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Understanding phenols function during tripartite interaction between legume- rhizobia and endophytic bacteria

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Abstract

Rhizobia can live inside the nodule plant under the control of several genes that regulate immunity. Five genes were identified that have a crucial role in immunity repression including *RSD*, *SymCRK*, *NAD1* and *NIN-16*. A mutation in these genes results in the formation of necrotic nodules characterised by the activation of the immune response and the induction of defence related proteins and with the accumulation of phenolic compounds (PCs). Little is known about phenolic accumulation during nodule defence response, our study aims to understand the regulation of these phenols inside the nodule and the role of PCs during the legume-rhizobium symbiosis. Analysis of RNAseq data was performed to identify the genes involved in the biosynthesis of PCs. As result, many gene families were determined to be up-regulated, and a conserved up-regulated PCs metabolic pathway was identified between *rsd*, *symCRK*, *nad1* and *nin-16* mutants. The phylogenetic analysis of two up-regulated genes family; chalcone synthase and caffeic acid O methyltransferase, indicates that probably one group of each family evolved to control the immune response in nodules. In addition, an alignment of three nucleotide sequences of three genes, two genes from the chalcone synthase family and one gene from the PR10 family in the 5'-UTR upstream region shows the presence of a conserved nucleotide block, indicating the presence of common transcription factors regulating PCs biosynthetic genes. To understand the role of PCs during immunity response, antimicrobial activity of selected PCs was tested on *Sinorhzibium medicae WSM419* and endophytic bacteria. The results show significant inhibition of *S. medicae WSM419*, which indicates high sensitivity of the symbionte to PCs. Our data support the hypothesis that PCs accumulation compromises the symbionte survival inside the nodule.

Key words: nodule, immunity, phenols, Sinorhizobium, symbiosis

Résumé

Les rhizobiums peuvent coloniser à l'intérieur des nodosités des plantes sous le contrôle de plusieurs gènes qui régulent l'immunité. Cinq gènes ont été identifiés comme jouant un rôle crucial dans la répression de l'immunité, notamment RSD, symCRK, NAD1 et NIN. Grâce à la régulation de ces gènes, les rhizobiums peuvent survivre à l'intérieur des nodules sans être endommagés. Une mutation de ces gènes entraîne souvent des mutants nécrotiques caractérisés par l'activation de la réponse immunitaire et l'induction de protéines de défense avec l'accumulation de composés phénoliques. Peu de connaissances sont disponibles sur l'accumulation des composés phénoliques dans la réponse de défense au sein des nodules. Notre étude vise à comprendre la régulation de ces phénols à l'intérieur des nodules. Une analyse de données RNAseq a été réalisée pour identifier les gènes impliqués dans la biosynthèse des composés phénoliques. En conséquence, plusieurs familles de gènes ont été identifiées comme étant surexprimées, et une voie métabolique conservée des flavonols a été identifiée. L'analyse phylogénétique de deux familles de gènes surexprimés, la chalcone synthase et la caféate O-méthyltransférase, indique qu'un groupe de chaque famille a probablement évolué et a été associé à la réponse immunitaire dans les nodules. De plus, un alignement des séquences nucléotidiques de trois gènes, deux de la famille de la chalcone synthase et un de la famille PR10 dans la région 5'-UTR en amont, montre la présence d'un bloc conservé, ce qui signifie qu'un groupe de facteurs de transcription est responsable de la régulation des composés phénoliques et de la protéine PR10 lors des réponses immunitaires. Afin de mieux comprendre le rôle des composés phénoliques pendant les réponses immunitaires, l'activité antimicrobienne a été testée sur les rhizobiums et les bactéries endophytes. Les résultats montrent une inhibition significative des rhizobiums, indiquant leur grande sensibilité, alors qu'aucune inhibition significative des bactéries endophytes n'a été observée. Ensemble, ces données renforcent l'hypothèse selon laquelle, au sein du nodule, la plante développe des mécanismes de défense tels que l'accumulation de composés phénoliques pour être capable d'éliminer le symbiosome dans différentes conditions.

الملخص

يمكن للبكتيرياالجذرية(الريزوبيوم) العيش داخل العقيدات النباتات تحت تحكم عدة جينات تنظم المناعة مما يوفر العيش RSD للريزوبيوم داخل العقيدات دون أي ضرر . تم تحديد خمس جينات تلعب دورًا مهما في قمع المناعة، بما في ذلك يؤدي الطفرات في هذه الجينات غالبًا إلى تفعيل الاستجابة المناعية وتحفيز .NIN و NAD1 و symCRKو البروتينات المتعلقة بالدفاع مع تراكم المركبات الفينولية.إن المعلومات المتوفرة حول تراكم الفينولات في استجابة الدفاع RNAseq داخل العقيدات قليلة. تهدف دراستنا إلى فهم تنظيم هذه الفينولات داخل العقيدات، لذا تم إجراء تحليل بيانات لتحديد الجينات المشاركة في انتاج المركبات الفينولية. ونتيجة لذلك، تم تحديد العديد من عائلات الجينات التي زادت تعبيرها، وتم التعرف على مسار أيضي محفوظ للفلافونول.ونتيجة لذلك تم تحديد العديد عائلات الجينات أنها عالية التعبير تساهم في إنتاج مركبات فينولية محددة مثلا الفلافونول. يشير التحليل الوراثي لعائلتين من الجينات ذات تعبير مرتفع؛ شالكون سينتاز و حمض الكافيك ترنسفيراز، إلى أن مجموعة واحدة على أقل من كل عائلة قد تطورت وارتبطت بالاستجابة المناعية في العقيدات. بالإضافة إلى ذلك، يُظهر مقارنة ثلاث تسلسلات نيوكليوتيدية لثلاثة جينات، اثنان من عائلة شالكون سينتاز الكالكور وواحد من عائلة جينات متعلقة بالدفاع 10 في منطقة الغير مستنسخة 5 العلوية، وجود عدة مسلسلات نكليونيدية محفوظة، مما يعني أن مجموعة من عوامل النسخ مسؤولة عن تنظيم المركبات الفينولية وبروتينات متعلقة بالدفاع أثناء الاستجابات المناعية. لفهم أفضل دور المركبات الفينولية خلال الاستجابة المناعية، تم اختبار النشاط المضاد للميكروبات على البكتيريا الجذرية والبكتيريا الداخلية. أظهرت النتائج تثبيطًا كبيرًا للبكتيريا الجذرية، مما يشير إلى حساسيتها العالية، في حين لم يُلاحظ تثبيط كبير للبكتيريا الداخلية. تدعم هذه البيانات الفرضية التي تفيد بأنه داخل العقيدة، تطور النبات آلية دفاع مثل تراكم المركبات الفينولية؛ لتتمكن من القضاء على السيمبيوزوم تحت ظروف مختلفة.

