Functional adaptation of the plant receptor-kinase gene *SYMRK* paved the way for the evolution of root endosymbioses with bacteria

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Functional adaptation of the plant receptor-kinase gene *SYMRK* paved the way for the evolution of root endosymbioses with bacteria

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Abbreviations

AM	Arbuscular Mycorrhiza
AR	Actinorhiza
bp	nucleic acid base pair(s)
BLAST	basic local alignment search tool
°C	degree(s) Celsius
CaMV	cauliflower mosaic virus
CC	coiled-coil domain
CCaMK	calcium/calmodulin dependent protein kinase
cDNA	complementary DNA
CEC	conserved extracellular region of predicted SYMRK proteins
DMI2	does not make infections 2
DNA	desoxyribonucleic acid
(e)GFP	(enhanced) green fluorescent protein
g/l	gram(s) per litre
h	hour(s)
IT	infection thread
LRR	leucine-rich repeat
LYK	LysM domain containing receptor-like kinase
ml	milliliter(s)
mm	millimeter(s)
mRNA	messenger RNA
n	number of tested items or specimens
NEC	N-terminal extracellular region of predicted SYMRK proteins
NFR	nod factor receptor
NIN	nodule inception
NLS	nuclear localization signal
nm	nanometre(s)
NORK	nodulation receptor kinase
NUP	nucleoporin
OD_{6oo}	optical density at 600 a wavelength of 600 nm
PCR	polymerase chain reaction
pg	picogram(s)
PIT	pre-infection thread
PK	protein kinase domain
PPA	pre-penetration apparatus
qRT-PCR	quantitative RT-PCR
RACE	rapid amplification of cDNA ends
RLS	rhizobium-legume symbiosis
RNA	ribonucleic acid
RNAi	RNA interference
RNS	root nodule symbiosis;
RT-PCR	polymerase chain reaction following reverse transcription of RNA

SbtM4	symbiosis induced subtilase gene 4 (SbtM type)
SYMRK	symbiosis receptor-like kinase
TM	transmembrane domain
WOC	week(s) of co-cultivation
WT	wild type; wild-type
µM	micromolar
µm	micrometer(s)

Species names and affiliations

Plants (Kingdom Plantae)

Full scientific name	**Abbreviation**	**Order**	**Common name**
Allocasuarina verticillata (Lam.) L.A.S. Johnson	*A. verticillata*	Fagales	
Alnus glutinosa (L.) Gaertn.	*A. glutinosa*	Fagales	alder
Arabidopsis thaliana (L.) Heynh.	*A. thaliana; Arabidopsis*	Brassicales	thale cress
Datisca glomerata (C. Presl) Baill	*D. glomerata; Datisca*	Cucurbitales	durango root
Lotus japonicus (Regel) K. Larsen	*L. japonicus; Lotus*	Fabales	birdsfoot trefoil
Lupinus albus L.	*L. albus*	Fabales	lupine
Lycopersicon esculentum Mill.	*L. esculentum*	Solanales	tomato
Oryza sativa L.	*O. sativa*	Poales	rice
Papaver rhoeas L.	*P. rhoeas*	Ranunculales	poppy
Tropaeolum majus L.	*T. majus; Tropaeolum*	Brassicales	nasturtium
Zea mays L.	*Z. mays*	Poales	maize

Fungi (Kingdom Fungi)

Full scientific name	**Abbreviation**
Glomus intraradices Schenck & Smith	*G. intraradices*

Bacteria (Kingdom Eubacteria)

Full scientific name	**Abbreviation**
Agrobacterium rhizogenes	*A. rhizogenes*
Frankia spec.	*Frankia*
Mesorhizobium loti	*M. loti*
Sinorhizobium meliloti	*S. meliloti*

Reference of the bacterial strains used in this study is provided in the materials and methods section (3.5, page 72).

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1 Summary

Combining a phylogenetic evolutionary approach with functional genetic analyses, this dissertation reveals breakthrough insights into the evolution of intracellular plant root symbioses with fungi or bacteria. Arbuscular Mycorrhiza (AM) with fungi is widespread among land plants today, and believed to date back to their earliest representatives. However, plant genes required for this symbiosis were confirmed only in legumes at the time this project started. The thesis provides evidence for a functional conservation of the legume symbiosis genes *CYCLOPS* and *SYMRK* in AM across dicot and monocot angiosperm lineages, and thus for the existence of a conserved genetic basis of AM in angiosperms. Rice *cyclops* mutants proved unable to form AM, and rice *CYCLOPS,* as well as *SYMRK* versions from rice and different dicot angiosperms were found to restore AM when transgenically introduced into legume mutants in which the orthologous genes are defective.

In legumes, at least seven "common symbiosis" genes—including *SYMRK* and *CYCLOPS*—are required not only for AM, but also for the more recently evolved root nodule symbiosis (RNS) with nitrogen-fixing rhizobia. RNS occurs in two major forms, legume-rhizobium symbiosis and Actinorhiza. RNS types differ in bacterial partner, intracellular infection pattern, and morphogenesis. This work identifies the receptor-like kinase gene *SYMRK* as the first plant gene known to be required for actinorhiza. *SYMRK* is shown to be necessary for Actinorhiza of the cucurbit *Datisca glomerata* and the Fagales tree *Casuarina glauca* with actinobacteria of the genus *Frankia,* providing the first functional evidence that all three types of plant root endosymbiosis, AM and both forms of RNS, are genetically and hence evolutionarily linked.

The overlap of AM and RNS genetic programs suggests a recruitment of symbiosis genes from AM during RNS evolution, but the molecular basis for this event is not clear. This work reveals that *SYMRK* exists in at least three distinct structural versions, of which the shorter forms from rice and tomato are sufficient for AM, but not for functional endosymbiosis with bacteria in the legume *Lotus japonicus*. Only the longest version, which is present in all RNS forming groups, can fully support RNS. All other common symbiosis genes are structurally conserved between monocot and dicot angiosperms, and rice *CYCLOPS* proved competent to support both AM and RNS in *Lotus japonicus*. The combined data support the idea that *SYMRK* sequence evolution was involved in the recruitment of a pre-existing signalling network from AM, paving the way for the evolution of intracellular root symbioses with nitrogen-fixing bacteria.

The key results of the study are discussed and interpreted in the light of a broader scientific context in the second chapter, which has been submitted as a review article.

2 Introduction and scientific context

Evolution of root endosymbiosis with bacteria: how novel are nodules?

The content of this chapter has been published as a review article. The manuscript and figures were prepared by the author of the thesis.

Markmann, K. and Parniske, M. (2009).
Evolution of root endosymbiosis with bacteria: how novel are nodules?
Trends in Plant Science: 14, 77-86.

2.1 Abstract

Plants form diverse symbioses with nitrogen-fixing bacteria to gain access to ammonium, a product of the prokaryote-exclusive enzyme nitrogenase. Improving the symbiotic effectiveness of crop plants like maize, wheat or rice is a highly topical challenge and could help reduce the need for energy-intense nitrogen fertilizer in staple food production. Root nodule symbiosis (RNS) constitutes one of the most productive nitrogen-fixing systems, but it is restricted to a small group of related angiosperms. Here, we review the genetic regulation of RNS and its interconnections with other plant symbiosis or plant developmental programs. Since RNS uses genetic programs that are widely conserved in land plants, we evaluate the prospects for a transfer to plants that are currently non-nodulating.

2.2 Root nodulation symbiosis: a rare but efficient source of nitrogen for plants

Limitation of water or nutrients such as nitrogen, potassium and phosphate restricts plant growth and development in many terrestrial ecosystems. Mutualistic symbioses of roots with fungi or bacteria help plants to cope with these constraints. Associative nitrogen fixation is found in plants as diverse as ferns (e.g. *Azolla*), gymnosperms (e.g. coralloid roots of cycads) and monocots, which can host endophytic diazotrophs such as *Azoarcus* or *Azospirillum*. Among the most sophisticated associations are intracellular (endo-) symbioses, where the microbial partner is accommodated within a living host cell [1]. This inter-organismic intimacy is based on complex molecular crosstalk between the partners and, except in parasitic associations where bacterial nitrogen fixation remains inefficient, allows efficient nutrient exchange to the benefit of both [1]. Root nodule symbiosis (RNS) effectively renders plants independent of other nitrogen supplies [2] but is restricted to only four related orders within the Eurosid clade of angiosperms [3]. RNS is characterized by two major evolutionary inventions: the intracellular uptake of bacteria and the formation of specialized organs, the root nodules [4,5]. The latter provide a suitable microenvironment for nitrogenase activity and allow

for a protected, controlled development of high bacterial population densities. Nodules thus resemble 'micro-fermenters' within the host plant that are optimized for symbiosis maintenance.

There are two main types of RNS that differ in bacterial partners, infection mechanisms and nodule organogenesis and morphology [4,5]. Legumes, including important agricultural crops such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*), interact with phylogenetically diverse nitrogen-fixing bacteria known as rhizobia [2,4]. Actinorhiza, a symbiosis with nitrogen-fixing actinobacteria of the genus Frankia [6], is formed by members of three rosid orders: the Fagales, Cucurbitales and Rosales [7]. Examples are alder (*Alnus spp*.), sea buckthorn (*Hippophae rhamnoides*) and the subtropical tree genus *Casuarina* [8].

Within the four orders where RNS occurs, its distribution is scattered [7,9]. It is an ongoing challenge to decipher the combination of molecular adaptations characterizing RNS-forming plants (i) relative to related species outside of the nodulating clade and (ii) relative to non-nodulating representatives within. The close kinship of taxa forming RNS prompted the proposition that a common ancestor could have acquired a genetic predisposition to evolve nodulation [3]. This predisposition, consisting of one or more genetic advances, consequently became a basis for the evolution of all types of RNS. This hypothesis predicts that the existing forms of RNS have overlapping genetic programs. However, experimental evidence for a genetic program shared between both types of RNS was obtained only recently [10,11]: post-transcriptional gene silencing demonstrated that the *SYMBIOSIS RECEPTOR-LIKE KINASE* (*SYMRK*) gene (also known as *DOES NOT MAKE INFECTIONS 2* [*DMI2*] in *Medicago truncatula* [barrel medic] and *NODULATION RECEPTOR KINASE* [*NORK*] in *Medicago sativa* [alfalfa]) [12,13], which is necessary for the interaction of legumes with rhizobia [12,13], is also required for actinorhiza with *Frankia* in the Fagales tree *Casuarina* glauca [10] and the cucurbit *Datisca glomerata* [11]. The common requirement of *SYMRK* is probably representative for other genes shared between RNS in legumes and actinorhiza (Figures 1, 2 and 3) and provides important support for the hypothesis that actinorhiza and legume RNS share a common genetic ancestry.

In this review, we discuss the genetic features that distinguish 'nodulators' from non-nodulating plants – and form the molecular prerequisite for the complex evolutionary achievement of nitrogen-fixing RNS.

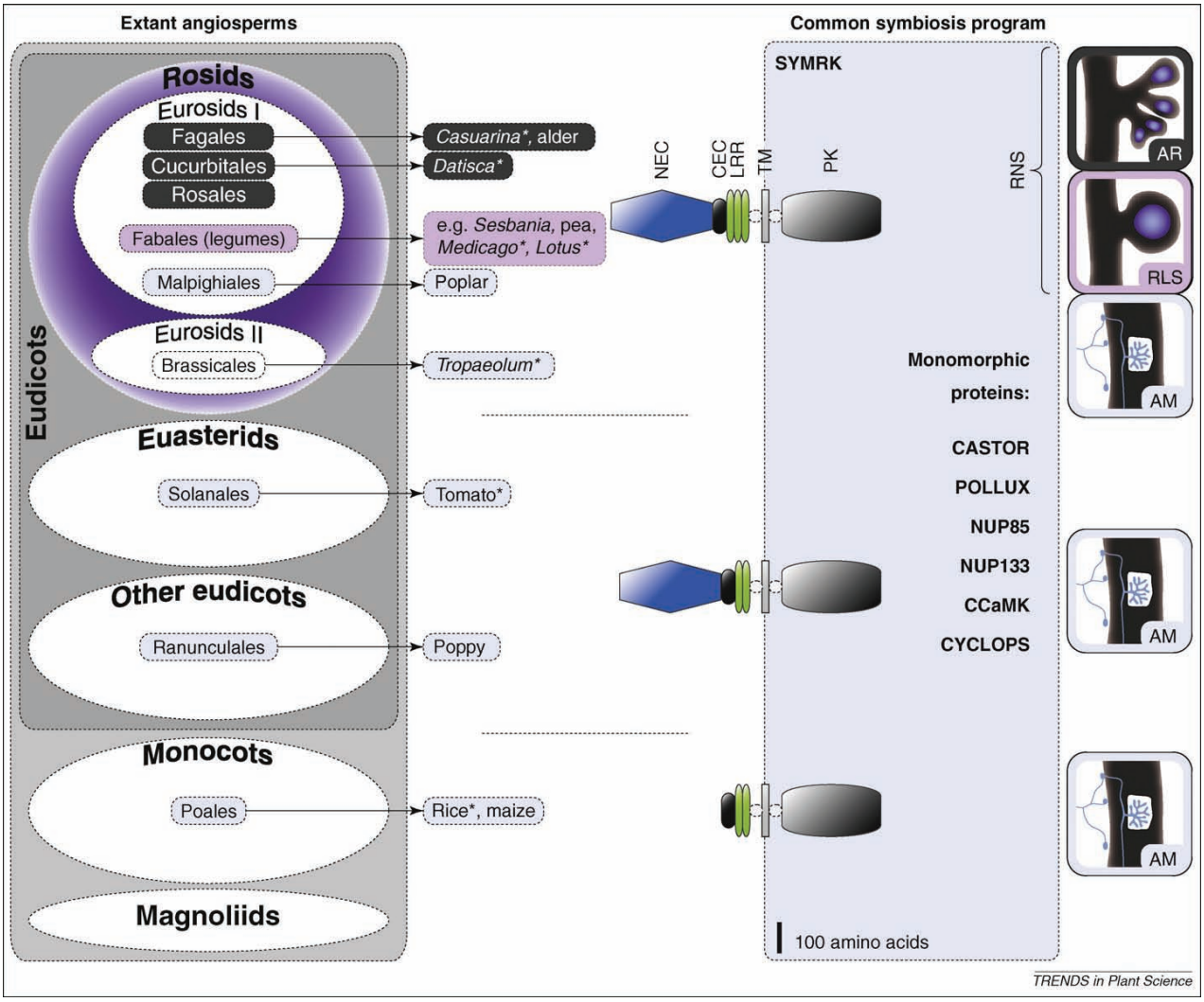
2.3 What are the key genetic inventions of the nodulating clade?

Among the prime candidates for adaptations specific to RNS-forming plants are genes involved in the perception of rhizobial signaling molecules, namely lipochito-oligosaccharide nodulation (Nod) factors. These receptors are required for the earliest host responses to symbiotic rhizobia and are involved in determining host–symbiont specificity [14–17]. They are likely to represent relatively recent achievements during the evolution of legume RNS. In the Japanese legume *Lotus japonicus*, a close relative of the Eurasian birdsfoot trefoil, perception of Nod factors depends on the receptor-like kinases (RLKs) NOD FACTOR RECEPTOR 1 (NFR1) and NFR5 and is highly specific in that Nod factors produced by different bacterial strains can be distinguished if they show minor alterations of their chemical structure [15–17]. NFR-predicted proteins contain lysine motif (LysM) domains in their predicted extracellular part, and these domains are involved in Nod factor recognition in *L. japonicus* [17]. Closely related genes mediate Nod-factor signaling in other legumes such as *M. truncatula* [14,18]. In an approach to induce a compatible interaction of rhizobial bacteria with an incompatible legume, *M. truncatula* was equipped with *L. japonicus* Nod factor receptor genes (*NFR1* and *NFR5*). Transgenic roots formed bacteria-containing nodules with the specific partner of *L. japonicus*, *Mesorhizobium loti* [17]. Although these nodules were not fully functional [17], the observations demonstrate that Nod factor receptors have a central role in defining host ranges in rhizobium–legume interactions. The Nod factor receptors are closely related to two receptors identified in the non-nodulating angiosperms *Arabidopsis thaliana* (thale cress) and rice (*Oryza sativa*), which are required for the defense-related perception of chitin oligomers, a chemical signature of fungi [19,20].

The high level of specificity of Nod-factor recognition and the necessity to evoke

symbiosis-related rather than defense-related downstream responses call for artificial transfer of compatible receptors to potential new hosts. Alternatively, infection systems that are likely to be independent of Nod-factor signaling, such as those employed by certain photosynthetic bradyrhizobia that induce nodules on aerial plant parts [21] or actinorhiza with *Frankia* [22], represent an interesting resource for further exploitation towards developing artificial host systems. Because these bacteria lack common *nod* genes required for Nod-factor synthesis in rhizobia [21,22], they must rely on alternative strategies to induce nodule organogenesis and infection. A key question is whether the Nod-factor receptors are specific to the legume branch of the nodulating clade or whether actinorhiza plants employ the same type of receptor for detection of *Frankia* symbionts. Key genes required for Nod-factor synthesis have not been detected in the fully sequenced *Frankia* genomes ([22]; Box 1). However, the exceptional genus *Parasponia* belongs to the Rosales that are normally nodulated by *Frankia* but is nodulated by rhizobia. *Parasponia* nodulation requires the rhizobial common nod genes, which suggests that LysM RLKs could be involved in RNS in this plant [23]. Although *Parasponia* might be an exception, it is possible that the adaption of LysM receptors for the recognition of symbiotic bacteria goes back to the 'predisposition event', but their involvement in actinorhizal symbiosis with *Frankia* is presently unclear.

Figure 1. Common endosymbiosis genes exhibit divergent patterns of structural and functional evolution. *SYMRK*, which might form the entry point to the common AM and RNS program for endosymbiosis with fungi or bacteria, has at least three distinct structural versions. All of these support AM symbiosis with fungi, but only the longest version is sufficient for functional RNS [11]. This longest *SYMRK* version has been found only in rosids and seems to be present in all species that form RNS, as well as in their relatives, such as poplar or *Tropaeolum*, that do not form RNS but form only AM symbioses [11]. This pattern suggests an involvement of *SYMRK* in a genetic adaptation that might have provided a molecular basis for the evolution of RNS within the rosid lineage. A common ancestor of the rosid clade (dark purple) possibly acquired a specific adaptation of *SYMRK*, allowing plant–bacterial interactions to access the conserved AM genetic program for endosymbiosis, which encompasses at least six further genes (*CASTOR* [34]; *POLLUX* [31,34]; *NUP85* [35]; *NUP133* [36]; *CCaMK* [32,37]; *CYCLOPS* [33,38]). In contrast to *SYMRK*, these are structurally conserved across dicot and monocot angiosperms (Figure 2). Potentially, the monomorphic components of the common symbiosis program can support RNS [26,28,38,39,47]. The small pictograms refer to the root endosymbiosis types supported by the respective structural version of *SYMRK*. Angiosperm orders shaded dark gray include members forming AM and actinorhizal RNS with *Frankia* (AR), those shaded light purple form AM and another form of RNS, rhizobium–legume symbiosis (RLS). Members of orders shaded light blue form AM but no RNS. The plant names (same color code as for orders) refer to species containing the respective structural versions of *SYMRK*. Asterisks (*) indicate the availability of data on the functional capabilities of the respective *SYMRK* genes [10,11]. Abbreviations: AM, arbuscular mycorrhiza; AR, actinorhiza; RLS, rhizobium–legume symbiosis; RNS, root nodulation symbiosis. Key to plant names: alder, *Alnus glutinosa*; *Casuarina*, *C. glauca*; *Datisca*, *D. glomerata*; *Lotus*, *L. japonicus*; maize, *Zea mays*; *Medicago*, *M. truncatula* and *M. sativa*; poplar, *P. trichocarpa*; poppy, *Papaver rhoeas*; *Tropaeolum*, *T. majus*; rice, *Oryza sativa*. Predicted protein regions are abbreviated as: CEC, conserved extracellular domain; LRR, leucine-rich repeat; NEC, N-terminal region of unknown function; PK, protein kinase domain; TM, transmembrane domain. Nomenclature of angiosperm phylogeny is based on Ref. [81].


Extant angiosperms
Common symbiosis program
Rosids
Eurosids I
Fagales
Cucurbitales
Rosales
Fabales (legumes)
Malpighiales
Eurosids II
Brassicales
Eudicots
Euasterids
Solanales
Other eudicots
Ranunculales
Monocots
Poales
Magnoliids
Casuarina*, alder
Datisca*
e.g. Sesbania, pea, Medicago*, Lotus*
Poplar
Tropaeolum*
Tomato*
Poppy
Rice*, maize
NEC
CEC
LRR
TM
PK
SYMRK
RNS
Monomorphic proteins:
CASTOR
POLLUX
NUP85
NUP133
CCaMK
CYCLOPS
AR
RLS
AM
AM
AM
100 amino acids
TRENDS in Plant Science

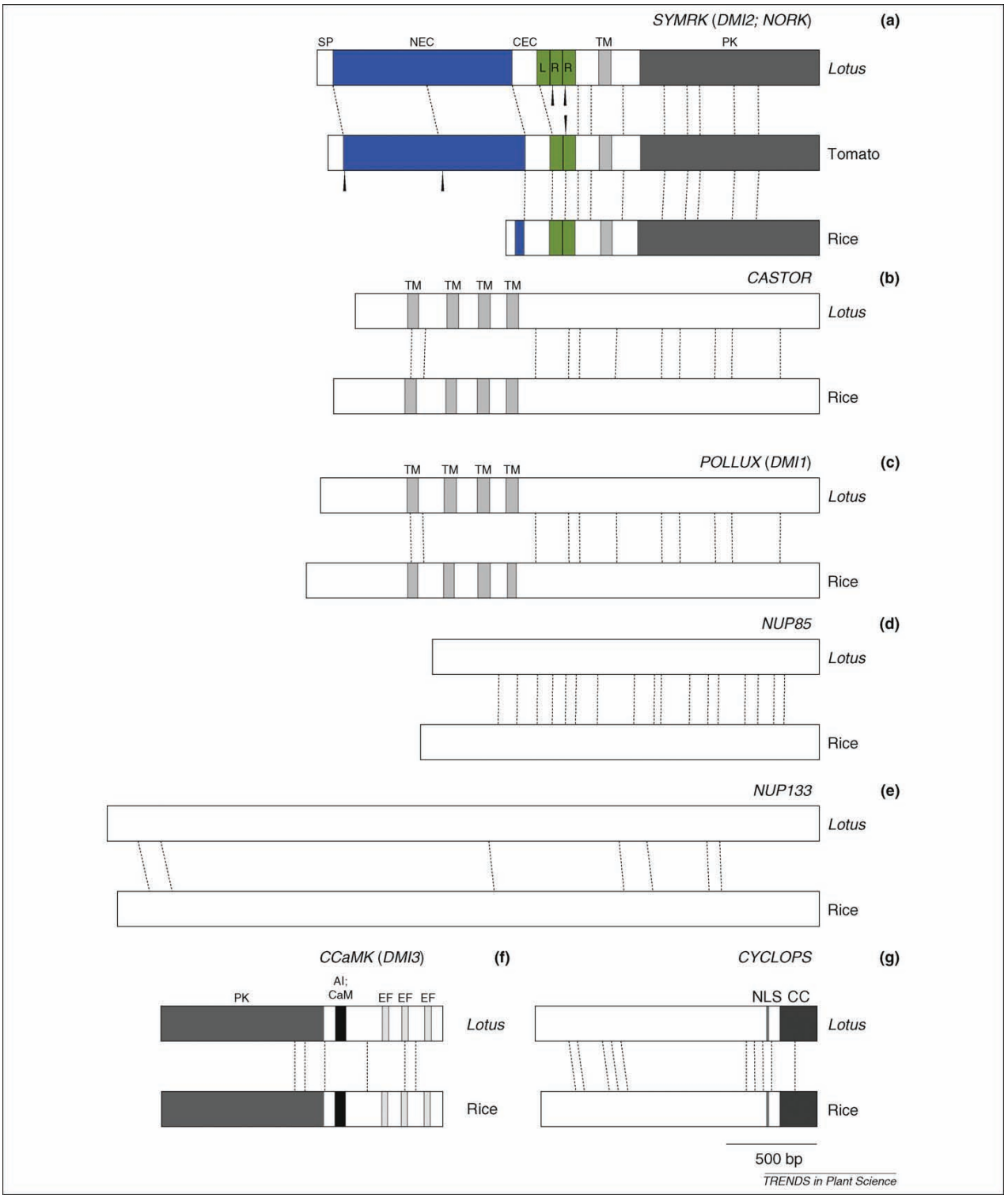

SYMRK (DMI2; NORK)
(a)
SP
NEC
CEC
TM
PK
L R R
Lotus
Tomato
Rice
CASTOR
(b)
TM TM TM TM
Lotus
Rice
POLLUX (DMI1)
(c)
TM TM TM TM
Lotus
Rice
NUP85
(d)
Lotus
Rice
NUP133
(e)
Lotus
Rice
CCaMK (DMI3)
(f)
PK
AI; CaM
EF EF EF
Lotus
Rice
CYCLOPS
(g)
NLS CC
Lotus
Rice
500 bp
TRENDS in Plant Science

Figure 2. The variation in gene structure and domain composition between SYMRK versions from different angiosperm lineages is exceptional among genes that are required for both AM and RNS symbioses (common symbiosis genes). The longest known version of *SYMRK* (a) contains three LRR motifs, all encoded by individual exons. Two shorter versions exist in other plant lineages, which form AM, but no RNS. *SYMRK* from the asterid tomato represents an intermediate version that only has two LRR encoding exons. The shortest known versions of *SYMRK* were isolated from monocots, such as rice [11]. In those regions of the gene that are present in all versions, intron positions and phases are conserved. Other common symbiosis genes (b–g) required for intracellular infection are structurally conserved between dicot and monocot angiosperms, such as Lotus and rice. Minor variations in exon lengths exist, but intron positions and phases are conserved in all cases. In *Lotus* CYCLOPS (g), two NLS motifs are predicted in silico, as compared to one in rice, but only one of the NLS from the Lotus ortholog was functional in a heterologous localization experiment [38]. Only this one, which corresponds to the one conserved in rice CYCLOPS, is depicted here. Key to plant names: *Lotus*, *L. japonicus*; tomato, *Lycopersicon esculentum*; rice, *Oryza sativa*. Filled boxes represent exons, or those regions of exons that are part of the coding sequence. Introns are not depicted, and dotted lines indicate the positions of exon–intron borders. Arrowheads indicate exon–intron borders that cannot be correlated between predicted orthologs with certainty. Predicted protein regions are abbreviated as: AI; CaM, autoinhibitory domain overlapping with calmodulin binding site; EF, calcium-binding EF hand; CC, coiled-coil region; CEC, conserved extracellular region; LRR, leucine-rich repeat motif; NEC, N-terminal extracellular region of unknown function; NLS, nuclear localization signal; PK, protein kinase domain; TM, transmembrane domain; SP, signal peptide. The annotations follow in silico predictions or published annotation of the depicted or homologous sequences where appropriate [11,13,37,38]. The genomic and coding sequences used for prediction of exon–intron structures were: (a) *SYMRK* (*DMI2*; *NORK*) [11–13], AP004579 and AF492655 (*Lotus*), AY940041 and AY935266 (tomato); AP003866 and AK099778 (rice); (b) *CASTOR* [34], AB162016 and AB162157 (*Lotus*), NC_008396 and AK068216 (rice); (c) *POLLUX* (*DMI1*) [31,34], AB162017 and AB162158 (*Lotus*), NC_008394 and AK072312 (rice); (d) *NUP85* [35], AP009253 and AB284835 (*Lotus*), NC_008394 and AK072636 (rice); (e) *NUP133* [36], AJ890252 and AJ890251 (*Lotus*), NC_008396 and AK073981 (rice); (f) *CCaMK* (*DMI3*) [32,37], AM230792 and AM230793 (*Lotus*), AC097175 and AK070533 (rice); (g) *CYCLOPS* [38], AP009158 and EF569221 (*Lotus*), AP008212 and EF569223 (rice). Coding sequences used for exon–intron structure determination are derived from cDNA, not from *in silico* predictions. Parts of the figure are modified from Ref. [11].

2.4 Bacterial uptake evolved by arbuscular mycorrhiza gene recruitment

RNS is genetically embedded in an ancient program for endosymbiosis that is widely conserved in plants today.The majority of land plants form arbuscular mycorrhiza (AM), a symbiosis with fungal symbionts of the phylum Glomeromycota [24]. AM hosts benefit from the extensive access of the fungal mycelia to soil compartments by exchanging photosynthates for water and nutrients such as phosphate [25]. AM-forming angiosperms have been shown to possess conserved genes required for intracellular accommodation of microbial symbionts [11,26–29]. Although originally evolved for symbiosis with fungi, components of this program are functional in RNS with bacteria and could potentially support RNS also in lineages that do not currently contain nodulating species. The 'common symbiosis program' is defined by genes that are required for both intracellular root symbioses with bacteria (RNS) and fungi (AM) [1]. In the model legume *L. japonicus*, seven common symbiosis genes have been identified [30], and for four of these, orthologs were isolated from other legumes such as *M. truncatula*, *M. sativa* and garden pea [12,31–33]. The RLK SYMRK (DMI2; NORK) functions among the earliest components of the common symbiosis program, near the point where rhizobium–legume signaling merges with the AM genetic program [12,13,16]. Further common symbiosis proteins include the two predicted cation channels CASTOR and POLLUX (*M. truncatula* DMI1) [31,34], two nuclear pore proteins (NUP85 [35] and NUP133 [36]), the calcium and calmodulin dependent kinase CCaMK (*M. truncatula* DMI3) [32,37] and the nuclear protein CYCLOPS [38] (*M. truncatula* INTERACTING PROTEIN OF DMI3 [IPD3]) [33].

The requirement of legume genes for both bacterial and fungal symbioses inspired the hypothesis that pre-existing AM genes were recruited during the evolution of RNS [1]. Indeed, predicted common symbiosis gene orthologs from non-legume dicots and from monocots were shown to be essential for AM in these lineages or supported AM if introduced in legume mutants deficient in the respective genes. Rice lines mutated in *CASTOR*, *POLLUX*, *CCaMK* (*DMI3*) or *CYCLOPS* (*IPD3*) were unable to establish

symbiosis with AM fungi [26,28,29,38,39], and transgenic introduction of *CASTOR*, *CCaMK* and *CYCLOPS* into corresponding legume mutants restored AM formation [26,28,38,39]. *SYMRK* genes from different dicot angiosperms and the monocot rice complemented the loss of AM in an *L. japonicus symrk* mutant [11]. This provides evidence for a conserved genetic framework of AM in angiosperms (Figure 1).

In contrast to RNS, plant organogenetic programs are not induced during AM [40]. However, in both symbioses the host plant accommodates the respective microbial symbiont inside living plant cells. Prior to AM fungal infection of host epidermal and cortical cells, characteristic nuclear movements and rearrangements of cytoskeleton and organelles occur [41,42]. Formation of the resulting cellular 'pre-penetration apparatus' was found to depend on the common symbiosis genes *DMI2* (*SYMRK*) and *DMI3* (*CCaMK*) in *M. truncatula* [42], indicating their involvement in preparing host cells for the intracellular invasion by fungal symbionts. Strikingly similar phenomena were observed in both rhizobium–legume [43,44] and actinorhizal [45] interactions, where cytoplasmic bridges, termed 'pre-infection threads', form in host cells before bacterial invasion. In both AM and RNS, these cytological structures dictate the path of microbial progression through infected host cells [41–45]. Consistent with this, mutant phenotypes of legumes impaired in common symbiosis genes involve either a total block or early arrest of intracellular microbial infection [30]. Together, these data strengthen the hypothesis [1] that AM gene recruitment during RNS evolution laid the basis for intracellular accommodation of bacteria. The recruitment event could have enabled the integration of receptor-mediated perception of bacterial partners into the pre-existing AM genetic program, which was a decisive step in the evolution of RNS. The genetic basis for this novel link might be identical to the enigmatic predisposition event at the root of the nodulating clade.

2.5 SYMRK is a likely determinant of the genetic predisposition for nodulation

Genetic adaptations conferring the predisposition to nodulate [3] have been acquired by

the ancestor of the monophyletic nodulating clade. Therefore, the corresponding features should be present in nodulating and non-nodulating members of this clade. At the same time, such adaptations should be absent in other angiosperm lineages. A corresponding pattern has so far only been detected for *SYMRK*, making this gene a prime candidate for carrying decisive sequence and functional alterations acquired by the ancestor of the nodulating clade. Among common symbiosis genes required for endosymbiotic infection in AM and RNS, contrasting evolutionary patterns are apparent (Figures 1 and 2). Unique structural and functional divergence was found between *SYMRK* genes isolated from different angiosperm lineages (Figures 1 and 2). At least three distinct versions of SYMRK exist, which differ in the domain configuration of the predicted products and in their potential to confer symbiotic ability [11]. Only the longest version, which is present in rosids including legumes, actinorhizal hosts and also non-nodulating species, fully supports both AM and RNS [11]. Both shorter *SYMRK* versions fully support AM but not RNS when transgenically introduced into a legume symrk mutant background [11]. The precise molecular features that are responsible for these differences remain to be determined. Interestingly, only the monocot version can support basic RNS related responses, including organogenesis induction and rare, aberrant infection events [11]. The precise extent to which non-rosid *SYMRK* versions can function in root interactions with bacteria remains to be determined. But the apparent correlation between the occurrence of the full-length *SYMRK* version in a monophyletic group including all RNS-forming lineages and the potential of this version, as opposed to the shorter ones, to support functional endosymbiosis with bacteria suggests that *SYMRK* is involved in the predisposition for nodulation. By contributing to the general ability of rosid lineages to evolve RNS, the longest *SYMRK* version is likely to represent one of the genetic advances that paved the way for RNS evolution in an ancestor of the nodulating clade [11].

In contrast to *SYMRK*, other common symbiosis genes exhibit a conserved exon–intron

structure within the coding sequence and similar predicted protein domains in hosts from dicot and monocot angiosperm lineages [27,32,34–36,38] (Figure 2). Examples are *CASTOR*, *CCaMK* and *CYCLOPS*, where this structural conservation coincides with conserved functionality in root endosymbioses. *CCaMK* from the monocots rice [46] and *Lilium longiflorum* [47] restored RNS when introduced into *ccamk* mutants of the legume *M. truncatula*, although later stages of symbiosis establishment were compromised when rice *CCaMK* was used for complementation [46]. In *castor*, *ccamk* and *cyclops* loss-of-function mutants from *L. japonicus*, rice versions of the respective genes fully restored both AM and RNS [28,38].

Potentially, with the exception of SYMRK [11], common symbiosis proteins from non-nodulating lineages can thus support RNS without major modification. The observed sequence adaptations of *SYMRK* might have been sufficient for the recruitment of the common symbiosis program in an ancestor of all nodulators.

2.6 Combining infection and organogenesis: what makes a 'predisposed rosid' a 'nodulator'?

Despite the scattered occurrence of nodulation within the rosid lineage, plant–bacterial endosymbioses have only been detected in association with nodule organogenesis. A known exception is *Gleditsia triacanthos* (honey locust), in which infection-thread formation and symbiotic nitrogen fixation do not involve the development of nodules [48]. In addition, organogenesis can be uncoupled from intracellular infection in nodulating legume species [47,49–52]. Mutants of *Sinorhizobium meliloti* exist that fail to induce root-hair reactions or intracellular infection in their host, *M. sativa*, but are able to induce nodule organogenesis [49]. By contrast, particular lines of *M. sativa*, *M. truncatula* and *L. japonicus* develop spontaneous nodules in the complete absence of rhizobia [47,49–52]. These data indicate that intracellular infection and nodule organogenesis are genetically separable processes in RNS. Consequently, genes involved in spatially coordinating and synchronizing nodule organogenesis with

bacterial infection are essential for an efficient RNS. Mutants in three independent loci were identified in *L. japonicus* that exhibit root nodule organogenesis in the absence of rhizobia. Two of the respective genes have been isolated [37,52]. Versions of CCaMK that are mutated in or lack the autoregulatory domain of the protein can induce the development of bacteria-free nodules with normal tissue organization in legumes forming either determinate [37] or indeterminate nodules [47]. CCaMK is presumed to be involved in deciphering nucleus-associated 'calcium spiking' [32,53], periodic fluctuations of calcium concentration that occur in response to rhizobia or isolated Nod factors [54]. Similar calcium-fluctuations of a distinct pattern occur in response to AM fungi [55], suggesting that calcium spiking might be involved in inducing different downstream responses in bacterial and fungal symbioses [55]. However, the observation that monocot orthologs can restore nodulation in *ccamk* loss-of-function mutants [28,46,47] rules out the possibility that legume-specific adaptations of *CCaMK* are essential for nodule organogenesis in this lineage.

In the *L. japonicus* intracellular infection program, *CCaMK* functions upstream of the common symbiosis gene *CYCLOPS* [38], which is required for intracellular infection but not for induction of nodule organogenesis. However, nodule organogenesis aborts at a primordial stage in *cyclops* mutants [38]. This developmental arrest can be circumvented by autoactive CCaMK, indicating that the initiation of organogenesis is partially parallel to the common symbiosis program for intracellular infection [38]. Importantly, these observations also suggest that in the wild-type situation, the progression of intracellular bacterial infection is a prerequisite for completion of nodule development [38].

RNS-related responses induced by an autoactive CCaMK version require several putative transcriptional regulators [37,47,56], which, in contrast to CCaMK itself, are not required for AM [56–59]. These include the GRAS type transcription factors NODULATION SIGNALING PATHWAY 1 (NSP1) [60,61] and NSP2 [61,62], the ETHYLENE RESPONSE FACTOR (ERF)-type AP2 protein ERF REQUIRED FOR NODULATION 1 (ERN1) [56] and NODULE INCEPTION (NIN) [59,63]. These all belong to families that contain close homologs in other angiosperms, including the

nonsymbiotic *A. thaliana* and the monocot rice [56,60,61,64]. It is possible that specific adaptations of the respective legume genes are required for their function in RNS because *NbNSP1* from *Nicotiana benthamiana* (tobacco) only partially complemented RNS in *M. truncatula nsp1* mutants [61]. Interestingly, these genes are required for both nodule organogenesis and infection-thread initiation (NSP1 [57,60]; NSP2 [58,62]; NIN [59]) or infection-thread development (ERN1 [56]). Their precise role during symbiosis establishment is unclear but potentially involves the regulation of distinct response patterns in root epidermal and cortical layers [65]. Several lines of evidence, most notably the discovery that a cytokinin receptor (*L. JAPONICUS* HISTIDINE KINASE 1 [LHK1] [52,66], *M. truncatula* CYTOKININ RESPONSE 1 [CRE1] [67]) has an important role in initiation of nodulation, indicate that cytokinin is a key trigger for nodule organogenesis, as discussed in two recent reviews [68,69]. In a possible scenario, Nod-factor signaling via the common symbiosis program initiates cytokinin production, which in turn induces transcriptional regulation of genes controlling progression of nodule organogenesis [68] in a cell-type-specific manner [69].

2.7 Cytokinin is a trigger for nodule organogenesis

Consistent with an essential role of cytokinin in initiating nodule organogenesis, an autoactive version of the cytokinin receptor LHK1 induces spontaneous nodule organogenesis in the absence of rhizobia [52]. By contrast, an *lhk1* loss-of-function allele (*hit1-1*) supports intracellular bacterial infection but strongly impairs the formation of nodules [66]. Experimental downregulation of the *M. truncatula* predicted ortholog of this gene, *CRE1*, led to loss of nodule organogenesis [67], indicating a role of cytokinin in the formation of both determinate and indeterminate nodules.

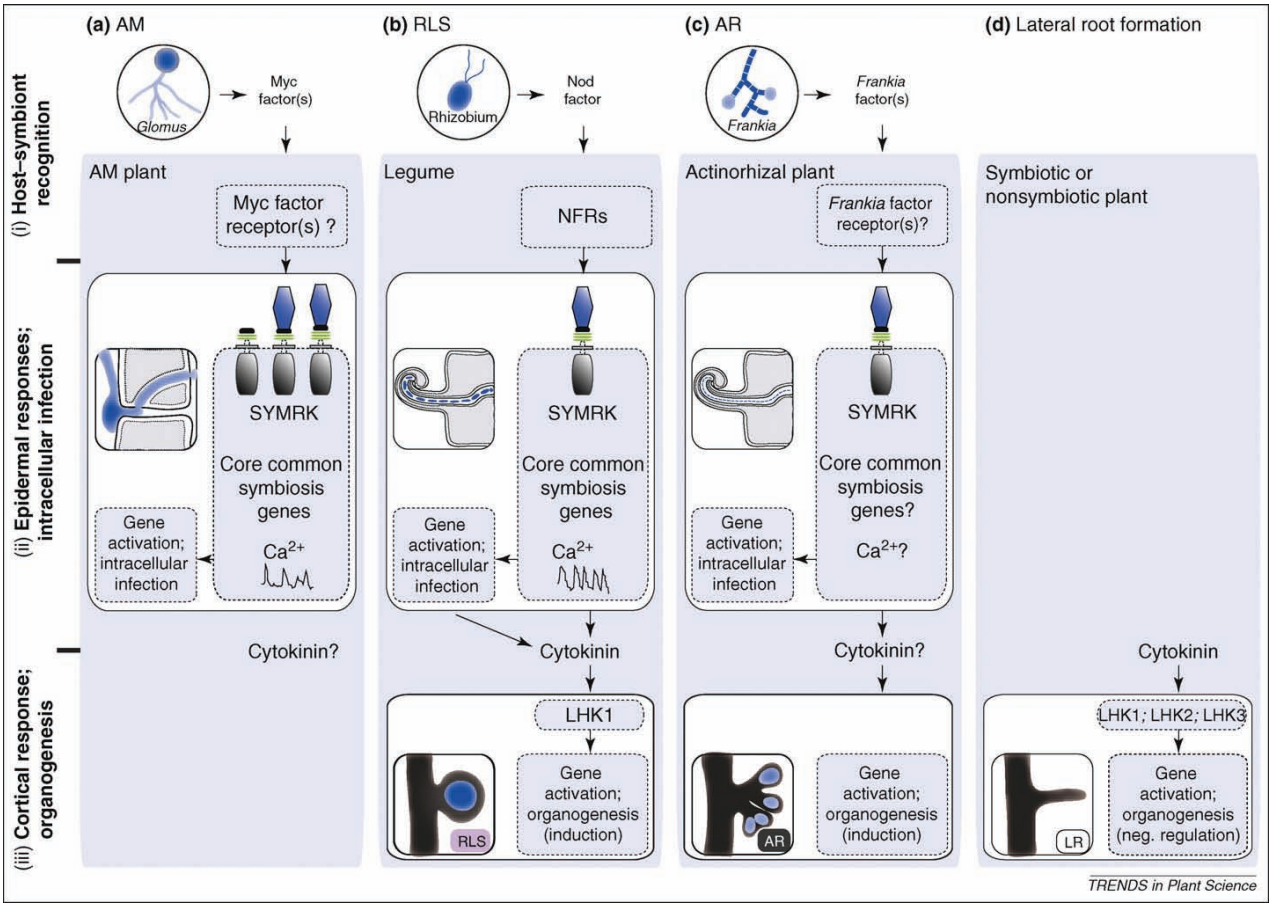
Both rhizobial and *Frankia* strains have been reported to secrete cytokinin [70,71], and Nod-factor-deficient, non-nodulating strains of *Sinorhizobium meliloti* regain the potential to induce nodule-like organs when manipulated to secrete *trans*-zeatin, a naturally occurring cytokinin [72]. These observations suggest a possible role of cytokinin secreted by bacterial symbionts during symbiosis establishment in the induction of nodule organogenesis in RNS.

Photosynthetic *Bradyrhizobium* strains were identified that lack the *nodABC* operon required for Nod-factor synthesis and were proposed to induce nodulation in a Nodfactor-independent manner by using purine derivatives, which could be related or identical to phytoactive cytokinins [21]. Together, these data suggest that bacterial symbionts might contribute to nodule organogenesis induction, or even circumvent the Nod-factor signaling pathway, by releasing cytokinin and activating the LHK1-mediated organogenesis program.

Presumed *LHK1* or *CRE1* orthologs are conserved in angiosperms, including *Arabidopsis* and rice [73,74]. Testing the potential of deregulated versions to autoinduce similar organogenetic responses in non-nodulating plants could reveal whether nodulating groups evolved a partially specific, LHK1-mediated cytokinin response [65] or whether they fully rely on pre-existing programs for organogenesis.

The direct involvement of cytokinin signaling in the regulation of nodule organogenesis relates RNS to fundamental developmental processes, such as lateral-root generation [67,73,75], regulation of root length [52,66,67,73] and vascular morphogenesis [52,76] (Figure 3). The evolution of nodule organogenesis might thus have involved the adaptation of pre-existing root developmental programs such that they respond to hormonal re-balancing of the root cortex induced by signals associated with endosymbiotic bacterial infection [68,77,78]. Consistent with this is the occurrence of nodules and nodule-like structures in diverse landplant lineages, indicating that the genetic tools for their formation are not limited to RNS-competent rosids. For example, nodules that are likely to have evolved as modified lateral roots similar to actinorhizal nodules occur naturally in some gymnosperms such as *Podocarpus spp*., where they are colonized by AM fungi and potentially provide a setting for bacterial nitrogen fixation [79]. Artificial induction of nodule-like structures was reported in rice after partially macerated root tissue was exposed to rhizobial bacteria [80]. Finally, nitrogen-fixing nodules differ greatly in their morphological and developmental characteristics between RNS-forming groups [4,5], giving further indication that a broad range of host genetic backgrounds can potentially support nodule formation.

Figure 3. Root nodulation symbiosis (RNS) with nitrogen-fixing bacteria is embedded in conserved genetic programs. Genes required for intracellular infection with (a) arbuscular mycorrhiza (AM) are shared with both main types of RNS, (b) rhizobium–legume symbiosis (RLS) and (c) actinorhiza (AR) with *Frankia*. Nodule organogenesis might be closely related to (d) lateral root formation. Three modules (i–iii) are apparent that define genetically separable response patterns in RNS. (i) Plant genes required for host–symbiont recognition in the different symbiosis types are likely to carry specific adaptations. Candidates are known only for RLS, where LysM kinases (*LjNFR*s in *Lotus japonicus*, and *MtLYK*s as well as *MtNFP* in *Medicago truncatula*) are required for the specific perception of rhizobial chito-oligosaccharide Nod factors (b) [14–18]. AM and AR are likely to employ other genes for recognition of fungal and *Frankia* signals, the chemical nature of which is not yet resolved ((a) and (c), respectively). (ii) The core module for intracellular infection is defined by at least seven genes that are required for endosymbiosis with both bacteria and fungi in legumes [30], and is likely to be conserved across AM-forming angiosperms [11,26,28,29,38,39,47]. *SYMRK* is likely to be active at the entry point of this program. The longest, probably rosid-specific version of the gene might carry an adaptation that specifically links receptor-mediated bacterial recognition to the common symbiosis program for endosymbiotic uptake. *SYMRK* is also required for AR, suggesting that the two forms of RNS rely on similar genetic programs [10,11]. Epidermal responses leading to intracellular infection involve distinct patterns of calcium oscillation in RLS and AM [54,55]. These calcium signals (referred to as ‘calcium spiking’) potentially represent a key trigger of AM- or RNS specific downstream gene activation. In RLS, activation of the common symbiosis program is assumed to induce the synthesis of cytokinin, which is assumed to act as a signal regulating organogenetic response in the root cortex [52,68,69]. The cytokinin receptor LHK1 (CRE1 in *M. truncatula*) has a central role in nodule organogenesis induction [52,66,67]. (iii) In the legume *L. japonicus*, continuous signaling from progressing intracellular infection, possibly via cytokinin, is necessary for maintenance and completion of nodule organogenesis [38] (b). Cytokinin balancing also has a central role in the induction of lateral roots (LRs), which structurally resemble actinorhizal nodules [5]. Response patterns are distinct in that a relative increase in cortical cytokinin levels induces nodulation but represses lateral-root formation [67,75]. Three histidine kinases (*At*LHK1, *At*LHK2 and *At*LHK3), including a putative ortholog of *Lj*LHK1 (*Mt*CRE1), are involved in regulating LR formation in the nonsymbiotic *Arabidopsis thaliana* [73] (d). It is possible that cytokinin induction of nodule organogenesis involves specific spatial induction patterns of cytokinin biosynthesis, molecular adaptations of *Lj*LHK1 (*Mt*CRE1) or downstream targets that are exclusive to RNS-forming species.


(a) AM
(b) RLS
(c) AR
(d) Lateral root formation
(i) Host–symbiont recognition
(ii) Epidermal responses; intracellular infection
(iii) Cortical response; organogenesis
Glomus
Myc factor(s)
Rhizobium
Nod factor
Frankia
Frankia factor(s)
AM plant
Myc factor receptor(s) ?
Legume
NFRs
Actinorhizal plant
Frankia factor receptor(s)?
Symbiotic or nonsymbiotic plant
SYMRK
Core common symbiosis genes
Ca2+
Gene activation; intracellular infection
Core common symbiosis genes?
Ca2+?
Cytokinin?
Cytokinin
LHK1
LHK1; LHK2; LHK3
RLS
AR
LR
Gene activation; organogenesis (induction)
Gene activation; organogenesis (neg. regulation)
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Box 1. Model systems for RNS transfer

Basal but efficient forms of RNS promise to involve a minimal set of host and bacterial genetic adaptations required for engineering artificial host systems. Developmental features of symbiosis establishment, such as infection mode, intracellular accommodation of the microbial symbiont or nodule organogenesis, depend on the host genotype [4,5], rendering the plant the prime target for such engineering efforts. Actinorhizal hosts [82] and legumes of the family Caesalpiniaceae, as well as some Fabaceae [4], retain their bacterial symbionts in tubular infection-thread-like structures lined with plant cell wall material. These have been referred to as 'fixation threads' [4] because they are the sites of bacterial nitrogen fixation in the respective lineages, and they might represent an ancient form of bacterial accommodation [4]. This is in contrast to many legumes of the families Mimosaceae and Fabaceae, including most fabacean crop legumes, where rhizobia are hosted within specialized membrane-bound compartments termed symbiosomes [83]. Because symbiosome formation poses stringent demands on the genetic compatibility of the symbiotic partners and plant control of the symbiosis, fixation thread symbioses like actinorhizas might be more suited as model systems for RNS transfer. Bacterial candidates for engineering artificial RNS systems should be compatible with a wide range of host genetic backgrounds. Promising examples include broad-host-range symbionts like *Rhizobium sp*. strain NGR234 [84] or *Frankia* bacteria [22], of which many strains can efficiently infect phylogenetically diverse hosts after either intra- or intercellular infection modes [85]. Most actinorhiza-forming *Frankia* bacteria [86], as well as certain rhizobia, such as *Azorhizobium caulinodans* [87] and symbiotic *Burkholderia* strains [88], have the capacity to subsist and fix nitrogen under both symbiotic and nonsymbiotic conditions. Although other factors can restrict nitrogen fixation within plant roots [89], such strains could provide a basis for artificially generating beneficial associations on a nonspecific or partially specific basis [90].

Box 2. Superficial details

In addition to Nod factors, other rhizobial molecules, such as exopolysaccharides and secreted effector proteins [49,91,92], can contribute to establishing fully compatible interactions with legumes. This equally applies to symbioses involving narrow- [49] and broad-host-range rhizobial strains like *Rhizobium sp*. strain NGR234 [91]. The possible existence of additional checkpoints for mutual compatibility in rhizobium–legume systems, potentially involving as yet unknown molecular components [17,91,92], might challenge a successful biotechnological transfer of fully compatible RNS. Deciphering the precise roles of bacterial surface molecules and secreted proteins in both legume and actinorhizal RNS represents an intriguing field for future research efforts.

2.8 Concluding remarks

The emergence of RNS seems to be based on the evolutionary concept of recruitment, in that many RNS-related processes rely on conserved genetic programs that also support pre-existing plant developmental processes and that later acquired additional roles in RNS. Its restricted occurrence in a few rosid plant orders, all part of a monophyletic clade, indicates that more than one specific molecular adaptation was necessary to make RNS evolution possible (Figure 3). These respective genetic changes probably served to link, or add to, pre-existing genetic programs, creating a novel trait of high ecological and economic value. The plant genes involved in these adaptations represent vantage points for efforts to transfer RNS to new hosts. Respective candidate proteins are involved (i) in direct host–microbiont crosstalk (for example, LysM receptor kinases such as *L. japonicus* NFRs or *M. truncatula* LYKs and NFP) and (ii) in mediating the molecular integration of plant–bacterial signaling into the host genetic background, thereby 'tapping' pre-existing plant programs for additional use in RNS. A possible example of such a 'recruiter protein' is the RLK SYMRK (DMI2; NORK), which probably contributed to the potential of AM hosts to form endosymbioses with bacteria [1], a capacity that later became associated with nodule organogenesis on independent evolutionary occasions [1,3], perhaps through the cytokinin receptor LHK1 (CRE1).

Efforts towards engineering novel host systems should focus on identifying the crucial specificities of RNS and aim to bring these together within target hosts. To achieve artificial forms of RNS efficient in symbiotic nitrogen fixation, more insights will further be required into the fine-tuning of host–bacterial recognition and compatibility determination (Box 2). In the past, genetic dissection of RNS has predominantly focused on legume model systems where the plant genetic programs involved are likely to be strongly derived. Less specialized forms of RNS, including actinorhizal associations with Frankia bacteria, combine features that render them more promising models for potential RNS transfer to new hosts (Box 1). Deciphering genetic determinants for the existing diversity of RNS within and beyond the legume lineage

will thus be an important step towards identifying a basic set of RNS genetic requirements and, ultimately, combining them in potential new systems.

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3 The study

Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria

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SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and *Frankia* bacteria. Proceedings of the National Academy of Sciences, USA: 105, 4928-4932.

* These authors contributed equally to the work.

Yano, K., Yoshida, S., Müller, J., Singh, S., Banba, M., Vickers, K., **Markmann, K.**, White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., Parniske, M. (2008).
CYCLOPS, a mediator of symbiotic intracellular accomodation. Proceedings of the National Academy of Sciences, USA: 105, 20540-20545.

3.1 Introduction

Nitrogen limits plant growth in many terrestrial ecosystems. Evolutionary adaptations to this constraint include symbiotic associations with bacteria that are capable of converting atmospheric nitrogen into ammonium. Extracellular associations of plants with diverse groups of nitrogen-fixing bacteria are phylogenetically widespread, but only a small group evolved the ability to accommodate bacteria endosymbiotically inside cell wall boundaries. Bacterial symbionts are confined within tubular structures called infection threads, which are surrounded by a host-derived membrane that is continuous with the plasma membrane, and bound by plant cell wall–like material [1, 2]. The bulk of host plants including all actinorhizal species retain the bacterial symbionts within these structures during the nitrogen-fixing stage of the symbiosis [1, 3]. In the most advanced forms found exclusively among legumes (Fabales) and *Gunnera* [4], symbiotic bacteria are delimited from the host cell cytoplasm only by a plant-derived membrane in the mature stage of the symbioses. In the respective legumes, they develop into bacteroids contained in organelle-like symbiosomes, where nitrogen fixation takes place (for a review, see [5]). Bacterial endosymbioses in both legumes and actinorhizal plants are typically associated with the formation of novel, root-derived plant organs, so-called nodules [6].

Nitrogen-fixing root nodule symbiosis (RNS) occurs in two major forms. Actinorhiza hosts belong to three eurosid orders (Figure 6) and nodulate with Gram-positive actinobacteria of the genus *Frankia* [7]. Legumes, on the contrary, enter specific interactions with members of a diverse group of Gram-negative bacteria, termed rhizobia. For almost a century, the extreme diversity in organ structure, infection mechanisms, and bacterial symbionts among nodulating plants obscured the fact that the nodulating clade is monophyletic, which was revealed by molecular phylogeny relatively recently [8]. The restriction of endosymbiotic root nodulation to a monophyletic group of four angiosperm orders (Figure 6) is coincident with a patchy occurrence within this clade. These observations led to the hypothesis that a genetic

change acquired by a common ancestor may predispose members of this lineage to evolve nodulation endosymbiosis with bacteria [8].

The molecular adaptations underlying the evolution of plant-bacterial endosymbioses are still a mystery, despite a substantial biotechnological interest in understanding the genetic differences between nodulating and non-nodulating plants. While the molecular communication between legumes and rhizobia has been studied in some detail, important clues are expected from the genetic analysis of the yet underexplored Actinorhiza.

