction with reactive oxygen species (ROS) in the defense of plants against pathogens (Thalineau *et al.*, 2016). NO is also produced during symbiotic interactions, and many studies have reported its presence during legume-rhizobia symbiosis. NO production is transiently induced in the roots of *Lotus japonicus* and *Medicago sativa* a few hours post-inoculation (hpi) with their bacterial partners (Nagata *et al.*, 2008; Fukudome *et al.*, 2016). Later, at 4 days post-inoculation (dpi), in *M. truncatula*, NO is produced at different sites during the infection process: in shepherd's crooks of root hairs, in infection threads, and in nodule

primordia (del Giudice *et al.*, 2011). The first evidence for the presence of NO in N₂-fixing nodules came from the detection of NO complexed with leghemoglobin (Lb) in several legumes (Maskall *et al.*, 1977; Mathieu *et al.*, 1998; Sánchez *et al.*, 2010). Baudouin *et al.*, (2006) showed that in *M. truncatula* mature nodules the presence of NO was associated with the N₂-fixing zone, but not the meristematic, infection and senescence zones. More recently, Cam *et al.*, (2012) observed that in *M. truncatula* nodules NO is produced between the N₂-fixing and senescence zones at the end of the symbiotic process. Considered together, these observations mean that NO is present throughout the whole symbiotic process and the question is raised of its physiological roles in different times and spaces of symbiotic interaction (Hichri *et al.*, 2015, 2016).

The toxic, signaling, or metabolic roles of NO depend on its concentration at the action site (Mur *et al.*, 2013b). Therefore, its concentration must be tightly controlled. In plants, NO removal was mainly ascribed to hemoglobins (Hbs) (Gupta *et al.*, 2011b). Plant Hbs, recently renamed phytoglobins (Phytogbs), have been classified into six categories including: Phytogb0 - nonsymbiotic hemoglobin (nsHb) present in algae, bryophytes and gymnosperms; Phytogb1 - class 1 nonsymbiotic hemoglobin (nsHb-1) and Phytogb2 - class 2 nonsymbiotic hemoglobin (nsHb-2) present in angiosperms; SymPhytogb - symbiotic hemoglobin (symHb) present in non-legume N₂-fixing plants; Lb - leghemoglobin (Lb) present in N₂-fixing legumes; and Phytogb3 - class 3 truncated hemoglobin (trHb) present in algae and land plants (Hill *et al.*, 2016). Based on their sequence, homology, and affinity for O₂, three types of Hbs were described in legumes and are expressed during N₂-fixing symbiosis: Phytogb1, Lb and Phytogb3 (Bustos-Sanmamed *et al.*, 2010). Due to their very high affinity for O₂ and NO (K_m 1-10 nM), Phytogb1 are capable of recovering traces of O₂ and NO to convert them to nitrate at very low O₂ concentrations (Gupta *et al.*, 2011b; Igamberdiev *et al.*, 2011). They were suggested to be responsible for maintaining the redox and energetic status of plant cells under hypoxia (Igamberdiev & Hill, 2009). In *L. japonicus* nodules, the overexpression of *LjHb1* reduces NO content and enhances the symbiotic N₂ fixation (Shimoda *et al.*, 2009), suggesting that reversible inhibition of nitrogenase is relieved by the scavenging of NO by LjHb1. Similarly, in the actinorhizal symbiosis between *Alnus firma* and *Frankia*, it was shown that Afns-Hb1, as a NO scavenger, could support the N₂ fixation (Sasakura *et al.*, 2006). Functional nodules are characterized by a microoxic environment, but energy is a requirement. In many root systems under microoxic conditions, NO production increases and is scavenged by Phytogb1 to generate ATP in a "Phytogb-NO" respiratory cycle (Gupta & Igamberdiev, 2011; Berger *et al.*, 2018b). In these conditions, the Phytogb-NO cycle contributes to the maintenance

of NAD(P)H/NAD(P)⁺ and ATP/ADP ratios in hypoxic cells and keeps their viability (Igamberdiev *et al.*, 2010). Accumulating data supports the existence of such a PhytoGb-NO cycle operation in legume nodules : a strong increase of LbNO complex formation is observed in nodules of soybean plants submitted to hypoxia (Meakin *et al.*, 2007; Sanchez *et al.*, 2010), and the inhibition of the PhytoGb-NO cycle strongly reduces the ATP/ADP ratio in *M. truncatula* nodules (Horchani *et al.*, 2011). Lbs accumulate at a millimolar concentration in the cytoplasm of infected nodular cells (Appleby, 1992). They are considered as markers of N₂-fixing symbiosis and their protein level correlates with the N₂-fixation activity of the nodules (Appleby, 1992). It is now accepted that Lbs buffer free O₂ in the nanomolar range, thus avoiding the inactivation of nitrogenase while maintaining a high flux of O₂ for respiration (Ott *et al.*, 2005). It has also been shown that deoxy-Lb binds to NO with high affinity to form stable complexes in soybean and that Lb could act as a scavenger of NO and peroxyxynitrite (Herold & Puppo, 2005). PhytoGb3 have been shown to be present and induced in *M. truncatula* (Vieweg *et al.*, 2005) and *Frankia* (Niemann & Tisa, 2008; Coats *et al.*, 2009) N₂-fixing symbiosis. Based on their expression pattern, they have been proposed to be involved in NO scavenging.

Although analyzed at certain time points of the N₂ -fixing symbiosis, neither kinetic analysis of NO production nor PhytoGbs expression has yet been performed on the entire symbiotic process. In this work, we first identify the different PhytoGb genes present in the *M. truncatula* genome. Then, we address the PhytoGbs expression and the production of NO from the first hours of symbiotic interaction up to eight weeks post-inoculation, when the interaction breaks down. Last, we investigate the relationship between NO and PhytoGb expression at two time points of the symbiosis, i.e. at 4 days and 3 weeks post-infection. Based on our data, we discuss the potential roles of NO and of PhytoGbs during the different stages of symbiosis.

Materials and methods

Plants growth and inoculation conditions

Medicago truncatula (cv Jemalong A17) were scarified, sterilized and germinated as in del Giudice *et al.*, (2011). Seedlings were cultivated and inoculated with *Sinorhizobium meliloti* 2011 strain either in Petri dishes as described in del Giudice *et al.*, (2011), or in planters as described in Horchani *et al.*, (2011). Cultures in Petri dishes were used for short-term experiments, between 0 and 14 days post-inoculation (dpi), while those in planters were used for long-term experiments between 0 and 8 week post-inoculation (wpi). Roots and/or nodules were harvested at various times of the kinetics. For short-term experiments, gene expression

and NO production were analyzed in the 2 cm-long root segments around the inoculation zone, while for long-term experiments they were analyzed only in the nodules.

Measurement of NO production

Detection of NO was performed using the 4,5-diaminofluorescein probe (DAF-2) (Horchani *et al.*, 2011). Segments of 2 cm roots or nodules were incubated in 1 ml of detection buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl) in the presence of 10 μ M DAF-2. The production of NO was measured with a spectrofluorimeter (Xenius, SAFAS, Monaco) every 30 min for 4 hours after addition of the probe. The production of NO was then normalized with the weight of the sample.

Nodule surface analysis

At 14 dpi, nodules from Petri dishes were harvested, numbered and placed on agar medium 1% (w/v) to be photographed. Nodule surfaces were analyzed using the ImageJ software. The image is transformed into a shade of gray (8 bit) and then black and white (binary). In this way nodules are transformed into a white surface that the software analyses by using the function "Analysis particles".

RNA isolation, reverse transcription and genes expressions

RNAs were isolated from 100 mg of frozen material ground in liquid N₂ using the RNazol following the manufacturer's recommendations (Sigma-Aldrich). RNA quality was checked and DNase treatment was carried out prior to the synthesis by GoScript reverse transcriptase (Promega) of the cDNAs. The RT-qPCR was made with Promega's Go-Taq qPCR master Mix kit (Promega A6001) according to manufacturer instructions. RT-qPCR data analysis were carried out using RqPCRBase, a R package working on R computing environment for analysis of quantitative real-time PCR data (Hilliou & Tran, 2013). The expression of the different genes was normalized against two housekeeping genes *Mtc27* (Van de Velde *et al.*, 2006) and *Mta38* (del Giudice *et al.*, 2011). RT-qPCR analyses were carried out in triplicate, using the primers reported in Table 2.1. The reference value "1" was attributed to the first time when the Ct of the analysed gene was significantly detectable. Comparative expression levels between genes are given on a log scale expressed as $40 - \Delta C_T$, where ΔC_T is the difference in qRT-PCR threshold cycle number between the respective gene and the reference gene; the number 40 was chosen because PCR run stops after 40 cycles (Bari *et al.*, 2006; Truong *et al.*, 2015).

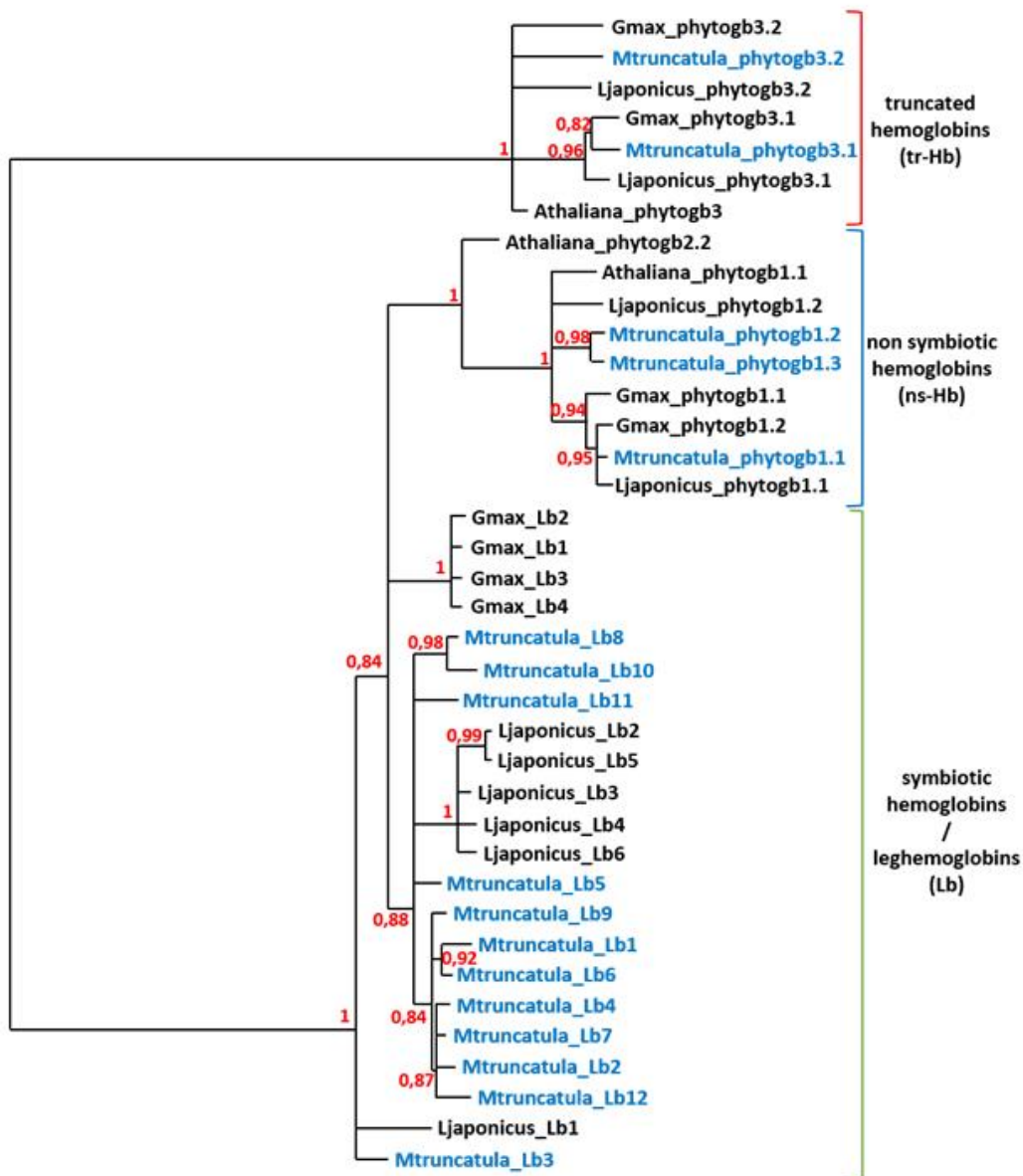


Figure 2.1: Phylogenetic tree of *Medicago truncatula*, *Lotus japonicus*, *Glycine max* and *Arabidopsis thaliana* phytoglobins.

Medicago Phytoglobin sequences were extracted from Noble database with Mt4.0 genome version. Phytoglobin sequences from *Glycin max*, *Lotus japonicus* and *Arabidopsis thaliana* were extracted from NCBI database. Phytoglobin protein sequences were aligned with MUSCLE program and the phylogenetic tree was constructed by maximum likelihood method using PhymL (<http://www.atgc-montpellier.fr/phymL/>). Nodes with bootstrap values less than 80% were collapsed into polytomies.

NO donor treatments

Plants in Petri dishes were treated with 0.5 mM of either diethylamine-NONOate (DEA-NO) or DEA sterile solutions. Two hundred µl of solution were added along the whole length of the roots, 2 h before inoculation with *S. meliloti*, and then every 24 h during 4 days. Control plants were treated with sterile water. After 4 days, plants were either analyzed for gene expression, or transferred on a NO donor free medium and grown for 10 additional days before measurement of nodule number, area and weight.

Induced senescence treatments

Induced senescence was performed on 4 wpi-old plants through either dark or nitrate treatments. Dark treated plants were let in the dark. Nitrate treatment was performed by adding 10 mM KNO₃ to the nutrient solution. Nodules were collected 48h later.

Phylogeny

The phylogeny data were obtained using the one-click mode of the website (Dereeper *et al.*, 2008) which includes a sequence alignment using the MUSCLE and Gblocks programs. Phylogenetic reconstruction was done with the PhyML program using the maximum likelihood method. *M. truncatula* sequences are listed in supplemental data (Table S1). Nodes with a robustness of less than 80% were pooled in the same phylogenetic subgroup.

Results

***Medicago truncatula* phytoglobins**

In the present work, we use the new nomenclature of plant Hbs, called phytoglobins (Phytogbs), that was internationally proposed (Hill *et al.*, 2016). Research in genomic and protein libraries (JCVI, <https://www.jcvi.org/medicago-truncatula-genome-database>; NCBI, <https://www.ncbi.nlm.nih.gov/>; UniProt, <https://www.uniprot.org/>) revealed that the *M. truncatula* genome contains 17 Phytogb encoding genes. Three of them encode for Phytogbs1, 12 encode for Lbs, and two for Phytogb3. There is currently, in the literature and databases, some confusion in the name and nomenclature of the *M. truncatula* Phytogb genes. Thus, we propose to homogenize their nomenclature. Affimetrix code, genes code, and Symbimix accession code (Roux *et al.*, 2014) of the 17 Phytogb genes are listed in Table S1. Phylogenetic analysis of protein sequences gives an overview of *M. truncatula* Phytogbs compared to two

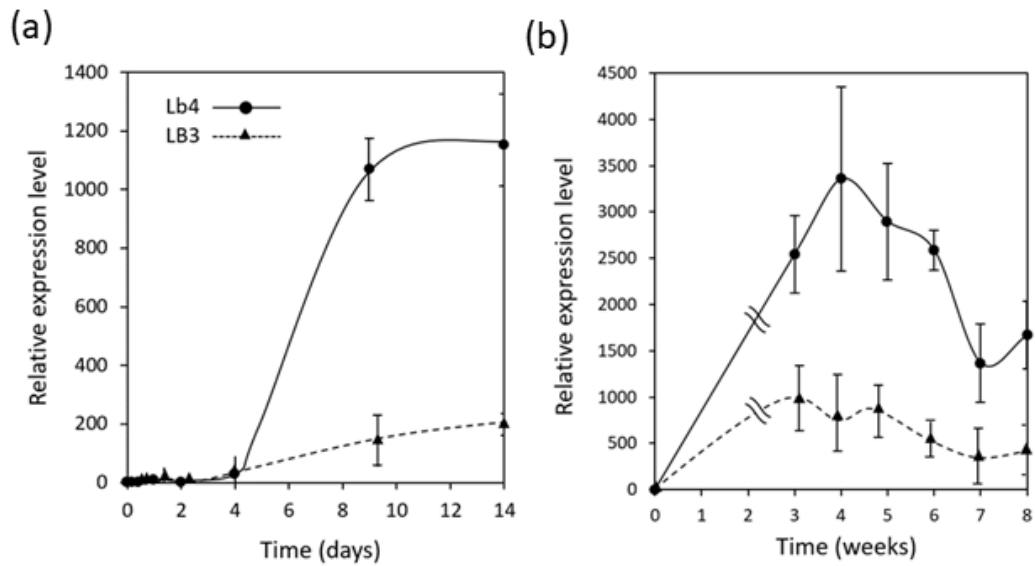


Figure 2.2: Expression of Lb genes during the symbiotic process

Expression analysis of *Lb3* and *Lb4* genes in roots and nodules during the symbiotic process. Short term kinetic 14 dpi (a), long-term kinetic 8 wpi (b). Values are means \pm SE of three biological replicates, each with three technical replicates.

legume plants, *G. max* or *L. japonicus*, and the non-legume plant *A. thaliana* (Fig. 2.1).

Whereas *G. max* and *L. japonicus* were found to possess 4 and 6 *Lbs* genes, respectively, *M. truncatula* possesses 12 *MtLb* genes. This large number of *Lbs* with distinct protein sequences (Fig. S1) and Affimetrix expression patterns (Fig. S2) highlights the still unresolved but different roles and locations of each of them within the *M. truncatula* nodule. The dendrogram (Fig. 2.1) shows that 11 of the 12 *Lbs* genes are phylogenetically closely related and come from relatively recent duplication processes (Anderson *et al.*, 1996). *Mtruncatula_Lb3* (*MtLb3*) which is different from the other *Phytogbs* is phylogenetically close to *Ljaponicus_Lb1* (Fig. 2.1) and the question arose as to whether *MtLb3* is a *Lb* or a *Phytogb1* gene. Based on protein sequence homologies (Fig. S1) and Affimetrix expression pattern (Fig. S2), we classified it as a *Lb*. Three *Phytogb1* genes were identified in *M. truncatula*, as compared with 2 in *G. max* and *L. japonicus*. The two truncated *Phytogb3* genes, previously identified as *MtTrHb1* and *MtTrHb2* by Vieweg *et al.*, (2005), are also present in the three legume species.

Phytoglobulin genes expression during the symbiotic process

In previous reports, *Phytogbs* expression has been investigated at specific time-points of the N₂-fixing symbiotic process, but not throughout the whole process from the inoculation to the nodule senescence. To this end, we used two types of *M. truncatula* cultures: a short-term culture from 0 to 14 dpi, and a long-term culture from 0 to 8 wpi.

The analysis of Affimetrix and Symbimix data (Fig. S2, Table S1) showed that the 12 *Lb* genes exhibit a similar expression pattern and are expressed in interzone II-III and zone III in 2 wpi-old nodules. Therefore, in order to avoid analyzing the expression of the 12 *Lb* genes, we used *Lb4*, whose expression is average among the different *Lbs*, as a representative *Lbs* marker (Fig. S2). *Lb4* expression remained close to the detection limit up to 2 dpi (Fig. 2.2a). Then, its expression strongly increased (3500 times) during nodule development to reach a maximum between 3 and 5 wpi, when the N₂-fixation activity of *M. truncatula* nodules is maximal, and finally decreased when the nodule enters in senescence between 6 and 8 wpi (Fig. 2.2b). To clarify the status of *Lb3*, its expression pattern was also analyzed during the symbiotic process (Fig. 2.2a ; 2.2b). Similarly to *Lb4*, it was expressed at very low levels up to 2 dpi, strongly increased to reach a maximum between 3 and 5 wpi, and then decreased towards 6-8 wpi. Such a pattern was an additional argument in favor of the classification of *Lb3* in the *Lb* type. Following inoculation, *Phytogb1.1* exhibited two transient overexpression peaks at 10 hpi and 4 dpi, a 5-fold increase between 1 and 5 wpi, and then a 16-fold increase after 8 wpi at

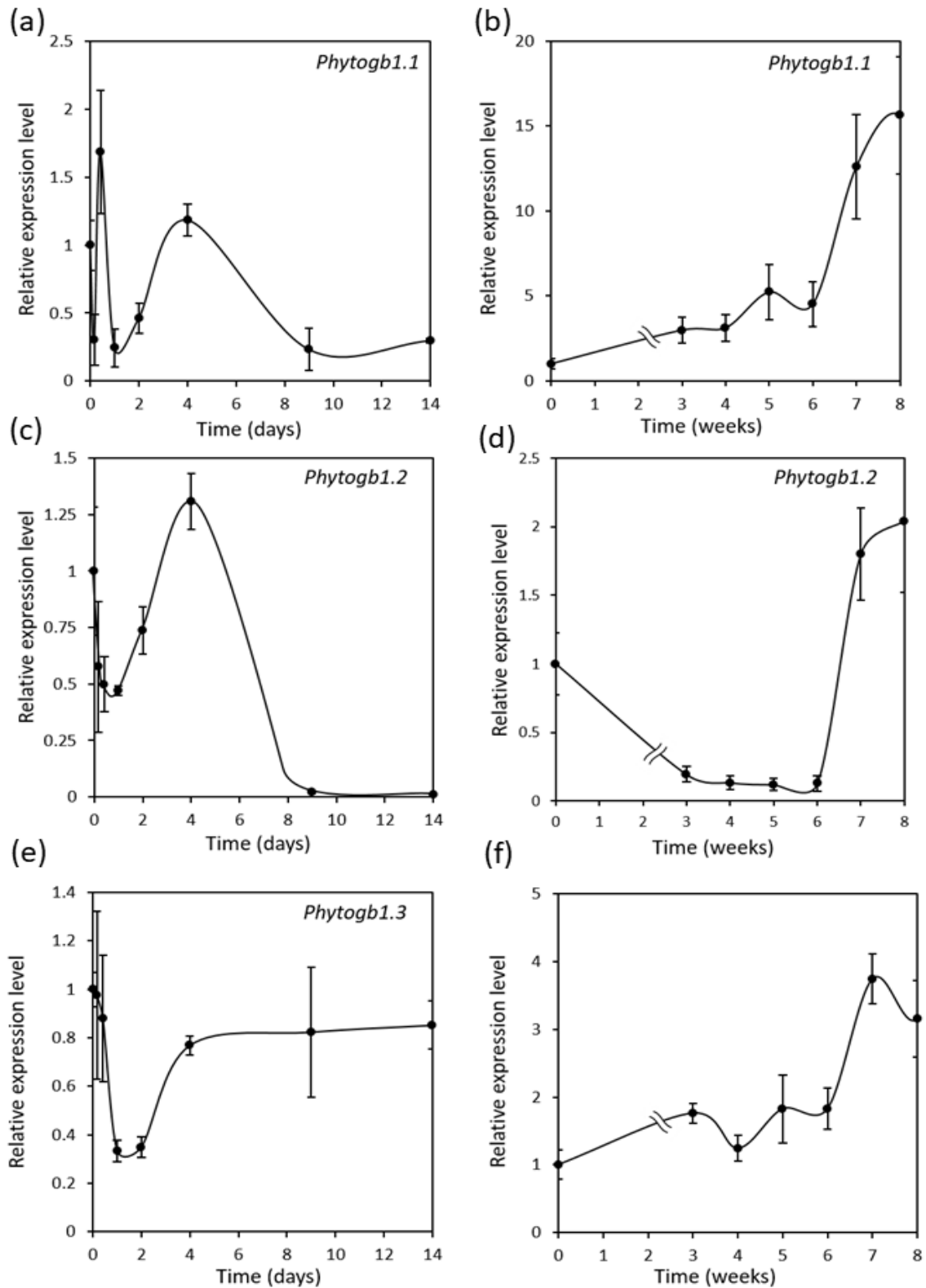


Figure 2.3: Expression of *PhytoGb1* genes during the symbiotic process

Expression analysis of *PhytoGb1* genes in roots and nodules during the symbiotic process. Short term kinetic 14 dpi (a, c, e), long-term kinetic 8 wpi (b, d, f). Expression of *PhytoGb1.1* (a,b) *PhytoGb1.2* (c,d) and *PhytoGb1.3* (e,f). Values are means \pm SE of three biological replicates, each with three technical replicates.

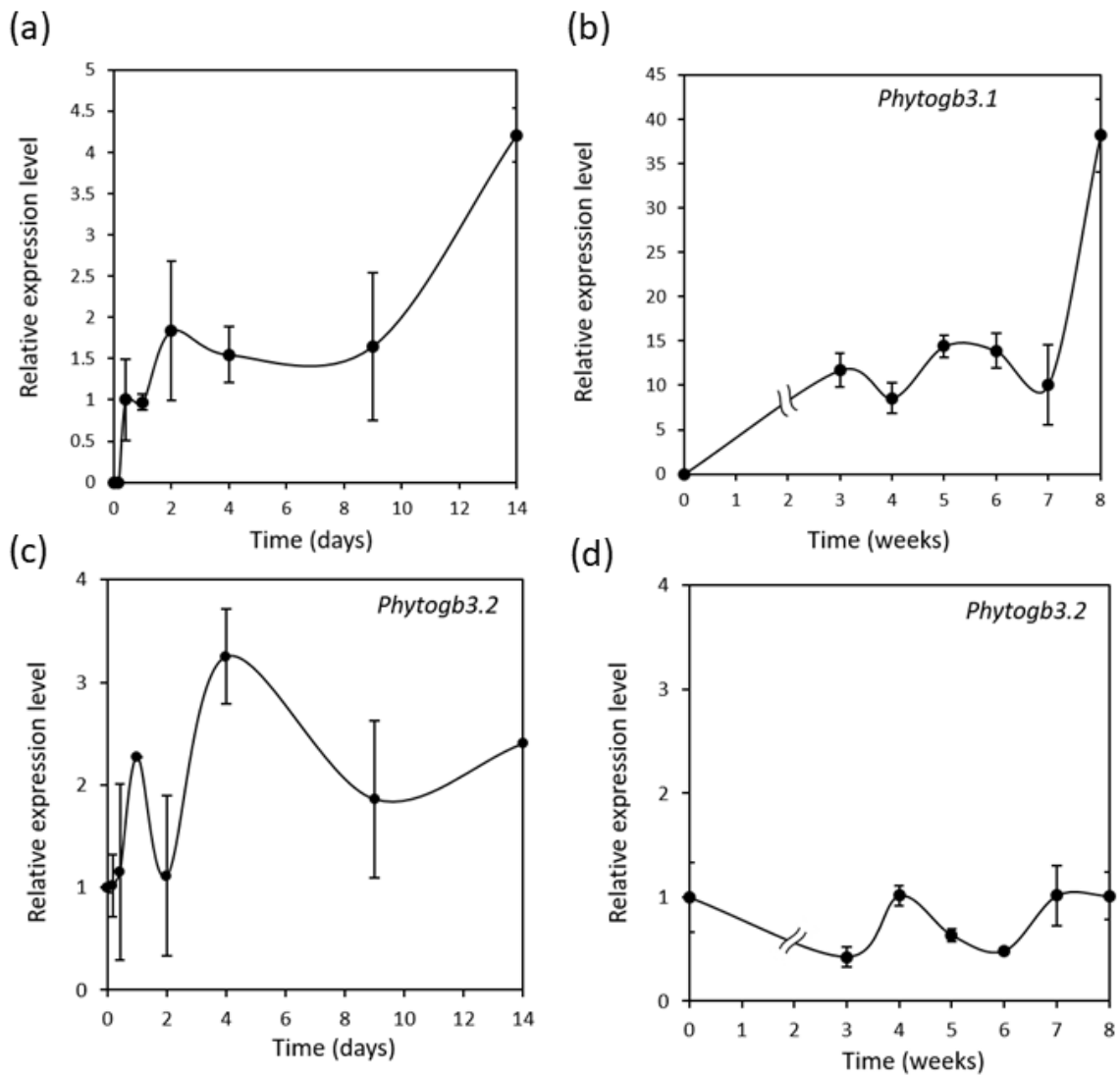


Figure 2.4: Expression of *Phytogb3* genes during the symbiotic process

Expression analysis of *Phytogb3* genes in roots and nodules during the symbiotic process. Short term kinetic 14 dpi (a, c), long-term kinetic 8 wpi (b, d). Expression of *Phytogb3.1* (a,b) and *Phytogb3.2* (c,d). Values are means \pm SE of three biological replicates, each with three technical replicates.

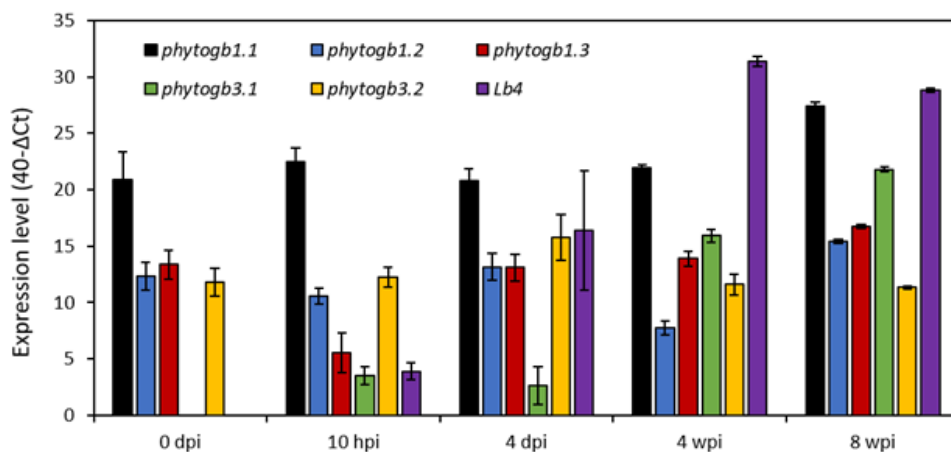


Figure 2.5: Expression of Phytoglobin genes at various times of the symbiotic process

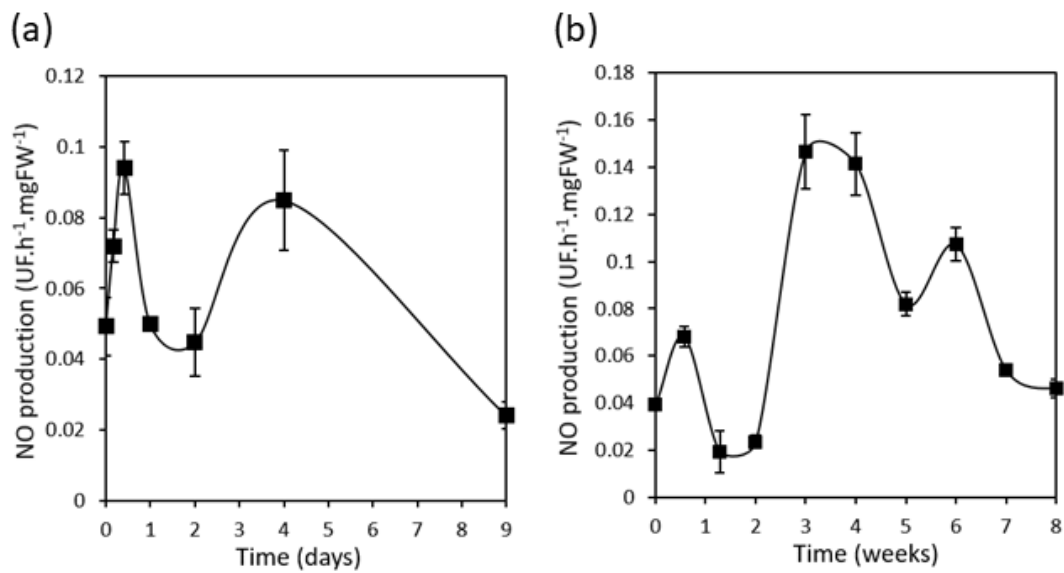


Figure 2.6: NO production during the symbiotic process. Short term kinetic 14 dpi (a), long-term kinetic 8 wpi (b). The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe. Values are means \pm SE of three biological replicates.

the setup of nodule senescence (Figs. 2.3a and b). After a decrease during the first hours of the interaction, *Phytogb1.2* expression transiently peaked at 4 dpi and finally strongly increased at 7-8 wpi in senescent nodules (Fig. 2.3c and d). The expression of *Phytogb1.3* only moderately changed up to 6 wpi and then peaked at 7 wpi when senescence is initiated (Fig. 2.3e and f). *Phytogb3.1* expression was undetectable in non-inoculated roots, but was rapidly induced at 10 hpi. Its expression remains steady up to 9 dpi, then increases to reach a plateau between 3 and 7 wpi, and finally increased strongly at 8 wpi (Fig. 2.4a and b). Except for a peak at 4 dpi, the expression of *Phytogb3.2* only moderately fluctuated and remained quite stable during the whole symbiotic process (Fig. 2.4c and d).

The expression level of *Phytogb* genes as compared to each other, before inoculation and at four time-points in the symbiosis, is represented in Fig. 2.5 using a logarithmic scale. In non-inoculated roots, *Phytogb1.1* is the most strongly expressed and it remains highly expressed throughout the symbiotic process. As expected, *Lb4* is the more highly expressed *Phytogb* in 4 wpi-old N₂-fixing nodules. It is interesting to note that, while *Phytogb1.2*, *1.3* and *3.2* are constitutively expressed in roots and nodules, *Phytogb3.1* is practically undetectable in non-inoculated roots and becomes one of the most expressed in mature nodules, suggesting that it plays a particular role in N₂-fixing nodules. Finally, it may be also noted that, with the exception of *Phytogb3.2*, all the *Phytogb* genes analyzed in this study were highly expressed in the senescent nodules at 8 wpi.

Considered globally, four periods may be distinguished from this kinetic: 1) around 10hpi, during the first hours of the interaction between the plant and the bacteria, characterized by an expression peak of *Phytogbs1.1*, 2) at 4 dpi, at early nodule development, characterized by an increase in the expression of all the *Phytogb* genes (with the exception of *Phytogb3.1*), 3) a period between 3 and 5 wpi, when nodule reaches maturity, marked by the strong expression of *Lbs* and 4) beyond 6 wpi, at the onset of nodule senescence, a period accompanied by a strong increase in the expression of *Phytogb1.1*, *1.2*, *1.3* and *3.1*, while that of *Lb genes* decrease.

NO is produced during the whole symbiotic process

NO production was analyzed from 0 to 8 wpi. As shown in Fig. 2.6, it shows four production peaks, at 10 hpi, 4 dpi, 3-4 wpi and 6 wpi, corresponding to the four periods characterized in relation to *Phytogbs* expression. Such pattern highlights the close relationship between the NO level and *Phytogbs* that has been reported in the literature

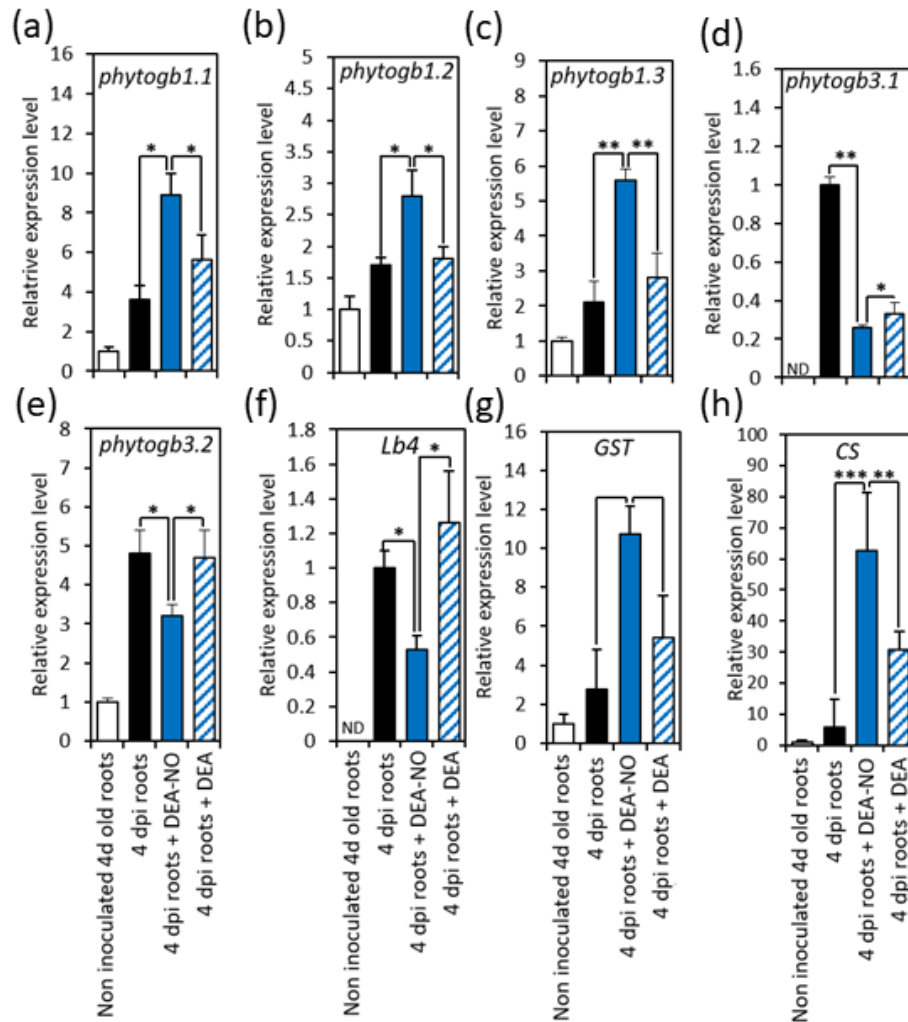


Figure 2.7: Phytoglobin and defense genes expression after 4 days of NO donor treatment. Plant roots were either inoculated with *S. meliloti* in the presence or absence of 0.5mM DEA/DEA-NO, or not inoculated (Control), and grown for four days before ARN extraction and analysis. Values are means \pm SE of three biological replicates, each with three technical replicates. Asterisks indicate statistical differences at P< 0.05 *, P<0.01 **, and P<0.001 ***, according to the Student's *t*-test.

(Hichri *et al.*, 2015; Berger *et al.*, 2018b), and raises the question of the reciprocal role played by NO and PhytoGBs in their respective regulation.

PhytoGB gene expression and nodulation phenotype in roots treated with NO donors.

Previous work showed that the addition of the NO scavenger cPTIO to *M. truncatula* roots during the first 4 days post-infection significantly reduces the production of nodules (del Giudice *et al.*, 2011), suggesting that NO plays a positive role in the set-up and the development of nodules. However, opposite results were observed in cPTIO-treated *L. japonicus* roots (Fukudome *et al.*, 2016). To clarify this contradiction, the effects of the NO donor DEA-NO were analyzed on PhytoGB gene expression and nodule setup. PhytoGB gene expression was analyzed immediately after the treatment in 4 dpi roots, and the effects of NO on nodule number, area, and weight were measured at 14 dpi. As reported in Fig. 2.7, a DEA-NO treatment up-regulates the expression of *PhytoGB1* genes, whereas it down-regulates that of *PhytoGB3* and *Lb4* genes. Considering that the induction of *PhytoGB3.1* and *Lb4* genes is associated with the establishment of N₂-fixation mechanisms during nodule development (Fig. 2.2 and 2.4), this suggests that an excess of NO inhibits and/or delays the process of nodule set up (Fukudome *et al.*, 2016). The expression of both glutathione S-transferase (GST) and chalcone synthase (CS), two genes known to be induced by NO in 4 dpi roots (Boscari *et al.*, 2013a) and used as positive controls, is up-regulated by DEA-NO treatment confirming the efficiency of the treatment. As compared with its control, a 4-day treatment with 0.5 mM DEA-NO triggers a decrease in nodule number per plant and in nodule growth, as measured by their surface area, but the total mass of nodules per plant remains unchanged (Table 2.2). These results mean that NO first negatively regulates the process of nodule establishment, but subsequently stimulates nodule development, probably through systemic regulation to compensate for their decreased number (Fergusson *et al.*, 2010).

PhytoGB genes expression and NO production in senescent nodules

After a study on the occurrence of NO production during the senescence of *M. truncatula* nodules, Cam *et al.* (2012) suggested that NO is a signal in developmental as well as stress-induced nodule senescence. The results of the present study show a transient NO production peak at 6 wpi (Fig 2.6b), which could be related to the beginning and the set-up of global nodule senescence. Thus, we aimed at analyzing the expression of PhytoGBs related with the production of NO during senescence. Previous work on nodulated *M. truncatula* plants report that a prolonged exposure to either dark or nitrate treatment triggers a rapid and global

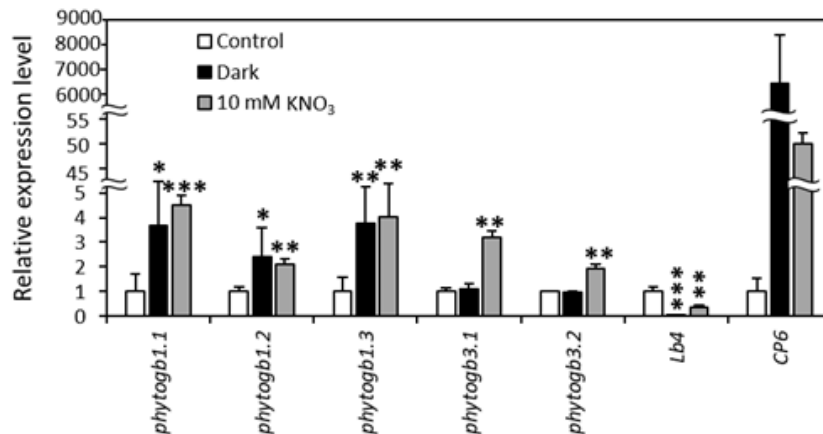


Figure 2.8: Phytoglobin and CP6 genes expression after induced-senescence treatment. Three wpi-old plants were treated for 48h with either darkness (black), or 10 mM KNO₃ (grey) treatment and compared to control plants (white). Gene expression levels are quantified relative to their level of expression in control (set to 1). Values are means \pm SE of three biological replicates, each with three technical replicates. Asterisks indicate statistical differences at P < 0.05 *, P < 0.01 **, and P < 0.001 ***, according to the Student's *t*-test.

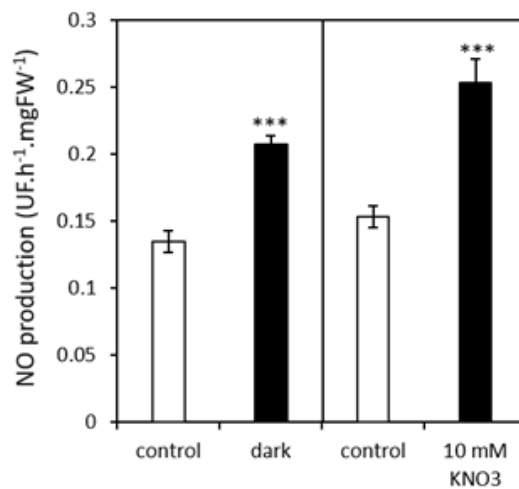


Figure 2.9: NO production after induced-senescence treatment. Three wpi-old plants were treated for 48h with either darkness, or 10 mM KNO₃ treatment and compared to control plants. The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe. Values are means \pm SE of three biological replicates, each with three technical replicates. Asterisks indicate statistical differences at P < 0.001 ***, according to the Student's *t*-test.

senescence of the whole nodule (Puppo *et al.*, 2005a; Perez Guerra *et al.*, 2010). Hence, we assessed the effects of a 2-day period of either darkness or 10 mM KNO₃ treatment on *Phytogb* gene expression and NO production in 4 wpi *M. truncatula* nodules.

The analysis of *Phytogb* gene expression after dark stress (Fig 2.8) shows that *Phytogb1* genes are induced by a factor 2.5 to 3.5 by stress, while *Phytogb3* genes are unaffected and that *Lb4* is strongly repressed (> 10-fold). Gene expression analysis also shows an induction of more than 6,000-folds of *MtCP6*, a cysteine protease marker gene for nodule senescence. As shown in Fig. S3, CP6 is strongly expressed during developmental senescence in 7-8 wpi-old nodules as compared to control roots during the symbiotic process. Treatment with KNO₃ induces the over-expression of *Phytogb1* and *Phytogb3* genes. As compared to dark treatment, the lower repression of *Lb4* (3-folds) and induction of *CP6* (45-folds) indicate that nodule senescence after nitrate stress is less marked than after dark stress. Interestingly, in response to both stress, NO production of nodules increases to the same extent, by a factor of about 1.6 (Fig. 2.9). These results show that the expression of *Phytogb1* genes and the production of NO increase similarly in response to both induced senescence treatments. However, the extent of nodular senescence, evaluated on the basis of the expression of both *CP6* and *Lb4*, is not the same as a function of either the darkness or the KNO₃ treatment (i.e. *CP6* is 125-fold more expressed in response to darkness than to nitrate), and the expression of *Phytogb3* genes is not regulated in the same way by darkness and nitrate.

Discussion

Since the discovery of leghemoglobins in soybean (Kubo, 1939), many reports have reviewed the different classes of *Phytogb*. The three *Phytogb* classes characterized in legumes are expressed in nitrogen-fixing nodules (Bustos-Sanmamed *et al.*, 2010). The first objective of this study was to make a phylogenetic survey of the *Phytogb* genes present in *Medicago truncatula*. We have identified 17 *Phytogb* genes composed by 12 *Lb*, 3 *Phytogb1* and 2 *Phytogb3*. *Lbs* are relatively close between them, but phylogenetically (Fig. 2.1) and structurally (Fig. S1) different from the other *Phytogb* types (Vinogradov *et al.*, 2006), suggesting different functions of the other *Phytogbs* in the symbiotic process. One particularity observed is the high number of *Lb* genes compared to other legumes species such as *G. max* or *L. japonicus* for which we identified maximum 6 genes. The number of legumes genomes fully sequenced remains weak and does not allow to draw the conclusion that difference could be

related to the type of nodules, determinate (Soybean and *L. japonicus*) or indeterminate (*M. truncatula*). The question of the role of the Lb diversity in legumes plants is not fully figured out even if it was proposed that abundance of Lbs could be one of the cornerstones necessary for the functioning of a “Phytogb/NO” cycle in the microaerobic conditions prevailing in nodules (Igamberdiev & Hill, 2004).

Regarding the NO, a number of studies have shown that it is produced at different stages of symbiosis (Hichri *et al.*, 2015), although no study specifically determined its production pattern during the entire symbiotic process. The second objective of this study was to analyze the expression of Phytogbs in parallel with the production of NO, from the rhizobial infection of *M. truncatula* roots to the set-up of nodule senescence, with the aim of 1) identifying the steps of the symbiotic process where NO is more strongly produced, and 2) describing the Phytogbs expression pattern and identifying those that would be involved in NO regulation. With respect to Phytogbs and NO, our results (Figs 2.2 to 2.6) show that four distinct periods can be considered: i) at 10 hpi, during the first hours of the interaction between the plant and the rhizobia, ; ii) at 4 dpi, corresponding to the early development of the nodule (Xiao *et al.*, 2014); iii) at 3-4 wpi, when the N₂-fixation zone ceases to grow and the nodule reaches the status of "mature N₂-fixing nodule" (Ferguson *et al.*, 2010), and iv) after 6 wpi, corresponding to the global onset of nodule senescence (Puppo *et al.*, 2005a).

The 10 hpi peak (Fig. 2.6) may be related to the NO produced by the first layers of epidermis cells (Hichri *et al.*, 2016). A similar short-term and transient NO production peak was observed at the *L. japonicus* root surface 4 hpi with *Mesorhizobium loti*, but not with the pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* with which NO was continuously produced for at least 24h (Nagata *et al.*, 2008). The decrease in NO level observed after its transient accumulation following infection with *M. loti* was assigned to *LjHBI* which gene expression was upregulated by the symbiont, but not by the pathogens (Nagata *et al.*, 2008). It was concluded that at early step of the symbiotic interaction the initial burst of NO induces the expression of *Phytogb1.1* that scavenges NO and down-regulates its level to lower plant defense response and allows the reception of the symbiont (Nagata *et al.*, 2009). At this stage in our study, *Mtphytogb1.1* is much more expressed than the other Phytogb genes (Fig. 2.5) and it is likely to be involved in the regulation of NO during very early interaction with rhizobia, as already demonstrated in *L. japonicus* (Shimoda *et al.*, 2009; Fukudome *et al.*, 2016). However, it should be noted that *Phytogb3.1* is highly expressed in 10 hpi roots as

compared to non-inoculated roots (Fig. 2.4a) which suggests that it could also be involved in NO regulation.