List of Abbreviations

PCs: phenolic compounds

DMSO: Dimethyl sulfoxide

NF: nod factor

Its: infection threads

CKs: cytokinin

flg22: flagellin peptide

ETI: effector-triggered immunity

LysM RLK: lysin motif receptor-like kinase

MAMP: microbe-associated molecular pattern

MTI: MAMP-triggered immunity

NF: Nod factor

Nop: nodulation outer protein

PRR: pattern recognition receptor

R resistance gene

ROS: reactive oxygen species

T3SS/T4SS: type III/type IV secretion system

BNF2DEFECTIVE IN NITROGEN FIXATION 2

symCRK: Symbiotic CYSTEINE-RICH RECEPTOR-LIKE KINASE

RSD; REGULATOR OF SYMBIOSOME DIFFERENTIATION

NAD: NODULES WITH ACTIVATED DEFENSE 1

NIN: NODULE INCEPTION

SMs: secondary metabolites

CCR: cinnamoyl-CoA reductase-like protein

4CL: 4-Coumarate-CoA ligase

CAD: (hydroxy)cinnamyl alcohol dehydrogenase

COM: caffeic acid O-methyltransferase

PAL: phenylalanine ammonia lyase

CHI: chalcone isomerase/ FI: flavanone isomesrase

F3H: flavanone 3-hydroxylase / FS: flavonol synthase

DFR: dihydroflavonol 4-reductase-like protein

CYPs450: cytochrome P450 family

ANR: Anthocyanidin reductase

CCoAOMT: caffeoyl-CoA 3-O-methyltransferase

CYPs450 C4H: cytochrome P450 family cinnamate4- hydroxylase

M17: *Pseudomonas aeruginosa*

M18: *Enterobacter sp*

BT37: *Streptococcus sp*

M50: *Lysinibacillus sp*

M67: *Bacillus amyloliquefaciens*

WSM419: *Sinorhizobium medicae*

Ga: Gallic acid

Ru: Rutin

Qu: Quercetin

Va: vanillin

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Part I. Literature Review

Chapter 1 Plant-microbes interactions world

1.1 Exploring the diverse spectrum of symbiotic relationships

In the rhizosphere which is the narrow zone around the plant roots but a highly dense vital area [1]. Plants can control microbiome communities by releasing root exudates [2]. These exudates can take different forms (sugars, fatty acid, secondary metabolites) [3] for different activities from suppressing pathogens attacks to attracting beneficial microbes [4]. Plants can shape a diverse spectrum of interactions which can be (Figure 1)

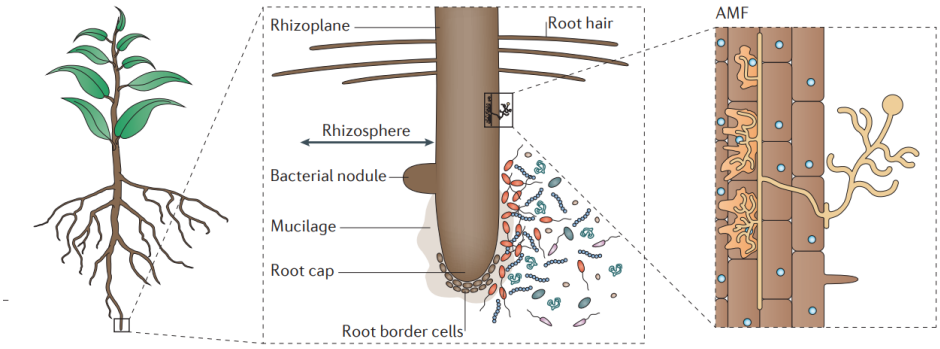

Figure 1. The rhizosphere. The rhizosphere, a zone surrounding plant root, Within this habitat, a variety of microorganisms live, including symbiotic bacteria and fungi like arbuscular mycorrhizal fungi(AMF) and saprophytic organisms.

Plants can perform different types of symbioses the most important are: the mycorhize, the beneficial interaction with plant promoting microorganisms and rhizobium symbiosis.

1.2 Mycorrhizal symbiosis

Mycorrhizal symbiosis is one of the most important evolved mutually beneficial interactions between plant roots and fungi, where 80% of terrestrial plants species have these beneficial relationships [5][6]. It provides a source of mineral nutrients such as phosphorus and nitrogen that are highly essential for plant growth [7]. In return, plants give a part of their photosynthesis process in in the form of photosynthates and fatty acids.[6] Plants release strigolactones (hormones) to stimulate fungal growth to spread its hypha and branching [8] mycorrhiza not only provides nutrients to plants but also assures protection from various stresses like salinity, drought, and pathogen attacks [9].

This symbiotic association can be classified in four categories: arbuscular mycorrhizas (AM), ericoid mycorrhizas (ERM), orchid mycorrhizas (ORM), ectomycorrhizas (ECM). [10], [11].

1.3 Plant growth promoting rhizobacteria

A wide range of mircobes inhabit the rhizosphere, with different life styles. One of the most beneficial interactions is associated with plant growth promoting rhizobacteria (PGPRs) [12], [13]. Several mechanisms by which PGPRs can promote plant development with, from open accessibility to nutrient uptake,[14] vitamin production, siderophores chelation [15], in a direct way [14] and alleviation of abiotic stressors such as aluminium toxicity [16], salinity, drought, and heat stresses [17], [18], [19], in an indirect way [14]. PGPRs can produce antibiotics, which makes it them acting as an effective biocontrol agent against phytopathogens [14]. Additionally, they could trigger induced systemic resistance mediated through salicylic and jasmonic acid[20] . Furthermore, regulation of plant metabolism involves modulating gene expression of phytohormones such as auxin, cytokinin and gibberellins [21],[12].

1.4 Endophytic bacteria symbiosis

Endophytic bacteria colonise most plant species ubiquitously without causing observed harm [22]. They provide a range of advantages to their host, from enhancing nutrient acquisition, phytohormones production [23], to stress resistance. Additionally, it leads to the secretion of a large gamma of bioactive compounds like alkaloids, flavonoids, and phenols [24]. Furthermore, endophytic bacteria can stimulate the immune system by increasing antioxidant levels as a response to protect cells from oxidation after an infection [25].

1.5 Legume-nitrogen fixing bacteria

Some plant species, like legumes (Fabaceae), can establish an interesting kind of symbiosis [22], where a very essential microelement like nitrogen, has a special organ to fix it in named the nodule, and this can only happen in the presence of a symbiont, which is the rhizobial bacteria [23]. This mutualistic interaction helps mostly the plant gain an available form of atmospheric nitrogen, like ammonia, and makes it a dominant source, which is usually difficult to uptake from soil [24]. This enables the host to grow quickly and healthily, which in turn gives its symbionts protection and nourishment. Besides, symbionts also contribute to host plant protection from diseases [25]. *Medicago truncatula* and *Lotus japonicus (Regel)* are two members of the Leguminosae family that are classified as legume models [26], [27], where *Sinorhizobium meliloti* is the rhizobial symbiont model. All of them are commonly employed

for the investigation of this symbiosis as hosts and symbionts, respectively. A complex signalling exchange mechanism is developed [28], and it is discussed in the next section.

Chapter 2 Legume-rhizobium symbiosis

2.1 The recognition phase of the legume-rhizobium symbiosis

In the nitrogen limiting conditions, legumes interact with soil nitrogen fixing bacteria that help the plant to achieve organic nitrogen with the formation of a new root organ which is nodule in which rhizobia fix the atmospheric nitrogen [22]. The interaction between the two partners begins first by the plants; in which they secret a specific aromatic secondary metabolite including flavonoid through their roots[23]. The flavonoids that are perceived by rhizobia bind and activate bacterial NodD proteins to induce the nod regulon expression that encoding for nod factor (NF) biosynthesis. NF interact with receptor-like kinases (RLKs) in the root epidermis cell [24], leading to the activation of signalling symbiotic pathway such as depolarization of host cell membrane, a production of reactive oxygen spices (ROS), and calcium spiking (increase of intracellular calcium (Ca^{+2})[25]. In parallel the expression of two key transcriptional regulators Nodule Inceptions (NIN) and ERF is activated for the nodule formation[26].

2.2 Formation of rhizobia home: Infection and nodule Organogenesis

After the bacteria colonized curled root hair, they infect the root hair through the formation of infection threads (ITs) [27] among them to release the targeted future symbiotic cell due to the reinitiation of the cell cycle by the NF and cytokinin (CKs) hormone action[28]. In the other hand, NF and CKs also involved in the organogenetic process through auxin transport inhibition. where the cells of root cortex become dedifferentiated leads to the formation of the nodule primordium which are the first cells that received rhizobia [29] [30].