Bacterial signalling molecules and corresponding plant receptors involved in RNS are known only for the legume–rhizobium interaction. *Frankia* signals may be biochemically distinct from rhizobial chito-oligosaccharide nodulation factors [9, 10], which would suggest an independent mechanism of host–symbiont recognition in Actinorhiza [11].

Phenotypic analysis of legume mutants has revealed a genetic link between RNS and Arbuscular Mycorrhiza (AM), which is a phosphate-scavenging association between plant roots and fungi belonging to the phylum Glomeromycota [12]. Fungi generally enter host root epidermal cells by forming swollen hyphal structures termed appressoria on the root surface, and penetrating directly into epidermal or outer cortical cells [13]. Hyphal entry may be eased by what is thought to be a host response involving the separation of epidermal cells at their antiklinal borders (“epidermal opening“) [14]. After intracellularly traversing the epidermal and outer cortical cell layers, hyphae re-enter the apoplast to proceed towards the inner cortex of the root. The key features of AM symbiosis, and sites of most pronounced plant-fungal intimacy during symbiosis development, are finely branched hyphal stuctures termed arbuscules [13]. Arbuscules are formed within inner cortical cells surrounding the central root vasculature, and are the presumed centres of exchange for nutrients between fungus and host [13, 15].

AM is widespread among land plants, where forms of AM are found in representatives of all major lineages [16]. Fossil evidence for ancient AM-like associations [17] suggests a role of this symbiosis in the colonization of land about 450 million years ago.

Whether the widesprad occurrence of this symbiosis is based on a common, conserved AM genetic program has long been unclear, as genes required for AM were known exclusively from legumes until recently [13, 18, 19]

The link of plant–fungal and plant–bacterial endosymbioses in legumes, which involves at least seven genes [20-24] termed "common symbiosis genes" [25], inspired the idea that during the evolution of bacterial endosymbiosis, genes were recruited from the pre-existing AM genetic program [26]. The molecular steps involved in the recruitment event are not clear, and key prerequisites for this scenario have not been verified.

3.2 Results

Structural and functional conservation of the common symbiosis gene *CYCLOPS* reveals a conserved AM genetic program

To gain insight into the evolution of root endosymbioses, we analyzed common symbiosis genes across angiosperm lineages with different symbiotic abilities. Many, including the calcium/calmodulin kinase gene *CCaMK* [22, 27], the gene *CYCLOPS* encoding a nuclear protein [19], or genes encoding the predicted cation channels CASTOR and POLLUX [20, 28, 29], are conserved in overall domain structure (Figure 2).

To test the functional stability of a structurally conserved representative, we analyzed *CYCLOPS*, which shows identical exon-intron structures in *Lotus* and rice [19] (Figure 4 A) and a 45% overall identity of the predicted proteins. The precise role of the CYCLOPS protein in symbiosis is still unknown. It co-localizes with and is phosphorylated by CCaMK [30], and *CYCLOPS* has thus been placed downstream of *CCaMK* in the common symbiosis genetic network leading to AM and RNS establishment [19].

Upon infection with the bacterial symbiont *M. loti*, *Lotus cyclops* mutants develop primordial swellings devoid of bacteria, with infection being arrested at the stage of

early infection thread elongation [19]. Although hyphae occasionally enterd the root cortex, infection with the AM fungus *G. intraradices* is frequently aborted within the epidermal and outer cortical layers. Aberrant hyphal structures similar to "balloon-like swellings" [31] associated with abortion of infection in legume mutants impaired in other common symbiosis genes like *SYMRK*, *CASTOR* and *POLLUX* [31, 32] accompanied the fungal growth arrest (Figure 5 A–B), suggesting defects in plant coordination of the intracellular infection process.

To determine whether the function of *CYCLOPS* in AM is conserved in the monocot lineage, we analyzed the AM phenotype of four independent rice *cyclops* mutant lines, each carrying a retrotransposon *Tos17* insertion within exon six of rice *CYCLOPS* (*OsCYCLOPS*) (Figure 4 A). Upon co-cultivation with *G. intraradices*, no arbuscules were seen in any of the mutant root systems tested (Figure 4 B). The fungus did, however, enter the root cortex, and abundant intraradical mycelium was observed (Figure 4 B). This indicates that *CYCLOPS* is required for arbuscule- and hence AM development in rice. The AM phenotype of these rice mutants resembles that apparent in *Lotus japonicus* (*Lotus*) *cyclops* mutants, where arbuscules rarely develop [31]. Aborted infection sites were present, but were also occasionally found in arbusculated control roots carrying wild type *OsCYCLOPS* alleles, and thus were not indicative for the mutant phenotype in this species. Despite the absence of arbuscules, likely the centres of nutrient exchange between host plant and fungus, few vesicles or intraradical spores were present in rice *cyclops* mutant roots. Such structures are formed by some AM fungi inter- or intracellularly within host roots, and are assumed to serve as storage organs and propagative modules [33]. A possible explanation for their presence in arbuscule-free *cyclops* roots is allocation of carbohydrate resources from chive nurse plants to parts of the same mycelium infecting rice roots. Alternatively, intraradical hyphae may mediate residual nutrient exchange in these cases. Vesicle numbers remained at a low basal level in homozygous mutant plants. In contrast, arbuscule and vesicle numbers were increased after three as compared to two weeks of co-cultivation with *G. intraradices* in plants carrying wild type alleles of *OsCYCLOPS* (Figure 4 B),

indicating continuous transfer of nutrients from the host plant to the fungus during progression of symbiotic infection.

The loss of AM in rice *cyclops* mutant lines reveals the functional conservation of a legume AM gene in the monocot rice, suggesting that the widespread occurrence of AM symbiosis may be based on a common, conserved genetic program.

Rice *CYCLOPS* can function in both AM and RNS in a legume

We tested whether CYCLOPS from rice is sufficient to function in root endosymbioses in a legume. Using *A. rhizogenes*-infection, we generated *Lotus* line EMS126 (*cyclops -3*) [19] roots carrying the *OsCYCLOPS* coding sequence under control of the *Lotus CYCLOPS* (*LjCYCLOPS*) promoter region. In these roots, both AM formation and RNS were restored (Table 2, Figure 5), indicating that the *CYCLOPS* gene of the non-nodulating monocot rice can interact with the *Lotus* symbiosis signalling context to support not only AM fungal, but also bacterial endosymbiosis.

The symbiosis receptor-like kinase gene *SYMRK* is structurally diverged in angiosperms

Contrasting to the conserved structure of *CYCLOPS* and other common symbiosis genes, we discovered exceptional diversification among genes encoding the symbiosis receptor kinase SYMRK in different plant species (Figure 6). While putative SYMRK kinase domains are conserved and contain characteristic sequence motifs discriminating them from related kinases (Figure 7), the predicted extracellular portion of SYMRK occurs in at least three versions of domain composition (Figure 6 and Table 1). The longest *SYMRK* version is present in all tested rosids, including nodulating and non-nodulating lineages. Comprising 15 exons, it encodes three leucine-rich repeat (LRR)

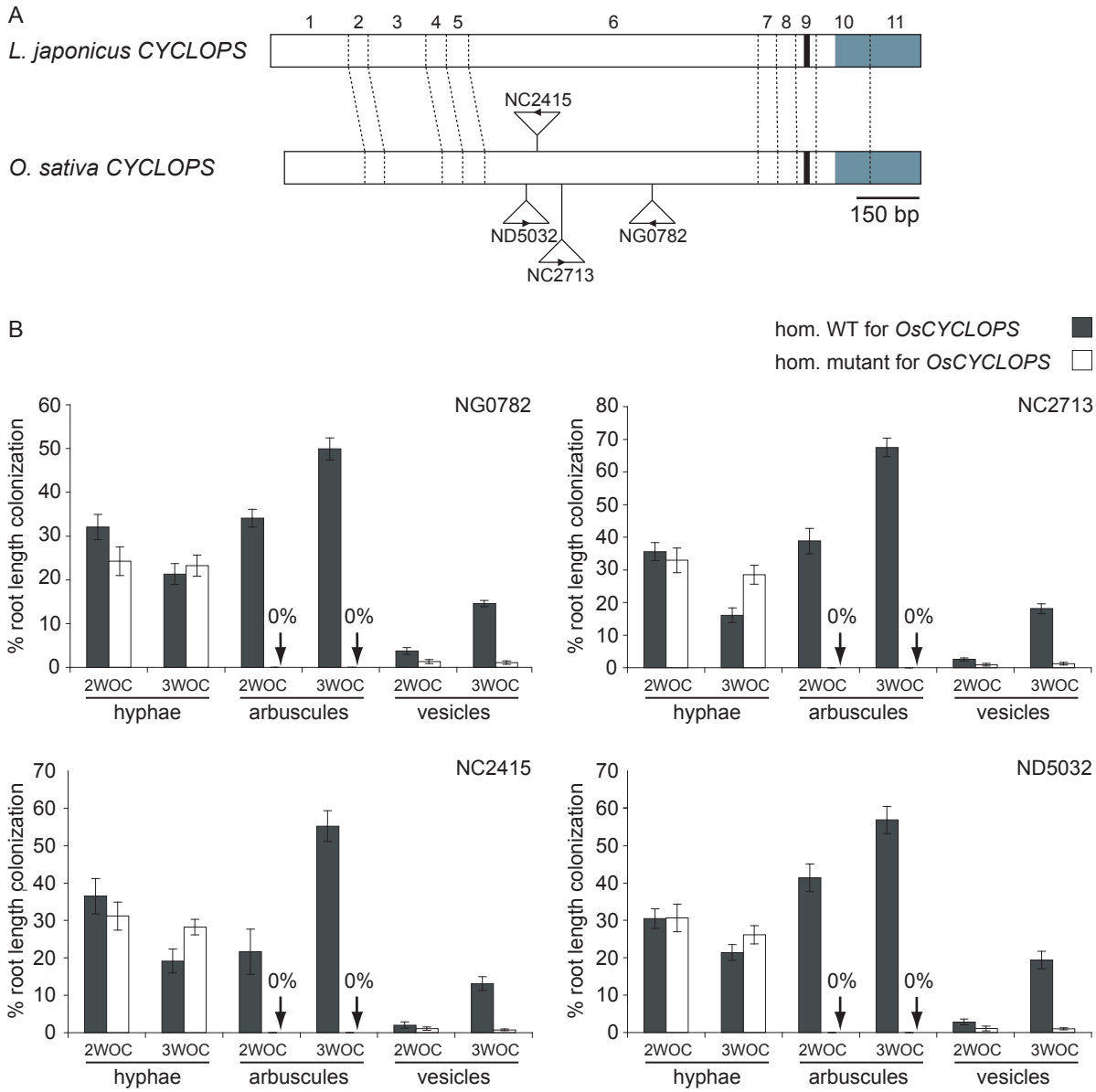


Figure 4. *CYCLOPS* is conserved in structure and AM function between between the rosid dicot *L. japonicus* (*Lotus*) and the monocot *O. sativa* (rice).
(A) Exon structures of *Lotus* and rice *CYCLOPS* genes are conserved. Exons are labelled 1-11. Only regions covering the coding sequences are represented. Dotted lines indicate intron positions, black bars represent predicted nuclear localization signals (NLSs) and grey bars coiled-coil regions in the conceptual amino acid sequences. Only one out of two predicted NLSs of *Lotus CYCLOPS* is depicted, as only this one proved functional in heterologous localization tests in tobacco [19]. Flags mark positions of Tos17 insertions in four rice *cyclops* lines analyzed. (B) Quantitative AM analysis in rice *cyclops* Tos17 insertion lines. For each of the lines, roots of twelve WT and twelve mutant plants were tested. An equal number of plants for each line was phenotyped after two and three WOC. Values are derived from 100 intersects randomly scored in four roots per plant. Intersects were scored as containing arbuscules, vesicles, or hyphae only. Standard errors are indicated. Hom., homozygous mutant plants; WOC, weeks of co-cultivation; WT, wild-type.

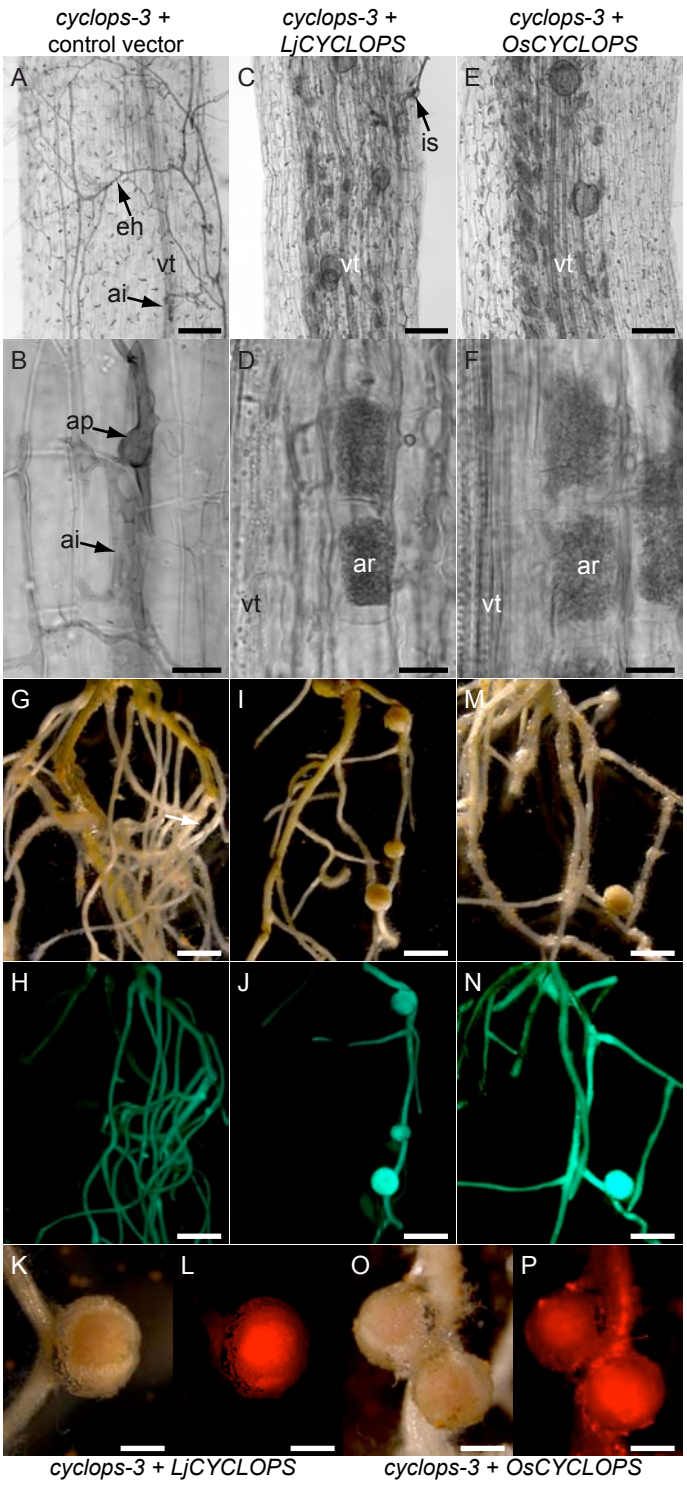


Figure 5. *O. sativa* (rice) *CYCLOPS* restores both fungal and bacterial endosymbioses in *L. japonicus* (*Lotus*) *cyclops-3* mutants. Transgenic roots were *A. rhizogenes*-induced and selected *via* fluorescence of eGFP encoded on the transfer-DNA. (A–B and G–H) *cyclops-3* roots transformed with a control vector lacking a *CYCLOPS* expression cassette. (C–D and I–L) *cyclops-3* roots carrying the *Lotus CYCLOPS* coding sequence and (E–F and M–P), the rice *CYCLOPS* coding sequence, both under control of *Lotus CYCLOPS* promoter region. (A–F) Roots co-cultivated with *G. intraradices* for three weeks. (A–B) Transgenic control root with extraradical mycelium but no intraradical fungal colonization or arbuscule formation. (A–B) Fungal infection attempts aborted within the epidermal or outer cortical tissue. (C–F) *cyclops-3* roots carrying the *Lotus CYCLOPS* (C–D) and rice *CYCLOPS* (E–F) coding sequences, showing dense fungal colonization (C and E) and arbuscule formation in the inner cortical tissue (D and F). (G–P) Root systems inoculated with a *DsRED* expressing strain of *M. loti*, and incubated for four weeks. (G–H) Transgenic control root without mature nodules. Small primordial swellings (arrow in G) do not contain bacteria (not shown). Such swellings are typically formed in *Lotus cyclops* mutant roots upon *M. loti* inoculation [19].
(I–P) *cyclops-3* roots carrying the *Lotus CYCLOPS* (I–L) and rice *CYCLOPS* (M–P) coding sequences, showing mature nodules. Nodules exhibit pink coloration in white light (K and O) and red fluorescence of the inner nodule tissue (L and P) indicating the presence of symbiosis-specific leghemoglobins and of *DsRED* expressing bacteria, respectively.
ai; aborted infection; ap, fungal appressorium; ar, arbus-cule; eh, extraradical hypha; h, intercellular hypha; Is, infection site; vt, root vasculature. Scale bars: (A, C and E) 0.1 mm; (B, D and F) 0.02 mm; (G-J) and (M-N), 2 mm; (K-L) and (O-P), 0.5 mm.

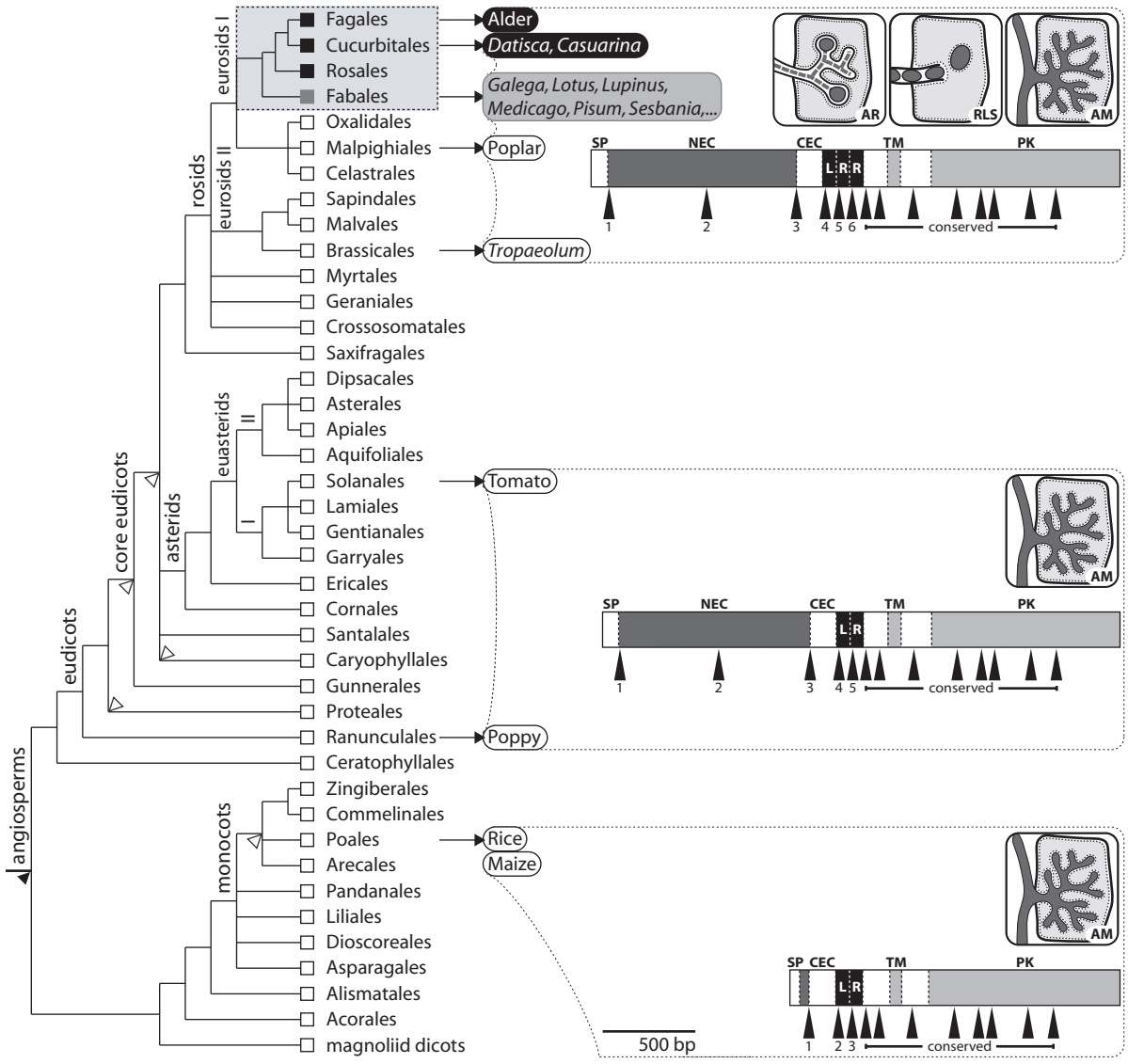


Figure 6. *SYMRK* exon-intron structure and root endosymbiotic abilities of angiosperm lineages. All putative *SYMRK* genes encode an N-terminal signal peptide, an extracellular region with two or three LRR motifs and one imperfect LRR, a transmembrane domain, and an intracellular serine/threonine protein kinase. *SYMRK* regions encoding putative kinase domains exhibit conserved intron positions and phases. Bars illustrate the exon-intron and predicted protein domain structure of representative *SYMRK* candidates. Positions of introns are indicated by black arrowheads. Predicted protein domains are SP, signal peptide; NEC, N-terminal extracellular region; CEC, conserved extracellular region preceeding LRRs; LR(R), leucine-rich repeats; TM, transmembrane domain; and PK, protein kinase domain. Names refer to species sampled and are shaded according to their root endosymbiotic capabilities: black, endosymbiosis with *Frankia* bacteria (Actinorhiza) and AM formation; gray, endosymbiosis with rhizobia and AM formation; white, AM formation only. Pictograms symbolize AR, Actinorhiza; RLS, Rhizobium-Legume Symbiosis; AM, Arbuscular Mycorrhiza. Dashed frames have no phylogenetic implications. The cladogram depicts relationships of angiosperm orders as deduced by molecular markers [34,35]. The four orders containing nodulating taxa are shaded light gray. Squares at the tips of branches indicate the presence of taxa with particular root endosymbiotic phenotypes (colour code is as for sampled plants). Filled and white wedges indicate branches where taxa on order and family level have been omitted, respectively. Popular species designations refer to Alder, *Alnus glutinosa*; Poplar, *P. trichocarpa*; Tomato, *L. esculentum*; Poppy, *P. rhoeas*; Rice, *O. sativa*; Maize, *Z. mays*.

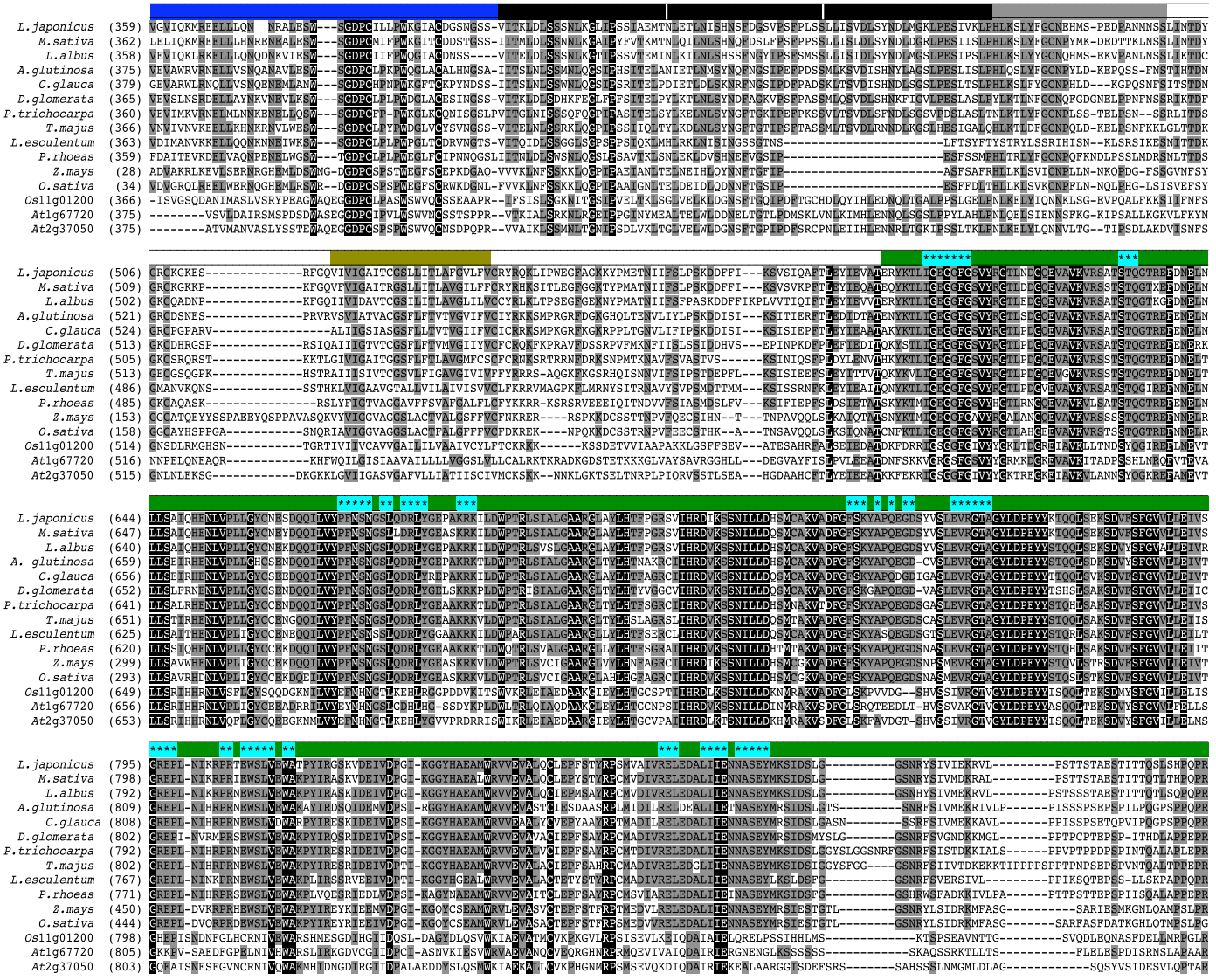


Figure 7. SYMRK kinase regions share several defining conserved amino acid motifs, which are absent in similar sequences in *A. thaliana* (*Arabidopsis*) and *O. sativa* (rice).
Black shading indicates amino acid residues identical in all sequences, residues found in at least 50% of the sequences are shaded gray. Bars delimit predicted SYMRK protein domains. Dark blue, conserved extracellular region (CEC); black, LRRs; gray, imperfect LRR; white, juxtamembrane regions; brown, transmembrane region; green, protein kinase domain. Light blue bars with stars mark some of the regions conserved among SYMRK candidates, but not in other homologous sequences in rice and *Arabidopsis.* Locus tags are indicated for similar sequences not regarded as SYMRK candidates. Sequences aligning with regions upstream of exon four of *LjSYMRK* are not included in the alignment.

motifs and an extended N-terminal domain of unknown function (NEC-domain, Figure 6 and Table 1). Outside of the eurosid clade, which encompasses all nodulating groups, one or more exons are absent from *SYMRK* coding sequences (Figure 6 and Table 1).

Table 1. *SYMRK* homologs, conceptual SYMRK proteins and the closest *A. thaliana* (*Arabidopsis*) sequences

Species	mRNA accession	AAs	Exons	LRRs	% AA identity/similarity		
					Overall	EC	IC
L. japonicus	AF492655 [24]	923	15	3	100/100	100/100	100/100
M. sativa	AJ491998 [24]	925	15	3	82/87	74/83	92/95
L. albus	AY935267 [36]	923	15[b]	3	79/86	72/83	89/92
A. glutinosa	AY935263 [36]	941	15[b]	3	61/75	51/69	78/88
C. glauca	EU273286 [37]	942	15	3	60/73	48/63	79/87
D. glomerata	AM271000 [36]	934	15	3	61/73	54/67	71/80
P. trichocarpa	AM851092[a]	933	15[c]	3	59/71	49/66	75/81
T. majus	AY935265 [36]	945	15[b]	3	59/72	50/65	74/84
L. esculentum	AY935266 [36]	903	14	2	54/68	45/65	72/81
P. rhoeas	AM270999 [36]	902	14[b]	2	54/69	43/59	75/86
Z. mays	DQ403195 [38]	579	12	2	55/69	30/49	74/86
O. sativa	XM_478749 [39]	576	12	2	55/69	33/51	72/84
A. thaliana	NM_105440 [40]	929	15	3	34/51	29/43	50/68
A. thaliana	NM_129261 [40]	933	15	3	33/50	27/42	50/69

Percentages of AA identity/similarity are relative to the *Lotus japonicus* SYMRK sequence and were obtained *via* BLASTX analysis using the NCBI Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/). AAs, amino acids; LRRs, predicted leucine-rich repeat domains; EC, extracellular region and PK, protein kinase domain of the conceptual proteins.

[a] The genomic sequence is available at http://genome.jgi-psf.org/Poptr1/Poptr1. home.html, the gene location is Poptr1/LG_VII:12333164-12338867.

[b] Only cDNA sequence information was obtained. The exon-intron structure was predicted based on splice site prediction and conserved intron position in other *SYMRK* genes.

[c] Only genomic sequence information was obtained. The exon-intron structure was predicted based on splice site prediction and conserved intron position in other *SYMRK* genes.

Genetic evidence indicates that SYMRK acts near a point of molecular convergence of AM and legume-rhizobium signalling [24, 41]. The presumed ability of its diverged extracellular domain to perceive symbiosis-related signals [24] renders it a prime target for investigating the molecular adaptations underlying the evolution of RNS.

The homogenous occurrence of "full-length" *SYMRK* genes among legumes, actinorhizal plants, and non-nodulating rosids raises the intriguing possibility that *SYMRK* is involved in a proposed genetic predisposition [8] of this clade to evolve RNS. An important prediction following from this hypothesis is the common requirement of a full-length *SYMRK* version for all types of RNS. Furthermore, non-nodulating members of this monophyletic clade would be expected to hold the same predisposition, and thus equally carry RNS-competent versions of *SYMRK* provided that such competence was not secondarily lost. To test this concept, we analyzed the functional capabilities of "full-length" *SYMRK* genes from symbiotically diverse rosids.

SYMRK is required for actinorhiza, suggesting a common genetic program for RNS

D. glomerata (*Datisca;* Cucurbitales) is a member of the only herbaceous acinorhizal genus [42], exhibiting faster growth and smaller statue than other actinorhizal model plants such as the Fagales tree species *Casuarina glauca* (*Casuarina*) or *Allocasuarina verticillata* [11]. It is thus well suited for laboratory work, but transgenic plants or roots for genetic analysis and symbiosis phenotyping have not been generated previously in this species. To investigate *SYMRK* function in Actinorhiza, we therefore established a hairy root transformation system for *Datisca.* We reduced root mRNA levels of *Datisca SYMRK* (*DgSYMRK*) *via* RNA-interference (RNAi). Quantitative PCR following reverse transcription showed a 36%–99% reduction of *DgSYMRK* transcript levels in knockdown roots (n = 16) compared with vector control roots (n = 16). Eight weeks after inoculation with *Frankia* bacteria, no nodules were detected on *DgSYMRK* RNAi roots (Figure 8 A–B). Instead, only small, primordial swellings formed on 16% of independent transformed roots (9/55). Nonsilenced control roots of the same plants and roots transformed with a binary vector lacking the silencing cassette (transgenic control roots) showed wild type–like nodules with lobed structure typical for *Datisca* (Figure 8 A–B). This result demonstrates that *SYMRK* is essential for Actinorhiza development in *Datisca*.

In a similar approach, in cooperation with the group of D. Bogusz (Institut de Recherche pour le Développement, Montpellier, France) we downregulated *SYMRK* in roots of the actinorhizal tree *Casuarina* using RNAi [37]. Similar to *Datisca*, nodulation with *Frankia* was strongly impaired in *Casuarina* [37]. In conjunction with the well-documented role of legume *SYMRK* in the interaction with rhizobia [24, 43], *SYMRK* thus represents a common genetic requirement for the two types of bacterial root endosymbiosis.

SYMRK function in AM is conserved across rosid lineages

In legumes, *SYMRK* is required for the infection and AM symbiosis establishment with glomeromycotan fungi [24, 32, 43]. To test whether *DgSYMRK* and *CgSYMRK* are also required for AM, we inspected *Datisca* and *Casuarina SYMRK* RNAi roots for AM formation with the fungus *G. intraradices. Datisca* wild-type roots of the same plants used for hairy root induction and independent transgenic control roots formed AM, with dense arbuscular colonization of inner cortical cells (Figure 8 C–D and E–F, respectively). In contrast, symbiotic development in *DgSYMRK* RNAi roots was strongly impaired. In 82% of independent transformed roots, no fungal infection was observed, despite the presence of extensive extraradical mycelium (Figure 8 G), with those roots exhibiting strong reduction levels of *DgSYMRK* being nonsymbiotic concerning both nodulation and AM formation. Occasional infection attempts occurred but typically were aborted in the outer cell layers (Figure 8 H). Similar results were obtained for *Casuarina* [37]. We conclude that *SYMRK* is involved in both bacterial and fungal endosymbioses not only in legumes, but also in the actinorhizal plants *Datisca* and *Casuarina*, all belonging to independent orders of rosid angiosperms.

Lotus SYMRK is sufficient for both RNS and AM in another legume, *Medicago truncatula*

The legume herbs *Medicago truncatula* (*Medicago*) and *Lotus japonicus* (*Lotus*) fall into two distinct cross-inoculation groups, meaning that they exhibit compatibility with different rhizobial strains [44]. They also display distinct types of nodule organogenesis, with *Medicago* forming zoned, indeterminate nodules with persistent tip meristem [45] and *Lotus* forming round, determinate nodules where the developmental stage of the infected tissue is uniform [46].

To determine whether *SYMRK* plays a role in regulating these differential parameters, we tested whether *Lotus SYMRK* (*LjSYMRK*) can mediate RNS in *Medicago*. The specific symbiont of *Lotus* is *Mesorhizobium loti*, whereas *Medicago* interacts with *Sinorhizobium meliloti*. *Medicago dmi2* 5P mutants exhibit a deletion in exon three of the *SYMRK* ortholog *DMI2*, leading to a frameshift and premature stop codon. *Dmi2* 5P plants form no infection threads or nodules upon inoculation with either rhizobial strain. Transgenic roots of these plants, and of wild-type control plants carrying *LjSYMRK*, formed infection threads and indeterminate, pink nodules typical for *Medicago* [45] with *S. meliloti* (Figure 9 A-J and Table 2). *LjSYMRK* can therefore fully restore RNS of *Medicago* with *S. meliloti*. This indicates that *SYMRK* is not directly involved in determining legume–rhizobium specificity, nor in mediating specific developmental processes during nodule organogenesis.

Medicago dmi2 5P mutants are also impaired in AM. No arbuscules were observed within two weeks of co-cultivation, with fungal infection being aborted at the root surface or after entry into epidermal cells (Figure 9 K–L and Table 2) *LjSYMRK* restored the AM defect in transgenic roots of this line (Figure 9 O–P and Table 2), demonstrating that *SYMRK* is sufficiently similar to *DMI2* to support both fungal and bacterial endosymbioses in *Medicago*.

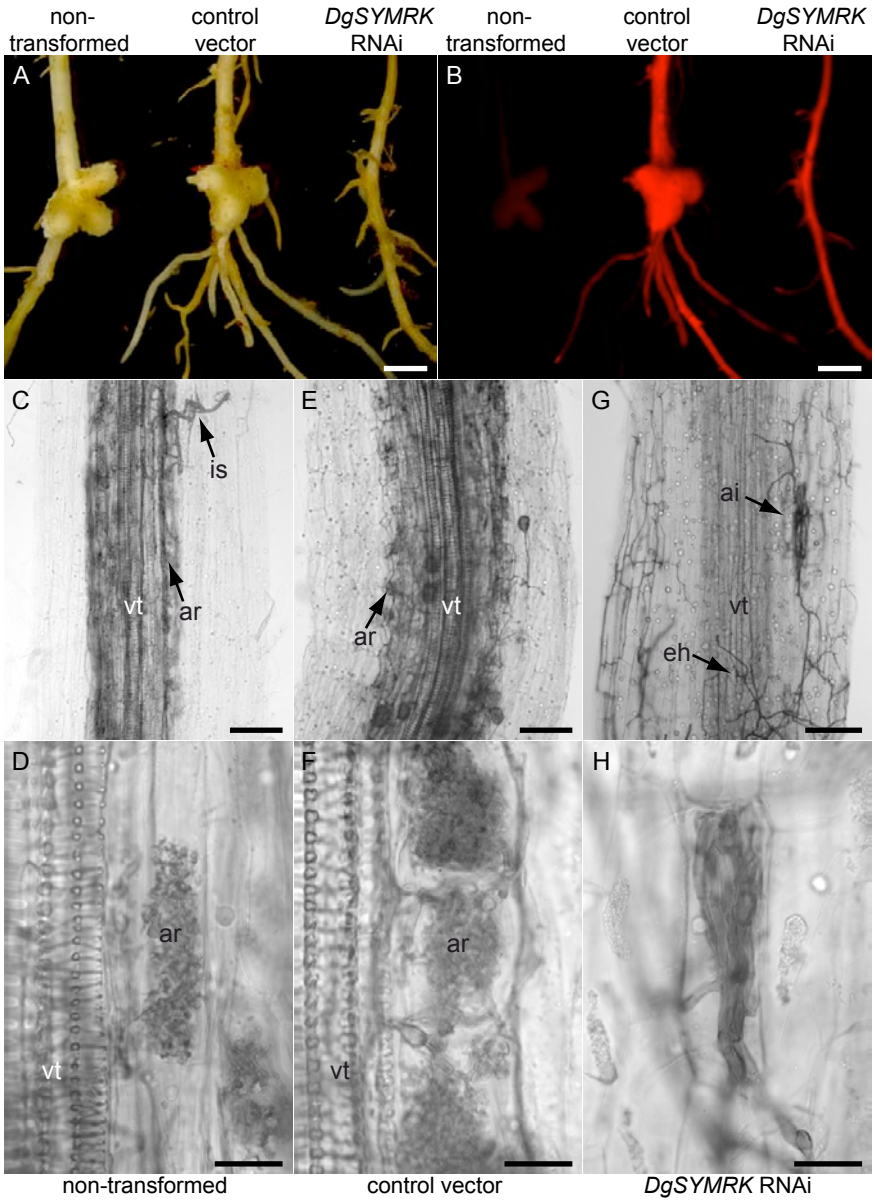


Figure 8. RNS and AM are impaired in *D. glomerata* (*Datisca*) *SYMRK* (*DgSYMRK*) knockdown roots. Co-transformed roots express *DsRED1* as a visible marker.
(A–B) Nodulated wild-type root (left), control root transformed with pRedRoot lacking the silencing cassette (middle), and non-nodulated DgRNAi knockdown root (right) (A) under white light and (B) with transgenic roots showing DsRED1 fluorescence.
(C–H) AM phenotype of *D. glomerata* (*Datisca*) wild type, transgenic control, and *DgSYMRK* knockdown roots. (C–D) Wild-type and (E–F) transgenic control roots are well colonized and show arbuscules in inner cortical cells. (G–H) Typical *DgSYMRK* knockdown root with no AM formation but extraradical hyphae (eh) and aborted fungal infection (ai). Such features were not seen in *Datisca* wild-type or transgenic control roots and are reminiscent of those observed on *L. japonicus symrk* mutant roots (Figure 10). Roots were inoculated simultaneously with *Frankia* bacteria and *G. intraradices* (eight weeks). Transgenic and regenerated nontransgenic roots of 27 (control) and 23 (*DgSYMRK* RNAi construct) plants from three independent experiments were tested. Independent transformed roots examined were n = 42 (control) and n = 55 (*DgSYMRK* RNAi).
ar, arbuscule; eh, extraradical hyphae; ai, aborted infection; is, wild-type infection site; vt, root vasculature.
Scale bars: (A–B) 2 mm; (C, E, and G) 0.1 mm; (D, F, and H) 0.02 mm.

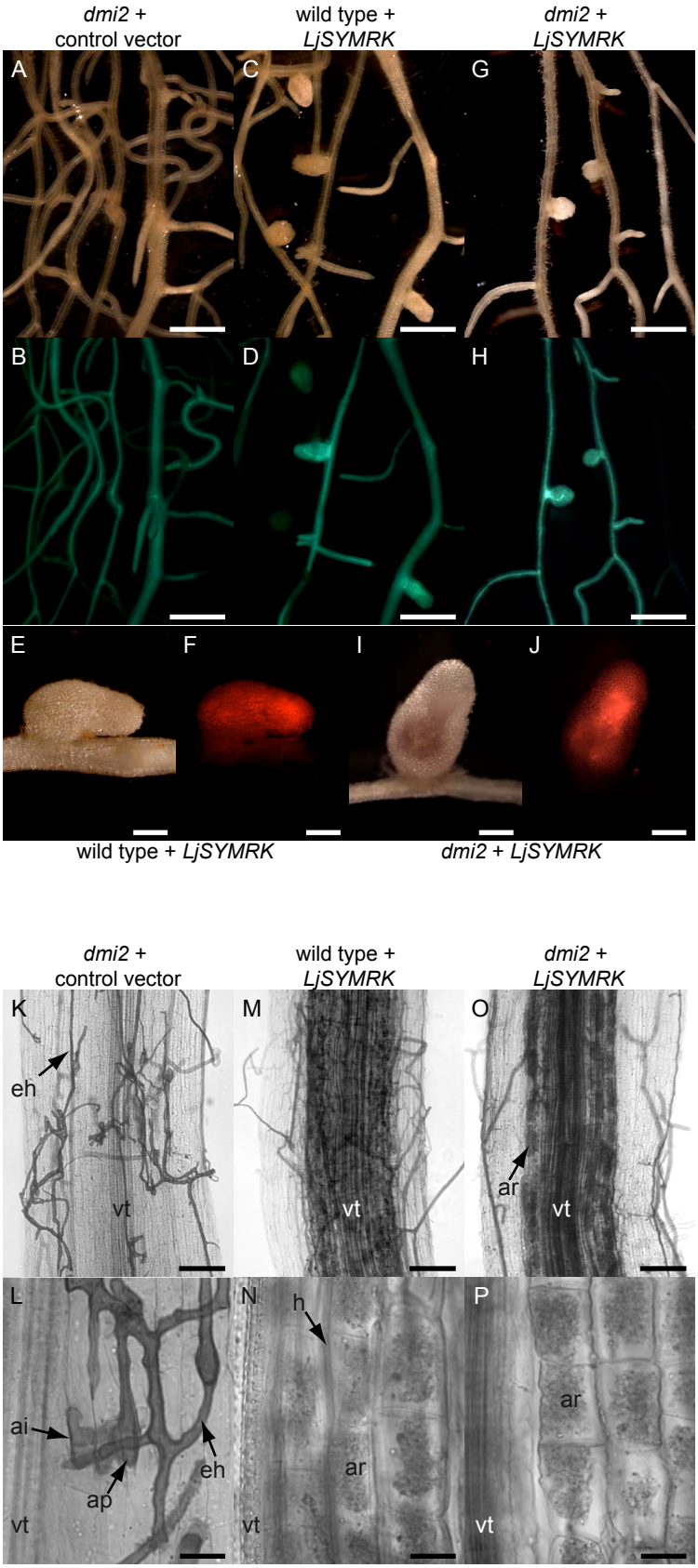


Figure 9. *Lotus japonicus* (*Lotus*) *SYMRK* (*LjSYMRK*) restores RNS and AM in *M. truncatula* (*Medicago*) *dmi2* 5P mutants.

Tranasgenic roots were *A. rhizogenes*-induced and selected *via* fluorescence of eGFP encoded on the transfer-DNA. (A–B and K–L) *Medicago dmi2* 5P roots transformed with the respective control vector lacking an *LjSYMRK* expression cassette. (C–F and M–N) *Medicago* wild-type and (G–J and O–P) *dmi2* 5P roots transformed with the *LjSYMRK* coding sequence controlled by the *LjSYMRK* promoter. (A–J) Roots at five weeks post inoculation with *S. meliloti* expressing *DsRED*. (A–B) Transgenic *dmi2* 5P roots carrying the control vector, showing no nodules. (C–D) Nodules on transgenic and nontransgenic roots of a wild-type plant transformed with *LjSYMRK* and (E–F) individual nodule containing *DsRED* expressing *S. meliloti* bacteria. (G–H) *dmi2* 5P root system with nodule formation confined to roots transformed with *LjSYMRK*. (I–J) Nodule on an *LjSYMRK* containing *dmi2* 5P root showing bacterial *DsRED* expression.

(K–P) Roots co-cultivated with *G. intraradices* for two weeks. (K–L) Transgenic *dmi2* 5P control roots lacking hyphal proliferation and arbuscule (ar) formation in the inner root cortex. Hyphal swellings in the root periphery (L) indicate abortion of fungal infections. Longer co-cultivation for three weeks or more allowed successful fungal infections of mutant roots, which was similarly reported for other *dmi2* mutant lines [47]. (M–N) Wild-type and (O–P) *dmi2* 5P roots transformed with *LjSYMRK* showing dense fungal colonization and arbuscule formation in the root inner cortex.

ai, aborted infection; ap, fungal appressorium; ar, arbuscule; eh, extraradical hypha; h, intercellular hypha; vt, root vasculature. Scale bars: (A–D and G–H) 2 mm; (E–F and I–J) 0.5 mm; (K, M, and O) 0.1 mm; (L, N, and P) 0.02 mm.

Table 2. Restoration of root symbioses in *dmi2* and *symrk* mutants upon transformation with different *SYMRK* versions

Plant genotype	Transgene	AM+	IT	Nod+	Nodules/ nodulated plant
dmi2 5P	marker only♦	0/21	-	0/17	0
dmi2 5P	*LjSYMRK*§	20/23	+	16/25	4.5
symrk-10	marker only♦	3/285	-	0/96	0
symrk-10	*LjSYMRK*§	40/45	+	32/39	4.9
symrk-10	ΔNEC-*LjSYRMK*§	16/31	+	0/22	0
symrk-10	*MtSYMRK*§	20/25	+	18/31	4.2
symrk-10	*CgSYMRK*§	27/48	+	11/63	4.1
symrk-10	*DgSYMRK*§	20/40	+	6/18	5.8
symrk-10	*TmSYMRK*§	20/28	+	14/21	2.6
symrk-10	*LeSYMRK*§	24/34	-	0/31	0
symrk-10	*OsSYMRK*§	38/40	+*	6**/43	1.5
cyclops-3	marker only♦	0/12	-	0/11	0
cyclops-3	*LjCYCLOPS*ϕ	19/24	+	14/21	3,1
cyclops-3	*OsCYCLOPS*ϕ	18/25	+	15/24	3,0

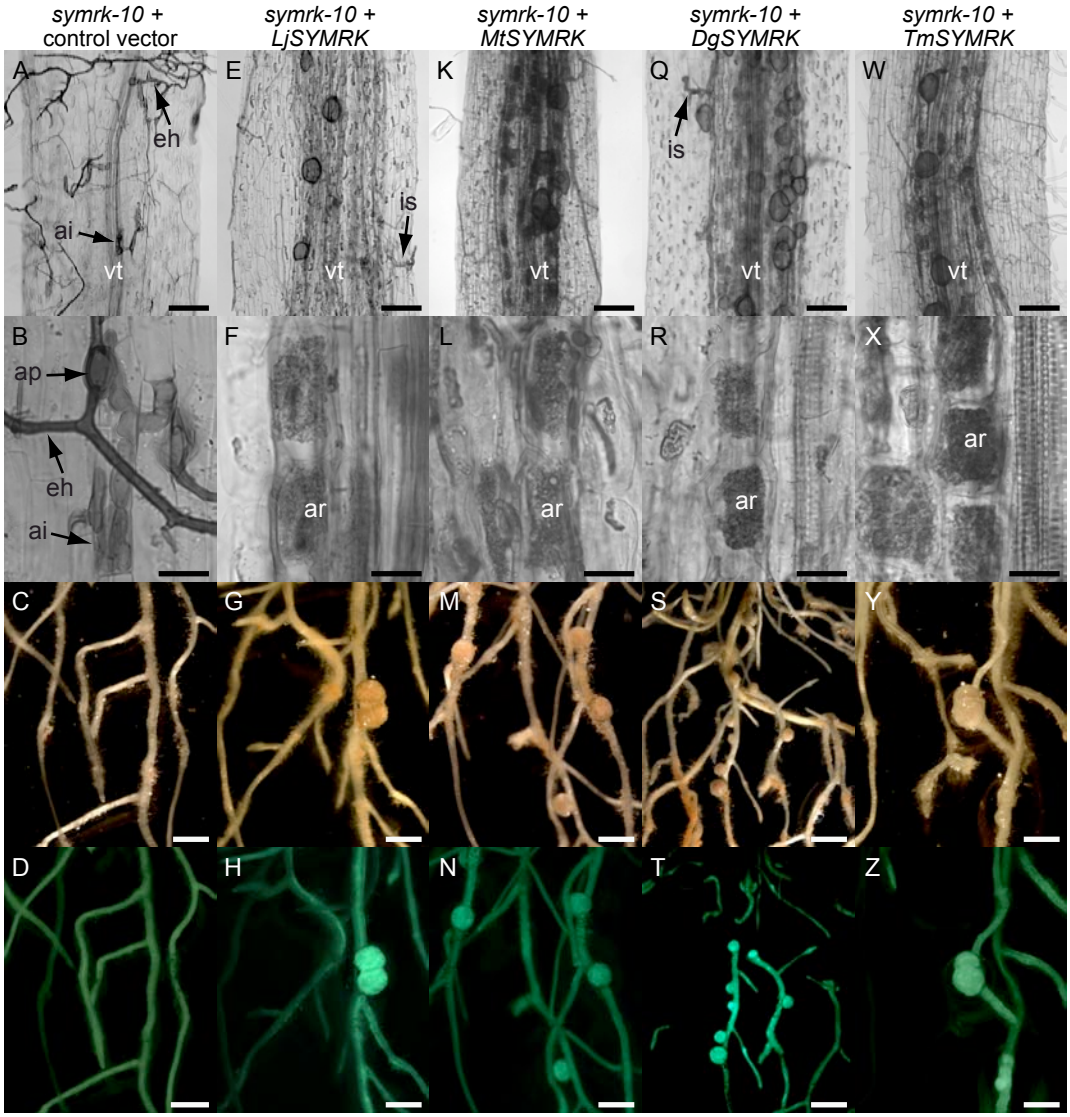
Numbers refer to *Agrobacterium rhizogenes*-transformed root systems. ♦ For negative controls, mutant plants were transformed with the respective binary vector lacking a *SYMRK* or *CYCLOPS* expression cassette. Constructs were under control of the *LjSYMRK* (§) or *LjCYCLOPS* (ϕ) promoter regions. Numbers are compiled results of one (*Medicago truncatula*) or two to three (*Lotus japonicus*) independent experiments. Wild type roots transformed with the same constructs formed wild type-like AM, ITs and nodules. IT, infection thread formation; Nod+, number of root systems showing nodules containing bacteria. *Infection threads were seen very rarely in these roots. **Nodule-like structures were small and showed, with one exception, no pinkish coloration under white light.

"Full-length" *SYMRK* versions from symbiotically distinct rosids can support both AM and RNS in *Lotus*

To analyze the symbiotic capabilities of "full-length" eurosid *SYMRK* genes from a legume (*MtDMI2*), two actinorhizal plants (*CgSYMRK* and *DgSYMRK*), and the non-nodulating, AM-forming *Tropaeolum majus* (*Tropaeolum*; Brassicales) (*TmSYMRK*), we tested their potential to function in the *Lotus* symbiosis signalling context. We introduced these genes, under the control of the *Lotus SYMRK* promoter region, into roots of *Lotus* line SL1951–6 (*symrk-10*), which carries a *symrk* mutant allele encoding a kinase-dead SYMRK version [48, 49]. Upon inoculation with *G. intraradices*, *symrk-*

10 roots form no AM, and fungal infections are typically associated with aberrant hyphal swellings and are aborted after entry into epidermal cells (Figure 10 A–B and Table 2). Interaction with *M. loti* is blocked at an early stage, and no infection threads or nodules form (Figure 10 C–D and Table 2). In *symrk-10* roots transformed with *MtDMI2*, *CgSYMRK*, *DgSYMRK* or *TmSYMRK* both AM and nodulation were restored, the latter involving the formation of infection threads and pink, bacteria-containing nodules (Figure 10 K–AB, Figure 11 and Table 2) that were indistinguishable from wild-type nodules. Although complemented AM roots often contained aborted fungal infection sites and poorly colonized regions, possibly reflecting varying levels of transgene expression or, in the case of heterologous *SYMRK* genes, suboptimal compatibility with the *Lotus* symbiosis signalling context, the decisive symbiotic structures such as wild type-like infection sites and arbuscules were present in these roots. In conclusion, consistent with a role of *SYMRK* in the predisposition to evolve RNS, we could not detect a functional diversification of the rosid *SYMRK* version linked to features differentiating actinorhizal and legume nodulation, or to the specific recognition of bacterial symbionts.