The presence of a second peak of NO production at 4dpi (Fig. 2.6) raises the question of whether NO is necessary or toxic for the establishment of symbiotic interaction and the nodule development. In the *M. truncatula* - *S. meliloti* interaction, both the scavenging of NO by c-PTIO and the overexpression in the plant partner of the bacterial flavohemoglobin *hmp* involved in NO detoxification led to a delayed nodulation phenotype (del Giudice *et al.*, 2011), indicating that NO is required for an optimal establishment of symbiotic interaction and nodule development. However, *L. japonicus* mutants affected on *LjGlb1-1* showed increased NO levels and reduced nodule numbers when compared to control roots, suggesting that NO was deleterious for nodule production (Fukudome *et al.*, 2016). The contradictory roles of NO in the establishment and development of symbiosis were explained by differences in either the symbiotic models, the timing of the observations in the symbiotic process, or the specific activity of either Hmp or PhytoGBs (del Giudice *et al.*, 2011; Fukudome *et al.*, 2016; Hichri *et al.*, 2015). Using the same model and timing as del Giudice *et al.*, (2011), we observed that treatments of *M. truncatula* roots during 4 dpi with the NO donor (DEA-NO) resulted in a decreased nodule number at 14 dpi (Fig. 2.8). These data clearly mean that, independently of the symbiotic model, it is the level of NO in plant roots that regulates the process of symbiosis. The NO production peak observed at 4 dpi (Fig. 2.5) suggests that NO is involved in the set-up of nodular meristem and nodule growth. This is in agreement with the observations of Boscari *et al.*, (2013a) who showed that in 4 dpi-old *M. truncatula* roots, a short-term treatment of 8 h with cPTIO leads to the induction of defense genes and the repression of cell cycle, protein synthesis, and proteolysis genes. This indicates that at this step NO down-regulates plant defense genes and up-regulates the mechanisms of cell division and growth. However, a long-term treatment from 0 to 4 dpi with the DEA-NO results in the up-regulation of *GST* and *CS* genes (Fig 2.7). In this case, the treatment with DEA-NO maintains a high level of NO, mimicking a pathogen attack (Delledonne *et al.*, 1998; Nagata *et al.*, 2008), and resulting in an up-regulation of the plant defense mechanisms. This means that, depending on the timing of the symbiosis, NO regulates the expression of plant defense genes differently: up-regulating them during the establishment of the interaction and down-regulating them at the beginning of the nodule growth. Note that, except *PhytoGB3.1*, all *PhytoGB* genes are induced at 4 dpi. The induction of *Lb* genes (Fig. 2.2) is consistent with nodule development and the beginning of the N₂-fixation activity (Xiao *et al.*, 2014). Moreover, the increased expression of *PhytoGB1* genes, particularly *PhytoGB1.1* (Fig. 2.3) suggests that these genes are also involved in NO regulation

during nodular development, via their loop of regulation by NO. Indeed, our results show that, directly or indirectly, NO up-regulates the expression of *Phytogb1* genes, whereas it down-regulates those of *Lb* and *Phytogb3* (Fig. 2.7).

The third peak of NO production at 3-4 wpi corresponds to the highest NO production in the symbiotic process (Fig. 2.5). The biological significance of NO in the mature nodule has been a matter of debate (Boscari *et al.*, 2013b; Hichri *et al.*, 2015, 2016). On one side, NO was reported to be a potent inhibitor of nitrogenase activity, and to inhibit *in vivo* N₂-fixing activity in soybean, *L. japonicus*, and *M. truncatula* nodules (Trinchant & Rigaud, 1982; Shimoda *et al.*, 2009; Kato *et al.*, 2010; Cam *et al.*, 2012). On another side, Kato *et al.*, (2010) showed that in the presence of 0.1 mM SNP, N₂-fixation is higher than after treatment with a lower (0.01 mM) or higher (10 mM) concentration, illustrating that low but significant NO levels are necessary to N₂-fixation. Otherwise, functional nodules are characterized by a microoxic environment, raising the question of energy supply within this organ. Accumulated data support the existence of a Phytogb-NO respiration in legume nodules exposed to hypoxia, in which both mitochondrial and bacterial ETCs are involved (Meakin *et al.*, 2007; Sánchez *et al.*, 2010; Horchani *et al.*, 2011). These studies reveal that NO is required for optimal N₂-fixation, but the regulation of its concentration and its detoxification represent critical aspects of nodule metabolism.

As expected, the expression of *Lbs* strongly increases in mature nodules (Fig. 2), but that of *Phytogb1.1* and *Phytogb3.1* also (Figs 2.3 and 2.4). One wonders what are their respective functions within N₂-fixing nodules? The presence of Lb-NO complexes, detected by EPR, in soybean and *L. japonicus* nodules *in vivo* clearly shows that Lbs are involved in the complexation of NO. It is not possible to differentiate between Lb-NO, Phytogb1-NO, and Phytogb3-NO spectra *in vivo*, but considering the intensity of the resonances of EPR spectra related to 'Phytogb-NO' complexes (Mathieu *et al.*, 1998), and the high expression of *Lbs* in mature nodules (Table S1, Fig. 2.5), it is likely that the large majority of the EPR signal was directly related to the presence of Lb-NO complexes. It may be also noted that the higher level of *Lb* gene expression observed in interzone II-III rather than in zone III (Table S1) is consistent with the fact that NO represses *Lb* gene expression (Fig. 2.8), and that it is mainly produced in zone III (Baudouin *et al.*, 2006). The ability of Lb to bind O₂ and NO to produce NO₃⁻ (Herold & Puppo, 2005) makes them good candidates to detoxify NO and participate in the regeneration of energy in the plant compartment through the functioning of the 'Phytogb-NO' respiration (Horchani *et al.*, 2011). Although much less expressed than *Lbs*, the significant expression of *Phytogb1.1*, *Phytogb1.3* and *Phytogb3.1* in mature nodules (Figs 2.2 & 2.3) suggests that each

of them has its own function in the N₂-fixing metabolism. In mature nodules of *L. Japonicus*, the overexpression of *LjHb1* (*LjGlb1.1*) results in decreased NO production and increased ARA (Shimoda et al., 2009), whereas nodules of mutants affected on *LjGlb1.1* show higher NO production and lower ARA (Fukudome et al., 2016). Our data suggest that *Phytogb1.1* and *Phytogb3.1*, in which expression is also regulated by NO (Fig. 2.7) and increased in mature nodules (Fig. 2.3), would play a similar role in *M. truncatula*. In this plant, both *Phytogb3* genes have been shown to be induced during symbiosis, *Phytogb3.1* being expressed in the infected cells of the N₂-fixing zone, and *Phytogb3.2* more predominantly in the vascular tissue of the nodule (Vieweg et al., 2005). Here we show that *Phytogb3.1* is expressed at significant levels in mature nodules (Fig. 2.5, Table S1) and its strong induction as compared to non-inoculated roots (Fig. 2.4) argues in favor of a particular role of this Phytogb, alongside Phytogb1.1 and Lbs, in N₂-fixing metabolism.

Why three Phytogb types in the N₂-fixation zone? The answer is probably related to the gradients of pO₂ and pNO in mature nodules and to the relative affinity of the different Phytogbs for O₂ and NO. In *Medicago* nodules, due to the presence of the O₂ barrier, the pO₂ decreases from 250 μM in the first layers of the epidermal cells to approximately 10-40 nM in the infected cells in the heart of zone III (Soupène et al., 1995). Although the pNO gradient has never been measured, several studies showed that NO level is higher at the center of *Medicago* nodules than at their periphery with a concentration gradient inverse to that of O₂ (Baudouin et al., 2006; del Giudice et al., 2011; Cam et al., 2012). High respiratory rates are needed, to sustain the energy consuming N₂-fixing metabolism and metabolite transport between roots and nodules. In *M. truncatula* nodules, the regeneration of ATP and the maintenance of the energetic state of the nodules depends on the functioning of the 'Phytogb-NO' respiration (Horchani et al., 2011). Thus, it is likely that the 'Phytogb-NO' respiration gradually substitutes for the O₂-dependent respiration to regenerate ATP as pO₂ decreases toward the center of the nodule. Phytogbs have very different relative affinities for O₂ with KmO₂ on the order of 2, 50-100 and 1500 nM for Phytogb1, Lbs and Phytogb3, respectively (Smagghe et al., 2009; Gupta et al., 2011b). We can therefore make the hypothesis that the role of the different Phytogbs is both to scavenge NO and to allow the functioning of the 'Phytogb-NO' respiration as a function of the pO₂ gradient within the nodule: the Phytogb3 acting in the weakly microoxic peripheral cells, Lbs in microoxic cells, and Phytogb1 acting in strongly microoxic cells in the center of the nodules.

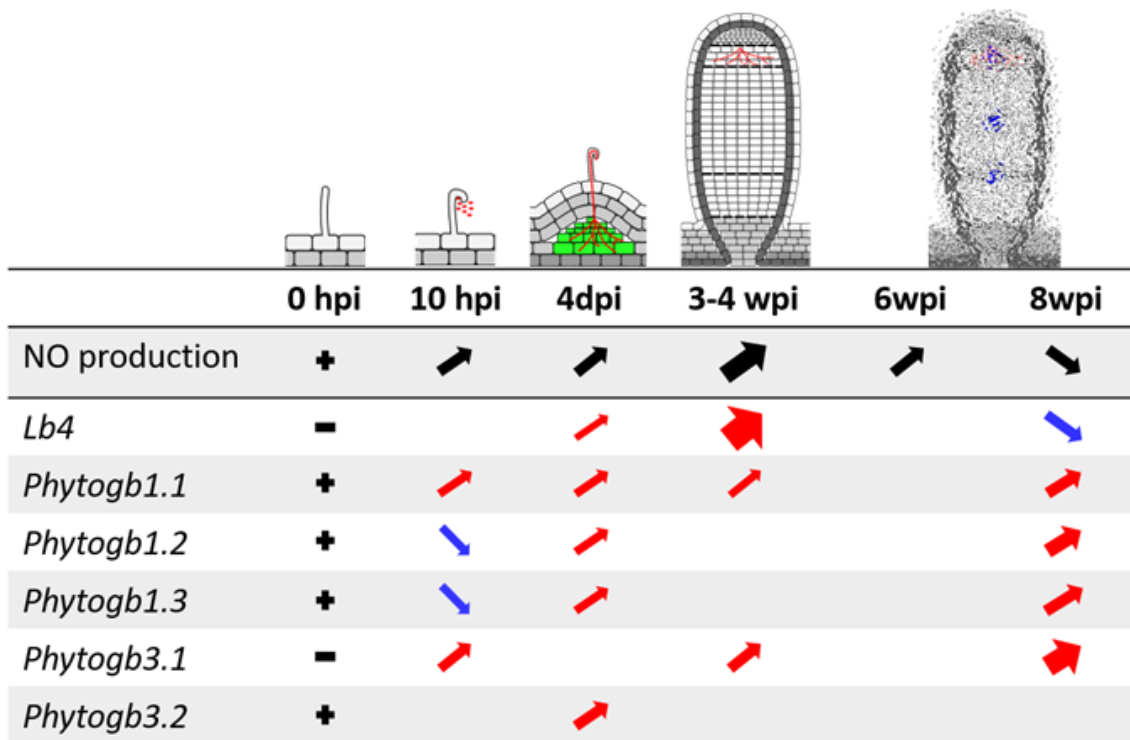


Figure 10: Synoptic representation of NO production and PhytoGb gene expression as a function of the different symbiosis steps. hpi; hour post-inoculation; dpi, day post-inoculation; wpi week post-inoculation. Either + or – indicates detectable or undetectable NO production/gene expression in non-inoculated roots. Blue and red arrows indicate respectively decreased and increased NO production/expression level. The width of the arrows symbolizes the extent of the changes observed.

Previously, NO was postulated as a positive regulator of nodule senescence (Cam *et al.*, 2012). In the present work, the peak of NO production observed at 6 wpi (Fig. 2.6), preceding the decreased expression of Lbs (Fig. 2.2), is in agreement with the assumption that NO could act as a regulator of senescence. The question arises as to whether NO either acts as a trigger for senescence, or is a consequence of the senescence process. Experiments of induced senescence, using either dark or 10 mM nitrate treatment, allow to put forward a beginning of answer. Indeed, both treatments result in a similar 60% increase in NO production (Fig. 2.9), but their effects on the expression of both *CP6* and *Phytogbs* genes are qualitatively and quantitatively different (Fig. 2.8): *Phytogb3* are induced only in response to nitrate but not to darkness, and *Lb4* and *CP6* are significantly more expressed in response to darkness than to nitrate. Thus, NO cannot be the trigger signal for senescence, but more certainly an element of the nodule response to the induced senescence treatments.

The high expression of *Phytogbs1* and *Phytogb3.1* (Figs 2.3 and 2.4) after 6 wpi was quite unexpected in a context where *Lb* expression and NO production decrease but remain globally high (Figs 2.2, 2.5 and 2.6). This strong expression of *Phytogb* genes may be related to oxidative stress and alteration of the redox state that occurs during nodule senescence (Puppo *et al.*, 2005a). Both ROS and NO have been suggested to be involved in the senescence of the nodule (Puppo *et al.*, 2013). NO can react with superoxide ($O_2^{\cdot-}$) to form peroxynitrite known to irreversibly inactivate proteins through selective tyrosine-nitration (Vandelle & Delledonne, 2011). *Lb* was shown to scavenge peroxynitrite, potentially precluding any damaging effect of this species in mature nodules (Herold & Puppo, 2005), and in soybean nodules *Lb* was found to be target of nitration *in vivo*, particularly during senescence (Navascues *et al.*, 2012). The induction of *Phytogb* genes in senescent nodules can therefore be interpreted as a means of renewing the *Phytogbs/Lbs* pool inactivated by the reactive nitrogen species in order to maintain the scavenging capacity of the latter while maintaining the N_2 -fixing capacity of the nodules.

In conclusion, this work shows the close relationship between NO production and *Phytogb* gene expression (Fig. 2.10) during the symbiotic process between *M. truncatula* and *S. meliloti*, and there is no doubt that NO level is regulated by the intervention of the different *Phytogbs* at each stage of the symbiosis. It remains to discover the particular role of each *Phytogb* in this regulation, but, it may be proposed that *Phytoglob1.1* plays a major role in this process. Otherwise, the control of NO in the nodule cannot be done only by the plant partner. Indeed, the *S. meliloti* flavohemoglobin, whose expression is induced both by microoxia and

NO, has been shown to be involved in NO degradation and to be essential in maintaining efficient nitrogen-fixing symbiosis (Meilhoc *et al.*, 2010; Cam *et al.*, 2012). Otherwise, the bacterial NO reductase (Nor) that catalyzes the reduction of NO into N₂O (nitrous oxide) in the denitrification pathway was shown to regulate NO level in *M. truncatula* – *S. meliloti* nodules (Meilhoc *et al.*, 2013). How the regulatory systems of the plant and the bacterial partners are coordinated to control NO is one of the main issues to decipher the toxic, signaling, and metabolic functions of NO at each stage of the symbiotic interaction.

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Author Contributions

A.Be., A.Bo. and R.B. planned and designed the research. A.Be. performed experiments, conducted fieldwork and analysed data. A.Be., A.Bo., A.P. and R.B. interpreted the data and wrote the manuscript.

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Table 1: Primer sequences for quantitative RT-PCR analysis

Name	code gene Mt4.0	Forward	Reverse	Reference
Mtc27	Medtr2g436620	TGAGGGAGCAACCAAATACC	GCGAAAACCAAGCTACCATC	Del guidice et al., 2011
Mta38	Medtr4g109650	TCGTGGTGGTGGTTATCAAA	TTCAGACCTTCCCATGACA	Del guidice et al., 2011
phytgb1.1	Medtr4g068860	CGGTAAAGTTCACGGTCAGAG	AAGTGCAAACCTTTGTCACCT	This work
phytgb1.2	Medtr4g068870	GGACAATGCCAATTGATAAGCAG	CTGGTGGAGCAATCTCAAGG	This work
phytgb1.3	Medtr0026s0210	TTCTCATGACATGTGAATCAGC	GTGACCACATTTCAGGTAATGC	This work
phytgb3.1	Medtr3g109420	CAACAGATACCATGCAAGCA	AAGCTGGGACCGATATACAG	This work
phytgb3.2	Medtr1g008700	AACTTTATAAGTTTCTTTTGTITG	GATAGACATATAGACGTTCAATCTT	This work
Lb3	Medtr1g090810	GTATAGCCTATGATGAAGTAGCAG	ATTTACTTACACAAGTGCTTGGA	This work
Lb4	Medtr1g011540	GAGCGAAGAATTGAGCACTGCT	TGCCTTCTTAATTGCAGTTGCC	This work
CP6	Medtr4g079800	CCTGCTGCTACTATTGCTGGATATG	CACTCGCATCAATGGCTACGG	Pierre et al., 2014
glutathione- S- transferase	Medtr7g071380	TTTGTTCACTAGTGAGAAATTTC	GAAGACTTTCATAACGAGCTTTAA	Boscari et al., 2013
chalcone synthase	Medtr2g035130	AAAGATGAAAGCCACCAGAG	AAACACCAAACCTCAAGTCCT	Boscari et al., 2013

Table 2: Nodule phenotype after 4 days of NO donor treatment. Plant roots inoculated with *S. meliloti* were treated with either 0.5 mM DEA-NO or 0.5mM DEA. After four days, plants were transferred on a NO donor free medium and grown for 10 additional days before measurement of nodule number, area and weight. Values are means \pm SE of three biological

replicates (with n > 140). Each data set was analyzed by one-way ANOVA followed by a Fisher test (P<0.05).

	Control	DEA-NO	DEA
Nodule number per plant	7.70 ± 0.43 a	5.61 ± 0.55 b	7.82 ± 0.39 a
Nodule size (mm ²)	46.92 ± 7.70 a	48.85 ± 5.60 b	51.95 ± 7.80 a
Nodule weight (mg/plant)	0.48 ± 0.045 a	0.95 ± 0.032 a	0.58 ± 0.086 a

Supplemental material

Table S1: Nomenclature, access code and symbionics expression of *Medicago truncatula* phytooglobins

Formal name	New nomenclature	code afyMetricx	code gene Mt4.0	Mt20120830-LIPM	DESEQ MEAN					
					FI	FIID	FIIP	I2	ZIII	
Leghemoglobins	Lb1	Mtr:27725.1.S1_s_at	Medtr5g080400	Mt0010_10133	18.8	65.8	515.5	126988.2	36232.2	
	Lb2	Mtr:40141.1.S1_at	Medtr5g066070	Mt0008_10574	112.8	905.6	4010	136478.7	137042.5	
	Lb3	Mtr:38572.1.S1_at	Medtr1g090810	Mt0039_00029	18.2	92.3	477	1413.4	1908.7	
	Lb4	Mtr:40134.1.S1_x_at	Medtr1g011540	Mt0002_10177	104.9	261.2	605.7	66480.1	38108.6	
	Lb5	Mtr:40138.1.S1_at	Medtr5g041610	Mt0012_10297	13.3	33.7	181.8	48344.6	24958.1	
	Lb6	Mtr:51231.1.S1_x_at	Medtr5g080440	Mt0010_10135	14.5	157.6	2343.9	111612	26444.4	
	Lb7	Mtr:31441.1.S1_at	Medtr5g081000	Mt0010_00186	104.1	1478.2	14585.5	185977	71579.4	
	Lb8	Mtr:8284.1.S1_s_at	Medtr1g090820	Mt0039_00027	21.2	197.8	5311.1	49348.4	14757.8	
	Lb9	Mtr:36094.1.S1_at	Medtr5g081030	Mt0010_00186	104.1	1478.2	14585.5	185977	71579.4	
	Lb10	Mtr:43465.1.S1_at	Medtr1g049330	Mt0002_11840	11.7	29.4	2188.5	52581.5	14066.4	
	Lb11	Mtr:36091.1.S1_at	Medtr7g110180	Mt0067_00016	1.5	1	84	766.9	145.9	
	Lb12	NC	Medtr5g080900	Mt0010_00174	0	0	0	19	4.8	
ns-Hb	phytoGb1.1	Mtr:10462.1.S1_at	Medtr4g068860	Mt0003_10767	2.4	1	2.2	152.1	167.5	
	phytoGb1.2	Mtr:29072.1.S1_s_at	Medtr4g068870	Mt0003_10768	20.6	5.1	1.8	7.9	11.7	
	phytoGb1.3	NC	Medtr002650210	Mt0218_10008	0	0.2	0	0	0	
tr-Hb	phytoGb3.1	Mtr:47990.1.S1_at	Medtr3g109420	Mt0007_00981	3.4	18.9	139.9	278.2	583.4	
	phytoGb3.2	Mtr:10341.1.S1_at	Medtr1g008700	Mt0002_00088	76.8	75.6	182.8	280.3	260.8	

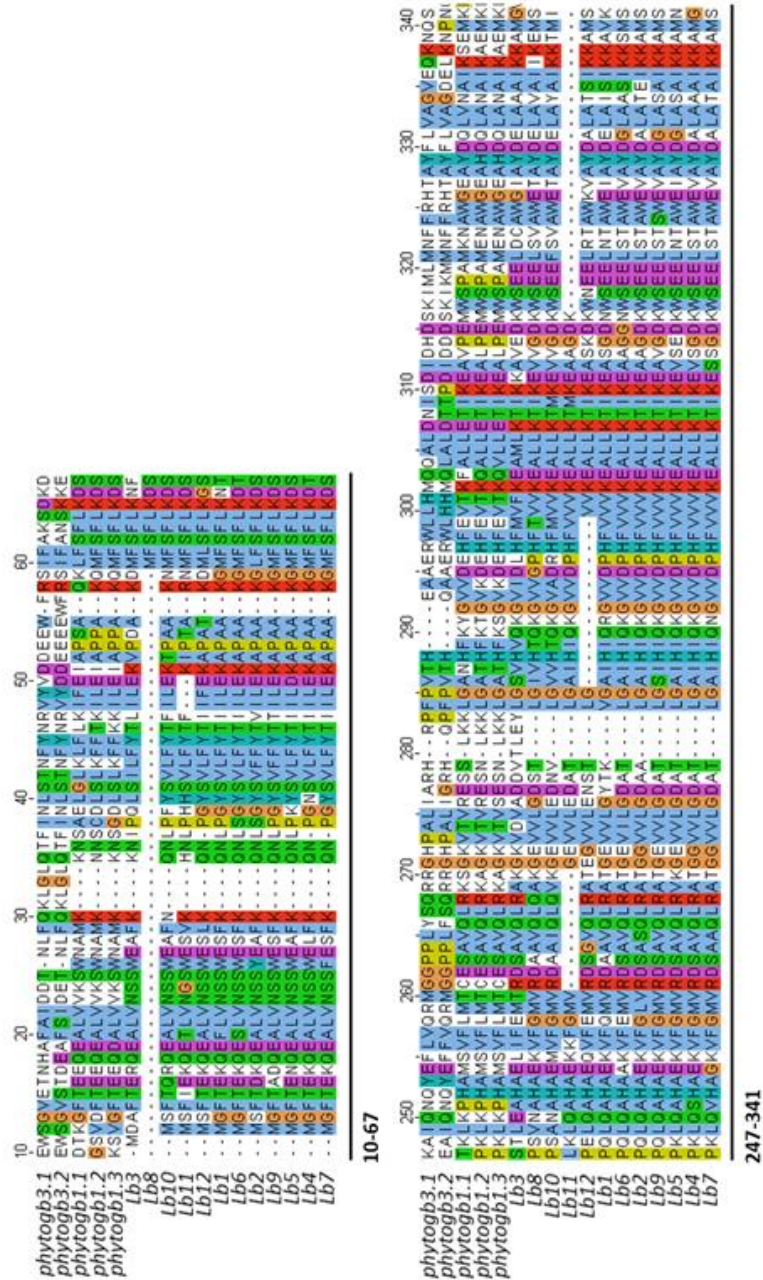


Figure S1: Multiple sequence alignment of *Medicago truncatula* Phytoglobins. Alignments of *M. truncatula* phytoglobin sequences computed by Muscle (<https://www.ebi.ac.uk/Tools/msa/muscle/>). ClustalX color code was used to highlight the protein segment properties: hydrophobic residue (AILMFW) in blue, acidic residue (DE) in magenta, basic residue (RK) in red, polar residue (QSNT) in green, other aromatic residue (YH) in dark blue and specific color for CP and G. Figure was generated using Jalview 2.7 (<http://www.jalview.org>).

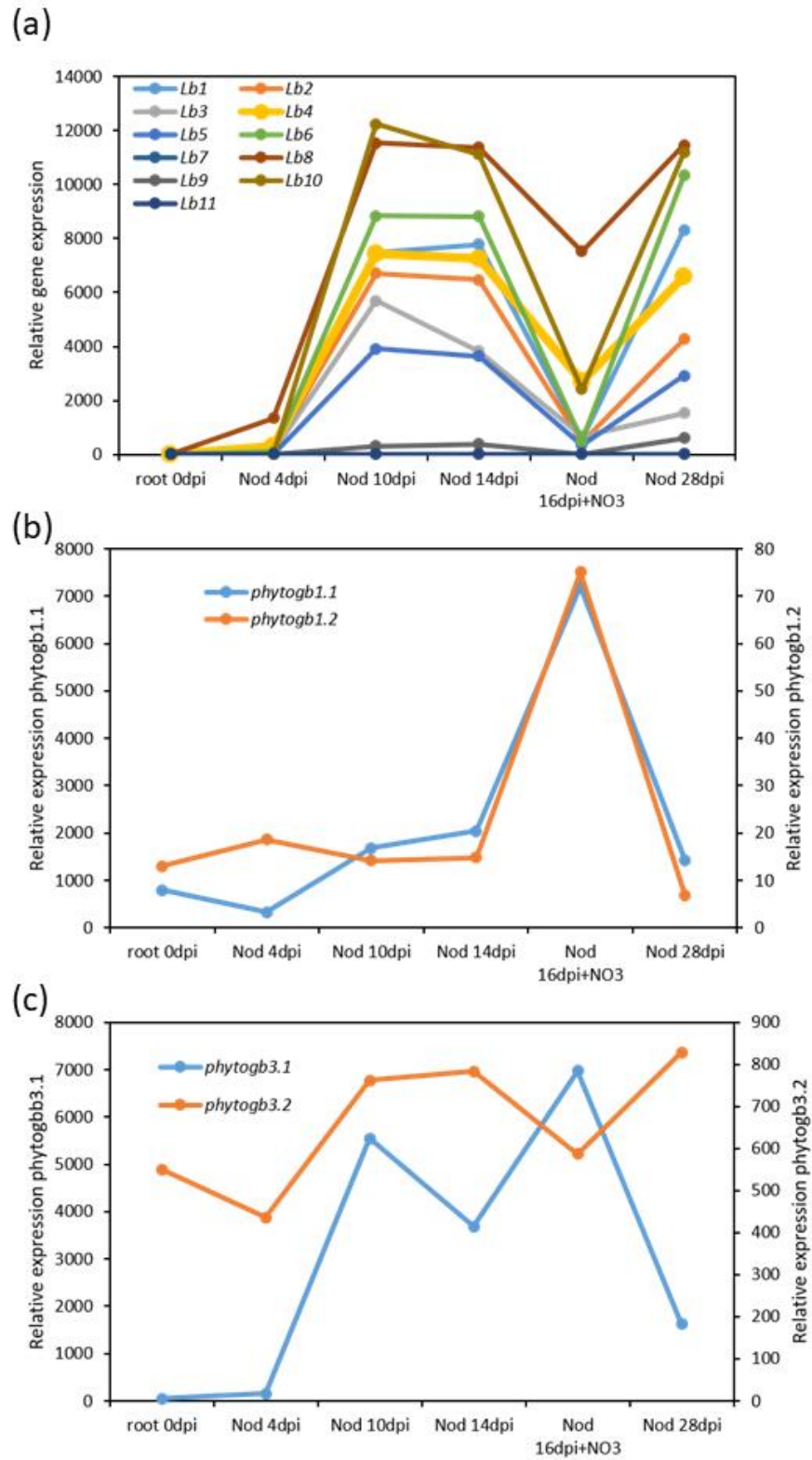


Figure S2: Microarray data *Medicago truncatula* phytoglobins genes

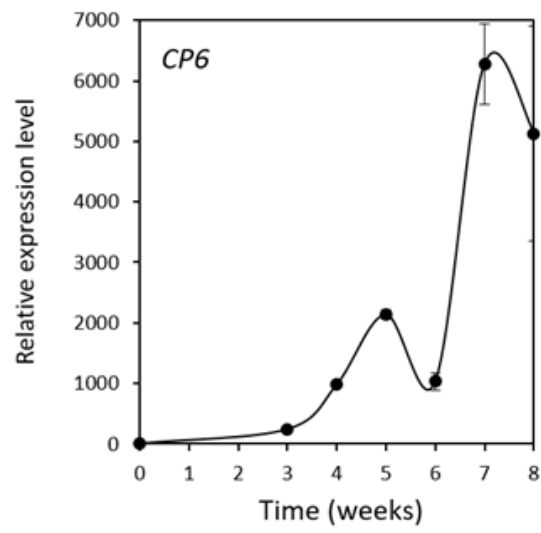


Figure S3 : Expression of *MtCP6* gene during the symbiotic process

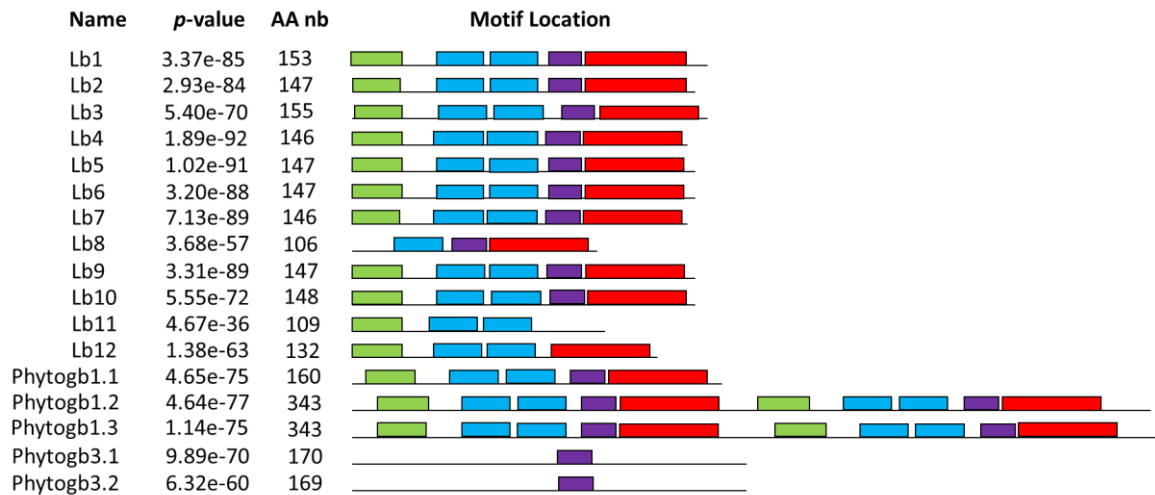


Figure 2.11: Modèles MEME des séquences primaires des phytoglobines de *M. truncatula*. Quatre motifs MEME sont identifiés par le logiciel sur les 17 protéines. La localisation de ces motifs, représentés par des rectangles de couleur sont alignés sur la séquence primaire des protéines schématisé par la ligne noire. La p-value de chaque modèle MEME des protéines est indiquée ainsi que la longueur en acides aminés.

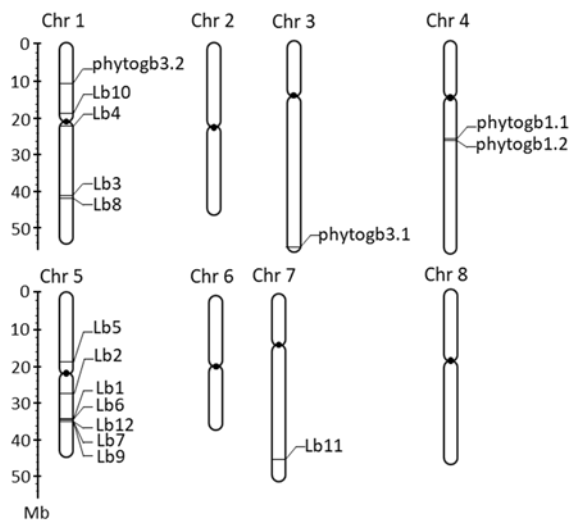
3. Résultats complémentaires et discussion

3.1. Analyse *in silico* des séquences protéiques et génomiques des phytoglobines de *M. truncatula*

En marge des résultats présentés dans la publication, nous avons cherché à analyser les séquences géniques et protéiques des différentes phytoglobines identifiées chez *M. truncatula*. L'arbre phylogénétique a permis de regrouper les phytoglobines en 3 classes, PhytoGb1, Lb et PhytoGb3. Afin de déterminer les différences entre les phytoglobines d'une même classe, une analyse de leur séquence par identification des motifs conservés a été réalisée. L'outil d'analyse MEME a été utilisé (<http://meme-suite.org>) pour identifier des motifs conservés au sein des séquences protéiques des phytoglobines de *M. truncatula*. Sur les 17 phytoglobines de *M. truncatula* analysées, 4 motifs hautement conservés, qui forment le domaine globine des PhytoGb, ont clairement été identifiés (Fig. 2.11). Les deux premiers motifs (en vert et bleu sur la Fig. 2.11) peuvent être attribués au début du domaine globine. Le troisième motif comporte l'histidine proximale (F8) responsable de la fixation de l'hème à la globine (motif violet). Le dernier motif, en rouge sur la Fig. 2.11, fait partie de la famille du domaine Mcr1 qui possède une activité NAD(P)H-flavin reductase (Marchler-Bauer *et al.*, 2017). Ce domaine est impliqué dans l'activité NO dioxygénase des phytoglobines (Smagghe *et al.*, 2008).

Sur les 12 séquences de Lbs, Lb8 ne possède pas le premier motif identifié (en vert sur la Fig. 2.11) et Lb11 ne possède pas le motif comportant l'histidine proximale ainsi que le dernier motif (violet et rouge sur la Fig 2.11). L'analyse de l'alignement des séquences protéiques (Fig S1) montre que Lb8 est plus courte d'une quarantaine d'acides aminés du côté N-terminal de la protéine, tandis que Lb11 est plus courte également d'une quarantaine d'acides aminés du côté C-terminal. L'absence de ces domaines pose la question du bon repliement de la structure tertiaire de ces deux Lbs, notamment à l'emplacement des hélices α A et H, respectivement placées du côté N et C-terminal de la protéine. De plus, l'absence de l'histidine F8 proximale, permettant la fixation du groupement hème chez Lb11 pose la question de son appartenance au groupe des hémoglobines. Les analyses de microarray (Benedito *et al.*, 2008) montrent que Lb11 est très faiblement exprimée dans les nodosités de *M. truncatula*, mais a un profil d'expression similaire aux autres Lbs (Fig S2). Ces observations, permettent de poser l'hypothèse que Lb11 serait un pseudo-gène de leghémoglobine (Vanin, 1985).

A



B

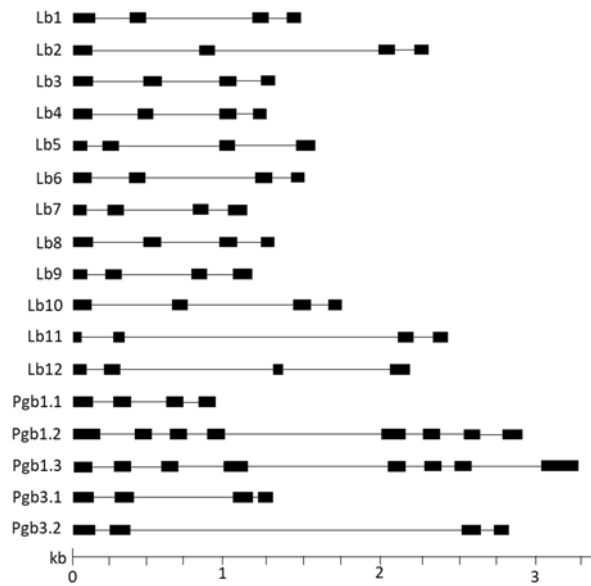


Figure 2.12 : Analyses génomiques des gènes des phytoglobines de *M. truncatula*

(A) Localisation des gènes des phytoglobines

(B) Structures exons-introns des gènes des phytoglobines

La séquence protéique de PhytoGb1.1 est très similaire à celle des Lb. En revanche, PhytoGb1.2 et PhytoGb1.3 possèdent une séquence deux fois plus longue que celle de PhytoGb1.1 et présentent une répétition des quatre motifs protéiques (Figure 2.11). Ce dédoublement de la séquence n'est pas observé chez les phytoglobines d'*A. thaliana*, ni chez des légumineuses telles que *G. max*, *L. japonicus* et *P. sativum* (<http://plantgrn.noble.org/LegumeIP/>), mais elle se retrouve chez *Trifolium subterraneum* et *Vicia faba* (<https://www.coolseasonfoodlegume.org/>). On peut poser l'hypothèse que la structure de ces 2 phytoglobines se rapproche d'un dimère composé des deux domaines globines pouvant fixer deux groupements hémiques. Il est possible que la capacité de fixation de l'O₂ ou du NO soit plus importante chez ces deux PhytoGb1 par rapport à certaines PhytoGb1 d'autres organismes grâce à un possible effet allostérique coopératif des deux sites de fixation (Bellelli & Brunori, 2011).

L'analyse des séquences d'acides aminés des PhytoGb3 indique que seul le motif contenant l'histidine proximale (violet) est conservé chez ces PhytoGb (Figure 2.11). Cette structure explique en partie pourquoi ces phytoglobines possèdent une faible affinité pour un ligand diatomique (O₂, NO) (Milani *et al.*, 2003). L'absence du motif Mcr1 suggère que ces phytoglobines ne possèdent pas d'activité NO dioxygénase par elles-mêmes. Cependant, une activité NO dioxygénase a été observée chez une tr-HbN de *M. tuberculosis* ne possédant pas de domaine réductase (Singh *et al.*, 2014). Il a été démontré que cette tr-HbN peut fixer une NADH-flavodoxine réductase (FdR) d'*Escherichia coli* et ainsi acquérir une activité NO dioxygénase (Singh *et al.*, 2014). Ceci permet de penser que les phytoGb3 pourraient se fixer au niveau du domaine FAD des NR et ainsi pouvoir oxyder le NO en nitrate (Chamizo-Ampudia *et al.*, 2017).

Chez *M. truncatula*, les gènes des Lbs sont retrouvés sur trois chromosomes différents (Fig. 2.12A), avec 4 gènes sur le chromosome 1, 7 sur le chromosome 5, et 1 sur le chromosome 7. Cinq gènes de Lbs sont localisés, très proches les uns des autres, dans une région du chromosome 5 de 265 kb. Ce cluster de gènes pourrait être à l'origine d'événements de duplication de gènes et ainsi expliquer le grand nombre de Lbs identifiés chez *M. truncatula* (Storz, 2016). Les PhytoGb1.1 et PhytoGb1.2 se retrouvent aussi dans une zone restreinte du chromosome 4, contrairement aux deux PhytoGb3 qui sont présentes sur deux chromosomes différents, le chromosome 1 et le chromosome 3 (Fig. 2.12A). Compte tenu de la proximité du gène de PhytoGb3.1 avec l'extrémité du chromosome, il est possible qu'un événement de crossing-over ait eu lieu entre le chromosome 1 et 3 au cours de l'évolution.

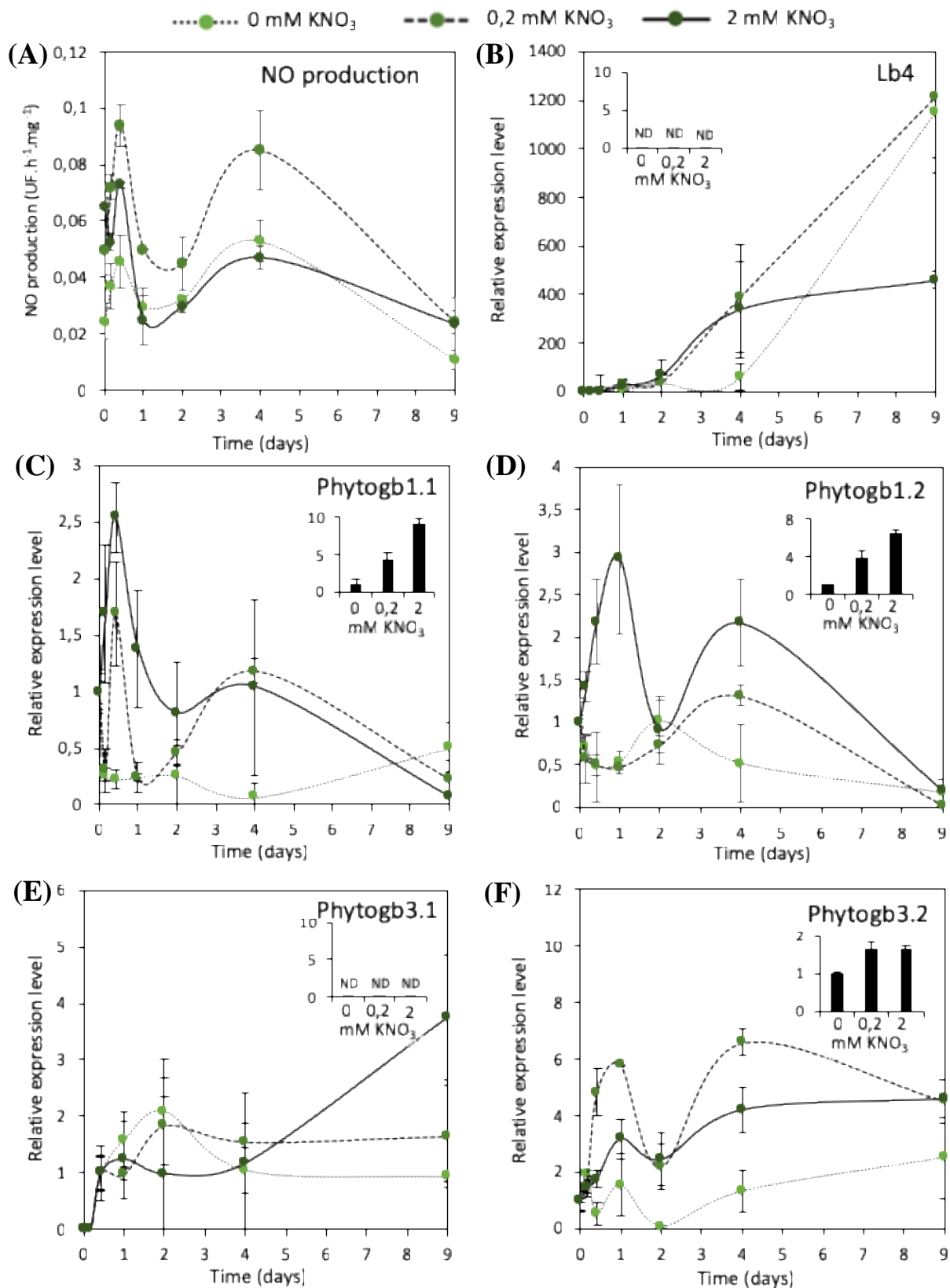


Figure 2.13 : Production de NO et Expression des gènes des phytoglobines au cours du processus symbiotique et selon la concentration en nitrate.

(A) Production de NO ; Profil d'expression du gène Lb4 (B) ; phytohb1.1 (C) ; phytohb1.2 (D) ; phytohb3.1 (E) et phytohb3.2 (F)

L'analyse de la structure « exon-intron » montre que la plupart des phytoglobines possèdent la même structure avec 4 exons séparés par 3 introns (Fig. 2.12B). Cette structure est retrouvée chez les phytoglobines de *L. japonicus* (Bustos-Sanmamed *et al.*, 2010) et est représentative du gène ancestral des hémoglobines (Hardison, 1998). Au sein d'un même gène, il existe une correspondance entre exons et domaines protéiques (de Souza *et al.*, 1998; Betts *et al.*, 2001). Cette correspondance est visible avec les *Phytogb1.2* et *1.3* avec la présence de 4 exons supplémentaires apportant un doublement du domaine globine (Fig. 2.12B). La proximité des gènes de phytoglobines sur le chromosome permet de poser l'hypothèse d'une fusion entre deux gènes de *phytgb1* pour n'en former plus qu'un.

3.2. Impact de la concentration en nitrate sur la production de NO et l'expression des phytoglobines

Les plantes ont la capacité de contrôler la symbiose fixatrice d'azote en réponse à la disponibilité en nitrate (Carroll & Mathews, 1990). Trois concentrations en nitrate (0, 0,2 et 2 mM) ont été utilisées pour étudier l'impact du nitrate sur la production de NO, ainsi que sur l'expression des phytoglobines, au cours des 9 premiers jours post-inoculation (Fig. 2.13). L'ajout d'une faible concentration en nitrate (0,2 mM) dans le milieu de culture permet d'obtenir une meilleure croissance des plantes et de promouvoir la nodulation par rapport à des plantes cultivées sans nitrate (résultats non montrés). Cependant, la présence d'une plus forte concentration en nitrate (2 mM) dans le milieu de culture inhibe d'environ 50 % la nodulation chez *M. truncatula* (A17) (Boscari A, communication personnelle).

Les mesures de production du NO obtenues aux trois concentrations de nitrate (Fig. 2.13A) montrent que les racines non-inoculées produisent d'autant plus de NO que la concentration en nitrate dans le milieu est élevée. Un tel effet de la nutrition nitraté sur la production de NO a déjà été rapportée dans la littérature (Mur *et al.*, 2013a). Au cours des premiers jours de la symbiose, les deux pics de production de NO observés à 10 hpi et 4 jpi se produisent indépendamment de la concentration en nitrate dans le milieu de culture (Fig. 2.13A). On peut cependant noter que la production de NO la plus forte est observée pour 0,2 mM de nitrate et qu'elle n'est pas proportionnelle à la fourniture en nitrate (Fig 2.13A).

L'expression des phytoglobines a été suivie pendant 9 jpi en présence de 0, 0,2 et 2 mM de nitrate. Dans les racines non-inoculées, *Lb4* et de *Phytogb3.1* ne sont pas exprimés quelle que soit la concentration en nitrate dans le milieu (Fig. 2.13B ;C). En revanche *Phytogb1.1*, *1.2*

et *Phytogb3.2* sont constitutivement exprimés dans les racines non inoculées, avec des niveaux d'expression dépendants de la présence de nitrate (Fig. 2.13D; E et F).

Au cours du processus symbiotique, la présence de nitrate affecte peu l'expression de *Lb4* pendant les 2 premiers jpi (Fig. 2.13B). Jusqu'à 4 jpi, *Lb4* reste peu exprimé en absence de nitrate, tandis qu'à 0.2 mM et 2 mM son expression est fortement induite. Au-delà de 4 jpi, l'expression de *Lb4* se stabilise en présence de 2 mM de nitrate, alors qu'elle augmente fortement en absence ou en présence de 0.2 mM de nitrate. Durant les premières heures de l'interaction, l'expression de *Phytogb1.1* dépend fortement de la présence de NO_3^- . En absence de nitrate, elle chute fortement de 80% et reste peu exprimée au cours des 9 jours qui suivent l'inoculation. En présence de 2 mM de nitrate, l'expression de *Phytogb1.1* augmente d'un facteur 2,5 au cours des 10 premières hpi puis retrouve son niveau initial, avant de chuter à 9 jpi. En revanche, c'est en présence de 0,2 mM de nitrate que les variations d'expression de *Phytogb1.1* sont les plus contrastées et que les 2 pics d'expression à 10 hpi et 4 dpi sont clairement visibles (Fig. 2.13C). De la même manière que pour *Phytogb1.1*, l'expression de *Phytogb1.2* est induite en présence de 2 mM de nitrate, avec 2 pics d'expression à 1 et 4 dpi, mais peu exprimé en son absence. Il est intéressant de noter qu'en présence de 0,2 mM de nitrate, *Phytogb1.2* est réprimé pendant les 2 premiers jpi, mais est induit à 4 dpi (Fig. 2.13D). La présence de nitrate a peu d'effet sur l'expression de *Phytogb3.1* jusqu'à 4 jpi. Au-delà de 4 jours, son expression est plus forte en présence de 2 mM de nitrate alors qu'elle se stabilise en présence de 0 et 0.2 mM de nitrate (Fig. 2.13C). En présence de 0,2 et 2 mM de nitrate, *Phytogb3.2* est plus fortement exprimé à 9 jpi, mais son expression varie peu durant le temps de l'expérience (Fig. 2.13F).

Les résultats obtenus montrent que si la production de NO varie en intensité, mais pas en profil, en fonction de la fourniture en nitrate, chaque phytoglobine est différemment régulée par le nitrate. Le profil d'expression de *Lb4* (marqueur de la fixation de l'azote) en fonction de la concentration en nitrate est cohérent avec le fait que le nitrate inhibe la fixation de l'azote. La régulation de la nodulation par le nitrate est connue aussi bien au niveau du processus d'infection, que du développement des nodosités et de leur activité fixatrice d'azote (Streeter *et al.*, 1988). En revanche, l'expression de *Phytogb3.1* dès les premières heures de la symbiose suggère que *Phytogb3.1* joue un rôle strictement relié au processus symbiotique, indépendamment du nitrate.

Par ailleurs, une corrélation est visible entre l'expression nitrate dépendante de la *NRI* et celles des *Phytogb1* (Fig Xx, 2.13C ;D). Dans la plupart des cas, il a été montré que les

Phytogl1.1 comme les *NR* sont régulés de façon identique par le nitrate ainsi que par le NO (Wang *et al.*, 2000a; Ohwaki *et al.*, 2005; Shimoda *et al.*, 2005; Bai *et al.*, 2016), ce qui renforce l'idée que les *NR* et les *Phytogl1.1* sont impliquées dans une boucle de régulation de la synthèse et de la dégradation du NO.

