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

Ariane Girardin

Université de Toulouse

THÈSE

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 :

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Rapporteurs :

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Pierre-Emmanuel Courty, Chargé de Recherches INRA (Dijon)

Autre(s) membre(s) du jury :

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Ma thèse en 180 secondes

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 8èmes 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 8èmes 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.

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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: SlLYK10), genes are indicated in italicized capitals (example: *SlLYK10*) and mutants are indicated in italicized lower case (example: *Sllyk10*).

AMS: Arbuscular Mycorrhiza Symbiosis

AMF: Arbuscular Mycorrhizal Fungus-i

AtCERK1: *Arabidopsis thaliana* Chitin Elicitor Receptor Kinase 1

BdLYR1: *Brachypodium distachyon* Lysin Motif Receptor-like Kinase LYR1

Cas9: CRISPR associated protein 9

CDS: Coding sequence

COs: chitooligosaccharides

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

CSSP: Common Symbiosis Signalling Pathway

ECR: Extracellular Region

ER: Endoplasmic Reticulum

ETI: Effectors-Triggered Immunity

GFP: Green Fluorescent Protein

GlcNAc: N-acetyl Glucosamine

GPI: Glycosylphosphatidylinositol

ICR: Intracellular Region

IT: Infection Thread

ITC: Isothermal Titration Calorimetry

KO: Knock-out

LCOs: lipo-chitooligosaccharides

LjLYS11: *Lotus japonicus* Lysin Motif Receptor-like Kinase 11

LjNFR1: *Lotus japonicus* Nod-Factor Receptor 1

LjNFR5: *Lotus japonicus* Nod-Factor Receptor 5

LRR: Leucine Rich Repeat

LysM: Lysin Motif

LysM-RLK: Lysin Motif Receptor-like Kinase

MAMPs: Microbe-Associated Molecular Patterns

MST: Microscale Thermophoresis

MTI: Microbe-Triggered Immunity

MtLYK3: *Medicago truncatula* Lysin Motif Receptor-like Kinase LYK3

MtLYR3: *Medicago truncatula* Lysin Motif Receptor-like Kinase LYR3

MtNFP: *Medicago truncatula* Nod-Factor Perception

OsCEBIP: *Oryzae sativa* Chitin Elicitor Binding Protein

PaNFP: *Parasponia andersonii* Nod-Factor Perception

PM: Plasma Membrane

PGN: Peptidoglycan

Pro35S: Promoter 35S of Cauliflower mosaic virus

RFP: Red Fluorescent Protein

RLS: Rhizobium Legume Symbiosis

RLK: Receptor-like kinase

RLP: Receptor-Like Protein

RLS: Rhizobium Legume Symbiosis

RNAi: RNA interference

RNS: Root Nitrogen fixing Symbiosis

ROS: Reactive Oxygen Species

SlLYK10: *Solanum lycopersicum* Lysin Motif Receptor-like Kinase 10

SP: Signal Peptide

TM: Trans-membrane domain

VIGS: Virus Induced Gene Silencing

Wpi: week(s) post-inoculation

WT: Wild Type

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

Abstract

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 glycine-rich loop and do not exhibit auto-phosphorylation activity *in vitro*. The latter are called dead-kinases.

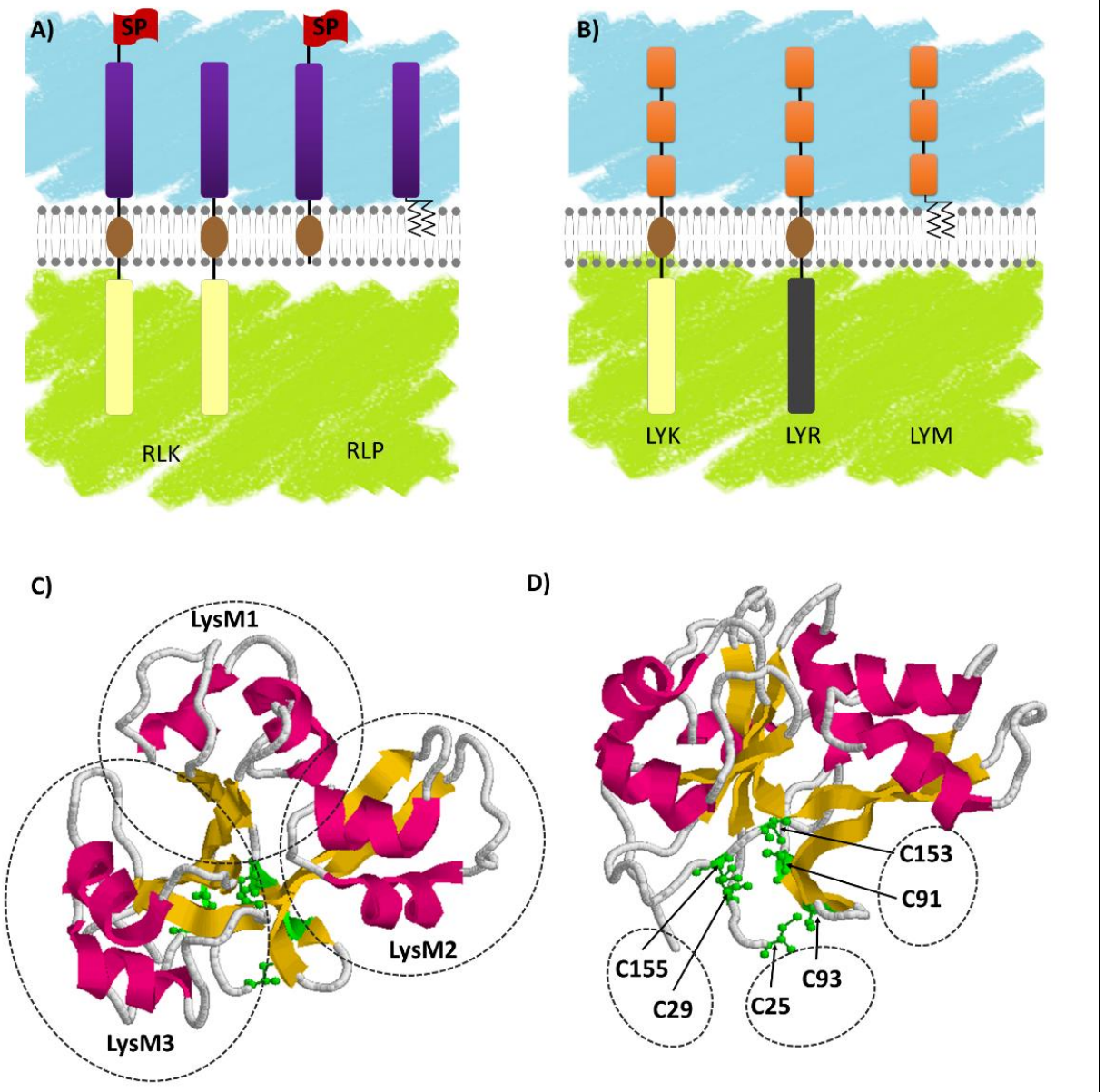

Fig. 1 LysM-RLK and LysM-RLP structure, synthesis and maturation. A) Receptor-like kinases (RLKs) are produced by ribosomes associated with the Endoplasmic Reticulum (ER). The extracellular region (ECR) preceded by a signal peptide (SP) is translocated into the ER lumen during the translation until the 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 of AtCERK1 ECR highlighting the cysteines involved in disulfide bridges (circled).

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 N-acetylmuramic 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.

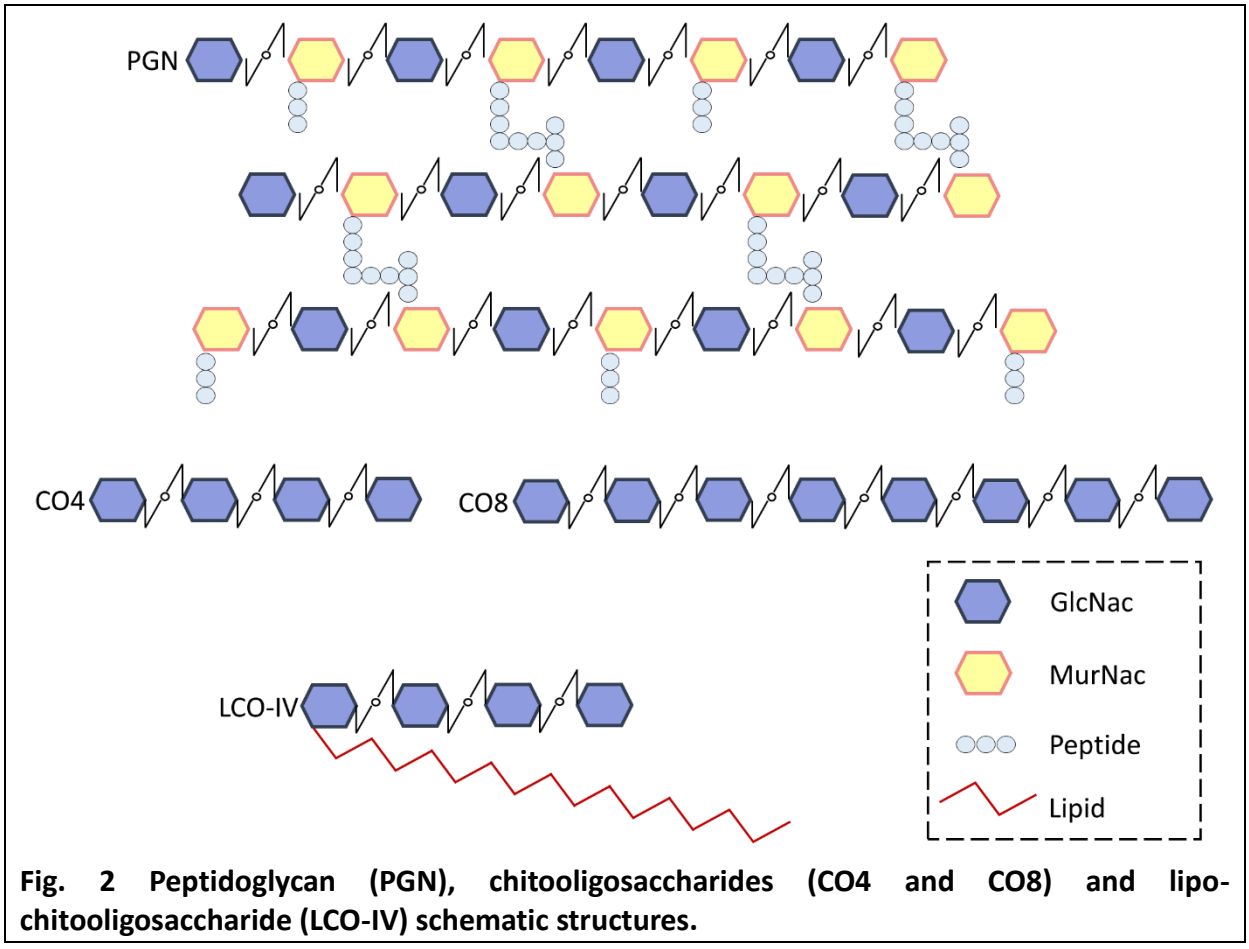

Fig. 2 Peptidoglycan (PGN), chitooligosaccharides (CO4 and CO8) and lipo-chitooligosaccharide (LCO-IV) schematic structures.

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 multi-function 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 (N_2) to ammonia (NH_3). 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 non-legumes. 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 (http://gpcr.biocomp.unibo.it/predgpi/pred.htm) 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 membrane-anchored 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 membrane-anchored 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.

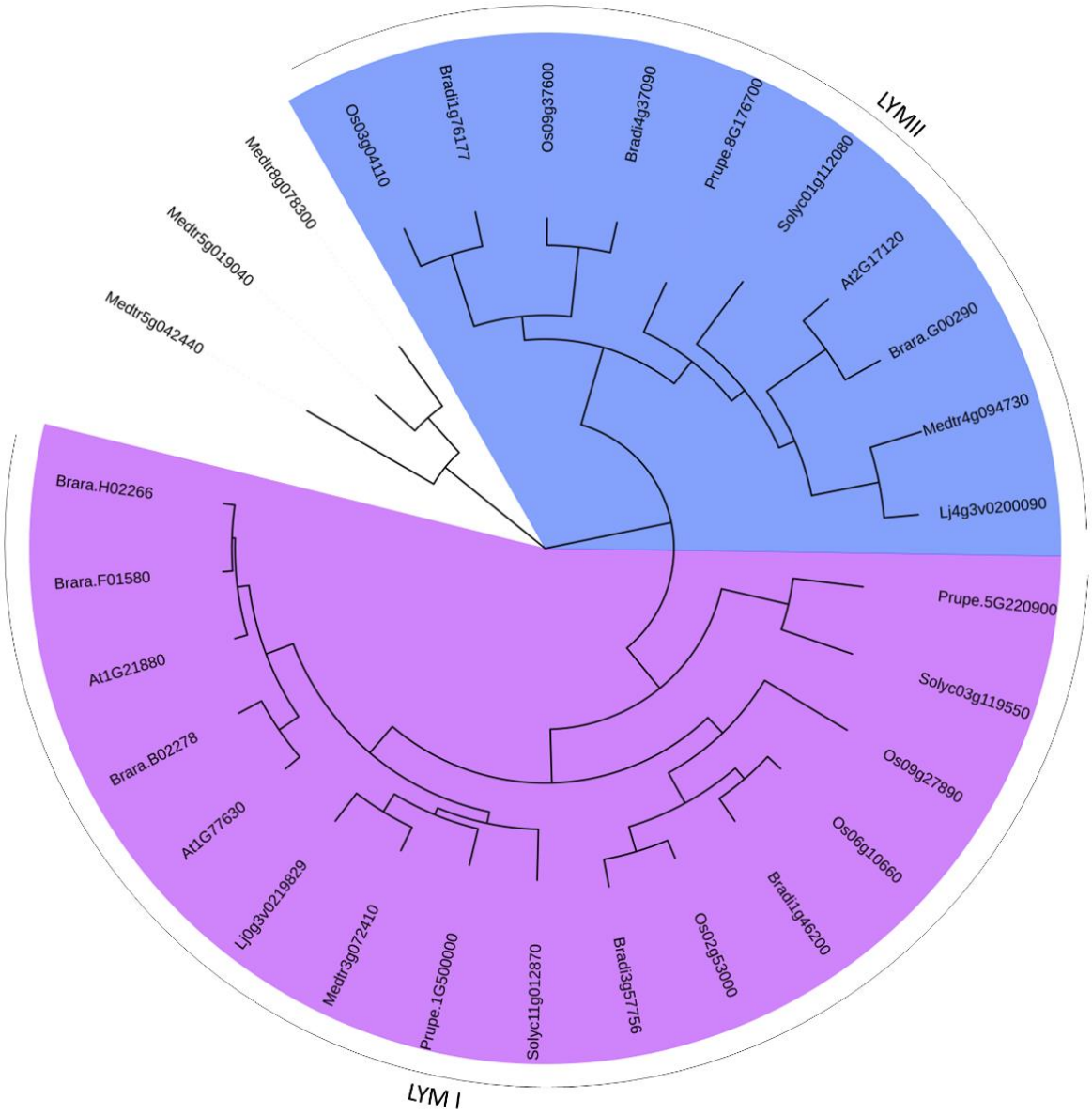

Fig. 3 PhyML phylogenetic tree of the LYM. Different phylogenetic groups are shown in different colors. ECR of 3 LYR proteins were used as outgroup sequences.

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 specifically bind PGN fragments (Willmann et al., 2011). The affinity of 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 N-acetylated 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.

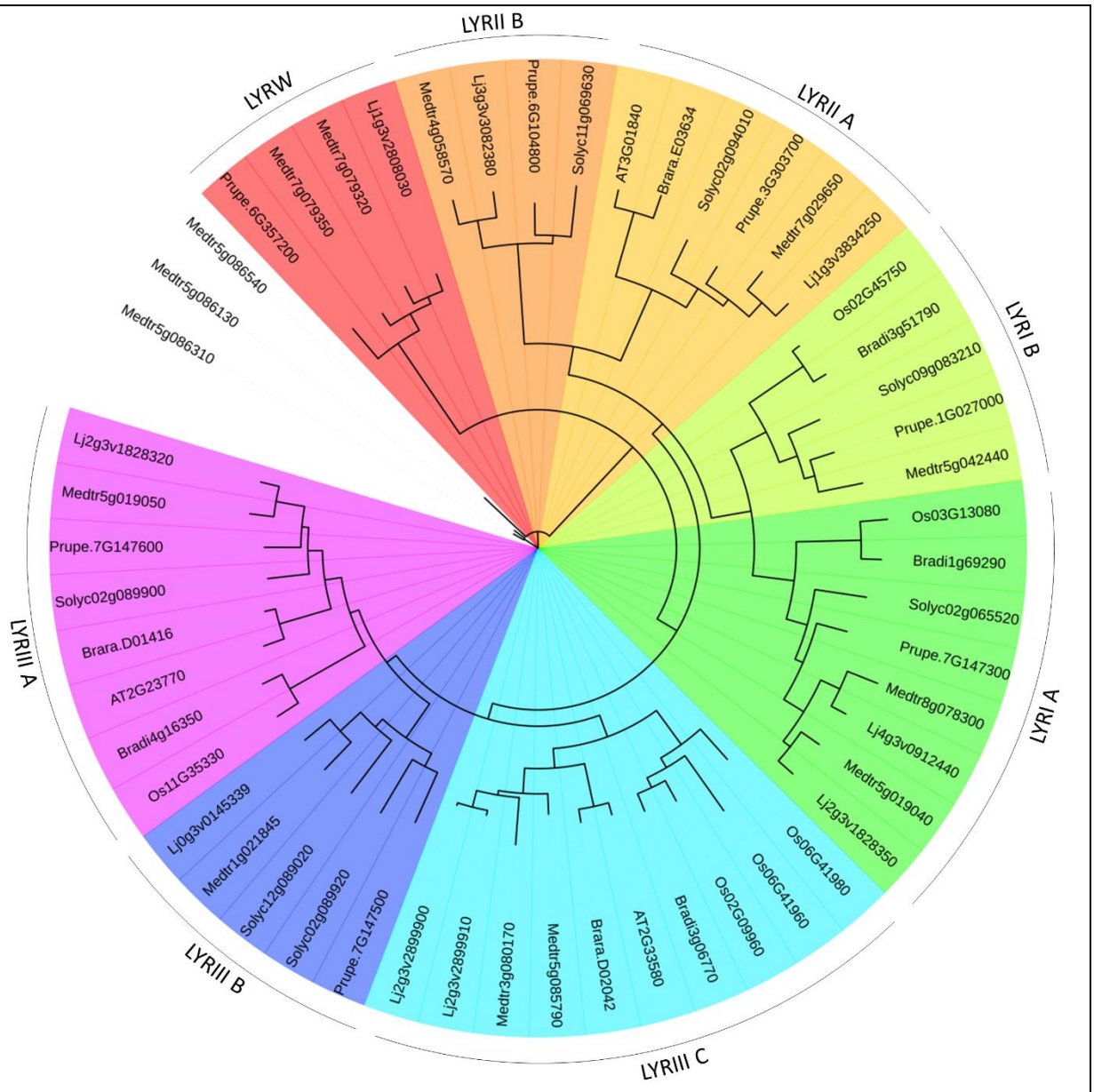

Fig. 4. PhyML phylogenetic tree of the LYR. Different phylogenetic groups are shown in different colors. Three LYK proteins were used as outgroup sequences.

Whereas *MtNFP* and *LjNFR5* are not essential for the establishment of the AMS in legumes (Ben Amor et al., 2003, Madsen et al., 2003), the tomato ortholog, *SlLYK10* (*Solyc02g065520*) has been shown to play a role in the AMS establishment (Buendia et al., 2016). Plant with decreased *SlLYK10* 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 groups (data not shown). This suggests that phylogeny of subgroup in the 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.

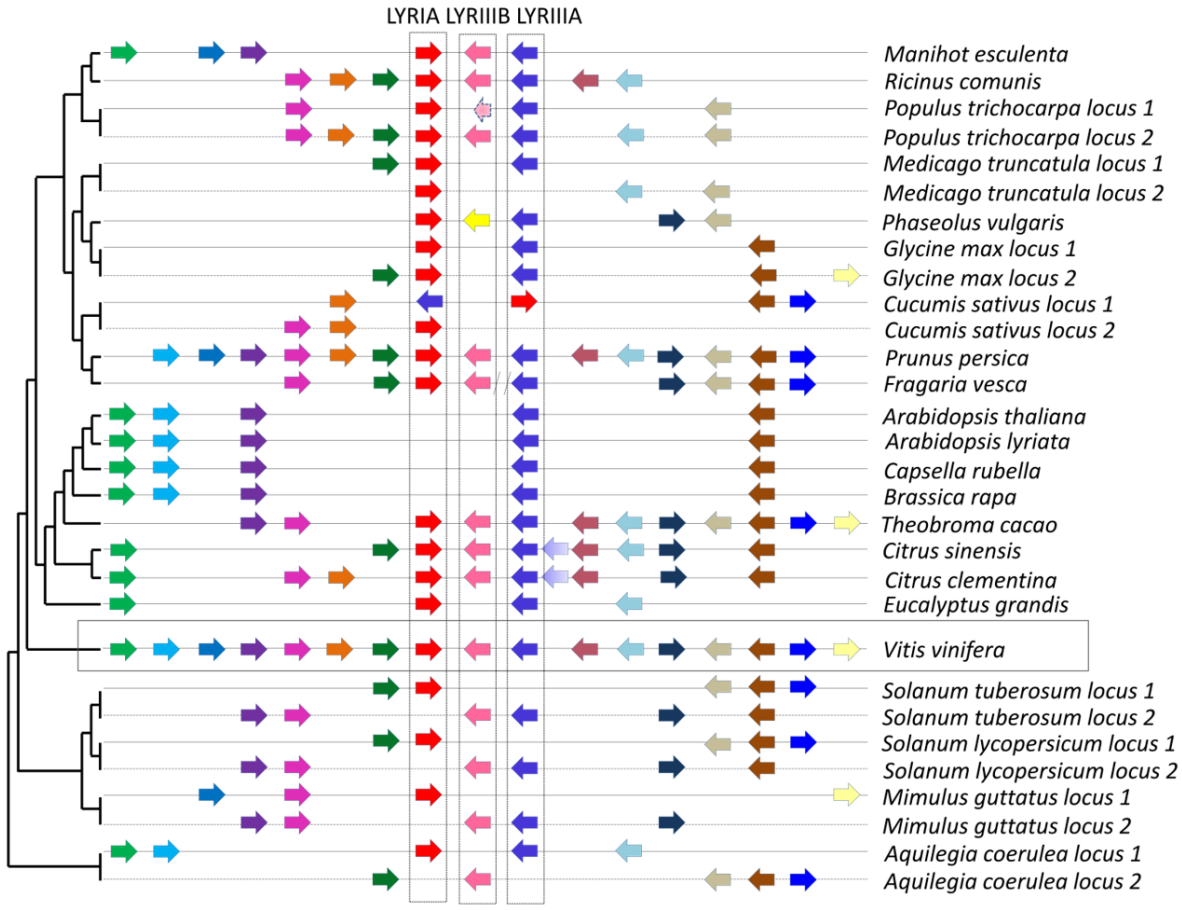

Fig. 5 Syntenic localization of the members of the phylogenetic groups LYRIA (red), LYRIIIB (pink) and LYRIIIA (blue). The orthologs are represented by similar color in the various genomes. Synteny was built by using the genome of *Vitis vinifera* as reference. The phylogenetic tree based on the plant 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. *Mtlyr4* 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*.

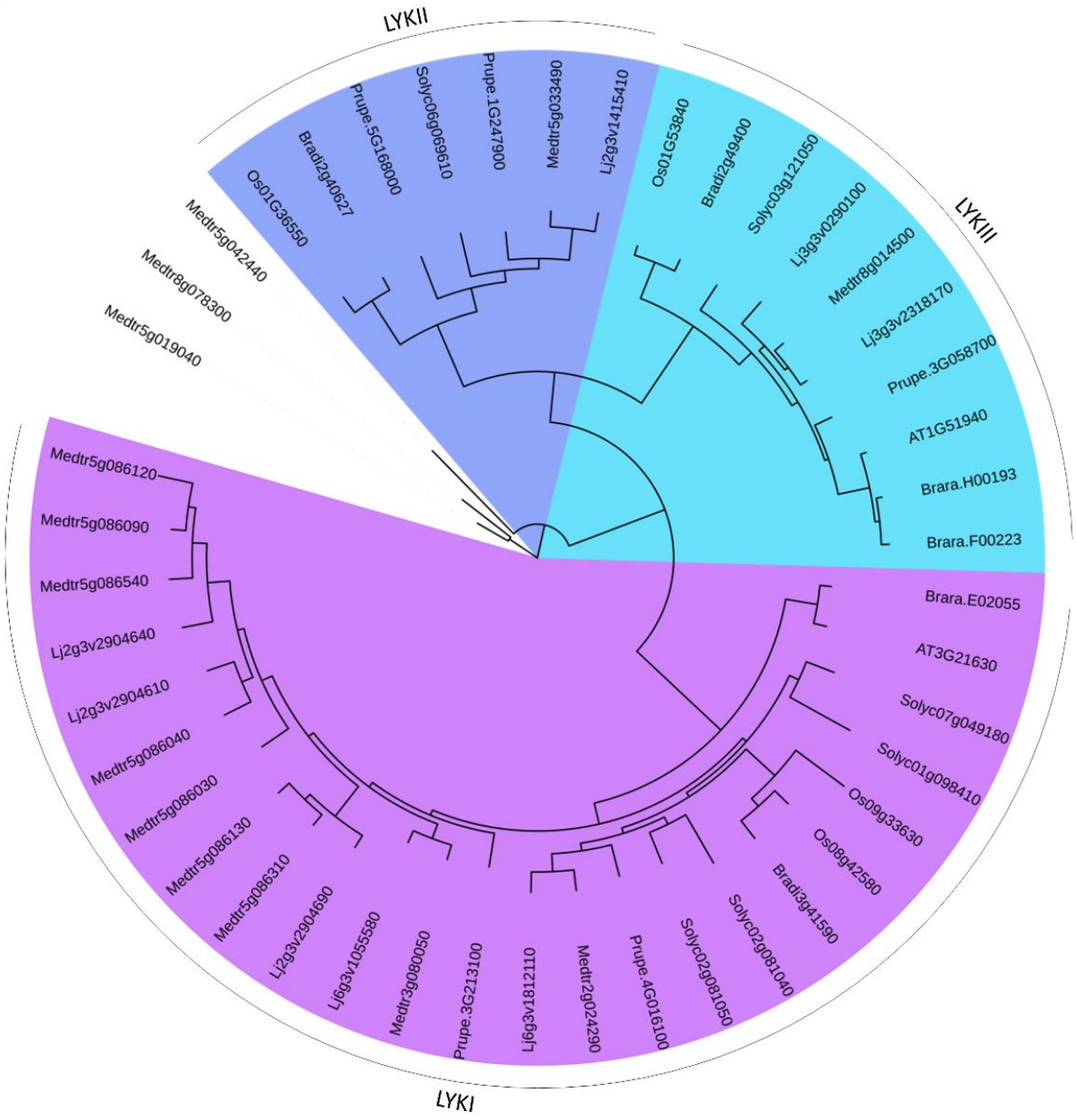

Fig. 6 PhyML phylogenetic tree of the LYK. Different phylogenetic groups are shown in different colors. Three LYR proteins were used as outgroup sequences.

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 µM. Similarly, other studies showed a low affinity binding (*Kd* of 44 µM and 455 µM 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 Ca^{2+} 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 µM for CO8, 227 µM 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.