Figure 10: *D. glomerata* (*Datisca*), *M. truncatula* (*Medicago*), and *T. majus* (*Tropaeolum*) *SYMRK* restore fungal and bacterial endosymbioses in *L. japonicus* (*Lotus*) *symrk-10* mutants.
Transgenic roots were induced by *A. rhizogenes* infection and identified *via* fluorescence of eGFP encoded on the transfer-DNA.
(A–D) *Lotus symrk-10* roots transformed with the respective control vector lacking a *SYMRK* expression cassette.
(E–AB) *Lotus symrk-10* roots transformed with *Lotus* (E–J), *Medicago* (K–P), *Datisca* (Q–V) and *Tropaeolum* (W–AB) *SYMRK* under control of the *LjSYMRK* promoter region.
(A, B, E, F, K, L, Q, R, W, and X) Roots co-cultivated with *G. intraradices* for three weeks. (A–B) Transgenic control roots devoid of intraradical hyphae or arbuscules, with aborted fungal infection structures within epidermal cells.
Roots carrying *Lotus* (E–F), *Medicago* (K–L), *Datisca* (Q–R), and *Tropaeolum* (W–X) *SYMRK* with dense fungal colonization of the inner root cortex (E, K, Q, and W) and arbuscule formation in inner cortical cells (F, L, R, and X).
(C, D, G–J, M–P, S–V, and Y–AB) Root systems inoculated with *M. loti* expressing *DsRED* at four weeks post inoculation. (C–D) Transgenic control roots showing no nodules. *symrk-10* root systems transformed with *Lotus* (G–J), *Medicago* (M–P), *Datisca* (S–V), and *Tropaeolum* (W–AB) *SYMRK* develop nodules on transgenic roots. Nodules exhibit pink coloration in white light, indicating the presence of symbiosis-specific leghemoglobins (I, O, U, and AA) and DsRED fluorescence in inner nodule tissue indicating the presence of *M. loti* (J, P, V, and AB).
ai, aborted infection; ap, fungal appressorium; ar, arbuscule; eh, extraradical hypha; is, infection site; vt, root vasculature. Scale bars: (A, E, K, Q, and W) 0.1 mm; (B, F, L, R, and X) 0.02 mm; (C, D, G, H, M, N, S, T, Y, and Z) 2 mm; (I, J, O, P, U, V, AA, and AB) 0.5 mm.


symrk-10 + control vector
symrk-10 + LjSYMRK
symrk-10 + MtSYMRK
symrk-10 + DgSYMRK
symrk-10 + TmSYMRK
A
E
K
Q
W
eh
ai
vt
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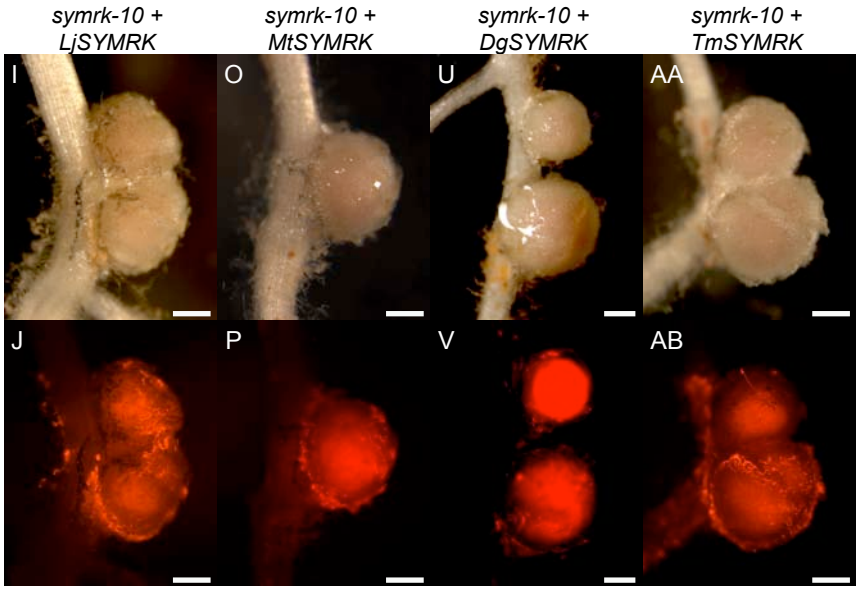
symrk-10 + LjSYMRK
symrk-10 + MtSYMRK
symrk-10 + DgSYMRK
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I
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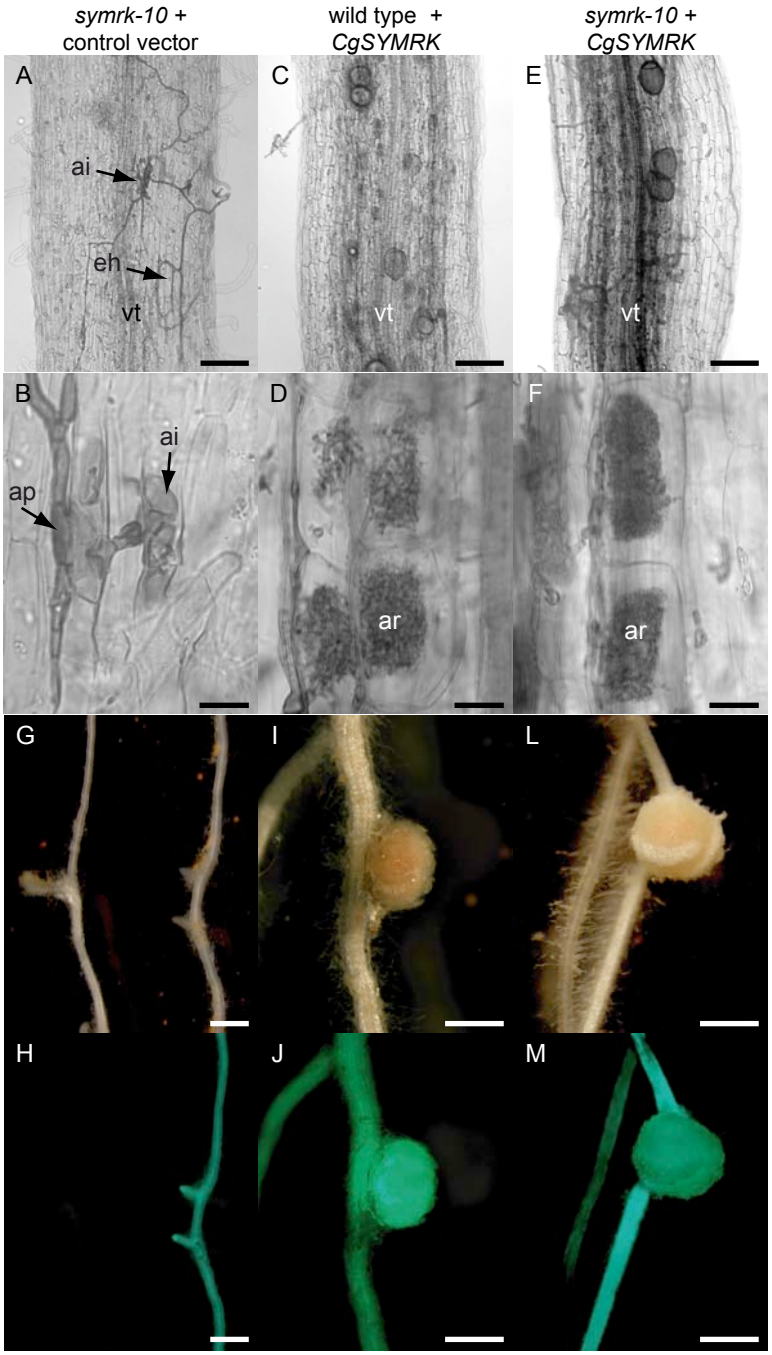


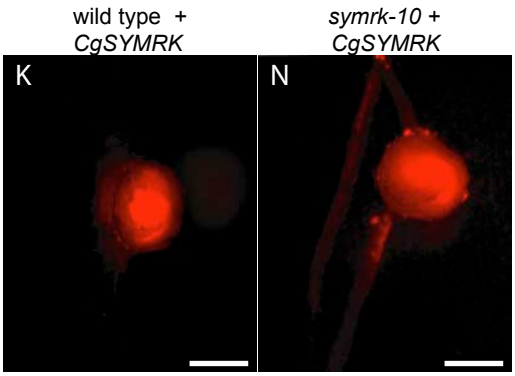


Figure 11. *SYMRK* of the actinorhizal plant *C. glauca* (*Casuarina*) (*CgSYMRK*) restores fungal and bacterial endosymbiosis in *L. japonicus symrk-10* mutants.
Transformation assay and selection were as in Figure 10. (A-B) and (G-H) *symrk-10* roots transformed with a control vector lacking a *SYMRK* expression cassette. (C-D) and (I-K) Wild-type roots and (E-F) and (L-N) *symrk-10* roots transformed with *CgSYMRK* under control of the *LjSYMRK* promoter region, respectively.
(A-F) Plants were co-cultivated with *G. intraradices* for three weeks. (A-B) Transgenic control mutant roots with no fungal colonization, and no arbuscule formation in the root cortex. Fungal infections are aborted in the epidermis or outer cortical cells. (C-D) Wild-type and (E-F) *symrk-10* roots with dense fungal colonization of the inner cortex (C and E), and arbuscule formation in inner cortical cells.
(G-N) Roots four weeks after inoculation with *M. loti* expressing *DsRED*. (G-H) Transgenic and non-transgenic *symrk-10* control roots without nodules. (I-K) Wild type and (L-N) *symrk-10* roots with nodules that show a pinkish coloration under (I and L) and DsRED fluorescence (K and N), indicating the presence of symbiotic leghemoglobins and *M. loti* bacteria, respectively.
ai, aborted infection; ap, fungal appressorium; ar, arbuscule; eh, extraradical hyphae; vt, root vasculature. Scale bars: (A, C and E) 0.1 mm; (B, D and F) 0.02 mm; (G-H) 1 mm; (I-N) 0.5 mm.

SYMRK versions of reduced length restore AM but not RNS in *Lotus*

SYMRK from the AM forming, but non-nodulating eudicots *Papaver rhoeas* (poppy) and *Lycopersicon esculentum* (tomato) represent intermediate length and domain composition with two LRR encoding exons only, but a full-length N-terminal domain (Figure 6 and Table 1). To explore the symbiotic capabilities of this version, we introduced the two-LRR encoding tomato *SYMRK* (*LeSYMRK*) genomic sequence fused to the *LjSYMRK* promoter into *Lotus symrk-10* transgenic roots. *LeSYMRK* restored AM symbiosis, but neither infection threads nor nodules developed upon inoculation with *M. loti* (Table 2 and Figure 12 E–L).

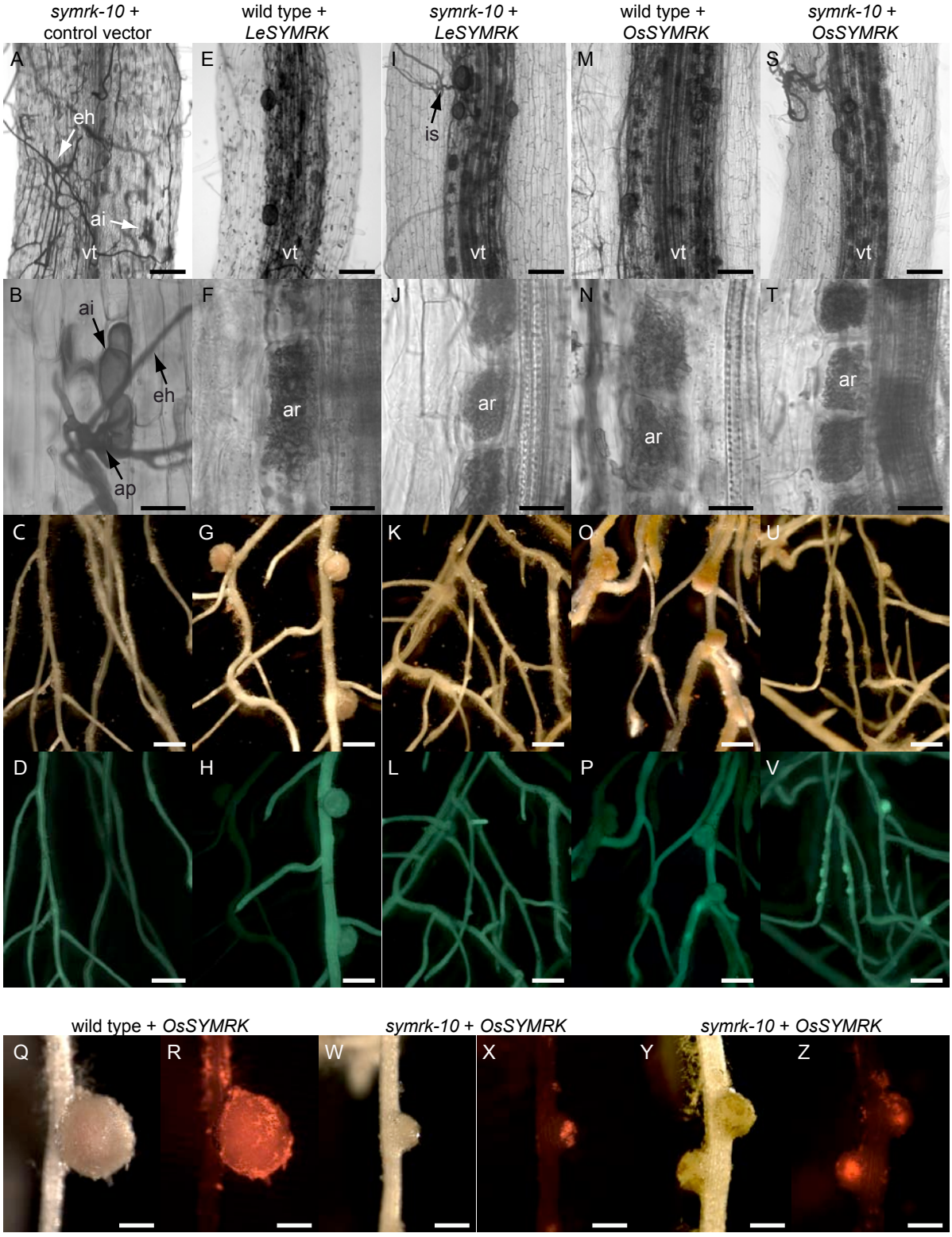
In hairy roots, which represent transgenic tissue generated on a non-transgenic background plant, morphological reactions of root hairs to symbiosis-related stimuli are difficult to monitor, as they may differ from wild-type roots. Similarly, expression level changes of symbiosis-regulated genes may be abnormal in transgenic roots on chimaeric plants. To further investigate to what extent the two-LRR *LeSYMRK* candidate can mediate symbiotic development in legumes, we therefore stably introduced its genomic sequence, including promoter region, into two *Lotus symrk* mutant lines, EMS61 (*symrk-7*) encoding a truncated SYMRK protein [24, 49] and *symrk-10.* Consistent with the observations on transgenic roots carrying the *LeSYMRK* genomic sequence fused to the *Lotus SYMRK* promoter, the *LeSYMRK* gene and promoter region restored AM symbiosis in these plants. In contrast, neither infection threads nor nodules developed upon infection with *M. loti* (Figure 13 and Table 3). The phenotypic rescue of AM co-segregated with *LeSYMRK* in mutant populations segregating the transgene (Figure 13 A).

Table 3. Summary of AM and RNS phenotyping results of *Lotus symrk-7* and *-10* mutant lines segregating the tomato *SYMRK* genomic region under control of the *tomato SYMRK* promoter.

Plant genotype	Transgene	AM+	IT	Nod+	Nodules/ nodulated plant
wild type	-	78/80	+	10/10	2.8
symrk-7	-	0/48	-	0/26	0
symrk-7	*LeSYMRK*	21/23	-	0/11	0
symrk-10	-	2/75	-	0/27	0
symrk-10	*LeSYMRK*	64/75	-	0/35	0

Plants were co-cultivated with *G. intraradices* for three weeks to test for AM formation, or with *M. loti* expressing *LacZ* for two weeks to analyze the interaction with rhizobial symbionts. AM-: no arbuscules present; AM+: both arbuscules and vesicles present; IT, infection thread formation. Four independent transgenic lines were tested, two in *symrk-7* and two in *symrk-10* background, and similar results were obtained in two (AM) or one (IT formation, nodulation) independent experiments.

Figure 12. *L. esculentum* (tomato) and *O. sativa* (rice) *SYMRK* restore AM symbiosis in *Lotus symrk-10* mutants, but cannot or only partially complement bacterial endosymbiosis formation.
Transformation assay and selection were as in Figure 2.7. (A–D) *Lotus symrk-10* roots transformed with the respective control vector lacking a *SYMRK* expression cassette. (E–L) *Lotus* wild-type (E–H) and *symrk-10* (I–L) roots transformed with *LeSYMRK*. (M–Z) *Lotus* wild-type (M–R) and *symrk-10* (S–Z) roots transformed with *OsSYMRK*.
(A, B, E, F, I, J, M, N, S, and T) Roots co-cultivated with *G. intraradices* for three weeks. (A and B) Transgenic *symrk-10* control root with extraradical mycelium but no intraradical fungal colonization or arbuscule formation. Swollen hyphal structures indicative of aborted fungal infections can be observed within epidermal cells (B and arrow in A). (E and F) Wild-type and (I and J) *symrk-10* roots transformed with *LeSYMRK*, showing fungal colonization of the inner root cortex (E, I) and arbuscule formation in inner cortical cells (F, J). (M and N) Wild-type and (S and T) *symrk-10* mutant roots transformed with *OsSYMRK*, similarly showing cortical AM colonization (M, S) and arbuscule formation (N, T).
(C, D, G, H, K, L, O–R, and U–Z) Root systems inoculated with *M. loti* expressing *DsRED* at four weeks post inoculation. (C and D) *symrk-10* root system with transgenic control roots, showing no nodules. (G and H) and (O–R) Wild-type root systems with *M. loti*–containing pink nodules on nontransgenic and on transgenic roots carrying *LeSYMRK* or *OsSYMRK*, respectively, indicating that these transgenes do not impair nodulation in transgenic wild-type roots. (K and L) *symrk-10* root system transformed with *LeSYMRK*, showing no nodules. In a single case, one nodule primordium was observed. (U–Z) *symrk-10* root system transformed with *OsSYMRK*, showing no fully developed nodules, but nodule primordia which are mostly noncolonized by bacteria, the latter proliferating on the primordial surface (W and X). In rare cases, small nodules were observed that contained bacteria, but, with one exception, showed no pinkish coloration in white light (Y and Z).
ai, aborted infection; ap, fungal appressorium; ar, arbuscule; eh, extraradical hypha; is, infection site; vt, root vasculature. Scale bars: (A, E, I, M, and S) 0.1 mm; (B, F, J, N, and T) 0.02 mm; (C, D, G, H, K, L, O, P, U, V) 2 mm; (Q, R, and W–Z) 0.5 mm.


symrk-10 + control vector
wild type + LeSYMRK
symrk-10 + LeSYMRK
wild type + OsSYMRK
symrk-10 + OsSYMRK
A
E
I
M
S
eh
ai
is
vt
B
F
J
N
T
ai
eh
ap
ar
C
G
K
O
U
D
H
L
P
V
wild type + OsSYMRK
symrk-10 + OsSYMRK
symrk-10 + OsSYMRK
Q
R
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Z

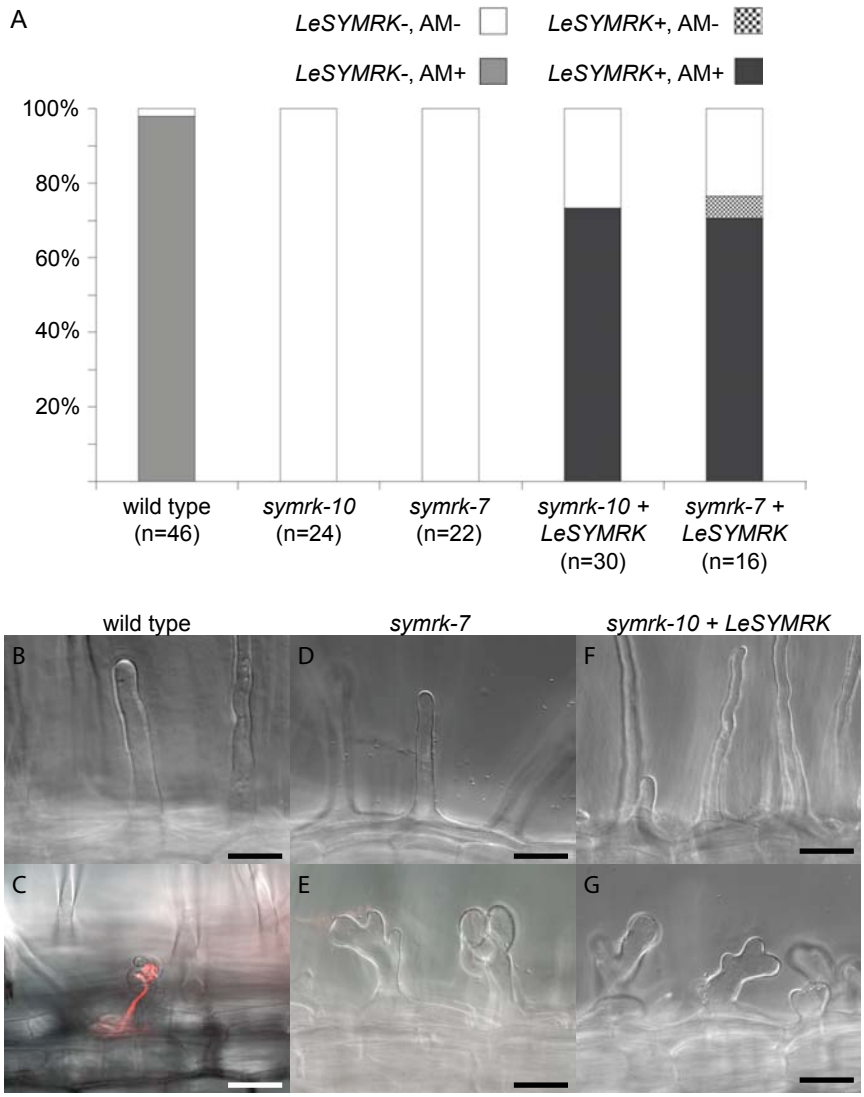

Figure 13. AM formation, but not RNS and IT formation are restored in *L. japonicus* (*Lotus*) *symrk-7* and *-10* mutant lines segregating the *L. esculentum* (tomato) *SYMRK* genomic region under control of the tomato *SYMRK* (*LeSYMRK*) promoter.
(A) Compilation of genotyping and AM phenotyping results for one representative line each in *symrk-7* and in *symrk-10* background. Plants were co-cultivated with *G. intraradices* for three weeks.
(B–G) Plants were cultivated on plates containing nitrogen-free medium for five days and mock-inoculated (B, D and F) or inoculated with *M. loti* MAFF expressing *DsRED* (C, E and G) to examine root hair reactions (3–6 days post inoculation). (B–C) Wild type plants, (D–E) *symrk-7* mutants, and (F–G) *symrk-7* mutants stably transformed with the *LeSYMRK* genomic region. (B, D and F) No root hair curling or strong deformations were observed in mock-inoculated roots of either line. (C) In wild type plants inoculated with *M. loti*, root hairs showed normal curling and IT formation, the majority of root hairs remained non-deformed. (E–G), The majority of root hairs displayed extensive branching and tip bulging in roots of control mutant and transgenic plants alike, and no entrapment of bacteria in curling root hairs was observed. Similar results were obtained in four independent experiments. A similar root hair response to inoculation with *M. loti* is characteristic for *Lotus symrk* mutants [24].
AM-: no arbuscules present; AM+: both arbuscules and vesicles present. Scale bars: 50 µM.

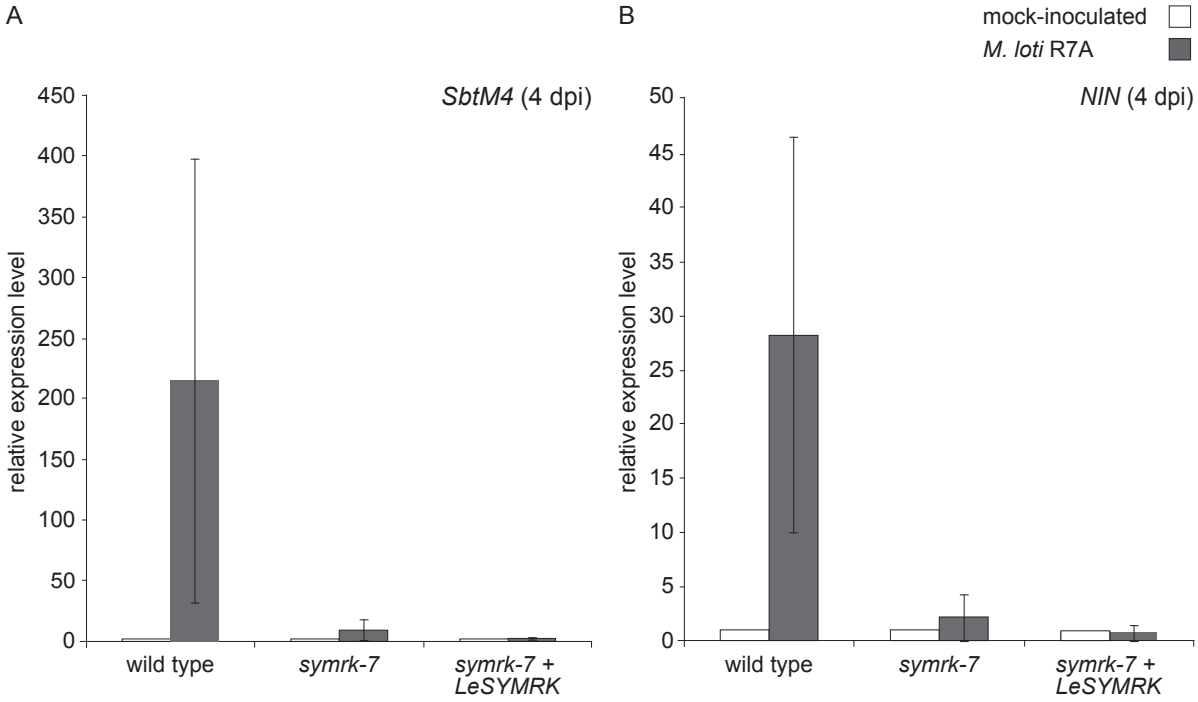

Figure 14. *NIN* and *SbtM4* expression are not induced in *M. loti* R7A induced *L. japonicus* (*Lotus*) *symrk-7* roots carrying tomato *SYMRK* (*LeSYMRK*).
Transgenic plants (*symrk-7 + LeSYMRK*) are homozygous for the *LeSYMRK* genomic region under control of the *LeSYMRK* promoter. Wild type and *symrk-7* control plants are non-transgenic. Wild type plants show a strong induction of both *NIN* (A) and *SbtM4* (B), which are known to be induced upon inoculation with *M. loti* in *Lotus*, while no significant induction could be detected in *symrk-7* controls or *symrk-7* plants transformed with *LeSYMRK*. Values are relative to mock-inoculated controls. Five days old seedlings were inoculated with *M. loti* R7A (OD_{600} = 0,01) and harvested at four days post inoculation (dpi) for RNA extraction and qPCR analysis. Values are averaged from three technical replicates of two independent biological replicates, derived from 6-8 bulked roots each. Error bars indicate standard errors of the mean.

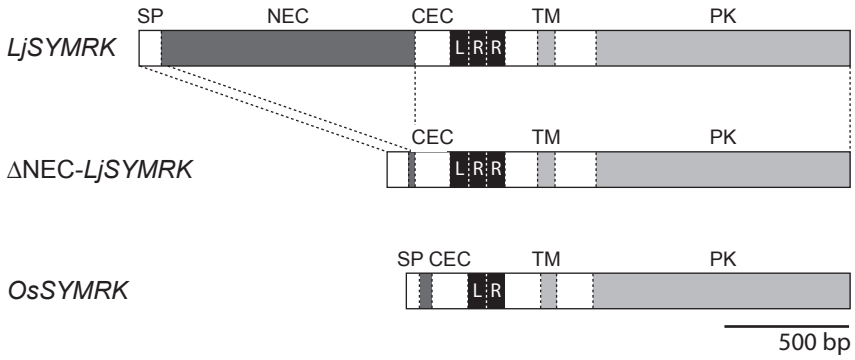

Figure 15. Structure of the *L. japonicus* (*Lotus*) *SYMRK* ΔNEC version (Δ*NEC-LjSYMRK*) compared to full-length *LjSYMRK* and *O. sativa SYMRK* (*OsSYMRK*).
The Δ*NEC-LjSYMRK* deletion construct lacks those parts of the NEC region that are absent in rice (*OsSYMRK*), but retains the three LRR-encoding exons. Bars illustrate the coding sequence of the respective *SYMRK* versions. Prediced protein domains and motifs are indicated. SP, signal peptide; NEC, N-terminal extracellular region; CEC, conserved extracellular region preceeding LRRs; LR(R), leucine-rich repeats; TM, transmembrane domain; and PK, protein kinase domain.

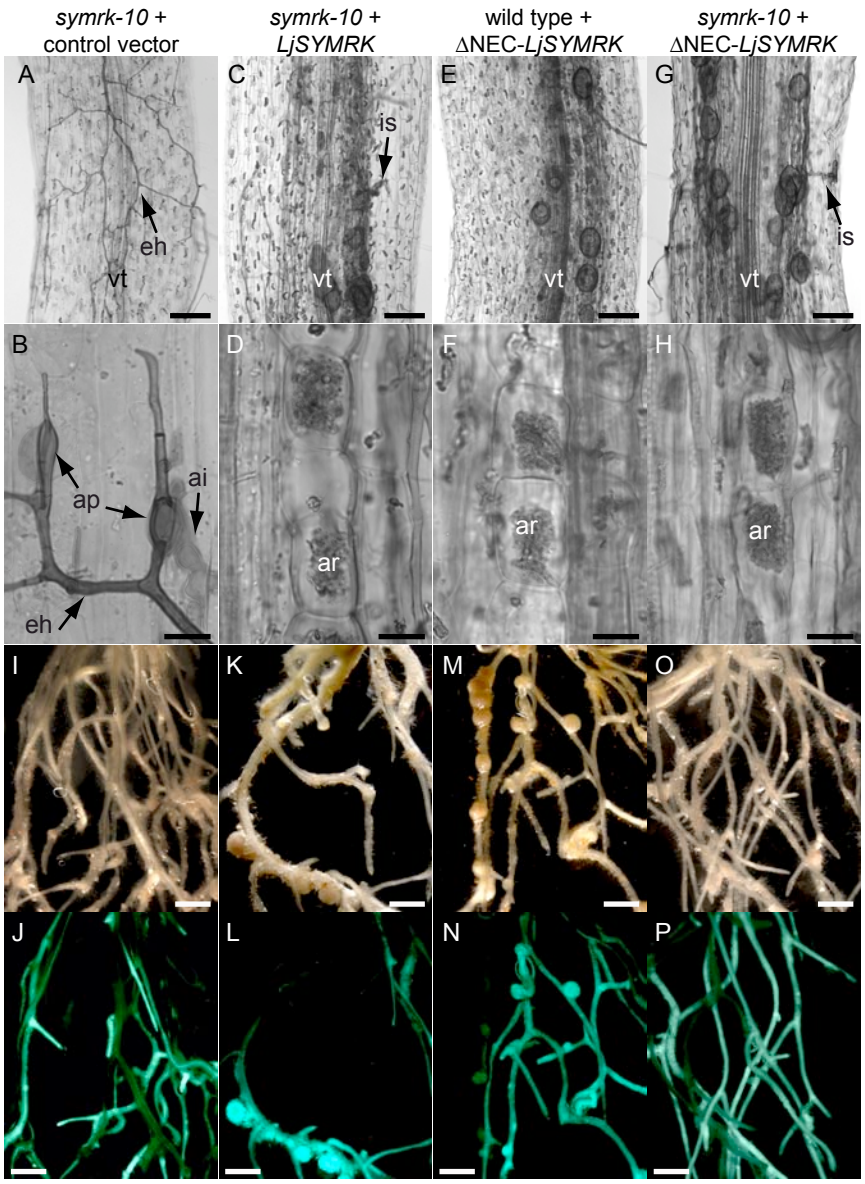

Figure 16. An *L. japonicus* (*Lotus*) *SYMRK* (*LjSYMRK*) deletion construct lacking the NEC region is sufficient for AM formation, but not for RNS in *Lotus*. Assay and selection were as in Figure 10.
(A–B and I–J) *Lotus symrk-10* roots transformed with a control vector lacking a *SYMRK* expression cassette. (C–D and K–L), *Lotus symrk-10* roots carrying the full length *LjSYMRK* cDNA under control of the *LjSYMRK* promoter region. (E–F and M–N) *Lotus* wild type and (G–H and O–P) *Lotus symrk-10* roots transformed with an *LjSYMRK* deletion construct lacking the NEC region (ΔNEC-*LjSYMRK*) (Figure 15).
(A–H) Transgenic roots co-cultivated with *G. intraradices*. (A–B) Transgenic control root with extraradical mycelium but no intraradical fungal colonization or arbuscule formation. Fungal infection attempts are aborted within the epidermal or outer cortical tissue. (C–H) *symrk-10* roots carrying the full *LjSYMRK* cDNA (C–D) and *Lotus* wild type (E–F) and *symrk-10* (G–H) roots carrying the ΔNEC-*LjSYMRK* version, with *G. intraradices* colonization and arbuscule formation in the inner cortical cells.
(I–P) Root systems inoculated with *M. loti*. (I–J) *symrk-10* root system with transgenic roots carrying a control vector, showing no nodules. (K–L) *symrk-10* root system with nodules on a root carrying the full *LjSYMRK* coding sequence. (M–N) *Lotus* wild type root system with nodules on transgenic roots carrying the reduced *LjSYMRK* version (fluorescent). Nodules are also apparent on a non-fluorescent root, not carrying the transgene. (O–P) *symrk-10* root system with roots carrying the reduced *LjSYMRK* version, forming no nodules.
ai; aborted infection; ap, fungal appressorium; ar, arbuscule; eh, extraradical hypha; is, infection site; vt, root vasculature. Scale bars: (A, C, E and G) 0,1 mm; (B, D, F and H) 0,02 mm; (I–P) 2 mm.

To test the responsiveness of *Lotus symrk* mutant plants carrying *LeSYMRK* at a more refined morphological and regulatory level, we isolated a *symrk-7* line homozygous for *LeSYMRK*. Upon inoculation with *Mesorhizobium loti,* root hairs of *Lotus symrk* mutant plants bulge and branch extensively, showing no infection pocket or infection thread formation [24]. Likewise, infection with a *DsRED* expressing strain of *M. loti* revealed no infection pockets or -threads in roots of *symrk-7* plants homozygous for *LeSYMRK*, and root hairs showed excessive branching and tip bulging similar to nontransgenic mutant controls (Figure 13 B–G). Root hairs of wild type plants displayed normal curling and infection thread formation, the majority of hairs remaining non-deformed (Figure 13).

Consistent with a complete block of responsiveness to rhizobial symbionts, induction of the RNS-responsive genes *NIN* [50] and *SbtM4* [51] in roots of *symrk-7* plants homozygous for *LeSYMRK* was at a similar minimal level as in nontransgenic mutant controls when co-cultivated with *M. loti* for four days (Figure 14). In contrast, both genes were strongly upregulated in roots of wild type control plants.

To test whether the LRR motif that is present in the rosid *SYMRK* version, but absent in *SYMRK* candidates from toamto, poppy and monocots is required for root endosymbioses in *Lotus*, we constructed deletion versions of *LjSYMRK*. Because the LRR located towards the N-terminus of the predicted protein (LRR1) shows high sequence similarity and is thus likely to be phylogenetically conserved between the versions, we generated deletion constructs lacking either one of the LRR2 or LRR3 encoding exons. Neither of these constructs was potent to restore AM or RNS in *Lotus symrk-10* mutants (not shown). These results indicate that no proteins capable of fulfilling normal functions in symbiosis were produced. To further analyze these regions, additional constructs could be generated and tested that rely on point mutations in the LRRs in question to reduce the risk of misfolding of the mutated proteins.

The AM-forming, non-nodulating monocots *Oryza sativa* (rice) and *Zea mays* (maize) harbour a “minimal“ *SYMRK* version encoding two LRRs only and a short N-terminal region, mainly covered by a single exon aligning with exon four of *LjSYMRK* (Figure 6

and Figure 7). To determine the role of *SYMRK* regions absent in monocot versions of the gene we generated an *LjSYMRK* version that structurally mimics rice *SYMRK* (*OsSYMRK*) in lacking sequence stretches which encode the N-terminal extracellular (NEC) region of the protein (ΔNEC-*LjSYMRK*, Figure 15). We tested whether this construct is sufficient for endosymbiosis establishment in *Lotus symrk-10* mutants. Although at lower rates than full-length *LjSYMRK* (Table 2), ΔNEC-*LjSYMRK* restored AM upon inoculation with *G. intraradices*, and wild type-like infection sites and arbuscules were formed in transgenic roots (Table 2 and Figure 16 A–B and E–H). Hence, ΔNEC-*LjSYMRK* is sufficient for AM formation.

In contrast, no infection threads or nodules were observed four weeks after inoculation with *M. loti* (Table 2 and Figure 16 I–J and M–P), whereas the introduction of the full-length *LjSYMRK* coding sequence restored both AM with fungi and RNS with bacteria (Table 2 and Figure 16 C–D and K–L). The NEC extension of *LjSYMRK* thus proved indispensable for bacterial endosymbiosis in *Lotus*. Similar to *LeSYMRK,* the reduced version of *LjSYMRK* thus selectively supports fungal, but not bacterial endosymbiosis in *Lotus*.

To determine whether the short *SYMRK* version of rice is sufficient for endosymbiosis formation in an RNS host, we introduced the *OsSYMRK* genomic sequence controlled by the *LjSYMRK* promoter into *Lotus symrk-10* roots. AM was fully restored in these roots, whereas nodulation with *M. loti* was impaired (Table 2 and Figure 12 M–Z). *Symrk-10* roots containing *OsSYMRK* exhibited primordial swellings generally devoid of bacteria after inoculation with *M. loti* (Figure 12 U–X). In rare cases, infection threads and small round nodules were observed, which contained bacterial colonies (Figure 12 Y–Z). Hence, similar to *LeSYMRK* and ΔNEC-*LjSYMRK*, *OsSYMRK* is compromised in supporting bacterial endosymbiosis in *Lotus*.

Successful complementation of *symrk-10* mutants with different *SYMRK* variants depended on the introduction of genomic sequence stretches. Although *Lotus* and *Tropaeolum SYMRK* coding sequence attached to the native *Lotus SYMRK* promoter region restored both AM and RNS in *Lotus symrk* mutant lines, no complementation

was observed using coding sequences of *Medicago* or rice. Also, complementation rates were lower and complementation was less reliable when using tomato *SYMRK* coding sequence as compared to genomic sequence (not shown). In the case of *Medicago DMI2*, retainment of the first intron was sufficient to achieve full complementation in *Lotus* for both AM and RNS. Where complementation of symbioses with fungi and bacteria was selective, namely in the intermediate tomato and minimal rice versions, we therefore generated constructs containing full genomic sequences for complementation assays to avoid that results were influenced by insufficient levels of correctly spliced *SYMRK* transcripts. However, for the ΔNEC-*LjSYMRK* deletion construct, only coding sequence was used. The results obtained with this construct should thus be interpreted with caution.

3.3 Discussion

An ancient genetic program for AM among angiosperms

The requirement of *CYCLOPS* for arbuscule formation, and thus for the establishment of functional AM in the monocot rice suggests a conserved role of this gene in monocot and dicot angiosperms. Rice *CYCLOPS*, and different versions of *SYMRK* from both dicot and monocot lineages were found competent to restore AM in *Lotus symrk* mutants, indicating that the role of *SYMRK* in AM is conserved despite the structural divergence evidenced by this gene. These data suggest a homologous nature of the AM genetic program in angiosperms. The recent finding that loss-of-function mutations in the rice version of *CCaMK* result in loss of AM symbiosis [18, 52], further supports the idea of widespread AM gene conservation. Interestingly, we found that the nonmycorrhizal legume *Lupinus albus* contains a full-length *SYMRK* version showing no apparent difference to other rosid *SYMRK* genes (Figure 7). To clarify whether the absence of AM in this species is in any way associated with the *SYMRK* gene, additional analyzes of the functional capabilities of this ortholog are required.

Syntenic and BLAST analyzes of the *Arabidopsis thaliana* (*Arabidopsis*) genome indicate that the lack of root symbiotic capability in this species is accompanied by a deletion of several symbiosis genes, including *SYMRK*, *CCaMK* and *CYCLOPS* [19, 29, 53]. Their conservation in AM forming species but absence in the asymbiotic *Arabidopsis* is consistent with an ancient and specific role in symbiosis. The analysis of plant progam components for AM and AM-like associations in non-angiosperm land plants, such as gymnosperms, bryophytes and pteridophytes (ferns) will be a future challenge.

A molecular link between the two types of RNS

In legumes, *SYMRK* is indispensable for root endosymbiosis with rhizobia and AM fungi [24, 43]. We show here that this endosymbiosis gene is also required for RNS in

the actinorhizal cucurbit *Datisca glomerata*, and the tree species *Casuarina glauca* (Fagales) [37]. *SYMRK* represents the first plant gene known to be necessary for Actinorhiza. Its common requirement for legume interaction with rhizobia and for Actinorhiza indicates a shared genetic basis of the two different types of RNS.

Interestingly, the modes of bacterial infection likely differ between these two actinorhizal species. *Frankia* infecting *Casuarina* and other actinorhizal Fagales, such as *Alnus* [54], enter the host root *via* intracellular root hair infection [55], an entry mode requiring *SYMRK* in legumes infected intracellularly by rhizobia [24, 43]. Among actinorhizal hosts, this infection mode is limited to Fagales species [7]. In the cucurbit genus *Datisca*, *Frankia* are assumed to penetrate intercellularly between epidermal and cortical cells of young roots [7]. The requirement of *SYMRK* for Actinorhiza may thus encompass actinorhizal hosts infected by both intra- and intercellular bacterial invasion. Further studies are required to determine the degree of overlap between the genetic systems of legume-rhizobium interaction and Actinorhiza, and define where the differences in symbiosis development, and determination of interaction partners find their genetic basis. It will be intriguing to determine how the fine-tuning of regulatory and response patterns differs between RNS forming groups, generating the present diversity of nodulation types with respect to organogenesis, ontogeny and cytology.

SYMRK is not involved in determining host-microbiont specificity

Ligands of the SYMRK extracellular domain have not been described to date, and the precise functions of SYMRK in AM and RNS are still unclear. In theory, the full-length, rosid version of SYMRK could exhibit features necessary for the specific recognition of bacterial symbionts. These features may not be required for AM formation, and may thus be missing in SYMRK versions of AM forming species outside the rosids where RNS with bacteria is absent, accounting for the inability of SYMRK versions from other non-eurosid lineages to support RNS in legumes.

We found that *SYMRK* genes from legumes of different cross-inoculation groups, and from other rosid lineages that form Actinorhiza with *Frankia* bacteria or are even unable to form RNS can support the specific interaction of a legume with its rhizobial symbiont. Thus, a combination of other factors, including nod factor receptor kinases [41, 44, 56], lectins [57] and perhaps yet-unknown additional components, is accountable for the fine-tuning of recognition specificity in plant–bacterial endosymbioses within the rosids.

A role of *SYMRK* in the predisposition to evolve RNS

Our survey of *SYMRK* sequences across angiosperms revealed at least three structurally distinct versions, and we could show that this polymorphism is functionally related to the root symbiotic capabilities of host plants. The variation in SYMRK domain composition is unique among the known common symbiosis genes (Figure 2). The congruence between the phylogenetic distribution of the "full-length" *SYMRK* version with the nodulating clade strongly suggests a link between *SYMRK* sequence evolution and the acquisition of endosymbiotic root nodulation with bacteria. The full-length version of SYMRK, which is required for RNS, is present in a monophyletic clade encompassing both nodulating and non-nodulating lineages. These facts make it a good candidate to be involved in a molecular predisposition for RNS proposed by Soltis *et al*. [8].

An attractive hypothesis is that *SYMRK* sequence divergence was a critical step in mediating the recruitment of the otherwise conserved common symbiosis pathway from the pre-existing AM genetic program. Recruitment was proposed to account for the genetic link of AM and nodulation in legumes [25, 26] and would make root–bacterial endosymbiosis as a whole a fascinating example for novel traits evolving on the basis of pre-existing genetic patterns. Further genetic adaptations would have been required for RNS involving nodule organogenesis to occur.

The decisive novelty that the molecular predisposition for RNS, by mediating the recruitment of AM genes, brought about may be the ability accomodate bacteria intracellularly. The common symbiosis genes defining the overlap of AM and RNS genetic programs play an apparent role in the intracellular infection process of fungi in AM, and of rhizobia in rhizobium-legume interactions. Supporting this hypothesis, a feature associated with endosymbiotic bacterial infection in both actinorhizal [58] and legume hosts [59], the formation of intracellular pre-infection threads (PITs) in host cells. These cytoplasmic structures resemble the pre-penetration apparatus (PPA) preceding fungal infection during AM formation [60, 61]. Forming in anticipation of bacterial symbionts, PITs are thought to coordinate the uptake of bacteria and determine the spatial progression of infection through the host cell [58, 59]. A similar role in guiding fungal transition through host cells in AM has been demonstrated for PPAs [60, 61]. These developmental similarities in AM, Actinorhiza, and legume-rhizobium infection may reflect a common genetic program for endosymbiosis establishment and symbiont uptake in all three types of interactions. In AM, PPAs are not formed in mutants that are defective in certain common symbiosis genes including *SYMRK* [60, 61]. It is therefore possible that a recruitment of AM symbiosis genes during the evolution of RNS facilitated the induction of intracellular accommodation structures in response to bacteria.

SYMRK domain function and evolution

Repetitive LRR modules have been implicated in the determination and evolution of novel recognition specificities of receptor proteins [62-64]. Interestingly, adaptive changes reflecting positive selective constraints can be traced in LRR– and NEC–encoding regions of *SYMRK* genes from different *Medicago* species [65]. Our functional comparison of eurosid *SYMRK* versions indicates that *SYMRK* is not involved in determining recognition specificity in nodulation. However, an extended *SYMRK* version containing a set of three LRR motifs, as present in eurosid *SYMRK* genes, is required for fully supporting RNS of *Lotus* with *M. loti*. Shorter *SYMRK* versions from

tomato or rice only suffice for AM. These functional differences may be caused by individual amino acid sequence polymorphisms, or alternatively, exons that are specifically required for bacterial endosymbiosis may be lacking in rice and tomato *SYMRK* versions. The ΔNEC-*Lj*SYMRK version of *Lotus* SYMRK, lacking the NEC region but exhibiting all three LRR motifs, was equally unable to restore RNS, while supporting AM in *Lotus symrk* mutants. These combined data indicate that both the NEC region and the LRRs are necessary for RNS establishment. At an overall structural level, exon acquisition from other genes encoding LRR or NEC-like domains [43, 66] or, alternatively, retainment of exons in eurosid *SYMRK* genes, may have been an integral genetic factor in the evolution of bacterial endosymbiosis in angiosperms.

The observation of small nodule-like structures on *Lotus symrk* mutant roots transformed with the *OsSYMRK* construct is counterintuitive, considering that the *LeSYMRK* and ΔNEC-*LjSYMRK* versions, which resemble legume *SYMRK* genes more closely, do not support such developmental responses.

A possible explanation for the different capabilities of tomato and rice *SYMRK* to support RNS in the complementation assays be that the nonmatching NEC region of *Le*SYMRK negatively interferes with RNS establishment, but not AM signalling in *Lotus*.

The *Lotus symrk* mutant line used for complementation assays, *symrk-10*, encodes a kinase-dead SYMRK protein [49]. This line was selected for the experiments due to its strong mutant phenotype, high rates of seed set and germination, and good growth and developmental properties as well as ease of transformability. It cannot be excluded that in *symrk-10* transgenic roots expressing rice *SYMRK*, *Lotus* mutant protein is produced which forms heterodimers with the shorter rice SYMRK version. These heterodimers would contain intact *Lotus* SYMRK extracellular domains, and rice SYMRK kinase domains, possibly leading to a complex of residual functionality in response to bacterial symbionts. Intermolecular phosphorylation has been demonstrated for SYMRK, and it is possible that SYMRK proteins form dimers or oligomers [49]. Similar effects may be blocked in the case of roots transformed with tomato *SYMRK* due to the presence of an

extracellular extension interfering with the extracellular domain of the *Lotus symrk* mutant protein. To obtain clarity about this possibility, and to confirm the degree of functionality of *OsSYMRK* in *Lotus* root endosymbiosis, the complementation assays performed in this study should be supplemented with similar assays using a different *symrk* mutant background, where the presence of mutant protein potentially exhibiting residual functionality can be excluded. Candidate mutant lines include such where the *SYMRK* coding sequence encodes a stop codon, like *Lotus symrk-19* [Perry *et al*., manuscript in preparation] or is interrupted by deletion (*Medicago* dmi2 5P) or insertion (*Lotus symrk-1* [24] or *symrk-3* [24]) of mutations close to the translation start site, or in regions of the gene encoding predicted extracellular sections of the protein, precluding proper membrane localization.

Like tomato *SYMRK*, the ΔNEC-*LjSYMRK* version of *Lotus SYMRK* restored AM only, with no evidence for organogenesis induction after inoculation of transgenic roots with *M. loti*. This is despite the higher similarity of its sequence to the native *Lotus SYMRK* at the sequence level as compared to rice *SYMRK*, and despite the presence of all three LRR motifs. The differences in competence of rice *SYMRK* and ΔNEC-*LjSYMRK* to support responsiveness to bacterial symbionts in *Lotus* are difficult to explain, but it should be pointed out that ΔNEC-*LjSYMRK* was generated using coding sequence only. The results obtained with ΔNEC-*LjSYMRK* should thus be interpreted with care, considering the importance of intronic sequences for efficient complementation with other *SYMRK* versions. Rice *SYMRK* supported the initiation of nodule orgaogenesis only when genomic sequence was used, while *OsSYMRK* coding sequence attached to the *LjSYMRK* promoter restored neither AM nor RNS in *Lotus symrk* roots. It is therefore possible that due to a lack of intronic regulatory sequences ΔNEC-*LjSYMRK* expression levels were too low to allow for responsiveness to rhizobia in transgenic roots. Lower levels of *SYMRK* may suffice for AM restoration, resulting in a certain degree of AM complemented roots. The experiment should thus be repeated using genomic sequence for construct generation.

It is not clear whether for the different *SYMRK* genes used in this study intronic sequences are directly involved in regulation of expression levels or transcript nuclear

export, or some *SYMRK* versions are processed by the *Lotus* splicing machinery in the absense of introns, thereby generating misspliced transcripts.

It is possible that regulatory sequences are also present in introns of *Lotus SYMRK*. In the case of *LjSYMRK*, which perfectly matches with the *Lotus* signalling context, reduced levels of native protein may suffice to support endosymbiosis establishment. On the contrary, reduced expression levels in combination with imperfect compatibility of heterologous or artificial *SYMRK* genes may result in an inability of these versions to restore endosymbioses.

The NEC domain encoded by *Lotus SYMRK* exons two and three, upstream of the conserved LRR flanking region (CEC), is present across eudicot plants (Figure 6). Its function outside the nodulating group is unknown. The proposed involvement of *SYMRK* in processes such as reduction of the touch sensitivity of root hairs [67] may rely on this domain thereby imposing selective constraints. The NEC domain shows possible overall relatedness but only a low level of similarity to sequences present in the rice genome, and to sequences other than *SYMRK* candidates in genomes of dicots like *Arabidopsis* [43]. The apparent divergence observed among these potentially homologous sequences of yet unknown function is consistent with a hypothetical role as a receptor domain.

It will be a future challenge to determine the contribution of individual SYMRK LRR units as well as of the NEC domain and to resolve at the amino acid level the features of SYMRK proteins involved in conferring endosymbiotic nodulation capacity.

Additional components required for nodulation

The diversity and scattered occurrence of nodulation symbioses within the eurosid lineage suggest multiple independent origins [68]. Only a subset of the plant species carrying the “full-length” version of *SYMRK* develop root nodules, yet *SYMRK* of the non-nodulating *Tropaeolum* proved competent to support RNS in *Lotus*. Hence, there must be additional genetic features distinguishing the nodulators. Candidate genes include those that express the legume LysM receptor kinases NFR1 and NFR5 [41, 56,

69], which are required for responsiveness to rhizobial lipo-chito-oligosaccharide nodulation factors, but not for AM formation. A potential relevance of LysM receptors in Actinorhiza, or the identity of alternative receptors perceiving yet unknown *Frankia* signals, remains to be determined.

3.4 Conclusions

Our findings indicate a monophyletic nature of the genetic program for AM in angiosperms, and suggest that AM-forming, non-nodulating representatives – including cereals like rice – share a basal set of endosymbiosis determinants. In legumes, certain AM genetic components are also necessary for the interaction with rhizobia [19-24], and we show here that this common endosymbiosis program encompasses actinorhizal nodulation. Together, these findings provide first functional evidence for the long-standing hypotheses that plant nodulation symbioses are of common ancestry [70], and that some of their signalling components were recruited from ancient AM functions [25, 26].

Endosymbiosis genes identified to date reflect differential evolutionary forces. Genes like *CYCLOPS* and *CCaMK* [30, 71] are highly conserved and can potentially function in plant-bacterial signalling also in non-nodulating lineages, as suggested by their ability to restore nodulation in respective legume mutants. In contrast, structurally and functionally distinct *SYMRK* versions exist in extant angiosperms with different root symbiotic abilities. The differential potential of tomato and rice *SYRMK,* and of the ΔNEC version of *Lotus SYMRK* to complement plant-fungal but not plant-bacterial interactions in *Lotus* suggests that *SYMRK* is directly involved in the evolution of bacterial endosymbioses and ultimately RNS.

3.5 Materials and methods

Isolation of *SYMRK* homologues

We used a PCR strategy employing degenerate primers to obtain *SYMRK* sequence information from diverse angiosperms, for which no genome or root-derived expressed sequence tag sequences were available. Degenerate primers for the isolation of *SYMRK* genes were positioned in regions of the coding sequence conserved among *SYMRK* candidates, but not in other similar *O. sativa* (rice) and *A. thaliana* (*Arabidopsis*) sequences. For primer sequences, see Table 4.

λ Zap cDNA libraries were available for isolation of *L. esculentum* (tomato) and *Alnus glutinosa* (alder) *SYMRK*. A cosmid clone carrying the *LeSYMRK* genomic region was isolated from a pooled tomato Cf2/9 library (kind gift of J.D.G. Jones, The Sainsbury Laboratory, United Kingdom) and shotgun sequenced.

For rapid amplification of complementary DNA ends (RACE) reactions, total RNA was extracted from roots of uninoculated seedlings or young plants using the Nucleospin RNA Plant kit (Macherey-Nagel) and DNaseI treated. Reverse transcription and 5′/3′RACE reactions were done using the SMART RACE kit (Clontech), following nested degenerate PCR reactions ([10 s 94 °C, 10 s 52 °C, 30 s 72 °C] × 35, 5 min 72 °C) to obtain initial sequence information.

Construct generation for mutant complementation and *SYMRK* RNAi experiments

For hairy root complementation assays, *SYMRK* cDNAs were amplified from complementary DNA (cDNA) preparations (Superscript II, Invitrogen) of uninoculated roots of the respective species. Binary transformation vectors were pCAMBIA 1302 or pK7WG2D,1 [72] derivatives. *D. glomerata* (*Datisca*), *L. japonicus* (*Lotus*), *Me. truncatula* (*Medicago*), and *T. majus* (*Tropaeolum*) *SYMRK* coding sequences were amplified from cDNA using primers DgSYMRK_EC_f with DgSYMRK_PK_r,

LjSYMRK_EC_f with LjSYMRK_PK_r, MtSYMRK_EC_f with MtSYMRK_PK_r, and TmSYMRK_EC_f with TmSYMRK_PK_r (Table 4), respectively.

The *LjSYMRK* delta NEC deletion construct (ΔNEC-*LjSYMRK*) was amplified as two fragments using primers LjSymRK_EC_f with LjSYMRK_EC_r and LjSYMRK_exon4_f with LjSymRK_PK_r (Table 4), which were then combined by overlapping PCR.

An *MtSYMRK* genomic segment containing intron one was amplified from total DNA using primers MtSYMRK_EC_f with MtSYMRK_01_r (Table 4) and ligated to the 3′ fragment of the *MtSYMRK* cDNA following BstB1 digestion of both. The genomic sequences of tomato and rice *SYMRK* were amplified from total DNA using primers LeSYMRK_EC_f with LeSYMRK_PK_r and OsSYMRK_EC_f with OsSYMRK_PK_r (Table 4), respectively. A 285-bp fragment amplified with primers polyA_NOS_f and polyA_NOS_r (Table 4) from pJawohl8 RNAi (kind gift of P. Schulze-Lefert, Max Planck Institute for Plant Breeding Research, Cologne, Germany) was used as terminater in all constructs. *SYMRK* genes or coding sequences were under control of 4,970 bp of genomic sequence preceding the *LjSYMRK* translation start site. For pK7WG2D constructs, the cauliflower mosaic virus (CaMV) 35S promoter driving the Gateway-cassette was deleted SalI(10124)-SalI(38). *C. glauca* (*Casuarina*) cDNA was amplified and cloned as described [37].

CYCLOPS coding sequences were amplified from cDNA preparations (Superscript II, Invitrogen) obtained from root material of uninoculated rice (*O. sativa* subspecies *japonica*, cultivar Nipponbare) or *L. japonicus* ecotype Gifu roots and cloned into a pCAMBIA 1301 derived vector. *Lotus* and rice *CYCLOPS* coding sequences were amplified using primers LjCYCL_ATG_f with LjCYCL_TAA_r and OsCYCL_ATG_f with OsCYCL_TAA_r (Table 4), respectively. The same 285 bp NOS (nopaline synthase) terminater fragment was used as for *SYMRK* constructs. *CYCLOPS* constructs were under control of 2271 bp genomic sequence preceding the *LjCYCLOPS* translation start site.

For *Datisca* RNAi experiments, a pRedRoot- [73] based binary vector encoding DsRED1 for visible selection was equipped with a pKANNIBAL [74] CaMV 35S promoter-driven hairpin construct containing 367-bp of 3′ coding and untranslated sequence of *DgSYMRK* in forward and reverse orientation, amplified with primers DgSYMRK_RNAi_f and DgSYMRK_RNAi_r (Table 4). For *Casuarina* RNAi experiments, construct generation was as described [37].

