

Understanding the molecular dialog between arbuscular mycorrhizal fungi and non-legume plants

Ariane Girardin

▶ To cite this version:

Ariane Girardin. Understanding the molecular dialog between arbuscular mycorrhizal fungi and non-legume plants. Cell Behavior [q-bio.CB]. Université Paul Sabatier - Toulouse III, 2017. English. NNT: 2017TOU30371. tel-03081199

HAL Id: tel-03081199 https://theses.hal.science/tel-03081199

Submitted on 18 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Université de Toulouse



En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par : Ariane Girardin

Le lundi 4 décembre 2017

Titre :

Understanding the molecular dialog between arbuscular mycorrhizal fungi and non-legume plants

ED SEVAB : Interactions plantes-microorganismes

Unité de recherche :

Laboratoire des Interactions Plantes Micro-organismes (LIPM)

Directeur(s) de Thèse :

Benoit Lefebvre, Chargé de Recherches INRA (Toulouse)

Rapporteurs :

Natalia Requena, Professeure de l'institut technologique de Karlsruhe (Allemagne) Pierre-Emmanuel Courty, Chargé de Recherches INRA (Dijon)

Autre(s) membre(s) du jury :

Matthieu Arlat, Professeur de l'Université Toulouse 3 Paul Sabatier



Université de Toulouse







En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Présentée et soutenue par : Ariane Girardin

Le lundi 4 décembre 2017

Titre :

Understanding the molecular dialog between arbuscular mycorrhizal fungi and non-legume plants

ED SEVAB : Interactions plantes-microorganismes

Unité de recherche :

Laboratoire des Interactions Plantes Micro-organismes (LIPM)

Directeur(s) de Thèse :

Benoit Lefebvre, Chargé de Recherches INRA (Toulouse)

Rapporteurs :

Natalia Requena, Professeure de l'institut technologique de Karlsruhe (Allemagne) Pierre-Emmanuel Courty, Chargé de Recherches INRA (Dijon)

Autre(s) membre(s) du jury :

Matthieu Arlat, Professeur de l'Université Toulouse 3 Paul Sabatier

Les racines ont des oreilles

Pour Aurélien, Aude, Franck et les séminaires internes du LIPM !

Qui n'a jamais rêvé d'avoir dans son jardin de belles cerises ? Avez-vous cependant pensé aux mycorhizes ? Ces petits champignons qui à la fois colonisent Le sol et les racines quand c'est la crise. Quand le besoin de trouver phosphate, eau et nitrate Se fait sentir pour la croissance de vos aromates Ils cherchent alors un associé en toute hâte. Cet associé est un champignon endomycorhizien. Le partenariat plante-champignon est vraiment très ancien, Il remonte à 400 millions d'années au moins ! Les théories scientifiques suggèrent Que ces champignons ont aidé les plantes à coloniser la Terre. En effet ce partenariat implique plus de 80 % des plantes terrestres, Qu'elles soient cultivées, sauvages ou sylvestres. Dans cette association, il n'y a pas de perdant, c'est une symbiose. Son nom de symbiose endomycorhizienne à arbuscule s'impose Puisque le champignon dans les racines forme de petites structures Qui ressemblent à des arbres. C'est le siège des échanges, pour sûr ! La plante donne au champignon des sucres tels que le glucose. En retour, le champignon lui fournit des nutriments. C'est la moindre des choses ! Il est clair que toute bonne association Nécessite une part de discussion Pour éviter une confusion. Au début de leur relation, La plante reconnait et accepte le champignon. Il ne faudrait pas qu'elle se leurre, Laisse entrer un pathogène et meure. Vous connaissez l'expression « les murs ont des oreilles » ? Eh ben pour les racines, c'est pareil ! Pour être capable de bien recevoir, Il faut tout d'abord percevoir L'inconnu qui se présente poliment Avec des molécules tout simplement.

Dans ma thèse, j'essaie de déterminer Comment plantes et champignons peuvent dialoguer. La génétique des champignons étant très compliquée Ma stratégie, du côté plante s'est orientée. Je tente de trouver les récepteurs dans les racines C'est-à-dire leurs oreilles qui captent les signes Des bons champignons qui les avoisinent. Et telle la reine de cœur dans Alice au pays des merveilles, Des racines, je coupe les oreilles ! Enfin pas vraiment ! En réalité, Je cherche à les muter. Je cherche à empêcher les plantes de comprendre les champignons. Si elles deviennent sourdes, c'est tout bon ! Entendez-moi bien, si j'ai ciblé le bon récepteur, Les plantes ne reconnaitront plus leurs champignons bienfaiteurs. J'espère que cette histoire ne s'arrêtera pas là, Et que ma modeste participation nous permettra De mieux comprendre les mécanismes du partenariat Que je viens de vous décrire. Penser maîtriser cette symbiose à plus long terme peut prêter à sourire Cette perspective est toutefois intéressante pour diminuer à l'avenir L'apport d'intrants Dans les champs.

> Moralité : Retenez que plantes et champignons sont bien malins Car en temps de disette, ils partagent leurs biens.

> > Ariane

Acknowledgments / Remerciements

First, I thank Prof. Natalia Requena and Dr. Pierre-Emmanuel Courty to have accepted to read this manuscript and to evaluate my work. I thank Prof Matthieu Arlat to have accepted to be part of the jury that will examine my PhD thesis. I am also grateful to Didier Reinhardt and Christophe Roux. They were part of my PhD committee and I really enjoyed the scientific discussion that enriched me during these two meetings.

I apologize to not-speaking French fellows as I will do the rest of the acknowledgments in French...

Je voudrais ensuite remercier Benoit Lefebvre qui a dirigé ma thèse. Merci à toi de m'avoir accordé ta confiance et de m'avoir permis de faire cette thèse. Je te remercie de m'avoir accompagnée, formée et conseillée durant ces quasis 4 ans passés au LIPM, même si j'avoue avoir parfois eu l'impression de me noyer sous l'avalanche d'idées au début de ma thèse ! Je te suis aussi très reconnaissante de m'avoir permis de montrer mon travail en congrès dès ma première année de thèse. J'ai adoré cet exercice, et j'ai tout autant apprécié de pouvoir repartir les deux années suivantes présenter mon travail en congrès. Tu m'as aussi permis de m'investir en dehors de ma thèse dans la vie du labo (création de l'AJS, organisation des 8^{èmes} journées des doctorants SPE, etc.). Enfin, je te remercie (et ce n'est pas le moindre des remerciements !) d'avoir relu et corrigé mon manuscrit de thèse.

Je remercie Marie Cumener qui m'a appris toutes les techniques de biochimie présentes dans ce manuscrit. Tu avais déjà des résultats montrant que BdLYR1 lie les LCOs. Je te remercie aussi de m'avoir offert ton amitié et m'avoir bien facilité la vie pendant mon stage. Et oui, l'air de rien, c'était un luxe de profiter de ta voiture pour faire les courses ! Je profite d'être dans la partie bioch pour remercier Virginie Gasciolli pour son soutien technique, toujours dans la bonne humeur, ainsi que Jean-Jacques Bono, grand maître du skatron et de l'HPLC (il arrive toujours à les remettre en marche...) pour ses conseils avisés pour l'interprétation des expériences de binding à l'équilibre. Je remercie aussi Christine qui a vraiment participé au bon déroulement de ma thèse par son écoute attentive et sa bienveillance. Bien sûr, je remercie aussi le reste des membres de l'équipe (présents et passés) : les non-perms Luis, Claudia, Mégane et Tongming et les permanents Clare, Julie, Sylvie, Sandra, Nicolas, Chrystel, Fréderic, Charles et Fabienne, gardienne du stock des COs et LCOs. J'aimerai particulièrement remercier Alexandra Henocq et Paul Gomez qui m'ont bien aidée dans mes manips lors de leurs stages.

Toujours au labo, j'aimerai particulièrement remercier Céline Remblière pour son investissement lors les campagnes de récolte d'embryons immatures de brachy et du reste de la transformation de brachy. Par la même occasion, je remercie Oumaya Bouchkabé-Coussa et Camille Soulat de l'IJPB de Versailles pour m'avoir montré la technique de prélèvement des embryons de *B. distachyon* et donné des conseils sur la transfo.

Je tiens particulièrement à remercier tous les membres du bureau de l'AJS. Ce fut une bonne expérience que de participer à la création de cette association. J'espère qu'elle perdurera dans le temps. Merci aussi à Vincent, Lucas, Pierre, Léa, Aude et Thomas. Ce fut un plaisir d'organiser les 8^{èmes} journées des doctorants SPE avec vous !

Je remercie tous les membres du LIPM. Je ne peux pas tous vous citer, mais travailler et interagir avec vous fut un plaisir.

J'aimerai remercier aussi Jérôme Crouzet qui travaille au Laboratoire de Stress, Défenses et Reproduction des Plantes (SDRP) de l'université de Reims. C'est grâce à toi que j'ai découvert le monde de la recherche lors de mon stage de M1 et surtout, tu m'as incitée à partir à Toulouse.

Je remercie mes amis, qu'ils aient été mes compagnons de thèse, Aude, Franck, Monica (ma colloc !) et Lucie ainsi que toute la bande de Reims et Sacha. Vous m'aurez bien soutenue.

Enfin, je remercie ma famille : Christophe et Annie mes parents, qui m'ont toujours encouragée et soutenue pour mes études comme dans la vie. Vous êtes d'une grande inspiration pour moi. Merci à Félix, mon frère. Je remercie aussi Aurélien, mon compagnon, ce ne fut pas facile tous les jours mais tu as été d'un soutien sans failles. Sans vous cette thèse n'aurait pas eu lieu.

Je souhaite aussi remercier la Région Occitanie et le département Santé des Plantes et Environnement (SPE) de l'INRA d'avoir financé mon doctorat.

Table of contents

Ma thèse en 180 se	econdes	4
Acknowledgments	/ Remerciements	6
Table of contents		9
List of figures		10
List of tables		13
Abbreviations		14
Introduction		17
Review		19
About Root endo-s	symbioses establishment	79
Objectives of my th	nesis	87
Chapter 1:	<i>SILYK10</i> regulates the AMS in tomato	89
Chapter 2:	SILYK10 binds LCOs with high affinity	113
Chapter 3:	AMS LysM-RLKs might have been directly recruited for the	e RLS 139
Discussion and	perspectives	163
Discussion		164
Short term perspec	ctives:	
Long term perspec	tives	
Conclusion		
Annexes		
Annex 1: B. distach	<i>ayon</i> genome editing by CRISPR-Cas9	
Annex 2: Genotyp	ing and phenotyping of putative <i>Bdlyr1</i> and <i>Bdlyk2</i> found	by TILLING 193
Annex 3: CDS of Ta	ILYR1A, TaLYR1B and TaLYR1D	
Annex 4: Protocol o	of Cross-linking between BdLYR1 and the photoactivatable LC	0 196
Résumé		
Abstract		

List of figures

Introduction

Fig. 1 Fig. 2	LysM-RLK and LysM-RLP structure, synthesis and maturation. Peptidoglycan (PGN), chitooligosaccharides (CO4 & CO8) and lipo-chitooligosacch	p.21 1aride
(LCO-I	V) schematic structures	p.24
Fig. 3	PhyML phylogenetic tree of the LYM.	p.29
Fig. 4	PhyML phylogenetic tree of the LYR.	p.34
Fig. 5	Syntenic localization of the members of the phylogenetic groups LYRIA (red), L	YRIIIB
(pink)	and LYRIIIA (blue).	p.38
Fig. 6	PhyML phylogenetic tree of the LYK.	p.41
Fig. 7 /	Amino acid sequence alignment of the members of the phylogenetic group LYKI	p.45
Fig. 8	LysM-RLK and/or LysM-RLP heterodimers.	p.49
Fig. 9	The hyphal network of AMF explores a larger soil area than plant roots.	p.79
Fig. 10) Steps of AMS establishment.	p.81
Fig. 11	L Arum and Paris type of root colonization.	p.81
Fig. 12	2 RLS establishment	p.83
Fig. 13	3 Common Symbiosis Signaling Pathway components and model.	p.84
Fig. S1	L MrBayes phylogenetic tree of the LYM.	p.71
Fig. S2	2 MrBayes phylogenetic tree of the LYR.	p.72
Fig. S3	3 MrBayes phylogenetic tree of the LYK.	p.73
Fig. S4	A MrBayes phylogenetic tree of the LYR using Gblock.	p.74

<u>Chapter 1</u>

Fig. 1 SICCaMK is essential for arbuscular mycorrhiza (AM) at 2 wpi with H	Rhizophagus
irregularis	p.94
Fig. 2 SICCaMK is essential for plant symbiotic gene expression at 2 wpi with H	Rhizophagus
irregularis.	p.95
Fig. 3 SILYK10 is the Medicago truncatula MtNFP ortholog.	p.96
Fig. 4 SILYK10 is essential for arbuscular mycorrhiza at 2 wpi with Rhizophagus irr	egularis
	p.97

Fig. 5 Strong silencing of SILYK10 correlates with the absence of fungal colonization and plant
symbiotic gene expression at 2 wpi with <i>Rhizophagus irregularis</i> p.98
Fig. 6 Silencing of SILYK10 reduces arbuscular mycorrhiza (AM) at 4 and 6 wpi with
Rhizophagus irregularisp.99
Fig. S1 Parameters tested to set up virus-induced gene silencing (VIGS); Si-Fi predictions.
p.105
Fig. S2 Tomato lysin motif receptor-like kinase (LysM-RLK) sequence identities; phylogenetic
tree based on extracellular regionsp.106
Fig. S3 Alignments of tomato LysM-RLK intracellular and extracellular regionsp.107-108
Fig. S4 Synteny of Solanum lycopersicum locus containing SILYK10; expression pattern of
<i>SILYK10</i> p.109
Fig. S5 Analysis of arbuscular mycorrhiza (AM) in SILYK10-silenced plants at 2 wpi with
Rhizophagus irregularisp.110
Fig. S6 Analysis of arbuscular mycorrhiza (AM) in SILYK10-silenced plants at 4 wpi with
Rhizophagus irregularis; quantification of Tobacco rattle virus (TRV)p.111

<u>Chapter 2</u>

Fig. 1 The proSILYK10::GUS fusion is mainly expressed in the epidermis and the outer co	rtex
before AMF colonization and in arbuscule-containing cells during colonizationp.	119
Fig. 2 SILYK10 is localized in the plasma membranep.	121
Fig. 3 SILYK10 has a high affinity for LCOsp.	122
Fig. 4 SILYK10 is selective for LCOs vs COsp.	123
Fig. S1 SILYK10 belongs to a phylogenetic group of LysM-RLKs that contains one gene in n	nost
plant species, two genes in most legumes and none in <i>Brassicaceae</i> p.	134
Fig. S2 The ProSILYK10::GUS fusion is mainly expressed in the epidermis and the outer co	rtex
p. p.	135
Fig. S3 In tomato SILYK10:YFP is localized in the plasma membranep.	136
Fig. S4 In N. benthamiana SILYK10c:YFP and SILYK7c:YFP are partially localized in the place	sma
p.	137

Chapter 3

Fig. 1 BdLYR1 has a high affinity for LCOs and is selective for LCOs versus COs.	p.145
Fig. 2 Bdlyr1-1 is able to establish the AMS.	p.146
Fig. 3 SILYK10 and BdLYR1 interact with MtLYK3 and activate the CSSP in <i>M. ti</i>	runcatula.p.148
Fig. 4 The promoter of <i>SILYK10</i> is transcribed in nodules in <i>M. truncatula</i> .	p.149
Fig. S1 Complementation of <i>Mtnfp-2</i> by <i>PsSYM10</i> and <i>BdLYR1</i> conserves host s	specificity.p.159
Fig. S2 BdLYR1 ¹⁻⁴⁶⁶ does not interact with MtLYK3 in <i>N. benthamiana</i> .	p.160

Discussion and perspectives

Fig. 1 BdLYR1 binds LCOs with different affinity depending on the LCO structure.	p.165
Fig. 2 Amino acid sequences of MtNFP and its orthologs show low conservation, e	specially in
the ECR	p.169
Fig. 3 BdLYR3 preferentially binds LCOs versus COs.	p.171
Fig. 4 LCOs induce lateral root growth in <i>B. distachyon</i> Bd21-3.	p.173
Fig. 5 PhLYK10 interacts with MtLYK3 in <i>N. benthamiana</i> .	p.175
Fig. 6 TaLYR1 binds LCOs with high affinity and selectivity versus COs.	p.180

<u>Annexes</u>

Fig. 1 DNA editing by CRISPR-Cas9.	p.186
Fig. 2 Representation of pRGEB32 used for genome edition of <i>B. distachyon</i>	p.187
Fig. 3 How to select an immature embryo of <i>B. distachyon</i> ?	p.189
Fig. 4 Dried calli.	p.190
Fig. 5 Transformation steps from selection to regeneration.	p.191

List of tables

Introduction

Table 1	p.56
Table S1	p.75-78
Chapter 1	
Table S1 Primers used for cloning.	p.103
Table S2 Gene accession numbers.	p.104
Chapter 2	
Table S1 List of primers used.	p.138
Chapter 3	
Table S1 List of primers used.	p.161
Annexes	
Annex 1 – Table 1 CIM medium.	p.189
Annex 1 – Table 2 Co-culture medium.	p.190
Annex 1 – Table 3 Selection medium.	p.191
Annex 1 – Table 4 Regeneration medium.	p.192
Annex 1 – Table 5 rooting medium.	p.192
Annex 2 – Table 1 Genotyping and phenotyping of <i>B. distachyon</i> mutageneized	d linesp.193

Abbreviations

All the abbreviations used in this manuscript are explained in the text or figure legends. In this section, I have included the abbreviations that I most frequently used. In the main text, the amino acids are abbreviated according to the international code with a single letter. Proteins are indicated in capitals with just before the first letters of the gender and species of the organism (example: SILYK10), genes are indicated in italicized capitals (example: *SILYK10*) and mutants are indicated in italicized lower case (example: *SILyK10*).

AMF: Arbuscular Mycorrhizal Fungus-i LjLYS11: Lotus AMF: Arbuscular Mycorrhizal Fungus-i Receptor-like Kinas	<i>japonicus</i> Lysin Motif		
Receptor-like Kinas			
AtCERK1: Arabidopsis thaliana Chitin	se 11		
Elicitor Receptor Kinase 1 LjNFR1: Lotus	japonicus Nod-Factor		
BdLYR1: Brachypodium distachyon Lysin	ignonicus Nod Factor		
Cos0: CDISDD associated protein 0 Receptor 5	Juponicus Nou-Factor		
LRR: Leucine Rich F	Repeat		
CDS: Coding sequence	LvsM: Lvsin Motif		
COs: chitooligosaccharides	otif Recentor-like Kinase		
CRISPR: Clustered Regularly Interspaced MAMPs: Microbe	-Associated Molecular		
CSSP: Common Symbiosis Signalling Patterns			
Pathway MST: Microscale Tl	hermophoresis		
ECR: Extracellular Region MTI: Microbe-Trigg	gered Immunity		
ER: Endoplasmic Reticulum MtLYK3: Medicago	o <i>truncatula</i> Lysin Motif		
ETI: Effectors-Triggered Immunity Receptor-like Kinas	se LYK3		
GFP: Green Fluorescent Protein MtLYR3: Medicago	o truncatula Lysin Motif		
GlcNAc: N-acetyl Glucosamine			
GPI: Glycosylphosphatidylinositol Perception			
ICR: Intracellular Region OsCEBIP: Oryzae	<i>sativa</i> Chitin Elicitor		
IT: Infection Thread Binding Protein			
ITC: Isothermal Titration Calorimetry PaNFP: Parasponic	PaNFP: Parasponia andersonii Nod-Factor		
KO: Knock-out Perception			

PM: Plasma Membrane	RNS: Root Nitrogen fixing Symbiosis
PGN: Peptidoglycan	ROS: Reactive Oxygen Species
Pro35S: Promoter 35S of Cauliflower mosaic virus	SILYK10: <i>Solanum lycopersicum</i> Lysin Motif Receptor-like Kinase 10
RFP: Red Fluorescent Protein	SP: Signal Peptide
RLS: Rhizobium Legume Symbiosis	TM: Trans-membrane domain
RLK: Receptor-like kinase	VIGS: Virus Induced Gene Silencing
RLP: Receptor-Like Protein	Wpi: week(s) post-inoculation
RLS: Rhizobium Legume Symbiosis	WT: Wild Type
RNAi: RNA interference	YFP: Yellow Fluorescent Protein

Introduction

Preamble

Most of the introduction of this PhD manuscript is written as a review that we plan to submit for publication. I added a part to describe more in details the arbuscular mycorrhizal symbiosis (AMS) and the rhizobium-legume symbiosis (RLS).

The aim of our review was to summarize all information from the literature concerning biological roles and biochemical properties of the Lysin Motif Receptor-Like Kinases/Proteins (LysM-RLKs/Ps) and to propose a nomenclature taking into account the diversity of LysM-RLKs/Ps in various plant species. We built phylogenetic trees of LysM-RLKs/Ps from various legumes, non-legumes dicots and monocots. We put a particular emphasis on the LysM-RLKs/Ps biochemical properties discussing their affinity for ligands in the light of the different methods used to measure it.

This review was a collaborative work with Luis Buendia who wrote the section on LYKs and made the figures, Tongming Wang who wrote the section on LYMs, and Ludovic Cottret who built the phylogenic trees. I wrote the section on LYRs and did the last updates.

LysM Receptor-Like Kinase and Receptor-Like Protein families: an update on phylogeny and functional characterizations.

Luis Buendia, Ariane Girardin, Tongming Wang, Ludovic Cottret and Benoit Lefebvre LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

<u>Abstract</u>

Lysin Motifs (LysM) are known to bind N-Acetyl Glucosamine (GlcNAc) containing molecules. Plant specific families of Receptor-Like Kinases (RLKs) and Receptor-Like Proteins (RLPs), containing 3 extracellular LysM have been shown to directly bind and/or to be involved in perception of lipo-chitooligosaccharides (LCO), chitooligosaccharides (CO) and peptidoglycan (PGN), three types of GlcNAc-containing molecules produced by microorganisms. These receptors are involved in microorganism perception by plants and can activate opposite plant responses leading either to symbiosis establishment or to defense responses against pathogens. LysM-RLK/Ps belong to multigenic families. Here, we provide a phylogeny of these families in 8 plant species including dicots and monocots and we discuss known or putative roles of the members in each of the identified phylogenetic groups. We also report in details and discuss the known biochemical functions of the LysM-RLK/Ps.

Abbreviations

AMF: Arbuscular Mycorrhizal Fungi; AMS: Arbuscular Mycorrhizal Symbiosis; ER: Endoplasmic Reticulum; ECR: Extracellular Region; GPI: Glycosylphosphatidylinositol; ICR: Intracellular Region; IT: Infection Thread; ITC: Isothermal Titration Calorimetry; LysM: Lysin Motif; MST: Microscale Thermophoresis; RL: Rhizobium Legume; RLK: Receptor-like kinase; RNS: Root Nitrogen fixing Symbiosis; SP: Signal Peptide. TM: transmembrane domain

Introduction

Plant receptor-like kinases and receptor-like proteins

Receptor-like kinases (RLKs) are cell surface proteins found in several eukaryotic organisms. They are transmembrane proteins with an Extracellular Region (ECR) containing a sensor domain, a transmembrane domain (TM) and an Intracellular Region (ICR) containing kinase domain participating to signal transduction (Fig. 1A). RLKs are involved in sensing the extracellular environment. They are found also in animals, but their number is particularly high in plants in which they have been mainly described to be involved in discrimination between beneficial and pathogenic microbes (for review Antolín-Llovera et al., 2012) and in cell/organ communication (for review Hazak and Hardtke, 2016, Belkhadir et al., 2014). Several RLKs were also shown to play a role during abiotic stresses (for review Ye et al., 2017). Plant RLKs are divided in subfamilies depending on their ECR (Shiu and Bleecker, 2003). Among these families, one bears three Lysin motifs (LysM) on the ECR. This subfamily is the main subject of this review.

Plant RLKs have a kinase domain belonging to the serine/threonine kinase family. However, it was shown that in addition to phosphorylate S/T residues, several plant RLKs can phosphorylate tyrosine residues (Klaus-Heisen et al., 2011, Oh et al., 2009). In various plant RLK subfamilies, several classes of kinase domain can be found. The canonical form contains a catalytic aspartate residue preceded by an arginine which is called RD kinase. A variant kinase domain found in plant RLKs lacks the R preceding the catalytic D and for this reason it is called non-RD kinase. Several non-RD kinases appear to have relatively weak kinase activity *in vitro* compared to the RD kinases, and their kinase activity is partially dispensable for their function (For review Schwessinger and Ronald, 2012). Kinase domain contains other essential conserved features among which a glycine-rich loop involved in nucleotide binding. Other variant kinase domains found in plant RLKs are lacking conserved features such as the glycinerich loop and do not exhibit auto-phosphorylation activity *in vitro*. The latter are called deadkinases.



Fig. 1 LysM-RLK and LysM-RLP structure, synthesis and maturation. A) Receptor-like kinases (RLKs) are produced by ribosomes associated with the Endoplasmic Reticulum (ER). The extracellular region (ECR) preceded by a signal peptide (SP) is translocated into the ER lumen during the translation until the transmembrane domain (TM, brown) is inserted in the lipid bilayer. The intracellular region (ICR) is then produced in the cytosol. SP is cleaved in the ER and the mature protein is transported through the secretory pathway to its final destination, mainly the plasma membrane. Receptor-like proteins (RLPs) are also produced by ribosome associated to the ER. After translocation, the SP is cleaved and the ECR is transferred to a glycosylphosphatidylinositol (GPI) anchor. The mature protein is then transported to its final destination. **B**) LysM-RLKs are composed of 3 lysin motifs (LysM, orange) in the ECR, a TM (brown) and an ICR bearing an active kinase (beige, LYK subfamily) or an inactive kinase (grey, LYR subfamily). LysM-RLPs (LYM) are composed of 3 LysM in the ECR attached to a GPI anchor. **C-D**) AtCERK1 3D structure resolved by (Liu et al., 2012b). Images were produced using the pdb file 4EBY: α -helices are indicated in pink, β -strands are indicated in yellow and cysteines are indicated in green. **C**) Orientation of AtCERK1 ECR highlighting the 3 LysMs (circled) packed together. **D**) Orientation

Another family of plant cell-surface proteins contains ECRs similar to those of RLKs but lacks ICR. Among these proteins, called Receptor-Like Proteins (RLPs), some contain only the ECR while others are produced as an ECR followed by a TM (Fig. 1A). The latter class of RLP can be further processed by cleavage of the ECR from the TM and transfer to a membrane embedded Glycosylphosphatidylinositol (GPI) anchor.

Plant RLKs and GPI-anchored RLPs are mainly found at the Plasma Membrane (PM), although they transiently accumulate in internal compartments of the secretory or endocytic pathways during their life cycle. Indeed, as integral surface proteins, they are produced in the Endoplasmic Reticulum (ER). RLKs are type I transmembrane proteins. They bear a Signal Peptide (SP) at their N-terminus (Fig 1A) allowing translocation of the ECR in the ER lumen during protein synthesis. SP is then cleaved, TM is embedded in the ER membrane and the RLKs follow the secretory pathway to the PM. From the PM they can be internalized after ligand perception through the endocytic pathway and they are ultimately degraded in the lytic vacuole (For review Beck et al., 2012).

Lysin motif receptor-like kinases

Lysin Motif Receptor-Like Kinases (LysM-RLKs) and Lysin Motif Receptor-Like Proteins (LysM-RLPs) are subfamilies of plant RLK/Ps which contains three LysM in their ECR (Fig. 1B). LysM is a ubiquitous protein domain of about 40 Amino Acids (AA) and found in most of the living organisms except in Archaea (Buist et al., 2008). Its name originates from its identification in bacterial autolysin proteins that hydrolyse bacterial Peptidoglycan (PGN) and lead to cell lysis. Although not conserved in term of primary sequence, LysM have highly conserved secondary and tertiary structures consisting in two α -helices stacking onto two antiparallel β -sheets as determined by X-Ray or NMR (Bateman and Bycroft, 2000, Bielnicki et al., 2006, Liu et al., 2012b, Sánchez-Vallet et al., 2013, Maxwell et al., 2013, Mesnage et al., 2014, Wong et al., 2014, Leo et al., 2015, Koharudin et al., 2015, Liu et al., 2016). Highly conserved cysteine (C) pairs separated by one AA (CXC) are found between the LysMs of all LysM-RLK/Ps. This C pairs are involved in disulfide bridges (Lefebvre et al., 2012, Liu et al., 2012b, Liu et al., 2016, Bozsoki et al., 2017) that pack together the three LysMs (Fig. 1C-D).

Two types of plant LysM-RLKs can be defined based on their kinase domains. The first type, named LYK (Fig. 1B), has a canonical RD kinase and shows *in vitro* autophosphorylation activities (Arrighi et al., 2006, Petutschnig et al., 2010, Madsen et al., 2011, Zeng et al., 2012). The second type, named LYR (Fig. 1B), carries an aberrant kinase lacking some conserved features such as the glycine-rich loop, and does not exhibit neither auto-phosphorylation nor trans-phosphorylation activities when tested *in vitro* (Arrighi et al., 2006, Madsen et al., 2011). Membrane anchored LysM-RLPs are also found in plants and are named LYM (Fig. 1B).

In this review, we summarize the currently known biological roles and biochemical functions of plant LysM-RLK/Ps. Most of the LysM-RLK/Ps that have been studied were shown to perceive structurally related N-Acetyl glucosamine (GlcNAc) containing molecules and/or to be involved in plant-microbe interactions including the establishment of defense responses or root endosymbioses.

Microbe-triggered immunity

One layer of plant defense against pathogenic microbes involves perception by plants of conserved microbial signatures also called Microbe-Associated Molecular Patterns (MAMPs), and consequently induction of the Microbe-Triggered Immunity (MTI). The defense responses mainly consist in basal resistance mechanisms such as cell wall reinforcement, stomatal closure and synthesis of antimicrobial compounds that can lead in some conditions to cell death. Many plant RLKs are involved in MAMP perception and signaling (for review Schwessinger and Ronald, 2012). Because MAMPs are conserved microbial signatures, they are not specific to pathogens but are also present in beneficial microbes. Pathogen specific signatures can also been perceived by plants. In most cases, these specific signatures are proteins called effectors. They are produced by pathogens to manipulate plant defense mechanisms or plant metabolism to promote the infection. Recognition of these proteins induces the Effectors-Triggered Immunity (ETI) that in most cases leads to cell death. In contrast to MTI, ETI is a pathogen-specific response. Few plant RLKs are involved in pathogen-specific signature perception (for review Schwessinger and Ronald, 2012).

Plant treatments with various MAMPs induce similar responses (such as alkalinisation of extracellular medium, Reactive Oxygen Species (ROS) production, MAP kinase phosphorylation and induction of defense-related gene transcription). These responses have been used to identify and characterize MAMPs. Chitin fragments are typical fungal MAMPs. Chitin is a long-chain GlcNAc polymer which is the major component of fungal cell walls.

Although chitin is insoluble, chitooligosaccharides (COs) are GlcNAc oligomers (Fig. 2) soluble at least up to a degree of polymerization of 8 GlcNAc residues. COs can be produced by chitin cleavage through the action of plant secreted chitinase. Chitin and COs are sometimes used indiscriminately in literature leading to confusion. For this reason, here we refer to chitin as long insoluble polymers and we also mention the degree of polymerization of CO (i.e. CO8 for 8 GlcNAc oligomers). CO8 have been shown to be the most active oligomer among COs for activation of the defense-related responses. PGN fragments are typical bacterial MAMPs. PGN is a major component of the bacterial cell walls. PGN is a polymer alternating GlcNAc and Nacetylmuramic acid (MurNAc) residues, branched with AA. As chitin, PGN is insoluble, while muropeptides (Fig. 2) are soluble PGN fragments. Chitin and PGN fragments are both perceived by LysM-RLK/Ps as detailed below.



Many other MAMPs do not contain GlcNAc. One of the best characterized bacterial MAMP is the flagellin. Various flagellin peptides are perceived by RLKs in animals and plants (for review Fliegmann and Felix, 2016). The flg22 peptide (originally identified in *Pseudomonas aeruginosa*, (Felix et al., 1999)) is perceived in *Arabidopsis thaliana* by AtFLS2, a Leucine Rich Repeat Receptor-Like Kinase (LRR-RLK, Chinchilla et al., 2006). Another well characterized bacterial MAMP is the elf18 peptide found in the bacterial Elongation Factor (EF-Tu) which is perceived in *A. thaliana* by another LRR-RLK called AtEFR (Zipfel et al., 2006).

Interestingly microbes secrete proteins called effectors to counteract MAMP perception by plant RLKs. For example, the bacterial effector AvrPtoB is a multi-domain and multifunction protein produced by several *Pseudomonas syringae* pathovars. It is known to contain an E3 ubiquitin ligase domain. AvrPtoB is injected in plant cells through the type III secretion system and targets several plant RLKs involved in MAMP perception, leading to their degradation or inactivation (For review Macho and Zipfel, 2015). Other examples are the fungal secreted effectors Avr4 and Ecp6 from *Cladosporium fulvum*, which have high affinity for COs and which are able to avoid plant perception of fungal COs by competing with plant CO receptors (for review Sánchez-Vallet et al., 2015).

Root endosymbioses

Plants also interact with many beneficial microbes. Arbuscular Mycorrhizal Fungi (AMF) can colonize the roots of most terrestrial plants by establishing an extended hyphal network in soil and by providing plant with mineral nutrients collected in the soil. Nitrogen fixing bacteria called Rhizobia and Frankia are able to trigger the formation of particular plant root organs called nodules, in phylogenetically related legumes and actinorhizal plants respectively. Inside the nodules, N-fixing bacteria can efficiently reduce gaseous atmospheric nitrogen (N₂) to ammonia (NH₃). For this reason these bacterial genera are extremely important for plant nutrition. Despite the differences in the nature of the microorganisms involved, the Arbuscular Mycorrhizal Symbiosis (AMS) and the Root Nitrogen fixing Symbioses (RNS) share commonalities. The RNS are considered to originate from more ancient AMS. Notably, plant genes that control a signaling pathway, termed the Common Symbiosis Signaling Pathway (CSSP), are required for the establishment of both AMS and RNS. CSSP activation leads to the production and decoding of oscillations in the calcium concentration (also called calcium spiking) in and around plant cell nuclei. Genes that code for components of the CSSP are only found in plants that can establish at least one of these symbioses (Delaux

et al., 2014). In such plants, mutations in the CSSP genes lead to an absence of AMF penetration at the root epidermis (for review Gutjahr and Parniske, 2013). In legume and actinorhizal plants, mutations in the CSSP genes also block nodule development and bacterial colonization of plant roots (for review Svistoonoff et al., 2014).

Lipo-chitooligosaccharides (LCOs) also called Nod-factors, are well known to be rhizobial secreted molecules essential for bacterial recognition by legumes and subsequently for rhizobial root colonization. Rhizobial LCOs have a core structure of 4 or 5 GlcNAc residues in which the terminal non-reducing sugar is substituted with an acyl chain (Fig. 2). In addition, Rhizobial LCOs bear decorations that are characteristic of bacterial strains and important for host specificity (For review, Fliegmann and Bono, 2015). AMF also secrete LCOs similar to those produced by rhizobia (Maillet et al., 2011), as well as short COs (mainly CO4 and CO5, Fig. 2, (Genre et al., 2013)) that might correspond to LCO precursors. Exogenous application of LCOs or short COs activate plant responses such as extracellular medium alkalinisation (Felle et al., 1996, Staehelin et al., 1994), calcium spiking (Oldroyd et al., 2001, Harris et al., 2003, Sun et al., 2015), or promotion of lateral root development (Olah et al., 2005, Sun et al., 2015, Herrbach et al., 2017) in various plant species including nodulating and non-nodulating plants. These plant responses have been shown to be CSSP-dependent. In addition, regulation of symbiosis-related gene transcription by treatment with LCOs have been shown in legumes (Combier et al., 2008, Camps et al., 2015, Hohnjec et al., 2015) but not yet reported in nonlegumes. The CO4 and CO5 are referred as Myc-COs or short chain COs and are currently considered to play a role in AMS establishment in contrast to the long chain COs, CO7 and CO8 described to be defense elicitors.

Phylogenetic analysis

Methodology

Studies that deal with functional characterization of LysM-RLK/Ps have been performed only within few species but which represent the genetic diversity of higher plants. This includes dicotyledons (*Arabidopsis thaliana*, *Medicago truncatula*, *Lotus japonicus* and *Solanum lycopersicum*) and a monocotyledon (*Oryza sativa*). Although several LysM-RLK/P phylogenetic trees have been published (Arrighi et al., 2006, Zhang et al., 2009, Shimizu et al., 2010, Buendia et al., 2016, Zeng et al., 2012, Lohmann et al., 2010) none of those phylogenies include all the listed species at the same time. Moreover naming of the LysM-RLK/Ps has been made independently in each species making the comparison between species even more complicated.

To discuss the evolution of LysM-RLK/Ps in higher plants, we have inferred phylogenetic trees using phyML (Fig. 3-4 and 6) and MrBayes (Fig. S1-4). In addition to the species mentioned above, we used the sequences of two additional dicotyledons (Prunus persica and Brassica rapa) and one more monocotyledon (Brachypodium distachyon). We also included new sequences obtained in the last version of the L. japonicus and M. truncatula genomes. Furthermore, we performed manual correction of many gene structure predictions (see notes in Table S1). Phylogenetic trees were inferred independently with the predicted protein sequences of the LYMs (Fig. 3 and S1), LYRs (Fig. 4 and S2) or LYKs (Fig. 6 and S3). Some proteins identified as putative LysM-RLK/Ps were not used for the phylogenetic analysis because they were truncated or their sequence/existence was uncertain (these proteins are indicated in italic in Table S1). We focused our analysis on the membrane-anchored LysM-RLPs (LYM, coding sequences containing a TM) and we did not considered the soluble LysM-RLPs containing 3 LysMs (called LYPII in Zhang et al., 2009). We used PredGPI (<u>http://gpcr.biocomp.unibo.it/predgpi/pred.htm</u>) to identify the GPI anchor sites in the LYMs. Orthologous genes based on the phylogenetic trees are arranged in Table S1 in lane with a color code. To reinforce the ortholog discrimination, we reported the intron-exon structure in Table S1 which is almost conserved among all the orthologs. Minor differences might be due to evolutionary changes or to residual errors in gene structure predictions.

We found a high variability in the number of LysM-RLKs (5 to 22) and membraneanchored LysM-RLPs (2 to 5) between the 8 plant species analyzed, with an expansion of the LysM-RLKs in dicotyledons (excepted in the Brassicaceae) versus monocotyledons and an expansion of membrane-anchored LysM-RLPs in monocotyledons versus in dicotyledons. Legumes showed the highest number of LysM-RLKs and the lowest number of membraneanchored LysM-RLPs. Although the number of gene is highly variable, phylogenetic groups can be distinguished with members in almost all species. We distinguished 2 phylogenetic groups of LYMs, 2 phylogenetic groups of LYRs, and 2 phylogenetic groups of LYKs common to dicotyledons and monocotyledons. Two additional phylogenetic groups of LYRs and several subgroups were found only in dicotyledons. For practical reasons, we propose to name the phylogenetic groups LYM, LYR and LYK with one number and eventually a letter when subgroup can be distinguished (Table S1). Below and in Table S1, we also reported the nomenclature proposed by Zhang et al in 2009. Most phylogenetic subgroups have one member in all species with few exceptions of duplications in particular species. Two phylogenetic groups (LYRI and LYKI) have however encountered many duplication events that explain most of the variability in the number of LysM-RLKs between species.

No intron was found in the 5' part of the genes coding the 3 LysMs, in either LYR, LYK or LYM. In contrast, strong differences of intron number were observed in the sequence coding the extracellular juxta membrane region and the intracellular region (0/1 in LYRs, 3/4 in LYMs and 9/12 in LYKs). This suggests independent combination of the sequence coding the 3 LysMs with either one coding a dead kinase for LYR ancestor, one coding an active kinase for LYK ancestor and one coding a site for GPI anchoring for LYM ancestor. This might have happened at the early time of plant evolution. Indeed, members of the phylogenetic groups LYRI and LYKI can be found in more ancient plant genera (Zhang et al., 2009) such as Physcomitrella (bryophytes) and Selaginella (lycophytes).

Biological roles and biochemical functions, as well as evolution of the number of members are discussed below for each phylogenetic group.

Description of the phylogenetic groups

LysM-RLP LYMI (LYP clade I)

One to three members can be found in the phylogenetic group LYMI (Fig. 3 and S1). Studies concerning members of this phylogenetic group have been only performed in A. thaliana and Oryza sativa species. They are involved in perception of PGN and resistance to bacterial pathogens.



different colors. ECR of 3 LYR proteins were used as outgroup sequences.

In *A. thaliana*, the two members of this phylogenetic group, *AtLYM1* (*At1g21880*) and *AtLYM3* (*At1g77630*) are required for the activation of PGN signaling (Willmann et al., 2011). In *Atlym1* or *Atlym3* knock-out mutants, transcriptional responses to PGN are abolished and growth of the bacterial pathogenic strain *P. syringae* pv. tomato DC3000 is increased. The double mutant has similar phenotypes than the single mutants suggesting a cooperative role of the two proteins rather than a redundant role. These two genes do not play a role in chitin perception. To demonstrate PGN binding, AtLYM1 and AtLYM3 ECR were produced in *Escherichia coli*, purified and pulled-down using insoluble PGN. Attachment of AtLYM1 or AtLYM3 ECR to insoluble PGN was decreased in presence of soluble PGN fragments (PGN hydrolyzed by sonication), but not in presence of CO6, CO8 or LCOs, showing that AtLYM1 and AtLYM3 for PGN is however not known.

In O. sativa, OsLYP4 (Os09g27890) and OsLYP6 (Os06g10660), two of the three members in the phylogenetic group LYMI, have been reported to play a similar role in PGN recognition but surprisingly also in CO recognition (Liu et al., 2012a). In plants with decrease expression of OsLYP4 or OsLYP6 by RNA interference (RNAi), responses to PGNs and to CO6 (transcriptional responses and callose deposition) were decreased and lesions due to bacterial (Xanthomonas oryzae or Xanthomonas oryzicola) or fungal (Magnaporthe oryza) pathogens were increased. In contrast, overexpression of the two proteins led to decreased lesions in presence of these pathogens. OsLYP4 and OsLYP6 ECR were also produced in E. coli, purified and pulled-down using insoluble PGN or insoluble chitin. Competition assays were performed using CO6 or soluble PGN fragments (PGN hydrolyzed by lysostaphin). These two types of molecules were able to inhibit OsLYP4 and OsLYP6 pull-down either by insoluble chitin or PGN, showing that the same binding site was responsible for CO and PGN fragment binding. The affinity of OsLYP4 and OsLYP6 for PGN or chitin is however not known. OsLYP5 (Os02g53000) is also a member of this phylogenetic group and OsLYP5 and OsLYP6 are the closest paralogs while OsLYP4 is slightly divergent (Fig. 3, Liu et al., 2012a). In contrast to what is mentioned in Liu et al, (2012a), we found a predicted transmembrane domain and GPI anchor site in OsLYP5. Using siRNA-Finder (Si-Fi, http://labtools.ipkgatersleben) we found that the construct used to silence OsLYP6 in Liu et al, (2012a) is predicted to target OsLYP5 as well. Thus an analysis of OsLYP5 role in PGN and chitin perception and an analysis of cross silencing of OsLYP5 by

OsLYP6 hairpin construct are required to clarify the differences between *A. thaliana* and *O. sativa* orthologs.

LysM-RLP LYMII (LYP clade III)

In dicotyledons, only one member was found in this phylogenetic group, while two members were present in monocotyledons (Fig. 3 and S1). Members of this phylogenetic group and especially rice OsCEBIP are among the best characterized LysM-RLK/Ps. They are involved in long chain CO perception and resistance to fungal pathogens.

In rice, knock-down of Chitin Elicitor Binding Protein (OsCEBiP, Os09g37600) by RNAi resulted in a decrease of the CO8-induced oxidative burst in a rice cell culture (Kaku et al., 2006) and in an increased of *M. oryzae* colonization in rice plants (Kishimoto et al., 2010). On the contrary, OsCEBiP overexpression led to a decrease of lesion sizes due to M. oryzae. Its role as a main actor in CO8 perception and responses was further confirmed in knock-out plants (Kouzai et al., 2014b). Although OsCEBIP was firstly described to have 2 LysM, elucidation of its 3D structure (Liu et al., 2016) unambiguously demonstrates that it bears 3 LysM as all LysM-RLK/Ps. OsCEBiP was originally purified from rice cell culture (Kaku et al., 2006) in which a binding site for CO8 had been characterized using a radiolabeled CO8 derivative with Kd of 5.4 or 29 nM in microsomal fraction (Shibuya et al., 1993) or PM fraction (Shibuya et al., 1996) respectively. Similarly, half incorporation of a radiolabeled photoactivatable CO8 derivative in microsomal fraction of a rice cell culture was of about 50 nM (Ito et al., 1997). Using a biotinylated CO8 derivative that can be crosslinked to proteins and detected by Western blotting (with antibodies raised against biotin), it has been shown that the CO8 binding site detected in rice cell culture disappears when OsCEBIP is silenced (Shinya et al., 2010) or knocked-out (Kouzai et al., 2014b). OsCEBIP was also expressed heterologously in a tobacco BY-2 cell culture and half saturation was found around 100 nM using the biotinylated CO8 derivative (Shinya et al., 2012). As shown for the CO8 binding site in rice cell culture (Shibuya et al., 1996), competition assays with different length of CO on OsCEBIP expressed in BY-2 cells demonstrated higher affinity for CO8 than for shorter COs (Shinya et al., 2010; Shinya et al., 2012). Note that all CO8 derivatives used in these studies have an opened GlcNAc at the reducing-end and were shown to have biological activities comparable to CO7. However, this does not impact the affinity deduced from the competition assays with unmodified COs. More recently, OsCEBIP ECR was expressed in insect cells and purified. Affinities of 3 µM for CO4 and 4 µM for CO8 were determined by Isothermal Titration

Calorimetry (ITC; Liu et al., 2016). The CO binding site was found on the second LysM both by Nuclear Magnetic Resonance (NMR) spectroscopy and modelling (Hayafune et al., 2014) and by X-Ray crystallography (Liu et al., 2016). By mutating Ile150 in LysM2 (named 122 in Hayafune et al., 2014 as numbering started after the SP), it has been demonstrated that Ile150 is critical for CO binding (Hayafune et al., 2014; Liu et al., 2016). Because the binding site on the second LysM is occupied by a CO3 (Hayafune et al., 2014); (Liu et al., 2016), it has been hypothesized that CO8 binding occurs through dimerization of OsCEBIP (Hayafune et al., 2014; Liu et al., 2016). CO8 was actually found to induce in vitro dimerization of the OsCEBIP ECR produced in E. coli (Hayafune et al., 2014), but not of the OsCEBIP ECR produced in insect cells (Liu et al., 2016). It has been also shown that OsCEBIP ECR is able to form homodimers in a yeast two hybrid system and that part of the OsCEBIP is found at a size corresponding to an homodimer in vivo in absence of COs using Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) and immunodetection (Shimizu et al., 2010). The requirement of dimerization to form a high affinity binding site might explain the low affinity for CO8 found in the OsCEBIP ECR produced in insect cells (Liu et al., 2016) and its inability to discriminate CO4 and CO8 (Liu et al., 2016), in contrast to the previous OsCEBIP biochemical characterization. Interestingly 100 nM of $(GlcN\beta1,4GlcNAc)_4$, an oligosaccharide alternating N-acetylated and non Nacetylated glucosamine (therefore carrying N-acetyl moieties only on one side of the polymer) was shown to inhibit CO8-induced OsCEBIP in vitro dimerization and ROS production in rice cells (at 0.1 nM of CO8, (Hayafune et al., 2014)). Then it is surprising that 100 nM of CO4 did not compete CO8 for these responses (Hayafune et al., 2014).

In wheat and Barley, orthologs of OsCEBIP were also shown to be involved in defense against pathogens. Wheat lines that were knockdown for *TaCEBIP* by Virus Induced Gene Silencing (VIGS) showed disease symptoms produced by the fungal pathogen *Mycosphaerella graminicola* line (mutated for an effector involved in virulence) which was reported not to be pathogenic on Wild Type (WT) wheat plants (Lee et al., 2014). Barley lines knock-down for *HvCEBIP* by VIGS also showed increased lesions due to *M. oryzae* (Tanaka et al., 2010). In these plant species, CO8 binding sites similar to those of rice were detected (Okada et al., 2002) although the corresponding proteins have not been characterized.
In *A. thaliana*, the only member of the phylogenetic group, AtLYM2 (*At2g17120*) is also a chitin-binding protein. Expressed in BY-2 cells, AtLYM2 showed similar binding to COs than OsCEBIP (Shinya et al., 2012). Surprisingly, it is not required for general responses to COs (Shinya et al., 2012; Narusaka et al., 2013). However, AtLYM2 was reported to be involved in defense against the fungal pathogens *Botrytis Cinerea* and *Alternaria brassicola* (Faulkner et al. 2013; Narusaka et al., 2013). This might occur through control of symplastic fluxes in response to COs (Faulkner et al., 2013).

In *M. truncatula,* the only member of the phylogenetic group LYMII, MtLYM2 (*Medtr4g094730*) was expressed in BY-2 cells and was reported to bind COs (Fliegmann et al., 2011) but its affinity and involvement in CO responses have not been characterized.

Although all orthologs of OsCEBIP seem to have similar CO binding properties and to be involved in basal resistance to pathogenic fungi, they appear to be involved in various mechanisms. These mechanisms have been characterized only in rice and Arabidopsis. Studies in additional plant species are then required to determine whether OsCEBIP orthologs are involved in defense mechanisms similarly to those found in rice or those found in *Arabidopsis*. Moreover the role of the second member of the phylogenetic group LYMII in monocotyledons needs to be determined.

LysM-RLK LYRI (LYK clade I)

Although absent in *A. thaliana* and *B.* rapa, all the plant species have at least two members in the phylogenetic group LYRI which is divided in two subgroups A and B, each of them containing in most cases one member (Fig. 4 and S2). Some legumes have the particularity to possess two genes in the subgroup A (Gough and Jacquet, 2013). Members of the phylogenetic group LYRIA and especially the legumes *MtNFP* (*Medtr5g019040*) / *LjNFR5* (*Lj2g3v1828350*) are also among the best characterized LysM-RLK/Ps. They are involved in LCO perception and establishment of root endosymbioses.

MtNFP and *LjNFR5* are required for the RNS in *M. truncatula* (Arrighi et al., 2006) and *L. japonicus*, (Madsen et al., 2003) respectively. Almost no LCO response is measured in plants mutated in *MtNFP* or *LjNFR5*. Knockdown of *MtNFP* together with expression data suggest that it is involved in rhizobia perception all along the infection process, including inside the nodules (Arrighi et al., 2006). Surprisingly, plants mutated in *MtNFP* have been also shown to be more sensitive to the pathogenic oomycete *Aphanomyces euteiches* (Rey et al., 2013) and the fungal pathogens *Colletotrichum trifolii* (Rey et al., 2013) and *Verticillium albo-atrum* (Ben

et al., 2013). Affinity for LCOs has been shown for LjNFR5 (Broghammer et al., 2012). LjNFR5 was expressed in a plant heterologous system (leaves of *Nicotiana benthamiana*), solubilized and purified to determine its affinity for LCOs with structures close to the main Nod-factor produced by *Mezorhizobium loti* (the symbiotic partner of *L. japonicus* in RNS). High affinity was measured by Surface Plasmon Resonance (SPR) and Microscale thermophoresis (MST), with *Kd* of 4 nM and 10 nM respectively. For SPR, a LCO derivative was immobilized on a chip and affinity determined using a range of LjNFR5 concentration. For MST, affinity was determined using a fluorescent LCO derivative and a range of protein concentration.



different colors. Three LYK proteins were used as outgroup sequences.