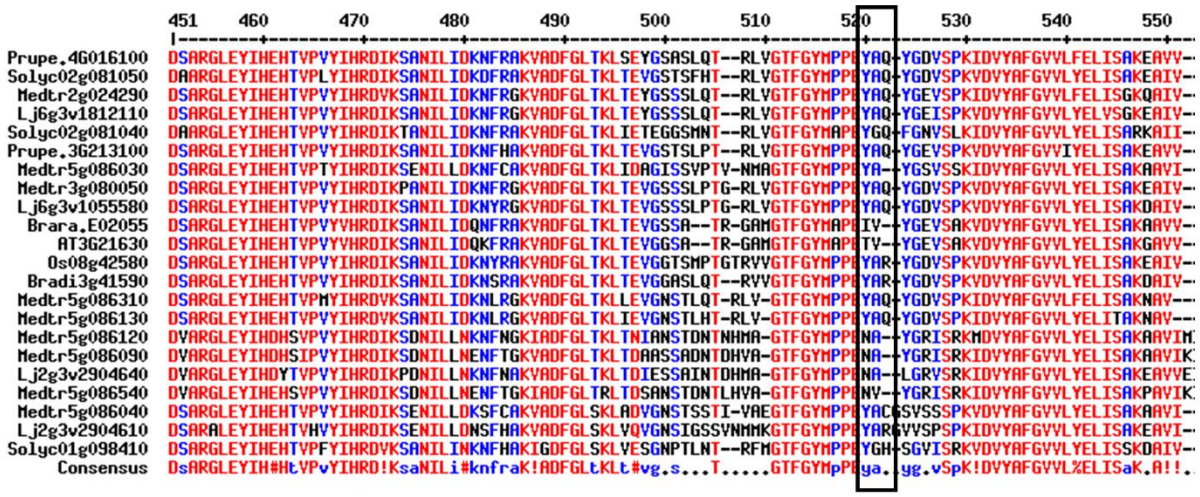

Fig. 7 Amino acid sequence alignment of the members of the phylogenetic group LYKI. The YAQ/R motifs is boxed in black.

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 *Ljepr3* 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 µM 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 µM) 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 (LYRIIIA) and MtLYK3 (LYKI, Fliegmann et al., 2016).

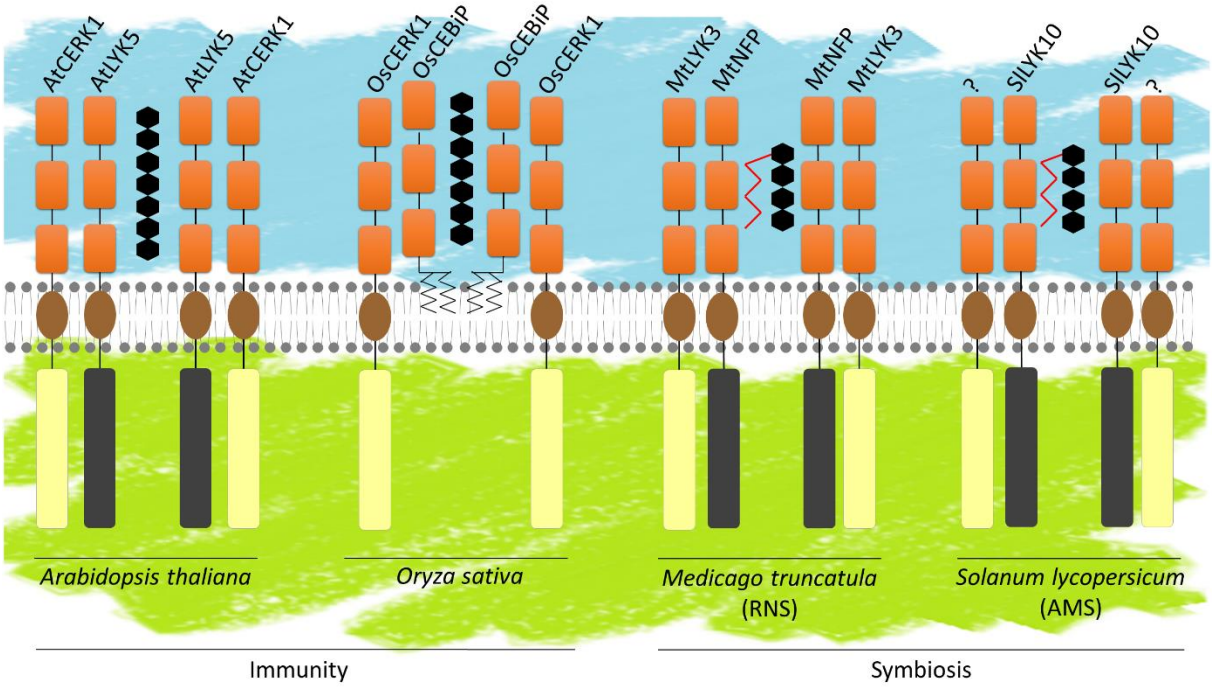

Fig. 8 LysM-RLK and/or LysM-RLP heterodimers. Known or hypothetical heterodimers involved in defense or symbiosis. Schematic representation of the LysM-RLKs/Ps as in Fig. 1. LysM-RLKs with beige ICR are LYKs, LysM-RLKs with grey ICR are LYRs.

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 µM and 4.5 µM 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 around 100 nM while using the OsCEBIP ECR produced in insect cells, the *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 Ca^{2+} 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, SlBti9 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^{-11}M 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 Nod-factor (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 Nod-factor 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 SlLYK10) 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 interaction	Ref	physical interaction	Ref
AtLYM3/AtCERK1	X	Willmann 2011		
AtLYM1/AtCERK1	X	Willmann 2011		
AtLYK4/AtCERK1	X	Wan 2012, Cao 2014		
AtLYK5/AtCERK1	X	Cao 2014	X	Cao 2014
OsLYP4/OsCERK1	X	Liu 2012a	X	Liu 2012a
OsLYP6/OsCERK1	X	Liu 2012a	X	Liu 2012a
OsCEBIP/OsCERK1	X	Liu 2016 Ao 2014 Kouzai 2014	X	Liu 2016 Hayafune 2014
TaCEBIP/TaCERK1	X	Lee 2014		
MtNFP/MtLYK3	X	Limpens 2003 arrighi 2006	X	
LjNFR5/LJNFR1	X	Madsen 2003 Radutoiu 2003	X	Madsen 2011
MtLYR3/MtLYK3			X	Fliegmann 2016

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Supplemental data

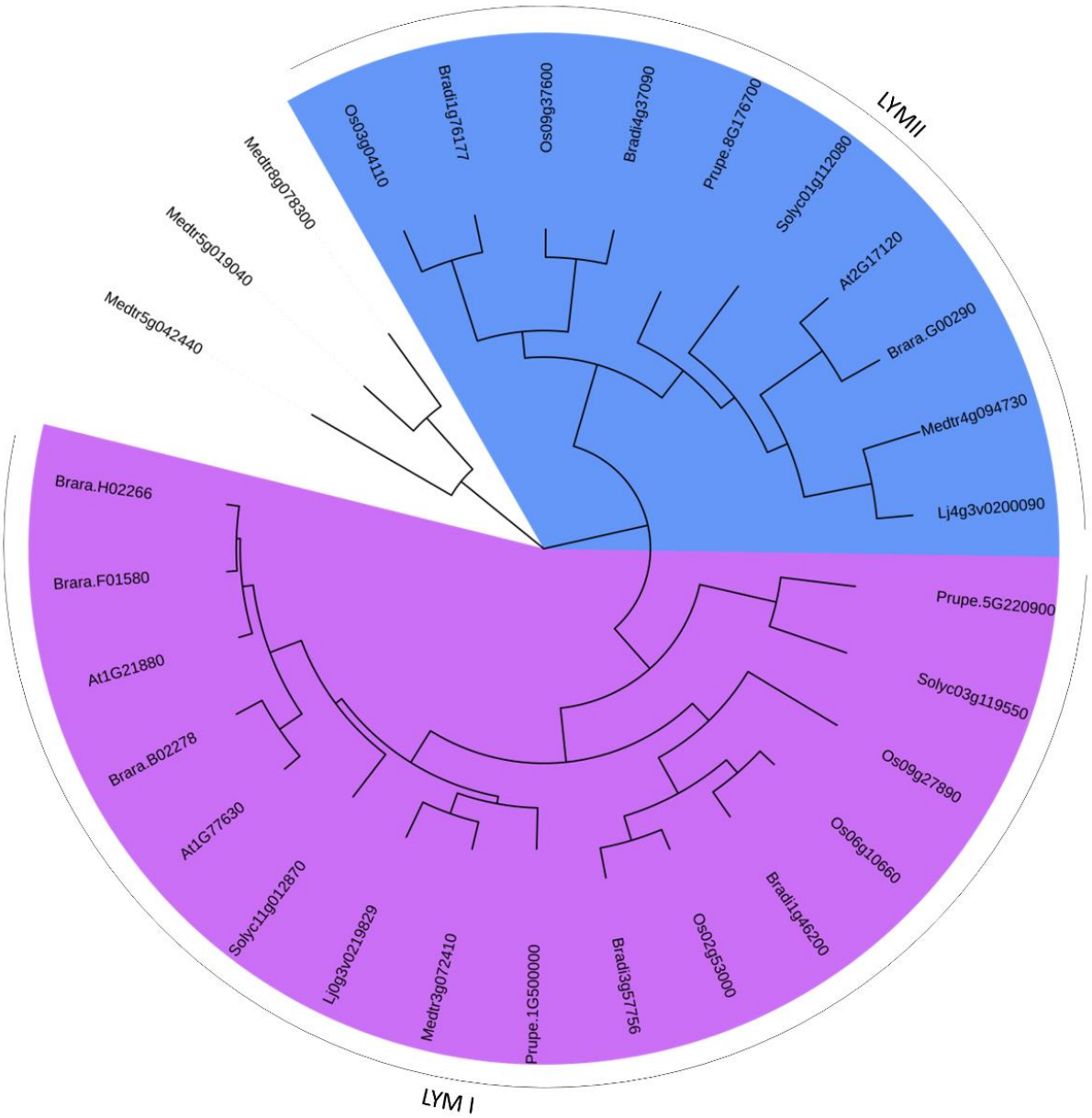

Fig. S1 MrBayes phylogenetic tree of the LYM. Different phylogenetic groups are shown in different colors. ECR of 3 LYR proteins were used as outgroup sequences.

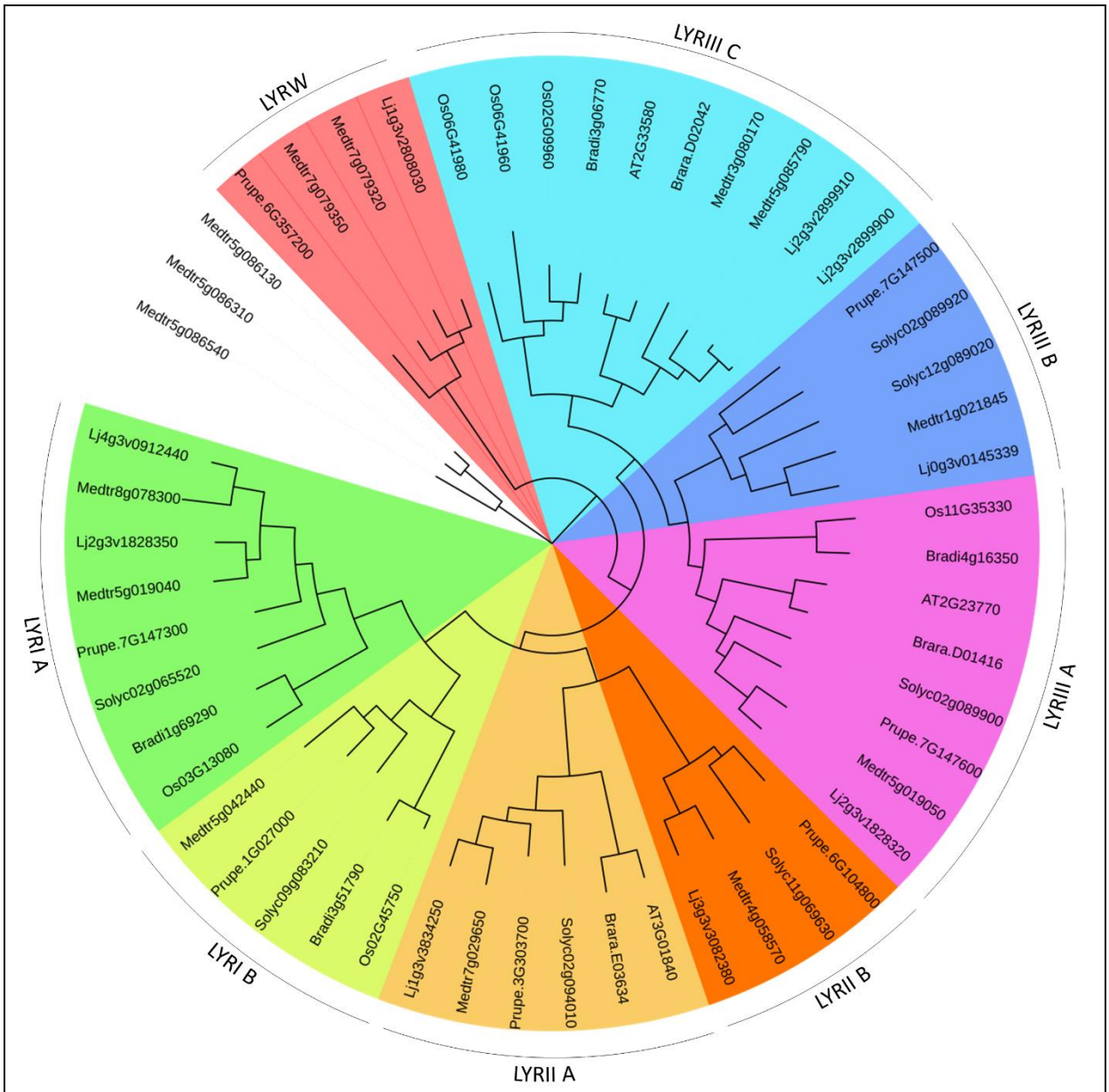

Fig. S2 MrBayes phylogenetic tree of the LYR. Different phylogenetic groups are shown in different colors. ECR of 3 LYK proteins were used as outgroup sequences.

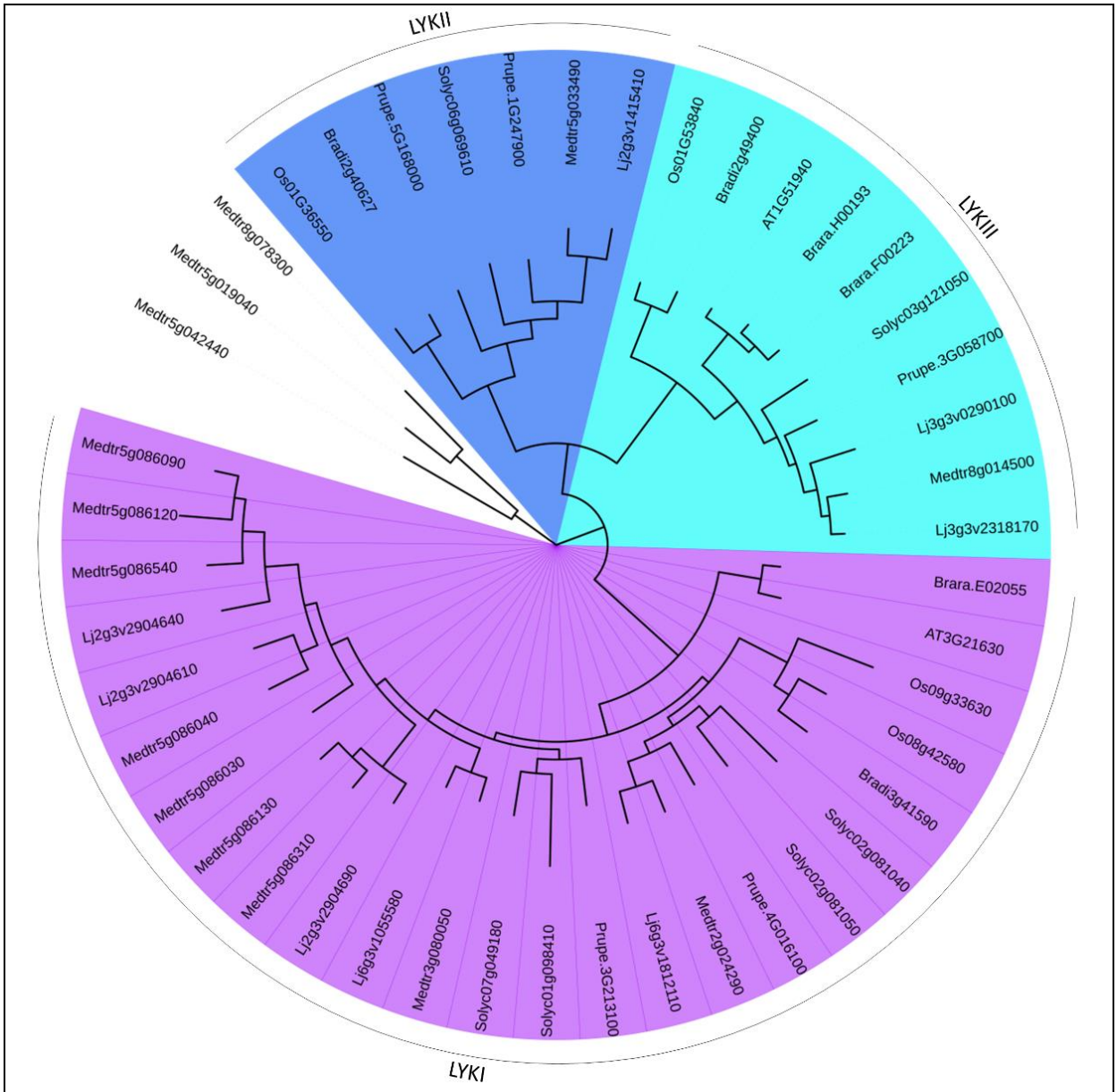

Fig. S3 MrBayes phylogenetic tree of the LYK. Different phylogenetic groups are shown in different colors. ECR of 3 LYR proteins were used as outgroup sequences.

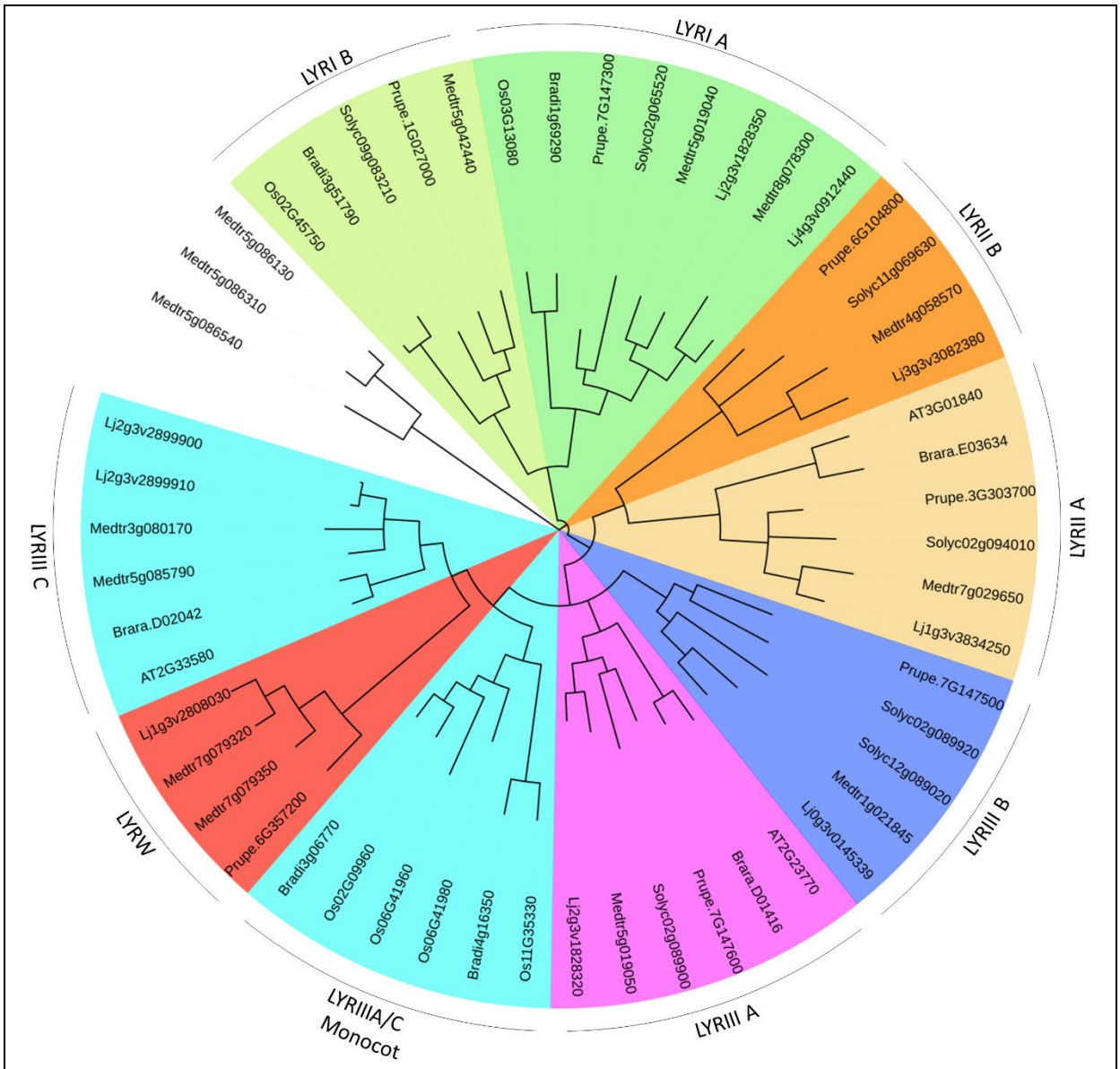

Fig. S4 MrBayes phylogenetic tree of the LYR using Gblock. Different phylogenetic groups are shown in different colors. ECR of 3 LYK proteins were used as outgroup sequences.

: truncated gene: no intracellular region

<u>nderlined :</u> <u>genes absent in previous phylogenetic analyses</u>

Italic : *genes not used in our phygenetic analysis*

* : prediction or sequence has been corrected

1	Last exon has been skipped 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
3	One short exon has been added according to the gene structure in other plant species and to symbimics data
4	One short exon has been added according to the gene structure in other plant species and to symbimics data
5	Start codon has moved according to the protein sequences in other plant species
6	The mRNA is annotated as two genes separated . Medtr5g086310 covers exon 1-6 and Medtr5g086330 covers 6-12
7	The mRNA can be found in two separated genes. Lj0g3v0102179 corresponds to exon 1 and Lj0g3v0124999 corresponds to exon 2
8	First short exon has been skipped according to the gene structure in other plant species
9	First short exon has been skipped according to the gene structure in other plant species
10	First short exon has been skipped. One short exon has been added and two intron borders have been moved according to the gene structure and protein sequences in other plant species
11	Start codon has moved according to the protein sequences in other plant species
12	A premature stop codon has been modified according to genomic sequences from other cultivars
13	Last exon has been skipped according to the gene structure in other plant species
14	Two exons have been added at the beginning of the gene according to the gene structure in other plant species
15	First exon has been skipped according to the gene structure in other plant species
16	Nucleotide A320 (from ATG) has been deleted accordingly to the sequence in *Solanum tuberosum*
17	Two intron have been added and one intron border has been modified according to the gene structure and protein sequences in other plant species
18	Start codon has moved according to the protein sequences in other plant species
19	Two exons have been added at the beginning of the gene according to shimizu et al., 2010
20	Five exons have been added at the beginning of the gene according to the gene structure and protein sequence in other plant species
21	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
LYPIII	**LYMI**	Medtr3g072410	LYM1	4	Lj0g3v0219829	4*[8]	Brara.F01580 Brara.H02266 Brara.B02278		4 2 3	At1g21880 At1g77630	AtLYM1 AtLYM3	4 4
?												
LYPI	**LYMII**	Medtr4g094730	LYM2	3	Lj4g3v0200090	3	Brara.G00290		3	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
LYPIII	**LYMI**	Prupe.1G500000	PpLYM1	4	Solyc11g012870	4	Bradi3g57756 Bradi1g46200	BdLYM1 BdLYM3	4 4	Os06g10660 Os02g53000 Os09g27890	OsLYP6 OsLYP5 OsLYP4	4 4 4
?		Prupe.5G220900	PpLYM3	4	Solyc03g119550	4*[16]						
LYPI	**LYMII**	Prupe.8G176700	PpLYM2	3	Solyc01g112080	3	Bradi1g76177 Bradi4g37090	BdLYM2 BdLYM4	3 3	Os03g04110 Os09g37600	OsCEBiP OsLYP3	3 3

	Clade			Medicago (Mt4)	Alternative name	Nb intron	Lotus (V3.0)	Alternative name	Nb intron	Peach (V2.1)	Alternative name	Nb intron	Tomato (ITAG2.3)	Alternative name	Nb intron
LYR	LysM-RLK I	LYRI	A	Medtr5g019040	NFP	0	Lj2g3v1828350	LjNFR5	0	Prupe.7G147300	PpLYR1	0	Solyc02g065520	SlLyk10	0
				Medtr8g078300	LYR1	0	Lj4g3v0912440	LjLYS11	0						
			B	Medtr5G042440	LYR8	1	*Lj0g3v0102179+3v0124999*		1*[7]	Prupe.1G027000	PpLYR2	1	Solyc09g083210	SlLyk9	1
													Solyc09g083200 #	SlLyk8	1
	LysM-RLK IV	LYRII	A	Medtr7g029650	LYR10	0	Lj1g3v3834250	LjLYS16	0	Prupe.3G303700	PpLYR6	0	Solyc02g094010	SlLyk2	1
			B	Medtr4g058570	LYR9	0	Lj3g3v3082380	LjLYS15	0	Prupe.6G104800	PpLYR7	0	Solyc11g069630	SlLyk15	0*[13]
	LysM-RLK II	LYRIII	A	Medtr5g019050	LYR3	0	Lj2g3v1828320	LjLYS12	0	Prupe.7G147600	PpLYR3	0*[9]	Solyc02g089900	SlLyk4	0
			B	Medtr1g021845	LYR2	0	Lj0g3v0145339		0	Prupe.7G147500	PpLYR4	0	Solyc02g089920	SlLyk7	0
													Solyc12g089020	SlLyk6	0*[12]
	LysM-RLK III		C	Medtr5g085790	LYR4	0	Lj2g3v2899910	LjLYS13	0						
				Medtr3g080170	LYR7	0	Lj2g3v2899900	LjLYS14	0						
		LYRIV		Medtr7g079350	LYR5	1*[1]	*absent in V3.0*	*LjLYS21*	1						
				Medtr7g079320	LYR6	1	Lj1g3v2808030	LjLYS20	1	Prupe.6G357200	PpLYR5	1			

	Clade			Brassica rapa (V1.3)	Alternative name	Nb intron	Arabidopsis (TAIR10)	Alternative name	Nb intron	Brachypodium (V3.1)	Alternative name	Nb intron	Rice (V7)	Alternative name	Nb intron
LYR	LysM-RLK I	LYRI	A							Bradi1g69290	Bd LYR1	0	Os03G13080	OsLysM-RLK2/OsLYK5/OsNFR5	0
			B							Bradi3g51790	Bd LYR2	1	Os02G45750		1
	LysM-RLK IV	LYRII	A	Brara.E03634		1	At3g01840	AtLyk2	1						
			B												
	LysM-RLK II	LYRIII	A	Brara.D01416		0	At2g23770	AtLyk4	0	Bradi4g16350	Bd LYR4	0	Os11G35330	OsLysM-RLK7 / OsLYK6	0
			B												
	LysM-RLK III		C	Brara.D02042		0	At2g33580	AtLyk5	0	Bradi3g06770	Bd LYR3	0	Os06G41960	OsLysM-RLK6 / OsLYK3	0
													Os06G41980	OsLysM-RLK5 / OsLYK2	0
													Os02G09960	OsLysM-RLK1 / OsLYK4	0
		LYRIV													

	Clade		Medicago (Mt4)	Alternative name	Nb intron	Lotus (V3.0)	Alternative name	Nb intron	Peach (V2.1)	Alternative name	Nb intron	Tomato (ITAG2.3)	Alternative name	Nb intron
LYK	LysM-RLK VI	LYKI	Medtr5g086540	LYK1	10*[2]	Lj2g3v2904640	LjLYS2	11						
			Medtr5g086120	LYK4	11*[3]									
			Medtr5g086090	LYK5	11*[4]									
			Medtr5g086040	LYK6	10	Lj2g3v2904610	LjLYS1	11						
			Medtr5g086030	LYK7	11*[5]									
			Medtr5g086310+5g086330	LYK2	11*[6]									
			Medtr5g086130	LYK3	11	Lj2g3v2904690	LjNFR1	11						
			Medtr3g080050	LYK9	12	Lj6g3v1055580	LjLYS6/LjCERK6	12	Prupe.3G213100	PpLYK2	11*[10]			
			Medtr2g024290	LYK8	11	Lj6g3v1812110	LjLYS7	11	Prupe.4G016100	PpLYK1	11	Solyc02g081050	SlLyk12	11
												Solyc02g081040	SlLyk11	11*[14]
												Solyc07g049180	SlLyk1 / SlBti9	11*[15]
												Solyc01g098410	SlLyk13	11
	LysM-RLK V	LYKII	Medtr5g033490	LYK10	9	Lj2g3v1415410	LjLYS3 / EPR3	9	Prupe.5G168000	PpLYK3	9	Solyc06g069610	SlLyk14	9
									Prupe.1G247900	PpLYK4	9			
		LYKIII	Medtr8g014500	LYK11	10	Lj3g3v2318170	LjLYS5	10	Prupe.3G058700	PpLYK5	10	Solyc03g121050	SlLyk3	10
						Lj3g3v0290100	LjLYS4	10						
	?													