Table 4. Oligonucleotide primer sequences

	Primer Name	Sequence 5´ to 3´
A	ALIEN_r	gaatccatagatctcatatattcagaagcrttrttytc
	EAM_r	aaagcaacttcaacaactctccacadngcytc
	EWA_f	ccaagacatgaatggtctctggtngartgggc
	GREP_f	ttctgctggaaattgttactggamgngarccnyt
B	Dg3RACE_f	ggcagtggcgtgtatcgaaccc
	Dg5RACEinn_r	ggactcacaagccaggccatcccagg
	Dg5RACEout_r	gttgggaggaagctcattgccatcgcc
	Le3RACE_f	tgcatggcgggacattgtaagaga
	Le5RACEinn_r	ccatgataccctcccttgatagt
	Le5RACEinn_r	cactctccacagcgcctca
	Pr3RACE_f	agaagcaatgtggagggtggtt
	Pr5RACEinn_r	ccacattgcttctgcattataccctgctt
	Pr5RACEout_r	caacttcaaccaccctccacattgctt
	Tm3RACE_f	catgcatggctgacattgttcgtg
	Tm5RACEinn_r	gtaacccccttttatggtcggat
	Tm5RACEout_r	gctacttctaccactcgccacatt
C	LjSYMRK_EC_f	TTATTTATCGATGatggagttaccagc
	LjSYMRK_EC_r	ctctcatcttctgaatcactcccactatgctctcaaacccttcagttgc
	LjSYMRK_exon4_f	gcaactgaagggtttgagagcatagtgggagtgattcagaagatgagag
	LjSYMRK_PK_r	ATTAAACCTAGGTAATAAGTCGACctatctcggctgtgggtgag
	MtSYMRK_EC_f	TTATTTATCGatgatggagttacaagttattaggatatttag
	MtSYMRK_01_r	cagaaatctatgtagtctttggtggc
	MtSYMRK_PK_r	ATTAAACCTAGGTAATAAGTCGACctatctcggttgagggtgtgac
	DgSYMRK_EC_f	TTATTTATCGatgatgatggaaggattgcataattg
	DgSYMRK_PK_r	ATTAAACCTAGGTAATAAGTCGACt catctgggttcaggaggagccaag
	TmSYMRK_EC_f	TTATTTATCGatgatgatggaaagactcgac
	TmSYMRK_PK_r	ATTAAACCTAGGTAATAAGTCGACctatcttggttcaggaggagtc
	LeSYMRK_EC_f	TTATTTATCGatggaagtagataattgctggaac
	LeSYMRK_PK_r	ATTAAACCTAGGTAATAAGTCGACcagcatttaccttggttgtggag
	OsSYMRK_EC_f	TTATTTATCGatggccgcccgcttcg
	OsSYMRK_PK_r	AAATAACCTAGGctaccccggaagcgaaggca
	LjCYCL_ATG_f	AATTAACCTAGGATCCatggaagggaggggttttctgg
	LjCYCL_TAA_r	AATAATCCCGGGttacatttttttcagtttctgatag
	OsCYCL_ATG_f	AATTAACCTAGGATCCatggagggcaggggtctgtctgag
	OsCYCL_TAA_r	AATAATCCCGGGttatgtgttggtatcagagacg
	polyA_NOS_f	AATAAACCTAGGatcagcttgcatgccggtcg
	polyA_NOS_r	AAATAAGTCGACctagagtcaagcagatcgttcaaac
D	DgSYMRK_RNAi_f	GGATCGATGGTACCatgcagaggcaatgtgga
	DgSYMRK_RNAi_r	GCTCTAGACTCGAGtcactctttcataatttcccaaaaggt

E	DgqPCR_Ubi_f	atgcagatytttgtgaagac
	DgqPCR_Ubi_r	accaccacgragacggag
	DgqPCR_SYMRK_f	tgtgtgctaaggtggcagactttgg
	DgqPCR_SYMRK_r	aactatggctcgtgtagtactcgggatc
	LjqPCR_Ubi_f	atgcagatcttcgtcaagaccttg
	LjqPCR_Ubi_r	acctcccctcagacgaag
	LjqPCR_NIN_f	tggatcagctagcatggaat
	LjqPCR_NIN_r	tctgcttctgctgttgtcac
	LjqPCR_SbtM4_f	atgtaagctatgctgctggaatagag
	LjqPCR_SbtM4_r	atgcaacagcaggggctag
F	OsSYMRK_Ex4_f	ctcaggtcgtggcgcgacggtgacccgtgc
	OsSYMRK_Ex5_r	ccacgctgatagataggccatgagg
	OsCYCL_In3_f	aggcattttcatcacccatc
	OsCYCL_Ex6_f	cacccagtcagactccaaca
	OsCYCL_Ex6_r	atgctgtaccaagccaaacc
	Tos17_f/r	attgttaggttgcaagttagttaaga

(A) Degenerate oligonucleotide primers used for identification of *SYMRK* candidates. (B) Nondegenerate primers used in 3´ and 5´RACE reactions to isolate full-length cDNA sequences of *SYMRK* homologs. (C) Nondegenerate primers used to generate *SYMRK* constructs for hairy root complementation experiments. Upper case characters in the sequences indicate restriction enzyme sites or artificial sequence framing these. (D) Nondegenerate primers used for the preparation of the *DgSYMRK* RNAi construct. (E) Degenerate and nondegenerate primers used for expression level analysis of the indicated genes via qPCR in *Datisca* (*Dg*) and *Lotus* (*Lj*). Primer orientation is indicated by _r, complementary and _f, forward. (F) Nondegenerate primers used for genotyping of *O. sativa* Tos17 insertion lines. Primer orientation is indicated by _r, complementary and _f, forward.

Biological material and phenotyping assays

AM and RLS phenotyping of *L. japonicus* whole plant transformormants

L. japonicus wild type ecotype B-129 Gifu and derived mutant lines *symrk-10* [48] and *symrk-7* [24, 75] as well as four independent transgenic lines carrying the *LeSYMRK* genomic region were tested, two in *symrk-7* and two in *symrk-10* background. For nodulation tests, plants were inoculated with *M. loti* R7A or, to visualize infection threads and confirm the presence of bacteria within nodules, *M. loti* R7A carrying pXLGD4 [76] as described [51]. For root hair response assays and gene expression analyzes, a *symrk-7* line homozygous for *LeSYMRK* was isolated. Root hair response assays were performed on plates containing ½ strength nitrogen-free B&D medium [77]

supplemented with 1% agar-agar and 0.1 μM aminoethoxyvinylglycine (AVG). Roots of three-days old seedlings were inoculated with *M. loti* MAFF 303099 expressing DsRED (kind gift of M. Hayashi), suspended in ½ strength liquid B&D medium to a final optical density at 600 nm (OD_{600}) of 0.01. Control plants were mock-inoculated with sterile medium. Plants were kept vertically and roots protected from light. Root hair responses were assayed at 3-6 days post inoculation. Growth conditions were 24°C constant at 16 h light/8 h dark cycles.

AM and RLS phenotyping of hairy roots in *L. japonicus* and *M. sativa*

L. japonicus wild-type ecotype B-129 Gifu and derived mutant lines *symrk-10* [48] and *cyclops-3* {Szczyglowski, 1998 #41; Yano, 2008 #344} were inoculated with *M. loti* R7A as described [24]. Growth conditions were 24°C constant at 16-h-light/8-h-dark cycles. For infection of *Agrobacterium rhizogenes*–induced transgenic roots in *Lotus*, *M. loti* MAFF 303099 expressing *DsRED* was applied at OD_{600} of 0.02 in translucent plastic boxes containing 300 ml Seramis (Mars) and 150 ml liquid Fahraeus Plant (FP) medium [78]. *A. rhizogenes*-transformed *M. truncatula* A17 wild type and *dmi2* 5P (kind gift of G. Oldroyd, John Innes Centre, United Kingdom) plants were inoculated with *S. meliloti* 1021 carrying pBHR-mRFP [79] (OD_{600} of 0.02) in planting pots containing Seramis, and fertilized with FP medium supplemented with 50 μM KNO_3 two times per week. For nodulation assays and simultaneous observation of infection thread (IT) formation in *L. japonicus* and *M. truncatula*, plants were harvested four and five weeks post inoculation, respectively. In *L. japonicus* plants transformed with *CgSYMRK*, nodulation was scored at four, five or 15 weeks post inoculation. Prior to inoculation, roots showing no eGFP fluorescence were removed in all cases. For AM phenotyping of *L. japonicus* and *M. truncatula*, plants were co-cultivated with *G. intraradices* BEG195 and harvested after three or two weeks of co-cultivation, respectively. Root systems were stained with acidic ink as described [32]. Prior to staining of *A. rhizogenes*–induced root systems, roots showing no eGFP fluorescence were removed. Roots were scored AM-positive (AM+) if symbiotic structures (arbuscules and vesicles) were present, as AM-negative (AM–) if no arbuscules were

present. Occasional *L. japonicus symrk* mutant roots showing vesicles not accompanied by arbuscules were scored AM–. Where complemented *L. japonicus symrk* mutant roots exhibited aborted infection sites in co-occurrence with successful infection and colonization events involving arbuscule and vesicle formation, roots were scored AM+.

AM and actinorhiza phenotyping of *D. glomerata SYMRK* knockdown roots

D. glomerata seeds and *Frankia* inoculum were a kind gift from K. Pawlowski (Department of Botany, Stockholm University, Sweden). *D. glomerata* was inoculated with compatible *Frankia* by potting in substrate with ~1 g/l crushed nodules and with *G. intraradices* BEG195 by adding substrate extracted from pots of inoculated *Allium schoenoprasum* plants. Growth conditions were 16 h light/8 h dark at 22°C and 60% relative humidity. Seeds of *T. majus* and *P. rhoeas* were purchased at Notcutts Garden Centres (UK). The ability to develop AM with *G. intraradices* was confirmed for all species involved in the study.

AM and actinorhiza phenotyping of *C. glauca SYMRK* knockdown roots

C. glauca hairy root phenotyping was done as described [38].

Genotyping and AM phenotyping of *Oscyclops* lines

All *O. sativa* plants were subspecies *japonica*, cultivar Nipponbare and grown as recommended at http://tos.nias.affrc.go.jp/~miyao/pub/tos17/. *Oscyclops* lines ND5032, NC2713, NC2415 and NG0782 were identified by screening a library of 42.700 rice mutant lines induced by retrotransposon *Tos17* [80]. For genotyping, primer *Tos17* was used with gene specific primers (Table 4). The presence and location of the insertions were confirmed by PCR and sequence analysis in all lines. For AM phenotyping, plants were co-cultivated with *G. intraradices* BEG195 and *Allium schoenoprasum* nurse plants for either two or three weeks. Quantification of AM colonization in rice roots followed the Magnified Line Intersect Method ([81], modified).

Plant transformation

L. japonicus hairy root and whole plant transformation

Transgenic roots on *Lotus symrk-10* and *cyclops-3* mutants were induced using *A. rhizogenes* strains AR1193 [82] and LBA1334 [83] as described by Díaz *et al*. [84] (modified). *L. japonicus symrk-7* and *symrk-10* lines carrying the *LeSYMRK* genomic region were obtained by *A. tumefaciens* Agl1 mediated transformation [85], using a pRK290 [86] based cosmid as binary vector.

M. truncatula hairy root transformation

Medicago seedlings were transformed as described at http://www.isv.cnrs-gif.fr/embo01/manuels/index.html (modified), using strain *A. rhizogenes* AR1193 [82].

D. glomerata hairy root transformation

Twelve-week-old *Datisca* plants were inoculated with *A. rhizogenes* strain LBA1334 [83] carrying the silencing construct by stem injection, and roots emerging at infection sites were covered with substrate. Three-week post inoculation roots were inspected for DsRED1 fluorescence. Nonfluorescent roots were removed, and plants were repotted and grown for eight weeks. After determination of the nodulation phenotype, individual fluorescent roots were divided into two halves. One half was stained for mycorrhiza visualization, the second used for total RNA extraction (RNeasy Plant Kit, Qiagen).

C. glauca hairy root transformation

C. glauca hairy roots were induced as described [87].

Quantitative PCR of reverse transcribed cDNA (qRT-PCR)

RNA was extracted from root tissues for expression level analyzes using the Nucleospin RNA Plant kit (Macherey-Nagel). Quantitative RT-PCR was performed with GeneAmp5700 (Applied Biosystems) using the SuperScript III Platinum Two-Step qRT-PCR-Kit (Invitrogen).

For determination of *SYMRK* expression levels in *Datisca symrk* knowckdown roots, a 123-bp *DgSYMRK* fragment was amplified using primers DgqPCR_SYMRK_f with DgqPCR_SYMRK_r (Table 4). As control, polyubiquitin cDNA was amplified using primers DgqPCR_Ubi_f with DgqPCR_Ubi_r (Table 4).

For expression level analysis of *NIN* [50] and *SbtM4* [51] in *Lotus symrk-7* roots carrying *LeSYMRK*, five days old seedlings of transgenic plants (*symrk-7* + *LeSYMRK*) homozygous for the *LeSYMRK* genomic region under control of the *LeSYMRK* promoter were mock-inoculated (controls) or inoculated with *M. loti* R7A (OD_{600} = 0.01). Roots were harvested at four days post inoculation for RNA extraction and qPCR analysis. RNA was extracted from 6-8 bulked roots per sample. Primers for fragment amplification were LjqPCR_Ubi_f with LjqPCR_Ubi_r for determining polyubiquitin levels as control, LjqPCR_NIN_f with LjqPCR_NIN_r and LjqPCR_SbtM4_f with LjqPCR_SbtM4_r for test genes *NIN* and *SbtM4*, respectively (Table 4).

Representative fragments were sequenced for identity confirmation.

Computational analysis

Databases used for BLAST sequence search and analysis included http://www.ncbi.nlm.nih.gov/BLAST/, http://www.arabidopsis.org/Blast/, http://www.gramene.org/Multi/blastview, and http://genome.jgi-psf.org/Poptr1/Poptr1.home.html. Sequence analysis and alignments were done using The Staden Package [88] v1.5.3 and Vector NTI v9.0. Protein domains and motifs were predicted using PROSITE [89], SIGNALP [90], TMHMM [91], and PredictNLS [92].

Accession Numbers

Sequences of *SYMRK* homologs were deposited at the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) under accession numbers AY935263 (*A. glutinosa*); AM271000, AM931079 (*D. glomerata* coding and genomic sequence,

respectively); AY935267 (*Lupinus albus*); AY935265 (*T. majus*); AY935266, AY940041 (*L. esculentum* coding and genomic sequence, respectively); AM270999 (*P. rhoeas*); AM851092 (*P. trichocarpa*). The accession number for pCAMBIA 1302 at GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) is AF234298.

The genomic/coding sequences used for prediction of exon-intron structures in Figures 6, 14 and 15 were:

SYMRK/DMI2/NORK [24, 36, 43]: AP004579/AF492655 (*L. japonicus*), AY940041/ AY935266 (*L. esculentum*), AP003866/AK099778 (*O. sativa*); *CYCLOPS* [19]: AP009158/EF569221 (*L. japonicus*), AP008212/EF569223 (*O. sativa*).

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5 Appendix

5.1 List of publications and author contributions

For unpublished data that are not yet contained in manuscripts, aspects not prepared by the author of this thesis are as follows:

Gábor Giczey (The Sainsbury Laboratory, United Kingdom; present affiliation: Quintiles, Hungary) isolated a cosmid carrying tomato *SYMRK* for transgenic complementation of *Lotus symrk* mutants, and Matthew Smoker (The Sainsbury Laboratory, United Kingdom) performed whole plant transformations.

For published data or unpublished manuscripts, the author of this thesis contributed as follows:

Markmann, K., Giczey, G. and Parniske, M. (2008).
Functional adaptation of a plant receptor kinase gene paved the way for the evolution of intracellular root symbioses with bacteria. PLoS Biology 6(3): e68.

The experiments presented in this publication have been performed, and the data acquired and analyzed by the author of the thesis, except for the identification of *SYMRK* genes from *Lupinus albus*, *Tropaeolum majus* and *Lycopersicon esculentum*, which were isolated by Gabor Giczey (The Sainsbury Laboratory, United Kingdom; present affiliation: Quintiles, Hungary) or by him and the thesis' author in cooperation. The supervisor of this thesis, Martin Parniske (The Sainsbury Laboratory, United Kingdom; present affiliation: University of Munich (LMU), Germany) identified the rice *SYMRK* candidate. Manuscript and figures were prepared by the thesis' author in cooperation with Martin Parniske.

Gherbi, H.*, **Markmann, K.***, Svistonoof, S., Estevan, J., Autran, D., Giczey, G., Auguy, F., Péret, B., Laplaze, L., Franche, C., Parniske, M. and Bogusz, D. (2008).
SymRK defines a common basis for plant root endosymbioses with AM fungi, rhizobia and *Frankia* bacteria. Proceedings of the National Academy of Sciences, USA 105(12): 4928-4932.
*These authors contributed equally to the work.

The author of the thesis designed, performed and analyzed the AM and RNS complementation experiments of *Lotus japonicus symrk* mutants with *Casuarina glauca SYMRK*. Hassen Gherbi (Institut de Recherche pour le Développement, France) and the thesis' author prepared the constructs used in this experiment in cooperation. The respective manuscript sections and figures were prepared by the author of the thesis, and the remainder of the manuscript was written by Hassen Gherbi and the thesis' author in cooperation.

Markmann, K. and Parniske, M. (2009).
Evolution of root endosymbiosis with bacteria: how novel are nodules?
Trends in Plant Science: 14, 77-86.

The author of the thesis prepared all text and figures of the review, with Martin Parniske providing advice and discussion.

Yano, K., Yoshida, S., Müller, J., Singh, S., Banba, M., Vickers, K., **Markmann, K.**, White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., Parniske, M. (2008).
CYCLOPS, a mediator of symbiotic intracellular accomodation. Proceedings of the National Academy of Sciences, USA: 105, 20540-20545.

All data, figures and text sections concerning the phenotyping of rice *CYCLOPS* insertion lines, and the complementation of *Lotus cyclops* mutants with rice and *Lotus CYCLOPS* genes were contributed by the author of this thesis. Rice *CYCLOPS* was originally isolated by Kate Vickers (The Sainsbury Laboratory, United Kingdom), who also retrieved the rice *cyclops* insertion mutant lines from the *Tos17* Mutant Panel Database (Rice Genome Resource Centre, Japan). Judith Müller isolated the homozygous mutant lines used in this study.

Perry, J., Welham, T., Brachmann, A., Charpentier, M., **Markmann, K**., Wang, T. and Parniske, M.
Mining the symbiotic component of the *Lotus japonicus* genome using classical genetics and thematic TILLING. Manuscript in preparation.

For this manuscript, AM and RNS phenotyping data of several *Lotus japonicus symrk* mutant alles were collected and analyzed by the author of the thesis.

5.2 Publications and manuscripts

The publications containing data stemming from the thesis presented here are attached below in the following order:

Markmann, K. and Parniske, M. (2009).
Evolution of root endosymbiosis with bacteria: how novel are nodules?
Trends in Plant Science: 14, 77-86.

Markmann, K., Giczey, G. and Parniske, M. (2008).
Functional adaptation of a plant receptor kinase gene paved the way for the evolution of intracellular root symbioses with bacteria. PLoS Biology 6(3): e68.

Gherbi, H.*, **Markmann, K.***, Svistonoof, S., Estevan, J., Autran, D., Giczey, G., Auguy, F., Péret, B., Laplaze, L., Franche, C., Parniske, M. and Bogusz, D. (2008).
SymRK defines a common basis for plant root endosymbioses with AM fungi, rhizobia and *Frankia* bacteria. Proceedings of the National Academy of Sciences, USA 105(12): 4928-4932.

Yano, K., Yoshida, S., Müller, J., Singh, S., Banba, M., Vickers, K., **Markmann, K.**, White, C., Schuller, B., Sato, S., Asamizu, E., Tabata, S., Murooka, Y., Perry, J., Wang, T., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., Parniske, M. (2008).
CYCLOPS, a mediator of symbiotic intracellular accomodation. Proceedings of the National Academy of Sciences, USA: 105, 20540-20545.

The following manuscript is in preparation and is therefore not attached:

Perry, J., Welham, T., Brachmann, A., Charpentier, M., **Markmann, K**., Wang, T. and Parniske, M.
Mining the symbiotic component of the *Lotus japonicus* genome using classical genetics and thematic TILLING. Manuscript in preparation.

Review

Cell PRESS

Evolution of root endosymbiosis with bacteria: how novel are nodules?

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Plants form diverse symbioses with nitrogen-fixing bacteria to gain access to ammonium, a product of the prokaryote-exclusive enzyme nitrogenase. Improving the symbiotic effectiveness of crop plants like maize, wheat or rice is a highly topical challenge and could help reduce the need for energy-intense nitrogen fertilizer in staple food production. Root nodule symbiosis (RNS) constitutes one of the most productive nitrogen-fixing systems, but it is restricted to a small group of related angiosperms. Here, we review the genetic regulation of RNS and its interconnections with other plant symbiosis or plant developmental programs. Since RNS uses genetic programs that are widely conserved in land plants, we evaluate the prospects for a transfer to plants that are currently non-nodulating.

Root nodule symbiosis: a rare but efficient source of nitrogen for plants

Limitation of water or nutrients such as nitrogen, potassium and phosphate restricts plant growth and development in many terrestrial ecosystems. Mutualistic symbioses of roots with fungi or bacteria help plants to cope with these constraints. Associative nitrogen fixation is found in plants as diverse as ferns (e.g. *Azolla*), gymnosperms (e.g. coralloid roots of cycads) and monocots, which can host endophytic diazotrophs such as *Azoarcus* or *Azospirillum*. Among the most sophisticated associations are intracellular (endo-) symbioses, where the microbial partner is accommodated within a living host cell [1]. This inter-organismic intimacy is based on complex molecular crosstalk between the partners and, except in parasitic associations where bacterial nitrogen fixation remains inefficient, allows efficient nutrient exchange to the benefit of both [1]. Root nodule symbiosis (RNS) effectively renders plants independent of other nitrogen supplies [2] but is restricted to only four related orders within the Eurosid clade of angiosperms [3]. RNS is characterized by two major evolutionary inventions: the intracellular uptake of bacteria and the formation of specialized organs, the root nodules [4,5]. The latter provide a suitable microenvironment for nitrogenase activity and allow for a protected, controlled development of high bacterial population densities. Nodules thus resemble 'micro-fermenters' within the host plant that are optimized for symbiosis maintenance.

There are two main types of RNS that differ in bacterial partners, infection mechanisms and nodule organogenesis and morphology [4,5]. Legumes, including important agricultural crops such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*), interact with phylogenetically diverse nitrogen-fixing bacteria known as rhizobia [2,4]. Actinorhiza, a symbiosis with nitrogen-fixing actinobacteria of the genus *Frankia* [6], is formed by members of three rosid orders: the Fagales, Cucurbitales and Rosales [7]. Examples are alder (*Alnus* spp.), sea buckthorn (*Hippophae rhamnoides*) and the subtropical tree genus *Casuarina* [8].

Within the four orders where RNS occurs, its distribution is scattered [7,9]. It is an ongoing challenge to decipher the combination of molecular adaptations characterizing RNS-forming plants (i) relative to related species outside of the nodulating clade and (ii) relative to non-nodulating representatives within. The close kinship of taxa forming RNS prompted the proposition that a

Glossary

Actinorhiza (AR): mutualistic association of plant roots with nitrogen-fixing bacteria of the genus *Frankia* (Actinobacteria). Actinorhiza is a form of RNS, involving the development of nodules on host roots, where high densities of symbiotic bacteria reside within plant cells. The term Actinorhiza also refers to infected nodule organs representing the physical manifestation of the symbiosis. Host plants include members of the angiosperm orders Fagales, Cucurbitales and Rosales.

Arbuscular mycorrhiza (AM): a phylogenetically widespread mutualistic symbiosis of plant roots with fungi of the phylum Glomeromycota. AM fungi form finely branched hyphal structures (arbuscules) within host cells and deliver phosphates and other nutrients to the plant in exchange for carbohydrates.

Determinate nodules: root nodules forming in response to symbiotic bacteria where cells of the tip meristem fully differentiate once the nodule has reached its full size. The developmental stage of the inner nodule tissue is therefore uniform. Determinate nodules are formed by different legume lineages, and examples of determinate-nodule-forming plants include *Lotus japonicus* and *Glycine max* (soybean).

Indeterminate nodules: symbiotic root nodules that retain their tip meristem and continuously develop new infected tissue. As a result, indeterminate nodules display a zonal composition, with different stages of symbiosis development contained in the same nodule. The nodule vasculature can be peripheral (legumes) or centrally localized (actinorhizal plants and *Parasponia*), leading to a stem-like or root-like tissue organization, respectively. Examples of indeterminate-nodule-forming legumes include: *Medicago truncatula* (barrel medic), *Medicago sativa* (alfalfa), *Pisum sativum* (garden pea) and *Sesbania rostrata*.

Rhizobium–legume symbiosis (RLS): RNS formed by legumes (Fabales) in association with diverse Gram-negative nitrogen-fixing bacteria termed rhizobia.

Root nodulation symbiosis (RNS): root symbiosis of plants with nitrogen-fixing bacteria, which are hosted inside specific root-derived organs (nodules). RNS supplies the host plant with bacterial-fixed nitrogen in exchange for carbohydrates. The two main forms of RNS are AR with *Frankia* bacteria and RLS with rhizobia.

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common ancestor could have acquired a genetic predisposition to evolve nodulation [3]. This predisposition, consisting of one or more genetic advances, consequently became a basis for the evolution of all types of RNS. This hypothesis predicts that the existing forms of RNS have overlapping genetic programs. However, experimental evidence for a genetic program shared between both types of RNS was obtained only recently [10,11]: post-transcriptional gene silencing demonstrated that the *SYMBIOSIS RECEPTOR-LIKE KINASE* (*SYMRK*) gene (also known as *DOES NOT MAKE INFECTIONS 2* [*DMI2*] in *Medicago truncatula* [barrel medic] and *NODULATION RECEPTOR KINASE* [*NORK*] in *Medicago sativa* [alfalfa]) [12,13], which is necessary for the interaction of legumes with rhizobia [12,13], is also required for actinorhiza with *Frankia* in the Fagales tree *Casuarina glauca* [10] and the cucurbit *Datisca glomerata* [11]. The common requirement of *SYMRK* is probably representative for other genes shared between RNS in legumes and actinorhiza (Figures 1, 2 and 3) and provides important support for the hypothesis that actinorhiza and legume RNS share a common genetic ancestry.

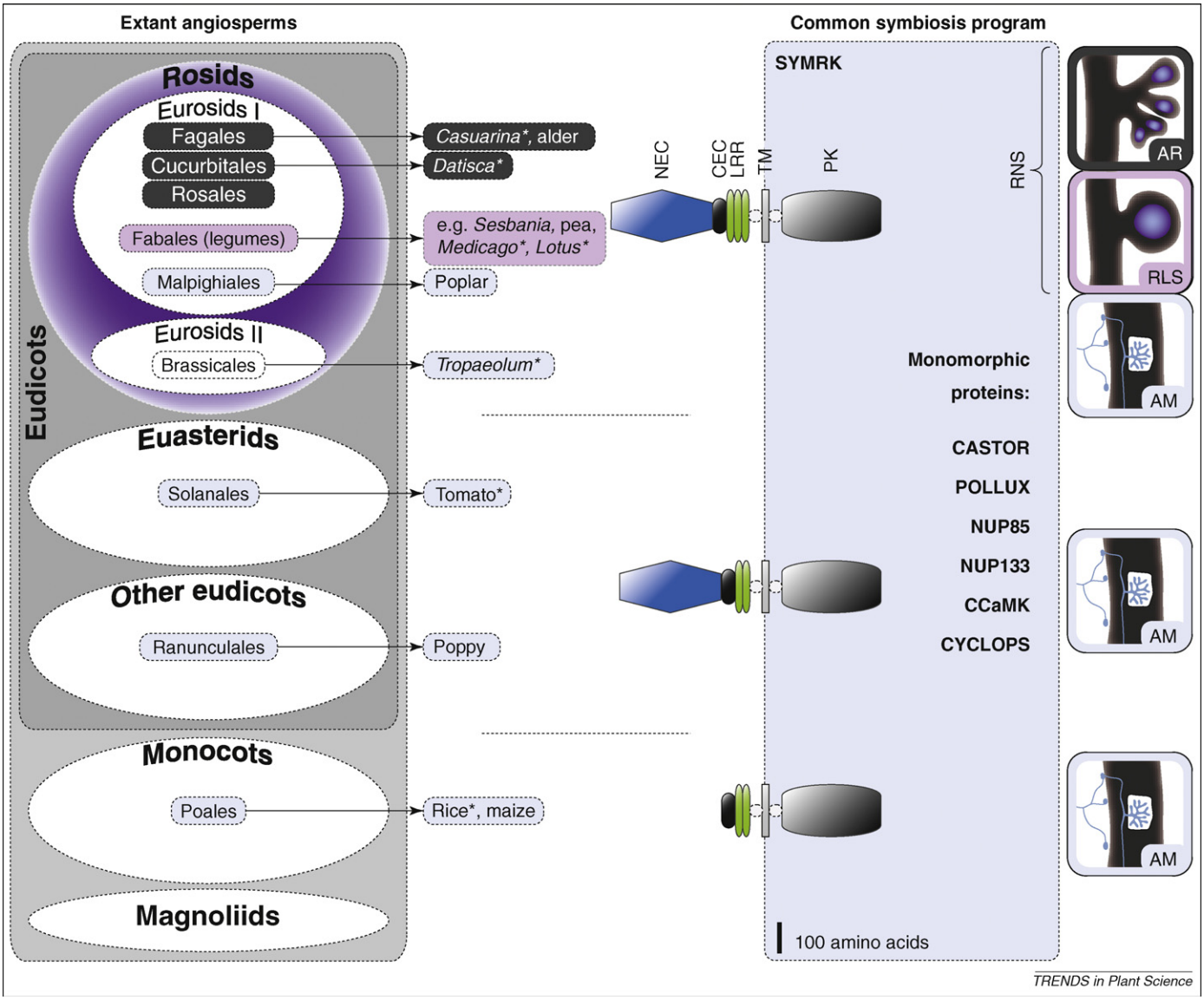

Figure 1. Common endosymbiosis genes exhibit divergent patterns of structural and functional evolution. *SYMRK*, which might form the entry point to the common AM and RNS program for endosymbiosis with fungi or bacteria, has at least three distinct structural versions. All of these support AM symbiosis with fungi, but only the longest version is sufficient for functional RNS [11]. This longest *SYMRK* version has been found only in rosids and seems to be present in all species that form RNS, as well as in their relatives, such as poplar or *Tropaeolum,* that do not form RNS but form only AM symbioses [11]. This pattern suggests an involvement of *SYMRK* in a genetic adaptation that might have provided a molecular basis for the evolution of RNS within the rosid lineage. A common ancestor of the rosid clade (dark purple) possibly acquired a specific adaptation of *SYMRK*, allowing plant–bacterial interactions to access the conserved AM genetic program for endosymbiosis, which encompasses at least six further genes (*CASTOR* [34]; *POLLUX* [31,34]; *NUP85* [35]; *NUP133* [36]; *CCaMK* [32,37]; *CYCLOPS* [33,38]). In contrast to *SYMRK*, these are structurally conserved across dicot and monocot angiosperms (Figure 2). Potentially, the monomorphic components of the common symbiosis program can support RNS [26,28,38,39,47]. The small pictograms refer to the root endosymbiosis types supported by the respective structural version of SYMRK. Angiosperm orders shaded dark gray include members forming AM and actinorhizal RNS with *Frankia* (AR), those shaded light purple form AM and another form of RNS, rhizobium–legume symbiosis (RLS). Members of orders shaded light blue form AM but no RNS. The plant names (same color code as for orders) refer to species containing the respective structural versions of *SYMRK*. Asterisks (*) indicate the availability of data on the functional capabilities of the respective *SYMRK* genes [10,11]. Abbreviations: AM, arbuscular mycorrhiza; AR, actinorhiza; RLS, rhizobium–legume symbiosis; RNS, root nodulation symbiosis. Key to plant names: alder, *Alnus glutinosa*; *Casuarina, C. glauca*; *Datisca, D. glomerata*; *Lotus, L. japonicus*; maize, *Zea mays*; *Medicago*, *M. truncatula* and *M. sativa*; poplar, *P. trichocarpa*; poppy, *Papaver rhoeas*; *Tropaeolum, T. majus*; rice, *Oryza sativa*. Predicted protein regions are abbreviated as: CEC, conserved extracellular domain; LRR, leucine-rich repeat; NEC, N-terminal region of unknown function; PK, protein kinase domain; TM, transmembrane domain. Nomenclature of angiosperm phylogeny is based on Ref. [81].

In this review, we discuss the genetic features that distinguish 'nodulators' from non-nodulating plants – and form the molecular prerequisite for the complex evolutionary achievement of nitrogen-fixing RNS.

What are the key genetic inventions of the nodulating clade?

Among the prime candidates for adaptations specific to RNS-forming plants are genes involved in the perception of rhizobial signaling molecules, namely lipochito-oligosaccharide nodulation (Nod) factors. These receptors are required for the earliest host responses to symbiotic rhizobia and are involved in determining host–symbiont specificity [14–17]. They are likely to represent relatively recent achievements during the evolution of legume RNS. In the Japanese legume *Lotus japonicus,* a close relative of the Eurasian birdsfoot trefoil, perception of Nod factors depends on the receptor-like kinases (RLKs) NOD FACTOR RECEPTOR 1 (NFR1) and NFR5 and is highly specific in that Nod factors produced by different bacterial strains can be distinguished if they show minor alterations of their chemical structure [15–17]. NFR-predicted proteins contain lysine motif (LysM) domains in their predicted extracellular part, and these domains are involved in Nod factor recognition in *L. japonicus* [17]. Closely related genes mediate Nod-factor signaling in other legumes such as *M. truncatula* [14,18]. In an approach to induce a compatible interaction of rhizobial bacteria with an incompatible legume, *M. truncatula* was equipped with *L. japonicus* Nod factor receptor genes (*NFR1* and *NFR5*). Transgenic roots formed bacteria-containing nodules with the specific partner of *L. japonicus*, *Mesorhizobium loti* [17]. Although these nodules were not fully functional [17], the observations demonstrate that Nod factor receptors have a central role in defining host ranges in rhizobium–legume interactions. The Nod factor receptors are closely related to two receptors identified in the non-nodulating angiosperms *Arabidopsis thaliana* (thale cress) and rice (*Oryza sativa*), which are required for the defense-related perception of chitin oligomers, a chemical signature of fungi [19,20].

The high level of specificity of Nod-factor recognition and the necessity to evoke symbiosis-related rather than defense-related downstream responses call for artificial transfer of compatible receptors to potential new hosts. Alternatively, infection systems that are likely to be independent of Nod-factor signaling, such as those employed by certain photosynthetic bradyrhizobia that induce nodules on aerial plant parts [21] or actinorhiza with *Frankia* [22], represent an interesting resource for further exploitation towards developing artificial host systems. Because these bacteria lack common *nod* genes required for Nod-factor synthesis in rhizobia [21,22], they must rely on alternative strategies to induce nodule organogenesis and infection. A key question is whether the Nod-factor receptors are specific to the legume branch of the nodulating clade or whether actinorhiza plants employ the same type of receptor for detection of *Frankia* symbionts. Key genes required for Nod-factor synthesis have not been detected in the fully sequenced *Frankia* genomes ([22]; Box 1). However, the exceptional genus *Parasponia* belongs to the Rosales that are normally nodulated by *Frankia* but is nodulated by rhizobia. *Parasponia* nodulation requires the rhizobial common *nod* genes, which suggests that LysM RLKs could be involved in RNS in this plant [23]. Although *Parasponia* might be an exception, it is possible that the adaption of LysM receptors for the recognition of symbiotic bacteria goes back to the 'predisposition event', but their involvement in actinorhizal symbiosis with *Frankia* is presently unclear.

Box 1. Model systems for RNS transfer

Basal but efficient forms of RNS promise to involve a minimal set of host and bacterial genetic adaptations required for engineering artificial host systems. Developmental features of symbiosis establishment, such as infection mode, intracellular accommodation of the microbial symbiont or nodule organogenesis, depend on the host genotype [4,5], rendering the plant the prime target for such engineering efforts. Actinorhizal hosts [82] and legumes of the family Caesalpiniaceae, as well as some Fabaceae [4], retain their bacterial symbionts in tubular infection-thread-like structures lined with plant cell wall material. These have been referred to as 'fixation threads' [4] because they are the sites of bacterial nitrogen fixation in the respective lineages, and they might represent an ancient form of bacterial accommodation [4]. This is in contrast to many legumes of the families Mimosaceae and Fabaceae, including most fabacean crop legumes, where rhizobia are hosted within specialized membrane-bound compartments termed symbiosomes [83]. Because symbiosome formation poses stringent demands on the genetic compatibility of the symbiotic partners and plant control of the symbiosis, fixation thread symbioses like actinorhizas might be more suited as model systems for RNS transfer.

Bacterial candidates for engineering artificial RNS systems should be compatible with a wide range of host genetic backgrounds. Promising examples include broad-host-range symbionts like *Rhizobium* sp. strain NGR234 [84] or *Frankia* bacteria [22], of which many strains can efficiently infect phylogenetically diverse hosts after either intra- or intercellular infection modes [85]. Most actinorhiza-forming *Frankia* bacteria [86], as well as certain rhizobia, such as *Azorhizobium caulinodans* [87] and symbiotic *Burkholderia* strains [88], have the capacity to subsist and fix nitrogen under both symbiotic and nonsymbiotic conditions. Although other factors can restrict nitrogen fixation within plant roots [89], such strains could provide a basis for artificially generating beneficial associations on a nonspecific or partially specific basis [90].

Bacterial uptake evolved by arbuscular mycorrhiza gene recruitment

RNS is genetically embedded in an ancient program for endosymbiosis that is widely conserved in plants today. The majority of land plants form arbuscular mycorrhiza (AM), a symbiosis with fungal symbionts of the phylum Glomeromycota [24]. AM hosts benefit from the extensive access of the fungal mycelia to soil compartments by exchanging photosynthates for water and nutrients such as phosphate [25]. AM-forming angiosperms have been shown to possess conserved genes required for intracellular accommodation of microbial symbionts [11,26–29]. Although originally evolved for symbiosis with fungi, components of this program are functional in RNS with bacteria and could potentially support RNS also in lineages that do not currently contain nodulating species. The 'common symbiosis program' is defined by genes that are required for both intracellular root symbioses with bacteria (RNS) and fungi (AM) [1]. In the model legume *L. japonicus*, seven common symbiosis genes have been ident-

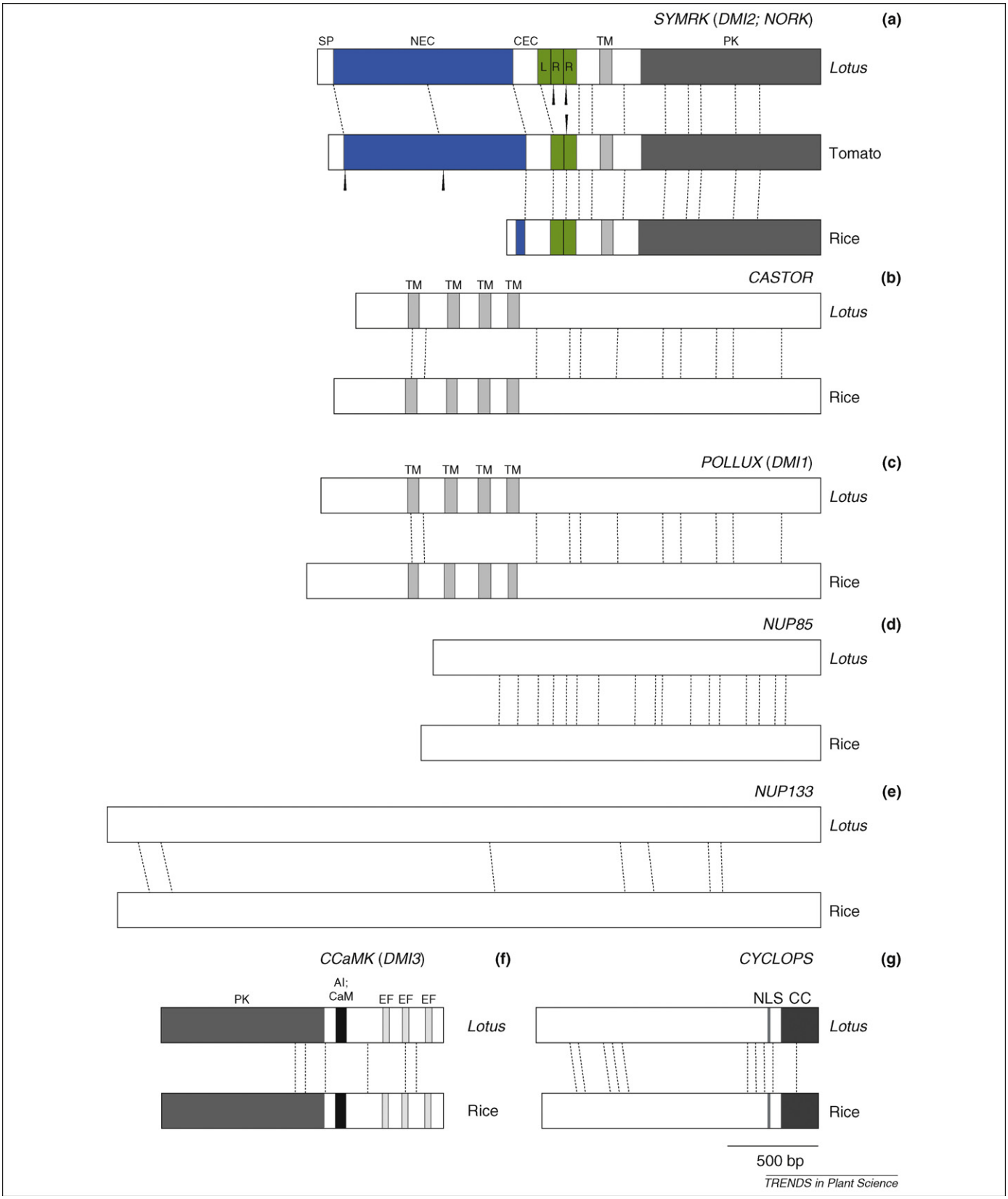

Figure 2. The variation in gene structure and domain composition between *SYMRK* versions from different angiosperm lineages is exceptional among genes that are required for both AM and RNS symbioses (common symbiosis genes). The longest known version of *SYMRK* **(a)** contains three LRR motifs, all encoded by individual exons. Two shorter versions exist in other plant lineages, which form AM, but no RNS. *SYMRK* from the asterid tomato represents an intermediate version that only has two LRR-encoding exons. The shortest known versions of *SYMRK* were isolated from monocots, such as rice [11]. In those regions of the gene that are present in all versions, intron positions and phases are conserved. Other common symbiosis genes **(b–g)** required for intracellular infection are structurally conserved between dicot and monocot angiosperms, such as *Lotus* and rice. Minor variations in exon lengths exist, but intron positions and phases are conserved in all cases. In *Lotus* CYCLOPS (g), two NLS motifs are predicted *in silico*, as compared to one in rice, but only one of the NLS from the *Lotus* ortholog was functional in a heterologous localization experiment [38]. Only this one, which corresponds to the one conserved in rice CYCLOPS, is depicted here. Key to plant names: *Lotus*, *L. japonicus*; tomato, *Lycopersicon esculentum*; rice, *Oryza sativa*. Filled boxes represent exons, or those regions of exons that are part of the coding sequence. Introns are not depicted, and dotted lines indicate the positions of

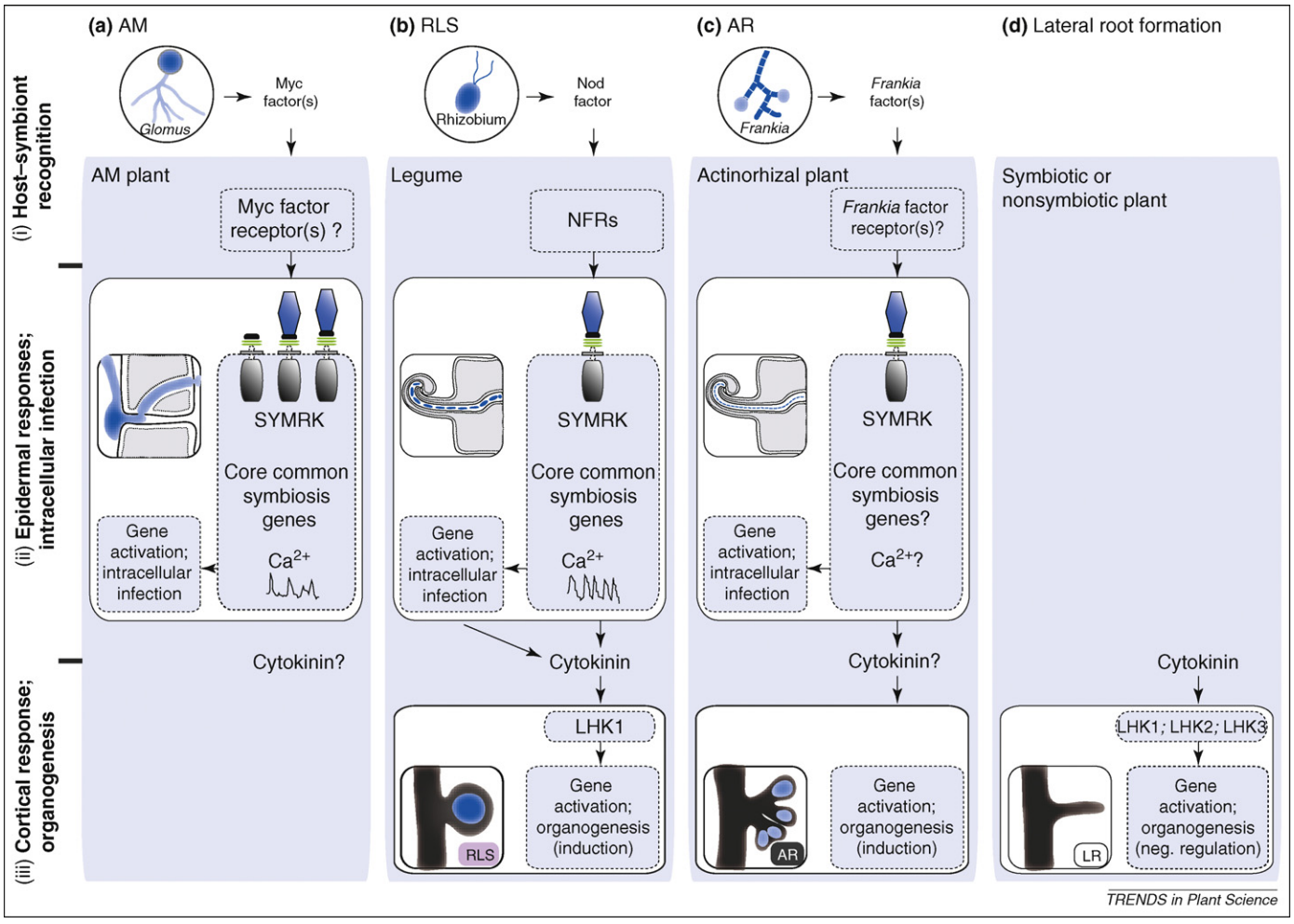

Figure 3. Root nodulation symbiosis (RNS) with nitrogen-fixing bacteria is embedded in conserved genetic programs. Genes required for intracellular infection with **(a)** arbuscular mycorrhiza (AM) are shared with both main types of RNS, **(b)** rhizobium–legume symbiosis (RLS) and **(c)** actinorhiza (AR) with *Frankia*. Nodule organogenesis might be closely related to **(d)** lateral root formation. Three modules (i–iii) are apparent that define genetically separable response patterns in RNS. (i) Plant genes required for host–symbiont recognition in the different symbiosis types are likely to carry specific adaptations. Candidates are known only for RLS, where LysM kinases (*LjNFR*s in *Lotus japonicus*, and *MtLYK*s as well as *MtNFP* in *Medicago truncatula*) are required for the specific perception of rhizobial chito-oligosaccharide Nod factors (b) [14–18]. AM and AR are likely to employ other genes for recognition of fungal and *Frankia* signals, the chemical nature of which is not yet resolved ((a) and (c), respectively). (ii) The core module for intracellular infection is defined by at least seven genes that are required for endosymbiosis with both bacteria and fungi in legumes [30], and is likely to be conserved across AM-forming angiosperms [11,26,28,29,38,39,47]. *SYMRK* is likely to be active at the entry point of this program. The longest, probably rosid-specific version of the gene might carry an adaptation that specifically links receptor-mediated bacterial recognition to the common symbiosis program for endosymbiotic uptake. SYMRK is also required for AR, suggesting that the two forms of RNS rely on similar genetic programs [10,11]. Epidermal responses leading to intracellular infection involve distinct patterns of calcium oscillation in RLS and AM [54,55]. These calcium signals (referred to as 'calcium spiking') potentially represent a key trigger of AM- or RNS-specific downstream gene activation. In RLS, activation of the common symbiosis program is assumed to induce the synthesis of cytokinin, which is assumed to act as a signal regulating organogenetic response in the root cortex [52,68,69]. The cytokinin receptor LHK1 (CRE1 in *M. truncatula*) has a central role in nodule organogenesis induction [52,66,67]. (iii) In the legume *L. Japonicus*, continuous signaling from progressing intracellular infection, possibly via cytokinin, is necessary for maintenance and completion of nodule organogenesis [38] (b). Cytokinin balancing also has a central role in the induction of lateral roots (LRs), which structurally resemble actinorhizal nodules [5]. Response patterns are distinct in that a relative increase in cortical cytokinin levels induces nodulation but represses lateral-root formation [67,75]. Three histidine kinases (*At*LHK1, *At*LHK2 and *At*LHK3), including a putative ortholog of *Lj*LHK1 (*Mt*CRE1), are involved in regulating LR formation in the nonsymbiotic *Arabidopsis thaliana* [73] (d). It is possible that cytokinin induction of nodule organogenesis involves specific spatial induction patterns of cytokinin biosynthesis, molecular adaptations of *Lj*LHK1 (*Mt*CRE1) or downstream targets that are exclusive to RNS-forming species.

ified [30], and for four of these, orthologs were isolated from other legumes such as *M. truncatula, M. sativa* and garden pea [12,31–33]. The RLK SYMRK (DMI2; NORK) functions among the earliest components of the common symbiosis program, near the point where rhizobium–legume signaling merges with the AM genetic program [12,13,16]. Further common symbiosis proteins include the two predicted cation channels CASTOR and POLLUX (*M. truncatula* DMI1) [31,34], two nuclear pore proteins (NUP85 [35] and NUP133 [36]), the calcium and calmodulin dependent

exon–intron borders. Arrowheads indicate exon–intron borders that cannot be correlated between predicted orthologs with certainty. Predicted protein regions are abbreviated as: AI; CaM, autoinhibitory domain overlapping with calmodulin binding site; EF, calcium-binding EF hand; CC, coiled-coil region; CEC, conserved extracellular region; LRR, leucine-rich repeat motif; NEC, N-terminal extracellular region of unknown function; NLS, nuclear localization signal; PK, protein kinase domain; TM, transmembrane domain; SP, signal peptide. The annotations follow *in silico* predictions or published annotation of the depicted or homologous sequences where appropriate [11,13,37,38]. The genomic and coding sequences used for prediction of exon–intron structures were: (a) *SYMRK* (*DMI2*; *NORK*) [11–13], AP004579 and AF492655 (*Lotus*), AY940041 and AY935266 (tomato); AP003866 and AK099778 (rice); (b) *CASTOR* [34], AB162016 and AB162157 (*Lotus*), NC_008396 and AK068216 (rice); (c) *POLLUX* (*DMI1*) [31,34], AB162017 and AB162158 (*Lotus*), NC_008394 and AK072312 (rice); (d) *NUP85* [35], AP009253 and AB284835 (*Lotus*), NC_008394 and AK072636 (rice); (e) *NUP133* [36], AJ890252 and AJ890251 (*Lotus*), NC_008396 and AK073981 (rice); (f) *CCaMK* (*DMI3*) [32,37], AM230792 and AM230793 (*Lotus*), AC097175 and AK070533 (rice); (g) *CYCLOPS* [38], AP009158 and EF569221 (*Lotus*), AP008212 and EF569223 (rice). Coding sequences used for exon–intron structure determination are derived from cDNA, not from *in silico* predictions. Parts of the figure are modified from Ref. [11].

kinase CCaMK (*M. truncatula* DMI3) [32,37] and the nuclear protein CYCLOPS [38] (*M. truncatula* INTERACTING PROTEIN OF DMI3 [IPD3]) [33].

The requirement of legume genes for both bacterial and fungal symbioses inspired the hypothesis that pre-existing AM genes were recruited during the evolution of RNS [1]. Indeed, predicted common symbiosis gene orthologs from non-legume dicots and from monocots were shown to be essential for AM in these lineages or supported AM if introduced in legume mutants deficient in the respective genes. Rice lines mutated in *CASTOR*, *POLLUX*, *CCaMK* (*DMI3*) or *CYCLOPS* (*IPD3*) were unable to establish symbiosis with AM fungi [26,28,29,38,39], and transgenic introduction of *CASTOR*, *CCaMK* and *CYCLOPS* into corresponding legume mutants restored AM formation [26,28,38,39]. *SYMRK* genes from different dicot angiosperms and the monocot rice complemented the loss of AM in an *L. japonicus symrk* mutant [11]. This provides evidence for a conserved genetic framework of AM in angiosperms (Figure 1).

In contrast to RNS, plant organogenetic programs are not induced during AM [40]. However, in both symbioses the host plant accommodates the respective microbial symbiont inside living plant cells. Prior to AM fungal infection of host epidermal and cortical cells, characteristic nuclear movements and rearrangements of cytoskeleton and organelles occur [41,42]. Formation of the resulting cellular 'pre-penetration apparatus' was found to depend on the common symbiosis genes *DMI2* (*SYMRK*) and *DMI3* (*CCaMK*) in *M. truncatula* [42], indicating their involvement in preparing host cells for the intracellular invasion by fungal symbionts. Strikingly similar phenomena were observed in both rhizobium–legume [43,44] and actinorhizal [45] interactions, where cytoplasmic bridges, termed 'pre-infection threads', form in host cells before bacterial invasion. In both AM and RNS, these cytological structures dictate the path of microbial progression through infected host cells [41–45]. Consistent with this, mutant phenotypes of legumes impaired in common symbiosis genes involve either a total block or early arrest of intracellular microbial infection [30]. Together, these data strengthen the hypothesis [1] that AM gene recruitment during RNS evolution laid the basis for intracellular accommodation of bacteria. The recruitment event could have enabled the integration of receptor-mediated perception of bacterial partners into the pre-existing AM genetic program, which was a decisive step in the evolution of RNS. The genetic basis for this novel link might be identical to the enigmatic predisposition event at the root of the nodulating clade.

SYMRK is a likely determinant of the genetic predisposition for nodulation

Genetic adaptations conferring the predisposition to nodulate [3] have been acquired by the ancestor of the monophyletic nodulating clade. Therefore, the corresponding features should be present in nodulating and non-nodulating members of this clade. At the same time, such adaptations should be absent in other angiosperm lineages. A corresponding pattern has so far only been detected for *SYMRK,* making this gene a prime candidate for carrying decisive sequence and functional alterations acquired by the ancestor of the nodulating clade. Among common symbiosis genes required for endosymbiotic infection in AM and RNS, contrasting evolutionary patterns are apparent (Figures 1 and 2). Unique structural and functional divergence was found between *SYMRK* genes isolated from different angiosperm lineages (Figures 1 and 2). At least three distinct versions of *SYMRK* exist, which differ in the domain configuration of the predicted products and in their potential to confer symbiotic ability [11]. Only the longest version, which is present in rosids including legumes, actinorhizal hosts and also non-nodulating species, fully supports both AM and RNS [11]. Both shorter *SYMRK* versions fully support AM but not RNS when transgenically introduced into a legume *symrk* mutant background [11]. The precise molecular features that are responsible for these differences remain to be determined. Interestingly, only the monocot version can support basic RNS-related responses, including organogenesis induction and rare, aberrant infection events [11]. The precise extent to which non-rosid *SYMRK* versions can function in root interactions with bacteria remains to be determined. But the apparent correlation between the occurrence of the full-length *SYMRK* version in a monophyletic group including all RNS-forming lineages and the potential of this version, as opposed to the shorter ones, to support functional endosymbiosis with bacteria suggests that *SYMRK* is involved in the predisposition for nodulation. By contributing to the general ability of rosid lineages to evolve RNS, the longest *SYMRK* version is likely to represent one of the genetic advances that paved the way for RNS evolution in an ancestor of the nodulating clade [11].