Whereas MtNFP and LiNFR5 are not essential for the establishment of the AMS in legumes (Ben Amor et al., 2003, Madsen et al., 2003), the tomato ortholog, SILYK10 (Solyc02q065520) has been shown to play a role in the AMS establishment (Buendia et al., 2016). Plant with decreased SILYK10 expression showed a delay and less efficient colonization by the AMF Rhizophagus irregularis. Interestingly, in Parasponia andersonii, which belongs to a unique group of non-legume species able to form both the RNS with rhizobia and the AMS, the putative ortholog, PaNFP plays a role in the establishment of both symbioses (Op den Camp et al., 2011). This suggests that an ancestral gene, involved in both LCO perception and AMS establishment has been recruited during the evolution for LCO perception in RNS establishment in legumes. In some legumes, a second member of the phylogenetic group LYRIA is found. MtLYR1 (Medtr8g078300) in M. truncatula and LjLYS11 (Lj4g3v0912440) in L. japonicus are the paralogs of MtNFP and LjNFR5 respectively. These genes originated from a duplication event in legumes. *MtLYR1* transcripts are detected in roots but not in nodules (Arrighi et al., 2006). During the AMS, MtLYR1 transcripts increased in roots and more particularly cortical cells colonized by AMF (Gomez and Harrison, 2009). So far no functional characterization of MtLYR1 has been published. In L. japonicus, LjLYS11 expression was not detected in roots and nodules but in cortical cells colonized by AMF. However Ljlys11 mutants and Ljlys11-Ljnfr5 double mutants are colonized by AMF similarly to WT plants (Rasmussen et al., 2016). Recently, a knock-out mutant in OsNFR5 (Os03G13080), the ortholog of MtNFP and LJNFR5 in rice, was generated (Miyata et al., 2016). This mutant has no clear phenotype on AMF colonization compared to WT although expression of AMS plant marker genes were reduced in the mutant compared to the WT, indicating a possible but weak role of OsNFR5 in AMS establishment.

In RNS, there is a strong host specificity that is known to depend at least in part on LCO structure. Indeed, rhizobia strains can produce major LCO structures with particular decorations. These decorations distinguish them from each other. Members of the phylogenetic group LYRIA from legumes are thus expected to have selectivity for LCO structure. This hypothesis is supported by genetic studies consisting in heterologous expression of orthologous genes from plant species interacting with rhizobia producing different LCO structures (Radutoiu et al., 2007, Bensmihen et al., 2011). In contrast, there is no strict host specificity for AMS, suggesting that legume LysM-RLKs might have acquired ability to discriminate LCO decorations while the ancestor protein involved in AMS did not

display this property. However, whether legume LysM-RLKs that binds Nod-factor have selectivity for LCO structure has not been demonstrated yet.

Altogether, current data suggest that genes belonging to the phylogenetic group LYRIA are involved in root endosymbioses. This is coherent with their absence in Brassicaceae that do not establish RNS or AMS. However, only partial or no inhibition of the AMS establishment was observed in plants with knock-down or knock-out for genes from the subgroup LYRIA in tomato, *P. andersonii*, rice and *L. japonicus* (Buendia et al., 2016, Op den Camp et al., 2011, Miyata et al., 2016, Rasmussen et al., 2016) suggesting a redundancy for the activation of the CSSP (which is required for AMS establishment) eventually through perception of other signals than LCOs.

All analyzed plant species have one gene in the subgroup LYRIB except in tomato where a double copy is present because the occurrence of a tandem duplication however one of the LYRIB copy displays a truncated intracellular domain, suggesting this protein is not functional. In contrast to most of LYRs, members of the phylogenetic group LYRIB have a unique intron. We found that in *L. japonicus*, the exons are splited in 2 different loci: *Lj0g3v0102179* corresponds to exon 1 and *Lj0g3v0124999* corresponds to exon 2. For this reason the lotus gene was not included in the phylogenetic analysis. To date, no biological role and no binding to GlcNAc-containing ligand have been found for the members of this phylogenetic group. Nevertheless, phylogenetic proximity to subgroup LYRIA and absence of member in *A. thaliana* and *B. rapa* make the members of the phylogenetic group LYRIB good candidates to play a role in AMS establishment in higher plants.

LysM-RLK LYRII (LYK clade IV)

This phylogenetic group was found only in dicotyledons and is divided in two subgroups here called A and B, each containing one member in the species analyzed except the group LYRIIB in which there is no members in Brassicaceae (Fig. 4 and S2). In contrast with the other phylogenetic groups, the number and even the position of introns vary between orthologs. Neither biological role nor biochemical function are known for these proteins. Because absent in the former versions of *M. trunctaula* genome, expression of these genes has not been analyzed by µarrays. Data in tomato and Arabidopsis indicate an expression in flowers for the members of the subgroup LYRIIA.

LysM-RLK LYRIII (LYK clades II and III)

In the phylogenetic group LYRIII, several gene duplications occurred. On the basis of the phylogenetic analysis, we divided this phylogenetic group in three subgroups (Fig. 4 and S2). However, these three subgroups were only detectable when the phylogeny was performed without Gblock (an algorithm for curing the alignment and that restricts the phylogeny analysis to conserved regions). In fact, when Gblock was integrated in the phylogeny analysis, the phylogenetic group LYRIII was divided between monocotyledonous and the dicotyledonous group members (Fig. S4). It is important to note that Gblock had no effect on the organization of the other phylogenetic group LYRIII is not robust.

More in details, all the species taken into account here carried the phylogenetic subgroup LYRIIIA. Only two of them have been partially characterized either for their biological role (*AtLYK4*, *At2g23770*) or for their biochemical properties (MtLYR3, Medtr5g019050), making the comparison between members of phylogenetic subgroup LYRIIIA difficult.

In *A. thaliana*, responses to long COs (CO6 and CO8) were decreased in *Atlyk4* knock-out mutants but were not totally abolished (Wan et al., 2012), showing that AtLYK4 plays a role in long CO perception. *AtLYK4* was also shown to play a positive role in defense against the fungal pathogen *Alternaria brassicicola* and the bacterial pathogen *P. syringae* (Wan et al., 2012). AtLYK4 was pulled down from *A. thaliana* solubilized membrane fraction using chitin beads and detected by mass spectrometry (Petutschnig et al., 2010, Wan et al., 2012). Affinity and selectivity of AtLYK4 for COs is thus unknown. Because the responses to long COs were not abolished in *Atlyk4* mutants, the authors suggested that an additional protein plays a role in long CO perception. However, the implication of *AtLYK4* in resistance to a bacterial pathogen questions the possible function of AtLYK4 as a CO binding and suggests a more general role in MAMP perception.

In M. truncatula, the biological role of MtLYR3 still remains unknown. However, Fliegmann et al. (2013) demonstrated that it has a high affinity for LCOs (Kd around 25 nM). The protein was expressed in N. benthamiana leaves and LCO binding assays were performed on membrane fractions by competition between radiolabeled LCOs at fixed concentration and ranges of concentration of various unlabeled LCOs or COs. MtLYR3 LCO binding site is specific for LCOs versus COs, however it does not discriminate LCO decorations on the GlcNAc backbone. Orthologs of MtLYR3 in other legumes including LjLYS12 (Lj2g3v1828320), display

the same affinity for LCOs that MtLYR3 except those of two *Lupinus* species incapable of forming the AMS (Malkov et al., 2016). The LCO binding properties of MtLYR3 and its legume orthologs are reminiscent of a binding site characterized in a *Medicago varia* cell culture (Gressent et al., 1999). Interestingly, genes from the phylogenetic groups LYRIA and LYRIIIA are located at the same locus, as neighbour genes in opposite orientation in most dicots (Fig. 5). LCO binding properties of the proteins from these two phylogenetic subgroups are likely the consequence of a tandem duplication of an ancestral gene coding a LCO binding protein.

There is at least one member of the phylogenetic subgroup LYRIIIB in the genome of the analyzed dicots, but no member in the Brassicaceae. These genes are located next to the genes from the phylogenetic groups LYRIIIB in most dicots except in legumes (Fig. 5). Neither biological role nor biochemical functions are known for these proteins.



Fig. 5 Syntenic localization of the members of the phylogenetic groups LYRIA (red), LYRIIIB (pink) and LYRIIIA (blue). The orthologs are represented by similar color in the various genomes. Synteny was built by using the genome of *Vitis vinifera* as reference. The phylogenetic tree based on the plant genomes is that of Phytozome v10.

Members of the phylogenetic subgroup LYRIIIC were not found in all the species analyzed here (they are absent in peach and tomato). AtLYK5 (At2g33580) is the better characterized member of this phylogenetic group. *AtLYK5* was shown to be involved in general response to long COs in *A. thaliana* and in defense against the fungus *A. brassicicola* (Cao et al., 2014). An *Atlyk5* knock-out mutant is strongly, although not fully, inhibited in responses to long COs (CO6 to CO8) elicitation. The double mutant *Atlyk4* and *Atlyk5* has completely abolished responses to COs. Affinity of AtLYK5 for COs was measured by Isothermic Titration Calorimetric (ITC) on AtLYK5 ECR produced in *E. coli*. An affinity of 1.72 μM was found for CO8 while no binding to CO4 was detected. Using mutated version of AtLYK5, it was shown that its CO binding activity is essential for its biological role. Key residues in AtLYK5 CO binding site were identified by comparison with Ecp6, a fungal secreted protein containing 3 LysM, which binds CO8 with high affinity (see below). Mutation in AtLYK5 Y128 and S206 led to inability of the tagged protein produced *in planta* to bind chitin beads and to the corresponding coding sequence to restore the ROS production in response to chitin when used to complement *Atlyk5* (Cao et al., 2014).

Two members of the phylogenetic subgroup LYRIIIC are found in legumes. In M. truncatula the MtLYR4 (Medtr5g085790) and MtLYR7 (Medtr3g080170) are found in different chromosome while in L. japonicus LjLYS13 (Lj2g3v2899910) and LjLYS14 (Lj2g3v2899900) are neighbor genes, suggesting independent duplication event in these species. Mt/yr4 mutants showed increase susceptibility to the fungal pathogen *B. cinerea* (Bozsoki et al., 2017). Consistently *Mtlyr4* mutants led to a loss of ROS production induced by CO4 or CO8. Furthermore, Mtlyr4 mutants showed a decrease MAPK 3/6 phosphorylation (a hallmark of MTI signaling) compared to WT when treated with 1 µM of CO8. LjLYS13 is expressed particularly in roots and up-regulated by CO8 treatment (Lohmann et al., 2010), suggesting a role in CO perception. LjLYS14 (Lj2g3v2899900) is expressed more ubiquitously and is also slightly induced by CO8. Interestingly, LjLYS13 and LjLYS14 expression is up-regulated in roots in presence of rhizobia but not in nodules (Lohmann et al., 2010). In contrast to Mtlyr4 mutants, ROS production was similarly induced by CO4 or CO8 in Ljlys13 or Ljlys14 mutants and WT plants (Bozsoki et al. 2017). This could be due to redundant function of LjLYS13 and LjLYS14. Since a double mutant could be hardly obtained by crossing single mutants because of the vicinity of the genes, it would be very informative to obtain the double mutant using the CRISPR-Cas9 technology. The difference in the responses of *Mtlyr4*, *LjLys13* and *Ljlys14* to

CO4 or CO8, reinforces the hypothesis that the duplication events in the phylogenetic subgroup LYRIIIC were independent in *M. truncatula* and *L. japonicus*.

In addition, MtLYR4 phosphorylation status was found to be affected by LCO treatment in a *MtNFP*-independent manner (Rose et al., 2012) suggesting the gene plays a role at the interface of symbiotic and defense processes. Although the effect of LCOs on MtLYR4 phosphorylation is CSSP-independent, the MtLYR4 phosphorylation status itself appears to be controlled by the CSSP (Rose et al., 2012). However, *Mtlyr4* mutants (as *Ljlys13* and *Ljlys14* mutants) were not affected in the RNS (Bozsoki et al., 2017).

LysM-RLK LYRW (not named in Zhang et al, 2009)

The phylogenetic group LYRW contains only members in legume species and in peach (Fig. 4 and S2), suggesting that this group emerged in a common ancestor to this phylogenetically related plant species. Two members of phylogenetic group LYRW were previously found in *M. truncatula* and were reported in *L. japonicus* (Lohmann et al., 2010) although only *LjLYS20* (*Lj1g3v2808030*) can be found in the current version of *L. japonicus* genome. Although the members of this phylogenetic group cluster with the other LYRs rather than with the LYKs used as outgroup, they are very different from the other LYRs. Actually, their kinase domains are closely related to RLKs from the WAK subfamily. They might represent a third independent apparition of LysM-RLKs through fusion of 3 LysM with another kinase domain than in the LYK or LYR group. For this reason, we named this group LYRW. Biological role and biochemical functions are currently unknown for these proteins.

LysM-RLK LYKI (LYK clade VI)

In the phylogenetic group LYKI the number of genes is highly variable between species (Fig. 6 and S3). Legumes display the highest number and diversity of members in this phylogenetic group (9 in *M. truncatula*, 5 in *L. japonicus*) whereas we only found 1 protein in Brassicaceae and in *B. distachyon*.



This phylogenetic group contains *AtLYK1/AtCERK1* (*At3g21630*) that has been widely studied and shown to be required for chitin responses. CO8-induced responses such as ROS production and MAP kinase phosphorylation were completely impaired in *Atcerk1* knock-out mutants (Miya et al., 2007, Wan et al., 2008). Moreover, the mutant was more susceptible to both fungal and bacterial pathogens. In addition, pre-treatment with crab shell chitin or purified CO8 decreased pathogen growth in the WT plants whereas this resistance was not induced in *Atcerk1* reinforcing the role of AtCERK1 in chitin perception (Miya et al., 2007, Wan et al., 2008).

Contrasted results have been obtained concerning the affinity of AtCERK1 for chitin and COs. High affinity for chitin (*Kd* of 2 nM) has been reported (Lizasa et al., 2009) for the full length protein fused to GFP, produced in yeast, solubilized and purified to measure binding on chitin beads using a range of protein concentration. However, in the same study competition assays on the chitin beads using CO5, CO6 or CO8 led to half-maximal inhibitory concentration (IC50) of about 100 μ M. Similarly, other studies showed a low affinity binding (*Kd* of 44 μ M and 455 μ M for CO8) using ITC with purified AtCERK1 ECR produced in insect cells and in *E. coli* respectively (Liu et al., 2012b, Cao et al., 2014). The huge differences between affinity reported for chitin and COs could be due to the methods used for affinity determination (quantification of AtCERK1:GFP fluorescence bound to chitin beads and ITC) or to differences in affinity for various degree of GlcNAc polymerization (chitin vs CO8).

Although AtCERK1 has been mainly studied for its role in chitin perception, it was originally showed that *Atcerk1* knock-out line was more sensitive to the pathogenic bacteria *P. syringae* (Miya et al., 2007, Wan et al., 2008). In addition, AtCERK1 was shown to be the target of the bacterial effector AvrPtoB, which is able to ubiquitinate and induce its degradation (Gimenez-Ibanez et al., 2009), suggesting that AtCERK1 is also involved in perception of bacterial MAMPs. Indeed, AtCERK1 has been shown to be involved in PGN perception (Willmann et al., 2011) although it does not appear to directly bind PGN (Petutschnig et al., 2010, Willmann et al., 2011).

OsCERK1 (Os08g42580) is also involved in chitin and PGN signaling (Shimizu et al., 2010, Ao et al., 2014). Similarly to what has been observed for the *Atcerk1* mutant, *Oscerk1* mutant displayed altered responses to chitin, soluble CO7-8 or PGN treatment (ROS production, apoplastic alkalinisation, genes regulation and callose deposition). As expected for a chitin-perception defective mutant, *Oscerk1* mutant is more susceptible to the fungal pathogen *M*.

oryzae (Kouzai et al., 2014a). However, unlike AtCERK1, OsCERK1 did not show any binding to insoluble colloidal chitin (Shinya et al., 2012). These functional differences between AtCERK1 and OsCERK1 were supported by the fact that OsCERK1 could not complement *Atcerk1* for CO8-induced ROS responses (Shinya et al., 2012). However, chimerical constructs with AtCERK1 ECR and OsCERK1 ICR were able to partially rescue ROS production, demonstrating that AtCERK1 binding properties are necessary for ROS response in *Arabidopsis*. Moreover, it has been recently published that *OsCERK1* is required for CO4 and CO5 perception as these molecules were unable to induce Ca²⁺ responses in *Oscerk1* whereas *Oscebip* and *Osnfr5* were still displaying calcium spiking. Unexpectedly, *Osnfr5* is normally colonized by *Rhizophagus irregularis* (Miyata et al., 2016), even if *OsNFR5* was able to complement *Ljnfr5* mutant for nodulation, indicating that probably the function is well conserved, at least concerning the ICR.

Interestingly, rice plants with decreased level of *OsCERK1* showed almost no fungal penetration at 6 weeks post inoculation (wpi) with AMF (Zhang et al., 2015). Similarly an *Oscerk1* knock-out line displayed a mycorrhizal phenotype (Miyata et al., 2014) with no root colonization at 15 days post inoculation (dpi), demonstrating a role of *OsCERK1* in early fungal colonization. Some penetration sites and arbuscules were observed at 30 dpi. This suggests that OsCERK1 is involved in perception or signaling of signals produced by AMF such as LCOs or short COs. OsCERK1 is therefore involved in a signaling pathway activated by various molecules (at least COs and PGN, and perhaps LCOs) leading to different biological responses such as defense against pathogens and symbiosis establishment.

In our and in previously published phylogenetic analyses (Fig. 6 and Shimizu 2010), it appears that there is another member of the phylogenetic group LYKI in rice, *OsRLK10* (*Os09g33630*) which is an *OsCERK1* paralog. This gene is not present in the monocotyledon *B. distachyon*. This protein does not contain the YAQ/R motif in the kinase (but rather only the AA sequence AR) which has been shown to be a hallmark of symbiotic function (Miyata et al., 2014), see below. It would be interesting to determine whether *OsRLK10* can be functionally redundant with *OsCERK1* for one or both of the *OsCERK1* functions. This would be particularly important considering that AMS establishment appears not completely abolished in *Oscerk1* knock-out mutant (Miyata et al., 2014).

Recently, Bozsoki et al. (2017) showed that the two orthologs in legumes *LjLYS6* (*Lj6g3v1055580*) and *MtLYK9* (*Medtr3g080050*) are involved in defense. *Ljlys6* and *Mtlyr9* knock-out mutants were impaired in defense. *Ljlys6* and *Mtlyr9* were more susceptible than WT plants to the fungal pathogen *B. cinerea*. Moreover, responses to a range of COs from CO4 to CO8 such as ROS production or MAPK3/6 phosphorylation were decreased compared to WT. To determine LjLYS6 affinity for COs, LjLYS6 ECR was produced in insect cells. After purification, it was deglycosylated and labelled with a fluorophore. Affinity for CO8, CO7, CO6 and CO5 was measured by MST using a range of CO concentration. LjLYS6 was found to have a lower affinity for COs with shorter chains, with a *Kd* of 38 μ M for CO8, 227 μ M for CO5 and no detectable binding of CO4. This affinity for CO8 is comparable to the affinity found for AtCERK1 ECR produced in insect cells and measured with CO8 by ITC (Liu et al., 2012b). Bozsoki et al. (2017) also made the crystal structure of LjLYS6 ECR and they found similar results that for AtCERK1 ECR (Liu et al., 2012b) although they could not observe LjLYS6 ECR bound to a CO.

In the phylogenetic group LYKI, there are two orthologous genes *LjNFR1* (*Lj2g3v2904690*) and *MtLYK3* (*Medtr5g086130*) that originates from a duplication event specific to legumes (De Mita et al., 2014). Both are involved in LCO (Nod-factor) perception in the RNS. *Ljnfr1* mutants were impaired in nodulation and in the earliest responses to LCOs (Radutoiu et al., 2003). Apoplast alkalinisation, occurring immediately after LCO application and later responses such as root hair deformation (few hours after LCO application), were not observed in *Ljnfr1* mutant lines. Whether *LjNFR1* also plays a role in AMS is a matter of debate. It has been shown that *Ljnfr1* mutant line displayed a lower colonization ratio compared to the WT 5 wpi (Zhang et al., 2015). Moreover, AMS marker genes and Myc-LCO-induced calcium spiking were reduced in an *Ljnfr1* mutant line compared to the WT (Zhang et al., 2015). In contrast, no difference in colonization ratio or fungal structure morphology was observed between a triple *Ljnfr1-Ljnfr5-Lys11* mutant line and the WT (Rasmussen et al., 2016). LjNFR1 LCO binding was analyzed with the same strategy as for LjNFR5 (phylogenetic group LYRIA). High affinity for LCO structures derivated from the *M. loti* main LCOs was found with a *Kd* of 4.9 nM using SPR and with a K*d* of 0.61 nM using MST (Broghammer et al., 2012).

A functional difference between LysM-RLKs of the phylogenetic group LYKI from *Arabidopsis* or from other plants was demonstrated. In all species except in Brassicaceae, there is at least one member of the phylogenetic group LYKI that contains a YAQ/R motif in the kinase domain while AtCERK1 and Brara.E02055 have respectively TV and IV as AA instead of YAQ or YAR at this position (Fig. 7). The YAQ/R motif has been demonstrated to be important for nodulation. Expression in an *Lfnfr1* mutant of a chimerical protein containing the LjNFR1 ECR and the AtCERK1 ICR was unable to restore nodulation (Nakagawa et al., 2011) while an LjNFR1-OsCERK1 chimera was (Miyata et al., 2014). Replacement in AtCERK1 of the TV AA by YAQ allowed the chimera LjNFR1-AtCERK1 (YAQ) to restore nodulation in *Ljnfr1*. This suggests that the YAQ/R motif is associated with a symbiotic either in the RNS as in MtLYK3 and LjNFR1 or in the AMS as in OsCERK1. Although they bear the YAQ motif, LjLYS6 and MtLYK9 were shown to be no essential in the RNS (Bozsoki et al., 2017) suggesting they rather play a role in the AMS.

	451	460	470	480	490	500	510	520	530	540	550
Prupe.46016100	DSARGL	EYIHEH	TYPYYIHRDIKS	ANILIDKNF	RAKYADFGLT	KLSEYGSASLO	TRLYGTF	GYMPPEYAQ	YGDYSPKIDY	AFGYVLFEL	ISAKEAVY-
Solyc02g081050	DAARGL	EYIHEH	TYPLYIHRDIKS	ANILIDKDF	RAKYADFGLT	KLTEVG STSFH	TRLYGTF	GYMPPEYAQ	YGDYSPKYDY	AFGVVLYEL	ISAKEAIY-
Medtr2g024290	DSARGL	EYTHEH	TYPYYIHRDYKS	ANILIDKNF	RGKYADFGLT	KLTEYGSSSLQ	TRLYGTF	GYMPPEYAQ	YGEVSPKIDY	AFGYYLFEL]	ISGKQAIV-
L_j6g3v1812110	DSARGL	EYIHEH	TYPYYIHRDIKS	ANILIDKNF	RGKYADFGLT	KLTEYGSSSLQ	TRLYGTF	GYMPPEYAQ	YGEISPKYDY	AFGYVLYEL!	SGKEAIY-
Solyc02g081040	DAARGL	EYIHEH	TVPVYIHRDIKT	ANILIDKNF	RAKYADFGLT	KLIETEGGSMN	TRLYGTF	GYMAPE YGQ	FGNYSLKIDY	(AFGVVLYEL)	ISARKAII-
Prupe.36213100	DSARGL	EYIHEH	TYPYYIHRDIKS	ANILIDKNF	HAKYADFGLT	KLTEVGSTSLP	TRLYGTF	GYMPPEYAQ	YGEVSPKYDY	AFGVVIYEL]	ISAKEAVY-
Medtr5g086030	DSARGL	EYIHEH	TYPTYIHRDIKS	ENILLOKNFO	CAKYADFGLT	KLIDAGISSYP	TY-NMAGTF	GYMPPEYA-	YGSYSSKIDY	AFGVVLYEL]	ISAKAAYI-
Medtr3g080050	DSARGL	EYIHEH	TVPVYIHRDIKP	ANILIDKNF	RGKYADFGLT	KLTEYGSSSLP	TG-RLYGTF	GYMPPEYAQ	YGDYSPKYDY	(AFGYVLYEL)	ISAKEAIY-
Lj6g3v1055580	DSARGL	EYIHEH	TVPVYIHRDIKS	ANILIDKNY	RGKYADFGLT	KLTEYGSSSLP	TG-RLYGTF	GYMPPEYAQ	YGDYSPKYDY	(AFGYVLYEL)	ISAKDAIV-
Brara,E02055	DSARGL	EYIHEH	TVPVYVHRDIKS	ANILIDQNFI	RAKYADFGLT	KLTEYGSSA	TR-GAMGTF	GYMAPE IV-	YGEYSAKYDY	(AFGVVLYEL)	esakaayy-
AT3621630	DSARGL	EYIHEH	TYPYYYHRDIKS	ANILIDQKF	RAKYADFGLT	KLTEYGGSA	TR-GANGTF	GYMAPE TY-	YGEYSAKYDY	/AFGYYLYELJ	esakgayy-
0s08g42580	DSARGL	EYIHEH	TYPYYIHRDIKS	ANILIDKNY	RAKYADFGLT	KLTEYGGTSMP	TGTRYYGTF	GYMPPEYAR	YGDYSPKYDY	(AFGYVLYEL)	ISAKEAIV-
Bradi3g41590	DSARGL	EYIHEH	TVPVYIHRDIKS	ANILIDKNS	RAKYADFGLT	KLTEYG GASLQ	TRYYGTF	GYMPPEYAR	YGDYSPKYDY	(AFGVVLYEL)	ISAKDAIV-
Medtr5g086310	DSARGL	EYIHEH	TYPHYIHRDYKS	ANILIDKNL	RGKYADFGLT	KLLEYGNSTLQ	T-RLY-GTF	GYMPPEYAQ	YGDYSPKIDY	/AFGYVLFEL]	ESAKNAY
Medtr5g086130	DSARGL	EYIHEH	TYPYYIHRDYKS	ANILIDKNLI	RGKYADFGLT	KLIEYGNSTLH	T-RLY-GTF	GYMPPEYAQ	YGDYSPKIDY	(AFGYYLYEL)	TAKNAY
Medtr5g086120	DVARGL	EYIHDH	SVPVYIHRDIKS	DNILLNKNFI	NGKIADFGLT	KLTNIANSTON	TNHMA-GTF	GYMPPENA-	YGRISRKHDYY	(AFGVVLYEL)	ISAKAAYIN
Medtr5g086090	DVARGL	EYIHDH	SIPVYIHRDIKS	DNILLNENF	TGKYADFGLT	KLTDAASSADN	TDHYA-GTF	GYMPPENA-	YGRISRKIDY	(AFGVVLYEL)	ISAKAAYIK
Lj2g3v2904640	DVARGL	EYIHDY	TYPYYIHRDIKP	DNILLNKNFI	NAKYADFGLT	KLTDIESSAIN	TDHMA-GTF	GYMPPENA-	LGRYSRKIDY	(AFGYYLYEL)	ESAKEAYYE
Medtr5g086540	DVARGL	EYIHEH	SYPYYIHRDIKS	DNILLNENF	TGKIADFGLT	RLTDSANSTDN	TLHYA-GTF	GYMPPENY-	YGRISRKIDY	/AFGVVLYEL]	<u>ISAKPAVIK</u>
Medtr5g086040	DSARGL	EYIHEH	TYPYYIHRDIKS	ENILLOKSF	CAKYADFGLS	KLADYGNSTSS	TI-YAEGTF	GYMPPEYAC	ISYSS <mark>SPKYDY</mark>	(AFGVVLYEL)	ISAKAAVI-
Lj2g3v2904610	DSARAL	EYIHEH	TYHYYIHRDIKS	ENILLONSFI	HAKYADFGLS	KLYQYGNSI GS	SYNMMKGTF	GYMPPE YAR	IVVSP <mark>SPKIDV</mark>	(AFGYYLYEL)	ISAKEAVI-
Solyc01g098410	DSARGL	EYIHEH	TYPFYIHRDYKS	ANILINKNFI	HAKIGDFGLS	KLYESGNPTLN	TRFMGTF	GYMPPEYGH	-SGYISRKYDY	/AFGVVLYEL]	ISSKDAIV-
Consensus	DsARGL	EYIH#H	LVPVYIHRD!Ks	aNILi#knfı	raK!ADFGLt	KLt#vg.s	TGTF	GYMpPEya.	yg.vSpK!DV	AFGYVL%EL3	[SaK.A!!.
				_	6 . 1		.				

Fig. 7 Amino acid sequence alignment of the members of the phylogenetic group LYKI. The YAQ/R motifs is boxed in black.

The LjNFR1 ortholog in *M. truncatula*, *MtLYK3*, has been also demonstrated to be involved in nodulation. *Mtlyk3* knock-down (Limpens et al., 2003) or missense (Smit et al., 2007) lines were impaired in nodule formation and in rhizobial colonization of *M. truncatula* roots but not in LCO responses such as apoplast alkalinisation or calcium spiking. This led to the hypothesis that the genetic control of the rhizobial colonization is different between *M. truncatula* and *L. japonicus* and that *MtLYK3* is involved LCO perception during rhizobial colonization but not in the LCO perception preceding colonization. However, a tandem duplication of *MtLYK3* has occurred in *M. truncatula*. Two genes *MtLYK3* and *MtLYK2*

(Medtr5g086310/Medtr5g086330) are *LjNFR1* orthologs (Fig. 6; De Mita et al., 2014). *MtLYK2* might be redundant with *MtLYK3*. Actually, MtLYK2 contains as MtLYK3, the YAQ motif in its kinase domain. Although *MtLYK2* is less expressed than *MtLYK3* in roots (Limpens et al., 2003), it is possible that the *MtLYK2* expression level in *Mtlyk3* mutant lines is enough to ensure the LCO responses preceding colonization but not the rhizobial colonization. This would explain the phenotypic difference between *Mtlyk3* and *Ljnfr1*. Complementation experiments have shown that a chimeric protein containing LjNFR1 ECR and MtLYK3 ICR can restore the absence of nodulation in a *Ljnfr1* mutant (Nakagawa et al., 2011) suggesting a conservation of the function of these legume LysM-RLKs.

Taken together, these data suggest a divergent evolution of the members of the phylogenetic group LYKI for which the ancestral protein might have a dual role in defense and the AMS. During the evolution, proteins have been specialized in pathogen recognition in Brassicaceae. In legumes, the genes experienced several duplication events, and members might have also been specialized in symbiosis establishment or in defense responses.

LysM-RLK LYKII (not named in Zhang et al, 2009)

In the Phylogenetic group LYKII, we found one ortholog in each species analyzed, except in Brassicaceae (Fig. 6 and S3). In the peach genome, gene duplication occurred and two copies are present. The only characterized member of the phylogenetic group LYKII is the L. japonicus member LjEPR3/LjLYS3 (Lj2g3v1415410) which has been shown to be implicated in the recognition of bacterial exopolysaccharides (EPS) and colonization by rhizobia (Kawaharada et al., 2015, Kawaharada et al., 2017). Knock-out or missense Ljepr3 mutants developed more nodules in presence of a rhizobial *M. loti* strain which produces an abnormal EPS structure (exoU) and which is almost unable to colonize L. japonicus (Kawaharada et al., 2015). However, the number of nodules found in this study was extremely low. In contrast to the *M. loti exoU*, a M. loti strain unable to produce EPS (exoB) was able to colonize L. japonicus, despite that WT plants inoculated with a *M. loti exoB* strain showed abnormal ITs (i.e.: branched or forming balloon-like structure at the epidermal-cortical cell boundary) and intercellular rhizobial colonization in nodules. Although in lower extent, this phenotype was also observed in the Ljepr3 mutants inoculated with WT M. loti. The quantitative phenotypic differences between Liepr3 and WT plants suggest that EPR3 is however not the only actor in EPS perception (Kawaharada et al., 2017). LjEPR3 expression is induced by Nod-factors and by rhizobia. In the presence of rhizobia, its expression pattern in roots corresponds to the zone susceptible to

rhizobial colonization, around the ITs and in the nodule primordia. This result suggests that *LjEPR3* is required all along the infection process (Kawaharada et al., 2017).

The ortholog in *M. truncatula MtLYK10* (*Medtr5g033490*) is also induced by Nod-factors and rhizobia and in lower extent by Myc-factors and during the AMS (Mt gene atlas, Mtr.25148.1.S1_at). Similarly, the orthologs in monocots (*Os01g36550* and *Bradi2g40627*) are induced during the AMS (Güimil et al., 2005), gene annotated *OsAM191*, and unpublished data). Absence of member of the phylogenetic group LYKII in Brassicaceae, together with the role of *LjEPR3* in rhizobial colonization and the induction of the expression of various orthologs in presence of root symbionts, suggest that the members of this phylogenetic group play a role in root endosymbioses. LjEPR3 ECR was expressed in insect cells by using a baculovirus system. Binding to EPS was measured by biolayer interferometry using purified LjEPR3 ECR (Kawaharada et al., 2015) and a *Kd* of 2.7 μ M was found. Currently, LjEPR3 is a unique example of a LysM-RLK/P binding a molecule not containing GlcNAc. Whether the recognition of EPS by LysM-RLK is specific to *L. japonicus* or legumes remains unknown. It is therefore of interest to study a putative role in AMS of non-legume members of the phylogenetic group LYKII and to determine their biochemical properties, especially their ability to bind EPS.

LysM-RLK LYKIII (LYK clade V)

In the phylogenetic group LYKIII, we identified at least one ortholog in each species, with duplications in *B. rapa* and in *L. japonicus* (Fig. 6 and S3). The only gene from this phylogenetic group that has been studied is *AtLYK3* (*At1g51940*) and it was shown to act as a negative regulator of plant immunity in *A. thaliana* (Paparella et al., 2014). T-DNA insertional mutant line displayed reduced symptoms in presence of the fungal pathogen *B. cinerea* or the bacterial pathogen *Pectobactirium carotovorus* when compared to the WT plants. In addition, basal expression in absence of pathogen of defense-related genes such as the plant defensin *PAD3* was higher in *Atlyk3* mutants than in WT plants. *AtLYK3* was also shown to be required for LCO perception in *A. thaliana* (Liang et al., 2013). The authors showed that LCO (at 100 nM) and CO4 (at 10 μ M) partially inhibit (about 25 %) responses to the MAMPs flg22 or CO8 in WT *A. thaliana*. LCO effect on the attenuation of flg22 responses seems to occur through degradation of AtFLS2. Effect of LCO on flg22-induced ROS production was not observed in an *Atlyk3* knock-out line and was stronger in an *AtLYK3* overexpressing line. Note that the data also suggest that in *Atlyk3* knock-out line, flg22-induced MAP kinase phosphorylation was

reduced in absence of LCO. No biochemical characterization of any member of the phylogenetic group LYKIII has been published.

Hetero-oligomeric complexes

Up to date, a model for ligand perception by LysM-RLK/Ps (including the symbiotic signals LCOs and CO4/5 or MAMPs such as CO8, chitin and PGN) involves hetero-oligomers composed of at least one LYR/LYM and one LYK (Fig. 8). It can be hypothesized that LYR or LYM, lacking active kinase domain, are the proteins that bind signal molecule with high affinity through their ECR. High affinity likely corresponds to Kd in the range of nM as measured for several LYRs and LYMs. Ligand binding to a LYR/LYM would induce i) interaction with a LYK, which possesses an active kinase, or ii) a change of conformation of the pre-existing LYR/LYM and LYK complex, leading to activation of the kinase of the LYK partner and signal transduction. Fitting this model, the LYMs OsCEBIP, OsLYP4, OsLYP6, AtLYM1, AtLYM2, AtLYM3 and MtLYM2 were found to bind PGN and/or COs and the LYRs AtLYK5, MtLYR3 and LjNFR5 were shown to bind COs or LCOs. Except for OsLYP4 and OsLYP6, these proteins showed selectivity for a single type of ligand. Moreover, when their affinity was measured, these proteins were found to have high affinity for ligand. In contrast, the LYKs AtCERK1 and OsCERK1 were found to be involved in perception of multiple signals and to have low or no affinity for GlcNAc containing ligands. Many genetic analyses actually suggest that LYMs/LYRs and LYKs interact since they show similar phenotypes in responses to molecules or microorganisms (Table 1). Supporting the requirement of heterodimeric receptors to bind a ligand and transduce the signal, changes in host range during RNS or ligand specific responses were obtained by expressing heterologously couples of LYR/LYM and LYK proteins. Co-expression of LjNFR5 (LYRIA) and LjNFR1 (LYKI) in *M. truncatula* or in *Lotus filicaulis* modified host range (Radutoiu et al., 2007), while single protein did not. Co-expression of chimeric LjNFR5–AtCERK1 and LjNFR1–AtCERK1 in A. thaliana led to production of ROS and expression of chitin-induced genes in response to LCO (Wang et al., 2014). Similarly, co-expression of chimeric OsCEBiP-LjNFR5 and OsCERK1-LjNFR1 in *L. japonicus* led to induction of LCO responsive genes in response to chitin and CO8 (Wang et al., 2014). Finally, physical interactions between LYMs/LYRs and LYKs have been demonstrated in planta. In rice cells, OsCEBIP (LYMII), OsLYP4 and OsLYP6 (LYMII) interact with OsCERK1 (LYKI) in presence of chitin (Shimizu et al., 2010, Ao et al., 2014). OsLYP4 and OsLYP6 also interact with OsCERK1 in presence of PGN (Ao et al., 2014). In A. thaliana, AtLYK5 (LYRII)

interacts with AtCERK1 (LYKI, (Cao et al., 2014) under CO elicitation (CO6, CO7 and CO8 but not CO5). In *M. truncatula* physical interaction between MtNFP (LYRIA) and MtLYK3 (LYKI) was shown in nodules (Moling et al., 2014) which contains rhizobia, although it does not demonstrate that the interaction requires a ligand. Physical interaction of LjNFR5 (LYRIA) with LjNFR1 (LYKI) was shown in absence of Nod-factors when expressed in heterologous system (Madsen et al., 2011). However, it has to be considered that it was in a context of high expression level. Recently, physical interaction that is modified in presence of LCO was shown for MtLYR3 (LYRIIA) and MtLYK3 (LYKI, Fliegmann et al., 2016).





The model of an RLK/P, showing high affinity for ligand and no kinase activity, interacting with another RLK without high affinity for ligand but bearing a kinase activity, is reminiscent of the perception of the MAMP peptides flg22 and elf18. Indeed, perception of these peptides occurs through high affinity binding to the LRR-RLKs AtFLS2 and AtEFR respectively, and subsequent complex formation with the LRR-RLK AtBAK1 (Chinchilla et al., 2007, Schwessinger et al., 2011). AtFLS2 and AtEFR have non RD kinases with weak activity (Schwessinger et al., 2011) compared to AtBAK1 which has a RD kinase and which is involved in multiple signaling pathways.

Although most data on LysM-RLK/Ps fit with our model, high affinity LCO binding on LjNFR1 (LYKI) questions it. Other high affinity ligand-binding proteins such as the LRR-RLK AtPERP1/2 which are receptors of endogenous peptides involved in wound signaling and innate immunity (Yamaguchi et al., 2006, Yamaguchi et al., 2010) and AtBRI1 which is the receptor of the Brassinosteroid hormone (He et al., 2000) have an active RD kinase.

Limitation in ligand binding assays

As reported above, the biochemical assays performed to characterize LysM-RLK/Ps have produced contrasted results. Such differences have also been found for a fungal effector Ecp6 which binds CO8. Using a recombinant Ecp6 purified from *Pichia pastoris Kd* between 3.7 μ M and 4.5 μ M were found by ITC (de Jonge et al., 2010, Mentlak et al., 2012) while a *Kd* of 1.3 nM or 380 nm were found by SPR using a CO8 immobilized or an effector-immobilized strategy respectively. Using a recombinant Ecp6 purified from mammalian cells, a *Kd* of 280 pM was found using ITC (Sánchez-Vallet et al., 2013). One reason explaining the differences between the results obtained with recombinant Ecp6 from yeast or mammalian cells might be the presence of yeast derived COs co-purified with the protein that biased the analysis (Sánchez-Vallet et al., 2013).

Differences in affinity for ligand of a single LysM-RLK/P found between studies might be due to various reasons including the production system, the region of the protein used (e.g. full length versus ECR) and the binding assay. Because the *E. coli* system is not efficient for the formation of disulfide bridges that were shown to be essential for the function of several LysM-RLKs including MtNFP (Lefebvre et al., 2012) and LjEPR3/LjLYS3 (Kawaharada et al., 2015), the affinity measured with ECR produced in *E. coli* might be underestimated. Indeed AtCERK1 ECR produced in insect cells showed a *Kd* for CO8 of 44 µM by ITC (Liu et al., 2012b) while AtCERK1 ECR produced in *E. coli* showed a *Kd* for CO8 of 455 µM (Cao et al., 2014) using the same binding assay. Expression of OsCEBIP full length protein in tobacco BY-2 cells suggested a *Kd* for CO8 was about 4 µM. It is thus essential to compare affinities of proteins or domains produced similarly and characterized using the same binding assay.

Although half incorporation in saturation experiments or half inhibition in competition experiments correspond to *Kd* in presence of a single binding site and appropriate receptor-ligand stoichiometry, proper *Kd* calculation requires either to know the ligand and the protein

concentrations (for radiolabeled ligand assays, ITC, MST) or to measure the kinetic of association and dissociation (for SPR). With insoluble chitin, PGN or uncharacterized mixtures, it is impossible to determine the molecular concentration of the ligands and thus to determine the *Kd* from saturation or competition experiments. The *Kd* found in the literature that are deduced from half incorporation or half inhibition experiments have thus to be considered with caution.

Another limitation to the current biochemical characterization and understanding of the LysM-RLK function is the lack of studies about specificity of the protein-ligand interaction. The controls used are often unrelated proteins and unrelated ligands. At this concern, it should be important to use other LysM-RLK/Ps and various structures of COs, LCOs or muropeptides as controls to correlate binding properties and biological functions.

Role of LysM-RLK/Ps in plant defense

Immune responses are central to plants which have to face to multiple biotic stresses and interact with a wide range of microorganisms from beneficial to pathogenic. Plant LysM-RLK/Ps are involved in the perception of GlcNAc-containing molecules that act as defense elicitors.

In fact, perception of chitin fragments is involved in plant resistance to fungal pathogens. This relies on LYMs, LYRs and LYKs which may act in same or parallel pathways. Actually, AtLYK5 (LYRIIIC) was found to have affinity for CO8 and was suggested to play a redundant role with AtLYK4 (LYRIIIA) for elicitation of defense responses through a signaling pathway depending on AtCERK1 (LYKI; Cao et al., 2014) and leading to ROS production. On the other hand, AtLYM2 (LYMII) was also shown to have affinity for CO8 and to play a role in resistance to fungal pathogens. However AtLYM2 is not required for the main ROS production in response to CO8 and has been proposed to play a role in plasmodesmata closure in response to COs. This occurs independently of AtCERK1 (Faulkner et al., 2013) rising the question of which co-receptor interacts with AtLYM2 for CO8 signaling. In rice, OsCEBIP, one of the AtLYM2 orthologs also has high affinity for chitin fragments but interacts with OsCERK1 (Shimizu et al., 2010) and is involved in the main ROS production in response to chitin fragments (Kaku et al., 2006). The differences found between Arabidopsis and rice for chitin perception point out the necessity of new studies on plant species in different phylogenetic clades to better understand how chitin perception has evolved in plants. Complementary roles

of *AtLYK5*, *AtLYK4* and *AtLYM2* show that genetic screens based on measurement at the plant or organ level of classical responses to MAMP (such as Ca²⁺ flux, ROS production, marker gene induction) allow to identify genes responsible of the widespread responses (intense and/ or produced in many cells). However, such screens do not permit to find genes controlling mechanisms in specific areas that could also play important roles in pathogen resistance. This encourages for screening mutant collections for a variety of responses to elicitors as well as to various pathogens.

Similarly to chitin fragment perception, PGN perception is involved in plant resistance to bacterial pathogens. Although less characterized than the chitin fragment perception, PGN perception relies on LYM which interacts with LYK for signaling.

The importance of LysM-RLKs in plant defense is highlighted by the fact that they are targeted by pathogen effectors. AtCERK1, which is a central actor for perception of chitin and PGN fragments in Arabidopsis, is the target of AvrPtoB, a *P. syringae* effector. AvrPtoB is able to ubiquitinate AtCERK1, inducing its degradation and suppressing MAMPS signaling (Gimenez-Ibanez et al., 2009). AvrPtoB is also able to interact with several LysM-RLKs in tomato which are AtCERK1 orthologs. Among them, SIBti9 displayed a reduced kinase activity in presence of AvrPtoB (Zeng et al., 2012). Some fungal effectors suppress plant immunity in another way, by competing with plant receptor for binding to chitin fragments. For instance, Mentlak and co-workers (2012) showed that the fungal effectors Ecp6 from C. fulvum and Slp1 from *M. oryzae* are able to bind COs with high affinity. When added exogenously, these effectors can compete for the binding of chitin fragments to OsCEBIP (de Jonge et al., 2010, Mentlak et al., 2012). In addition, they appear to have higher affinity than OsCEBIP for CO8 (de Jonge et al., 2010, Mentlak et al., 2012, Sánchez-Vallet et al., 2013). Interestingly, these effectors are composed of three LysM as the LysM-RLK/Ps. However they might originate from an independent association of LysM, since the loops between the LysMs are different and the highly conserved CXC motifs found in LysM-RLK/Ps are not conserved although disulfide bridges are also involved in packing together the LysM. The association of three LysM in different proteins for binding chitin fragments thus represents an example of convergent evolution. Fungal effectors with LysM are found in many fungi (Bolton et al., 2008) including pathogens with different lifestyles or host ranges suggesting that competing chitin binding to plant receptors in order to avoid MTI activation is a widespread strategy in fungi. Several proteins containing LysM were show to play a role in fungal pathogenicity: Mg3LysM in

Mycosphaerella graminicola (Marshall et al., 2011), ChELP1 and ChELP2 in *Colletotrichum higginsianum ogenesis* (Takahara et al., 2016) and Vd2LysM in *Verticillium dahlia* (Kombrink et al., 2017).

Role of LysM-RLK/Ps in symbioses

The role of LysM-RLKs in the RNS has been unambiguously determined. Members of the phylogenetic group LYRIA and LYKI, act together for perception of LCOs produced by rhizobia (Nod-factors). They are required for the earliest responses to LCOs, for rhizobial colonization and for nodule development. Although demonstrated for L. japonicus LysM-RLKs, evidence for LCO binding to the M. truncatula LysM-RLKs involved in LCO perception are still lacking, questioning the similarity between these two legumes species for LCO perception. In addition, differences in sensitivity of root hairs for responses to LCOs have been detected between these species. Half of the root hairs showed calcium spiking in *M. truncatula* roots treated by up to 10⁻¹³ M of S. meliloti Nod-factors (Oldroyd et al., 2001, Sun et al., 2015) while no root hairs showed calcium spiking in *L. japonicus* roots treated with 10⁻¹¹M of *M. loti* Nod-factors (Sun et al., 2015). In contrast, most root hairs showed calcium spiking in *L. japonicus* roots treated with 10⁻⁹ M of *M. loti* Nod-factors (Oldroyd et al., 2001, Sun et al., 2015). Moreover, S. meliloti, a rhizobial symbiont of M. truncatula, produces a LCO-IV(C16:2,S) as major Nodfactor (Lerouge et al., 1990) while M. loti, a rhizobial symbiont of L. japonicus, produces LCO-V(C16:1,Cb,Fuc,Ac) as a major Nod-factor (Bek et al., 2010). A double insaturation on the fatty acid and a sulfate groups are two LCO properties which are rare among the variety of Nodfactor structures produced by rhizobia. Altogether, this suggests differences in LCO receptors between these two species.

The role of LysM-RLKs in AMS still remains unclear. By analogy to RNS, the current hypothesis is that LysM-RLKs are fungal LCO receptors and are required for AMF colonization. Until now, two MtNFP/LjNFR5 orthologs (PaNFP and SILYK10) have been identified as putative Myc-LCO receptor as AMF colonization is impaired in plants in which their expression is silenced (Op den Camp et al., 2011, Buendia et al., 2016). However, no data about binding properties to Myc-LCO is available.

Evidences for short CO perception were already published in the 90s. A CO4/CO5 binding site was found in a tomato cell culture (Baureithel et al., 1994). Few years later, classical responses to MAMPs such as apoplast alkalinisation were found in several *Solanaceae* (including tobacco and tomato) and in rice cell cultures. However, no response was observed in an Arabidopsis cell culture, indicating that CO4/5 binding site does not exist in this plant species (Felix et al., 1999). Several years after, short COs (CO4 and CO5) were shown to induce in *M. truncatula* calcium spiking which is currently considered a hallmark of symbiotic responses. Moreover CO4 and CO5 synthesis is stimulated by strigolactones (Genre et al., 2013), an plant hormone which is known to promote AMF colonization. Recently, it has been shown that the LysM-RLK *OsCERK1* which is required for AMF colonization, is involved in CO4 perception (Carotenuto et al., 2017), likely through controlling the signal transduction.

In conclusion, the role of LysM-RLKs in AMS has only started to be explored. The importance of Myc-factors (LCOs and short COs) for AMS establishment remains unclear as no LCO or short CO binding protein has been identified yet. Differences between plant species in the mechanism of perception and in the responses to Myc-LCOs and Myc-COs might complicate this research field. More studies on LysM-RLKs from various plant species could help to better understand how plants perceive AMF and distinguish them from other microorganisms.

Dual roles of LysM-RLKs in symbioses and defense

Symbiotic signals are structurally related to defense elicitors like chitin and PGN fragments. Consequently, it is logical to think that receptors should share similarities in terms of tridimensional structure and operating mode. Several questions arise from this statement. Under an evolutionary point of view, have symbiotic organisms exploited defense pathways or *vice versa* and are symbiotic receptors derived from PAMP receptors? How can a plant deal with symbiotic partners that produce both symbiotic signals and MAMPs? In the complexity of the rhizosphere microbiome, how can plants distinguish and adapt their responses when surrounded by microbial communities composed of thousands of different microorganisms? Can some pathogens use the symbiotic pathway to overcome plant defenses? Even if most of these questions remain unanswered, several evidences suggest strong overlaps between symbiosis and defense.

Nod-factors transiently induce defense genes that are also induced by flg22 and chitin fragments (mix of CO2 to CO8), including PR proteins, peroxydases and transcription factors. Chitin fragments were able to transiently induce symbiotic genes independently of Nod-factor receptors, indicating that they are perceived by others receptors activating symbiotic gene expression through the CSSP (Nakagawa et al., 2011). Interestingly, co-expression of two symbiotic LysM-RLKs, MtNFP and MtLYK3 or LjNFR5 and LjNFR induces defense responses and cell death, suggesting that these proteins can interfere with signaling pathways involved in defense mechanisms (Pietraszewska-Bogiel et al., 2013; Madsen et al., 2011).

Some LysM-RLKs display dual role in both symbiosis and defense. *OsCERK1* was first shown to play an essential role in signal transduction after chitin or PGN elicitation and in a second time to play a role in AMF colonization (Miyata et al., 2014, Zhang et al., 2015). Similarly MtNFP which is essential for LCO signaling and rhizobial colonization plays a role in resistance to several pathogens (fungi and oomycetes) (Rey et al., 2013, Ben et al., 2013). Further studies on other plant species phylogenetically distant from the classical plant models used in the studies mentioned above could help to better understand how MAMPs perception and symbiotic signal perception have evolved through time.

Liang *et al.* (2013) demonstrated that *A. thaliana* is able to perceive LCOs that can interfere with responses induced by flg22. It is still undetermined whether effect of LCOs is occurring i) by direct regulation of MAMP perception or signaling pathways or ii) because of a competition/desensitization of common actors involved in LCO and MAMP signaling pathways; specially because high amount of LCO and flg22 were used in this study. Observation of LCOs effects in *A. thaliana* was surprising since this plant is not able to establish AMS and has lost the LYRI group members, known to be involved in LCO perception. An explanation to a cross-talk between symbiosis and defense is that plants perceive MAMPs produced by their symbiotic partners, and need to turn-off their defenses, at least locally for symbiosis establishment. Thus, we can expect that in plants able to establish the RNS and/or the AMS, symbiotic signals have a direct effect on defense mechanisms by down-regulating them. To better understand the role of LysM-RLKs in the cross-talks between defense and symbioses, similar analyses to those performed by Liang *et al.* have to be performed in plants that establish the RNS and/or the AMS.