2.3 Nodule structure, development and Function

During legume rhizobia symbiosis, two nodule types are formed, the determinate and indeterminate nodules. the determinate nodules are characterized by a spherical shape due to the nonpersistent nature of the meristem in some legume plants such as *Phaseolus vulgaris*, *Glycine max*. while the indeterminate nodules formed by plants like Medicago and Pisum species have an elongated shape due to a persistent meristem located in the nodule apex also characterized by different zones[31] [32].

2.3.1 The zones of indeterminate nodules

First zone is the apical meristem zone I (ZI) is necessary for the growth of the organ to give it its final elongated shape. Then there is infection zone or zone II (ZII), the rhizobia in this zone

infect the host cell. The next zone is characterized by the fixation of the atmospheric nitrogen by differentiated bacteroid, which is the fixation zone, or zone III (ZIII). Finally, a senescent zone, zone IV (ZIV), we observed the death of bacteroid with the host cell. Interestingly, there is one more interzone between (ZII) and (ZIII), where the bacteroid go through a terminal differentiation (TDB) before the nitrogen fixation [33] [34].

2.3.2 Terminal Bacteroid Differentiation

In the point where the rhizobia arrived to the symbiotic cells, the bacteroid is endocytosed to form the symbiosome that contain a bacteroid surrounded by peri-bacteroid membrane (PBM), the two last are separated with peri-bacteroid space (PBS) [35]. The Inverted Repeat Lacking Clade (IRLC) family synthesis NODULE-SPECIFIC CYSTEINE-RICH (NCR) peptides in the nodule to begin TDB. The TDB resulting a bacteroid that is incapable to live outside the cell host because: genome endoreduplication associated with the increased of the endosymbiont size and the inhibition of bacterial cell division [36] [37].

2.3.3 The functional nodule

After bacteroid differentiation, the symbiosome are now capable to express sensitive nitrogenase enzyme that fixing atmospheric nitrogen under lower concentration of oxygen O2 [38]. The regulation of O2 concentration in the infected cell plant due to legume protein, the leghemoglobin which known to be a marker of healthy functional nodules [39]. Nitrogenase convert the nitrogen N2 to ammonia NH3 + through the following equation[40]:

$$N_2 + 8H^+ + 8e^- + 16Mg\text{-}ATP \rightarrow 2NH_3^+ + 2H^+ + 16Mg\text{-}ADP + 16\ Pi$$

When the nodule is old, or nitrogen is added to the medium or the plant is under stress; a senescence process is activated where start dying before the degradation of host cell. This process is viewed can be observed in zone IV [33].

Chapter 3 Immunity during Legume-rhizobium symbiosis

3.1 Introduction to plant immune system

Plants are surrounded by diverse microorganisms; some of them are pathogens affect the growth and survival of the plants[41]. As result plants develop several immune responses including pattern-triggered immunity (PTI), effector-triggered immunity (ETI) to inhibit the pathogen invasion [42].

The immune system is initiated by the binding of microbe/ pathogen -associated molecular patterns (MAMPs, PAMPs) including flagellin, the surface polysaccharides lipopolysaccharides (LPSs), capsular polysaccharides (CPSs), and exopolysaccharides (EPSs), with Pattern Recognition Receptors (PRRs) at the plant cell membrane [43], the interaction between them leading to the activation of the defence response (MTI) which characterised rapid cellular responses such as the production of ROS, calcium influx, and induction of immune signalling (MAPK)[44]. More defence-related proteins are produced by the host cell, such as antimicrobial or PR proteins and hydrolytic enzymes [45] [46]. Pathogenic microbes have developed another strategy to escape PTI through virulence proteins secretion (effectors) that are injected into the host cell cytoplasm using type III, IV, and VI secretion systems. In parallel, the plants evolved another immunity line to recognise and control bacterial effectors via R genes, these genes induce Effector Triggered immunity (ETI)[47] [48]. In a symbiotic relationship, the plant's innate immune system controls the selection of beneficial partners; in other hand, rhizobia modulate immune responses to successful infection.

3.2 Immune Regulation in Early Symbiotic Interactions

Rhizobia can activate plant innate immunity during initial stages of symbiosis; for example, the inoculation of rhizobium with Medicago truncatula as well as soybean showing induction of defense-related genes only in the early inoculation hours; after the expression of defense-related genes were downregulated it means the suppression of plant immunity[49] [50][51]. Interestingly, a study suggests that not all host plant induce the defence-related genes [52] [53]. The rhizobia develop some strategies to inactivate the immunity, through the production of exopolysaccharide (EPS), EPS supress the MAMP-triggered immunity (MTI) by chelating calcium [54] [49]. Moreover, rhizobia like S. meliloti produce modified MAMP in this case the flg22 peptide, to be unable to trigger MTI in the plant, because the unmodified flg22 peptide can be recognized by the plant and as resultant activate MTI [55]. In addition, rhizobia NFs can recognize LysM RLKs and supress MTI. Additionally, group of rhizobia inject symbiotic

effectors protein (for example, Nop proteins) in the host cell to block defense response and promote infection [55] [56].

3.3 Immunity response inside mature nodule

Several genes were identified in *Medicago littoralis* are responsible of bacteroid survival inside the host plant such as *DEFECTIVE IN NITROGEN FIXATION 2 (DNF2)* [57], *REGULATOR OF SYMBIOSOME DIFFERENTIATION (RSD)* [58], *CYSTEINE-RICH RECEPTOR-LIKE KINASE (SymCRK)* [59], *NODULES WITH ACTIVATED DEFENSE 1 (NAD1)*[60] *,and NODULE INCEPTION (NIN)* [61]. these genes encode for proteins with different functional and They are corresponding, respectively to a phospholipase-C phosphatidyl-dependent protein-like, C2H2 transcription factor (TF), a cysteine-rich receptor kinase, transmembrane protein and a symbiotic TF, the five genes are known as NODULINS. The genes act together to regulate nodule defense. In addition, some studies about TDB process show plants that have mutations in DNF2 [62] , RSD [58], symCRK [63] and NAD1[60] [64] stop bacteroid development in TDB process. Interestingly, DNF2, SymCRK, and RSD act successively during the development of nodules to downregulate immunity [65]. Moreover, the signal responsible for defence activation in dnf2, symCRK, and rsd nodules is different. DNF2 repress immunity due to environmental factors while SymCRK and RSD inhibits defense responses because of bacterial infection and/or bacteroid differentiation [65] [57]. Mutations in NODULINS genes activate a strong defence response in nodules that do not fix nitrogen and characterized by induction of defense genes expression and accumulation of phenolic compounds leading to necrotic cell death [61]**.**

Chapter 4 plant phenolic compounds.

4.1 Introduction to plant secondary metabolites

secondary metabolites (SM) are small organic molecules of natural compounds characterize of molecular masses less than 3000 da these metabolites are not necessary for growth and reproduction like primary metabolites [66] [67]. Plants produce diverse group of plant secondary metabolites (PSM) with complex chemical composition play an essential role plants defense mechanisms, response to environmental stresses, and in organismal interactions [68]. PSM are classified in three major families including terpenoids, non-protein nitrogen compounds, and phenolic compounds [69]

4.2 Plant phenolic compounds

Phenolic compounds (PCs) are a class of the most abundant secondary metabolites of plants [70], widely distributed in plant kingdom, with more than 8,000 phenolic identified [71], with diverse structures and function from higher plants varying from simple molecules such as phenolic acids to complexes structures such as tannin[72]. PCs are consisting of an aromatic ring, containing one or more hydroxyl substituents [73][74], they are classified in several groups with different properties including phenolic acids, flavonoids, stilbenes, tannins and lignans[75].