	Clade		Brassica rapa (V1.3)	Alternative name	Nb intron	Arabidopsis (TAIR10)	Alternative name	Nb intron	Brachypodium (V3.1)	Alternative name	Nb intron	Rice (V7)	Alternative name	Nb intron
LYK	LysM-RLK VI	LYKI	Brara.E02055	Brara.E02056	11	At3g21630	AtLyk1/AtCERK1	11	Bradi3g41590	Bd LYK1	11*[17]	Os08g42580	OsLysM-RLK9/CERK1	11*[19]
												Os09g33630	OsLysM-RLK10	12*[20]
	LysM-RLK V	LYKII							Bradi2g40627	Bd LYK2	9*[18]	Os01G36550	OsLysM-RLK3/LYK1	9*[21]
		LYKIII	Brara.H00193	Brara.H00194	10*[11]	At1g51940	AtLyk3	10	Bradi2g49400	Bd LYK3	10	Os01G53840	OsLysM-RLK4	10
			Brara.F00223	Brara.F00224	8									
	?											*Os11g34624*	*OsLysM-RLK8*	*6*

About Root endo-symbioses establishment

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 micro-organisms. 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.

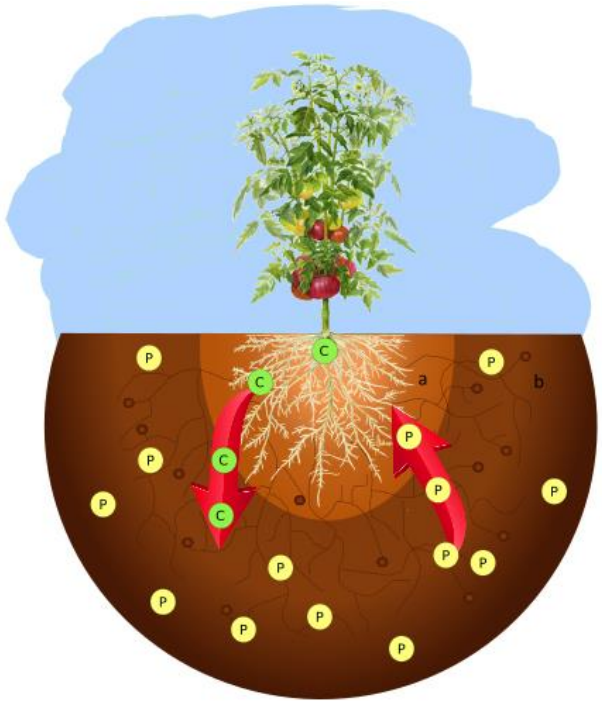

Fig. 9 The hyphal network of AMF explores a larger soil area than plant roots. Roots of plants can explore a restrained soil area (a) that will be rapidly deprived in mineral nutrients such as phosphate (P). In contrast, AMF can explore a larger soil volume (b) and collect nutrients such as P that will 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, lipo-chitooligosaccharides (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).

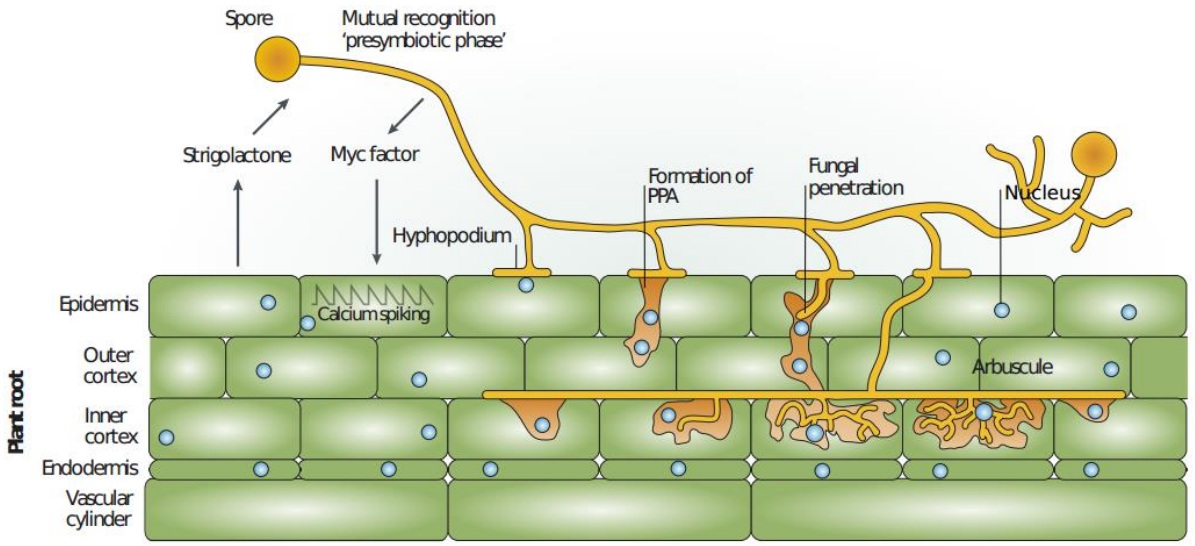

Fig. 10 Steps of AMS establishment. Plant roots exude signals such as strigolactones which induce spore germination and hyphal branching. Fungi produce Myc-Factors that induce in and around the nuclei oscillations of calcium concentration, called calcium spiking. At the epidermis, AMF form special types 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).

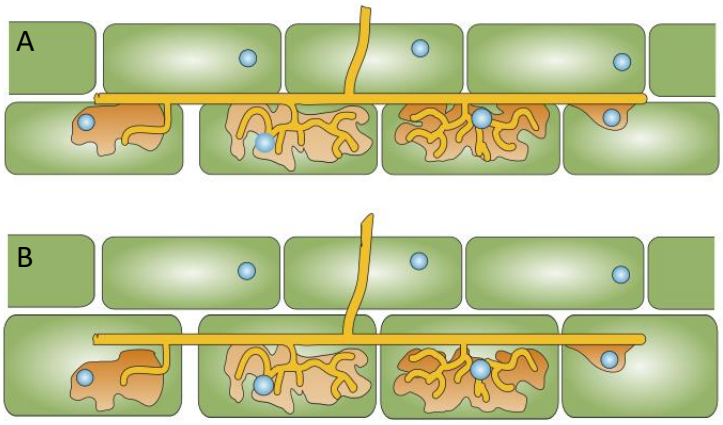

Fig. 11 *Arum* and *Paris* type of root colonization. A) *Arum* type: the hypha spreads between cortical cells. **B)** *Paris* type: the hypha spreads from cell to cell.

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.

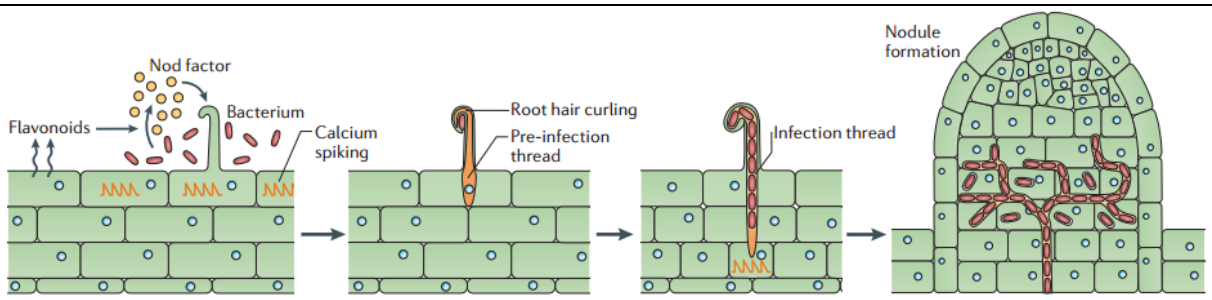

Fig. 12 RLS establishment. Flavonoids are secreted by the plant root and perceived by rhizobia in the rhizosphere. They induce the production of nodulation factors (Nod factors) that are recognized by the plant. Nod factor perception activates the symbiosis signaling pathway, leading to calcium spiking. 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). Ca^{2+} spiking is a secondary messenger of the CSSP. MtDMI2/LjSYMRK is an LRR-RLK that acts upstream the Ca^{2+} spiking and might be activated by Nod-factor and Myc-factor receptors. Ca^{2+} spiking is controlled by potassium importer in the nucleus (MtDMI1/ LjCASTOR and LjPOLLUX) and generated by calcium release in the nucleus/cytoplasm through the Ca^{2+} channels MtCNGC15 a, b and c (Charpentier et al., 2016) and by Ca^{2+} uptake from the nucleus/cytoplasm through a calcium pump, the ATPase MtMCA8 (Capoen et al., 2011). These Ca^{2+} 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.

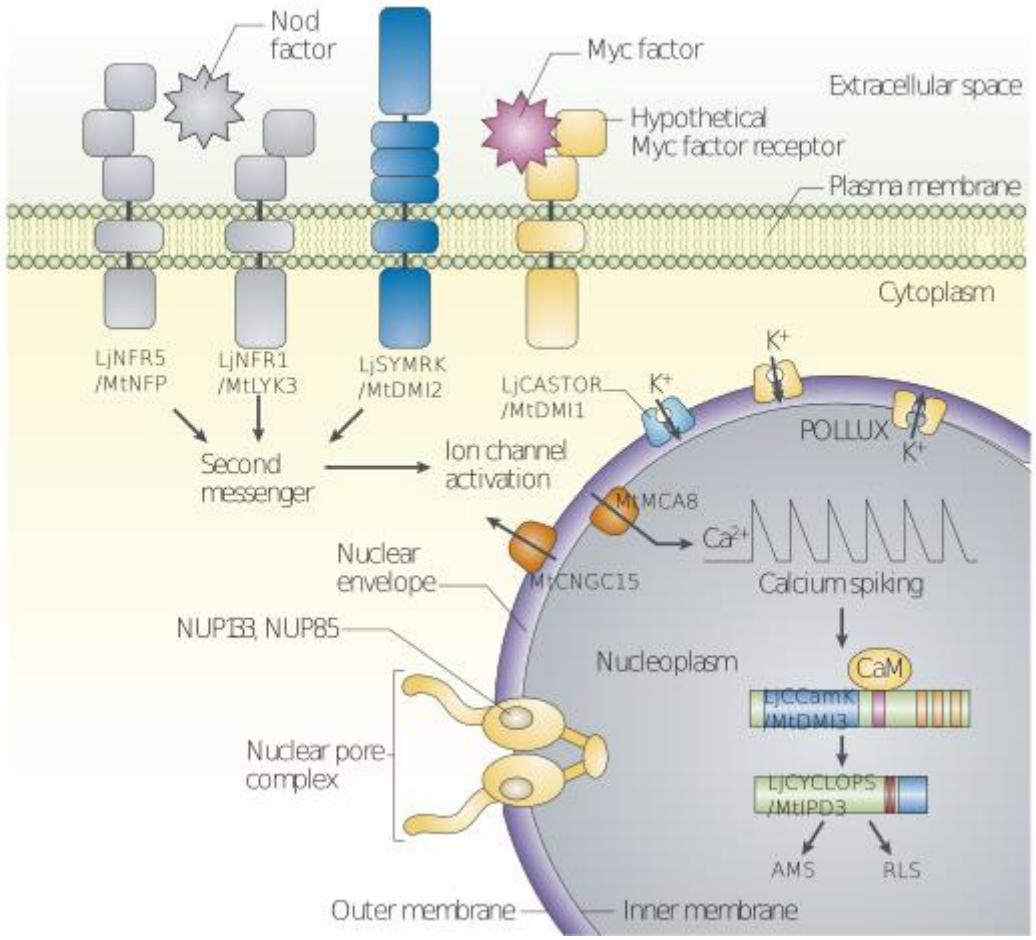

Fig. 13: Common Symbiosis Signaling Pathway components and model. Perception of AMF or rhizobial signals triggers early signal transduction, which is mediated by at least seven shared components. The symbiosis receptor kinase LjSYMRK/MtDMI2 acts upstream of the Nod factor- and Myc factor-induced calcium signatures (Ca^{2+} spiking) occurring in and around the nucleus. Ca^{2+} 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 Ca^{2+} 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).

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Objectives of my thesis

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 AMS have evolved to be involved in LCO perception in the frame of the RLS. During my PhD I mainly studied *SlLYK10* 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.

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 SlLYK10 and BdLYR1 LCO receptors?

ii) Are *SlLYK10* and *BdLYR1* important for the AMS establishment?

iii) Can *SlLYK10* 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:

SlLYK10 regulates the AMS in tomato

Preamble

SlLYK10 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 *SlLYK10* might play a role in AMS. To demonstrate this hypothesis, *SlLYK10* 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.

New Phytologist

The LysM receptor-like kinase SlLYK10 regulates the arbuscular mycorrhizal symbiosis in tomato

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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 calmodulin-dependent protein kinase (CCaMK), lysin motif receptor-like kinase (LysM-RLK), *NFP*, *NFR5*, *Solanum lycopersicum*, *Solyc02 g065520*, tomato.

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 SlLYK10, whose orthologs in legumes are essential for nodulation, but not for AM, and SlCCaMK, 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 *SlLYK10* 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.

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 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 calmodulin-dependent 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

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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-LCO-induced 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 $MgCl_2$, 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 (OD_{600}) = 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 10 s 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 × modified Long Ashton medium (7.5 μM NaH_2PO_4) 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 transcription-polymerase 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-α*) 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.ipk-gatersleben.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 *SlPDS* was used in further experiments as a control for VIGS efficiency. The average efficiency of *SlPDS* VIGS, as measured by the percentage of plants with observable bleached leaves at 3 wk after *A. tumefaciens* inoculation, was 53.8% (± 17.4%, $n = 45$).

Silencing of *SlCCaMK* 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 *SlCCaMK* by the pTRV2-*SlCCaMK* 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 *SlCCaMK* 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 *t*-test *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-*SlCCaMK*-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

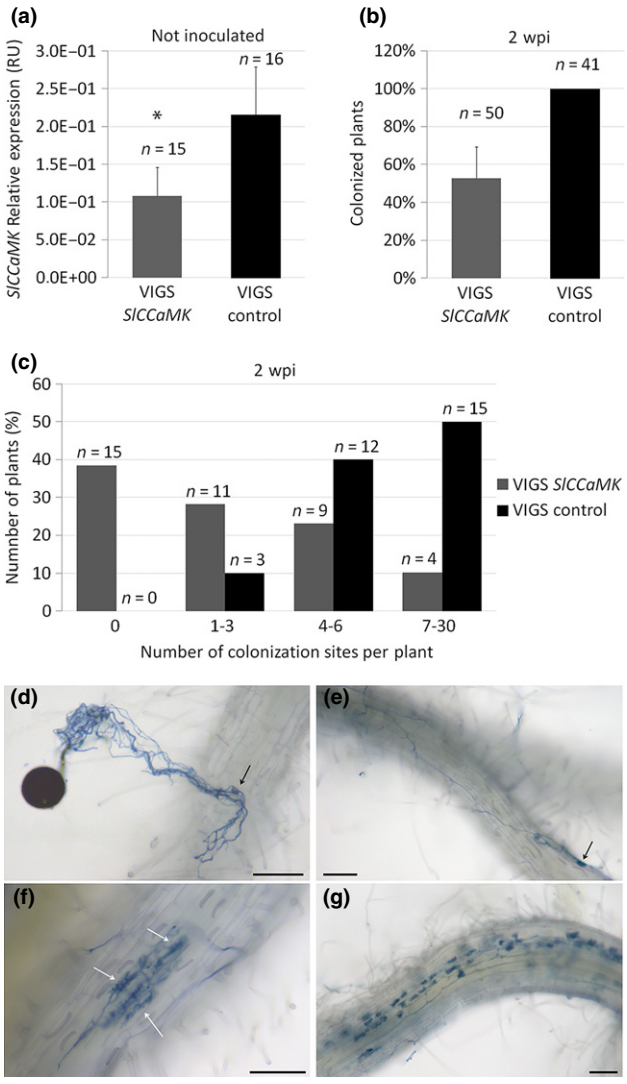

Fig. 1 *SlCCaMK* is essential for arbuscular mycorrhiza (AM) at 2 wk post-inoculation (wpi) with *Rhizophagus irregularis*. (a) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), of *SlCCaMK* in *Solanum lycopersicum* roots inoculated with *Agrobacterium tumefaciens* bearing pTRV2-*SlCCaMK* 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 *SlCCaMK* on AM. Entire root systems of plants inoculated with pTRV2-*SlCCaMK* 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-*SlCCaMK*. 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.

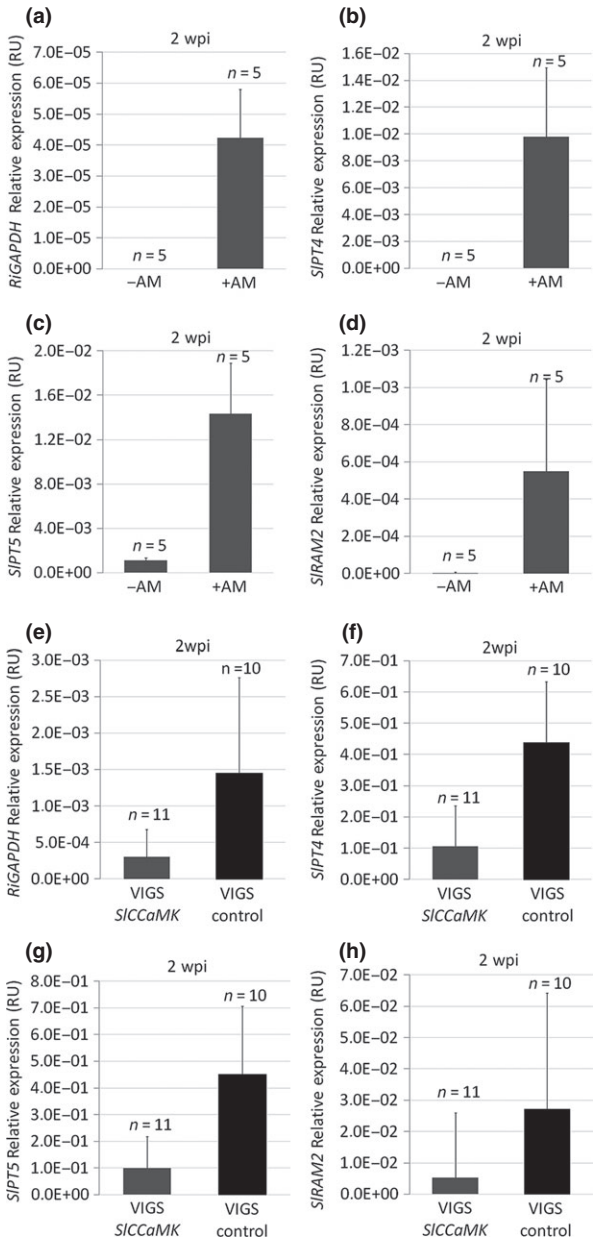

Fig. 2 *SlCCaMK* is essential for plant symbiotic gene expression at 2 wk post-inoculation (wpi) with *Rhizophagus irregularis*. (a–d) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), of the indicated genes in wild-type (WT) *Solanum lycopersicum* 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) *SlPT4*, (c) *SlPT5* and (d) *SlRAM2* are specifically detected in roots inoculated with *R. irregularis*. (e–h) Analysis of the effect of silencing of *SlCCaMK* on arbuscular mycorrhiza (AM). Relative expression, measured by qRT-PCR, of the indicated genes in roots inoculated with *Agrobacterium tumefaciens* carrying pTRV2-*SlCCaMK* 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, *SlPT4* and *SlPT5* (f, g) the degree of arbuscule formation, and *SlRAM2* (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.

SlLYK10 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 SlSYMRK, 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 *MtNFP/MtLYR1* 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

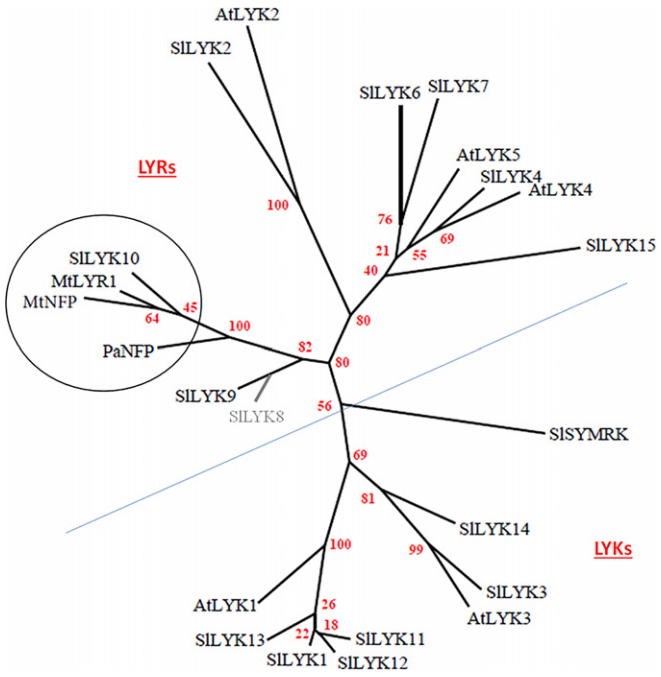

Fig. 3 *SlLYK10* is the *Medicago truncatula MtNFP* ortholog. Intracellular region amino acid sequences of all lysin motif receptor-like kinases (LysM-RLKs) from *Solanum lycopersicum* and *Arabidopsis thaliana*, plus *M. truncatula* MtNFP and MtLYR1 and *Parasponia andersonii* PaNFP (see Supporting Information Table S1 for accession numbers), were used to construct a phylogenetic tree. SlSYMRK, 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).

SlLYK10 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

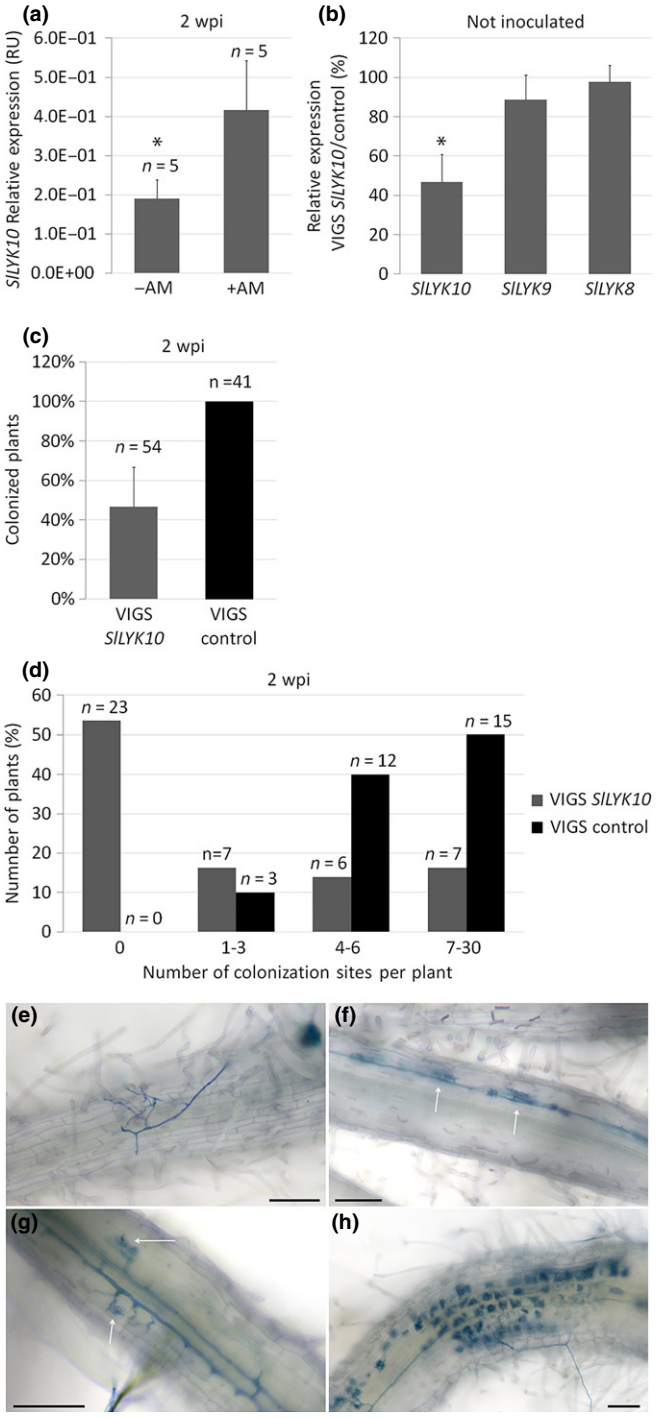

Fig. 4 *SlLYK10* is essential for arbuscular mycorrhiza (AM) at 2 wk post-inoculation (wpi) with *Rhizophagus irregularis*. (a) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), of *SlLYK10* in wild-type (WT) *Solanum lycopersicum* roots inoculated (+ 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-*SlLYK10* specifically silences *SlLYK10*. Relative expression in roots inoculated with pTRV2-*SlLYK10* vs control pTRV2 of *SlLYK10* 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 qRT-PCR. Mean and standard error (SE) between three biological replicates are shown, corresponding to a total of 19 pTRV2-*SlLYK10*-inoculated and 10 control roots. A significant difference was detected for the *SlLYK10* transcript level in pTRV2-*SlLYK10*-inoculated roots compared with control roots (*, $P < 0.05$). (c–h) Analysis of the effect of silencing of *SlLYK10* on AM. Entire root systems of plants inoculated with pTRV2-*SlLYK10* 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-*SlLYK10*: 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.