Table 1

	genetic	Ref	physical	Ref
	interaction		interaction	
AtLYM3/AtCERK1	Х	Willmann 2011		
AtLYM1/AtCERK1	Х	Willmann 2011		
AtLYK4/AtCERK1	Х	Wan 2012,		
		Cao 2014		
AtLYK5/AtCERK1	Х	Cao 2014	Х	Cao 2014
OsLYP4/OsCERK1	Х	Liu 2012a	х	Liu 2012a
OsLYP6/OsCERK1	Х	Liu 2012a	х	Liu 2012a
OsCEBIP/OsCERK1	Х	Liu 2016	Х	Liu 2016
		Ao 2014		Hayafune 2014
		Kouzai 2014		
TaCEBIP/TaCERK1	Х	Lee 2014		
MtNFP/MtLYK3	Х	Limpens 2003	х	
		arrighi 2006		
LjNFR5/LJNFR1	Х	Madsen 2003	X	Madsen 2011
		Radutoiu 2003		
MtLYR3/MtLYK3			X	Fliegmann 2016

References

Antolín-Llovera, M., Ried, M. K., Binder, A. and Parniske, M. (2012) 'Receptor kinase signaling pathways in plant-microbe interactions', *Annu Rev Phytopathol*, 50, pp. 451-73.

Ao, Y., Li, Z., Feng, D., Xiong, F., Liu, J., Li, J. F., Wang, M., Wang, J., Liu, B. and Wang, H. B. (2014) 'OsCERK1 and OsRLCK176 play important roles in peptidoglycan and chitin signaling in rice innate immunity', *Plant J*.

Arrighi, J. F., Barre, A., Ben Amor, B., Bersoult, A., Soriano, L. C., Mirabella, R., de Carvalho-Niebel, F., Journet, E. P., Ghérardi, M., Huguet, T., Geurts, R., Dénarié, J., Rougé, P. and Gough, C. (2006) 'The Medicago truncatula lysin [corrected] motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes', *Plant Physiol*, 142(1), pp. 265-79.

Bateman, A. and Bycroft, M. (2000) 'The structure of a LysM domain from E. coli membrane-bound lytic murein transglycosylase D (MltD)', *J Mol Biol*, 299(4), pp. 1113-9.

Baureithel, K., Felix, G. and Boller, T. (1994) 'Specific, high affinity binding of chitin fragments to tomato cells and membranes. Competitive inhibition of binding by derivatives of chitooligosaccharides and a Nod factor of Rhizobium', *J Biol Chem*, 269(27), pp. 17931-8.

Beck, M., Heard, W., Mbengue, M. and Robatzek, S. (2012) 'The INs and OUTs of pattern recognition receptors at the cell surface', *Current Opinion in Plant Biology*, 15(4), pp. 367-374.

Bek, A. S., Sauer, J., Thygesen, M. B., Duus, J., Petersen, B. O., Thirup, S., James, E., Jensen, K. J., Stougaard, J. and Radutoiu, S. (2010) 'Improved characterization of nod factors and genetically based variation in LysM Receptor domains identify amino acids expendable for nod factor recognition in Lotus spp', *Mol Plant Microbe Interact*, 23(1), pp. 58-66.

Belkhadir, Y., Yang, L., Hetzel, J., Dangl, J. L. and Chory, J. (2014) 'The growth-defense pivot: crisis management in plants mediated by LRR-RK surface receptors', *Trends Biochem Sci*, 39(10), pp. 447-56.

Ben Amor, B., Shaw, S. L., Oldroyd, G. E. D., Maillet, F., Penmetsa, R. V., Cook, D., Long, S. R., Denarie, J. and Gough, C. (2003) 'The NFP locus of Medicago truncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation', *Plant Journal*, 34(4), pp. 495-506.

Ben, C., Toueni, M., Montanari, S., Tardin, M. C., Fervel, M., Negahi, A., Saint-Pierre, L., Mathieu, G., Gras, M. C., Noël, D., Prospéri, J. M., Pilet-Nayel, M. L., Baranger, A., Huguet, T., Julier, B., Rickauer, M. and Gentzbittel, L. (2013) 'Natural diversity in the model legume Medicago truncatula allows identifying distinct genetic mechanisms conferring partial resistance to Verticillium wilt', *J Exp Bot*, 64(1), pp. 317-32.

Bensmihen, S., de Billy, F. and Gough, C. (2011) 'Contribution of NFP LysM domains to the recognition of Nod factors during the Medicago truncatula/Sinorhizobium meliloti symbiosis', *PLoS One*, 6(11), pp. e26114.

Bielnicki, J., Devedjiev, Y., Derewenda, U., Dauter, Z., Joachimiak, A. and Derewenda, Z. S. (2006) 'B. subtilis ykuD protein at 2.0 A resolution: insights into the structure and function of a novel, ubiquitous family of bacterial enzymes', *Proteins*, 62(1), pp. 144-51.

Bolton, M. D., van Esse, H. P., Vossen, J. H., de Jonge, R., Stergiopoulos, I., Stulemeijer, I. J., van den Berg, G. C., Borrás-Hidalgo, O., Dekker, H. L., de Koster, C. G., de Wit, P. J., Joosten, M. H. and Thomma, B. P. (2008) 'The novel Cladosporium fulvum lysin motif effector Ecp6 is a virulence factor with orthologues in other fungal species', *Mol Microbiol*, 69(1), pp. 119-36.

Bozsoki, Z., Cheng, J., Feng, F., Gysel, K., Vinther, M., Andersen, K. R., Oldroyd, G., Blaisea, M., Radutoiu, S. and Stougaard, J. (2017) 'Receptor-mediated chitin perception in legume roots is functionally separable from Nod factor perception', *Proc Natl Acad Sci U S A*, 201706795.

Broghammer, A., Krusell, L., Blaise, M., Sauer, J., Sullivan, J. T., Maolanon, N., Vinther, M., Lorentzen, A., Madsen, E. B., Jensen, K. J., Roepstorff, P., Thirup, S., Ronson, C. W., Thygesen, M. B. and Stougaard, J. (2012) 'Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding', *Proc Natl Acad Sci U S A*, 109(34), pp. 13859-64.

Buendia, L., Wang, T. M., Girardin, A. and Lefebvre, B. (2016) 'The LysM receptor-like kinase SILYK10 regulates the arbuscular mycorrhizal symbiosis in tomato', *New Phytologist*, 210(1), pp. 184-195.

Buist, G., Steen, A., Kok, J. and Kuipers, O. P. (2008) 'LysM, a widely distributed protein motif for binding to (peptido)glycans', *Mol Microbiol*, 68(4), pp. 838-47.

Camps, C., Jardinaud, M. F., Rengel, D., Carrère, S., Hervé, C., Debellé, F., Gamas, P., Bensmihen, S. and Gough, C. (2015) 'Combined genetic and transcriptomic analysis reveals three major signalling pathways activated by Myc-LCOs in Medicago truncatula', *New Phytol*, 208(1), pp. 224-40.

Cao, Y., Liang, Y., Tanaka, K., Nguyen, C. T., Jedrzejczak, R. P., Joachimiak, A. and Stacey, G. (2014) 'The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1', *Elife*, 3.

Carotenuto, G., Chabaud, M., Miyata, K., Capozzi, M., Takeda, N., Kaku, H., Shibuya, N., Nakagawa, T., Barker, D. G. and Genre, A. (2017) 'The rice LysM receptor-like kinase OsCERK1 is required for the perception of short-chain chitin oligomers in arbuscular mycorrhizal signaling', *New Phytol*, 214(4), pp. 1440-1446.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. (2006) 'The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception', *Plant Cell*, 18(2), pp. 465-76.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D., Felix, G. and Boller, T. (2007) 'A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence', *Nature*, 448(7152), pp. 497-500.

Combier, J. P., Küster, H., Journet, E. P., Hohnjec, N., Gamas, P. and Niebel, A. (2008) 'Evidence for the involvement in nodulation of the two small putative regulatory peptideencoding genes MtRALFL1 and MtDVL1', *Mol Plant Microbe Interact*, 21(8), pp. 1118-27.

de Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M. H. and Thomma, B. P. (2010) 'Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants', *Science*, 329(5994), pp. 953-5.

De Mita, S., Streng, A., Bisseling, T. and Geurts, R. (2014) 'Evolution of a symbiotic receptor through gene duplications in the legume-rhizobium mutualism', *New Phytologist*, 201(3), pp. 961-972.

Delaux, P., Varala, K., Edger, P., Coruzzi, G., Pires, J. and Ane, J. (2014) 'Comparative Phylogenomics Uncovers the Impact of Symbiotic Associations on Host Genome Evolution', *Plos Genetics*, 10(7).

Faulkner, C., Petutschnig, E., Benitez-Alfonso, Y., Beck, M., Robatzek, S., Lipka, V. and Maule, A. J. (2013) 'LYM2-dependent chitin perception limits molecular flux via plasmodesmata', *Proc Natl Acad Sci U S A*, 110(22), pp. 9166-70.

Felix, G., Duran, J. D., Volko, S. and Boller, T. (1999) 'Plants have a sensitive perception system for the most conserved domain of bacterial flagellin', *Plant J*, 18(3), pp. 265-76.

Felle, H. H., Kondorosi, E., Kondorosi, A. and Schultze, M. (1996) 'Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals', *Plant Journal*, 10(2), pp. 295-301.

Fliegmann, J. and Bono, J. J. (2015) 'Lipo-chitooligosaccharidic nodulation factors and their perception by plant receptors', *Glycoconj J*, 32(7), pp. 455-64.

Fliegmann, J. and Felix, G. (2016) 'Immunity: Flagellin seen from all sides', *Nat Plants*, 2(9), pp. 16136.

Fliegmann, J., Jauneau, A., Pichereaux, C., Rosenberg, C., Gasciolli, V., Timmers, A. C., Burlet-Schiltz, O., Cullimore, J. and Bono, J. J. (2016) 'LYR3, a high-affinity LCO-binding protein of Medicago truncatula, interacts with LYK3, a key symbiotic receptor', *FEBS Lett*, 590(10), pp. 1477-87.

Fliegmann, J., Uhlenbroich, S., Shinya, T., Martinez, Y., Lefebvre, B., Shibuya, N. and Bono, J.-J. (2011) 'Biochemical and phylogenetic analysis of CEBiP-like LysM domaincontaining extracellular proteins in higher plants', *Plant Physiology and Biochemistry*, 49(7), pp. 709-720.

Genre, A., Chabaud, M., Balzergue, C., Puech-Pages, V., Novero, M., Rey, T., Fournier, J., Rochange, S., Becard, G., Bonfante, P. and Barker, D. G. (2013) 'Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in Medicago truncatula roots and their production is enhanced by strigolactone', *New Phytologist*, 198(1), pp. 179-189.

Gimenez-Ibanez, S., Hann, D. R., Ntoukakis, V., Petutschnig, E., Lipka, V. and Rathjen, J. P. (2009) 'AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants', *Curr Biol*, 19(5), pp. 423-9.

Gomez, S. K. and Harrison, M. J. (2009) 'Laser microdissection and its application to analyze gene expression in arbuscular mycorrhizal symbiosis', *Pest Manag Sci*, 65(5), pp. 504-11.

Gough, C. and Jacquet, C. (2013) 'Nod factor perception protein carries weight in biotic interactions', *Trends in Plant Science*, 18(10), pp. 566-574.

Gressent, F., Drouillard, S., Mantegazza, N., Samain, E., Geremia, R. A., Canut, H., Niebel, A., Driguez, H., Ranjeva, R., Cullimore, J. and Bono, J. J. (1999) 'Ligand specificity of a highaffinity binding site for lipo-chitooligosaccharidic Nod factors in Medicago cell suspension cultures', *Proceedings of the National Academy of Sciences of the United States of America*, 96(8), pp. 4704-4709. Gutjahr, C. and Parniske, M. (2013) 'Cell and Developmental Biology of Arbuscular Mycorrhiza Symbiosis', *Annual Review of Cell and Developmental Biology, Vol 29,* 29, pp. 593-617.

Gutjahr, C. and Paszkowski, U. (2013) 'Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis', *Front Plant Sci*, 4, pp. 204.

Güimil, S., Chang, H. S., Zhu, T., Sesma, A., Osbourn, A., Roux, C., Ioannidis, V., Oakeley, E. J., Docquier, M., Descombes, P., Briggs, S. P. and Paszkowski, U. (2005) 'Comparative transcriptomics of rice reveals an ancient pattern of response to microbial colonization', *Proc Natl Acad Sci U S A*, 102(22), pp. 8066-70.

Harris, J. M., Wais, R. and Long, S. R. (2003) 'Rhizobium-Induced calcium spiking in Lotus japonicus', *Mol Plant Microbe Interact*, 16(4), pp. 335-41.

Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., Arima, S., Squeglia, F., Ruggiero, A., Tokuyasu, K., Molinaro, A., Kaku, H. and Shibuya, N. (2014) 'Chitininduced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwichtype dimerization', *Proc Natl Acad Sci U S A*, 111(3), pp. E404-13.

Hazak, O. and Hardtke, C. S. (2016) 'CLAVATA 1-type receptors in plant development', Journal of Experimental Botany, 67(16), pp. 4827-4833.

He, Z., Wang, Z. Y., Li, J., Zhu, Q., Lamb, C., Ronald, P. and Chory, J. (2000) 'Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1', *Science*, 288(5475), pp. 2360-3.

Herrbach, V., Chirinos, X., Rengel, D., Agbevenou, K., Vincent, R., Pateyron, S., Huguet, S., Balzergue, S., Pasha, A., Provart, N., Gough, C. and Bensmihen, S. (2017) 'Nod factors potentiate auxin signaling for transcriptional regulation and lateral root formation in Medicago truncatula', *J Exp Bot*, 68(3), pp. 569-583.

Hohnjec, N., Czaja-Hasse, L. F., Hogekamp, C. and Küster, H. (2015) 'Pre-announcement of symbiotic guests: transcriptional reprogramming by mycorrhizal lipochitooligosaccharides shows a strict co-dependency on the GRAS transcription factors NSP1 and RAM1', *BMC Genomics*, 16(1), pp. 994.

lizasa, E., Mitsutomi, M. and Nagano, Y. (2009) 'Direct binding of a plant LysM receptorlike kinase, LysM RLK1/CERK1, to chitin in vitro', *J Biol Chem*, 285(5), pp. 2996-3004.

Ito, Y., Kaku, H. and Shibuya, N. (1997) 'Identification of a high-affinity binding protein for N-acetylchitooligosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labeling', *Plant J*, 12(2), pp. 347-56.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E. and Shibuya, N. (2006) 'Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor', *Proc Natl Acad Sci U S A*, 103(29), pp. 11086-91.

Kawaharada, Y., Kelly, S., Nielsen, M. W., Hjuler, C. T., Gysel, K., Muszyński, A., Carlson, R. W., Thygesen, M. B., Sandal, N., Asmussen, M. H., Vinther, M., Andersen, S. U., Krusell, L., Thirup, S., Jensen, K. J., Ronson, C. W., Blaise, M., Radutoiu, S. and Stougaard, J. (2015) 'Receptor-mediated exopolysaccharide perception controls bacterial infection', *Nature*, 523(7560), pp. 308-12.

Kawaharada, Y., Nielsen, M. W., Kelly, S., James, E. K., Andersen, K. R., Rasmussen, S. R., Füchtbauer, W., Madsen, L. H., Heckmann, A. B., Radutoiu, S. and Stougaard, J. (2017) 'Differential regulation of the Epr3 receptor coordinates membrane-restricted rhizobial colonization of root nodule primordia', *Nat Commun*, 8, pp. 14534.

Kishimoto, K., Kouzai, Y., Kaku, H., Shibuya, N., Minami, E. and Nishizawa, Y. (2010) 'Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus Magnaporthe oryzae in rice', *Plant J*, 64(2), pp. 343-54.

Klaus-Heisen, D., Nurisso, A., Pietraszewska-Bogiel, A., Mbengue, M., Camut, S., Timmers, T., Pichereaux, C., Rossignol, M., Gadella, T. W. J., Imberty, A., Lefebvre, B. and Cullimore, J. V. (2011) 'Structure-Function Similarities between a Plant Receptor-like Kinase and the Human Interleukin-1 Receptor-associated Kinase-4', *Journal of Biological Chemistry*, 286(13), pp. 11202-11210.

Koharudin, L. M., Debiec, K. T. and Gronenborn, A. M. (2015) 'Structural Insight into Fungal Cell Wall Recognition by a CVNH Protein with a Single LysM Domain', *Structure*, 23(11), pp. 2143-54.

Kombrink, A., Rovenich, H., Shi-Kunne, X., Rojas-Padilla, E., van den Berg, G. C., Domazakis, E., de Jonge, R., Valkenburg, D. J., Sánchez-Vallet, A., Seidl, M. F. and Thomma, B. P. (2017) 'Verticillium dahliae LysM effectors differentially contribute to virulence on plant hosts', *Mol Plant Pathol*, 18(4), pp. 596-608.

Kouzai, Y., Mochizuki, S., Nakajima, K., Desaki, Y., Hayafune, M., Miyazaki, H., Yokotani, N., Ozawa, K., Minami, E., Kaku, H., Shibuya, N. and Nishizawa, Y. (2014a) 'Targeted gene

disruption of OsCERK1 reveals its indispensable role in chitin perception and involvement in the peptidoglycan response and immunity in rice', *Mol Plant Microbe Interact*, 27(9), pp. 975-82.

Kouzai, Y., Nakajima, K., Hayafune, M., Ozawa, K., Kaku, H., Shibuya, N., Minami, E. and Nishizawa, Y. (2014b) 'CEBiP is the major chitin oligomer-binding protein in rice and plays a main role in the perception of chitin oligomers', *Plant Mol Biol*, 84(4-5), pp. 519-28.

Lee, W. S., Rudd, J. J., Hammond-Kosack, K. E. and Kanyuka, K. (2014) 'Mycosphaerella graminicola LysM effector-mediated stealth pathogenesis subverts recognition through both CERK1 and CEBiP homologues in wheat', *Mol Plant Microbe Interact*, 27(3), pp. 236-43.

Lefebvre, B., Klaus-Heisen, D., Pietraszewska-Bogiel, A., Herve, C., Camut, S., Auriac, M.-C., Gasciolli, V., Nurisso, A., Gadella, T. W. J. and Cullimore, J. (2012) 'Role of N-Glycosylation Sites and CXC Motifs in Trafficking of Medicago truncatula Nod Factor Perception Protein to Plasma Membrane', *Journal of Biological Chemistry*, 287(14), pp. 10812-10823.

Leo, J. C., Oberhettinger, P., Chaubey, M., Schütz, M., Kühner, D., Bertsche, U., Schwarz, H., Götz, F., Autenrieth, I. B., Coles, M. and Linke, D. (2015) 'The Intimin periplasmic domain mediates dimerisation and binding to peptidoglycan', *Mol Microbiol*, 95(1), pp. 80-100.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C. and Denarie, J. (1990) 'Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal', *Nature*, 344(6268), pp. 781-4.

Liang, Y., Cao, Y., Tanaka, K., Thibivilliers, S., Wan, J., Choi, J., Kang, C., Qiu, J. and Stacey, G. (2013) 'Nonlegumes Respond to Rhizobial Nod Factors by Suppressing the Innate Immune Response', *Science*, 341(6152), pp. 1384-1387.

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T. and Geurts, R. (2003) 'LysM domain receptor kinases regulating rhizobial Nod factor-induced infection', *Science*, 302(5645), pp. 630-633.

Liu, B., Li, J. F., Ao, Y., Qu, J. W., Li, Z. Q., Su, J. B., Zhang, Y., Liu, J., Feng, D. R., Qi, K. B., He, Y. M., Wang, J. F. and Wang, H. B. (2012a) 'Lysin Motif-Containing Proteins LYP4 and LYP6 Play Dual Roles in Peptidoglycan and Chitin Perception in Rice Innate Immunity', *Plant Cell*, 24(8), pp. 3406-3419.

Liu, S., Wang, J., Han, Z., Gong, X., Zhang, H. and Chai, J. (2016) 'Molecular Mechanism for Fungal Cell Wall Recognition by Rice Chitin Receptor OsCEBiP', *Structure*, 24(7), pp. 1192-200.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J. M. and Chai, J. (2012b) 'Chitin-induced dimerization activates a plant immune receptor', *Science*, 336(6085), pp. 1160-4.

Lohmann, G. V., Shimoda, Y., Nielsen, M. W., Jørgensen, F. G., Grossmann, C., Sandal, N., Sørensen, K., Thirup, S., Madsen, L. H., Tabata, S., Sato, S., Stougaard, J. and Radutoiu, S. (2010) 'Evolution and regulation of the Lotus japonicus LysM receptor gene family', *Mol Plant Microbe Interact*, 23(4), pp. 510-21.

Macho, A. P. and Zipfel, C. (2015) 'Targeting of plant pattern recognition receptortriggered immunity by bacterial type-III secretion system effectors', *Curr Opin Microbiol,* 23, pp. 14-22.

Madsen, E. B., Antolín-Llovera, M., Grossmann, C., Ye, J., Vieweg, S., Broghammer, A., Krusell, L., Radutoiu, S., Jensen, O. N., Stougaard, J. and Parniske, M. (2011) 'Autophosphorylation is essential for the in vivo function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5', *Plant J*, 65(3), pp. 404-17.

Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N. and Stougaard, J. (2003) 'A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals', *Nature*, 425(6958), pp. 637-40.

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E. A., Driguez, H., Bécard, G. and Dénarié, J. (2011) 'Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza', *Nature*, 469(7328), pp. 58-63.

Malkov, N., Fliegmann, J., Rosenberg, C., Gasciolli, V., Timmers, A. C., Nurisso, A., Cullimore, J. and Bono, J. J. (2016) 'Molecular basis of lipo-chitooligosaccharide recognition by the lysin motif receptor-like kinase LYR3 in legumes', *Biochem J*, 473(10), pp. 1369-78.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K. E., Thomma, B. P. and Rudd, J. J. (2011) 'Analysis of two in planta expressed LysM effector homologs from the fungus Mycosphaerella graminicola reveals novel functional properties and varying contributions to virulence on wheat', *Plant Physiol*, 156(2), pp. 756-69.

Maxwell, K. L., Fatehi Hassanabad, M., Chang, T., Paul, V. D., Pirani, N., Bona, D., Edwards, A. M. and Davidson, A. R. (2013) 'Structural and functional studies of gpX of

Escherichia coli phage P2 reveal a widespread role for LysM domains in the baseplates of contractile-tailed phages', *J Bacteriol*, 195(24), pp. 5461-8.

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., Terauchi, R., Nishizawa, Y., Shibuya, N., Thomma, B. P. and Talbot, N. J. (2012) 'Effector-mediated suppression of chitin-triggered immunity by magnaporthe oryzae is necessary for rice blast disease', *Plant Cell*, 24(1), pp. 322-35.

Mesnage, S., Dellarole, M., Baxter, N. J., Rouget, J. B., Dimitrov, J. D., Wang, N., Fujimoto, Y., Hounslow, A. M., Lacroix-Desmazes, S., Fukase, K., Foster, S. J. and Williamson, M. P. (2014) 'Molecular basis for bacterial peptidoglycan recognition by LysM domains', *Nature Communications*, 5.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H. and Shibuya, N. (2007) 'CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis', *Proc Natl Acad Sci U S A*, 104(49), pp. 19613-8.

Miyata, K., Hayafune, M., Kobae, Y., Kaku, H., Nishizawa, Y., Masuda, Y., Shibuya, N. and Nakagawa, T. (2016) 'Evaluation of the Role of the LysM Receptor-Like Kinase, OsNFR5/OsRLK2 for AM Symbiosis in Rice', *Plant Cell Physiol*.

Miyata, K., Kozaki, T., Kouzai, Y., Ozawa, K., Ishii, K., Asamizu, E., Okabe, Y., Umehara, Y., Miyamoto, A., Kobae, Y., Akiyama, K., Kaku, H., Nishizawa, Y., Shibuya, N. and Nakagawa, T. (2014) 'The Bifunctional Plant Receptor, OsCERK1, Regulates Both Chitin-Triggered Immunity and Arbuscular Mycorrhizal Symbiosis in Rice', *Plant Cell Physiol*, 55(11), pp. 1864-72.

Moling, S., Pietraszewska-Bogiel, A., Postma, M., Fedorova, E., Hink, M. A., Limpens, E., Gadella, T. W. and Bisseling, T. (2014) 'Nod factor receptors form heteromeric complexes and are essential for intracellular infection in medicago nodules', *Plant Cell*, 26(10), pp. 4188-99.

Nakagawa, T., Kaku, H., Shimoda, Y., Sugiyama, A., Shimamura, M., Takanashi, K., Yazaki, K., Aoki, T., Shibuya, N. and Kouchi, H. (2011) 'From defense to symbiosis: limited alterations in the kinase domain of LysM receptor-like kinases are crucial for evolution of legume-Rhizobium symbiosis', *Plant J*, 65(2), pp. 169-80.

Narusaka, Y., Shinya, T., Narusaka, M., Motoyama, N., Shimada, H., Murakami, K. and Shibuya, N. (2013) 'Presence of LYM2 dependent but CERK1 independent disease resistance in Arabidopsis', *Plant Signal Behav*, 8(9).

Oh, M. H., Wang, X. F., Kota, U., Goshe, M. B., Clouse, S. D. and Huber, S. C. (2009) 'Tyrosine phosphorylation of the BRI1 receptor kinase emerges as a component of brassinosteroid signaling in Arabidopsis', *Proceedings of the National Academy of Sciences of the United States of America*, 106(2), pp. 658-663.

Okada, M., Matsumura, M., Ito, Y. and Shibuya, N. (2002) 'High-affinity binding proteins for N-acetylchitooligosaccharide elicitor in the plasma membranes from wheat, barley and carrot cells: conserved presence and correlation with the responsiveness to the elicitor', *Plant Cell Physiol*, 43(5), pp. 505-12.

Olah, B., Briere, C., Becard, G., Denarie, J. and Gough, C. (2005) 'Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in Medicago truncatula via the DMI1/DMI2 signalling pathway', *Plant Journal*, 44(2), pp. 195-207.

Oldroyd, G. E., Mitra, R. M., Wais, R. J. and Long, S. R. (2001) 'Evidence for structurally specific negative feedback in the Nod factor signal transduction pathway', *Plant J*, 28(2), pp. 191-9.

Op den Camp, R., Streng, A., De Mita, S., Cao, Q., Polone, E., Liu, W., Ammiraju, J. S., Kudrna, D., Wing, R., Untergasser, A., Bisseling, T. and Geurts, R. (2011) 'LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia', *Science*, 331(6019), pp. 909-12.

Paparella, C., Savatin, D. V., Marti, L., De Lorenzo, G. and Ferrari, S. (2014) 'The Arabidopsis LYSIN MOTIF-CONTAINING RECEPTOR-LIKE KINASE3 regulates the cross talk between immunity and abscisic acid responses', *Plant Physiol*, 165(1), pp. 262-76.

Petutschnig, E. K., Jones, A. M., Serazetdinova, L., Lipka, U. and Lipka, V. (2010) 'The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in Arabidopsis thaliana and subject to chitin-induced phosphorylation', *J Biol Chem*, 285(37), pp. 28902-11.

Pietraszewska-Bogiel, A., Lefebvre, B., Koini, M. A., Klaus-Heisen, D., Takken, F. L. W., Geurts, R., Cullimore, J. V. and Gadella, T. W. J. (2013) 'Interaction of Medicago truncatula Lysin Motif Receptor-Like Kinases, NFP and LYK3, Produced in Nicotiana benthamiana Induces Defence- Like Responses', *Plos One*, 8(6).

Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Gronlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N. and Stougaard, J. (2003) 'Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases', *Nature*, 425(6958), pp. 585-92.

Radutoiu, S., Madsen, L. H., Madsen, E. B., Jurkiewicz, A., Fukai, E., Quistgaard, E. M., Albrektsen, A. S., James, E. K., Thirup, S. and Stougaard, J. (2007) 'LysM domains mediate

lipochitin-oligosaccharide recognition and Nfr genes extend the symbiotic host range', *EMBO J*, 26(17), pp. 3923-35.

Rasmussen, S. R., Füchtbauer, W., Novero, M., Volpe, V., Malkov, N., Genre, A., Bonfante, P., Stougaard, J. and Radutoiu, S. (2016) 'Intraradical colonization by arbuscular mycorrhizal fungi triggers induction of a lipochitooligosaccharide receptor', *Sci Rep,* 6, pp. 29733.

Rey, T., Nars, A., Bonhomme, M., Bottin, A., Huguet, S., Balzergue, S., Jardinaud, M. F., Bono, J. J., Cullimore, J., Dumas, B., Gough, C. and Jacquet, C. (2013) 'NFP, a LysM protein controlling Nod factor perception, also intervenes in Medicago truncatula resistance to pathogens', *New Phytologist*, 198(3), pp. 875-886.

Rose, C. M., Venkateshwaran, M., Volkening, J. D., Grimsrud, P. A., Maeda, J., Bailey, D. J., Park, K., Howes-Podoll, M., den Os, D., Yeun, L. H., Westphall, M. S., Sussman, M. R., Ané, J. M. and Coon, J. J. (2012) 'Rapid phosphoproteomic and transcriptomic changes in the rhizobia-legume symbiosis', *Mol Cell Proteomics*, 11(9), pp. 724-44.

Schwessinger, B. and Ronald, P. C. (2012) 'Plant innate immunity: perception of conserved microbial signatures', *Annu Rev Plant Biol*, 63, pp. 451-82.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A. and Zipfel, C. (2011) 'Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1', *PLoS Genet*, 7(4), pp. e1002046.

Shibuya, N., Ebisu, N., Kamada, Y., Kaku, H., Cohn, J. and Ito, Y. (1996) 'Localization and binding characteristics of a high-affinity binding site for N-acetylchitooligosaccharide elicitor in the plasma membrane from suspension-cultured rice cells suggest a role as a receptor for the elicitor signal at the cell surface', *Plant and Cell Physiology*, 37(6), pp. 894-898.

Shibuya, N., Kaku, H., Kuchitsu, K. and Maliarik, M. J. (1993) 'Identification of a novel high-affinity binding site for N-acetylchitooligosaccharide elicitor in the membrane fraction from suspension-cultured rice cells', *FEBS Lett*, 329(1-2), pp. 75-8.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H. and Shibuya, N. (2010) 'Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice', *Plant J*, 64(2), pp. 204-14.

Shinya, T., Motoyama, N., Ikeda, A., Wada, M., Kamiya, K., Hayafune, M., Kaku, H. and Shibuya, N. (2012) 'Functional characterization of CEBiP and CERK1 homologs in arabidopsis

and rice reveals the presence of different chitin receptor systems in plants', *Plant Cell Physiol*, 53(10), pp. 1696-706.

Shinya, T., Osada, T., Desaki, Y., Hatamoto, M., Yamanaka, Y., Hirano, H., Takai, R., Che, F. S., Kaku, H. and Shibuya, N. (2010) 'Characterization of Receptor Proteins using Affinity Cross-linking with Biotinylated Ligands', *Plant and Cell Physiology*, 51(2), pp. 262-270.

Shiu, S. H. and Bleecker, A. B. (2003) 'Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis', *Plant Physiol*, 132(2), pp. 530-43.

Smit, P., Limpens, E., Geurts, R., Fedorova, E., Dolgikh, E., Gough, C. and Bisseling, T. (2007) 'Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling', *Plant Physiol*, 145(1), pp. 183-91.

Staehelin, C., Granado, J., Muller, J., Wiemken, A., Mellor, R. B., Felix, G., Regenass, M., Broughton, W. J. and Boller, T. (1994) 'Perception of Rhizobium nodulation factors by tomato cells and inactivation by root chitinases', *Proc Natl Acad Sci U S A*, 91(6), pp. 2196-2200.

Sun, J., Miller, J. B., Granqvist, E., Wiley-Kalil, A., Gobbato, E., Maillet, F., Cottaz, S., Samain, E., Venkateshwaran, M., Fort, S., Morris, R. J., Ané, J. M., Dénarié, J. and Oldroyd, G. E. (2015) 'Activation of symbiosis signaling by arbuscular mycorrhizal fungi in legumes and rice', *Plant Cell*, 27(3), pp. 823-38.

Svistoonoff, S., Hocher, V. and Gherbi, H. (2014) 'Actinorhizal root nodule symbioses: what is signalling telling on the origins of nodulation?', *Current Opinion in Plant Biology*, 20, pp. 11-18.

Sánchez-Vallet, A., Mesters, J. R. and Thomma, B. P. (2015) 'The battle for chitin recognition in plant-microbe interactions', *FEMS Microbiol Rev*, 39(2), pp. 171-83.

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D. J., Thomma, B. P. and Mesters, J. R. (2013) 'Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization', *Elife*, 2, pp. e00790.

Takahara, H., Hacquard, S., Kombrink, A., Hughes, H. B., Halder, V., Robin, G. P., Hiruma, K., Neumann, U., Shinya, T., Kombrink, E., Shibuya, N., Thomma, B. P. and O'Connell, R. J. (2016) 'Colletotrichum higginsianum extracellular LysM proteins play dual roles in appressorial function and suppression of chitin-triggered plant immunity', *New Phytol*, 211(4), pp. 1323-37.

Tanaka, S., Ichikawa, A., Yamada, K., Tsuji, G., Nishiuchi, T., Mori, M., Koga, H., Nishizawa, Y., O'Connell, R. and Kubo, Y. (2010) 'HvCEBiP, a gene homologous to rice chitin receptor
CEBiP, contributes to basal resistance of barley to Magnaporthe oryzae', *BMC Plant Biol*, 10, pp. 288.

Wan, J., Tanaka, K., Zhang, X., Son, G., Brechenmacher, L., Tran, H. and Stacey, G. (2012) 'LYK4, a Lysin Motif Receptor-Like Kinase, Is Important for Chitin Signaling and Plant Innate Immunity in Arabidopsis', *Plant Physiology*, 160(1), pp. 396-406.

Wan, J., Zhang, X. C., Neece, D., Ramonell, K. M., Clough, S., Kim, S. Y., Stacey, M. G. and Stacey, G. (2008) 'A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis', *Plant Cell*, 20(2), pp. 471-81.

Wang, W., Xie, Z. P. and Staehelin, C. (2014) 'Functional analysis of chimeric lysin motif domain receptors mediating Nod factor-induced defense signaling in Arabidopsis thaliana and chitin-induced nodulation signaling in Lotus japonicus', *Plant Journal*, 78(1), pp. 56-69.

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M.-A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J.-J., Cullimore, J. V., Jehle, A. K., Goetz, F., Kulik, A., Molinaro, A., Lipka, V., Gust, A. A. and Nuernberger, T. (2011) 'Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection', *Proceedings of the National Academy of Sciences of the United States of America,* 108(49), pp. 19824-19829.

Wong, J., Alsarraf, H., Kaspersen, J. D., Pedersen, J. S., Stougaard, J., Thirup, S. and Blaise, M. (2014) 'Cooperative binding of LysM domains determines the carbohydrate affinity of a bacterial endopeptidase protein', *Febs Journal*, 281(4), pp. 1196-1208.

Yamaguchi, Y., Huffaker, A., Bryan, A. C., Tax, F. E. and Ryan, C. A. (2010) 'PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis', *Plant Cell*, 22(2), pp. 508-22.

Yamaguchi, Y., Pearce, G. and Ryan, C. A. (2006) 'The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells', *Proc Natl Acad Sci U S A*, 103(26), pp. 10104-9.

Ye, Y. Y., Ding, Y. F., Jiang, Q., Wang, F. J., Sun, J. W. and Zhu, C. (2017) 'The role of receptor-like protein kinases (RLKs) in abiotic stress response in plants', *Plant Cell Reports*, 36(2), pp. 235-242.

Zeng, L., Velásquez, A. C., Munkvold, K. R., Zhang, J. and Martin, G. B. (2012) 'A tomato LysM receptor-like kinase promotes immunity and its kinase activity is inhibited by AvrPtoB', *Plant J*, 69(1), pp. 92-103. Zhang, X., Dong, W., Sun, J., Feng, F., Deng, Y., He, Z., Oldroyd, G. E. and Wang, E. (2015) 'The receptor kinase CERK1 has dual functions in symbiosis and immunity signalling', *Plant J*, doi: 10.1111/tpj.12723(81), pp. 258-267.

Zhang, X. C., Cannon, S. B. and Stacey, G. (2009) 'Evolutionary genomics of LysM genes in land plants', *BMC Evol Biol*, 9, pp. 183.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D., Boller, T. and Felix, G. (2006) 'Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacteriummediated transformation', *Cell*, 125(4), pp. 749-60.

Supplemental data









#: truncated gene: no intracellular region

inderlined : genes absent in previous phylogenetic analyses

Italic : genes not used in our phygenetic analysis

- *: prediction or sequence has been corrected
- Last exon has been skipped according to the gene structure in other plant species and to symbimics data -
- One short exon has been added according to the gene structure in other plant species and to symbimics data 2
- One short exon has been added according to the gene structure in other plant species and to symbimics data m
 - One short exon has been added according to the gene structure in other plant species and to symbimics data 4
 - Start codon has moved according to the protein sequences in other plant species ഹ
- The mRNA is annotated as two genes separated . Medtr5g086310 covers exon 1-6 and Medtr5g086330 covers 6-12 9
- The mRNA can be found in two separated genes. Lj0g3v0102179 corresponds to exon 1 and Lj0g3v0124999 corresponds to exon 2
 - - First short exon has been skipped according to the gene structure in other plant species ∞
- First short exon has been skipped according to the gene structure in other plant species б
- First short exon has been skipped. One short exon has been added and two intron borders have been moved 10

according to the gene structure and protein sequences in other plant species

- Start codon has moved according to the protein sequences in other plant species 11
- A premature stop codon has been modified according to genomic sequences from other cultivars 12
- Last exon has been skipped according to the gene structure in other plant species 13
- Two exons have been added at the beginning of the gene according to the gene structure in other plant species 14
- First exon has been skipped according to the gene structure in other plant species 15
- Nucleotide A320 (from ATG) has been deleted accordingly to the sequence in Solanum tuberosum 16
- Two intron have been added and one intron border has been modified 17

according to the gene structure and protein sequences in other plant species

- Start codon has moved according to the protein sequences in other plant species 18
- Two exons have been added at the beginning of the gene according to shimizu et al., 2010 19
- Five exons have been added at the beginning of the gene according to the gene structure and protein sequence in other plant species 20
 - One exon has been added according to the gene structure in other plant species

	Clade	Medicago (Mt4)	Alternative name	Nb introns	Lotus (V3.0)	Nb introns	Brassica rapa (V1.3)	Alternative name	Nb introns	Arabidopsis (TAIR10)	Alternative name	Nb introns
ГАРІІІ	LYMI	Medtr3g072410	LYM1	4	Lj0g3v0219829	4* ⁸	Brara.F01580 Brara.H02266		4	At1g21880	AtLYM1	4
							Brara.B02278		3	At1g77630	AtLYM3	4
ć												
ГЛРІ	ГАМІІ	Medtr4g094730	LYM2	£	Lj4 <u>g</u> 3v0200090	£	<u>Brara.G00290</u>		ß	At2g17120	AtLYM2	3
	Clade	Peach (V2.1)	Alternative name	Nb introns	Tomato (ITAG2.3)	Nb introns	Brachypodium (V3.1)	Alternative name	Nb introns	Rice (V7)	Alternative name	Nb introns
ГАРШ	ГАМІ	Prupe.1G500000	PpLYM1	4	Solyc11g012870	4	Bradi3g57756 Bradi1e46200	BdLYM1 BdLYM3	4 4	Os06g10660 Os02 #53000	OsLYP6 OslYP5	4
									r	Os09g27890	OsLYP4	4
ć		Prupe.5G220900	PpLYM3	4	Solyc03g119550	4* ¹⁶						
ГЛРІ	ГАМІІ	Prupe.8G176700	PpLYM2	ε	Solyc01g112080	Ω	Bradi1g76177 Bradi4g37090	BdLYM2 BdLYM4	ოო	Os03g04110 Os09g37600	OsCEBiP OsLYP3	ოო

Nb intron	0		1	1	T	0* ¹³	0	0 0* ¹²			Nb intron	0		1			0		000	
Alternative name	SILyk10		63ILYAK9	SILyk8	SILyk2	<u>SILyk15</u>	SILYk4	צור¢אפ צור¢א∠			Alternative name	OsLysM-RLK2/OsLYK5/OsNFR5					OsLysM-RLK7 / OsLYK6		OsLysM-RLKG / OSLYK3 OsLysM-RLK5 / OSLYK3 OSLysM-RLK1 / OSLYK3	
Tomato (ITAG2.3)	Solyc02g065520		Solyc09g083210	Solyc09g083200 #	Solyc02g094010	<u>Solyc11g069630</u>	Solyc02g089900	Solyc02g089920 Solyc12g089020			Rice (V7)	Os03G13080		<u>0s02G45750</u>			Os11G35330		Os06G41960 Os06G41980 Os02G09960	
Nb intron	0		1		0	0	_{6*} 0	0		1	Nb intron	0		1			0		0	
Alternative name	PpLYR1		PpLYR2		PpLYR6	PpLYR7	PpLYR3	PpLYR4		PpLYR5	Alternative name	<u>Bd LYR1</u>		<u>Bd LYR2</u>			Bd LYR4		Bd LYR3	
Peach (V2.1)	Prupe.7G147300		Prupe.1G027000		Prupe.3G303700	Prupe.6G104800	Prupe.7G147600	Prupe.7G147500		Prupe.6G357200	Brachypodium (V3.1)	<u>Bradi1g69290</u>		Bradi3g51790			<u>Bradi4g16350</u>		Bradi3 <u>g06770</u>	
Nb intron	0	0	1*7		0	0	0	0	0	1 1	Nb intron				1		0		0	
Alternative name	LjNFRS	LjLYS11	124999		LjLYS16	LjLYS15	LjLYS12		LjLYS13 LjLYS14	1)LYS21 LJLYS21	Alternative name				AtLyk2		AtLyk4		AtLyk5	
Lotus (V3.0)	Lj2g3v1828350	Lj4g3v0912440	Lj0q3v0102179+3v0		Lj1g3v3834250	Lj3g3v3082380	Lj2g3v1828320	Lj0g3v0145339	Lj2 <u>g</u> 3v2899910 Lj2g3v2899900	<i>absent in V3.0</i> Lj1g3v2808030	Arabidopsis (TAIR10)				At3g01840		At2g23770		At2g33580	
Nb intron	0	0	7		0	0	0	0	0 0	1^{*1} 1	Nb intron				1		0		0	
Alternative name	NFP	LYR1	<u>LYR8</u>		<u>LYR10</u>	LYR9	LYR3	LYR2	LYR4 LYR7	LYR5 LYR6	Alternative name									
Medicago (Mt4)	Medtr5g019040	Medtr8g078300	<u>Medtr5G042440</u>		<u>Medtr7g029650</u>	Medtr4g058570	Medtr5g019050	Medtr1g021845	Medtr5g085790 Medtr3g080170	Medtr7g079350 Medtr7g079320	Brassica rapa (V1.3)				<u>Brara.E03634</u>		<u>Brara.D01416</u>		Brara.D02042	
	YRI A		В		YRII A	Β	rriii A	۵	0	/RIV		YRI A		8	VRII A	Β	rriii A	8	0	/RIV
Clade		RLK			RLK L			RLK	RLK	5	Clade		LysM-RLK LysM-RLK LysM-RLK LysM-RLK LysM-RLK				RLK			
		LysM-	-		LysM-	≥		LysM- II	LysM III								LysM III			
								LYR				LVR								

Clade Medicago (Mt4) Altern	lade Medicago (Mt4) Altern	Medicago (Mt4) Altern	Altern	lative name	Nb intron	Lotus (V3.0)	Alternative name	Nb intron	Peach (V2.1)	Alternative name	Nb intron	Tomato (ITAG2.3)	Alternative name	Nb intron
LYKI Medtr5g086540 LYK1 10*2 Lj2g3v2904640 Medtr5g086120 LYK4 11*3	LYKI Medtr5g086540 LYK1 10*2 LJ2g3v2904640 Medtr5g086120 LYK4 11*3	Medtr5g086540 LYK1 10*2 Lj2g3v2904640 Medtr5g086120 LYK4 11*3	LYK1 10* ² Lj2g3v2904640 LYK4 11* ³	10* ² Lj2g3v2904640 11* ³	Lj2g3v2904640		۲Ì۲۸25	11						
Medtr5g086090 LYK5 11* ⁴ Medtr5g086040 LYK6 10 Lj2g3v2904610 Medtr5g086030 LYK7 11* ⁵	Medtr5g086090 LYK5 11* ⁴ Medtr5g086040 LYK6 10 Lj2g3v2904610 Medtr5g086030 LYK7 11* ⁵	Medtr5g086090 LYK5 11* ⁴ Medtr5g086040 LYK6 10 Lj2g3v2904610 Medtr5g086030 LYK7 11* ⁵	LYK5 11* ⁴ LYK6 10 Lj2g3v2904610 LYK7 11* ⁵	11** 10 Lj2g3v2904610 11* ⁵	Lj2g3v2904610		LjLYS1	11						
LysM- Ri K vi Medtr5g086310+5g086330 LYK2 11* ⁶ Ri K vi Medtr5g086130 LYK3 11 Li2g3v2904690	Medtr5g086310+5g086330 LYK2 11*6 Medtr5g086130 LYK3 11 Li2g3v2904690	Medtr5g086310+5g086330 LYK2 11* ⁶ Medtr5g086130 LYK3 11 Li2g3v2904690	LYK2 11* ⁶ LYK3 11 LI2 <u>R</u> 3v2904690	11* ⁶ 11 Li2 <u>e</u> 3v2904690	Li2 <u>e</u> 3v2904690		Linfr1	11						
Medtr3g080050 LYK9 12 Lj6g3v105558	Medtr3g080050 LYK9 12 Lj6g3v105558	Medtr3g080050 LYK9 12 Lj6g3v105558	LYK9 12 Lj6g3v105558	12 Lj6g3v105558	Lj6g3v105558	0	LjLYS6/LjCERK6	12	Prupe.3G213100	PpLYK2	11^{*10}			
Medtr2g024290 LYK8 11 Lj6g3v181211	Medtr2g024290 LYK8 11 Lj6g3v181211	Medtr2g024290 LYK8 11 Lj6g3v181211	LYK8 11 Lj6g3v181211	11 Lj6g3v181211	Lj6g3v181211	0	Ljt YS7	11	Prupe.46016100	PpLYK1	11	Solyc02g081050 Solyc02g081040 Solyc07g049180 Solyc01g098410	SILYK12 SILYK11 SILYK13 SILYK13	$11 \\ 11*^{14} \\ 11*^{15} \\ 11 \\ 11$
LYKII Medtr5g033490 LYK10 9 Lj2g3v1415. LysM- 1415. <	LYKII <u>Medtr5g033490</u> <u>LYK10</u> 9 Lj2g3v1415.	<u>Medtr5g033490</u> <u>LYK10</u> 9 Lj2g3v1415.	<u>LYK10</u> 9 Lj2g3v1415	9 Lj2g3v1415 [,]	Lj2g3v1415 [,]	410	LjLYS3 / EPR3	6	Prupe.5G168000 Prupe.1G247900	<u>PpLYK3</u> <u>PpLYK4</u>	6 6	Solyc06g069610	SiLyk14	б
RLK V LYKII Medtr8g014500 LYK11 10 Lj3g3v23181 1333 Li3g3v02901 Li3g3v02901 Li3g3v02901 Li3g3v02901	LYKIII Medtr8g014500 LYK11 10 Lj3g3v23181 Li3g3v02901 Li4t1 10 Lj3g3v02901	I <u>Medtr8g014500</u> LYK11 10 Lj3g3v23181 Lj3g3v02901	<u>LYK11</u> 10 Lj3g3v23181 Lj3g3v02901	10 Lj3g3v23181 Lj3g3v02901	Lj3g3v23181 Lj3g3v02901	70 00	rjlys5 Ljlys5	10 10	Prupe.3G058700	<u>PpLYK5</u>	10	Solyc03g121050	SILyka	10
2														
Clade Brassica rapa (V1.3) Alternative name Nb Arabidopsis (TAII intron	lade Brassica rapa (V1.3) Alternative name Nb Arabidopsis (TAII intron	Brassica rapa (V1.3) Alternative name Nb Arabidopsis (TAII intron	Alternative name Nb Arabidopsis (TAII	Nb Arabidopsis (TAII intron	Arabidopsis (TAI	310)	Alternative name	Nb intron	Brachypodium (V3.1)	Alternative name	Nb intron	Rice (V7)	Alternative name	Nb intron
LysM- RLK VI Brara F02055 Brara F02056 11 At3271630	LYKI Brana F02056 11 At3021630	Riara F02055 Brara F02055 11 At3e271630	Brara F02056		05916954V		AHVK1/AACERK1	5	Bradi3g41590	Bd LYK1	11* ¹⁷	Os08g42580 Os09g33630	OsLysM-RLK9/CERK1 OsLysM-RLK10	⁰¹ *10
LysM-	LYKII								<u>Bradi2g40627</u>	Bd LYK2	9* ¹⁸	Os01G36550	OsLysM-RLK3/LYK1	9* ²¹
RLK V LYKIII Brara.H00193 Brara.H00194 10* ¹¹ At1g51940 Brara.F00223 Brara.F00224 8	LYKIII Brara.H00193 Brara.H00194 At1g51940 Brara.F00223 Brara.F00224 8	l <u>Brara.H00193</u> <u>Brara.H00194</u> 10* ¹¹ At1g51940 <u>Brara.F00223</u> <u>Brara.F00224</u> 8	Brara.H00194 10*11 At1g51940 Brara.F00224 8	10 ^{*11} At1g51940 8	At1g51940		AtLyk3	10	Bradi2g49400	Bd LYK3	10	Os01G53840	OsLysM-RLK4	10
i i i i i i i i i i i i i i i i i i i												Os11g34624	OsLysM-RLK8	9

Arbuscular mycorrhizal symbiosis in brief

Plant metabolism relies on mineral nutrient and water. However, plant are sessile organisms and cannot move to access nutrients and water. Nutrient and water uptake occurs via their roots. This uptake largely relies on symbiotic interactions with soil-born microorganisms. One of the most ancient and spread symbioses is the arbuscular mycorrhizal symbiosis (AMS) that is established between the roots of more than 80 % of land plants and fungi belonging to the *Glomeromycota* phylogenetic group. These fungi are ubiquitous and can colonize a broad range of plant species. The AMS likely exists since the colonization of land by plants about 450 million years ago (Remy et al., 1994; Smith and Smith, 2011). The arbuscular mycorrhizal fungi (AMF) are obligate biotroph meaning they need to colonize plant roots to accomplish their life cycle. The plant provides fungi with carbohydrates issued from the photosynthesis (Smith and Smith, 2011) as well as with fatty acids that AMF are unable to synthesize (Bravo et al., 2017). In return, AMF provide plants with nutrients (such as phosphate) they collect through their extra-radicular hyphal network (Fig. 9). Nutrient exchanges between the plant and the AMF take place in the inner cortex of roots in which AMF develop highly branched structures called arbuscules (Fig. 10). Arbuscules are surrounded by a peri-arbuscular membrane separating the AMF from the plant cell cytoplasm.



Fig. 9 The hyphal network of AMF explores a larger soil area than plant roots. Roots of plants can explore a restrained soil area (a) that will be rapidly deprived in mineral nutrients such as phosphate (P). In contrast, AMF can explore a larger soil volume (b) and collect nutrients such as P that will be delivered to plants in exchange of carbohydrates and lipids (C). AMS establishment is tightly controlled by the plant and for example, in high phosphate containing soils plant does not establish AMS. AMS establishment first relies on a molecular dialog between plant and AMF (Fig. 10). The AMF spore is activated by plant exudates, in which strigolactones, plant hormones, have been identified to activate hyphal metabolism and branching (Parniske, 2008). The plant likely perceives AMF through signal molecules secreted by AMF, called Myc-Factors that activate in plant cells a signaling cascade involving oscillation of calcium concentration around and into the nucleus. In AMF exudates, lipochitooligosaccharides (LCOs) and short chito-oligosaccharides (COs), that are described in details in the first part of the introduction, have been identified as potential Myc-Factors (Maillet et al., 2011; Genre et al., 2013). When the AMF hypha encounters the root epidermis it forms a structure called hyphopodium. The cells in contact with the hyphopodium will prepare the way for hypha penetration inside the root. This includes a rearrangement of the cell cytoskeleton leading to the formation of a pre-penetration apparatus (PPA). The hypha will cross the epidermal and outer cortex cell layers and reach the inner cortex where it will form the arbuscules (Parniske, 2008; Gutjahr and Parniske, 2013).