In contrast to *SYMRK*, other common symbiosis genes exhibit a conserved exon–intron structure within the coding sequence and similar predicted protein domains in hosts from dicot and monocot angiosperm lineages [27,32,34–36,38] (Figure 2). Examples are *CASTOR*, *CCaMK* and *CYCLOPS*, where this structural conservation coincides with conserved functionality in root endosymbioses. *CCaMK* from the monocots rice [46] and *Lilium longiflorum* [47] restored RNS when introduced into *ccamk* mutants of the legume *M. truncatula*, although later stages of symbiosis establishment were compromised when rice *CCaMK* was used for complementation [46]. In *castor*, *ccamk* and *cyclops* loss-of-function mutants from *L. japonicus*, rice versions of the respective genes fully restored both AM and RNS [28,38].

Potentially, with the exception of SYMRK [11], common symbiosis proteins from non-nodulating lineages can thus support RNS without major modification. The observed sequence adaptations of *SYMRK* might have been sufficient for the recruitment of the common symbiosis program in an ancestor of all nodulators.

Combining infection and organogenesis: what makes a 'predisposed rosid' a 'nodulator'?

Despite the scattered occurrence of nodulation within the rosid lineage, plant–bacterial endosymbioses have only been detected in association with nodule organogenesis. A known exception is *Gleditsia triacanthos* (honey locust), in which infection-thread formation and symbiotic nitro-

gen fixation do not involve the development of nodules [48]. In addition, organogenesis can be uncoupled from intracellular infection in nodulating legume species [47,49–52]. Mutants of *Sinorhizobium meliloti* exist that fail to induce root-hair reactions or intracellular infection in their host, *M. sativa*, but are able to induce nodule organogenesis [49]. By contrast, particular lines of *M. sativa*, *M. truncatula* and *L. japonicus* develop spontaneous nodules in the complete absence of rhizobia [47,49–52]. These data indicate that intracellular infection and nodule organogenesis are genetically separable processes in RNS. Consequently, genes involved in spatially coordinating and synchronizing nodule organogenesis with bacterial infection are essential for an efficient RNS.

Mutants in three independent loci were identified in *L. japonicus* that exhibit root nodule organogenesis in the absence of rhizobia. Two of the respective genes have been isolated [37,52]. Versions of CCaMK that are mutated in or lack the autoregulatory domain of the protein can induce the development of bacteria-free nodules with normal tissue organization in legumes forming either determinate [37] or indeterminate nodules [47]. CCaMK is presumed to be involved in deciphering nucleus-associated 'calcium spiking' [32,53], periodic fluctuations of calcium concentration that occur in response to rhizobia or isolated Nod factors [54]. Similar calcium-fluctuations of a distinct pattern occur in response to AM fungi [55], suggesting that calcium spiking might be involved in inducing different downstream responses in bacterial and fungal symbioses [55]. However, the observation that monocot orthologs can restore nodulation in *ccamk* loss-of-function mutants [28,46,47] rules out the possibility that legume-specific adaptations of *CCaMK* are essential for nodule organogenesis in this lineage.

In the *L. japonicus* intracellular infection program, *CCaMK* functions upstream of the common symbiosis gene *CYCLOPS* [38], which is required for intracellular infection but not for induction of nodule organogenesis. However, nodule organogenesis aborts at a primordial stage in *cyclops* mutants [38]. This developmental arrest can be circumvented by autoactive CCaMK, indicating that the initiation of organogenesis is partially parallel to the common symbiosis program for intracellular infection [38]. Importantly, these observations also suggest that in the wild-type situation, the progression of intracellular bacterial infection is a prerequisite for completion of nodule development [38].

RNS-related responses induced by an autoactive CCaMK version require several putative transcriptional regulators [37,47,56], which, in contrast to CCaMK itself, are not required for AM [56–59]. These include the GRAS-type transcription factors NODULATION SIGNALING PATHWAY 1 (NSP1) [60,61] and NSP2 [61,62], the ETHYLENE RESPONSE FACTOR (ERF)-type AP2 protein ERF REQUIRED FOR NODULATION 1 (ERN1) [56] and NODULE INCEPTION (NIN) [59,63]. These all belong to families that contain close homologs in other angiosperms, including the nonsymbiotic *A. thaliana* and the monocot rice [56,60,61,64]. It is possible that specific adaptations of the respective legume genes are required for their function in RNS because *NbNSP1* from *Nicotiana benthamiana* (tobacco) only partially complemented RNS in *M. truncatula nsp1* mutants [61].

Interestingly, these genes are required for both nodule organogenesis and infection-thread initiation (NSP1 [57,60]; NSP2 [58,62]; NIN [59]) or infection-thread development (ERN1 [56]). Their precise role during symbiosis establishment is unclear but potentially involves the regulation of distinct response patterns in root epidermal and cortical layers [65].

Several lines of evidence, most notably the discovery that a cytokinin receptor (*L. JAPONICUS* HISTIDINE KINASE 1 [LHK1] [52,66], *M. truncatula* CYTOKININ RESPONSE 1 [CRE1] [67]) has an important role in initiation of nodulation, indicate that cytokinin is a key trigger for nodule organogenesis, as discussed in two recent reviews [68,69].

In a possible scenario, Nod-factor signaling via the common symbiosis program initiates cytokinin production, which in turn induces transcriptional regulation of genes controlling progression of nodule organogenesis [68] in a cell-type-specific manner [69].

Cytokinin is a trigger for nodule organogenesis

Consistent with an essential role of cytokinin in initiating nodule organogenesis, an autoactive version of the cytokinin receptor LHK1 induces spontaneous nodule organogenesis in the absence of rhizobia [52]. By contrast, an *lhk1* loss-of-function allele (*hit1-1*) supports intracellular bacterial infection but strongly impairs the formation of nodules [66]. Experimental downregulation of the *M. truncatula* predicted ortholog of this gene, *CRE1*, led to loss of nodule organogenesis [67], indicating a role of cytokinin in the formation of both determinate and indeterminate nodules.

Both rhizobial and *Frankia* strains have been reported to secrete cytokinin [70,71], and Nod-factor-deficient, non-nodulating strains of *Sinorhizobium meliloti* regain the potential to induce nodule-like organs when manipulated to secrete *trans*-zeatin, a naturally occurring cytokinin [72]. These observations suggest a possible role of cytokinin secreted by bacterial symbionts during symbiosis establishment in the induction of nodule organogenesis in RNS.

Photosynthetic *Bradyrhizobium* strains were identified that lack the *nodABC* operon required for Nod-factor synthesis and were proposed to induce nodulation in a Nod-factor-independent manner by using purine derivatives, which could be related or identical to phytoactive cytokinins [21]. Together, these data suggest that bacterial symbionts might contribute to nodule organogenesis induction, or even circumvent the Nod-factor signaling pathway, by releasing cytokinin and activating the LHK1-mediated organogenesis program.

Presumed *LHK1* or *CRE1* orthologs are conserved in angiosperms, including *Arabidopsis* and rice [73,74]. Testing the potential of deregulated versions to autoinduce similar organogenetic responses in non-nodulating plants could reveal whether nodulating groups evolved a partially specific, LHK1-mediated cytokinin response [65] or whether they fully rely on pre-existing programs for organogenesis.

The direct involvement of cytokinin signaling in the regulation of nodule organogenesis relates RNS to fundamental developmental processes, such as lateral-root generation [67,73,75], regulation of root length [52,66,67,73] and vascular morphogenesis [52,76] (Figure 3). The evolution of nodule organogenesis might thus have involved the adaptation of pre-existing root developmental programs such that they respond to hormonal re-balancing of the root cortex induced by signals associated with endosymbiotic bacterial infection [68,77,78]. Consistent with this is the occurrence of nodules and nodule-like structures in diverse land plant lineages, indicating that the genetic tools for their formation are not limited to RNS-competent rosids. For example, nodules that are likely to have evolved as modified lateral roots similar to actinorhizal nodules occur naturally in some gymnosperms such as *Podocarpus* spp., where they are colonized by AM fungi and potentially provide a setting for bacterial nitrogen fixation [79]. Artificial induction of nodule-like structures was reported in rice after partially macerated root tissue was exposed to rhizobial bacteria [80]. Finally, nitrogen-fixing nodules differ greatly in their morphological and developmental characteristics between RNS-forming groups [4,5], giving further indication that a broad range of host genetic backgrounds can potentially support nodule formation.

Concluding remarks

The emergence of RNS seems to be based on the evolutionary concept of recruitment, in that many RNS-related processes rely on conserved genetic programs that also support pre-existing plant developmental processes and that later acquired additional roles in RNS. Its restricted occurrence in a few rosid plant orders, all part of a monophyletic clade, indicates that more than one specific molecular adaptation was necessary to make RNS evolution possible (Figure 3). These respective genetic changes probably served to link, or add to, pre-existing genetic programs, creating a novel trait of high ecological and economic value. The plant genes involved in these adaptations represent vantage points for efforts to transfer RNS to new hosts. Respective candidate proteins are involved (i) in direct host–microbiont crosstalk (for example, LysM receptor kinases such as *L. japonicus* NFRs or *M. truncatula* LYKs and NFP) and (ii) in mediating the molecular integration of plant–bacterial signaling into the host genetic background, thereby 'tapping' pre-existing plant programs for additional use in RNS. A possible example of such a 'recruiter protein' is the RLK SYMRK (DMI2; NORK), which probably contributed to the potential of AM hosts to form endosymbioses with bacteria [1], a capacity that later became associated with nodule organogenesis on independent evolutionary occasions [1,3], perhaps through the cytokinin receptor LHK1 (CRE1).

Efforts towards engineering novel host systems should focus on identifying the crucial specificities of RNS and aim to bring these together within target hosts. To achieve artificial forms of RNS efficient in symbiotic nitrogen fixation, more insights will further be required into the fine-tuning of host–bacterial recognition and compatibility determination (Box 2). In the past, genetic dissection of RNS has predominantly focused on legume model systems where the plant genetic programs involved are likely to be strongly derived. Less specialized forms of RNS, including actinorhizal associations with *Frankia* bacteria, combine features that render them more promising models for potential RNS transfer to new hosts (Box 1). Deciphering genetic determinants for the existing diversity of RNS within and beyond the legume lineage will thus be an important step towards identifying a basic set of RNS genetic requirements and, ultimately, combining them in potential new systems.

Box 2. Superficial details

In addition to Nod factors, other rhizobial molecules, such as exopolysaccharides and secreted effector proteins [49,91,92], can contribute to establishing fully compatible interactions with legumes. This equally applies to symbioses involving narrow- [49] and broad-host-range rhizobial strains like *Rhizobium sp.* strain NGR234 [91]. The possible existence of additional checkpoints for mutual compatibility in rhizobium–legume systems, potentially involving as yet unknown molecular components [17,91,92], might challenge a successful biotechnological transfer of fully compatible RNS. Deciphering the precise roles of bacterial surface molecules and secreted proteins in both legume and actinorhizal RNS represents an intriguing field for future research efforts.

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Functional Adaptation of a Plant Receptor-Kinase Paved the Way for the Evolution of Intracellular Root Symbioses with Bacteria

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Nitrogen-fixing root nodule symbioses (RNS) occur in two major forms—Actinorhiza and legume-rhizobium symbiosis—which differ in bacterial partner, intracellular infection pattern, and morphogenesis. The phylogenetic restriction of nodulation to eurosid angiosperms indicates a common and recent evolutionary invention, but the molecular steps involved are still obscure. In legumes, at least seven genes—including the symbiosis receptor-kinase gene *SYMRK*—are essential for the interaction with rhizobia bacteria and for the Arbuscular Mycorrhiza (AM) symbiosis with phosphate-acquiring fungi, which is widespread in occurrence and believed to date back to the earliest land plants. We show that *SYMRK* is also required for Actinorhiza symbiosis of the cucurbit *Datisca glomerata* with actinobacteria of the genus *Frankia*, revealing a common genetic basis for both forms of RNS. We found that *SYMRK* exists in at least three different structural versions, of which the shorter forms from rice and tomato are sufficient for AM, but not for functional endosymbiosis with bacteria in the legume *Lotus japonicus*. Our data support the idea that *SYMRK* sequence evolution was involved in the recruitment of a pre-existing signalling network from AM, paving the way for the evolution of intracellular root symbioses with nitrogen-fixing bacteria.

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Introduction

Nitrogen limits plant growth in many terrestrial ecosystems. Evolutionary adaptations to this constraint include symbiotic associations with bacteria that are capable of converting atmospheric nitrogen into ammonium. Extracellular associations of plants with diverse groups of nitrogen-fixing bacteria are phylogenetically widespread, but only a small group evolved the ability to accommodate bacteria endosymbiotically inside cell wall boundaries. Bacterial symbionts are confined within tubular structures called infection threads, which are surrounded by a host-derived membrane that is continuous with the plasma membrane, and bound by plant cell wall–like material [1,2]. The bulk of host plants including all actinorhizal species retain the bacterial symbionts within these structures during the nitrogen-fixing stage of the symbiosis [1,3]. In the most advanced forms found exclusively among legumes (Fabales) and *Gunnera* [4], symbiotic bacteria are delimited from the host cell cytoplasm only by a plant-derived membrane in the mature stage of the symbioses. In the respective legumes, they develop into bacteroids contained in organelle-like symbiosomes, where nitrogen fixation takes place (for a recent review, see [5]). Bacterial endosymbioses in both legumes and actinorhizal plants are typically associated with the formation of novel plant organs, so-called nodules, which are root-derived in the majority of cases [6]. Nitrogen-fixing root nodule symbiosis (RNS) occurs in two major forms. Actinorhiza hosts belong to three eurosid orders (Figure 1) and nodulate with Gram-positive actinobacteria of the genus *Frankia* [7]. Legumes, on the contrary, enter specific interactions with members of a diverse group of Gram-negative bacteria, termed rhizobia. For almost a century, the extreme diversity in organ structure, infection mechanisms, and bacterial symbionts among nodulating plants obscured the fact that the nodulating clade is monophyletic, which was revealed by molecular phylogeny relatively recently [8]. The restriction of endosymbiotic root nodulation to a monophyletic group of four angiosperm orders (Figure 1) is coincident with a patchy occurrence within this clade. These observations led to the hypothesis that a genetic change acquired by a common ancestor may predispose members of this lineage to evolve nodulation endosymbiosis [8].

The molecular adaptations underlying the evolution of plant-bacterial endosymbioses are still a mystery, despite a substantial biotechnological interest in understanding the genetic differences between nodulating and non-nodulating plants. While the molecular communication between legumes and rhizobia has been studied in some detail, important clues are expected from the genetic analysis of the yet underexplored Actinorhiza.

Bacterial signalling molecules and corresponding plant

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Abbreviations: AM, Arbuscular Mycorrhiza; LRR, leucine-rich repeat; NEC, N-terminal extracellular region of predicted SYMRK proteins; PIT, pre-infection thread; PPA, pre-penetration apparatus; RNS, root nodule symbiosis; RACE, rapid amplification of cDNA ends; RNAi, RNA interference;

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Author Summary

As an adaptation to nutrient limitations in terrestrial ecosystems, most plants form Arbuscular Mycorrhiza (AM), which is a symbiotic relationship between phosphate-delivering fungi and plant roots that dates back to the earliest land plants. More recently, a small group including the legumes and close relatives has evolved the ability to accommodate nitrogen-fixing bacteria intracellularly. The resulting symbiosis is manifested by the formation of specialized root organs, the nodules, and comes in two forms: the interaction of legumes with rhizobia, and the more widespread Actinorhiza symbiosis of mostly woody plants with *Frankia* bacteria. The symbiosis receptor kinase SYMRK acts in a signalling pathway that legume plants require to trigger the development of nodules and the uptake of fungi or bacteria into their root cells. Here we show that the induction of Actinorhiza nodulation also relies on SYMRK, consistent with the idea that both types of nodulation evolved by recruiting common signalling genes from the pre-existing AM program. We observed that *SYMRK* from different land plant lineages differs significantly in exon composition, with a "full-length" version in the nodulating clade and shorter *SYMRK* genes in plants outside this lineage. Only the most complete *SYMRK* version was fully functional in nodulation, suggesting this gene played a central role in the recruitment event associated with the evolution of intracellular root symbioses with bacteria.

receptors involved in RNS are known only for the legume–rhizobium interaction. *Frankia* signals may be biochemically distinct from rhizobial chito-oligosaccharide nodulation factors [9], which would suggest an independent mechanism of host–symbiont recognition in Actinorhiza.

Phenotypic analysis of legume mutants has revealed a genetic link between RNS and Arbuscular Mycorrhiza (AM), which is a phosphate-scavenging association between plant roots and fungi belonging to the phylum Glomeromycota [10]. AM is widespread among land plants, where forms of AM are found in representatives of all major lineages. Fossil evidence for ancient AM-like associations [11] suggests a role of this symbiosis in the colonization of land about 450 million years ago.

The link of plant–fungal and plant–bacterial endosymbioses in legumes, which involves at least seven genes [12–16] termed "common symbiosis genes" [17], inspired the idea that during the evolution of bacterial endosymbiosis, genes were recruited from the pre-existing AM genetic program [18]. However, the molecular steps involved are not clear.

Results

To gain insight into the evolution of nitrogen-fixing root nodulation, we analysed common symbiosis genes across angiosperm lineages with different symbiotic abilities. Many, including the calcium/calmodulin kinase gene *CCaMK* [14,19], or genes encoding the predicted cation channels CASTOR and POLLUX [12,20,21], are conserved in overall domain structure. We discovered exceptional diversification among genes encoding the symbiosis receptor kinase SYMRK in different species (Figure 1). While putative SYMRK kinase domains are conserved and contain characteristic sequence motifs discriminating them from related kinases (Figure S1), the predicted extracellular portion of SYMRK occurs in at least three versions of domain composition (Figure 1 and Table 1). The longest *SYMRK* version is present in all tested eurosids, including nodulating and non-nodulating lineages. Comprising 15 exons, it encodes three leucine-rich repeat (LRR) motifs and an extended N-terminal domain of unknown function (NEC-domain, Figure 1 and Table 1). Outside of the eurosid clade, which encompasses all nodulating groups, one or more exons are absent from *SYMRK* coding sequences (Figure 1 and Table 1).

Genetic evidence indicates that SYMRK acts near a point of molecular convergence of AM and legume-rhizobium signalling [16,22]. The presumed ability of its diverged extracellular domain to perceive symbiosis-related signals [16] renders it a prime target for investigating the molecular adaptations underlying the evolution of RNS.

The homogenous occurrence of "full-length" *SYMRK* genes among legumes, actinorhizal plants, and non-nodulating eurosids raises the intriguing possibility that *SYMRK* is involved in the proposed genetic predisposition [8] of this clade to evolve nodulation. An important prediction following from this hypothesis is the common requirement of a full-length *SYMRK* version for all types of RNS. Furthermore, also non-nodulating members of this monophyletic clade may carry nodulation-competent versions of *SYMRK*. To test this concept, we analysed the functional capabilities of "full-length" *SYMRK* genes from symbiotically diverse eurosids.

SYMRK Is Required for Actinorhiza and AM in *Datisca glomerata*

To investigate *SYMRK* function in Actinorhiza, we reduced root mRNA levels of *D. glomerata* (*Datisca*) *SYMRK* (*DgSYMRK*) via RNA-interference (RNAi). Quantitative PCR following reverse transcription showed a 36%–99% reduction of *DgSYMRK* transcript levels in knockdown roots ($n = 16$) compared with vector control roots ($n = 16$). Eight weeks after inoculation with *Frankia* bacteria, no nodules were detected on *DgSYMRK* RNAi roots (Figure 2A and 2B), except for small, primordial swellings on 16% of independent transformed roots (9/55). Nonsilenced control roots of the same plants and roots transformed with a binary vector lacking the silencing cassette (transgenic control roots) showed wild type–like nodules with lobed structure typical for *Datisca* (Figure 2A and 2B). This result demonstrates that *SYMRK* is essential for Actinorhiza development in *Datisca*. In conjunction with the well-documented role of legume *SYMRK* in the interaction with rhizobia [16,23], *SYMRK* thus represents a common genetic requirement for the two types of bacterial root endosymbiosis.

To test whether *DgSYMRK* is also required for AM, we inspected *DgSYMRK* RNAi roots for AM formation with the fungus *Glomus intraradices* (*Glomus*). *Datisca* wild-type roots of the same plants used for hairy root induction and independent transgenic control roots formed AM, with dense arbuscular colonization of inner cortical cells (Figure 2C–2F). In contrast, symbiotic development in *DgSYMRK* RNAi roots was strongly impaired. In 82% of independent transformed roots, no fungal infection was observed, despite the presence of extensive extraradical mycelium (Figure 2G), with those roots exhibiting strong reduction levels of *DgSYMRK* being nonsymbiotic concerning both nodulation and AM formation. Occasional infection attempts occurred but typically were aborted in the outer cell layers (Figure 2G and 2H). We conclude that similar to the situation in legumes, *SYMRK* of

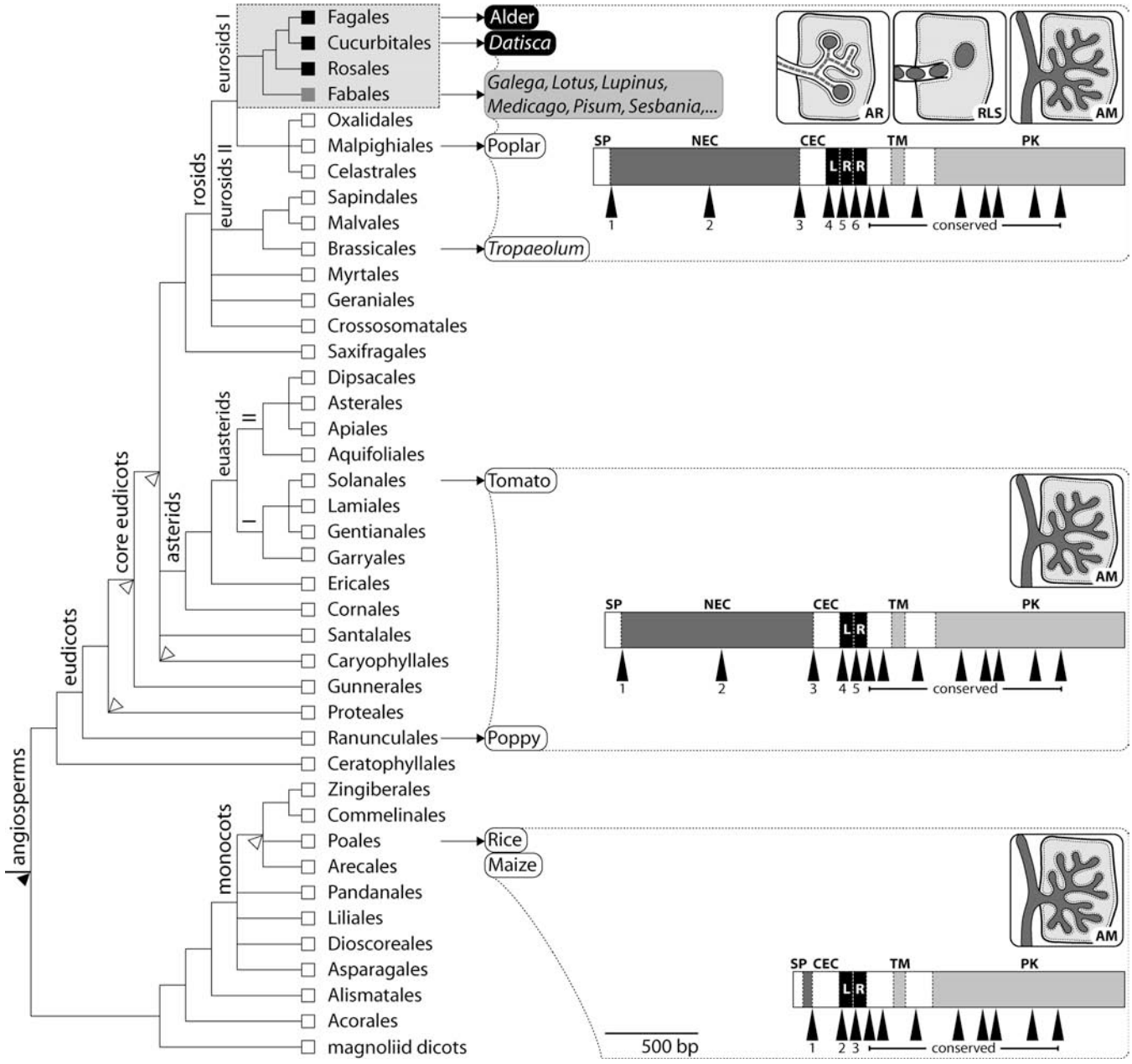

Figure 1. *SYMRK* Exon-Intron Structure and Root Endosymbiotic Abilities of Angiosperm Lineages

All putative *SYMRK* genes encode an N-terminal signal peptide, an extracellular region with two or three LRR motifs and one imperfect LRR, a transmembrane domain, and an intracellular serine/threonine protein kinase. *SYMRK* regions encoding putative kinase domains exhibit conserved intron positions and phases. Bars illustrate the exon-intron and predicted protein domain structure of representative *SYMRK* candidates. Positions of introns are indicated by black arrowheads. Predicted protein domains are SP, signal peptide; NEC, N-terminal extracellular region; CEC, conserved extracellular region preceeding LRRs; LR(R), leucine-rich repeats; TM, transmembrane domain; and PK, protein kinase domain. Names refer to species sampled and are shaded according to their root endosymbiotic capabilities: black, endosymbiosis with *Frankia* bacteria (Actinorhiza) and AM formation; gray, endosymbiosis with rhizobia and AM formation; white, AM formation only. Pictograms symbolize AR, Actinorhiza; RLS, Rhizobium-Legume Symbiosis; AM, Arbuscular Mycorrhiza. Dashed frames have no phylogenetic implications. The cladogram depicts relationships of angiosperm orders as deduced by molecular markers [53,54]. The four orders containing nodulating taxa are shaded light gray. Squares at the tips of branches indicate the presence of taxa with particular root endosymbiotic phenotypes (colour code is as for sampled plants). Filled and white wedges indicate branches where taxa on order and family level have been omitted, respectively. Popular species designations refer to Alder, *Alnus glutinosa*; Poplar, *Po. trichocarpa*; Tomato, *Ly. esculentum*; Poppy, *P. rhoeas*; Rice, *O. sativa*; Maize, *Z. mays*.

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Table 1. *SYMRK* Homologs, Conceptual SYMRK Proteins, and the Closest *Arabidopsis* Sequences

Species	mRNA Accession	AAs	Exons	LRRs	Percent AA Identity/Similarity		
					Overall	EC	IC
L. japonicus	AF492655 [16]	923	15	3	100/100	100/100	100/100
Me. truncatula	AJ491998 [16]	925	15	3	82/87	74/83	92/95
Lu. albus	AY935267[a]	923	15[c]	3	79/86	72/83	89/92
Al. glutinosa	AY935263[a]	941	15[c]	3	61/75	51/69	78/88
D. glomerata	AM271000[a]	934	15	3	61/73	54/67	71/80
Po. trichocarpa	AM851092[b]	933	15[d]	3	59/71	49/66	75/81
T. majus	AY935265[a]	945	15[c]	3	59/72	50/65	74/84
Ly. esculentum	AY935266[a]	903	14	2	54/68	45/65	72/81
P. rhoeas	AM270999[a]	902	14[c]	2	54/69	43/59	75/86
Z. mays	DQ403195 [55]	579	12	2	55/69	30/49	74/86
O. sativa	XM_478749 [56]	576	12	2	55/69	33/51	72/84
A. thaliana	NM_105440 [57]	929	15	3	34/51	29/43	50/68
A. thaliana	NM_129261 [57]	933	15	3	33/50	27/42	50/69

Percentages of AA identity/similarity are relative to the *L. japonicus SYMRK* sequence and were obtained via BLASTX analysis using the NCBI Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/).

AAs, amino acids; LRRs, predicted leucine-rich repeat domains; EC, predicted extracellular region and IC, predicted intracellular region of the conceptual proteins.

[a] This publication.

[b] The genomic sequence is available at http://genome.jgi-psf.org/Poptr1/Poptr1.home.html, the gene location is Poptr1/LG_VII:12333164–12338867.

[c] Only cDNA sequence information was obtained. The exon-intron structure was predicted based on splice site prediction and conserved intron position on other SYMRK genes.

[d] Only genomic sequence information was obtained. The exon-intron structure was predicted based on splice site prediction and conserved intron position on other SYMRK genes.

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the actinorhizal plant *Datisca* is involved in both bacterial and fungal endosymbioses.

SYMRK Does Not Mediate Specificity in Legume–Rhizobium Recognition

To determine whether *SYMRK* plays a role in the specific recognition of rhizobia by legume hosts, we tested whether *L. japonicus* (*Lotus*) *SYMRK* (*LjSYMRK*) can mediate nodulation in another legume, which interacts with a different rhizobial partner. The specific symbiont of *Lotus* is *Mesorhizobium loti*, whereas *Medicago truncatula* (*Medicago*) interacts with *Sinorhizobium meliloti*. *Medicago dmi2* 5P mutants exhibit a deletion in exon three of the *SYMRK* ortholog *DMI2*, leading to a frameshift and premature stop codon. *Dmi2* 5P plants form no infection threads or nodules upon inoculation with either rhizobial strain. Transgenic roots of these plants, and of wild-type control plants carrying *LjSYMRK*, formed infection threads and indeterminate, pink nodules typical for *Medicago* [24] with *S. meliloti* (Figure S2 and Table 2). *LjSYMRK* can therefore fully restore nodulation of *Medicago* with *S. meliloti*, indicating that *SYMRK* is not directly involved in determining legume–rhizobium specificity.

Medicago dmi2 5P mutants are also impaired in AM. No arbuscules were observed within 2 wk of co-cultivation, with fungal infection being aborted at the root surface or after entry into epidermal cells (Figure S2 and Table 2). *LjSYMRK* restored the AM defect in transgenic roots of this line (Figure S2 and Table 2), demonstrating that *SYMRK* is sufficiently similar to *DMI2* to support both fungal and bacterial endosymbioses in *Medicago*.

"Full-Length" *SYMRK* Versions from Symbiotically Distinct Eurosids Can Support Both AM and RNS in *Lotus*

To analyze the symbiotic capabilities of "full-length" eurosid *SYMRK* genes from a legume (*MtDMI2*), an actinorhizal plant (*DgSYMRK*), and the non-nodulating, AM-forming *Tropaeolum majus* (*Tropaeolum*; Brassicales) (*TmSYMRK*), we tested their potential to function in the *Lotus* symbiosis signalling context. We introduced these genes, under the control of the *Lotus SYMRK* promoter region, into roots of *Lotus* line SL1951–6 (*symrk-10*), which carries a *symrk* mutant allele encoding a kinase-dead SYMRK version [25,26]. Upon inoculation with *Glomus*, *symrk-10* roots form no AM, and fungal infections are typically associated with aberrant hyphal swellings and are aborted after entry into epidermal cells (Figure 3A and 3B, and Table 2). Interaction with *M. loti* is blocked at an early stage, and no infection threads or nodules form (Figure 3C and 3D, and Table 2). In *symrk-10* roots transformed with *MtDMI2*, *DgSYMRK* or *TmSYMRK* both AM and nodulation were restored, the latter involving the formation of infection threads and pink, bacteria-containing nodules (Table 2 and Figure 3) that were indistinguishable from wild-type nodules. In conclusion, consistent with a role of *SYMRK* in the predisposition to evolve RNS, we could not detect a functional diversification of the eurosid *SYMRK* version linked to features differentiating actinorhizal or legume nodulation, or to the specific recognition of bacterial symbionts. Thus, other factors, such as nod factor receptor kinases [22,27,28] or yet-unknown additional components, are likely accountable for the fine-tuning of recognition specificity in plant–bacterial endosymbioses within the eurosids.

SYMRK Versions of Reduced Length Restore AM but Not RNS in *Lotus*

SYMRK from the non-nodulating eudicots *Papaver rhoeas* (poppy) and *Lycopersicon esculentum* (tomato) represent intermediate length and domain composition (Figure 1 and Table 1). To explore the symbiotic capabilities of this version, we introduced the two-LRR encoding *LeSYMRK* genomic sequence fused to the *LjSYMRK* promoter into *Lotus symrk-10* transgenic roots. *LeSYMRK* selectively restored AM symbiosis, whereas neither infection threads nor nodules developed upon inoculation with *M. loti* (Table 2 and Figure S3E–S3L).

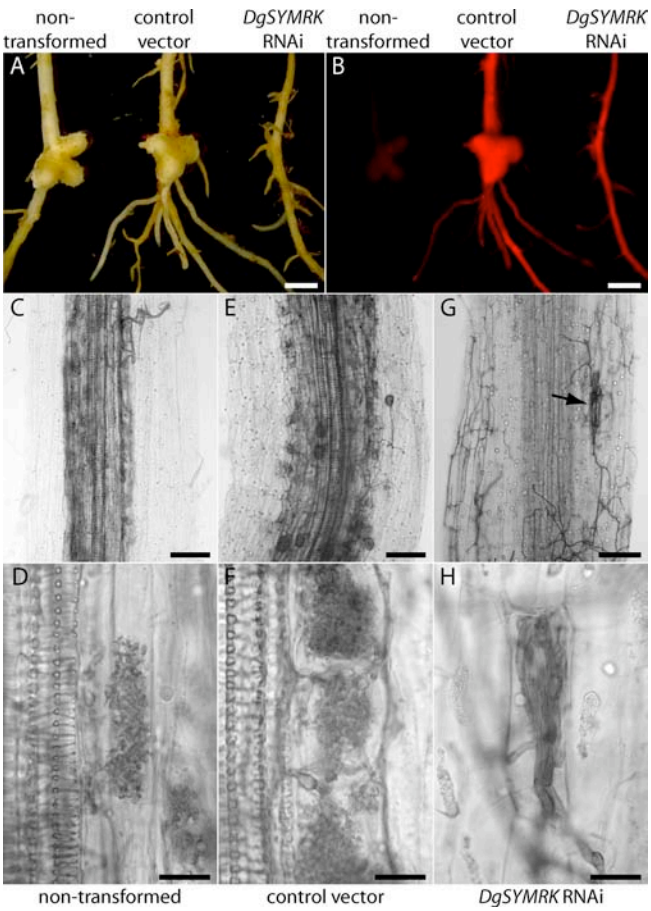

Figure 2. Nodulation and AM Formation Are Impaired in *DgSYMRK* Knockdown Roots

Co-transformed roots express *DsRED1* as visible marker.

(A and B) Nodulated wild-type root (left), control root transformed with pRedRoot lacking the silencing cassette (middle), and non-nodulated DgRNAi knockdown root (right) (A) under white light and (B) with transgenic roots showing DsRED1 fluorescence.

(C–H) AM phenotype of *D. glomerata* (*Datisca*) wild type, transgenic control, and *DgSYMRK* knockdown roots. (C and D) Wild-type and (E and F) transgenic control roots are well colonized and show arbuscules in inner cortical cells. (G and H) Typical *DgSYMRK* knockdown root with no AM formation but extraradical mycelium and aborted fungal infections (H and arrow in G). Such features were not seen in *Datisca* wild-type or transgenic control roots and are reminiscent of those observed on *L. japonicus symrk* mutant roots (Figure 3). Roots were inoculated simultaneously with *Frankia* bacteria and *G. intraradices* (8 wk). Transgenic and regenerated nontransgenic roots of 27 (control) and 23 (*DgSYMRK* RNAi construct) plants from three independent experiments were tested. Independent transformed roots examined were $n = 42$ (control) and $n = 55$ (*DgSYMRK* RNAi).

Scale bars: (A and B) 2 mm; (C, E, and G) 0.1 mm; (D, F, and H) 0.02 mm.

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A reduced *SYMRK* version is present in the AM-forming, non-nodulating monocots *Oryza sativa* (rice) and *Zea maize* (maize), encoding two LRRs only and a short N-terminal region, mainly covered by a single exon aligning with exon four of *LjSYMRK* (Figure 1 and Figure S1). To determine whether the short *SYMRK* version of rice, *OsSYMRK*, is sufficient for endosymbiosis formation in *Lotus*, we introduced the *OsSYMRK* genomic sequence controlled by the *LjSYMRK* promoter into *symrk-10* roots. AM formation was fully restored in these roots, whereas nodulation with *M. loti* was impaired (Table 2 and Figure S3M–S3Z). *OsSYMRK*-containing *symrk-10* roots inoculated with *M. loti* exhibited primordial swellings generally devoid of bacteria (Figure S3U–S3X). In rare cases, infection threads and small round nodules were observed, which contained bacterial colonies (Figure S3Y and S3Z). Hence, similar to *LeSYMRK*, *OsSYMRK* is compromised in supporting bacterial endosymbiosis in *Lotus*.

Discussion

A Molecular Link between the Two Types of RNS

In legumes, *SYMRK* is indispensable for root endosymbiosis with rhizobia and AM fungi [16,23]. We show here that this endosymbiosis gene is also required for nodulation in the actinorhizal plant *Datisca*. *SYMRK*, which is likewise essential for Actinorhiza formation of the tree species *Casuarina glauca* (Fagales) [29], represents the first known plant gene required for Actinorhiza, indicating a shared genetic basis of the two different types of RNS. A future task will be to determine whether further endosymbiosis genes acting in concert with *SYMRK* in legumes are also required for Actinorhiza.

An Ancient Genetic Program for AM among Angiosperms

The ability of different *SYMRK* versions from both dicot and monocot lineages to restore AM in *Lotus* indicates a homologous nature of the AM genetic program in angiosperms. This is consistent with the observation that loss-of-function mutations in the rice version of the legume symbiosis gene *CCaMK* results in loss of AM symbiosis [30]. In *Arabidopsis*, the absence of root symbiotic capability is accompanied by a deletion of several symbiosis genes, including *SYMRK* and *CCaMK* [21,31,32].

A Role of *SYMRK* in the Predisposition to Evolve RNS

Our survey of *SYMRK* sequences across angiosperms revealed at least three structurally distinct versions, and we could show that this polymorphism is functionally related to the root symbiotic capabilities of host plants. The variation in SYMRK domain composition is exceptional among the known common symbiosis genes. The congruence between the phylogenetic distribution of the "full-length" *SYMRK* version with the nodulating clade strongly suggests a link between *SYMRK* sequence evolution and the acquisition of endosymbiotic root nodulation with bacteria. An attractive hypothesis is that *SYMRK* sequence divergence was a critical step in mediating the recruitment of the otherwise conserved common symbiosis pathway from the pre-existing AM genetic program. Recruitment was proposed to account for the genetic link of AM and nodulation in legumes [17,18] and would make root–bacterial endosymbiosis as a whole a fascinating example for novel traits evolving on the basis of pre-existing genetic patterns.

A common feature associated with endosymbiotic bacterial infection in both actinorhizal [33] and legume hosts [34] is the formation of intracellular pre-infection threads (PITs) in host cells. These cytoplasmic structures resemble the pre-penetration apparatus (PPA) preceding fungal infection during AM formation [35]. Forming in anticipation of bacterial symbionts, PITs are thought to coordinate the uptake of bacteria and determine the spatial progression of infection through the host cell [33,34]. A similar role in guiding fungal transition through host cells in AM has been demonstrated for PPAs [35]. These developmental similarities in AM,

Table 2. Restoration of Root Symbioses in *Medicago dmi2* and *Lotus symrk* Mutants Transformed with Different *SYMRK* Versions

Plant Genotype	Transgene	AM+	IT	Nod+	Nodules/Nodulated Plant
dmi2 5P	marker only[a]	0/21	–	0/17	0
dmi2 5P	*LjSYMRK*	20/23	+	16/25	4.5
symrk-10	marker only[a]	3/251	–	0/62	0
symrk-10	*LjSYMRK*	40/45	+	32/39	4.9
symrk-10	*MtSYMRK*	20/25	+	18/31	4.2
symrk-10	*DgSYMRK*	20/40	+	6/18	5.8
symrk-10	*TmSYMRK*	20/28	+	14/21	2.6
symrk-10	*LeSYMRK*	24/34	–	0/31	0
symrk-10	*OsSYMRK*	38/40	+[b]	6[c]/43	1.5

Numbers refer to *Ag. rhizogenes*-transformed root systems of *Me. trunculata* (*Medicago*) *dmi2* 5P or *L. japonicus* (*Lotus*) *symrk-10* mutants.
Constructs were under control of the *LjSYMRK* promoter region. Numbers are compiled results of one (*Medicago*) or two to three (*Lotus*) independent experiments. Wild-type roots transformed with the same constructs formed wild type–like AM, ITs, and nodules. IT, infection thread formation; Nod+, number of root systems showing nodules containing bacteria.
[a] Plants were transformed with the respective binary vector lacking a *SYMRK* expression cassette.
[b] Infection threads were seen very rarely in these roots.
[c] Nodule-like structures were small and showed, with one exception, no pinkish coloration under white light.
doi:10.1371/journal.pbio.0060068.t002

Actinorhiza, and legume-rhizobium infection may reflect a common genetic program for endosymbiosis establishment and symbiont uptake in all three types of interactions. In AM, PPAs are not formed in mutants that are defective in certain common symbiosis genes [35]. It is therefore possible that a recruitment of AM symbiosis genes during the evolution of RNS facilitated the induction of intracellular accommodation structures in response to bacteria.

SYMRK Domain Function and Evolution

Repetitive LRR modules have been implicated in the determination and evolution of novel recognition specificities of receptor proteins [36–38]. Interestingly, adaptive changes reflecting positive selective constraints can be traced in LRR– and NEC–encoding regions of *SYMRK* genes from different *Medicago* species, but these do not correlate with shifts in rhizobial specificity [39]. Our functional comparison of eurosid *SYMRK* versions indicates that *SYMRK* is not involved in determining recognition specificity in nodulation. However, an extended *SYMRK* version containing a set of three LRR motifs, as present in eurosid *SYMRK* genes, is required for fully supporting nodulation symbiosis of *Lotus* with *M. loti*. Shorter *SYMRK* versions from tomato or rice only suffice for AM. These functional differences may be caused by individual amino acid sequence polymorphisms, or alternatively, exons that are specifically required for bacterial endosymbiosis may be lacking in rice and tomato *SYMRK* versions.

At an overall structural level, exon acquisition from other genes encoding LRR or NEC-like domains [23,40] or, alternatively, retainment of exons in eurosid *SYMRK* genes, may have been an integral genetic factor in the evolution of bacterial endosymbiosis in angiosperms. The observation of small nodule-like structures on *Lotus symrk* mutant roots transformed with the *OsSYMRK* construct is counterintuitive, considering that the *LeSYMRK* version, which resembles the legume version more closely, does not support such developmental responses. One possible explanation may be that the nonmatching NEC region of *Le*SYMRK negatively interferes with nodulation, but not AM signalling in *Lotus*.

The NEC domain encoded by *Lotus SYMRK* exons two and three, upstream of the conserved LRR flanking region (CEC), is present across eudicot plants (Figure 1). Its function outside the nodulating group is unknown. The proposed involvement of *SYMRK* in processes such as reduction of the touch sensitivity of root hairs [41] may rely on this domain thereby imposing selective constraints. The NEC domain shows possible overall relatedness but only a low level of similarity to sequences present in the rice genome, and to sequences other than *SYMRK* candidates in genomes of dicots like *Arabidopsis* [23]. The apparent divergence observed among these potentially homologous sequences of yet unknown function is consistent with a hypothetical role as a receptor domain.

It will be a future challenge to determine the contribution of individual SYMRK LRR units as well as of the NEC domain and to resolve at the amino acid level the features of SYMRK proteins involved in conferring endosymbiotic nodulation capacity.

Additional Components Required for Nodulation

The diversity and scattered occurrence of nodulation symbioses within the eurosid lineage suggest multiple independent origins [42]. Only a subset of the plant species carrying the "full-length" version of *SYMRK* develop root nodules, yet *SYMRK* of the non-nodulating *Tropaeolum* proved competent to support nodulation in *Lotus*. Hence, there must be additional genetic features distinguishing the nodulators. Candidate genes include those that express the legume LysM receptor kinases NFR1 and NFR5 [22,27,43], which are required for responsiveness to rhizobial lipo-chito-oligosaccharide nodulation factors, but not for AM formation. A potential relevance of LysM receptors in Actinorhiza, or the identity of alternative receptors perceiving yet unknown *Frankia* signals, remains to be determined.

Materials and Methods

Isolation of *SYMRK* homologues. We used a PCR strategy employing degenerate primers to obtain *SYMRK* sequence information from diverse angiosperms, for which no genome or root-derived expressed sequence tag sequences were available. Degenerate primers for the isolation of *SYMRK* genes were positioned in regions of the coding sequence conserved among *SYMRK* candidates, but not in other similar *O. sativa* (rice) and *A. thaliana* (*Arabidopsis*) sequences. For primer sequences, see Table S1.

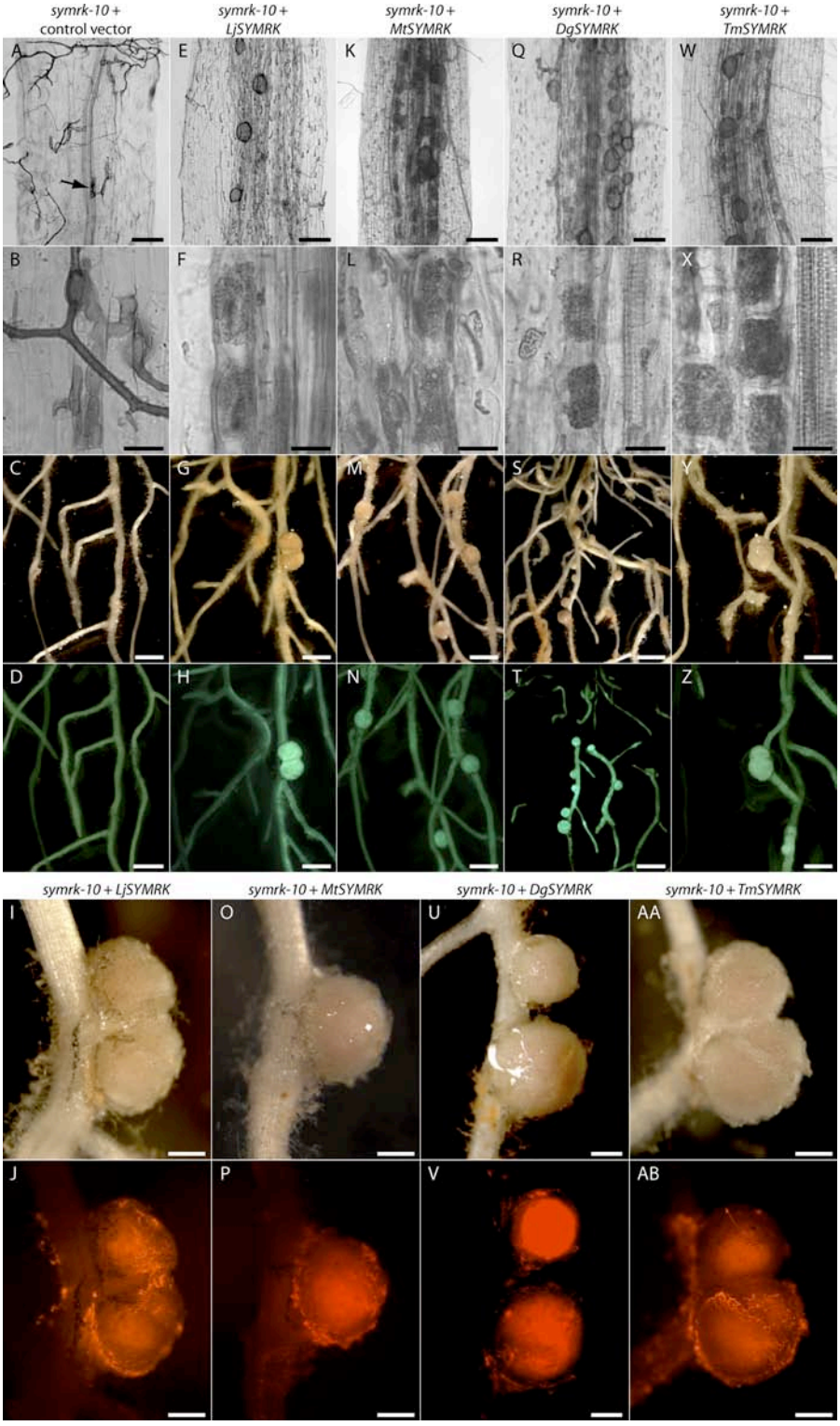

Figure 3. *Datisca*, *Medicago*, and *Tropaeolum SYMRK* Restore Fungal and Bacterial Endosymbioses in *Lotus symrk-10* Mutants

Transgenic roots were identified via fluorescence of eGFP encoded on the transfer-DNA.
(A–D) *L. japonicus* (*Lotus*) *symrk-10* roots transformed with the respective control vector lacking a *SYMRK* expression cassette.
(E–AB) *Lotus symrk-10* roots transformed with *Lotus* (E–J), *Me. truncatula (Medicago)* (K–P), *D. glomerata (Datisca)* (Q–V) and *T. majus (Tropaeolum)* (W–AB) *SYMRK* under control of the *LjSYMRK* promoter region.
(A, B, E, F, K, L, Q, R, W, and X) Roots co-cultivated with *G. intraradices* for 3 wk. (A and B) Transgenic control roots devoid of intraradical hyphae or arbuscules, with aborted fungal infection structures within epidermal cells (B and arrow in A). Roots carrying *Lotus* (E and F), *Medicago* (K and L), *Datisca* (Q and R), and *Tropaeolum* (W and X) *SYMRK* with dense fungal colonization of the inner root cortex (E, K, Q, and W) and arbuscule formation in inner cortical cells (F, L, R, and X).
(C, D, G–J, M–P, S–V, and Y–AB) Root systems inoculated with *M. loti* expressing *DsRED* for 4 wk. (C and D) Transgenic control roots showing no nodules. *symrk-10* root systems transformed with *Lotus* (G–J), *Medicago* (M–P), *Datisca* (S–V), and *Tropaeolum* (W–AB) *SYMRK* develop nodules on transgenic roots.

Nodules exhibit pink coloration in white light, indicating the presence of symbiosis-specific leghemoglobins (I, O, U, and AA) and DsRED fluorescence in inner nodule tissue indicating the presence of *M. loti* (J, P, V, and AB).
Scale bars: (A, E, K, Q, and W) 0.1 mm; (B, F, L, R, and X) 0.02 mm; (C, D, G, H, M, N, S, T, Y, and Z) 2 mm; (I, J, O, P, U, V, AA, and AB) 0.5 mm.
doi:10.1371/journal.pbio.0060068.g003

λ Zap cDNA libraries were available for isolation of *Ly. esculentum* (tomato) and *Alnus glutinosa* (alder) *SYMRK*. A cosmid clone carrying the *LeSYMRK* genomic region was isolated from a pooled tomato Cf2/9 library (kind gift of J.D.G. Jones, The Sainsbury Laboratory, United Kingdom) and shotgun sequenced.

For rapid amplification of cDNA ends (RACE) reactions, total RNA was extracted from roots of uninoculated seedlings or young plants and DNaseI treated. RT and 5′/3′RACE reactions were done using the SMART RACE kit (Clontech), following nested degenerate PCR reactions ([10 s 94 °C, 10 s 52 °C, 30 s 72 °C] × 35, 5 min 72 °C) to obtain initial sequence information.

Construct generation for mutant complementation and *Datisca SYMRK* RNAi experiments. For hairy root complementation assays, *SYMRK* cDNAs were amplified from root cDNA preparations (Superscript II, Invitrogen) of the respective species. Binary transformation vectors were pCAMBIA 1302 or pK7WG2D,1 [44] derivatives. *L. japonicus* (*Lotus*), *Me. truncatula* (*Medicago*), *D. glomerata* (*Datisca*), and *T. majus* (*Tropaeolum*) *SYMRK* coding sequences were amplified from complementary DNA using primers LjSYMRK__EC__f with LjSYMRK__PK__r, MtSYMRK__EC__f with MtSYMRK__PK__r, DgSYMRK__EC__f with DgSYMRK__PK__r and TmSYMRK__EC__f with TmSYMRK__PK__r (Table S1), respectively. An *MtSYMRK* genomic segment containing intron one was amplified from total DNA using primers MtSYMRK__EC__f with MtSYMRK__01__r (Table S1) and ligated to the 3′ fragment of the *MtSYMRK* cDNA following BstB1 digestion of both. The genomic sequences of tomato and rice *SYMRK* were amplified from total DNA using primers LeSYMRK__EC__f with LeSYMRK__PK__r and OsSYMRK__EC__f with OsSYMRK__PK__r (Table S1), respectively. A 285-bp fragment amplified with primers polyA__NOS__f and polyA__NOS__r (Table S1) from pJawohl8 RNAi (kind gift of P. Schulze-Lefert, Max Planck Institute for Plant Breeding Research, Cologne, Germany) was used as terminater in all constructs. *SYMRK* genes or coding sequences were under control of 4,970 bp of genomic sequence preceding the *LjSYMRK* translation start site. For pK7WG2D constructs, the cauliflower mosaic virus (CaMV) 35S promoter driving the Gateway-cassette was deleted SalI(10124)-SalI(38).

For *Datisca* RNAi experiments, a pRedRoot- [45] based binary vector encoding DsRED1 for visible selection was equipped with a pKANNIBAL [46] CaMV 35S promoter-driven hairpin construct containing 367-bp of 3′ coding and untranslated sequence of *DgSYMRK* in forward and reverse orientation, amplified with primers DgSYMRK__RNAi__f and DgSYMRK__RNAi__r (Table S1).

Biological material and phenotyping assays. *L. japonicus* wild-type ecotype B-129 Gifu and derived mutant line *symrk-10* [25] were inoculated with *M. loti* R7A as described [16]. Growth conditions were 24 °C constant at 16-h-light/8-h-dark cycles. For infection of *Agrobacterium rhizogenes*–induced transgenic roots in *Lotus*, *M. loti* MAFF 303099 expressing *DsRED* was applied at a final optical density at 600 nm (OD_{600}) of 0.02 in translucent plastic boxes containing 300 ml Seramis (Mars) and 150 ml liquid Fahraeus Plant (FP) medium [47]. *Ag. rhizogenes*-transformed *Me. truncatula* A17 wild type and *dmi2* 5P (kind gift of G. Oldroyd, John Innes Centre, United Kingdom) plants were inoculated with *S. meliloti* 1021 carrying pBHR-mRFP [48] (OD_{600} of 0.02) in planting pots containing Seramis, and fertilized with FP medium supplemented with 50 μM KNO_3 two times per week. For nodulation assays and simultaneous observation of infection thread (IT) formation in *Lotus* and *Medicago*, plants were harvested 4 and 5 wk post inoculation, respectively. Prior to inoculation, roots showing no eGFP fluorescence were removed. For AM phenotyping of *Lotus* and *Medicago*, plants were co-cultivated with *G. intraradices* BEG195 and harvested after 3 or 2 wk of co-cultivation, respectively. Root systems were stained with acidic ink as described [49]. Prior to staining of *Ag. rhizogenes*–induced root systems, roots showing no eGFP fluorescence were removed. Roots were scored AM-positive (AM+) if symbiotic structures (arbuscules and vesicles) were present, as AM-negative (AM–) if no arbuscules were present. Occasional *Lotus symrk* mutant roots showing vesicles not accompanied by arbuscules were scored AM–. Where complemented *Lotus symrk* mutant roots exhibited aborted infection sites in co-occurrence with successful infection and colonization events involving arbuscule and vesicle formation, roots were scored AM+. *Datisca* seeds and *Frankia* inoculum were a kind gift from K. Pawlowski (Department of Botany, Stockholm University, Sweden). *Datisca* was inoculated with compatible *Frankia* by potting in substrate with ~1 g/l crushed nodules and with *G. intraradices* BEG195 by adding substrate extracted from pots of inoculated *Allium schoenoprasum* plants. Growth conditions were 16 h light/8 h dark at 22 °C and 60% relative humidity. Seeds of *T. majus* and *P. rhoeas* were purchased at Notcutts Garden Centres (UK). The ability to develop AM with G. intraradices was confirmed for all species involved in the study.

***Lotus* transformation.** Transgenic roots on *Lotus symrk-10* mutants were induced using *Ag. rhizogenes* strains AR1193 [50] and LBA1334 [51] as described by Díaz et al. [52] (modified).