4.3 Biosynthesis of phenolic compounds

The initial step in this pathway involves the deamination of phenylalanine to trans-cinnamic acid, catalyzed by the enzyme phenylalanine ammonia lyase (PAL) (Figure 2). Subsequently, the hydroxylation of trans-cinnamic acid by cinnamate-4-hydroxylase (C4H) yields p-coumaric acid. The introduction of a hydroxyl group at the 3-position of p-coumaric acid by coumaryl 3-hydroxylase (C3H) results in caffeic acid. Methylation of caffeic acid by caffeic acid O-methyltransferase (COMT) produces ferulic acid. Additionally, ferulic acid can undergo hydroxylation at the 5-position by ferulic 5-hydroxylase (F5H), followed by methylation by COMT, to yield sinapic acid that will be directed to lignin production. Alternatively, p-coumaric acid can be activated to its CoA ester by 4-coumarate: CoA ligase (4CL), leading to the synthesis of coumarins and stilbenes[76][77].

Another major branch of the phenylpropanoid pathway involves the biosynthesis of flavonoids (Figure 2). P-coumaroyl-CoA is converted to naringenin chalcone by chalcone synthase (CHS) and subsequently to naringenin by chalcone isomerase (CHI). Naringenin is now a key

precursor for the biosynthesis of various flavonoid subclasses. One branch leads to the formation of flavones through the action of flavone synthase (FNS). Another branch involves the conversion of naringenin to dihydroflavonols by flavanone 3-hydroxylase (F3H). Dihydroflavonols can be further modified to produce flavonols, anthocyanidins, and flavan-3-ols through the sequential action of flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR), and leucocyanidin reductase (LAR), respectively. Anthocyanidins, the precursors of anthocyanin pigments, are formed by the action of anthocyanidin synthase (ANS).[78][79]

4.4 Plant Phenolic compounds against biotic and abiotic stress

Plants are exposed many abiotic as well as biotic stress conditions, and they are facing these conditions through the accumulation of PCs. Each type of stress characterizes by production of specific PCs. Light stress activates the biosynthesis pathway of phenolic acids and flavonoids in plants [80]. Additionally, Temperature stress caused by high or low temperature is responsible of anthocyanins accumulation [81] and lignin accumulation[82]. Moreover, plants accumulate PCs such as flavonoids in contract to oxidative stress caused by salinity [83]. Finally, plants produce and accumulate PCs at the infection sites to slow down and stop the growth of microbial pathogens [84][85].

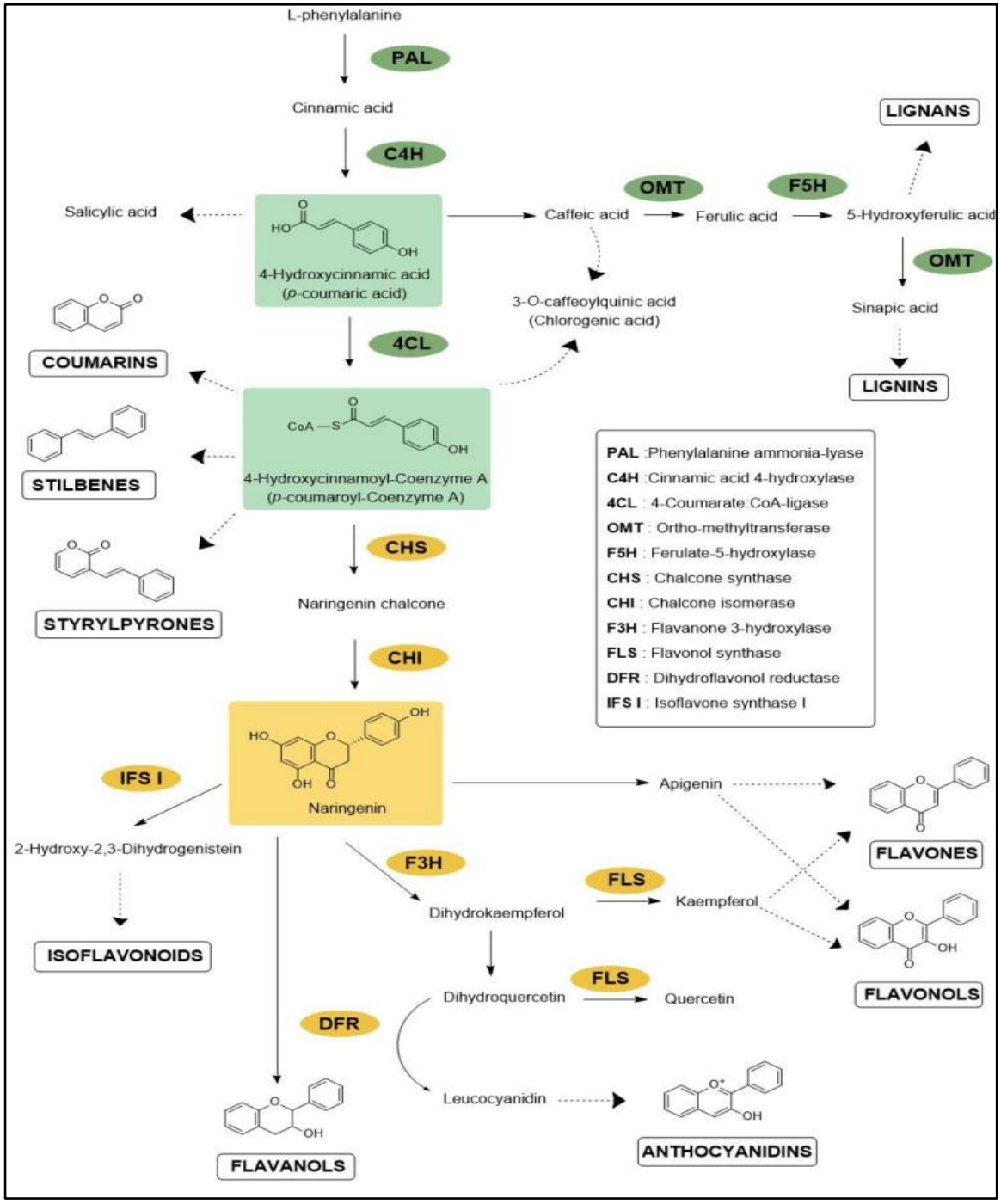

Figure 2. Main steps of the phenylpropanoid and flavonoid pathways. The figure shows the biosynthesis step of phenolic compounds in plants. The enzymes that involved in the biosynthesis are: cinnamoyl-CoA reductase-like protein (CCR), 4-Coumarate-CoA ligase (4CL), (hydroxy)cinnamyl alcohol dehydrogenase (CAD), caffeic acid O-methyltransferase (COMT), phenylalanine ammonia lyase (PAL), chalcone isomerase (CHI) and flavanone isomerase (FI), flavanone 3-hydroxylase (F3H) and flavonol synthase (FS), dihydroflavonol 4-reductase-like protein (DFR), cinnamic acid 4- hydroxylase (C4H), ferulated 5-hydroxylase (F5H), isoflavone synthase 1(IFS1)

Introduction to the Problematic

Interaction between legume and soil nitrogen fixing bacteria named rhizobia is characterized by the formation of new organ known as nodule. Inside the nodule, rhizobia convert atmospheric nitrogen to organic form for their host.