In the inner cortex, the AMF spread along the root axis. Depending on the plant species and the AMF species the fungal hyphae spread in the intercellular space between cortical cells, (*Arum* type), or from cell to cell (*Paris* type, Fig. 11). Between these two types of colonization mode a wide range of possibilities exist (Dickson, 2004).



Fig. 10 Steps of AMS establishment. Plant roots exude signals such as strigolactones which induce spore germination and hyphal branching. Fungi produce Myc-Factors that induce in and around the nuclei oscillations of calcium concentration, called calcium spiking. At the epidermis, AMF form special types of appressoria called hyphopodia. As a consequence of sequential chemical and mechanical stimulation, plant cells produce a pre-penetration apparatus (PPA). Membrane invagination inside the PPA allows the fungus to cross the epidermis and outer cortex cell layers. AMF then grows laterally along the root axis inside or between the inner cortex cells (See Fig.3). AMF also form in these cells branched structures called arbuscules where nutrient exchanges take place. Growth of extraradical hypha will allow secondary penetration of root. Finally, spores will be produced outside of the plant root (Adapted from Parniske 2008).



Rhizobium legume symbiosis in brief

The Rhizobium-legume symbiosis (RLS) is established between bacteria of the rhizobium type and plants that belong in most of the cases to the legume family. The RLS appeared more recently than the AMS in the plant history, about 60 million years ago (Kistner and Parniske, 2002). The RLS allows plants to access a new nitrogen source: the gaseous atmospheric dinitrogen that is reduced by rhizobia in ammonium (NH_4^+). In exchange, plants provide carbohydrates to the bacteria. As for establishment of the AMS, establishment of the RLS relies on a molecular dialog between plant hosts and rhizobia (Fig. 12; for review about the RLS establishment, see Oldroyd and Downie, 2008). Plants secrete in their root exudates flavonoids that are perceived by rhizobia and that activate Nod gene expression, allowing bacterial production of nodulation factors (Nod-factors). Nod-factors are LCOs that are essential for RLS establishment in most legumes. In contrast to the AMS, the RLS shows high level of host specificity for rhizobia strains. Nod-factors are considered as the key element of this specificity. Indeed variations in the LCO structure between rhizobial strains seems to be determinant for the plant to recognize its rhizobial partner (Dénarié et al., 1996; Downie, 2010). Nod-factors are perceived by plant receptors of the multigenic Lysin-Motif Receptor-Like Kinase (LysM-RLKs) family, previously described in details. Nod-factor perception activates a signaling pathway similar to that activated by Myc-factors. In the most evolved legumes such as the models Medicago truncatula and Lotus japonicus, root hairs curl and entrap a rhizobium cell. Then an infection thread is formed by plasma-membrane invagination allowing bacteria to colonize root hairs and reach the cortex (Fig. 12). Plant cells prepare bacterial penetration through cell rearrangements similar to those leading to the PPA in the AMS are observed before membrane invagination. At the same time cortical cells start to multiply, leading to the formation of a primordium that is going to form the nodule, a new organ that emerges from the root. The bacteria multiply in the infection thread that branches in the nodule. Bacteria enclose by a membrane (symbiosome) are then released in the cytoplasm of nodule cells and differentiate into bacteroids, the nitrogen-fixing form.

82



Fig. 12 RLS establishment. Flavonoids are secreted by the plant root and perceived by rhizobia in the rhizosphere. They induce the production of nodulation factors (Nod factors) that are recognized by the plant. Nod factor perception activates the symbiosis signaling pathway, leading to calcium spiking. Rhizobia are entrapped by root hairs that curl around bacteria. Infection threads are invaginations of the plant cell plasma membrane that are initiated at the site of root hair curling and allow rhizobia colonization first of the root and later of the nodule. Nodules initiate below the site of bacterial infection through the formation of a meristem in the cortex. The infection threads grow towards the emergent nodules and ramify within the nodules. The bacteria are released from the infection threads into membrane-bound compartments inside the cells of the nodules. After release, the bacteria differentiate into a nitrogen-fixing state. From Oldroyd, 2013.

The common symbiosis signaling pathway

The same signaling pathway is activated by Nod-factors and Myc-factors and is required for establishment of both symbioses. This pathway (summarized in Fig. 13) has thus been called the Common Symbiosis Signaling Pathway (CSSP). Ca²⁺ spiking is a secondary messenger of the CSSP. MtDMI2/LjSYMRK is an LRR-RLK that acts upstream the Ca²⁺ spiking and might be activated by Nod-factor and Myc-factor receptors. Ca²⁺ spiking is controlled by potassium importer in the nucleus (MtDMI1/ LjCASTOR and LjPOLLUX) and generated by calcium release in the nucleus/cytoplasm through the Ca²⁺ channels MtCNGC15 a, b and c (Charpentier et al., 2016) and by Ca²⁺ uptake from the nucleus/cytoplasm through a calcium pump, the ATPase MtMCA8 (Capoen et al., 2011). These Ca²⁺ oscillations are decoded by a nuclear calcium and calmodulin dependent protein kinase called MtDMI3/LjCCamK that directly interacts with MtIPD3/LjCYCLOPS, a transcription factor. All these genes are present in plants able to establish at least one of the two root endo-symbioses, the AMS or the RLS (Delaux et al., 2014). This led to the hypothesis that during evolution, the pre-existing signaling pathway involved in AMS establishment was recruited for RLS establishment.



Fig. 13: Common Symbiosis Signaling Pathway components and model. Perception of AMF or rhizobial signals triggers early signal transduction, which is mediated by at least seven shared components. The symbiosis receptor kinase LjSYMRK/MtDMI2 acts upstream of the Nod factor- and Myc factor-induced calcium signatures (Ca²⁺ spiking) occurring in and around the nucleus. Ca²⁺ spiking involves calcium release and uptake from/in a storage compartment (probably the nuclear envelope/endoplasmic reticulum) through the channel MtCNGC15 a, b and c and the pump MtMCA8 respectively. The potassium-permeable channels LjCASTOR and LjPOLLUX (MtDMI1) might compensate for the resulting charge imbalance. The nucleoporins NUP85 and NUP133 are required for Ca²⁺ spiking. The calcium and calmodulin-dependent protein kinase (LjCCaMK/MtDMI3) forms a complex with LjCYCLOPS/MtIPD3 that might decode the symbiotic calcium signatures (Modified from Parniske 2008).

<u>Reference</u>

Bravo A, Brands M, Wewer V, Dörmann P, Harrison MJ (2017) Arbuscular mycorrhizaspecific enzymes FatM and RAM2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. New Phytol 214: 1631–1645

Capoen W, Sun J, Wysham D, Otegui MS, Venkateshwaran M, Hirsch S, Miwa H, Downie JA, Morris RJ, Ane J-M, et al (2011) Nuclear membranes control symbiotic calcium signaling of legumes. Proc Natl Acad Sci 108: 14348–14353

Charpentier M, Sun J, Martins TV, Radhakrishnan G V, Findlay K, Soumpourou E, Thouin J, Véry A, Sanders D, Morris RJ, et al (2016) Symbiotic Calcium Oscillations. Science (80-) 352: 1102–5

Delaux PM, Varala K, Edger PP, Coruzzi GM, Pires JC, Ané JM (2014) Comparative Phylogenomics Uncovers the Impact of Symbiotic Associations on Host Genome Evolution. PLoS Genet. doi: 10.1371/journal.pgen.1004487

Dénarié J, Debellé F, Promé JC (1996) Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu Rev Biochem 65: 503–535

Dickson S (2004) The Arum-Paris continuum of mycorrhizal symbioses. New Phytol 163: 187–200

Downie JA (2010) The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiol Rev 34: 150–170

Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, Fournier J, Rochange S, Bécard G, Bonfante P, et al (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol 198: 190–202

Gutjahr C, Parniske M (2013) Cell and Developmental Biology of Arbuscular Mycorrhiza Symbiosis. Annu Rev Cell Dev Biol 29: 593–617

Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7: 511–518

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, et al (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469: 58–63

Oldroyd GED (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11: 252–263

Oldroyd GED, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59: 519–546

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6: 763–775

Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci U S A 91: 11841–11843

Smith SE, Smith FA (2011) Roles of Arbuscular Mycorrhizas in Plant Nutrition and Growth: New Paradigms from Cellular to Ecosystem Scales. Annu Rev Plant Biol 62: 227–250

Context of my PhD

The team in which I did my PhD is called symbiotic signals. The historical and currently main research of this team is on symbiotic signal perception and signaling in the frame of the RLS. This is done on the model legume *M. truncatula* by genetic and biochemical approaches. My PhD is part of a new research line in this team, developed by Benoit Lefebvre, on symbiotic signal perception and signaling in the frame of the AMS using non-legume models.

Objectives of my PhD

AMS establishment is an active process from the plant. Indeed root cells prepare themselves to receive the AMF. Thus, communication between the plant and the AMF is necessary for the plant to perceive its symbiotic partner. Because LCOs are essential in the RLS establishment and because LCOs can activate the CSSP in non-nodulating plants, it has been hypothesized that LCOs produced by the AMF could be symbiotic signals perceived by the plant and involved in the AMS establishment.

The main objective of my PhD thesis was to determine whether perception of AMF LCOs is important for the AMS establishment. To understand the role of LCOs in the AMS, we decided to identify the LCO receptors in plants with an *a priori* approach. By analogy with the RLS, we decided to study orthologs in non nodulating plants of LysM-RLKs known to be involved in LCO perception and required for the RLS establishment. I focused my investigations on non-nodulating plants to avoid any cross-talk between signaling for the RLS and for the AMS establishment and also to reduce the chances to have genetic redundancy between the LysM-RLKs studied since more *LysM-RLK* duplication events occurred in legumes than in non-nodulating plants. I also investigated how genes putatively involved in LCO perception in the frame of the RLS. During my PhD I mainly studied *SILYK10* from *Solanum lycopersicum* and *BdLYR1* from *Brachypodium distachyon* which are orthologs of *MtNFP*, a gene essential for the RLS in *Medicago truncatula*. The objective of studying these two species was to compare the role of LCOs in the AMS establishment in dicots and monocots.

87

To determine the role of LCOs in the AMS establishment and the evolution of LCO perception in legumes, I aimed to answer the following questions:

i) Are SILYK10 and BdLYR1 LCO receptors?

ii) Are SILYK10 and BdLYR1 important for the AMS establishment?

iii) Can *SILYK10* and *BdLYR1* coding sequence complement the absence of nodulation in an *Mtnfp* mutant?

iv) Does *MtNFP* promoter region have evolved compared to *SlLYK10* and *BdLYR1* promoter regions for an expression in nodules?

Our results led us to hypothesize that the ancestor of *MtNFP* was already functional for RLS and was directly recruited during evolution for a role in the RLS establishment.

Chapter 1: *SILYK10* regulates the AMS in tomato

Preamble

SILYK10 belongs to the LYRIA phylogenetic group of the LysM-RLK family that contains members shown to play positive role in RLS establishment (*MtNFP, PaNFP* and *LjNFR5*) and to bind LCOs (LjNFR5). *PaNFP* was also shown to play a role in AMS establishment in *Parasponia andersonii*. Moreover *Arabidopsis thaliana*, unable to form the AMS, has no member in the LYRIA group. By analogy to the legume and *P. andersonii* LysM-RLKs, we though that *SILYK10* might play a role in AMS. To demonstrate this hypothesis, *SILYK10* was knock-down by Virus Induced Gene Silencing (VIGS). Plant were then inoculated with spores of the AMF *Rhizophagus irregularis* and root colonization was quantified both by counting colonization sites and by qRT-PCR on a fungal house-keeping gene and on plant AMS marker genes.

When I started my PhD, most of the results were obtained. The article was in revision. I worked to answer to the reviewer's comments, performing mainly the qRT-PCR experiments. Our work was published in New Phytologist in 2016.



The LysM receptor-like kinase SILYK10 regulates the arbuscular mycorrhizal symbiosis in tomato

Luis Buendia^{1,2}*, Tongming Wang^{1,2}*, Ariane Girardin^{1,2}* and Benoit Lefebvre^{1,2}

¹Laboratoire des Interactions Plantes-Microorganismes, INRA, UMR441, Castanet-Tolosan F-31326, France; ²Laboratoire des Interactions Plantes-Microorganismes, CNRS, UMR2594, Castanet-Tolosan F-31326, France

Author for correspondence: Benoit Lefebvre Tel: +33561285322 Email: benoit.lefebvre@toulouse.inra.fr

Received: 5 August 2015 Accepted: 10 October 2015

New Phytologist (2016) 210: 184-195 doi: 10.1111/nph.13753

Key words: arbuscular mycorrhiza/ mycorrhizal (AM), calcium- and calmodulindependent protein kinase (CCaMK), lysin motif receptor-like kinase (LysM-RLK), NFP, NFR5. Solanum lycopersicum. Solyc02 g065520, tomato.

Introduction

The arbuscular mycorrhizal (AM) symbiosis between Glomeromycota fungi and the majority of land plants, including the legumes and Solanaceae, but not the Brassicaceae, allows better nutrient and water uptake for plants and could be important for sustainable low-input agriculture. The establishment of this symbiosis involves communication between partners. Plants produce strigolactones, a class of hormones (Gomez-Roldan et al., 2008; Umehara et al., 2008), which stimulate AM fungal metabolism, germination and presymbiotic growth (Akiyama et al., 2005; Besserer et al., 2008). The AM fungus Rhizophagus irregularis produces lipochitooligosaccharides (called Myc-LCOs; Maillet et al., 2011) and short chitooligosaccharides (called Myc-COs; Genre et al., 2013). In legumes, Myc-LCOs and Myc-COs activate a plant signaling pathway essential for the establishment of the symbiosis. Following these early signaling events, fungi form hyphopodia at the surface of the root epidermis and plants form a prepenetration apparatus (Genre et al., 2008) which allows fungal penetration through the root epidermis, followed by intercellular colonization of the cortex. Finally, highly branched structures, called arbuscules, are developed in cortical cells and are surrounded by a plant plasma membrane-derived

Summary

• Most plants have the ability to establish a symbiosis with arbuscular mycorrhizal (AM) fungi, which allows better plant nutrition. A plant signaling pathway, called the common symbiosis signaling pathway (CSSP), is essential for the establishment of both AM and root nodule symbioses. The CSSP is activated by microbial signals. Plant receptor(s) for AM fungal signals required for the activation of the CSSP and initial fungal penetration are currently unknown.

• We set up conditions to use virus-induced gene silencing (VIGS) in Solanum lycopersicum to study the genes potentially involved in AM.

• We show that the lysin motif receptor-like kinase SILYK10, whose orthologs in legumes are essential for nodulation, but not for AM, and SICCaMK, a component of the CSSP, are required for penetration of the AM fungus Rhizophagus irregularis into the roots of young tomato plants.

• Our results support the hypothesis that the SILYK10 ancestral gene originally played a role in AM and underwent duplication and neofunctionalization for a role in nodulation in legumes. Moreover, we conclude that VIGS is an efficient method for fast screening of genes playing major roles in AM.

> periarbuscular membrane. Nutrient exchange takes place principally in these cells (for a review, see Gutjahr & Parniske, 2013). Among the proteins involved in these exchanges, members of the phosphate transporter family are specifically expressed during AM symbiosis in various plants (Harrison et al., 2002; Paszkowski et al., 2002; Nagy et al., 2005; Maeda et al., 2006; Chen et al., 2014), localize exclusively in periarbuscular membranes (Rausch et al., 2001; Harrison et al., 2002; Maeda et al., 2006; Kobae & Hata, 2010; Chen et al., 2014) and are required for AM (Maeda et al., 2006; Javot et al., 2007).

> The signaling pathway essential for the establishment of AM is also essential for the establishment of the nitrogen-fixing root nodule (RN) symbioses in legumes with rhizobia bacteria, and in actinorhizal plants with Frankia bacteria (Svistoonoff et al., 2014). It has thus been called the common symbiosis signaling pathway (CSSP). CSSP activation leads to the production and decoding of calcium oscillations in and around the nucleus. Among the proteins participating in the CSSP and downstream of the calcium oscillations is a calcium- and calmodulindependent protein kinase (CCaMK). This protein is encoded by a single gene, which is found only in plants which undergo AM and/or nodulation (Delaux et al., 2014). Mutations in genes participating in the CSSP are blocked in the fungal penetration of the root epidermis (for a review, see Gutjahr & Parniske, 2013). Downstream of the CSSP, genes have been identified in

^{*}These authors contributed equally to this work.

Medicago truncatula to play specific roles in AM, but not RN, symbiosis (Gobbato *et al.*, 2012; Wang *et al.*, 2012). These genes, *RAM1* and *RAM2*, have orthologs in nonlegume species that undergo AM. *RAM1* encodes a transcription factor and *RAM2*, which is a direct target of RAM1, encodes a glycerol-3-phosphate acyl transferase producing a signal required for fungal penetration (Gobbato *et al.*, 2012; Wang *et al.*, 2012).

Most rhizobia depend on the production of LCOs (called Nod factors; for a review, see Gough & Cullimore, 2011) to establish RN symbiosis. It is considered that RN symbioses have evolved more recently than AM symbiosis, by recruiting signals and the CSSP, pre-existing for AM. Plasma membrane receptors able to perceive microbial symbiotic signals are expected to activate the CSSP. Lysin motif receptor-like kinases (LysM-RLKs) and lysin motif receptor-like proteins (LysM-RLPs) belong to a plant-specific multigenic family. Some members of this family have been shown to bind structurally related N-acetylglucosamine-containing molecules, such as LCOs, chitin oligomers or peptidoglycans (Kaku et al., 2006; Willmann et al., 2011; Broghammer et al., 2012; Fliegmann et al., 2013) and/or are involved in defense or symbioses (Gust et al., 2012). MtNFP in M. truncatula and its orthologs in other legumes are essential for the establishment of RN, but not AM, symbiosis (Radutoiu et al., 2003; Arrighi et al., 2006; Indrasumunar et al., 2010; Zhang et al., 2014). However, MtNFP plays a partial role in Myc-LCOinduced lateral root formation (Maillet et al., 2011), suggesting partial redundancy for Myc-LCO perception in this species.

In contrast with legumes, an ortholog of *MtNFP* in *Parasponia* andersonii, called *PaNFP*, has been shown to play a dual role in both RN and AM symbioses (Op den Camp *et al.*, 2011). *Parasponia* species are unique nonlegumes establishing the RN symbiosis with rhizobia. In *P. andersonii* plants with reduced levels of *PaNFP*, *R. irregularis* was able to penetrate the roots and colonize the cortex, but not to form fully developed arbuscules. As the *P. andersonii* genome is not yet available, the LysM-RLK family in this plant is unknown.

In order to clarify the role in AM of *MtNFP* orthologs in nonlegumes and to extend this role in non-nodulating plants, we used virus-induced gene silencing (VIGS) to knock down *SlLYK10*, the ortholog in tomato, a plant with a sequenced genome, and found that *SlLYK10* is involved in the establishment of AM.

Materials and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L. cv Marmande) was used for the VIGS experiments. Seeds were surface sterilized with a bleach solution (2.5% of active chloride) and 0.1% of tween20 for 20 min. Seeds were rinsed three times with sterile water and incubated for 24 h in a shaken flask with sterile water. The seeds were germinated in square Petri dishes with agar medium for 7 d in a growth chamber (25°C, 16 h : 8 h, day : night photoperiod).

Plasmid construction

pTRV1 and pTRV2-*NbPDS* vectors (Liu *et al.*, 2002a) were obtained from Dr S. P. Dinesh-Kumar (UC Davis, Davis, CA, USA). pTRV2-*NbPDS* was digested by *Eco*RI and *Xho*I, and the *NbPDS* fragment was replaced by a 613-bp PCR-amplified fragment of *SlLYK10* or a 478-bp PCR-amplified fragment of *SlCCaMK*, both containing *Eco*RI and *Xho*I sites (see Supporting Information Table S1 for primer sequences). Control pTRV2 was made by PCR by removing the *NbPDS* fragment and creating overlapping *Eco*RI and *Xho*I sites as follows: GAATTCTCGAG. pTRV1, pTRV2-*NbPDS*, pTRV2-*SlLYK10*, pTRV2-*SlCCaMK* and control pTRV2 were introduced into *Agrobacterium tumefaciens* strain C58 GV3101 pmp90.

Plant vacuum infiltration

Agrobacterium tumefaciens strains were grown on Luria-Bertani (LB) agar plates for 72 h at 28°C, with appropriate antibiotics. The bacterial cells were harvested by scraping the plates and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.6, 150 µM acetosyringone). Strains containing pTRV1 were mixed in a 1:1 ratio with strains containing the pTRV2 derivatives [final optical density at 600 nm (OD₆₀₀) = 0.5 of each) and kept for 3-4 h at room temperature before infiltration. One-week-old plantlets were immerged into the bacterial suspension and placed in a vacuum chamber. The vacuum was maintained at 50 kPa twice for 10s and plantlets were left in the suspension for 30 min. Infiltrated plantlets were planted in vermiculite (Agravermiculite fine grade, Pull Rhenen BV, Rhenen, the Netherlands) or calcinated clay (Attapulgite, Oil Dri US Special, Damolin, Fur, Denmark) in 7-cm square pots (four plants per pot) or in perforated 50-ml tubes (one plant per tube), irrigated first with $0.5 \times \text{modified}$ Long Ashton medium (7.5 μ M NaH₂PO₄) and then with water. The pots and tubes were placed in plastic propagators and grown at 21°C (16 h: 8 h, day: night photoperiod).

Rhizophagus irregularis inoculation

Rhizophagus irregularis DAOM197198 spore stock solution (Agronutrition, Carbonne, France) was diluted to a final concentration of 250 spores ml^{-1} in modified Long Ashton medium. Each plantlet received 2 ml of inoculum, 1 wk after *A. tumefaciens* infiltration for VIGS experiments or directly after planting for wild-type (WT).

Root ink staining and mycorrhizal phenotype analysis

Entire root systems, except for Fig. S5(e) where half root systems were used, were harvested, rinsed and stained by an ink–vinegar protocol: 10 min at 95°C in 10% (w/v) KOH, 3 min in a 5% ink–vinegar solution (Vierheilig *et al.*, 1998).

For the quantification of AM, either the number of colonization sites was counted on entire root systems or randomized root fragments were analyzed following the mycocalc method (Trouvelot *et al.*, 1986). An area with several neighboring arbuscules was considered as a single colonization site. These were often on lateral roots and linked to a spore. For mycocalc, 30 root fragments per plant were observed on eight plants inoculated with pTRV2-*SlLYK10* and eight plants inoculated with control pTRV2.

RNA extraction and quantitative reverse transcriptionpolymerase chain reaction (qRT-PCR) analyses

Entire root systems, except for Fig. S5(e) where half root systems were used, were harvested, frozen in liquid nitrogen and ground in 2-ml tubes with a 4-mm steel bead using a MM400 mill (Retsch, Haan, Germany). Total RNA was isolated using an RNAeasy plant mini kit (Qiagen), except that the DNAse treatment was performed using the Turbo DNase kit (Ambion) after elution from columns. The integrity and quantity of the RNA preparations were checked using Agilent RNA 6000 Nano Chips (Agilent Technologies, Santa Clara, CA, USA). Reverse transcription was performed with SuperScript II (Invitrogen, Santa Clara, CA, USA) and polyT primer (for fungal and plant mRNA) or primer 305 (for viral RNA; see Table S1 for primer sequences) on at least 350 ng of total RNA. For the quantification of SlCCaMK and SlLYK10 cDNA, primers (see Table S1 for primer sequences) were designed outside of the gene regions cloned in pTRV2. Primer specificity was verified on agarose gel for the presence of single amplicons. LightCycler 480 SYBR Green I Master was used to achieve qRT-PCR on a LightCycler 480 (Roche). Transcript levels were quantified with absolute standard curves. Control of genomic DNA contamination was performed using primers in an SlCCaMK intron. Relative expression levels were calculated using Solyc03g111010 (SlGAPDH) or Solyc06g009960 (SlEF1-a) as reference genes. Results were similar and only values calculated with Solyc03g111010 are shown, except for Fig. S5(e), where only Solyc06g009960 was used. Technical duplicates were made for each biological replicate.

In silico sequence analyses

Alignments were made using MAFFT (http://mafft.cbrc.jp/ alignment/server) on amino acid sequences of either extracellular or intracellular regions without the transmembrane domains. Phylogenetic trees and bootstraps were calculated by PhyML, and trees were drawn by TreeDyn (http://www.phylogeny.fr). The WAG amino acid substitution model was used, which was optimal by ProtTest3.2 (Darriba et al., 2011) compared with JTT or Dayhoff. Gaps were removed from the alignment by PhyML (56.8% and 57.9% of the extracellular and intracellular region alignments, respectively). Gamma distribution parameters of 3.524 and 1.484, and proportions of invariable sites of 0.051 and 0.023, were used by PhyML for extracellular and intracellular region tree building, respectively. The number of bootstraps was 100. siRNA-Finder (Si-Fi) was obtained at 'http://labtools.ipkgatersleben.de'.

Statistical analyses

Statistical analysis of the difference of the means between two groups was performed using Student's test (*t*-test) when variances were equal and Student's test with Welch correction (Welch *t*test) when variances were unequal (*P* values are given in the text). Homogeneity of variances was tested using Levene's test.

Results

Establishment of a protocol for tomato VIGS compatible with the analysis of root AM

Tomato is widely used as a plant model to study AM. VIGS, which works especially well in Solanaceae, has been proven to be efficient for studies on defense or development, but has not yet been used for the study of AM in tomato. VIGS should theoretically allow a rapid screen for the determination of genes essential or playing major roles in AM. Indeed, it has been shown in pea that VIGS can lead to the silencing of genes with known roles in AM to levels sufficient to produce symbiotic phenotypes (Grønlund et al., 2010, 2013). Based on the available protocols for VIGS in tomato (Ryu et al., 2004; Yan et al., 2012), we set up conditions compatible with the growth of plants in low phosphate, inoculation with AM fungi, easy access to roots and a limited size of the root system to facilitate AM analysis. Following the optimization of several parameters (Fig. S1a), we observed that plant growth was reduced and root colonization by R. irregularis was higher at 2 wk post-inoculation (wpi) when the plants were grown in vermiculite compared with calcinated clay. In order to perform VIGS in this work, we used Tobacco rattle virus (TRV), a bipartite RNA virus. Corresponding cDNAs had been modified previously and introduced into two binary vectors (pTRV1 and pTRV2) by Liu et al. (2002b). Modified TRV was reconstituted in each inoculated plant through T-DNA injection by A. tumefaciens, and spread autonomously inside each plant. For the establishment of optimal conditions for silencing, we used the construct pTRV2-NbPDS which targets the phytoene desaturase gene (PDS; Liu et al., 2002a). PDS is involved in carotenoid biosynthesis, and silencing of this gene leads to photobleaching of leaves (Kumagai et al., 1995). In our experiments, the first bleaching symptoms appeared after 12 d, suggesting that the gene had already been silenced, as the depletion of carotenoids takes a few days. Optimized parameters for VIGS were selected based on the maximal number of plants with leaves showing bleaching symptoms observed at 2 wk after A. tumefaciens inoculation (Fig. S1a). VIGS of SIPDS was used in further experiments as a control for VIGS efficiency. The average efficiency of SIPDS VIGS, as measured by the percentage of plants with observable bleached leaves at 3 wk after A. tumefaciens inoculation, was 53.8% (\pm 17.4%, n=45).

Silencing of *SICCaMK* demonstrates that VIGS can be used to study AM in tomato

In order to test the efficiency of VIGS on the genes involved in AM in the root, we introduced into the pTRV2 plasmid a DNA

fragment of the S. lycopersicum CCaMK (Table S2), whose orthologs are essential for the establishment of AM in various plants (Levy et al., 2004; Tirichine et al., 2006; Gutjahr et al., 2008; Chen et al., 2009; Sinharoy & DasGupta, 2009; Svistoonoff et al., 2013). The software Si-Fi was used to predict the number of efficient siRNA targets that can be produced by the plant gene fragment introduced into pTRV2. A good correlation between VIGS efficiency and Si-Fi prediction has been shown previously (Lee et al., 2014). Si-Fi predicted efficient targeting of SICCaMK by the pTRV2-SICCaMK construct (Fig. S1b). Indeed, in a sample of 15 plants inoculated with an A. tumefaciens strain containing pTRV2-SlCCaMK, but not inoculated with R. irregularis, the SICCaMK transcript level was significantly reduced (t-test P value: 3.0E-06) by c. 50% compared with control plants inoculated with an A. tumefaciens strain containing the pTRV2 vector (Fig. 1a).

In five independent VIGS experiments, fungal colonization and development of arbuscules were observed by microscopy after ink staining of entire root systems from individual plants. On average, 47% of plants inoculated with the A. tumefaciens strain containing pTRV2-SlCCaMK showed no apparent colonization by R. irregularis at 2 wpi (Fig. 1b). In comparison, all control plants were colonized by R. irregularis. As the level of silencing is expected to be different in each plant, ranging from no silencing to maximum silencing, the corresponding phenotypes are expected to range from WT to no colonization. In order to quantify AM in the population, the number of fungal colonization sites was counted in two of these experiments. A drastic reduction (80%) in the average number of colonization sites was observed in pTRV2-SlCCaMK-inoculated plants compared with controls (Welch ttest P value: 6.4E-06). The distribution of the number of colonization sites per plant is shown by classes in Fig. 1(c). Variability in the number of colonization sites was found for control plants, as always observed for AM in plants. The pTRV2-SICCaMK-inoculated plants showed a clear difference in distribution compared with control plants.

Microscopic observation showed that, among the 47% of plants inoculated with pTRV2-*SlCCaMK* which were not colonized, most of the roots were devoid of any extraradical fungal hyphae. In some roots, fungal hyphae were attached to the root and hyphopodia were observed, but without penetration (Fig. 1d,e). In rare cases, roots of pTRV2-*SlCCaMK*-inoculated plants showed limited colonization, manifested by poor growth of hyphae and by arbuscules that were not fully developed (Fig. 1f). In all the other roots, despite changes in the number of colonization sites, fungal hyphae developed normal arbuscules as in control roots (Fig. 1g).

In an additional experiment, the development of AM was quantified by qRT-PCR in root extracts. The amount of fungi was estimated by measuring the relative level of a fungal housekeeping gene (*RiGADPH*), which can only be detected in plants inoculated by *R. irregularis* (Fig. 2a). A reduction (79%) in *RiGADPH* transcripts was observed in pTRV2-*SlCCaMK*-inoculated plants compared with control plants (Fig. 2e). In the same root extracts, the degree of arbuscule formation was estimated by



Fig. 1 SICCaMK is essential for arbuscular mycorrhiza (AM) at 2 wk postinoculation (wpi) with Rhizophagus irregularis. (a) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (gRT-PCR), of SICCaMK in Solanum lycopersicum roots inoculated with Agrobacterium tumefaciens bearing pTRV2-SICCaMK or control pTRV2. Entire root systems were harvested at 3 wpi with A. tumefaciens without R. irregularis inoculation. Mean and standard error (SE) between individual roots are shown. A significant difference was detected (*, P < 0.05). RU, relative units; VIGS, virus-induced gene silencing. (b-g) Analysis of the effect of silencing of SICCaMK on AM. Entire root systems of plants inoculated with pTRV2-SICCaMK or control pTRV2 were stained by an ink-vinegar protocol and observed under a binocular stereomicroscope for AM at 2 wpi with R. irregularis. The number of plants analyzed (n) is shown above the bars (a-c). (b) Percentage of plants colonized by R. irregularis. Histogram bars represent the mean of five independent experiments. Relative SE between the means of each experiment is shown. (c) Number of colonization sites per plant. The percentages of plants in the indicated colonization classes are shown. Histogram bars represent the mean of two independent experiments. (d-f) Images of roots inoculated with pTRV2-SICCaMK. Most of the roots were devoid of any fungal hypha (not shown). In a rare case, fungal hyphae and hyphopodia (black arrows) were observed on roots (d, e). In other rare cases, fungal hyphae penetrated into the roots, but showed limited growth and arbuscules were not fully developed (f, white arrows). (g) Image of roots inoculated with the pTRV2 control: all roots were colonized by R. irregularis and arbuscules were fully developed. Bars, 100 µm.



Fig. 2 SICCaMK is essential for plant symbiotic gene expression at 2 wk post-inoculation (wpi) with Rhizophagus irregularis. (a-d) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (gRT-PCR), of the indicated genes in wild-type (WT) Solanum lycopersicum roots inoculated (+ AM) or not (-AM) with R. irregularis. Entire root systems were harvested at 2 wpi with R. irregularis. Mean and standard error (SE) between individual roots are shown. The graphs show that (a) R. irregularis RiGAPDH and tomato (b) SIPT4, (c) SIPT5 and (d) SIRAM2 are specifically detected in roots inoculated with R. irregularis. (e-h) Analysis of the effect of silencing of SICCaMK on arbuscular mycorrhiza (AM). Relative expression, measured by gRT-PCR, of the indicated genes in roots inoculated with Agrobacterium tumefaciens carrying pTRV2-SICCaMK or control pTRV2, before inoculation with R. irregularis. Entire root systems were harvested at 3 wpi with A. tumefaciens and 2 wpi with R. irregularis. Mean and SE between individual roots are shown. Relative expression of RiGAPDH (e) reflects the amount of fungus, SIPT4 and SIPT5 (f, g) the degree of arbuscule formation, and SIRAM2 (h) the activation of the common symbiosis signaling pathway by *R. irregularis*. The number of plants analyzed (*n*) is shown above the bars. RU, relative units; VIGS, virus-induced gene silencing.

measuring the transcript level of tomato mycorrhizal-induced phosphate transporters *SlPT4* and *SlPT5* (Chen *et al.*, 2014; Fig. 2b,c). Similar reductions (76% and 78%, respectively) in the transcript levels of *SlPT4* and *SlPT5* (Fig. 2f,g) were observed in pTRV2-*SlCCaMK*-inoculated plants compared with control plants. Finally, to assess whether downstream targets of the CSSP were affected by the silencing of *SlCCaMK*, we measured, in the same root extracts, the level of *SlRAM2*, the ortholog of *M. truncatula RAM2*. *SlRAM2* is induced during AM symbiosis in tomato (Fig. 2d) as in *M. truncatula*. The *SlRAM2* transcript level was also decreased by 80% in pTRV2-*SlCCaMK*-inoculated plants compared with control plants (Fig. 2h).

Altogether, these results show that the silencing of *SlCCaMK* through VIGS leads to unsuccessful formation of AM.

SILYK10 is the ortholog of *M. truncatula* MtNFP/MtLYR1 LysM-RLKs

Members of the LysM-RLK family are expected to be involved in the perception of signals produced by AM fungi and to play roles in AM. We first reinvestigated the LysM-RLK family in the tomato genome and found one extra gene in addition to the 13 genes previously described by Zeng et al. (2011). This gene was named SlLYK15 (Table S2) according to previous nomenclature. Despite differences in the percentage of amino acid (16-86%) and nucleotide (38-91%) identities between these paralogs (Fig. S2a,b), all these LysM-RLKs have well-conserved structural elements. Three lysin motifs (LysM) are separated by highly conserved cysteine pairs (Fig. S3a) which, in MtNFP, are involved in disulfide bridges and essential for activity (Lefebvre et al., 2012). Phylogenetic trees were built with either intracellular (Fig. 3) or extracellular (Fig. S2c) regions of the complete LysM-RLK families from tomato and Arabidopsis thaliana, MtNFP and MtLYR1 from M. truncatula, and PaNFP from P. andersonii. In the tree based on the intracellular regions (Fig. 3), two defined subfamilies of LysM-RLKs were found as in M. truncatula (Arrighi et al., 2006): the LYR subfamily containing proteins with an intracellular kinase domain missing the well-conserved glycine-rich loop (Fig. S3b) and predicted to be inactive, and the LYK subfamily containing proteins with predicted active kinases. LYR and LYK proteins were almost equidistant from the outgroup SISYMRK, another RLK belonging to the leucine-rich repeat (LRR) family, showing high divergence in the intracellular regions of the two LysM-RLK subfamilies. The topology of the tree based on the extracellular regions was predicted with less certainty (Fig. S2c), but, overall, only a few differences were seen compared with the trees based on the intracellular region. In both trees, MtNFP, MtLYR1 and PaNFP clearly grouped in a clade with SlLYK10. In tomato, one protein was found in this clade, as in all nonlegume plants undergoing AM symbiosis (Gough & Jacquet, 2013; Delaux et al., 2014). Orthology of SlLYK10 with MtNFPIMtLYR1 was confirmed by microsynteny (Fig. S4a). Microsynteny analysis also suggested that the absence of an SlLYK10 ortholog in Arabidopsis might be a result of a deletion of this and surrounding genes (Fig. S4a). Tomato transcriptomic data (The Tomato



Fig. 3 *SlLYK10* is the *Medicago truncatula MtNFP* ortholog. Intracellular region amino acid sequences of all lysin motif receptor-like kinases (LysM-RLKs) from *Solanum lycopersicum* and *Arabidopsis thaliana*, plus *M. truncatula* MtNFP and MtLYR1 and *Parasponia andersonii* PaNFP (see Supporting Information Table S1 for accession numbers), were used to construct a phylogenetic tree. SISYMRK, a leucine-rich repeat RLK was used as an outgroup. SlLYK8 (in gray) was not used to build the tree because it has a truncated kinase. Its position on the tree is based on the analysis of the extracellular regions (Fig. S2c). Numbers at the branches correspond to bootstrap values. The clade containing SlLYK10 is circled. The blue line separates the LYR and LYK subfamilies.

Genome Consortium, 2012) showed that *SlLYK10* is mainly expressed in roots, with some expression in other organs, particularly in opened flowers (Fig. S4b).

SILYK10 is involved in AM

We first analyzed by qRT-PCR the expression of *SlLYK10* in roots during AM symbiosis. A small (2.2-fold), but significant (*t*-test *P* value: 4.7E-03), increase in *SlLYK10* transcript level was detected in roots at 2 wpi with *R. irregularis* compared with non-inoculated roots (Fig. 4a).

In order to determine whether *SlLYK10* plays a role in AM, we introduced in pTRV2 a DNA fragment of *SlLYK10*, which was predicted by Si-Fi to efficiently target *SlLYK10* (Fig. S1b). We measured the level of *SlLYK10* transcript by qRT-PCR in plants subjected to pTRV2-*SlLYK10* or control pTRV2, but not inoculated with *R. irregularis*. On average, *SlLYK10* transcript levels were significantly (*t*-test *P* value: 1.8E-04) reduced by *c*. 55% in pTRV2-*SlLYK10*-inoculated plants relative to control plants (Fig. 4b). Cross-silencing of other LysM-RLKs by the pTRV2-*SlLYK10* construct is highly unlikely as Si-Fi does not predict any efficient hit in the *SlLYK10* paralogs (Fig. S1b). Indeed, identity between genes of the LysM-RLK family is low (Fig. S2b). However, we measured, by qRT-PCR, the expression

of the closest *SlLYK10* paralogs, *SlLYK9* and *SlLYK8*, in the same root extracts. In contrast with the decrease in *SlLYK10* transcript levels, the transcript levels of *SlLYK9* and *SlLYK8* were not significantly affected by the pTRV2-*SlLYK10* construct (Fig. 4b).

In five independent VIGS experiments, an average of 53% of plants inoculated with pTRV2-*SlLYK10* showed no colonization by *R. irregularis* at 2 wpi (Fig. 4c). The average number of fungal colonization sites, counted in two of these experiments, was drastically reduced (*c.* 70%) at 2 wpi in plants inoculated with pTRV2-*SlLYK10* compared with control plants (Welch *t*-test *P* value: 1.2E-10). The number of colonization sites per plant (Fig. 4d) showed a clear difference in distribution between pTRV2-*SlLYK10*-inoculated and control plants.

Of the 53% of pTRV2-*SlLYK10*-inoculated plants that showed no colonization with *R. irregularis*, most were also devoid of fungal hyphae on the surface of the roots. In rare cases, spores and extraradical hyphae were observed around the roots without penetration (Fig. 4e). In other rare cases, some of the colonized pTRV2-*SlLYK10*-inoculated roots showed limited growth of hyphae and arbuscules that were not fully developed (Fig. 4f,g). In all the other roots, despite changes in the number of colonization sites, fungal hyphae developed normal arbuscules as in control roots (Fig. 4h).

In an independent experiment, mRNA was extracted from roots at 2 wpi with *R. irregularis.* Transcript levels of *RiGAPDH* (reflecting the amount of AM fungi in the roots, Fig. S5a), *SlPT4* and *SlPT5* (reflecting the degree of arbuscule formation in the roots, Fig. S5b,c) and *SlRAM2* (reflecting CSSP-dependent gene regulation, Fig. S5d), detected by qRT-PCR, were reduced by *c.* 66%, 53%, 54% and 70%, respectively, in plants silenced for *SlLYK10* compared with control plants.

In two other independent experiments, we analyzed the AM phenotype and *SlLYK10* transcript levels in individual roots at 2 wpi with *R. irregularis*. In the first experiment, colonization was observed by microscopy after ink staining in one half of the root system and the *SlLYK10* transcript level was quantified by qRT-PCR in the second half of the root system (Fig. S5e). Absence of colonization was observed in roots with decreased *SlLYK10* transcript levels. In the second experiment, *SlLYK10*, *RiGAPDH*, *SlPT4*, *SlPT5* and *SlRAM2* transcript levels were quantified by qRT-PCR within individual entire root systems (Fig. 5a–e). *RiGAPDH*, *SlPT4*, *SlPT5* and *SlRAM2* transcripts were virtually undetectable in the roots with decreased *SlLYK10* transcript levels, suggesting an absence of colonization in these roots. In both experiments, a clear correlation between *SlLYK10* silencing and the absence of AM was found.

In order to investigate the effect of *SlLYK10* silencing at later time points after inoculation with *R. irregularis*, roots were analyzed at 4 and 6 wpi. At 4 wpi, the average number of fungal colonization sites was still drastically reduced (*c.* 70%) in plants inoculated with pTRV2-*SlLYK10* compared with control plants (Welch *t*-test *P* value: 3.9E-07). A difference in the distribution of the number of colonization sites per plant was still observed in the two sets of plants (Fig. 6a); however, the number of pTRV2-*SlLYK10*-inoculated plants without colonization was reduced compared with that at 2 wpi (Fig. 4d). To analyze AM in more



detail, the frequency of mycorrhiza (F), the intensity of the mycorrhizal colonization (M) and the arbuscule abundance (A) in the root systems were all calculated according to Trouvelot *et al.* (1986), and were all strongly reduced (Fig. S6a). At 6 wpi, all plants inoculated with pTRV2-*SlLYK10* were colonized. Individual colonization sites were no longer distinguishable at this stage. We quantified AM by qRT-PCR, measuring *RiGAPDH*, *SlPT4*, *SlPT5* and *SlRAM2* transcript levels, and found that the levels of all of these molecular markers were reduced (Fig. 6b–e), suggesting that fungal abundance and arbuscule development were still

Fig. 4 SILYK10 is essential for arbuscular mycorrhiza (AM) at 2 wk postinoculation (wpi) with Rhizophagus irregularis. (a) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (gRT-PCR), of SILYK10 in wild-type (WT) Solanum lycopersicum roots inoculated (+ AM) or not (-AM) with R. irregularis. Samples were the same as in Fig. 2(a–d). A significant difference was detected (*, P < 0.05). RU, relative units. (b) pTRV2-SILYK10 specifically silences SILYK10. Relative expression in roots inoculated with pTRV2-SILYK10 vs control pTRV2 of SILYK10 and its closest paralogs. Entire root systems were harvested at 3 wpi with Agrobacterium tumefaciens without R. irregularis inoculation and transcript levels were measured by gRT-PCR. Mean and standard error (SE) between three biological replicates are shown, corresponding to a total of 19 pTRV2-SILYK10-inoculated and 10 control roots. A significant difference was detected for the SILYK10 transcript level in pTRV2-SILYK10-inoculated roots compared with control roots (*, P < 0.05). (c-h) Analysis of the effect of silencing of SILYK10 on AM. Entire root systems of plants inoculated with pTRV2-SILYK10 or control pTRV2 were stained by an ink-vinegar protocol and observed under a binocular stereomicroscope for AM at 2 wpi with R. irregularis. The number of plants analyzed (*n*) is shown above the bars (a-d). (c) Percentage of plants colonized by R. irregularis at 2 wpi. Histogram bars represent the mean of five independent experiments. Relative SE between the means of each experiment is shown. (d) Number of colonization sites per plant. The percentages of plants in the indicated colonization classes are shown. Histogram bars represent the mean of two independent experiments. (e-g) Images of roots inoculated with pTRV2-SILYK10: most of the roots were devoid of any fungal hypha. In a rare case, fungal hyphae were observed on roots (e). In other rare cases, fungal hyphae penetrated the roots, but showed limited growth and arbuscules were not fully developed (f, g, white arrows). (h) Image of roots inoculated with pTRV2 control: all roots were colonized by R. irregularis and arbuscules were fully developed. Bars, 100 µm.

reduced in plants inoculated with pTRV2-*SlLYK10* compared with control plants.

To assess SlLYK10 silencing efficiency by VIGS over time, we first quantified the amount of TRV viral RNA in roots at 2 and 6 wpi with R. irregularis by qRT-PCR using a specific primer for reverse transcription of viral RNA on total RNA extracted from roots. We verified the linearity of TRV2 quantification by qRT-PCR using a dilution of the plasmid pTRV2-SlLYK10 and primers in the sequence coding the coat protein (Fig. S6b). We found that the amount of TRV RNA2 was reduced in roots at 6 wpi compared with 2 wpi with R. irregularis (Fig. 6f). We also measured the SlLYK10 silencing level in roots at 2 and 6 wpi with R. irregularis. SlLYK10 transcript levels still appeared to be decreased in pTRV2-SlLYK10-inoculated plants compared with control plants at both time points (Fig. 6g). The difference between 2 and 6 wpi was not significant (*t*-test *P* value: 3.1E-01). Higher variability of SlLYK10 transcript levels was observed between individuals in plants inoculated with both virus (pTRV2-SlLYK10 or pTRV2-control) and R. irregularis compared with plants inoculated with virus only. This could perhaps be caused by the regulation of SlLYK10 expression by R. irregularis, thus making it difficult to analyze the degree of SlLYK10 silencing in plants inoculated with R. irregularis.

Discussion

In this work, we set up conditions in order to use VIGS in tomato to study the role of genes involved in AM. Following the



Fig. 5 Strong silencing of *SILYK10* correlates with the absence of fungal colonization and plant symbiotic gene expression at 2 wk post-inoculation (wpi) with *Rhizophagus irregularis*. (a–e) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), of the indicated genes in *Solanum lycopersicum* roots inoculated with pTRV2-*SILYK10* or control pTRV2. Entire root systems were harvested at 3 wpi with *Agrobacterium tumefaciens* and 2 wpi with *R. irregularis*. Histogram bars represent values for individual root systems. Relative expression of *R. irregularis RiGAPDH* (b) reflects the amount of fungus, *SIPT4* and *SIPT5* (c, d) the degree of arbuscule formation, and *SIRAM2* (e) the activation of the common symbiosis signaling pathway by *R. irregularis*. RU, relative units; VIGS, virus-induced gene silencing.

testing of several parameters (Fig. S1a), conditions were chosen which proved to be efficient for the reproducible silencing of either the *SlPDS* gene in leaves or symbiotic genes in roots. We found that LysM-RLK, *SlLYK10* and *SlCCaMK* are essential for the colonization of roots of young tomato plants by the AM fungus *R. irregularis.*

Direct genetic evidence has shown previously that *CCaMK* is essential for AM in legumes (Levy *et al.*, 2004; Tirichine *et al.*, 2006; Sinharoy & DasGupta, 2009), actinorhizal plants (Svistoonoff *et al.*, 2013) and rice (Gutjahr *et al.*, 2008; Chen *et al.*, 2009). Moreover, indirect evidence (complementation of *M. truncatula Mtdmi3* mutants) has suggested that *CCaMK* from basal plants (liverworts and hornworts; Wang *et al.*, 2010) can play a similar role to *CCaMK* from higher plants (*M. truncatula* and rice; Godfroy *et al.*, 2006). Using VIGS, we show here that, as expected, *SlCCaMK* is also essential for AM in tomato. As in other plants, it is required at the early stage of fungal penetration into the epidermis.

We observed an absence of AM at 2 wpi with *R. irregularis* in plants with highly reduced levels of SlLYK10. In contrast with SlCCaMK, such an early role of SlLYK10 in AM was not evident. Phylogenetic analysis indicates that SlLYK10 is an ortholog of P. andersonii, PaNFP. In P. andersonii plants with silenced PaNFP, penetration of AM fungi in roots and cortical cell invasion were normal, but arbuscule development was blocked (Op den Camp et al., 2011). By contrast, our work has revealed a role of SlLYK10 at the early stage of fungal penetration into the epidermis, suggesting that SILYK10 is involved in the perception of the fungal signal(s) required for plants to allow fungi to penetrate the roots, probably through activation of the CSSP. Indeed, the decrease in SlRAM2 expression on R. irregularis inoculation in plants with silenced SlLYK10 might reflect a decrease in CSSP activation. Similar to its ortholog in P. andersonii, SlLYK10 might also play a role in tomato in arbuscule development in addition to its role in fungal root penetration, as, in some roots, abnormal arbuscules were observed at 2 wpi (Fig. 4f,g). Such a phenotype might represent an intermediate phenotype in roots with lower levels of SlLYK10 silencing. The phenotypical differences between the two species could thus be a result of a lower degree of silencing in *P. andersonii* than in tomato. Alternatively, the establishment of AM in *P. andersonii* might only partially rely on *PaNFP* because of functional redundancy with other gene(s), or because of different mechanisms for the establishment of AM.

We still observed a decrease in colonization and arbuscule abundance at 4 and 6 wpi with *R. irregularis* in plants inoculated with pTRV2-*SlLYK10* compared with control plants, but the number of pTRV2-*SlLYK10*-inoculated plants colonized by *R. irregularis* increased over time. Previous work has shown that VIGS phenotypes are stable over time (Senthil-Kumar & Mysore, 2011). In our conditions, we detected less viral TRV RNA in tomato roots at 6 wpi with *R. irregularis* (7 wpi with TRV mediated by *A. tumefaciens*) than at 2 wpi with *R. irregularis* (3 wpi with TRV), but still a decrease in *SlLYK10* transcript level at 7 wpi with TRV. In this experiment, we cannot exclude that the presence of *R. irregularis* affects the level of the virus in the roots. Although this question has been poorly studied, work on



other viruses has shown either an increase (Miozzi *et al.*, 2011) or a decrease (Maffei *et al.*, 2014) in viral titer during AM symbiosis. A residual *SlLYK10* transcript level in pTRV2-*SlLYK10*inoculated plants might lead to a delay in colonization by *R. irregularis* compared with control plants. Alternatively, an

New Phytologist

Fig. 6 Silencing of SILYK10 reduces arbuscular mycorrhiza (AM) at 4 and 6 wk post-inoculation (wpi) with Rhizophagus irregularis. (a) Number of colonization sites per plant (Solanum lycopersicum). Entire root systems of plants inoculated with pTRV2-S/LYK10 or control pTRV2 were stained by an ink-vinegar protocol and observed under a binocular stereomicroscope for AM at 4 wpi with R. irregularis. The percentages of plants in the indicated colonization classes are shown. Histogram bars represent the mean of two independent experiments. (b-e) Expression of symbiotic markers. Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (gRT-PCR), of the indicated genes in roots inoculated with pTRV2-S/LYK10 or control pTRV2. Entire root systems were harvested at 7 wpi with Agrobacterium tumefaciens and 6 wpi with R. irregularis. Mean and standard error (SE) between individual roots are shown. (f) Quantification by gRT-PCR of Tobacco rattle virus (TRV) RNA2 in root systems inoculated with pTRV2-S/LYK10 or control pTRV2 at the indicated time after inoculation with R. irregularis. Histogram bars represent the mean number of cycles for detection of the TRV RNA2 (crossing point (cp), right y-axis). SEs are shown. Black triangles represent the absolute quantification calculated as 2^{-cp} (left yaxis). (g) Relative expression, measured by gRT-PCR, of SILYK10 in roots inoculated with pTRV2-SILYK10 or control pTRV2. Entire root systems were harvested at 2 and 6 wpi with R. irregularis. Mean and SE between individual root systems are shown. Number of plants analyzed (n) is shown above the bars. RU, relative units; VIGS, virus-induced gene silencing.