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 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

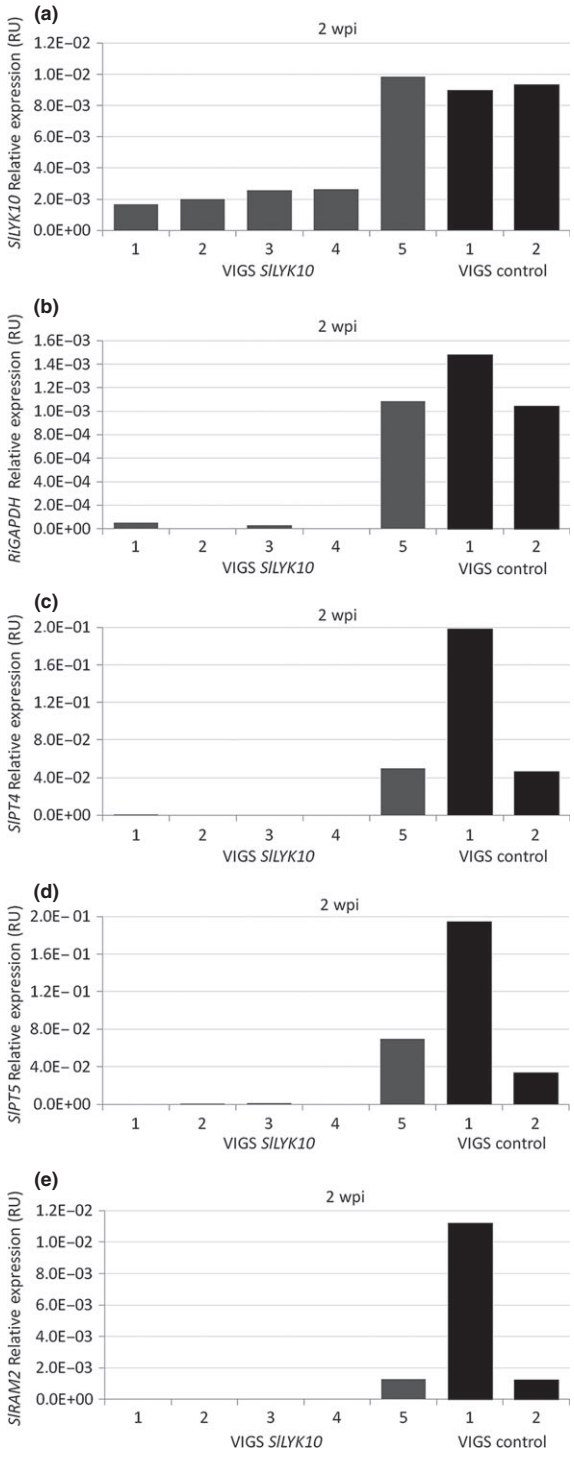

Fig. 5 Strong silencing of *SlLYK10* correlates with the absence of fungal colonization and plant symbiotic gene expression at 2 wk post-inoculation (wpi) with *Rhizophagus irregularis*. (a–e) Relative expression, measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), of the indicated genes in *Solanum lycopersicum* roots inoculated with pTRV2-*SlLYK10* 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, *SlPT4* and *SlPT5* (c, d) the degree of arbuscule formation, and *SlRAM2* (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 SlLYK10 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

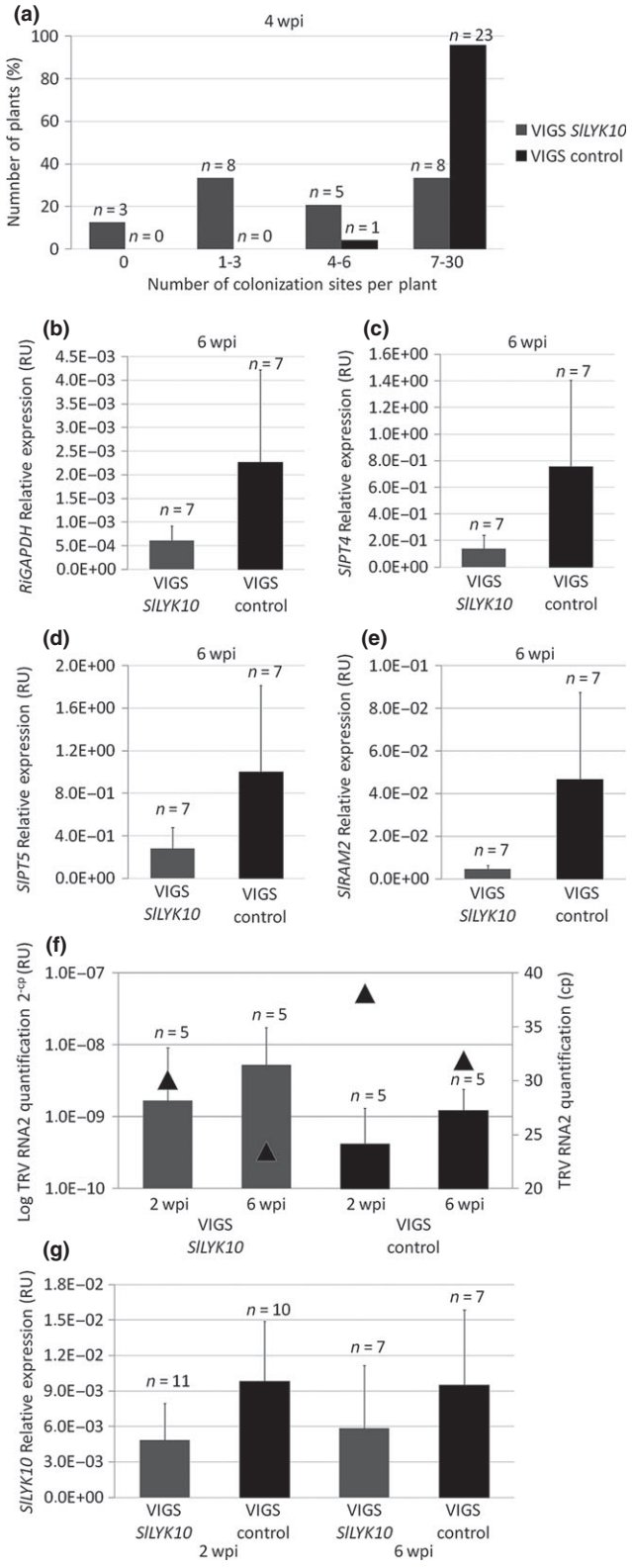

Fig. 6 Silencing of *SlLYK10* reduces arbuscular mycorrhiza (AM) at 4 and 6 wk post-inoculation (wpi) with *Rhizophagus irregularis*. (a) Number of colonization sites per plant (*Solanum lycopersicum*). Entire root systems of plants inoculated with pTRV2-*SlLYK10* or control pTRV2 were stained by an 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 (qRT-PCR), of the indicated genes in roots inoculated with pTRV2-*SlLYK10* 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 qRT-PCR of *Tobacco rattle virus* (TRV) RNA2 in root systems inoculated with pTRV2-*SlLYK10* 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 *y*-axis). (g) Relative expression, measured by qRT-PCR, of *SlLYK10* in roots inoculated with pTRV2-*SlLYK10* 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.

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 *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 co-receptor 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 SlLYK10, 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 SlLYK10 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-amino-acid 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): SlLYK1 and SlLYK12. 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.

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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

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

Table S1

Purpose	Sequence	Primer name
Cloning in TRV2	GGAATTCCTTCTTAGTTTATGAATATGC	VIGS SlLYK10 F
Cloning in TRV2	CCGCTCGAGCACCTGAAGTCCAAGACGGTCC	VIGS SlLYK10 R
Cloning in TRV2	GAGAATTCACCATTAAAGATTATGG	VIGS SlCCamK F
Cloning in TRV2	CCGCTCGAGCTTCAAACTCCAACAATGTTGC	VIGS SlCCamK R
qRT-PCR	GGGTGAGGGGAGAGTTAGCA	SlCCamK F
qRT-PCR	CTGCTGCACGAAACTTACGC	SlCCamK R
qRT-PCR[(1)]	CGTCCTCCAACGTAACTGCT	SlLYK10 F1
qRT-PCR[(1)]	GTGGACGGGCACGATATGAA	SlLYK10 R1
qRT-PCR[(2)]	AGTCCGGTAATAGCACGTTG	SlLYK10 F2
qRT-PCR[(2)]	TCAGATTAGTTATCGTTCATGTGGC	SlLYK10 R2
qRT-PCR	TTACACGGCCAGCATCTTCA	SlLYK8 F
qRT-PCR	GCCCACAAACCCCTAAACCA	SlLYK8 R
qRT-PCR	ACAGAGCTAGAGCACCAGAGT	SlLYK9 F
qRT-PCR	ACAAGAACACGTGATGGGGA	SlLYK9 R
qRT-PCR	GCTGCTGGTGATGATCCTGT	SlGAPDH F
qRT-PCR	GCGTTTGTGGTCCGAGAGAA	SlGAPDH R
qRT-PCR	CCAGGGGCATCAATCACAGT	SlEF1-α F
qRT-PCR	AGTAAGCCTGGGTGCTTGAC	SlEF1-α R
qRT-PCR	TCTTCTCTCCGGGTTAGTACG	SlCCamK intron F
qRT-PCR	TCGCGGAAAAATTCGTTTTGGA	SlCCamK intron R
qRT-PCR	CGGGCAGAATGAGACACAGATG	SlPT4 F
qRT-PCR	GAAGATAGAAAGCACAAGGCGTAGT	SlPT4 R
qRT-PCR	GCAGAACGAGACGCAGATGAA	SlPT5 F
qRT-PCR	TGCTGAATTTGATAAACTTGCCAA	SlPT5 R
qRT-PCR	AAGCCAGGAGTACTTGTTGGTAA	SlRAM2 F
qRT-PCR	TGGACTAAACGACCATCATGGA	SlRAM2 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
SlCCamK	*Solyc01g096820*		
SlRAM2	*Solyc04g005840*		
AtLYK1	*At3g21630*	*CERK1*	LYK
AtLYK2	*At3g01840*		LYR
AtLYK3	*At1g51940*		LYK
AtLYK4	*At2g23770*		LYR
AtLYK5	*At2g33580*		LYR
SlLYK1	*Solyc07g049180*	*Bti9*	LYK
SlLYK2	*Solyc02g094010*		LYR
SlLYK3	*Solyc03g121050*		LYK
SlLYK4	*Solyc02g089900*	Tandem duplication with *SlLYK7*	LYR
SlLYK6	*Solyc12g089020*	Kinase partly truncated	LYR
SlLYK7	*Solyc02g089920*	Tandem duplication with *SlLYK4*	LYR
SlLYK8	*Solyc09g083200*	Kinase truncated Tandem duplication with *SlLYK9*	LYR
SlLYK9	*Solyc09g083210*	Tandem duplication with *SlLYK8*	LYR
SlLYK10	*Solyc02g065520*		LYR
SlLYK11	*Solyc02g081040*	Tandem duplication with *SlLYK12*	LYK
SlLYK12	*Solyc02g081050*	Tandem duplication with *SlLYK11*	LYK
SlLYK13	*Solyc01g098410*		LYK
SlLYK14	*Solyc06g069610*		LYK
SlLYK15	*Solyc11g069630*		LYR
MtNFP	*Medtr5g019040*	*NFR5*	LYR
MtLYR1	*Medtr8g078300*		LYR
PaNFP	HQ705608		LYR
SlSYMRK	*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*	*SlPDS*	21
pTRV2-*SlCCaMK*	*SlCCaMK*	98
pTRV2-*SlLYK10*	*SlLYK10*	225
pTRV2-*SlLYK10*	*SlLYK9*	0
pTRV2-*SlLYK10*	*SlLYK8*	0
pTRV2-*SlLYK10*	*SlLYK2*	0
pTRV2-*SlLYK10*	*SlLYK6*	0
pTRV2-*SlLYK10*	*SlLYK4*	0
pTRV2-*SlLYK10*	*SlLYK7*	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)

Identity (Amino Acids)

SlLYK8	85.79												
SlLYK6	30.75	23.41											
SlLYK7	29.24	25.00	46.86										
SlLYK4	31.24	25.47	38.98	40.10									
SlLYK14	26.77	16.82	24.15	21.74	24.56								
SlLYK1	28.03	20.63	23.31	22.93	21.79	29.98							
SlLYK13	27.74	20.73	23.45	23.40	24.14	28.85	58.46						
SlLYK15	23.39	17.47	28.68	26.06	25.89	20.34	24.05	23.17					
SlLYK12	26.40	17.11	24.48	23.02	23.36	29.91	61.58	54.59	22.05				
SlLYK11	26.87	17.09	22.56	23.47	21.48	28.25	58.60	54.31	22.51	60.56			
SlLYK2	25.91	18.82	26.63	25.43	25.41	23.54	23.76	22.66	23.58	23.05	21.77		
SlLYK3	25.96	17.68	24.90	23.37	24.75	37.02	30.78	30.57	20.25	30.67	28.87	21.20	
SlLYK10	35.37	30.60	27.89	26.85	26.11	24.40	23.94	23.85	23.49	23.02	23.00	22.90	22.87
	SlLYK9	SlLYK8	SlLYK6	SlLYK7	SlLYK4	SlLYK14	SlLYK1	SlLYK13	SlLYK15	SlLYK12	SlLYK11	SlLYK2	SlLYK3

(b)

Identity (Nucleotides)

SlLYK8	90.44												
SlLYK2	52.86	51.34											
SlLYK6	51.20	47.76	47.46										
SlLYK4	49.42	46.87	47.78	54.71									
SlLYK7	48.58	46.15	46.28	63.10	57.05								
SlLYK12	44.69	40.15	42.63	44.71	44.85	43.64							
SlLYK15	46.50	42.03	44.38	53.07	51.61	50.85	42.16						
SlLYK1	44.76	41.24	42.67	44.64	42.92	43.68	66.87	42.03					
SlLYK11	44.23	39.34	42.43	44.93	43.59	44.09	71.67	42.05	64.73				
SlLYK13	42.85	39.54	42.89	44.15	43.52	43.26	64.14	40.59	71.24	63.44			
SlLYK3	43.35	38.40	40.69	42.84	42.24	42.07	50.17	40.00	49.01	49.75	50.00		
SlLYK14	44.88	42.03	41.07	44.81	42.76	42.88	49.52	42.04	49.91	49.32	49.33	59.56	
SlLYK10	58.99	56.66	53.21	47.13	46.84	44.59	43.92	43.69	43.49	42.64	42.55	41.37	41.88
	SlLYK9	*SlLYK8*	*SlLYK2*	*SlLYK6*	*SlLYK4*	*SlLYK7*	*SlLYK12*	*SlLYK15*	*SlLYK1*	*SlLYK11*	*SlLYK13*	*SlLYK3*	*SlLYK14*

(c)

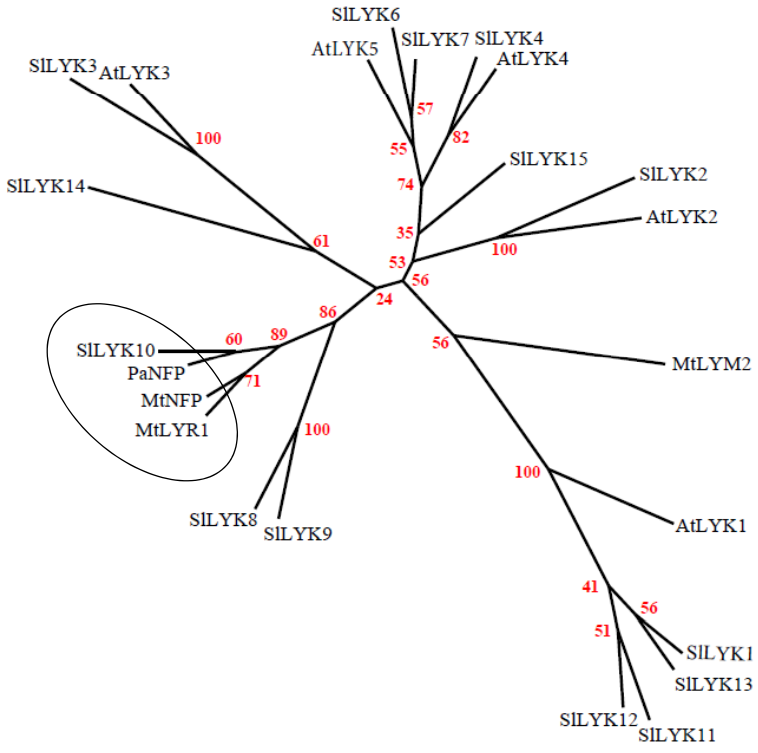
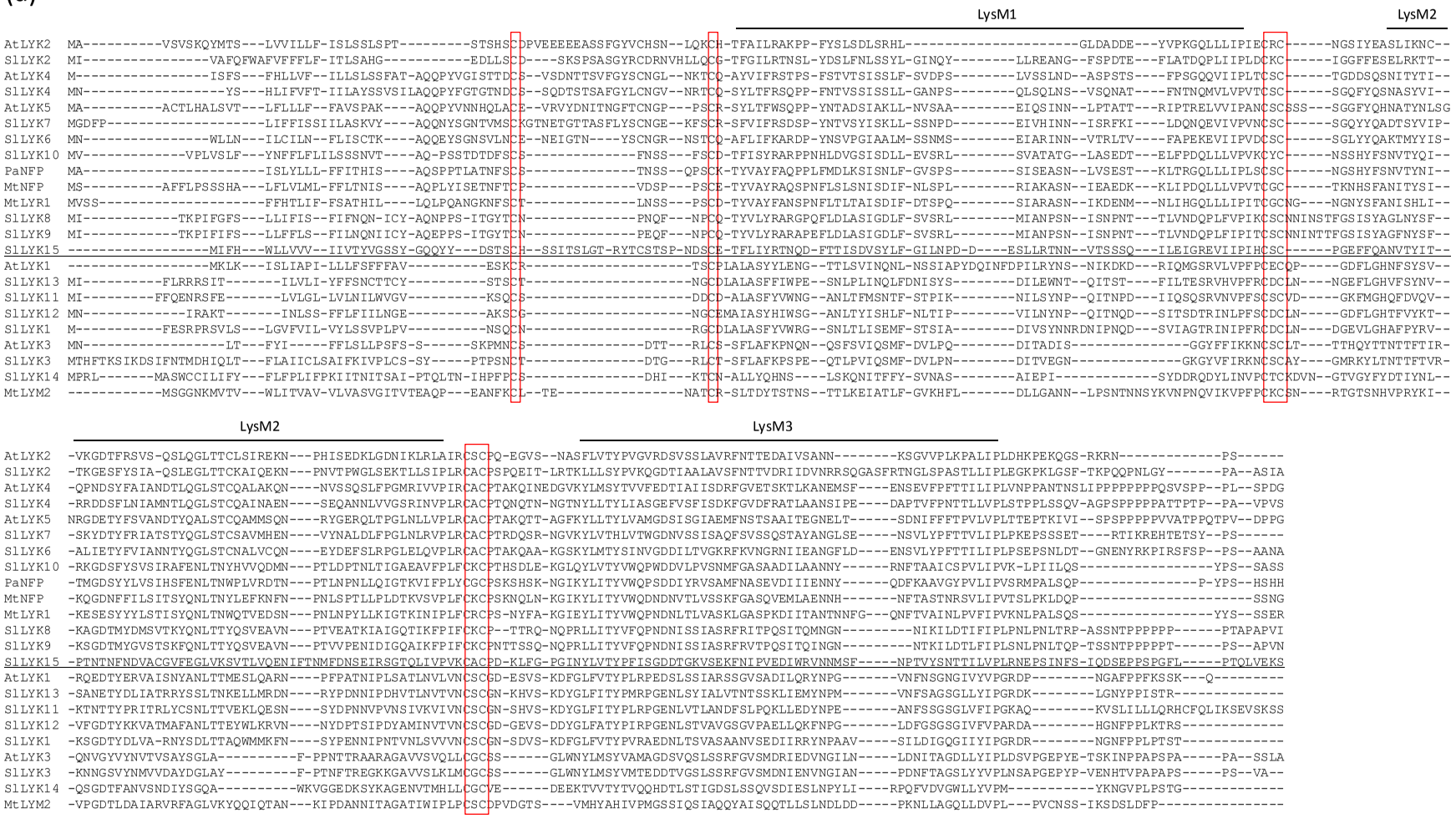


Fig. S2. (a) Amino acid identities between tomato LysM-RLK proteins. Identities between SlLYK10 and other tomato LysM-RLKs are highlighted (b) Nucleotide identities between tomato LysM-RLK genes. Identities between *SlLYK10* and other tomato LysM-RLKs are highlighted (c) Extracellular region amino acid 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 SlLYK10 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

Fig. S3

(a)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LysM2
LysM2
LysM3
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SlLYK2
AtLYK4
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SlLYK6
SlLYK10
PaNFP
MtNFP
MtLYR1
SlLYK8
SlLYK9
SlLYK15
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AtLYK3
SlLYK3
SlLYK14
MtLYM2

Fig. S3

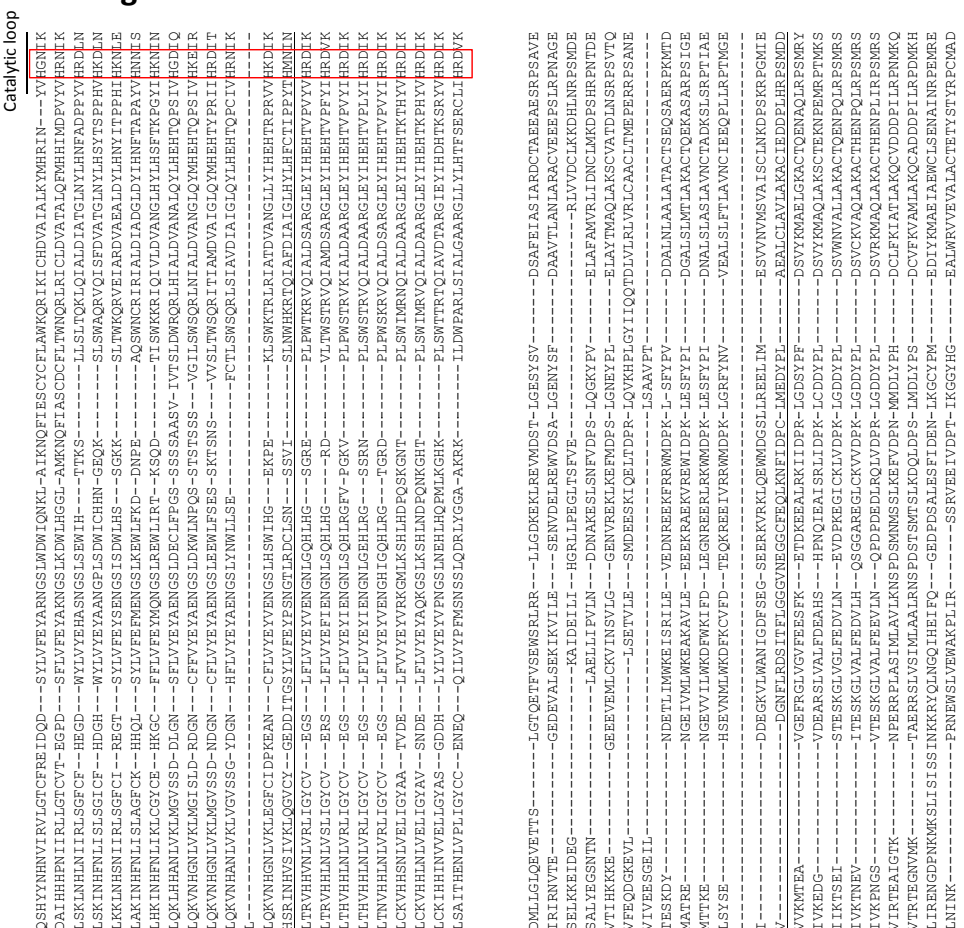


Fig. S3. (a) Alignment corresponding to the extracellular regions used for tree in Fig. S2c is shown. Members of LYR (upper part) and LYK (lower part) are separated by a line. Conserved cystein and cystein pairs are boxed in red. (b) Alignment corresponding to the intracellular regions used for tree 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.

Fig. S4

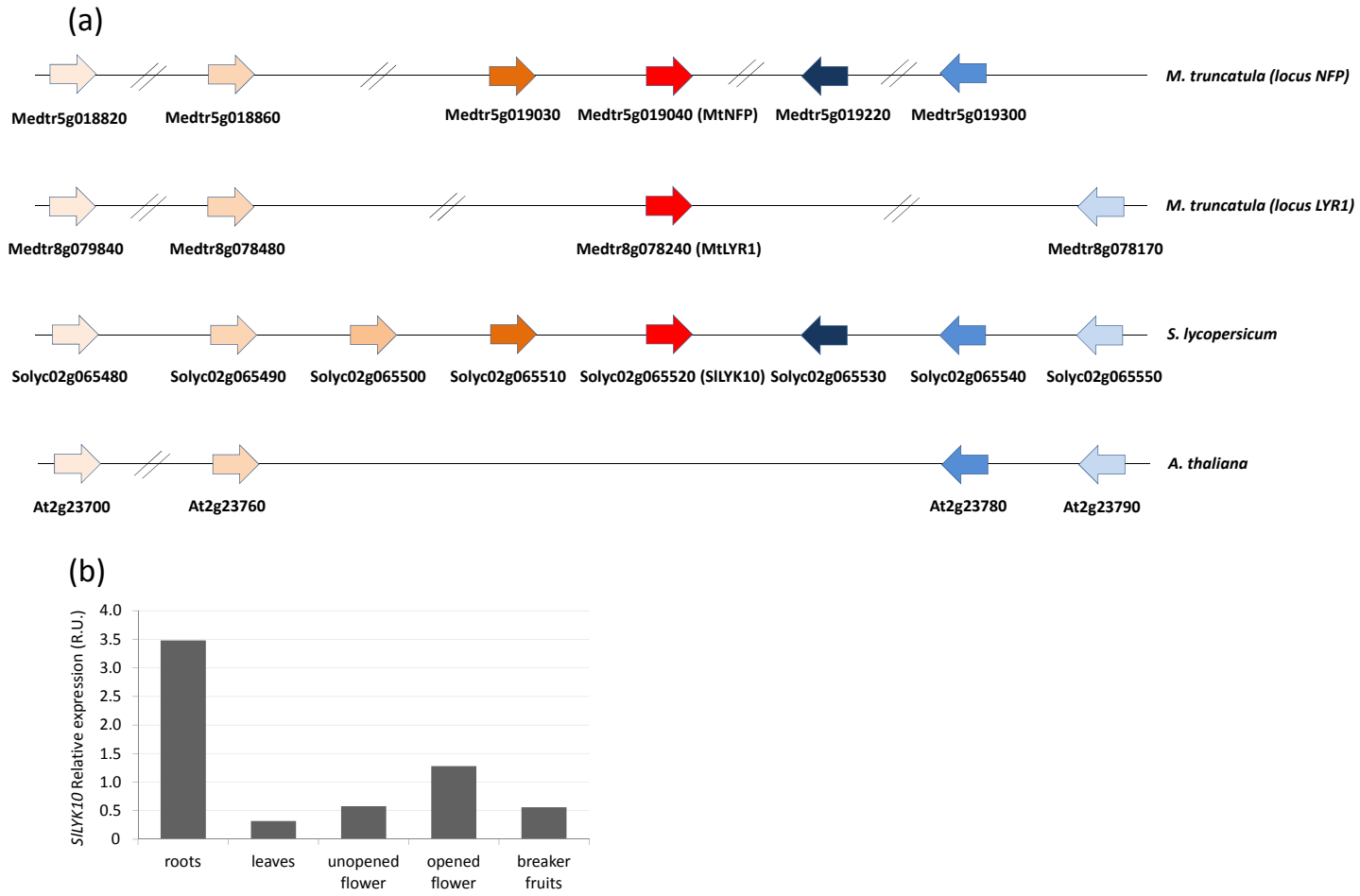


Fig. S4. (a) Synteny of *S. lycopersicum* locus containing *SlLYK10* with *M. truncatula* and *A. thaliana* orthologous loci. Same color is used for orthologous genes. Slashes represent the presence of additional gene(s) without orthologs in *S. lycopersicum* locus. (b)Tissue-specific expression patterns 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

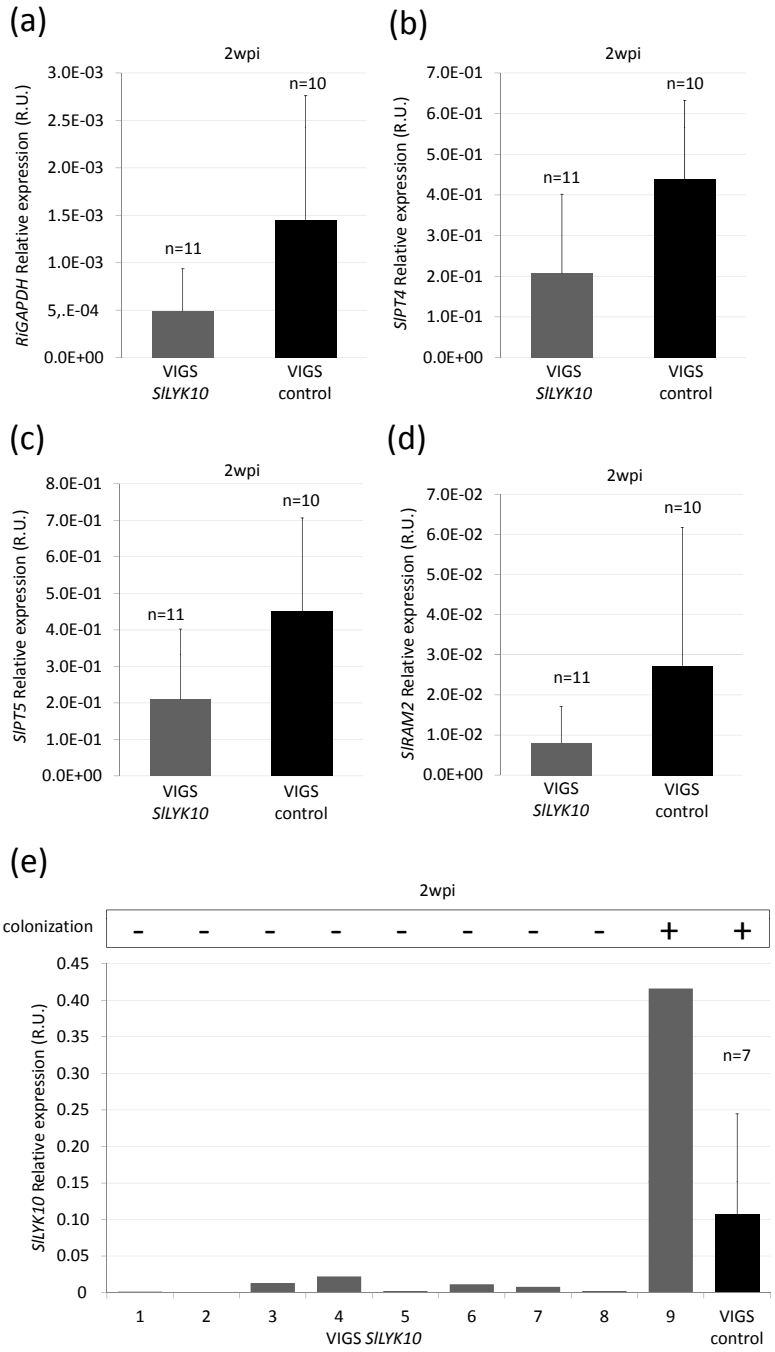
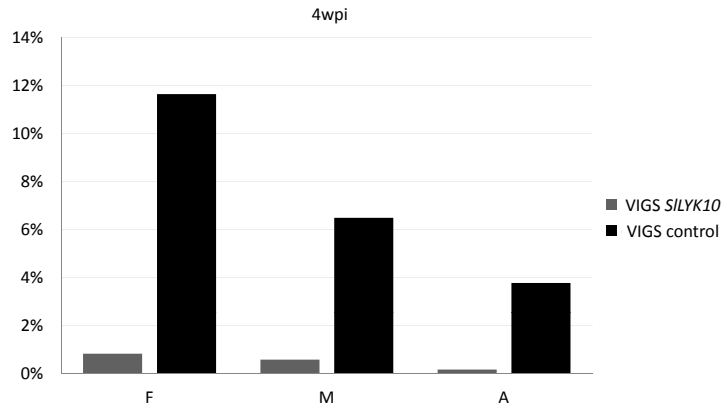


Fig. S5. (a-d) Relative expression measured by qRT-PCR, of the indicated genes in roots inoculated with pTRV2-*SlLYK10* or control pTRV2. Relative expression of *RiGAPDH* (a) reflects the amount of fungus, *SlPT4* and *SlPT5* (b-c) the degree of arbuscule formation, *SlRAM2* (d) the activation of the 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) *SlLYK10* relative expression in individual pTRV2-*SlLYK10* 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 *SlLYK10* which correlates with absence of AM.