***Medicago* transformation.** *Medicago* seedlings were transformed as described at http://www.isv.cnrs-gif.fr/embo01/manuels/index.html (modified), using strain *Ag. rhizogenes* AR1193 [50].

***Datisca* transformation.** Twelve-wk-old *Datisca* plants were inoculated with *Ag. rhizogenes* strain LBA1334 [51] carrying the silencing construct by stem injection, and roots emerging at infection sites were covered with substrate. Three-wk post inoculation roots were inspected for DsRED1 fluorescence. Nonfluorescent roots were removed, and plants were repotted and grown for 8 wk. After determination of the nodulation phenotype, individual fluorescent roots were divided into two halves. One half was stained for mycorrhiza visualization, the second used for total RNA extraction (RNeasy Plant Kit, Qiagen). Quantitative RT-PCR was performed with GeneAmp5700 (Applied Biosystems) using the SuperScript III Platinum Two-Step qRT-PCR-Kit (Invitrogen). A 123-bp *DgSYMRK* fragment was amplified using primers DgqPCR__SYMRK__f with DgqPCR__SYMRK__r (Table S1). As control, polyubiquitin cDNA was amplified using primers DgqPCR__Ubi__f with DgqPCR__Ubi__r (Table S1). Representative fragments were sequenced for identity confirmation.

Computational analysis. Databases used for BLAST sequence search and analysis included http://www.ncbi.nlm.nih.gov/BLAST/, http://www.arabidopsis.org/Blast/, http://www.gramene.org/Multi/blastview, and http://genome.jgi-psf.org/Poptr1/Poptr1.home.html.

Supporting Information

Figure S1. SYMRK Kinase Regions Share Several Defining Conserved Amino Acid Motifs, Which Are Absent in Similar Sequences in *Arabidopsis* and Rice

Black shading indicates amino acid residues identical in all sequences, residues found in at least 50% of the sequences are shaded gray. Bars delimit predicted SYMRK protein domains. Dark blue, conserved extracellular region (CEC); black, LRRs; gray, imperfect LRR; white, juxtamembrane regions; brown, transmembrane region; green, protein kinase domain. Light blue bars with stars mark some of the regions conserved among SYMRK candidates, but not in other homologous sequences in rice and *A. thaliana*. Locus tags are indicated for similar sequences not regarded as SYMRK candidates. Sequences aligning with regions upstream of exon 4 of *LjSYMRK* are not included.

Found at doi:10.1371/journal.pbio.0060068.sg001 (96 KB DOC).

Figure S2. *LjSYMRK* Restores Nodulation and AM Formation in *Medicago dmi2* 5P Mutants

Transformation assay and selection were as in Figure 3. (A, B, K, and L) *Me. truncatula* (*Medicago*) *dmi2* 5P roots transformed with the respective control vector lacking an *LjSYMRK* expression cassette.
(C–F, M, and N) *Medicago* wild-type and (G–J, O and P) *dmi2* 5P roots transformed with the *LjSYMRK* coding sequence controlled by the *LjSYMRK* promoter.
(A–J) Roots inoculated with *S. meliloti* expressing *DsRED* for 5 wk. (A and B) Transgenic *dmi2* 5P roots carrying the control vector, showing no nodules. (C and D) Nodules on transgenic and nontransgenic roots of a wild-type plant transformed with *LjSYMRK* and (E and F) individual nodule containing *DsRED* expressing *S. meliloti* bacteria. (G and H) *dmi2* 5P root system with nodule formation confined to roots transformed with *LjSYMRK*. (I and J) Nodule on an *LjSYMRK* containing *dmi2* 5P root showing bacterial DsRED expression.
(K–P) Roots co-cultivated with *G. intraradices* for 2 wk. (K and L) Transgenic *dmi2* 5P control roots lacking hyphal proliferation and

arbuscule formation in the inner root cortex. Hyphal swellings in the root periphery (L and arrow in K) indicate abortion of fungal infections. Longer co-cultivation for 3 wk or more allowed for successful fungal infections of mutant roots, which was similarly reported for other *dmi2* mutant lines [58]. (M and N) Wild-type and (O and P) *dmi2* 5P roots transformed with *LjSYMRK* showing dense fungal colonization of the root inner cortex accompanied by arbuscule formation.
Scale bars: (A–D and G–H) 2 mm; (E–F and I–J) 0.5 mm; (K, M, and O) 0.1 mm; (L, N, and P) 0.02 mm.

Found at doi:10.1371/journal.pbio.0060068.sg002 (1.8 MB PDF).

Figure S3. Tomato and Rice *SYMRK* Restore AM Symbiosis in *Lotus symrk-10* Mutants, but Cannot or Only Partially Complement Bacterial Endosymbiosis Formation

Transformation assay and selection were as in Figure 3. (A–D) *Lotus symrk-10* roots transformed with the respective control vector lacking a *SYMRK* expression cassette. (E–L) *Lotus* wild-type (E–H) and *symrk-10* (I–L) roots transformed with *LeSYMRK*. (M–Z) *Lotus* wild-type (M–R) and *symrk-10* (S–Z) roots transformed with *OsSYMRK*.
(A, B, E, F, I, J, M, N, S, and T) Roots co-cultivated with *G. intraradices* for three weeks. (A and B) Transgenic *symrk-10* control root with extraradical mycelium but no intraradical fungal colonization or arbuscule formation. Swollen hyphal structures indicative of aborted fungal infections can be observed within epidermal cells (B and arrow in A). (E and F) Wild-type and (I and J) *symrk-10* roots transformed with *LeSYMRK*, showing fungal colonization of the inner root cortex (E, I) and arbuscule formation in inner cortical cells (F, J). (M and N) Wild-type and (S and T) *symrk-10* mutant roots transformed with *OsSYMRK*, similarly showing cortical AM colonization (M, S) and arbuscule formation (N, T).
(C, D, G, H, K, L, O–R, and U–Z) Root systems inoculated with *M. loti* expressing *DsRED* for 4 wk. (C and D) *symrk-10* root system with transgenic control roots, showing no nodules. (G and H) and (O–R) Wild-type root systems with *M. loti*–containing pink nodules on nontransgenic and on transgenic roots carrying *LeSYMRK* or *OsSYMRK*, respectively, indicating that these transgenes do not impair nodulation in transgenic wild-type roots. (K and L) *symrk-10* root system transformed with *LeSYMRK*, showing no nodules. In a single case, one nodule primordium was observed. (U–Z) *symrk-10* root system transformed with *OsSYMRK*, showing no fully developed nodules, but nodule primordia which are mostly noncolonized by bacteria, the latter proliferating on the primordial surface (W and X). In rare cases, small nodules were observed that contained bacteria, but, with one exception, showed no pinkish coloration in white light (Y and Z).
Scale bars: (A, E, I, M, and S) 0.1 mm; (B, F, J, N, and T) 0.02 mm; (C, D, G, H, K, L, O, P, U, V) 2 mm; (Q, R, and W–Z) 0.5 mm.

Found at doi:10.1371/journal.pbio.0060068.sg003 (3.8 MB PDF).

Table S1. Primer Sequences

Found at doi:10.1371/journal.pbio.0060068.st001 (106 KB DOC).

Accession Numbers

Sequences of *SYMRK* homologs were deposited at the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) under accession numbers AY935263 (*Al. glutinosa*); AM271000, AM931079 (*D. glomerata* coding and genomic sequence, respectively); AY935267 (*Lupinus albus*); AY935265 (*T. majus*); AY935266, AY940041 (*Ly. esculentum* coding and genomic sequence, respectively); AM270999 (*P. rhoeas*); AM851092 (*Po. trichocarpa*). The GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) accession number for pCAMBIA 1302 is AF234298.

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Author contributions. KM, GG and MP conceived and designed the experiments, performed the experiments, and analyzed the data. KM and MP wrote the paper.

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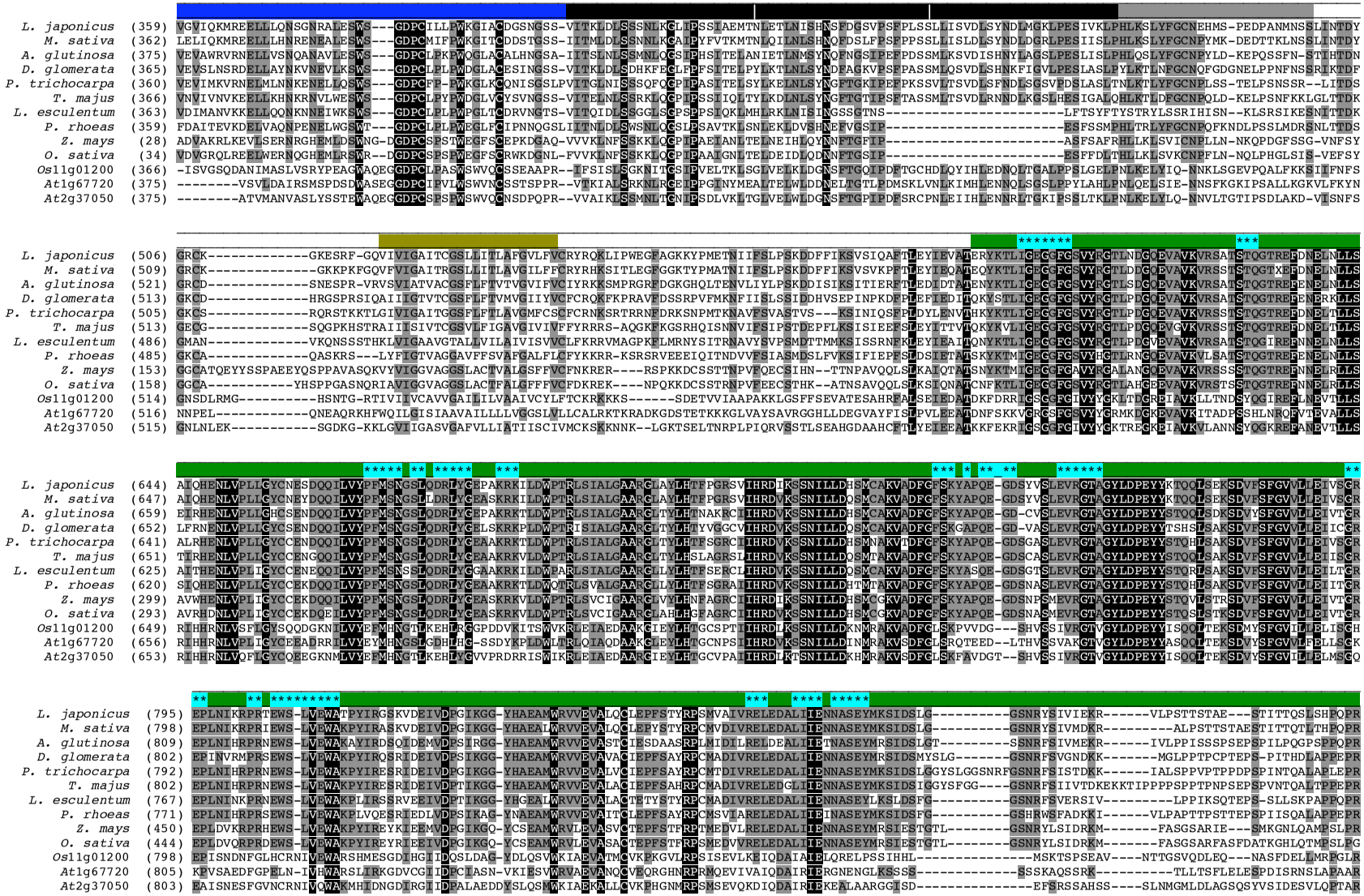
L. japonicus (359) VGVIQKMREELLLQNSGNRALESWS---GDPCILLEWKGIACDGSNGSS-VITKLDLSSSNLKCLIPSSIAEMTNLETLNISHNSFDGSVPSFPLSSLLISVDLSYNDLMGKLPESIVKLPHLKSLYFGCNEHMS-PEDPANMNSSLINTDY
M. sativa (362) LELIQKMREELLLHNRENEALESWS---GDPCMIFEWKGITCDDSTGSS-IITMLDLSSNNLKCAIPYFVTKMTNLQILNLSHNQFDSLFPSFPPSSLLISLDLSYNDLDGRLPESIISLPHLKSLYFGCNFPYMK-DEDTTKLNSSLINTDY
A. glutinosa (375) VEVAWRVRNLELLVSNQANAVLESWS---GDPCLKEFWQGLACALHNGSA-IITSLNLSSMNLQGSIPHSITELANIETLNMSYNQFNGSIPEFPDSSMLKSVDISHNYLAGSLPESLISLPHLQSLYFGCNPYLD-KEPQSSFN-STTHTDN
D. glomerata (365) VEVSLNSRDELLAYNKVNEVLKSWS---GDPCLPLEWDGLACESINGSS-VITKLDLSDHKFEGLFPFSITELPYLKTLNLSYNDFAGKVPSFPASSMLQSVDLSHNKFIGVLPESLASLPYLKTLNFGCNQFGDGNELPPNFNSSRIKTDF
P. trichocarpa (360) VEVIMKVRNELMLNNKENELLQSWS---GDPCFP-EWKGLKCQNISGSLPVITGLNISSSQFQGPIPASITELSYLKELNLSYNGFTGKIPEFPKSSVLTSVDLSFNDLSGSVPDSLASLTNLKTLYFGCNPLSS-TELPSNSSR--LITDS
T. majus (366) VNVIVNVKEELLKHNKRNVLWESWS---GDPCLPYPWDGLVCYSVNGSS-VITELNLSSRKLQGPIPSSIQLTYLKDLNLSYNGFTGTIPSFTASSMLTSVDLRNNDLKGSLHESIGALQHLKTLDFGCNPQLD-KELPSNFKKLGLTTDK
L. esculentum (363) VDIMANVKKELLQQNKNNEIWKSWS---GDPCLPLEWPGLTCDRVNGTS-VITQIDLSSGGLSGPSPPSIQKLMHLRKLNISLNGSSGTNS----------------------LFTSYFTYSTRYLSSRIHISN--KLSRSIKESNITTDK
P. rhoeas (359) FDAITEVKDELVAQNPENELWGSWT---GDPCLPLEWEGLFCIPNNQGSLIITNLDLSWSNLQGSLPSAVTKLSNLEKLDVSHNEFVGSIP----------------------ESFSSMPHLTRLYFGCNPQFKNDLPSSLMDRSNLTTDS
Z. mays (28) ADVAKRLKEVLSERNRGHEMLDSWNG-DGDPCSPSTWEGFSCEPKDGAQ-VVVKLNFSSKKLQGPIPAEIANLTELNEIHLQYNNFTGFIP----------------------ASFSAFRHLLKLSVICNPLLN-NKQPDGFSSG-VNFSY
O. sativa (34) VDVGRQLREELWERNQGHEMLRSWR--DGDPCSPSPWEGFSCRWKDGNL-FVVKLNFSSKKLQGPIPAAIGNLTELDEIDIQDNNFTGSIP----------------------ESFFDLTHLLKLSVKCNPFLN-NQLPHGLSIS-VEFSY
Os11g01200 (366) -ISVGSQDANIMASLVSRYPEAGWAQEGGDPCLPASWSWVQCSSEAAPR--IFSISLSGKNITGSIPVELTKLSGLVELKLDGNSFTGQIPDFTGCHDLQYIHLEDNQLTGALPPSLGELPNLKELYIQ-NNKLSGEVPQALFKKSIIFNFS
At1g67720 (375) --------VSVLDAIRSMSPDSDWASEGGDPCIPVLWSWVNCSSTSPPR--VTKIALSRKNLRGEIPPGINYMEALTELWLDDNELTGTLPDMSKLVNLKIMHLENNQLSGSLPPYLAHLPNLQELSIE-NNSFKGKIPSALLKGKVLFKYN
At2g37050 (375) --------ATVMANVASLYSSTEWAQEGGDPCSPSPWSWVQCNSDPQPR--VVAIKLSSMNLTGNISSDLVKLTGLVELWLDGNSFTGPIPDFSRCPNLEIIHLENNRLTGKIPSSLTKLPNLKELYLQ-NNVLTGTIPSDLAKD-VISNFS
L. japonicus (506) GRCK-------------GKESRF-GQVIVIGAITCGSLLITLAFGVLFVCRYRQKLIPWEGFAGKKYPMETNIIFSLPSKDDFFIKSVSIQAETLEYIEVATERYKTLIGEGCFGSVYFGTLNDGQEVAVKVRSATSTQGTREFDNELNLLS
M. sativa (509) GRCK-------------GKKPKFGQVFVIGAITRGSLLITLAVGILFFCRYRHKSITLEGFGGKTYPMATNIIFSLPSKDDFFIKSVSVKPFTLEYIEQATEQYKTLIGEGCFGSVYFGTLDDGQEVAVKVRSSTSTQGTKEFDNELNLLS
A. glutinosa (521) GRCD-------------SNESPR-VRVSVIATVACGSFLFTVTVGVIFVCIYRKKSMPRGRFDGKGHQLTENVLIYLPSKDDISIKSITITERFTLEDIDTATENYKTLIGEGCFGSVYFGTLSDGQEVAVKVRSATSTQGTREFENELNLLS
D. glomerata (513) GKCD-------------HRGSPRSIQAIIIGTVTCGSFLFTVMVGIIYVCFCRQKFKPRAVFDSSRPVFMKNFIISLSSIDDHVSEPINPKDFPLEFIEDITQKYSTLIGEGCFGSVYFGTLPDGQEVAVKVRSATSTQGTREFENRKLLS
P. trichocarpa (505) GKCS-------------RQRSTKKTLGIVIGAITGGSFLFTLAVGMFCSCFCRNKSRTRRNFDRKSNPMTKNAVFSVASTVS---KSINIQSFPLDYLENVTHKYKTLIGEGCFGSVYFGTLPDGQEVAVKVRSSTSTQGTREFDNELTLLS
T. majus (513) GECG-------------SQGPKHSTRAIIISIVTCGSVLFIGAVGIVIVFFYRRRS-AQGKFKGSRHQISNNVIFSIPSTDEPFLKSISIEEFSLEYITTVTQKYKVLIGEGCFGSVYFGTLPDGQEVGVKVRSSTSTQGTREFDNELTLLS
L. esculentum (486) GMAN-------------VKQNSSSTHKLVIGAAVGTALLVILAIVISVVCLFKRRVMAGPKFLMRNYSITRNAVYSVPSMDTTMMKSISSRNEKLEYIEAITQNYKTLIGEGCFGSVYFGTLPLGVEVAVKVRSATSTQGIREFNNELNLLS
P. rhoeas (485) GKCA-------------QASKRS---LYFIGTVAGGAVFFSVAFGALFLCFYKKRR-KSRSRVEEEIQITNDVVFSIASMDSLFVKSIFIEPFSLDSIETATSKYKTMIGEGCFGSVYFGTLRNGQEVAVKVLSATSTQGTREFENELNLLS
Z. mays (153) GGCATQEYYSSPAEEYQSPPAVASQKVYVIGGVAGGSLACTVALGSFFVCFNKRER----RSPKKDCSSTTNPVFQECSIHN--TTNPAVQQLSLKAIQTATSNYKTMIGEGCFGAVYFGALANGQEVAVKVRSSSTQGTREFNNELRLLS
O. sativa (158) GGCA-----------YHSPPGASNQRIAVIGGVAGGSLACTFALGFFFVCFDKREK----NPQKKDCSSTRNPVFEECSTHK--ATNSAVQQLSLKSIQNATCNFKTLIGEGCFGSVYFGTLAHGEEVAVKVRSTSTQGTREFNNELRLLS
Os11g01200 (514) GNSDLRMG----------HSNTG-RTIVIIVCAVVGAILILVAAIVCYLFTCKRKKKS------SDETVVIAAPAKKLGSFFSEVATESAHRFALSEIEDATDKFDRRIGSGGFGTIVYGKLTDGREIAVKLLTNDSYQGIREFLNEVTLLS
At1g67720 (516) NNPEL------------QNEAQRKHFWQILGISIAAVAILLLLVGGSLVLLCALRKTKRADKGDSTETKKKGLVAYSAVRGGHLLDEGVAYFISLPVLEEATDNFSKKVGRGSFGSVYYGRMKDGKEVAVKITADPSSHLNRQFVTEVALLS
At2g37050 (515) GNLNLEK-----------SGDKG-KKLGVIIGASVGAFVLLIATIISCIVMCKSKKNNK--LGKTSELTNRPLPIQRVSSTLSEAHGDAAHCFTLYEIEEATKKFEKRIGSGGFGIVYYGKTREGKEIAVKVLANNSYQGKREFANEVTLLS
L. japonicus (644) AIQHENLVPLLGYCNESDQQILVYPFMSNGSLQDRIYGEPAKRKILDWPTRLSIALGAARGLAYLHTFPGRSVIHRDIKSSNILLDHSMCAKVADFGFSKYAPQE-GDSYVSLEVRGTAGYLDPEYYKTQQLSEKSDVFSFGVVLLEIVSGR
M. sativa (647) AIQHENLVPLLGYCNEYDQQILVYPFMSNGSLLDRIYGEASKRKILDWPTRLSIALGAARGLAYLHTFPGRSVIHRDVKSSNILLDQSMCAKVADFGFSKYAPQE-GDSYVSLEVRGTAGYLDPEYYKTQQLTEKSDVFSFGVVLLEIVSGR
A. glutinosa (659) EIRHENLVPLLGHCSENDQQILVYPFMSNGSLQDRIYGEPAKRKTLDWPTRLSIALGAARGLTYLHTNAKRCIIHRDVKSSNILLDHSMCAKVADFGFSKYAPQE-GD-CVSLEVRGTAGYLDPEYYSTQQLTDKSDVYSFGVVLLEIVTGR
D. glomerata (652) LFRNENLVPLLGYCSENDQQILVYPFMSNGSLQDRIYGELSKRKPLDWPTRISIALGAARGLTYLHTYVGGCVIHRDVKSSNILLDQSMCAKVADFGFSKGAPQE-GD-VASLEVRGTAGYLDPEYYTSHSLSAKSDVFSFGVVLLEIICGR
P. trichocarpa (641) ALRHENLVPLLGYCCENDQQILVYPFMSNGSLQDRIYGEAAKRKTLDWPTRLSIALGAARGLTYLHTFSGRCIIHRDVKSSNILLDHSMNAKVTDFGFSKYAPQE-GDSGASLEVRGTAGYLDPEYYSTQHLSAKSDVFSFGVVLLEIVSGR
T. majus (651) TIRHENLVPLLGYCCENGQQILVYPFMSNGSLQDRIYGEAAKRKVLDWPTRLSIALGAARGLTYLHSLAGRSLIHRDVKSSNILLDQSMTAKVADFGFSKYAPQE-GDSCASLEVRGTAGYLDPEYYSTQQLTAKSDVFSFGVVLLEIISGR
L. esculentum (625) AITHENLVPLIGYCCENEQQILVYPFMSNSSLQDRIYGCAAKRKILDWPARLSIALGAARGLLYLHTFSERCLIHRDVKSSNILLDQSMCAKVADFGFSKYASQE-GDSGTSLEVRGTAGYLDPEYYSTQRLSAKSDVFSFGVVLLEILTGR
P. rhoeas (620) SIQHENLVPLLGYCCEKDQQILVYPFMSNGSLQDRIYGEAAKRKTLDWPTRLSVALGAARGLLYLHTFSGRAIIHRDVKSSNILLDHTMTAKVADFGFSKYAPQE-GDSNASLEVRGTAGYLDPEYYSTQHLSAKSDVFSFGVVLLEIITGR
Z. mays (299) AVWHENLVPLIGYCCEKDQQILVYPFMSNGSLQDRIYGEASKRKVLDWPTRLSVCIGAARGLVYLHNFAGRCIIHRDIKSSNILLDHSMCGKVADFGFSKYAPQE-GDSNPSMEVRGTAGYLDPEYYSTQVLSTRSDVFSFGVVLLEIVTGR
O. sativa (293) AVRHDNLVPLIGYCCEKDQEILVYPFMSNGSLQDRIYGEASKRKVLDWPTRLSVCIGAARGLAHLHGFAGRCIIHRDVKSSNILLDHSMCGKVADFGFSKYAPQE-GDSNASMEVRGTAGYLDPEYYSTQSLSTKSDVFSFGVVLLEIVTGR
Os11g01200 (649) RIHHRNLVSFLGYSQQDGKNILVYEFMHNGTLKEHIRGGPDDVKITSWVKRLEIAEDAAKGIEYLHTGCSPTIIHRDLKSSNILLDKNMRAKVADFGLSKPVVDG---SHVSSIVRGTVGYLDPEYYISQQLTEKSDVYSFGVVLLELISGH
At1g67720 (656) RIHHRNLVPLIGYCEEADRRILVYEYMHNGSLGDHLHG-SSDYKPLDWLTRVQIAQDAAKGLEYLHTGCNPSIIHRDVKSSNILLDINMRAKVSDFGLSRQTEED--LTHVSSVAKGTVGYLDPEYYASQQLTEKSDVYSFGVVLFELLSGK
At2g37050 (653) RIHHRNLVQFLGYCQEEGKNMLVYEFMHNGTLKEHIYGVVPRDRRISWIKRLEIAEDAARGIEYLHTGCVPAIIHRDLKTSNILLDKHMRAKVSDFGLSKFAVDGT--SHVSSIVRGTVGYLDPEYYISQQLTEKSDVYSFGVVLLELMSGQ
L. japonicus (795) EPLNIKRPRTEWS-LVEWATPYIRGSKVDEIVDPGIKGG-YHAEAMWRVVEVALQCLEPFSTYRPSMVAIVRELEDALIIENNASEYMKSIDSLG----------GSNRYSIVIEKR------VLPSTTSTAE---STITTQSLSHPQPR
M. sativa (798) EPLNIKRPRIEWS-LVEWAKPYIRASKVDEIVDPGIKGG-YHAEAIWRVVEVALQCLEPYSTYRPCMVDIVRELEDALIIENNASEYMKSIDSLG----------GSNRYSIVMDKR----------ALPSTTSTAESTITTQTLTHPQPR
A. glutinosa (809) EPLNIHRPRNEWS-LVEWAKAVIRDSQIDEMVDPSIRGG-YHAEAMWRVVEVASTCIESDAASRPLMIDILRELEDEALIIETNASEYMRSIDSLGT---------SSNRFSIVMEKR------IVLPPISSSPSEPSPILPQGPSPPQPR
D. glomerata (802) EPINVRMPRSEWS-LVEWAKPYIRQSRIDEIVDPSIKGG-YHAEAMWRVVEVAVACIEPFSAYRPCMADIVRELEDALIIENNASEYMRSIDSMYSLG-------GSNRFSVGNDKK-------MGLPPTPCPTEPS-PITHDLAPPEPR
P. trichocarpa (792) EPLNIHRPRNEWS-LVEWAKPYIRESRIDEIVDPGIKGG-YHAEAMWRVVEVALVCIEPFSAYRPCMTDIVRELEDALIIENNASEYMKSIDSLGGYSLGGSNRFGSNRFSISTDKK------IALSPPVPTPPDPSPINTQALAPLEPR
T. majus (802) EPLNIHRPRNEWS-LVEWAKPYIRESRIDEIVDPTIKGG-YHAEAMWRVVEVALAGIEPFSAHRPCMADIVRELEDGLIIENNASEYMKSIDSIGGYSFGG----GSNRFSIIVTDKEKKTIPPPPSPPTPNPSEPSPVVNTQALTPPEPR
L. esculentum (767) EPLNINKPRNEWS-LVEWAKPLIRSSRVEEIVDPTIKGG-YHGEAIWRVVEVALAGTETYSTYRPCMADIVRELEDALIIENNASEYLKSLDSFG----------GSNRFSVERSIV---------LPPIKSQTEPS-SLLSKPAPPQPR
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O. sativa (444) EPLDVQRPRDEWS-LVEWAKPYIREYRIEEIVDPGIKGQ-YCSEAMWRVLEVASACTEPFSTFRPSMEDVVRELEDALIIENNASEYMRSIESTGTL--------GSNRYLSIDRKM-------FASGSARFASFDATKGHLQTMPSLPG
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At1g67720 (805) KPVSAEDFGPELN-IVHWARSLIRKGDVCGIIDPCIASN-VKIESVWRVAEVANQCVEQRGHNRPRMQEVIVAIQDAIRIIERGNENGLKSSSS----------SSSKAQSSRK----------TLLTSFLELESPDISRNSLAPAAR
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Figure S1

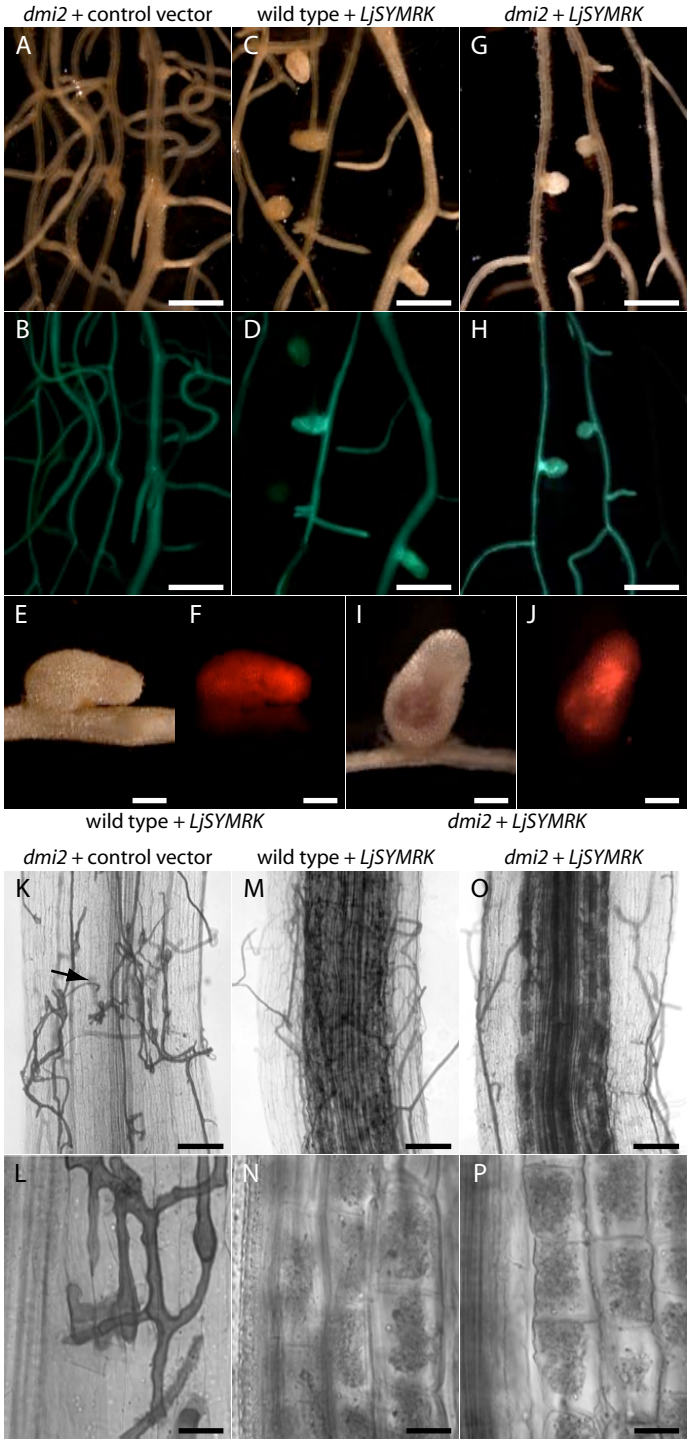
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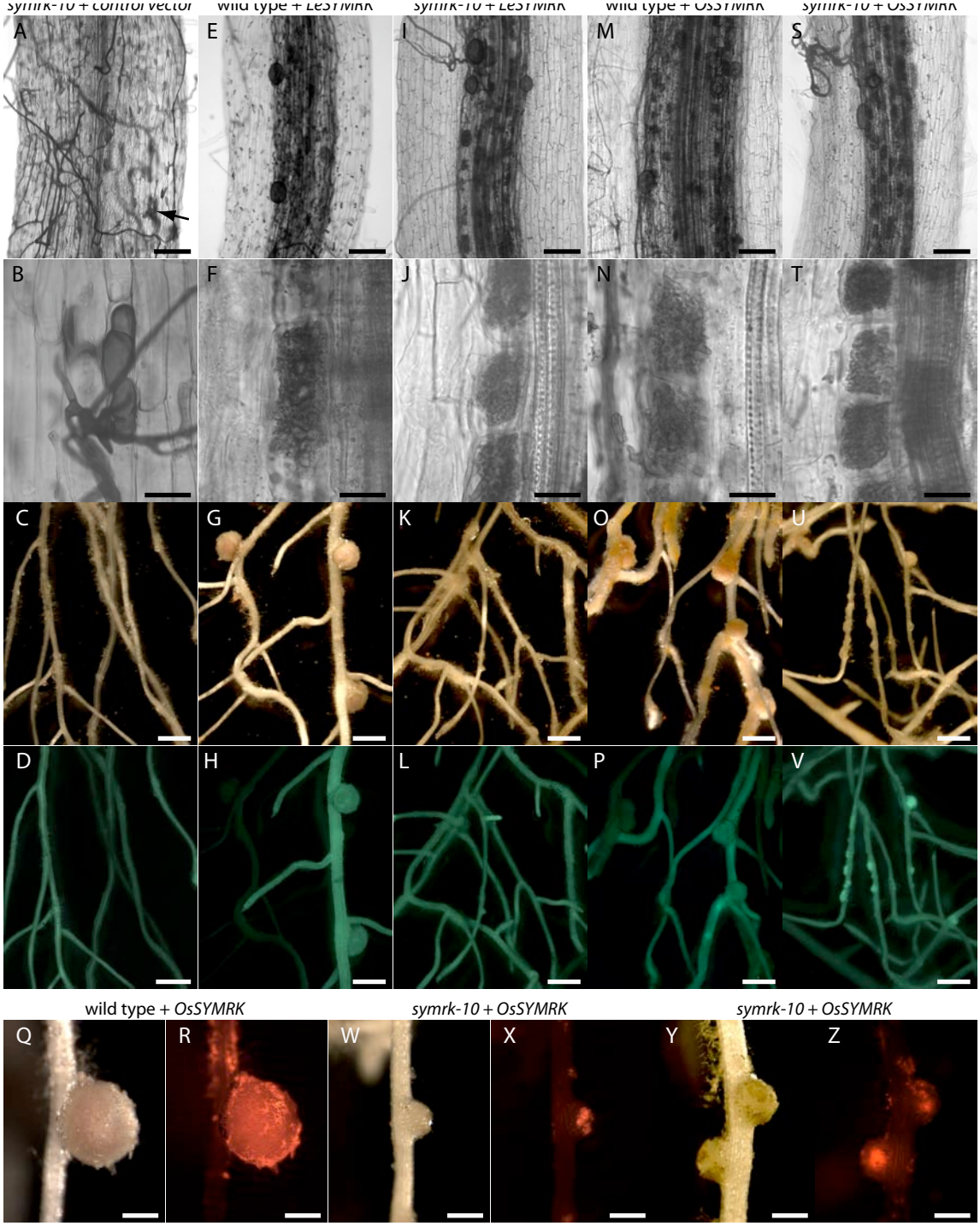
Figure S2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M
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B
F
J
N
T
C
G
K
O
U
D
H
L
P
V
wild type + OsSYMRK
symrk-10 + OsSYMRK
symrk-10 + OsSYMRK
Q
R
W
X
Y
Z

Figure S3

SymRK defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and *Frankia* bacteria

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Root endosymbioses vitally contribute to plant nutrition and fitness worldwide. Nitrogen-fixing root nodulation, confined to four plant orders, encompasses two distinct types of associations, the interaction of legumes (Fabales) with rhizobia bacteria and actinorhizal symbioses, where the bacterial symbionts are actinomycetes of the genus *Frankia*. Although several genetic components of the host–symbiont interaction have been identified in legumes, the genetic basis of actinorhiza formation is unknown. Here, we show that the receptor-like kinase gene *SymRK*, which is required for nodulation in legumes, is also necessary for actinorhiza formation in the tree *Casuarina glauca*. This indicates that both types of nodulation symbiosis share genetic components. Like several other legume genes involved in the interaction with rhizobia, *SymRK* is also required for the interaction with arbuscular mycorrhiza (AM) fungi. We show that *SymRK* is involved in AM formation in *C. glauca* as well and can restore both nodulation and AM symbioses in a *Lotus japonicus symrk* mutant. Taken together, our results demonstrate that *SymRK* functions as a vital component of the genetic basis for both plant–fungal and plant–bacterial endosymbioses and is conserved between legumes and actinorhiza-forming Fagales.

actinorhizal symbioses | *Casuarina glauca* | mycorrhizae | signaling

Root endosymbioses are associations between plants and soil microorganisms involving intracellular accommodation of microbes within host cells. The most widespread of these associations is arbuscular mycorrhiza (AM), which is formed by the majority of land plants with fungi belonging to the phylum Glomeromycota (1). In contrast, nitrogen-fixing nodulation symbioses of plant roots and bacteria are restricted to four orders of eurosid dicots (2). Actinorhiza, formed by members of the Fagales, Rosales, and Cucurbitales with Gram-positive *Frankia* bacteria, differs from the interaction of legumes with Gram-negative rhizobia in several morphological and cytological aspects (3). Although these differences suggest independent regulatory mechanisms, the close relatedness of nodulating lineages indicates a common evolutionary basis of root nodulation symbioses (2). In the legume–rhizobia interaction, among the key factors mediating recognition between the plant and the bacteria are Nod factors (NFs). NFs are bacterial lipochitooligosaccharides with an *N*-acetylglucosamine backbone (4). The perception of NFs induces a series of responses in host roots, including ion flux changes and membrane depolarization, rhythmic calcium oscillations in and around the nucleus (calcium spiking), cytoskeletal modifications and root hair curling, and activation of cortical cell divisions (5). Extensive mutant screenings performed in legumes led to the identification of several loci involved in this signaling cascade, and recently most of the corresponding genes were identified by map-based approaches (6). In *Lotus japonicus*, two genes, *NFR1* and *NFR5* encoding receptor-like serine/threonine kinases with LysM domains, are assumed to be involved in NF perception, because the corresponding mutants are impaired in the earliest NF responses (7). Several downstream components of the NF signaling cascade, including the leucine-rich-repeat receptor kinase gene *L. japonicus SymRK* (*DMI2*/*NORK* in *Medicago truncatula* and *M. sativa*, respectively) (8, 9), are dually involved in AM and nodulation symbiosis. *SymRK* is likely active near the junction of fungal and rhizobial signaling cascades (8). This makes it a particularly interesting candidate for studying a possible role of legume symbiosis genes in *Casuarina glauca*, which similarly forms AM, but in contrast to legumes interacts not with rhizobia but with *Frankia* bacteria to form actinorhiza.

In actinorhizal symbioses, very little is known about signaling mechanisms involved in plant-bacteria recognition. Analyses of the genome of three *Frankia* strains (10), the biochemical characterization of a *Frankia* root hair-deforming factor whose chemical structure is unknown (11), and the failure of *Frankia* DNA to complement rhizobial *nod* gene mutants (12) suggest that *Frankia* symbiotic signals are structurally different from rhizobial NFs. No plant genes involved in the perception and transduction of *Frankia* symbiotic signals have been identified to date, mostly due to the lack of genetic tools in actinorhiza-forming plants. Here, we isolate *CgSymRK*, a predicted *SymRK* gene from the actinorhizal tree *C. glauca*, and analyze its role in root endosymbioses. Our results reveal that *SymRK* is required for both AM and actinorhiza formation in *C. glauca*, indicating shared genetic mechanisms between fungal and bacterial root endosymbioses in *C. glauca* and legumes.

Results

Isolation of *C. glauca SymRK*. A *C. glauca SymRK* candidate, *CgSymRK*, was isolated by using a degenerate priming approach based on similarity with legume *SymRK* sequences. The gene is 7,280 bp long and contains 15 putative exons, encompassing a 2,829-bp coding sequence. Intron positions and phases are identical to *SymRK* genes of *L. japonicus* and other legumes, including *Medicago truncatula*, *Pisum sativum*, and *Sesbania rostrata*. The predicted protein of 941 aa contains an N-terminal

Author contributions: H.G. and K.M. contributed equally to this work; H.G., K.M., S.S., J.E., D.A., G.G., F.A., B.P., L.L., C.F., M.P., and D.B. designed research; H.G., K.M., S.S., J.E., D.A., G.G., F.A., B.P., L.L., and C.F. performed research; H.G., K.M., S.S., J.E., D.A., G.G., F.A., B.P., L.L., C.F., M.P., and D.B. analyzed data; and H.G., K.M., S.S., L.L., C.F., M.P., and D.B. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EU294188 (*CgSymRK* genomic) and EU273286 (*CgSymRK* CDS)].

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This article contains supporting information online at www.pnas.org/cgi/content/full/0710618105/DC1.

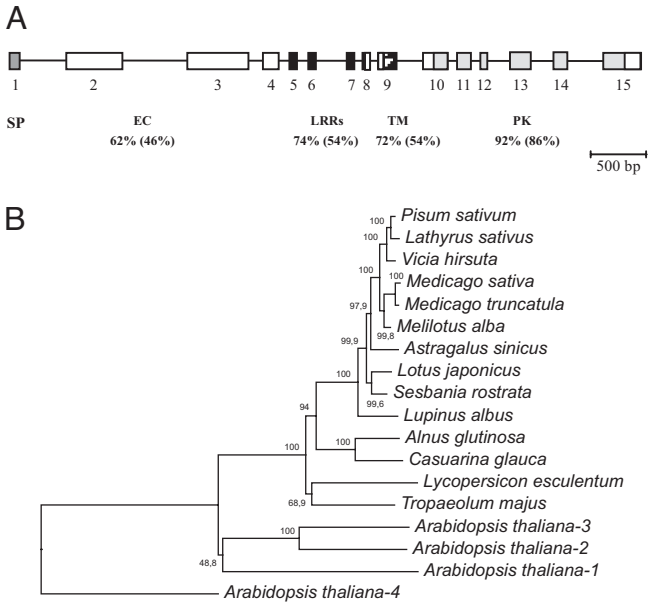

Fig. 1. *C. glauca SymRK* gene. (*A*) Genomic structure of *CgSymRK* with indicated predicted protein domains. Exons are depicted as boxes, introns as a black line. SP, predicted signal peptide; EC, extracellular domain; LRR leucine-rich repeat motifs; TM, transmembrane domain; PK, protein kinase domain. Percentages of similarity and identity between *Cg*SYMRK and *Lj*SYMRK are indicated below each predicted protein domain. (*B*) Distance tree of predicted SYMRK protein sequences based on a CLUSTALW alignement. Numbers above the branches represent the percentages of 1,000 bootstrap replications.

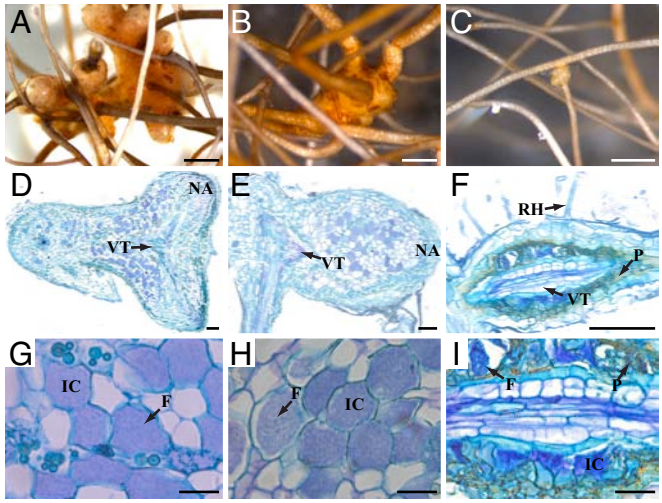

Fig. 2. Knockdown phenotype of *CgSymRK* after *Frankia* inoculation. (*A*) Nontransgenic nodule consisting of multiple lobes 10 weeks postinoculation (wpi). A nodular root develops at the apex of each nodule lobe. (*B*) Nodule on a hairy root transformed with a control vector at 10 wpi. Nodule morphology is similar to wild-type nodules. (*C*) Nodule-like structure formed on *CgSymRK* knockdown (RNAi) roots 10 wpi. Nodule lobes are small and do not branch to form a multilobed structure. (*D* and *E*) Sections of wild-type and transgenic control nodules. Each nodule lobe exhibits a central vascular bundle and cortical parenchyma infected with *Frankia*. (*F*) Section of a nodule-like structure observed on an RNAi plant showing few small infected cells and abnormal accumulation of polyphenols in the endodermis. (*G*) Closeup of area in *D*, showing both infected and uninfected cortical cells. Infected cells are hypertrophied and filled with *Frankia*. (*H*) Closeup of area in *E*. As in nontransgenic nodules, hypertrophied cortical cells are filled with *Frankia*. (*I*) Closeup of area in *F*. Infected cells are few and small compared with cells in nontransgenic and transgenic control nodules. IC, infected cell with *Frankia*; RN, root nodule; NA, nodule apex; VT, vascular tissue; P, polyphenol droplets; RH, root hair. [Scale bars: 1 mm (*A–C*); 100 μm (*D–F*); 25 μm (*G–I*).]

region of unknown function, three leucine-rich repeat motifs, a transmembrane region, and a serine/threonine protein kinase (Fig. 1*A*). The SYMRK kinase domain is highly conserved between legumes and actinorhizal plants. However, SYMRK extracellular regions are conserved between the two actinorhizal plants *C. glauca* and *A. glutinosa* but highly variable between legumes and actinorhizal plants (data not shown). Both actinorhizal proteins cluster together in a phylogenetic distance tree in the same subgroup as the legume SYMRK (Fig. 1*B*). Southern blot experiments suggested that only one *SymRK* gene exists in *C. glauca* (data not shown). In *C. glauca* roots infected with *Frankia*, real-time expression analysis revealed very little change in *CgSymRK* transcript abundance within 2 weeks after inoculation [supporting information (SI) Fig. 5*A*]. However, we cannot rule out that localized changes in *CgSymrk* expression might occur. *CgSymRK* expression was three times higher in 3-week-old nodules than in uninoculated roots (SI Fig. 5*B*).

***CgSymRK* Is Necessary for Actinorhizal and AM Symbioses in *C. glauca*.** To investigate the role of *SymRK* in root endosymbioses in *C. glauca*, we reduced *CgSymRK* expression levels in *Agrobacterium*-induced hairy roots by using RNAi. In parallel, control plants bearing nontransgenic and hairy roots transformed with a control vector comprising the *GFP* reporter gene but lacking the RNAi cassette were analyzed. A total of 78 RNAi composite plants and 48 transgenic control composite plants showing high GFP fluorescence in hairy roots were analyzed in two independent experiments. At 3 weeks after inoculation, plants transformed with the control vector began to develop nodules that were similar in size and shape to those produced on nontransgenic roots (Fig. 2*A* and *B*). As observed in ref. 13, nodulation of transgenic control hairy roots was reduced compared with nontransgenic controls. In *CgSymRK* RNAi roots, the frequency of nodulated root systems was 50% less than in transgenic control root systems (SI Table 1). Nodulated RNAi roots showed strong alterations in nodule development compared with control roots. We observed a gradient of phenotypes ranging from aborted prenodules (Fig. 2*C* and *F*) to nodules usually consisting of one thin lobe, whereas mature nodules in transgenic and nontransgenic control roots were multilobed (Fig. 2 *A* and *B* and *D* and *E*). On *CgSymRK* RNAi root nodules, the nodular roots, which are unbranched roots exhibiting negative geotropism growing at the apex of each nodule lobe, often behaved like adventitious roots exhibiting normal root growth and branching (data not shown). These aberrant nodules and nodular roots were never seen on transgenic or nontransgenic control roots. Histological analysis of 10 aberrant symbiotic structures of *CgSymRK* RNAi roots revealed an accumulation of phenolic compounds (Fig. 2*F*) and the presence of small infected cells in the cortex (Fig. 2*I*) contrasting with the hypertrophied infected cells observed in nontransgenic and transgenic control nodules (Fig. 2 *G* and *H*). We tested the ability of *CgSymRK* RNAi nodules to fix nitrogen via acetylene reduction activity (ARA) assays. *CgSymRK* RNAi nodules exhibited a quasinull ARA compared with transgenic control nodules (SI Fig. 6). To test the efficacy of *CgSymRK* knockdown in RNAi roots, *CgSymRK* expression was tested by quantitative RT-PCR (qPCR) in subcultivates of five *CgSymRK* RNAi roots. A 52–76% reduction of *CgSymRK* mRNA levels was observed in RNAi roots compared with transgenic control roots (SI Fig. 7). Taken together, our results indicate that a reduction in *CgSymRK* expression results in severe impairment in actinorhiza formation and symbiotic nitrogen fixation.

In legumes, *SymRK* has been shown to play also a crucial role in the establishment of AM symbiosis (14). To investigate whether *CgSymRK* is also involved in AM formation in *C. glauca*, RNAi hairy roots plants were generated and cultivated in pots

containing *Glomus intraradices* inoculum. Plants were harvested after 4 or 8 weeks, and GFP fluorescence was checked in transformed roots. Seventeen control and 21 RNAi plants showing GFP fluorescence and six nontransformed root systems were subjected to AM analysis. In nontransgenic control plants, fungal structures such as intraradical hyphae, vesicles, and arbuscules (SI Figs. 8 and 9 *A* and *B*) were observed at high frequencies ranging from 22% to 52% total root length colonization. Similar structures were observed at relatively high frequencies in roots of most transgenic control plants, 4 or 8 weeks after inoculation (SI Figs. 8 and 9 *C* and *D*). The slight reduction of colonization compared with nontransgenic control roots might be linked to modifications of hormonal balance. In contrast, most plants transformed with the *CgSymRK* RNAi construct showed very weak levels of AM colonization, and four composite plants showed a complete absence of intraradical structures 4 weeks after inoculation (SI Fig. 8). The absence of intracellular colonization was not due to an absence of inoculum, because extraradical hyphae were very often observed. Some RNAi roots showed extensive development of extraradical mycelium, usually growing along the epidermal cells and forming appressoria, which were frequently associated with abnormal, swollen hyphal structures (SI Fig. 9 *E* and *F*). Most fungal penetration attempts aborted, resulting in very low levels of intraradical colonization (SI Fig. 8). However, on the rare occasions where penetration succeeded, intraradical hyphae, arbuscules, and vesicles morphologically similar to those found in transgenic and nontransgenic control roots were observed (SI Fig. 9 *G* and *H*). Compared with control roots, colonized patches were generally smaller, spreading over few cells near the entry point and never succeeding in colonizing the whole root. These results indicate that *CgSymRK* knockdown strongly affects early steps of the AM interaction, especially fungal penetration into the root cortex, thereby revealing a conservation of *SymRK* function in AM between legumes and *C. glauca*.

***CgSymRK* Can Restore Root Endosymbioses in a Legume *symrk* Mutant.** To test whether *CgSymRK* can function in root endosymbioses in a legume, we introduced its coding sequence linked to the *L. japonicus SymRK* promoter region into *Agrobacterium rhizogenes*-induced roots of *L. japonicus symrk-10* (15) mutants. Interaction with AM fungi is usually aborted in *L. japonicus symrk* mutants at the epidermal level (14), with few hyphae invading the root cortex and no arbuscules developing within 3–6 weeks of exposure to fungal inoculum.

Similarly, after 3 weeks of cocultivation with *G. intraradices*, *symrk-10* roots transformed with a vector lacking a *SymRK* expression cassette (control vector) formed no AM, and typical hyphal swellings formed in epidermal cells indicating abortion of fungal infections (SI Table 2; Fig. 3 *A* and *B*). In contrast, wild-type (Fig. 3 *C* and *D*) and *symrk-10* (Fig. 3 *E* and *F*) plants transformed with *CgSymRK* developed AM (SI Table 2), involving the formation of wild-type-like cortical arbuscules (Fig. 3*F*) and infection sites in the complemented mutants. Similar results were obtained with wild-type and *symrk-10* mutant plants transformed with an *LjSymRK* expression cassette controlled by the same promoter region (SI Table 2). These results demonstrate that *CgSymRK* can complement the mycorrhization defect of *L. japonicus symrk* mutants.

Wild-type *L. japonicus* plants respond to inoculation with their rhizobial symbiont *Mesorhizobium loti* by root hair curling, infection thread formation, and nodule development. *L. japonicus symrk* mutants, in contrast, show no normal curling reaction of root hairs, and bacteria are unable to induce infection thread or nodule formation (8). This was equally the case in *symrk-10* roots transformed with a control vector (SI Table 2; Fig. 4 *A* and *B*), whereas *L. japonicus* wild-type and *symrk-10* roots carrying *CgSymRK* formed wild-type-like infection threads and nodules (Table 2; Fig. 4 *C–E* and *F-H*, respectively). These exhibited bacteria-filled and noninfected host cells (Fig. 4 *I* and *J*). *L. japonicus* wild-type and *symrk-10* roots transformed with a *L. japonicus SymRK* expression cassette equally formed infection threads and infected nodules (SI Table 2; Fig. 4 *K* and *L*, respectively). The observation that *SymRK* from the actinorhiza-forming plant *C. glauca* can restore not only the interaction with AM fungi but also with *M. loti* bacteria in *L. japonicus symrk* mutants indicates that this gene is highly conserved in its function in both AM and nodulation symbioses, whereas the specificity of recognition of bacterial partners is *SymRK*-independent.

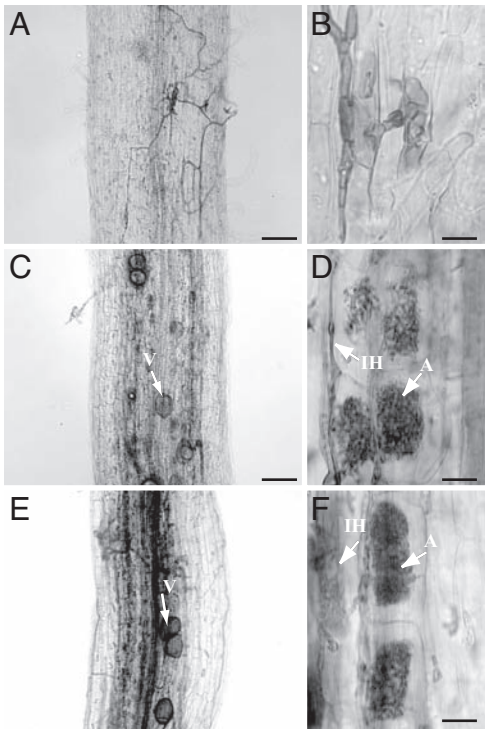

Fig. 3. AM formation in *L. japonicus symrk-10* mutants complemented with the *CgSymRK* coding sequence under control of the *LjSymRK* promoter, after 3 weeks of cocultivation with *G. intraradices*. Cleared roots with fungal structures are stained with acidic ink. (*A* and *B*) *symrk-10* roots transformed with a control vector. (*A*) Noncolonized root with extraradical mycelium and aborted infection structure (arrow). (*B*) Fungal appressorium and entry point associated with aborted infection structure within host epidermal cell. (*C* and *D*) Wild-type and (*E* and *F*) *symrk-10* roots transformed with *CgSymRK* linked to the *LjSymRK* promoter. Fungal hyphae grow through epidermis and exodermis and form arbuscules and vesicles in the inner root cortex. A, arbuscule; IH, intraradical hyphae; V, vesicle. [Scale bars: 100 μm (*A*, *C*, and *E*); 20 μm (*B*, *D*, and *F*).]