Inside the host cell their some genes were identified as responsible of the nodule immunity control and essential for maintaining bacteroid survival within the host cells. Knocking out of these genes induces strong immunity response with the death of bacteroïds and accumulation of phenolic compounds. Our study aims to understand a part of the metabolic defence regulation which includes the biosynthesis of phenolic compounds during defence response through the followed step:

- Determination of expression profile for each mutants
- Identification of the conserved pathway of phenols biosynthesis between the mutant
- Determination of history evolution of two upregulated genes families
- Research for common transcription factors controlling phenols biosynthetic gene families
- Examinate the antimicrobial activity of phenolic compounds against rhizobia and endophytic bacteria

Part II. Materials and methods

1. RNA sequencing data analyses

To determine the different expression patterns of phenols (PC) biosynthetic pathway. RNAseq data were firstly collected for each mutant using data described in the corresponding studies: *rsd* [58] *nad1* [60] *nin16* [61]. *symCRK* data was obtained from SYMUNITY lab (Institute of Plant Science Saclay) and correspond to RNAseq analysis of *symCRK* Vs. WT nodules of plant cultivated in vitro and inoculated with Sinorhizobium medicae WS419. The analysis was performed on 21 days post inoculation nodules. The data were then analyzed using TBtools software (a Toolkit for Biologists integrating various biological data-handling tools) [86].

2. Venn Diagram Construction

To identify the common and specific genes encoding phenols biosynthesis enzyme, comparison of the expression levels between mutant and corresponding control of the studided genes were performed using Excel software. The genes showing at least two folds induction in the mutants compared to the WT were considered as up-regulated. The commonly and specifically induced genes were then isolated and a Venn diagram was generated based on the obtained results [87] [88]

3. Phylogenetic analysis

To understand the evolutionary history of selected genes families recruited during phenol bioynhteis, we used the Molecular Evolutionary Genetics Analysis X (MEGAX) software [89] to create a phylogenetic tree. The phylogenetic analysis starts with the research of the protein homologous in the Phytozome database [90]. Once the homologous proteins are determined, the protein sequences of each homologous are downloaded from the (NCBI Protein) database and transformed into FASTA form. The FASTA document of protein sequences was used to build the phylogenetic tree using Neighbor-Joining method [91] with the bootstrap test (1000 replicates located next to the branches) [92].

4. Genomic Sequence Alignment

For the alignment analysis, we used three up regulated genes. two genes from CHS family and one gene from another family such as PR10 gene which known to their binding ability to different ligands such as flavonoid [93]. The nucleotides sequences were first downloaded from Phytozome database [90]. Then The sequences were aligned using CLUSTALW algorithm in

the BioEdit software [94]. The sequences were from the region 5' untranslated region (UTR), 500 base pairs upstream of the start codon.

5. Antimicrobial activity of PCs

To determine the role of the PCs during immunity responses within plants. PCs from different classes were used; from the flavonoids class like rutin (Ru) [95] and quercetin (Qu) [96]; from the phenolic acid class as gallic acid (Ga) [97]; and at least vanillin (Va) as phenolic aldehyde. These PCs are then tested in six different strains; *Sinorhizobium medicae* strain WSM419 [98] as well as five endophytic bacteria that were identified in the lab and they are as follows: *Pseudomonas aeruginosa* (M17), *Enterobacter sp* (M18), *Lysinibacillus sp* (M50), *Bacillus amyloliquefaciens* (M67) and *Streptococcus sp* (BT37)

6. Bacterial strains and culture conditions

Sinorhizobium medicae strain WSM419 [98] and endophytic bacterial strains *Pseudomonas aeruginosa*, *Enterobacter sp*, *Lysinibacillus sp*, *Bacillus amyloliquefaciens* and *Streptococcus sp* were cultivated in yeast extract broth (YEB)-agar medium [99] for 48h of grown on agar plate at 30°C.

7. Chemical preparation

Stock solutions for each PCs were prepared based on their solubility. The concentration of each solution is 0.01 g/ml for gallic acid and vanillin, 0.03 g/ml for quercetin and 0.13 mg/ml for rutin. All PCs were dissolving in sterile water except quercetin which is diluted in sterile DMSO.

8. Inhibition test of PCs

The disc diffusion method was used for evaluating the antimicrobial activity of PCs [100]. After 48h incubation, the bacterial strains were transferred to sterile physiological serum for the adjustment of the optical density at (OD600=0.1). The inoculum suspension of each bacterium was spread onto the agar plate surface using a sterile cotton swab. After 30 minutes, a sterile paper disc containing 15µl or 35µl of PC was placed on the surface of the inoculated Petri plates. Each Petri plate contains one PC with one of the six bacteria was incubated for 24h to 48h at 30°C. after incubation period the diameter of inhibition zone are measured [101] [102].

Part III. Results and Discussion

1. Mutant showing nodules defense response overexpress of PC synthetic genes

PCs are known to be one of the most groups in plants secondary metabolites that have diverse functions against abiotic and biotic stress [103]. Different studies describe their biosynthesis under different conditions [104], but not enough studies show their production during a nodule immune response in symbiosis.

To determine the group of enzymes involved in the biosynthesis of PCs during the immune response in *M. truncatula* nodules. First, we identified the *M. truncatula* enzymes involved in the different steps of the biosynthesis chain of PCs in plants as described in the Figure 2. This enzymes are: cinnamoyl-CoA reductase-like protein (CCR), 4-Coumarate-CoA ligase (4CL), (hydroxy)cinnamyl alcohol dehydrogenase (CAD), caffeic acid O-methyltransferase (COMT), phenylalanine ammonia lyase (PAL), chalcone isomerase (CHI) and flavanone isomerase (FI), flavanone 3-hydroxylase (F3H) and flavonol synthase (FS), dihydroflavonol 4-reductase-like protein (DFR), cytochrome P450 family (CYPs450), Anthocyanidin reductase (ANR), caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) , cytochrome P450 family cinnamate4-hydroxylase (CYPs450 C4H:), cinnamic acid 4- hydroxylase (C4H)

Next, we determined which enzymes are stimulated during the immune response in nodules, for that, RNAseq analysis of four mutants of *M. truncatula* showing defense response in the nodule (*symCRK*, *nad1*, *nin-16* and *rsd)* was performed (Figure 2).

Among the 98 identified enzymes M. truncatula genome, in the *symCRK* nodules, 30 enzymes are down regulated from different enzyme families including 13 COMT enzymes, 3 enzymes from 4CL and CCR family, 6 enzymes from CAD family, and one enzyme from ANR, DFR, CYPs450, CHI, FI families. While 68 enzymes showing up-regulation which are 2 enzymes from DFR and 4CL, 14 enzymes from COMT, 16 enzymes from CHS/SS,4 enzymes from PAL, CAD, CHI, FI, CCoAOMT families, 3enzymes from CCR, 5 enzymes from CYPs450, 9 enzymes from F3H, FS (Figure 2A). In *nad1* mutant, 23 enzymes from diverse families (6 enzymes from COMT, 2 enzymes from 4CL, CAD and PAL, 2 enzymes from CAD, 5 enzymes from CHI, FI, 3 enzymes from CCR and PAL), one enzyme from CHS/SS family) were down regulated (Figure 2B). In contrast, 55 enzymes were up regulated from 8 enzymes from COMT, 6 enzymes from FS/F3H, 7 enzymes from 4CL and CYPs450f family, 3 enzymes from PAL, DFR, and CAD family, 13 enzymes from CHS/SS families, and one enzyme from ANR and CCR family (Figure 2B). The *nin-16* nodule shows 51 enzymes with different expression

profiles, where 5 enzymes are down regulated (2 enzymes from FS/F3H and 4CL family, and one enzyme from COMT), and 46 enzymes (5 enzymes from PAL family, DFR, 12 enzymes from CHS/SS family, 4CL, 3 enzymes from CCoAOMT, 4CL and CCR families , 4 enzymes from FS/F3H family 3 from CHI, FI, CHS and CAD family, one enzymes from COMT, DFR, CHI, C4H) are up regulated (Figure 2C). Finally, only few enzymes were detected in the *rsd* mutant, with 16 enzymes showed down regulation(3 enzymes from CHS and 4CL family, 4 enzymes from DFR family, 2 enzymes from COMT, and one enzyme from the families CAD, PAL, F3H/FS. and one enzyme is up regulated from the DFR family (Figure 2D). The up-regulated genes in each mutant are probably a part of the PCs biosynthesis in *M. truncatula* and explain the accumulation of PCs compounds in studied mutants [60] [61] [59] [58]. The various number of up and down regulated gene between the different mutants could be explained by the experimental designs, which were different between the studies.