Par ailleurs, il a été montré que *NR* et les *Phytogl1* sont co-régulés chez le maïs suite à un traitement avec du nitrate (Trevisan *et al.*, 2011). Chez *A. thaliana*, il a été montré que les ARNm de *NR* et *Phytogl1* sont co-localisés sur le site d'accumulation du NO ce qui indique que *NR* et *Phytogl1* sont deux éléments de régulation de l'homéostasie du NO et de sa signalisation (Stöhr & Strelau, 2006). Nos observations vont dans le même sens et suggèrent que le même type de régulation a lieu au début du processus symbiotique.

3.3. Impact des différents donneurs de NO sur le phénotype de nodulation

Afin d'analyser l'impact du NO sur la nodulation au cours des premières étapes de la symbiose, trois différents couples de donneurs de NO ont été utilisés en parallèle avec leur contrôle : le DEA-NO (dont les effets sont rapportés dans la publication), le SNP et le GSNO. Le traitement du donneur de NO sur les racines a été fait toutes les 24h pendant 4 jours suivant l'inoculation. L'effet du traitement a été observé à 14 jpi.

Pour rappel, le DEA-NO provoque une diminution du nombre de nodosités (Fig. 2.14A), et une augmentation de leur taille (Fig. 2.14B). Le SNP, qui produit le cation nitrosonium NO^+ , induit une augmentation du nombre de nodosités de la même manière que son contrôle FeCN_6 (Fig. 2.14A), alors que seul le SNP provoque une augmentation de la taille des nodosités (Fig. 2.14B). Le GSNO, qui produit également du NO^+ , provoque une diminution du nombre de nodosités de la même façon que son contrôle GSH (Fig. 2.14A), et seulement un traitement avec le GSNO provoque une augmentation de la taille des nodosités (Fig. 2.14B)

Ainsi pour ces 2 donneurs de NO, il apparaît que les effets observés sont d'avantage liés à leurs contrôles respectifs qu'au NO qu'ils libèrent. Plusieurs explications des effets du FeCN_6 et du GSH peuvent être avancées. L'un comme l'autre sont connus pour inhiber certaines activités enzymatiques. Il a été montré que le FeCN_6 inhibe le transport d'électron du photosystème II (Drechsler & Neumann, 1982), la NADH oxydoréductase (Seddon & McVittie, 1974) et la glutamine synthétase (Communication personnelle R. Brouquisse). Il a été également montré que le GSH est un inhibiteur de la catalase (Sun & Oberley, 1989), du transport du sulfate (Rennenberg *et al.*, 1988) et qu'il induit la réponse hypersensible (Hiruma *et al.*, 2013).

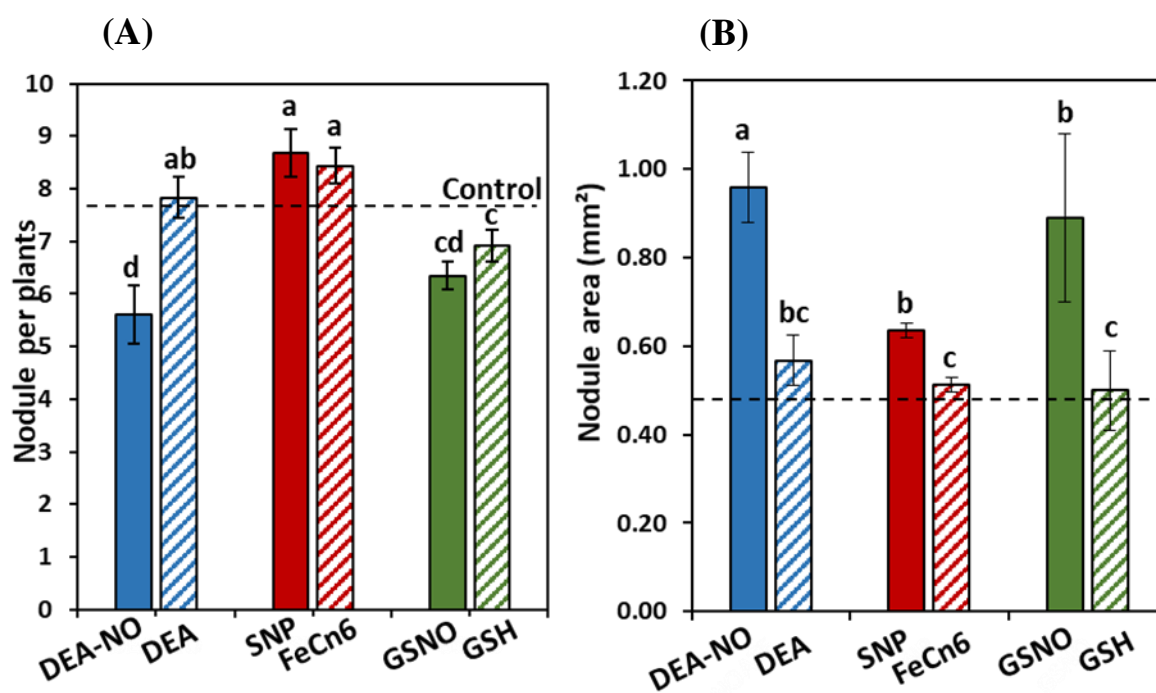


Figure 2.14 : Effet des donneurs de NO et de leurs contrôles sur les nodosités
 Les nodosités sont dénombrées à 14 jpi sur des plantes traitées par DEA-NO/DEA, SNP/FeCN6 et GSNO/GSH (A). L'aire des nodosités correspond à la moyenne des nodosités obtenues (B).

On peut également penser que ces effets sont liés aux propriétés oxydo-réductrices de ces composés. En effet, le FeCN₆ est un composé oxydant alors que le GSH est un composé réducteur. L'inhibition du nombre de nodosités par le GSH pourrait s'expliquer par l'induction des réponses de défense comme cela a été observé chez l'orge (Maughan & Foyer, 2006), ce qui se traduirait par une diminution des événements de nodulation. Ceci permet de supposer que le GSH ou l'apport de composés réducteurs peut être responsable de la diminution du développement de la nodosité. De la même manière, on peut penser que l'apport de composés oxydant peut engendrer une augmentation du nombre de nodosités comme cela est observé avec le FeCN₆. Cependant, il est difficile de conclure sur cet aspect sans analyse supplémentaire telle qu'un contrôle en présence de DTT ou de GSH oxydé. L'analyse des effets du NO sur la croissance des nodosités montre que les 3 donneurs de NO (SNP, DEA-NO et GSNO) induisent la croissance des nodosités comparativement à leurs contrôles respectifs. L'impact des donneurs de NO sur l'aire de la nodosité (et donc sur la croissance) indique que les mécanismes d'initiation de la nodulation et de la croissance de la nodosité sont différents. En effet, l'apport de composés réducteurs ou oxydants n'influence pas la croissance de la nodosité contrairement à l'initiation de la nodulation. Le processus de nodulation semble donc plus sensible à l'état redox de la cellule que le processus de développement nodulaire.

En résumé, l'ensemble des résultats obtenus dans cette deuxième partie, sur la caractérisation des phytoglobines de *M. truncatula*, la production et l'impact du NO sur la symbiose, montre qu'il existe une forte relation entre les phytoglobines et la régulation du niveau de NO au cours du processus symbiotique. Il apparaît que chaque phytoglobine possède une expression propre en fonction de l'état de développement de la nodosité, ce qui suggère que chacune d'entre elles est impliquée dans la régulation du NO à un temps donné du processus symbiotique. Le parallèle entre l'expression de *Phytogl1.1*, la production de NO, et la spécificité d'expression de *Phytogl3.1* au cours de la symbiose nous ont poussé à analyser plus précisément leur fonction dans le processus symbiotique. Le dernier chapitre des résultats, est consacré à caractériser plus précisément le rôle de *Phytogl1.1* et *Phytogl3.1* lors du développement de la symbiose, son fonctionnement et lors de la sénescence de la nodosité.

Chapitre 3 :

Rôles de PhytoGb1.1 au cours des premières étapes, du fonctionnement et de la sénescence de la nodosité

Contexte et objectifs du travail: Characterization of *Medicago truncatula phytoGb1.1* in relation with nitric oxide production during the nitrogen-fixing symbiosis with *S. meliloti*

Les résultats obtenus dans le chapitre précédent ont permis de suivre la production de NO et l'expression des gènes des phytoglobines au cours du processus symbiotique (Berger et al., 2019). Ces travaux ont montré que deux phytoglobines PhytoGb1.1 et PhytoGb3.1 sont régulés de façon parallèle à la production de NO. Au cours de la symbiose, il a été montré chez le lotier que la surexpression de *LjPgb1.1* diminue le niveau de NO dans les racines et les nodosités tout en améliorant la fixation de l'azote et les événements de nodulation (Shimoda et al., 2009 ; Fukudome et al., 2016). Le rôle de PhytoGb1.1 dans les premières étapes, ainsi que dans la nodosité mature a été beaucoup étudié (Nagata et al., 2008 ; 2009 ; Shimoda et al., 2005 ; 2009 ; Fukudome et al., 2016). Cependant peu de données sont disponibles sur le rôle de *PhytoGb1.1* de *M. truncatula* lors du développement de la nodosité, ainsi que lors du processus de sénescence.

L'objectif de ce chapitre est de déterminer le rôle de PhytoGb1.1 dans la régulation du NO au cours du processus symbiotique, notamment lors du développement de la nodosité, au sein de la nodosité mature mais également lors de la sénescence. Pour réaliser cette caractérisation fonctionnelle dans les racines et les nodosités, une approche utilisant des racines transgéniques a été utilisée. Deux types de constructions ont été utilisés, l'une permettant la sous expression et la surexpression de *PhytoGb1.1* sous le contrôle du promoteur 35s, et l'autre permettant les mêmes modifications de l'expression de *PhytoGb1.1* sous le contrôle d'un promoteur spécifique de la zone de fixation des nodosités matures, NCR001. Grâce à ces constructions, nous avons analysé l'impact de PhytoGb1.1 sur la production de NO, le phénotype de nodulation, l'activité nitrogénase et l'expression de gènes marqueurs de l'infection (*Enod20*), de la réponse de défense (*GST*, *CS*), du développement nodositaire (*Cre1*), du fonctionnement de la nodosité (*Lb*), de la sénescence (*CP6*) et du métabolisme azoté (*NR1/2/3*, *GS1a*).

2. Publication N°3

Characterization of *Medicago truncatula* phytohemoglobin1.1 in relation with nitric oxide production during the nitrogen-fixing symbiosis with *S. meliloti*

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Supporting information: 2 Figures, 2 Table.

Summary

- The nitrogen-fixing symbiosis between legumes and rhizobia leads to the formation of a new root organ called nodule. In legumes nodules, three types of phytoglobins were characterized: phytoglobin1, leghemoglobin and phytoglobin3. They are known to buffer oxygen (O₂) and to scavenge nitric oxide (NO). During the symbiotic process between *M. truncatula* and *S. meliloti*, Phytoglobin1.1 (*Phytogb1.1*) of *M. truncatula* was shown to be induced during nodule development, in mature nodule and during senescence process. The aim of this study is to analyse the function of *Phytogb1.1* in relation to NO regulation during these different steps.
- By using transformed roots/nodules overexpressing and silencing *Phytogb1.1*, we investigated the impact of *Phytogb1.1* modification on NO production, nodulation phenotype, nitrogenase activity, measured through its acetylene reducing activity (ARA), and the expression of genes related to the defence response, nodule establishment and development, and hypoxia and nitrogen metabolism.
- Our results reveal that *Phytogb1.1* regulates NO level in inoculated roots and nodules, and indirectly guide lines the infection step and nodule development potentially *via* the regulation of NO level.
- In mature nodules, the overexpression of *Phytogb1.1* improves ARA, expands the nodule lifespan and delays senescence. On the contrary, the down-expression of *Phytogb1.1* triggers a decline in ARA and leads to an early senescence process.

Keywords: Hemoglobin, nitric oxide, nitrogen-fixing symbiosis, *Medicago truncatula*, phytoglobin, *Sinorhizobium meliloti*.

Introduction

Plant hemoglobins (Hbs) were first investigated with the discovery of symbiotic leghemoglobins (Lb) in soybean root nodules (Kubo, 1939). Thereafter, others hemoglobins have been characterized based on their structural and biochemical properties (Arredondo-Peter *et al.*, 1998; Garrocho-Villegas *et al.*, 2007; Gupta *et al.*, 2011b). Recently, plant Hbs were renamed phytoglobins (Phytogbs) (Hill *et al.*, 2016), and have been classified into six categories including: Phytogb0 - nonsymbiotic hemoglobin (nsHb) present in algae, bryophytes and gymnosperms; Phytogb1 - class 1 nonsymbiotic hemoglobin (nsHb-1) present in angiosperms; Phytogb2 - class 2 nonsymbiotic hemoglobin (nsHb-2) present in angiosperms; SymPhytogb - symbiotic hemoglobin (symHb) present in non-legume N₂-fixing plants; Lb - leghemoglobin (Lb) present in N₂-fixing legumes; and Phytogb3 - class 3 truncated hemoglobin (trHb) present in algae and land plants (Hill *et al.*, 2016).

In legumes, Phytogb1, Lb and Phytogb3 classes were described and shown to be expressed during N₂-fixing symbiosis ((Bustos-Sanmamed *et al.*, 2010; Berger *et al.*, 2018a).

Lb, that evolved from Phytogb2, are known to play a key role to supply bacteroid and mitochondria with O₂ for energy regeneration process and to keep free O₂ away from the oxygen-sensitive-nitrogenase enzyme (Watts *et al.*, 2001; Garrocho-Villegas *et al.*, 2007; Smaghe *et al.*, 2009 ; Appleby, 1984). They are a high affinity for O₂ and NO (K_m ~ 150 nM) (Gupta *et al.*, 2011).

Phytogb3 (Phytogb3) have only been found late in plants (Watts *et al.*, 2001) They are characterized by their low concentration (in the order of nM) and their low affinity for O₂ and NO (K_m ~ 1500 nM) . (Watts *et al.*, 2001). The main role of Phytogb3 in plants remains unclear.

Phytogb1 (Phytogb1) are present in both monocotyledons and dicotyledons, and have a very high affinity for O₂ (K_m ~ 1-2 nM) and nitric oxide (NO) (Duff *et al.*, 1997). Several reports indicate that Phytogb1 are involved in the regulation of intracellular levels of NO (Igamberdiev & Hill, 2004; Hebelstrup *et al.*, 2012; Hill, 2012). *Phytogb1* genes are overexpressed during various stresses such as hypoxia, osmotic and saline stress (Trevaskis *et al.*, 1997; Lira-Ruan *et al.*, 2001; Zhao *et al.*, 2008), but also following various treatments with either nitrate, nitrite, NO, salicylic acid; jasmonic acid, ethylene or H₂O₂ (Wang *et al.*, 2000b; Sakamoto *et al.*, 2004; Ohwaki *et al.*, 2005; Qu *et al.*, 2006; Sasakura *et al.*, 2006). Sowa *et al.* (1998) first reported that maize cells overexpressing barley *Phytogb1* are able to maintain ATP level under hypoxic conditions more efficiently than wild-type cells. In corn cells, the level of NO was shown to be inversely related to *Phytogb1* expression levels (Dordas *et al.*, 2004),

suggesting that *Phytogl* was able to catabolize NO. This function is related to its NO dioxygenase activity (Igamberdiev & Hill, 2004; Perazzolli *et al.*, 2004).

NO is produced during symbiotic interactions (Hichri *et al.*, 2016), and different studies have reported its presence during legume-rhizobia symbiosis (Hichri *et al.*, 2015, 2016).

In the *M. truncatula* – *S. meliloti* symbiosis, it was shown to be produced during the whole symbiotic process with production peaks during the first hours of the interaction between the plant and the bacteria, at early nodule development, in the N₂-fixing nodules and at the onset of nodule senescence (Baudouin *et al.*, 2006; Nagata *et al.*, 2008; del Giudice *et al.*, 2011; Cam *et al.*, 2012; Berger *et al.*, 2018a).

In *M. Truncatula*, del Giudice *et al.*, (2011) observed NO production at the infection pocket and the infection thread (at 4 dpi). Moreover, these authors analyzed the effect of a low NO level on nodulation, by using either *S. meliloti* strain that overexpressed hmp or a NO scavenger (cPTIO). This decrease of NO level caused nodulation delay and inhibited gene expression involved in nodule development (*MtCRE1/MtCCS52A*) (del Giudice *et al.*, 2011). In the same way, the transcriptomic analysis of 4 dpi *M. truncatula* roots treated with cPTIO showed that NO depletion resulted in a lack of activation of genes involved in cell cycle and protein synthesis (Boscari *et al.*, 2013a).

Similar reduction of nodule number was observed after the treatment of soybean with the NOS inhibitor L-NNA during early interaction with *B. japonicum*, a phenotype reverted by the addition of the NO-donor DETA-NO (Leach *et al.*, 2010). A nodulation delayed was also observed, on *L. japonicus*, during a treatment with NO donor (SNP or SNAP) which inhibits the elongation of the infection thread (Fukudome *et al.*, 2016) suggesting that a high NO level inhibits nodulation.

NO appeared also, as a key regulator of the carbon and nitrogen metabolism within the nodule (Hichri *et al.*, 2016). Cytosolic glutamine synthetase (GS1) is a key enzyme in NH₄⁺ entrapment and nitrogen metabolism, as it allows the synthesis of glutamine (Gln) following the condensation of NH₄⁺ with glutamate (Glu) (Silva & Carvalho, 2013). In *M. truncatula*, GS1a is responsible for 90% of total nodule activity (Carvalho *et al.*, 2000) and was inactivated by NO via Tyr-nitration (Melo *et al.*, 2011). In addition to GS1a, NO was reported to be a potent inhibitor of nitrogenase activity as measured *in vitro* (Trinchant & Rigaud, 1982). However, ARA was more substantial in *L. japonicus* nodules in the presence of 0.1 mM SNP (NO donor) than either in the absence or in the presence of higher (1 mM) concentration of SNP, indicating that low but significant NO concentrations are beneficial to nitrogen fixation (Kato *et al.*, 2010). NO has also been demonstrated to play a beneficial metabolic function for the maintenance of

the energy status under hypoxic conditions in roots (Igamberdiev & Hill, 2009; Igamberdiev *et al.*, 2010; Gupta & Igamberdiev, 2011) and in nodule (Horchani *et al.*, 2011). NO is involved in the cycle named phytohemoglobin-NO (Phytohb-NO) respiration allowing the regeneration of ATP in nodule. The Phytohb-NO cycle is divided into 4 steps including (1) NO_3^- reduction to NO_2^- by cytosolic NR, (2) NO_2^- transport from the cytosol to the mitochondrial matrix, (3) NO_2^- reduction to NO by the ETC, and (4) NO diffusion to the cytosol and oxidation to NO_3^- by Phytohbs (Igamberdiev & Hill, 2004; Hichri *et al.*, 2016).

Interestingly, an increase of NO levels in *M. truncatula* nodules, resulting either from the use of *S. meliloti* strains deficient in NO degradation (*hmp*, *norB*, *nnrS1*) or from the exogenous addition of a NO donor, led to a premature senescence of the nodules, the severity of which correlated with the NO levels inside the nodules (Cam *et al.*, 2012; Meilhoc *et al.*, 2013; Blanquet *et al.*, 2015).

Taken together, these observations indicate that depending on the step of the symbiosis, NO may act either as an inhibitor, a regulatory signal of a beneficial metabolic intermediate. Subsequently, the NO regulation should be an important aspect for all these mechanisms. During the symbiotic process, NO removal was mainly ascribed to phytohemoglobins.

In *Phytohb1* mutants of *L. japonicus* containing a high NO level, exhibited an alteration of the infection process due to a defect infection thread (Fukudome *et al.*, 2016). In detached nodules of *L. japonicus* overexpressing *LjHb1* display reduced contents of NO compared to control nodules, leading to significantly higher ARA activity within these nodules (Shimoda *et al.*, 2009), suggesting that reversible inhibition of nitrogenase is relieved by the scavenging of NO by *LjHb1*. Similarly, in the actinorhizal symbiosis between *Alnus firma* and *Frankia*, it was shown that Afns-Hb1, as a NO scavenger, could support the N_2 fixation (Sasakura *et al.*, 2006). Recently, using *M. truncatula/S. meliloti* model, Berger *et al.* (2018a) showed that the NO production observed throughout the symbiotic process in *M. truncatula* correlated with the expression of a phytohemoglobin *Phytohb1.1*. All together, these observations indicated that NO homeostasis is essential at several stage of symbiosis and that *Phytohb1.1* could be regulated the NO level during the different step of the symbiotic process.

The present work aimed at deciphering the relationship between *Phytohb1.1* and NO during early nodule development and in mature nodule. To this end we first designed and generated transgenic *M. truncatula* roots either overexpressing or silencing *Phytohb1.1* under the control of the 35s promoter. Using these plants we investigated the nodulation phenotype, NO production and genes expression at the onset of nodule organogenesis. Then, using a the NCR001 promoter, which is specific of N_2 -fixing zone (Mergaert *et al.*, 2003b) we generated

nodules over and silencing *Phytogl.1.1*. Using these nodules we studied the impact of *Phytogl.1.1* expression modulation on the nitrogen fixation activity and on the nodule senescence process. Based on our data we conclude that *Phytogl.1.1* is key regulator of NO production during the first step of interaction to lead the infection and the development of nodule but also maintain the good physiological condition inside mature nodule and delayed senescence.

Materials and methods

Plants growth and inoculation conditions

Medicago truncatula (cv Jemalong A17) were scarified, sterilized and germinated as in del Giudice *et al.*, (2011). Seedlings were cultivated and inoculated with *Sinorhizobium meliloti* 2011 strain either in Petri dishes as described in del Giudice *et al.*, (2011), or in planters as described in Horchani *et al.*, (2011). Roots and/or nodules were harvested at either 4 dpi, 14 dpi or 3wpi depending on analysis.

Plasmid constructions

For overexpression construction *Phytogl.1.1* genes was amplified for the complete cDNA of *M. truncatula* by PCR. This sequence was introduced in the pK7WG2D under the control of 35S promoter (renamed 35s::*Phytogl.1.1*) by simple Gateway reaction according to the manufacturer's instructions (Invitrogen, <http://www.invitrogen.com>). To construct the plasmid for the overexpression of *Phytogl.1.1* under the control of NCR001 (noted NCR::*Phytogl.1.1*) a multiple Gateway reaction was used to insert the promoter in front of the *Phytogl.1.1* sequence.

For the RNAi construct, a common region of ~ 200 bp, found in the target genes *Phytogl.1.1* was amplified from the complete cDNA of *M. truncatula* by PCR using the couple primers RNAi-*Phytogl.1.1*. This sequence was introduced into the pK7GWIW2D vector (Karimi *et al.*, 2002) (renamed RNAi::*Phytogl.1.1*) and into the pK7GWIWG5D(II) (Horchani *et al.*, 2011) (named NCR::*Phytogl.1.1*). All the primers, sequences, and vectors are provided in Table S1 and Table S2, respectively.

Roots transformation by *A. rhizogenes*

The constructions 35s::*Phytogl.1.1* and RNAi::*Phytogl.1.1* were introduced into *A. rhizogenes* strain *Arqual* (Quandt & Hynes, 1993). *M. truncatula* plants were transformed with

A. rhizogenes according to Boisson-Dernier *et al.*, (2001). Control plants were transformed with *A. rhizogenes* containing the pK7GWIGW2D or the pK7WG2d empty vectors. Transformed plants were selected based on the fluorescent marker GFP.

The constructions NCR::*Phytogb1.1* and NCR-RNAi::*Phytogb1.1* were introduced into *A. rhizogenes* strain *Arqual* (Quandt & Hynes, 1993). *M. truncatula* plants were transformed with *A. rhizogenes* according to (Vieweg *et al.*, 2005). Control plants were transformed with *A. rhizogenes* containing the empty vector. Selection of hairy roots based on the fluorescent marker took place 21 d after transformation. The roots were rapidly examined with a fluorescence stereomicroscope (Leica MZFL III), and the composite plants harboring transgenic roots were used for the inoculation with the appropriate rhizobial strain.

Nodule surface analysis

At 14 dpi, nodules from Petri dishes were harvested, numbered and placed on agar medium 1% (w/v) to be photographed. Nodule surfaces were analyzed using the ImageJ software (<https://imagej.nih.gov/ij/>). The image was transformed into a shade of gray (8 bit) and then black and white (binary). Nodules pictures were transformed into a white surface and nodule areas were calculated by using the "Analysis particles" function of the software.

Measurement of NO production

Detection of NO was performed using the 4,5-diaminofluorescein probe (DAF-2) (Horchani *et al.*, 2011). Segments of 2 cm roots or nodules were incubated in 1 ml of detection buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl) in the presence of 10 μ M DAF-2. The production of NO was measured with a spectrofluorimeter (Xenius, SAFAS, Monaco) every 30 min for 4 hours after addition of the probe. The production of NO was then normalized with the weight of the sample.

RNA isolation, reverse transcription and gene expression analysis

RNAs were isolated from 100 mg of frozen material ground in liquid N₂ using the RNAzol following the manufacturer's recommendations (Sigma-Aldrich). RNA quality was checked and DNase treatment was carried out prior to the synthesis by GoScript reverse transcriptase (Promega) of the cDNAs. The RT-qPCR and data analysis were made as described in Berger *et al.* (2018a). RT-qPCR analyses were carried out in triplicate, using the primers reported in Table 1. The reference value "1" was attributed to the first time when the amplification value (Ct) of the analysed gene was significantly detectable.

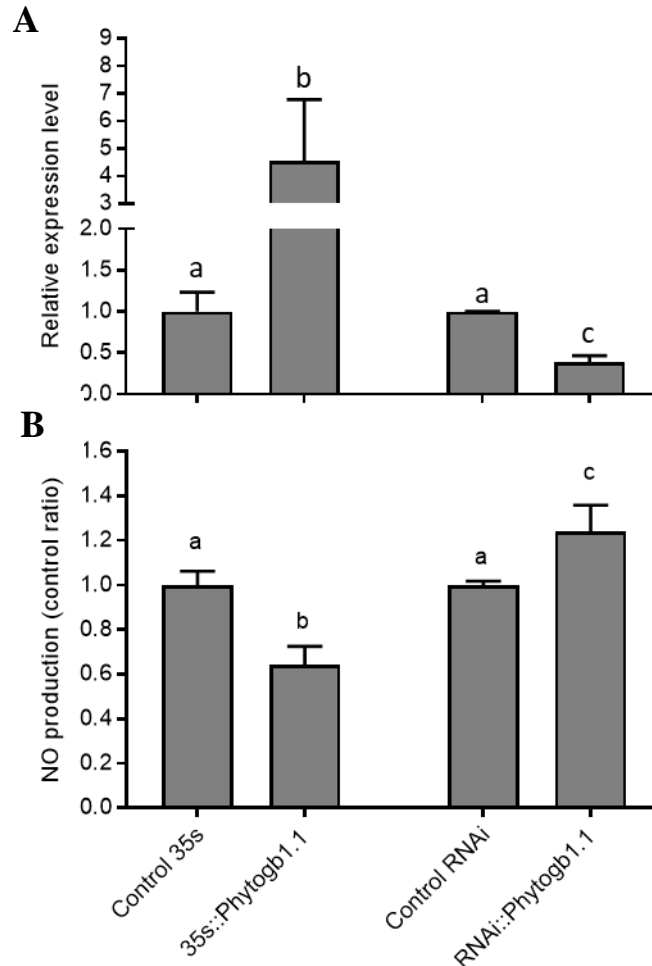


Figure 3.1: Relative expression level of PhytoGb1.1 and NO production in roots of control plant, 35s::PhytoGb1.1 and RNAi::PhytoGb1.1 at 4 dpi. (a)

Expression analysis of *PhytoGb1.1* genes in control plant and transformed root plant overexpressing (35s::PhytoGb1.1) or underexpressing *PhytoGb1.1* (RNAi::PhytoGb1.1) at 4 dpi. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

(b) Analysis of NO production in roots of control plant and transformed root plant overexpressing (35s::PhytoGb1.1) or underexpressing *PhytoGb1.1* (RNAi::PhytoGb1.1) at 4 dpi. The fluorescence intensity of the NO production was measured using DAF-2 fluorescent probe. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

Nitrogen-fixing capacity

Nitrogenase activity of nodules was determined *in vivo* by measuring acetylene reducing activity (ARA), as previously described by Hardy *et al.* (1968). Nodulated roots were harvested and incubated at 30 °C for 1 h in rubber-capped tubes containing a 10% acetylene atmosphere. Ethylene concentrations were determined by gas chromatography (Agilent GC 6890N, Agilent Technologies, Santa Clara, CA, USA) equipped with a GS-Alumina (30 m × 0.534 mm) separating capillary column. Two biological replicates have been performed with five technical replicates or each replicate.

Induced senescence treatments

Induced senescence was initiated on 3 wpi-old plants with a 10 mM nitrate treatment for two days (Berger *et al.*, 2018a).

Results

***Phytogl.1.1* expression modulates NO production in transformed root and influences the nodulation phenotype, at 4 dpi, during the onset of nodule development.**

In order to analyse the modification of *Phytogl.1.1* expression during the nodule development, two transformed roots of *M. truncatula* were generated. The first one, 35s::*Phytogl.1.1* transformed roots, overexpressed *Phytogl.1.1* under the control of the constitutive CaMV 35s promoter. The second one, RNAi::*Phytogl.1.1* transformed roots, silenced *Phytogl.1.1* expression. At 4 days post-infection (dpi), the 35s::*Phytogl.1.1* transformed roots showed a 4.5-fold enhanced expression of *Phytogl.1.1* as compared with the control plants in (Fig. 3.1A), while RNAi::*Phytogl.1.1* transformed roots showed a 2.5-fold decrease in *Phytogl.1.1* expression (Fig. 3.1A).

NO production was monitored in transformed roots and control plants in response to *S. meliloti* by using cell permeable fluorescent dye 4-amino-5-methylamino-2',7'-difluorescein (DAF-2). As compared to the control, our data showed that NO production in 35s::*Phytogl.1.1* was 1.6-fold decreased in 4 dpi roots (Fig. 3.1B), while it was 1.3-fold increased in RNAi::*Phytogl.1.1* 4 dpi roots (Fig. 3.1B). It may be concluded that NO level is controlled by the presence of *Phytogl.1.1*.

In order to investigate the effect of *Phytogl.1.1* either over or under expression on the nodulation process, nodule number and size were analysed in transformed versus control inoculated roots. As reported in Table 1, the nodule number per plants was lower both in 35s::*Phytogl.1.1* and in

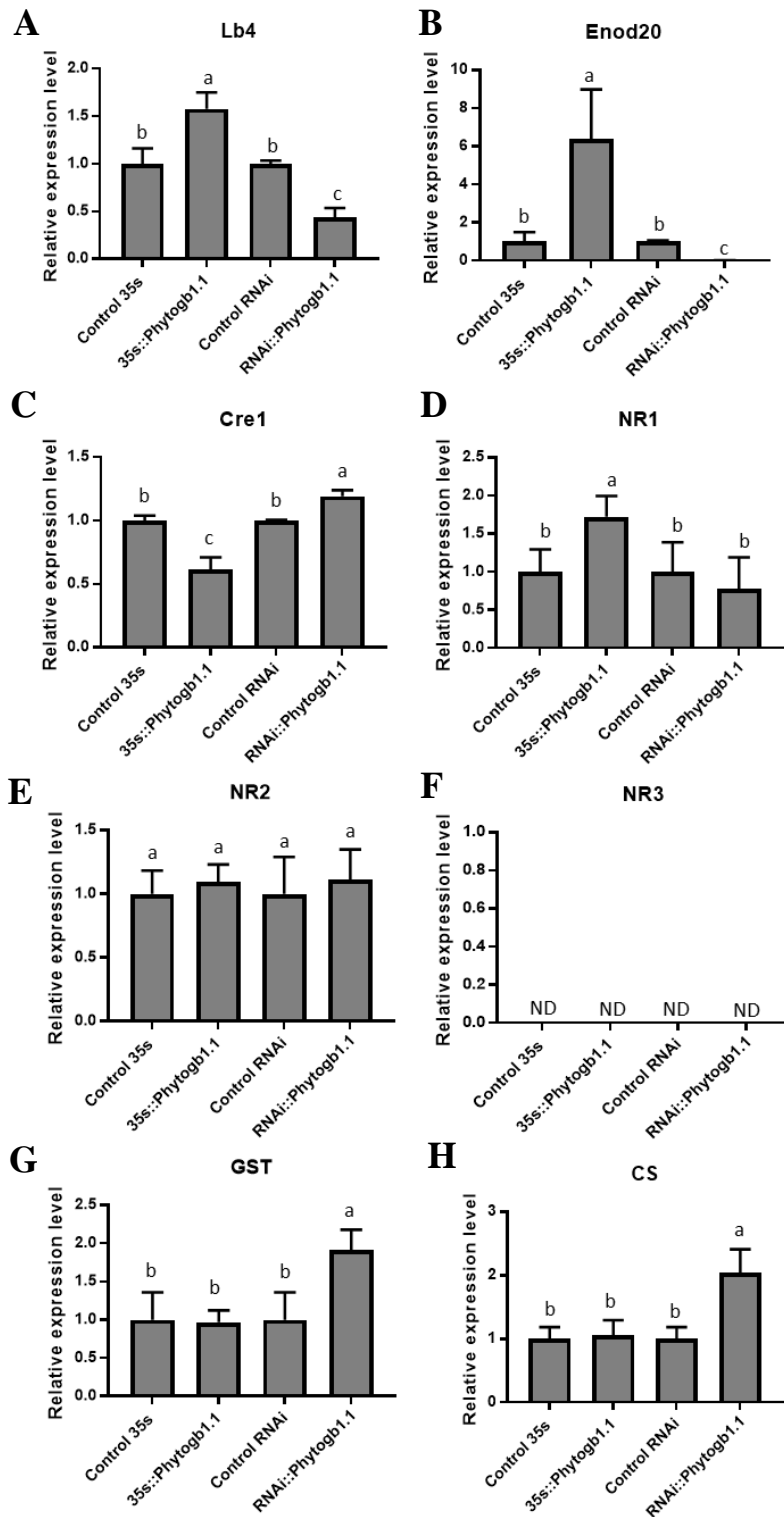


Figure 3.2: Relative gene expression level in 4 dpi-old roots of control, 35s::Phytogb1.1 and RNAi::Phytogb1.1 plants. Expression analysis of (a) *Lb4* genes (b) *Enod20*, (c) *Cre1*, (d) *NR1*, (e) *NR2*, (f) *NR3* (g) *GST* and (h) *CS* genes, in control plant and transformed root plant overexpressing (35s::Phytogb1.1) or underexpressing *Phytogb1.1*(RNAi::Phytogb1.1) at 4 dpi. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

RNAi::*Phytogl.1* roots as compared to control roots. However, no change was observed on nodule size between control and transformed plants (Tab. 3.1).

Modulation of gene expression in *Phytogl.1* transformed roots at 4 dpi.

To go further in the understanding of the role of *Phytogl.1* during nodule establishment and development, we analysed the expression of genes involved various symbiotic processes in both control and transformed roots (Fig 3.2).

Lbs are considered to be markers of nodule development and functioning as their expression level and/or protein level correlate with the N₂-fixing activity of the nodules (Appleby, 1992; Berger *et al.*, 2018a). We previously showed that *Lb* expression is detectable in 4 dpi-old roots of *M. truncatula* (Berger *et al.*, 2018a). The analysis of Affimetrix and Symbimix data (Fig. S1, S2) showed that the 12 *MtLb* genes exhibit a similar expression pattern during the symbiotic process (Roux *et al.*, 2014). Therefore, in order to avoid analyzing the expression of the 12 *Lb* genes, we used *Lb4*, whose expression is average among the different *Lbs*, as a representative *Lbs* marker (Fig. S1). Figure. 3.2A, shows that the expression of *Lb4* is higher in 35s::*Phytogl.1* and lower in RNAi::*Phytogl.1* compared to the control roots, supporting that *Lb4* could be regulated by *Phytogl.1* (Fig. 3.2A).

Enod20 and *Cre1* have been shown to be markers of bacterial infection event (Vernoud *et al.*, 1999), and nodule organogenesis (Frugier *et al.*, 2008), respectively. *Enod 20* expression was up regulated in 35s::*Phytogl.1* roots, and down regulated in RNAi::*Phytogl.1* roots (Fig. 3.2B) suggesting that infection process is regulated in the same way as, and possibly by, *Phytogl.1*. On the other hand, *Cre1* was down regulated in 35s::*Phytogl.1* roots, and up regulated in RNAi::*Phytogl.1* roots (Fig. 3.2C) suggesting a reverse regulation to that of *Enod20* and possibly a opposite regulation between the infection process and the nodule development. Nitrate reductase (NR) has been shown to be the entrance of nitrogen in the nitrogen metabolism (Campbell, 1999) and the main NO sources during the symbiotic process (Horchani *et al.*, 2011). In *Medicago truncatula* genome databases, 3 sequences encoding NR have been identified, called *NR1*, *NR2* and *NR3*. They correspond respectively to inducible, constitutive and symbiosis specific isoforms (Benedito *et al.*, 2008; Roux *et al.*, 2014).

In the present study, *NR1* was induced in 35s::*Phytogl.1*, but its expression was not modified in RNAi::*Phytogl.1*, whereas *NR2* expression was not impacted by *Phytogl.1* modification, and the expression of *NR3* was not detectable at this stage of the symbiotic process (Fig. 3.2 D,E,F). Two additional genes expression were carried out to determine the impact of *Phytogl.1* expression modification on plant defence response, namely glutathione S-

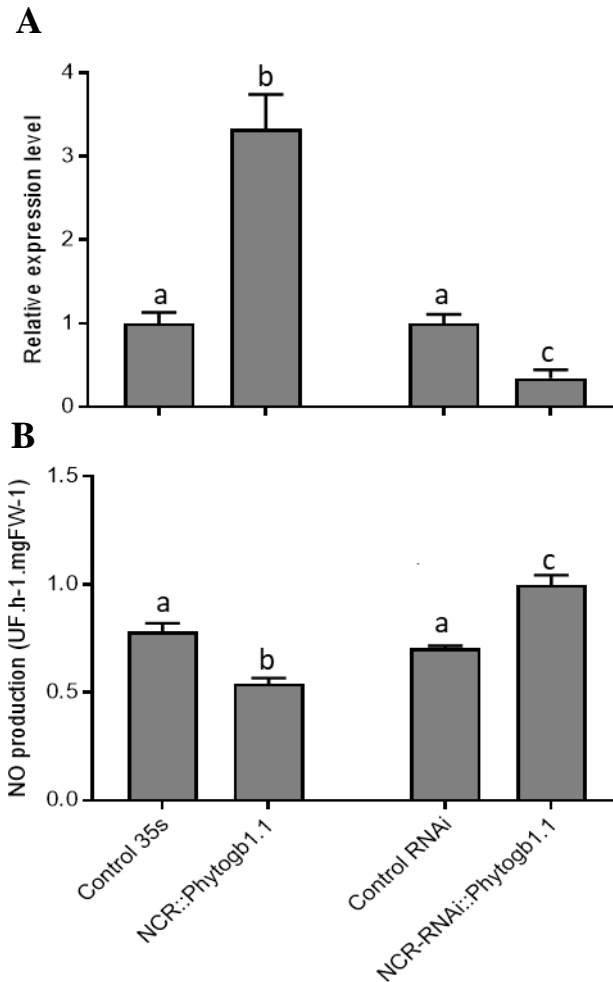


Figure 3: Relative expression level of *Phytogl.1* and NO production in nodules of control plant, NCR::*Phytogl.1* and NCR-RNAi::*Phytogl.1* at 3 wpi. (a) Expression analysis of *Phytogl.1* genes in control plant and nodule plant overexpressing (NCR::*Phytogl.1*) or underexpressing *Phytogl.1*(NCR-RNAi::*Phytogl.1*) at 3 wpi. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$) (b) Analysis of NO production in nodule of control plant and nodule plant overexpressing NCR::*Phytogl.1*) or underexpressing *Phytogl.1*(NCR-RNAi::*Phytogl.1*) at 3 wpi. The fluorescence intensity of the NO production was measured using DAF-2 fluorescent probe. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

transferase (GST) and chalcone synthase (CS) (Boscari *et al.*, 2013a). Both genes were found to be induced in RNAi::*Phytogl.1*, while their expression were unchanged in 35s::*Phytogl.1* (Fig. 3.2G,H), which suggest that their expression is up regulated by increased NO level, but not repressed under low NO.

***Phytogl.1* expression modulated NO production in nodule of transformed root at 3wpi.**

In order to analyse the modification of *Phytogl.1* expression in mature nodule, and especially in the N₂-fixing zone, we designed new constructions to over and down express *Phytogl.1* in the nodule. To this end, we used the zone III specific promoter NCR001 (Mergaert *et al.*, 2003b) in place to the 35s promoter and named them NCR::*Phytogl.1* for the overexpression construct and NCR-RNAi::*Phytogl.1* for the silencing construct. These constructs have the advantage of modifying the expression of *Phytogl.1*, specifically in the zone 3 of the nodule. Moreover, these constructs do not impact the nodule formation and development. The expression of *Phytogl.1* was verified by RT-qPCR with both constructions (Fig 3.3A). It resulted that *Phytogl.1* was 3.3-fold more expressed in NCR::*Phytogl.1* transformed nodules, and 3-fold less expressed in NCR-RNAi::*Phytogl.1* nodules as compared to their respective controls (Fig 3.3A). NO production was analysed in transformed and control 3 wpi-old nodules by using the DAF-2 fluorescent probe. As expected, NO production in NCR::*Phytogl.1* was lower (~ 1.47 times) compared to the control (Fig 3.3B), while NO production in NCR-RNAi::*Phytogl.1* was higher (~ 1.42 times) than the control (Fig 3.3B).

***Phytogl.1* expression modulated symbiotic nitrogen fixation**

Nitrogenase activity, measured as ARA, was measured in nodules of transformed and control plants at 3 wpi (Table 3.2). NCR::*Phytogl.1* nodules exhibited a 34% higher ARA compared to the nodule of control plants, while RNAi-NCR::*Phytogl.1* nodules exhibited a 30% reduced ARA compared to control ones.

Modulation of gene expression in *Phytogl.1* transformed nodules at 3 wpi.

The expression of genes involved in N₂-reduction and assimilation, hypoxia and senescence, was analysed in both control and transformed nodules at 3 wpi (Fig 3.4). The expression of *Lb4* was found to be induced in NCR::*Phytogl.1* nodules and reduced in NCR-RNAi::*Phytogl.1* ones as compared to their respective controls (Fig 3.4A), indicating that *Lb4* expression and N₂-fixation activity were regulated in the same way. The expression of genes encoding for NR were investigated together with that of glutamine synthetase 1a (GS1a) involved in the

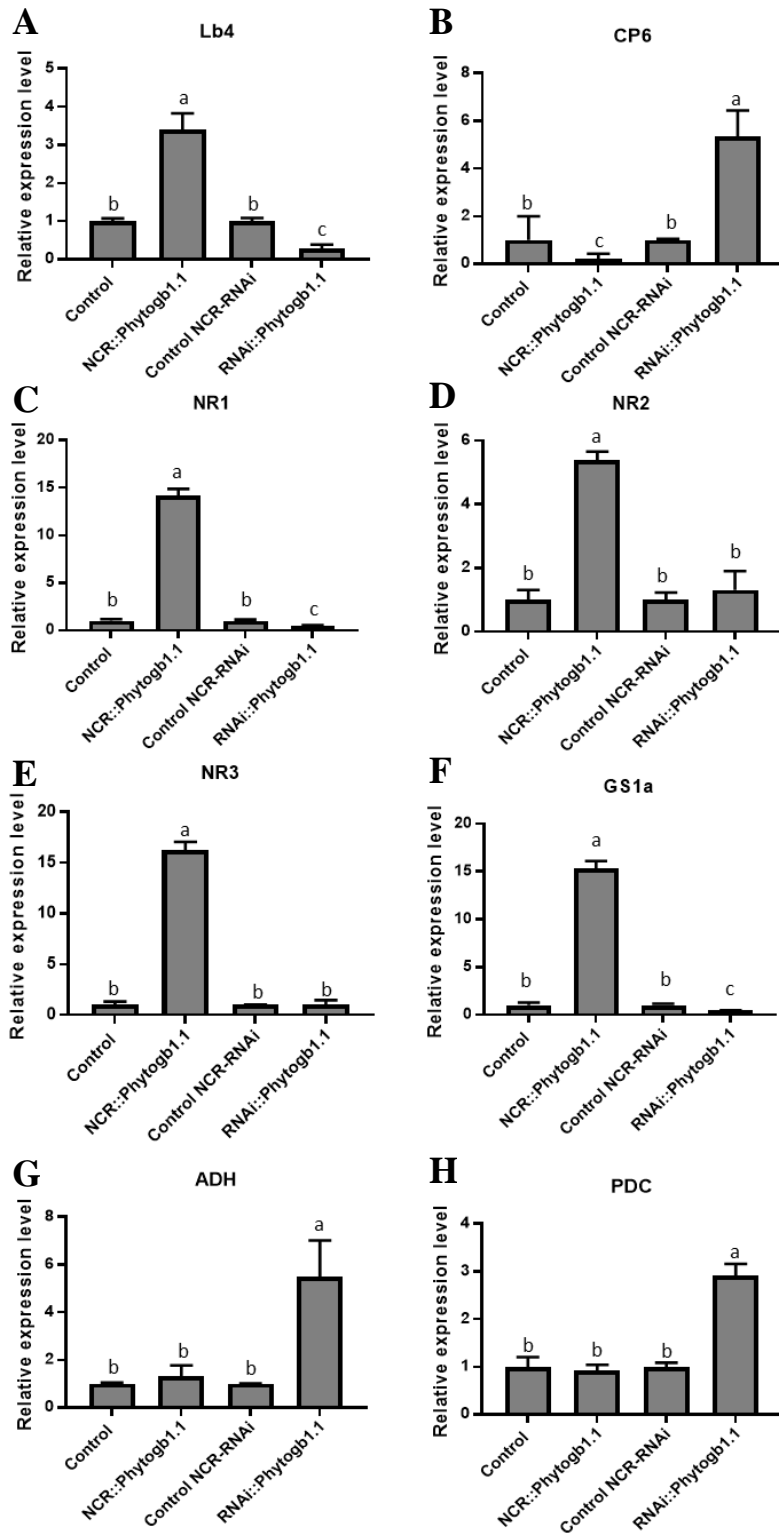


Figure 3.4: Relative expression genes analysis at 3 wpi in nodule of control plant, NCR::Phytogb1.1 and NCR-RNAi::Phytogb1.1. Expression analysis of (a) *Phytogb1.1* and *Lb4* genes (b) *NR1*, *NR2*, *NR3* and *GS1a* genes (c) *ADH* and *PDC* (d) *CP6* genes, in control plant and nodule plant overexpressing (NCR::Phytogb1.1) or underexpressing *Phytogb1.1*(NCR-RNAi::Phytogb1.1) at 3 wpi. Value are means \pm SE of three biological replicates, each with three technical replicates. Asterisks indicate statistical difference according to the Student's t-test

assimilation of reduced nitrogen in nodules (Groat & Vance, 1981). The 3 *NR* genes and that of *GS1a* were induced in NCR::*Phytogl1.1* nodules, while only *NR1* and *GS1a* were repressed in NCR-RNAi::*Phytogl1.1* nodules, (Fig 3.4C-F). As mature nodule exhibit a microoxic environment, we quantified the expression of two genes that was known to be overexpressed during hypoxia stress, alcohol dehydrogenase (*ADH*) and pyruvate decarboxylase (*PDC*). In our study, the expression of this two genes was increased in NCR-RNAi::*Phytogl1* nodules, whereas their expression was unchanged in (Fig 3.4G,H). We also analysed the expression of cysteine protease (*CP6*) gene which was shown to be a reliable senescence marker in *M. truncatula* nodules (Van de Velde *et al.*, 2006; Pierre *et al.*, 2014). *CP6* was found to be down-regulated in NCR::*Phytogl1.1* nodules, and strongly up-regulated in NCR-RNAi::*Phytogl1.1* one as compared to their controls (Fig 3.4B) suggesting that overexpression of *Phytogl1.1* delayed the senescence while the downregulation promoted it.

Phytogl1.1 an actor of NO regulation and nitrogen fixation during nodule senescence

To investigate the effects of *Phytogl1.1* expression on the senescence process, control and transformed plants were either submitted or not to a 10 mM KNO₃ treatment for two days (Berger *et al.*, 2018a). We conducted experiments with plants at 3 wpi without nitrate (-NO₃⁻) and with plants at 3 wpi and 2 days of nitrate treatment (+NO₃⁻). In control nodules, *CP6* expression was strongly induced (~24 fold) after nitrate treatment (Fig. 3.5B), while *Lb4* expression was 7-fold repressed (Fig. 3.5A), confirming that nitrate treatment triggered nodule senescence. In NCR::*Phytogl1.1* nodules, *CP6* and *Lb4* were respectively up and down-regulated, as observed in control nodules. However, their respective either induction or repression factors by nitrate (6 and 2.2-fold, respectively) were lower than in the control nodules. Similar trend was observed in NCR-RNAi::*Phytogl1.1* nodules, but the induction factor for *CP6* expression was more important (~134-fold) than in control ones (Fig. 3.5B). This observation suggests that the overexpression of *Phytogl1.1* delayed nodule senescence, while its repression promoted it.