Discussion

There are three major types of root endosymbioses in angiosperms. These include the arbuscular mycorrhiza symbiosis with fungi and nitrogen-fixing root nodulation of legumes and actinorhiza-forming plants. In recent years, there has been a tremendous increase in knowledge of the molecular mechanisms responsible for NF perception and signal transduction in legumes (6). Genetic approaches in model legumes led to the identification of several components and the definition of a signaling cascade (5). Part of this signaling cascade is also involved in transduction of the symbiotic signal in AM symbioses (5). This gave rise to the hypothesis that the evolutionarily recent legume–rhizobia symbiosis reuses some of the molecular mechanisms of the more ancient AM symbiosis (16). This common signaling pathway includes the receptor kinase *SymRK*/*DMI2*. So far, nothing is known about the symbiotic signals and their perception during actinorhizal symbioses. Available data indicate only that the *Frankia* symbiotic signal is likely chemically

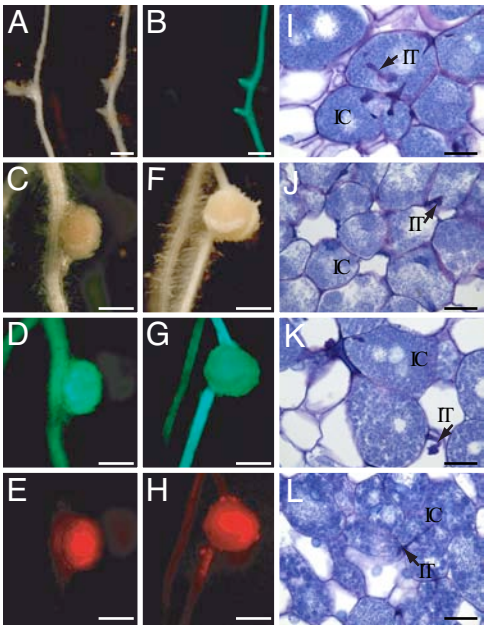

Fig. 4. Nodulation in *L. japonicus symrk-10* mutants complemented with the *CgSymRK* coding sequence under control of the *LjSymRK* promoter, 8 weeks after inoculation with *M. loti* MAFF expressing *DsReD*. Transgenic roots carried an *sGFP* reporter gene. (*A*, *C*, and *F*) Roots and nodules under white light. (*B*, *D*, and *G*) Transgenic roots and nodules showing GFP fluorescence. (*E* and *H*) Red fluorescence of bacterial DsRED. (*A* and *B*) *symrk-10* root transformed with the control vector, showing no nodules. (*C–E*) Transgenic wild-type root carrying the *CgSymRK* coding sequence. Nodules contain *DsReD*-expressing bacteria (*E*). (*F–H*) *symrk-10* mutant root transformed with the *CgSymRK* coding sequence, carrying wild-type-like nodules. (*I–L*) Semithin sections of nodules stained with toluidine blue. (*I* and *J*) Nodules on *symrk-10* mutant and wild-type roots complemented with the *CgSymRK* coding sequence, respectively. (*K* and *L*) Nodules on *symrk-10* mutant and wild-type roots complemented with the *LjSymRK* coding sequence, respectively. Infection threads (IT) are contained within bacteria-infected cells (IC). [Scale bars: 500 μm (*A–H*); 25 μm (*I–L*).]

different from NFs (10–12). Here, we report the isolation and characterization of *CgSymRK*, a *SymRK*/*DMI2* homolog from the actinorhizal tree *C. glauca*. Our data demonstrate that *CgSymRK* is functionally equivalent to *LjSymRK* in symbiosis formation in *L. japonicus*. In our experience, both AM and nodulation symbiosis formation in hairy roots of *L. japonicus* can vary in efficiency, particularly in complemented mutant tissue. Despite the differences in numbers of rescued root systems between the symbiosis types (*CgSymRK*) and constructs, phenotypic analyses clearly suggest that both symbiosis types can be fully supported by *CgSymRK* in *L. japonicus*.

Moreover, we were able to show that *CgSymRK* is necessary for functional symbiosis with *Frankia*. We therefore conclude that *CgSymRK* is probably a component of the signaling pathway involved in the perception and the transduction of yet-unknown *Frankia* factors. As in legumes (17, 18) *CgSymRK* expression level remained constant during root infection and increased in mature nodules compared with noninoculated roots. The reduction in the number of nodulated plants obtained by RNAi is less pronounced than the one obtained in legumes (18, 19); however, it clearly indicates that *CgSymRK* is involved during the early stages of *Frankia* root hair infection. A second symbiotic defect was observed downstream of this infection with striking differences in the nodule morphology and tissue organization relative to the control. *CgSymRK* RNAi hairy roots mostly developed small nonfixing nodule-like structures. Light microscopy revealed that the nodule apical meristem was absent, and we did not observe the gradient of infection and differentiation in the cortex that is present in transgenic and nontransgenic control nodules. Cortical cells also seemed to be less infected, and infected cells were smaller than those of control nodules. In addition, we observed the formation of dense deposits of polyphenols in *CgSymRK* RNAi nodules. These data suggest that the loss of *CgSymRK* function also affects *C. glauca–Frankia* symbiotic interaction after bacterial penetration. This is consistent with qPCR results that indicate an enhancement of *CgSymRK* expression in mature nodules.

We also analyzed the role of *CgSymRK* in the *G. intraradices–C. glauca* interaction. Hairy roots of *C. glauca* carrying the *CgSymRK* RNAi construct were able to form arbuscules and vesicles morphologically similar to those found in control plants, suggesting that *CgSymRK* is not involved in the formation of these late symbiotic structures. However, most RNAi plants showed a significant decrease in fungal colonization. At the root surface, hyphae developed abundant appressoria, but these colonization attempts rarely succeeded, pointing to a role of *CgSymRK* during hyphal penetration. Similar results were shown for *L. japonicus symrk* (14) and *M. truncatula dmi2* mutants (20, 21). This work report a role of *SymRK* in AM symbiosis formation in a nonlegume plant.

In summary, our data indicate that *SymRK* is involved in the symbiotic signal transduction pathway leading to actinorhizal symbioses. Our results demonstrate that, in *C. glauca* as in legumes, *SymRK* is involved in the establishment of both nitrogen-fixing nodule and AM symbioses, thus supporting the hypothesis that signaling genes have been recruited from the more ancient AM symbiosis during the evolution of nitrogen-fixing symbioses. It will now be essential to compare signal transduction pathways involved in endosymbiotic accommodation of AM fungi, rhizobia, and *Frankia* to develop strategies for the transfer of nodulation to nonnodulated plants.

Materials and Methods

Plant, Bacterial, and Fungal Material. *C. glauca* seeds were provided by Carter Seeds and grown as described in ref. 22. *L. japonicus* ecotype B-129 Gifu and *L. japonicus symrk* mutant *symrk-10* from the same ecotype (15) were grown for transformation as described in ref. 23. *C. glauca* and *L. japonicus* plants were transformed with *A. rhizogenes* strains A4RS (24) and AR1193 (25). For nodulation phenotyping of *C. glauca*, plants were inoculated with *Frankia* strain CcI3. *C. glauca* mycorrhization experiments were performed in pots containing an autoclaved mixture of quartz sand and soil (4:1). Plants were transferred from *in vitro* cultures and grown for 4–8 weeks in a growth chamber and watered with a modified Hoagland solution (22) containing 10 μM phosphate. *G. intraradices* inoculum was prepared by extracting spores from *in vitro* cultures of *G. intraradices* (26). One Petri dish showing extensive sporulation was kept at 4°C for at least 3 weeks and used to inoculate 2 liters of sand:soil mixture. For nodulation phenotyping of *L. japonicus*, composite plants were grown in plastic pots with 300 ml of Seramis substrate (Mars) and 150 ml of FP medium (27) and inoculated with *Mesorhizobium loti* strain MAFF expressing *DsReD* (M. Hayashi, personal communication). To test for AM formation, plants were cocultivated with *G. intraradices* BEG195 in chive nurse pots as described (14) and harvested after 3 weeks. Transgenic roots were selected via GFP fluorescence and stained with acidic ink as described (14) for visualization of fungal structures.

Identification and Cloning of *CgSymRK* Sequences and Phylogenetic Analysis. Amplification of *CgSymRK* was conducted on a cDNA library prepared from *C. glauca* uninfected roots by using the degenerated primers *SymRKdeg-5* (5′-CCAAGACATGAATGGTCTCTGGTNGARTGGGC-3) and *SymRKdeg-3* (5′-GAATCCATAGATCTCATATATTCAGAAGCRTTRTTYTC-3). The amplified fragment was cloned into a pGEM-T easy vector (Promega) and sequenced. cDNA fragments were obtained by RACE-PCR on a root cDNA library by using the Marathon cDNA amplification kit (Clontech), and the CDS was amplified by using primers *CgSymRKATG* (5′-ATGATGGAGGGATTGCATAAT-3′) and *CgSymRKSTOP* (5′-TCCTCCACAGCCAAGATAA -3′). The *CgSymRK* genomic sequence was obtained by using a Genome Walker kit (Clontech) and cloned in a pGEM-T easy vector. For the phylogenetic analysis, sequences (with GenBank accession numbers in parentheses) from *Alnus glutinosa* (62946487), *Sesbania rostrata* (56412259), *Melilotus alba* (21698802), *Pisum sativum* (21698794), *Lathyrus sativus* (89213719), *Vicia hirsuta* (21698800), *Medicago truncatula* (21698783), *Lotus japonicus* (21622628), *Medicago sativa* (21698781), *Lupinus albus* (62946493), *Tropaeolum*

majus (62946489), *Astragalus sinicus* (61723807), and *Lycopersicon esculentum* (62944413) were retrieved from GenBank via BLASTP search performed with the *L. japonicus*-predicted SYMRK sequence. Sequences of SYMRK homologs in *Arabidopsis thaliana* (−: At5g48740; −2: At2g37050; −3 At1g67720) described in ref. 28 were also included. *A. thaliana* (−: At3g25560), an NSP-interacting kinase (29), was used to root the tree. The alignment was performed by using the CLUSTALW and Neighbor-Joining algorithms in the CLC-Free Workbench 4 software package (CLC bio).

***Agrobacterium rhizogenes*-Mediated Transformation, RNAi and Complementation.** *C. glauca* and *L. japonicus* hairy root transformation was performed by following standard procedures (13, 23). To produce the knockdown construct, 365 bp corresponding to the kinase domain of *CgSymRK* sequence were amplified from genomic DNA by using *CgRNAi*-5 (5′-GGGAGCTGGAGGATGCTTTGA-3′) and *CgRNAi-3* (5′-TAAGTAGTAGTAGGTGGGGAGATTATTC-3′) primers containing 5′ XhoI or BamHI restriction sites for *CgRNAI-5* and KpnI or ClaI for *CgRNAI-3*. Amplified fragments (XhoI-*CgRNAi*-KpnI and BamHI-*CgRNAi*-ClaI) were then cloned into pKannibal (30) downstream of the CaMV 35S promoter, and the RNAi cassette was then cloned into the pHKN29 binary vector (31). This vector also contains the GFP gene under the control of the CaMV 35S promoter. For functional complementation, the *CgSymRK* coding sequence was fused to *LjSymRK* promoter (2,415-bp) and terminator (315-bp) regions and transferred into the pHKN29 binary vector. For complementation of *L. japonicus symrk* mutants with *LjSymRK*, the binary vector pCAMBIA 1302 was equipped with 4,970 bp of the *LjSymRK* promoter region fused to the *LjSymRK* coding sequence and a 285-bp NOS terminator fragment amplified from pJawohl8 RNAi [kind gift of P. Schulze-Lefert (Max Planck Institute, Cologne, Germany)] by using primers TNOS-5 5′-AATAAACCTAGGATCAGCTTGCATGCCGGTCG-3′ and TNOS-3 5′-AAATAAGTCGACCTAGAGTCAAGCAGATCGTTCAAAC-3′.

qPCR and Acetylene Reduction Assay. Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen). RNAs were quantified with Quant-iT Ribogreen RNA Reagent (Invitrogen). One hundred nanograms of total RNA was reverse-transcribed by using SuperScriptIII H^- reverse transcriptase (Invitrogen) and oligo$(dT)_{12–18}$. qPCR was performed by using the FullVelocity SYBR Green QPCR Master Mix (Stratagene). The primers used were *qCgSymRKFor1* 5′-GCAGGAGGTAGCAGTGAAGGTTC-3′ and *qCgSymRKRev2* 5′-GCGATCTTGAAGCGAGCCATTAG-3′. The FullVelocity cycling PCR program on an MX 3005P (Stratagene) was as follows: 1 cycle at 95°C for 5 min, 40 cycles at 95°C for 10 s and 60°C for 30 s, ended by 1 cycle at 95°C for 1 min, 60°C for 30 s, and 95°C for 30 s. Reactions were performed in triplicate, and the comparative threshold-cycle method was used to quantify *CgSymRK* expression (32). The results were standardized with *CgUbi* expression levels (32). The acetylene reduction assay was performed according to ref. 33.

Histochemical Analysis and Microscopy. *C. glauca* nodules were fixed as described in ref. 34. *L. japonicus* nodules were fixed in a solution containing 50% EtOH, 4% formaldehyde, and 5% acetic acid and dehydrated in 70% EtOH. Samples were embedded in Technovit 7100 resin (Heraeus Kulzer), and thin sections (6 μm) were cut with a microtome (Microm HM355S), stained with toluidine blue (0.01%), and mounted in Clearium Mountant (Surgipath). Visualization of AM in *L. japonicus* was performed as described in ref. 14. To visualize AM in *C. glauca*, roots were cleared for 1–2 days in 10% KOH at 90°C, rinsed, and stained for at least 1 h with a 0.05% trypan blue/5% acetic acid solution at 60°C and destained in water as described (35). Samples were viewed under a DMRB microscope (Leica). Colonization was assessed with a microscope by using the gridline intersect method (35) on at least 100 intersections per sample.

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Table 1. Reduced nodulation in *CgSymRK* RNAi composite plants

Root systems (genotype)	Nodulated/total root systems analyzed	% nodulated root systems
Nontransgenic	59/63	94
Transgenic (GFP+)	24/48	50
CgSymRK RNAi (GFP+)	21/78	27

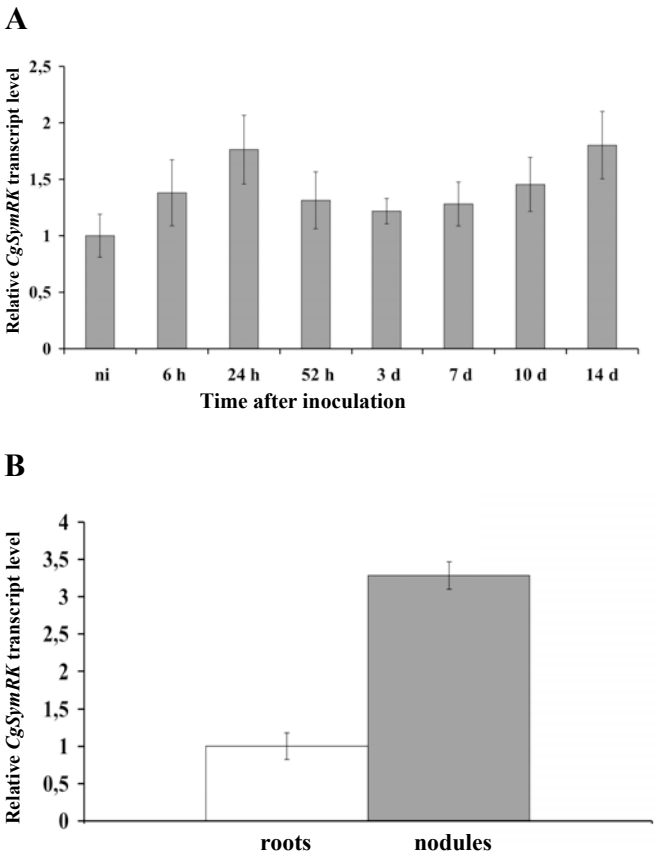
Nodulation was scored 12 weeks after inoculation with *Frankia.*

Table 2. Complementation of Nodulation and AM formation in *Lotus symrk* mutants carrying *CgSYMRK*

Root systems (genotype)	Nodulated/total root systems analyzed	AM+/total root systems analyzed
symrk-10 + control vector (GFP+)	0/34	0/34
wild type + CgSymRK (GFP+)	21/23	31/31
symrk-10 + CgSymRK (GFP+)	11/63	27/48
wild type + LjSymRK (GFP+)	11/11	16/17
symrk-10 + LjSymRK (GFP+)	8/20	7/22

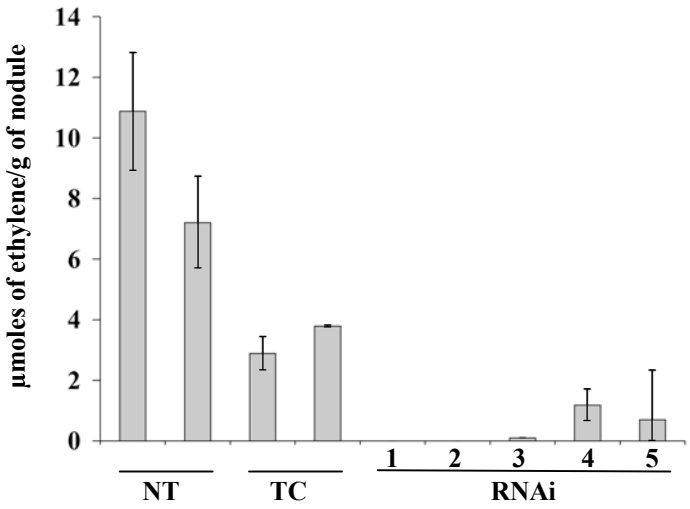
Nodulation was scored 4, 8, or 15 weeks after inoculation with M. loti, and AM after 3 weeks of cocultivation with G. intraradices. Results are compiled from two independent experiments.

SI Figure 5



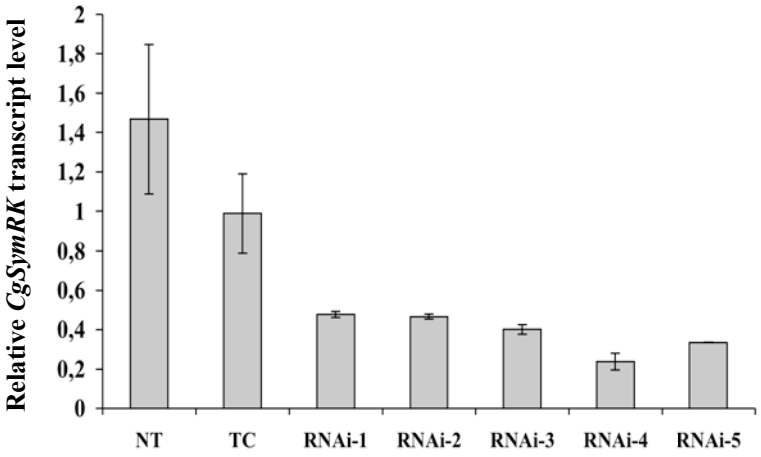
SI Fig. 5. *CgSymRK* mRNA levels in *C. glauca* roots and nodules determined by quantitative RT-PCR. *CgSymRK* expression levels in (A) inoculated roots and (B) mature nodules (grey bar) are given relative to non-inoculated roots. *CgUbi* was used as reference gene. ni, non inoculated roots. Error bars indicate standard errors of the mean of three technical replicates.

SI Figure 6



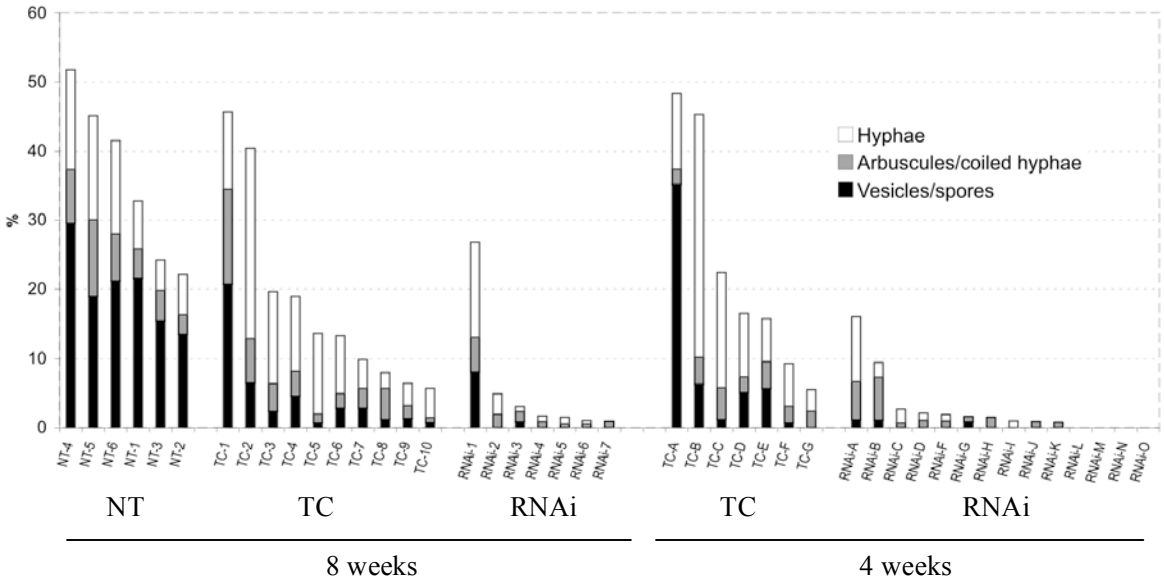
SI Fig. 6. Acetylene reduction activity (ARA) assays to assess the effects of *CgSymRK* RNAi on nitrogen fixation. Values obtained with two nontransgenic control root systems, two transgenic control root systems and five *CgSymRK* RNAi root systems are compared. NT: non-transgenic control; TC: transgenic control; 1-5: *CgSymRK* RNAi root systems with small unilobed nodules (1-3) and nodules showing 2-3 lobes each (4-5). Error bars indicate standard errors of the mean.

SI Figure 7



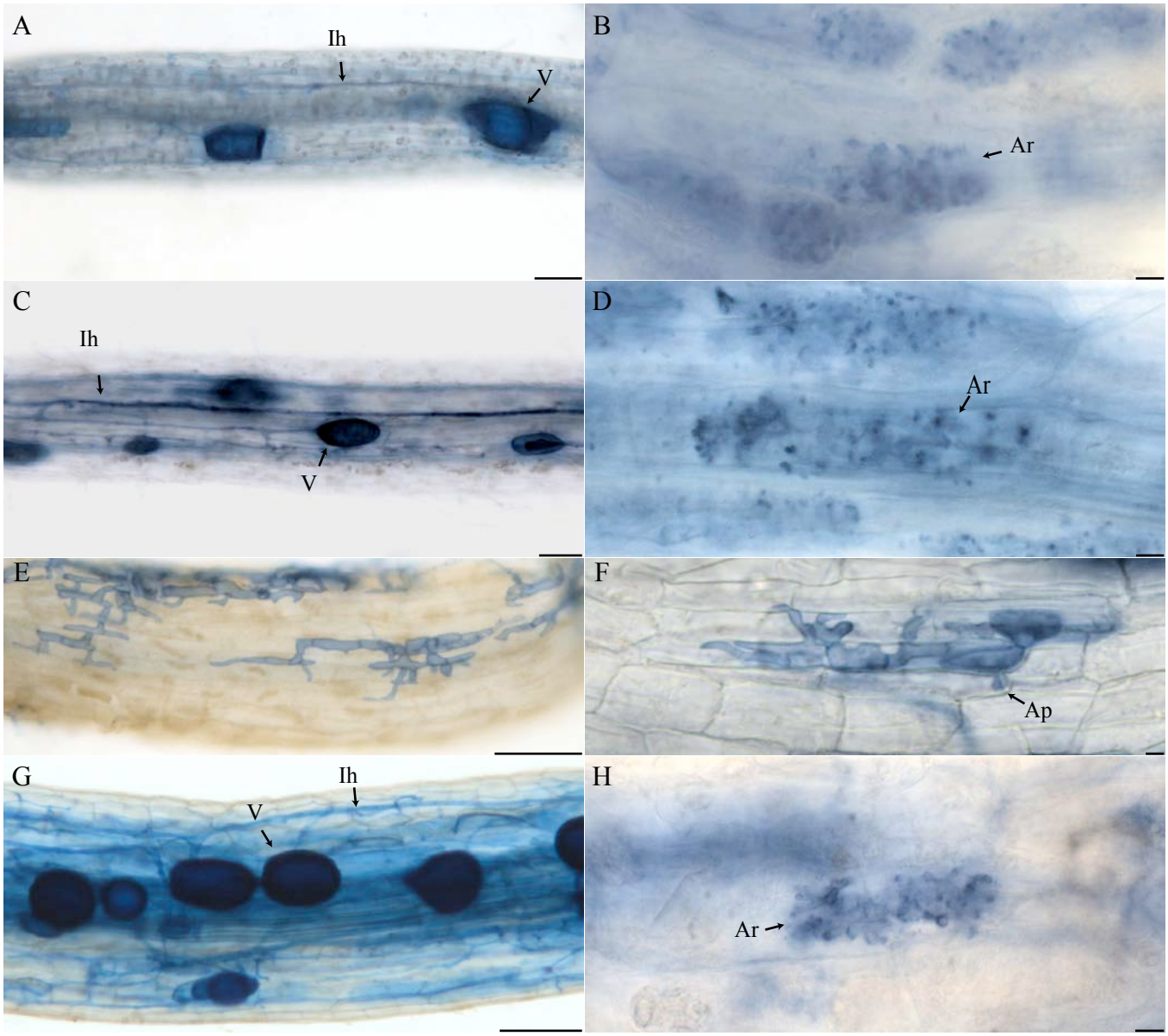
SI Fig. 7. Quantification of *CgSymRK* mRNA levels in 5 independent *CgSymRK* RNAi plants determined by real-time qPCR. *CgUbi* was used as reference. The average of two independent non-transgenic control roots and three transgenic control roots is shown. Expression levels are presented relative to transgenic control roots. All error bars indicate standard errors of the mean of 3 technical replicates on different samples. NT, non-transgenic control roots; TC, transgenic control roots. RNAi, *CgSymRK* RNAi plants.

SI Figure 8



SI Fig. 8. Intraradical colonization of *C. glauca* roots 4 or 8 weeks after inoculation with *G. intraradices*. Presence of intraradical hyphae, arbuscules/coiled hyphae and vesicles was assessed in roots from non-transgenic control (NT) and composite plants transformed with the control vector (TC) or with a *CgSymRK* RNAi vector (RNAi). Each bar represents quantitative analysis of one root system.

SI Figure 9



SI Fig. 9. Colonization of *C. glauca* roots by *G. intraradices* 8 weeks after inoculation. (A-B) Non-transgenic control roots and (C-D) roots transformed with the control vector show high colonization levels, arbuscules (Ar) vesicles (V) and intraradical hyphae (Ih) are present at high frequencies. (E-H) *CgSymRK* knockdown (RNAi) roots. Overall colonization levels are very low, but extensive extraradical hyphal growth can be observed on few roots (E). Hyphae tend to develop at the root surface following the epidermal cells forming appressoria (Ap) and aberrant hyphal swellings. (G-H) Penetration succeeds rarely, but can lead to the formation of vesicles (G) and arbuscules (H) morphologically similar to those formed in control plants. Scale bars: A, C, E, G : 50 µm ; B, D, F, H : 5 µm.

CYCLOPS, a mediator of symbiotic intracellular accommodation

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The initiation of intracellular infection of legume roots by symbiotic rhizobia bacteria and arbuscular mycorrhiza (AM) fungi is preceded by the induction of calcium signatures in and around the nucleus of root epidermal cells. Although a calcium and calmodulin-dependent kinase (CCaMK) is a key mediator of symbiotic root responses, the decoding of the calcium signal and the molecular events downstream are only poorly understood. Here, we characterize *Lotus japonicus cyclops* mutants on which microbial infection was severely inhibited. In contrast, nodule organogenesis was initiated in response to rhizobia, but arrested prematurely. This arrest was overcome when a deregulated *CCaMK* mutant version was introduced into *cyclops* mutants, conferring the development of full-sized, spontaneous nodules. Because *cyclops* mutants block symbiotic infection but are competent for nodule development, they reveal a bifurcation of signal transduction downstream of CCaMK. We identified *CYCLOPS* by positional cloning. CYCLOPS carries a functional nuclear localization signal and a predicted coiled-coil domain. We observed colocalization and physical interaction between CCaMK and CYCLOPS in plant and yeast cell nuclei in the absence of symbiotic stimulation. Importantly, CYCLOPS is a phosphorylation substrate of CCaMK in vitro. *Cyclops* mutants of rice were impaired in AM, and rice *CYCLOPS* could restore symbiosis in *Lotus cyclops* mutants, indicating a functional conservation across angiosperms. Our results suggest that *CYCLOPS* forms an ancient, preassembled signal transduction complex with CCaMK that is specifically required for infection, whereas organogenesis likely requires additional yet-to-be identified CCaMK interactors or substrates.

BiFC | map-based cloning | plant-microbe symbiosis | protein phosphorylation | protein–protein interaction

Legume plants can establish endosymbiotic interactions with nitrogen-fixing rhizobia and phosphate-delivering arbuscular mycorrhiza (AM) fungi. Plant root hairs form a tight curl in which rhizobia are entrapped. From this closed infection pocket, the bacteria are guided by plant membrane-delimited infection threads (ITs) into the root nodule, a specialized organ developed by the plant to provide an optimized environment for nitrogen fixation (1). AM fungal hyphae are guided through epidermal and cortical cells toward the inner cortex (2), where arbuscules, highly branched intracellular symbiotic structures, are formed (3). Intracellular infection by rhizobia and AM fungi is preceded by an exchange of specific signaling molecules. Rhizobia produce lipochito-oligosaccharides (Nod factors) that activate host plant responses including root hair deformation, and preinfection thread formation, which are structures that determine the path of IT growth through the root (4), and initiation of cortical cell division (1). One of the earliest plant responses to stimulation by Nod factors is Ca^{2+}-spiking, which consists of perinuclear oscillations of calcium concentration in root cells (5). In the legume *Lotus japonicus*, a shared genetic program defined by seven "common symbiosis genes" (6), is required for the establishment of both symbioses (7). An LRR-receptor kinase SYMRK (8, 9), the ion channel-like proteins CASTOR and POLLUX (10, 11), and the nucleoporins NUP85 and NUP133 (12, 13) are all required for the generation of Ca^{2+}-spiking, whereas a calcium and calmodulin-dependent protein kinase (CCaMK) is not, suggesting that CCaMK functions downstream of Ca^{2+}-spiking (14–16). CCaMK is composed of a kinase domain, a calmodulin-binding site and 3 EF hand motifs (16). Its catalytic activity is modulated by either free, or calmodulin (CaM)-bound Ca^{2+} ions (16, 17), suggesting that CCaMK converts the Ca^{2+} oscillation signal into a protein phosphorylation read-out. Deregulation of CCaMK by either a point mutation in the autophosphorylation site, or the deletion of the C-terminal regulatory domain, results in spontaneous nodule formation in the absence of rhizobia, demonstrating that CCaMK is a central regulator of the nodule organogenesis program (16, 17). *CCaMK* is also required for root hair curling and IT formation upon rhizobial infection and arbuscule formation during AM (7, 18, 19). However, how CCaMK differentially activates infection- *vs.* organogenesis-related pathways is still unclear.

CYCLOPS has been positioned downstream of *CCaMK*, because *cyclops* mutants exhibit impaired AM and rhizobial infection, but retain Ca^{2+}-spiking and formation of nodule primordia (7, 20–23). In this work, we describe the map-based cloning of *CYCLOPS*, which we found to encode a nuclear-localized protein with a coiled-coil motif. CYCLOPS is an ortholog of *Medicago truncatula* IPD3, which was recently identified as *I*nteracting *P*rotein of *DMI3* (the *M. truncatula* CCaMK ortholog) (24). We show that CYCLOPS specifically interacts with kinase-active CCaMK in planta and is phosphorylated by CCaMK in vitro, suggesting that CYCLOPS is a phosphorylation

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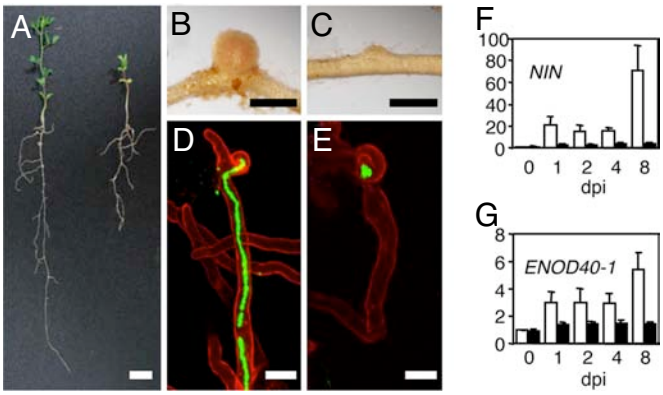

Fig. 1. Phenotypic characteristics of the *cyclops-3* mutant. (*A*) Growth phenotype in nitrogen-limiting conditions of ecotype Gifu WT (*Left*) and *cyclops-3* (*Right*). (*B*) WT nodule. (*C*) *cyclops-3* nodule primordium. (*D*) Infection thread within WT root hair. (*E*) Aborted infection within curled root hair of *cyclops-3*. (*A–C*) Plants were grown for 1 month after inoculation with *M. loti* MAFF303099. (*D* and *E*) Plants were grown for 2 weeks after inoculation with *M. loti* BN02 expressing GFP. [Scale bars: (*A*) 1.0 cm; (*B* and *C*) 1.0 mm; (*D* and *E*) 20 μm.] (*F* and *G*) Quantitative RT-PCR analysis of *NIN* (*F*) and *ENOD40-1*(*G*) expression in noninoculated (0 days) WT (white columns) and *cyclops-3* (black columns) roots, and at 1, 2, 4, and 8 dpi with *M. loti* MAFF303099. Fold increases in expression are shown relative to WT roots at 0 dpi (*F* and *G*). Mean values ± SD are shown.

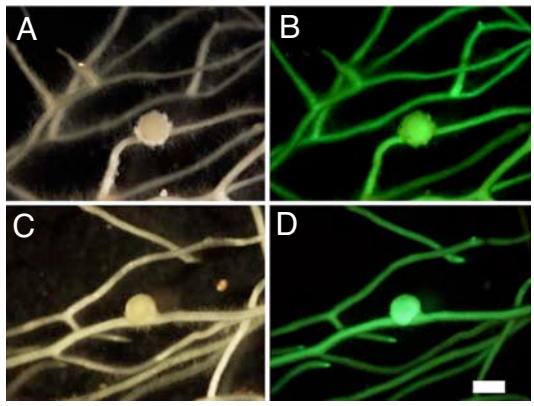

Fig. 2. Spontaneous nodule formation on *cyclops-4* and WT *L. japonicus* hairy roots transformed with 35S promoter-driven gain-of-function CCaMK^{T265D}. CCaMK^{T265D} was introduced into *cyclops-4* or WT hairy roots. (*A–D*) Spontaneous nodule development on *cyclops-4* (*A* and *B*) and WT roots (*C* and *D*) induced by CCaMK^{T265D}. Transformed roots were visualized by GFP fluorescence (*B* and *D*). Nodules were observed 8 weeks after transformation. (Scale bar, 1 mm.)

target of CCaMK. Our data position CYCLOPS on an infection-specific branch of the signaling network downstream of CCaMK.

Results

***cyclops* Mutants Abort Infection by Rhizobia and AM Fungi.** In forward genetic screens, we previously identified an allelic series of mutants impaired in the interaction with both rhizobia and AM fungi [supporting information (SI) Table S1]. Phenotypic analysis revealed that in contrast to WT *L. japonicus* plants (Fig. 1*A*), which developed mature nodules upon inoculation with *M. loti* under nitrogen-limiting conditions (Fig. 1*B*) on *cyclops-3* mutant roots, nodule development was prematurely arrested, and only nodule primordia were observed (Fig. 1 *A* and *C*; Table S1). In WT plants, intracellular ITs developed and grew through the root hair toward the nodule primordium (Fig. 1*D*). On *cyclops-3* mutant roots, curled root hair tips were colonized by rhizobia, however no ITs were observed (Fig. 1*E*). Because of this characteristic mutant phenotype, the corresponding gene was called *CYCLOPS*. In rare cases ITs were initiated, but elongation was aborted within root hairs (22). On *cyclops* mutant roots, the passage of AM fungal hyphae through the outer cell layers was characterized by abnormal hyphal swellings forming within epidermal or outer cortical cells, indicating an impairment of the intracellular infection process (Fig. S1 *A* and *B*). Where infection events were successful, hyphal growth proceeded toward the inner root cortex, and apoplastic growth along the root axis led to longitudinal spread of the fungal infection. Despite fungal colonization of the root cortex, arbuscules were almost completely absent from *cyclops* mutants (Fig. S1 *A* and *B*, Table S2) (7). These data indicate that *CYCLOPS* is required for fungal infection of the outer cortical cell layers and for arbuscule development.

Expression of *NIN* and *ENOD40–1* Is Impaired in *cyclops* Mutants. In *L. japonicus*, transcription of the *NIN* and *ENOD40–1* genes is induced within a few hours during the symbiotic interaction with *M. loti* (25, 26). The transcript levels of these genes are useful markers for the activity of symbiotic signal transduction processes (26, 27). In the WT, biphasic induction kinetics were observed for both genes upon inoculation with *M. loti* (Fig. 1 *F* and *G*). The first phase occurred at, or before, 1 day after inoculation (dpi), and the second between 4–8 dpi. This two-phase induction most likely reflects epidermal and cortical expression of *NIN* and *ENOD40–1*, as reported previously (28). *NIN* induction was still detectable, but at 8 dpi was 17-fold reduced in *cyclops-3* compared to the WT (Fig. 1*F*). In contrast, slight variations in *ENOD40–1* transcript levels in *cyclops-3* mutant roots were not statistically significant (Fig. 1*G*). These data indicate that *CYCLOPS* is required for full transcriptional activation of *NIN* and *ENOD40–1*.

***CYCLOPS* Is Dispensable for Nodule Organogenesis.** In contrast to previously identified common symbiosis mutants, *cyclops* mutants retain the ability to initiate cortical cell division. However, it is unclear whether *CYCLOPS* is necessary for the progression of nodule organogenesis beyond the primordium stage. To answer this question, we took advantage of a derivative of CCaMK conferring spontaneous nodulation. CaMV 35S promoter-driven expression of CCaMK^{T265D}, in which the predicted autophosphorylation site Thr-265 was replaced by Asp, resulted in spontaneous nodule formation in the absence of rhizobia in the WT as well as the *cyclops-4* background (Fig. 2 *A–D* and Table S3). The size of spontaneous nodules formed on *cyclops-4* roots did not differ significantly ($P < 0.01$) from those formed on WT roots (Fig. 2 *A* and *C*, Table S3). None of the negative controls including WT and *cyclops-4* plants transformed with WT *CCaMK* developed spontaneous nodules (Table S3). These results indicate that *CYCLOPS* is dispensable for nodule organogenesis and suggest that the developmental arrest observed in *cyclops* mutants is an indirect consequence of the aborted bacterial infection.

Map-Based Cloning of *CYCLOPS*. The *CYCLOPS* gene was isolated from *L. japonicus* by using a map-based approach (Fig. S2 *A–C*). Comparison with corresponding cDNA clones originating from nodulated roots (29) revealed that *CYCLOPS* is composed of 11 exons encoding a protein of 518 aa (Figs. S2*D* and S3*A*). Point mutations or single nucleotide deletions result in frame shifts and/or premature stop codons in *cyclops-2*, *-3*, *-4*, and *-5* (Table S1). *Cyclops-1* carries a transposon insertion in intron 10, resulting in a sequence insertion between exons 10 and 11 of the cDNA (Fig. S2*D*, Table S1). Hairy root transformation of *cyclops-3* with the genomic *CYCLOPS* sequence, including the native promoter,

resulted in the development of WT-like nodules upon inoculation with *M. loti* (Fig. S4). Similarly, the introduction of *CYCLOPS* cDNA fused to the native promoter into the same mutant line restored WT-like nodulation, as well as AM formation (Fig. S1, Table S2). The identification of genetic lesions in each of the 5 independent *cyclops* alleles together with the transgenic complementation of the mutant phenotype is unequivocal evidence for the correct identification of the *CYCLOPS* gene.

Within the conceptual *CYCLOPS* gene product, a coiled-coil motif in the C-terminal 67 aa residues and 2 nuclear localization signals (NLSs) were predicted (Figs. S3*A* and S5). However, no overall similarity to proteins of known function was identified by interrogation of public databases. Genomic DNA gel blot analysis and searches in the complete genome sequence (30) indicated that *CYCLOPS* is a single copy gene in *L. japonicus* and that single copy *CYCLOPS*-related sequences occur in other legume species including *Medicago truncatula* and *Pisum sativum* (data not shown). A combination of database searches and PCR amplification of cDNA from the respective species identified putative *CYCLOPS* orthologs in *M. truncatula* (*IPD3*) (24), *P. sativum*, and *Oryza sativa* (Fig. S5).

***CYCLOPS* Expression and Subcellular Localization.** By quantitative RT-PCR, *CYCLOPS* mRNA was detectable in uninfected roots and increased in abundance between 4–8 dpi with *M. loti* (Fig. S6*A*), which is after the initiation of IT development and cortical cell division. *CYCLOPS* mRNA accumulated in mature nodules, whereas the mRNA level in roots harvested 3 weeks after inoculation, nodules on which were removed, was comparable to uninfected roots (Fig. S6*B*). In situ hybridization of mature nodules indicated that *CYCLOPS* mRNA was present in cells of the central tissue (Fig. S6 *C* and *D*). *CYCLOPS* promoter activity during nodule development was monitored in *L. japonicus* hairy roots transformed with fusions of the *CYCLOPS* promoter to the beta-glucuronidase (GUS) reporter gene. Upon treatment with *M. loti*, strong GUS activity was observed in dividing cortical cells during early stages of nodule development (Fig. S6*E*). In agreement with the in situ hybridization results, strong expression was detected in the central tissue of mature nodules at later stages (Fig. S6*F*). *CYCLOPS* mRNA was not detectable in plant shoots, regardless of whether the plants were inoculated with *M. loti* or not (Fig. S6*B*).

To determine the subcellular localization of CYCLOPS, we transiently expressed GFP-CYCLOPS fusion constructs driven by the 35S promoter in *Nicotiana benthamiana* leaf epidermis cells (Fig. S7). GFP-CYCLOPS and a C-terminal truncation lacking the coiled-coil motif (GFP-CYCLOPS 1–449) were exclusively detected in the cell nucleus (Fig. S7 *A* and *B*), whereas CYCLOPS deletion mutants lacking either the second or both predicted NLS (GFP-CYCLOPS 1–421 and 1–366) were distributed randomly in the cytosol and nucleus, similar to the pattern observed with GFP alone (Fig. S7 *C–E*), indicating that at least the NLS proximal to the C terminus is functional. Because CCaMK was also localized in the nucleus (31), we tested for possible colocalization with CYCLOPS in *L. japonicus* root cells. Fusion constructs of CCaMK-GFP and RFP-CYCLOPS, both under the control of the CaMV 35S promoter, were introduced into WT *L. japonicus* roots via *A. rhizogenes* transformation. Transformed hairy roots exhibited GFP and RFP fluorescence in the same nuclei (Fig. S8 *A–D*). Similar results were obtained with protoplast cells derived from *L. japonicus* root tissue (data not shown). The observed spatiotemporal expression pattern of *CYCLOPS* and its nuclear colocalization with CCaMK are consistent with the findings obtained for the *M. truncatula* CYCLOPS ortholog IPD3 (24).

CYCLOPS Interacts With CCaMK in the Nucleus. In yeast two-hybrid (Y2H) analysis, full-length CYCLOPS fused to the GAL4 DNA binding domain (BD) showed strong autoactivation in yeast (data not shown). Consequently, a CYCLOPS deletion derivative BD-fusion (residues 1–259) or full-length CYCLOPS fused to the GAL4 activation domain (AD), both lacking autoactivation (data not shown), were used for subsequent experiments. We detected a strong interaction between CYCLOPS and CCaMK (Fig. 3*A* and *B*). A CCaMK point-mutation in the autophosphorylation site (T265I) that causes nodule development in the absence of rhizobia (16) did not disrupt interactions with CYCLOPS (Fig. 3*A*). However, 2 kinase-defective CCaMK mutants, G30E (as in the *ccamk-3* mutant) (16) and a point-mutant of a catalytic lysine residue (K44A) (32), as well as the K44A/T265I double mutant, did not interact with CYCLOPS (Fig. 3*A*), suggesting that kinase activity of CCaMK is required for interaction with CYCLOPS. The analysis of deletion constructs revealed that the 2 C-terminal EF-hand motifs of CCaMK are not required for a strong interaction with CYCLOPS (Fig. 3*A*). A *CYCLOPS* deletion series delimited the region required for CCaMK interaction between CYCLOPS residues 81–366 (Fig. 3*B*). Using CYCLOPS 1–259 as bait revealed that CYCLOPS forms homodimers, and that the region between residues 81–366 is necessary for self-interaction (Fig. 3*B*).

To confirm CYCLOPS protein interactions in planta, we performed bimolecular fluorescence complementation (BiFC) in transiently transformed *N. benthamiana* epidermis cells (33, 34). Strong fluorescence was observed in the nucleus when CYCLOPS and CCaMK fused to the C- and N-terminal half of YFP, respectively, were coexpressed (Fig. 3 *C* and *E*). Consistent with the requirement of kinase-active CCaMK for interaction in Y2H assays (Fig. 3*A*), no fluorescence was observed in cells cotransformed with constructs encoding CYCLOPS and CCaMK kinase-dead mutants (K44A and G30E) (Fig. 3*D* and data not shown). Self-interaction of full-length CYCLOPS was also observed in planta (Fig. 3*F*).

CCaMK Phosphorylates CYCLOPS In Vitro. Their strong interaction in plant and yeast cells prompted us to analyze whether CCaMK can phosphorylate CYCLOPS in in vitro kinase assays. CYCLOPS, $CYCLOPS^{1-449}$, and $CYCLOPS^{81-366}$, which interacted with CCaMK in the Y2H assay, were all phosphorylated by CCaMK (Fig. 3*G*, *Left Image*). The N-terminal truncation $CYCLOPS^{255-518}$, did not interact in the Y2H assay and was not phosphorylated by CCaMK (Fig. 3*G*, *Left Image*). The relatively stronger phosphorylation of $CYCLOPS^{81-366}$ may indicate additional CCaMK phosphorylation sites that are inaccessible in the full-length protein. Ca^{2+} alone increased autophosphorylation of CCaMK, whereas full-length CYCLOPS phosphorylation was stimulated by Ca^{2+}/CaM (Fig. 3*G*, *Right Image*). These results demonstrate that CYCLOPS is a substrate of CCaMK in vitro.

Rice *CYCLOPS* Is Indispensable for AM and Restores Rhizobial and Fungal Symbiosis in *Lotus cyclops-3*. Alignment of the nucleotide and protein sequences of *L. japonicus CYCLOPS* and rice (*Oryza sativa*) *OsCYCLOPS* revealed a 45% overall amino acid sequence identity, and a conserved exon–intron structure (Figs. S3*A* and S5). To elucidate the function of CYCLOPS in rice, we analyzed the AM phenotype of 4 independent *Tos17* lines (35), which each carry an insertion within exon 6 of *OsCYCLOPS* (Fig. S3*A*). Upon cocultivation with *G. intraradices*, arbuscules were not observed in any of the mutant root systems tested, despite the presence of abundant intraradical mycelium (Fig. S3*B*), indicating that *OsCYCLOPS* is required for arbuscule and hence, AM development in rice. Hyphal swellings within epidermal and outer cortical cells, resembling aborted infection sites observed in *L. japonicus cyclops* mutant roots, were also present in rice *cyclops* mutant roots, but were not indicative of the mutant phenotype in this species as they similarly occurred in rice

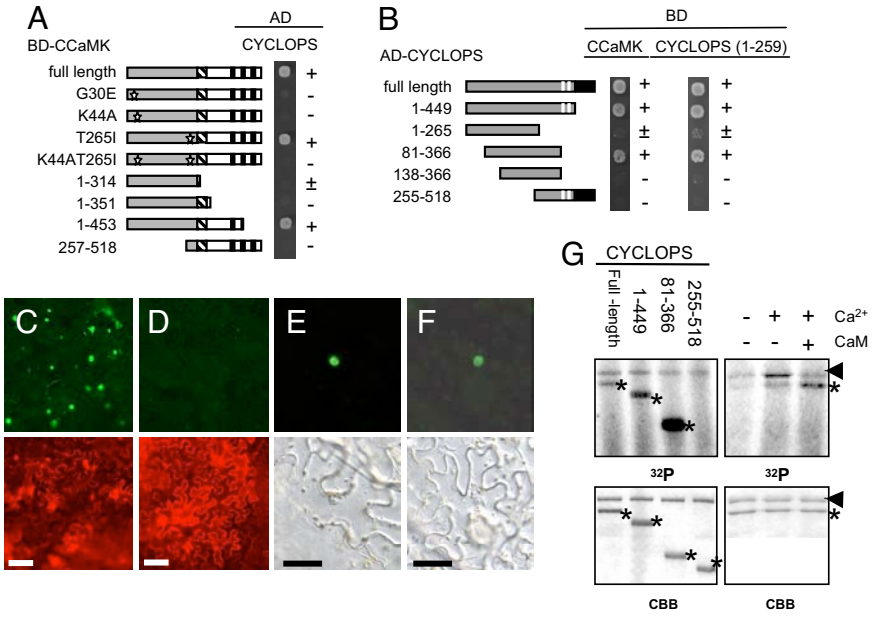

Fig. 3. CYCLOPS–CCaMK interactions and in vitro phosphorylation. (*A* and *B*) Detection of protein–protein interactions by Y2H analysis. Representative yeast growth on the selection medium (-leu, -trp, -his, -ade) is shown, and interaction results are indicated on the right. (+, growth; −, no growth; ±, very weak growth; AD, fused to the activation domain of GAL4; BD, fused to the DNA binding domain of GAL4). (*A*) Schematic illustrations of CCaMK derivatives (gray box, kinase domain; slashed box, calmodulin binding domain; black box, EF-hand; star, amino acid replacement) and their interaction with CYCLOPS. (*B*) Schematic illustrations of CYCLOPS derivatives (black box, coiled-coil motif; white box, NLS) and interaction of CYCLOPS derivatives with CCaMK and CYCLOPS$^{1-259}$. (*C–F*) Detection of protein–protein interactions in *N. benthamiana* leaves by BiFC. YFP fluorescence of leaves cotransformed with CCaMK-YFP^{N} (N-terminal half of YFP) and YFP^{C} (C-terminal half of YFP)-CYCLOPS (*C*) or CCaMK K44A-YFP^{N} and YFP^{C}-CYCLOPS (*D*). *Lower Images* in (*C*) and (*D*) show RFP fluorescence of the corresponding area resulting from cotransformation with a 35S/RFP construct as a positive control for successful transformation. [Scale bars, 100 μm (*C* and *D*).] YFP fluorescence of a leaf epidermis cell cotransformed with CCaMK-YFP^{N} and YFP^{C}-CYCLOPS (*E*) or CYCLOPS-YFP^{N} and YFP^{C}-CYCLOPS (*F*). *Lower Images* in (*E*) and (*F*) show the cell architecture (differential interference contrast) of the same cell. [Scale bars, 50 μm (*E* and *F*).] (*G*) In vitro phosphorylation of CYCLOPS by CCaMK. (*Left Images*) Phosphorylation of full-length CYCLOPS and derivatives in the presence of calcium (Ca^{2+}) and calmodulin (CaM). (*Right Images*) Phosphorylation of full-length CYCLOPS in the presence (+) or absence (−) of Ca^{2+} and CaM. Arrowheads indicate MBP-CCaMK and asterisks indicate 6×His-CYCLOPS or derivatives. (*Upper Images*) Autoradiographs of kinase assays (^{32}P) and (*Lower Images*) Coomassie staining (CBB) of the same gels.

segregants of the same lines carrying wild-type alleles of *OsCYCLOPS* (data not shown). By using *A. rhizogenes* infection, we generated *L. japonicus cyclops-3* hairy roots carrying a fusion of the *OsCYCLOPS* coding sequence to the *Lotus CYCLOPS* promoter region. In these roots, both AM and nodulation by *M. loti* were restored (Table S2, Fig. S1), demonstrating that the *OsCYCLOPS* gene of nonnodulating rice can support not only AM fungal, but also bacterial endosymbiosis.

Discussion

In this study, we identified *CYCLOPS* by map-based cloning. *CYCLOPS* encodes a plant-specific protein with a short C-terminal coiled-coil domain and a functional NLS. *CCaMK* and *CYCLOPS* are both required for the intracellular accommodation of bacteria and AM fungi. These phenotypes position *CCaMK* and *CYCLOPS* on a pathway required for the induction of plant infection structures. In contrast to other common symbiosis genes, both genes act downstream of the generation of Ca^{2+}-spiking (23). CCaMK has previously been localized to the nucleus and the presence of calcium-binding EF-hands and a calmodulin-binding domain made it a prime candidate to be the direct receiver of the calcium signal during spiking (14–16). We found that CYCLOPS and CCaMK colocalize within the nucleus, and we could observe physical interaction between the 2 proteins in yeast, as well as in plant cell nuclei, as was described for the *M. truncatula* orthologs IPD3 and DMI3 (24). Moreover, CYCLOPS was found to be a substrate for the CCaMK kinase in vitro. It is likely that the molecular mechanism by which CCaMK and CYCLOPS mediate the induction of infection structures involves the activation of symbiosis-related genes downstream (7). In a hypothetical signaling pathway, the perinuclear calcium spiking induced by rhizobial Nod factors or signals from AM fungi (36) modulate CCaMK kinase activity and hence the phosphorylation status of CYCLOPS (Fig. 4). Phosphorylation of CYCLOPS may, in turn, influence the transcription of infection-related plant genes. The identity of signaling components that function directly downstream of CYCLOPS and CYCLOPS-regulated genes remains to be identified.

Although the physical interaction of CCaMK and CYCLOPS and their requirement for infection are clear cut, there are significant phenotypic differences between corresponding mutants. In *ccamk* loss-of-function mutants, root-hair curling and cortical cell division were abolished upon symbiotic interaction with *M. loti* (14–16, 20, 21), whereas both responses were detected in *cyclops* mutants. Moreover, the introduction of gain-of-function CCaMK into the *cyclops* mutant background complemented the lack of organogenesis of full-sized (spontaneous) nodules, demonstrating that CYCLOPS is not required for nodule organogenesis. The interaction in Y2H and BiFC occurred in the absence of calcium spiking, indicating that CYCLOPS and CCaMK form a preassembled, stimulus-independent complex. This complex is likely to contain additional signaling components required for nodule initiation. The loss of CYCLOPS may weaken other interactions within the complex. Such reduced stability of the CCaMK complex may explain the reduction of spontaneous nodule numbers in the *cyclops* mutant background (Table S3).

Our data reveal a bifurcation of signal transduction at or below CCaMK. One CYCLOPS-dependent pathway leads to IT formation and AM infection, whereas a second CYCLOPS-independent pathway mediates nodule organogenesis and root-hair curling (Fig. 4). These 2 pathways may have additional

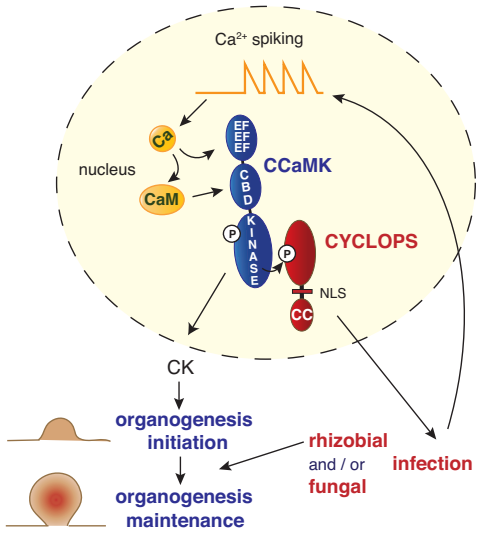

Fig. 4. Model for the function of CYCLOPS and CCaMK. Upon Ca^{2+}-spiking generated in the nucleus, CCaMK is activated by binding of calcium (Ca) to the C-terminal EF hands, leading to autophosphorylation (P) and/or binding of calcium-activated calmodulin (CaM) to the CaM binding domain (CBD). Subsequently, signal transduction initiated by CCaMK proceeds via different routes. In the root cortex, CCaMK induces and maintains nodule organogenesis which requires a sustained, elevated concentration of cytokinin (CK). In cells subject to microbial invasion, CCaMK signals via CYCLOPS to mediate infection. Infection thread development and ramification appears to be required for nodule progression, providing a continuous supply of morphogenetic signal, the Nod factor. (NLS, nuclear localization signal; CC, coiled-coil domain.)

tissue-specific components because a mutant version of *CCaMK*, *snf1*, encoding a deregulated kinase, spontaneously induces the expression of *NIN* in the cortical but not the epidermal cell layer, where nodule organogenesis or infection are respectively initiated (16). The second and *CYCLOPS*-independent pathway proceeds via hypothetical additional phosphorylation target(s) of CCaMK and likely involves the activation of cytokinin synthesis. Cytokinin is subsequently perceived in a cell nonautonomous fashion by the cytokinin receptor LHK1 (37), an event leading to the induction of cell division in hormonally predetermined cells (38).