SlLYK10-independent pathway might lead to this delayed colonization by the AM fungus. Tomato plants with knock-out of *SlLYK10* are required to clarify the role of *SlLYK10* in the late colonization by *R. irregularis*, and to study the role of *SlLYK10* in the perception of fungal signals.

In any case, it is clear that both *SlLYK10* and *PaNFP* play roles in AM. As *S. lycopersicum* and *P. andersonii* are distant species in the dicots, it can be hypothesized that the ancestral gene plays a role in AM. In legumes, this gene has been duplicated. *MtNFP*, one of the duplicated genes, has apparently been neofunctionalized for a role in nodulation, whereas the other, *MtLYR1*, has been speculated to be involved in AM, perhaps partially redundantly with *MtNFP* (Op den Camp *et al.*, 2011; Young *et al.*, 2012). To date, no role has been reported for *MtLYR1* or its orthologs in other legumes. Redundancy might explain the difficulty of finding major roles for LysM-RLK(s) in AM in legumes. For this reason, the clear role of *SlLYK10* in AM in tomato is particularly interesting and will stimulate studies on related genes in other species.

Many RLKs and RLPs function as heterodimers, consisting of a binding protein with an inactive or low active kinase and a coreceptor with an active kinase. This is the case for the two LRR RLKs, FLS2 and BAK1 (Chinchilla *et al.*, 2007), and the LysM-RLP and LysM-RLK pairs of CEBIP and OsCERK1 (Shimizu *et al.*, 2010) or AtLYM1/3 and AtCERK1 (Willmann *et al.*, 2011). Consistent with this idea, both LYR and LYK are required for RN symbiosis: NFP and LYK3 in *M. truncatula* (Limpens *et al.*, 2003; Arrighi *et al.*, 2006) and their orthologs in other legumes. By analogy, PaNFP and SILYK10, which belong to the LYR subfamily, might interact with a member of the LYK subfamily. Indeed, it has been shown recently that OsCERK1, which belongs to the LYK subfamily, regulates AM in rice (Miyata *et al.*, 2014; Zhang *et al.*, 2014). OsCERK1 also plays a role in the perception of pathogen-associated molecular patterns, such as

chitin and peptidoglycan (Ao et al., 2014), although it does not appear to bind these molecules. OsCERK1 has thus been proposed to be a co-receptor which interacts with different protein partners to activate different signaling pathways. OsCERK1 orthologs in tomato and P. andersonii might thus interact with SILYK10 and PaNFP to play a role in AM. In tomato, gene duplication has occurred leading to several orthologs of OsCERK1, namely SlLYK1, SlLYK11, SlLYK12 and SlLYK13 (Figs 3, S2c). SlLYK1 plays a role in defense (Zeng et al., 2011) as OsCERK1. Previous studies have suggested that a three-aminoacid motif (YAQ in dicots and YAR in monocots) in the kinase domain of OsCERK1, and its orthologs in other plants, is required for function in root endosymbioses (Nakagawa et al., 2011; Miyata et al., 2014). This motif is absent in AtLYK1. Two of the tomato orthologs of OsCERK1 contain the YAQ/R motif in their kinase (Fig. S3a) and are expressed in roots (The Tomato Genome Consortium, 2012): SILYK1 and SILYK12. Functional redundancy is thus highly probable between these proteins for a role in AM.

In conclusion, VIGS has efficiently demonstrated the role of *SlLYK10* in the colonization of tomato roots by *R. irregularis*, and can be used as a tool for the fast screening of genes playing major roles in AM as in other plant–microbe interactions. This study and previous work in *P. andersonii* suggest that a LysM-RLK gene, ancestral to *SlLYK10*, is involved in the regulation of AM. Studies of orthologs in additional species will be required to determine how conserved this role is in plants able to establish AM.

Acknowledgements

This work was funded by the French Agence Nationale de la Recherche (contract ANR-2010-JCJC-1602-01 LCOinNON-LEGUMES) and by the 'Laboratoire d'Excellence (LABEX)' entitled TULIP (ANR-10-LABX-41; ANR-11-IDEX-0002). A.G.'s fellowship was funded by Région Midi-Pyrénées and INRA Department of Plant Health and Environment (SPE). T.W.'s fellowship was funded by the Chinese Scholarship Council (CSC). We thank Professor Dinesh-Kumar for pTRV1 and pTRV2-*NbPDS* plasmids. We thank Julie Cullimore, Clare Gough and Frédéric Debellé for critical reading of the manuscript, and Paul Gomes for technical help.

Author contributions

B.L. designed the research. L.B., T.W., A.G. and B.L. performed the experiments and analyzed the data. B.L. wrote the manuscript.

References

- Akiyama K, Matsuzaki K, Hayashi H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435: 824–827.
- Ao Y, Li Z, Feng D, Xiong F, Liu J, Li JF, Wang M, Wang J, Liu B, Wang HB. 2014. OsCERK1 and OsRLCK176 play important roles in peptidoglycan and chitin signaling in rice innate immunity. *Plant Journal* 80: 1072–1084.
- Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Gherardi M, Huguet T et al. 2006.

The *Medicago truncatula* lysin motif-receptor-like kinase gene family includes *NFP* and new nodule-expressed genes. *Plant Physiology* **142**: 265–279.

- Besserer A, Becard G, Jauneau A, Roux C, Sejalon-Delmas N. 2008. GR24, a synthetic analog of strigolactones, stimulates the mitosis and growth of the arbuscular mycorrhizal fungus *Gigaspora rosea* by boosting its energy metabolism. *Plant Physiology* 148: 402–413.
- Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ et al. 2012. Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proceedings of the National Academy of Sciences, USA 109: 13859– 13864.
- Chen A, Chen X, Wang H, Liao D, Gu M, Qu H, Sun S, Xu G. 2014. Genomewide investigation and expression analysis suggest diverse roles and genetic redundancy of Pht1 family genes in response to Pi deficiency in tomato. *BMC Plant Biology* 14: 61.
- Chen C, Fan C, Gao M, Zhu H. 2009. Antiquity and function of *CASTOR* and *POLLUX*, the twin ion channel-encoding genes key to the evolution of root symbioses in plants. *Plant Physiology* 149: 306–317.
- Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JD, Felix G, Boller T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448: 497–500.
- Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* 27: 1164–1165.
- Delaux PM, Varala K, Edger PP, Coruzzi GM, Pires JC, Ane JM. 2014. Comparative phylogenomics uncovers the impact of symbiotic associations on host genome evolution. *PLoS Genetics* **10**: e1004487.
- Fliegmann J, Canova S, Lachaud C, Uhlenbroich S, Gasciolli V, Pichereaux C, Rossignol M, Rosenberg C, Cumener M, Pitorre D et al. 2013. Lipochitooligosaccharidic symbiotic signals are recognized by LysM receptor-like kinase LYR3 in the legume *Medicago truncatula*. ACS Chemical Biology 8: 1900–1906.
- Genre A, Chabaud M, Balzergue C, Puech-Pages V, Novero M, Rey T, Fournier J, Rochange S, Becard G, Bonfante P *et al.* 2013. Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca²⁺ spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytologist* **198**: 190–202.
- Genre A, Chabaud M, Faccio A, Barker DG, Bonfante P. 2008. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both *Medicago truncatula* and *Daucus carota. Plant Cell* 20: 1407–1420.
- Gobbato E, Marsh JF, Vernié T, Wang E, Maillet F, Kim J, Miller JB, Sun J, Bano SA, Ratet P et al. 2012. A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Current Biology* 22: 2236–2241.
- Godfroy O, Debellé F, Timmers T, Rosenberg C. 2006. A rice calcium- and calmodulin-dependent protein kinase restores nodulation to a legume mutant. *Molecular Plant–Microbe Interactions* 19: 495–501.
- Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pages V, Dun EA, Pillot JP, Letisse F, Matusova R, Danoun S, Portais JC *et al.* 2008. Strigolactone inhibition of shoot branching. *Nature* 455: 189–194.
- Gough C, Cullimore J. 2011. Lipo-chitooligosaccharide signalling in endosymbiotic plant–microbe interactions. *Molecular Plant–Microbe Interactions* 24: 867–878.
- Gough C, Jacquet C. 2013. Nod factor perception protein carries weight in biotic interactions. *Trends in Plant Science* 18: 566–574.
- Grønlund M, Albrechtsen M, Johansen IE, Hammer EC, Nielsen TH, Jakobsen I. 2013. The interplay between P uptake pathways in mycorrhizal peas: a combined physiological and gene-silencing approach. *Physiologia Plantarum* 149: 234–248.
- Grønlund M, Olsen A, Johansen EI, Jakobsen I. 2010. Protocol: using virusinduced gene silencing to study the arbuscular mycorrhizal symbiosis in *Pisum sativum. Plant Methods* 6: 28.
- Gust AA, Willmann R, Desaki Y, Grabherr HM, Nurnberger T. 2012. Plant LysM proteins: modules mediating symbiosis and immunity. *Trends in Plant Science* 17: 495–502.
- Gutjahr C, Banba M, Croset V, An K, Miyao A, An G, Hirochika H, Imaizumi-Anraku H, Paszkowski U. 2008. Arbuscular mycorrhiza-specific signaling in

rice transcends the common symbiosis signaling pathway. *Plant Cell* 20: 2989–3005.

Gutjahr C, Parniske M. 2013. Cell and developmental biology of arbuscular mycorrhiza symbiosis. *Annual Review of Cell and Developmental Biology* 29: 593–617.

Harrison M, Dewbre G, Liu J. 2002. A phosphate transporter from *Medicago truncatula* involved in the acquisiton of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell* 14: 2413–2429.

Indrasumunar A, Kereszt A, Searle I, Miyagi M, Li D, Nguyen CD, Men A, Carroll BJ, Gresshoff PM. 2010. Inactivation of duplicated nod factor receptor 5 (NFR5) genes in recessive loss-of-function non-nodulation mutants of allotetraploid soybean (*Glycine max* L. Merr.). *Plant and Cell Physiology* 51: 201–214.

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ. 2007. A Medicago truncatula phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proceedings of the National Academy of Sciences, USA 104: 1720– 1725.

Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E, Shibuya N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences, USA* 103: 11086–11091.

Kobae Y, Hata S. 2010. Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. *Plant and Cell Physiology* 51: 341–353.

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK. 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences*, USA 92: 1679– 1683.

Lee WS, Rudd JJ, Hammond-Kosack KE, Kanyuka K. 2014. Mycosphaerella graminicola LysM effector-mediated stealth pathogenesis subverts recognition through both CERK1 and CEBiP homologues in wheat. Molecular Plant-Microbe Interactions 27: 236–243.

Lefebvre B, Klaus-Heisen D, Pietraszewska-Bogiel A, Herve C, Camut S, Auriac MC, Gasciolli V, Nurisso A, Gadella TW, Cullimore J. 2012. Role of Nglycosylation sites and CXC motifs in trafficking of *Medicago truncatula* Nod factor perception protein to the plasma membrane. *Journal of Biological Chemistry* 287: 10812–10823.

Levy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ane JM, Lauber E, Bisseling T *et al.* 2004. A putative Ca²⁺ and calmodulindependent protein kinase required for bacterial and fungal symbioses. *Science* 303: 1361–1364.

Limpens E, Franken C, Smit P, Willemse J, Bisseling T, Geurts R. 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302: 630–633.

Liu Y, Schiff M, Dinesh-Kumar SP. 2002a. Virus-induced gene silencing in tomato. *Plant Journal* 31: 777–786.

Liu Y, Schiff M, Marathe R, Dinesh-Kumar SP. 2002b. Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. *Plant Journal* **30**: 415–429.

Maeda D, Ashida K, Iguchi K, Chechetka SA, Hijikata A, Okusako Y, Deguchi Y, Izui K, Hata S. 2006. Knockdown of an arbuscular mycorrhiza-inducible phosphate transporter gene of *Lotus japonicus* suppresses mutualistic symbiosis. *Plant and Cell Physiology* 47: 807–817.

Maffei G, Miozzi L, Fiorilli V, Novero M, Lanfranco L, Accotto GP. 2014. The arbuscular mycorrhizal symbiosis attenuates symptom severity and reduces virus concentration in tomato infected by tomato yellow leaf curl Sardinia virus (TYLCSV). *Mycorrhiza* 24: 179–186.

Maillet F, Poinsot V, Andre O, Puech-Pages V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A *et al.* 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469: 58–63.

Miozzi L, Catoni M, Fiorilli V, Mullineaux PM, Accotto GP, Lanfranco L. 2011. Arbuscular mycorrhizal symbiosis limits foliar transcriptional responses to viral infection and favors long-term virus accumulation. *Molecular Plant– Microbe Interactions* 24: 1562–1572.

Miyata K, Kozaki T, Kouzai Y, Ozawa K, Ishii K, Asamizu E, Okabe Y, Umehara Y, Miyamoto A, Kobae Y *et al.* 2014. The bifunctional plant

receptor, OsCERK1, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice. *Plant and Cell Physiology* **55**: 1864–1872.

- Nagy R, Karandashov V, Chague W, Kalinkevich K, Tamasloukht M, Xu G, Jakobsen I, Levy A, Amrhein N, Bucher M. 2005. The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. *Plant Journal* 42: 236–250.
- Nakagawa T, Kaku H, Shimoda Y, Sugiyama A, Shimamura M, Takanashi K, Yazaki K, Aoki T, Shibuya N, Kouchi H. 2011. From defense to symbiosis: limited alterations in the kinase domain of LysM receptor-like kinases are crucial for evolution of legume–*Rhizobium* symbiosis. *Plant Journal* 65: 169– 180.

Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JS, Kudrna D, Wing R, Untergasser A *et al.* 2011. LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume *Parasponia. Science* 331: 909–912.

Paszkowski U, Kroken S, Roux C, Briggs SP. 2002. Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences, USA* 99: 13324–13329.

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N *et al.* 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425: 585– 592.

Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N, Bucher M. 2001. A phosphate transporter expressed in arbuscule-containing cells in potato. *Nature* 414: 462–470.

Ryu CM, Anand A, Kang L, Mysore KS. 2004. Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *Plant Journal* 40: 322–331.

Senthil-Kumar M, Mysore K. 2011. Virus-induced gene silencing can persist for more than 2 years and also be transmitted to progeny seedlings in *Nicotiana benthamiana* and tomato. *Plant Biotechnology Journal* 9: 797–806.

Shimizu T, Nakano T, Takamizawa D, Desaki Y, Ishii-Minami N, Nishizawa Y, Minami E, Okada K, Yamane H, Kaku H *et al.* 2010. Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant Journal* 64: 204–214.

Sinharoy S, DasGupta M. 2009. RNA interference highlights the role of CCaMK in dissemination of endosymbionts in the Aeschynomeneae legume *Arachis. Molecular Plant–Microbe Interactions* 22: 1466–1475.

Svistoonoff S, Benabdoun FM, Nambiar-Veetil M, Imanishi L, Vaissayre V, Cesari S, Diagne N, Hocher V, de Billy F, Bonneau J et al. 2013. The independent acquisition of plant root nitrogen-fixing symbiosis in Fabids recruited the same genetic pathway for nodule organogenesis. PLoS ONE 8: e64515.

Svistoonoff S, Hocher V, Gherbi H. 2014. Actinorhizal root nodule symbioses: what is signalling telling on the origins of nodulation? *Current Opinion in Plant Biology* 20: 11–18.

The Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485: 635–641.

Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandal N, Albrektsen AS, Kawaguchi M *et al.* 2006. Deregulation of a Ca²⁺/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441: 1153–1156.

Trouvelot A, Kough J, Gianinazzi-Pearson V. 1986. Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson V, Gianinazzi S, eds. *Physiological and genetical aspect of mycorrhizae*. Paris, France: INRA Press, 217–221.

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K et al. 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature* 455: 195–200.

Vierheilig H, Coughlan AP, Wyss U, Piche Y. 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology* 64: 5004–5007.

Wang B, Yeun LH, Xue JY, Liu Y, Ane JM, Qiu YL. 2010. Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of

mycorrhizas in the colonization of land by plants. *New Phytologist* **186**: 514–525.

Wang E, Schornack S, Marsh JF, Gobbato E, Schwessinger B, Eastmond P, Schultze M, Kamoun S, Oldroyd GE. 2012. A common signaling process that promotes mycorrhizal and oomycete colonization of plants. *Current Biology* 22: 2242–2246.

Willmann R, Lajunen HM, Erbs G, Newman MA, Kolb D, Tsuda K, Katagiri F, Fliegmann J, Bono JJ, Cullimore JV et al. 2011. Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proceedings of the National Academy of Sciences, USA 108: 19824–19829.

Yan HX, Fu DQ, Zhu BZ, Liu HP, Shen XY, Luo YB. 2012. Sprout vacuuminfiltration: a simple and efficient agroinoculation method for virus-induced gene silencing in diverse solanaceous species. *Plant Cell Reports* 31: 1713–1722.

Young ND, Debelle F, Oldroyd GE, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H et al. 2012. The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature 480: 520–524.

Zeng L, Velasquez AC, Munkvold KR, Zhang J, Martin GB. 2011. A tomato LysM receptor-like kinase promotes immunity and its kinase activity is inhibited by AvrPtoB. *Plant Journal* 69: 92–103.

Zhang X, Dong W, Sun J, Feng F, Deng Y, He Z, Oldroyd GE, Wang E. 2014. The receptor kinase *CERK1* has dual functions in symbiosis and immunity signalling. *Plant Journal* 81: 258–267.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Parameters tested to set up virus-induced gene silencing (VIGS); Si-Fi predictions.

Fig. S2 Tomato lysin motif receptor-like kinase (LysM-RLK) sequence identities; phylogenetic tree based on extracellular regions.

Fig. S3 Alignments of tomato lysin motif receptor-like kinase (LysM-RLK) intracellular and extracellular regions.

Fig. S4 Synteny of *Solanum lycopersicum* locus containing *SlLYK10*; expression pattern of *SlLYK10*.

Fig. S5 Analysis of arbuscular mycorrhiza (AM) in *SlLYK10-*silenced plants at 2 wk post-inoculation (wpi) with *Rhizophagus irregularis.*

Fig. S6 Analysis of arbuscular mycorrhiza (AM) in *SlLYK10-*silenced plants at 4 wk post-inoculation (wpi) with *Rhizophagus irregularis*; quantification of *Tobacco rattle virus* (TRV).

Table S1 Primers used for cloning and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Table S2 Gene accession numbers

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.


Supporting Information Tables S1-S2 and Fig. S1–S6

Table S1

Purpose	Sequence	Primer name
Cloning in TRV2	GGAATTCCTTCTTAGTTTATGAATATGC	VIGS SILYK10 F
Cloning in TRV2	CCGCTCGAGCACCTGAAGTCCAAGACGGTCC	VIGS SILYK10 R
Cloning in TRV2	GAGAATTCACCATTAAAGATTATGG	VIGS SICCamK F
Cloning in TRV2	CCGCTCGAGCTTCAAACTCCAACAATGTTGC	VIGS SICCamK R
qRT-PCR	GGGTGAGGGGAGAGTTAGCA	SICCamK F
qRT-PCR	CTGCTGCACGAAACTTACGC	SICCamK R
qRT-PCR ⁽¹⁾	CGTCCTCCAACGTAACTGCT	SILYK10 F1
qRT-PCR ⁽¹⁾	GTGGACGGGCACGATATGAA	SILYK10 R1
qRT-PCR ⁽²⁾	AGTCCGGTAATAGCACGTTG	SILYK10 F2
qRT-PCR ⁽²⁾	TCAGATTAGTTATCGTTCATGTGGC	SILYK10 R2
qRT-PCR	TTACACGGCCAGCATCTTCA	SILYK8 F
qRT-PCR	GCCCACAAACCCCTAAACCA	SILYK8 R
qRT-PCR	ACAGAGCTAGAGCACCAGAGT	SILYK9 F
qRT-PCR	ACAAGAACACGTGATGGGGA	SILYK9 R
qRT-PCR	GCTGCTGGTGATGATCCTGT	SIGAPDH F
qRT-PCR	GCGTTTGTGGTCCGAGAGAA	SIGAPDH R
qRT-PCR	CCAGGGGCATCAATCACAGT	SIEF1-α F
qRT-PCR	AGTAAGCCTGGGTGCTTGAC	SIEF1-α R
qRT-PCR	TCTTCTCCCGGGTTAGTACG	SICCamK intron F
qRT-PCR	TCGCGGAAAAATTCGTTTTGGA	SICCamK intron R
qRT-PCR	CGGGCAGAATGAGACACAGATG	SIPT4 F
qRT-PCR	GAAGATAGAAAGCACAAGGCGTAGT	SIPT4 R
qRT-PCR	GCAGAACGAGACGCAGATGAA	SIPT5 F
qRT-PCR	TGCTGAATTTGATAAACTTGCCAA	SIPT5 R
qRT-PCR	AAGCCAGGAGTACTTGTTGGTAA	SIRAM2 F
qRT-PCR	TGGACTAAACGACCATCATGGA	SIRAM2 R
qRT-PCR	GACGTCTCAGTTGTTGATTTA	RiGAPDH F
qRT-PCR	TTTGGCATCAAAAATACTAGA	RIGAPDH R
qRT-PCR	GCAGTTTCCAGATAAGAAGGTG	TRV2 CP F
qRT-PCR	GCTGAAGCGTTCTGAATCACA	TRV2 CP R
RT-PCR TRV2	GGGCGTAATAACGCTTACG	Primer 305

⁽¹⁾qRT-PCR on VIGS plants, ⁽²⁾qRT-PCR on WT plants

Table S1. Primers used for cloning the gene fragments in pTRV2 for VIGS and for qRT-PCR are shown from 5' to 3'.

Table S2

Name	ID	Other name, remark	Subfamily
SICCamK	Solyc01g096820		
SIRAM2	Solyc04g005840		
AtLYK1	At3g21630	CERK1	LYK
AtLYK2	At3g01840		LYR
AtLYK3	At1g51940		LYK
AtLYK4	At2g23770		LYR
AtLYK5	At2g33580		LYR
SILYK1	Solyc07g049180	Bti9	LYK
SILYK2	Solyc02g094010		LYR
SILYK3	Solyc03g121050		LYK
SILYK4	Solyc02g089900	Tandem duplication with SILYK7	LYR
SILYK6	Solyc12g089020	Kinase partly truncated	LYR
SILYK7	Solyc02g089920	Tandem duplication with SILYK4	LYR
SILYK8	Solyc09g083200	Kinase truncated Tandem duplication with <i>SILYK9</i>	LYR
SILYK9	Solyc09g083210	Tandem duplication with SILYK8	LYR
SILYK10	Solyc02g065520		LYR
SILYK11	Solyc02g081040	Tandem duplication with SILYK12	LYK
SILYK12	Solyc02g081050	Tandem duplication with SILYK11	LYK
SILYK13	Solyc01g098410		LYK
SILYK14	Solyc06g069610		LYK
SILYK15	Solyc11g069630		LYR
MtNFP	Medtr5g019040	NFR5	LYR
MtLYR1	Medtr8g078300		LYR
PaNFP	HQ705608		LYR
SISYMRK	Solyc02g091590	DMI2	
MtLYM2	Medtr4g094730	CEBIP	

Table S2. Genes used for phylogenetic analyses and their accession numbers from *S. lycopersicum* ITAG2.3 assembly, *A. thaliana* TAIR annotation release 10, *M. truncatula* Mt4.0 assembly and EMBL database.

Fig. S1

(a)

Parameters tested	Importance for VIGS efficiency	Chosen parameter
A. tumefaciens strains (LBA4404 virGN54D or C58 GV3101 pmp90)	++	GV3101 pmp90
Plant inoculation method (vacuum infiltration, agrodrench*, or combination)	-	Combination
Bacteria solution final OD (0.5, 1 or 2)	Necrosis	0.5
Substrate (calcinated clay or vermiculite)	-	Vermiculite
Age of plantlets before A. tumefaciens inoculation (3 days or 7 days)	++	7 days
Infiltration medium composition (acetosyringone concentration, Silwet77)	+	150μM acetosyringone , no Silwet77
Cut of cotyledons, roots or both	Necrosis	No cut
Lighting condition (neon tubes, lamps)	+	Neon
Parameters tested	Importance for AM	Chosen parameter
Substrate (calcinated clay or vermiculite)	++	Vermiculite
Number of R. irregularis spores per plant (100, 200, 500 or 1000)	+	500
Irrigation (Long Ashton medium followed by water or Long Ashton medium)	-	Water

*Ryu CM, Anand A, Kang L, Mysore KS. 2004. Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *Plant Journal* 40: 322-331

(b)

VIGS construct	Target	Effective hits
pTRV2-NbPDS	SIPDS	21
pTRV2-SICCaMK	SICCaMK	98
pTRV2-SILYK10	SILYK10	225
pTRV2-SILYK10	SILYK9	0
pTRV2-SILYK10	SILYK8	0
pTRV2-SILYK10	SILYK2	0
pTRV2-SILYK10	SILYK6	0
pTRV2-SILYK10	SILYK4	0
nTRV2-SILYK10	SILYK7	0

Fig. S1. (a) Parameters tested to set up condition for using VIGS to study AM are listed. Importance of the parameters are summarized by - (no effect), + low effect and ++ (strong effect). (b) Specificity of silencing as predicted by siRNA-Finder (si-Fi) software.

Fig. S2

(a) SILYK8	85.79					Identity (/	Amino Ac	ids)					
SILYK6 SILYK7 SILYK4	30.75 29.24 31.24	23.41 25.00 25.47	46.86 38.98	40.10									
SILYK14	26.77	16.82	24.15	21.74	24.56								
SILYK1	28.03 27 74	20.63	23.31 23.45	22.93 23.40	21.79 24 14	29.98 28.85	58 46						
SILYK15	23.39	17.47	28.68	26.06	25.89	20.34	24.05	23.17					
SILYK12	26.40	17.11	24.48	23.02	23.36	29.91	61.58	54.59	22.05				
SILYK11	26.87	17.09	22.56	23.47	21.48	28.25	58.60 23.76	54.31 22.66	22.51	60.56 23.05	21 77		
SILYK3	25.96	17.68	24.90	23.37	24.75	37.02	30.78	30.57	20.25	30.67	28.87	21.20	
SILYK10	<mark>35.37</mark> SILYK9	30.60 SILYK8	27.89 SILYK6	26.85 SILYK7	26.11 Silyka	24.40 SILYK14	23.94 SILYK1	23.85 SILYK13	23.49 SILYK15	23.02 SILYK12	23.00 SILYK11	22.90 SILYK2	22.87 SILYK3
(b)						Identity (Nucleotic	les)					
SILYK8	90.44					,,							
SILYK2	52.86	51.34	47.46										
SILYKO SILYKA	51.20 49.42	47.76	47.46	54.71									
SILYK7	48.58	46.15	46.28	63.10	57.05								
SILYK12	44.69	40.15	42.63	44.71	44.85	43.64	12.10						
SILYK15 SILYK1	46.50 44.76	42.03 41.24	44.38 42.67	53.07 44.64	51.61 42.92	50.85 43.68	42.16	42.03					
SILYK11	44.23	39.34	42.43	44.93	43.59	44.09	71.67	42.05	64.73				
SILYK13	42.85	39.54	42.89	44.15	43.52	43.26	64.14	40.59	71.24	63.44	50.00		
SILYK3 SILYK14	43.35 44.88	38.40 42.03	40.69 41.07	42.84 44.81	42.24	42.07	50.17 49.52	40.00	49.01 49.91	49.75	50.00 49.33	59.56	
SILYK10	<mark>58.99</mark>	56.66	53.21	47.13	46.84	44.59	43.92	43.69	43.49	42.64	42.55	41.37	41.88
	SILYK9	SILYK8	SILYK2	SILYK6	SILYK4	SILYK7	SILYK12	SILYK15	SILYK1	SILYK11	SILYK13	SILYK3	SILYK14
(c)													
SILY	(K3 A 4T	VV2		At		SILYK7	SILYK	4 YK4					
	All					57	/						
SILYK	14-		100		5	5	SIL	YK15	SIL	YK2			
			\sim	<u>61</u>	:	35	100	\leq	Atl	LYK2			
					24	3 56	100						
	SILYK	10	60	<u>*</u>		56				MtLYM	2		
	ر ۱	PaNFP MtNFI	71				\backslash						
				/ ¹⁰⁰			100	h					
		S	ILYK8	' SILYK9				\backslash		AtLYK	1		
								41	K				
								5.	1	SILVE	1		
									$ \rangle$	SILYKI	13		
								SILY	K12 V SIL	YK11			

Fig. S2. (a) Amino acid identities between tomato LysM-RLK proteins. Identities between SILYK10 and other tomato LysM-RLKs are highlighted (b) Nucleotide identities between tomato LysM-RLK genes. Identities between *SILYK10* and other tomato LysM-RLKs are highlighted (c) Extracellular region amino acid sequences of all LysM-RLKs from *S. lycopersicum* and *A. thaliana* plus *M. truncatula* MtNFP and MtLYR1 and *P. andersonii* PaNFP, (See Table S2 for accession numbers) were used to construct a phylogenetic tree. MtLYM2, a LysM receptor-like protein (Fliegmann *et al.*, 2011) was used as an outgroup. Numbers at the branches correspond to bootstrap values. The clade containing SILYK10 is surrounded.

Fliegmann J, Uhlenbroich S, Shinya T, Martinez Y, Lefebvre B, Shibuya N, Bono JJ. 2011. Biochemical and phylogenetic analysis of CEBiP-like LysM domain-containing extracellular proteins in higher plants. *Plant Physiology and Biochemistry* **49**: 709-720

LysM2	 	QGS-RKRN
LysM1	<pre>vvbebebasserstordenutl.doc -rpdil.rmsit.vsvpl.ersil.buskilt</pre>	USMJ USMJ USENDED AND AND AND AND AND AND AND AND AND AN
	<pre>KLIXK2 MASTGNKQYMTSLUVILLE-ISLSSLSFTAOPYCISTTD5 SLIXK4 MASTGNKQYMTSLUVILLE-ISLSSLSFT-AOPYCISTTD5 LLXK4 MA</pre>	LySM2 ALIXY2

Fig. S3

(q)	Gly I	catalytic loop Catalytic loop
AtLYK2 SlLYK2	TVGTTTPRKPVLEIYAFEELEKATENFSSSNHIK	GSVY FGSL-KGKDLA IKOVNADEKRFDFGLINDOSHYNHNVIRVLGTCFREIDOSYLVFEYARNGSLMDMIDNKL-AIKNOFIESCYCFLAMKORIKICHDVA IALKYMHRINVVHGNIK OSVEHGRI-GGKMLAIKOMETSSISKID-FGLFNDAIHHHNIIRLGTCYT-EGPDSFLVFEYAKNGSLKDMLHGGL-AMKNOFIASCDCFLYMNORLAICLDVAALDFMHHINDPYVYHNUK
AtLYK4	GLSGMVVESLKVYKFHELQSATSDFTSSSSIG	SGY1GKI-NGDGAMIKKIEGNASEEVNLJSKINHLN1IRLSGFCFHEGDWYLVYEHASNGSLSEMIHTTKSLLSLTGKLQIALDIATGLNYLHNFADFPYVHRDLN
Sllyk5	SLSS-IAQSVKVYKFEEVKAATENFSPTCLIK GLKS-ATESLTLVRFNDLOSATSNFSDENRTK-	SEVYRGT1-GGPPAITKNGYDVSKETNLISKTNHNTLESECG7-HDB1WTYPYEXANGPLSDVDTHH-GED20ESURAGYDYGSEDVAGTAVZHAYENEPEHYKIN SEVYRGT1-GGDPAAITKNGYDSSKETNLISKTNHNTLESECG7-HDB1WTYPYEXANGPLSDVDTHH-GED20SURAGADYDSED20
SLLYK7	SIVG-EGOMIKVYEFEELLAATENFSSRKKFG	BCVFKGVL-RCKLMAIKEMSTDISKEVKFLAKINHFNLISLAGFCKHHQLSYLVFEFMENGSLKEMLFKDDNFEAQSWNCRIRIALDIADGLDYHNFTAFAYVHNIS
SLLYK6	EIAN-IDHVPKVFKFKELKHATONFDSKNRIK	Serveder-Stefrawkin IMVSRBVILIMINHNILICOCYGFEKGFEKGFEKMLIETKODFEKGL-IVIDVEKEKIICU-VLDVARGHEHYLISEKEKIIKINI Serveder-Steframkers of the served served of the served served served served of the served ser
PANFP	GVSV-YLGKPIMYEIKMIMEATMNFNDQYKIG	SEYTAMI AGSTAVKAKENTE-MILIQKVHGAUVCAGES-FESTASSESDAMINGSESDAMANGSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESDAMANGSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSESTASSES
MtNFP Wtive1	GVSG-YVSKPTMYEIDAIMEGTTNLSDNCKIG	ESYYKANI-DGRVLAVKKIKKDASEELKILQKVNHGNLVKLMSVSSD-NDGNCELVYEYAENGSLEEMLFSSS-SKTSNSVVSLFWSQRITIAMDVAIGLQYMHEHFYPRII <mark>HRD</mark> I Vouvovar och and
S11.YK8	EVSN-IVSEPTVIEVGMIMKATMNINEMCELG DISD-CI.DEVEMVEMEOI.WEATNGFDEECI.TO-	
SILYK9	DVSE-CLDKYKMYKMEQIWEATDGFDEGCLIQ	SEVYRGT-DESVPATIKKWWANELKTLOKVNHONLVKLEGFCIDFKEANCELVYEVYENGSLHSMIHGEKPEKLSWKTRLRIATDVANGLL/YHEHTRPRVVHKDIK
SILYK1	DLFAGIKYTLGEYNIDELTNATCDFSEETKVS	NUVFRGCV-DKVEVLIKKIREEDTRQVIDVHSRINHVSIVKLQGVCYGEDDITGSYLVEEVPSNGTLRDCLSNSLVMHKRTQIAFDIAIGLHYLHFCTLEPYTHNIN
AtLYK1	GIAAISVDKSVEFSLEELAKATDNFNLSFKIG	COEPANY YAEL -ROEKAA IKKUMAA SKOL TAEKULTIKVIHVULUPLICYVV EGS LEUVERYURGNLGOHIHO SGE EL WITKUO IALDSARGLEYHIEHUVYVVHED IK
CITVV11	GL= - SGLAV DNSVEFSIQELAESIDNESISNEC	
SILYK12	RPTGTTVDKSVEFSYEELAKATNNFSMENKIC	SOFILTPYCKIL. FCREARATINGTOODS. ALLE ALLAVITHAHIMINGTOODS. SALATATINGTOODS. ALLAND
SlLYK1	VLSGITVDKSVEFTYEELATATNDFSIANKIG	ÖGGESAVYYAEL-RGEKAAIKKMDMEATREFL-AELKVLTNVHHLNIVRLIGYCVEGSLELVYEYVENGHIGGTGRDPLPMSKRVOTALDSARGLEYIHEHTVPYYIR
At LYK3	ALGDGMFEIEKPMVFTYEEIRAATDEFSDSNLLG	ggnysyfgll-regevavkrutatktrefa-aemkulgvuhenlineligvatvuetevvrevrgmlkshlhddgskgntbesminrngialdarglevihentryv <mark>h</mark> rdik
SILYK3	VIGTDVFDMEKPLVFAYEDILSSTDGFSDSNLLG	HGT VEST YAIL-RNDEVAIKRMTATKTKEFT-AEMKVLCKVHHLNUVELIGYAVSNDELEUVYEYAQKGSLKSHLNDPQNKGHTPLSWIMRVQIALDAAGLEYIHEHTKPHYV <mark>H</mark> RD
SLLYKI	KMPVFESERPVIYSLEEIAEATSDFDESKIIG SMDTTMMKSISSRNFKLEYIEAITQNYKTLIG	AGGYSSYYYGTI-CKQEMAIKKWISNGSKEFM-AELKVLCKIHHINVPELGZAGCDDHLILVEEVPRGSIABELLAPMAKGHKELSWTFRTQIAVITARGIEXIEDHTKSRYHEDIK 3GGFSVFRGTLPDGVEVAVKVRSATSTQGIREFN-NELMLSAITHENLVPLIGYCCENEQQILVYPFMSNSSLQDRLYGGA-AKRKLDMPARLSIALGARGLLYLHTESERCLIHEDUK
0	atalytic loop Activation seg	ment
AtLYK2	SRNIFINEDLRGKVGNFGMSKCVTNELA	DEBILFAYGIIVMEVLSGOTPDMLGLOEVETTSLGTQETFVSEMSRLRRLLGDKEKLREVMDST-LGESYSVDSAFEIASIARDCTAEEAESRPSAVE
S1LYK2	SRNILLDEEFKAKVGNFGMARCVEDDVA	Kgylafeckkoeiiafsidifafsultevusgotpiriknvtegedevalsekikvile senvdelrewusa-lgenysfdavtlanlaracveeepsirenage
AtLYK4	SNNVFLDLEFRAKIGSLGSARSTTEDFVLTKI	JVEGTRGYLAPEXLEHGLVSTKLDVYAFGVVLLEIVTGREASELKKEIDEGKAIDEILI-+GRLLPEGLTSFVE
SlLYK4	GDNILLDGDLRAKIANFGLARSADGQEGEFALTRI	HIVGTOGYMA PEYLENGLYSPKLDYYLGYLLLEILTGKEYSALYEGSNINLALLI-PLALALALALALALALALALALALALALALALALALALA
AtLYK5	STNILLDSNFRAKIANFGVARILDEGDLDLQLTRU SMCTTTMMUTANETYTCHEGEEE	PVBC5051A BEVINUT 15K KUNCUPERCVATUELLSGERAVTI HKKRGEEBVENLCNTNNUGGERVERLKERMDS-LGBEVELLELTATMAGLAKSCVATUEVDUS 19K VIO TTO MUNOMA DE ATATATATATATATATATATATATATATATATATATAT
STLYK6	SNS LLLNRHLRAK ISNES LARSAN NEGKVTSSMK. SDN TT T DNN TD AK TTNESVA KF TDTS CMSML	F EGNUNGY MEYLLEGVERLING VERLING VERLING KOMMUNUNGY MEYLLENG VERLING KOMMUNUNGY MEYLLEGVERLING VERLING V
OVITITO	TONITIT DEPERATINE SAMAGED IS THE TOTAL SAMA SAMA SAMA SAMA SAMA SAMA SAMA SA	- 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1
PANFP	TSNTLDSRFKAKTANFSMARSAASA	
MUNFP	T SN I L L G SN FKAK I AN F GMART STNS	MURKI DYFEREN D
MtLYR1	SSNILLDSKFKAKIANFSVARTTKNP	VITKVDVLGYGNVIMELITGKKELSYSE
S1LYK8		
S1LYK9	TSNILLDSNMRAKVANFGLAKSGCNAITM	HIVGTOGY LAPEYLTDGIVSTKMDVESFGVVLLELVSGKEAIDEGKVVMANIGDFSEG-SEEKKVRKLOEMMDGSLLREELIMESVVNVMSVAISCLNKDPSKRPGMIE
SILYK1	SKNVFLTANWRAKLAT FGAKGGTE PATS RDI G	I GS I GGMTA PEHLVHGSVSEVDD FA FGVVLLE LI SGKRDVDGNFLRDS TFFLGGGVNRGGGFROLKNTI DC-LMEDY PLAALCLAVLA KACTEDDFLHR BMDD
AtLYK1	SANILIDOKFRAKVADFGLTKLTEVGGSATRG-	AMCTFGYMAPETV-Y GEVSAXVDYYATGVVLYELLSAKGAVVKMTEAVGEFRLVVVFEESFYETD/GEAALKT11DPR-LGDSYPFDSYYKMAELGKACTOBNAOLRESMRY
SILYKI	SANILINKNFHAKIGDFGLSKLVESGNPTLNT	R PRGTEGYMP PEYGHSGVI SRKVDVYA FGVVLYELISSKDAIVKEDGVDEARSLVALEDEAHSHPNQIEAISRLID PK-LCDDY PLDSVY KMAQLAKSCTEKN PEMR PTMKS
SILYKI	TANILIDKNFRAKVADFGLTKLIETEGGSMNT	XLVGTFGYMA FYGGGGNVSLKIDVYA FGVVLYELI SARKAI IKTSEISTESKGLVGLFEDVLNEVDPKEGICKLVDPK-LGDDY PLDSVMNVALLAKACTGENPOLRPSMRS
SILYK12	SANILIDKDFRAKVADFGLTKLTEVGSTSFHT	xlvGtfgympe <mark>ya</mark> QyGpvSfkvDvYaFGvVlyEllSakealvkTnev1TeSKGLvALfeDvLHOSGGAReGLCKVvDFK-LGDDYPLDSvCKvAQLAKACTHENPQLRFSMRS
SILYK1	TANILIDKNFHAKVADFGLTKLTEVGSSSLQT	RIVGTFGYMPFE <mark>YA</mark> QYGDVSPKVDVYAFGVVLYELLSAKEALVKPNGSVTESKGLVALFEEVLNPPPDEDLRQLVDFR-LGDVPLDSVRKMAQLAKACTHENPLIRPSMRS
At LYK3	TSNILLDEAFRAKISDFGLAKLVEKTGEGEISVTI	KVVGTYGYLAPBYLSDGLATSKSDIYAFGVVLFEIISGREAVIRTEAIGTKNPBRRPLASIMLAVLKNSPDSMNMSSLKEFVDPN-MMDLYPHDCLFKIATLAKQCVDDDFILRPNMKQ
SILYK3	T SN I L L D D S F R A K I S D F G L S K L M G I T N D A E A S A T I	XVVGTYGYVA PEYLRDGLATKKTDVYA FGVVLFEMLTGERATTRTEGNVMKTABRRSLVSIMLAALRNS PDSTSWTSLKDQLDFS-LIMDLY PSDCVFKVAMLAKQCADD PILR PDMKH
SLLYKL	TSN I LLUETMKAKVADFGLAKLVGKTNEDEFLAT. SSN I LLUDQSMCAKVADFGFSKYASQEGD-SGTSLI	KLYGTPESYKELQITTKTDY FAFGYVLAELI TGKKALIRENGPRIKEKSLISISSINKKY DLNGQIHELEQGEDPDSALESELDEN-LKGY EMEDIY SVRGTAGYLDPEYYSTQRLSKSDVFSFGYVLLELLTGREPLNINKPRNEMSLISISSINKKYDLTSRVBELVDPT-IKGYHGELIY
At LYK2	IAERVSRLVDDDEDEEDEAV	XAVESTIISXAVESTIIS
S1LYK2	IVKKLSRLVEEKLLEGEEQL-	ESCKPLFKAEATSTSM
AtLYK4	NVMSLSKILAATQNWEES-	
SlLYK4	IVQSVSRIMTATHSWETSF	SISUSSENERT
At LYK5	VLTTLSMIVSSSIDWEPSDI	
Sllyk7	VISTLLKIQSDVQN	
SILYK6		
DANED	T AF'NLCF'LAQSSE'E-MY GPSWT'SGE.	
M+NFD	TYPE SHOULD BE SHOWN THE SHOW THE SHOULD BE SHOULD BE SHOULD BE SHOWN THE SHOW THE S	2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 2012 - 201
Mt LYR1	VVLSLSLLTQPSPT-LLEVSWTYGI	2012
S1LYK8		

Fig. S3. (a) Alignment corresponding to the extracellular regions used for tree in Fig. S2c is shown. Members of LYR (upper part) and LYK (lower part) are separated by a line. Conserved cystein and cystein pairs are boxed in red. (b) Alignment corresponding to the intracellular regions used for tree in Fig. 2 is shown. Members of LYR (upper part) and LYK (lower part) are separated by a line. Features of the kinase domain are boxed in red. In most of the LYR proteins, the highly conserved Glycine-rich loop is absent and the HRD motif in the catalytic loop is degenerated. In the LYK proteins, YAQ motif is shown in red.

-GLVQGR-----NLMSGR--GIVQGR-

----MDVGS--FYGNQGMI----

---WEATL--AGNSQVFS------WEATL--AGNSQVFS-----MEASL--GGDSLVFS-

--SSIE---

SILYKI1 IVVALMTISST--SILYK12 IVVALMTISS---SILYK1 IVVALMTISS---ALLYK3 VVISLSQILL---ALLYK3 VVISLSQILL---SILYK14 VVVSLSQIKL----SLIYMKI IVVELSQIKL----SLIYMKI VVELSQIKL----

---SSTE-

--- IENNASE-

---STAD----STED-----SSVE-

---SHA-

Atlyki ivvalstlfs-silyki3 vvvalmalss-I UVALSTLFS-

---STED-

-WDIGS--FYEN

---STGN-----DDVGN--FQNE-DLV--

--VSEEGLS

---LFTD-

IVYALSKSID-

SILYK9 SILYK15

I IKVLARMYHKLVLKMILFIDHDJ

--HLMSGR-

-SLQEWCDFQYLY -- PRQVTA-R-----SLMSGR---HLISGR-

Fig. S3



Fig. S4. (a) Synteny of *S. lycopersicum* locus containing *SlLYK10* with *M. truncatula* and *A. thaliana* orthologous loci. Same color is used for orthologous genes. Slashes represent the presence of additional gene(s) without orthologs in *S. lycopersicum* locus. (b)Tissue-specific expression patterns of *SlLYK10* analyzed by RNAseq (data extracted from The tomato genome consortium, 2012) in tomato cultivar Heinz.

The tomato genome consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485**: 635-641



Fig. S5. (a-d) Relative expression measured by qRT-PCR, of the indicated genes in roots inoculated with pTRV2-*SILYK10* or control pTRV2. Relative expression of *RiGAPDH* (a) reflects the amount of fungus, *SIPT4* and *SIPT5* (b-c) the degree of arbuscule formation, *SIRAM2* (d) the activation of the CSSP by *R. irregularis*. Entire root systems were harvested 3wpi with *A. tumefaciens* and 2wpi with *R. irregularis*. Mean and standard errors of transcript levels between individual roots are shown. (e) *SILYK10* relative expression in individual pTRV2-*SILYK10* inoculated and control roots. Total RNA was isolated from half root systems of individual plants 2wpi with *R. irregularis* and gene expression was analyzed by qRT-PCR. Average transcript level for control plants is shown with standard error between individual roots. The second half of roots systems were stained by an ink-vinegar protocol and AM observed with a binocular stereomicroscope. On the top of the bars, (-) represents no observation of colonization and (+) observation of colonization. VIGS induces a strong silencing of *SILYK10* which correlates with absence of AM.



Fig. S6. (a) Estimation of mycorrhizal colonization at 4wpi with the mycocalc method. F is the frequency of mycorrhiza in the root system; M the intensity of the mycorrhizal colonization in the root system and A the arbuscule abundance in the root system. (b) Quantification by qRT-PCR, of the plasmid pTRV2-*SILYK10.* The graph shows linearity between cycle quantification (cp) and plasmid quantities from 1fg to 100ng.

Fig. S6

Chapter 2: SILYK10 binds LCOs with high affinity

Preamble

In the previous chapter, we showed that *SlLYK10* is involved in AMS establishment. *SlLYK10*-silenced plants were almost not colonized at early stage of colonization (2 wpi with *R. irregularis*). As *SlLYK10* is a LysM-RLK that belong to the LYRIA phylogenetic group of the LysM-RLK family and because in this phylogenetic group, LjNFR5 was previously shown to bind LCO with high affinity, we hypothesized that SlLYK10 is a LCO receptor in tomato. To prove it, we expressed SlLYK10:YFP in an heterologous system (*N. benthamiana* leaves) allowing massive protein production and disulfide bridges formation. With this system, we could measure for the first time high affinity for LCOs on a protein involved in the AMS. Moreover, we analyzed the expression pattern of *SlLYK10* during the AMS by transforming tomato roots with a *pSlLYK10*:*GUS* construct expressing the *B-glucuronidase* (a reporter gene) under the control of the *SlLYK10* promoter. We observed *SlLYK10* expression in arbuscule-containing cells. These results bring strong evidences supporting the fact that LCO produced by AMF are important for AMS establishment.

I participated to these results mainly during the second year of my PhD. I performed the biochemical experiments on SILYK10 and I made the construct p*SILYK10::GUS*. I also participated to the writing the manuscript.

This manuscript was submitted to Plant Physiology at the beginning of 2017 and a revised version, presented here, will be resubmitted soon.

Binding properties of SILYK10 suggest a role of lipo-chitooligosaccharides in arbuscular mycorrhiza

Ariane Girardin[#], Tongming Wang[#], Luis Buendia, Virginie Gasciolli, Jean-Jacques Bono and Benoit Lefebvre

LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

Abstract

Lipo-chitooligosaccharides (LCOs) are signaling molecules produced by rhizobial bacteria and arbuscular mycorrhizal fungi (AMF) that activate the plant Common Symbiosis Signaling Pathway (CSSP) essential for establishment of symbioses with these microorganisms. Data suggest that activation of the CSSP by rhizobial LCOs evolved from the preexisting mechanisms involved in arbuscular mycorrhiza (AM). Although LCOs are essential for establishment of the rhizobial symbiosis, it is not known whether fungal Myc-LCOs play a similar role in AM. We previously showed that the tomato Lysin Motif-Receptor-Like kinase 10 (SILYK10), a putative receptor for Myc-LCOs is involved in AM establishment. Here we show that *SILYK10* is expressed in the root epidermis before fungal penetration, and later in cortical cells colonized by AMF. Moreover, we determined that SILYK10 has a high affinity and selectivity for LCOs. Our results suggest that Myc-LCOs are involved in different steps of AMF colonization through binding to the SILYK10 receptor.

Introduction

Arbuscular mycorrhiza (AM) is a symbiosis between Glomeromycota fungi and the majority of land plants, in which fungi provide plants with nutrients acquired from the soil in exchange for carbohydrates from photosynthesis. To colonize roots, arbuscular mycorrhizal fungi (AMF) first cross epidermal and outer cortical cells. Fungal hyphae then spread inter- or intra-cellularly within roots. Inside inner root cortical cells, AMF form highly branched structures called arbuscules that are surrounded by plant peri-arbuscular membranes, and in which most nutrient exchange takes place. Establishment of AM relies on a plant signaling pathway called the Common Symbiosis Signaling Pathway (CSSP). The CSSP is also required in legumes for the establishment of a symbiosis with nitrogen fixing bacteria called rhizobia. Lipo-chitooligosaccharides (LCOs) produced by rhizobia are signaling molecules required for bacterial recognition by legume roots through activation of the CSSP and for subsequent bacterial colonization (Murray, 2011). Rhizobial LCOs have a core structure of 4 or 5 N-acetyl glucosamine (GlcNAc) units in which the terminal non-reducing sugar is substituted with an acyl chain. These LCOs also bear decorations that are characteristic of each bacterial strain and important for host specificity (Fliegmann and Bono, 2015). It is hypothesized that the rhizobia-legume symbiosis (RLS) has evolved from the preexisting AM symbiosis by recruiting the same receptors and the CSSP. Indeed, AMF produce LCOs, called Myc-LCOs (Maillet et al., 2011). Major Myc-LCOs identified up to now consist of 4 or 5 GlcNAc, either sulfated or not on the reducing end, and with acyl chains of 16 or 18 carbons. Exogenous application of Myc-LCOs both activates the CSSP in various plant species (Sun et al., 2015, Camps et al., 2015), and increases the level of AMF root colonization (Maillet et al., 2011). However, whether Myc-LCOs play a role in establishment of AM is not known. In addition, short chain chitooligosaccharides (COs) are produced by AMF and exogenous application of Myc-COs also activates the CSSP (Genre et al., 2013) although differences have been osberved in plant responses to Myc-LCO and Myc-CO depending on the species analyzed (Miyata et al., 2014; Sun et al., 2015). Both Myc-LCOs and Myc-COs might thus be involved in partner recognition during AM.