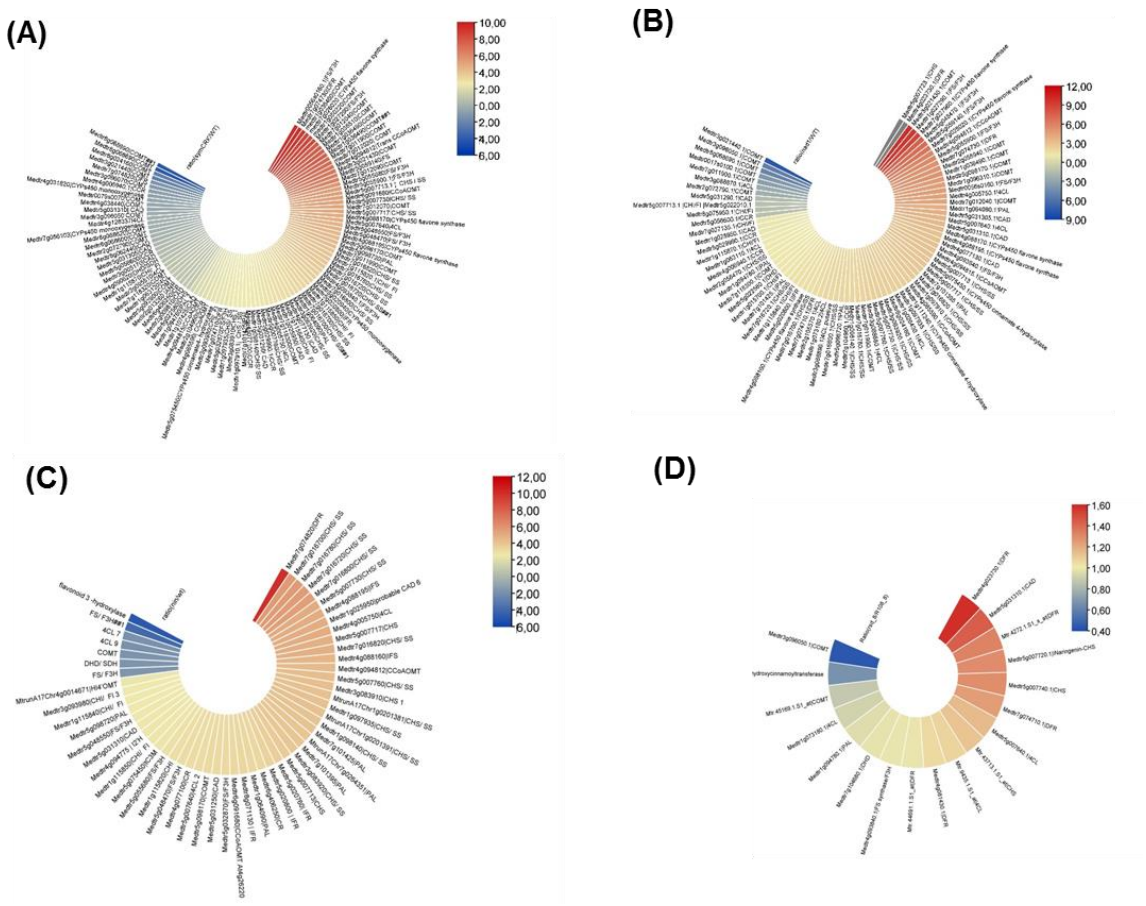

Figure 2. Differential gene expression analysis of RNA-seq. Heatmap summarizing the up-regulated and down regulated genes encoding for enzymes involved in the biosynthesis of PCs *in M. truncatula*. (A) *symCRK* mutant. (B) *nad1* mutant. (C) *nin16* mutant. (D) *rsd* mutant. The Heatmap colours indicate the log2 of rate (mutant/wild type) where a higher expression level corresponds to high colour intensity. The enzymes of the PCs biosynthesis are (CCR : cinnamoyl-CoA reductase-like protein, 4CL : 4-Coumarate-CoA ligase, CAD : (hydroxy)cinnamyl alcohol dehydrogenase, COMT: caffeic acid O-methyltransferase, PAL: phenylalanine ammonia lyase, CHI: chalcone isomerase/ FI: flavanone isomesrase, F3H: flavanone 3-hydroxylase / FS: flavonol synthase, SDH: shikimate dehydrogenase/ DHD: 3-dehydroquinate dehydratase, DHS:3-dehydroquinate synthase, DFR: dihydroflavonol 4-reductase-like protein,CYPs450: cytochrome P450 family, ANR: Anthocyanidin reductase, CCoAOMT, caffeoyl-CoA 3-O-methyltransferase , CYPs450 C4H: cytochrome P450 family cinnamate4- hydroxylase).

2. A common genetic programme is activated for PCs biosynthesis observed in studied mutants

The identified up-regulated genes in the different mutants are used next compared to determine specifically induced genes for each mutant and commonly stimulated genes between different mutants. The comparison of the expression pattern between the mutants reveals five specific induced genes in *symCRK* mutant (Medtr1g094780|PAL, Medtr7g012070COMT, Medtr7g118300.1COMT, Medtr1g107425CAD, Medtr5g029990.1CCR.), while *nin16* had only two genes (Medtr7g074820|DFR and Medtr6g406250|CR) and in the other hand in *nad1* mutant nine specific genes were identified (Medtr3g088890.14CL, Medtr2g105570.14CL, Medtr1g073180.24CL, Medtr3g088880.14CL, Medtr7g074730.1DFR, Medtr1g027290.1FS/F3H, Medtr1g026430.14CL, Medtr8g024160.1COMT, Medtr5g031305.1CAD) (Figure 3).

Comparison of commonly induced genes between studied mutants reveals sixteen common genes between *symCRK* and *nad 1*, In contrast, *nad1* and *nin-16* shared only two commonly upregulated genes. However, *symCRK* and *nin-16* have eleven commonly upregulated genes. Finally, twenty-two common genes across the three mutants have been observed. These results indicated a conservation of the biosynthesis pathway between *M. truncatula* mutants and possibly resulting the production of Flavones, Flavonols and Flavanols [105] [106]. However, the low number of specifically induced gene confort the hypothesis of a common genetic program involved in the stimulation of defense response during the symbiosis.