We previously showed that NO production was increased after senescence induction by nitrate treatment (Berger *et al.*, 2018a). As *Phytogl1.1* regulate NO level in nodule (Fig. 3.3B), we also investigated NO production in response to nitrate treatment. In control NCR::*Phytogl1.1* and NCR-RNAi::*Phytogl1.1* nodules, NO production was increased after senescence induction, but to different extent (Fig 3.6). In presence of nitrate, NO production was lower in NCR::*Phytogl1.1* nodules as compared to the control once, while it was higher in NCR-RNAi::*Phytogl1.1* nodules.

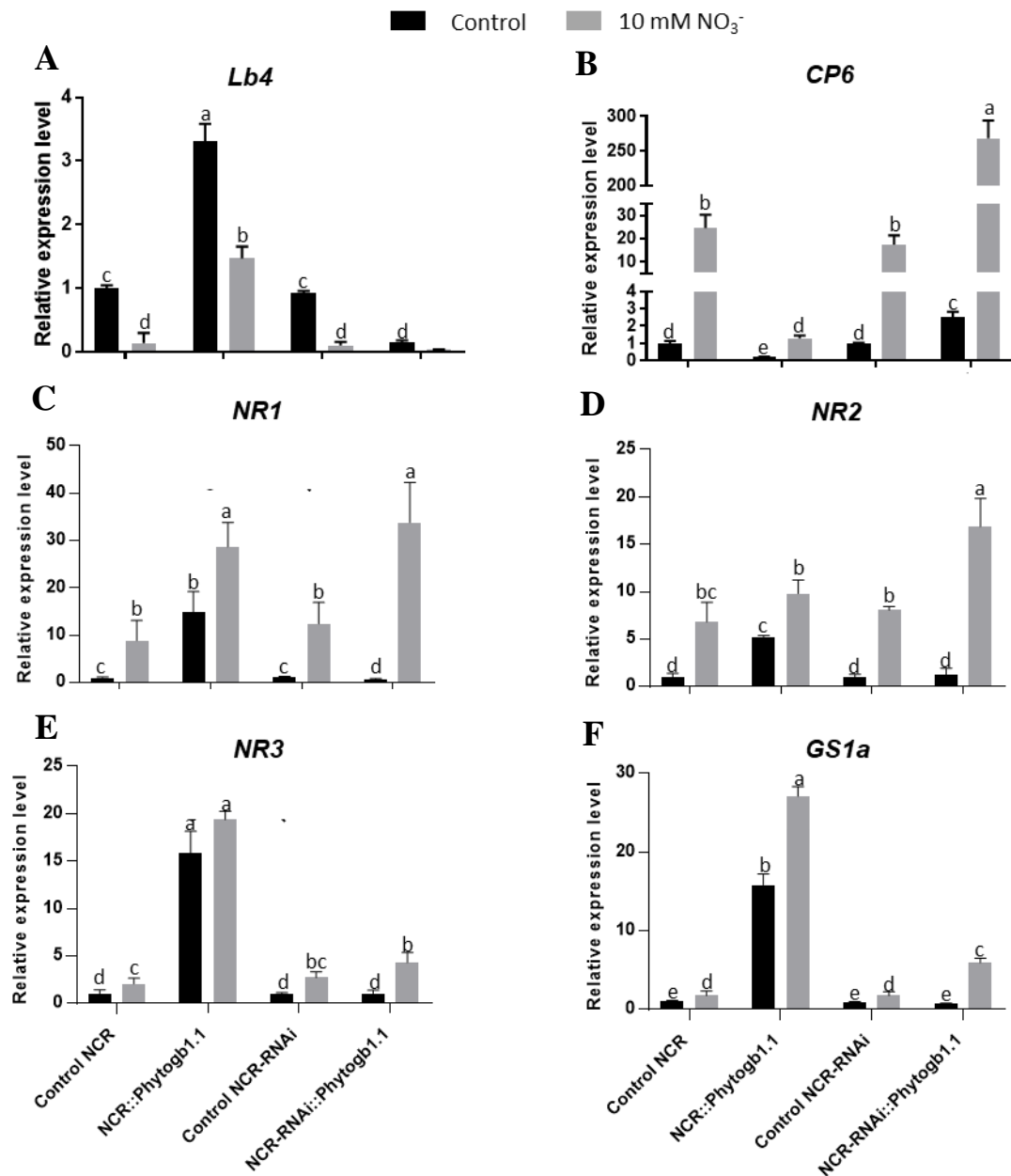


Figure 3.5: Effect of nitrate treatment on nitrogen metabolism genes expression. Expression analysis of (a) *Lb4* (b) *CP6*, (c) *NR1* (d) *NR2*, (e) *NR3* and (f) *GS1a* genes, in control plant and nodule plant overexpressing (NCR::*Phytogb1.1*) or underexpressing *Phytogb1.1*(NCR-RNAi::*Phytogb1.1*) at 3 wpi treated or not with 10 mM of nitrate. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

In addition, to check the hypothesis that *Phytogl1.1* delayed the nodule senescence, we measured the nitrogen fixation capacity of controlled and transformed plants after senescence induction (Tab 3). Even if the ARA was strongly reduced in nitrate treated plants (Tab 3), it remained higher in NCR::*Phytogl1.1* nodules, and lower in NCR-RNAi::*Phytogl1.1* nodules, than in control once (Table 3). These data are consistent with the fact that *Lb4* expression was proportionally less repressed by nitrate in NCR::*Phytogl1.1* nodules compared to control NCR once (Fig. 3.5A), whereas its expression was below the detection threshold after nitrate treatment in NCR-RNAi::*Phytogl1.1* nodules (Fig. 3.5A). Conversely, after nitrate treatment, *CP6* expression was proportionally less induced in NCR::*Phytogl1.1* nodules, and more induced in NCR-RNAi::*Phytogl1.1* nodules, compared to control nodules (Fig. 3.5B).

As nitrate treatment has been shown to induce senescence of nodules, it is also known that such treatment induces the expression of several genes, particularly those of nitrogen metabolism (Wang et al., 2000). In addition, another effect of nitrate treatment to the nodule is the switch from a nitrogen-fixing to a nitrate assimilation metabolism in the roots (Yashima et al., 2005). In order to investigate the influence of *Phytogl1.1* modification on nitrogen metabolism shift, we analysed some genes of nitrogen metabolism. As shown in Fig. 3.5, the three *NR* genes (*NR1*, *NR2*, *NR3*) and the glutamine synthetase (*GS1a*) genes were induced by a factor 8, 6, 2 and 2, respectively, in control nodules after nitrate treatment. In NCR::*Phytogl1.1* nodules the induction factor of these genes by nitrate were less than 2 for the four genes (Fig. 3.5C-F). In the opposite way, their nitrate induction factors are 28, 14, 4 and 8, respectively in NCR-RNAi::*Phytogl1.1* nodules.

Discussion

In a previous work, we showed that *Phytogl1.1* expression correlates with NO production level during *M. truncatula* – *S. meliloti* symbiotic process (Berger et al., 2018a). In the present work, we first generated overexpressing and silencing *Phytogl1.1* transformed *M. truncatula* roots under the control of a constitutive promoter (35s) to investigate the role of *Phytogl1.1* in the regulation of NO level and to analyse its potential involvement during the nodulation process. As reported in Fig. 3.1B the overexpression of *Phytogl1.1* decreased NO production while its down-expression increased NO production (Fig. 3.1B). This confirms that *Phytogl1.1* negatively regulates NO level as reported in previous studies carried out in *Lotus japonicus* (Nagata et al., 2008; Shimoda et al., 2009, Fukudome et al., 2016). Interestingly, our data show that, when compared to control conditions, both higher and lower NO levels inhibit the nodulation.

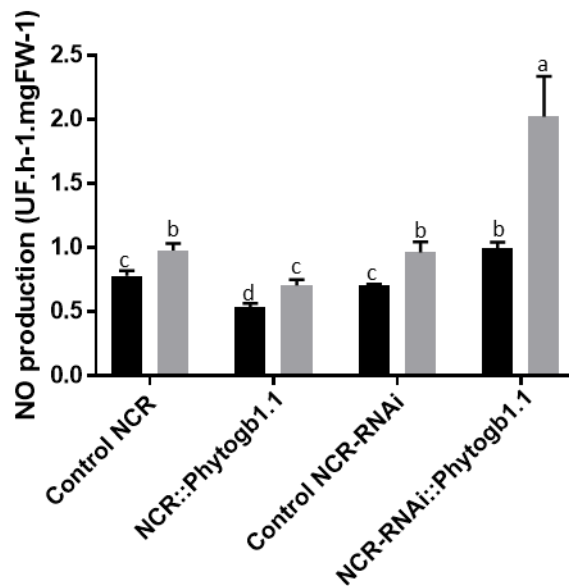


Figure 3.6: Effect of nitrate treatment on NO production. Analysis of NO production in nodule of control plant and nodule plant overexpressing NCR::*Phytogb1.1*) or underexpressing *Phytogb1.1*(NCR-RNAi::*Phytogb1.1*) at 3 wpi. The fluorescence intensity of the NO production was measured using DAF-2 fluorescent probe. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

These results are in agreement with those obtained with the *L. japonicus* knockout mutant of *LjGlb1* (Fukudome *et al.*, 2016) showing that high NO production inhibits the nodulation by affecting the infection thread. However, they also confirm the observation of Pii *et al.* (2008) and del Guidice *et al.* (2011) reporting that nodulation is inhibited by a decrease in NO level, when plants are treated with the NO scavenger cPTIO. This clearly means that an excess as well as a lack of NO impair nodule establishment, and that NO concentration needs to be tightly regulated and balanced at the site of nodule initiation for a successful establishment of the symbiotic relationship.

A transitory NO production peak was observed during symbiotic process between *M. truncatula* and *S. meliloti* at 10 hpi (Berger *et al.*, 2019). A similar short-term and transient NO production peak was observed at the *L. japonicus* root surface when inoculated with its compatible symbiont *Mesorhizobium loti*, but not with the pathogens *Ralstonia solanacearum* and *Pseudomonas syringae* with which high NO production level was maintained for at least 24 hpi (Nagata *et al.*, 2008). In the symbiotic infection with *M. loti*, the decrease in NO level following its transient accumulation was assigned to *LjPhytogb1.1* (*LjHB1*) which gene expression was up-regulated by the symbiont, but not by the pathogens (Nagata *et al.*, 2008). The up-regulation of *Phytogb1.1* may be linked to the NO production peak, since *Phytogb1.1* expression has been shown to be positively regulated by NO in both *L. japonicus* (Shimoda *et al.*, 2005; Bustos-Sanmamed *et al.*, 2011) and *M. truncatula* (Berger *et al.*, 2018a). In *G. max*, a large number of plant immunity related genes were shown to be induced within 12 hours post-inoculation by *B. japonicum*, but their expression gradually returned to background level within 24 h of inoculation (Libault *et al.*, 2010). Both *L. japonicus* and *M. truncatula* exhibited a similar induction of defense gene expression shortly after treatment with their symbiotic rhizobia, and then a reduction to resting levels after the establishment of symbiosis (Stacey *et al.*, 2006; Jones *et al.*, 2008). In RNAi::*Phytogb1.1* *M. truncatula* roots, we observed that higher NO level (Fig. 3.1B) correlates with an increase in defense reactions, *via* the induction of *GST* and *CS* genes (Fig. 3.2G and 3.2H). This observation is in good agreement with our previous data showing that NO donor treatment induces *GST* and *CS* gene expression (Berger *et al.*, 2018a). Thus, high defense reactions may be linked to high NO level resulting of *Phytogb1.1* down-regulation. Considered together, in the case of a symbiotic interaction, these data can be interpreted as follows: 1) the interaction between the plant and the symbiont triggers an increase in NO level and the induction of defense reactions in the plant roots, 2) the induction of *Phytogb1.1* by NO makes it possible to reduce NO to its initial level, which in return has the effect of lowering the defense reactions, allowing the reception of the symbiont and initiating the symbiotic process.

Why *Phytogl1.1* is not induced by NO in response to a pathogenic attack remains to be determined.

In *35s::Phytogl1.1* roots, the high expression of *Enod20* gene (Fig. 3.2B), a marker of early host cell infection (Vernoud *et al.*, 1999), suggests a high number of infection events. This could be explained by the low level of NO in *35s::Phytogl1.1* and, indirectly by the lower level of defense gene expression (Feechan *et al.*, 2005). Conversely, the very low expression level of *Enod20* in *RNAi::Phytogl1.1* roots exhibiting high NO level, argues in favour of a negative regulation of this gene by NO (Fig 3.2B). This observation is consistent with results obtained in an *A. thaliana* NR-deficient double mutant (*nia1/nia2*) with a low NO level (Vitor *et al.*, 2013). This mutant failed to exhibit a hypersensitive response, and was shown to be prone to infection by *P. syringae* (Modolo *et al.*, 2006; Oliveira *et al.*, 2009) and by the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* or *Botrytis cinerea* (Perchepped *et al.*, 2010; Rasul *et al.*, 2012).

The induction and repression of *Cre1* expression in the presence of high and low NO level respectively (Fig 3.2C) indicates that high NO promotes nodule development, while low NO inhibits it. Indeed, *Cre1*, that encodes a cytokinin receptor and is considered as a nodule development marker, has already been shown to be a NO responsive gene (Ferrarini *et al.*, 2008 ; del Giudice *et al.*, 2011). This gene regulates the early symbiotic interaction between *M. truncatula* and *S. meliloti*, by modulating the formation of nodule primordia. During the first days following inoculation with symbiotic rhizobia, del Giudice *et al.* (2011) reported a specific production of NO in the pericycle, endodermis and dividing cortical root cells, a block of cells named ‘controlled area’ where the nodule primordium is initiated. Consequently, NO could control the nodulation process by regulating cytokinin perception that is supposed to be a ‘secret agent’ of the symbiotic interaction (Frugier *et al.*, 2008). Thus, our results could partly explain the importance of the decrease of NO level observed between 10 hpi and 4 dpi (Berger *et al.*, 2018a). The first peak (10 hpi) of NO production is responsible for the defense genes induction. Then, NO level must be down regulated to allow the rhizobium to infect the plant cells before going up at 4 dpi to stimulate the growth and the development of the nodule regulation (del Giudice *et al.*, 2011). Consequently, one essential role of *Phytogl1.1* would be to adjust the NO level between these two crucial steps of the symbiotic process. This control is necessary because although NO is a key element of plant disease resistance, it is also involved in cell growth (Del Rio, 2015). Moreover, these results indicate that NO acts, at specific concentrations, on different mechanisms according to the timing of the nodulation process.

In the second part of our study, we analyzed the effect of *Phytogl.1* over and down-expression in nodules under the control of the NCR001 promoter which is specifically expressed in the N₂-fixing zone of mature nodules (Mergaert et al., 2003). Lower NO level (Fig. 3.3B), higher *Lb4* expression (Fig. 3.4A) and higher nitrogenase activity (Tab. 2) were measured in *Phytogl.1* overexpressing nodules, while opposite effects were observed in *RNAi::Phytogl.1* nodules (Fig. 3.3B; Fig 3.4A; Tab. 3.2). NO is known to be a potent inhibitor of nitrogenase (Trinchant & Rigaud, 1982; Kato et al., 2010), and to repress the expression of the bacterial *nifH* and *nifD* (Sanchez et al., 2010). However, ARA is more substantial in *L. japonicus* nodules in the presence of 0.1 mM SNP (NO donor) than either in the absence or in the presence of higher (1 mM) concentration of SNP, indicating that low but significant NO concentration is beneficial to nitrogen fixation (Kato et al., 2010).

The induction and repression of *Lb4* in *NCR::Phytogl.1* and *NCR-RNAi::Phytogl.1* nodules, respectively, (Fig 3.4A) is in good agreement with previous observations showing that NO-donor treatment represses *Lb4* expression in 4 dpi *M. truncatula* roots (Berger et al., 2018a). However, NO is certainly not the only regulator of *Lb* expression as their expression dramatically increased between 1 and 3 wpi, as well as NO production (Berger et al., 2018a). *Lbs* as well as *Phytogl.1* have the ability to bind O₂ and NO to produce NO₃⁻ (Herold & Puppo, 2005), which makes them good candidates to participate in the regeneration of energy through the functioning of the “Pgb-NO” respiration in the plant compartment (Horchani et al., 2011). This respiration is a substitute for the O₂-dependent respiration as pO₂ in nodule environment was low to prevent nitrogenase inhibition (Soupène et al., 1995). However, high respiratory rates are needed to sustain the energy consuming N₂-fixing metabolism and metabolite transport between roots and nodules (Bergersen & Appleby, 1981). Consequently, in *NCR::Phytogl.1* nodules exhibiting a high nitrogenase activity, it is not surprising to find high *NR* gene expression (Fig 3.C-E) in relation to increased Pgb-NO respiration, and high *GS1a* expression (Fig. 3.4F) to improve the assimilation of ammonia produced by nitrogenase (Robertson et al., 1975). The induction of *NR1/2* is correlated with the overexpression of *Phytogl.1* (Fig. 3.5) during treatment with nitrate. It is recognized that when genes are co-regulated, they are involved in the same cellular processes (Osterman & Overbeek, 2003). *NR* and *Pgb* are involved in Pgb-NO respiration (Dordas et al., 2004, Igamberdiev et al., 2005, 2010). Overexpression of these two genes supports this alternative respiration and thus produces enough energy to maintain the nitrogen-fixing activity (Udvardi & Day, 1997).

Interestingly, in *NCR::Phytogl.1* overexpressing nodules the senescence marker *CP6* (Van de Velde et al., 2006 ; Berger et al., 2019) was repressed (Fig. 4b), indicating a possible delay of

the senescence process. At the contrary, *CP6* was strongly induced in NCR-RNAi::*Phytogl.1* nodules (Fig. 4b). Pierre *et al.* (2014) reported that early expression of *CP6* in *M. truncatula* mature nodules results in early senescence, whereas RNAi suppression of *CP6* expression delays senescence and sustains nitrogen fixation. Altogether, our results dealing with nitrogenase activity, as well as *Lb4* and *CP6* expression indicate that *Phytogl.1* overexpression extends the nodule's lifespan. To test this hypothesis, we triggered senescence in nodules overexpressing or under-expressing *Phytogl.1* by using a 48h treatment in the presence of 10 mM nitrate. Indeed, previous work has shown that a treatment with 10 mM nitrate on *M. truncatula* 3 wpi-old nodules caused a 60% increase in NO production, a decrease in *Lb4* expression and an increase in *CP6* (Berger *et al.*, 2018). Our present data show that, in nodules over-expressing *Phytogl.1*, the inhibitory factor of ARA by nitrate treatment (83%) is lower than that observed in control nodules (91%, Control NCR) (Tables 2 and 3). The same observation can be made for the repression factor of *Lb4* (54% versus 90%, Fig. 3.5A), and for the induction factor of *CP6* (600% versus 2500%, Fig. 3.5B). In other words, the overexpression of *Phytogl.1* in the nodules makes it possible to limit the increase of the level of NO consecutive to the nitrate treatment, to prolong the nodule fixation activity and to delay the senescence. Conversely, following nitrate treatment, in nodules under-expressing *Phytogl.1*, ARA is more strongly inhibited (93% versus 91%), the expression of *Lb4* is completely repressed and the expression of *CP6* is proportionally more strongly induced (10 000% versus 1800%) than in their respective controls (Tables 2 and 3, Fig. 3.5A and B). These results confirm the observations of Cam *et al.* (2012) who showed that, in nodules of *M. truncatula* induced by lines over-expressing the bacterial flavo-hemoprotein hmp, the decrease in the level of NO leads to an increase in nitrogen fixation, a delay in nodular senescence and decreased expression of senescence markers.

It is known that NO and ROS are involved in the senescence process of nodosity (Puppo *et al.*, 2013). For example, NO reacts with the superoxide anion to form peroxynitrite which inactivates many proteins via nitration of tyrosines (Vandelle and Delledonne, 2011). It has been suggested that Lbs may react with peroxynitrite generated during senescence and thus protect mature nodules from the toxic effects of this RNS (Navascues *et al.*, 2012). Our results are therefore consistent with the hypothesis that the induction of Phytogbs in the mature nodules of *M. truncatula* can be interpreted as a mechanism of renewal of the pool of Phytogbs inactivated by RNS, in order to maintain a low level of RNS and thus maintain nitrogen binding capacity (Berger *et al.*, 2018).

Moreover, it is interesting to note that nitrate treatment induces *NRI*, *NR2* and *GS1a* induction in *Phytoglob1.1* over-expressing and silencing nodule (Fig. 3.5). The induction of these genes, linked to the assimilation of nitrogen via the reduction of nitrate, confirms a shift from the fixation and symbiotic assimilation of nitrogen, to the assimilation of nitrogen from nitrate in nodule (Yashima *et al.*, 2005). Plant growth under symbiotic nitrogen fixation is lower than growth using sufficient fertilizer (Terpolilli *et al.*, 2008). It was shown that, the growth rate of plants relying on symbiotic nitrogen fixation is approximately between 60% and 90% of the growth of plants supplied with sufficient amounts of fertilizer (Terpolilli *et al.*, 2008). There are evidences to indicate that when plants have the “choice” between nitrogen nutrition and N₂-fixation, the former is “favoured” (Wery *et al.*, 1986).

Conclusion

In conclusion, our results indicate that regulation of NO by *Phytoglob1.1* between 10 hpi and 4 dpi is an important step to lead the infection but also to the onset of nodule development. Moreover, we also demonstrate that overexpression of *Phytoglob1.1* improves the symbiosis metabolism by increasing the nitrogenase activity but also by delaying nodule senescence after nitrate treatment. On the contrary, the silencing of *Phytoglob1.1* is deleterious for the symbiosis activity and promotes the shift between nitrogen acquisitions from symbiotic process to nitrogen acquisition from root, after senescence induction by nitrate. Finally, regarding our results it appeared that NO production is a secondary effect of senescence induction.

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Table 1: Number and size of nodules in control plant and Phyto**g**b1.1 transformed plant at 14 dpi. Values are means ± SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test (P<0.05)

Constructs	Nodule number	Nodule size (mm ²)
Control	14.3 ± 1.09 (a)	0.7 ± 0.03 (a)
35S::Phyto g b1.1	6.7 ± 0.51 (b)	0.8 ± 0.04 (a)
RNAi::Phyto g b1.1	6.6 ± 0.48 (b)	0.8 ± 0.02 (a)

Table 2: Nitrogenase activity in control plant and Phyto**g**b1.1 transformed plant at 3 wpi. Nitrogenase activity (estimated as ARA) was measured at 3 wpi and normalised per nodule fresh weight. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

Constructs	ARA (/mg nodule)
Control NCR	15.8 \pm 1.10 (a)
NCR::Phyto g b1.1	21.2 \pm 1.44 (b)
Control RNAi-NCR	16.7 \pm 1.69 (a)
NCR-RNAi::Phyto g b1.1	11.0 \pm 0.81 (c)

Table 3: Nitrogenase activity in control plant and Phyto**g**b1.1 transformed plant at 3 wpi after 2 days of 10 mM nitrate treatment. Nitrogenase activity (estimated as ARA) was measured at 3 wpi and normalised per nodule fresh weight. Values are means \pm SE of three biological replicates, each with three technical replicates. Each data set was analyzed by one-way ANOVA followed by a Fisher test ($P < 0.05$)

Constructs	ARA + NO₃⁻ (/mg nodule)
Control NCR	1.4 \pm 0.09 (a)
NCR::Phyto g b1.1	3.5 \pm 0.58 (b)
Control RNAi-NCR	1.3 \pm 0.13 (a)
NCR-RNAi::Phyto g b1.1	0.7 \pm 0.13 (c)

Supporting information

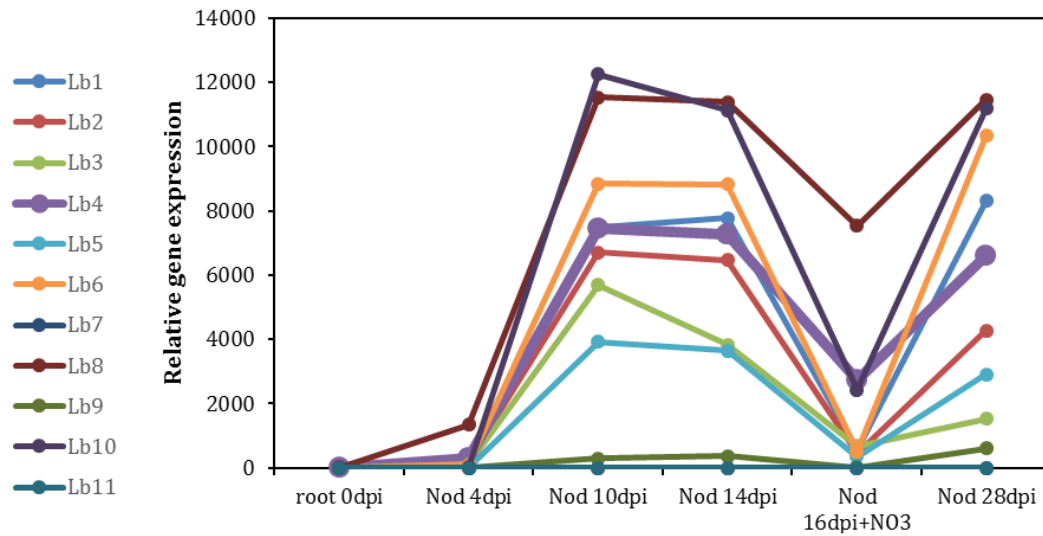


Figure S1 : Microarray data of *M. truncatula* leghémoglobine (Benedito et al., 2008)

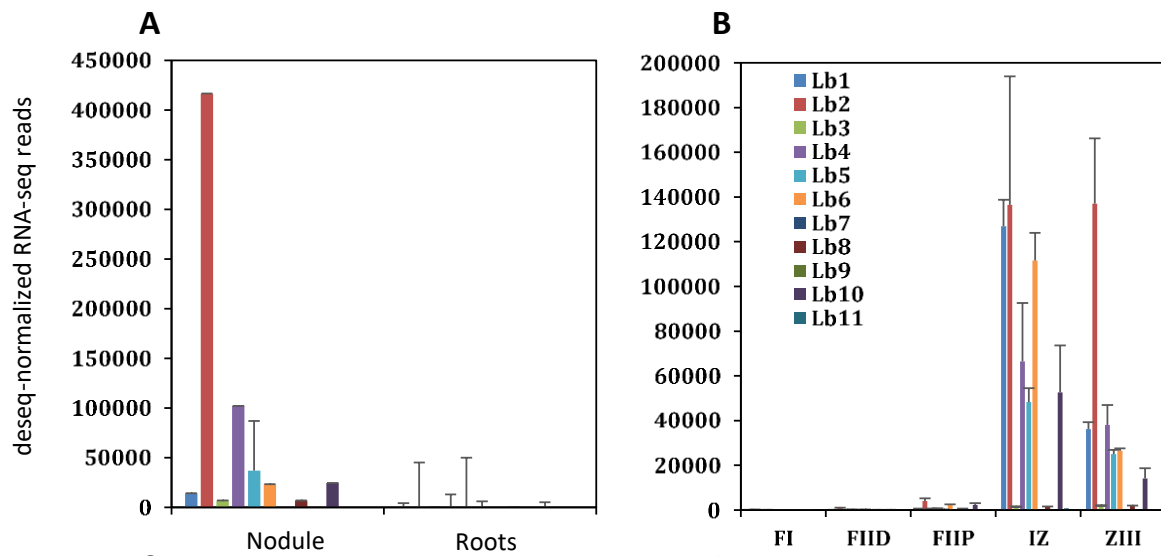


Figure S2 : Symbiosis expression of *M. truncatula* leghemoglobins. (a) Expression in roots and nodules ; (b) Repartition in different zones of nodule (FI, zone 1; FIID, zone 2 distal; FIIP, zone 2 proximal; IZ, interzone; ZIII, zone 3)

Table S1: List of primers

Genes	Code gene	Description	Amorces sequences 5'-3'	Efficaciency	References
Mtc27	Medtr2g436620	housekeeping gene	F : GTGGGAGGTTGAGGGAAAGT; R : TTGAAGTCCTTGAGCTTGC	97%	del Guidice et al., 2011
a38	Medtr4g109650	housekeeping gene	F : TCGTGGTGGTGGTTATCAAA ; R : TTCAGACCTTCCCATTGACA	92.50%	del Guidice et al., 2011
ENOD20	Medtr8g145270	early noduline	F : TCAACTCCAATTCTCATCC; R : AATCTGAAGGTGACGGTG	95%	del Guidice et al., 2011
CRE1	Medtr8g106150	cytokinin receptor histidine kinase	F : CTCTTGCCATCTTGTTCCTCAA; R : GTGCATAGGCCACTCCACTAA	91%	del Guidice et al., 2011
phytob1.1	Medtr4g068870	no symbiotic hemoglobin	F : GGACAATGCCAATTGATAAGCAG ; R : CTGGTGGAGCAATCTCAAGG	90%	Berger et al., 2018
lb 4	Medtr1g011540	leghemoglobine	F : GAGCGAAGAATTGAGCACTGCT ; R : TGCCTCTTAATTGCAAGTTGCC	88.50%	Berger et al., 2018
GS1	Medtr3g065250	glutamine synthetase isoforme 1	F : CTTGACCTCTCCGAAACCA; R : CTTGGGAAGCTGTGAAGGG	93%	Berger et al., 2018
CP6	Medtr4g079800	cysteine protease	F : CCTGCTGCTACTATTGCTGGATATG; R : CACTCGCATCAATGGCTACGG	97%	Pierre et al., 2014
nr1	Medtr3g073180	Nitrate reductase isoforme 1	F : GTTCAGTTTGAGTAAAGCC; R : ATACATACAGCGTCTACTC	91%	Horchani et al., 2011
nr2	Medtr5g089820	Nitrate reductase isoforme 2	F : CCACCTATGATCAATTTGCTG ; R : TCTATTACTTGCCCTAGAACAC	97%	Horchani et al., 2011
nr3	Medtr3g073150	Nitrate reductase isoforme 3	F : GCATGGGATCTGGCTAATAACAC ; R : TTATTCTTAGGGTCTGGTCTAGAG	93%	Boscari et al., 2013
nir	Medtr4g086020	Nitrite Reductase	F : AAATGGTAAGGCTACTGAAGG ; R : CTACAATAGGCACCAAGTCC	96%	Boscari et al., 2013
GST	Medtr7g065600	glutathion S-transferase	F : TTTGTTCACTAGTGAGAAATTTCC; R : GAAGACTTTTCATAACGAGCTTTAA	115%	Boscari et al., 2013
CS	Medtr1g124600	chalcone synthase	F : AAAGA5TAAATCCACCCAGAG; R : AAACACCAAACCTCAAGTCTC	120%	Boscari et al., 2013
ADH	Medtr3g089940	alcool déhydrogénase	F : GGGACTATGTTCTCAATCTGG; R : TAGGTACCAAATGTCACAGTCTC	100%	Rovere et al., 2019
PDC	Medtr2g015560	pyruvate decarboxylase	F : GCCCCGCGTTAAGATCAAC; R : CCAAGTTATTCACCACTGCCT	98%	Rovere et al., 2019

Table S2: List of plasmids

Plasmides	
pDONR207	Invitrogen
pK7WG2D	Invitrogen
pK7GWIWG2D	Karimi <i>et al.</i> , 2002
pK43rollDGFP	Karimi <i>et al.</i> , 2002
pENTR-T35S	Karimi <i>et al.</i> , 2002
pDONR P4-P1r-NCR01	Horchani <i>et al.</i> , 2011
pK7WG2D-Phytob1.1	This work
pK7GWIWG2D-Phytob1.1	This work
pdonr p4-p1r-ncr001-Phytob1.1	This work

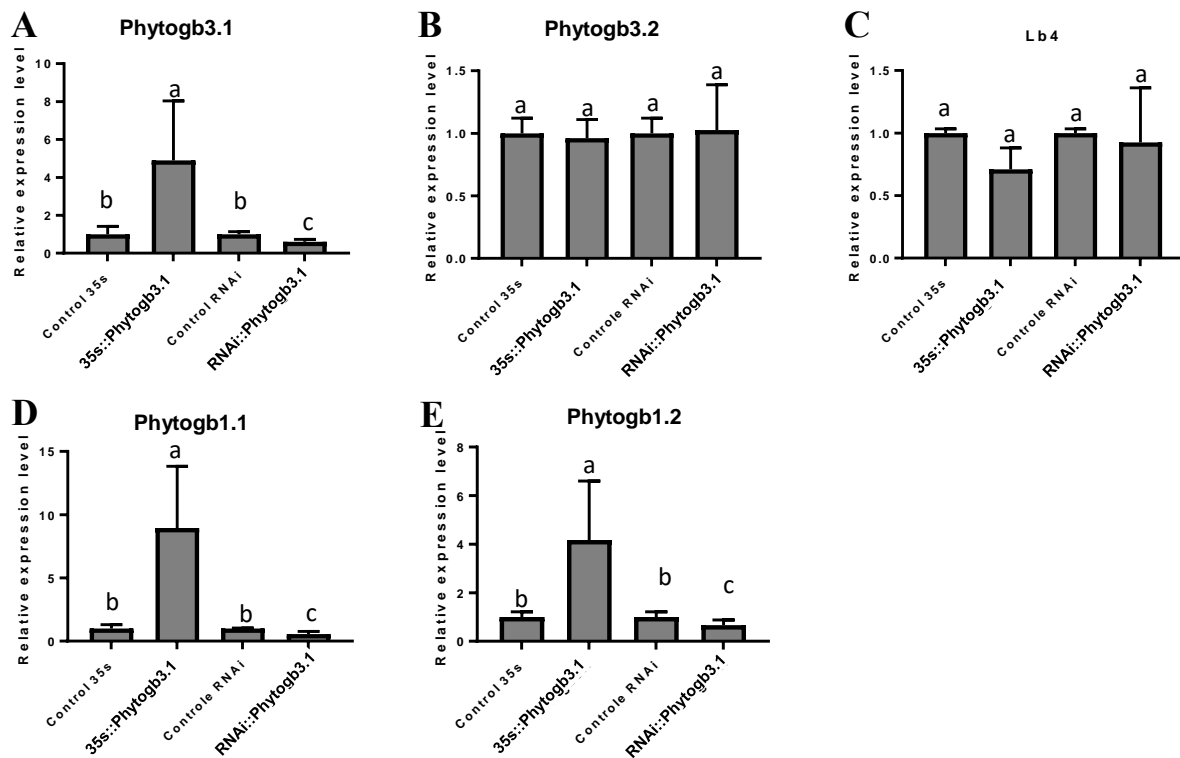


Figure 3.7 : Niveaux d'expression relative des gènes de *PhytoGb* dans des racines contrôle, 35s::*PhytoGb1.1* et RNAi::*PhytoGb1.1* à 4 jpi. L'expression des différents gènes ; *PhytoGb3.1* (a), *PhytoGb3.2* (b), *Lb4* (c), *PhytoGb1.1* (d) et *PhytoGb1.2* (e) est normalisée relativement à deux gènes de référence. Les expériences sont réalisées en triplicat sur trois expériences indépendantes. Les barres d'erreurs correspondent à l'erreur standard (n=3) et une ANOVA à une dimension est utilisée pour analyser les données. Les moyennes sont comparées par un test de Fisher avec un intervalle de confiance à 95%.

3. Résultats complémentaires et discussion

3.1. Analyse de l'état énergétique des nodosités transformées sur *Phytogb1.1*

Des analyses sur l'état énergétique des nodules par mesure du ratio ATP/ADP, sont actuellement en cours de réalisation pour déterminer l'impact de la modification de l'expression de *Phytogb1.1* sur le métabolisme énergétique des nodosités. Les résultats obtenus sur les nodosités de 3 spi montrent que la surexpression de *Phytogb1.1* aboutit à une meilleure fixation de l'azote (Tab 3.2), une induction de l'expression de *Lb4* (Fig. 3.4a) et une répression de l'expression de *CP6* (Fig. 4b). Au vu de ces résultats, il est possible de d'attendre un rapport ATP/ADP plus élevé dans les racines sur exprimant *Phytogb1.1* par rapport au contrôle, et l'inverse dans les racines sous exprimant *Phytogb1.1*. Ces résultats apporteraient des données physiologiques et confirmeraient l'implication de *Phytogb1.1* dans le maintien du métabolisme énergétique de la nodosité.

En parallèle de ce travail, une analyse fonctionnelle a été réalisée sur le gène *Phytogb3.1* lors des premières étapes de la symbiose à 4 jpi. Ce gène, comme *Phytogb1.1* est régulé de façon parallèle à la production de NO. L'analyse fonctionnelle de *Phytogb3.1* est réalisée selon la même approche méthodologique de surexpression et de sous expression sous le contrôle du promoteur constitutif 35s.

3.2. Impact de la modulation de *Phytogb3.1* lors du développement de la nodosité à 4 jpi

3.2.1. *Phytogb3.1* impact l'expression des *Phytogb1* dans les racines nodulées

La validation des constructions 35s::*Phytogb3.1* et RNAi::*Phytogb3.1* montre respectivement une induction de l'expression de *Phytogb3.1* de 5 fois et une diminution de 2.5 fois (Fig. 3.7A). Cependant, l'analyse des autres gènes de phytoglobine montre une induction de l'expression de *Phytogb1.1* et *Phytogb1.2* respectivement d'un facteur 9 et 4, dans les racines 35s::*Phytogb3.1* (Fig. 3.7D, 3.7E), sans que l'expression de *Phytogb3.2* et *Lb4* ne soit modifiée (Fig. 3.7B, 3.7C). De plus, la sous-expression de *Phytogb3.1*, semble aussi impacter l'expression des *Phytogb1* (Fig. 3.7D, 3.7E), mais ne provoque pas de modification significative de l'expression des autres phytoglobines. Il est donc difficile de pouvoir conclure sur le rôle distinct de *Phytogb3.1* à cause de son impact sur l'expression des *Phytogb1*. Néanmoins un certain nombre de points peuvent être soulignés dans les résultats suivants.

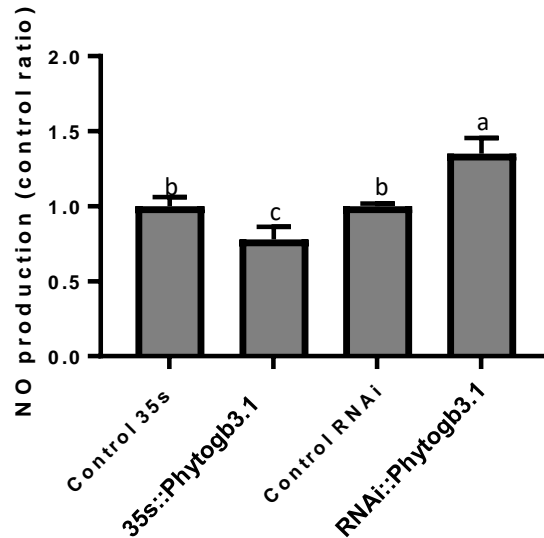


Figure 3.8 : Mesure de la production de NO dans des racines 35s::Phytogb3.1, RNAi::Phytogb3.1 et contrôle. Les mesures sont effectuées à 4 jpi. Les données correspondent à la moyenne de 4 expériences biologiques indépendantes réalisées en triplicat. Les barres d'erreur correspondent à l'erreur standard et une ANOVA à une dimension est utilisée pour analyser les données. Les moyennes sont comparées par un test de Fisher avec un intervalle de confiance à 95%.

Tableau 3.4 : Analyse du phénotype de nodulation sur les racines 35s::Phytogb3.1, RNAi::Phytogb3.1 et contrôle. Le nombre de nodosité et leur taille sont mesurés à 14 jpi. L'ensemble des données correspondent à la moyenne de 3 expériences biologiques indépendantes. Une ANOVA à une dimension est utilisée pour analyser les données. Les moyennes sont comparées par un test de Fisher avec un intervalle de confiance à 95%

Construction	Nombre de nodosités	Taille des nodosités (mm ²)
Contrôle 35s	14.3 ± 1.09 (a)	0.7 ± 0.03 (a)
35S::Phytogb3.1	8.62 ± 0.75 (b)	0.8 ± 0.04 (b)
Contrôle RNAi	14.2 ± 1.38 (a)	0.7 ± 0.05 (a)
RNAi::Phytogb3.1	10.73 ± 0.86 (b)	0.9 ± 0.04 (b)

3.3. Modification de la production de NO dans les racines nodulées

La surexpression et la sous expression de *Phytogb3.1* entraînent, respectivement une baisse et une augmentation similaire (1.3 fois) de la production de NO (Fig 3.8) qui dans les deux cas se traduit par une baisse du nombre de nodosités (Tab 3.3). Ces résultats sont cohérents avec ceux obtenus avec *Phytogb1.1*. Il est cependant impossible d'attribuer la responsabilité directe de ces modifications à *Phytogb3.1*, puisque les mêmes effets sont observés chez les racines des plantes modifiées sur *Phytogb1.1*. De même, il est difficile de conclure quant au rôle de *Phytogb3.1* sur la croissance de la nodosité puisque sa surexpression ainsi que sa sous-expression augmentent la taille des nodosités.

On peut cependant noter que, quelles que soient les constructions (*Phytogb1.1/Phytogb3.1*), les variations de production de NO sont faibles et ne sont pas corrélées avec les facteurs d'induction de ces gènes. En effet, une forte surexpression de *Phytogb1.1* ou *Phytogb3.1* (fig. 3.7) ne provoque pas une baisse similaire de la production de NO (Fig. 3.8). Une hypothèse a été posée sur la présence d'un niveau de NO différent dans les racines transformées avec *A. rhizogenes* par rapport à des racines non transformées. Des analyses préliminaires de production de NO sur des racines de plantes sauvages et des racines de plantes composites (35S::LacZ) ont été réalisées. La production de NO a été mesurée au sein des deux types de plantes à plusieurs zones de la racine : l'apex racinaire, la partie centrale et la partie supérieure de la racine située sous le collet (Fig. 3.9A), sachant que la partie centrale de la racine correspond à la zone de la racine où va se développer majoritairement l'infection et que l'apex racinaire est une zone de production de NO avérée. Les résultats montrent que le niveau de NO est 2 à 4 fois plus important dans l'apex et la zone centrale des racines sauvages respectivement, comparé aux racines transgéniques. En revanche, on ne constate aucune différence au niveau de la partie supérieure des deux types racinaires (Fig. 3.9B). On peut observer également un gradient de niveau de NO au sein des racines sauvages. Ce gradient paraît absent dans les racines de plantes composites.

Ces résultats obtenus sont en accord avec l'hypothèse d'un niveau de NO plus faible dans des racines transgéniques. Il est donc important de garder à l'esprit cette différence dans l'analyse des résultats sur la production de NO obtenus avec l'utilisation des racines transgéniques. Dans le cas de nos résultats, la faible augmentation de la production de NO, observée dans les racines RNAi, vient probablement du fait que la production de NO dans ces racines est naturellement plus faible. Mais si la production de NO est plus faible dans les racines transgéniques pourquoi n'observe-t-on pas une production beaucoup plus faible dans les racines sur-exprimant les *Pgb* ?

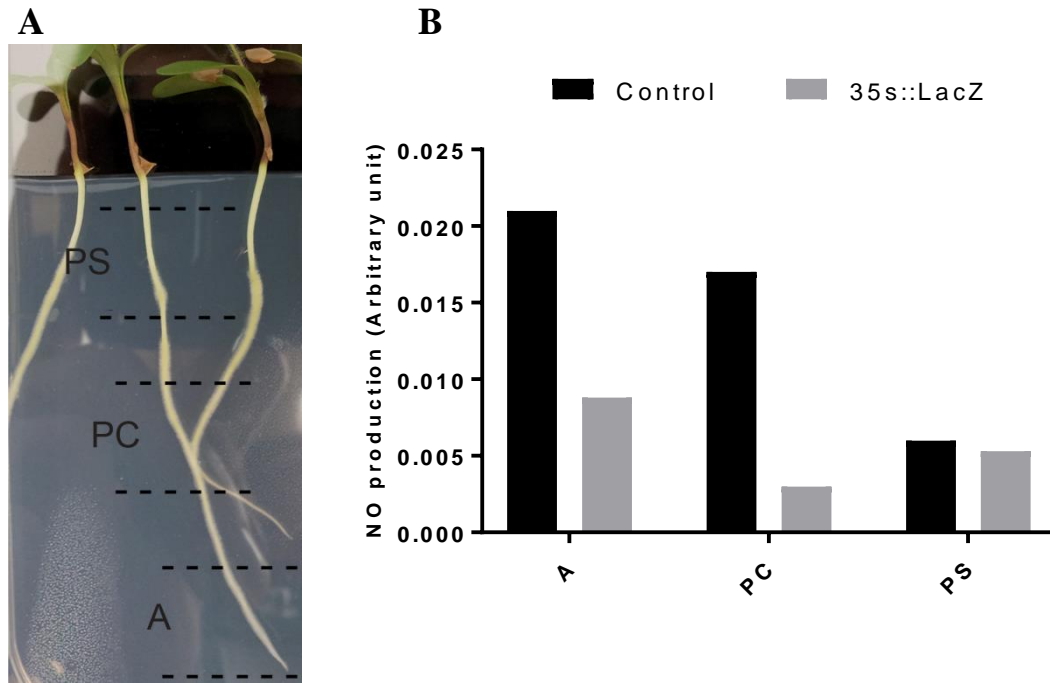


Figure 3.9: Photo d'une racine de *M. truncatula* et mesure du niveau de NO. PS = Partie supérieure ; PC = Partie centrale ; A = Apex racinaire. B. Mesure du niveau de NO dans les différentes parties de racines non inoculées de plantes sauvages et transgéniques. (Résultats préliminaires)

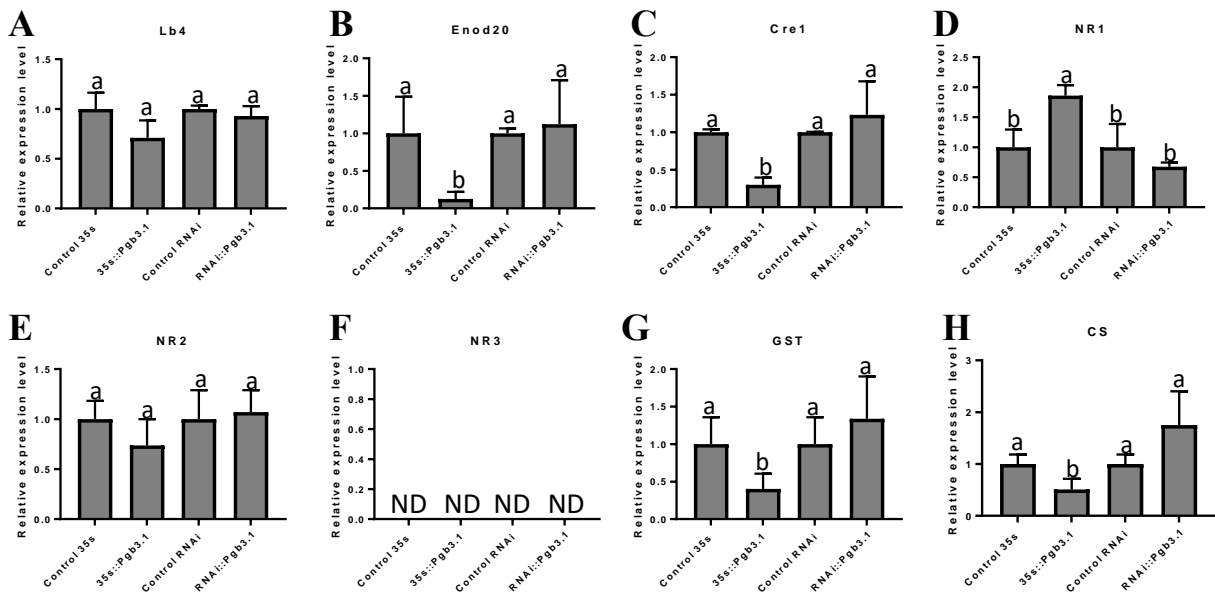


Figure 3.10 : Niveaux d'expression relative des gènes dans des racines contrôle, 35s::Phytogb3.1 et RNAi::Phytogb3.1 à 4 jpi. L'expression des différents gènes ; Lb4 (a), Enod20 (b), Cre1 (c), NR1 (d) NR2 (e), NR3 (f), GST (g) et CS (h) est normalisée relativement à deux gènes de référence. Les expériences sont réalisées en triplicat sur trois expériences indépendantes. Les barres d'erreurs correspondent à l'erreur standard (n=3) et une ANOVA à une dimension est utilisée pour analyser les données. Les moyennes sont comparées par un test de Fisher avec un intervalle de confiance à 95%.