Plant proteins of the Lysin Motif Receptor-Like Kinase (LysM-RLKs) multigenic family are good candidates to be fungal signal receptors involved in AM. LysM-RLKs are composed of 3 extracellular LysMs, a transmembrane domain (TM) and an intracellular region (ICR)

containing a kinase-like domain. Several LysM-RLKs have been shown to control AM (Op den Camp et al., 2011; Miyata et al., 2014, Zhang et al., 2014; Buendia et al., 2016). However, ligand binding properties of these LysM-RLKs have not been determined so far. Other LysM-RLKs have the ability to bind GlcNAc containing molecules (Kaku et al., 2006; Willmann et al., 2011, Cao 2014) including LCOs (Broghammer et al., 2012; Fliegmann et al., 2013; Malkov et al., 2016), but no role in AM has so far been shown for these LysM-RLKs. One phylogenetic group of the LysM-RLK family (Supplemental Fig. S1) is of particular interest since it contains at least one gene in plant species that establish AM, but none in the Brassicaceae, a family of plants that do not establish AM (Gough and Jacquet 2013). Among these genes, the legume Medicago truncatula MtNFP and Lotus japonicus LjNFR5 are essential for the RLS but not for AM (Radutoiu et al., 2003; Arrighi et al., 2006; Indrasumunar et al., 2010) and LjNFR5 has been reported to directly bind rhizobial LCOs (Broghammer et al., 2012). In non-legumes, a clear role in AM was found for a likely MtNFP/LjNFR5 ortholog in Parasponia andersonii (PaNFP; Op den Camp et al., 2011) but not in rice (OsNFR5; Miyata et al., 2016). We recently showed that SILYK10, belonging to this phylogenetic group, is required in tomato for efficient root colonization by AMF (Buendia et al., 2016). Reduction of SILYK10 level of expression resulted in significantly lower levels of AM colonization. Here, we study the expression pattern of *SILYK10* during the symbiotic process and characterize its binding properties towards different LCOs. Our results suggest that LCO perception trough SILYK10 is involved in AM establishment in tomato.

<u>Results</u>

SILYK10 is expressed in root cells before and during invasion by AMF

To better understand the role of *SILYK10* in AM, we produced *Agrobacterium rhizogenes* mediated transgenic tomato roots expressing the GUS reporter under the control of the *SILYK10* promoter region. Transgenic roots were grown either still attached to shoots in pots, or as root organ cultures (ROCs) *in vitro*. Strongest GUS activity was observed in un-inoculated lateral roots of independently transformed roots grown in both conditions (Fig. 1A, Supplemental Fig. S2A). These parts of root systems are known to be a preferential site of AMF penetration (Gutjahr and Paszkowski 2013). Root tips of un-inoculated lateral roots showed a reduced level or no GUS activity. Transverse and longitudinal sections (Fig. 1B, Supplemental Fig. S2B), showed GUS activity in the epidermis and the outer cortex.

In ROC lines inoculated with AMF spores, strongest GUS staining was observed in some, but not all, arbuscule-containing cells (Fig. 1C-D). Formation of new arbuscules occurs in cells surrounding those already containing arbuscules. Mature arbuscules collapse after a few hours or days leading to continuous renewal of arbuscules (Kobae and Hata 2010; Kobae et al., 2016). Strong GUS staining appeared restricted to certain arbuscule-containing cells suggesting that *SILYK10* expression was associated with particular stages of arbuscule development.



Fig. 1 The pro*SILYK10::*GUS fusion is mainly expressed in the epidermis and the outer cortex before AMF colonization and in arbuscule-containing cells during colonization. A) Representative image of transgenic tomato roots attached to shoots in the absence of AM fungi. Lateral roots show maximal GUS activity (magenta). B) Transversal section in a root segment showing GUS staining in A. Maximal GUS activity (magenta) is found in the epidermis and the outer cortex. C) Representative image of transgenic tomato ROC lines colonized by AM fungi. Maximal GUS activity (blue, upper panel) is found in arbuscule-containing cells (WGA staining, green, lower panel). D) Close-up of images from C. Arbuscule-containing cells are marked by an asterisk (*). Peripheries of cells with strongest GUS staining are drawn in white in GUS and WGA staining images to confirm that strong GUS staining is associated with arbuscule-containing cells. Bars represent 500 μm in A, 50 μm in B-C.

SILYK10 is an LCO binding protein

In order to determine the subcellular localization of SILYK10, we introduced a translational fusion SILYK10:YFP in tomato roots using Agrobacterium rhizogenes. Fluorescence was observed in the cell periphery in epidermal cells of roots containing the p35S::SILYK10:YFP construct while no fluorescence was detected in the control roots (Fig. 2A-B). Co-localization with FM4-64 confirmed that this pattern corresponds to a plasma membrane (PM) localization as expected for a RLK (Fig. S3), In order to characterize the biochemical function of SILYK10, we aimed to produce it in a eukaryotic system allowing the formation of disulfide bridges. Indeed, LysM-RLKs have 3 LysMs packed together by disulfide bridges (Liu et al., 2012; 2016) that are essential for their function (Lefebvre et al., 2012; Kawaharada et al., 2015). We used Agrobacterium tumefaciens mediated transient expression in leaves of Nicotiana benthamiana to produce high quantities of the SILYK10:YFP translational fusion. Microscope observation revealed that the protein was localized in undefined cytoplasmic structures in N. benthamiana leaves (Fig. 2B). We observed similar mislocalization for other tomato LysM-RLKs expressed in *N. benthamiana*, while we did not face this problem for LysM-RLKs of phylogenetically more distant species, including MtNFP (Lefebvre et al., 2010). We thus made chimeras (further referred to as SILYK10c, Fig. 2C) composed of the extracellular region (ECR) of SILYK10 and the TM/ICR of MtNFP as we previously showed that such chimeras have LCO binding properties that are similar to full length proteins (Fliegmann et al., 2013). Although a fraction of SILYK10c:YFP was localized in the endoplasmic reticulum of *N. benthamiana* leaf cells (Fig. 2D), both co-localization with a PM marker and the analysis of SILYK10c:YFP N-glycan maturation (Supplemental Fig. S4A and C) indicated that most of the protein reached the PM. SILYK10c:YFP was furthermore immunodetected in the membrane fraction extracted from N. benthamiana leaves (Fig. 2E).



Fig.2 SILYK10 is localized in the plasma membrane. A) Confocal images of epidermal cells from tomato ROC lines expressing SILYK10:YFP (left panel) or ROC control (right panel) acquired with similar microscope settings. **B)** Confocal images of epidermal cells from *Nicotiana benthamiana* leaf expressing SILYK10:YFP showing localization partly in undefined compartments. **C)** Schematic view of the chimeric constructs SILYK10c and SILYK7c. In orange is represented the extracellular region (ECR) composed of the 3 LysMs of SILYK10 or SILYK7. In gray is represented the transmembrane domain (TM) and the intracellular region (ICR) that both originate from MtNFP. **D)** Confocal images of epidermal cells from *N. benthamiana* leaves expressing SILYK10c:YFP (upper panel) or SILYK7c:YFP (lower panel) acquired with similar microscope settings. **E)** Immunodetection of YFP-fusion proteins using anti-GFP antibodies in 10 μg of membrane fraction proteins prepared from *N. benthamiana* leaves expressing the indicated proteins or untransformed leaves. SILYK10c:YFP and SILYK7c:YFP have an expected molecular mass of about 100 kDa including 6 and 5 putative N-glycans respectively. Bars represent 20 μm.

In order to determine the affinity of SILYK10 for LCOs, we performed radioligand binding experiments using a radiolabeled LCO (³⁵S-LCO) purified from the rhizobial strain *Rhizobium tropici* and various unlabeled synthetic Myc-LCOs and COs. The ³⁵S-LCO was obtained by radiolabeling LCO-V(C18:1,NMe) with ³⁵S as previously described (Gressent et al., 2004). Strong specific binding of the ³⁵S-LCO to membrane fractions was detected from extracts of leaves expressing SILYK10c:YFP, but not for untransformed leaves (Fig. 3A). By adopting the same procedure, we made a chimeric construct with another tomato LysM-RLK, *SILYK7* (SILYK7c:YFP), belonging to another phylogenetic group (Supplemental Fig. S1). SILYK7c:YFP was slightly less expressed than SILYK10c:YFP but showed a similar localization pattern in *N. benthamiana* leaves (Fig. 2D and Fig. S4A-C). However, no LCO specific binding was associated to the membrane fraction containing SILYK7c:YFP (Fig. 3A), excluding artefactual detection of LCO binding sites and suggesting that not all tomato LysM-RLKs bind LCOs.

The affinity of SILYK10c:YFP for LCO-V(C18:1,NMe,S) was determined by cold saturation experiments using this ³⁵S-LCO and the corresponding unlabeled molecule. Scatchard plot analysis revealed a single class of binding site (Fig. 3B) exhibiting a high affinity (K_d of 19 nM +/- 4 nM (n=3)).



Fig. 3 SILYK10 has a high affinity for LCOs. A) Binding activity of membrane fractions incubated with radiolabeled LCO-V(C18:1,NMe,³⁵S) in the absence or the presence of 1 μ M of unlabeled LCO-V(C18:1,NMe,S) allowing the determination of total and non specific binding respectively and by difference the specific binding. Means and standard errors (SE) between n replicates are shown. B) Scatchard plot analysis of a cold saturation experiment using LCO-V(C18:1,NMe,S) and membrane fraction containing SILYK10c:YFP. The plot is representative of experiments performed on prepared membrane fractions from 3 independent batches of agro-infiltrated N. benthamiana leaves.

To determine the selectivity of SILYK10c:YFP towards known Myc-LCO and CO structures, we performed competition assays between the ³⁵S-LCO and an excess (1 μ M) of unlabeled competitors. All Myc-LCOs were able to compete the binding of the ³⁵S-LCO to the membrane fraction containing SILYK10c:YFP (Fig. 4A). The affinities of SILYK10c:YFP for the sulfated and non-sulfated Myc-LCOs, LCO-IV(C16:0,S) and LCO-IV(C16:0), were further determined by competitive binding experiments using the ³⁵S-LCO and corresponding unlabeled LCO-IV(C16:0,S) or LCO-IV(C16:0) (Fig. 4B), which revealed a *K*_i of 192 nM +/- 52 nM (n=3) and of 354 nM +/- 60 nM (n=3), respectively. In contrast to LCOs, CO4 and CO8 were much less efficient competitors, showing that the SILYK10 LCO binding site exhibits lower affinity for the latter molecules with *K*_is higher than 1 μ M.



Fig. 4 SILYK10 is selective for LCOs vs COs. A) Membrane fractions containing SILYK10c:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,³⁵S) in presence of $1 \,\mu M$ of the indicated unlabeled molecules used as competitors. specific binding Non was determined in the presence of 1 μM LCO-V(C18:1,NMe,S). Bars represent the percentage of specific binding (means and SE) obtained in the presence of an excess of competitor (1 µM) for 3 experiments performed on membrane fractions prepared from independent batches of N. benthamiana agro-infiltrated leaves. B) Competitive inhibition radiolabeled of LCO-V(C18:1,NMe,³⁵S) to membrane fraction containing SILYK10c:YFP in presence of a range of concentration of Myc-LCO-IV(C16:0,S), black circles and Myc-LCO-IV(C16:0), white circles.

Discussion

In this study, we report the spatio-temporal expression pattern and the LCO binding property of a LysM-RLK that controls AM. *SILYK10* expression in the epidermis and the outer cortex of lateral roots is consistent with a role in perceiving a fungal signal required for crossing these cell layers, and coherent with the absence of fungal penetration 2 weeks post inoculation (wpi) in *SILYK10* silenced plants (Buendia et al., 2016). Furthermore, SILYK10 affinity for LCOs strongly suggests that SILYK10 is involved in LCO perception, and thus that Myc-LCO perception by SILYK10 plays a positive role in AMF penetration in tomato roots. *SILYK10* expression in arbuscule-containing cells is also coherent with our previous observation of incorrectly developed arbuscules in some plants down-regulated for *SILYK10* expression (Buendia et al., 2016) and suggests that Myc-LCO perception by SILYK10 also plays a positive role in arbuscule development.

Data about the expression patterns of *SILYK10* orthologs are only available in legumes. Although one *SILYK10* ortholog is found in most plant species that establish AM, two genes are found in several legumes (Gough and Jacquet 2013), including *MtNFP* and *MtLYR1* in *M. truncatula* and *LjNFR5* and *LjLYS11* in *L. japonicus*. It was hypothesized that *MtNFP/LjNFR5* and *MtLYR1/LjLYS11* are derived from the duplication of an ancestral gene involved in AM and that *MtNFP/LjNFR5* have been neofunctionalized for an essential role in the RLS (Madsen et al., 2003; Arrighi et al., 2006). The *SILYK10* expression pattern might represent that of the ancestral legume gene involved in AM, and it is partly reminiscent of the expression profiles of its dual orthologs in legumes. *MtLYR1/LjLYS11* are also expressed in arbuscule-containing cells (Gomez et al., 2009; Rasmussen et al., 2016), and *MtNFP* but not *MtLYR1/LjLYS11*, is expressed in lateral roots of un-inoculated plants (Arrighi et al., 2006; Rasmussen et al., 2016; *M. truncatula* Gene Expression Atlas, http://mtgea.noble.org/v3). A difference with *SILYK10* is the strong expression of *MtNFP* in root hairs, which might have been acquired in legumes together with the rhizobial root hair infection process.

Although widely speculated, no data demonstrate the role of LCOs in AM establishment. Myc-LCOs have been shown to be biologically active molecules (Maillet et al., 2011; Czaja et al; 2012; Sun et al., 2015; Camps et al., 2015) but this does not prove that they play a role in AM. Furthermore, LysM-RLKs have been identified with roles in AM (Op den Camp et al., 2011; Miyata et al., 2014, Zhang et al., 2014; Buendia et al., 2016), but none of these proteins had

previously been shown to bind LCOs. In this study, we unambiguously demonstrate that SILYK10 is a high affinity LCO binding protein using a radiolabeled LCO binding assays. Our binding assay on membrane fractions allows the accurate measurement of the specific binding of the radiolabeled ligand as long as the detection of a significant amount of bound ligand is not hampered by dissociation during the washing step, separating ligand bound to the membrane fraction from free ligand. We found that LCO-V(C18:1,NMe,S), which has high affinity for SILYK10, fulfilled this requirement. Using LCO-V(C18:1,NMe,S) as the radiolabeled ligand, we found that SILYK10 recognized the Myc-LCO structures published in Maillet et al., 2011. A slightly lower affinity was found for the non-sulfated Myc-LCO compared to sulfated Myc-LCO but such difference is likely not biologically relevant for RLK that often discriminate ligand through difference in affinity with Kd ranging from pM to μ M. Moreover, we cannot exclude that this slight difference is due to difference in hydrophobicity between the nonsulfated LCO. In contrast SILYK10 exhibited a higher affinity for LCO-V(C18:1,NMe,S) compared to the known Myc-LCO structures, indicating that such LCOs or related structures could potentially represent additional Myc-LCOs. Analysis of Myc-LCOs from different AMF and determination of their affinities to SILYK10 would help to understand the specificity of LCO perception for establishing AM.

Our results suggest that Myc-LCO perception by SILYK10 plays a positive role in AM establishment in tomato. However, data indicate that *SILYK10* orthologs have variable roles depending on the plant species. It was shown that in *P. andersonii* plants with reduced levels of *PaNFP* expression, arbuscules do not develop correctly (Op den Camp et al., 2011). In a rice *Osnfr5* knockout line, a significant decrease of AM marker gene expression was measured by qRT-PCR, but no reduction was observed in the number of AMF colonization sites 2 wpi. Whether PaNFP and OsNFR5 are able to bind LCOs and whether LCO perception is affected in these knockdown/knockout plants need to be determined to conclude about the role of LCOs in AM establishment in these plants species. In legumes, although specifically expressed in arbuscule-containing cells, *LjLYS11* is not required for AM establishment in *L. japonicus* (Rasmussen et al., 2016). Also, there is no apparent redundancy between *MtNFP/LjNFR5* and *MtLYR1/LjLYS11* since a *L. japonicus* line mutated in both *LjNFR5* and *LjLYS11* is colonized by AMF similarly to the WT (Rasmussen et al., 2016). Moreover, despite almost completely abolished CSSP-dependent biological responses to both rhizobial LCOs and Myc-LCOs, including changes to root architecture, gene transcription and nuclear Ca²⁺ concentrations

(Ben Amor et al., 2003; Maillet et al., 2011; Sun et al., 2015; Hohnjec et al., 2015), M. truncatula nfp mutant lines can still be colonized apparently normally by AMF (Ben Amor et al., 2003; Zhang et al., 2014). Altogether, the data reported in legumes suggest that LCO perception is not as critical for AM establishment as for RNS establishment, and that another signal is involved and possibly redundant with LCOs for AM establishment in these plants. Indeed nuclear Ca²⁺ oscillations that are dependent on the CSSP, can still be measured in a *M*. truncatula nfp mutant line after treatment with CO4 (Genre et al., 2013; Sun et al., 2015) suggesting that CO receptors are redundant with LCO receptors for CSSP activation and possibly for AM establishment. In tomato SILYK10 silenced plants, no AMF colonization was found 2 wpi but plants were colonized 6 wpi (Buendia et al., 2016). It is thus also possible that CO perception is partly redundant with LCO perception for AM establishment in tomato. However, AMF colonization and LCO perception need to be analyzed in SILYK10 knockout plants to determine more precisely the role of SILYK10 in AM establishment and to evaluate possible redundancy between LCO and other signal for CSSP activation. In any case, to interpret the differences found between species, it can be hypothesized that the level of redundancy between LCO and CO receptors for AM establishment varies between plants and/or AMF species or between stages of the AM symbiosis. In addition, we cannot exclude that other AM fungal signals are involved in AM establishment.

Conclusion

We found that SILYK10 is able to bind LCOs with high affinity. The spatio-temporal expression pattern of *SILYK10*, together with our previous results showing that this gene is involved in for both AMF penetration and arbuscule development, suggest that LCOs control AM establishment in tomato.

Material and methods

Cloning

11.8 kbp corresponding to the non-coding region between *Solyc02g065510* and *Solyc02g065520 (SILYK10)*, including the *SILYK10 5' UTR* was amplified by PCR (Primers in Supplemental Table 1) and cloned in transcriptional fusion with a *GUS* reporter containing a plant intron, in a pCambia 2200 modified for Golden gate cloning and containing a pUbi::DsRed reporter (Fliegmann et al., 2016).

The extracellular regions of SILYK10 and SILYK7, as well as the TM/ICR of MtNFP were amplified by PCR (Primers in Supplemental Table 1) and cloned in translational fusion with YFP under the control of Pro35S by Golden gate cloning in a similar pCambia 2200 modified for Golden gate without the DsRed reporter, essentially as in Fliegmann et al., 2013.

Tomato Agrobacterium rhizogenes mediated transformation

Tomato (*Solanum lycopersicum* cv Marmande) seeds were surface sterilized and germinated *in vitro* for 7 to 10 d until cotyledons were fully expanded. Plantlets were cut at the hypocotyl level, immerged in a *A. rhizogenes* suspension at OD_{600nm} 0.3 and put on Petri dishes containing MS medium with agar. After 3 d at 25°C, the plantlets were transferred to Petri dishes with a similar medium supplemented with 50 mg/l kanamycin and 200 mg/l cefotaxim until emergence of transgenic roots. Transgenic roots containing the ProSILYK10::GUS constructs (expressing the DsRed reporter) or those expressing SILYK10:YFP were selected by fluorescence microscopy. Plantlets were grown in a plant growth chamber in pots containing vermiculite and watered with modified Long Ashton solution as described in Buendia et al., 2016. ROC lines derived from transformed roots were grown in dark, on Petri dishes with MS medium supplemented with 50 mg/l kanamycin.

Inoculation with AMF

Sterilized *Gigantea gigaspora* spores, harvested from a leek nurse culture, were pregerminated 5 d on M medium (Becard and Fortin 1988) in a 3% CO₂ incubator at 32°C. Two spores and one fragment of a transgenic tomato ROC line were then co-cultured on a Petri dish containing M medium supplemented with 50 mg/l kanamycin. Petri dishes were placed vertically with ROC lines above the fungal spores at 25°C in dark for 4 weeks.

After overnight GUS staining (using X-Gluc or Magenta-Gluc), AMF were stained by treating root tissues with 100% ethanol for 4 h, then with 10% (w/v) KOH for 8 min at 95°C, and finally with 0.2 M PBS pH 7.2, triton-X100 0.01% (v/v), 10 μ g/ml WGA CF488A conjugate (Biotum, Fremont, CA, USA) overnight.

Transient Expression in N. benthamiana

Leaves of *N. benthamiana* were infiltrated with *A. tumefaciens* strains allowing expression of Pro35S::SILYK10-YFP, Pro35S::SILYK10c-YFP or Pro35S::SILYK7c-YFP as described in Mbengue et al., 2010. Leaves were harvested 3 d after infiltration. *PMA4:GFP* and *HDEL:GFP* constructs are described in Lefebvre et al., 2004.

Microscopy

Tomato ROC were incubated at room temperature 5 minutes in water with 1 μ g / ml of DAPI or 20 μ M of FM4-64 before confocal imaging. Fluorescence in tomato ROC and *N. benthamiana* leaves were imaged using a SP8 confocal microscope (Leica, Wetzlar Germany). After GUS and WGA staining, roots were imaged using an Axiozoom V16 microscope (Zeiss, Jena, Germany). Automatic delimitation and drawing of cells strongly expressing GUS was performed with ImageJ.

Membrane fraction preparation

Approximately 20 g of leaves were homogenized at 4°C in a blender in the presence of 40 ml of extraction buffer (25 mM Tris, pH 8.5, 0.47 M sucrose, 5 mM EDTA, 10 mM DTT, 0.6% PVPP and protease inhibitors (0.1 mM AEBSF, and 1 mg/mL each of leupeptin, aprotinin, antipain, chymostatin, and pepstatin). Samples were centrifuged for 15 min at 3000 g, and then the supernatant was recentrifuged for 30 min at 45000 g. The pellet (membrane fraction) was first washed in 5 ml and then resuspended in 2 ml of binding buffer (25 mM Na-Cacodylate pH 6, 250 mM sucrose, 1 mM CaCl2, 1 mM MgCl2 and protease inhibitors). After each extraction, amount of SILYK10c:YFP or SILYK7c:YFP was quantified by immunoblotting in 10 µg of membrane fraction proteins.

LCO binding assays

LCO-V(C18:1 Δ 11,NMe) and LCO-V(C18:1 Δ 11,NMe,S) were purified from the rhizobial strain *Rhizobium tropici*. The synthetic LCO aryl-azido derivative is described in Fliegmann et al., 2013. Synthetic Myc-LCOs, LCO-IV(C18:1 Δ 9), LCO-IV(C18:1 Δ 9,S), LCO-IV(C16:0) and LCO-IV(C16:0,S), are described in Maillet et al., 2011. Labeling of LCO-V(C18:1 Δ 11,NMe)was performed as described in Fliegmann et al., 2013. LCO binding assays on membrane fraction (containing 20 or 40 µg protein) were performed as in Fliegmann et al., 2013 using between 1 and 2 nM of radiolabeled LCO and ranges of unlabeled LCO or CO between 1 nM to 1 µM.

PNGaseF treatment and immunoblotting

PNGaseF treatment, SDS-PAGE, transfer to nitrocellulose membranes and Western blotting were performed as described in Lefebvre et al., 2012 except that anti-GFP (AMS Biotechnology, Abingdon, UK.) was used to detect protein fused with YFP.

Acknowledgements

We thank Marie Cumener for help with initial LCO binding assays on SILYK10. We thank Fabienne Maillet for LCO purification from rhizobia, Véréna Poinsot for LCO analysis by mass spectrometry, Mireille Chabaud for *G. gigaspora* spore production and Julie Cullimore, Clare Gough and Christine Hervé for critical reading of the manuscript.

References

Arrighi JF, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet EP, Ghérardi M, Huguet T, Geurts R, Dénarié J, Rougé P and Gough C (2006) The Medicago truncatula lysin motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. Plant Physiol 142: 265-279

Becard G and Fortin JA (1988) Early events of vesicular arbuscular mycorrhiza formation on ri t-dna transformed roots. New Phytol 108: 211-218

Ben Amor B, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Denarie J and Gough C (2003). The NFP locus of Medicago truncatula controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J 34: 495-506 Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ, Roepstorff P, Thirup S, Ronson CW, Thygesen MB and Stougaard J (2012) Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proc Natl Acad Sci USA 109: 13859-13864

Buendia L, Wang TM, Girardin A and Lefebvre B (2016) The LysM receptor-like kinase SILYK10 regulates the arbuscular mycorrhizal symbiosis in tomato. New Phytol 210: 184-195

Camps C, Jardinaud MF, Rengel D, Carrère S, Hervé C, Debellé F, Gamas P, Bensmihen S, Gough C (2015) Combined genetic and transcriptomic analysis reveals three major signalling pathways activated by Myc-LCOs in Medicago truncatula. New Phytol 208: 224-40

Cao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A and Stacey G (2014). The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1. Elife 3: e03766

Czaja LF, Hogekamp C, Lamm P, Maillet P, Martinez EA, Samain E, Dénarié J, Küster H and Hohnjec N (2012). Transcriptional responses toward diffusible signals from symbiotic microbes reveal MtNFP- and MtDMI3-dependent reprogramming of host gene expression by arbuscular mycorrhizal fungal lipochitooligosaccharides. Plant Physiol 159: 1671-1685

Fliegmann J. and Bono JJ (2015) Lipo-chitooligosaccharidic nodulation factors and their perception by plant receptors. Glycoconj J 32: 455-464

Fliegmann J, Canova S, Lachaud C, Uhlenbroich S, Gasciolli V, Pichereaux C, Rossignol M, Rosenberg C, Cumener M, Pitorre D, Lefebvre B, Gough C, Samain E, Fort S, Driguez H, Vauzeilles B, Beau JM, Nurisso A, Imberty A, Cullimore J and Bono JJ (2013) Lipochitooligosaccharidic symbiotic signals are recognized by lysm receptor-like kinase LYR3 in the legume Medicago truncatula. ACS Chem Biol 8: 1900-1906

Fliegmann J, Jauneau A, Pichereaux C, Rosenberg C, Gasciolli V, Timmers AC, Burlet-Schiltz O, Cullimore J and Bono JJ (2016) LYR3, a high-affinity LCO-binding protein of Medicago truncatula, interacts with LYK3, a key symbiotic receptor. FEBS Lett 590: 1477-1487

Genre A, Chabaud M, Balzergue C, Puech-Pages V, Novero M, Rey T, Fournier J, Rochange S, Becard G, Bonfante P and Barker DG (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in Medicago truncatula roots and their production is enhanced by strigolactone. New Phytol 198: 179-189

Gomez SK, Javot H, Deewatthanawong P, Torres-Jerez I, Tang Y, Blancaflor EB, Udvardi MK and Harrison MJ (2009) Medicago truncatula and Glomus intraradices gene expression in

cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. BMC Plant Biol 9: 10

Gough C and Jacquet C (2013) Nod factor perception protein carries weight in biotic interactions. Trends Plant Sci 18: 566-574

Gressent F, Cullimore J, Ranjeva R and Bono JJ (2004) Radiolabeling of lipochitooligosaccharides using the NodH sulfotransferase: a two-step enzymatic procedure. BMC Biochem 5: 4

Gutjahr C and Paszkowski U (2013) Multiple control levels of root system remodeling in arbuscular mycorrhizal symbiosis. Frontiers Plant Sci 4: 204

Hohnjec N., Czaja-Hasse LF, Hogekamp C and Küster H (2015). Pre-announcement of symbiotic guests: transcriptional reprogramming by mycorrhizal lipochitooligosaccharides shows a strict co-dependency on the GRAS transcription factors NSP1 and RAM1. BMC Genomics 16: 994.

Indrasumunar A, Kereszt A, Searle I, Miyagi M, Li D, Nguyen CD, Men A, Carroll BJ and Gresshoff PM (2010) Inactivation of duplicated nod factor receptor 5 (NFR5) genes in recessive loss-of-function non-nodulation mutants of allotetraploid soybean (Glycine max L. Merr.). Plant Cell Physiol 51: 201-214

Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E and Shibuya N (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc Natl Acad Sci USA 103, 11086-11091.

Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszyński A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, Vinther M, Andersen SU, Krusell L, Thirup S, Jensen KJ, Ronson CW, Blaise M, Radutoiu S and Stougaard J (2015) Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature 523: 308-312

Kobae Y and Hata S (2010) Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. Plant Cell Physiol 51: 341-353

Kobae Y, Ohmori Y, Saito C, Yano K, Ohtomo R and Fujiwara T (2016) Phosphate Treatment Strongly Inhibits New Arbuscule Development But Not the Maintenance of Arbuscule in Mycorrhizal Rice Roots. Plant Physiol 171: 566-579

Lefebvre B, Klaus-Heisen D, Pietraszewska-Bogiel A, Herve C, Camut S, Auriac MC, Gasciolli V, Nurisso A, Gadella TWJ and Cullimore J (2012) Role of N-Glycosylation Sites and

CXC Motifs in Trafficking of Medicago truncatula Nod Factor Perception Protein to Plasma Membrane. J Biol Chem 287: 10812-10823

Lefebvre B, Timmers T, Mbengue M, Moreau S, Herve C, Toth K, Bittencourt-Silvestre J, Klaus D, Deslandes L, Godiard L, Murray JD, Udvardi MK, Raffaele S, Mongrand S, Cullimore J, Gamas P, Niebel A and Ott T (2010) A remorin protein interacts with symbiotic receptors and regulates bacterial infection. Proc Natl Acad Sci USA 107: 2343-2348

Lefebvre B, Batoko H, Duby G, Boutry M (2004) Targeting of a Nicotiana plumbaginifolia H+ -ATPase to the plasma membrane is not by default and requires cytosolic structural determinants. Plant Cell 16:1772-1790

Liu S, Wang J, Han Z, Gong X, Zhang H and Chai J (2016) Molecular Mechanism for Fungal Cell Wall Recognition by Rice Chitin Receptor OsCEBiP. Structure 24: 1192-1200

Liu T. Liu Z, Song C, Hu Y, Han Z, She J, Fan F, Wang J, Jin C, Chang J, Zhou JM and Chai J (2012) Chitin-induced dimerization activates a plant immune receptor. Science 336: 1160-1164

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 637-40

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, Martinez EA, Driguez H, Bécard G and Dénarié J (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469: 58-63

Malkov N, Fliegmann J, Rosenberg C, Gasciolli V, Timmers AC, Nurisso A, Cullimore J and Bono JJ (2016). Molecular basis of lipo-chitooligosaccharide recognition by the lysin motif receptor-like kinase LYR3 in legumes. Biochem J 473: 1369-1378

Mbengue M, Camut S, de Carvalho-Niebel F, Deslandes L, Froidure S, Klaus-Heisen D, Moreau S, Rivas S, Timmers T, Hervé C, Cullimore J, and Lefebvre B (2010) The Medicago truncatula E3 ubiquitin ligase PUB1 interacts with the LYK3 symbiotic receptor and negatively regulates infection and nodulation. Plant Cell 22, 3474–3488

Miyata K, Kozaki T, Kouzai Y, Ozawa K, Ishii K, Asamizu E, Okabe Y, Umehara Y, Miyamoto A, Kobae Y, Akiyama K, Kaku H, Nishizawa Y, Shibuya N and Nakagawa T (2014). The Bifunctional Plant Receptor, OsCERK1, Regulates Both Chitin-Triggered Immunity and Arbuscular Mycorrhizal Symbiosis in Rice. Plant Cell Physiol 55: 1864-1872

Miyata K, Hayafune M, Kobae Y, Kaku H, Nishizawa Y, Masuda Y, Shibuya N and Nakagawa T (2016) Evaluation of the Role of the LysM Receptor-Like Kinase, OsNFR5/OsRLK2 for AM Symbiosis in Rice. Plant Cell Physiol. In press

Murray JD (2011) Invasion by invitation: rhizobial infection in legumes. Mol Plant-Microbe Interact 24: 631-639

Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JS, Kudrna D, Wing R, Untergasser A, Bisseling T and Geurts R (2011) LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia. Science 331: 909-912

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N and Stougaard J (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425: 585-592

Rasmussen SR, Füchtbauer W, Novero M, Volpe V, Malkov N, Genre A, Bonfante P, Stougaard J and Radutoiu S (2016) Intraradical colonization by arbuscular mycorrhizal fungi triggers induction of a lipochitooligosaccharide receptor. Sci Rep 6: 29733

Sun J, Miller JB, Granqvist E, Wiley-Kalil A, Gobbato E, Maillet F, Cottaz S, Samain E, Venkateshwaran M, Fort S, Morris RJ, Ané JM, Dénarié J and Oldroyd GE (2015) Activation of symbiosis signaling by arbuscular mycorrhizal fungi in legumes and rice. Plant Cell 27: 823-838

Willmann R, Lajunen HM, Erbs G, Newman MA, Kolb D, Tsuda K, Katagiri F, Fliegmann J, Bono JJ, Cullimore JV, Jehle AK, Goetz F, Kulik A, Molinaro A, Lipka V, Gust AA and Nuernberger T (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proc Natl Acad Sci USA 108, 19824-19829

Zhang X, Dong W, Sun J, Feng F, Deng Y, He Z, Oldroyd GE and Wang E (2014). The receptor kinase CERK1 has dual functions in symbiosis and immunity signalling. Plant J 81: 258-267

Supplemental Data



Medicago truncatula MtNFP and MtLYR1, *Lotus japonicus* LjNFR5 and LjLYS11, *Parasponia andersonii* PaNFP and *Oryza sativa* OsNFR5 were used to construct a phylogenetic tree as described in *Buendia et al., 2016.* SlLYK8 was not used to build the tree because it has a truncated kinase. Numbers at the branches correspond to bootstrap values. The phylogenetic group containing *SlLYK10* is circled. An arrow points *SlLYK7*.



Fig. S2 The Prosiler R102: GUS fusion is mainly expressed in the epidermis and the outer cortex before AMF colonization. A) Representative image of tomato transgenic ROC lines grown in absence of AM fungi. Lateral roots show maximal GUS activity (magenta). B) Longitudinal section in a root segments showing GUS staining. Maximal GUS activity (magenta) is found in the epidermis (e) and the outer cortex (oc) but not in the inner cortex (ic) or in the vessels (v). Bars represent 5000 μ m in A and 50 μ m in B.



Fig. S3 In tomato SILYK10 localized at the plasma membrane. A) Confocal images of epidermal cells from tomato ROC lines expressing SILYK10:YFP and treated with FM4 64 (which labels the plasma membrane after treatment before being internalized) and DAPI (which labels the nucleus) showing that SILYK10:YFP is localized in the plasma membrane. Bars represent 50 μ m.




In N. benthamiana SILYK10c and SILYK7c are partially localized in the plasma membrane. A) Confocal images of epidermal cells from N. benthamiana leaves expressing combinations of PMA4:GFP (a plasma membrane (PM) marker) or HDEL:GFP (an endoplasmic reticulum (ER) SILYK10c:YFP marker) together with or SILYK7c:YFP. Fluorescence detected in the GFP and YFP channels is shown in red and green respectively, to facilitate the visualization of the overlay. Bars represent 10 µm. B) Leaves were grinded and fractionated. Proteins were immunodetected in the total extract (T), the 100 000g pellet (P) and the supernatant (S) using anti-GFP antibodies (to follow the YFP fusions), anti-BIP antibodies (to follow an ER marker) or anti-H⁺-ATPase antibodies (to follow a PM marker). SILYK10c:YFP and SILYK7c:YFP were enriched in the pellet corresponding to the

membrane fraction but were also detected in the supernatant that likely contains some ER membrane. **C)** Proteins were incubated (+) or not (-) with PNGaseF. Higher band after incubation indicates that protein N-glycans are insensitive to the PNGaseF and shows that protein exit the ER and were processed in the Golgi apparatus. Lower band after incubation indicates that protein N-glycans are sensitive to the PNGaseF digestion and shows that they did not exit the ER. Part of SILYK10c:YFP and SILYK7c:YFP are insensitive to PNGaseF while the positive control, a mutated form of MtNFP lacking disulfide bridges and known to be retained in the ER (Lefebvre et al, 2012) is completely digested by PNGaseF. The Ponceau staining shows that the equal amount of proteins were used for SDS-PAGE.

Supplemental Table 1: List of primers used

Pro SILYK10 For	GGTCTCTAAATGGGTTATAGAGCTGTAATGC
Pro SILYK10 Rev	GGTCTCATTTGCGATGCAAAGCTTAGATAAC
GUS For	GGTCTCTCAAAATGTTACGTCCTGTAGAAACCC
GUS Rev	AAAGGTCTCGCGTATCATTGTTTGCCTCCCTGCTG
SILYK10c For	GGTCTCGCAAAATGGTAGTTCCTCTTGTGTCCTTG
SILYK10c Rev	GGTCTCGTAAGTCCATGCTTGGATTTTCTACTGCTTGC
SILYK7c For	GGTCTCGCAAAATGGGTGATTTTCCACTTATCTTTTCATAAGTTC
SILYK7c Rev	GGTCTCGTAAGGCCGTATGATATTTTGTGCCTAGAAGG

Chapter 3: AMS LysM-RLKs might have been directly recruited for the RLS

Preamble

In parallel to the study of SILYK10, I analyzed its ortholog in *B. distachyon* BdLYR1. *B. distachyon* is the plant with the lowest number of LysM-RLKs among the mycorrhizal plant species with sequenced genomes. I characterized the LCO binding properties of BdLYR1 and I identified a *B. distachyon* line mutated in *BdLYR1* leading to an early stop codon in the *BdLYR1* sequence coding for the ICR.

We also wanted to understand whether the legume LysM-RLKs involved in RLS establishment were duplicated and neo-functionalized as suggested in the literature or whether the ancestral LysM-RLKs were directly recruited for a role in the RLS during evolution. Thus, I introduced *SILYK10* or *BdLYR1*, which are orthologs of the legume *MtNFP* in the non-nodulating *Mtnfp-2* mutant. We observed that *BdLYR1* was able to restore nodulation in *Mtnfp-2*. We were thus wondering whether the function of *MtNFP* and in particularly its expression pattern in nodules originates from a gain of cis element that would be absent in non-legumes orthologs. I expressed the *β-glucuronidase* reporter under the control of the promoter of *MtNFP*, *SlLYK10* or *BdLYR1*. I observed that *SlLYK10* promoter is regulated as *MtNFP* promoter during the RLS in *M. truncatula*. We thus think that *MtNFP* ancestor was already functional for the RLS and that pre-existing LysM-RLKs involved in the AMS were directly recruited for the RLS during evolution.

I started this part at the beginning of my PhD and I conducted it all along the three years, in parallel of the studies presented in the chapter 1 and 2. I performed most of the experiments presented in this chapter. I organized the results I obtained in an article format although additional experiments will be required for publication. We are currently thinking to share our results with other groups, which obtained data on *BdLYR1*, orthologs to build a publishable manuscript.

MtNFP ancestor was probably already functional for arbuscular mycorrhizal symbiosis and directly recruited together with the CSSP.

Ariane GIRARDIN¹, Marie CUMENER¹, Virginie GASCIOLLI¹, Richard SIBOUT², Marion DALMAIS³, Abdel BENDAHMANE³, Jean Jacques BONO¹ and Benoit LEFEBVRE¹ Affiliations: ¹INRA-CNRS, Castanet-tolosan, France; ²INRA-AgroParisTech, Versailles, France; ³Univ. Paris 11-Univ. Evry-Univ. Paris7-CNRS-INRA, Evry, France

<u>Abstract</u>

Root endosymbioses formed between soil-born microorganisms and plant roots often lead to a better mineral nutrition of the plant in exchange of carbohydrates produced during photosynthesis provided to micro-organisms. The arbuscular mycorrhizal symbiosis (AMS) is a widespread and ancient root endosymbiosis formed between fungi belonging to the Glomeromycota monophyletic group and at least 80 % of land plants. Another root endosymbiosis is the Rhizobium legume symbiosis (RLS) during which a new organ is formed, the nodule, where atmospheric nitrogen is fixed by rhizobial bacteria. Despite their differences, AMS and RLS share similarities for their early establishment. Indeed AM fungi (AMF) and rhizobia secrete same kind of signal molecules called Lipo-chitooligosaccharides (LCO) which perception, activates a signaling pathway shared by AMS and RLS: the common symbiosis signaling pathway (CSSP). The current hypothesis concerning the apparition of the RLS is that during evolution, the CSSP and the plant machinery allowing AMF colonization were directly recruited for the RLS. In contrast, it is considered that a Lysin Motif Receptor-Like Kinase (LysM-RLK) involved in the perception of LCOs produced by AMF was duplicated in legumes and that one of the copies was neo-functionalized to perceive LCOs produced by rhizobia. Here, we show that SILYK10 and BdLYR1 that are orthologs in Solanum lycopersicum and Brachypodium distachyon of MtNFP a LysM-RLK essential for RLS establishment in Medicago truncatula, bind LCO with high affinity. We also show that BdLYR1, under the control of a strong expression promoter can complement the absence of nodulation in *Mtnfp-2*. Finally, we show that the promoter of *SILYK10* has a similar expression pattern as the promoter of MtNFP when introduced in M. truncatula. These results bring new insights concerning the hypothesis of the LysM-RLK recruitment for the RLS during evolution.

Introduction

Root endosymbioses are beneficial associations between soil microorganisms and the roots of the plants. Today, the most studied root endosymbiosis is established between nitrogen-fixing bacteria (called rhizobia) and plants almost restricted to the *Fabaceae* family (legumes). In the rhizobium-legume symbiosis (RLS), bacteria strains are often colonizing specifically one host species. Bacterial signal molecules involved in plant recognition and host specificity were called Nod-factors and identified as lipo-chitooligosaccharides (LCO, (Dénarié et al., 1996). Nod-factors are composed of a core of 4/5 N-acetyl glucosamine (GlcNAc) backbone that bears a fatty acid on the non-reducing sugar. The specificity of interaction between rhizobia and their host legume relies on decorations displayed by Nod-factors as well as length and saturation of the acyl moiety. (Fliegmann and Bono, 2015). The Major Nod-factor produced by *Sinorhizobium meliloti* (also called *Ensifer meliloti*) a symbiont of *Medicago truncatula*, is a LCO composed of 4 GlcNAc, a C16:2 acyl chain, a sulfate group and an acetate group: LCO-IV(C16:2,S,Ac).

In legumes Nod-factors are perceived by proteins of the Lysin Motif Receptor-Like Kinase (LysM-RLK) multigenic family (Gough and Cullimore, 2011). LysM-RLKs have already been shown to bind GlcNAc containing ligand as chitooligosaccharides (CO) or LCOs (Broghammer et al., 2012; Fliegmann et al., 2013; Cao et al., 2014; Malkov et al., 2016). They are transmembrane proteins composed in their extracellular region (ECR) of 3 lysin motifs (LysM) involved in the binding of GlcNAc containing ligands, and in their intracellular region (ICR) of a domain with homology to kinase. The LysM are separated by a pair of cysteines (CXC) (Liu et al., 2012; Liu et al., 2016), creating disulfide bridges necessary for the LysM-RLK functions (Lefebvre et al., 2012; Kawaharada et al., 2015). LysM-RLKs are divided in 2 classes depending on whether their ICR has an active kinase domain (LYK) or has an aberrant kinase domain (LYR) (Gust et al., 2012). In M. truncatula and Lotus japonicus, two model legumes, it was shown that heterodimers of LysM-RLKs are involved in Nod-factor perception. These heterodimers are composed of a LYR, MtNFP or LjNFR5, and a LYK, MtLYK3 or LjNFR1 (Madsen et al., 2011; Moling et al., 2014). Mutants in one of these genes cannot establish the RLS (Radutoiu et al., 2003; Arrighi et al., 2006; Smit et al., 2007). The LCO perception through LysM-RLKs activates a signaling pathway required in the RLS establishment. This pathway includes cytosolic and nuclear oscillations of calcium concentration also called calcium spiking that can be measured as a LCO perception response in plants (Ehrhardt et al., 1992).

Interestingly, the arbuscular mycorrhiza symbiosis (AMS), a widespread root endosymbiosis involving soil fungi belonging to the *Glomeromycota* phylum and at least 80% of land plants, shares the early signaling pathway with the RLS. That is why this pathway was called common symbiosis signaling pathway (CSSP). LCOs have also been found in AM fungi (AMF) exudates together with related structures: short chain COs that are only composed of 4/5 GlcNAc. It has been shown that both LCOs and short COs can induce in non-legumes as in legumes responses that depend on the CSSP, such as increase in lateral root number and calcium spiking (Maillet et al., 2011; Genre et al., 2013; Sun et al., 2015).

Despite their differences (host spectrum, type of microorganism involved, etc.) the RLS and AMS share the signal molecules (at least LCOs) and the CSSP. The facts that the AMS is an ancient root endosymbiosis that probably helped plants to colonize lands 450 million years ago (Remy et al., 1994; Heckman, 2001), while the RLS appeared more recently, about 65 million years ago (Kistner and Parniske, 2002) led to the hypothesis that during evolution the LysM-RLKs and the signaling pathway involved in AMS establishment, were recruited for the RLS (De Mita et al., 2013). However, gene duplications occurred in the LysM-RLKs in most legumes (Gough and Jacquet, 2013; De Mita et al., 2013). For example, there are two paralogs MtNFP/MtLYR1 and LjNFR5/LjLYS11 in legumes while only one gene is found in non-legumes that form the AMS, PaNFP in Parasponia andersonii, SILYK10 in Solanum lycopersicum or OsNFR5 in Oryza sativa. It was suggested that the ancestral gene had a role in the AMS and that in legumes, following the duplication, one copy (MtNFP/LjNFR5) was neo-functionalized for a role in the RLS (Madsen et al., 2003; Young et al., 2012; De Mita et al., 2013) while the second copy kept the ancestral role in the AMS. Supporting this hypothesis, in P. andersonii which belongs to the only gender establishing the RLS outside the legume family, silencing PaNFP prevented both the RLS and the AMS establishment (Op den Camp et al., 2011). Furthermore, in tomato, a non-legume plant that only forms the AMS, we showed that silencing SILYK10, affected the AMS establishment (Buendia et al., 2016). Finally, MtLYR1 and LjLYS11 are expressed in arbuscule-containing cells (Gomez et al., 2009; Rasmussen et al., 2016) while *Mtnfp* and *Ljnfr5* mutants are not affected in AMF colonization. However *LjLYS11* mutants were also colonized by AMF as WT plants suggesting that even if involved in AMS, *LjLYS11* is not essential and might have a redundant function with (an)other gene(s).

In this study, we characterized *BdLYR1* the ortholog of *MtNFP/MtLYR1* in *Brachypodium distachyon*, a monocotyledon. We established that BdLYR1 is a functional ortholog of SlLYK10 by measuring its affinity for LCOs and of MtNFP by trans-complementation experiments in chimeric roots of *M. truncatula* in an *Mtnfp* mutant. We also analyzed the transcriptional activity of *SlLYK10* promotor region in *M. truncatula* during nodulation. The results led us to propose a new hypothesis concerning the evolution of the symbiotic *MtNFP LysM-RLK* orthologs in legumes.

<u>Results</u>

BdLYR1 has high affinity and selectivity for LCOs

First, we wanted to determine the ability of BdLYR1 to bind LCOs. We produced BdLYR1 with a YFP tag (BdLYR1:YFP) in *N. benthamiana* leaves, an heterologous expression system allowing massive protein expression, with correct conformation, specially through the formation of disulfide bridges between the LysM that are essential for LysM-RLK functions (Lefebvre et al., 2012; Kawaharada et al., 2015). As shown in Fig. 1A, BdLYR1:YFP localized in cell periphery of *N. benthamiana* leaf epidermal cells, consistent with its putative role as a plasma membrane (PM) LCO receptor. We were able to measure LCO binding on membrane fraction extracted from leaves expressing BdLYR1:YFP (Fig. 1B). We then determined the affinity of BdLYR1:YFP for LCO by cold saturation using a radiolabeled LCO-V(C18:1,NMe,³⁵S) and a range of concentration of the same unlabeled LCO-V(C18:1,NMe,S). We found a *Kd* of 9 nM +/- 2 nM (n=3), showing that BdLYR1:YFP has high affinity for this LCO (Fig. 1C). In order to determine whether BdLYR1 LCO binding site has selectivity for LCOs versus COs, we performed competition assays using 1 μ M of unlabeled CO4 or CO8 (Fig. 1B). We observed that CO4 or CO8 poorly compete binding of LCO-V(C18:1,NMe,³⁵S) on BdLYR1 in comparison to the competition measured in presence of 1 μ M LCO-V(C18:1,NMe,S).



Fig. 1 BdLYR1 has a high affinity for LCOs and is selective for LCOs vs COs. A) Confocal image showing BdLYR1:YFP expressed in epidermal cells of *N. benthamiana*. Scale bar represents 50 μ m. **B)** Membrane fractions containing BdLYR1:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,³⁵S) in presence of 1 μ M of the indicated unlabeled molecules used as competitors. Non specific binding was determined in the presence of 1 μ M LCO-V(C18:1,NMe,S). Bars represent the percentage of specific binding (means and SE) obtained in 3 experiments performed on membrane fractions prepared from independent batches of *N. benthamiana* agro-infiltrated leaves. **C)** Scatchard plot analysis of a cold saturation experiment using LCO-V(C18:1,NMe,S) and membrane fraction containing BdLYR1:YFP. The plot is representative of experiments performed on membrane fraction specific not specified from 3 independent batches of *N. benthamiana* fractions of experiments performed on membrane fraction containing BdLYR1:YFP. The plot is representative of experiments performed on membrane fraction containing BdLYR1:YFP. The plot is representative of agro-infiltrated *N. benthamiana* leaves.

Bdlyr1-1 mutant is still able to establish AMS

After showing BdLYR1 has high affinity for LCOs, we wanted to know its role during AMS. We obtained a line mutated in *BdLYR1 (Bdlyr1-1)* by TILLING screen of a NaN₃ mutagenized population. In this mutant, a change in the coding sequence (CDS) of G1401 to A led to the apparition of a stop codon. The corresponding protein is truncated of the major part of its ICR (Fig. 2A). We measured the number of colonization sites per root 3 weeks after inoculation (wpi) with *Rhizophagus irregularis* in a segregating population containing either the WT or mutated version of *BdLYR1* (Fig. 2B). We observed no significant difference in the colonization site number between control plants and *Bdlyr1-1* plants.



Fig. 2 *Bdlyr1-1* is able to establish the AMS. A) Schematic representation of the protein BdLYR1. The upper protein is BdLYR1 WT and the lower represents BdLYR1¹⁻⁴⁶⁶, with in white the part lacking due to the stop mutation in the gene. Red boxes represent the LysMs, green ball the transmembrane domain, and orange box, the ICR containing an aberrant kinase domain. B) Colonization by *R. irregularis* was measured by counting infection points 3 wpi in roots of WT and *Bdlyr1-1* plants. Box plot represent the values obtained in 3 independent biological replicates containing each about 10 individuals. Statistical analysis made by Wilcoxon test that gave a p-value of 0.068.