Fig. S6

(a)



(b)

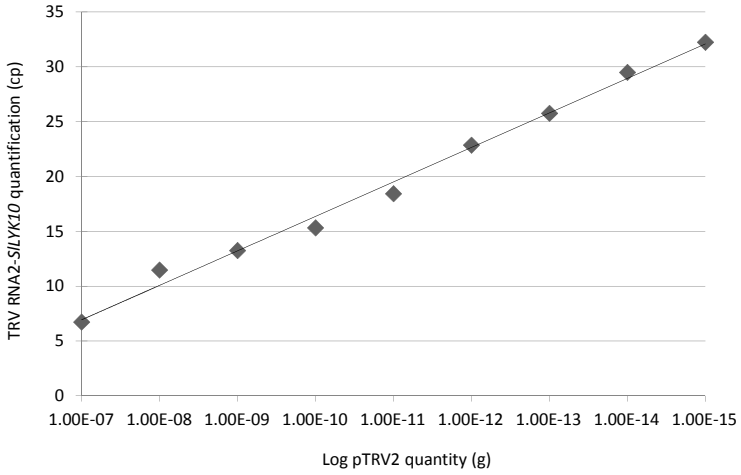


Fig. S6. (a) Estimation of mycorrhizal colonization at 4wpi with the mycocalc method. F is the frequency of mycorrhiza in the root system; M the intensity of the mycorrhizal colonization in the root system and A the arbuscule abundance in the root system. (b) Quantification by qRT-PCR, of the plasmid pTRV2-*SlLYK10.* The graph shows linearity between cycle quantification (cp) and plasmid quantities from 1fg to 100ng.

Chapter 2:

SlLYK10 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 p*SlLYK10::GUS* construct expressing the *β-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 SlLYK10 and I made the construct p*SlLYK10::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 SlLYK10 suggest a role of lipo-chitooligosaccharides in arbuscular mycorrhiza

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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 (SlLYK10), a putative receptor for Myc-LCOs is involved in AM establishment. Here we show that *SlLYK10* is expressed in the root epidermis before fungal penetration, and later in cortical cells colonized by AMF. Moreover, we determined that SlLYK10 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 SlLYK10 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 *SlLYK10,* belonging to this phylogenetic group, is required in tomato for efficient root colonization by AMF (Buendia et al., 2016). Reduction of *SlLYK10* level of expression resulted in significantly lower levels of AM colonization. Here, we study the expression pattern of *SlLYK10* during the symbiotic process and characterize its binding properties towards different LCOs. Our results suggest that LCO perception trough SlLYK10 is involved in AM establishment in tomato.

Results

SlLYK10 is expressed in root cells before and during invasion by AMF

To better understand the role of *SlLYK10* in AM, we produced *Agrobacterium rhizogenes* mediated transgenic tomato roots expressing the GUS reporter under the control of the *SlLYK10* 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 *SlLYK10* expression was associated with particular stages of arbuscule development.

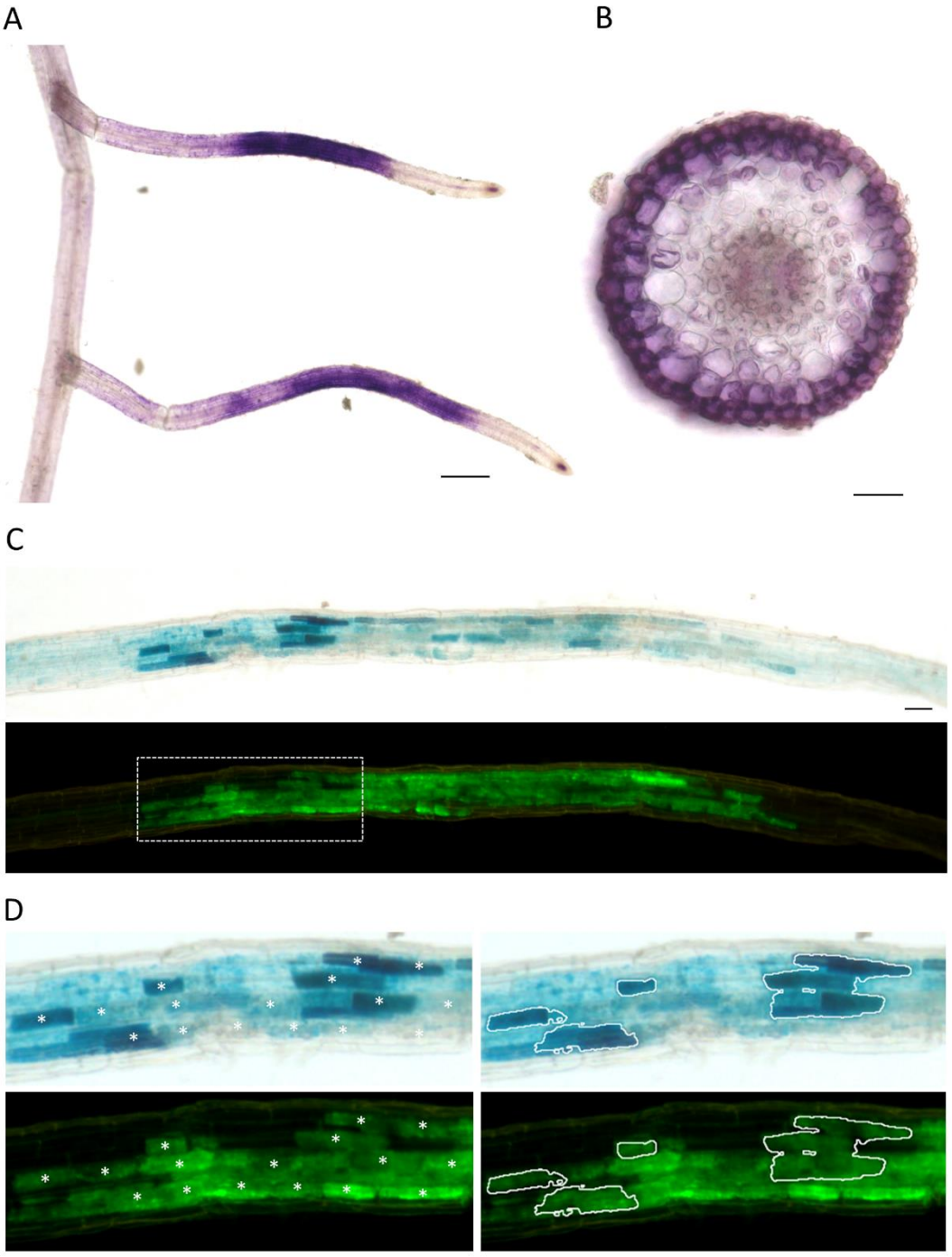

Fig. 1 The pro*SlLYK10::*GUS fusion is mainly expressed in the epidermis and the outer cortex before AMF colonization and in arbuscule-containing cells during colonization. A) Representative image of transgenic tomato roots attached to shoots in the absence of AM fungi. Lateral roots show maximal GUS 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.

SlLYK10 is an LCO binding protein

In order to determine the subcellular localization of SlLYK10, we introduced a translational fusion SlLYK10:YFP in tomato roots using *Agrobacterium rhizogenes*. Fluorescence was observed in the cell periphery in epidermal cells of roots containing the *p35S::SlLYK10: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 SlLYK10, 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 SlLYK10: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 SlLYK10c, Fig. 2C) composed of the extracellular region (ECR) of SlLYK10 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 SlLYK10c: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 SlLYK10c:YFP N-glycan maturation (Supplemental Fig. S4A and C) indicated that most of the protein reached the PM. SlLYK10c:YFP was furthermore immunodetected in the membrane fraction extracted from *N. benthamiana* leaves (Fig. 2E).

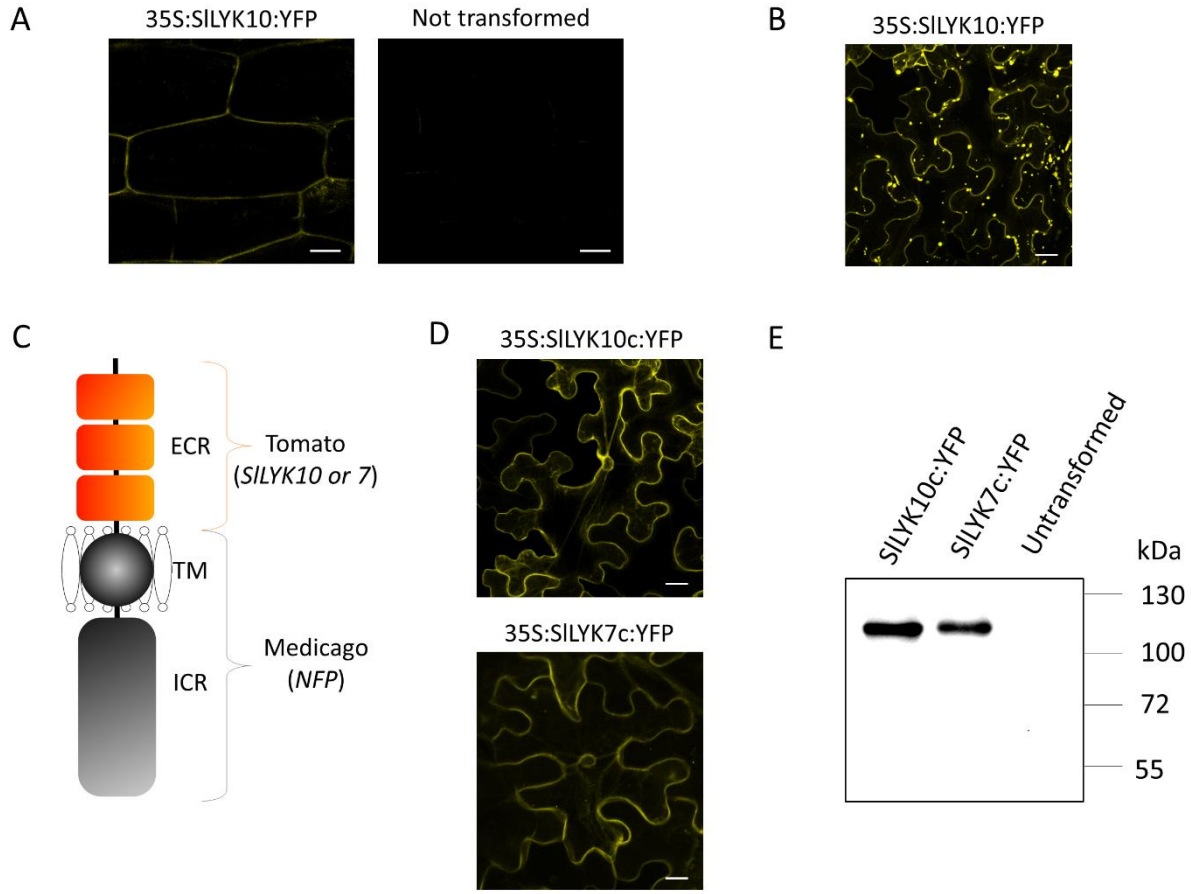

Fig.2 SlLYK10 is localized in the plasma membrane. A) Confocal images of epidermal cells from tomato ROC lines expressing SlLYK10:YFP (left panel) or ROC control (right panel) acquired with similar microscope settings. **B)** Confocal images of epidermal cells from *Nicotiana benthamiana* leaf expressing *S*lLYK10:YFP showing localization partly in undefined compartments. **C)** Schematic view of the chimeric constructs SlLYK10c and SlLYK7c. In orange is represented the extracellular region (ECR) composed of the 3 LysMs of SlLYK10 or SlLYK7. 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 SlLYK10c:YFP (upper panel) or SlLYK7c: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. SlLYK10c:YFP and SlLYK7c: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 SlLYK10 for LCOs, we performed radioligand binding experiments using a radiolabeled LCO (^{35}S-LCO) purified from the rhizobial strain *Rhizobium tropici* and various unlabeled synthetic Myc-LCOs and COs. The ^{35}S-LCO was obtained by radiolabeling LCO-V(C18:1,NMe) with ^{35}S as previously described (Gressent et al., 2004). Strong specific binding of the ^{35}S-LCO to membrane fractions was detected from extracts of leaves expressing SlLYK10c:YFP, but not for untransformed leaves (Fig. 3A). By adopting the same procedure, we made a chimeric construct with another tomato LysM-RLK, *SlLYK7* (SlLYK7c:YFP), belonging to another phylogenetic group (Supplemental Fig. S1). SlLYK7c:YFP was slightly less expressed than SlLYK10c: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 SlLYK7c:YFP (Fig. 3A), excluding artefactual detection of LCO binding sites and suggesting that not all tomato LysM-RLKs bind LCOs.

The affinity of SlLYK10c:YFP for LCO-V(C18:1,NMe,S) was determined by cold saturation experiments using this ^{35}S-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)).

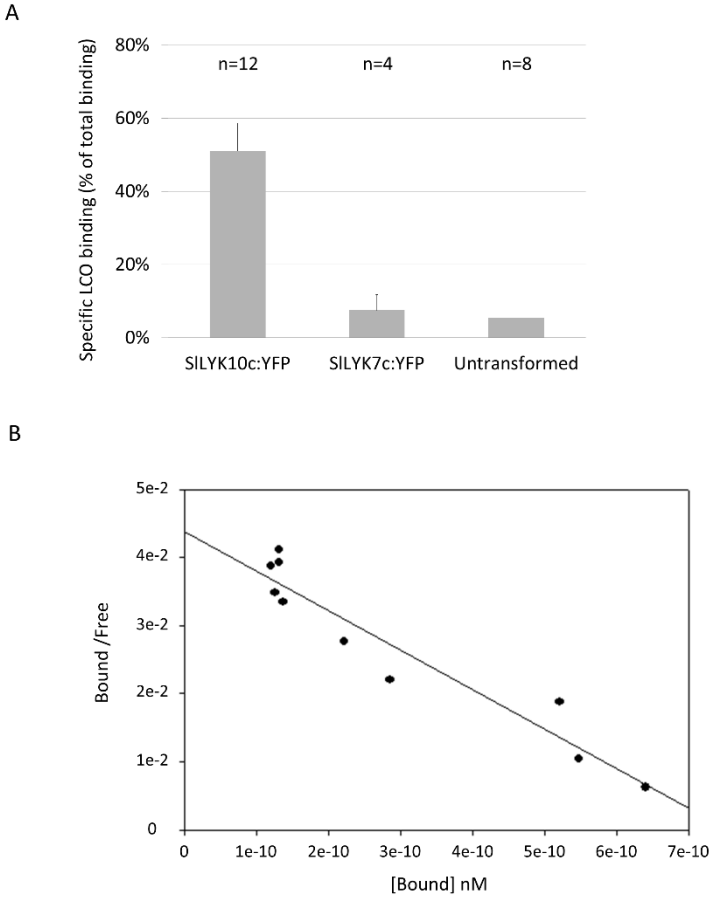

Fig. 3 SlLYK10 has a high affinity for LCOs. A) Binding activity of membrane fractions incubated with radiolabeled LCO-V(C18:1,NMe,^{35}S) in the absence or the presence of 1 µM of unlabeled LCO-V(C18:1,NMe,S) allowing the determination of total and non specific binding respectively and by difference 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 SlLYK10c:YFP. The plot is representative of experiments performed on membrane fractions prepared from 3 independent batches of agro-infiltrated *N. benthamiana* leaves.

To determine the selectivity of SlLYK10c:YFP towards known Myc-LCO and CO structures, we performed competition assays between the ^{35}S-LCO and an excess (1 µM) of unlabeled competitors. All Myc-LCOs were able to compete the binding of the ^{35}S-LCO to the membrane fraction containing SlLYK10c:YFP (Fig. 4A). The affinities of SlLYK10c: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 ^{35}S-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 SlLYK10 LCO binding site exhibits lower affinity for the latter molecules with K_is higher than 1 µM.

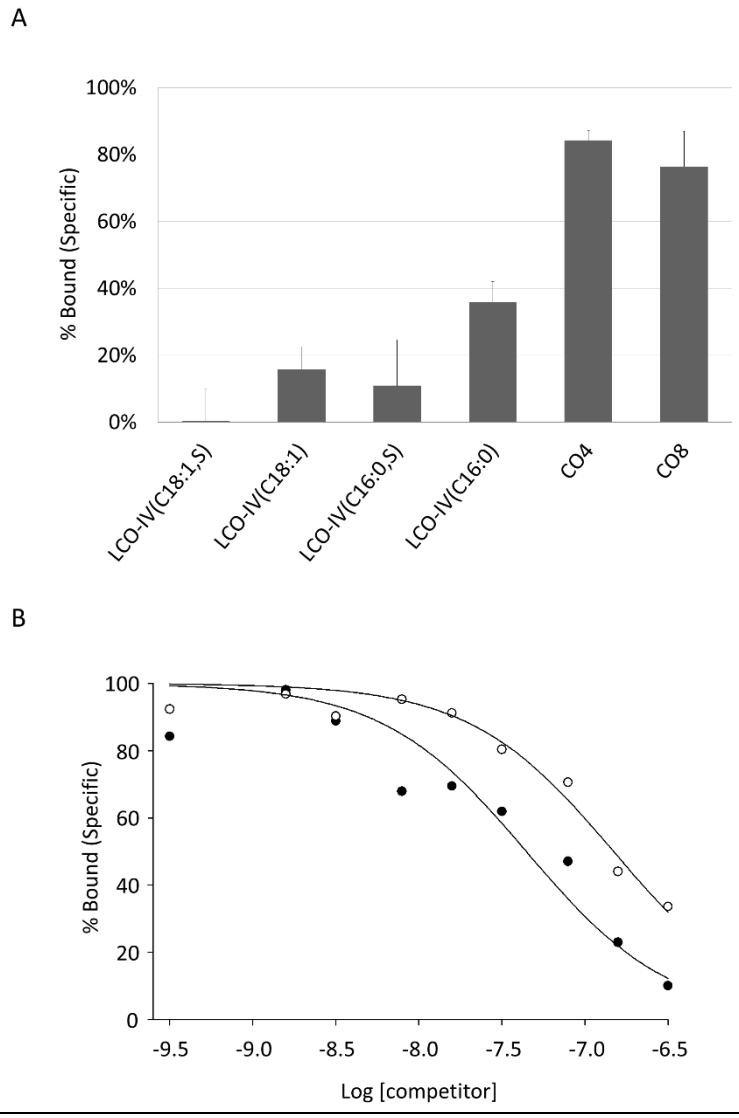

Fig. 4 SlLYK10 is selective for LCOs vs COs. A) Membrane fractions containing SlLYK10c:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,^{35}S) in presence of 1 µM of the indicated unlabeled molecules used as competitors. Non specific binding was determined in the presence of 1 µM LCO-V(C18:1,NMe,S). 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 of radiolabeled LCO-V(C18:1,NMe,^{35}S) to membrane fraction containing SlLYK10c: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. *SlLYK10* 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 *SlLYK10* silenced plants (Buendia et al., 2016). Furthermore, SlLYK10 affinity for LCOs strongly suggests that SlLYK10 is involved in LCO perception, and thus that Myc-LCO perception by SlLYK10 plays a positive role in AMF penetration in tomato roots. *SlLYK10* expression in arbuscule-containing cells is also coherent with our previous observation of incorrectly developed arbuscules in some plants down-regulated for *SlLYK10* expression (Buendia et al., 2016) and suggests that Myc-LCO perception by SlLYK10 also plays a positive role in arbuscule development.

Data about the expression patterns of *SlLYK10* orthologs are only available in legumes. Although one *SlLYK10* 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 *SlLYK10* 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 *SlLYK10* 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 SlLYK10 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 SlLYK10, fulfilled this requirement. Using LCO-V(C18:1,NMe,S) as the radiolabeled ligand, we found that SlLYK10 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 µM. Moreover, we cannot exclude that this slight difference is due to difference in hydrophobicity between the non-sulfated LCO. In contrast SlLYK10 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 SlLYK10 would help to understand the specificity of LCO perception for establishing AM.

Our results suggest that Myc-LCO perception by SlLYK10 plays a positive role in AM establishment in tomato. However, data indicate that *SlLYK10* 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 Ca^{2+} 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 Ca^{2+} 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 *SlLYK10* 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 *SlLYK10* knockout plants to determine more precisely the role of *SlLYK10* 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 SlLYK10 is able to bind LCOs with high affinity. The spatio-temporal expression pattern of *SlLYK10*, 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 (SlLYK10)*, including the *SlLYK10 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 SlLYK10 and SlLYK7, 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 ProSlLYK10::GUS constructs (expressing the DsRed reporter) or those expressing SlLYK10: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 pre-germinated 5 d on M medium (Becard and Fortin 1988) in a 3% CO_2 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 µg/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::SlLYK10-YFP, Pro35S::SlLYK10c-YFP or Pro35S::SlLYK7c-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 µg / ml of DAPI or 20 µM 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 SlLYK10c:YFP or SlLYK7c:YFP was quantified by immunoblotting in 10 µg of membrane fraction proteins.

LCO binding assays

LCO-V(C18:1Δ11,NMe) and LCO-V(C18:1Δ11,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(C18:1Δ9), LCO-IV(C18:1Δ9,S), LCO-IV(C16:0) and LCO-IV(C16:O,S), are described in Maillet et al., 2011. Labeling of LCO-V(C18:1Δ11,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 SlLYK10. 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.

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Supplemental Data

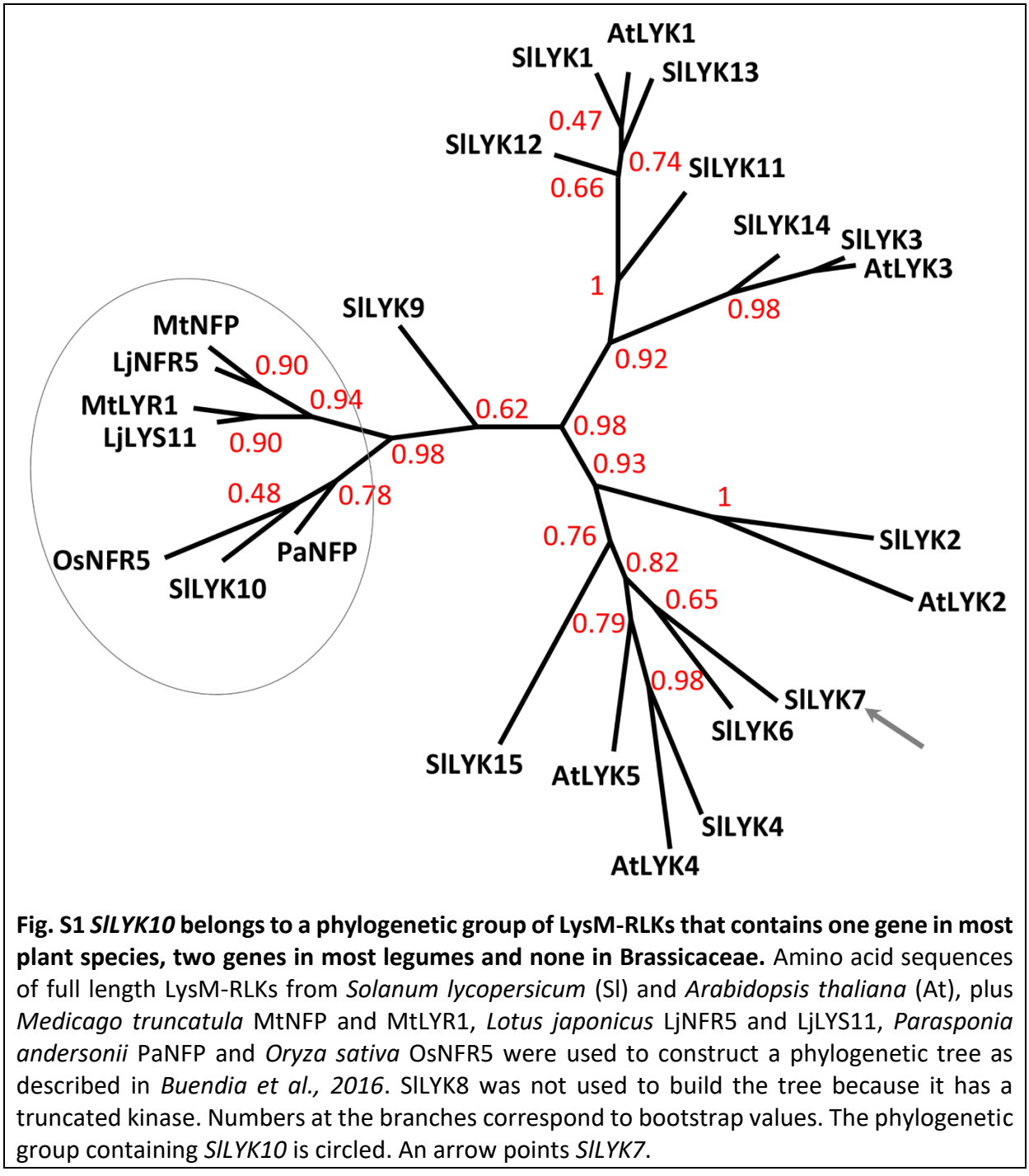


Fig. S1 *SlLYK10* belongs to a phylogenetic group of LysM-RLKs that contains one gene in most plant species, two genes in most legumes and none in Brassicaceae. Amino acid sequences of full length LysM-RLKs from *Solanum lycopersicum* (Sl) and *Arabidopsis thaliana* (At), plus *Medicago truncatula* MtNFP 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*.