Cela peut s'expliquer en partie par une surexpression du gène codant pour la NR (*NR1*) en parallèle de la surexpression des gènes *Phytogb1.1* et *Phytogb3.1*. L'impact de ces deux enzymes, l'une impliquée dans la production de NO (NR) (Horchani *et al.*, 2011) et l'autre dans son catabolisme (Pgb) (Sasakura *et al.*, 2006 ; Fukudome *et al.*, 2016) limite probablement les variations observées.

3.4. Expression des gènes à 4 jpi

Dans les racines 35s::*Phytogb3.1*, l'expression du gène *NR1* augmente d'un facteur 1.9 par rapport au contrôle, sans modifier significativement l'expression de *NR2* (Fig 3.10D ; 3.10E). Par ailleurs, dans les racines RNAi::*Phytogb3.1* l'expression de *NR1* et *NR2* n'est pas modifiée (Fig 3.10D ; 3.10E). Concernant les gènes marqueur de la nodulation, l'expression de *Enod20* et *Cre1* diminue respectivement de 7 et 3 fois dans les racines 35s::*Phytogb3.1* (Fig 3.10B ; 3.10C), mais ne sont pas modifiés dans les racines RNAi::*Phytogb3.1* (Fig 3.10B ; 3.10C). Enfin, la surexpression de *Phytogb3.1* diminue l'expression des gènes de défenses *GST* et *CS* alors qu'aucune modification de leur expression n'est visible dans les racines RNAi::*Phytogb3.1* (Fig 3.10G ; 3.10H).

L'induction de *NR1*, lors de la surexpression de *Phytogb3.1* (Fig 3.10D), du même ordre de grandeur que ce qui a été observé dans les racines qui sur-expriment *Phytogb1.1* (Fig 3.2) est cohérente avec le fait que *NR1* est positivement régulé par le NO (Chamizo-Ampudia *et al.*, 2016). En revanche, la répression de *Enod20* et *Cre1* ainsi que *GST* et *CS* dans les racines 35s::*Phytogb3.1* est difficile à interpréter dans la mesure où ces gènes ne sont pas inversement régulés dans les racines RNAi::*Phytogb3.1*. De plus, aucun parallèle logique ne peut être fait avec les résultats obtenus avec les racines modifiées sur *Phytogb1.1*.

Il conviendra donc d'approfondir l'analyse fonctionnelle de *Phytogb3.1*, en lien avec *Phytogb1.1*, en observant les répercussions de leur dérégulation sur des pas de temps plus courts, à une échelle tissulaire, voire cellulaire, et en analysant d'autres gènes liés au développement. De plus l'analyse fonctionnelle de *Phytogb3.1* au sein de la nodosité mature permettra d'apporter des informations complémentaires à celles obtenues avec *Phytogb1.1*.

Conclusions & Perspectives

Les principaux objectifs de cette thèse ont été, d'une part, de caractériser et étudier le rôle des NR et des phytoglobines dans la régulation de l'homéostasie du NO lors de la symbiose entre *M. truncatula* et *S. meliloti* et, d'autre part, d'analyser le rôle du NO dans le développement et le fonctionnement du processus symbiotique.

Les résultats obtenus au cours de cette thèse ont permis de mettre évidence 4 pics de production de NO à des étapes clés de l'interaction symbiotique : 1) 10 hpi, l'infection par *S. meliloti*, 2) 4 dpi, le début de l'organogénèse du primordium nodulaire, 3) 3-4 spi, lors de l'optimum de fonctionnement de la nodosité mature et 4) 6 spi, lors du déclenchement du processus de sénescence. En outre, mes travaux montrent une parfaite corrélation entre l'expression des NR, l'activité NR globale et les différents pics de production de NO. De plus les résultats obtenus permettent de déterminer que, parmi les 3 NRs de *M. truncatula*, NR1 est impliquées majoritairement dans la production de NO au sein de la nodosité. Par ailleurs, une des caractéristiques particulières de *M. truncatula* est de posséder une isoforme de NR spécifique de la nodosité, NR3 (Roux *et al.*, 2014) qui est fortement induite lors de la période de sénescence. L'utilisation de mutants de *M. truncatula* ciblés sur chaque isoforme de NR permettra de confirmer et distinguer la production de NO spécifique à chaque isoforme.

Des mutants sur les 3 NR sont disponibles au laboratoire depuis peu. Ils ont été recherchés dans les collections de mutants (par insertion de transposon *tnt1*) réalisées par la fondation Noble (Samuel Roberts Noble Foundation, Ardomore, USA). Cependant, ces lignées mutantes présentent un nombre élevé d'insertions du transposon *tnt1*, nécessitant par conséquent plusieurs rétrocroisements avec une lignée de type sauvage pour nettoyer leur fond génétique. L'obtention de ces lignées mutées sur chacune des 3 NR de *M. truncatula* permettra de réaliser une caractérisation complète de ces enzymes jouant un rôle majeur dans l'assimilation de l'azote et la signalisation NO. Ces mutants devraient aussi permettre d'obtenir des informations supplémentaires sur le rôle respectif de ces trois NR au cours du processus symbiotique. En particulier, caractérisation du mutant ciblé contre NR3 permettra de comprendre son rôle dans la nodosité et particulièrement lors de la sénescence.

Très peu de données sont disponibles sur la régulation de la NR au cours de la symbiose. Cependant, une étude récente montre que la NR peut être régulée par l'intermédiaire d'une Mitogen Activated Protein Kinase, la MAPK6 (Guo *et al.*, 2019). De plus, il a été montré chez *M. truncatula* que l'induction de la MAPK6 lors des premières heures suivant l'inoculation par *S. meliloti* était plus faible que son induction par *PstDC3000*, un agent pathogène (Chen *et al.*, 2017). (Chen *et al.*, 2017). Cette régulation permettrait de diminuer la production de NO en

inhibant l'activation de la NR par phosphorylation *via* la MAPK6 (Chen *et al.*, 2017). L'étude et l'identification de cette régulation permettrait de mieux comprendre la régulation de la production de NO au cours des premières étapes de l'interaction.

De plus, l'utilisation des différents mutants en parallèle avec des mutants bactériens impliqués dans le métabolisme du NO (i.e. *napA*, *nirK*) devraient permettre de déterminer quel partenaire, plante ou bactérie, est responsable de quel pic de production de NO.

Les résultats obtenus au cours de cette thèse ont également permis d'identifier et classer toutes les phytoglobines de *M. truncatula* avec 3 *Phytogb1*, 12 *Lbs* et 3 *Phytogb3*. Les cinétiques d'expression, notamment celle de *Phytogb1.1* et *Phytogb3.1*, ont montré une forte corrélation avec la cinétique de production de NO. De plus, les données de surexpression et de sous expression de ces gènes ont permis de confirmer leur implication dans la régulation négative du niveau de NO lors des premières étapes de la symbiose ainsi que dans la nodosité mature.

Cependant, il reste à confirmer que la modulation de l'expression des gènes de ces phytoglobines impacte directement la production de NO via leurs activités NO dioxygénase. A ce jour, aucune donnée n'existe sur la quantité de protéine ou l'activité biologique *in vivo* spécifique de chaque phytoglobine de *M. truncatula*. De plus, il serait donc important de déterminer la part relative de chacune de ces phytoglobines dans l'activité NO dioxygénase totale mesurée tout au long du processus symbiotique. Dans le cas où un tel projet devrait voir le jour, l'utilisation de la spectrométrie de masse permettra de distinguer et quantifier les différentes phytoglobines présentes chez *M. truncatula*. L'approche expérimentale consistera à cloner chaque gène de phytoglobine, à l'exprimer en système hétérologue et à purifier la phytoglobine correspondante. Une fois purifiée, l'activité NO dioxygénase de chacune d'elle pourra être mesurée et les constantes cinétiques vis-à-vis de l'O₂ et du NO. L'analyse par spectrométrie de masse de chaque phytoglobine permettra d'obtenir un ou plusieurs « peptides signatures » spécifiques de chacune. Ces peptides signatures serviront ensuite à identifier les phytoglobines dans un échantillon d'extrait protéique total de racines ou de nodosités. Les résultats seront analysés et normalisés avec les peptides signatures pour quantifier chacune des phytoglobines. Cette quantification permettra de faire le lien entre les mesures d'expression de gènes et la production de NO. Ceci étant, un tel projet représente un investissement en temps et en argent qu'il est, raisonnablement, difficile à envisager à l'échelle d'un laboratoire de recherche publique.

La répartition spatiale de chaque phytoglobine au sein de la nodosité est aussi un élément important à prendre en compte. D'une part, les phytoglobines possèdent des affinités pour l'O₂

très différentes, de l'ordre de 2, 50-100 et 1500 nM, respectivement, pour *Phytogb1*, *Lbs* et *Phytogb3* (Gupta *et al.*, 2011b). D'autre part, Soupène *et al.* (1995) ont montré qu'à cause de la présence d'une barrière à l'oxygène, la pO_2 diminue de 250 μM dans les premières couches de l'épiderme à 10-40 nM dans les cellules au cœur de la zone 3. Par conséquent, et comme cela a été discuté dans le chapitre 2, il est possible d'émettre l'hypothèse que la répartition des phytoglobines est dépendante de la pO_2 au sein de la nodosité. Les données Symbimics permettent de déterminer la localisation de l'expression majoritaire des phytoglobines selon les 4 zones de la nodosité (Roux *et al.*, 2014). Cependant, leur répartition au sein de la zone de fixation n'a jamais été démontrée. Une analyse microscopique à l'aide de constructions « promoteur-gène rapporteur » permettra de déterminer leur localisation au sein de la zone de fixation et de vérifier si les phytoglobines sont réparties uniformément ou séquentiellement au sein cette zone.

Par ailleurs, des valeurs de pO_2 de l'ordre de 10-40 nM posent la question de la régénération de l'énergie. Il a été montré qu'au sein des nodosités, se produit une respiration alternative, dite « Pgb-NO », pour permettre la régénération de l'ATP (Horchani *et al.*, 2011 ; Gupta & Igamberdiev, 2011 ; Berger *et al.*, 2018). Notre hypothèse est qu'il existe dans la nodosité, un gradient de NO inverse de celui du gradient de l' O_2 lié à la mise en place et au fonctionnement de la respiration « Pgb-NO ». Bien que ce gradient de pNO n'ait jamais été mesuré, un plus fort niveau de NO est observé au centre des nodosités relativement à la périphérie (Baudouin *et al.*, 2006 ; Cam *et al.*, 2012). L'utilisation de micro-électrode à NO et O_2 , permettra de mesurer les gradients de ces deux gaz sans perturber l'environnement micro-oxique de la nodosité.

Sur la base des affinités respectives des phytoglobines pour l' O_2 et le NO, on peut émettre l'hypothèse que les *Phytogb3* se retrouveraient en périphérie de la nodosité dans des cellules faiblement hypoxiques, les *Lbs* au sein des cellules intermédiaires et les *Phytogb1* dans des cellules fortement hypoxiques, au centre de la nodosité.

L'étude de *Phytogb1.1* a permis de mettre en évidence son rôle dans la régulation négative du NO lors du développement de la nodosité, mais également au sein de la nodosité mature et sénescence (chapitre 3). Les résultats ont montré, d'une part, que la surexpression de *Phytogb1.1* permet de réguler la concentration en NO durant les premières étapes de la symbiose pour favoriser l'infection, mais aussi pour induire le développement du primordium nodulaire. D'autre part, nos résultats indiquent que dans la nodosité mature la surexpression de *Phytogb1.1* permet de maintenir l'activité de fixation de l'azote et donc le fonctionnement de

la nodosité et ainsi de retarder le processus de sénescence. A l'inverse, la sous expression de *Phytogb1.1* impacte négativement le développement de la nodosité via l'augmentation des réponses de défenses de la plante et compromet son fonctionnement via la diminution de l'activité nitrogénase.

Au-delà de l'interaction symbiotique, on peut aussi imaginer que la modulation du niveau de NO via la dérégulation de *Phytogb1.1* pourrait être utile lors des interactions plante/pathogène. Il serait intéressant de tester l'interaction de racine de *M. truncatula* sous exprimant *Phytogb1.1* (avec un niveau élevé de NO) avec un pathogène (par ex : *A. euteiches*). L'objectif serait d'analyser la réponse au pathogène et d'observer le phénotype de résistance de la plante dans ce contexte de fort niveau de NO. Les résultats obtenus seront comparés à ceux obtenus par Thalineau *et al.* (2016) qui montrent que *M. truncatula* est plus résistant à *A. euteiches* chez des racines sur exprimant la *GSNOR* et possédant une quantité de SNO plus importante.

Parallèlement aux expériences sur *Phytogb1.1*, il sera nécessaire de poursuivre l'analyse de la caractérisation de *Phytogb3.1* au sein de la nodosité mature et sénescente. Peu de données sont disponibles quant au rôle de *Phytogb3.1* dans la symbiose, mais l'analyse d'expression des gènes montre une forte induction de *Phytogb3.1* au cours de la sénescence qui pourrait refléter l'implication de *Phytogb3.1* dans ce processus. De plus, l'hypothèse d'une interaction potentielle de *Phytogb3.1* et de la NR à récemment été posée (Chamizo-Ampudia *et al.*, 2017). L'hypothèse est que *Phytogb3.1* dépourvue de domaine réductase se lie à la NR, reçoit le pouvoir réducteur lié aux électrons du groupe FAD de la NR permettant ainsi l'acquisition d'une activité NO dioxygénase. Dans ce cas, l'interaction *Phytogb3.1*-NR pourrait jouer un rôle dans le rétrocontrôle de la production de NO en diminuant le pouvoir réducteur disponible pour la production du nitrite ou du NO par la NR (Chamizo-Ampudia *et al.*, 2017). Cette hypothèse a été posée chez *Chlamydomonas reinhardtii* et il sera intéressant de la vérifier chez *M. truncatula*. L'existence d'une telle interaction pourra être analysé par co-immunoprécipitation, stratégie double hybride ou Biacore (résonance plasmonique de surface).

Pour conclure, le travail réalisé au cours de cette thèse, a permis de mettre en évidence la production transitoire du NO et de sa régulation par les NR et les phytoglobines au cours du processus symbiotique. Cependant, peu de données sont disponibles sur les mécanismes d'action du NO dans la régulation transcriptionnelle des gènes qu'il régule. Récemment, des travaux du laboratoire ont mis en évidence un facteur de transcription (ERF-B2.1) sensible au NO et qui pourrait activer l'expression de *Phytogb1.1* lors d'un stress hypoxique. Ce facteur de

transcription, de la famille des ERF-VII (Ethylene Response factor) a été décrit comme un régulateur clé de la réponse au stress hypoxique chez *A. thaliana* (Licausi *et al.*, 2011; Gibbs *et al.*, 2015). Des travaux sont actuellement menés dans l'équipe pour déterminer le motif de reconnaissance de ce facteur de transcription sur la séquence d'ADN du promoteur de *Phytogl.1*. L'identification d'un motif *cis*-régulation spécifique de la signalisation NO permettrait de mieux comprendre la transduction du signal dans la cellule et ainsi détecter les gènes qui peuvent potentiellement être induit par le NO lors d'un stress abiotique (hypoxique, hydrique, etc...) et biotique (interaction symbiotique ou pathogène).

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Annexes

Annexe 1 :

Liste des gènes et codes d'accès ayant permis l'analyse phylogénétique

Espèce	Gènes	code d'accèsion
<i>M. truncatula</i>	Lb1	Medtr5g080400
	Lb2	Medtr5g066070
	Lb3	Medtr1g090810
	Lb4	Medtr1g011540
	Lb5	Medtr5g041610
	Lb6	Medtr5g080440
	Lb7	Medtr5g081000
	Lb8	Medtr1g090820
	Lb9	Medtr5g081030
	Lb10	Medtr1g049330
	Lb11	Medtr7g110180
	Lb12	Medtr5g080900
	phytob1.1	Medtr4g068860
	phytob1.2	Medtr4g068870
	phytob1.3	Medtr0026s0210
	phytob3.1	Medtr3g109420
phytob3.2	Medtr1g008700	
<i>G. max</i>	Lb1	NP_001235928.1
	Lb2	NP_001235423.1
	Lb3	NP_001235248.1
	Lb4	NP_001235248.2
	phytob1.1	XP_006590912.1
	phytob1.2	KRH29528.1
	phytob3.1	KRH16206.1
	phytob3.2	KRH62000.1
<i>L. japonicus</i>	Lb1	BAE46737.1
	Lb2	BAE46736.1
	Lb3	Q9FEP8.1
	Lb4	AFK43403.1
	Lb5	Q9FEP8.2
	Lb6	BAB18106.1
	phytob1.1	BAE46739.1
	phytob1.2	BAE46740.1
	phytob3.1	AFk37393.1
	phytob3.2	BT137598.1
<i>A. thaliana</i>	phytob1.1	NP_179204.1
	phytob1.2	NP_187663.1

Annexe 2 :


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SPECIAL ISSUE

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Pathways of nitric oxide metabolism and operation of phytooglobins in legume nodules: Missing links and future directions

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Pathways of nitric oxide metabolism and operation of phytohemoglobins in legume nodules: Missing links and future directions

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Abstract

The interaction between legumes and rhizobia leads to the establishment of a beneficial symbiotic relationship. Recent advances in legume–rhizobium symbiosis revealed that various reactive oxygen and nitrogen species including nitric oxide (NO) play important roles during this process. Nodule development occurs with a transition from a normoxic environment during the establishment of symbiosis to a microoxic environment in functional nodules. Such oxygen dynamics are required for activation and repression of various NO production and scavenging pathways. Both the plant and bacterial partners participate in the synthesis and degradation of NO. However, the pathways of NO production and degradation as well as their crosstalk and involvement in the metabolism are still a matter of debate. The plant-originated reductive pathways are known to contribute to the NO production in nodules under hypoxic conditions. Nonsymbiotic haemoglobin (phytohemoglobin; Pgb) possesses high NO oxygenation capacity, buffers, and scavenges NO. Its operation, through a respiratory cycle called Pgb–NO cycle, leads to the maintenance of redox and energy balance in nodules. The role of Pgb–NO cycle under fluctuating NO production from soil needs further investigation for complete understanding of NO regulatory mechanism governing nodule development to attain optimal food security under changing environment.

KEYWORDS

mitochondria, nitrate reductase, nitric oxide, nitrite, phytohemoglobin, rhizobium

1 | INTRODUCTION

Nitric oxide (NO) is a gaseous reactive nitrogen species present in all living organisms. In plants, NO is ubiquitous and is involved in many cellular processes. It participates in the regulation of main plant development stages, from germination to senescence, and in a number of physiological processes such as stomatal closure, flowering, organization of root architecture, or iron homeostasis (Corpas & Barroso, 2015; Domingos, Prado, Wong, Gehring, & Feijo, 2015). NO is also involved in plant response to many abiotic stresses such as hypoxia, salinity, osmotic stress, contamination by heavy metals, heat and cold, as well as in the biotic “plant-microbe” interactions, where it has been shown to act as a signalling molecule in the induction of cell death, to regulate defence genes expression, or to interact with reactive oxygen

species (ROS) during plant defence against pathogens (Thalineau et al., 2016). NO is also produced during symbiotic interactions, particularly in the nitrogen-fixing symbiosis between legumes and bacteria of rhizobium type (Hichri et al., 2015; Hichri, Boscari et al., 2016). The interaction between legumes and rhizobia leads to the establishment of a symbiotic relationship characterized by the formation of a new root organ, called nodule, which provides a niche for the bacterial nitrogen fixation. In nodules, bacteria differentiate into bacteroids that have the ability to fix atmospheric nitrogen via nitrogenase activity (Oldroyd & Downie, 2008). Nitrogen fixation within nodules requires a microaerophilic environment because nitrogenase is inhibited by traces of oxygen (Appleby, 1992). Thus, nodule development occurs under changing oxygen conditions, from a normoxic environment during the establishment of symbiosis to a microoxic (hypoxic)

environment in functional nodules. During the last decade, many studies have shown that NO is produced throughout the symbiotic process: from the first hours of the interaction between plant and bacteria (Nagata et al., 2008), during the organogenesis of nodules (del Giudice et al., 2011), in mature nitrogen-fixing nodules (Baudouin, Pieuchot, Engler, Pauly, & Puppo, 2006; Pauly et al., 2006), and during the entry into nodule senescence (Cam et al., 2012).

Both the plant and the bacterial partners participate in the synthesis and degradation of NO (Hichri, Meilhoc et al., 2016). NO production pathways include plant nitrate reductase (NR), nitrite (NO_2^-): NO reductase activity of the mitochondrial electron transport chain (ETC), putative plant NO synthase (NOS)-like enzyme, as well as bacterial inner and periplasmic NRs and NOS. NO degradation pathways include plant nonsymbiotic haemoglobins (phytoglobins [Pgbs]) and possibly symbiotic haemoglobins (leghaemoglobins [Lbs]), bacterial flavohaemoglobins, and NO reductase. The balance between NO production and degradation keeps NO concentration at the required amount for the regulation of various processes in nodules. The pathways of NO production and degradation, as well as the involvement and the role of each partner in the metabolism of NO, are still a matter of debate. The chemical nature, concentration, cellular environment, and compartmentation of NO indeed strongly influence its biological effects. As a result, NO has been differently described as a signalling molecule, a toxic byproduct, or a beneficial metabolic intermediate of symbiosis. Thus, it has been reported that during the symbiotic process, NO regulates the expression of many genes, among which genes involved in cortical cell dedifferentiation, nodule organogenesis, and repression of plant defence reactions, consequently favouring the establishment of the interaction between the plant and the rhizobium (Boscari, Del Giudice et al., 2013). Other studies have highlighted NO inhibitory effects on bacterial nitrogenase activity and expression (Sánchez et al., 2010), symbiotic nitrogen fixation in nodules (Kato, Kanahama, & Kanayama, 2010; Sasakura et al., 2006; Shimoda et al., 2009), and growth of rhizobia in free culture (Meilhoc, Cam, Skapski, & Bruand, 2010). On the contrary, it has also been demonstrated that in the microoxic nodules, NO takes part in the maintenance of the energy state (Horchani et al., 2011) and the regulation of nitrogen metabolism (Melo, Silva, Ribeiro, Seabra, & Carvalho, 2011). All these observations raise the question of the origin, the role, and the fate of NO in the nitrogen-fixing symbiosis. This review provides an overview of the source and degradation pathways of NO and its functions throughout the symbiotic process. A special focus is given on coordination between the mitochondrial ETC and NR in increasing energy efficiency. We further analyse NO interactions with ROS and raise a number of lines to decipher its multifaceted roles in regulating the symbiotic cooperation between legumes and rhizobia.

2 | NO SOURCES DURING SYMBIOSIS: CURRENT STATUS

Each legume establishes a specific association with a particular symbiotic rhizobium. Using cell-permeable NO-fluorescent probes, NO production was shown to be transiently induced in the roots of *Lotus*

Highlights

- Nitric oxide (NO) plays an important role in legume–rhizobium symbiosis.
- Different degrees of hypoxia in nodules lead to activation and repression of various NO production and scavenging pathways in plant and bacterial partners.
- Plant-originated reductive pathways contribute to NO production in nodules whereas phytoglobin scavenges NO.
- This review highlights pathways of NO production and degradation as well as their crosstalk and involvement in metabolism of nodules.

japonicus and *Medicago sativa* 4-hr post-inoculation with their cognate symbionts (Nagata et al., 2008). NO was also detected 4 days of post-infection, in different *Medicago truncatula* cell types such as shepherd's crooks of root hair, infection threads, and nodule primordia (del Giudice et al., 2011). At later stages of the symbiosis, NO production was associated with the N_2 -fixing zone (Baudouin et al., 2006), or at the frontier between N_2 -fixing and senescence zones (Cam et al., 2012) of *M. truncatula*–*Sinorhizobium meliloti* mature nodules. Nevertheless, the source of NO in establishment of functional nodules is still under debate; hence, we summarize here some data describing these possible sources. Some studies attribute the NO production during nodule formation to the roots and others to the bacteroids (Table 1). Regarding the plant origin, various pathways that produce NO have been reported. These are divided into the oxidative and the reductive reactions (Gupta, Fernie et al., 2011). The well-characterized NR, mitochondrial ETC, and plasma membrane-associated NR are all reductive NO-production pathways in nature, whereas NO production from L-arginine, via the NOS-like enzyme, polyamine, and hydroxylamine, is the oxidative routes. NR is located in the cytosol and plasma membrane and uses nitrate (NO_3^-) as a substrate to produce nitrite. Nitrite can also be used by NR as a low-affinity substrate and further reduced to NO. Both steps use NADH or NADPH as reducing power. Nitrite is a limiting factor for NO production (Planchet, Jagadis Gupta, Sonoda, & Kaiser, 2005). Under hypoxic conditions, such as those prevalent in microoxic nodules, NR produces NO (Gupta, Stoimenova, & Kaiser, 2005). Not only NR but also the mitochondrial electron transport is capable to reduce nitrite to NO. Cytochrome c oxidase (COX; Complex IV) and Complex III (bc_1) are the sites of NO production in ETC (Figure 1). Inhibition of the Mitochondrial Complex III with myxothiazol and inhibition of Complex IV with KCN indeed lead to the abolishment of NO production in mitochondria (reviewed in Gupta & Igamberdiev, 2011). Nitrite is the terminal electron acceptor, whereas either NADH or NADPH provides electrons via ubiquinone to the mitochondrial ETC. The affinity for nitrite of the mitochondrial nitrite: NO reductase reaction is quite low (K_m 175 μM ; Gupta et al., 2005), indicating that the reaction requires a significant accumulation of nitrite. In contrast, the K_i value of oxygen inhibition of NO

TABLE 1 Pathway-specific information during nodule formation and the role of NO scavenging pathways in specific interactions

(A) Pathway-specific information during nodule formation		
Interaction	Source of nitric oxide	Reference
1. <i>Medicago truncatula</i> - <i>Sinorhizobium meliloti</i>	Both plant and bacterial nitrate reductase and electron transfer chains are involved in NO synthesis	Horchani et al., 2011
2. <i>Lupinus albus</i> - <i>Bradyrhizobium</i>	Nitric oxide synthase (plant origin)	Cueto et al., 1996
3. Soybean- <i>Bradyrhizobium japonicum</i> nodules	Periplasmic nitrate reductase (Nap) from bacterial origin	Sánchez et al., 2010
4. <i>M. truncatula</i> - <i>S. meliloti</i>	Nitrate reductases (plant origin)	Cam et al., 2012
5. <i>B. japonicum</i> and soybean root nodules interaction	Periplasmic nitrate reductase (bacterial origin)	Meakin et al., 2007
6. <i>M. truncatula</i> - <i>S. meliloti</i>	Nitric oxide synthase (bacterial origin)	Baudouin et al., 2006
7. <i>B. japonicum</i> and Soybean root nodules	Nitric oxide synthase (plant origin)	Keyster, Klein, & Ludidi, 2010
8. Soybean- <i>B. japonicum</i> nodules	Nitric oxide synthase (plant origin)	Leach, Keyster, Du Plessis, & Ludidi, 2010
9. <i>M. truncatula</i> - <i>S. meliloti</i>	Nitrate reductases and nitric oxide synthase (both plant and bacterial)	Meilhoc, Boscari, Bruand, Puppo, & Brouquisse, 2011
10. <i>M. truncatula</i> - <i>S. meliloti</i>	Nitrate reductases (bacterial origin)	Boscari, Del Giudice et al., 2013
11. Soya bean nodules- <i>S. meliloti</i>	Nitrate reductase, product of the nap genes (bacterial origin)	Meilhoc et al., 2010
12. <i>M. truncatula</i> - <i>S. meliloti</i>	Either plant or the bacteria is involved in this NO synthesis	Del Giudice et al., 2011
13. <i>Lotus japonicus</i> - <i>Mesorhizobium loti</i>	Nitrate-independent nitrate reductase from plant	Kato, Okamura, Kanahama, & Kanayama, 2003
14. <i>M. truncatula</i>	Putative NOS gene from plant	Pauly et al., 2011
15. Legume-rhizobium and mycorrhizal symbioses	Nitrate reductases (plant origin) <i>M. truncatula</i> genome possess 3 NR genes, <i>MtNR3</i> being only expressed during the nodulation process. <i>MtNR1</i> and <i>MtNR2</i> are strongly induced during nodulation process	Puppo et al., 2013
16. Soybean- <i>B. japonicum</i> nodules	Nitric oxide synthase	Mathieu, Moreau, Frendo, Puppo, & Davies, 1998
17. <i>M. truncatula</i> - <i>S. meliloti</i>	Nitric oxide synthase either from plant or bacterial origin	Del Giudice et al., 2011
(B) NO scavenging pathways and their role in specific interactions		
Interaction	Nitric oxide scavenger	Reference
1. <i>L. japonicus</i> - <i>M. loti</i>	Class 1 Hb (<i>L. japonicus</i>)	Shimoda et al., 2009
2. <i>L. japonicus</i> - <i>M. loti</i>	Nonsymbiotic and truncated haemoglobin (<i>L. japonicus</i>)	Bustos-Sanmamed et al., 2011
3. <i>M. truncatula</i> - <i>S. meliloti</i>	Hmp flavohaemoglobin (<i>S. meliloti</i>)	Del Giudice et al., 2011
4. <i>M. truncatula</i> - <i>S. meliloti</i>	Flavohaemoglobin Hmp (<i>S. meliloti</i>)	Meilhoc et al., 2013
5. <i>M. truncatula</i> - <i>S. meliloti</i>	Flavohaemoglobin Hmp (<i>S. meliloti</i>)	Cam et al., 2012
6. <i>M. truncatula</i> - <i>S. meliloti</i>	Flavohaemoglobin (Hmp; <i>S. meliloti</i>)	Blanquet et al., 2015
7. <i>L. japonicus</i> - <i>M. loti</i>	Class 1 Hb (<i>L. japonicus</i>)	Nagata et al., 2008
8. <i>Alnus firma</i> - <i>Frankia</i>	AfHb1 (<i>A. firma</i>)	Sasakura et al., 2006
9. Soybean- <i>B. japonicum</i>	Nitrosylhaemoglobin (<i>B. japonicum</i>). Lb has a major role in detoxifying NO and nitrite produced by bacteroidal denitrification in response to flooding conditions	Sánchez et al., 2010
10. Soybean	Oxylegghaemoglobin from Soybean nodules	Herold & Puppo, 2005
11. <i>M. truncatula</i> - <i>S. meliloti</i>	Flavohaemoglobins (<i>hmp</i>) from <i>S. meliloti</i>	Meilhoc et al., 2010
12. <i>L. japonicus</i> - <i>M. loti</i>	Haemoglobin LjGlb1-1 from <i>L. japonicus</i>	Fukudome et al., 2016

production by the mitochondrial ETC is about 0.6 μM , which corresponds to the magnitude order of the K_m of COX, hence indicating that the production of NO in the ETC occurs when oxygen drops to very low levels.

It has been found that the production of NO under normoxic and hypoxic conditions has a role in the regulation of respiratory oxygen consumption, thereby it can help in oxygen homeostasis (Benamar et al., 2008; Gupta, Fernie et al., 2011). Consequently, the NO produced by NR and mitochondria can help to maintain microaerobic

conditions. Horchani et al. (2011) found that NR and mitochondrial ETC are involved in NO production in *M. truncatula*-*S. meliloti* nodules. By using RNAi plants of *M. truncatula* NR1/2 with reduced NR activity, or in the presence of tungstate, a NR inhibitor, they observed reduced NO production compared with controls. However, in both cases, the addition of nitrite increased the production of NO. In contrast, mitochondrial ETC inhibitors suppressed NO production, either in the presence or in the absence of added nitrite, suggesting that in *M. truncatula* nodules nitrite is reduced to NO at the ETC level.

mutant strains impaired nitrogenase or nitrite reductase activities but had no effect on NO production, suggesting that the bacterial denitrification is in fact only a minor source of NO in nodules (Baudouin et al., 2006). More recently, Horchani et al. (2011) demonstrated by using the fluorescence of the 4,5-diaminofluorescein (DAF) probe that two thirds of the NO generated by *M. truncatula*-*S. meliloti* nodules are produced via plant NR and mitochondrial ETC and one third by the bacteroid denitrification pathway.

3 | COORDINATED OPERATION OF NR AND MITOCHONDRIA IN GENERATION OF NO IN NODULES

Hypoxia is known to positively modulate the activity of NR (Planchet et al., 2005). The reduction of nitrate to nitrite catalysed by NR leads to an increased production of nitrite, which is subsequently reduced to NO by the mitochondrial ETC, when oxygen concentration falls below 1% (Gupta & Igamberdiev, 2011, 2016). In animal systems, it was shown that in the process of reduction of the COX by electrons from the haem *a*, the copper atom obtains an electron and then transfers it to Fe_{e3} (Brunori et al., 2006). These two reactions can result in nitrite reduction, and in summary, nitrite reduction to NO formation is linked to the oxidation of iron by nitrite after its binding to the fully reduced centre (Castello et al., 2008; Gupta & Igamberdiev, 2011). The reduction of nitrite to NO occurs with a decrease of pH by 0.5 units (Castello, David, McClure, Crook, & Poyton, 2006). Hypoxic conditions are characterized by a pH decrease, which is a common condition observed under oxygen deprivation. This decreased pH is known to activate plant NR, which can generate nitrite (Kaiser & Brendle-Behnisch, 1995). In plants, the pH of the aerobic cytoplasm is generally found to be around 7.4–7.6, and the onset of oxygen deprivation causes its fall below 7, whereas a longer duration of the hypoxic period leads to further drop in pH. These observations mean that the ability of COX to function as a nitrite: NO reductase in hypoxic cells or mitochondria is mediated by the changes in intracellular pH and suggests that the reaction may be linked to proton pumping and cytosolic acidosis (Castello et al., 2006).

The high *K_m* of nitrite: NO reductase reaction for nitrite suggests that elevated levels of nitrite are required for mitochondria to reduce nitrite to NO (Gupta et al., 2005). The nitrite pool depends on either plant NR or periplasmic NR activities and indirectly depends on soil NO₃⁻ levels. Under nearly anoxic conditions (0.12- μ M oxygen), higher levels of NO are observed in mitochondria (Gupta et al., 2005; Gupta & Igamberdiev, 2011). These hypoxic conditions stimulate NR and produce nitrite that can act as a substrate for the mitochondrial ETC to produce NO. In yeast mitochondria, it was shown that at 0.5 μ M O₂, COX5a gene is repressed, and COX5b is induced, which can strongly modify NO production (Castello et al., 2006; Castello et al., 2008). Under hypoxic conditions, the inhibition of COX can also lead to a leakage of electrons that can be used by Complex III to reduce nitrite to NO. This reaction was observed in animal and plant mitochondria (Gupta et al., 2005; Kozlov, Staniek, & Nohl, 1999).

Horchani et al. (2011) well addressed the coordinate role of plant NR and mitochondrial ETC in NO production in *M. truncatula*-*S. meliloti*

nodules. They observed that NO production was inhibited by NR inhibitor tungstate and by abolition of NR expression, because NA-interference (RNAi) double knockdown *MtNR1/2 Medicago* plants exhibited decreased NR activities and NO levels. Very interestingly, the suppressed NO production was reversed by nitrite addition suggesting that apart from NR, the nitrite-dependent NO plays an important role in NO production. The nitrite-dependent NO production was abolished by ETC inhibitors, indicating that mitochondrial ETC was the site of nitrite reduction to NO (Horchani et al., 2011). Thus, in *M. truncatula* nodules, nitrate may be reduced to NO in a two-step mechanism involving successively NR and ETC.

Alternative oxidase (AOX) is a stress-induced protein in plant mitochondria that plays a role in NO and ROS homeostasis. AOX is implicated in the regulation of the cellular NO level. Electron flow through AOX reduces leakage of electrons to nitrite and the accumulation of NO (Cvetkovska & Vanlerberghe, 2012). When COX is inhibited by NO, the AOX pathway contributes to the minimization of the nitrite-dependent NO synthesis. AOX could play a role in buffering of ROS levels during nodulation. Although NO production in *M. truncatula* nodules was insensitive to AOX inhibitor propyl gallate (Horchani et al., 2011), anoxic NO production by purified *M. truncatula* root mitochondria was found to be inhibited by propyl gallate (unpublished data). This discrepancy probably comes from the fact that propyl gallate does not penetrate nodules properly and suggests that AOX is a potential site of NO production in nodules. This hypothesis is supported by the 5- to 20-fold induction of AOX gene in the nodules compared with the roots of *M. truncatula* (Benedito et al., 2008; Boscari, Meilhoc et al., 2013). However, an opposite trend was observed in isolated mitochondria from soybean nodules where the AOX-dependent respiration was low (Kearns, Whelan, Young, Elthon, & Day, 1992). Because AOX is supposed to play a role in keeping NO levels low, the expression of AOX in nodular development and its role deserve a deeper investigation.

As mitochondria are involved in energy production in nodules under hypoxic conditions, their protection is vital for the symbiosis establishment. Several lines of evidence revealed that addition of nitrate leads to the protection of mitochondria in wheat and rice seedlings (Vartapetian et al., 2003). Mitochondria ultrastructure and integrity were indeed retained under hypoxia when nitrate was supplied (Vartapetian & Polyakova, 1999). Previously, it was thought that nitrate acts as a terminal electron acceptor, but recent work revealed that nitrite rather than nitrate protects mitochondria (Gupta, Lee, & Ratcliffe, 2017), and this protection can be attributed to nitrite reduction to NO, supporting the anoxic NADH and NADPH oxidation and resulting in the increased mitochondrial integrity, enhancement of membrane potential, increased ATP synthesis, decreased production of ROS, and decreased lipid peroxidation. Mitochondrial components of the ETC are documented to be organized in supercomplexes with various configurations and stoichiometries. The formation of supercomplexes fine-tunes the energy metabolism and ATP yield upon environmental conditions modification (Schägger, 2001). A recent report by Gupta et al. (2017) revealed that nitrite addition can facilitate formation of the supercomplex I + III. However, the supercomplex formation in nodule mitochondria has not been investigated so far.

4 | NITRITE TRANSPORT TO MITOCHONDRIA

Under hypoxia, mitochondria undergo various morphological, biochemical, and metabolic changes (Shingaki-Wells, Millar, Whelan, & Narsai, 2014). Under these conditions, nitrite can be substituted for O_2 as a terminal electron acceptor of the ETC and consequently contributes to low rates of ATP synthesis by mitochondria. Nitrite is reduced to NO at the level of the COX or Complex III (Gupta & Kaiser, 2010; Igamberdiev & Hill, 2004; Planchet et al., 2005; Stoimenova, Igamberdiev, Gupta, & Hill, 2007). The produced NO freely diffuses from mitochondria into the cytosol where it is oxygenized to nitrate by nonsymbiotic haemoglobins (Pgbs). Class 1 Pgbs are induced at low oxygen conditions and possess extremely high avidity to O_2 ($K_d \sim 2$ nM), which makes them functional at oxygen levels of two orders of magnitude lower than the K_m of COX to oxygen (Gupta, Hebelstrup et al., 2011; Igamberdiev, Bykova, & Hill, 2011; Shankar et al., 2018). The NO_3^- is then reduced to NO_2^- by NR and subsequently transported to mitochondria where it is again reduced within the ETC (Horchani et al., 2011; Igamberdiev & Hill, 2009; Stoimenova et al., 2007). This cycle, known as the Pgb-NO cycle (Figure 1) thus makes possible to maintain the energy and redox state of the hypoxic plant cell by contributing to the regeneration of ATP and by limiting the accumulation of NADH and NADPH pools in the cytosol (Horchani et al., 2011; Stoimenova et al., 2007).

Although the different steps of the Pgb-NO cycle have been characterized, only the transport of NO_2^- from the cytosol to the mitochondrial matrix remains equivocal (Gupta & Kaiser, 2010; Stoimenova et al., 2007). The passive diffusion of NO_2^- through the external and internal mitochondrial membranes to the mitochondrial matrix is unlikely as nitrite is essentially under its anionic form at physiological pH. Nitrite entry into the mitochondria is consequently likely to occur via active transport, as described for the transport of nitrite through chloroplast membranes (Rexach, Fernández, & Galván, 2000). However, to date, no specific mitochondrial nitrite transporter has ever been described in the literature, regardless of the kingdom. Its identification and characterization constitute the missing link that would definitively confirm the existence and the significance of the Pgb-NO cycle in the maintenance of the energy state in hypoxic cells.

Nitrite uptake has been characterized in bacteria (Jia, Tovell, Clegg, Trimmer, & Cole, 2009; Moir & Wood, 2001), fungi (Wang et al., 2008), yeast (Machín et al., 2004), and algae (Galván, Quesada, & Ferna, 1996; Rexach et al., 2000) and occurs via bispecific NO_3^-/NO_2^- transporters or via NO_2^- -specific transporters (Galván et al., 1996; Machín et al., 2004; Wang et al., 2008). However, little is known about the transport of NO_2^- in higher plants so far. Nitrite uptake in the roots of *Arabidopsis* and sunflower (*Helianthus annuus* L.) follows Michaelis-Menten kinetics, which argues in favour of the existence of NO_2^- transporters (Agüera, de la Haba, Fontes, & Maldonado, 1990; Kotur, Siddiqi, & Glass, 2013). In plants, the family of NO_3^- transporters is subdivided into three subfamilies: Nitrate Transporter 1 (NRT1/Peptide Transporter [NPF]), NRT2 (Major Facilitator Superfamily), and NRT3, which aid in stabilization and transport activity of members of the NRT2 subfamily (Krapp, 2015; Wang, Hsu, & Tsay, 2012; Wirth et al., 2007). Very few NO_3^- carriers from the NPF,

NRT2, and NRT3 families with NO_2^- transport activity have been identified or characterized to date in plants. These candidates include cucumber (*Cucumis sativus*) CsNitr1-L (later renamed CsNPF3.2), which is a chloroplast nitrite transporter (Pike et al., 2014; Sugiura, Georgescu, & Takahashi, 2007), *Arabidopsis* AtNRT2.1, AtNAR2.1–2 (Kotur et al., 2013) and AtNPF3.1 (Pike et al., 2014), and grapevine (*Vitis vinifera*) VvNFP3.2 (Pike et al., 2014). However, none of these carriers has a mitochondrial targeting sequence nor has it been associated with mitochondria. Alternatively, the plant inner membrane anion channel that is active under low levels of ATP may be involved in the transport of nitrite to mitochondria (Trono, Laus, Soccio, & Pastore, 2014), but to date, no activity of nitrite transport has been associated with it. Recently, a Major Facilitator Superfamily family transporter has been identified from mitochondria of *M. truncatula* roots. Preliminary results show that its overexpression leads to an increase in NO production in nodules of *M. truncatula* and that nitrite transport activity may be associated with its heterologous expression in *Xenopus* eggs (unpublished data). However, the location of this transporter and its functional characterization in vivo remain to be demonstrated. Likewise, an orthologue of this putative *Medicago* nitrite transporter has been identified in chickpea and is currently under analysis (unpublished data). These data will constitute a crucial basis to better understand and later improve the Pgb-NO cycle operation in crop plants facing hypoxia.

5 | THE ROLE OF PHYTOGLOBINS IN NO BALANCE

Symbiotic Lbs constitute the most abundant haemoglobins present in nodules of leguminous plants. They belong to the Class 2 Pgbs characterized by a lower affinity for oxygen compared with Class 1 Pgbs and have a K_d at the submicromolar range, which is at least two orders of magnitude higher than oxygen affinity of Class 1 Pgbs. This characteristic allows them to fulfil the function of quenching oxygen and delivering it to rhizobium in low concentration. The function of these proteins in NO metabolism is limited under low oxygen conditions because of significant differences in avidity to oxygen and NO as compared with Class 1 Pgbs. However, at oxygen amounts that can saturate COX, Lbs may contribute to the scavenging of NO. Lbs play a role in maintaining a suitable microaerobic environment to allow O_2 respiration and production of energy without inactivation of the nitrogenase (Ott et al., 2005). Lbs bind NO to form the LbNO complex (Mathieu et al., 1998). High levels of NO and O_2 can inactivate nitrogenase, thus Lb binding to NO and O_2 is necessary for the protection of the nitrogenase (Herold & Puppo, 2005). Operation of both Class 1 Pgbs and Lbs in nodules may result in fine adjustments of concentrations of oxygen and NO to achieve high metabolic rates of nitrogen fixation.

In recent years, nonsymbiotic haemoglobins such as Class 1 Pgbs have emerged as important players in the legume-rhizobia symbiosis. A key feature of these Pgbs is their involvement in NO metabolism. Class 1 Pgb can react with NO to form nitrate at very low oxygen concentrations of two orders of magnitude lower than K_m of COX (Gupta, Hebelstrup et al., 2011; Igamberdiev et al., 2011). In this reaction, the

oxidized ferrous form of Pgb is converted to the deoxidized ferric (methaemoglobin) form that can be reduced back to the ferrous form by a methaemoglobin reductase (Igamberdiev, Bykova, & Hill, 2006). The application of NO donor (*S*-nitroso-*N*-acetyl-D,L-penicillamine) and NO scavenger (c-PTIO) to *L. japonicus* roots resulted correspondingly in the induction and repression of *LjHB1* (Nagata et al., 2008; Shimoda et al., 2005). These observations led the authors to hypothesize that the transient peak of NO during early steps of the symbiotic interaction resulted from downregulation of Class 1 Pgb to increase NO burst and then up regulation to bring back the level of NO to physiological range to allow the symbiont into the roots (Murakami et al., 2011). The Class 1 Pgb encoding genes are also up-regulated in mature root nodules of *L. japonicus* and induced by NO, cold, and hypoxia (Bustos-Sanmamed et al., 2011).

Uchiumi's team reported an increase in the number of nodules on *L. japonicus* transgenic hairy roots overexpressing either *LjHb1* or *Alnus firma AfHb1*, underlining the importance of Pgb in nodulation (Shimoda et al., 2009). Overexpression of *Pgb1* enhances symbiotic nitrogen fixation, suggesting that reversible inhibition of nitrogenase is relieved by scavenging of NO by Pgb1. Functional nodules are characterized by a microoxic environment, but for various processes, energy is a requirement. Under hypoxic conditions, the produced NO is scavenged by Pgb1 to generate ATP in the Pgb-NO cycle (Figure 1, reviewed in Gupta & Igamberdiev, 2011). One possible candidate for methaemoglobin reductase is the cytosolic monodehydroascorbate reductase having affinity also to Pgb (Igamberdiev et al., 2006). Another possible candidate for methaemoglobin reductase is ferredoxin NADP⁺ oxidoreductase (Jokipii-Lukkari et al., 2016). Operation of Pgb-NO cycle is particularly important for reoxidation of NADH and NADPH when oxygen concentrations are not sufficient to saturate COX and support operation of mitochondria with oxygen. In these conditions, the Pgb-NO cycle contributes to the maintenance of NADH/NAD⁺, NADPH/NADP⁺, and ATP/ADP ratios in hypoxic cells and thus keeps their viability (Igamberdiev, Bykova, Shah, & Hill, 2010). As reported above, recent evidence suggests that the Pgb-NO cycle operates in nodules under hypoxia where both mitochondrial and bacterial ETCs are involved (Horchani et al., 2011).

6 | SIGNALLING ROLE OF NO AND THE INTERPLAY BETWEEN ROS, NO, AND ANTIOXIDANTS IN NODULATION

Increasing evidence supports the critical role of NO in the recognition, signalling, and immunity processes during the symbiotic association between legumes and rhizobia. At several key stages of this interaction, from early interaction steps between the plant and the bacterial partners to N₂-fixing and senescence steps in mature nodules, a specific production of NO has been highlighted (Hichri et al., 2015). NO production was particularly observed in dividing root cortical cells at the onset of the nodule organogenesis (del Giudice et al., 2011). The presence of NO in nodule primordia exhibits high similarity with the local NO increase observed in lateral root primordia (Correa-Aragunde, Graziano, & Lamattina, 2004; Lanteri, Graziano, Correa-Aragunde, & Lamattina, 2006). In these studies, authors reported that NO could

modulate the expression of cell cycle regulatory genes. It was notably observed in *M. truncatula* that NO scavenging triggers the downregulation of plant genes involved in nodule development, such as *MtCRE1* and *MtCCS52A* (del Giudice et al., 2011). It is noteworthy that nodulation efficiency and lateral roots formation are finely tuned according to the nitrate availability (Streeter & Wong, 1988; Zhang, Jennings, Barlow, & Forde, 1999) and that nitrogen nutrient availability influences NO production. Furthermore, transcriptomic analysis of 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO)-treated inoculated roots of *M. truncatula* showed that NO could be involved in the transcriptional regulation of many genes involved in cell cycle and protein synthesis in nodule primordia (Boscari, Del Giudice et al., 2013), which reinforces the hypothesis that NO plays a similar function in nodule and lateral root organogenesis. Additionally, the expression of a number of genes involved in terpene, flavonoid, and phenylpropanoid pathways and genes encoding PR-proteins and cytochrome P450 were significantly affected by cPTIO. These observations indicate that NO could be involved in the repression of plant defence reactions, thereby favouring the establishment of the beneficial plant-microbe interaction (Boscari, Del Giudice et al., 2013).