MtNFP orthologs can complement absence of nodulation in Mtnfp

We wanted to know whether BdLYR1 and SlLYK10 are functional orthologs of MtNFP. It was previously shown that MtNFP and MtLYK3, when co-expressed in N. benthamiana leaves, induce cell death (Pietraszewska-Bogiel et al., 2013). Hence, we tested whether co-expression of BdLYR1:YFP or SILYK10:YFP with MtLYK3:YFP induces cell death (Fig. 3A). We observed no lesion when BdLYR1:YFP, SlLYK10:YFP or MtLYK3:YFP were expressed alone in N. benthamiana leaves, but we observed cell death when we co-expressed both BdLYR1:YFP or SlLYK10:YFP with MtLYK3:YFP. Consecutively, we tested complementation M. truncatula Mtnfp-2 by BdLYR1 or SILYK10 (Fig. 3B). Mtnfp-2 mutant is not able to produce nodule in presence of rhizobia (Arrighi et al., 2006). We introduced in roots of Mtnfp-2 plants (through Agrobacterium rhizogenes) either an empty vector (EV) or the LysM-RLK CDS in translational fusion with YFP and under the CaMV 35S promoter (p35S::MtNFP:YFP, p35S::BdLYR1:YFP, p35S::SILYK10:YFP). In addition, we introduced PsSYM10, the NFP ortholog from Pisum sativum (p35S::PsSYM10:YFP) or AtCERK1 (p35S::AtCERK1:YFP) an unrelated LysM-RLK from the LYK subfamily as negative control. Expression of *MtNFP* or *PsSYM10* was able to restore the nodule formation in the presence of S. meliloti. No significant difference was found in the number of nodules in roots expressing MtNFP or PsSYM10. Although with less efficiency, BdLYR1 expression restored nodule formation in Mtnfp-2. In contrast, no nodule was observed on *Mtnfp-2* roots expressing *AtCERK1* or containing the empty vector (EV). Introduction of p35S::SILYK10:YFP did not restore nodulation in Mtnfp-2, however when we quantified the protein expression by immunoblotting, we detected much less SILYK10:YFP than BdLYR1:YFP or MtNFP:YFP proteins (Data not shown). We further analyzed the nodule colonization by rhizobia in the nodules from roots expressing MtNFP:YFP, PsSYM10:YFP or BdLYR1:YFP (Fig. 3C). By microscopy, we observed that S. meliloti bearing an X-GAL reporter gene colonized the cells of the nodules.

At the contrary of *M. truncatula*, pea establishes RLS with rhizobial strains that produce un-sulfated LCOs. Hence, we were curious to know whether p355::*PsSYM10:YFP* or p355::*BdLYR1:YFP* would conserve the specificity for sulfated LCOs to restore nodulation in *Mtnfp-2* complemented plants. Thus we inoculated with a mutated *S. meliloti* strain unable to produce sulfated LCOs (*S. meliloti* Δ *NodH*) on plants complemented by *MtNFP* as control, *PsSYM10* or *BdLYR1* (Fig. S1). We observed that introduction of p355::*PsSYM10:YFP* or p355::*BdLYR1:YFP* in *Mtnfp2* did not modified the nodule number per plants compared to

plants complemented by p35S::MtNFP:YFP. As p35S::BdLYR1:YFP could restore nodulation when introduced in *Mtnfp-2* mutant, we used the complementation to test the functionality of the truncated protein produced in the *Bdlyr1-1* mutant. Thus we introduced in *Mtnfp-2* p35S::BdLYR1¹⁻⁴⁶⁶:YFP (Fig. 3B). This gene could not restore nodulation in *Mtnfp-2*. We also tested whether BdLYR1¹⁻⁴⁶⁶:YFP could induce cell death in co-expression with MtLYK3 in *N*. *benthamiana* (Fig. S2). At the contrary of co-expression of BdLYR1 or MtNFP with MtLYK3, BdLYR1^{1-W467*} co-expression with MtLYK3 did not induce cell death.



Fig. 3 SILYK10 and BdLYR1 interact with MtLYK3 and activate the CSSP in *M. truncatula*. **A)** BdLYR1, SILYK10 and MtLYK3 did not induce cell death when expressed separately in *N. benthamiana* leaves, but when BdLYR1 or SILYK10 were co-expressed with MtLYK3, cell death was induced. Images are representative of at least 3 biological replicates. **B)** Roots of *Mtnfp-2* mutant were transformed via *A. rhizogenes* with empty vector (EV), p355::*AtCERK1:YFP*, p355::*MtNFP:YFP*, p355::*PsSYM10:YFP*, p355::*BdLYR1:YFP* or p355::*BdLYR1¹⁻⁴⁶⁶:YFP*. WT plants are A17 not transformed. Nodule number was counted 4 wpi. Different statistical classes were established according a Kruskal-Wallis test (with α = 0.05) and are indicated by different letters. **C)** Nodules from complementation of *Mtnfp-2* by *MtNFP* (left), *PsSYM10* (middle) or *BdLYR1* (right) were harvested 4 wpi, cut and stained (in blue) in order to reveal bacteria containing the β-galactosidase. Scale bar: 1 mm.

pSILYK10 has the same transcriptional activity that pMtNFP during nodulation in M. truncatula

MtNFP is expressed in nodule primordia and later in the infection zone of mature nodules (Arrighi et al., 2006). We asked whether during evolution, the promoter of *MtNFP* ancestor acquired properties for expression in nodules. To answer this question, we produced *M. truncatula* roots expressing *B-glucuronidase* (*GUS*) under the control of p*SlLYK10*, p*BdLYR1* or p*MtNFP*. In non-inoculated conditions, p*SlLYK10*, p*BdLYR1* and p*MtNFP* transcription was in the root hair growing zone of lateral roots (data not shown), although p*BdLYR1* transcription was extremely weak. After inoculation with rhizobia, GUS staining (in magenta) appeared specifically in the nodule primordia and later in the apex of mature nodules in roots containing the p*SlLYK10::GUS* or p*MtNFP::GUS* constructs (Fig4). No GUS staining was observed in nodules of roots containing the p*BdLYR1::GUS* construct.



Fig. 4 The promoter of *SILYK10* **is transcribed in nodules in** *M. truncatula***.** A17 roots were transformed via *A. rhizogenes* with *pMtNFP::GUS* or *pSILYK10::GUS*. Roots were inoculated with a *S. meliloti* strain containing the β -galactosidase, and harvested and stained to visualize expression of promoters (magenta) and to visualize the bacteria (blue) 6 dpi or 4 wpi. Arrows in the upper panel point infection threads. Scale bar: 200 µm.

Discussion

We found that BdLYR1 has high affinity for LCOs (Kd of 9 nM +/- 2 nM) as its orthologs in tomato SlLYK10 and in *L. japonicus* LjNFR5 (Girardin et al., in revision; Broghammer et al., 2012). The LCO binding site of BdLYR1, as the LCO binding site of SlLYK10, is specific for LCOs versus COs. Therefore, it is very likely that BdLYR1 is a functional ortholog of SlLYK10 and is involved in LCO perception for establishment of the AMS in *B. distachyon* as SlLYK10 in tomato.

However, in contrast to tomato or *P. andersonii* (a non-legume able to establish both the RLS and AMS) in which silencing of *BdLYR1* orthologs (*SlLYK10* and *PaNFP* respectively) led to a drastic decrease of AMF colonization and/or arbuscule formation (Op den Camp et al., 2011; Buendia et al., 2016), *BdLYR1* is not essential for AMS establishment in *B. distachyon*. *Bdlyr1-1* mutant is not affected in AMF colonization and arbuscule development. This mutant produces a protein that lacks most of the ICR. We found that this truncated protein cannot induce cell death when co-expressed with MtLYK3 in *N. benthamiana* and cannot complement absence of nodulation in *Mtnfp-2* showing that it is not functional. Similarly, Miyata et al., 2016 reported that *OsNFR5*, the *BdLYR1* ortholog in rice, is not essential for AMS establishment in rice. *Osnfr5* showed a reduction of AMS marker gene expression measured by qRT-PCR, but no difference with WT plants in colonization and arbuscule formation was observed by microscopy. In addition, Rasmussen et al., 2016 recently showed that a double *Ljlys11-Ljnfr5* mutant is not affected in AMS establishment.

CSSP activation is required for both AMS and RLS establishment. CSSP activation for RLS establishment relies on *MtNFP* or orthologs in legumes. It appears that CSSP activation for AMS establishment does not rely only on *MtNFP* orthologs in non-legumes although we showed that at *BdLYR1* is a functional ortholog of *MtNFP*. Moreover the level of requirement on *MtNFP* orthologs for CSSP activation during AMS establishment appears to vary between non-legumes species. One hypothesis to explain the difference observed between species, is that in *Poaceae* and *Fabaceae*, there is a higher level of redundancy than in *Solanaceae* or in *P. andersonii* between the MtNFP ortholog and genes and other signals involved in symbiotic partner perception and subsequently activation of the CSSP.

BdLYR1 and PsSYM10 when expressed in *M. truncatula* roots can restore nodule formation and colonization by rhizobia in *Mtnfp-2*. It is interesting to observe that complementation of *Mtnfp-2* by *PsSYM10* and *BdLYR1* not only restored nodulation but kept the specificity for *S. meliloti* strain producing sulfated LCOs. This is surprising since the rhizobial partner of pea does not produce sulfated LCOs. We would have expected that PsSYM10 recognize non-sulfated LCOs (produced by *S. meliloti* $\Delta NodH$) and does not recognize the sulfated LCOs, (produced by *S. meliloti*). One hypothesis to explain these results is that the sulfate group on *S. meliloti* Nod-factors is not recognize by MtNFP but by another protein from *M. truncatula* and that MtNFP ortholog are able to interact with such protein and MtLYK3 to activate the CSSP in response to sulfated *S. meliloti* Nod-factors.

BdLYR1 can partially complement nodule formation in the Mtnfp-2 mutant. This result suggests that the CDS of BdLYR1 is functional to activate the CSSP in presence of S. meliloti in *M. truncatula*, leading to nodule formation and bacterial colonization of the nodules. This partly challenges the theory that during evolution, the ancestor of the MtNFP type LysM-RLKs was neo-functionalized for a role in nodulation. Although the efficiency of nodulation is highest using *MtNFP* for complementation, the function provided by MtNFP can be replaced by the CDS of an ortholog. We could not show complementation of *Mtnfp-2* by *SlLYK10* probably because SILYK10 is poorly expressed in M. truncatula, even under control of p35S. We also observed a low expression level and miss-localization of SILYK10:YFP in N. benthamiana leaves (Girardin et al., in revision). We believe that MtNFP orthologs need to be expressed at a minimum level in order to complement *Mtnfp-2*. This is supported by the fact that *PsSYM10* cannot complement nodulation in *Mtnfp-2* under *pNFP* promoter (Bensmihen et al., 2011) while it complements nodulation in *Mtnfp-2* under the p35S promoter. Altogether, this suggests that the endogenous gene MtNFP has a better efficiency for nodule initiation but that its function, activation of the CSSP for nodule organogenesis and bacterial colonization, is conserved in the CDS of its orthologs.

As the experiment of complementation suggested there was no neo-functionalization of the CDS of the *MtNFP* ancestor during evolution for a role in RLS, we analyzed whether the promoter of *MtNFP* ancestor was neo-functionalized for an expression in nodules. We found that the *BdLYR1* promoter (p*BdLYR1*) does not allow GUS expression in *M. truncatula* nodules. This could be due to the highest GC content sequence of p*BdLYR1* compared to p*MtNFP* (45 % for p*BdLYR1* and 35 % for p*MtNFP*), preventing *M. truncatula* transcription factors and/or

the polymerase complex to recognize the sequence of pBdLYR1. On the other hand, the *SlLYK10* promoter (p*SlLYK10*; 35 % of GC content) has a similar expression pattern as p*MtNFP* (Arrighi et al., 2006) in *M. truncatula* roots, including in nodules. It suggests that all cis element required for nodule expression were already present on the promoter sequence of the *MtNFP* ancestor before the apparition of RLS. Nevertheless, we cannot exclude that as the CDS, the promoter sequence of the *MtNFP* ancestor has evolved to improve efficiency of the RLS in legumes.

We found that p*SlLYK10* is expressed in arbuscule-containing cells in tomato (Girardin et al., in revision) as *MtLYR1* and *LjLYS11* (Gomez et al., 2009; Rasmussen et al., 2016) in *M. truncatula* and *L. japonicus* respectively, while *MtNFP* and *LjNFR5* were not detected in arbuscule-containing cells (Gough, personal communication; Rasmussen et al., 2016). It would be interesting to test whether *pSlLYK10* is transcribed in arbuscule-containing cells in *M. truncatula*. If so, it would suggest that its ancestor in legumes before duplication had the ability to be expressed in arbuscule-containing cells and in nodules, and that following duplication MtNFP/LjNFR5 ancestor was specialized and improved for RLS establishment and lost the ability to be expressed in arbuscule-containing cells, while MtLYR1/LjLYS11 ancestor was specialized for AMS establishment and lost ability to be expressed in arbuscule of the absence of nodulation in an *Ljnfr5* mutant by *LjLYS11* under the control of the p*355*, but not under the control of *pLjNFR5* (Rasmussen et al., 2016), suggesting again a functional redundancy between the genes but with a difference in efficiency for CSSP activation.

Conclusion

We found that BdLYR1 is a high affinity LCO binding protein but a non-functional *Bdlyr1* mutant has no AMS phenotype. More importantly, we showed that a non-legume ortholog of *MtNFP* can restore nodulation in the *Mtnfp-2* mutant or that promoter of *SlLYK10* has the same expression pattern that p*MtNFP* in nodules. This suggests that non-legume orthologs are functional for RLS establishment in legume. It implies that LysM-RLKs involved in the AMS might have been directly recruited for a role in the RLS and that biotechnological development of the RLS in a non-legume species does not require the introduction of a RLS specific MtNFP/LjNFR5 type of LysM-RLK. Nevertheless, *MtNFP* may have been improved during evolution, enabling a more efficient nodule organogenesis.

Materiel and methods

Cloning

2.5 kbp corresponding to the non-coding region before the CDS of *BdLYR1* (*Bradi1g69290*), was amplified by PCR (Primers in Supplemental Table 1) from *B. distachyon* Bd21-3 genomic DNA and cloned in transcriptional fusion with a *GUS* reporter containing a plant intron, in a pCambia 2200 modified for Golden gate cloning and containing a *pUbi::DsRed* reporter (Fliegmann et al., 2016). For *SlLYK10* (*Solyc02g065520*) promoter expression analysis, the same construct as in Girardin et al. was used.

BdLYR1 CDS was amplified by PCR (Primers in Table S1) from Bd21-3 genomic DNA and cloned in translational fusion with YFP under the control of p*35S* by Gateway cloning in a pBIN vector (Lefebvre et al., 2010).

Agrobacterium rhizogenes mediated transformation of Medicago truncatula and nodulation assays

M. truncatula Jemalong A17 or *Mtnfp-2* line (Arrighi et al al., 2006) seedlings were cut at the extremity of the root. Seedlings were put on Farhaeus medium supplemented with kanamycin (20 mg / l). A drop of *A. rhizogenes* ARQUA1 (containing empty vector or the genetic constructs: p35S::*MtNFP:YFP*, p35S::*BdLYR1:YFP*, p35S::*SlLYK10:YFP*, p35S::*AtCERK1:YFP*, p35S::*BdLYR1¹⁻⁴⁶⁶:YFP*, p35S::*PsSYM10:YFP*, p*MtNFP::GUS*, p*SlLYK10::GUS* or p*BdLYR1::GUS*) suspension at OD₆₀₀ 0.03 was put on the cut. Seedlings were incubated at 21°C for 1 week and transferred at 25°C for 1 or 2 weeks. Then plants were transferred in pots (4 plants per pot) containing attapulgite (Sorbix) in plastic propagator.

For nodulation assays, plants were inoculated (4 days after transfer) with *S. meliloti* or the mutant *S. meliloti* Δ *NodH*. Both strains are pXLGD4 that harbor a plasmid containing the hemA-lacZ. 2.5 ml of bacterial suspension diluted in water at OD₆₀₀ 0.025 were used to inoculate each plant. Plant were then watered with sterilized water or with liquid Farheus medium once a week.

For promoter expression analyses, about 10 plants for each of the 2 biological replicates were analyzed at 6 dpi or 4 wpi. For complementation assays, between 2 and 4 biological replicates were analyzed at 4 wpi. Root and nodule staining as well as nodule sections were performed as in Mbengue et al., 2010. Kruskal-Wallis tests were used for the statistical analysis of nodule number per plant.

AM assays on Brachypodium distachyon

A *B. distachyon* Bd21-3 population mutagenized by NaN₃ was screened by TILLING to identify lines with mutations in *BdLYR1* (*Bradi1g69290*). A line was found to contain a stop codon in the CDS of *BdLYR1* and named *Bdlyr1-1*. AMS assays were performed by inoculating plantlets with 200 spores of *Rhizophagus irregularis* (DAOM197198, Agronutrition) per plant, in falcon tube system described in Maillet et al., 2011. Plants were harvested. On 3 biological replicates, root systems were stained 3 wpi by ink as in Vierheilig et al., 1998.

Transient Expression in N. benthamiana

Leaves of *N. benthamiana* were infiltrated with *A. tumefaciens* strains allowing expression of p35S::SILYK10:YFP, p35S::BdLYR1:YFP or p35S::MtLYK3:YFP as described in Mbengue et al., 2010. Leaves were harvested 3 dpi.

Membrane fraction preparation

Approximately 20 g of leaves were homogenized at 4°C in a blender in the presence of 40 ml of extraction buffer (25 mM Tris, pH 8.5, 0.47 M sucrose, 5 mM EDTA, 10 mM DTT, 0.6% PVPP and protease inhibitors (0.1 mM AEBSF, and 1 mg/mL each of leupeptin, aprotinin, antipain, chymostatin, and pepstatin). Samples were centrifuged for 15 min at 3000 g, and then the supernatant was recentrifuged for 30 min at 45000 g. The pellet (membrane fraction) was first washed in 5 ml and then resuspended in 2 ml of binding buffer (25 mM Na-Cacodylate pH 6, 250 mM sucrose, 1 mM CaCl2, 1 mM MgCl2 and protease inhibitors). Protein concentration in the membrane fraction was measured with BCA Protein Assay Kit (Pierce[™]). BdLYR1:YFP was detected by immunoblotting on 10 µg of membrane fraction proteins using rabbit anti-GFP antibodies as in Lefebvre et al., 2012.

LCO binding assays

LCO-V(C18:1 Δ 11,NMe) and LCO-V(C18:1 Δ 11,NMe,S) were purified from the rhizobial strain *Rhizobium tropici*. Synthetic Myc-LCOs, LCO-IV(C18:1 Δ 9), LCO-IV(C18:1 Δ 9,S), LCO-IV(C16:0) and LCO-IV(C16:O,S), are described in Maillet et al., 2011. CO4 and CO8 were produced as described in Fliegmann et al., 2013. Labeling of LCO-V(C18:1 Δ 11,NMe) by ³⁵S was performed as described in Fliegmann et al., 2013. LCO binding assays on membrane fraction (containing 5 or 10 µg protein) were performed as in Fliegmann et al., 2013 using between 1 and 2 nM of radiolabeled LCO and ranges of unlabeled LCO or CO between 1 nM to 1 µM.

Acknowledgments

We thank Fabienne Maillet and Sébastien fort providing us LCOs and COs, Alexandra Henocq for her help with the experiments of trans-complementation and on the characterization of *Bdlyr1* mutant, Sylvie Camut for her help on trans-complementation of *M. truncatula*, and Ton Timmers for the nodule cuts and colorations.

References

Ben Amor B, Shaw SL, Oldroyd GED, Maillet F, Penmetsa RV, Cook D, Long SR, Dénarié J, Gough C (2003) The *NFP* locus of *Medicago truncatula* controls an early step of Nod factor signal transduction upstream of a rapid calcium flux and root hair deformation. Plant J 34: 495–506

Arrighi J-F, Barre A, Ben Amor B, Bersoult A, Soriano LC, Mirabella R, de Carvalho-Niebel F, Journet E-P, Ghérardi M, Huguet T, et al (2006) The *Medicago truncatula* lysin motifreceptor-like kinase gene family includes *NFP* and new nodule-expressed genes. Plant Physiol 142: 265–279

Bensmihen S, de Billy F, Gough C (2011) Contribution of NFP LysM domains to the recognition of Nod factors during the Medicago truncatula/*Sinorhizobium meliloti* symbiosis. PLoS One. doi: 10.1371/journal.pone.0026114

Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ, et al (2012) Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proc Natl Acad Sci 109: 13859– 13864

Buendia L, Wang T, Girardin A, Lefebvre B (2016) The LysM receptor-like kinase SILYK10 regulates the arbuscular mycorrhizal symbiosis in tomato. New Phytol 210: 184–195

Cao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A, Stacey G (2014) The kinase LYK5 is a major chitin receptor in *Arabidopsis* and forms a chitin-induced complex with related kinase CERK1. Elife 3: 1–19

Dénarié J, Debellé F, Promé JC (1996) Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu Rev Biochem 65: 503–535

Ehrhardt DW, Atkinson EM, Long SR (1992) Depolarization of alfalfa root hair membrane potential by Rhizobium meliloti Nod factors. Science 256: 998–1000

Fliegmann J, Bono JJ (2015) Lipo-chitooligosaccharidic nodulation factors and their perception by plant receptors. Glycoconj J 32: 455–464

Fliegmann J, Canova S, Lachaud C, Uhlenbroich S, Gasciolli V, Pichereaux C, Rossignol M, Rosenberg C, Cumener M, Pitorre D, et al (2013) Lipo-chitooligosaccharidic symbiotic signals are recognized by LysM receptor-like kinase LYR3 in the legume *Medicago truncatula*. ACS Chem Biol 8: 1900–1906

Fliegmann J, Jauneau A, Pichereaux C, Rosenberg C, Gasciolli V, Timmers ACJ, Burlet-Schiltz O, Cullimore J, Bono JJ (2016) LYR3, a high-affinity LCO-binding protein of *Medicago truncatula*, interacts with LYK3, a key symbiotic receptor. FEBS Lett 590: 1477–1487

Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, Fournier J, Rochange S, Bécard G, Bonfante P, et al (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol 198: 190–202

Gomez SK, Javot H, Deewatthanawong P, Torres-Jerez I, Tang Y, Blancaflor EB, Udvardi MK, Harrison MJ (2009) *Medicago truncatula* and *Glomus intraradices* gene expression in cortical cells harboring arbuscules in the arbuscular mycorrhizal symbiosis. BMC Plant Biol 9: 10

Gough C, Cullimore J (2011) Lipo-chitooligosaccharide signaling in endosymbiotic plantmicrobe interactions. Mol Plant Microbe Interact 24: 867–878

Gough C, Jacquet C (2013) Nod factor perception protein carries weight in biotic interactions. Trends Plant Sci 18: 566–574

Gust A a., Willmann R, Desaki Y, Grabherr HM, Nürnberger T (2012) Plant LysM proteins: Modules mediating symbiosis and immunity. Trends Plant Sci 17: 495–502

Heckman DS (2001) Molecular Evidence for the Early Colonization of Land by Fungi and Plants. Science (80-) 293: 1129–1133

Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K, Minami E, Shibuya N (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc Natl Acad Sci U S A 103: 11086–11091

Kawaharada Y, Kelly S, Nielsen MW, Hjuler CT, Gysel K, Muszyński A, Carlson RW, Thygesen MB, Sandal N, Asmussen MH, et al (2015) Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature 523: 308–312

Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7: 511–518

Lefebvre B, Klaus-heisen D, Pietraszewska-bogiel A, Hervé C, Camut S (2012) Role of N -Glycosylation Sites and CXC Motifs in Trafficking of *Medicago truncatula* Nod Factor Perception Protein to Plasma. J Biol Chem 287: 10812–10823

Liu S, Wang J, Han Z, Gong X, Zhang H, Chai J (2016) Molecular Mechanism for Fungal Cell Wall Recognition by Rice Chitin Receptor OsCEBiP Recognition by Rice Chitin Receptor OsCEBiP. Structure 24: 1–9

Liu T, Liu Z, Song C, Hu Y, Han Z, She J, Fan F, Wang J, Jin C, Chang J, et al (2012) Chitin-Induced Dimerization Activates a Plant Immune Receptor. Science (80-) 336: 1160–1164

Madsen EB, Antolín-Llovera M, Grossmann C, Ye J, Vieweg S, Broghammer A, Krusell L, Radutoiu S, Jensen ON, Stougaard J, et al (2011) Autophosphorylation is essential for the in vivo function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J 65: 404–417

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, et al (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425: 637–640

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, et al (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469: 58–63

Malkov N, Fliegmann J, Rosenberg C, Gasciolli V, Timmers ACJ, Nurisso A, Cullimore J, Bono J-J (2016) Molecular basis of lipo-chitooligosaccharide recognition by the lysin motif receptor-like kinase LYR3 in legumes. Biochem J 473: 1369–1378

Mbengue M, Camut S, de Carvalho-Niebel F, Deslandes L, Froidure S, Klaus-Heisen D, Moreau S, Rivas S, Timmers T, Hervé C, et al (2010) The *Medicago truncatula* E3 Ubiquitin Ligase PUB1 Interacts with the LYK3 Symbiotic Receptor and Negatively Regulates Infection and Nodulation. Plant Cell 22: 3474–3488

De Mita S, Streng A, Bisseling T, Geurts R (2013) Evolution of a symbiotic receptor through gene duplications in the legume – rhizobium mutualism. New Phytol 961–972

Miyata K, Hayafune M, Kobae Y, Kaku H, Nishizawa Y, Masuda Y, Shibuya N, Nakagawa T (2016) Evaluation of the role of the LysM receptor-like kinase, OsNFR5/OsRLK2 for AM symbiosis in rice. Plant Cell Physiol 57: 2283–2290

Moling S, Pietraszewska-Bogiel A, Postma M, Fedorova E, Hink MA, Limpens E, Gadella TWJ, Bisseling T (2014) Nod Factor Receptors Form Heteromeric Complexes and Are Essential for Intracellular Infection in Medicago Nodules. Plant Cell 26: 4188–4199

Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JSS, Kudrna D, Wing R, Untergasser A, et al (2011) LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume *Parasponia*. Science 331: 909–912

Pietraszewska-Bogiel A, Lefebvre B, Koini MA, Klaus-Heisen D, Takken FLW, Geurts R, Cullimore J V., Gadella TWJ (2013) Interaction of *Medicago truncatula* Lysin Motif Receptor-Like Kinases, NFP and LYK3, Produced in *Nicotiana benthamiana* Induces Defence-Like Responses. PLoS One 8: 16–18

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, et al (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425: 585–592

Rasmussen SR, Füchtbauer W, Novero M, Volpe V, Malkov N, Genre A, Bonfante P, Stougaard J, Radutoiu S (2016) Intraradical colonization by arbuscular mycorrhizal fungi triggers induction of a lipochitooligosaccharide receptor. Sci Rep 6: 1–12

Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci U S A 91: 11841–11843

Smit P, Limpens E, Geurts R, Fedorova E, Dolgikh E, Gough C, Bisseling T (2007) Medicago LYK3, an entry receptor in rhizobial nodulation factor signaling. Plant Physiol 145: 183–191

Sun J, Miller JB, Granqvist E, Wiley-Kalil A, Gobbato E, Maillet F, Cottaz S, Samain E, Venkateshwaran M, Fort S, et al (2015) Activation of Symbiosis Signaling by Arbuscular Mycorrhizal Fungi in Legumes and Rice. Plant Cell 27: 823–838

Vierheilig H, Coughlan AP, Wyss URS, Recherche C De (1998) Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi. Appl Environ Microbiol 64: 5004–5007

Willmann R, Lajunen HM, Erbs G, Newman M -a., Kolb D, Tsuda K, Katagiri F, Fliegmann J, Bono J-J, Cullimore J V., et al (2011) *Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proc Natl Acad Sci 108: 19824–19829

Young ND, Debellé F, Oldroyd GED, Geurts R, Steven B, Udvardi MK, Benedito V a, Mayer KFX (2012) The *Medicago* Genome Provides Insight into the Evolution of Rhizobial Symbioses. Nature 480: 520–524

Supplemental data







Table S1: Primers used for cloning			
Used for	Primer	5'- 3' sequence	
Promoter of	ProBd69290GG For	GGTCTCTAAATGCTCTGAAGTAAGGCTGAGTAC	
BdLYR1	ProBd69290GG Rev	GGTCTCATTTGCCTGTGTGTGTGTGCGAAGCG	
Promoter of <i>SILYK10</i>	ProSl65520GG For	GGTCTCTAAATGGGTTATAGAGCTGTAATGC	
	ProSl65520GG Rev	GGTCTCATTTGCGATGCAAAGCTTAGATAAC	
CDS of BdLYR1	BdLYR1 BP For	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAACACCGCCGTTTCGG	
	BdLYR1 BP Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCGGGCGGCCACCTCATTGAC	
CDS of <i>Bdlyr1</i>	BdLYR1 BP For	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAACACCGCCGTTTCGG	
	BdLYR1∆IC BP Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTCCACTTGTCGAGGGAACCCT	

Discussion and perspectives

Discussion

Are LCOs important for AMS establishment?

We showed that SILYK10 and BdLYR1, that belong to the phylogenic group LYRIA (*cf.* Introduction), are proteins with high affinity for LCOs. Both have similarly higher affinity for LCO-V(C18:1,NMe,S), in the 10 nM order than for the published LCOs. We also observed that both SILYK10 and BdLYR1 have lower affinity for LCO-IV(C16:2,S) (Fig. 1A-B). Actually, LCO-IV(C16:2,S) is the major LCO produced by *S. meliloti*, the symbiotic partner of *M. truncatula* in the RLS. We first performed binding assays using a LCO-IV(C16:2,³⁵S) and we could not measure radioactivity bound to membrane fractions containing BdLYR1 or SILYK10, showing that SILYK10 and BdLYR1 have not enough affinity for LCO-IV(C16:2,S) to used it as a radiolabeled LCO in our assay. On another side, SILYK10 and BdLYR1 do not seem to have the same binding properties concerning their ability to discriminate sulfated and non-sulfated LCOs. Indeed, I showed that SILYK10 has almost the same affinity for LCO-IV(C16:0,S) and LCO-IV(C16:0) in the 2nd chapter (Girardin et al., in revision). By contrast, we observed by competition of a photoactivatable radiolabeled LCO that BdLYR1 has highest affinity for sulfated LCOs than for non-sulfated LCOs (Fig. 1A).

In order to characterize the BdLYR1 LCO binding properties, I mainly used rhizobial LCOs while we expect this protein to perceive fungal LCOs. A major difference between rhizobial and AMF LCOs is the position of the insaturation in the fatty acid chain. Indeed, LCOs with an acyl chain of 18C produced by rhizobia are mainly C18:1Δ11 (vaccenic acid) whereas AMF produce LCOs with C18:1Δ9 acyl chain (oleic acid). To determine whether the position of the insaturation (in the fatty acid moiety of LCOs) was important for LCO recognition by BdLYR1, I performed competition assays on BdLYR1 using a low concentration of the radiolabeled LCO-V(C18:1,NMe,³⁵S) and a range of concentrations of LCO-V(C18:1Δ11, Fuc/MeFuc) purified from *Rhizobium fredii* or of LCO-V(C18:1Δ9, Fuc/MeFuc) synthesized by our chemist collaborators (Group of Sebastien Fort at the CERMAV in Grenoble, France). I could not determine the affinity of BdLYR1 for these LCOs mainly because I obtained aberrant values with the highly diluted concentrations of the molecules in the competition assays. This could be due to the non-specific interactions of the molecules with the tubes and tips used to prepare the dilutions, leading to strong depletion of the molecules at low concentration.



affinity depending on the LCO structure. A) Crosslinking of immunopurified BdLYR1:YFP with a radiolabeled photoactivatable LCO derivative in competition with a range of concentration of the indicated unlabeled LCO. For each test, autoradiography is shown in the upper part and western blotting is shown in the lower part. Both were performed on the same nitrocellulose membrane. **B)** Membrane fractions containing BdLYR1:YFP were incubated with radiolabeled LCO-V(C18:1Δ11,

NMe,³⁵S) in presence of 1 μ M of the indicated unlabeled molecules used as competitors. Nonspecific binding was determined in the presence of 1 μ M LCO-V(C18:1 Δ 11,NMe,S). Bars represent the percentage of specific binding (means and SE) obtained in the presence of an excess of competitor (1 μ M) for at least 3 experiments performed on membrane fractions prepared from independent batches of *N. benthamiana* agro-infiltrated leaves. LCO-V(C16:1,Cb,Fuc,Ac) and LCO-IV(C16:2,Ac,S) are the major LCO produced by *M. loti* and *S. meliloti* respectively. *M. loti* is the symbiotic partner of *L. japonicus* and *S. meliloti* is the symbiotic partner of *M. truncatula*.

0

LCO-V (C18:1Δ11, Fuc/MeFuc)

CO-VIII

CO-IV

LCO-V (C18:1Δ9, Fuc/MeFuc) LCO-V (C16:1,Cb,Fuc,Ac) LCO-IV (C16:2, Ac, S) However BdLYR1 appears to have similar high affinity for these LCOs. Indeed, binding of LCO-V(C18:1,NMe,³⁵S) on BdLYR1 was fully inhibited in presence of 50 nM of unlabeled LCO-V(C18:1Δ11, Fuc/MeFuc) or LCO-V(C18:1Δ9, Fuc/MeFuc) suggesting a *Kd* for these molecules lower than 50 nM (data not shown). We also showed that these LCOs can compete as LCO-V(C18:1,NMe,S) the binding on BdLYR1 of radiolabeled LCO-V(C18:1,NMe,³⁵S) and the photoactivatable radiolabeled LCO (Fig. 1A-B). Thus, I think the place of the insaturation in the acyl chain is not important for LCO recognition by BdLYR1 and that our results obtained with LCOs produced with rhizobia can be extrapolated to AMF LCOs bearing the same decorations.

We decided to use LCO structures such as LCO-V(C18:1,NMe,S) or LCO-V(C18:1 Δ 9, Fuc/MeFuc) different from those published by Maillet et al, in 2011 since studies from Guillaume Bécard's group (LRSV, France) showed that additional and even more abundant LCO structures are produced by AMF. These results were communicated during the iMMM2015 by Guillaume Bécard (Bécard, 2015) and during the iMMM2017 by Virginie Puech-Pagès (Bascaules et al., 2017). They found that LCO-IV/V(C18:1 Δ 9, Fuc/MeFuc) is one of the most abundant LCO structure in AMF exudates. They also found a larger range of LCO structures produced by ectomycorrhizal fungi (EMF) than by AMF. It would be interesting to measure the affinity of SILYK10 and BdLYR1 for LCO structures produced by EMF to determine whether SILYK10 and BdLYR1 have specificity for LCO structures produced by AMF. It would also be interesting to characterize the LysM-RLKs in plants that establish EMS and to measure their affinity for EMF and AMF LCOs. Thus, we could determine whether the clade LYRIA is also involved in LCO perception for EMF establishment or not. On another hand, LCOs could also be produced by other microorganisms such as pathogenic fungi or even by plants. If such LCOs are found, it would be interesting to test the affinity of SILYK10 and BdLYR1 for these LCOs to validate whether they specifically recognize AMF LCOs or whether they are involved in broader roles.

We showed that SILYK10 and BdLYR1 have high affinity for some LCO structures. But, what is their role in the AMS? In the 1st chapter of my PhD thesis, we showed that *SILYK10* silencing strongly affects AMF penetration in tomato roots 2 wpi (Buendia et al., 2016). Hence, we could conclude that LCO perception by SILYK10 is important for AMS establishment in tomato. However, the importance of LCOs in AMS establishment cannot be generalized to all the land plants. Indeed, *Bdlyr1* mutants are colonized as control plants by AMF 3 wpi. Similarly, rice *Osnfr5* mutants (the ortholog of *BdLYR1*) were colonized by AMF as WT plants, except a

slight decrease of AMS marker genes observed by qRT-PCR (Miyata et al., 2016). Although LCO binding has not been shown for OsNFR5, this suggests that LCO perception in monocots is not as important as in tomato for AMS establishment. However, before concluding on the role of LCOs in the AMS establishment in *B. distachyon*, we have to confirm that *Bdlyr1* mutants are impaired in LCO perception (it will be discuss later).

Nevertheless, the fact that Bdlyr1 mutants are colonized at the same level as control plants could be explained by the fact that other signals than LCOs are involved in AMS establishment. Indeed, it was shown that short COs can induce calcium spiking in M. truncatula (Genre et al., 2013), in L. japonicus and in rice (Sun et al., 2015). Furthermore, at the iMMM2017 Giles Oldroy (Feng et al., 2017) showed that long COs (such as CO8, before believed to have a role only in defense elicitation) can induce calcium spiking in *M. truncatula*. Moreover a double mutant in Mtlyk9-Mtnfp shows a decrease in AMF colonization (iMMM2017: Feng et al., 2017). This suggests that two receptor complexes (one containing MtLYK9 and the second one MtNFP) control AMS establishment. This reinforces the hypothesis that *MtNFP* is important for the AMS in addition to the RLS, which was suggested by the decreased in responses to Myc-LCOs in *Mtnfp* mutants shown by Maillet et al. in 2011. It also suggests that there is redundancy and/or additional effects of LCO and CO perception on AMS establishment. The possible role of CO8 in AMS establishment is supported by the study of Miyata et al., 2014 that showed that a mutant in OsCERK1 (belonging to the LYKI phylogenetic group and involved in plant defense) shows defect in AMF colonization compared to WT and perceives LCOs as WT but not CO8. Hence it is possible that in B. *distachyon* COs might be more important than LCOs for AMS establishment, as maybe in rice. Thus it is important to determine whether BdLYR1 (and SlLYK10) can bind COs and to identify LysM-RLKs with high affinity for COs. Actually, the binding assay we currently use to determinate the affinity of a protein for LCOs relies on radiolabeling of a LCO. Thus we only can observe the competition of unlabeled COs or LCOs for the binding of the radiolabeled LCO on the LCO binding site. We cannot exclude that in the LCO binding LysM-RLKs, it exists another site, which could bind COs with highest affinity than LCOs. To determine CO binding properties of BdLYR1, SlLYK10 or other proteins, we could use the Nanotemper technology (microscale thermophoresis) that relies on fluorescent molecules (it could be LysM-RLKs or its ligand for example) and that our lab recently acquired. This new technology will allow us to identify the CO receptors in B. distachyon and in tomato. Indeed, SILYK10 silencing affected

AMF colonization in tomato but 6 wpi all plants were colonized (even if they did not reach the colonization level of control plants). This could be also due to a redundancy between LCOs and COs in AMS establishment. Moreover, AMF can also secrete effectors. The only one to play a role in AMS interferes with plant defense (Kloppholz et al., 2011) but AMF effector could activate the CSSP as it was found for some rhizobial strains (Okazaki et al., 2013).

To understand the respective roles of LCOs and COs in AMS establishment, we have started a collaboration with J.M. Ané (Wisconsin University, Madison, USA) to introduce a calcium reporter (Geco) in stable lines of *B. distachyon* 21-3. On our side, we have also introduced this calcium reporter in tomato and wheat. This reporter will be useful to measure LCO and CO responses in "WT" plants and to compare the responses between monocotyledons and dicotyledons. It will be also an interesting tool to demonstrate, after crossing Geco plants with *Sllyk10* or *Bdlyr1* mutated plants, whether these mutants still perceive LCOs and COs or not.

Another question is what LysM-RLKs become after they perceive their ligand? Are they degraded, or relocated as FIS2 after ligand perception (Beck et al., 2012)? We tried to understand whether BdLYR1 or SILYK10 were degraded or relocated by expressing them in *N. benthamiana* and observing by confocal microscopy what happens after leave disc incubation in a solution containing LCOs but we could not conclude. If we really want to address this question I think we should over-express SILYK10:YFP and BdLYR1:YFP in tomato roots and *B. distachyon* roots respectively. However, SILYK10:YFP was hardly visible by confocal microscopy in tomato roots and we should use a more sensitive tag to observe the proteins in tomato roots or in *B. distachyon* roots and to follow their behavior after treatment with LCOs or COs.
Is LCO binding important for MtNFP activity?

It is very interesting to observe that several orthologs of *MtNFP* we tested for LCO binding showed high affinity for LCOs, but not MtNFP itself. If we look at the amino acid sequence of MtNFP and its orthologs, there is low conservation of the sequence between all the proteins (Fig. 2). Actually, LysM-RLKs are more conserved on their secondary and tertiary structures than on their primary structure. The disulfide bridges between the CXC motifs separating each LysM are conserved in all proteins, indeed they are essential for LysM-RLK functions.

						Ly	/sM1						Lysi	/ 12
	1	10	20	30	40	50	60	70	80	90	100	110	120	130
Ht.NFP PsSYM10 S1LYK10 PhLYK10 BdLYR1 TaLYR1 Consensus	HSAFF MAIFF MYVP MVAP MEHRI MDPR	LPSSSHALFI LPSSSHALFI LYSLFYNFFI LASLFCIVFI RFGCPLLLLA RFLCCLLLA 1.s1fi	LVLMLFFLTN LALM-FFYTN LFLI-LSSSN LFLI-LSSSN LLVVVLIFRC LALAFRR	ISAQPLYISEI ISAQPLQLSGI YTAQP-SSTDI YTAQT-SIADF SEAQDVANGTE CGAQGAANGTO AQ	INFTCPVDSP INFSCPVDSP IDFSCSFNSS IDFLCSVNSS IRFACLVPAP IRFACLVPAP	PSCETYVRY PSCETYVTYI FSCDTFISYI LSCDTYVTYI 	RAQSPNFLSLS FARSPNFLSLT RARPPNHLDVG RARPP-YLDVG RTQSPGFLGLG RTQAPGSLDLG raq.PLdlg	NISDIFNLSP INISDIFDISP SISDLLEYSR SISDLLGYSR SISDLFGYSR AISDLFGYSR SISDLFGYSR	LRIAKASNIE LSIAKASNIE LSYATATGLA LNIAKATGLA AMIASANNLT AMIASANGLS 1.!A.A.gl.	REDKKLIF DEDKKLYE SEDTELFF SEDTELFF AKDGYLLF LDREGAALLF aedL.F	PDQLLLVPYTC GQVLLIPYTC PDQLLVPYKC PDQLLVPYKC PDQPLLVPYKC PDQPLLVPYKC PDQPLLVPYKC dQ.LL!PY.C	GCTKNHSFAN GCTRNRYFAN YCNSSHYFSN YCNGSHYFSN GCTGNRSFAN GCTGNRSFAN GCTGNRSFYN gCtgnrsF.N	ITYSIKQGDI FTYTIKLGDU YTYQIRKGDS YTYQIKKGES YTYPIQDGDI YTYPIRSGDI VTYPIRSGDI VTY.IG#	FFILSI YFIVST FYSVSI FYSASI YYALAL FYALAL %%.1s.
							LysM3	3						
	131	140 +	150 +	160	170	180	190	200	210 +	220 +	230	240 +	250	260 I
Ht.NFP PsSYM10 S1LYK10 PhLYK10 BdLYR1 TaLYR1 Consensus	TSYQN TSYQN RAFEN GAFEN TAFEN TGFEN La%#N	LTNYLEFKN LTNYVEMEN LTNYHVVQD LTNYHVVQD LTDFNIMQQ LTDFNIMQQ LTTPDVIQE LTNy.v.q#	FNPNLSPTLL FNPNLSPNLL HNPTLDPTNL HNPTLDPTNL LNPQAPATRL LNPQAYFNKL .NP.1.pt.L	PLDTKVSVPLF PPEIKVVVPLF TIGAEAVFPLF TVGTEAVFPLL QAPEEVTVPLF NVSQLVTVPLF	CKCPSKNQL CKCPSKNQL CKCPTHSDL CKCPASSDL CRCPTQAER CRCPTPAER CRCPTPAER	NKGIKYLITY SKGIKHLITY EKGLQYLYTY EKGLQYIYTY RAGGIRYHITY RSGGAQLLYTY L.KG.qyl!TY	(VHQDNDNYTL (VHQANDNYTR (VHQPHDDVLP (VHQPGDDVLP (VHQPGDDVLP (LHRPEDDHST (HHRPVDTHSE (VHqp,Ddv.)	VSSKFGASQV RVSSKFGASQV PVSNHFGASAA PVSTHFGASAA VSTLMNSSKS VSKLMNSSAS VS.fgaSa.	EMLAENN-H- DMFTENN-Q- DILAANN-YR DILEANN-YR DIAEANN-YR DIAEANNYTT AIAAYNNYSA di.aaNN	NFTASTNRS\ NFTASTNYPJ NFTAAICSP\ NFTAAICLP\ EFTSPTAQPI DFTSTTMLPI #FTa.t.p,	/LIPYTSLPKL (LIPYTKLPYI /LIPYKLPIIL /VIPYKSPIIL (LIPYSQPPKL (LIPYARPPYL ,1IPY,.pp,1	DQPSSNGRKS DQPSSNGRKN QSYPSSNSS- QSYPSSTSST PPLRYDASAD PPLQYGASAS	SSQNLf STQKPf RKSKH(RKFKH GSGANKR(GSGANKR(TGDPGVSKRf k.f	LIIGIS FIIGIS HIVITV RILIVV RGYAVA SGATVA
										In	tracellula	r Domain		
	261	270	280	290	300	310	320	330	340	350	360	370	380	390
HtNFP PsSYM10 S1LYK10 PhLYK10 BdLYR1 TaLYR1 Consensus	LGSAF LGCAF LGING LSING AGVAG ASIAG lgiag	-FILVLTLS -FVVVLTLS -LLAVFSFC -VVLVCSLC SLVAFAALC SLIAVAALC ,Ivav,,1c	L-VYVYCLKH L-VYVYCLKH LHVYHRHLEE VHVYHRLLYE VAAFAYRRYR AAIFAYRRYR V%.y.ly.	KRLNRSTS KRLNRSTS KRRSNLAHNSS KRRT-LARNTS KKKETYYQLG EKKATY-HSAS Kr1S	SSETAD LAETAD TLETSDLFH TLESSDLIQ PYATPKLSH RFASPRFCF et.dl	ITK ITK IHKHQQQHNY(NQNAY(.k		KLLS KLLS DHKNIQDKLLP SKIIQDKLLP IMNGGGDKIIT (INGG-DKLLT dK11.	GYSGYYSKPT GYSGYYSKPT GYSGYIGKPI GYSGYLGKPI SYSQFIDKPI SYSQFIDKPY gYSg%i.KPi	HYEIDAINEG HYEHDAINEG IYDLKIINEG HYDLKIINEG YFGADEINEG IFGTAEINEG .%IMEa	TTNLSDNCKI THNLSENCKI TVDLSERYRI THTLSERYRI THNLDERCRI THNLDERCRI THNLDERCRI	GESYYKANID GESYYKANID GGSYYKATIN GGSYYKAVID GSSYYRAKLD GSSYYRAKLE G.Syyka#	GRVLAVKKI GRVLAVKKI DQVYAVKK-F NQVLAVKK-T GEMFAVKPA GEVFAVKPA g.v.AVKk.F	KDASEE KDASEE KQASEE GDVSAE GDVSAE GDVSAE k#aSeE
	391	400	410	420	430	440	450	460	470	480	490	500	510	520
HLNFP PsSYM10 S1LYK10 PhLYK10 BdLYR1 TaLYR1 Consensus	I LKILQ LKILQ LTILQ LKYLQ HRHTQ LRHHQ \$1Q	KYNHGNLYKI KYNHGNLYKI KLHHANLYKI KLHHANLYKI MYNHASLIKI MYNHANLIRI KYNHANLIRI	LMGYSSDNDG LMGYSSDNDG LMGYSSDDLG LMGISSDNHG LAGISFGTEG LAGISIGADG LAGISIGADG	NC-FLYYEYAB NC-FLYYEYAB NS-FLYYEYAB NF-FLYYEYYB DYTFLYYEFAB DYTFLYYEFAB #.,FLYYE%aB	NGSLEEHLF NGSLDEHLF NGSLDECLF NGSLDKHLF KGSLDKHLY KGSLDKHLY EnGSL#kwL%	SESSKT SESSKT PGSSSS PRPSPS QKPPSSLPSS QKPPSSLPSS (psss)	-SNSVYSL -SNSVYSL -SAASVIVTSL -GSVLLL SSSSSSSSVDTL SSSSSDTL _SSSSDTL	THSORITIAN THSORITYAY DHRORLHIAL GHIORLHIAL SHNORLGIAF SHNORLGIAF .H.OR1.!A.	DVATGLQYNH DVAVGLQYNH DVANALQYLH DVANALQYLH DVANGLLYNH DVANGLLYNH DVANGLLYNH	EHTYPRIIH EHTYPRIIH EHTQPSIVHO EHSQPSIVHO EHTLPSNVHO EHTQPSNVHO EHTQPSIVHO	RDITTSNILLG RDITTSNILLD DITTCNILD RDIRTSNILLD RDIRTSNILLD RDYRARNILLT RDYRARNILLT RDYRARNILLT	SNFKAKIANF SNFKAKIANF SRFKAKIAGF SRFKAKIAGF ADFRAKISNF ADFRARISNF \$.FKAKIANF	GMARTS-TNS SMARTS-TNS STARHA-TNS STARHA-ANS SYATPAMADF SVATPAMADF s.Ar.a.a#s	HIMPKID HMPKID HMPKID LMLKVD LMLKVD ADTSSD AATSSD
	521	530	540	550	560	570	580	590	600	610	620	630	640	647
MENFP PsSYM10 S1LYK10 PhLYK10 BdLYR1 TaLYR1 Consensus	VFAFG VFAFG VFAFG VFAFG VFAFG VFAFG VFAFG	VYLIELLTG VYLIELLTG IYLLELLSG LYLLELLSG LLILELLSG LLYLELLSG IVIIELLSG	KKAHTTKE-N KKAITTHE-N KKATESKDYN KQSHETKD-N RRAMEARY-G RTAMEARY-G k.amen	GEVYILAKDFA GEVYILAKDFA DETLIAAKEIS GEILILAKEIS AEIGALARDIF AEIGALARDIF .Ei.i\$Akki.	IKIFDLEGNR IKIFDLEGNR SRILEVEDNR Kgileaednr Ravleagdkr Ravleagdkr !l#aednr	REERLRKHMDF REESLRKHMDF REEKFRRHMDF REEKFRRHMDF RDARLRKHMDF RDAKLRKHMDF R#ek1RkHMDF R#ek1RkHMDF	PKLESFYPIDN PKLENFYPIDN PKL-SFYPYDD PNL-SFYPYDD PALGSEFHNDF PALGDEYYLDF P+L.sf%p.D.	IALSLASLAVN IALSLASLAVN IALNLAALATA IALSLAALANA IALSLAGMARA IALSLAGMARA ALSLAGMARA	CTADKSLSRP CTADKSLSRP CTSEQSAERP CTSEKSTDRP CTEEDAARRP CTEEDAARRP CTEEDAARRP CT.#.sa.RP	TIAEIYLCLS SIAEIYLCLS KHTDIVFNLC KHTDIVFNLS KHADVVFSLS KHADVVFSLS kna#!Vf.Ls	SLLNQPSSEPH SLLNQSSSEPH SFLAQSSFE-H SFLTQSSFE-H SHLVQPLPVGD SHLVQPLPVGD S.L.Qps.e.H	LERSLTSGLD LERSLTSGLD YGPSHTSGEA YGRTSISGEA AFEKLHQYSS AFEKLHQPSS	AEATHYVTS VEATHYVTSJ EEIVQIVSP EEIVQVISP EDNMGIVNE EENIRIVNE e#!!	I TAR VAR TAR TAR AAR AAR AR
Fig. 2 /	\ mir	no acio	d sequ	ences	of Mtl	NFP an	d its o	rtholog	gs shov	v low	conser	vation	, espe	cially
in the l	ECR.	The 3	LysM	and the	e ICR a	ire indi	icated l	by lines	s. The C	CXC mo	otif sep	arating	g the L	,sMs
is squa	red.		-								·			

Despite the protein sequences of MtNFP orthologs are divergent, they can complement lack of nodulation of the mutant Mtnfp-2. I showed in the 3rd chapter of my PhD thesis that p35S::PsSYM10:YFP and p35S::BdLYR1:YFP could restore the nodulation in the M. truncatula mutant Mtnfp-2 that is not able to establish the RLS. I also showed that Mtnfp-2 plants complemented by PsSYM10 (that is not expected to have specificity for sulfated LCOs since it perceives Rhizobium leguminosarum LCOs that are not sulfated) shows specificity for S. meliloti producing sulfated LCO. These plants have default in nodulation as plants complemented by MtNFP when inoculated with a strain that produces non-sulfated LCO (S. *meliloti* Δ *NodH*). This experiment suggests that for RLS establishment, it is not MtNFP that brings the selectivity for sulfated LCOs but another protein. Thus MtNFP might interact with a protein which has high affinity for the LCO of S. meliloti. This unknown partner could be specific to *M. truncatula* and absent from *L. japonicus*. Indeed, *M. truncatula* is particularly responsive to LCOs. LCOs at 10⁻¹² M triggers responses (such as root hair deformation or calcium spiking) in *M. truncatula* whereas in *L. japonicus* responses are triggered at a 10⁻⁰⁹ M (Sun et al., 2015). Such a difference in sensitivity could be explained by the recruitment in *M*. truncatula of the LCO binding protein with very high affinity for LCOs. Moreover, in contrast to MtNFP, LjNFR5 was shown to bind LCOs (Broghammer et al., 2012). The lack of LCO binding on MtNFP could be due to the fact that this unknown partner is lacking in our LCO binding assays. To confirm this hypothesis, we could try to complement absence of RLS in an Ljnfr5 mutant by *MtNFP*. I expect that it would not work if MtNFP is not be able to bind alone LCOs. A complementation of Ljnfr5 mutant by BdLYR1 might restore RLS as BdLYR1 can bind LCO-V(C16:1,Cb,Fuc,Ac) that is the major LCO produced by *M. loti* (Bek et al., 2010), although its affinity for *M. loti* LCO was not determined (Fig. 1B). However, it is highly possible that the affinity of BdLYR1 for LCO-V(C16:1,Cb,Fuc,Ac) is lower than the affinity of LjNFR5 for this LCO. Then, BdLYR1 would not be able to complement *Ljnfr5* mutant.