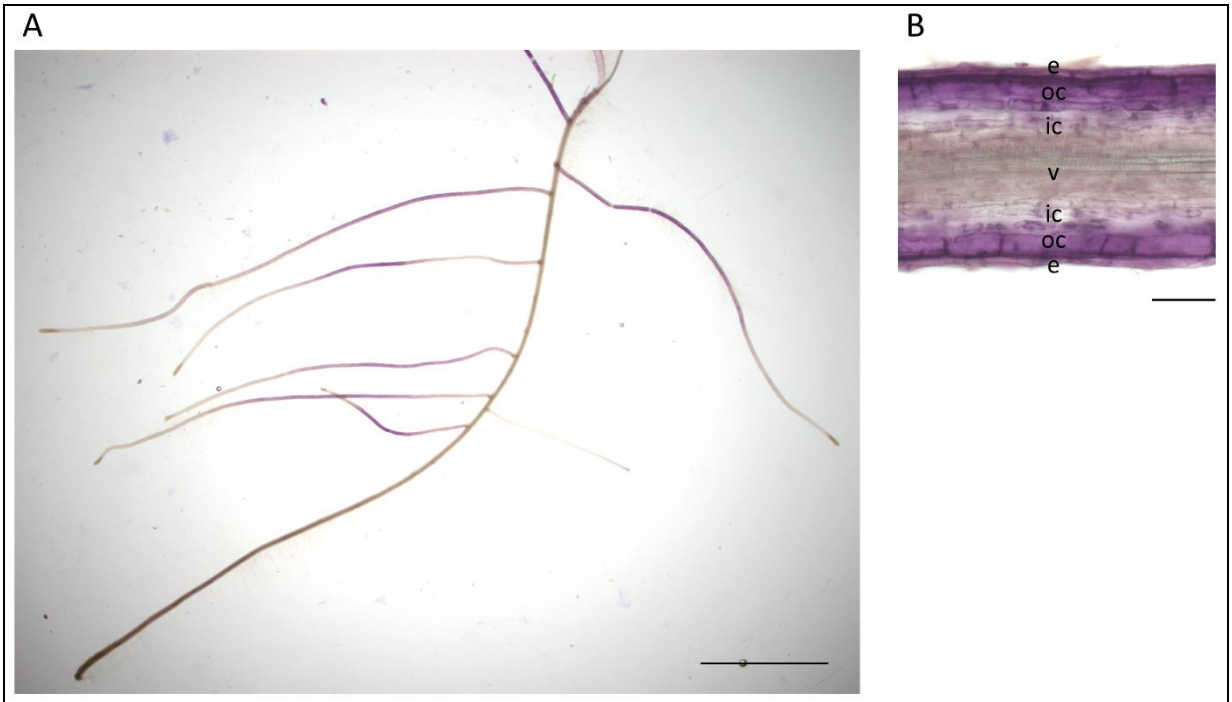


Fig. S2 The Pro*SlLYK10::*GUS fusion is mainly expressed in the epidermis and the outer cortex before AMF colonization. A) Representative image of tomato transgenic ROC lines grown in absence of AM fungi. Lateral roots show maximal GUS activity (magenta). **B)** Longitudinal section in a root segments 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 µm in A and 50 µm in B.

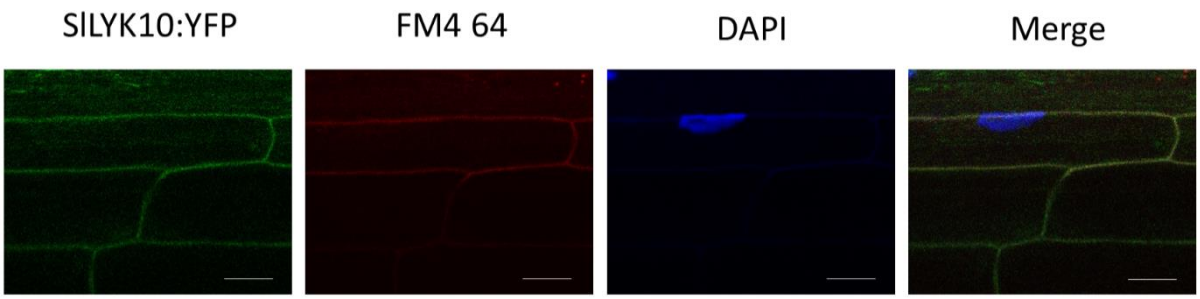
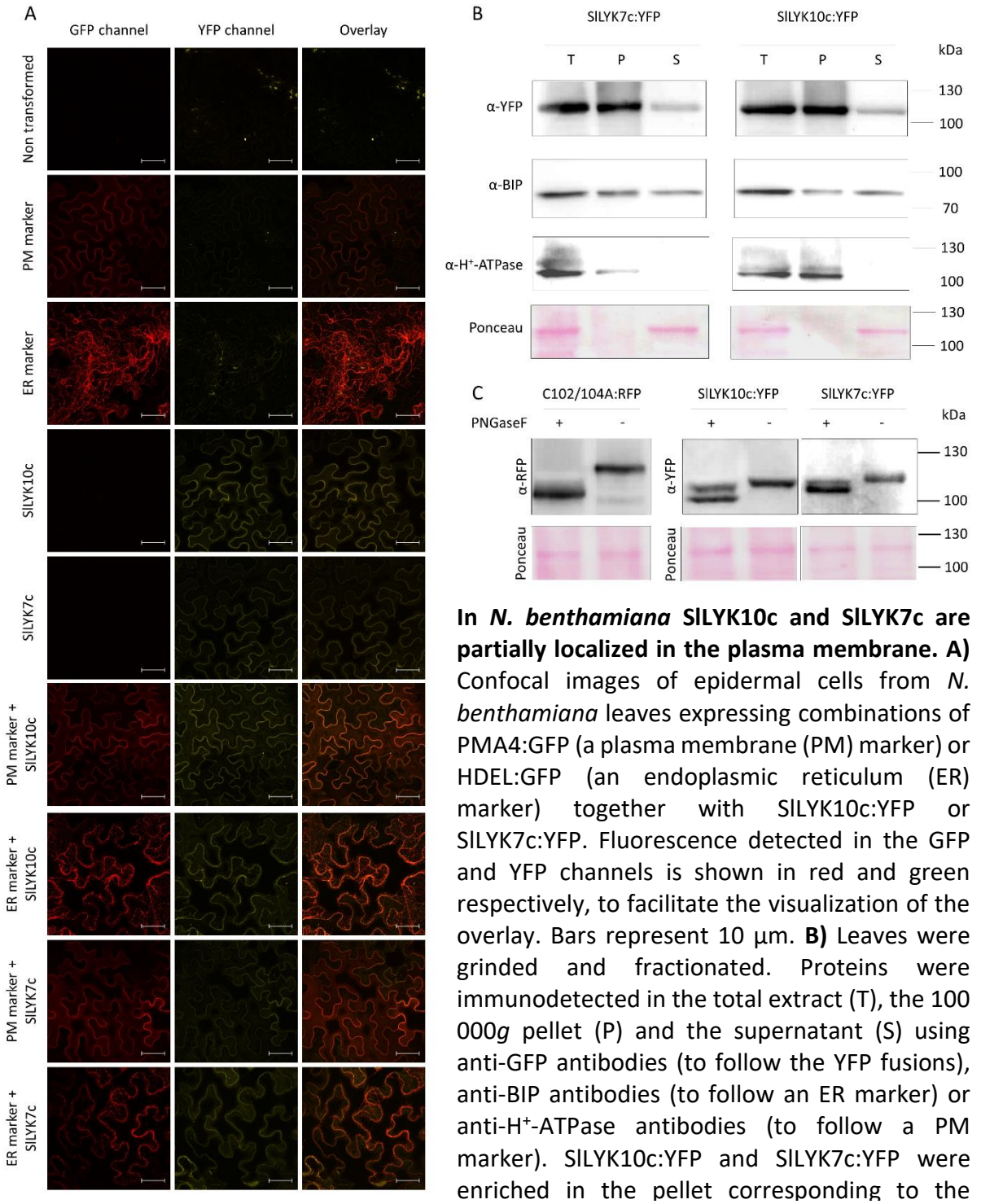


Fig. S3 In tomato SlLYK10 localized at the plasma membrane. A) Confocal images of epidermal cells from tomato ROC lines expressing SlLYK10:YFP and treated with FM4 64 (which labels the plasma membrane after treatment before being internalized) and DAPI (which labels the nucleus) showing that SlLYK10:YFP is localized in the plasma membrane. Bars represent 50 µm.



In *N. benthamiana* SlLYK10c and SlLYK7c 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) marker) together with SlLYK10c:YFP or SlLYK7c: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 100 000*g* 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). SlLYK10c:YFP and SlLYK7c: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 SlLYK10c:YFP and SlLYK7c: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 SlLYK10 For	GGTCTCTAAATGGGTTATAGAGCTGTAATGC
Pro SlLYK10 Rev	GGTCTCATTTGCGATGCAAAGCTTAGATAAC
GUS For	GGTCTCTCAAAATGTTACGTCCTGTAGAAACCC
GUS Rev	AAAGGTCTCGCGTATCATTGTTTGCCTCCCTGCTG
SlLYK10c For	GGTCTCGCAAAATGGTAGTTCCTCTTGTGTCCTTG
SlLYK10c Rev	GGTCTCGTAAGTCCATGCTTGGATTTTCTACTGCTTGC
SlLYK7c For	GGTCTCGCAAAATGGGTGATTTTCCACTTATCTTTTTCATAAGTTC
SlLYK7c Rev	GGTCTCGTAAGGCCGTATGATATTTTGTGCCTAGAAGG

Chapter 3:

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

Preamble

In parallel to the study of SlLYK10, 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 *SlLYK10* 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.

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Abstract

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 SlLYK10 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 *SlLYK10* 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, SlLYK10* 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 *SlLYK10*, 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.

Results

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,^{35}S) 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 µM of unlabeled CO4 or CO8 (Fig. 1B). We observed that CO4 or CO8 poorly compete binding of LCO-V(C18:1, NMe, ^{35}S) on BdLYR1 in comparison to the competition measured in presence of 1 µM LCO-V(C18:1,NMe,S).

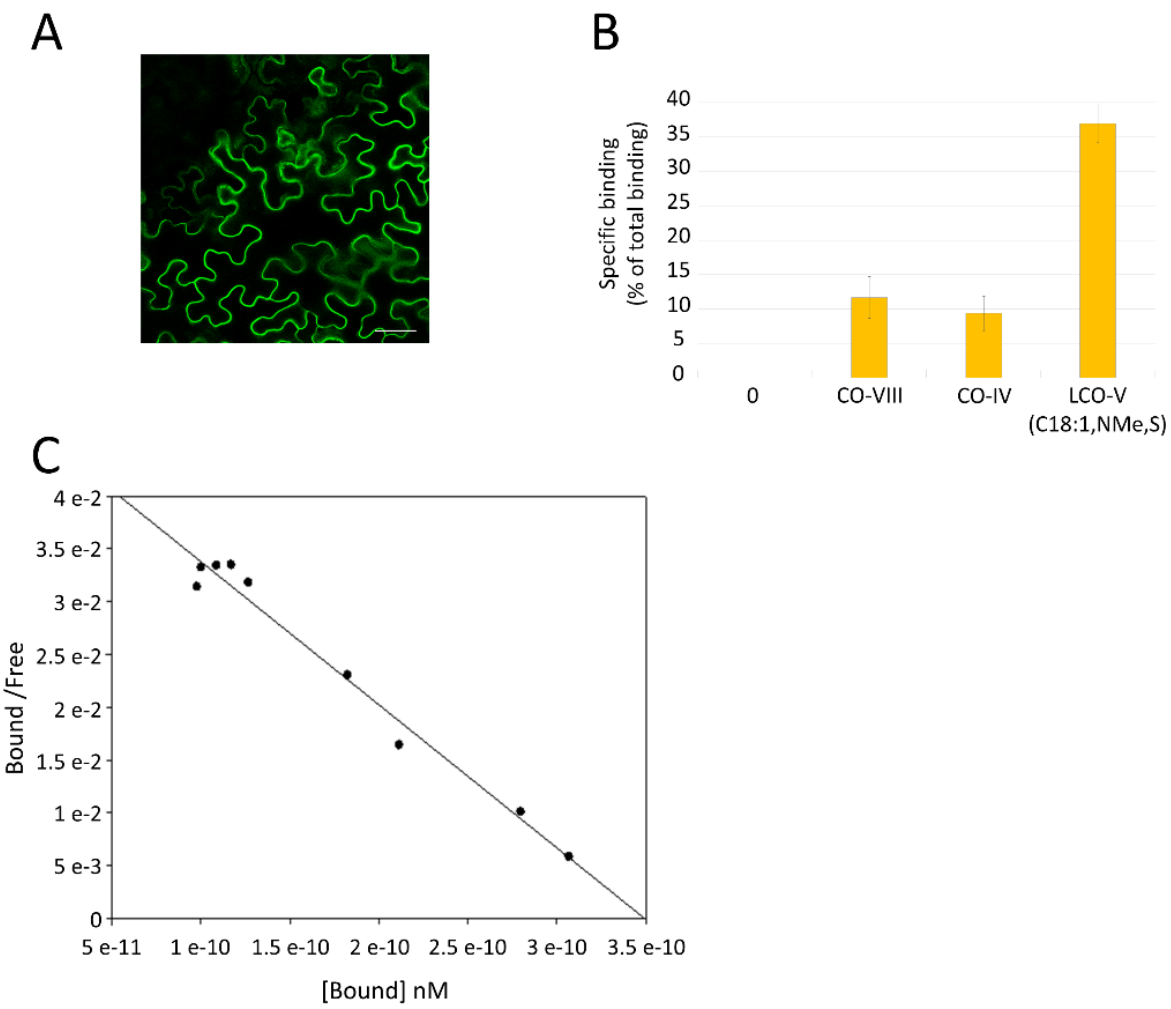

Fig. 1 BdLYR1 has a high affinity for LCOs and is selective for LCOs vs COs. A) Confocal image showing BdLYR1:YFP expressed in epidermal cells of *N. benthamiana*. Scale bar represents 50 µm. **B)** Membrane fractions containing BdLYR1:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,^{35}S) in presence of 1 µM of the indicated unlabeled molecules used as competitors. Non specific binding 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 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 fractions prepared from 3 independent batches 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 NaN_3 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.

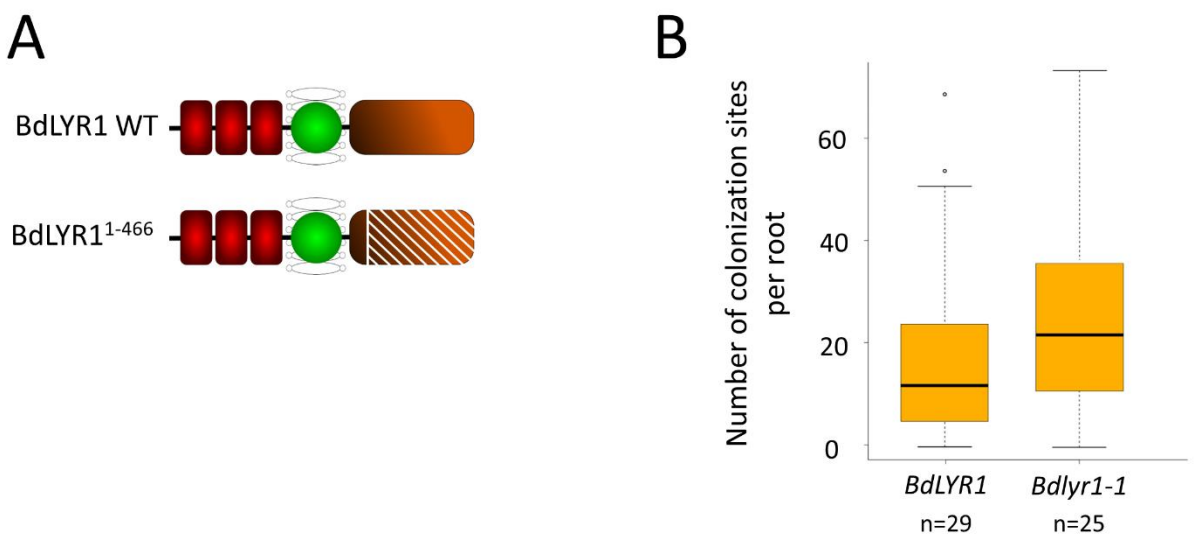

Fig. 2 *Bdlyr1-1* is able to establish the AMS. A) Schematic representation of the protein BdLYR1. The upper protein is BdLYR1 WT and the lower represents BdLYR1$^{1-466}$, with in white the part lacking due to the stop mutation in the gene. Red boxes represent the LysMs, green ball the transmembrane 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 *SlLYK10: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 *SlLYK10* (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 (p*35S::MtNFP:YFP*, p*35S::BdLYR1:YFP,* p*35S::SlLYK10:YFP*). In addition, we introduced *PsSYM10*, the *NFP* ortholog from *Pisum sativum* (p*35S::PsSYM10:YFP*) or *AtCERK1* (p*35S::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 p*35S::SlLYK10:YFP* did not restore nodulation in *Mtnfp-2,* however when we quantified the protein expression by immunoblotting, we detected much less SlLYK10: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 p*35S::PsSYM10:YFP* or p*35S::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 ΔNodH*) on plants complemented by *MtNFP* as control, *PsSYM10* or *BdLYR1* (Fig. S1). We observed that introduction of p*35S::PsSYM10:YFP* or p*35S::BdLYR1:YFP* in *Mtnfp2* did not modified the nodule number per plants compared to

plants complemented by p*35S::MtNFP:YFP.* As p*35S::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* p*35S::BdLYR1*$^{1\text{-}466}$*:YFP* (Fig. 3B). This gene could not restore nodulation in *Mtnfp-2.* We also tested whether BdLYR1$^{1\text{-}466}$: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$^{1\text{-}W467*}$ co-expression with MtLYK3 did not induce cell death.

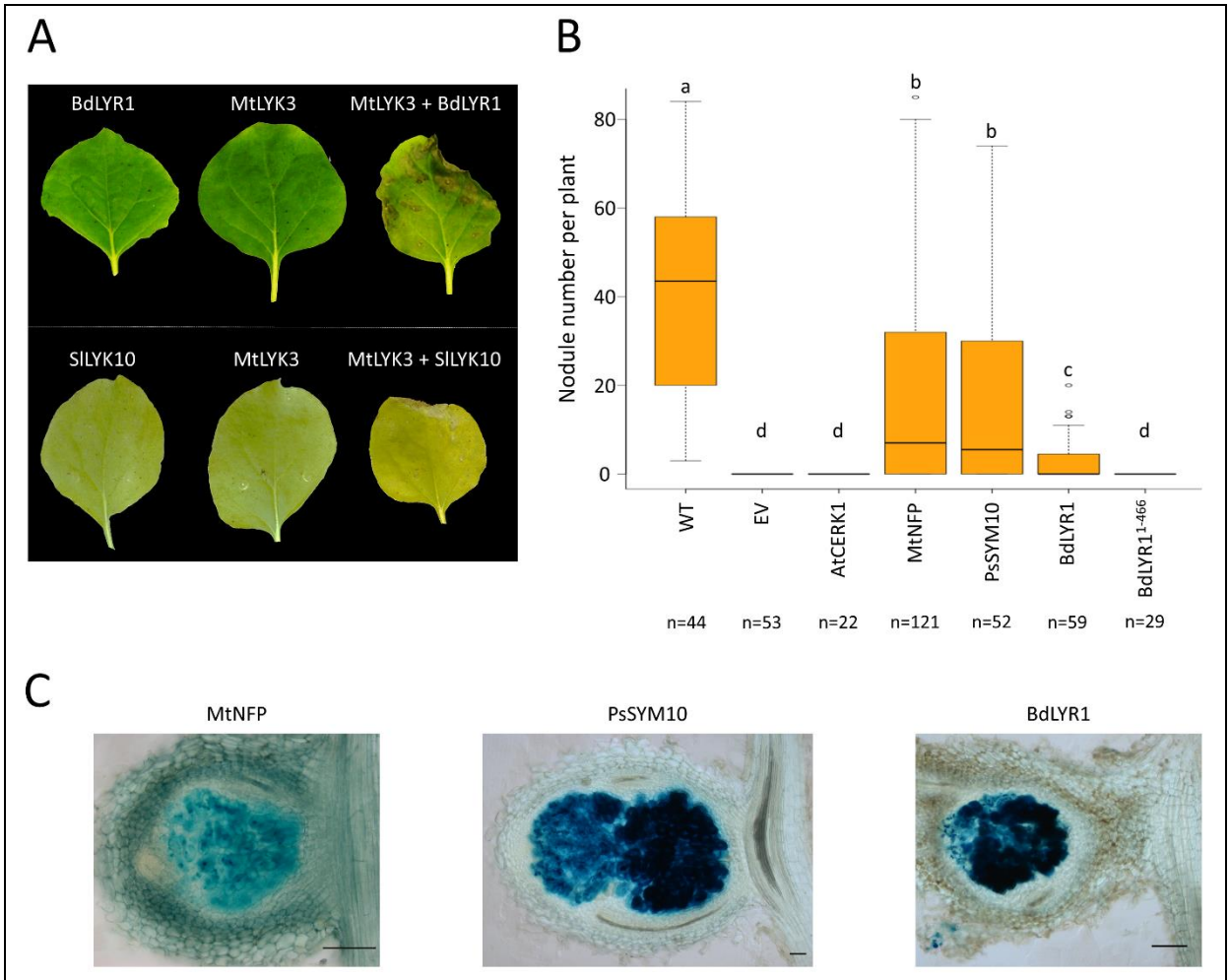

Fig. 3 SlLYK10 and BdLYR1 interact with MtLYK3 and activate the CSSP in *M. truncatula*. A) BdLYR1, SlLYK10 and MtLYK3 did not induce cell death when expressed separately in *N. benthamiana* leaves, but when BdLYR1 or SlLYK10 were co-expressed with MtLYK3, cell death was induced. Images are representative of at least 3 biological replicates. **B)** Roots of *Mtnfp-2* mutant were transformed via *A. rhizogenes* with empty vector (EV), p*35S::AtCERK1:YFP*, p*35S::MtNFP:YFP,* p*35S::PsSYM10:YFP,* p*35S::BdLYR1:YFP* or p*35S::BdLYR1*$^{1\text{-}466}$*:YFP*. WT plants are A17 not transformed. Nodule number was counted 4 wpi. Different statistical classes were established according a Kruskal-Wallis test (with $\alpha = 0.05$) 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.

p*SlLYK10 has the same transcriptional activity that* p*MtNFP 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 *β-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.

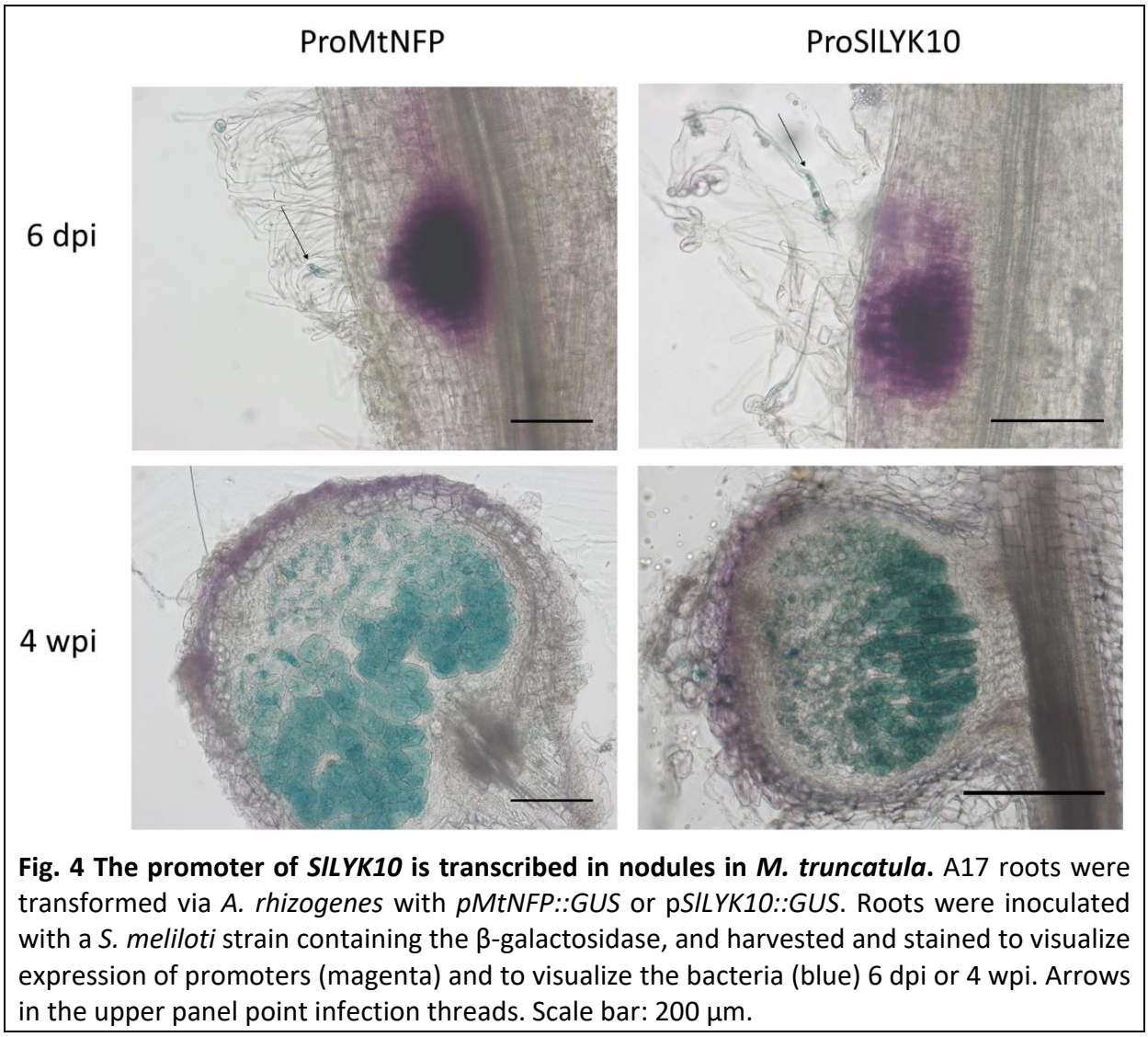

Fig. 4 The promoter of *SlLYK10* is transcribed in nodules in *M. truncatula*. A17 roots were transformed via *A. rhizogenes* with *pMtNFP::GUS* or p*SlLYK10::GUS*. Roots were inoculated with a *S. meliloti* strain containing the β-galactosidase, and harvested and stained to visualize expression of promoters (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 ΔNodH*) 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 *SlLYK10* is poorly expressed in *M. truncatula*, even under control of p*35S*. We also observed a low expression level and miss-localization of SlLYK10: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 p*35S* 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 p*BdLYR1*. 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 p*SlLYK10* 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 nodules. This is supported by the complementation of the absence of nodulation in an *Ljnfr5* mutant by *LjLYS11* under the control of the p*35S*, 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: p*35S::MtNFP:YFP*, p*35S::BdLYR1:YFP,* p*35S::SlLYK10:YFP,* p*35S::AtCERK1:YFP*, p*35S::BdLYR1*$^{1\text{-}466}$*:YFP,* p*35S::PsSYM10:YFP*, p*MtNFP::GUS*, p*SlLYK10::GUS* or p*BdLYR1::GUS*) suspension at OD_{600} 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. melilotiΔNodH*. Both strains are pXLGD4 that harbor a plasmid containing the hemA-lacZ. 2.5 ml of bacterial suspension diluted in water at OD_{600} 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 NaN_3 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 p*35S::SlLYK10:YFP*, p*35S::BdLYR1:YFP* or p*35S::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(C18:1Δ11,NMe) and LCO-V(C18:1Δ11,NMe,S) were purified from the rhizobial strain *Rhizobium tropici*. Synthetic Myc-LCOs, LCO-IV(C18:1Δ9), LCO-IV(C18:1Δ9,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(C18:1Δ11,NMe) by ^{35}S 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.