The signalling role(s) of NO in mature nodules is not fully understood. NO may have a metabolic role, as it has been suggested that, under the microaerobic conditions prevailing in nodules, a nitrate-NO respiratory pathway contributes to energy supply (Horchani et al., 2011). However, NO has been reported to be a potent inhibitor of nitrogenase activity in soybean (*Glycine max*) and Lotus (*L. japonicus*; Kato et al., 2010; Trinchant & Rigaud, 1982). NO has also been proposed to play a signalling role in the control of the *M. truncatula* nodule senescence process (Cam et al., 2012). Using a biosensor *S. meliloti* strain, authors suggested that NO levels were the highest in the most proximal zone of the nodule, consistent with the hypothesis that NO could play a signalling function at the onset of senescence. Interestingly, an increase of NO levels in *M. truncatula* nodules, resulting either from the use of *S. meliloti* strains deficient in NO degradation (*hmp*, *norB*, and *nnrS1*) or from the exogenous addition of a NO donor, led to a premature senescence of the nodules, the severity of which correlated with the NO levels inside the nodules (Blanquet et al., 2015; Cam et al., 2012; Meilhoc, Blanquet, Cam, & Bruand, 2013). In contrast, overexpression of the *hmp* gene led to a decrease in NO levels and a delay in nodule senescence, whether developmental or dark-induced (Cam et al., 2012). This work demonstrates the pivotal role of the bacterial NO detoxification response in the prevention of early nodule senescence and hence the maintenance of an efficient symbiosis. Taken together, these results highlight the contrasted roles that NO might have at the different steps of symbiosis and underline the importance not only of plant Pgb1s and bacterial NO-degrading proteins but also of NR enzymes in maintaining a balanced NO level for symbiosis maintenance or breakdown.

Data concerning ROS and NO production at the different stages of the interaction have been acquired (Figure 2; Puppo, Pauly, Boscari, Mandon, & Brouquisse, 2013). Different studies showed that ROS and NO present a fine-tuned spatio-temporal modulation that plays a critical role in signalling and immunity in the mutualistic associations between legumes and rhizobia (Damiani, Pauly, Puppo, Brouquisse, & Boscari, 2016; del Giudice et al., 2011; Puppo et al., 2013).

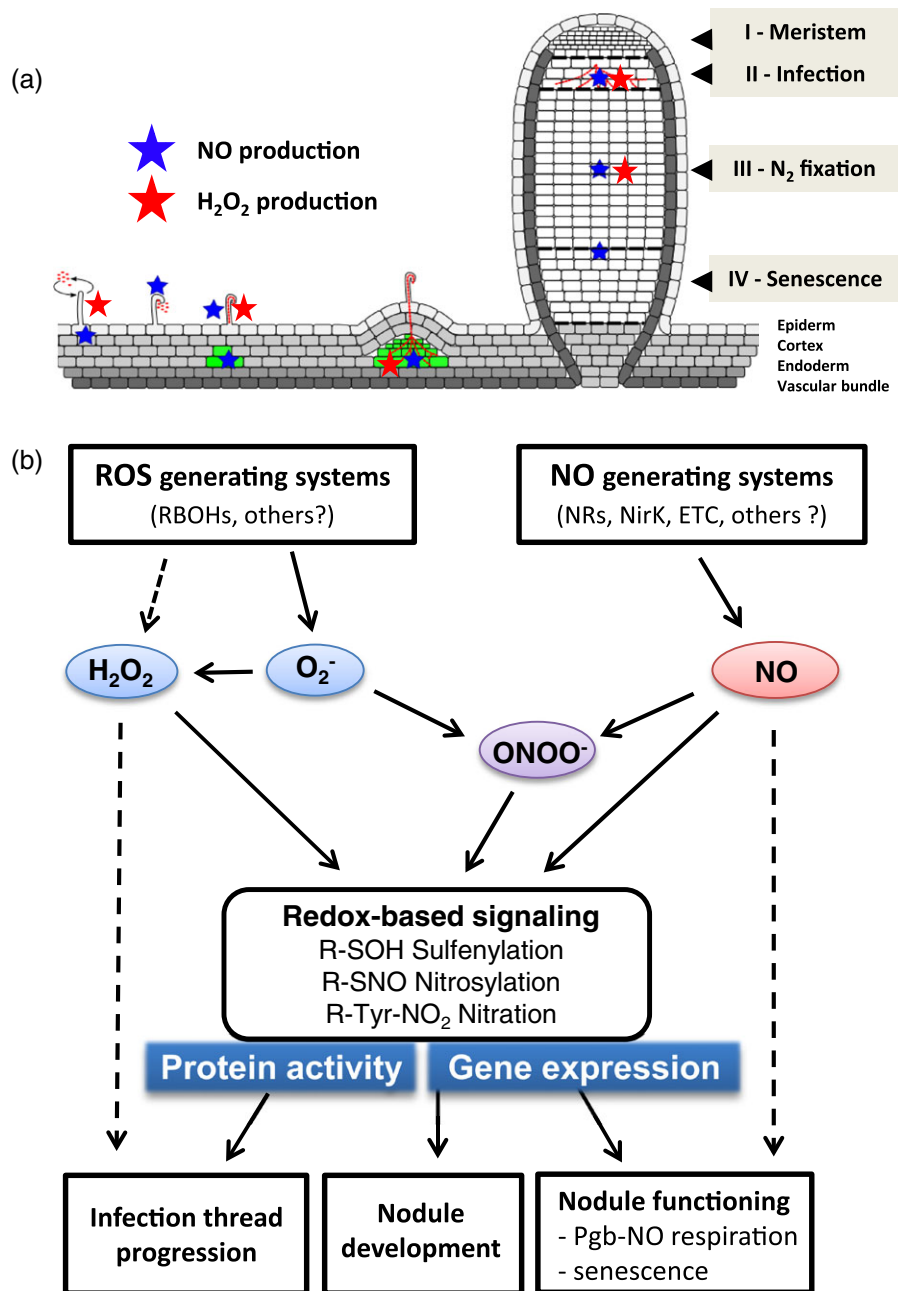


FIGURE 2 General scheme summarizing the localization and the roles of H₂O₂ and nitric oxide (NO) in the nodulation process. (a) Spatial production of NO (blue stars) and H₂O₂ (red stars) are indicated. Green cells refer to nodule primordium. Free rhizobia (points) and infection thread (lines) are labelled in red colour. Functional nodule zones (I to IV) are indicated with arrows. (b) Scheme of NO and reactive oxygen species (ROS) sources, signalling pathways, and crosstalks that modulate essential steps of the symbiotic process. Redox-based regulation of the symbiotic process is partly mediated by NO- and ROS-dependent post-translational modifications (S-sulfenylation, S-nitrosylation, and Tyr-nitration) that regulate protein activity and gene expression. NRs = nitrate reductases [Colour figure can be viewed at wileyonlinelibrary.com]

Transcriptomics analyses of *M. truncatula* inoculated roots treated with either a NO scavenger (cPTIO) or a NADPH oxidase inhibitor (diphenyleneiodonium) reveal a strong overlap in the signalling pathways triggered by either molecule (Andrio et al., 2013; Boscardi, Meilhoc et al., 2013). Amongst the 316 genes commonly regulated by both molecules, cell wall formation and development processes are mainly down-regulated, whereas gene families involved in plant defence and secondary metabolism are up-regulated (Puppo et al., 2013). Moreover, *NR1*, *NiR*, and *ns-Hb1* genes were strongly up-regulated in transcriptomic analysis of diphenyleneiodonium-treated *M. truncatula* plants, suggesting that H₂O₂ could control the

transcriptional regulation of enzyme involved in NO homeostasis in *M. truncatula* roots. These results point to a significant crosstalk between ROS and reactive nitrogen species (RNS) during the establishment of the symbiotic process. Such a crosstalk is further supported by the finding of both S-sulfenylated and S-nitrosylated proteins post-translational modification during nitrogen (N₂)-fixing symbiosis (Oger, Marino, Guigonis, Pauly, & Puppo, 2012; Puppo et al., 2013), linking ROS/NO production to a redox-based regulation of the symbiotic process (Figure 2). The fact that these molecules act synergistically has not been fully determined yet. However, recent work demonstrated the contribution of the bacterial NO to the modulation of post-translational

modification of target proteins in *M. truncatula* nodule (Blanquet et al., 2015). The peroxyxynitrite (ONOO⁻), formed from the direct interaction of NO with O₂^{•-}, impacts protein activities through Tyr nitration. Thus, cytosolic glutamine synthetase (GS1), a key enzyme in the nitrogen assimilatory process, is inactivated by Tyr nitration in *M. truncatula* mature nodules (Melo et al., 2011). This modulation of GS1 activity was interpreted as a potential metabolic response to the inhibition of symbiotic N₂-fixation by RNS (Boscarì, Meilhoc et al., 2013).

7 | DOES SOIL N STATUS AND NO INFLUENCE NODULATION?

Human-driven N deposition is one of the major global environmental changes that has strong impacts on ecosystem functioning (Bobbink & Hicks, 2014). Major causes of human-assisted N deposition are excessive use of N fertilizers, excessive use of fossil fuel for vehicles, burning of crop remains, and industry, which raises the levels of NO. Human-driven N deposition enhances NO efflux largely due to increase in NH₄⁺, which is strongly influenced by soil moisture content, available N and temperature (Vourlitis, DeFotis, & Kristan, 2015). According to one estimate, human was responsible for 80% production of NO (Bobbink & Hicks, 2014). N exists in both organic and inorganic forms in soil. The relative abundances of different nitrogen forms in the soil are a function of plant and soil community composition, as well as abiotic conditions, litter chemistry, and overall decomposition rates (Suding et al., 2005). Nearly all plant species uptake inorganic (NH₄⁺ and NO₃⁻) soil nitrogen, but some plants can also uptake and utilize organic nitrogen directly in the form of amino acids. Soils are recognized as an important source of NO as a part of N cycle through biological processes, and the world amount of NO emitted from soils to the atmosphere was estimated to be between 13 and 21 10¹² g·N·year⁻¹ (Davidson & Kingerlee, 1997). The nitrification processes, carried out by autotrophic nitrosobacteria and nitrobacteria and by heterotrophic nitrifiers (bacteria and fungi), globally oxidize NH₄⁺ into NO₃⁻, whereas the denitrification processes, carried out mainly by bacteria but also fungi, lead to the reduction of NO₃⁻ into N₂. NO is produced as part of these two processes. The chemical decomposition of NO₂⁻ (chemodenitrification) is also a source of NO in low pH soils. An important factor for NO production is the presence of N in the soil, and a rapid increase in NO emission is usually observed immediately after fertilizer application (Ludwig, Meixner, Vogel, & Forstner, 2001). However, other parameters such as oxygen concentration, soil moisture, temperature, pH, and vegetation type can significantly influence NO emissions that annually range throughout the globe from less than 0.01 to 30 kg·N·ha⁻¹·year⁻¹ (Ludwig et al., 2001; Pilegaard, 2013).

Under controlled laboratory conditions, NO was detected at the root surface of the plant partner within the first few hours of interaction with bacteria (Nagata et al., 2008), and later in the infection thread and the nodular cortex (del Giudice et al., 2011), but not in the bacterial partner. Currently, no simultaneous analysis of the NO produced, respectively, by the soil and the plant during the symbiotic process has been carried out, hence the difficulty of evaluating the impact of the NO emitted by the soil in the recognition and establishment of the symbiotic interaction. However, a number of experiments carried

out in the presence of either NO donors or NO scavengers show that the supply or deprivation of NO can influence the dynamics of the symbiotic process. Thus, treatment of *M. truncatula* roots in interaction with *S. melliloti* with 1-mM cPTIO inhibits nodulation as long as cPTIO is present in the medium (del Giudice et al., 2011), suggesting that NO is needed for symbiosis. In contrast, in the *L. japonicus*-*Mesorhizobium loti* symbiosis, the overexpression of Pgb LjHb1 (LjGlb1) or AfHb1 results in a decrease in NO level and an increase in nodule number (Shimoda et al., 2009), whereas *L. japonicus* lines mutated for LjGlb1 show higher NO production and lower infection and nodule number (Fukudome et al., 2016). Similarly, experiments carried out in peas (*Pisum sativum*) in symbiosis with *Rhizobium leguminosarum* show that 2-mM sodium nitroprusside, a NO donor, affects the adhesion and the penetration of rhizobia in roots and that this effect is reversed by the addition of 2-μM equine erythrocyte haemoglobin (Glyan'ko, Mitanova, & Vasil'eva, 2008). These observations argue for an inhibition of the symbiosis establishment by NO. In other experiments, treatments of soybean roots (*G. max*) with 1-mM Nω-nitro-L-arginine (L-NNA), a NOS inhibitor, lead to 70% reduction in NO, and treatment with 200-μM NONOate diethylenetriamine (DETA/NO), a NO donor, reduced the number and weight of nodules during its interaction with *B. japonicum* (Leach, Keyster, Du Plessis, & Ludidi, 2010). Interestingly, this phenotype is reversed by the simultaneous addition of L-NNA and DETA/NO (Leach et al., 2010). Considered together, these observations mean that (a) not only the presence of NO is necessary for the establishment of the symbiotic interaction, but the contribution of exogenous NO influences the dynamics of the symbiotic process and that (b) an excess like a lack of NO disturbs the establishment of the symbiosis, and therefore, NO concentration in the target tissues must be very finely regulated to allow the proper progress of the symbiosis. To date, the extent to which soil NO can influence nodulation is still largely unknown because so far little research has been done. However, data from the literature strongly suggest that soil NO may be a key regulator of the establishment and functioning of the symbiosis, and more attention deserves to be brought to it.

8 | FUTURE PROSPECTS

1. NR and mitochondrial ETC are major NO-producing enzymatic systems in nodules. The mechanism of activation of these pathways during the various developmental stages nevertheless needs further investigation.
2. The presence of NOS-like or polyamine oxidase activities, that is, oxidative NO-production pathways, has been shown in mature nodule extracts. Because mature root nodules have a low oxygen environment, the operation of these pathways under low oxygen needs further studies as well.
3. The Pgb-NO cycle has been demonstrated to generate ATP, and this process can also occur in nodules. However, it is not known to what extent this hypoxia-generated ATP contributes to nodule development.
4. Generation of plant and bacterial mutant lines producing differential sources of NO will help to investigate the effects of NO levels

at different stages of the symbiosis process. It will indeed allow understanding the regulatory role of NO during nodule development and establishment of optimal symbiosis.

5. Although the different stages of the Pgb-NO cycle have been characterized, the missing link is yet the transport of NO_2^- from the cytosol to the mitochondrial matrix, which remains to be elucidated. Once this transporter is identified, it will be interesting to test the impact of the nitrite transporter on the acceleration of the Pgb-NO cycle to produce more ATP for hypoxia survival and optimal nodule function.
6. Soil emits high levels of NO that depends on various factors such as N form, presence of nitrifying and denitrifying bacteria, invasive species decomposition, and ammonification. The remaining question is how NO pathways function in nodules in the background of soil NO.
7. To date, hundreds of S-nitrosylated plant and bacterial proteins have been identified in response to plant legume symbiosis. The analysis and characterization of S-nitrosylated proteins are crucial for the understanding of the specific signalling function of NO in nodule development.
8. The N-end rule of protein degradation has been shown to play a role in the hypoxic survival. How this pathway functions in the nodules experiencing different levels of oxygen and NO throughout their development is a priority in the future research.

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Annexe 3 :

Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis

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1 **Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis**

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13
14
15 **Short title:** Nitric oxide in nitrogen-fixing symbiosis

16
17 **Highlights:**

- 18 • Nitric oxide (NO) is produced throughout the nitrogen-fixing symbiosis
19 • NO performs specific signalling and/or metabolic functions during symbiosis
20 • Both plant phytohemoglobins and bacterial hemoproteins play a central role in the control
21 of NO level.

22 **Abstract**

23

24 The interaction between legumes and Rhizobium bacteria leads to the establishment of a
25 symbiotic relationship characterized by the formation of new organs, called nodules, which
26 have the ability to fix atmospheric nitrogen (N₂) via nitrogenase activity. Significant nitric
27 oxide (NO) production was evidenced at different stages of the symbiotic process. This
28 suggests that NO performs specific signalling and/or metabolic functions during symbiosis.
29 NO modulates the expression of many during nodule development. NO production is
30 increased in hypoxic nodules and it is supposed to be linked, via a phytohemoglobin-NO respiration
31 process, with improved capacity of the nodules to maintain their energy status under hypoxic
32 conditions. Otherwise, NO is known to be a potent inhibitor of nitrogenase and other plant
33 and bacterial enzymes. Other data suggest that NO is a developmental signal involved in the
34 induction of nodule senescence. Hence, the questions is raised of the signalling/metabolic
35 functions versus toxic effects of NO, and of the regulation of NO levels compatible with
36 nitrogen fixation metabolism. The present review analyses the different apparently
37 paradoxical roles of NO at various stages of the symbiosis, and discuss the role of plant
38 phytohemoglobins and bacterial hemoproteins in the control of NO level.

39

40

41 **Key words:** Hypoxia, Legumes, Nitric oxide, Nitrogen-fixing symbiosis, Phytohemoglobin,
42 Rhizobium.

43 **1 – Introduction**

44 The symbioses between plants and microorganisms improve the growth capacities of plants
45 under nutrient-limiting conditions (Martin *et al.*, 2017). Thus, the symbioses between plants
46 and mycorrhizae allow a facilitated supply of water, phosphate and mineral nitrogen. It is
47 estimated that as many as 90% of all plants depend on mycorrhizae to survive, and
48 mycorrhizae probably enabled plants to colonize land around 450 million years ago
49 (Lanfranco *et al.*, 2016). Other symbiotic interactions allowed the biological fixation of
50 atmospheric nitrogen in plants. Sixty five million years ago, the legume family has developed
51 a mutually beneficial relationship with soil bacteria, the Rhizobiaceae, which allow to reduce
52 atmospheric nitrogen (N_2) to ammonia (NH_3), an assimilable form of nitrogen, under
53 conditions of nutritional deficiency (Wang *et al.*, 2018). In exchange of nitrogen, the plant
54 provides the bacteria with energy in the form of photosynthates and an ecological niche for
55 their development.

56 This symbiosis is achieved within a neofomed organ, called the nodule (Oldroyd *et al.*,
57 2011). The formation of the nodule results from the cross-recognition between the two
58 symbiotic partners. In a first step, flavonoid compounds released by the roots attract the
59 rhizobia. In return, the bacteria produce nodulation factors (Nod factors) which, if recognized
60 by the plant, lead to the infection of the plant by the bacteria and the development of new
61 nodule primordia. The specific recognition of Nod factors by nodule receptors is essential for
62 all responses induced in the host (Via *et al.*, 2016). The Nod factors induce the curvature of
63 the root hairs in a “shepherd's crook” structure inside which the bacteria divide and form a
64 micro-colony. The lysis of the plant wall and the formation of an infection threads (an intra-
65 cellular channel) allow bacteria to enter the plant and subsequently to be internalized in plant
66 cells by endocytosis. The bacteria surrounded by the plant plasma membrane are called the
67 symbiosomes.

68 The cells infected by the bacteria derive from root inner cortex cells that dedifferentiated and
69 then multiplied to form the nodule meristem. In a further step, the cells differentiate to
70 accommodate the symbiosomes. Inside the symbiosomes, bacteria also differentiate into
71 nitrogen-fixing bacteroids (Haag *et al.*, 2013). One of the key elements of nitrogen-fixing
72 symbiosis is the production of nitrogenase, a bacterial enzyme complex that catalyzes the
73 reduction of N_2 to NH_3 . As the nitrogenase is irreversibly denatured by oxygen, it is necessary
74 for the reaction to occur in a micro-aerobic environment. This environment is produced within
75 the nodule by the setup of an oxygen barrier in the outer cell layers of the nodule and by the
76 synthesis of leghemoglobin (Lb), a hemoprotein with a high oxygen affinity, by the plant

77 partner. Both processes decrease the oxygen diffusion at the level of the nodule internal
78 cortex. These structural and biochemical modifications lead to a deep transformation in the
79 nodule metabolism.

80 Legumes produce two types of nodules depending on the persistence of the nodule meristem
81 (Hirsch, 1992). In the case of a persistent meristem, the nodules are of indeterminate type.
82 They contain four zones (Fig 1): the meristematic zone which allows the growth of the nodule
83 (Zone I), the infection zone where the cells differentiate and are infected by the bacteria (Zone
84 II), the N₂-fixing zone where the bacteria differentiated in bacteroids and nitrogenase reduces
85 N₂ into NH₃ (Zone III), and the senescence zone where there is a disruption in the symbiotic
86 interaction (Zone IV). This type of nodule is generally found in legumes grown in temperate
87 environments such as alfalfa, pea or clover. In the case of a transient meristem, the nodules
88 are of the determined type with a round shape and all the infected cells always at the same
89 stage of development (meristem, infection, fixation or senescence; Fig. 1). This type of nodule
90 is generally found in tropical legumes such as soybean or lotus.

91 The nitrogen-fixing symbiosis therefore results from a plant-bacterium interaction and the
92 formation of a new organ with a significant metabolic reprogramming. A characteristic
93 common to these events is their regulation by the modification of the redox state of the cells.
94 Antioxidant defences of plant and bacterial partners play an important role in the
95 establishment and functioning of nodules (Chang *et al.*, 2009; Frendo *et al.*, 2013). The
96 production of reactive oxygen species (ROS) has been observed at different stages of
97 symbiosis and its role has also been demonstrated (Puppo *et al.*, 2013; Montiel *et al.*, 2016).
98 Another player in redox regulation is nitric oxide (NO). NO is a gaseous reactive nitrogen
99 species (RNS) and ROS which has been demonstrated to be a key regulator of plant
100 development such as root development, stomatal aperture, photomorphogenesis, and
101 flowering (Sanz *et al.*, 2015). In plants, NO is also involved in plant responses to abiotic
102 stresses including hypoxia, heavy metal, cold, drought and salt stress (Fancy *et al.*, 2017).
103 Furthermore, NO is also implicated in the plant responses to biotic interactions, such as the
104 hypersensitive response to pathogens (Domingos *et al.*, 2015) and the symbiotic processes
105 (Hichri *et al.*, 2015, 2016). This review focuses on the role of NO either as a signal, a
106 metabolic intermediate or a toxic compound in root nodule development and functioning as
107 well as its importance in the different stages of nitrogen-fixing symbiosis.

108

109 **2 – Nitric Oxide Homeostasis and roles in symbiotic interaction**

110 The presence of NO during legume-rhizobia symbiosis has been reported for about 15 years.
111 NO level is transiently increased in the roots of *Lotus japonicus* during the first hours of the
112 interaction (Nagata *et al.*, 2008; Fukudome *et al.*, 2016). In *Medicago truncatula* roots, NO
113 production was observed at 4 days post-inoculation (dpi) in shepherd's crooks of root hairs,
114 infection threads, and nodule primordia (del Giudice *et al.*, 2011). The presence of NO was
115 detected in the N₂-fixing zone of *M. truncatula* mature nodules (Baudouin *et al.*, 2006) and in
116 the interzone III-IV, between the N₂ fixing and senescence zones, at the end of the symbiotic
117 process (Cam *et al.*, 2012). Recently, using the *M. truncatula* model, NO production was
118 monitored throughout the symbiotic process, from the first hours of interaction with
119 *Sinorhizobium meliloti* to 8 weeks post-infection (wpi) (Berger *et al.*, 2018a). Four production
120 peaks, corresponding to four physiological stages of symbiosis, were observed: 1) during the
121 first hours of the interaction (10 hpi), 2) at the onset of nodule organogenesis (4 dpi), 3) when
122 nodules reach in maturity (3-4 wpi) and 4) at the beginning of senescence process (6 wpi)
123 (Fig. 2).

124 **NO production pathways.** On plants side, several NO production pathways, either reducing or
125 oxidative, have been identified (Gupta *et al.*, 2011a). Oxidative pathways include NO
126 production from polyamines or arginine, which involve respectively polyamine oxidase and
127 NOS-like enzymes (Mur *et al.*, 2013). The denomination “NOS-like” was adopted due to the
128 similarities with the functioning of animal NOS, including the same substrate and the
129 sensitivity to animal NOS inhibitors (Corpas *et al.*, 2009). This is the case during the
130 interaction between *Lupinus albus*/*Bradyrhizobium sp.* and *M. truncatula*/*S. meliloti* where
131 the addition of L-NMMA (L-N-monomethyl arginine) inhibits the production of NO (Cueto *et al.*,
132 1996; Baudouin *et al.*, 2006). However, despite the detection of NOS-like activity, gene
133 encoding an animal NOS orthologue has not been identified to date in the genomes of
134 sequenced land plants (Jeandroz *et al.*, 2016). A Hydroxylamine-mediated NO pathway has
135 been postulated in plants (Rümer *et al.*, 2009), but its significance remains to be demonstrated
136 and there is no data on its possible presence in legumes. Several reductive NO production
137 pathways have been described in plants, involving nitrite (NO₂⁻) as substrates. A non-
138 enzymatic conversion of NO₂⁻ to NO was reported to occur in the apoplast of barley aleurone
139 layers (Bethke *et al.*, 2004), but the others are enzymatically catalysed. The main reductive
140 pathways in plants involve either nitrate reductase (NR), plasma membrane bound nitrite:NO
141 reductase (NiNOR), xanthine oxidoreductase (XOR) or the mitochondrial electron transfer
142 chain (ETC) (Gupta *et al.*, 2011b). In N₂-fixing symbiosis, the pathway involving NR is the
143 best characterized. The higher plants contain from one to three NR enzymes with different

144 electron donor and different tissue specificity (Caboche and Rouzé, 1990). *M. truncatula*
145 contains 3 isoforms, one of which being nodule-specific (*MtNR3*). Horchani *et al.* (2011)
146 demonstrated that the decrease of NO production in RNAi *M. truncatula MtNR1/2* double
147 knockdown was related to the decrease of the plant NR activity. These observations were
148 confirmed by using tungstate (Tg), a NR inhibitor, as it showed that in mature nodule Tg
149 treatment inhibited NO production (Horchani *et al.*, 2011). These authors further
150 demonstrated, by using a pharmacological approach with ETC inhibitors, that ETC is
151 involved in the reduction of NO_2^- in NO. Thus, in nodule, nitrate (NO_3^-) is reduced in NO via
152 a two-step pathway, involving successively NR and mitochondrial ETC (Horchani *et al.*,
153 2011). On the bacterial side, rhizobia participate also in NO production. A NOS-like activity
154 has been identified in *S. meliloti*, but no corresponding gene has been identified in the genome
155 (Pii *et al.*, 2007). Denitrification pathway is the complete reduction of NO_3^- to N_2 , via
156 intermediates NO_2^- , NO and nitrous oxide (N_2O), thanks to the action of the enzymes nitrate
157 reductase (Nap), nitrite reductase (Nir), NO reductase (Nor) and N_2O reductase (Nos)
158 respectively (van Spanning *et al.*, 2007). In mature nodules, the denitrification pathway was
159 shown to contribute for 30% (Horchani *et al.*, 2011) to over 90% (Meakin *et al.*, 2007;
160 Sánchez *et al.*, 2010) of NO production in bacteria.

161 ***NO effects and roles in plant and bacterial cells.*** The cellular environment may greatly
162 influence the chemical form of NO and consequently its biochemical and biological effects.
163 During the symbiotic interaction NO has been described as signal molecule, as a metabolite
164 but also a toxic compound (Mur *et al.*, 2013). An increased number of reports on the
165 occurrence of NO during the whole symbiotic process suggest an important signaling role of
166 this molecule (Hichri *et al.*, 2016, and references therein). Using *M. truncatula*, two
167 transcriptional studies led to identify NO responsive genes either nodule organogenesis
168 (Boscari *et al.*, 2013a) or in developing and mature nodules (Ferrarini *et al.*, 2008). In these
169 studies, the authors analyzed by either RNA-seq or cDNA AFLP the effects of either a NO
170 supply via NO donor treatments (sodium nitroprussiate, SNP, and S-nitrosoglutathione,
171 GSNO) or a NO depletion via NO scavenger treatment (carboxy-PTIO) on inoculated roots.
172 They showed that more than 2000 genes are NO-regulated during the symbiotic process,
173 including genes involved in nodule development that are normally induced by the symbiont
174 (Boscari *et al.*, 2013a). All these findings suggest that NO participates in signal transduction
175 in plant-symbiont interaction (Ferrarini *et al.*, 2008; Boscari *et al.*, 2013a). In free-living
176 bacteria, NO is known to be a toxic molecule (Meilhoc *et al.*, 2010), but in a symbiotic
177 interaction it also contributes as a signal molecule. In *S. meliloti*, transcriptomic analyses

178 showed that NO regulates the expression of approximately 100 genes, 70% of them being also
179 regulated under microaerobic conditions (Bobik *et al.*, 2006; Meilhoc *et al.*, 2010). Most of
180 these genes are regulated by the two-component system FixLJ (Gilles-Gonzalez *et al.*, 2008;
181 Meilhoc *et al.*, 2011). The component FixL is a membrane-bound sensor for diatomic gas as
182 O₂, CO and NO, which is related with the response regulator component FixJ (Mesa *et al.*,
183 2008). Another regulator, named NnrR (nitrite and nitric oxide reductase regulator), is also
184 involved in the NO response in denitrifying bacteria (Bobik *et al.*, 2006; Meihloc *et al.*,
185 2010). NnrR is a member of the Crp/FNR family of transcription factors. It has been
186 described to induce the expression of the *nir* and *nor* gene clusters coding respectively for
187 nitrite reductase and NO reductase (Stern and Zhu, 2014). It should be noted that, in *S.*
188 *meliloti*, both system FixLJ and NnrR do not regulate the whole set of NO induced genes,
189 suggesting that other regulators remain to be identified (Meilhoc *et al.*, 2011).

190 The biological activity of NO is particularly mediated through redox-dependent protein
191 modifications such as S-nitrosylation, tyrosine nitration (Tyr-nitration) and metal-
192 nitrosylation (Besson-Bard *et al.*, 2008). S-nitrosylation and metal-nitrosylation reactions are
193 reversible modifications. They consist of the addition of NO on the sulfhydryl group of a
194 cysteine residue to form an S-nitrosothiol (SNO), or on a transition metal of metalloproteins.
195 Tyr-nitration, on the other hand, affects the tyrosine residues of peptides by the irreversible
196 addition of a nitro group (Stamler *et al.*, 2001; Besson-Bard *et al.*, 2008). Several key proteins
197 in nodule primary metabolism, or involved in stress response were reported to be S-
198 nitrosylated, indicating a crucial regulatory role of NO in the carbon and nitrogen metabolism
199 within the nodule (Puppo *et al.*, 2013). A recent study reports the regulation of *M. truncatula*
200 glutathione peroxidase 1 by NO through S-nitrosylation and Tyr-nitration modifications
201 (Castella *et al.*, 2017). Similar regulation was observed through the inactivation of the *M.*
202 *truncatula* glutamine synthetase 1a (MtGS1a) via Tyr-nitration (Melo *et al.*, 2011). During the
203 nodule senescence, Lbs have also been shown to be inhibited by NO *via* either metal
204 nitrosylation (Mathieu *et al.*, 1998), heme nitration (Navascues *et al.*, 2012) or Tyr-nitration
205 (Sainz *et al.*, 2015).

206 Several studies have highlighted the toxic or inhibitory effect of NO during the symbiosis
207 process. NO was reported as potent inhibitor of nitrogenase activity *in vitro* (Trinchant and
208 Rigaud, 1982) and this was confirmed by *in vivo* studies (Sasakura *et al.*, 2006; Kato *et al.*,
209 2010). NO is known to inhibit soybean bacterial nitrogenase activity and expression (Sánchez
210 *et al.*, 2010), and to inhibit the growth of rhizobia in free culture (Meilhoc *et al.*, 2010). It was
211 also shown to negatively affect the life-time of the symbiotic interaction by triggering nodule

212 senescence (Cam *et al.*, 2012). This negative effect was observed with high NO nodule level
213 obtained either by using *S. meliloti* mutant strains deficient in the degradation of NO (*hmp*,
214 *norB*, *nnrSI*), or by treating nodules with NO donors (Cam *et al.*, 2012; Meilhoc *et al.*, 2013;
215 Blanquet *et al.*, 2015). Conversely, by using plants that over-expressed *hmp*, a decrease NO
216 levels was observed that leads to a delay of nodule senescence (Cam *et al.*, 2012).

217 In contrast, NO has also been demonstrated to play a beneficial metabolic function for the
218 maintenance of the energy status under hypoxic conditions (Igamberdiev and Hill, 2009;
219 Igamberdiev *et al.*, 2010; Gupta and Igamberdiev, 2011). Under these conditions, NO is
220 involved in the cycle named phytoglobin-NO (Phytogb-NO) respiration allowing the
221 regeneration of ATP under hypoxia. Stoimenova *et al.* (2007) demonstrated that under both
222 anaerobic and reducing conditions, purified mitochondria of barley and rice roots are able to
223 use NO_2^- as the terminal acceptor of ETC to reduce it in NO. This step allows the generation
224 of a proton gradient necessary for the production of ATP. The Phytogb-NO cycle is divided
225 into 4 steps including (1) NO_3^- reduction to NO_2^- by cytosolic NR, (2) NO_2^- transport from the
226 cytosol to the mitochondrial matrix, (3) NO_2^- reduction to NO by the ETC, and (4) NO
227 diffusion to the cytosol and oxidation to NO_3^- by Phytogbs (Igamberdiev and Hill, 2004;
228 Hichri *et al.*, 2016). Taken together, these observations indicate that NO effects and functions
229 depend on its level at its action site (Mur *et al.*, 2013). Therefore, NO concentration should be
230 tightly controlled in time and space to avoid toxic effect, and lead the signaling and metabolic
231 functions.

232 **NO turnover and detoxification.** In plants, NO removal was mainly ascribed to Phytogbs
233 (Hill, 2012), previously named hemoglobins (Hbs) (Hill *et al.*, 2016). Phytogbs, have been
234 classified into six categories including: Phytogb0 - nonsymbiotic hemoglobin (nsHb) present
235 in algae, bryophytes and gymnosperms; Phytogb1 - class 1 nonsymbiotic hemoglobin (nsHb-
236 1) and Phytogb2 - class 2 nonsymbiotic hemoglobin (nsHb-2) present in angiosperms;
237 SymPhytogb - symbiotic hemoglobin (symHb) present in non-legume N_2 -fixing plants; Lb -
238 leghemoglobin (Lb) present in N_2 -fixing legumes; and Phytogb3 - class 3 truncated
239 hemoglobin (trHb) present in algae and land plants (Hill *et al.*, 2016). Based on their
240 sequence, homology, and affinity for O_2 , three types of Phytogb were described in legumes
241 and are expressed during N_2 -fixing symbiosis: Phytogb1, Lb and Phytogb3 (Bustos-
242 Sanmamed *et al.*, 2010). Phytogb1 have a very high affinity for oxygen (K_m 1-10 nM), higher
243 than mammalian Hbs (Gupta *et al.*, 2011c). They are present in both monocotyledons and
244 dicotyledons. In *A. thaliana*, one gene codes for a *Phytogb1*, while 2 are present in *G. max*
245 and *L. japonicus*, and 3 in *M. truncatula* (Garrocho-Villegas *et al.*, 2007; Bustos-Sanmamed

246 *et al.*, 2010; Berger *et al.*, 2018a). They are mainly expressed in seeds, roots and stems
247 (Arredondo-Peter *et al.*, 1998; Hill, 1998). In their physiological state, PhytoGBs are mainly in
248 oxidized form even in presence of low oxygen level. In this form they are able to bind NO to
249 oxidize it into NO₃⁻ thanks to NADH consumption. The overexpression of *PhytoGB1* in *L.*
250 *japonicus* was shown to decrease NO level (Shimoda *et al.*, 2009), indicating that it is
251 involved in NO regulation level. Lbs are nodule specific PhytoGBs present in high
252 concentration (order of mM) and have a high affinity for oxygen (K_m 100-200 nM). Lbs are
253 encoded by many genes in legumes. Thus, 12, 4 and 6 genes have been identified in *M.*
254 *truncatula*, *G. max* and *L. japonicas*, respectively (Berger *et al.*, 2018a). Although generally
255 specific to legumes, Lbs are also found in the nodules of non-leguminous plants, such as the
256 Ulmaceae *Parasponia andersonii* in symbiosis with *Rhizobium*, and the actinorhizal
257 *Casuarina glauca* in symbiosis with the actinomycete *Frankia* (Appleby *et al.*, 1988). They
258 are known to play a role in oxygen scavenging to avoid the nitrogenase inhibition and oxygen
259 transport towards the mitochondria (Ott *et al.*, 2005). In addition, Lbs are also able of binding
260 NO with a very high affinity and detoxifying it to nitrate (Herold and Puppo, 2005). PhytoGB3
261 possess a low affinity for oxygen (K_m ~ 1500 nM), giving them a role in the regulation of
262 oxygen at low ambient oxygen concentrations. PhytoGB3 derive from truncated Hbs (tr-Hbs)
263 found in eubacteria, protozoan cyanobacteria, algae and plants (Vuletich and Lecomte, 2006).
264 Phylogenetic analyses show that bacterial tr-Hbs can be subdivided into three sub-classes (I,
265 II and III), called trHbN, trHbO and trHbP (Wittenberg *et al.*, 2002). In *Frankia*, tr-Hbs were
266 shown to be regulated either by nitrate and NO (trHbN), or by oxygen (trHbO), and would
267 play a role in the adaptation to low oxygen concentration (Coats *et al.*, 2009). In
268 *M.truncatula*, the two *PhytoGB3* genes do not have the same expression and localization
269 patterns. *MtTrHb1* was expressed in the infected cells of the N₂-fixing zone of root nodules,
270 whereas the *MtTrHb2* promoter was predominantly expressed in the nodule vascular tissue
271 (Vieweg *et al.*, 2005). However, the main role of PhytoGB3 in plants remains unclear. Other
272 enzymes participate to NO detoxification/degradation *in planta*, such as GSNO reductase
273 (GSNOR) and glutathione peroxidase (Gpx). GSNOR is able to reduce GSNO to oxidized
274 glutathione (GSSG) and ammoniac by using a reducing power provided by NADH. The
275 GSNO is known to act as a reservoir of NO bioactivity in plant cells (Liu *et al.*, 2001;
276 Sakamoto *et al.*, 2002). In addition to releasing NO, GSNO can also transfer a NO group on
277 specific cysteine residues of target proteins (Pawloski *et al.*, 2001). The modulation of the
278 cellular S-nitrosothiol-containing proteins (SNO-proteins) plays an important role in many
279 signalisation processes such as the immune response (Begara-Morales and Loake, 2016), and

280 in the nitrogen, carbon and energy metabolism in nodules (Puppo *et al.*, 2013). This
281 regulation involves the intervention of proteins from the superfamily of thioredoxins (Meyer
282 *et al.*, 2008).

283 From bacterial side, several proteins are involved in NO detoxification. Two proteins, *Hmp*
284 coding for a flavohemoglobin, and *Nor* coding for NO reductase, were shown to be major
285 NO-detoxifying enzymes (Cam *et al.*, 2012; Meihloc *et al.*, 2013). Cam *et al.*, (2012) showed
286 that *Hmp* is essential for maintaining NO levels compatible with symbiosis, as they observed
287 a higher NO level correlated to a lower N₂ fixation and earlier senescence in nodule obtained
288 with *Hmp* mutants. On the other hand, by using *norC* mutants, several reports showed a
289 significant increase in NO level when nodules were subjected to flooding or nitrate treatment
290 (Sánchez *et al.*, 2010; Gómez-Hernández *et al.*, 2011; Meilhoc *et al.*, 2013). More recently,
291 two others genes *nnrS1* and *nnrS2* were proposed to be involved in NO degradation under
292 both in free living and symbiotic conditions (Blanquet *et al.*, 2015). In the pathogenic bacteria
293 *Vibrio cholerae* and *Neisseria meningitidis*, NnrS is involved in the resistance to NO (Stern *et*
294 *al.*, 2012; Arai *et al.*, 2013), and similar role was suggested in symbiotic nodules (Blanquet *et*
295 *al.*, 2015). Consequently, denitrifying bacteria as *S. meliloti*, possess potentially at least four
296 NO detoxification systems (*Hmp*, *Nor*, *NnrS1* and *NnrS2*). During symbiotic interaction,
297 these proteins have different localization and expression patterns in the different zones of the
298 nodule (Meilhoc *et al.*, 2013; Roux *et al.*, 2014). The reasons of such NO-regulating system
299 redundancy in rhizobia are still unclear, but this underlines the importance of the regulation in
300 time and space of NO on the bacterial side (Torres *et al.*, 2016).

301

302 **3 – Symbiosis establishment**

303 NO level increases in the plant partner within the first hours of the interaction with the
304 symbiont (Nagata *et al.*, 2008; Berger *et al.*, 2018a). In the roots of *Lotus japonicus* and *M.*
305 *sativa*, a transient NO overproduction of few hours was observed 4 hours post inoculation
306 (hpi) (Nagata *et al.*, 2008), and 10 hpi in *M. truncatula* roots (Berger *et al.*, 2018a). This
307 transient increase in NO production is to be contrasted with the continuous NO production for
308 at least 24 hours observed following inoculation by the pathogens *Ralstonia solanacearum*
309 and *Pseudomonas syringie* (Nagata *et al.*, 2008). NO in synergy with ROS is known to trigger
310 an hypersensitive response and to activate plant defence genes expression (Delledonne *et al.*,
311 1998; Durner *et al.*, 1998). These observations suggest that, after the first peak of NO which
312 marks the entry into interaction of the two partners, the decline in NO production in the
313 symbiotic process leads to decrease the defence reactions of the plant to allow the entry of the

314 symbiont in roots (Murakami *et al.*, 2011; Berger *et al.*, 2018a). In the plant-pathogen
315 interaction, the prolonged NO production and resistance responses are accompanied by either
316 the suppression or the maintenance of a low PhytoGb expression level (Wally *et al.*, 2013),
317 whereas in the symbiotic interaction the decrease in NO production is concomitant with the
318 overexpression of PhytoGlb genes, mainly *PhytoGb1.1*, suggesting that PhytoGbs down-
319 regulate NO level to decrease the defence response (Berger *et al.*, 2018a). It should be noted
320 that the increase in NO production during the first hours of the interaction is not visible when
321 legumes are inoculated with non-compatible rhizobia. This means that the recognition
322 between the two partners is highly specific of a compatible symbiotic interaction (Nagata *et*
323 *al.*, 2008). This specific recognition comes partly from the lipochitooligosaccharide (LCO)
324 motives of the rhizobia Nod factors (Murakami *et al.*, 2011).

325 Later in the symbiotic process NO was found to be produced in shepherd's crooks of root hair
326 and in the infection thread (del Giudice *et al.*, 2011). PhytoGb1 mutants of *L. japonicus*
327 containing a high NO level exhibit an alteration of the infection process due to a defective
328 formation of infection thread (Fukudome *et al.*, 2016). The same phenotype is observed
329 during NO donor treatments (SNP or SNAP) which inhibit the elongation of the infection
330 thread (Fukudome *et al.*, 2016), suggesting that a high NO level inhibits nodulation. These
331 results are contradictory with that of del Giudice *et al.* (2011) and Pii *et al.* (2007) who
332 observed a nodulation delay when plants are either inoculated with an *hmp*⁺⁺ *S. meliloti* strain
333 or treated with the NO scavenger cPTIO. These observations seem to be in apparent
334 opposition regarding the positive or negative role of NO in the nodulation process, and the
335 question arose as to whether this contradiction was related either to the symbiotic model, to
336 the stages of symbiosis analyzed or to the duration of treatments. This point is developed in
337 the next paragraph.

338

339 **4 – Nodule development**

340 Nodule primordial development starts with the first divisions of the pericycle and endodermis
341 cells in the outer cortex, i.e. 24 hpi in the *M. truncatula* model, and ends when the nodule
342 becomes mature and reaches a maximum N₂ fixation activity, i.e. 3-4 wpi for *M. truncatula*
343 nodules (Ferguson *et al.*, 2010; Xiao *et al.*, 2014). In the *M. truncatula* – *S. meliloti* symbiosis
344 a second peak of NO production occurs during this period of time with a maximum at 4 dpi
345 (Berger *et al.*, 2018a). During the formation of the *M. truncatula* nodule primordium del
346 Giudice *et al.* (2011) reported a specific NO production in the pericycle, endodermis and
347 dividing cortical cells, a block of cells named 'controlled area' (Xiao *et al.*, 2014). Cell

348 division of the “controlled area’ is associated with an increase in auxin concentration in these
349 cells (Mathesius *et al.*, 1998). Analyzing the sequence of events during nodule formation,
350 Xiao *et al.* (2014) postulated that Nod factor signaling induces the decrease of PIN proteins,
351 in the plasma membrane of ‘controlled area’ cells. PIN proteins are transmembrane carriers
352 involved in the efflux of auxin (Adamowski and Friml, 2015). The reduction of PIN carriers
353 would increase the cellular concentration in auxin and trigger the entry into division of the
354 cells in the ‘controlled area’. Interestingly, NO has been shown to repress the expression of
355 PIN genes in Arabidopsis and rice roots (Liu *et al.*, 2015; Sun *et al.*, 2017). Therefore, it could
356 be assumed that the NO produced in the ‘controlled area’ could down-regulate the expression
357 of the PINs carriers in legume plants, resulting in auxin accumulation and division of nodular
358 primordium cells. In this scenario, the down-regulation of *Phytogb1* genes that was observed
359 in *M. truncatula* early in the formation of nodule primordium (Berger *et al.*, 2018a) would
360 participate to the increase in NO level observed in the controlled area. The spatiotemporal
361 analysis of the expression of the different PIN and *Phytogb* genes in the controlled area would
362 be an interesting issue to confirm this hypothesis.

363 In an attempt to determine the function of NO production during nodule development, several
364 studies have developed approaches to modulate NO production. The scavenging of NO
365 through either the use of cPTIO, or the overexpression in the plant partner cells of the
366 bacterial flavohemoglobin *hmp* involved in NO detoxification (Poole and Hughes, 2000), led
367 to a delayed nodulation phenotype in the *M. truncatula* - *S. meliloti* interaction (del Giudice *et al.*,
368 2011). Pii *et al.* (2007) also reported that *Medicago* plants with elevated NO level
369 generate more nodules than control plants, whereas cPTIO treatment reduces the number of
370 nodules. Both studies indicate that NO is required during the nodule development. However,
371 the overexpression of either *LjHb1* or *Alnus firma AfHb1* in *L. japonicus* transgenic hairy
372 roots reduced NO production and triggered an increase in the number of nodules compared to
373 those in control roots (Shimoda *et al.*, 2009). In the same way, *LjGlb1-1* mutant of *L.*
374 *japonicus* showed increased NO levels and reduced nodule numbers when compared to
375 control roots (Fukudome *et al.*, 2016) indicating that an increase in NO level following the
376 mutation of *Phytogb* genes was deleterious for nodule production. This apparent discrepancy
377 about the roles of NO in the establishment and development of symbiosis was first explained
378 by differences in either the symbiotic models (i.e. *M. truncatula* versus *L. japonicus*) or the
379 specific activity of either *Hmp* or *Phytogbs* (del Giudice *et al.*, 2011; Fukudome *et al.*, 2016;
380 Hichri *et al.*, 2015). Using the same symbiotic model and timing as del Giudice *et al.* (2011),
381 Berger *et al.* (2018a) recently demonstrated that treatments of *M. truncatula* roots with NO

382 donors during 4 dpi resulted in a decreased nodule number, indicating that independently of
383 the symbiotic model, it is the level of NO that regulates the nodule development process,
384 emphasizing the need of setting-up a NO concentration range for successful establishment of
385 the symbiotic relationship.

386 Using RNA-Seq approach a transcriptomic analysis was performed with *M. truncatula*
387 inoculated roots treated with c-PTIO to identify genes potentially regulated by NO during
388 nodule primordium development (Boscari *et al.*, 2013a). A comparison of NO-depleted and
389 control inoculated roots revealed differential patterns of expression for more than 2000 genes.
390 NO depletion prevented the up-regulation of many genes usually induced by inoculation with
391 the microsymbiont. Many of these genes encode TFs and proteins involved in nodule
392 development, such as cyclin-like proteins, peptidases or ribosomal protein families, most of
393 which are required for the dedifferentiation of cortical cells and the induction of cell division
394 during nodule formation. In contrast, a number of genes involved in secondary metabolism
395 (terpene, flavonoid and phenylpropanoid), PR-proteins and cytochrome P450 were
396 significantly induced by cPTIO. The most striking finding was, upon cPTIO treatment, the
397 up-regulation of defense genes normally repressed by inoculation with rhizobia, indicating
398 that, at this stage of the symbiotic process, NO could be involved in the repression of plant
399 defense reactions, thereby favoring the setup of the nodule primordium and the development
400 of the nodule. This potential way of NO action is similar to that proposed to occur in
401 mycorrhizal symbiosis (Espinosa *et al.*, 2014), but differs markedly from the signaling
402 functions of NO in pathogenic interactions, in which NO cooperates with reactive oxygen
403 species (ROS) to induce hypersensitive cell death (Delledonne *et al.*, 1998). This means that,
404 depending on the timing of the symbiosis, NO differently regulates the plant defense
405 response: up-regulating it during the establishment of the interaction, and down-regulating it
406 at the beginning of nodule development.