Another approach, in a longer perspective, to understand whether LCO binding property is important to complement nodulation in *Mtnfp-2* could be to try to complement *Mtnfp-2* mutants by expressing a version of BdLYR1 unable to bind LCO (mutations in the residues conferring the high affinity of BdLYR1 for LCOs but conserving the conformation of the protein).

Other LysM-RLKs, non orthologs of MtNFP, can bind LCOs

MtNFP orthologs (LYRIA) are not the only LysM-RLKs that can bind LCOs. Another group of LysM-RLKs belonging to the clade LYRIII (*cf.* Introduction) contains members that were shown to bind LCOs. MtLYR3 binds various LCO structure with similarly high affinity (*Kd* of about 25 nM) (Fliegmann et al., 2013; Malkov et al., 2016). Recently, in the team we also observed that NbLYK4 and PpLYR3 the orthologs in *N. benthamiana* and in *Prunus persica* (peach) respectively, bind LCOs with high affinity (*Kd* comprised between 20 nM and 100 nM for all LCO structures tested) and their LCO binding sites have a weak affinity for COs (in the μ M range). Finally at the beginning of my PhD thesis, I started to study BdLYR3 (*Bd3g06770*) a putative ortholog of MtLYR3 in *B. distachyon*. I was able to show that BdLYR3 binds LCOs and that the LCO binding site has selectivity for LCOs versus COs (Fig. 3). However, I have not determine its affinity for several LCO structures.



Fig. 3 BdLYR3 preferentially binds LCOs versus COs. Crosslinking of immunopurified BdLYR3c:YFP by a radiolabeled and photoactivatable LCO derivative in competition with 2 μ M of indicated unlabeled LCOs or COs. Autoradiography was performed on the same nitrocellulose membrane used for western blotting (WB).

Intriguingly, the clade LYRIII also contains AtLYK4. An *Atlyk4* KO mutant was decreased in responses to long CO and in defense to fungal and bacterial pathogens (Wan et al., 2012). In *A. thaliana*, the clade LYRIII contains another member, called AtLYK5 which seems to specifically bind long CO (*Kd* for CO8 measured by ITC on ECR produced in insect cells of 1.72 μ M) and mutations in *AtLYK5* negatively impacted defense responses against the fungal pathogen *A. brassicicola* (Cao et al., 2014). The possibility of a role of LCO both in AMS and defense will be discussed further, in the long term perspectives.

Short term perspectives:

I organized the results I have obtained on *BdLYR1* as a publication on the evolution of LysM-RLK between non-legumes and legumes. However, additional experiments are required to bring the data in a publishable state. So I will discuss here experiments we need to perform to achieve this study. I will also discuss short term perspectives regarding the work we have done on *SlLYK10*.

Does BdLYR1 play a role in AMS?

I showed in the third chapter of my PhD thesis that *Bdlyr1-1* mutant (obtained through a TILLING screen) is colonized as control plants by *R. irregularis*. Additional mutant alleles of *BdLYR1* would be useful to confirm that *BdLYR1* is not involved in AMS establishment as *SlLYK10*. I actually transformed *B. distachyon* (embryogenic calli issued from immature embryos) with an *A. tumefaciens* strain containing a CRISPR-Cas9 construct targeting *BdLYR1* (*cf.* Annex 1). Only a few plants were regenerated. Unfortunately, although they integrated the T-DNA containing the CRISPR-Cas9 construct, no genome edition was found in these plants.

The AMS phenotype of the available *Bdlyr1-1* mutant should be more tightly analyzed. I only counted the number of infection site per plant 3 wpi. It would be interesting to look carefully at the shape of the arbuscules in the *Bdlyr1-1* plants. Also, qRT-PCR analysis of AMS marker genes on at least 3 replicates of 10 plants per condition (WT+/- AMF and *Bdlyr1-1* +/- AMF) can be performed. This could reveal a quantitative role of the gene in AMS establishment and/or in arbuscule development as found for *SlLYK10*, *PaNFP* or *OsNFR5*.

I also demonstrated that BdLYR1 has high affinity for LCOs, however, to demonstrate that BdLYR1 is involved in LCO perception, we need to determine whether *Bdlyr1-1* plants are affected in LCO perception. A way to measure it is to quantify lateral root number in response to LCOs on *Bdlyr1-1* plants. Indeed, LCO perception induces the formation of lateral roots in *B. distachyon* WT (Fig. 4) as in M. truncatula (Olah et al., 2005). A weakness of this experiment is that lateral root induction by LCOs is a highly variable response and experiments will require a lot of individuals.



Fig. 4 LCOs induce lateral root formation in *B. distachyon* **Bd 21-3**. Two examples of lateral root induction bioassay. Plantlets were grown *in vitro* on gelified culture medium supplemented or not with LCOs:

(—) Control; (—) Mix LCO-IV(C18:1,S) and LCO-IV(C16,S) at 10⁻⁷ M; (—) Mix LCO-IV(C18:1,S) and LCO-IV(C16,S) at 10⁻⁹ M; (—) LCO-V(C18:1,NMe,S) at 10⁻⁷ M; (—) LCO-V(C18:1,NMe,S) at 10⁻⁹M. Lateral roots were counted 6 to 10 days after seedlings were transferred on the supplemented medium. Bars represent mean and SD of the number of lateral root per plant. Each condition contains at least 60 individuals. A general linear model was performed with the software Statgraphics. Statistical differences are indicated by letters.

Another way to determine whether *Bdlyr1-1* is still able to perceive LCOs is to perform qRT-PCR analysis on LCO perception marker genes. With the aim to identify such genes, we performed an RNAseq on *B. distachyon* plants treated or not with LCOs, COs and both LCOs and COs at 10⁻⁷ M. In this experiment, expression of only a few genes was found to be regulated by LCOs and/or COs. However, we might have identified one potential LCO perception marker gene that could be used to determine whether *Bdlyr1-1* is affected in LCO perception. Induction of this marker gene by LCOs will have to be tested in a *B. distachyon* line mutated in a CSSP gene to determine whether the LCO induction is CSSP dependent. Crossing *Bdlyr1-1* with the GECO line would be an alternative way to measure CSSP activation by LCOs in *Bdlyr1-1*. These experiments are critical to determine whether BdLYR1 can activate the CSSP in responses to LCOs and/or whether an additional receptor (likely a LysM-RLK in *B. distachyon*) that binds LCOs with high affinity is redundant for activation of the CSSP in response to LCOs.

Finally, to achieve the characterization of the role of *BdLYR1* in AMS, we need its expression pattern at least in non-colonized and colonized roots. We transformed *B. distachyon* with a construct allowing expression of the *β-glucuronidase* (*GUS*) reporter gene under the control of the promoter of *BdLYR1* (p*BdLYR1::GUS*) and obtained a few plants containing the constructs that are currently producing seeds. If we find expression in

arbuscule as observed for *BdLYR1* orthologs *MtLYR1*, *LjLYS11* and *SlLYK10*, it would suggest a role in arbuscule development and reinforce the hypothesis of a functional redundancy with other receptors activating the CSSP.

Was an ancestral LysM-RLK involved in the AMS establishment recruited for the RLS establishment during evolution?

In the chapter 3 of my thesis, I showed that p355::BdLYR1 and p355::PsSYM10 could complement the lack of nodulation of the mutant *Mtnfp-2*. However, the experiment of complementation of nodulation in *Mtnfp-2* by *MtNFP*, *PsSYM10*, *BdLYR1*, *AtCERK1* and *Bdlyr1*¹⁻⁴⁶⁶ lacks immunoblotting to verify that the various proteins were expressed at similar levels. This is required to exclude the possibility that *AtCERK1* and *Bdlyr1*¹⁻⁴⁶⁶ cannot complement nodulation in *Mtnfp-2* due to a weak or no protein production in *M. truncatula* roots, as it might be the case for *SlLYK10*. In addition, one more biological replicate would reinforce the conclusion on the absence of *Mtnfp-2* nodulation complementation by *AtCERK1* and *Bdlyr1*. Furthermore, *AtCERK1* belongs to the LYK (*cf.* introduction), then, it would be interesting to rather use *BdLYR3* or *BdLYR4* that belong to the LYR group as negative controls to complement absence of nodulation in *Mtnfp-2* in *M. truncatula*. They are phylogenetically closer to *BdLYR1* and *MtNFP* than *AtCERK1*, but do not belong to the phylogenetic group of *BdLYR1* and *MtNFP* (*cf.* introduction). Moreover, I found that BdLYR3 binds LCOs (Fig. 3) thus it would be particularly interesting to test whether this gene is functionally redundant with BdLYR1.

Concerning the fact that *SILYK10* expression pattern is similar to *MtNFP* expression pattern during RLS in *M. truncatula*, I would like to make an additional biological replicate of this experiment even if I am confident with this result. Moreover, it would be very interesting to have data about the expression pattern of *MtNFP* and *SILYK10* during AMS in *M. truncatula*.

Interestingly, we observed in the team (PhD thesis of Tongming Wang, 2017 and collaboration with Didier Reinhardt, Fribourg University, Swisserland) that a transposon insertion in the ortholog of *SlLYK10* in *Petunia hybrida* (*PhLYK10*) leads to a decrease of AMF colonization of the roots and arbuscule development 4 wpi. This result confirms the role of *SlLYK10* in AMS. I tested co-expression of *MtLYK3:YFP* and *PhLYK10:YFP* in tobacco and as for all *MtNFP* orthologs tested, the co-expression led to lesions on the leaves (Fig. 5).



Fig. 5 PhLYK10 interacts with MtLYK3 in N. benthamiana. PhLYK10 and MtLYK3 do not induce cell death when expressed separately in Ν. *benthamiana*, but when they are co-expressed, cell death is induced. Images are representative of 3 biological replicates. Lower panel is a close-up.

Additionally, we observed by microscopy that *PhLYK10* seems to be better localized and more expressed than *SlLYK10* in *N. benthamiana* leaves, hence *PhLYK10* could be better expressed in *M. truncatula* than *SlLYK10*. If it is the case, we should complement RLS in *Mtnfp-2* with p355::*PhLYK10*:*YFP* to determine whether *Solanaceae* orthologs of *MtNFP* can restore nodulation in *Mtnfp-2* mutants. We should also express *GUS* under the promoter of *PhLYK10* is regulated as the promoter of *MtNFP* in *M. truncatula* during symbioses. With these two experiments, we would have argues to claim that the CDS of *MtNFP* ancestor was not neo-functionalized during evolution and that the regulator cis elements on its promoter required for expression in the nodule where already present in the ancestor.

One more experiment I would have like to perform is to test the expression pattern of *MtNFP* and its ortholog during infection of *M. truncatula* by a pathogen. Indeed, *MtNFP* was shown to play a role in defense against various pathogens (Ben et al., 2013; Rey et al., 2013). It would be an interesting information to know whether *MtNFP* and orthologs expression is relocalized upon pathogen infection, as for rhizobial colonization.

Confirming the role of SILYK10 and BdLYR1 in AMS

In the 2nd chapter of my PhD thesis, I demonstrated that SILYK10 has high affinity for LCOs and in the 1st chapter of my PhD thesis, we demonstrated that *SILYK10* is involved in AMS establishment. Hence, we could conclude that LCO perception *via* SILYK10 is important for AMS establishment. To corroborate this, we first have to confirm the phenotype we observed by VIGS (Virus Induced Gene Silencing) on *SILYK10* in tomato. We could, in a first instance, edit *SILYK10* with CRISPR-Cas9 in order to confirm defect of AMF colonization in another *SIlyk10* allele.

On another hand, we have to confirm that SILYK10 binding properties are important for AMS establishment to validate that LCOs are important for AMS. Thus, it would be interesting to complement a KO line (obtained by CRISPR-Cas9 for example) with a construct coding for a mutated version of SILYK10 that would be impaired in it LCO binding capacity but not in its conformation, so that could still interact with partners for signal transduction. Ability to interact with partner could be tested by co-expression in *N. benthamiana* leaves with MtLYK3 and observation of necrosis apparition. This would clearly show that LCOs binding through SILYK10 is important for AMS establishment in tomato.

Another way to show the importance of LCO binding by SILYK10 in the AMS would be to demonstrate whether *Sllyk10* mutants are impaired in LCO perception. To demonstrate it, we could adapt the lateral root quantification bioassay on tomato. In the same idea, we could perform RNAseq analyses on WT and *Sllyk10* mutants, treated or not with LCO (mixture of AMF LCOs at 10⁻⁷ M), COs (mixture of CO4 and CO5 at 10⁻⁷ M and CO8 at 10⁻⁷ M) and spore exudates. This could allow us to identify genes regulated by LCO or CO perception and give some clues about LCO / COs perception in tomato.

What are the residues responsible of the binding activity of SILYK10 and BdLYR1?

Even if we know the affinity of SILYK10 and BdLYR1 for some LCO structures, we do not know which residues are responsible of the binding properties of these proteins. We do not know whether the LCO binding sites of SILYK10 and BdLYR1 are the same. Identifying the LCO binding site would be helpful to predict, based on their sequences, the ability of other LysM-RLKs to bind LCOs.

First, to identify which LysM(s) is (are) responsible of the LCO binding in SILYK10 or BdLYR1 we could perform swaps between the LysM of SILYK10 and SILYK7 (we showed that the later does not bind LCOs, Chapter 2) or between BdLYR1 and BdLYR4 (that does not bind LCOs). It would allow to identify which LysM(s) is responsible for the binding and whether it is the same in SILYK10 and BdLYR1. Then, by comparing these LysMs with the one that have high affinity for GlcNAc containing ligand identified through X-ray crystallography (OsCEBIP or ECP6 (Liu et al., 2016; Sánchez-Vallet et al., 2013)) we might find putative residues involved in binding of the GlcNAc moiety. We could produce proteins mutated in these residues and perform binding assays to show whether the targeted residues are involved or not in the LCO binding site.

Are hetero-complexes of LysM-RLKs involved in AMF perception?

In plants, LysM-RLKs seems to be a major component for microbe perception. They are found in all the green lineage. LysM-RLKs are involved in defense responses and in symbioses establishment, probably in both case through GlcNAc containing ligand perception. In *A. thaliana*, AtLYK5 (and maybe AtLYK4, both belonging to the clade of LYRIII that has inactive kinase, *cf.* Introduction) interacts with AtCERK1 (that belongs to the clade LYKI and has an active kinase) for long CO perception and activation of defense against pathogens (Cao et al., 2014). In *L. japonicus* and *M. truncatula*, it was recently shown that the orthologs of *AtCERK1*, respectively *LjLYS6* (renamed *LjCERK6*) and *MtLYK9* were involved in defense and resistance against the fungal pathogen *Botrytis cinerea* (Bozsoki et al., 2017). LjCERK6 probably interacts with LjLYS13 and LjLYS14 (which are LYRs), and MtLYK9 with MtLYR4 to transduce the signal, although no interaction has yet been shown between MtLYR4, LjLYS13 or LjLYS14 and COs.

Concerning symbioses, interactions between LysM-RLKs were only shown in legumes and validated for the RLS. Again, hetero-complexes are formed between LYRs and LYKs: MtNFP/LjNFR5 and MtLYK3/LjNFR1 interact together and this interaction seems to be responsible of the activation of the CSSP, resulting in RLS establishment (Madsen et al., 2011; Moling et al., 2014).

Altogether, it suggests that pathogen and rhizobia perception goes through a heterodimer of LysM-RLKs, leading us to presume that in the AMS LysM-RLKs also function as heterodimers composed of a LYR that directly binds the symbiotic signal and a LYK that transduces the signal *via* its active kinase. To validate this hypothesis, we could identify the SILYK10 and BdLYR1 co-receptor LysM-RLKs by testing physical interactions through yeast two hybrid or co-immunoprecipitations in heterologous system as *N. benthamiana*. Once interactors are found we could look for or create mutant in the corresponding genes. Another strategy to identify co-receptors involved in the AMS (particularly adapted to *B. distachyon* which only has 4 LYMs, 4 LYRs and 3 LYKs) is to edit all the LysM-RLKs by CRISPR-Cas9. If we have enough regenerating transformants, we should obtain lines individually mutated in each LysM-RLKs in addition of plants with combinations of mutated genes in several or in all LYMs, LYRs or LYKs to identify which LYMs, LYRs and LYKs are important for AMS and for defense.

Are LysM-RLKs at the interface between root endo-symbioses and plant defense?

We now have numerus evidences indicating that LysM-RLKs play overlapping roles in root endo-symbioses establishment and in plant defense. OsCERK1 (LYKI group) was initially shown to play a role with its partner OsCEBIP (LYMII group) in the perception of fungal pathogens (via chitin fragment perception). But recently, it was shown that *Oscerk1* mutants, but not *Oscebip* mutants, display a reduce number of colonization sites (Miyata et al., 2014; Zhang et al., 2015) implying an overlapping role of *OsCERK1* in both defense and AMS establishment. This dual role could be explain by the fact that OsCERK1 does not directly bind GlcNAc containing ligand, but is the partner (that transduces the signal through its active kinase) of binding proteins for different GlcNAc containing ligands: OsCEBIP in the case of defense signaling and an unknown co-receptor, probably belonging to the LYR, in the case of AMS signaling.

More interestingly, in our team, Tongming Wang showed in his thesis that NbLYK4, a LYR that directly binds LCOs with a high affinity, plays a role in both defense and AMS

establishment in *N. benthamiana*. Silenced plants for *NbLYK4* (*Nblyk4*) are more colonized by *R. irregularis* 4 wpi than WT, but also by the bacterial pathogen *Pseudomonas syringae*. Moreover, LCO treatment on WT plants reduced the effect of flg22 on the growth of plantlets but not in *Nblyk4* plants indicating a possible crosstalk between the roles of *NbLYK4* in AMS and defense. It is to note that *NbLYK4* is the ortholog of *AtLYK4* shown to be involved in defense against bacterial and fungal pathogens but for which no ligand binding has been demonstrated yet.

In addition, a recent study (Bozsoki et al., 2017) showed that *MtLYK9* and *MtLYR4* are involved in fungal pathogen perception. Even if single mutants are not impaired in AMS nor in RLS establishment compare to WT, double mutants in *MtLYK9-MtNFP* show a decrease in AMF colonization (iMMM2017: Feng et al., 2017). Furthermore *MtNFP* that is necessary for RLS establishment also seems to play a role in defense against the oomycete pathogen *Aphanomyces euteiches*, but also to the fungal pathogens *Colletotrichum trifolii* (Rey et al., 2013) and *Verticillium albo-atrum* (Ben et al., 2013). Thus, I think it would be interesting to test on robust mutants of *Sllyk10* and *Bdlyr1* whether they are more susceptible to fungal or bacterial pathogens.

Molecular dialog between AMF and plants may go through other components than LysM-RLKs and LCOs/COs

A major question concerning the establishment of AMS, and particularly concerning the molecular dialog between AMF and plants remains to determine whether LCOs and COs are important symbiotic signals and whether other fungal signals are involved in AMS establishment. This work partially addresses this question: LCOs seem to be involved in AMS establishment in tomato and in petunia. However these mutants in LCO receptors were not totally blocked in AMS establishment. Moreover, we observed in *B. distachyon* that mutation in a potential LCO receptor did not blocked AMS establishment. It could be because other LCO receptors play a redundant role in AMS, or it could be because other signals (like COs or AMF effectors for example) are involved in AMS establishment. One way to find out new AMF signals could be by searching for other genes involved in early steps of AMS establishment by screening mutagenized populations of *Sllyk10* tomato mutants or *Bdlyr1 B. distachyon* mutants for a decrease of fungal penetration (or no colonization at all). Then we would have

to identify genetic determinants of the colonization defect and characterize there genetic and biochemical functions in the AMS.

Towards transfer in crops

In parallel of the biochemical characterization of SILYK10 and BdLYR1, I also started the characterization of the ortholog in wheat. I worked on hexaploid wheat, so I cloned the 3 paralog genes, named *TaLYR1A*, *TaLYR1B* and *TaLYR1D* (cloned sequences in Annex 3). We performed binding experiments to verify whether TaLYR1A, B and D could bind LCO-V(C18:1,NMe,S). All the three protein could bind LCO-V(C18:1,NMe,S) (Fig 6). As membrane fraction expressing TaLYR1D showed highest LCO binding activity, we used it to determine its affinity for LCO-V(C18:1,NMe,S) by cold saturation with the radiolabel LCO-V(C18:1,NMe,³⁵S). We could determine TaLYR1D has a *Kd* of 13.8 nM for LCO-V(C18:1,NMe,S), in the same range than for SILYK10 or BdLYR1. Hence, TaLYR1D has high affinity and is a biochemical ortholog of SILYK10 and BdLYR1. We also performed competition assays with 1 μ M of CO4 or CO8 and we could observe that the LCO binding site of TaLYR1D do not have high affinity for COs. All these experiments were performed once and need to be repeated. We also have to find out whether TaLYR1A and TaLYR1B have the same affinity for LCOs than TaLYR1D. Then it would be interesting to know if wheat mutants in *Talyr1* are impaired or not in AMS establishment or in LCO perception in order to conclude on the role of LCO in AMS in monocots and dicots.



Fig. 6 TaLYR1 binds LCOs with high affinity and selectivity versus COs. A) Membrane fractions containing TaLYR1A:YFP, TaLYR1B:YFP or TaLYR1D:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,³⁵S) in absence (dark orange) or in presence (light orange) of 1 μ M of LCO-V(C18:1,NMe,S). B) Scatchard plot analysis of a cold saturation experiment using the radiolabeled LCO-V(C18:1,NMe,³⁵S) and the unlabeled LCO-V(C18:1,NMe,S) on membrane fraction containing BdLYR1:YFP. C) Membrane fraction containing TaLYR1D:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,³⁵S) in presence of 1 μ M of the indicated unlabeled competitor. In A) and C) Bars represent mean and SD between technical replicates.

Conclusion

During my thesis, we showed that SILYK10 in tomato, i) plays a role in AMS in tomato (silencing this gene decreased tomato colonization by AMF), and ii) binds LCOs with high affinity (with a *Kd* in the nM range). These two results strongly suggest that LCO perception is important for AMS establishment in tomato. On the other hand we also showed that BdLYR1 binds LCOs with a similar affinity but a mutant in this gene in *B. distachyon* was not affected in AMF colonization. Interestingly, in the legume *M. truncatula*, MtNFP was never shown to bind LCOs and *Mtnfp* mutants are not affected in the AMS. The different importance of these orthologs in AMS raises the question whether perception mechanisms involved in AMS establishment in different plant species are different. Actually, it could rely more on CO perception in monocotyledons than in dicotyledons. In legumes we cannot exclude higher functional redundancy between the receptors as the LysM-RLK family has enlarged through many duplication events.

In this PhD thesis, I also showed that CDS of *MtNFP* orthologs were able to activate the CSSP in *M. truncatula*, leading to RLS establishment in the *Mtnfp-2* mutant impaired in nodulation. I showed that the cis elements in the promoter of *SlLYK10* are sufficient to drive expression in nodules of *M. truncatula*. This suggests that ancestors of the legume MtNFP and orthologs were already functional for RLS apparition.

References

Bascaules A, Jargeat P, Cope K, Ané J-M, Martin FM, Bécard G, Puech-Pagès V (2017) Production of lipochitooligosaccharides and short chitooligosaccharides by ectomycorrhizal fungi. iMMM2017. p Talk3

Bécard G New aspects on Myc-LCOs and their role as molecular signals in the AM symbiosis. iMMM2015

Beck M, Zhou J, Faulkner C, MacLean D, Robatzek S (2012) Spatio-Temporal Cellular Dynamics of the Arabidopsis Flagellin Receptor Reveal Activation Status-Dependent Endosomal Sorting. Plant Cell 24: 4205–4219

Bek AS, Sauer J, Thygesen MB, Duus JØ, Petersen BO, Thirup S, James E, Jensen KJ, Stougaard J, Radutoiu S (2010) Improved characterization of nod factors and genetically based variation in LysM Receptor domains identify amino acids expendable for nod factor recognition in Lotus spp. Mol Plant Microbe Interact 23: 58–66

Ben C, Toueni M, Montanari S, Tardin M-C, Fervel M, Negahi A, Saint-Pierre L, Mathieu G, Gras M-C, Noël D, et al (2013) Natural diversity in the model legume *Medicago truncatula* allows identifying distinct genetic mechanisms conferring partial resistance to *Verticillium* wilt. J Exp Bot 64: 317–332

Bozsoki Z, Cheng J, Feng F, Gysel K, Vinther M, Andersen KR, Oldroyd G, Blaise M, Radutoiu S, Stougaard J (2017) Receptor-mediated chitin perception in legume roots is functionally separable from Nod factor perception. Proc Natl Acad Sci 201706795

Broghammer A, Krusell L, Blaise M, Sauer J, Sullivan JT, Maolanon N, Vinther M, Lorentzen A, Madsen EB, Jensen KJ, et al (2012) Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proc Natl Acad Sci 109: 13859– 13864

Buendia L, Wang T, Girardin A, Lefebvre B (2016) The LysM receptor-like kinase SILYK10 regulates the arbuscular mycorrhizal symbiosis in tomato. New Phytol 210: 184–195

Cao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A, Stacey G (2014) The kinase LYK5 is a major chitin receptor in *Arabidopsis* and forms a chitin-induced complex with related kinase CERK1. Elife 3: 1–19

Feng F, Sun J, Luginbuehl L, Oldroyd GED (2017) How plants recognize and feed mycorrhizal fungi. iMMM2017. p Talk1

Fliegmann J, Canova S, Lachaud C, Uhlenbroich S, Gasciolli V, Pichereaux C, Rossignol M, Rosenberg C, Cumener M, Pitorre D, et al (2013) Lipo-chitooligosaccharidic symbiotic signals are recognized by LysM receptor-like kinase LYR3 in the legume *Medicago truncatula*. ACS Chem Biol 8: 1900–1906

Genre A, Chabaud M, Balzergue C, Puech-Pagès V, Novero M, Rey T, Fournier J, Rochange S, Bécard G, Bonfante P, et al (2013) Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. New Phytol 198: 190–202

Kloppholz S, Kuhn H, Requena N (2011) A secreted fungal effector of glomus intraradices promotes symbiotic biotrophy. Curr Biol 21: 1204–1209

Lefebvre B, Klaus-heisen D, Pietraszewska-bogiel A, Hervé C, Camut S (2012) Role of N -Glycosylation Sites and CXC Motifs in Trafficking of *Medicago truncatula* Nod Factor Perception Protein to Plasma. J Biol Chem 287: 10812–10823

Liu S, Wang J, Han Z, Gong X, Zhang H, Chai J (2016) Molecular Mechanism for Fungal Cell Wall Recognition by Rice Chitin Receptor OsCEBiP Recognition by Rice Chitin Receptor OsCEBiP. Structure 24: 1–9

Madsen EB, Antolín-Llovera M, Grossmann C, Ye J, Vieweg S, Broghammer A, Krusell L, Radutoiu S, Jensen ON, Stougaard J, et al (2011) Autophosphorylation is essential for the in vivo function of the Lotus japonicus Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J 65: 404–417

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, et al (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature 469: 58–63

Malkov N, Fliegmann J, Rosenberg C, Gasciolli V, Timmers ACJ, Nurisso A, Cullimore J, Bono J-J (2016) Molecular basis of lipo-chitooligosaccharide recognition by the lysin motif receptor-like kinase LYR3 in legumes. Biochem J 473: 1369–1378

Miyata K, Hayafune M, Kobae Y, Kaku H, Nishizawa Y, Masuda Y, Shibuya N, Nakagawa T (2016) Evaluation of the role of the LysM receptor-like kinase, OsNFR5/OsRLK2 for AM symbiosis in rice. Plant Cell Physiol 57: 2283–2290

Miyata K, Kozaki T, Kouzai Y, Ozawa K, Ishii K, Asamizu E, Okabe Y, Umehara Y, Miyamoto A, Kobae Y, et al (2014) The bifunctional plant receptor, OsCERK1, regulates both chitin-

triggered immunity and arbuscular mycorrhizal symbiosis in rice. Plant Cell Physiol 55: 1864– 1872

Moling S, Pietraszewska-Bogiel A, Postma M, Fedorova E, Hink MA, Limpens E, Gadella TWJ, Bisseling T (2014) Nod Factor Receptors Form Heteromeric Complexes and Are Essential for Intracellular Infection in Medicago Nodules. Plant Cell 26: 4188–4199

Okazaki S, Kaneko T, Sato S, Saeki K (2013) Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. Proc Natl Acad Sci U S A 110: 17131–6

Oláh B, Brière C, Bécard G, Dénarié J, Gough C (2005) Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in Medicago truncatula via the DMI1/DMI2 signalling pathway. Plant J 44: 195–207

Rey T, Nars A, Bonhomme M, Bottin A, Huguet S, Balzergue S, Jardinaud MF, Bono JJ, Cullimore J, Dumas B, et al (2013) NFP, a LysM protein controlling Nod factor perception, also intervenes in Medicago truncatula resistance to pathogens. New Phytol 198: 875–886

Sánchez-Vallet A, Saleem-Batcha R, Kombrink A, Hansen G, Valkenburg DJ, Thomma BPHJ, Mesters JR (2013) Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. Elife 2013: 1–16

Sun J, Miller JB, Granqvist E, Wiley-Kalil A, Gobbato E, Maillet F, Cottaz S, Samain E, Venkateshwaran M, Fort S, et al (2015) Activation of Symbiosis Signaling by Arbuscular Mycorrhizal Fungi in Legumes and Rice. Plant Cell 27: 823–838

Wan J, Tanaka K, Zhang X-C, Son GH, Brechenmacher L, Nguyen THN, Stacey G (2012) LYK4, a Lysin Motif Receptor-Like Kinase, Is Important for Chitin Signaling and Plant Innate Immunity in Arabidopsis. Plant Physiol 160: 396–406

Wang T (2017) Role of fungal symbiotic signal perception in non-nodulating dicotyledons. Université Toulouse 3 Paul Sabatier

Zhang X, Dong W, Sun J, Feng F, Deng Y, He Z, Oldroyd GED, Wang E (2015) The receptor kinase CERK1 has dual functions in symbiosis and immunity signalling. Plant J 81: 258–267

Annexes

CRISPR-Cas9

About CRISPR-Cas9

CRISPR-Cas9 is a new tool for reverse genetics and genomics. It functions with a bacterial endonuclease, Cas9, and a RNA that guides Cas9 to the double strand DNA that Cas9 will cut (Fig. 1A). To edit the *LysM-RLKs* of *B. distachyon*, I chose to use the system described in (Xie et al., 2015) (Fig. 1B). Indeed, this system was improved for monocot as *Cas9* is under the control of a promoter of maize ubiquitin which expression in monocot is higher than p*355*. Also the selection for transgenesis is hygromycin, one of the recommended selections for *B. distachyon* together with paromomycin sulfate (Vogel and Hill, 2008). The system developed by Xie et al., 2015 allows to introduce multiple guide RNAs in a single construct and the cloning (Golden Gate type) is fast and easy to perform.



Fig. 1 DNA editing by CRISPR-Cas9. A) The guide RNA (gRNA) is divided in 2 parts, one part (also called the spacer) is composed of 20 bases that hybridizes with the targeted DNA. The other part (also called the scaffold) which is always the same RNA sequence has a secondary structure recognized by Cas9. The endonuclease Cas9 is anchored on the DNA target by the gRNA and will cut the 2 DNA strands. When the cell tries to repair the double break, it makes mistakes as base deletion or insertion, leading to frame shifts. **B)** System of Xie et al (2015). A polycistronic tRNA-gRNAs allows to produce a single RNA molecule containing multiple gRNAs. tRNA are cleaved by the endogenous RNAse P and Z that specifically recognize tRNA secondary structure, releasing the gRNAs. Adapted from Xie et al. (2015).

Constructs and sequences of tRNA/gRNA for the CRISPR-Cas9 editing

Plasmids constructed to transform *B. distachyon* to edit *BdLYR1*, all the *LYRs* and *BdLYK1* are shown in Fig. 2. gRNA sequences used in these constructs are shown below.



In yellow: Sequencing primers; In green: tRNA; In pink: Spacer part of the gRNA; In blue: Scaffold part of the gRNA; In grey: Terminator.

>pRGEB32-BdLYR1 TATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGCAACAAAGCACCAG TGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA<mark>CGCTT</mark> GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA<mark>AAG(</mark> <mark>GCTA</mark>GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA C<mark>TTTTTTTTTTTG</mark>TTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG <mark>TGC</mark>TTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCATGCCTGCAG<mark>GTCCAC</mark> ATTCGGGTCAAG >pRGEB32-BdLYRs :<mark>GTAGTGGGCCATGAAGCC</mark>TTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGACAACAAAGACTAG TATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGC<mark>AACAAAGCACCAG</mark> TGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCCGGGTTCGATTCCCGGCTGGTGCA<mark>CGCTTCGCAACACACAC</mark>A AGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC<mark>AACAAA</mark> CACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA<mark>ACGCCCACCAA</mark>(GGAAGTGTTTTAGAG

CAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA <mark>CTCGA</mark>
CGCCAACAGCCTCAC GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGA
CTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCgATTCCCGGCTGGTG
CA <mark>GACTCCCCGTGGTGCCGCTC</mark> GTTTTAgAGCTAGAAATAGCAAGTTAAAATAAgGcTAGTCCGTTATCAACTTGAAAAAGTG
${\tt GCACCGAGTCGGTGC} {\tt TTTTTTTTTTTTTTT} {\tt gtTTTAGagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaa}$
agtggcaccgagtcggtgcttttttgttttagagctagaaatagcaagttaaaataaggctagtccgtttttagcgcgtgcat
gcctgcag <mark>gtccacaaattcgggtcaag</mark>
>pRGEB32-BdLYK1
TCGTAGTGGGCCATGAAGCC TTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGACAACAAAGACTAG
TATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGC <mark>AACAAAGCACCAG</mark>
TGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA <mark>CTTGTTTTATAGACGAAGA</mark>
AGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC <mark>AACAAA</mark>
CCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCA <mark>GATGGCACTTCA</mark>
TCAGCTGC GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
CTTTTTTTTTT GTTTGCTGTCGCTCATCC <mark>GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA</mark>
AAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTG

Brachypodium distachyon *stable transformation via* Agrobacterium tumefaciens AGL1 *method*

This method is adapted from the unpublished method used by Oumaya Bouchkabé-Coussa, at IJPB Versailles. This method consists in embryogenic callus transformation. Calli are from immature embryos of *Brachypodium distachyon* Bd21-3 and are transformed with *Agrobacterium tumefaciens*. Transgenic plants are regenerated by somatic embryogenesis.

The method is delicate and several steps are critical. These critical points are detailed below and concern the following steps: growth conditions culture of the plants, choice of immature embryos, choice of calli for transformation, choice of the selection, and stage of isolation of the somatic embryo for rooting.

Plant production

Sterilize Bd21-3 seeds (30 sec in EtOH 75%, 3 washes, 5 min in bleach 2.5%, 3 washes) and put them on agar gel supplemented with 1mM GA3. Put at 4°C in the dark for 2 to 4 weeks. Put the seeds at 25°C in the dark for 24 hours. Plant 1 plantlet per pot (soil:attapulgite 1:1). Put the plants in a growth chamber (16h light, light intensity of 320 µmol.m⁻².s⁻², hygrometry 60% and 25°C). Plants will grow 4 to 5 weeks before apparition of spikes. Control cautiously the spikes during the 10 to 12 days after their apparition: they have to be full but still green and flexible.

<u>Critical points</u>: Bd21-3 does not flower in short days unless it has been vernalizes at least for 2 weeks. And plants must be watered with fertilizer once a week to obtain high yield of embryogenic calli.

Embryogenic calli production

From this step, all the procedure must be performed in sterile conditions. Sterilize immature spikes for 30 min (800 ml H2O, 1.5g bleach tablet, 0.1% Triton 100-X). Wash the spikes with sterile water 3 times. Leave the spikes in water to avoid desiccation. Under a binocular loupe remove the spiklets, and with forceps and scalpel, remove the lemma. Press the seed in its middle in order to eject the embryo (Fig. 3). Put the embryo on cal inducing medium (CIM) (Table 1). Put 15 to 20 embryo per petri dish. Seal the boxes with parafilm and put them at 28°C in the dark for 6 weeks in total, but refresh the medium first after 3 weeks and then after 2 weeks.

<u>Critical points</u>: only the very first development stages of embryo can produce embryogenic calli (Fig. 3B). The CIM must be at room temperature before transferring the embryos on it.



Fig. 3 How to select an immature embryo of *B. distachyon***? A)** Monocot flower structure. **B)** The 4 embryos at the left will produce embryogenic calli. The 2 embryos on the right are too mature and will not produce embryogenic calli. **C)** The callus on the left is embryogenic in contrast to the callus on the right.

	Table 1: CIM mediu	m
	Stock concentration	Volume or mass (for 1 L)
MS (M0222.0050 Duchefa)	4405.19 mg/L	4.4 g
Sucrose	30 g/L	30 g
MES	14%	5 mL
ВСР	0.16%	5 mL
CuSO ₄	0.6 mg/mL	0.6 mL
Phytagel	2 g/L	2 g
2,4-D	2.5 mg/mL	1 mL
milliQ water		Qsp 1 L

Embryogenic calli transformation

Grow *A. tumefaciens* on a petri dish. Harvest the by scratching and resuspend in 5 ml of co-culture medium (Table 2) in a 50 ml falcon. Do not vortex to resuspend the bacteria. Dilute to an OD_{600nm} from 0.6 to 0.8 in 20 ml of co-culture medium. Put the calli in the bacterial suspension for 5 min. Discard the liquid and roll the calli on sterilized wathmann paper. Once the calli are dry, put 5 calli per petri dish on a wathmann paper for 3 days (from Thursday evening to Monday morning) at 22°C-23°C in the dark. At the end of the co-culture, calli must be completely dry (Fig. 4).



Fig. 4 Dried calli (3 days after co-culture with *A. tumefaciens* AGL1).

٦	Table 2: Co-culture medium	
Solution	Concentration	Volume (for 200 ml)
CIM (liquid) without 2,4D &		200 mL
BCP		
Acetosyringone	30 mg/mL	400 μL
2,4D	2.5 mg/mL	200 μL
Pluronic 10%	10 μL/ mL	2 mL

Selection

Transfer the dry calli on selection medium (Table 3). Seal the petri dishes with parafilm and put them in the dark at 28°C for 3 weeks. Control carefully the calli every 2-3 days to avoid bacterial contaminations.

Tabl	e 3: Selection medium	
Solution	Concentration	Volume or mass (1L)
sucrose	30 g/L	30 g
MS (M0222.0050 Duchefa)	4405.19 mg/L	4.4 g
MES	14%	5 mL
ВСР	0.16%	5 mL
CuSO ₄	0.6 mg/ mL	0.6 mL
Phytagel // Bacto-Agar	2 g/L // 8 g/L	2 g // 8 g
milliQ water	Qsp 1 L	
Autoclave and when the medium is o	cold enough, add the following	filtered solutions:
2, 4 D	2,5 mg/mL	1 mL
Timentin*	250 mg/mL	1 mL
Hygromycin**//Paromomycin	40 mg/mL // 400 mg/mL	1 mL

*Timentin: Tircarcillin Disodium Mixture 15:1 & Potassium Clavulanate n°: T0190.0025

**Hygromycin (with phytagel) and paromomycin sulfate (with BactoAgar) are recommended selections for *Brachypodium distachyon*.

Regeneration

Select the calli surviving onto the selection medium and transfer them on regeneration medium (Fig. 5 and Table 4). On hygromycin, non-transformed cells turn brown, transfer them with healthy cells. On paromomycin, non-transformed cells become aqueous, do not transfer them on regeneration medium.



Fig. 5 Transformation steps from selection to regeneration. A) The callus on the right is transgenic in contrast to the callus on the left. B) Regenerated plantlet 3 weeks after transfer on regeneration medium. C) Rooting in tube 2 weeks after transfer on rooting medium. D) Transformed plant 3 weeks after its transfer in growth chamber.

Та	ble 4: Regeneration medium	
Solution	Concentration	Volume or Masse (1L)
Maltose	30 g/L	30 g
MS (M0222.0050 Duchefa)	4405.19 mg/L	4.4 g
MES	14 %	5 mL
ВСР	0.16 %	5 mL
Phytagel	2 g/L	2 g
milliQ water	Qsp 1 L	
Autoclave and when the mediur	n is cold enough, add the followir	ng filtered solutions::
Kinetin*	0,2 mg/mL	1 mL
Timentin	250 mg/mL	1 mL
Hygromycin // Paromomycin	40 mg/mL // 400 mg/mL	1 mL

*Kinetin dissolves with some drops of NaOH, then you can dilute it in water.

Rooting

When regenerated plantlets are big enough (1-2 cm) and when root starts to appear, transfer plantlets in a magenta box or a glass tube with rooting medium (Fig. 5 and Table 5).

-	Table 5: rooting medium	
Solution	Concentration	Volume or Masse (1 L)
sucrose	10 g/L	10 g
MS (M0222.0050 Duchefa)	4405.19 mg/L	4.4 g
ВСР	0.16 %	5 mL
MES	14 %	5 mL
Phytagel	2 g/L	2 g
milliQ water		Qsp 1 L
Phytagel	2 g/L	2 g
Autoclave and when the medium	is cold enough, add the following	ng filtered solutions:
IBA	0.25 mg/L	500 μL

Transfer in growth chamber

When plantlets show a root of 2-3 cm, transfer it in pot with soil. Put it in cover plastic propagators for 4 days and then remove by steps the cover.

Bibliography

Vogel J, Hill T (2008) High-efficiency Agrobacterium-mediated transformation of Brachypodium distachyon inbred line Bd21-3. Plant Cell Rep 27: 471–478

Wang K, Bragg JN, Anderton A, Nieu R, Vogel JP (2014) Agrobacterium protocols: Third edition. Agrobacterium Protoc Third Ed 1: 1–368

Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci 112: 3570–3575

	Table 1:	genotyping and p	henotyping	summary of B. dis	tachyon I	nutagen	eized lines.	
				Genotype			Phenotype	
Mutated gene	Seed line	Sequencing priemer	WT	Heterozygote	mutant	AMS	Binding to LCO- V(C18:1,NMe,S)	Lateral root induction by LCO
	3732	Seg Rev	27/27	0/27	0/27			
	4287		34/34	0/34	0/34			
	6107		35/35	0/35	0/35			
	6001		3/10	5/10	2/10	+	+	2
	4252		4/7	1/7	2/7	+	+	2
	4914 (M3)		19/19	0/19	0/19			
00007-11-0	3774 (M3)		21/21	0/21	0/21			
067608TD9	(EM) 6603		35/35	0/35	0/35			
(BOLYKI)	6039 (M2)		38/38	0/38	0/38			
	4914 (M2)	Til For	22/22	0/22	0/22			
	5525 (M3)	Till2 For	7/15	0/15	0/15	ć	5	2
	5525 (M3)	Till2 Rev	16/25	1/25	0/25	ę	5	2
	5525 (M2)	Till2 Rev	14/20	0/20	0/20	ę	5	7
	5525 (M4)	Till2 Rev	29/125	33/125	25/125	+	5	2
	3774 (M2)	Til Seq For 2	3/6	1/6	2/6	ę	5	5
	7754	Seg Rev	8/23	3/23	12/23	٤	5	5
	8653	Til For	14/17	3/17	1/17	ç	2	2
	8122	Seq Rev	21/21	0/21	0/21			
Bd2g40627	7889	Til For	14/14	0/14	0/14			
(BdLYK2)	4974	Til For	1/6	1/6	4/6	٤	2	2
	8511	Til For	4/7	1/7	3/7	5	2	2
	3718	Til Rev	4/8	0/8	4/8	5	2	2
	5570	Til Rev	2/13	0/13	11/13	2	2	2
	Lines containin	g only WT plants						
+	same phenotyp	oe as WT plants						

Annex 2: Genotyping and phenotyping of putative *Bdlyr1* and *Bdlyk2* found by TILLING screen

>TaLYR1 A

 $\tt CTCCGGCGACACCTTCTACGCGCTCGCGCTCACCGGCTTCGAGAACCTCACCACCCCCGACGTCATCCAGGAGCTCAACCCGC$ GCCGGGGCGCAGCTGCTCGTCACCTACATGTGGCGCCCTGTCGACACCATGCCCGAGGTGAGCAACCTGATGGGCTCCGACGT CGGCGCCATCGCTGCGGTGAACAACGTCAGCGCCGACTTCACCTCCACGACGATGCTGCCGATGCTGGTCCCGGTGGCGCGGC CGCCGGTGCTTCCTCCGCTGCAATATGGCGCGCGAGCCCGAGCACCGGCGATCCCCGGAGCCAGCAAGGGCTTCTCGGGCGCCACC GTCGCGGCGAGCATCGCGGGGGTCTCTCGTCGCCGTCGCTGCCCTGTGCACGGCGATCTTCGCGTACCGGAGGTACCGCGAGAA GAAGGCCACGGTGCACTCGGCGTCCAGGTTCGCGAGCCCGAGGTTTTGCTTCAACCAGAACGCTTACGGGATTCAGAACAGCA GTTCCATCGCTCGCATGATCAACGGAGGGGACAAGCTGCTCACCAGCGTGTCGCAGTTCATCAACAAGCCTATCATCTTCGGT ACAGAGAGATCATGGAGGCGACGATGAACTTGGACGAACGGTGCAGGATCGGCAGCTCCTACTACCGGGCCAAGCTGGAAGG CGAGGTGTTCGCGGTGAAGCCGGCGAAAGGCGACGTGTCGGCGGAGCTGAGGATGATGCAGATGGTCAACCACGCCAACCTCA AAGTGGCTGTACCAGAAGCCTCCGTCCTCGCTGCCGTCGTCGAGCTCATCGGCGGACACGCTCTCGTGGAACCAGAGGCTGGG GGAACATCCTCCTCACCGCGGACTTCAGGGTCAGGATATCTAACTTCTCCGTGGCCACGCCGGCGATGGCCGACGCCGCGGCG GGAGATCGGCATGCTGTGGAGGGACATCCGGGCGGTGCTGGAGGCCGGGGACAAGAGGGACGCCAAGCTGAGGAAGTGGATGG ACCCCGCCCTTGGGGACGAGTACTACTTGGATGCGGCACTCAGCCTGGCCGGCATGGCGAGGGCCTGCACGGAGGAGGACGACGCG GCGCGGCCGAAGATGGCCGACGTCGTGTTCAGCCTTTCAATGCTGGTGCAGCCGTCACCGGTGGGCGACGCGTTCGAGAA GCTATGGCAGCCCAGCTCGGAGGAGAACATTAGGATTGTCAATGAAGTGGCAGCCAGATGA

>TaLYR1 B

ACCTCGGCGCCATCTCGGACCTCTTCGGCGTGAGCCGGGCCATGATCGCCAGCGCCAACGGCCTCAGCCTCGCCGCGAGGAC GCGGCGCTGCTGCCCGACCAGCCGCTGCTCGTGCCCGTCCGGTGCGGCTGCACCGCCACCGCTCCTTCGTCAACGTCACCTA CCCCATCCACTCCGGCGACACCTTCTACGCGCTCGCGCTCACCGGCTTCGAGAACCTCACCACCCCCGACGTCATCCAGGAGC TCAACCCGCAGGCGGTTTTCAACAAGCTCAATGTCTCGCAGCTGGTCACCGTGCCGCTCTTCTGCCGGTGCCCCACGCCGGCG GAGCGGAGCGCCGGGGTGCAGCTGCACGTCACCTACATGTGGCGACCTGTCGACACCATGTCCGAGGTGAGCAAGCTGATGAA CTCCAGTGCGAGCGCCATCGCTGCGGCCAACAACGTCACTGCCGACTTCACCTCCACGACGATGCTGCCGATGCTGATCCCGG TGGCGCGGCCGGCGGTGCTTCCTCCGCTGCGCTATGGTGCGAGCCCGAGCACCGGCGATCTCGGAGCTAGCAAGGGCTTCTCG CCGCAAGAAGAAGGCCACGGTGCACTCGGCGTCCAGGTTCGCGAGGCCCGAGGTTTTGCTTCAACCAGAACGCCTACGGGATTC AGAGCAGCAGTTCCATCGCTCGCATGATCAACGGAGGGGGGCAAGCTGCTCACCAGTGTCACAGTTCATCAACAAGCCTATC ATCTTCGGCACAGCGGAGATCATGGAGGCGACGATGAACTTGGACGAACGGTGCAGGATCGGCACCTCCTACTACCGGGCCAA GCTAGAAGGCGAGGTGTTCGCGGTGAAGCCGGCGAAAGGCGACGTGTCGGCGGAGCTGAGGATGATGCAGATGGTCAACCACG ${\tt CCAACCTCATCAGGCTGGCCGGCATATCCATCGGCGCGGATGGGGACTACACNTTCCTCGTGTACGAATTCGCCGAGAAGGGC}$ GCTGGGCATCGCGTTCGACGTCGCCAACGGCCTGCTGTACATGCACGAGCACACTCAGCCGAGCATGGTGCACGGCGACGTCC GCGCACGGAACATCCTCCTCACCGCAGACTTCAGGGCCAGGATATCCAACTTCTCCGTGGCCACGCCGGCGATGGCCGACGCC CGGCGCGGAGATCGGCATGCTGTGGAGGGACATCCGGGCGGTGCTGGAGGCCGGGGACAAGAGGGACGCCAAGCTGAGGAAGT GGATGGACCCCGCCCTTGGGGACGAGTACTACTTGGATGCGGCACTCAGCCTGGCCGGCATGGCCAGGGCTTGCACGGAGGAG GACGCGGCGGCGGCGGCCGAAGATGGCCGACGTCGTGTTCAGCCTGTCAATGCTGGTGCAGCCGTTACCGGTGGGCGACGGGTT CGAGAAGCTATGGCAACCCAGCTCGGAGGAGAACATTAGGATTGTCAATGAAGTGGCAGCCAGA

>TaLYR1 D

Annex 4: Protocol of Cross-linking between BdLYR1 and the photoactivatable LCO

Photoactivatable LCO aryl-azido derivative was performed as described in Fliegmann et al., 2013. For photoaffinity labeling, 400 µl of membrane fractions (10 µg/µl protein) were centrifuged for 30 min at 100000 g and the pellets resuspended in 8 ml of 25 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.2% dodecyl maltoside. After 30 min at 4°C and 30 min centrifugation at 100000 g, solubilized BdLYR1:YFP was purified using GFP-TrapM (ChromoTek, Munich, Germany). After washing, magnetic beads were incubated in binding buffer with 1 nM ³⁵S-LCO aryl-azido derivative in the presence or absence of 1 µM of LCO-V(C18:1,NMe,S) for 1 h at 4°C. Samples were illuminated 10 min at 365 nm with a 15 W UV tube at a distance of 4 cm. After SDS-PAGE and transfer, autoradiography of the nitrocellulose membrane was performed with a Phosphorimager.