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Supplemental data

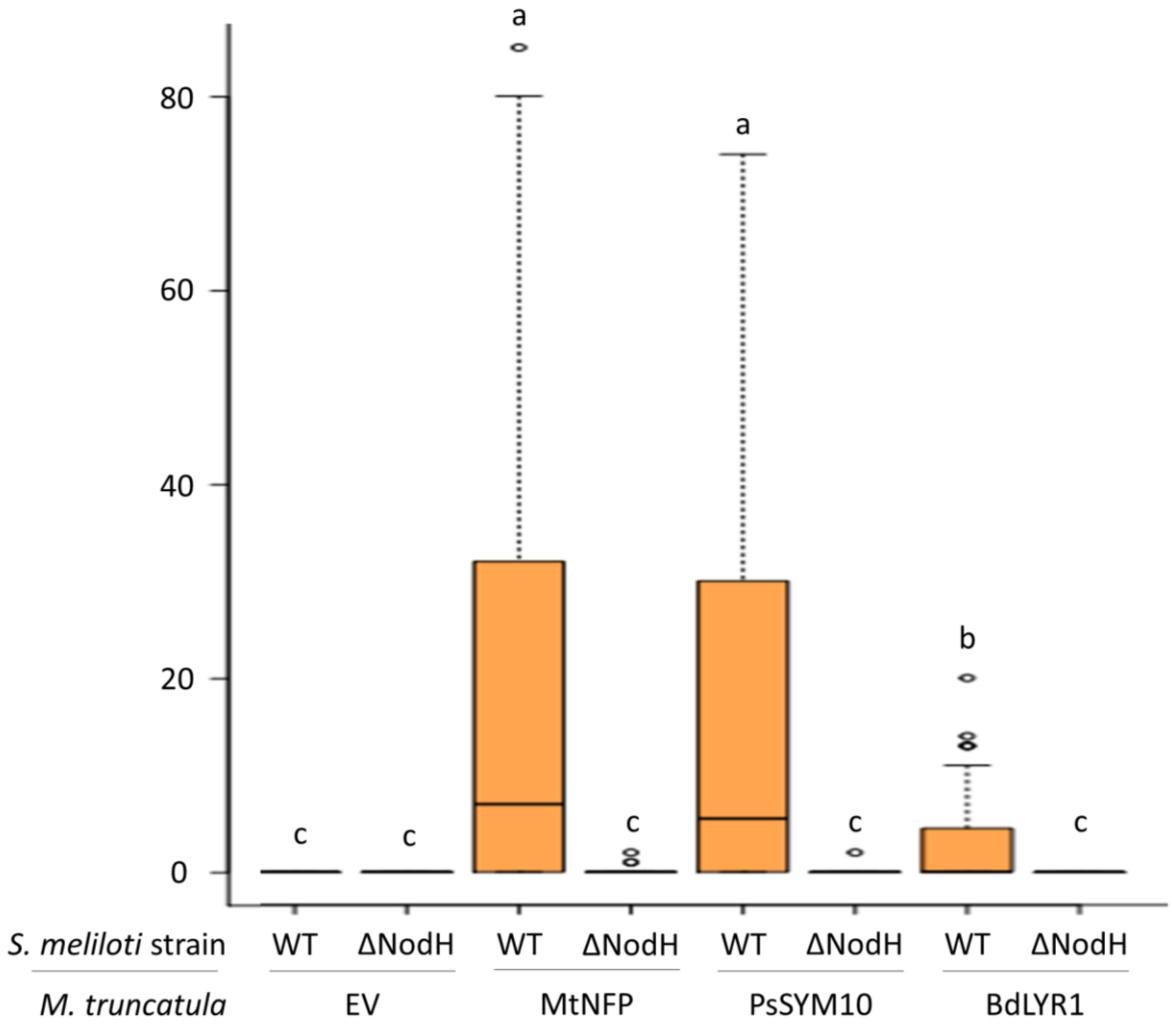

Fig. S1 Complementation of *Mtnfp-2* by *PsSYM10* and *BdLYR1* conserves host specificity. Roots of *Mtnfp-2* mutant were transformed via *A. rhizogenes* with an empty vector (EV), p*35S::MtNFP,* p*35S::PsSYM10,* p*35S::BdLYR1*. Nodule number was counted 4 wpi with a *S. meliloti* strain producing a non sulfated LCOs (ΔNodH). Different statistical classes, after Kruskal-Wallis test (with $\alpha = 0.05$), are indicated by different letters.

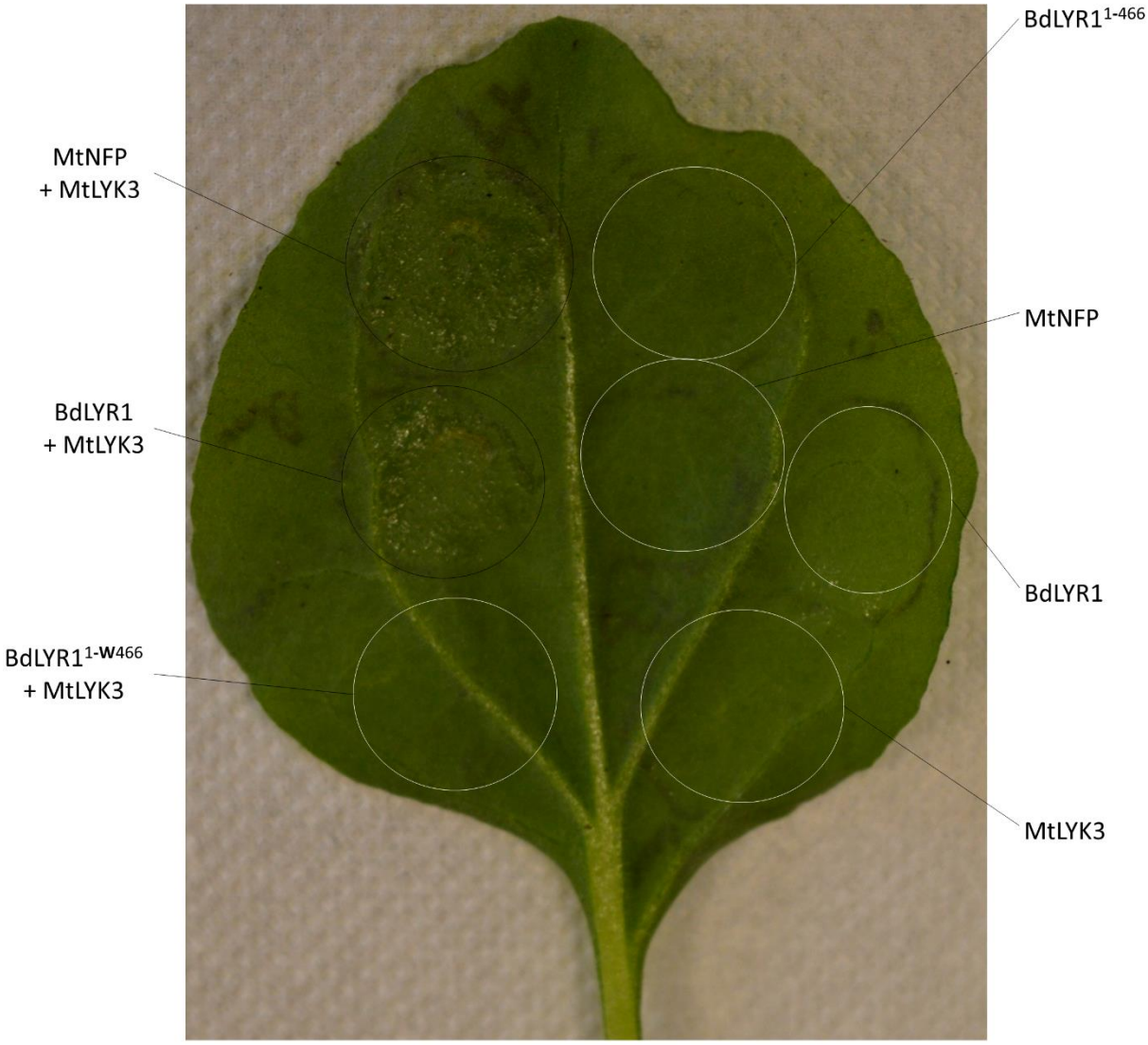

Fig. S2 BdLYR1$^{1-466}$ does not interact with MtLYK3 in *N. benthamiana*. BdLYR1, BdLYR1$^{1-466}$, MtNFP and MtLYK3 did not induce cell death when expressed separately in *N. benthamiana*, but when MtNFP or BdLYR1 were co-expressed with MtLYK3, cell death was induced. BdLYR1$^{1-466}$ co-expressed with MtLYK3 did not induce cell death. Image is representative of at least 3 biological replicates.

Table S1: Primers used for cloning		
Used for	Primer	5'- 3' sequence
Promoter of *BdLYR1*	ProBd69290GG For	GGTCTCTAAATGCTCTGAAGTAAGGCTGAGTAC
	ProBd69290GG Rev	GGTCTCATTTGCCTGTGTGTGTGTTGCGAAGCG
Promoter of *SlLYK10*	ProSl65520GG For	GGTCTCTAAATGGGTTATAGAGCTGTAATGC
	ProSl65520GG Rev	GGTCTCATTTGCGATGCAAAGCTTAGATAAC
CDS of *BdLYR1*	BdLYR1 BP For	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAACACCGCCGTTTCGG
	BdLYR1 BP Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCGGGCGGCCACCTCATTGAC
CDS of *Bdlyr1*	BdLYR1 BP For	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAACACCGCCGTTTCGG
	BdLYR1ΔIC BP Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTCCACTTGTCGAGGGAACCCT

Discussion and perspectives

Discussion

Are LCOs important for AMS establishment?

We showed that SlLYK10 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 SlLYK10 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,^{35}S) and we could not measure radioactivity bound to membrane fractions containing BdLYR1 or SlLYK10, showing that SlLYK10 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, SlLYK10 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 SlLYK10 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,^{35}S) 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.

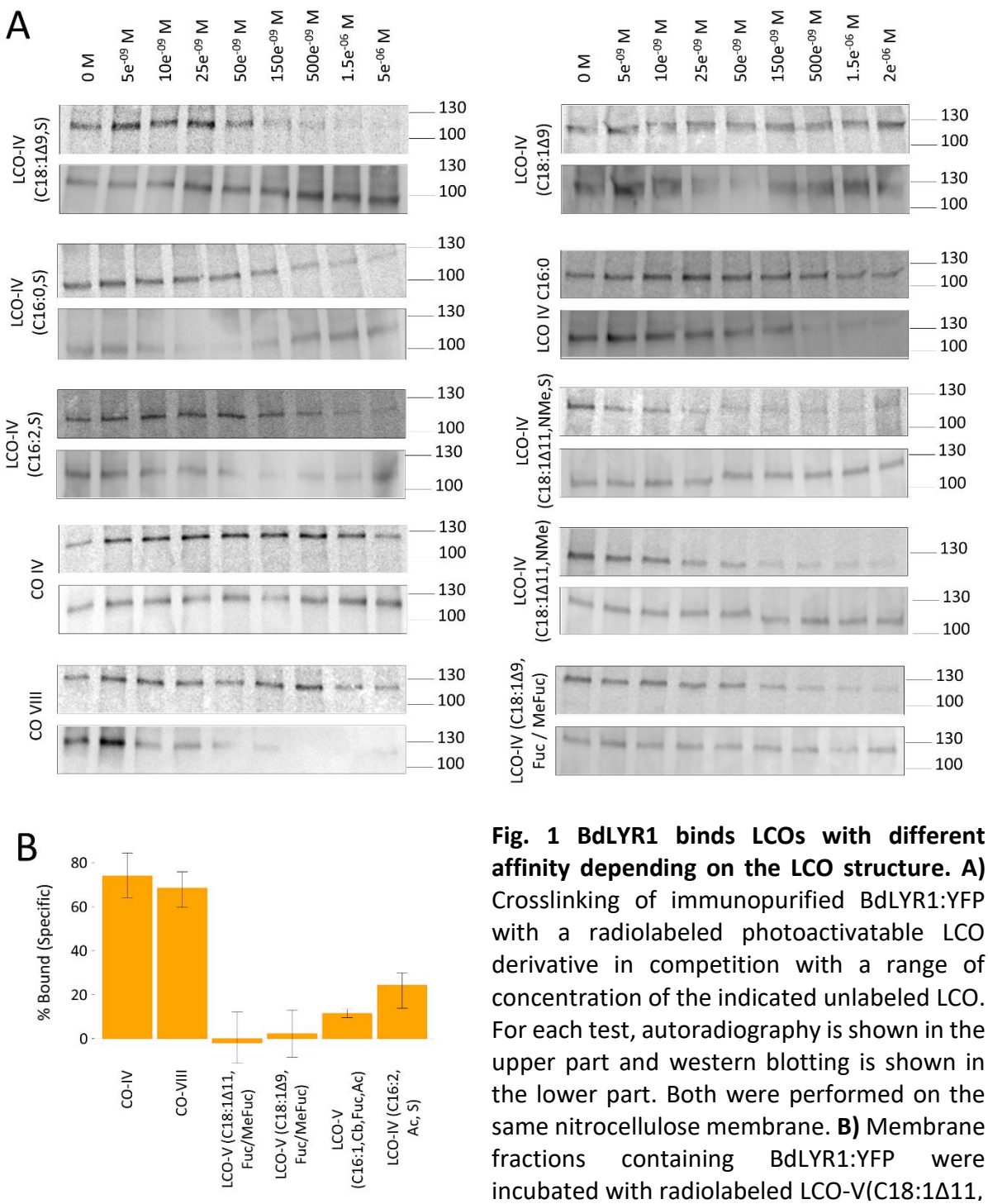

Fig. 1 BdLYR1 binds LCOs with different affinity depending on the LCO structure. A) Crosslinking of immunopurified BdLYR1:YFP with a radiolabeled photoactivatable LCO derivative in competition with a range of concentration of the indicated unlabeled LCO. For each test, autoradiography is shown in the 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,^{35}S) in presence of 1 µM of the indicated unlabeled molecules used as competitors. Non-specific binding was determined in the presence of 1 µM LCO-V(C18:1 Δ11,NMe,S). Bars represent the percentage of specific binding (means and SE) obtained in the presence of an excess of competitor (1 µM) 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*.

However BdLYR1 appears to have similar high affinity for these LCOs. Indeed, binding of LCO-V(C18:1,NMe,^{35}S) 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,^{35}S) 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(C18:1Δ9, 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(C18:1Δ9, 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 SlLYK10 and BdLYR1 for LCO structures produced by EMF to determine whether SlLYK10 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 SlLYK10 and BdLYR1 for these LCOs to validate whether they specifically recognize AMF LCOs or whether they are involved in broader roles.

We showed that SlLYK10 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 *SlLYK10* silencing strongly affects AMF penetration in tomato roots 2 wpi (Buendia et al., 2016). Hence, we could conclude that LCO perception by SlLYK10 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, *SlLYK10* 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 FlS2 after ligand perception (Beck et al., 2012)? We tried to understand whether BdLYR1 or SlLYK10 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 SlLYK10:YFP and BdLYR1:YFP in tomato roots and *B. distachyon* roots respectively. However, SlLYK10: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.

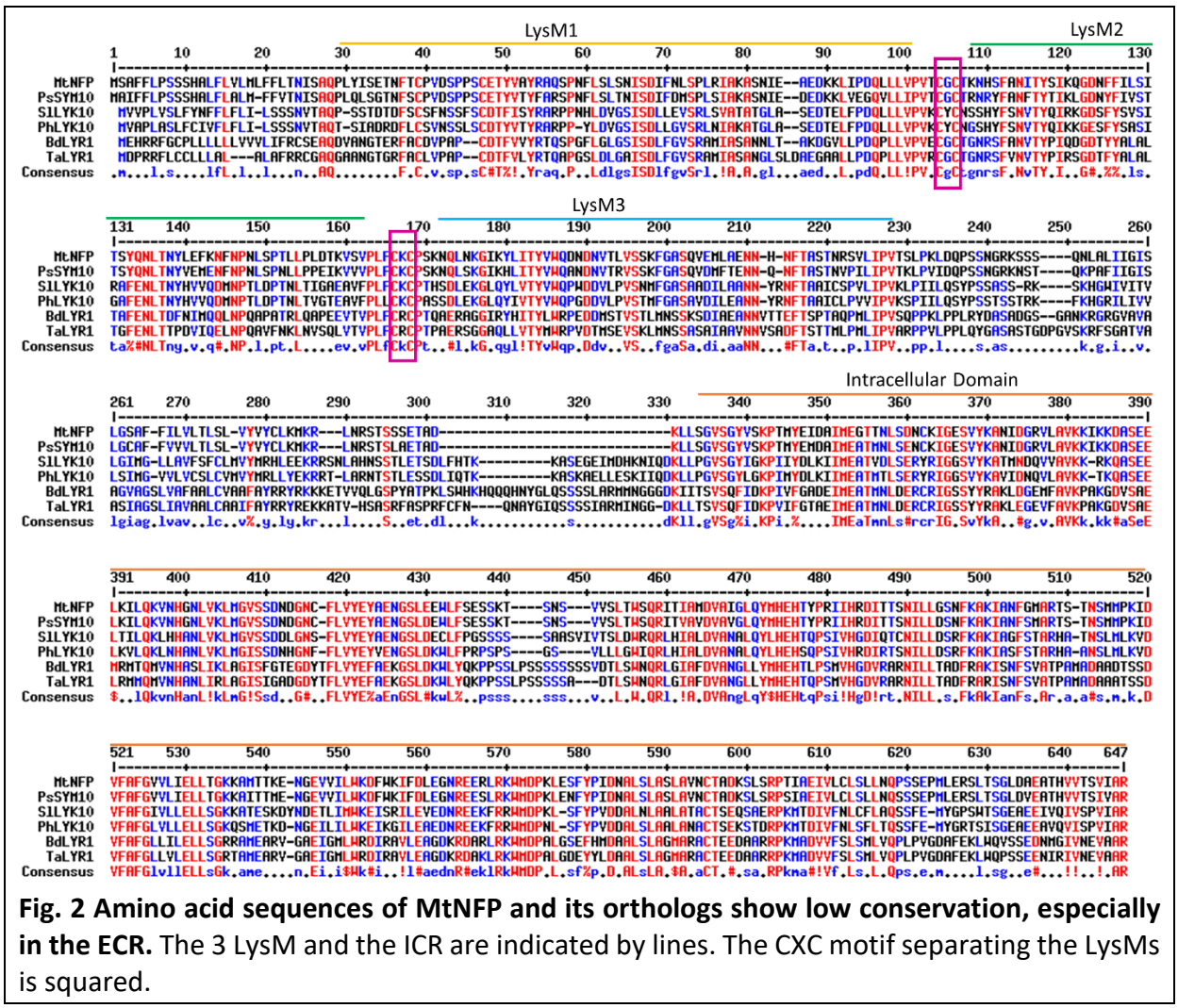

Fig. 2 Amino acid sequences of MtNFP and its orthologs show low conservation, especially in the ECR. The 3 LysM and the ICR are indicated by lines. The CXC motif separating the LysMs is squared.

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 p*35S::PsSYM10:YFP* and p*35S::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 µM 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.

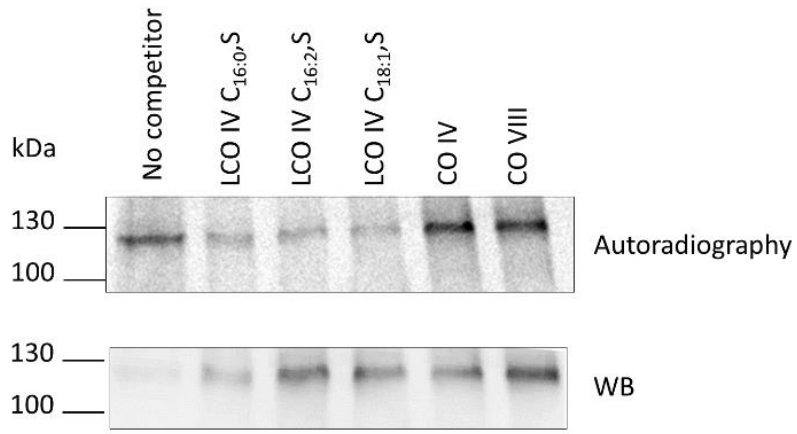

Fig. 3 BdLYR3 preferentially binds LCOs versus COs. Crosslinking of immunopurified BdLYR3c:YFP by a radiolabeled and photoactivatable LCO derivative in competition with 2 µM of indicated unlabeled LCOs or COs. Autoradiography was performed on the same nitrocellulose membrane used for western blotting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

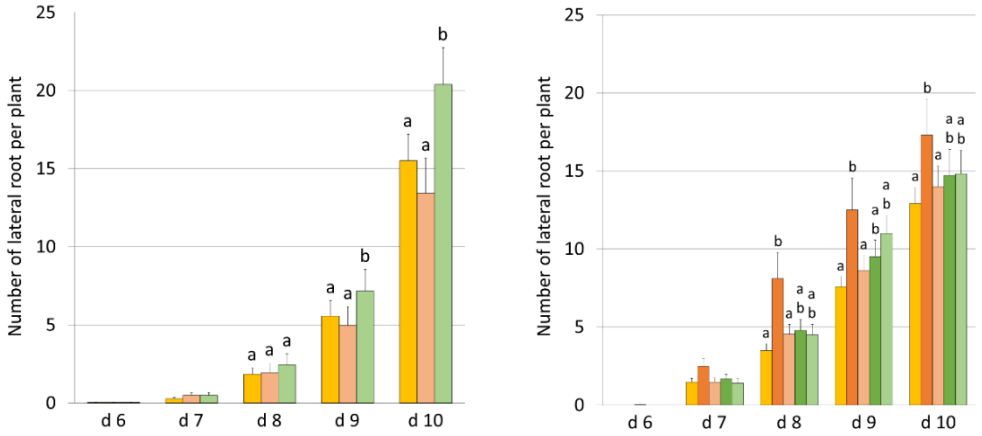

Fig. 4 LCOs induce lateral root formation in *B. distachyon* Bd 21-3. Two examples of lateral root induction bioassay. Plantlets were grown *in vitro* on gelified culture medium supplemented or not with LCOs:
(■) Control; (■) Mix LCO-IV(C18:1,S) and LCO-IV(C16,S) at 10^{-7} M; (■) Mix LCO-IV(C18:1,S) 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^{-9}M. 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 p*35S::BdLYR1* and p*35S::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 *SlLYK10* 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 *SlLYK10* 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).

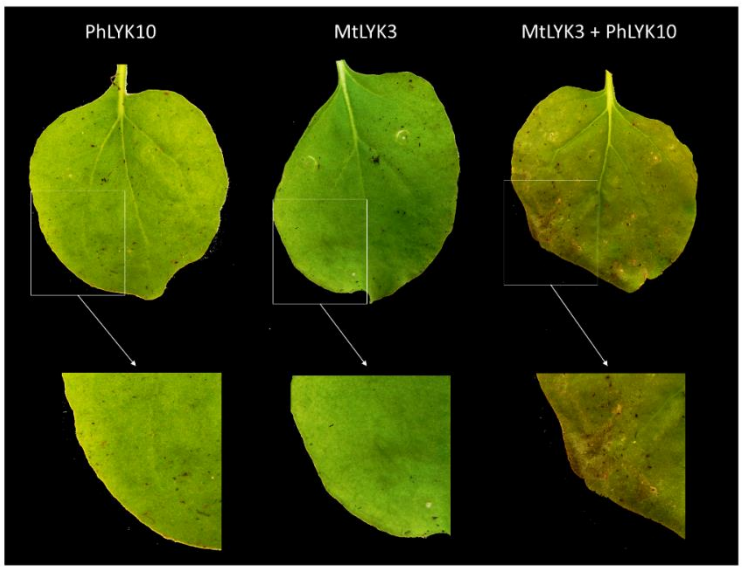

Fig. 5 PhLYK10 interacts with MtLYK3 in *N. benthamiana*. PhLYK10 and MtLYK3 do not induce cell death when expressed separately in *N. benthamiana*, but when they are co-expressed, cell death is induced. Images are representative of 3 biological replicates. Lower panel is a close-up.

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 p*35S::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* in *M. truncatula* during RLS and AMS to observe whether 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 SlLYK10 and BdLYR1 in AMS

In the 2nd chapter of my PhD thesis, I demonstrated that SlLYK10 has high affinity for LCOs and in the 1st chapter of my PhD thesis, we demonstrated that *SlLYK10* is involved in AMS establishment. Hence, we could conclude that LCO perception *via* SlLYK10 is important for AMS establishment. To corroborate this, we first have to confirm the phenotype we observed by VIGS (Virus Induced Gene Silencing) on *SlLYK10* in tomato. We could, in a first instance, edit *SlLYK10* with CRISPR-Cas9 in order to confirm defect of AMF colonization in another *Sllyk10* allele.

On another hand, we have to confirm that SlLYK10 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 SlLYK10 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 SlLYK10 is important for AMS establishment in tomato.

Another way to show the importance of LCO binding by SlLYK10 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.

Long term perspectives

What are the residues responsible of the binding activity of SlLYK10 and BdLYR1?

Even if we know the affinity of SlLYK10 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 SlLYK10 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 SlLYK10 or BdLYR1 we could perform swaps between the LysM of SlLYK10 and SlLYK7 (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 SlLYK10 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 SlLYK10 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 SlLYK10 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,^{35}S). We could determine TaLYR1D has a *Kd* of 13.8 nM for LCO-V(C18:1,NMe,S), in the same range than for SlLYK10 or BdLYR1. Hence, TaLYR1D has high affinity and is a biochemical ortholog of SlLYK10 and BdLYR1. We also performed competition assays with 1 µM 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.

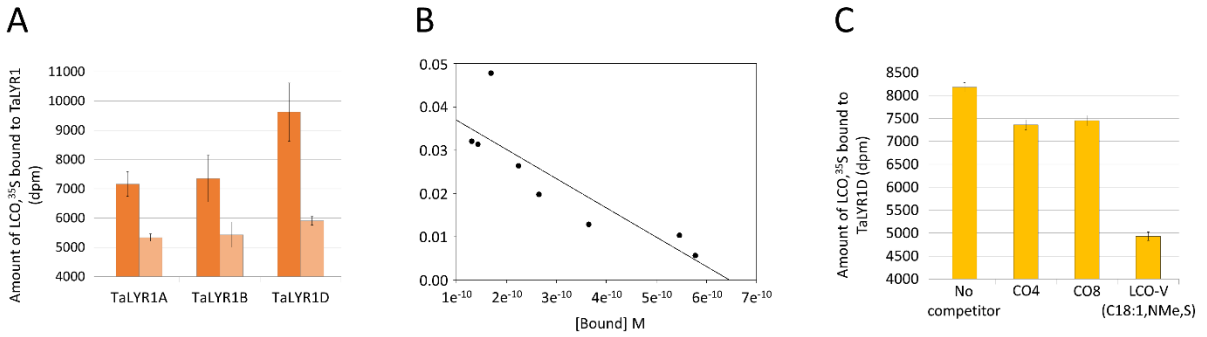

Fig. 6 TaLYR1 binds LCOs with high affinity and selectivity versus COs. A) Membrane fractions containing TaLYR1A:YFP, TaLYR1B:YFP or TaLYR1D:YFP were incubated with radiolabeled LCO-V(C18:1,NMe,^{35}S) in absence (dark orange) or in presence (light orange) of 1 µM of LCO-V(C18:1,NMe,S). **B)** Scatchard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

Conclusion

During my thesis, we showed that SlLYK10 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.

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Annexes

Annex 1: *B. distachyon* genome editing by CRISPR-Cas9

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*35S*. 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.