407

408 **5 – Nitrogen-fixing nodules**

409 The main function of the mature nodule is to reduce and assimilate N₂ to provide it to the
410 plant. Considering that this process is expensive in energy, the number of nodules and their
411 N₂-fixing capacity need to be tightly regulated to maintain a balance between the reduced
412 nitrogen production required for plant development and the supply of carbon substrates to
413 bacteroids. Various lines of evidence suggest that NO is a key player in this regulation. NO is
414 highly present in the N₂-fixing zone of the nodules of *L. japonicus* and *M. truncatula*, mainly
415 in bacteroid-containing cells, but not in meristematic, infection, and senescence zones

416 (Baudouin *et al.*, 2006; Shimoda *et al.*, 2009). The biological significance of NO in mature
417 nodules has been a matter of debate over the last few years and may be particularly related to
418 the microoxic status of the nodule (reviewed in Boscari *et al.*, 2013b; Hichri *et al.*, 2015,
419 2016). On the one hand, NO was reported to be a potent inhibitor of nitrogenase activity as
420 measured *in vitro* (Trinchant and Rigaud, 1982). In nodules of soybean plants subjected to
421 flooding, the increase in NO production is associated with a more than three-fold reduction in
422 the expression of bacterial *nifH* and *nifD*, and this inhibition is partially reversed by the
423 application of the NO scavenger c-PTIO, which illustrates the inhibitory role of NO on the
424 expression of nitrogenase genes (Sánchez *et al.*, 2010). Furthermore, using both
425 pharmacological approach, with NO-donors and scavengers, and molecular approach with
426 transgenic plants with modified NO levels, several studies report that NO inhibits *in vivo* N₂-
427 fixing activity in soybean, *L. japonicus*, and *M. truncatula* nodules (Trinchant and Rigaud,
428 1982; Shimoda *et al.*, 2009; Kato *et al.*, 2010; Cam *et al.*, 2012). On the other hand, ARA is
429 more substantial in *L. japonicus* nodules in the presence of 0.1 mM SNP than either in the
430 absence or in the presence of higher (1 mM) concentration of SNP, indicating that low but
431 significant NO concentration is beneficial to nitrogen fixation (Kato *et al.*, 2010). Functional
432 nodules are characterized by a microoxic environment, raising the question of energy supply
433 within this organ. In plant roots submitted to hypoxia, the “Phytogb-NO respiration” cycle
434 occurs, where nitrite is used as a final electron acceptor instead of O₂ to be reduced to NO,
435 which allows cell energy status retention (Gupta and Igamberdiev, 2011; Berger *et al.*,
436 2018b). Accumulated data support the existence of such a Phytogb-NO respiration in
437 microoxic *M. truncatula* nodules, in which both NR and mitochondrial ETC are involved in
438 NO production and in the maintenance of the nodule energy state (Horchani *et al.*, 2011).
439 Similarly, in nodules of *M. sativa* plants submitted to hypoxia the maintenance of the
440 ATP/ADP ratio of the root and nodule system is due to the establishment and the functioning
441 of a Phytogb-NO respiration (Aridhi, Brouquisse, personal communication) indicating that the
442 production of NO is involved in energy regenerating processes.

443 At tissue level, root aerenchyma formation is a strategy implemented by a number of plants
444 for delivering oxygen from aerial organs to the roots in hypoxic soils or under long-term
445 flooding (Armstrong, 1980; Yamauchi *et al.*, 2013). Arikado (1954) first showed that soybean
446 plants submitted to soil flooding develop secondary aerenchymas in the roots and the nodules.
447 Similarly, secondary aerenchymas have been shown to cover the surface of soybean nodules
448 in flooded soils and in hypoxic rhizosphere conditions (Pankhurst and Sprent, 1975; Parsons
449 and Day, 1990). This phenomenon has also been discovered in flood-tolerant legumes such

450 as *Sesbania aculeata* (Scott and Wager, 1988), *Sesbania rostrata* (Saraswati *et al.*, 1992;
451 Shiba and Daimon, 2003), *Neptunia oleracea* (Metclafe, 1931), *Melilotus siculus* (Teakle *et*
452 *al.*, 2011; Verboven *et al.*, 2012; Konnerup *et al.*, 2018), and *Viminaria juncea* (Walker *et al.*,
453 1983). Several lines of evidence showed that ethylene is a predominant mediator that
454 promotes the development of aerenchymas in roots (Jackson and Armstrong, 1999). Recently,
455 Wany *et al.* (2017) found that hypoxically induced NO is important for induction of the
456 ethylene biosynthetic genes encoding ACC synthase and ACC oxidase, and for the
457 development of aerenchymas in wheat roots under hypoxia. Interestingly, in *M. truncatula*
458 developing nodules, several ACC synthase genes were shown to be under the control of NO
459 (Boscari *et al.*, 2013a), suggesting that NO could likely controls the formation and
460 development of aerenchymas in legume nodules subjected to flooding or long-term hypoxic
461 conditions.

462 The signaling or regulation functions of NO pass through post-translational modifications of
463 proteins such as S-nitrosylation and Tyrosine nitration. Puppo *et al.* (2013) reported that in *M.*
464 *truncatula* mature nodules, about 80 S-nitrosylated proteins from both the plant and the
465 bacterial partner have been identified. Many of these proteins were enzymes related to
466 glycolysis (fructokinase, fructose-bisphosphate aldolase, enolase, glyceraldehyde-3-P
467 dehydrogenase, phosphoenolpyruvate carboxylase), to the tricarboxylic acid cycle (succinate
468 dehydrogenase, citrate synthase, isocitrate dehydrogenase, malate dehydrogenase), energy
469 metabolism (mitochondrial ATPase β -subunit) and to nitrogen assimilation (nitrogenase nifK
470 and nifH subunits), emphasizing the crucial regulatory roles of NO in the modulation of
471 enzymatic activity. Interestingly, 27 proteins, mainly related to carbon, nitrogen, and energy
472 metabolism, were found to be both sulfenylated and nitrosylated (Puppo *et al.*, 2013),
473 underlining the key role of other redox players than NO, such as glutathione (GSH) and H₂O₂,
474 in the redox control of primary metabolism. Such a regulation at the cross-talk between NO,
475 ROS and GSH was recently demonstrated on the *M. truncatula* glutathione peroxidase 1
476 (MtGpx1) which activity is reversibly inhibited by the NO-donors diethylamine-NONOate
477 and S-nitrosoglutathione through S-nitrosylation and S-glutathionylation, but irreversibly
478 inhibited by peroxynitrite through Tyr-nitration (Castella *et al.*, 2017). In nodules, the
479 regulation and potential inhibition of nitrogenase activity by NO is believed to occur through
480 S-nitrosylation, as suggested by computational prediction of S-nitrosylation protein sites (Xue
481 *et al.*, 2010). Its activity during N₂-fixation process generates ammonium, which at high
482 concentration becomes toxic and compromises plant growth (Li *et al.*, 2014). Cytosolic
483 glutamine synthetase (GS1) is also a key enzyme in nitrogen metabolism as it assimilates

484 ammonium. In *M. truncatula*, *MtGS1a* is responsible for 90% of the total nodule GS activity
485 (Carvalho *et al.*, 2000). In N₂-fixing nodules, *MtGS1a* can be inactivated by NO through
486 tyrosine nitration, but not by S-nitrosylation (Melo *et al.*, 2011). This post-translational
487 modification irreversibly abolishes *MtGS1a* enzymatic activity and affects N₂-fixation.
488 Glutamate is the substrate of GS, but also a precursor for the biosynthesis of the glutathione
489 (GSH), which is both a major antioxidant compound and the synthesis precursor of S-
490 nitrosoglutathione (GSNO). Consequently, NO-mediated inactivation of the GS activity may
491 promote glutamate redirection for GSH and GSNO synthesis (Melo *et al.*, 2011).
492 Interestingly, the plastidial *MtGS2a*, which share a high degree of sequence homology and a
493 remarkably conserved active site fold with *GS1a*, is reversibly inactivated by S-nitrosylation,
494 indicating that the differential localization of the isoenzymes in a specific plant tissue is
495 probably involved in the NO-mediated regulation of GS activity (Melo *et al.*, 2011). In
496 addition, it has been reported that GS nitration is enhanced in *hmp* mutant nodules which
497 present higher NO levels (Silva and Carvalho, 2013). As *hmp* mutant nodules exhibited
498 premature senescence (Cam *et al.*, 2012), it is tempting to associate NO-induced GS
499 inhibition and nodule senescence.

500 The extent to which NO-related processes such as PhytoGb-NO respiration, aerenchymas
501 formation, or inhibition of N₂-fixation metabolism interferes with each other is currently
502 unknown. This highlights the fact that NO level needs to be tightly regulated and its
503 detoxification represents a critical aspect of nodule metabolism. In this context, the
504 involvement of PhytoGb in the control of NO has been particularly emphasized. In *L.*
505 *japonicus* and *M. truncatula* mature nodules, the expression of the three PhytoGb classes
506 (*PhytoGb1*, *Lbs* and *PhytoGb3*) strongly increases as compared to non nodulated roots (Bustos-
507 Sanmamed *et al.*, 2010; Berger *et al.*, 2018a) and the question of their respective functions
508 within the N₂-fixing process was raised. The presence of Lb-NO complexes, detected by EPR,
509 in soybean and cowpea nodules *in vivo* (Maskall *et al.*, 1977; Mathieu *et al.*, 1998; Meakin *et al.*,
510 2007; Sánchez *et al.*, 2010) clearly shows that *Lbs* are involved in the complexation of
511 NO. It is not possible to differentiate between Lb-NO, *PhytoGb1*-NO, and *PhytoGb3*-NO
512 spectra *in vivo*, but considering the intensity of the resonances of EPR spectra related to
513 'PhytoGb-NO' complexes (Mathieu *et al.*, 1998), and because *Lbs* are much more expressed
514 than *PhytoGbs1* and *PhytoGbs3* in mature nodules (Berger *et al.*, 2018a), it is likely that the
515 large majority of the EPR signal was directly related to the presence of Lb-NO complexes.
516 The ability of *Lbs* to bind O₂ and NO to produce NO₃⁻ (Herold and Puppo, 2005) makes them
517 good candidates to detoxify NO and participate in the regeneration of energy in the plant

518 compartment through the functioning of the 'Phytogb-NO' respiration (Horchani *et al.*, 2011).
519 Although much less expressed than *Lbs*, the significant expression of *Phytogb1.1*, *Phytogb1.3*
520 and *Phytogb3.1* in *M. truncatula* nodules (Berger *et al.*, 2018a) suggests that each of them has
521 its own function in the N₂-fixing metabolism. In mature nodules of *L. Japonicus*, the
522 overexpression of *LjHb1* (*LjPhytogb1.1*) results in decreased NO production and increased
523 ARA (Shimoda *et al.*, 2009), whereas nodules of mutants affected on *LjPhytogb1.1* show
524 higher NO production and lower ARA compared to WT-control (Fukudome *et al.*, 2016).
525 These data indicate that, in mature nodules, *Phytogb1.1* is involved in the regulation of the
526 NO level, in order to avoid that an excess of NO inhibits nitrogen fixation and the energy and
527 carbon metabolism of the two symbiotic partners. *Phytogb3* (tr-Hb) have been shown to be
528 expressed in soybean and *M. truncatula* nodules (Lee *et al.*, 2004; Vieweg *et al.*, 2005; Berger
529 *et al.*, 2018a), as well as in actinorhizal nodules of *Datisca glomerata* (Pawlowski *et al.*,
530 2007). In *M. truncatula* nodules, both *Phytogb3* genes have been shown to be induced in
531 response to symbiosis, *Phytogb3.1* being expressed in the infected cells of the N₂-fixing zone,
532 and *Phytogb3.2* more predominantly in the vascular tissue of the nodule (Vieweg *et al.*,
533 2005). The high expression of *Phytogb3.1* in *M. truncatula* nodules (Vieweg *et al.*, 2005;
534 Berger *et al.*, 2018a) and of *tr-Hb1* in *D. glomerata* (Pawlowski *et al.*, 2007) nodules as
535 compared to non-inoculated roots argues in favor of a specific role of this *Phytogb*, alongside
536 *Phytogb1.1* and *Lbs*, in N₂-fixing metabolism.

537 Why three *Phytogb* types in the N₂-fixation zone? The answer is probably related to
538 the gradients of O₂ and NO concentration in mature nodules and to the relative affinity of the
539 different *Phytogbs* for O₂ and NO. In *Medicago* nodules, due to the presence of the O₂ barrier,
540 the pO₂ decreases from 250 μM in the first layers of the epidermal cells to approximately 10-
541 40 nM in the infected cells in the heart of zone III (Appleby, 1992; Soupène *et al.*, 1995).
542 Although the pNO gradient has never been measured, a number of studies show that NO level
543 is higher at the center of nodules than at their periphery with a concentration gradient inverse
544 to that of O₂ (Baudouin *et al.*, 2006; del Giudice *et al.*, 2011; Cam *et al.*, 2012). High
545 respiratory rates are needed, in both plant and bacterial partners, to sustain the energy
546 consuming N₂-fixing metabolism and metabolite transport between roots and nodules. In
547 normoxic plants, ATP synthesis is routinely performed through the O₂-dependent respiration.
548 In microoxic *M. truncatula* nodules, it has been shown that the regeneration of ATP and the
549 maintenance of the energetic state of the nodules depends on the functioning of the '*Phytogb*-
550 NO' respiration (Horchani *et al.*, 2011). Thus, it is likely that the '*Phytogb*-NO' respiration
551 gradually substitutes for the O₂-dependent respiration to regenerate ATP as pO₂ decreases

552 toward the center of the nodule. Phytogbs have very different relative affinities for O₂ with
553 Km_{O₂} on the order of 2, 50-100 and 1500 nM for Phytogb1, Lbs and Phytogb3, respectively
554 (Smaghe *et al.*, 2009; Gupta *et al.*, 2011c). Thus, it may be hypothesized that the role of the
555 different Phytogbs is both to scavenge NO and to allow the functioning of the 'Phytogb-NO'
556 respiration as a function of the pO₂ gradient within the nodule: the Phytogb3 acting in the
557 weakly microoxic peripheral cells, Lbs in microoxic cells, and Phytogb1 acting in strongly
558 microoxic cells in the center of the nodules.

559 Plant Phytogbs could be expected to be sufficient for controlling NO level in nodules.
560 However, four proteins involved in the control of NO level have also been also identified in
561 the bacterial partner: the flavohemoglobin Hmp, the respiratory NO reductase Nor and the
562 two proteins of the NnrS family, NnrS1 and NnrS2 (Fig. 2). Using *M. truncatula* nodules
563 induced with either WT, hmp null mutant strain (*hmp*) or hmp over-expressing strain
564 (*hmp++*) of *S. meliloti*, Cam *et al.* (2012) demonstrated that hmp controls NO level in mature
565 nodule and is essential in maintaining efficient nitrogen-fixing symbiosis. Similarly, using the
566 same strategy, norB (Meilhoc *et al.*, 2013), and both NnrS1 and NnrS2 (Blanquet *et al.*, 2015)
567 have been shown to be also involved in the control of NO level in *M. truncatula* nodules. In
568 *B. japonicum*, as well as in *Rhizobium etli*, Nor was shown to be involved in nodule NO
569 degradation (Gómez-Hernández *et al.*, 2011; Sánchez *et al.*, 2011). Therefore, not only plant
570 but also bacterial proteins control NO level inside nodules and it may be anticipated that other
571 plant and bacterial proteins such as glutathione peroxidase, thioredoxins or GSNO reductase
572 might indirectly participate to NO detoxification/degradation/signalisation *in planta* (Lee *et al.*,
573 2010). The question is raised as to whether why so many different systems to reduce NO
574 level? Both the plant and the bacterial partners have been shown to produce NO in mature
575 nodules (Sánchez *et al.*, 2010; Horchani *et al.*, 2011). Considering first, that NO is a highly
576 diffusible gas, and second, that it is highly reactive, efficient NO scavenging systems are
577 probably necessary in the different cellular compartments of the nodule to balance its different
578 signaling, metabolic and toxic effects and to maintain the symbiotic N₂ fixation.

579

580 **6 – Nodule senescence**

581 Nodule senescence is characterized by the breakdown of the interaction between the
582 two partners. In *M. truncatula*, the first signs of natural senescence appear around 3-4 wpi
583 with the formation of zone IV, (Puppo *et al.*, 2005; Van de Velde *et al.*, 2006), but their N₂-
584 fixing capacity starts to decline 6 weeks after their initiation (Cam *et al.*, 2012).
585 Environmental stress like prolonged darkness or nitrate treatments can also trigger premature

586 senescence ((Matamoros *et al.*, 1999; Perez Guerra *et al.*, 2010; Pierre *et al.*, 2014). In *M.*
587 *truncatula* nodules NO production was found to increase both at the beginning of N₂-fixation
588 decline and when nodules are submitted to environmental stress (Berger *et al.*, 2018a).
589 Interestingly, an increase of NO levels in *M. truncatula* nodules, resulting either from the use
590 of *S. meliloti* strains deficient in NO degradation (*hmp*, *norB*, *nnrS1*) or from the exogenous
591 addition of a NO donor, led to a premature senescence of the nodules, the severity of which
592 correlated with the NO levels inside the nodules(Cam *et al.*, 2012; Meilhoc *et al.*, 2013;
593 Blanquet *et al.*, 2015). Conversely, nodules induced by *S. meliloti* strains overexpressing
594 either *hmp* or *nnrS1* showed a low NO level and present a delayed senescence. Dark-induced
595 senescence was also delayed in nodules induced by *hmp*-overexpressing strains, suggesting
596 that NO is also involved in the regulation of stress-induced senescence (Cam *et al.*, 2012).
597 A typically visible nodule senescence sign is its color, changing from pink to green resulting
598 from Lb inactivation and degradation. Lb has been shown to be subjected to NO-induced
599 post-translational modifications such as metal nitrosylation (Mathieu *et al.*, 1998); heme
600 nitration (Navascues *et al.*, 2012) and Tyr-nitration (Sainz *et al.*, 2015). As mentioned in the
601 previous paragraph, many enzymes involved in carbon, nitrogen and energy metabolism, such
602 as nitrogenase, GS, TCA and glycolysis enzymes, have been shown to be also modified and
603 inhibited by either NO or NO-derivatives (Sánchez *et al.*, 2010; Melo *et al.*, 2011; Puppo *et*
604 *al.*, 2013), and NO was postulated to be a signal in developmental as well as stress-induced
605 senescence (Cam *et al.*, 2012). Recently, Berger *et al.* (2018)a observed that, in early
606 senescent *M. truncatula* nodules a peak of NO production occurs concomitant with a decrease
607 in Lb gene expression, and the question arose as to whether NO either acts as a trigger for
608 senescence, or is a consequence of the senescence process. By comparing the effects of two
609 stress-induced senescences (using either dark or 10 mM nitrate treatment), the authors put
610 forward a beginning of answer (Berger *et al.*, 2018)a. Indeed, both treatments result in a
611 similar increase in NO production, but their effects on the expression of both CP6, a cysteine-
612 endopeptidase known to be a marker of nodule senescence (Pierre *et al.*, 2014), and Phyto**g**s
613 genes are qualitatively and quantitatively different. Since the same increase in NO does not
614 produce the same effects on gene expression, the authors suggest that NO cannot be the
615 trigger signal for senescence, but more certainly an element of the nodule response to the
616 induced senescence treatments.

617 Interestingly, in *M. truncatula*, Berger *et al.* (2018)a observed that two weeks after the onset
618 of nodule senescence, the expression of class 1 and 3 Phyto**g** genes, corresponding to non-
619 symbiotic and truncated Hbs, strongly increases in a context where Lbs expression and NO

620 production decrease but remain globally high. Such a strong expression of *Phytogb1* and 3
621 genes is possibly related to the oxidative stress and alteration of the redox state that occurs
622 during nodule senescence (Puppo *et al.*, 2005). Indeed, NO has been suggested to be involved
623 in the senescence of the nodule as it can react with superoxide ($O_2^{\cdot-}$) to form peroxynitrite, a
624 reactive nitrogen species known to irreversibly inactivate proteins through the selective
625 nitration of Tyr residues (Vandelle and Delledonne, 2011). Lb was shown to scavenge
626 peroxynitrite, potentially precluding any damaging effect of this species in mature nodules
627 (Herold and Puppo, 2005), and in soybean nodules Lb was found to be target of nitration *in*
628 *vivo*, particularly during senescence (Navascues *et al.*, 2012). The induction of *Phytogb* genes
629 in senescent nodules could therefore be interpreted as a means of renewing the *Phytogbs/Lbs*
630 pool inactivated by the reactive nitrogen species in order to maintain the scavenging capacity
631 of the latter while maintaining the nitrogen fixing capacity of the nodules.

632

633 **7 – Future prospects**

634 Considered together, the data presented in this review show that NO is produced all along the
635 N_2 -fixing symbiosis, from the first hours of the interaction between the plant and the bacteria
636 to the breakdown of the association between the two partners. Its various spatial accumulation
637 during the time of the symbiotic interaction, i.e. in root surface cells, infection thread, nodule
638 cortex, N_2 -fixation zone, underlines the diversity of the physiological environments wherein it
639 is produced. Understanding the control mechanisms of how much, where and when NO is
640 produced is a challenging issue for the next years. Indeed, NO has been shown to modulate
641 the expression of many genes, including genes involved in defence response and in cell
642 division, during nodule development. NO production has been shown to be increased in
643 hypoxic nodules and this production was supposed to be linked, via a phytoglobin-NO
644 respiration process, with improved capacity of the nodules to maintain their energy status
645 under hypoxic conditions showing its role in nodule response to abiotic stresses. In the same
646 time, NO was shown to be a potent inhibitor of bacterial nitrogenase and of other plant and
647 bacterial enzymes involved in N_2 fixation. Other data suggest that NO involved in the
648 induction of nodule senescence. Hence, the question was raised of the toxic effects versus
649 signalling/metabolic functions of NO, and of the regulation of NO levels compatible with N_2 -
650 fixation. Regarding this point, the number of plant *Phytogbs* and of bacterial detoxication
651 systems (*Hmp*, *Nor*, *NnrS*) acting together to control NO level has been particularly
652 evidenced.

653 However, a number of points deserve to be investigated in the future. First, NO detection
654 techniques need to be refined. Regardless of the problem of quantification of NO, it would be
655 necessary to be able to precisely analyze NO production within the nodules without affecting
656 the nodule structure, nor to break the microoxia prevailing inside the fixation zone.
657 In N₂-fixing nodules, the two symbiotic partners produce NO. Another challenge will be, on
658 the one hand, to discriminate the origin of the NO between the plant and the bacterial partner,
659 and on the other hand, to understand how the NO produced by one partner regulates or
660 influences the signaling and the metabolism of the other partner during the interaction. The
661 use of bacterial mutant strains for NO synthesis and degradation pathways (denitrification
662 pathway, hmp, NnrS) has already shown that the bacterial partner can regulate the functioning
663 of the plant partner. The availability of legumes, particularly of Lotus and Medicago species,
664 either mutated on, or overexpressing, phytohemoglobins and NRs, should soon make it possible to
665 verify how the plant partner influences the behavior of the rhizobia in nodules. On the other
666 hand, if the diversity of NO production and degradation systems in both partners is becoming
667 clear, it remains to determine the spatial and temporal role of each of the actors in NO
668 regulation during the symbiotic process. Finally, regardless of the need to keep NO at a low
669 level to reduce its toxic effects, the question of the simultaneous roles of NO as both a
670 regulatory signal for gene expression, a modulator of protein activity and a metabolic
671 intermediate in maintaining the energetic state of nodules remains open: In what ways do
672 post-translational modifications of proteins mediate the NO signaling referral? How important
673 is the redox state of cells in this signaling? How are the different functions of the NO
674 performed in the same cell? From this point of view, deciphering the interactions between
675 NO, ROS and hormones remains a challenging issue for the future.

676

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683

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1043

1044 **Figures**

1045

1046 **Figure 1:** NO production sites at various steps of the legume-Rhizobium symbiosis, and in
1047 mature indeterminate and determinate nodules. During symbiosis establishment NO
1048 production (yellow stars) is increased at the root surface following inoculation with rhizobia,
1049 in shepherd's crook of root hair, infection thread and nodule primordia. In mature nodule, NO
1050 detection is associated with infection zone (Zone II), N₂-fixing zone (Zone III) and inter-zone
1051 III-IV of indeterminate nodules, and with N₂-fixing zone of determinate nodules. En,
1052 endodermis; Co, cortex; dpi, day post-inoculation; Ep, epidermis; Vb, vascular bundle; wpi,
1053 week post-inoculation.

1054

1055 **Figure 2:** NO production during the symbiotic process between *Medicago truncatula* and
1056 *Sinorhizobium meliloti*. The fluorescence intensity of the NO production was measured using
1057 the DAF-2 fluorescent probe. Data are adapted from Berger et al., 2018. Each NO production
1058 peak is related with a physiological stage of the symbiotic process.

1059

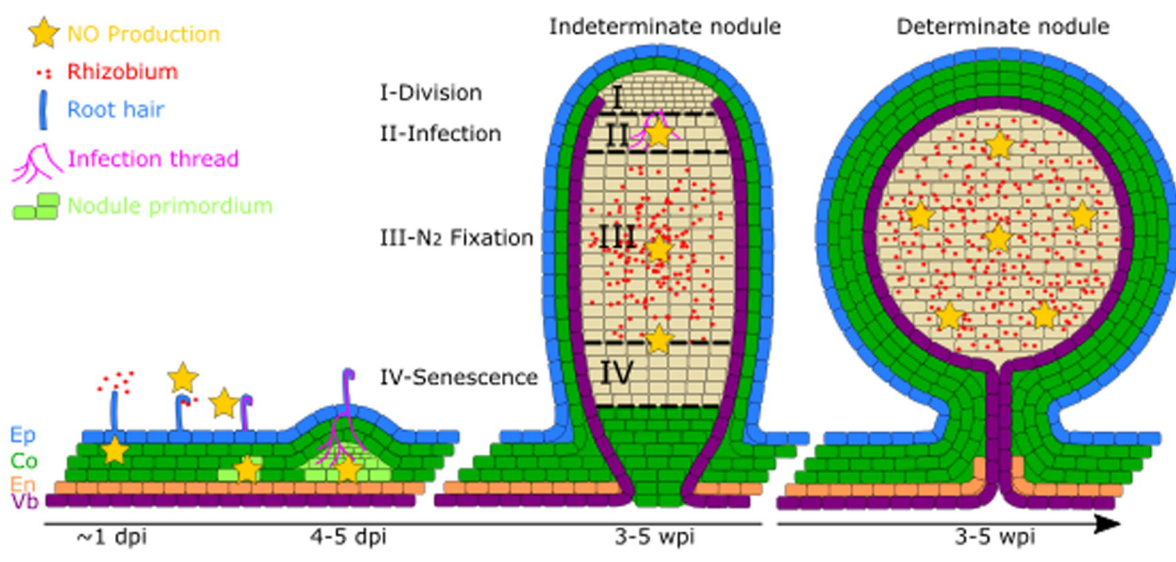
1060 **Figure 3:** Representation of the different NO production (A) and degradation (B) pathways in
1061 nodule. On the plant side NO may be produced by reductive pathways via the reduction of
1062 nitrite (NO_2^-) by nitrate reductase (NR), xanthine oxydoreductase (XOR), nitrite-NO
1063 reductase (NiNOR) and the mitochondrial electron transfert chain (ETC) at cytochrome
1064 oxydase (COX) and cytochrome bc1 level. Oxidative pathways involve potential NO
1065 production via polyamine oxydase (PA oxydase), nitric oxide synthase-like (NOS like) and
1066 hydroxylamine oxidase (HA oxydase). NO may be metabolized by complexing with
1067 superoxide anion ($\text{O}_2^{\cdot-}$) to form peroxyntirite (ONOO^-) or with oxygen (O_2) to produce N_2O_3
1068 and nitric oxides (NO_x). NO may either be oxidized in NO_3^- by phytooglobins (Pgb), or
1069 interact with glutathion (GSH) and proteins (RSH) to form respectively S-nitrosogluthation
1070 (GSNO) and S-nitrosylated proteins (RS-NO). GSNO is reduced by GSNO reductase
1071 (GSNOR) to form glutathion disulfide (GSSG) and ammoniac (NH_3). RS-NO is reduced bach
1072 to RSH through the action of thioredoxin reductase (TrxR) at the expense of reduced
1073 thioredoxin ($\text{Trx}(\text{SH})_2$). On the bacterial side, NO formation involves the denitrification
1074 pathway via nitrate reductase (NapA) and nitrite reductase (NirK), and hypothetically NOS-
1075 like enzymes. NO degration/detoxification occurs via either the denitrification pathway
1076 through nitric oxide reductase (NorC), the flavohemoglobin (Hmp), or the NnrS pathway.
1077 DH, dehydrogenase; Nos, nitrous oxide reductase; PBS peribacteroid space; MIS,
1078 mitochondrial intermembrane space; NAD(P)H DH, NAD(P)H dehydrogenase.

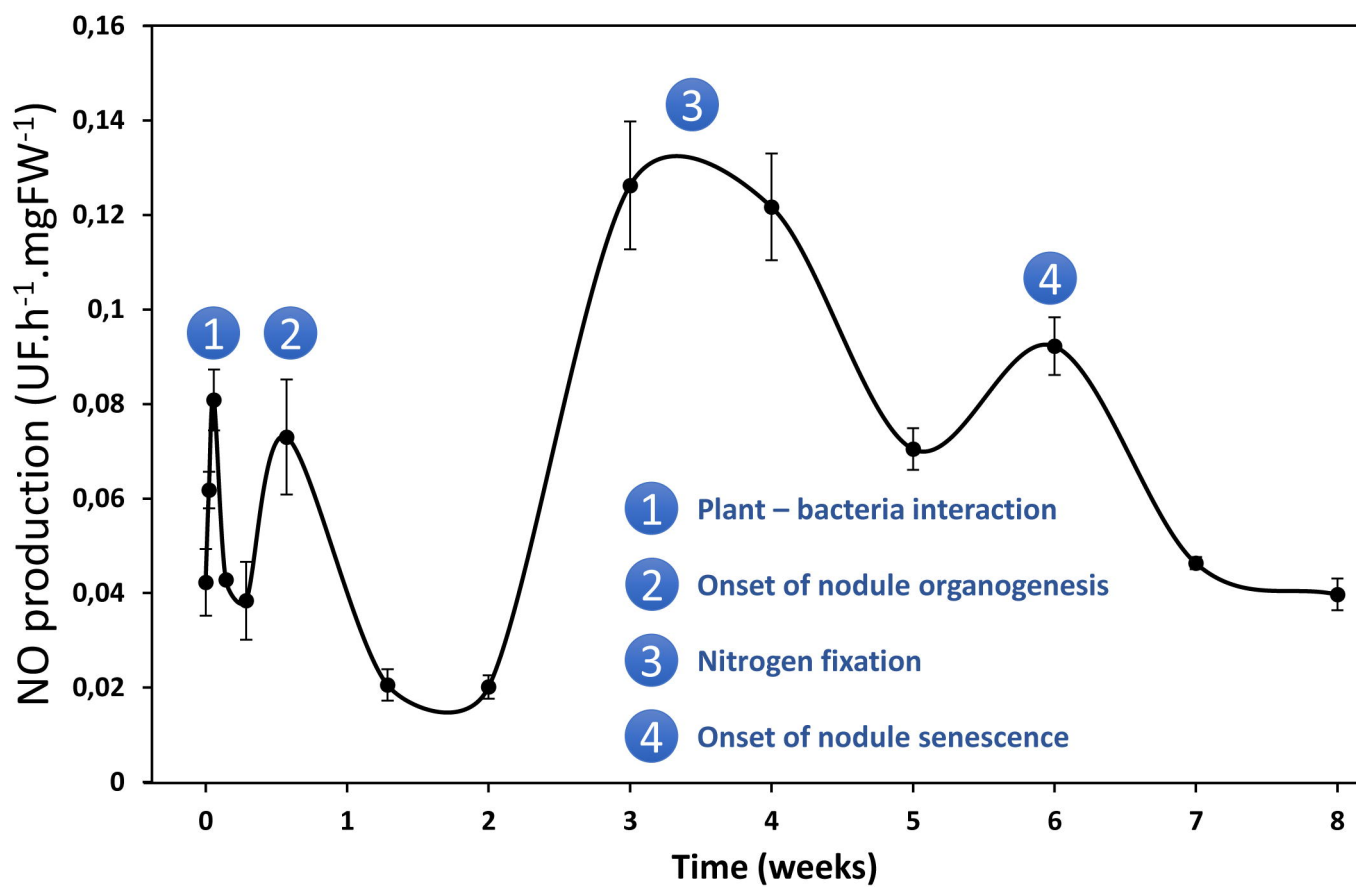
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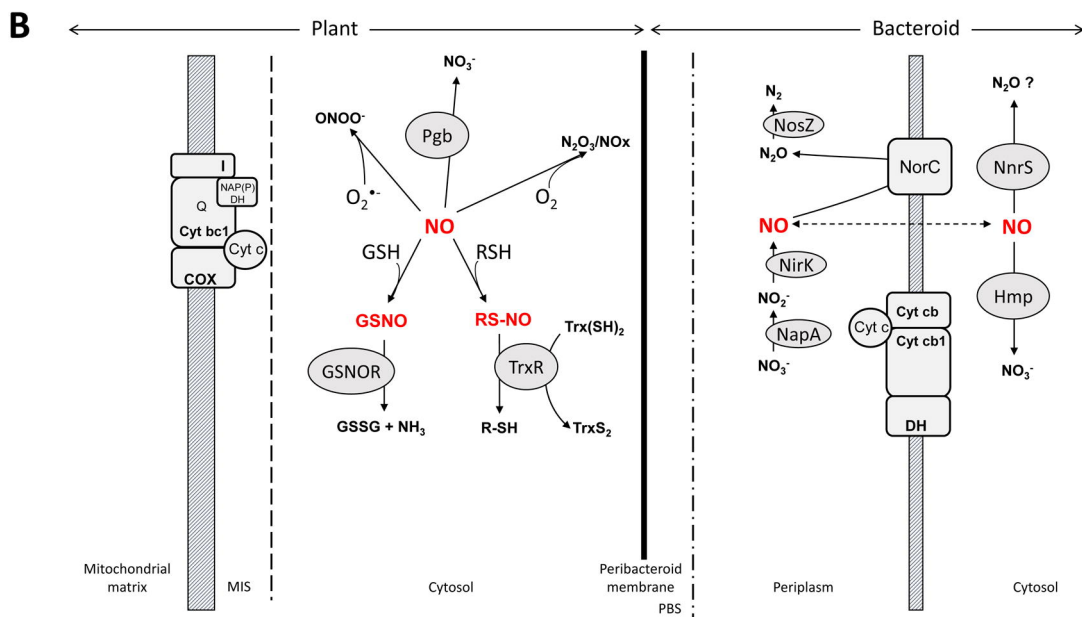
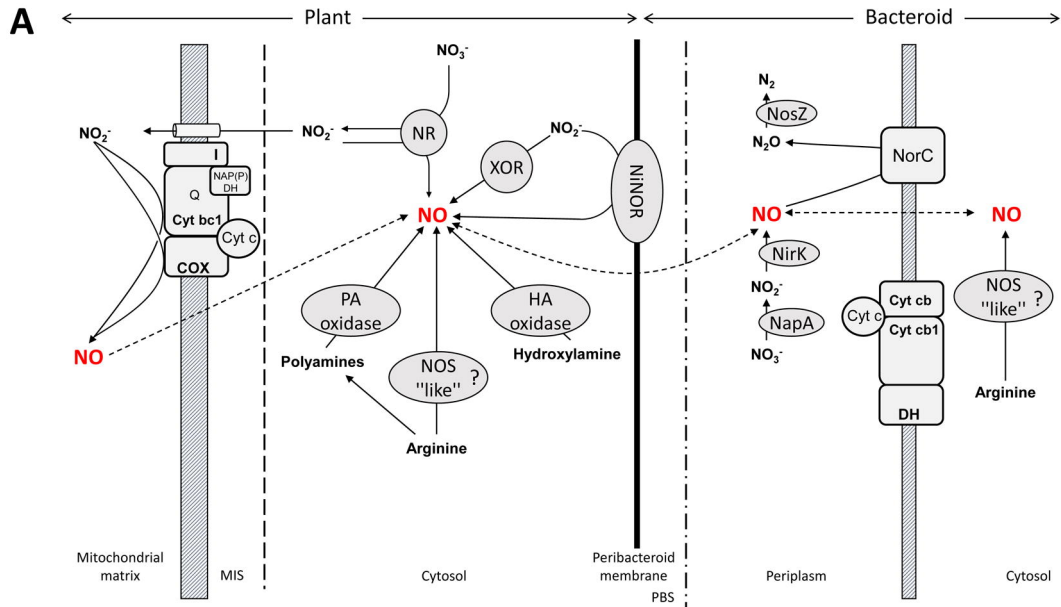
1080 **Figure 4:** Synoptic representation of NR and Phytoighb gene expression as a function of the
1081 symbiosis step. hpi; hour post-inoculation; dpi, day post-inoculation; wpi week post-
1082 inoculation. + and – indicate detectable or non-detectable gene expression in non-inoculated
1083 roots, respectively. Blue and red arrows indicate decreased and increased expression level,
1084 respectively. The width of the arrows symbolizes the extent of the changes observed.

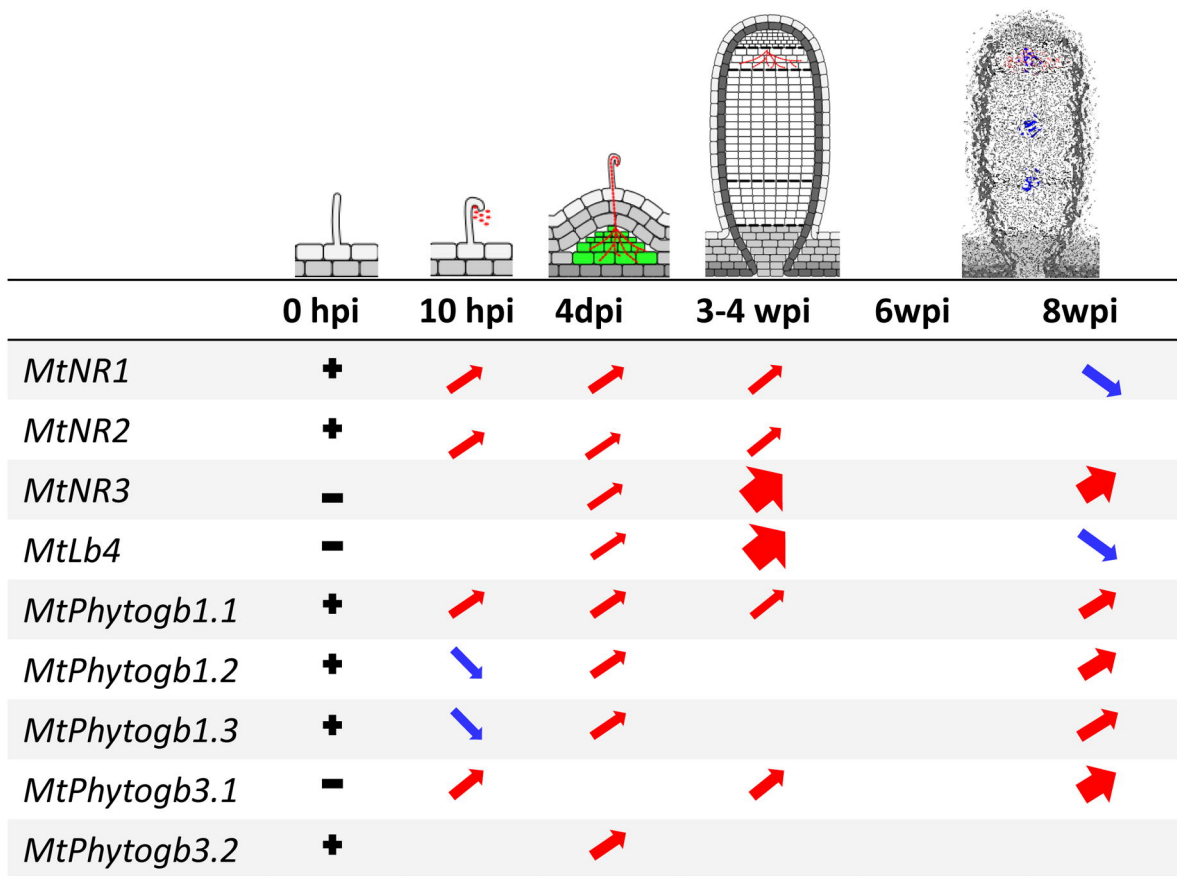
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Annexe 4:



Cross-Regulation between N Metabolism and Nitric Oxide (NO) Signaling during Plant Immunity

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Cross-Regulation between N Metabolism and Nitric Oxide (NO) Signaling during Plant Immunity

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Plants are sessile organisms that have evolved a complex immune system which helps them cope with pathogen attacks. However, the capacity of a plant to mobilize different defense responses is strongly affected by its physiological status. Nitrogen (N) is a major nutrient that can play an important role in plant immunity by increasing or decreasing plant resistance to pathogens. Although no general rule can be drawn about the effect of N availability and quality on the fate of plant/pathogen interactions, plants' capacity to acquire, assimilate, allocate N, and maintain amino acid homeostasis appears to partly mediate the effects of N on plant defense. Nitric oxide (NO), one of the products of N metabolism, plays an important role in plant immunity signaling. NO is generated in part through Nitrate Reductase (NR), a key enzyme involved in nitrate assimilation, and its production depends on levels of nitrate/nitrite, NR substrate/product, as well as on L-arginine and polyamine levels. Cross-regulation between NO signaling and N supply/metabolism has been evidenced. NO production can be affected by N supply, and conversely NO appears to regulate nitrate transport and assimilation. Based on this knowledge, we hypothesized that N availability partly controls plant resistance to pathogens by controlling NO homeostasis. Using the *Medicago truncatula/Aphanomyces euteiches* pathosystem, we showed that NO homeostasis is important for resistance to this oomycete and that N availability impacts NO homeostasis by affecting S-nitrosothiol (SNO) levels and S-nitrosoglutathione reductase activity in roots. These results could therefore explain the increased resistance we noted in N-deprived as compared to N-replete *M. truncatula* seedlings. They open onto new perspectives for the studies of N/plant defense interactions.

Keywords: nitrogen metabolism, plant immunity, *Aphanomyces euteiches*, *Medicago truncatula*, nitric oxide homeostasis

NITROGEN AND THE PLANT IMMUNE RESPONSE

Plants are under the constant threat of pathogen attacks that limit their survival and are major yield-limiting factors. In response to these attacks, plants activate multiple defense reactions both at the site of infection and systemically, which in many cases lead to resistance. These reactions include massive transcriptional reprogramming, cell wall reinforcement, synthesis of

antimicrobial metabolites, and production of pathogenesis-related (PR) proteins. These events are mediated by a variety of rapidly mobilized molecules, such as second messengers, e.g., Ca^{2+} , protein kinases, reactive oxygen species (ROS), or reactive nitrogen species (RNSs), including nitric oxide (NO). Although these defense responses have been widely studied, it has become increasingly obvious over the past years that a plant's capacity to mobilize them is greatly affected by its physiological status (Snoeijsers et al., 2000) and its development (Develey-Riviere and Galiana, 2007).

Nutrients are important for the growth and development of plants and microorganisms. Among them, nitrogen (N) can affect the fate of an interaction between a plant and a pathogen (Dordas, 2008). No general rules can be drawn about modification of resistance by N. Although we know that N lack or excess, along with the nature of available N in soil, can modulate plant resistance (Huber and Watson, 1974), the underlying mechanisms remain unclear. Recent works indicate that plants' capacity to acquire and assimilate N could partly explain nutrition effects on plant defense. N is taken up by the roots mostly in the form of nitrate (NO_3^-) in aerobic soils and ammonium (NH_4^+) in flooded wetlands or acidic soils. Ammonium taken up directly from the soil or resulting from the reduction of NO_3^- and nitrite (NO_2^-) by nitrate reductase (NR) and nitrite reductase (NiR), respectively, is assimilated *via* the glutamine synthetase (GS)/glutamate synthase cycle (Xu et al., 2012). The uptake of mineral N from the soil and the subsequent distribution to the whole plant is driven by nitrate transporters from the multigenic *NRT2* and *NPF* families and by ammonium transporters from the *AMT* family (Krapp, 2015). The contribution of several of these transporters to plant defense has recently been highlighted in *Arabidopsis thaliana*. For instance, induction of *AMT1.1* expression was evidenced upon infection by the bacterium *Pseudomonas syringae* or the fungus *Erysiphe cichoracearum* (Liu et al., 2010). The role of specific transporters was demonstrated using plant mutants: *nrt2* (deficient in the expression of the *NRT2.1* and *NRT2.2* genes) and *nrt2.6-1* mutants displayed altered sensitivity to the bacterial phytopathogens *P. syringae* and *E. amylovora* (Camanes et al., 2012; Dechorgnat et al., 2012). Besides N uptake into plants and its subsequent allocation, several results indicate that N assimilation and particularly amino acid homeostasis can impact plant-pathogen interactions (Zeier, 2013; Luna et al., 2014). Conversely, pathogen attacks are correlated with modulation of the expression of genes or of the activity of enzymes involved in N assimilation such as NR or GS2, in N remobilization such as GS1, and in amino acid metabolism [reviewed by Fagard et al. (2014)]. Whether these changes in N metabolism reflect the manipulation of host metabolism by the pathogen or result from the modulation of plant defenses is not always clear. Interestingly, some members of the GLR glutamate receptor family were recently proposed to play a role as amino acid sensors during plant defense, perhaps by sensing changes in extracellular amino acids caused by pathogen infection (Forde and Roberts, 2014). Crosstalk between N metabolism and phytohormones can also interfere with plant stress responses

and could be considered as a mechanism involved in the partitioning of available resources between defense and growth. For instance, N limitation induced the accumulation of salicylic acid (SA) in *A. thaliana* leaves (Yaeno and Iba, 2008). Conversely, ethylene/jasmonic acid signaling coordinated the upregulation of the nitrate transporter *NRT1.8* (*AtNPF7.2*) and the downregulation of *NRT1.5* (*AtNPF7.3*) genes to tune NO_3^- reallocation in plants from the shoot to the roots under stress conditions (Zhang et al., 2014). Finally, experiments on rice showed that N-induced susceptibility to *Magnaporthe oryzae* is genotype-dependent, and may be linked to N use efficiency (Ballini et al., 2013). These interesting data raise the question of the genetic control of N effects on plant immunity. The identification of the corresponding QTLs will permit to uncover new molecular actors of N-controlled resistance to pathogens.

NITRIC OXIDE AND N METABOLISM

The role of NO in plant defense is widely accepted. NO is involved in transcriptional regulation of defense genes encoding PR proteins or proteins involved in phytoalexin synthesis, SA accumulation, and post-translational protein modifications (Wendehenne et al., 2014). NO is a nitrogen species produced *via* a variety of pathways in plants (reviewed by Gupta et al., 2011c). Briefly, these pathways can be classified into two groups according to nitrogen-containing precursors: the L-arginine-dependent pathway (oxidative pathway), and the NO_2^- -dependent pathway (reductive pathway). NO_2^- -dependent NO synthesis involves NR which reduces NO_2^- to NO both *in vitro* and *in vivo* in specific physiological contexts (Yamasaki and Sakihama, 2000); alternatively, formation of NO through the reduction of NO_2^- by the mitochondrial respiratory chain can also be observed, particularly in roots (Gupta et al., 2011a; Horchani et al., 2011). Finally, NO can be produced by an apoplasmic non-enzymatic conversion of NO_2^- to NO at acidic pH, in the presence of reductants such as ascorbic acid (Bethke et al., 2004).

Several pathways involved in NO transformation and turnover and balancing the bioavailability of this molecule have been identified (Leitner et al., 2009). Firstly, NO can react with reduced glutathione to produce S-nitrosoglutathione (GSNO), a low-molecular-weight S-nitrosothiol (SNO) that is more stable than NO and considered to be a mobile reservoir of NO. The cellular level of GSNO is enzymatically regulated primarily by GSNO reductase (GSNOR), which catalyzes the reduction of GSNO to oxidized glutathione and ammonium. Importantly, Yun et al. (2016) recently reported that NO and GSNO have additive functions in plant immunity but also in plant development. NO and GSNO might have distinct or overlapping molecular targets, thus allowing differential control of key cellular processes belonging to both defense and development. Secondly, besides their O_2 binding properties, hemoglobins (Hbs) can metabolize NO into NO_3^- and therefore are also considered as NO and NO_2^- concentration modulators (Gupta et al., 2011b). Finally, NO rapidly reacts with superoxide (O_2^-) to

form peroxynitrite (ONOO^-), an oxidizing and nitrating RNS produced for instance in plant cells during immune responses (Vandelle and Delledonne, 2011). These molecules associated with NO turnover also play a role in the plant immune response. For instance, GSNO plays a key role in mediating the structural and functional changes of NPR1, a key transcription coactivator of plant immunity (Tada et al., 2008).