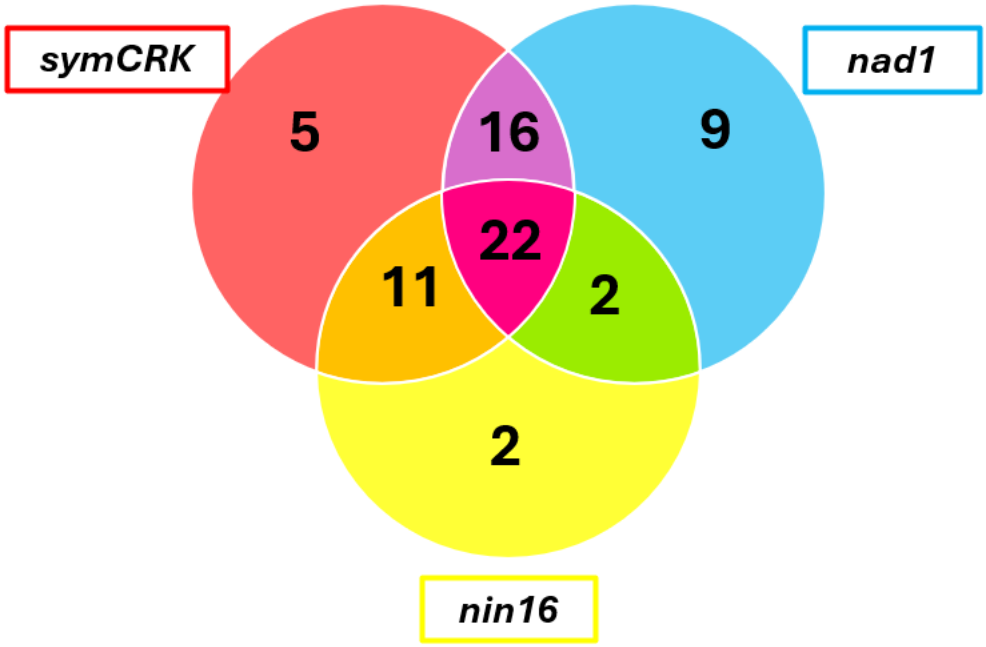

Figure 3. Venn diagrams summarizing the total number of the upregulated genes across three mutants of the plant M. truncatula. Whereas each colour represents the specific or the common genes between three mutants *symCRK*, *nad1*, *nin-16*.

3. Analysis of commonly stimulated PCs biosynthesis genes reveals a conservation of the complete pathway of PCs synthesis during nodule defense response

To characterize the biosynthetic pathways controlling PCs synthesis during nodule defense response, functional analysis of identified commonly up-regulated genes were performed. The table 1 shows obtained results.

The commonly up-regulated genes are two 4CL, two PAL, one CHI/FI, one COMT, twelve CHS/SS, one FS/F3H, one UFGT and two CYP450.

This observation indicate a conservation of the full pathways for synthesis of PCs in the nodules, interestingly, CHS/SS seems the most commonly expressed family, underling the importance of these genes during nodule defence response.

Table 1. Protein encoding different common genes between three mutants

Two Common genes			Three common genes
SymCRK-nad1	Nad1-nin16	SymCRK-nin16	SymCRK-Nad1-Nin16
Medtr7g012040.1|COMT Medtr7g011990.1|COMT Medtr1g036490.1|COMT Medtr3g021430|COMT Medtr1g096310.1|COMT Medtr2g055940.1|COMT Medtr1g097910.1 |CHS/ SS Medtr4g077130 |CAD Medtr4g085590.1|CCoAOMT Medtr4g094815.1|CCoAOMT Medtr7g074710.1 |DFR Medtr0056s0160.1|FS/F3H Medtr4g093840.1|FS/F3H Medtr5g055900.1|FS/F3H Medtr5g059140.1|FS/F3H Medtr2g104960|CCR	Medtr7g101395|PAL Medtr5g031310|CAD	Medtr7g101425.1 | PAL Medtr1g115820.1 | CHI/FI Medtr3g093980.1 | CHI/ FI Medtr1g115850.1 |CHI/FI Medtr1g025950|probable CAD 6 Medtr5g031250|CAD Medtr6g091680|CCoAOMT At4g26220 Medtr3g083910|CHS 1 Medtr4g077100|CCR Medtr5g032870|FS/F3H Medtr5g048550|FS/F3H	Medtr4g005750 |4CL Medtr5g007640|4CL Medtr1g064090.1 | PAL Medtr5g098720.1 | PAL Medtr1g115840.1 | CHI/FI Medtr5g098170.1|COMT Medtr5g007713.1 | CHS / SS Medtr7g016720|CHS/ SS Medtr7g016700|CHS/ SS Medtr7g016800|CHS/ SS Medtr1g098140|CHS/ SS Medtr7g016780|CHS/ SS Medtr5g007760|CHS/ SS Medtr5g007730|CHS/ SS Medtr3g083920|CHS/ SS Medtr1g097935|CHS/ SS Medtr7g016820.1|CHS/SS Medtr5g007717.1|CHS/SS Medtr5g048470.1 |FS/ F3H Medtr5g090620| UFGT Medtr5g075450.1 | CYPs450 C4H Medtr1g111240.1|CYPs450 C4H

4. Phylogenetic analyses reveals specifically evolved group of chalcone synthase and caffeic acid O methyltransferase controlling PCs synthesis during nodule defense

To better understand the evolution history of enzymes involved in the biosynthesis of PCs in nodule, a phylogenetic analysis of two most represented type of enzymes during nodule defense, chalcone synthase and caffeic acid O-methyltransferase was conducted (Figure 4).

Chalcone synthase contains two distinct gene clusters with different evolutionary histories. First cluster mainly contain up-regulated genes (12 from 13). While in the second cluster contain only 3 among 8 up-regulated genes and the other genes are mostly down regulated (Figure 4A). Similarly, the second family shows one a sub-branch including up-regulated genes

(Figure 4B), and the rest are downregulated (Figure 4B). Interestingly, the two group of each gene family are distributed in whole *M. truncatula* genome (from the chromosome 1 to 8). As an example, the chromosome 3 contain upregulated genes as well as down regulated genes from the two families of enzymes. The phylogenetic result indicates that the evolution of PCs biosynthetic genes results from gene duplication and transfer, which explaining the presence of similar up-regulated genes in various chromosomes.

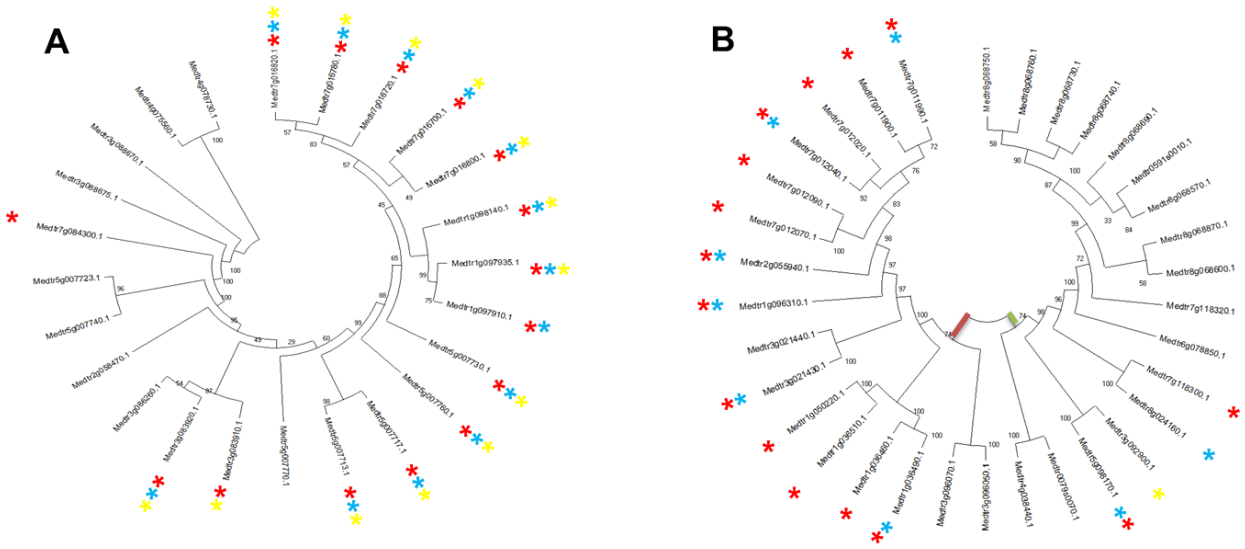

Figure 4. phylogenetic tree of two upregulated enzymes families. (A) phylogenetic tree of chalcone stilbene synthase with 24 orthologs in plant M. truncatula. (B) phylogenetic tree of caffeic acid O methyltransferase with 33 orthologs in plant M. truncatula. The phylogenetic tree was obtained using the MEGA 5.0, a Bootstrap analysis was conducted with 1000 replicates.