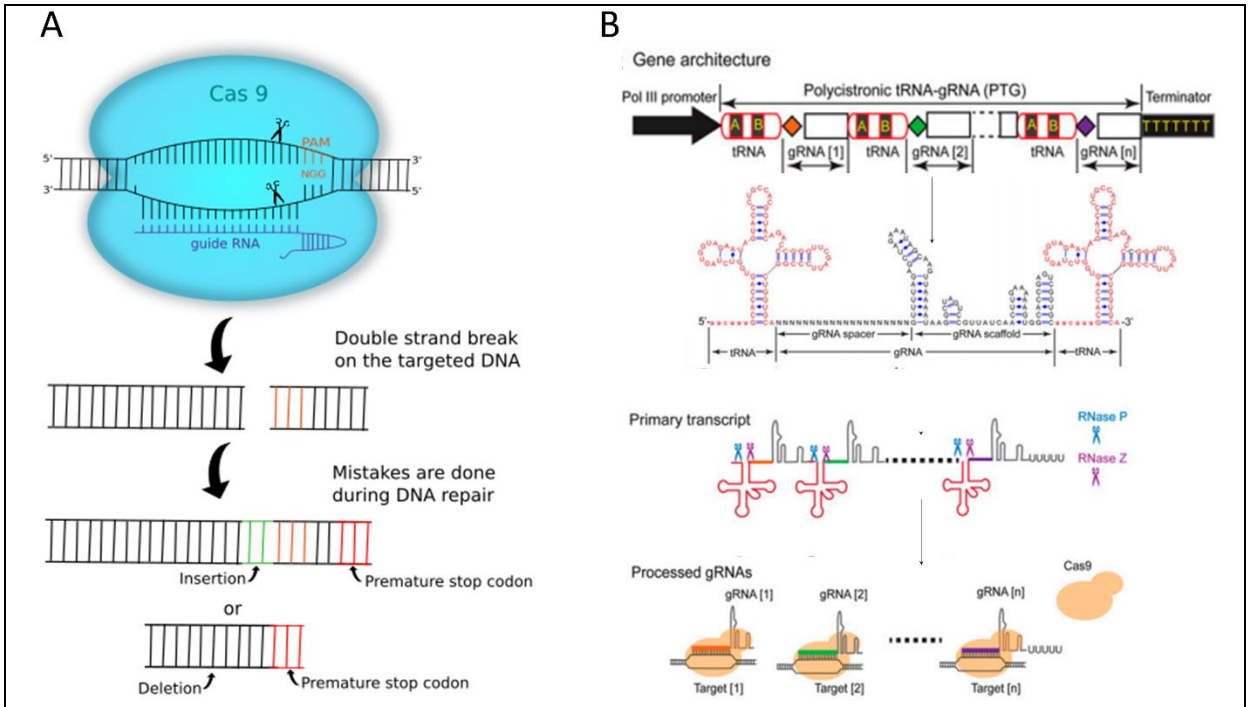

Fig. 1 DNA editing by CRISPR-Cas9. A) The guide RNA (gRNA) is divided in 2 parts, one part (also called the spacer) is composed of 20 bases that hybridizes with the targeted DNA. The other part (also called the scaffold) which is always the same RNA sequence has a secondary structure recognized by Cas9. 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.

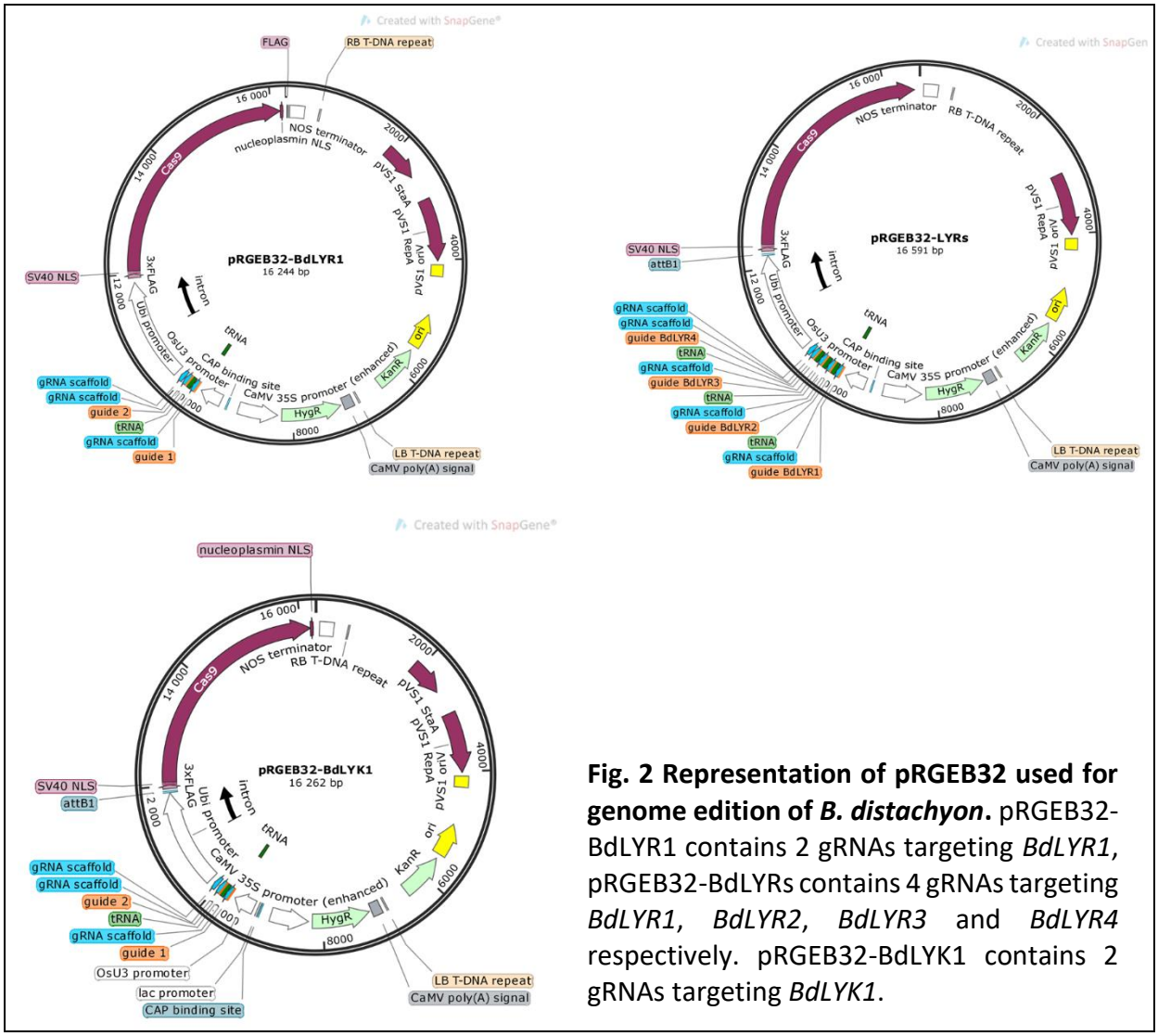

Fig. 2 Representation of pRGEB32 used for genome edition of *B. distachyon*. pRGEB32-BdLYR1 contains 2 gRNAs targeting *BdLYR1*, pRGEB32-BdLYRs contains 4 gRNAs targeting *BdLYR1*, *BdLYR2*, *BdLYR3* and *BdLYR4* respectively. pRGEB32-BdLYK1 contains 2 gRNAs targeting *BdLYK1*.

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
TCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGACAACAAAGACTAG
TATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGCAACAAAGCACCAG
TGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCACGCTTCGCAACACACACAC
AGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAA
GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAAAGCTTCCTCCG
CTGCGCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
CTTTTTTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCG
GTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCATGCCTGCAGGTCCACA
AATTCGGGTCAAG
>pRGEB32-BdLYRs
TCGTAGTGGGCCATGAAGCCTTTCAGGACATGTATTGCAGTATGGGCCGGCCCATTACGCAATTGGACGACAACAAAGACTAG
TATTAGTACCACCTCGGCTATCCACATAGATCAAAGCTGATTTAAAAGAGTTGTGCAGATGATCCGTGGCAACAAAGCACCAG
TGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCACGCTTCGCAACACACACAC
AGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAA
GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAACGCCCACCAAG
CTGGAAGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG

CAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCACTCGA
CGCCAACAGCCTCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGA
GTCGGTGCAACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCgATTCCCGGCTGGTG
CAGACTCCCCGTGGTGCCGCTCGTTTTAgAGCTAGAAATAGCAAGTTAAAATAAgGcTAGTCCGTTATCAACTTGAAAAAGTG
GCACCGAGTCGGTGCTTTTTTTTTTTgtTTTAGagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaa
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gcctgcaggtccacaaattcgggtcaag
>pRGEB32-BdLYK1
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TGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCACTTGTTTTATAGACGAAGA
AGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCAACAAA
GCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAGACCCGGGTTCGATTCCCGGCTGGTGCAGATGGCACTTCA
TCAGCTGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG
CTTTTTTTTTTGTTTGCTGTCGCTCATCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTG
CATGCCTGCAGGTCCACAAATTCGGGTCAAG

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.

Critical points: 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.

Critical points: 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.

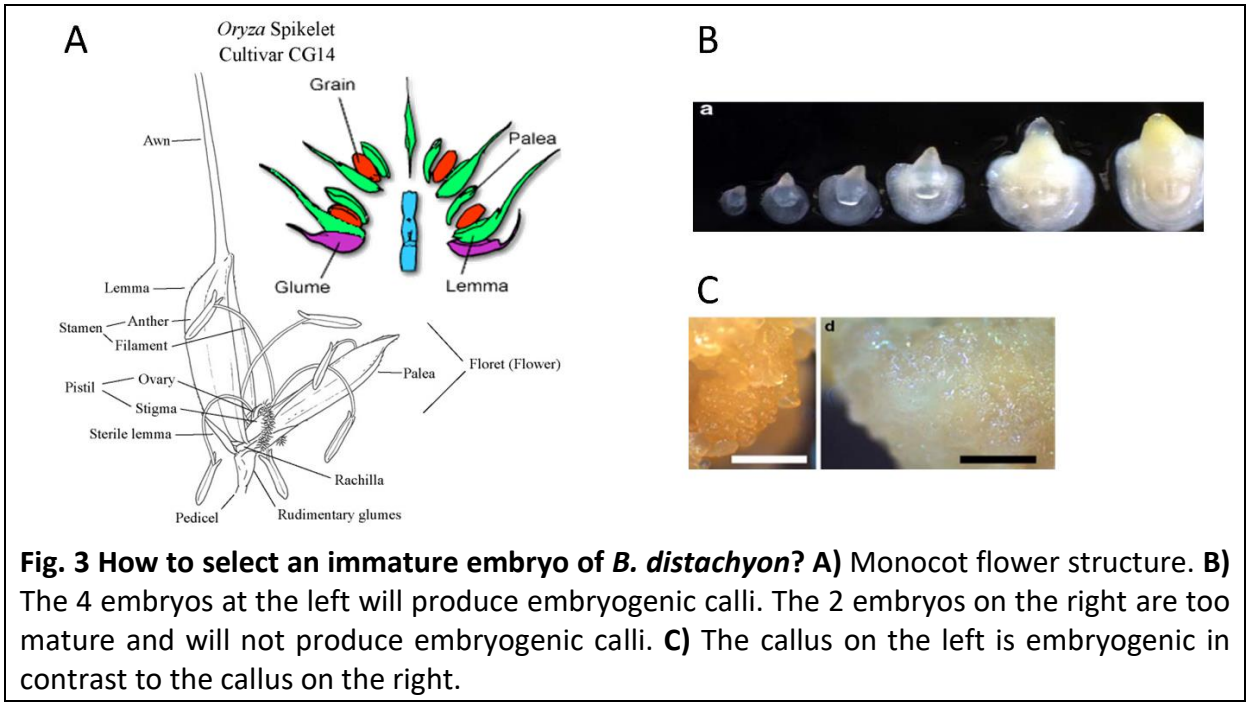

Fig. 3 How to select an immature embryo of *B. distachyon*? A) Monocot flower structure. **B)** The 4 embryos at the left will produce embryogenic calli. The 2 embryos on the right are too mature and will not produce embryogenic calli. **C)** The callus on the left is embryogenic in contrast to the callus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

Table 1: CIM medium		
	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
BCP	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).

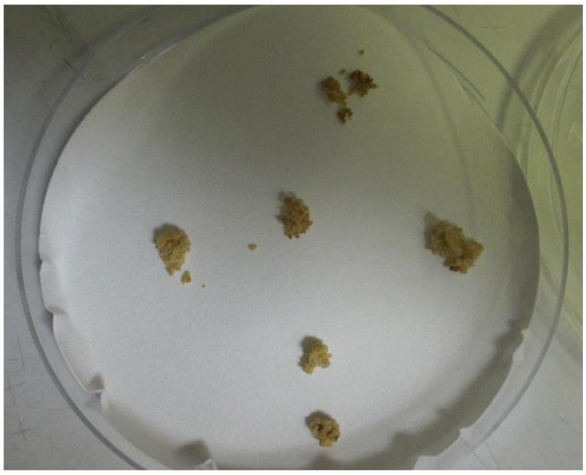

Fig. 4 Dried calli (3 days after co-culture with *A. tumefaciens* AGL1).

Table 2: Co-culture medium		
Solution	Concentration	Volume (for 200 ml)
CIM (liquid) without 2,4D & BCP		200 mL
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.

Table 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
BCP	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 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.

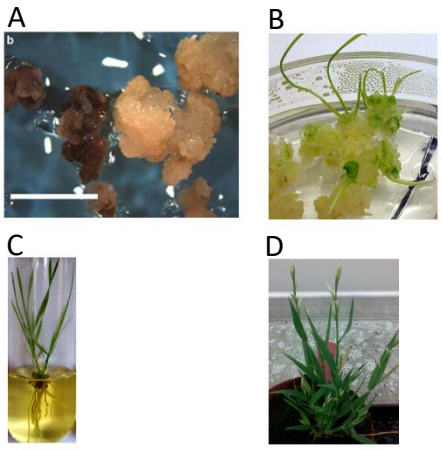

Fig. 5 Transformation steps from selection to regeneration. A) The callus on the right is transgenic in contrast to the callus on the left. **B)** Regenerated plantlet 3 weeks after transfer on regeneration medium. **C)** Rooting in tube 2 weeks after transfer on rooting medium. **D)** Transformed plant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

Table 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
BCP	0.16 %	5 mL
Phytagel	2 g/L	2 g
milliQ water	Qsp 1 L	
Autoclave and when the medium is cold enough, add the following 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
BCP	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 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.

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

Table 1: genotyping and phenotyping summary of *B. distachyon* mutageneized lines.

Mutated gene	Seed line	Sequencing priemer	Genotype			Phenotype		
			WT	Heterozygote	mutant	AMS	Binding to LCO-V(C18:1,NMe,S)	Lateral root induction by LCO
Bd1g69290 (BdLYR1)	3732	Seq 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	+	+	?
	4252		4/7	1/7	2/7	+	+	?
	4914 (M3)		19/19	0/19	0/19			
	3774 (M3)		21/21	0/21	0/21			
	6039 (M3)		35/35	0/35	0/35			
	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	?	?	?
	5525 (M3)	Till2 Rev	16/25	1/25	0/25	?	?	?
	5525 (M2)	Till2 Rev	14/20	0/20	0/20	?	?	?
	5525 (M4)	Till2 Rev	29/125	33/125	25/125	+	?	?
	3774 (M2)	Til Seq For 2	3/6	1/6	2/6	?	?	?
Bd2g40627 (BdLYK2)	7754	Seq Rev	8/23	3/23	12/23	?	?	?
	8653	Til For	14/17	3/17	1/17	?	?	?
	8122	Seq Rev	21/21	0/21	0/21			
	7889	Til For	14/14	0/14	0/14			
	4974	Til For	1/6	1/6	4/6	?	?	?
	8511	Til For	4/7	1/7	3/7	?	?	?
	3718	Til Rev	4/8	0/8	4/8	?	?	?
	5570	Til Rev	2/13	0/13	11/13	?	?	?

Lines containing only WT plants same phenotype as WT plants

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Annex 3: CDS of TaLYR1A, TaLYR1B and TaLYR1D

>TaLYR1_A
ATGGACCCCCGCCGCTTCCTCTGCTGCCTCCTGCTCGCCCTCGCCCTCGCCTCCCGCCGCTGCGCCGCGCAGGGCGGCGCCGG
GCGCTTCGCGTGCCTCGTGCCGGCCCCGTGCGACACGTTCGTCCTGTACCGCACGCAGGCCCCGGGGTCCCTCGACCTCGGCG
CCGTCTCGGACCTCTTCGGCGTGAGCCGGGCCATCATCGCCAGCGCCAACGGCTTCGGCCTCGACGCCGAGGGCGCGGCGCTG
CTGCCCGACCAGCCGCTGCTCGTGCCCGTCCGCTGCGGCTGCACCGGCGGCCGCTCCTTCGTCAACGTCACCTACCCCATCCG
CTCCGGCGACACCTTCTACGCGCTCGCGCTCACCGGCTTCGAGAACCTCACCACCCCCGACGTCATCCAGGAGCTCAACCCGC
AGGCGGTCTTCAACAAGCTCAATGTCTCGCAGCTCGTCACCGTGCCGCTCTTCTGCCGCTGCCCCACGCCGGCCGAGCGGGCC
GCCGGGGCGCAGCTGCTCGTCACCTACATGTGGCGCCCTGTCGACACCATGCCCGAGGTGAGCAACCTGATGGGCTCCGACGT
CGGCGCCATCGCTGCGGTGAACAACGTCAGCGCCGACTTCACCTCCACGACGATGCTGCCGATGCTGGTCCCGGTGGCGCGGC
CGCCGGTGCTTCCTCCGCTGCAATATGGCGCGAGCCCGAGCACCGGCGATCCCGGAGCCAGCAAGGGCTTCTCGGGCGCCACC
GTCGCGGCGAGCATCGCGGGGTCTCTCGTCGCCGTCGCTGCCCTGTGCACGGCGATCTTCGCGTACCGGAGGTACCGCGAGAA
GAAGGCCACGGTGCACTCGGCGTCCAGGTTCGCGAGCCCGAGGTTTTGCTTCAACCAGAACGCTTACGGGATTCAGAACAGCA
GTTCCATCGCTCGCATGATCAACGGAGGGGACAAGCTGCTCACCAGCGTGTCGCAGTTCATCAACAAGCCTATCATCTTCGGT
ACAGAGGAGATCATGGAGGCGACGATGAACTTGGACGAACGGTGCAGGATCGGCAGCTCCTACTACCGGGCCAAGCTGGAAGG
CGAGGTGTTCGCGGTGAAGCCGGCGAAAGGCGACGTGTCGGCGGAGCTGAGGATGATGCAGATGGTCAACCACGCCAACCTCA
TCAGGCTGGCCGGCATATCCATCGGCGCAGACGGGGACTACACCTTCCTCGTGTACGAGTTCGCCGAGAAGGGCTCGCTCGAC
AAGTGGCTGTACCAGAAGCCTCCGTCCTCGCTGCCGTCGTCGAGCTCATCGGCGGACACGCTCTCGTGGAACCAGAGGCTGGG
CATCGCGTTCGACGTCGCCAACGGCCTGCTCTACATGCACGAGCACACTCAGCCGAGCATGGTGCACGGCGACGTCCGCGCAC
GGAACATCCTCCTCACCGCGGACTTCAGGGTCAGGATATCTAACTTCTCCGTGGCCACGCCGGCGATGGCCGACGCCGCGGCG
ACGAGCAGCGACGTGTTCGCCTTCGGCCTGCTGGTCCTCGAGCTTCTCTCCGGCAGGACGGCCATGGAGGCGCGCGTCGGCGC
GGAGATCGGCATGCTGTGGAGGGACATCCGGGCGGTGCTGGAGGCCGGGGACAAGAGGGACGCCAAGCTGAGGAAGTGGATGG
ACCCCGCCCTTGGGGACGAGTACTACTTGGATGCGGCACTCAGCCTGGCCGGCATGGCGAGGGCCTGCACGGAGGAGGACGCG
GCGCGGCGGCCGAAGATGGCCGACGTCGTGTTCAGCCTTTCAATGCTGGTGCAGCCGTCACCGGTGGGCGACGCGTTCGAGAA
GCTATGGCAGCCCAGCTCGGAGGAGAACATTAGGATTGTCAATGAAGTGGCAGCCAGATGA

>TaLYR1_B
ATGGACCCCCGCCGCTTCCTCTGCCACCTCTTGCTCGCCCTCGCCCTCGCCTTCCGCCGCTGCGACGCGCAGGGCGCCGGCAA
CGGCACCGGGCGCTTCGCCTGCCTCGTGCCGGCCCCGTGCGACACGTTCGTCCTGTACCGCACGCAGTCCCCGGGGTCCCTCG
ACCTCGGCGCCATCTCGGACCTCTTCGGCGTGAGCCGGGCCATGATCGCCAGCGCCAACGGCCTCAGCCTCGCCGCCGAGGAC
GCGGCGCTGCTGCCCGACCAGCCGCTGCTCGTGCCCGTCCGGTGCGGCTGCACCGGCAACCGCTCCTTCGTCAACGTCACCTA
CCCCATCCACTCCGGCGACACCTTCTACGCGCTCGCGCTCACCGGCTTCGAGAACCTCACCACCCCCGACGTCATCCAGGAGC
TCAACCCGCAGGCGGTTTTCAACAAGCTCAATGTCTCGCAGCTGGTCACCGTGCCGCTCTTCTGCCGGTGCCCCACGCCGGCG
GAGCGGAGCGCCGGGGTGCAGCTGCACGTCACCTACATGTGGCGACCTGTCGACACCATGTCCGAGGTGAGCAAGCTGATGAA
CTCCAGTGCGAGCGCCATCGCTGCGGCCAACAACGTCACTGCCGACTTCACCTCCACGACGATGCTGCCGATGCTGATCCCGG
TGGCGCGGCCGCCGGTGCTTCCTCCGCTGCGCTATGGTGCGAGCCCGAGCACCGGCGATCTCGGAGCTAGCAAGGGCTTCTCG
GGTGCCACCGTAGCGGCCAGCATCGCGGGGTCTCTCGTCGCGGTCGCCGCCTTGTGCGCGGCGATCTTCGCGTACCGGAGGTA
CCGCAAGAAGAAGGCCACGGTGCACTCGGCGTCCAGGTTCGCGAGCCCGAGGTTTTGCTTCAACCAGAACGCCTACGGGATTC
AGAGCAGCAGTTCCATCGCTCGCATGATCAACGGAGGGGACAAGCTGCTCACCAGTGTGTCACAGTTCATCAACAAGCCTATC
ATCTTCGGCACAGCGGAGATCATGGAGGCGACGATGAACTTGGACGAACGGTGCAGGATCGGCACCTCCTACTACCGGGCCAA
GCTAGAAGGCGAGGTGTTCGCGGTGAAGCCGGCGAAAGGCGACGTGTCGGCGGAGCTGAGGATGATGCAGATGGTCAACCACG
CCAACCTCATCAGGCTGGCCGGCATATCCATCGGCGCGGATGGGGACTACACNTTCCTCGTGTACGAATTCGCCGAGAAGGGC
TCGCTCGACAAGTGGCTGTACCAGAAGCCTCCGTCCTCGCTGCCGTCGTCGAGCTCAGTGGATACGCTCTCGTGGAACCAGAG
GCTGGGCATCGCGTTCGACGTCGCCAACGGCCTGCTGTACATGCACGAGCACACTCAGCCGAGCATGGTGCACGGCGACGTCC
GCGCACGGAACATCCTCCTCACCGCAGACTTCAGGGCCAGGATATCCAACTTCTCCGTGGCCACGCCGGCGATGGCCGACGCC
GCGGCGACGAGCAGCGACGTGTTCGCCTTCGGCCTACTGGTCCTCGAGCTTCTCTCCGGCAGGATGGCCATGGAGGCGCGCGT
CGGCGCGGAGATCGGCATGCTGTGGAGGGACATCCGGGCGGTGCTGGAGGCCGGGGACAAGAGGGACGCCAAGCTGAGGAAGT
GGATGGACCCCGCCCTTGGGGACGAGTACTACTTGGATGCGGCACTCAGCCTGGCCGGCATGGCCAGGGCTTGCACGGAGGAG
GACGCGGCGCGGCGGCCGAAGATGGCCGACGTCGTGTTCAGCCTGTCAATGCTGGTGCAGCCGTTACCGGTGGGCGACGGGTT
CGAGAAGCTATGGCAACCCAGCTCGGAGGAGAACATTAGGATTGTCAATGAAGTGGCAGCCAGA

>TaLYR1_D
ATGGACCCCCGCCGCTTCCTCTGCTGCCTCTTGCTCGCCCTCGCCCTCGCCTTCCGCCGCTGCGGCGCGCAGGGCGCCGCCAA
CGGCACCGGGCGCTTCGCGTGCCTCGTGCCGGCCCCGTGCGACACGTTCGTCCTGTACCGCACGCAGGCCCCGGGGTCCCTCG
ACCTCGGCGCCATCTCGGACCTCTTCGGCGTGAGCCGGGCCATGATCGCCAGCGCCAACGGCCTCAGCCTCGACGCCGAGGGC
GCGGCGCTGCTGCCCGACCAGCCGCTGCTCGTGCCCGTCCGCTGCGGCTGCACCGGCAACCGCTCCTTCGTCAACGTCACCTA
CCCCATCCGCTCCGGCGACACCTTCTACGCGCTCGCGCTCACCGGCTTCGAGAACCTCACCACCCCCGACGTCATCCAGGAGC
TCAACCCGCAGGCGGTCTTCAACAAGCTCAATGTCTCGCAGCTGGTCACCGTGCCGCTCTTCTGCCGGTGCCCCACGCCGGCG
GAGCGGAGCGGCGGGGCGCAGCTGCTCGTCACCTACATGTGGCGACCTGTCGACACCATGTCCGAGGTGAGCAAGCTGATGAA
CTCTAGCGCGAGCGCGATCGCTGCGGTGAACAACGTCAGCGCCGACTTCACCTCCACGACGATGCTGCCGATGCTGATCCCGG
TGGCGCGGCCGCCGGTGCTTCCTCCGCTGCAATATGGCGCGAGCGCGAGCACCGGCGATCCCGGAGTTAGCAAGCGCTTCTCG
GGTGCCACCGTCGCGGCGAGCATCGCCGGGTCTCTCATCGCGGTCGCCGCCTTGTGCGCGGCGATCTTCGCGTACCGGAGGTA
CCGCGAGAAGAAGGCCACGGTGCACTCGGCGTCCAGGTTCGCGAGCCCGAGGTTTTGTTTCAACCAGAACGCCTACGGGATTC
AGAGCAGCAGTTCCATCGCGCGCATGATCAACGGAGGGGACAAGCTGCTCACCAGCGTGTCGCAGTTCATCGACAAGCCTGTC
ATCTTCGGCACAGCGGAGATCATGGAAGCGACGATGAACTTGGACGAACGGTGCAGGATCGGCAGCTCCTACTACCGGGCCAA

GCTAGAAGGCGAGGTGTTCGCGGTGAAGCCGGCGAAAGGCGACGTGTCGGCGGAGCTGAGGATGATGCAGATGGTCAACCACG
CCAACCTCATCAGGCTGGCCGGCATATCCATCGGCGCCGATGGGGACTACACCTTCCTCGTGTACGAGTTCGCCGAGAAGGGC
TCGCTCGACAAGTGGCTGTACCAGAAGCCTCCGTCCTCGCTGCCGTCGTCGAGCTCATCAGCGGACACGCTCTCGTGGAACCA
GAGGCTGGGCATCGCGTTCGACGTCGCCAACGGCCTACTATACATGCACGAGCACACTCAGCCGAGCATGGTGCACGGTGACG
TCCGCGCACGGAACATCCTCCTCACCGCGGACTTCAGGGCCAGGATATCCAACTTCTCCGTGGCCACGCCGGCGATGGCCGAC
GCCGCGGCGACGAGCAGCGACGTGTTCGCCTTCGGCCTACTTGTCCTCGAGCTTCTCTCCGGCAGGACGGCCATGGAGGCGCG
CGTCGGCGCGGAGATCGGCATGCTATGGAGGGACATCCGGGCGGTGCTGGAGGCCGGGGACAAGAGGGACGCCAAGCTGAGGA
AGTGGATGGACCCCGCCCTTGGGGACGAGTACTACTTGGATGCGGCACTCAGCCTGGCCGGCATGGCGAGGGCTTGCACGGAG
GAGGACGCGGCGCGGCGGCCGAAGATGGCCGACGTGGTGTTCAGCCTCTCGATGCTGGTGCAGCCGTTGCCGGTGGGCGACGC
GTTCGAGAAGCTATGGCAGCCCAGCTCGGAGGAGAACATTAGGATTGTCAATGAAGTGGCAGCCAGATGA

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 ^{35}S-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.