Nitric oxide is partly produced through NR, dependent on its substrate/product $\text{NO}_3^- / \text{NO}_2^-$ as well as on L-arginine and polyamines. As a result, cross-regulation between NO signaling and N supply/metabolism is expected. Several lines of evidence show that NO production is likely to be affected by N supply. In a physio-pathological context, plant NO production is dependent on the form of N supply. Besson-Bard et al. (2008) and Gupta et al. (2013) showed that tobacco cell suspensions or leaves from plants grown on ammonium instead of nitrate as an N source emitted less NO when elicited by cryptogeiin or *P. syringae*. Thus these data highlight the determining role of the N source on the rate of NO synthesis. Modifications of the intracellular concentration of diverse intermediates of N metabolism such as amino acids or polyamines also result in the modulation of NO production. For instance, exogenously added polyamines induced rapid NO biosynthesis in *A. thaliana* (Tun et al., 2006). In the same manner, overexpression of the Asparagine synthetase 1 gene significantly enhanced the NO burst (Hwang et al., 2011). Finally, N nutrition could also impact important redox molecules associated with NO homeostasis. Nitrate deprivation led to altered levels of ROSs in *A. thaliana* and tobacco (Schachtman and Shin, 2007; Besson-Bard et al., 2008). Pathogen-induced expression of the nitrate transporter *NRT2.6* was also correlated with ROS accumulation (Dechorgnat et al., 2012). Concentrations of antioxidant molecules such as glutathione (GSH) were altered (decreased in shoots and increased in roots) in *A. thaliana* and barley plants exposed to N deficiency (Kandlbinder et al., 2004; Kovacik et al., 2014).

Reciprocally, NO and derived RNS could participate in the regulation of N metabolism. NO can control physiological processes by modifying gene transcription. By analyzing available literature and databases, we identified interesting candidates likely to contribute to the crosstalk between N metabolism and NO among the numerous NO-regulated genes. Transcriptomic studies highlighted the up- or down-regulation of transcripts encoding N transporters (Ahlfors et al., 2009; Corti Monzon et al., 2014; Trevisan et al., 2015) or N assimilation/remobilization genes (Ferrarini et al., 2008; Ahlfors et al., 2009; Xu et al., 2013; Begara-Morales et al., 2014; Corti Monzon et al., 2014; Zeng et al., 2014; Trevisan et al., 2015) and amino acid metabolism-related genes (Ferrarini et al., 2008; Xu et al., 2013) upon modulation of NO homeostasis by treatment with NO donors, NO scavengers, or using mutants affected in NO homeostasis. Physiological studies identified NO as a regulator of N uptake in *Chlamydomonas reinhardtii*, possibly through the control of the expression of the nitrate or ammonium (*AMT1.1* and *AMT2.2*) transporters. In *A. thaliana*, the expression of the high affinity nitrate transporter *NRT2.1* was down-regulated by NO donors and in a *GSNOR* knock-out mutant, but the expression

of the low-affinity nitrate transporter *NRT1.1* remained unaltered (Frunghillo et al., 2014), suggesting a switch from high- to low-affinity nitrate transport. By contrast, the expression of *NRT2.1* was up-regulated through an NO-dependent process in *A. thaliana* roots exposed to cadmium (Besson-Bard et al., 2009). In addition to NO-mediated transcriptional regulation, many of NO biological functions arise as a direct consequence of chemical reactions between proteins and NO/RNS. Metal-nitrosylation, S-nitrosylation, and tyrosine nitration are notably emerging as main NO-dependent post-translational protein modifications (Astier and Lindermayr, 2012). Among the soluble proteins identified as S-nitrosylated or Tyr-nitrated, possible candidates contributing to the NO/N metabolism interplay are mainly involved in both N assimilation/remobilization and amino acid metabolism (Table 1). Post-translational inhibition of high-affinity ammonium and high-affinity $\text{NO}_3^- / \text{NO}_2^-$ transporters by NO was highlighted in *C. reinhardtii* (Sanz-Luque et al., 2013). However, whether the reversible effect of NO was linked to S-nitrosylation of the transporters or to an indirect effect of NO leading to other post-translational modifications of the transporters remains to be determined (Sanz-Luque et al., 2013). In that same study, NO also inhibited NR activity reversibly, but not NiR or GS activity. This post-translational effect of NO on N transporters and NR might mediate the fast inhibition of N uptake and assimilation by ammonium in *C. reinhardtii*. More recently, inhibition of NR activity by NO was proposed to be partly mediated by a truncated hemoglobin THB1 whose gene expression is highly induced by NO (Sanz-Luque et al., 2015).

In higher plants, NO produced by denitrification in the rhizosphere of forest soils impacts N uptake without affecting gene expression patterns of putative N transporters, suggesting post-translational modification of these transporters (Dong et al., 2015). NR is also highly regulated by complex transcriptional and post-translational mechanisms. Studies on different models using NO donors, NO synthase inhibitors, or the scavenger cPTIO indicate that NO modulates NR activity. Results are sometimes contradictory. NR activity in leaves was inhibited under high NO concentrations (Rosales et al., 2011, 2012; Frunghillo et al., 2014), but was enhanced in cabbage (Du et al., 2008). Moreover, the inhibition or activation of NR by NO in tomato roots could depend on the NO_3^- concentration (Jin et al., 2009). The mechanisms explaining these effects of NO on NR are poorly understood. Regulation of NR by NO could occur through transcriptional downregulation of the NR *NIA* genes in *Chlamydomonas* and *A. thaliana* (de Montaigu et al., 2010). A direct interaction of NO with NR is possible, as S-nitrosylation of NR was evidenced in poplar exposed to cold stress (Cheng et al., 2015). Glutamine synthetase 2 is a second key enzyme of plant N metabolism involved in the synthesis of essentially nitrogenous compounds *via* Gln production. Interestingly, GS1 and GS2 were identified as molecular targets of NO (Table 1). GS activity was inhibited by Tyr nitration in root nodules of *Medicago truncatula*. This post-translational modification may mediate channeling of glutamate to boost plant antioxidant defenses (Melo et al., 2011) in response to NO. This interesting feature does not seem to be shared across the plant kingdom

TABLE 1 | Examples of S-nitrosylated or Tyr-nitrated proteins involved in N and amino acid metabolism.

Functions	Post-translational modifications	Identified Proteins	Conditions	Reference
Amino acid metabolism	Tyrosine nitration	Methionine synthase	–	Lozano-Juste et al., 2011
		S-nitrosylation	Asparagine synthase 3	Biotic stress
	S-nitrosylation	Glutamate decarboxylase	Biotic stress	Maldonado-Alconada et al., 2011
		EPSP synthase	Biotic stress	Astier et al., 2012
		Acetohydroxy acid isomeroreductase (Val and Ile synthesis)	Biotic stress	Astier et al., 2012
		Aspartate aminotransferase	Biotic stress	Astier et al., 2012
		Cysteine synthase	Abiotic stress	Puyaubert et al., 2014
		Alanine glyoxylate aminotransferase	Abiotic stress	Puyaubert et al., 2014
		Glutamate glyoxylate aminotransferase	Abiotic stress	Puyaubert et al., 2014
		Glutamine synthetase 2	Biotic stress	Cecconi et al., 2009; Lozano-Juste et al., 2011
Nitrogen metabolism	Tyrosine nitration	Glutamine synthetase 1	Rhizobium-legume symbiosis	Melo et al., 2011
		S-nitrosylation	Argininosuccinate synthase	Biotic stress
	Nitrite reductase	<i>atgsnar1-3</i>		Hu et al., 2015
	Glutamate synthase	Abiotic stress	Puyaubert et al., 2014	
	Glutamate dehydrogenase 1	Biotic stress	Maldonado-Alconada et al., 2011	
	Glutamate dehydrogenase 2	Biotic stress	Maldonado-Alconada et al., 2011	

since GS activity was not affected by the NO donor DEA-NONOate in the alga *Chlamydomonas* (Sanz-Luque et al., 2013).

ROLE OF NO/RNS IN THE MODULATION OF THE IMMUNE RESPONSE BY N NUTRITION: FIRST EXPERIMENTAL EVIDENCE

Altogether, these data indicate that N supply has an impact on plant immunity and NO/RNS signaling and lead us to wonder about the role of NO/RNS in the modulation of the immune response by N nutrition. In the present work, we used an *in vitro* pathosystem composed of the legume *M. truncatula* challenged with the soil-borne root pathogen *Aphanomyces euteiches*. This oomycete is considered as the most limiting factor for legume production. Resistance of *M. truncatula* roots includes protection of the central cylinder against pathogen invasion, associated with frequent pericycle cell divisions, lignin deposition, and accumulation of soluble phenolic compounds (Djébalí et al., 2009). First investigations of the biochemical processes underlying the expression of this resistance showed modulation of H₂O₂ levels and of the activity of antioxidant enzymes (Djébalí et al., 2009, 2011). Interestingly, in the *M. truncatula* A17 genotype, resistance against *A. euteiches* was significantly enhanced in response to NO₃⁻ starvation as compared to sufficient N conditions (Thalineau et al., unpublished). Based on the current literature, we hypothesized that NO could play a role in this N-induced modulation of *M. truncatula* defense responses against *A. euteiches*. We therefore first assessed whether changes in NO homeostasis could

indeed affect *M. truncatula* resistance to *A. euteiches*. Secondly, we determined whether NO homeostasis could be modulated by N nutrition during the *M. truncatula*-*A. euteiches* interaction. We considered NO homeostasis as the maintenance of a functional NO concentration in a specific condition, through a balance between its biosynthesis (e.g., NR activity) and turnover pathways (e.g., interactions with GSH or O₂^{·-} to form GSNO or ONOO⁻, respectively).

MATERIALS AND METHODS

Plant Growth and Inoculation by *A. euteiches*

We used the *M. truncatula* Jemalong-A17 genotype. *M. truncatula* seeds were scarified according to Djébalí et al. (2009). After stratification overnight at 4°C, they were germinated in phytochambers with 16 h light under 350 μmol m⁻² s⁻¹ photons at 23°C / 8 h night at 21°C. One day after germination, the seedlings were transferred to 12 cm × 12 cm square Petri dishes containing modified M medium (Bécard and Fortin, 1988). This modified medium was sugar-free, enriched in phosphate (1.3 mM final concentration), and contained either 3.2 mM nitrate (complete medium) or no nitrate (NØ medium). The Petri dishes were sealed with parafilm and the roots were protected from light with aluminum foil, and then placed vertically in the culture chamber (16 h light under 350 μmol m⁻² s⁻¹ photons at 23°C/8 h night at 21°C) for 7 days. The strain *Aphanomyces euteiches* Drechs ATCC 201684 was used to inoculate the seeds one day after germination. Zoospores were produced as described in Rey et al. (2013), and each root was inoculated with 500 zoospores.

Agrobacterium rhizogenes Root Transformation

The pENTR4 vector carrying the MtNR1 or the MtNR2 fragment (Horchani et al., 2011) was recombined with the pK7GWIWG2d vector using LR clonase II enzyme mix (Invitrogen, France) to create RNA interference expression vectors. The MtGSNOR gene (*M. truncatula* Gene code Medtr7g099040) (1,143 bp) was amplified using *M. truncatula* cDNA as a template and the specific primers GSNOR-F 5'-AAAAGCAGGCTTCACCATGGCATCGTCGACTGAAGGT-3' and GSNOR-R 5'-AGAAAAGCTGGGTGTCAATGCAATGCAAGCACAC containing the corresponding attB recombination sites. The PCR product was recombined into the pDONR entry vector (Invitrogen) and checked by sequencing. The pDONR vector carrying the MtGSNOR gene was recombined with pK7WG2d plasmids¹ to create the overexpression vector. The constructs pK7GWIGW2d-MtNR1-2/GFP (RNAi::MtNIA1/2) and pK7WG2d-MtGSNOR/GFP (35S::GSNOR) were introduced into *A. rhizogenes* strain Arqual (Quandt et al., 1993). *M. truncatula* plants were transformed with *A. rhizogenes* according to Boisson-Dernier et al. (2001). Control plants were transformed with *A. rhizogenes* containing the pK7GWIGW5D or the pK7WG2d empty vectors. Hairy roots were selected based on the fluorescent marker GFP 21 days after transformation.

RNA Extraction, Reverse Transcription, and Quantitative PCR on Transformed Roots

Total RNA was extracted from transformed roots using TRIzol[®] Reagent (Life Technologies) according to the manufacturer's recommendations. To carry out the qPCR reaction, RNAs (0.5–1 µg) were reverse-transcribed in a final volume of 20 µL in the presence of RNasin (Promega, Charbonnières, France), and oligo(dT)₁₅, with M-MLV reverse transcriptase (Promega, Charbonnières, France), as recommended by the manufacturer.

Quantitative PCR was performed on reverse-transcribed RNAs from four independent biological replicates per condition and from two independent plant cultures. Quantitative PCR reactions were performed in an ABI PRISM 7900 sequence detection system (Applied Biosystems[®], Saint-Aubin, France), in a final volume of 15 µL containing Absolute SYBR green ROX Mix (Thermo Scientific, Surrey, UK), 0.3 µM of gene-specific primers, and 5 µL of cDNA template diluted 60-fold. The reference gene used for normalization was *MtEF1α*. Relative expression was expressed as $2^{-\Delta Ct \text{ test genes} - \text{reference gene}}$. The primers used for the qPCR all displayed a high amplification efficiency (90–100%). They were the following:

MtGSNORforward 5'-GTGACTGGGCGTGTATGGAA-3'
 MtGSNORreverse 5'-TGCAAGCACACAACGAAGAC-3'
 MtNIA1forward 5'-TGTTCCACAGGCTTCTCCAGATCA-3'
 MtNIA1reverse 5'-CATACAGCGTCTACTCAGCGACA-3'

MtNIA2forward 5'GCAAACCGGACGGAGGATGA-3'
 MtNIA2reverse 5'CCGTGATGAATCCCACACTATATTC-3'
 MtEF1αforward 5'-AAGCTAGGAGGTATTGACAAG-3'
 MtEF1αreverse 5'-ACTGTGCAGTAGTACTTGGTG-3'

Inoculation of Transformed Root Cultures with *A. euteiches*

Roots were cultured on Shb10 medium (Boisson-Dernier et al., 2001) and transferred on modified Fahraeus medium enriched in ammonitrate (1 mM NH₄NO₃ final) one day before inoculation. Inoculation of the root cultures with *A. euteiches* strain ATCC 201684 was carried out by adding 10 mL of an *A. euteiches* zoospore suspension containing 80,000 zoospores.mL⁻¹ in sterilized Volvic (Colditz et al., 2007) water. Zoospore production was initiated as described in Rey et al. (2013). Control root cultures were inoculated with 10 mL of sterile Volvic water. After 4 h of incubation in the dark, the zoospore solution was drained off the roots, and the Petri dishes were placed back into the growth room and left there for 7 days in the dark.

Assessing Infection Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Assessment of *A. euteiches* development in roots was performed by ELISA, using rabbit polyclonal serum raised against *A. euteiches*, and a mouse anti-rabbit IgG alkaline phosphatase conjugate as described by Slezack et al. (1999), on protein extracts from roots from pooled plants. Alkaline phosphatase activity was monitored by recording the increase in absorbance at 405 nm for 2–3 h, and was expressed as the slope of the resulting curve per mg of root fresh weight.

Hydrogen Peroxide Quantification

H₂O₂ concentration was measured using an Amplex Red[®]/peroxidase-coupled fluorescence assay adapted from Ashtamker et al. (2007). Roots were ground on ice and in the dark, in 1 mL of KRPG buffer (145 mM NaCl; 5.7 mM K₂HPO₄; 4.86 mM KCl; 0.54 mM CaCl₂; 1.22 mM MgSO₄; 5.5 mM glucose; pH 7.35) with 10 µM Amplex Red[®] and 0.2 U/mL of Horse Radish Peroxidase (HRP) per 100 mg of fresh weight. Catalase, an H₂O₂ scavenger, was used as a control. After 10 min of incubation at 4°C with catalase (1 unit/µL), 10 µM Amplex Red[®] and 0.2 U/mL of HRP were added to the samples. After centrifugation (10,000×g, 15 min, 4°C), 100 µL of supernatant were used to quantify resorufin (λ_{ex} = 560 nm; λ_{em} = 584 nm) by spectrofluorimetry (Mithras, Berthold Technology). The relative fluorescence units were converted into µmol of H₂O₂ mg⁻¹ root fresh weight on the basis of a standard curve established from known concentrations of H₂O₂.

Nitric Oxide and Peroxynitrite Quantification

ONOO⁻ and NO concentrations were determined using A17 or transformed roots ground on ice and in the dark, with 1 mL of Tris-HCl (10 mM, pH 7.5), KCl (10 mM) buffer

¹<https://gateway.psb.ugent.be/>

with 5 μM aminophenyl fluorescein (APF) or 10 μM 4,5-diaminofluorescein (DAF), respectively, per 100 mg of fresh weight. Epicatechin, an ONOO⁻ scavenger, was used as a control. After 10 min of incubation at 4°C with epicatechin (1 mM), APF was added to the samples at a final concentration of 5 μM . cPTIO, an NO scavenger, was used as a control. After 10 min of incubation at 4°C with cPTIO (500 μM), DAF was added to the samples at a final concentration of 10 μM .

After centrifugation (10,000 \times g, 15 min, 4°C), 100 μL of supernatant were used to quantify ONOO⁻ or NO ($\lambda_{\text{ex}} = 485 \text{ nm}$; $\lambda_{\text{em}} = 535 \text{ nm}$) by spectrofluorimetry (Mithras, Berthold Technology).

S-nitrosothiol Quantification

S-nitrosothiol quantification was performed using the Saville–Griess assay (Gow et al., 2007). A17 roots or transformed roots were ground, on ice and in the dark, in extraction buffer (1 mL/100 mg of fresh weight, 0.1 M Tris-HCl, pH 7.5; 1 mM PMSF). After centrifugation (10,000 \times g, 15 min, 4°C), 100 μL of supernatant were incubated with 100 μL of buffer A (0.5 M HCl; 1% sulfanilamide) or 100 μL of buffer B (0.5 M HCl; 1% sulfanilamide; 0.2% HgCl₂). After incubation (15 min at room temperature), 100 μL of Griess reagent[(0.5 M HCl; 0.02% N-(1-naphthyl)-ethylenediamine dihydrochloride)] were added. After 15 min, SNOs were quantified by measuring absorbance at 540 nm. A standard curve was obtained using different concentrations of GSNO.

Nitrate Determination

Nitrate determination was performed according to Miranda et al. (2001), based on the reduction of nitrate to nitrite by vanadium and colorimetric detection at 540 nm of nitrite in the presence of sulfanilamide and N-1-naphthylethylenediamine. Approximately 100 mg of 7-day-old plant roots were collected, flash-frozen in liquid N₂, and ground into powder. Three hundred micro liter of ultra-pure water were added to 20 mg of frozen sample, thoroughly vortexed, and incubated with occasional mixing for 15 min on ice. After centrifugation 15 min at 13,000 \times g and 4°C, the supernatant was recovered and used for nitrate determination.

Nitrate Reductase Activity Measurements

Transformed root samples were frozen in liquid nitrogen and ground using pestle and mortar. Extraction was performed in MOPS buffer (1 mL per 100 mg of fresh weight, 50 mM MOPS-KOH buffer, pH 7.6; 5 mM NaF; 1 μM Na₂MoO₄; 10 μM FAD; 1 μM leupeptin, 0.2 g/g FW polyvinylpyrrolidone; 2 mM β -mercaptoethanol; 5 mM EDTA). After centrifugation (20,000 \times g, 5 min, 4°C), the supernatant was used to measure NR activity. The reaction mixture consisted of 50 mM MOPS-KOH buffer, pH 7.6, containing 1 mM NaF, 10 mM KNO₃, 0.17 mM NADH, and 5 mM EDTA. After incubation 15 min at 30°C, the reaction mixture was stopped by adding an equal volume of sulfanilamide (1% w/v in 3 N HCl) followed by N-naphthylethylenediamine dihydrochloride (0.02%, w/v), and

the A₅₄₀ was measured. A standard curve was obtained based on different concentrations of nitrite.

GSNOR Activity Measurements

To measure GSNOR activity, roots were ground in liquid nitrogen and proteins were extracted in 50 mM Tris-HCl buffer, pH 8, 0.5 mM EDTA, and 1 mg/mL of a protease inhibitor cocktail (1 mL of buffer per 100 mg of fresh weight). GSNOR activity was assayed from the rate of NADH oxidation by measuring the decrease in absorbance at 340 nm at 25°C using 25 μg of proteins in a total volume of 200 μL of extraction buffer containing 350 μM NADH with or without 350 μM GSNO. GSNO reductase activity was determined by subtracting NADH oxidation values in the absence of GSNO from values in the presence of GSNO. All samples were protected from light during the assay and tested for linearity. A standard curve was obtained using different concentrations of NADH.

Statistical Analyses

Statistical analyses were performed using one- or two-way analysis of variance (ANOVA) followed by Fisher's test. Data were considered as significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

NO Homeostasis Participates in the *M. truncatula* Immune Response

To investigate the putative role of NO homeostasis in the *M. truncatula*/*A. euteiches* interaction, roots were transformed to inactivate the NR-encoding *MtNIA1/2* genes or to overexpress GSNOR-encoding genes. Quantification of gene transcripts in transformed roots using RT-qPCR confirmed that the two *NIA* genes were repressed (**Figure 1A**) while GSNOR was overexpressed (**Figure 1B**). To perform functional validation of the different constructs, we quantified NO and SNO levels in transformed roots. The two genetic manipulations modulated NO or SNO levels (**Figure 1**). SNO levels remained unchanged in *RNAi::MtNIA1/2* roots as compared to the controls, whereas NO levels clearly decreased (**Figure 1A**). This was in accordance with the downregulation of NR, a major enzymatic source of NO. Conversely, NO levels in the *35S::GSNOR* roots did not significantly change, but SNO significantly increased as compared to control roots (**Figure 1B**). This was surprising because in most previous experiments a negative correlation was described between SNO levels and GSNOR activity (Feechan et al., 2005; Rusterucci et al., 2007; Yun et al., 2011). However, it is interesting to note that in pea (a legume closely related to *M. truncatula*), higher SNO levels induced by wounding were correlated with higher GSNOR activity (Corpas et al., 2008).

We studied the impact of these genetic transformations on the *M. truncatula*/*A. euteiches* interaction. ELISA tests using antibodies raised against *A. euteiches* (Slezack et al., 1999) were performed to quantify the presence of the pathogen in roots. In *RNAi::MtNIA1/2* roots (**Figure 2A**), *A. euteiches* colonization was significantly greater than in control transformed roots

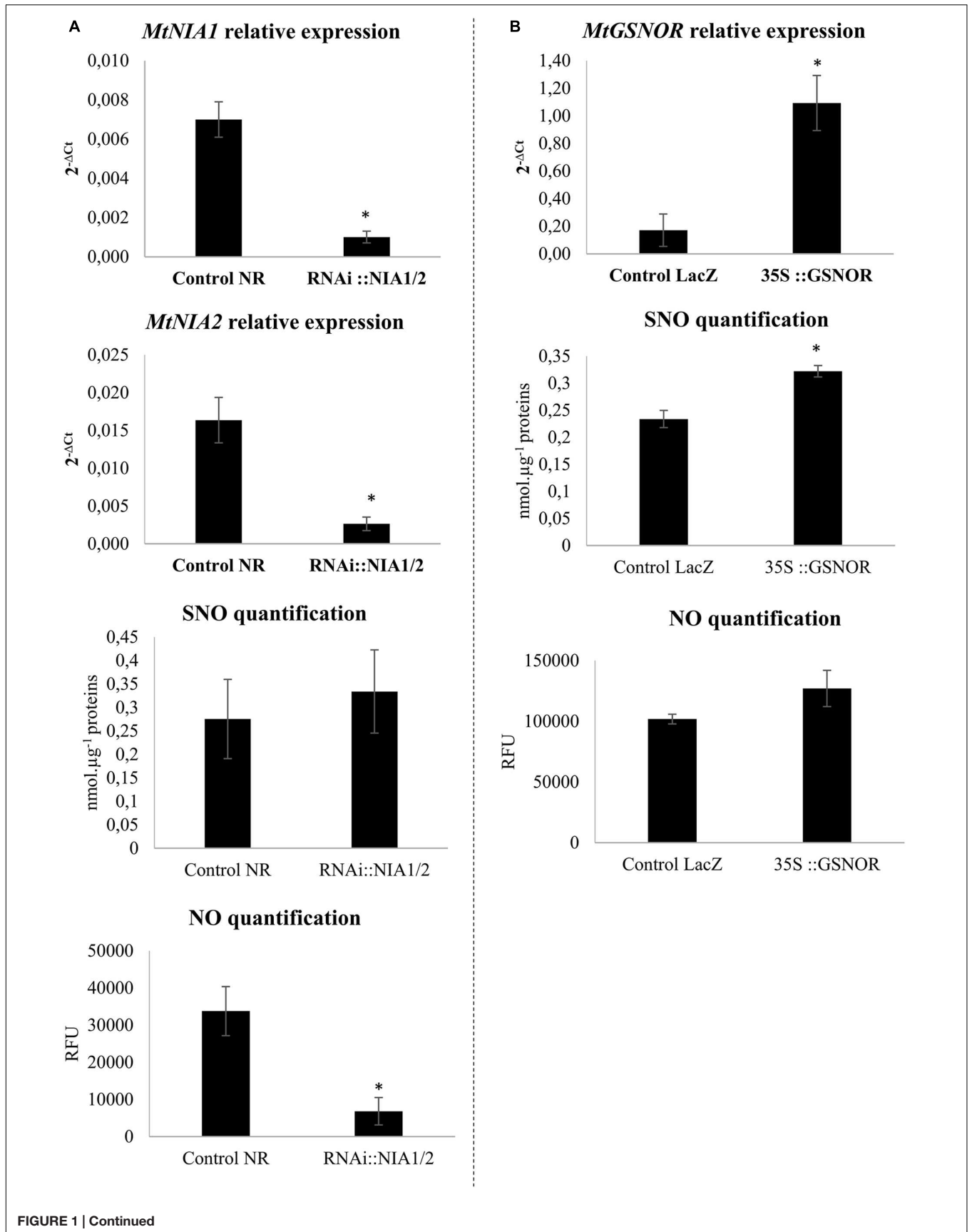
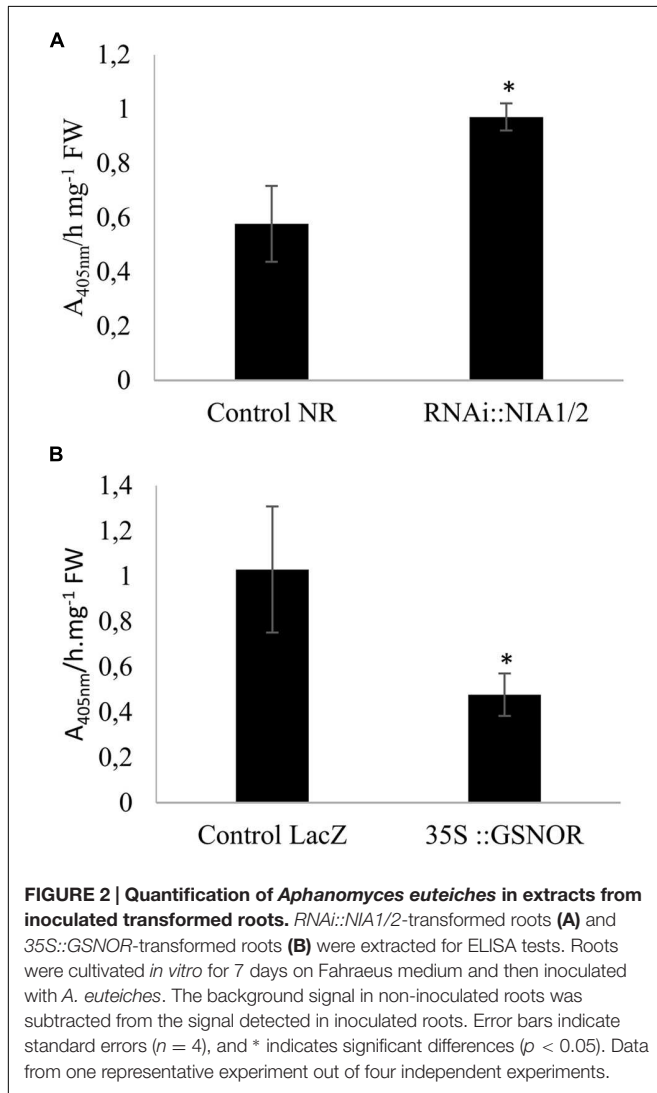


FIGURE 1 | Continued

Transformed root validation. (A) Transcript levels of *MtNIA1* and *MtNIA2* in *RNAi::NIA1/2*-transformed roots were compared to control transformed roots (control NR). SNO quantification using the Saville–Griess assay and NO quantification using the fluorophore DAF (10 μ M). Control NR and *RNAi::NIA1/2*-transformed roots extracts were pre-incubated or not with 500 μ M cPTIO as an NO scavenger. **(B)** Transcript levels of *MtGSNOR* in *35S::GSNOR*-transformed roots were compared to control transformed roots (control LacZ). SNO quantification using the Saville–Griess assay and NO quantification using the fluorophore DAF (10 μ M). Control LacZ and *35S::GSNOR*-transformed roots extracts were pre-incubated or not with 500 μ M cPTIO as an NO scavenger. Error bars indicate standard errors ($n = 4$ for transcripts and NO levels; $n = 8$ for SNO levels), and * indicates significant differences ($p < 0.05$).

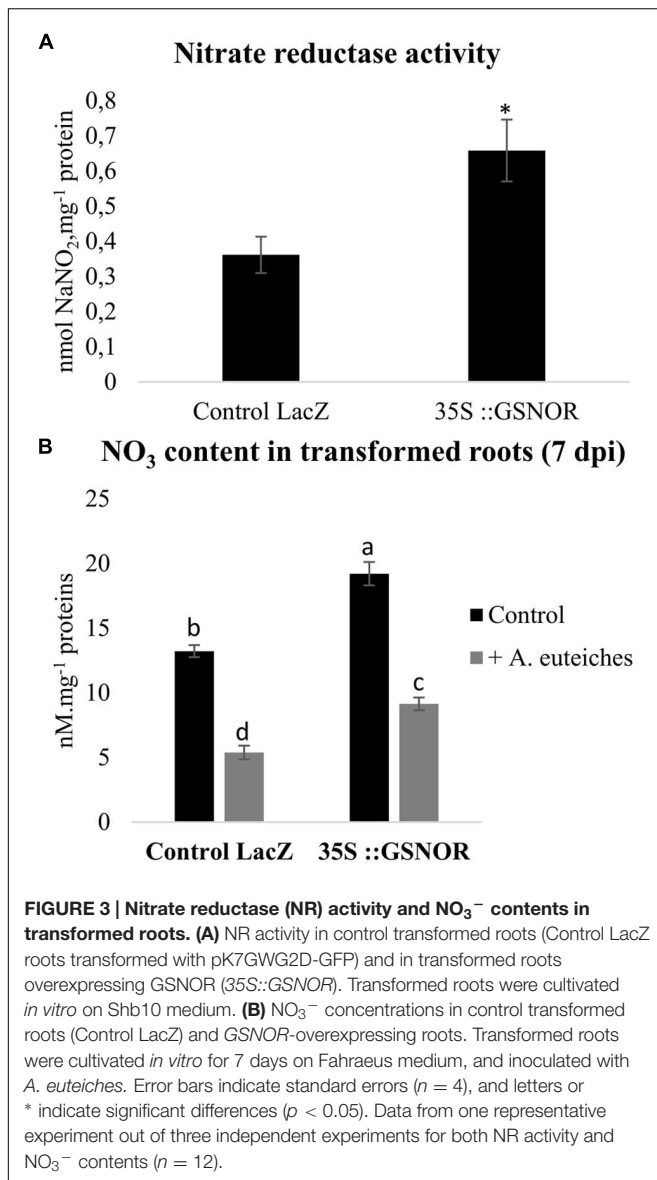


(Control NR roots). These data reaffirm the role of the NR enzyme in the plant immune response. In *A. thaliana*, the NR-deficient double mutant (*nia1 nia2*) failed to exhibit a hypersensitive response and was hyper-susceptible to *P. syringae* (Modolo et al., 2006; Oliveira et al., 2009) and to the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* or *Botrytis cinerea* (Perchepied et al., 2010; Rasul et al., 2012). Although these effects were attributed to the substantially reduced NO levels in this mutant, a side effect of N metabolism on plant defense cannot be excluded as NR stands at the crossroads between N metabolism and NO production.

Our results using *GSNOR*-transformed roots showed that pathogen levels were lower in *GSNOR*-overexpressing roots (Figure 2B) than in control transformed roots (Control LacZ roots). *GSNOR* could therefore be considered as a positive regulator of *M. truncatula* resistance to *A. euteiches*. Previous works already investigated the physiological roles of *GSNOR* in plant-pathogen interactions, using transgenic *A. thaliana* plants (Feechan et al., 2005; Rusterucci et al., 2007; Yun et al., 2011). Results are sometimes contradictory, as modulation of *AtGSNOR* expression enhanced or decreased plant disease resistance depending on the pathosystem. *GSNOR* could play a significant role in plant immunity because GSNO is considered as a mobile reservoir of NO, is more stable than NO, and is a transnitrosylation agent of proteins. The contrasted results obtained in our study with NR and *GSNOR* constructs could be attributed to the specific roles of the corresponding proteins in NO homeostasis. NR is involved in NO synthesis, whereas the primary role of *GSNOR* is to regulate GSNO contents. The recent results from Yun et al. (2016) confirm that GSNO and NO may play distinct roles in plant immunity by acting on different molecular targets. In addition, *GSNOR* indirectly affects NO, GSH, ROS, and total intracellular nitrosothiol (SNO) levels, indicating that *GSNOR* might be more globally involved in the regulation of the cell redox state (Espunya et al., 2006; Yun et al., 2011).

Nitric Oxide partly regulates N metabolism. Therefore we also investigated the effects of *GSNOR* overexpression on root NO_3^- contents and NR activity in transformed roots. *GSNOR* overexpression increased basal NO_3^- content and NR activity (Figures 3A,B). Modulation of N metabolism by GSNO and NO in *A. thaliana* has been described (Frunghillo et al., 2014), and was explained by the effect of NO and GSNO on NR activity and on the expression of the *AtNRT2.1* high-affinity NO_3^- transporter gene. Similarly to our data, that study shows that *GSNOR* overexpression is correlated with higher NR activity and NO_3^- content. Interestingly, we noted that pathogen colonization reduced NO_3^- concentrations in roots by approximately 65%, suggesting an effect of *A. euteiches* on nitrate transport and/or NO_3^- assimilation. Although we found a higher NO_3^- content in *35S::GSNOR*-infected roots than in control infected roots, the amplitude of the pathogen-induced decrease in NO_3^- level was not impacted in *35S::GSNOR* roots, suggesting that this process is independent of GSNO homeostasis. This reduced level of NO_3^- is unlikely to result from consumption of NO_3^- by the pathogen: data mining of the *A. euteiches* database revealed that no homologs of the NR, NIR, and NO_3^- transporter (NRT2) genes were detected in the genome of this pathogen², confirming

²<http://www.polebio.lrsv.ups-tlse.fr/aphano/>



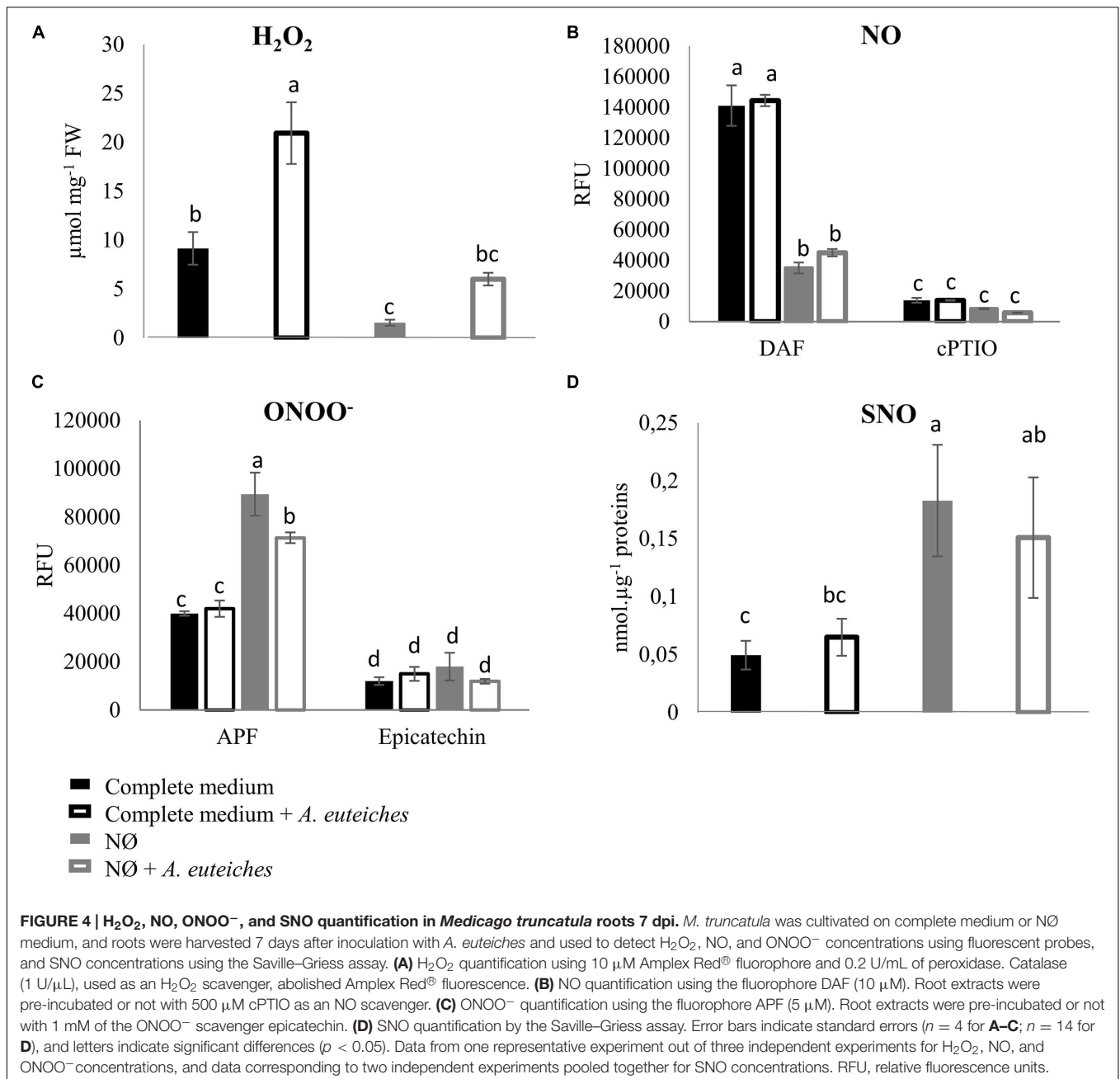
earlier observations that NO₃⁻ is unfavorable for *A. euteiches* development (Huber and Watson, 1974). Alternatively, we cannot exclude that the decreased NO₃⁻ content in infected roots could be due to nitrate leakage from the roots related to developing necrosis.

Effect of N Nutrition on NO/H₂O₂/ONOO⁻ Accumulation and SNO Contents

To analyze the role of N availability on NO, H₂O₂, and ONOO⁻ accumulation, *M. truncatula* plants were cultivated in complete medium or NO₃⁻-deficient medium (NØ), and inoculated or not with *A. euteiches*. The NO scavenger cPTIO and the ONOO⁻ scavenger epicatechin were used as controls to check the specificity of the fluorescence probes. We observed that NO₃⁻ deficiency caused a significant increase in ONOO⁻ content

on NØ medium (Figure 4C), whereas NO and H₂O₂ levels decreased (Figures 4A,B), highlighting a link between NO₃⁻ content and production of these reactive species. A clear effect of pathogen colonization was only evidenced for H₂O₂ contents (Figure 4A), and this increase was abolished on NØ. Surprisingly, although NO production is considered as a common response to pathogens, no increase in NO levels was detected in response to *A. euteiches* (Figure 4B). More generally, whereas NO, ROS, or ONOO⁻ production has been widely described in response to pathogens, the literature does not give a clear picture of the cross-talks between these molecules. For instance, we observed a negative correlation between NO and ONOO⁻ contents in response to NO₃⁻ deficiency, but in other models high NO levels are often correlated with high ONOO⁻ levels (Abramowski et al., 2015; Kulik et al., 2015). These conflicting observations raise some questions. Are these discrepancies due to plant models or due to the difficulty in measuring and precisely localizing these molecules? Differences in the stability of these molecules or their specific scavenging by plants during pathogen attack could explain why we did not detect changes in ONOO⁻ or NO levels in response to *A. euteiches*. Moreover, NO could also be used by the pathogen to activate its own metabolism, an important step in plant infection by fungi (Sedlářová et al., 2016).

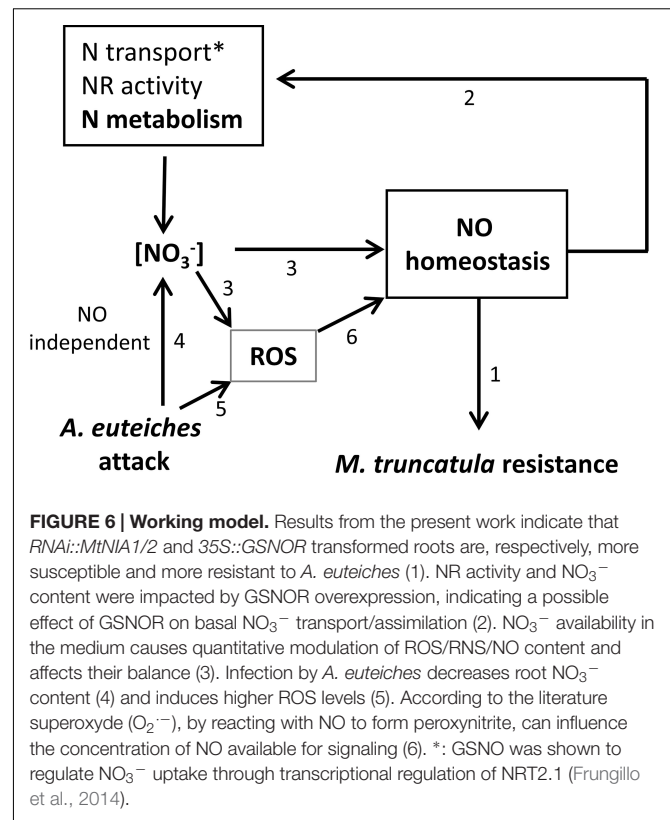
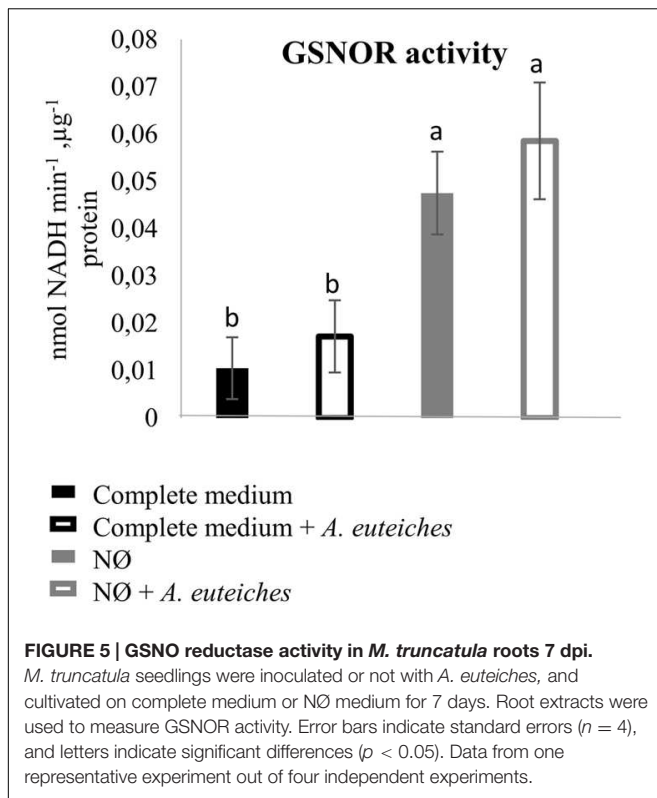
We also measured root SNO levels and GSNOR activity in the biological conditions of interest. Root SNO contents, determined using the Saville–Griess method, significantly increased on NØ medium as compared to the complete medium (Figure 4D). In response to *A. euteiches*, no significant change in SNO levels was highlighted (Figure 4D). Therefore, on NØ medium, the SNO content evolved in an opposite way to the NO content, similarly to the ONOO⁻ content. This result is in accordance with results reported in *Helianthus annuus* (Chaki et al., 2011), and can be attributed to the fact that NO is the source for ONOO⁻ and SNO. By contrast, a high NO content can be correlated with a high SNO content when plants are grown on culture medium containing NO₃⁻ (Abramowski et al., 2015; Pietrowska et al., 2015). Our data also suggest that NO₃⁻ nutrition impacts the overall balance between NO, ONOO⁻, and SNO. Regarding GSNOR, no changes in its activity was detected upon inoculation, in line with the absence of change in SNO levels in infected roots. In the roots of plants cultivated on NØ (Figure 5), higher GSNOR activity was correlated with higher SNO levels, confirming the positive correlation between GSNOR activity and SNO levels observed in 35S::GSNOR-transformed *Medicago* roots (Figure 1) and in pea, a closely related legume (Corpas et al., 2008). The positive or negative correlation between GSNOR activity and SNO levels or between NO and SNO levels depending on plant species and experimental conditions can be explained by several hypotheses. The SNO level is regulated through nitrosylation and denitrosylation; GSNOR, by controlling the level of GSNO, indirectly affects the level of S-nitrosylation. However, the TRX (thioredoxin)/NTR (NADPH-dependent TRX reductase) enzymatic system also controls S-nitrosylation (Kneeshaw et al., 2014). Interestingly, these activities were also identified in roots and activated by NO, leading to denitrosylation of specific proteins (Correa-Aragunde et al., 2015). Thus, these results, together with our study, illustrate the complex relationships



between NO production/GSNOR activity and total SNO levels. Abiotic stresses also increase GSNOR activity (Kubienova et al., 2014), and this appears to be the case for *M. truncatula* plants under NO₃⁻ deficiency. Higher GSNOR activity in N-deprived roots (Figure 5) could lead to a physiological state inducing higher resistance to *A. euteiches*, as observed in the 35S::GSNOR-transformed roots (Figure 2B). This could partly explain the enhanced resistance to this oomycete on NØ medium despite the low levels of NO in the roots. Thus, altogether our data highlight the possible positive and non-redundant roles of NO (Figures 1A and 2A) and SNO (Figures 1B, 2B, and 4D) in mediating *M. truncatula* resistance to *A. euteiches*.

CONCLUSION AND NEW HYPOTHESES

The results obtained in the present study are summarized in Figure 6. We have demonstrated, using transformed roots affected in genes involved in NO synthesis (*NIA* genes) and turnover (*GSNOR* gene), that deregulation of NO homeostasis has an effect on *M. truncatula* resistance against *A. euteiches*, as observed in other pathosystems (1). In addition, it appears that the modulation of NO homeostasis (through GSNOR overexpression) impacts NR activity and NO₃⁻ content, indicating possibly an effect of GSNOR (or GSNO) on basal NO₃⁻ transport/assimilation



and confirming the results of Frungillo et al. (2014) (2). In return, NO_3^- availability in the medium can affect NO homeostasis by modulating ROS/RNS/NO contents and their balance (3). Finally, infection by *A. euteiches* decreases root NO_3^- content (4) and induces higher ROS levels (5). Altogether these results highlight the close interplay occurring between N nutrition and NO homeostasis as well as the involvement of NO in the modulation of plant resistance by N nutrition.

Future work should take into account the role of N availability on NO-mediated plant molecular responses. Thus, the study of the specific role of GSNO in this process through the identification S-nitrosylated/denitrosylated proteins under different N availability conditions and N sources seems promising. A focus will be made on proteins involved in the plant immune response (1), but also on the feedback regulation of N metabolism by NO because NO could control NO_3^- availability and therefore plant resistance (2) (Figure 6). Investigations using foliar pathogens and other plant models will lead to a possible generalization of this phenomenon. More generally, plant N use efficiency can be affected by NO since NO controls not only N metabolism but also plant root growth and architecture changes in response to NO_3^- (Manoli et al., 2014; Sun et al., 2015). Recent data show that plant N use efficiency and N-induced susceptibility to pathogens may be linked (Ballini et al., 2013). Consequently future studies should also focus on candidate proteins involved in root development. Finally, experiments conducted with plant genotypes differing in their resistance levels will permit to

study the quantitative effect of NO/ROS production on plant defense.

AUTHOR CONTRIBUTIONS

ET, H-NT, ABo, and SJ conceived and designed the research; ET, H-NT, CF, ABo, and ABe carried out the experiments and analysis/interpretation of data; ET, H-NT, DW, and SJ wrote the manuscript. All authors contributed to the discussion and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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