5. PCs biosynthetic up-regulated genes share similar sequence in the putative promoter location

To identify potential cis-regulatory element between PCs biosynthetic genes, the 500 nucleotides up-stream the 5'untranslated region was compared between three different genes involved in PCs metabolisms and up-regulated in studied mutants: two CS/SS and one Pathogenesis-related Protein 10. The alignment of three nucleotide sequences shows a higher degree of conservation between the three gene sequences in different point of up-stream 5'-UTR region (Figure 5). This result supports the conclusion that probably there is one group of transcription factors that regulate the expression of the two gene families. As an example, the

MYB TF family have been reported its role in the regulation of PCs as well as PR10 genes on plants [107] [93].

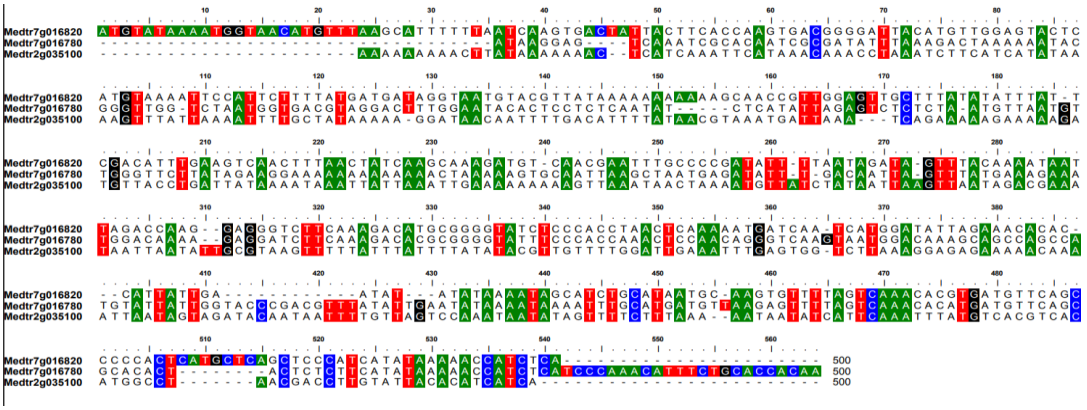

Figure 5. Pre analysis of cis element regulatory in the 500-nucleotides upstream region of three different genes. The comparison of two chalcone synthase sequences (Medtr7g016820, Medtr7g016780) and one PR10 sequence (Medtr2g035100).

6. The symbiont of M. truncatula is more sensitive to PCs than endophytic bacteria

In order to study the physiological function of PCs during the nodule immune response, antimicrobial test was carried out using four PCs including Ga, Ru, Qu, and Va were tested on five endophytic bacteria, M17, M18, BT37, M50, M67, or on *Sinorhizobium medicae WSM419*, the symbionte of *M. truncatula*.

No inhibition profile of PCs on the five studied endophytic bacteria was observed, by contrast, *S. medicae WSM419* show differential inhibition across the four PC, interestingly, the higher inhibition is observed when the gallic acid was used (Figure 6).

Taken together, these results indicate that the *M. truncatula* maybe produce PCs during immunity response to eliminate the bacteroid within nodule.

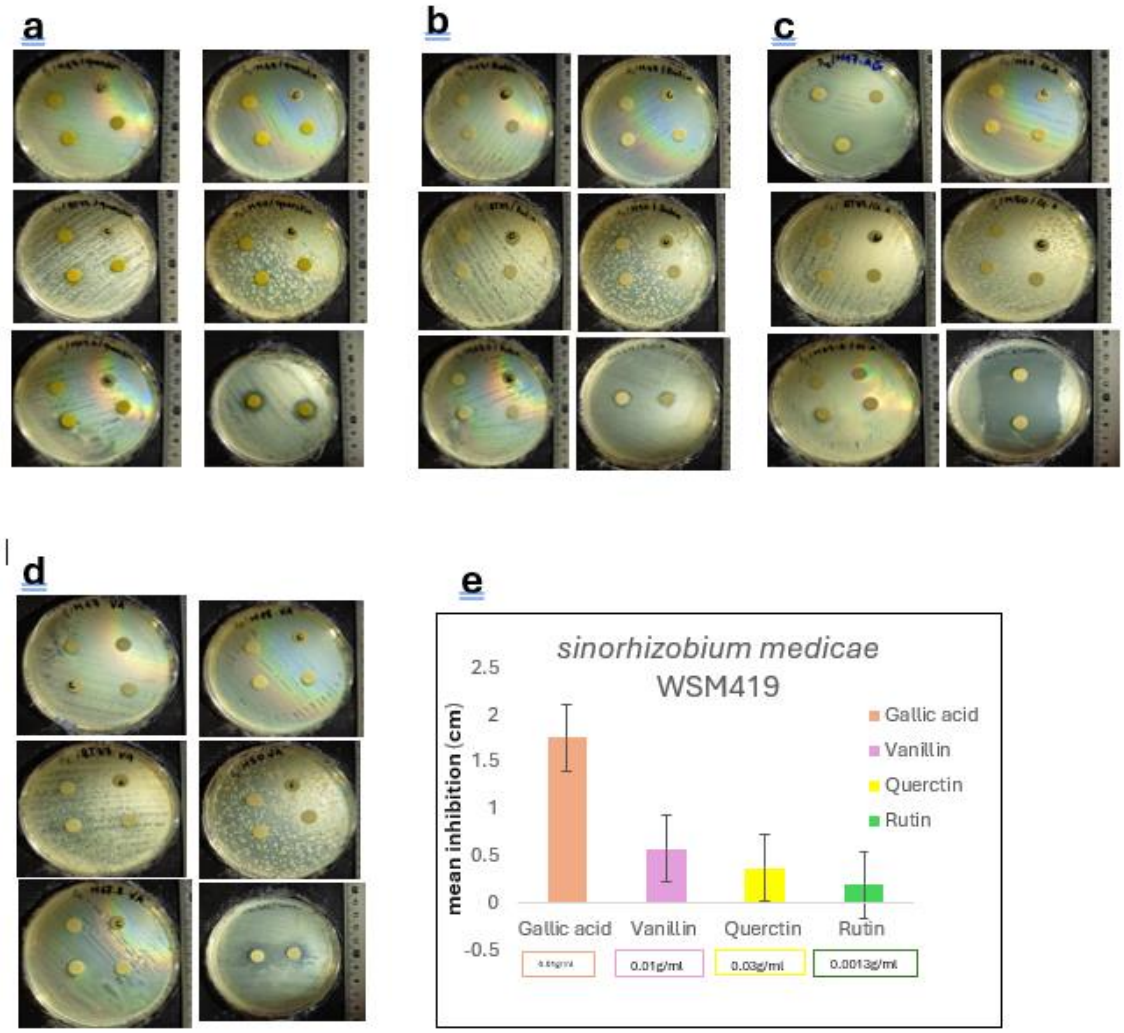

Figure 6. analysis of bacterial sensibility to different PCs. Pictures showing the inhibition zone of four PCs against six bacterial strains (M17, M18, BT37, M50, M67, WSM419) tested by disc diffusion method. (A), (b), (c) and (d) correspond respectively to quercetin, rutin, gallic acid and vanillin. (e) Measured inhibition profile of four PCs against the WSM419 bacteria

Conclusion

In this work we study the PCs synthesis during nodule defense response and the role of PCs during the defences. We find the presence of a common genetic network controlling the PCs production during nodule defences. This network seems to be controlled by common transcription factors and involved a specific group of genes. Finally, the produce PCs inhibits *S. mediacae* growth, indicating a role of these compounds during nodule defences.

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