If *CYCLOPS* is not required for nodule organogenesis, why do *cyclops* mutants not develop mature nodules upon rhizobial infection? Nodule meristem progression mediated by CCaMK may require the continuous and sufficient supply of bacterial morphogenetic signals such as Nod factors being released by rhizobia into the developing ITs that are not provided in *cyclops* mutants. Thus, the continuation of nodule morphogenesis would rely on continuous IT development that is itself dependent on *CYCLOPS*. This hypothesis is consistent with the fact that the expression domain of *CYCLOPS* is congruent with the anticipated developmental region explored by ITs in the developing nodule, as shown in our *CYCLOPS* promoter-GUS expression analysis (Fig. S6).

The nodule primordia phenotype of *cyclops* resembles *M. truncatula hcl* mutants, which induce limited cortical cell divisions in response to rhizobia, and in which spontaneous nodules form on transformation of gain-of-function CCaMK (39). However, in contrast to *cyclops* mutants, loss-of-function *hcl* mutants show an aberrant root-hair response toward rhizobia, resulting either in extensive root-hair deformation without curling, or extensive curling and a lack of bacteria within curled root hairs (40). *HCL* encodes the LysM-type receptor-like kinase LYK3, which is a putative ortholog of the *L. japonicus* Nod factor receptor NFR1 (27, 41).

Infection and nodule organogenesis are spatially and temporally coordinated, and the CCaMK–CYCLOPS complex may contribute to this coordination. When CCaMK versions in which the C-terminal regulatory domain was deleted were introduced into the *M. truncatula dmi3-1* (*ccamk*) mutant background, spontaneous nodules formed in the absence of rhizobia. Importantly, when these transgenic roots were inoculated with rhizobia, the developing nodules were not infected (17). This specific restoration of the organogenesis, but not the infection program, may be explained by our observation that the site between the CaM-binding domain and the second EF hand is required for the *L. japonicus* CCaMK–CYCLOPS interaction in yeast (Fig. 3*A*), a domain that is lacking in the *M. truncatula* DMI3 1–311 or 1–326 C-terminal deletions.

Interestingly, the allelic series of symbiosis-defective *cyclops* mutants exclusively comprises frame shifts or premature stop codons, suggesting that they represent loss-of-function alleles (Table S1). No mutants with single amino acid substitutions leading to missense mutations were recovered from forward screens for nodulation defective mutants (42). This is an exceptional bias suggesting that amino acid substitutions are either largely tolerated without significantly impacting CYCLOPS function, or lethal.

Amino acid sequences with high overall similarity to CYCLOPS were only found in legume plants. The C-terminal region of CYCLOPS, comprising the second NLS and the coiled-coil motif, is conserved in a wide range of plant species, including the moss *Physcomitrella patens*, the primitive angiosperm *Amborella trichopoda*, and higher plants like *Vitis vinifera* and *Populus trichocarpa*. No sequence with significant similarity to *CYCLOPS* was identified in the genome of the asymbiotic plant *Arabidopsis thaliana*, in which other common symbiosis genes like *SYMRK* or *CCaMK* are also absent (43).

The symbiosis between plants and AM fungi dates back to the earliest land plants (44). Our analysis of rice *cyclops* mutants has demonstrated the importance of *OsCYCLOPS* for the establishment of AM. The finding that *CYCLOPS* is conserved in AM-forming angiosperms is consistent with an ancient and specific role of *CYCLOPS* in symbiosis. The observed intraradical colonization, but lack of intracellular arbuscules in *cyclops* mutants, indicates a function predominantly serving the intracellular accommodation of the AM fungi in both *L. japonicus* and rice. The evolution of plant-derived structures supporting bacterial infection (infection threads) was a critical step during the evolution of root nodule symbiosis (6). In *L. japonicus cyclops* mutants, arbuscule development and the initiation or elongation of ITs, are aborted (7, 22, this study). This is consistent with the idea that both symbioses share a common genetic program and hence an evolutionary history. Genetic and structural considerations strongly suggest that the IT evolved from the pre-penetration apparatus observed in response to AM fungi (2). The additional and perhaps later invention of nodule organogenesis relies on yet unidentified CCaMK-downstream components that act independently of CYCLOPS.

Materials and Methods

Plant Material, Inoculation, and Growth Conditions. *L. japonicus* ecotype Gifu WT and *cyclops* mutants identified in genetic screens and through TILLING (42) (Table S1) were grown and inoculated with *M. loti* MAFF303099 or BN02 as described (45).

Expression Analysis. RNA was quantified by real-time PCR, or detected by in situ hybridization of root sections with *CYCLOPS* antisense and sense probes as described in (46) and *SI Materials and Methods*.

Plasmid Construction. Detailed information is provided in *SI Materials and Methods*.

Transformation of *CYCLOPS* and *CCaMK* Constructs. T-DNA constructs (described in *SI Materials and Methods*) were introduced by hairy root transformation as described (47), plants were cocultivated with rhizobia or *G. intrara-*

dices BEG195, and nodulation or AM formation was scored as described in *SI Materials and Methods*.

Subcellular Localization, BiFC Analysis, and Microscopy. T-DNA constructs (described in *SI Materials and Methods*) were introduced into *N. benthamiana* leaf cells by *A. tumefaciens*-mediated transient transformation, and leaf cells were analyzed with an epifluorescence microscope (for detailed description, see *SI Materials and Methods*). For colocalization analysis, 35S/CCaMK-GFP and 35S/RFP-CYCLOPS T-DNA constructs (described in *SI Materials and Methods*) were introduced into *L. japonicus* WT plants by hairy root transformation, and the localization of the corresponding proteins was assessed by confocal laser scanning microscopy.

Yeast Two-Hybrid Analysis. Y2H analysis was carried out according to standard procedures (Stratagene Product Manual #235702; pBD-GAL4 Cam Phagemid Vector Kit) by using the yeast strain AH109 (Clontech). Synthetic drop-out media (-trp, -leu, -his, 1 mM 3-amino-1,2,4-triazole and -trp, -leu, -his, -ade) were used for selection of protein interactions.

In Vitro Kinase Assays. Protein purification and kinase assays were done as described (16) with modifications (see *SI Materials and Methods*).

Genotyping and AM Phenotyping of *Oscyclops* Lines. *O. sativa* subspecies *japonica*, cv. Nipponbare, was grown as recommended at http://tos.nias.affrc.go.jp/~miyao/pub/tos17/. The *Oscyclops* mutant lines ND5032, NC2713, NC2415, and NG0782 were identified by interrogation of a rice-mutant sequence tag library induced by retrotransposon *Tos17* (35). For genotyping, primer *Tos17* was used with gene-specific primers (Table S4). The presence and location of the insertions were confirmed by PCR and sequence analysis. For AM phenotyping, plants were cocultivated with *G. intraradices* BEG195 and *Allium schoenoprasum* nurse plants for 2 or 3 weeks and AM colonization was quantified as described in (48).

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Supporting Information

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SI Materials and Methods

Expression Analysis. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen), followed by DNase I treatment. Quantitative RT-PCR was performed with the GeneAmp5700 (Applied Biosystems) by using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). The relative expression value was normalized by using *EF-1*. Amplification efficiencies of each product were 0.82 (*CYCLOPS*), 0.91 (*NIN*), 0.81 (*ENOD40–1*), and 0.91 (*EF-1*). Experiments were carried out 3 times with 3 independent samples. Primer sequences for the amplification of *CYCLOPS*, *NIN*, *ENOD40–1*, and *EF-1* are listed in Table S4.

In Situ Hybridization. A cDNA fragment of *CYCLOPS* (493 bp) was amplified (see Table S4 for primer sequences) and cloned into the pGEM-T Easy Vector (Promega). Plasmids digested with SpeI were used as templates for T7 RNA Polymerase (antisense probe), or were digested with NcoI for transcription via SP6 RNA Polymerase (sense probe). Hybridization of 10-μm root sections was performed as described (1). Signals were detected with an Olympus BX-50 microscope.

Plasmid Construction. For promoter-*GUS* analysis (Fig. S6 *E* and *F*) and complementation tests (Fig. S4), a Gateway compatible destination vector, pCYCLOPS-GW, was constructed as follows: HPTII in pCAMBIA1300 was replaced with GFP and an AscI linker was introduced into the SmaI site (2, 3). A genomic sequence of the *CYCLOPS* promoter region (3,788 bp) was amplified by PCR, digested with BamHI and AscI, and ligated with the vector. The transcript terminator (968 bp) was amplified by PCR, followed by digestion with AscI and SacI and ligation with the vector. The reading frame cassette A of the Gateway vector conversion system (Invitrogen) was introduced into the AscI site of the vector. A GUS entry clone was constructed as follows: The GUSPlus gene in pCAMBIA1305.1 was amplified by 2 rounds of PCR and the fragment introduced into pDONR/Zeo (Invitrogen) via Gateway BP reaction (Invitrogen). An entry clone carrying the *CYCLOPS* genomic coding sequence was constructed by amplification of the coding region (4,054 bp) by 2 rounds of PCR, followed by introduction of the PCR product into pDONR/Zeo (Invitrogen) via the Gateway BP reaction (Invitrogen). Each fragment was then transferred from the entry vectors into pCYCLOPS-GW by Gateway LR reactions (Invitrogen).

For the RFP-CYCLOPS and CCaMK-GFP fusion constructs, *CYCLOPS* and *CCaMK* cDNA were amplified by PCR and cloned into pENTR-D Topo or pENTR-SD-D Topo (Invitrogen). The CYCLOPS entry clone was transferred into pK7WGR2 (4) by Gateway LR reactions. The CCaMK entry clone was transferred into pK7FWG2 (4) by Gateway LR reactions.

Constructs used for GFP fusions (Fig. S7), Y2H analysis (Fig. 3 *A* and *B*), BiFC (Fig. 3 *C–F*), and protein purification (Fig. 3*G*) were made by using Gateway technology (Invitrogen). Full-length and truncated *CYCLOPS* or *CCaMK* cDNA fragments were amplified by PCR and cloned into pENTR-D Topo or pENTR-SD-D Topo (Invitrogen). Point mutations were introduced by PCR as described previously (5). Resulting entry clones were transferred to destination vectors by Gateway LR reactions. The following destination vectors were used: For N-terminal GFP fusions, a Gateway-compatible pAMPAT-MCS derivative (GenBank accession AY436765); for Y2H, modified pBD-GAL4 Cam (Stratagene) and pGAD424 (Clontech) vectors in which Gateway cassettes were introduced; for BiFC, pSPYNE 35S GW and pGWB735/1 vectors (unpublished, gift from Dr. Thomas Lahaye, University of Halle, Halle, Germany); for CYCLOPS protein purification from *E. coli*, pDEST17 (Invitrogen).

For the complementation analysis of *cyclops-4* with gain-of-function CCaMK^{T265D}, the binary vector backbone encoding the CaMV 35S promoter was constructed as follows: The PCR fragment of multi cloning sites (XbaI-StuI-SpeI-BamHI-KpnI-AscI) flanked by the 35S promoter and the Nos terminator, was digested with HindIII and SacI. The fragment was inserted into the HindIII-SacI site of pHKN29r (2, 3), which resulted in p35S-GFP. The 35S promoter and the Nos terminator were amplified with pBI121 (6) as a template. The primers for PCR were: HindIII-*35S-f* and *35S-MCS-r* for 35S promoter, and *MCS-Nos-f* and *Nos-SacI- r* for Nos terminator amplification. The gain-of-function mutation of CCaMK (CCaMK^{T265D}) was constructed as follows: The mutation was introduced by PCR using 2 primer combinations, followed by PCR ligation of 2 fragments with the outermost primer combination. The primers for PCR were: XbaI-CCaMK-f and CCaMK-TD-r for the 5′ region of *CCaMK*, and *CCaMK-TD-f* and *CCaMK-AscI-r* for the 3′ region of *CCaMK*. All primer sequences are shown in Table S4. The final PCR product was digested with XbaI and AscI, and inserted in the XbaI-AscI site of p35S/GFP.

For hairy root complementation of *Ljcyclops-3* with rice *OsCYCLOPS*, *CYCLOPS* coding sequences were amplified from cDNA preparations (SuperScript II, Invitrogen) obtained from uninoculated root material of *O. sativa* subspecies *japonica*, cultivar Nipponbare, or *L. japonicus* ecotype Gifu roots and cloned into a pCAMBIA 1301 derived vector. *L. japonicus* and rice *CYCLOPS* coding sequences were amplified by using primers LjCYCL_ATG_f with LjCYCL_TAA_r and OsCYCL_ATG_f with OsCYCL_TAA_r, respectively. A 285-bp fragment amplified with primers polyA_NOS_f and polyA_NOS_r from pJawohl8 RNAi (kind gift of P. Schulze-Lefert, MPI for Plant Breeding Research, Cologne, Germany) was used as the terminator in all constructs. *CYCLOPS* coding sequences were fused to a 2,271 bp genomic sequence, preceding the *LjCYCLOPS* transcription start site and containing the promoter. Primer sequences are listed in Table S4.

Plant Transformation and Microscopy. The *CYCLOPS* promoter-GUS construct, the *LjCYCLOPS* and *OsCYCLOPS* genome sequence constructs, *CCaMK-GFP*, *RFP-CYCLOPS*, *CCaMK* WT, and *CCaMK^{T265D}* cDNA constructs were introduced into *L. japonicus* WT ecotype Gifu or *cyclops* mutant hairy roots by *Agrobacterium rhizogenes* AR1193 (7) according to (8) with minor modifications. *L. japonicus cyclops* mutants identified in genetic screens and through TILLING (9) are listed in Table S1. Plant growth conditions and inoculation with *M. loti* MAFF303099 or BN02 were described previously (10). Signals were detected with an Olympus SZX12 stereomicroscope (Figs. S4 and S6 *E* and *F*), a Leica SP5 confocal laser scanning microscope (Fig. S8), or a Leica MZFLIII stereomicroscope (Fig. 2).

Transformation of *cyclops-4* with Gain-of-Function CCaMK^{T265D} and Plant Cultivation Conditions. Seven-day-old *L. japonicus* Gifu WT or *cyclops-4* seedlings were uprooted and inoculated with *Agrobacterium rhizogenes* strain LBA1334 (11) harboring a WT CCaMK or a gain-of-function CCaMK^{T265D} construct, both

fused to the CaMV 35S promoter. After 5 days of cocultivation, plants were transferred to HRE medium, and after 6 more days, they were transferred to vermiculite. After 8 weeks, hairy roots were examined, and the number of spontaneous nodules was analyzed.

Subcellular Localization, BiFC Analysis, and Microscopy. For transient transformation of *N. benthamiana* leaves (12), *Agrobacterium tumefaciens* strains GV3101 pMP90 (13) and AGL1 (14) were used for GFP-fusions and BiFC experiments, respectively. *Agrobacterium* strains carrying a plasmid encoding the P19 silencing suppressor (15) and CaMV 35S promoter-driven RFP were cotransformed for enhancing expression and as a transformation control, respectively. Leaf cells were observed with a Leica DMI4000B inverse fluorescence microscope with GFP (excitation: BP 470/40, bichromatic mirror 500; emission: BP 525:50) and YFP (excitation: BP 500/20, dichromatic mirror 515; emission: BP 535:30) filter sets (Figs. 3 *C–F* and S7). Images were taken either with a Leica DFC300FX or DFC350FX R2 digital camera.

In Vitro Kinase Assays. CYCLOPS and CYCLOPS derivatives (1–449, 81–366 and 255–518) were expressed from pDEST17 (Invitrogen) as N-terminal 6xHis-tagged fusion proteins in *E. coli* strain Rosetta pLacI (Novagen). Expression products were affinity purified via nickel-agarose (Qiagen) under denaturing conditions by using 8 M urea (CYCLOPS, CYCLOPS 1–449 and 81–366), or native conditions (CYCLOPS 255–518), respectively. Denatured proteins were refolded by stepwise dialysis. Dialysis was carried out against buffer A (20 mM Pipes, 200 mM KCl, 250 mM arginine, 10% glycerol, 1 mM DTT, pH 7.9) containing 6 M, 4 M, 2 M, 1 M, and 0 M urea. Dialysed protein samples were centrifuged (50,000 × *g*, 30 min, 4 °C) and the supernatant was used for kinase assays. Protein elutions obtained from native purification (CYCLOPS 255–518) were combined and transferred into buffer A by PD-10 column (GE Healthcare). Purification of maltose binding protein (MBP) tagged CCaMK and in vitro kinase assays were described previously (5). Each reaction was carried out by using 1-μg MBP-CCaMK protein and 2-μg full-length 6xHis-CYCLOPS, or derivatives, as substrate. Phosphorylation reactions were performed at 25 °C for 30 min in the presence of either 4 mM EGTA or 0.1 mM $CaCl_2$ with or without 0.5 μM bovine calmodulin (Sigma).

Complementation Analysis With *OsCYCLOPS*. For analysis of root nodule symbiosis complementation, *A. rhizogenes*-induced transgenic roots of *L. japonicus* WT Gifu and of the *cyclops-3* mutant line (Table S1) (transformed with the *OsCYCLOPS*, or *LjCYCLOPS* coding sequence, respectively, each fused to the *LjCYCLOPS* promoter) were inoculated with *M. loti* MAFF303099 expressing *DsRED*. After 4 weeks of cocultivation (WOC), plants were analyzed for the simultaneous presence of nodules and ITs. For AM complementation analysis, transformed plants were cocultivated with *G. intraradices* BEG195, and harvested after 3 WOC; transformed roots were stained as described (16). Roots were scored "AM+", if symbiotic structures (arbuscules and vesicles) were present, and as "AM−", if no arbuscules and no vesicles were observed. Roots were considered complemented and scored AM+, when aborted infection sites in co-occurrence with successful infection, and fungal colonization events, accompanied by arbuscule and vesicle formation, were observed.

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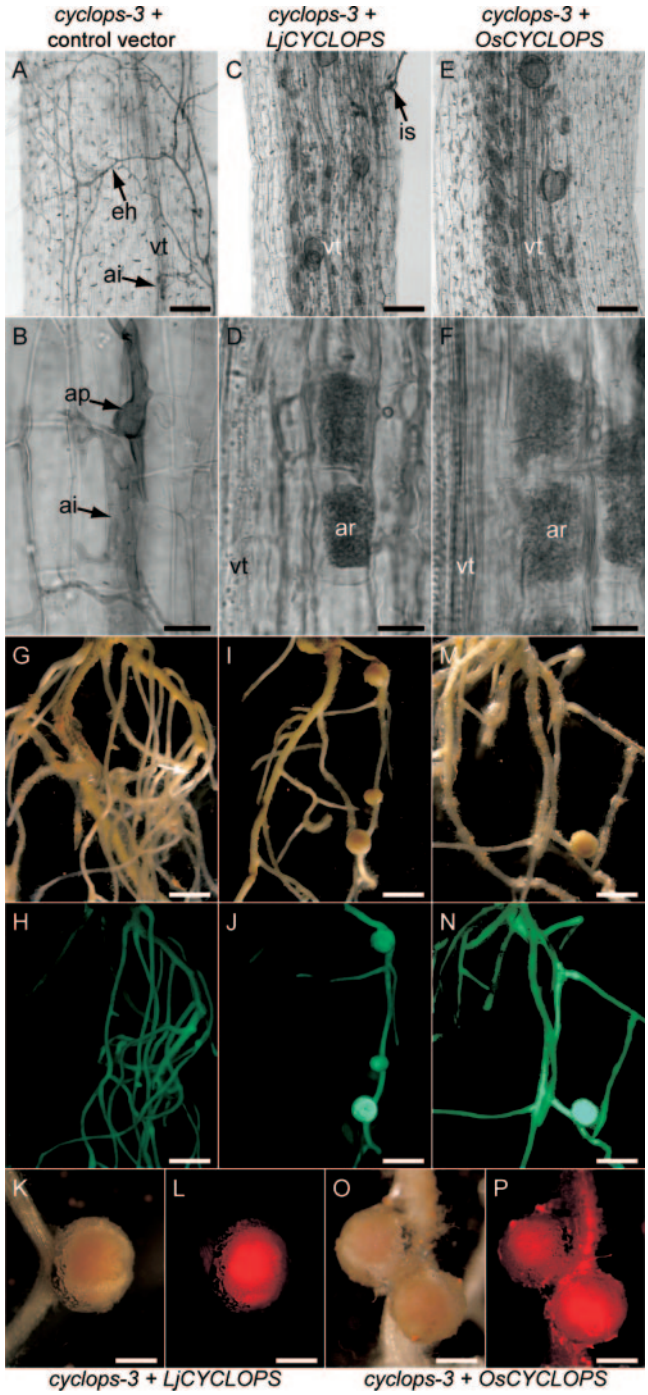

Fig. S1. *OsCYCLOPS* restores both fungal and bacterial endosymbioses in *Lotus cyclops-3* mutants. Transgenic roots were *A. rhizogenes* induced and selected via fluorescence of eGFP encoded on the transfer DNA. (*A*, *B*, *G*, and *H*) *cyclops-3* roots transformed with a control vector lacking a *CYCLOPS* expression cassette. (*C*, *D*, and *I–L*) *cyclops-3* roots carrying the *LjCYCLOPS* coding sequence and (*E*, *F*, and *M–P*), the *OsCYCLOPS* coding sequence, both under control of the *LjCYCLOPS* promoter region. (*A–F*) Roots cocultivated with *G. intraradices* for 3 weeks. (*A* and *B*) Transgenic control root with extraradical mycelium, but no intraradical fungal colonization or arbuscule formation. (*A and B*) Fungal infection attempts aborted within the epidermal or outer cortical tissue. (*C–F*) *cyclops-3* roots carrying the *LjCYCLOPS* (*C* and *D*) and *OsCYCLOPS* (*E* and *F*) coding sequences, showing dense fungal colonization (*C* and *E*) and arbuscule formation in the inner cortical tissue (*D* and *F*). (*G–P*) Root systems inoculated with a *DsRED* expressing strain of *M. loti* and incubated for 4 weeks. (*G* and *H*) Transgenic control root without mature nodules. Small primordial swellings (arrow in *G*) do not contain bacteria (not shown). Such swellings are typically formed in *Ljcyclops* mutant roots upon *M. loti* inoculation (17, 20). (*I–P*) *cyclops-3* roots carrying the *LjCYCLOPS* (*I–L*) and *OsCYCLOPS* (*M–P*) coding sequences, showing mature nodules. Nodules exhibit pink coloration in white light (*K* and *O*) and red fluorescence of the inner nodule tissue (*L* and *P*) indicating the presence of symbiosis-specific leghemoglobins and of *DsRED*- expressing bacteria, respectively. (ai, aborted infection; ap, fungal appressorium; ar, arbuscule; eh, extraradical hypha; is, infection site; vt, root vasculature.) [Scale bars: 0.1 mm (*A*, *C*, and *E*); 0.02 mm (*B*, *D*, and *F*); 2 mm (*G–J* and *M–N*); 0.5 mm (*K–L* and *O–P*).]

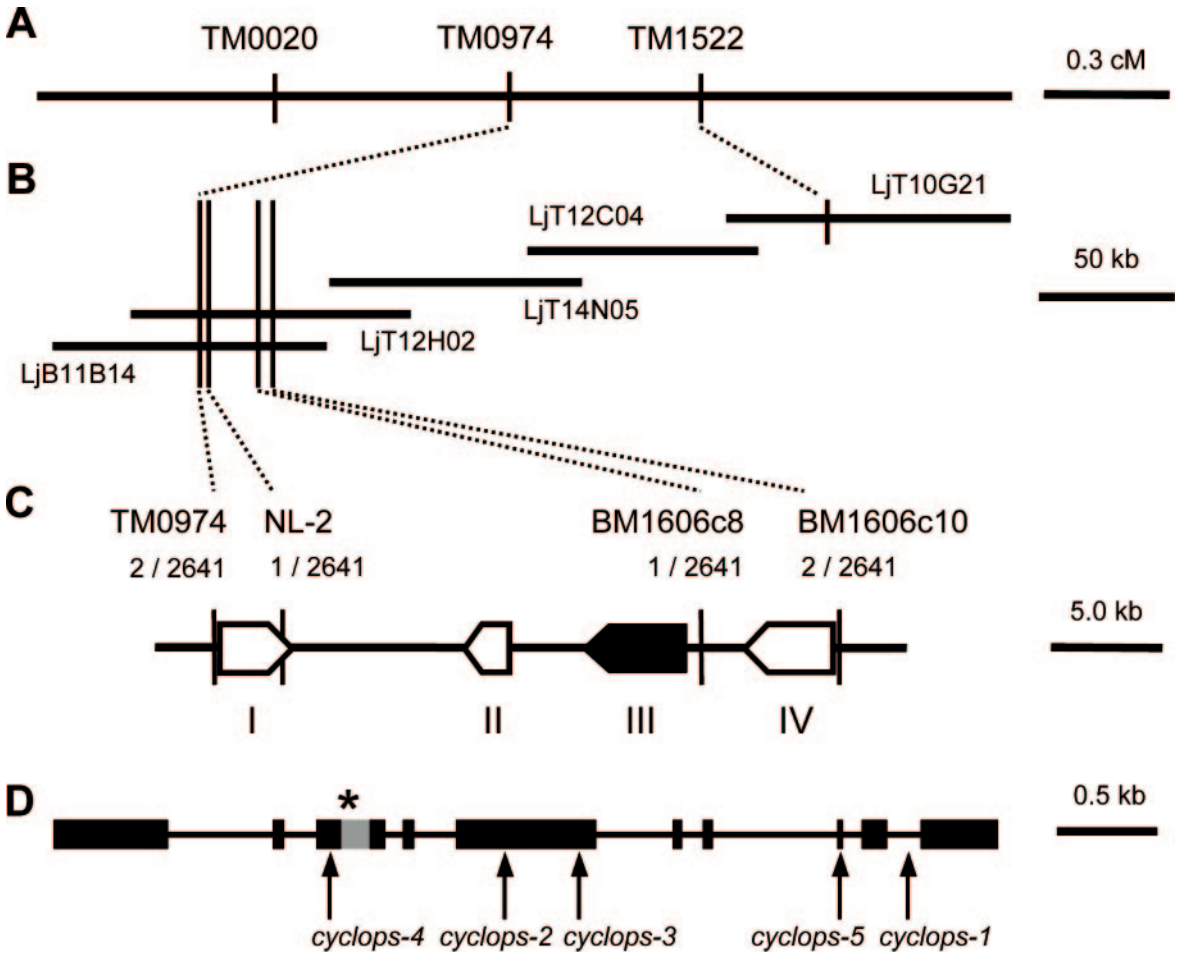

Fig. S2. Positional cloning of *CYCLOPS*. (*A*) Genetic map of the *CYCLOPS* locus on linkage group 2. TM0020, TM0974, and TM1522 are SSR markers. (*B*) Physical map of the *CYCLOPS* locus. LjB11B14 is a BAC clone, LjT12H02, LjT14N05, LjT12C04, and LjT10G21 are TAC clones. Two F_2 populations from *cyclops-3* × MG-20 (458 plants) and *cyclops-4* × MG-20 (2,183 plants) were analyzed for mapping. (*C*) Candidate genes in the *CYCLOPS* locus delimited by recombination events. (I, NBS-LRR type disease resistance protein; II, hypothetical protein; III, CYCLOPS; IV, ATP-dependent Clp protease-like protein.) Positions of markers are indicated together with the number of recombinant plants in the mapping population. (*D*) Gene structure of *CYCLOPS*. Black boxes indicate exons. Gray box indicates alternative splicing (retention of intron 3) identified through sequence analysis of cDNA clones and RT-PCR across exon borders. The asterisk indicates the position of a premature stop codon in the splice variant. Arrows indicate the positions of mutations in *cyclops* alleles.

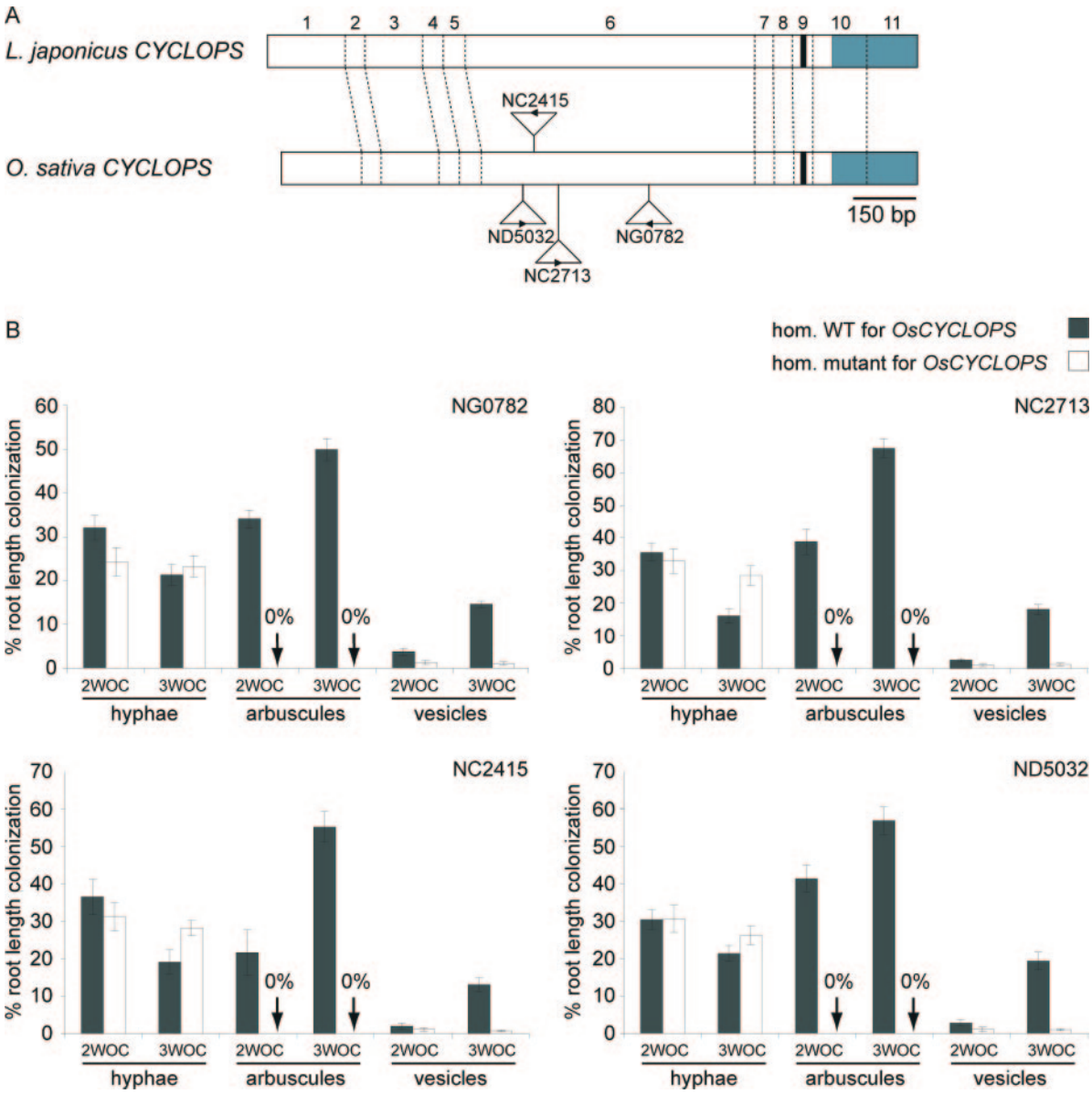

Fig. S3. Characterization of *O. sativa cyclops Tos17* insertion lines. (*A*) Exon structures of *L. japonicus* (*Lj*) *CYCLOPS* and rice (*Os*) *CYCLOPS* genes are conserved. Exons are labeled 1–11. Only regions covering the coding sequences are represented. Dotted lines indicate intron positions, black bars represent predicted nuclear localization signals (NLSs) and gray bars are coiled-coil regions in the conceptual amino acid sequences. Only the functionally confirmed NLS (Fig. S7) of *L. japonicus* CYCLOPS is depicted. The positions of *Tos17* insertions in the 4 rice *cyclops* lines are indicated. (*B*) AM quantification in rice *cyclops* insertion lines after 2 and 3 weeks of coculture (WOC) with *G. intraradices*. For each of the lines and at each time point, roots of 12 WT and 12 mutant plants were tested. Values are derived from 100 intersects randomly scored in 4 roots per plant. Intersects were scored as containing arbuscules, vesicles, or intraradical hyphae only. Mean values ± SD are shown. (Hom., homozygous; WT, wild-type plants.)

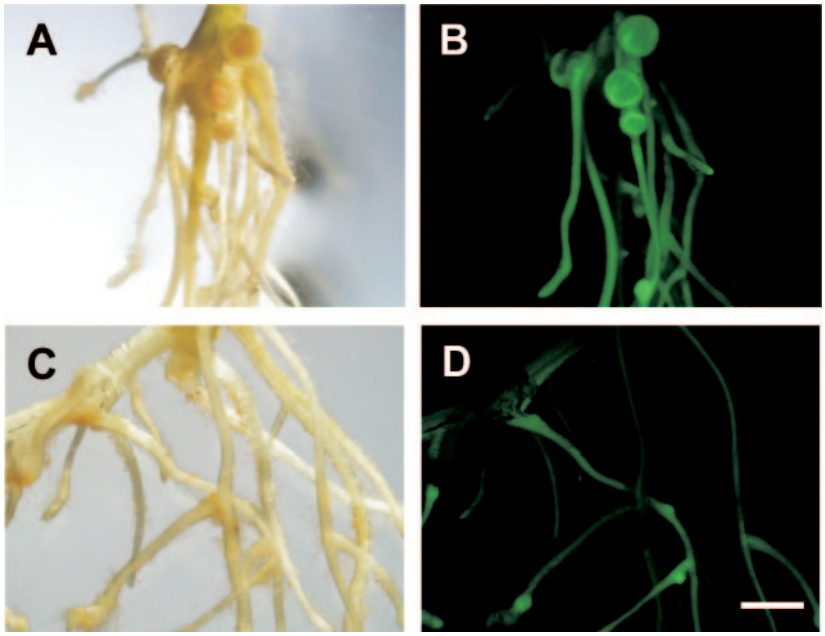

Fig. S4. Complementation of *cyclops-3* with the *CYCLOPS* genomic sequence. Hairy roots were induced via *A. rhizogenes*-mediated transformation, and inoculated with *M. loti* MAFF303099. (*A* and *B*) A vector containing the *CYCLOPS* genomic sequence including its own promoter was introduced into mutant plants homozygous for the *cyclops-3* allele. Nodule development was observed in 27 of 30 transformed plants, with an average number of 4.0 ± 1.8 fully developed, WT-like nodules per plant. (*C* and *D*) The *cyclops-3* mutant was transformed with an empty vector. None of the 29 transformed plants developed WT-like nodules. (*B* and *D*) GFP fluorescence indicating transformed hairy roots. (Scale bar, 2 mm.)

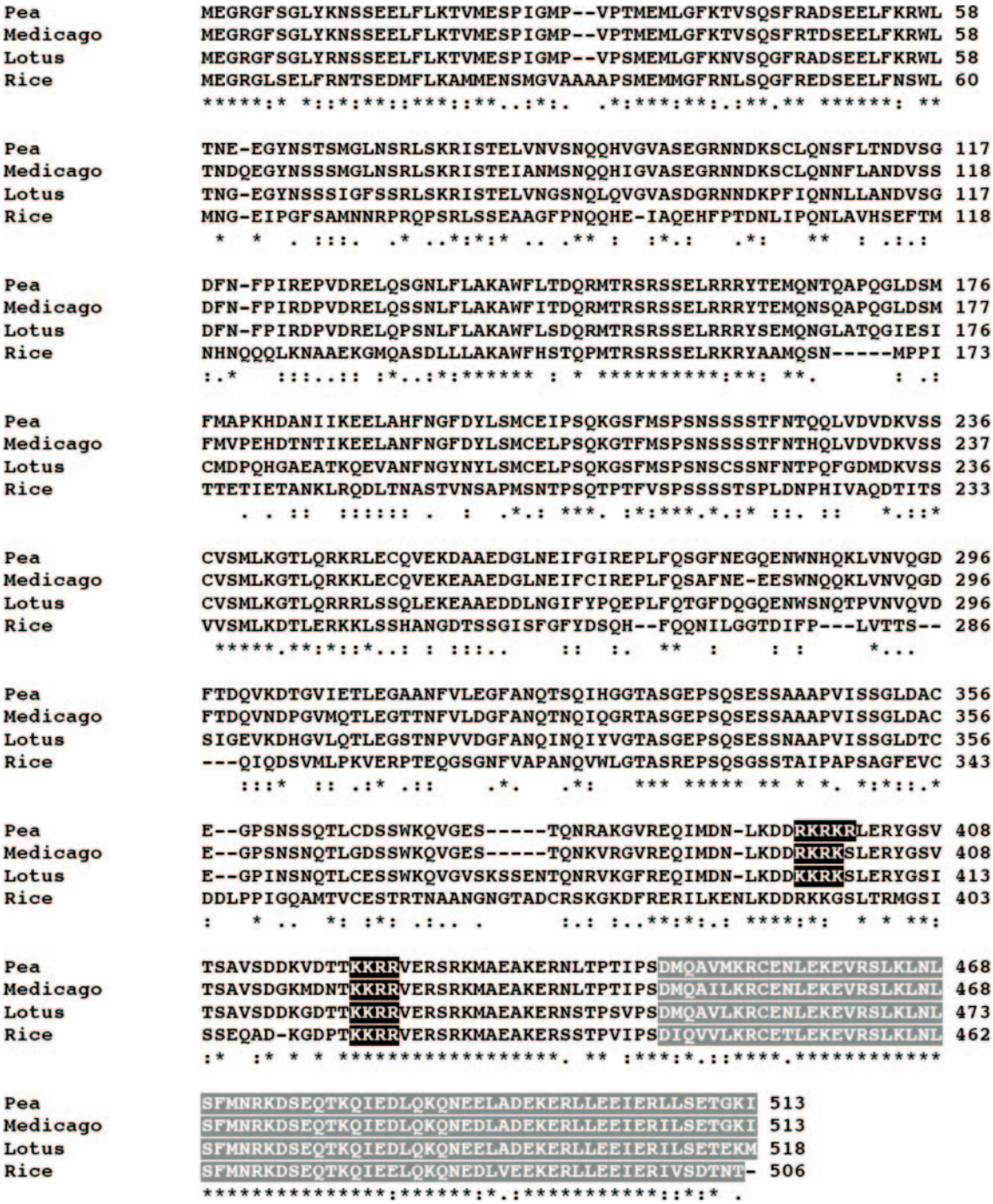

Fig. S5. Alignment of the *Lotus japonicus*, *Medicago truncatula*, *Pisum sativum* (pea), and *Oryza sativa* (rice) CYCLOPS proteins. Accession numbers are as follows: LjCYCLOPS, ABU63668; MtCYCLOPS, ABU63671; PsCYCLOPS, ABU63669; OsCYCLOPS, ABU63670. Alignment was done with CLUSTALW (http://align.genome.jp/). Residues that are identical among all 4 protein sequences (*), conserved substitutions (:), and semiconserved substitutions (.) are indicated. *Medicago*, pea, and rice CYCLOPS comprise 513, 513, and 506 residues and have overall 78%, 79%, and 47% sequence identity with *L. japonicus* CYCLOPS, respectively. Two predicted nuclear localization signals (NLSs) identified by PSORT (http://www.psort.org/) are highlighted in black. Note that the 1st NLS of pea CYCLOPS is overlapped by 2 redundant sequences of 4 amino acid residues (RKRK and KRKR). Only 1 NLS was predicted in rice CYCLOPS. A predicted coiled-coil motif at the C terminus of the protein (http://www.ch.embnet.org/software/COILS_form.html) (21) is highlighted in gray (threshold value is 0.999 with 28 residues scan).

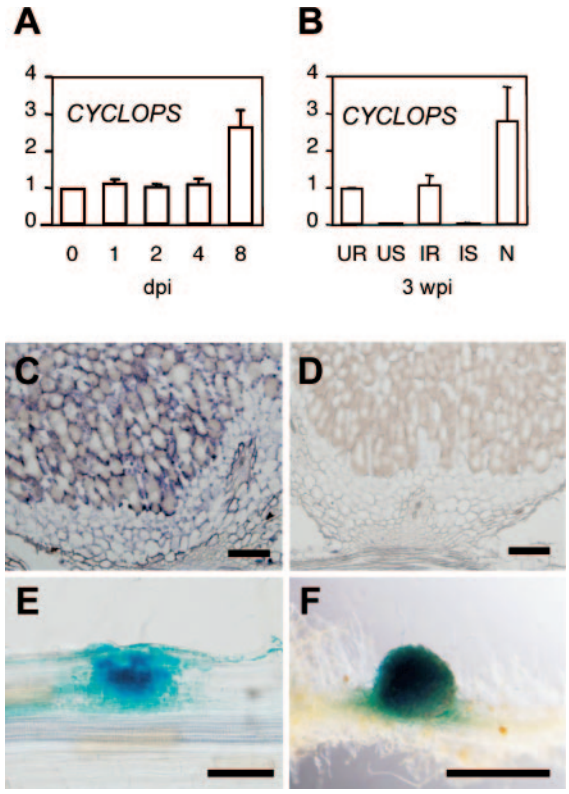

Fig. S6. Expression of *CYCLOPS* in response to *M. loti* MAFF303099. Quantitative RT-PCR analysis of (*A*) *CYCLOPS* expression in WT roots, noninoculated (0 days) and at 1, 2, 4, and 8 days post inoculation (dpi). (*B*) *CYCLOPS* expression in uninoculated WT *L. japonicus* roots (UR) and shoots (US), and in inoculated shoots (IS), infected roots without nodules (IR), and nodules (N) 3 weeks post inoculation (wpi). Fold increases in expression are shown relative to uninoculated roots (*A* and *B*). Mean values ± standard deviations are shown. (*C* and *D*) In situ hybridization of nodules with *CYCLOPS* antisense probe (*C*) and sense probe (*D*). (Scale bars, 100 μm.) (*E* and *F*) Activity of the *CYCLOPS* promoter fused to the *GUS* reporter gene in WT roots. (*E*) Dividing cortical cells in the root inoculated with *M. loti*. (*F*) Mature nodule. [Scale bars, 100 μm (*E*) and 1.0 mm (*F*).]

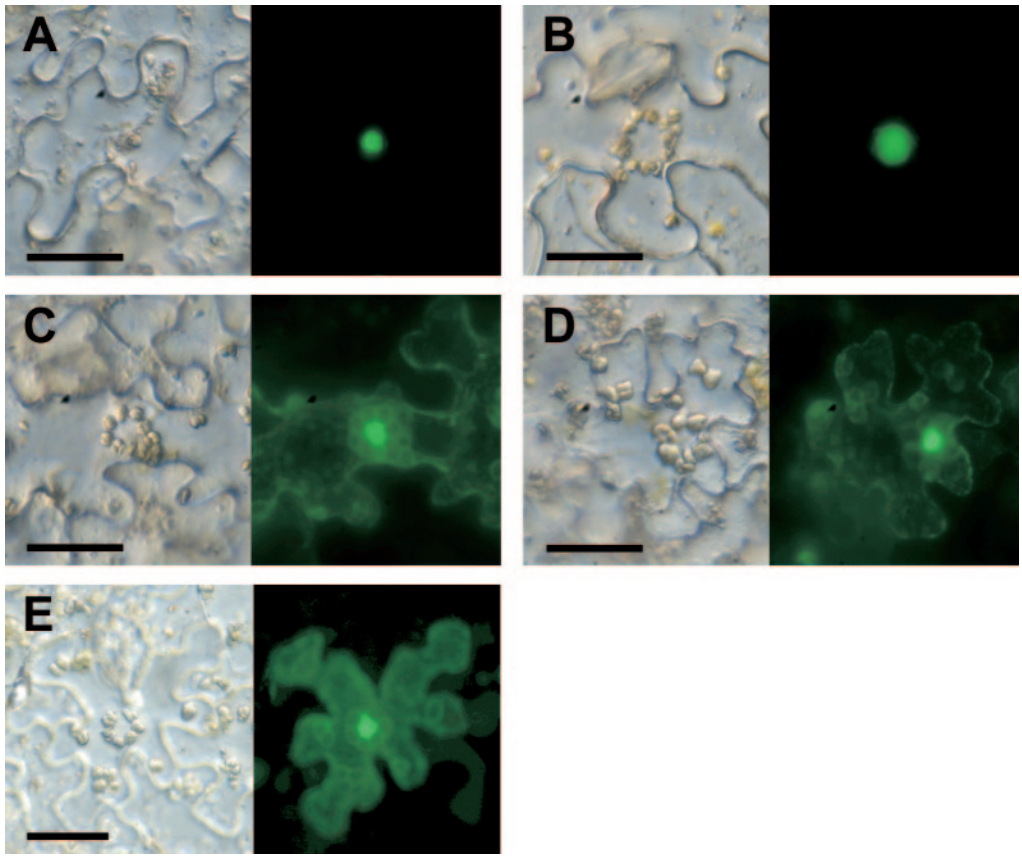

Fig. S7. Subcellular localization of CYCLOPS. Fusions of GFP to the N terminus of either full-length or C-terminally truncated CYCLOPS derivatives were transiently expressed in *N. benthamiana leaves* via *A. tumefaciens* transformation. (*A*) GFP-CYCLOPS (full-length). (*B*) GFP-CYCLOPS 1–449, lacking the coiled-coil motif. (*C*) GFP-CYCLOPS 1–421 lacking the second NLS. (*D*) GFP-CYCLOPS 1–366 lacking both NLSs. (*E*) Expression of free GFP. *Left Images* show the cell architecture (differential interference contrast); *Right Images* show GFP fluorescence of the same cell. (Scale bars, 50 μm.)

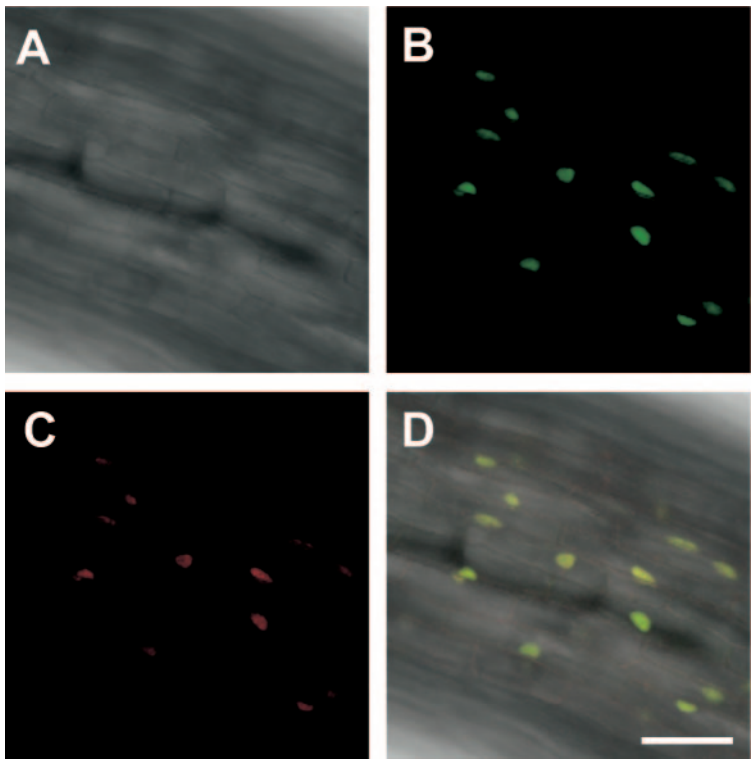

Fig. S8. Colocalization of CYCLOPS and CCaMK in *L. japonicus* root nuclei. *L. japonicus* WT seedlings were cotransformed with 2 *Agrobacterium* strains carrying either a CCaMK-GFP or RFP-CYCLOPS T-DNA construct. Cotransformed hairy roots were selected by fluorescence of both fusion proteins. (*A*) Laser transmission image. (*B*) CCaMK-GFP. (*C*) RFP-CYCLOPS. (*D*) Merged image of *A*–*C*. (Scale bar, 50 μm.)

Table S1. *cyclops* mutant alleles

Allele	Line	Previous allele	Ref(s)	Mutation		Phenotype	
				cDNA	Amino acid	Nod*	AM†
cyclops-1	10512.9	*sym6-1*	(17, 18)	Insertion between exons 10 and 11	+15 residues after R478	np	–
cyclops-2	1962-124	*sym6-2*	(18)	Deletion A737	+17 residues after L245	np	–
cyclops-3	EMS126	*sym30*, *sym6-3*	(17, 18, 19)	G1112A	W371 stop	np	–
cyclops-4	N-4	*sym82*	(20)	C319T	Q107 stop	np	–
cyclops-5	SL1347-2		(9, ‡)	Deletion G1281	+29 residues after K427	np	–

Complementation between *cyclops-1* and *cyclops-2* (18), or *cyclops-3* (17) was previously described.
*''np'' indicates nodule primordia with arrested infection within curled root hairs.
†''–'' indicates defects in AM symbiosis.
‡The *cyclops-5* allele was identified in this work through TILLING (9).

Table S2. Restoration of root symbioses in the *Lotus cyclops-3* mutant

Plant genotype	Transgene	AM+	IT	Nod+	Nodules/ nodulated plant
cyclops-3	marker only*	0/12	–	0/11	0
WT	*LjCYCLOPS*	12/12	+	30/32	5.0
cyclops-3	*LjCYCLOPS*	19/24	+	14/21	3.1
WT	*OsCYCLOPS*	14/14	+	16/16	7.3
cyclops-3	*OsCYCLOPS*	18/25	+	15/24	3.0

AM+, root systems containing arbuscules; IT, infection thread formation; Nod+, roots with fully developed nodules. Numbers refer to *A. rhizogenes*-transformed root systems. Numbers are compiled results of 2 independent experiments. Constructs were equipped with the *L. japonicus* (*Lj*) or rice (*Os*) *CYCLOPS* coding sequence and were under the control of the *LjCYCLOPS* promoter region.
*Plants were transformed with the respective binary vector lacking a *CYCLOPS* expression cassette.

Table S3. Spontaneous nodule formation with gain-of-function CCaMK

Genotype	Construct	Inoculation	Nod+/total*	Nod/plant†	Nod size, cm†
WT	T265D	–	27/28	7.19 ± 4.58	0.690 ± 0.159
cyclops-4	T265D	–	10/66	1.80 ± 1.03	0.699 ± 0.180
WT	T265T	–	0/44	0	–
cyclops-4	T265T	–	0/34	0	–

Spontaneous nodules (Nod) were developed without inoculation. Nod were scored 8 weeks after transplanting.
*Number of plants with spontaneous nodules per number of transformed plants.
†Average ± SD is shown.

Table S4. Primer sequences

Target sequence		Primer sequence
Expression analysis:		
CYCLOPS	forward	5′-GCTGGCAGATGAAAAAGAGC-3′
	reverse	5′-GCGTGTTTGAGCACAACATT-3′
NIN	forward	5′-TGGATCAGCTAGCATGGAAT-3′
	reverse	5′-TCTGCTTCTGCTGTTGTCAC-3′
ENOD40-1	forward	5′-TCACCATGGAGGTATGCTCA-3′
	reverse	5′-TTTGGAACAGCACAAGTTGG-3′
EF-1	forward	5′-GCAGGTCTTTGTGTCAAGTCTT-3′
	reverse	5′-CGATCCAGAACCCAGTTCT-3′
Plasmid construction:		
CYCLOPS promoter	forward	5′-ATGGATCCCCCAGAGATCCCTGATGATG-3′
	reverse	5′-ATGGCGCGCCTTCCAATTGAAGCTGTTGTTTTC-3′
CYCLOPS transcript terminator	forward	5′-ATGGCGCGCCTATGAGAATCAATGTTGTGCTCAAACACG-3′
	reverse	5′-ATGAGCTCTGTTAATTTTGTTAGCTCAAGTGG-3′
GUSPlus 1st PCR	forward	5′-AAAAAGCAGGCTACCATGGTAGATCTGAGGGTAA-3′
	reverse	5′-AGAAAGCTGGGTTCACACGTGATGGTGATGGT-3′
GUSPlus 2nd PCR	forward	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′
	reverse	5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′
CYCLOPS genomic sequence 1st PCR	forward	5′-AAAAAGCAGGCTTGGAAATGGAAGGGAGGGG-3′
	reverse	5′-AGAAAGCTGGGTTCATTACATTTTTTCAGTTTCTGATAGAATTC-3′
CYCLOPS genomic sequence 2nd PCR	forward	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′
	reverse	5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′
In situ hybridization:		
CYCLOPS cDNA (493 bp)	forward	5′-CTGCGAAAGCTCATGGAAAC-3′
	reverse	5′-GCGTGTTTGAGCACAACATT-3′
Primer name		**Primer sequence**
Complementation analysis of *cyclops-4* with gain-of-function CCaMK:		
HindIII-35S-f	forward	5′-TCCAGTAAGCTTTCCCCAGATTAGCCTTTTCA-3′
35S-MCS-r	reverse	5′-CCTAGGTACCGGATCCACTAGTAGGCCTTCTAGAGTCCCCCGTGTTCT-3′
MCS-Nos-f	forward	5′-AGTGGATCCGGTACCTAGGCGCGCGCCATCGATCGTTCAAACATTTGGC-3′
Nos-SacI-r	reverse	5′-AATCGGAGCTCGATCTAGTAACATAGATGACACCG-3′
XbaI-CCaMK-f	forward	5′-GCTCTAGAATGGGATATGATCAAACCAGAAAG-3′
CCaMK-TD-r	reverse	5′-CCTTCCAATCCTTCTCATAGAAAC-3′
CCaMK-TD-f	forward	5′-TCAGTTTCTATGAGAAGGATTGGA-3′
CCaMK-AscI-r	reverse	5′-AGGCGCGCCCTATGATGGACGAAGAGAAGAGA-3′
Genotyping of *O. sativa Tos17* insertion lines:		
OsCYCL_In3_f	forward	5′-AGGCATTTTCATCACCCATC-3′
OsCYCL_Ex6_f	forward	5′-CACCCAGTCAGACTCCAACA-3′
OsCYCL_Ex6_r	reverse	5′-ATGCTGTACCAAGCCAAACC-3′
Tos17_f/r	for/rev	5′-ATTGTTAGGTTGCAAGTTAGTTAAGA-3′
***OsCYCLOPS* cross-species complementation analysis*:**		
LjCYCL_ATG_f	forward	5′-aattaacctaggatccATGGAAGGGAGGGGGTTTTCTGG-3′
LjCYCL_TAA_r	reverse	5′-aataatcccgggTTACATTTTTTCAGTTTCTGATAG-3′
OsCYCL_ATG_f	forward	5′-aattaacctaggatccATGGAGGGCAGGGGTCTGTCTGAG-3′
OsCYCL_TAA_r	reverse	5′-aataatcccgggTTATGTGTTGGTATCAGAGACG-3′
polyA_NOS_f	forward	5′-aataaacctaggATCAGCTTGCATGCCGGTCG-3′
polyA_NOS_r	reverse	5′-aaataagtcgacCTAGAGTCAAGCAGATCGTTCAAAC-3′

*Lower case characters indicate engineered sequences including restriction sites.

5.3 Curriculum Vitae

CURRICULUM VITAE

KATHARINA MARKMANN

PERSONAL DATA

Date of Birth	Nov. 02, 1976
Place of Birth	Telgte (North-Rhine-Westphalia, Germany)

PRIMARY AND SECONDARY EDUCATION

1983-1987	Everwordt-Grundschule Freckenhorst
1987-1996	Gymnasium Laurentianum Warendorf
Aug.-Dec. 1993	Niles North High School (Chicago, Illinois, USA)
June 1996	Abitur (Final examination)

HIGHER EDUCATION

Oct. 1996- Jan. 2003	Philipps-Universität Marburg Degree program: Biology (diploma); main subjects: Mycology, Ecology, Cell Biology/Applied Botany
July 1998	Pre-Diploma examination; average: 1.0
Sept. 1998-June 1999	Wilfrid Laurier University (Waterloo, Ontario, Canada); courses in Biology and Anthropology (student exchange program)
Dec. 2001-Nov. 2002	Diploma thesis (Dept. of Mycology, Prof. Dr. Gerhard Kost); average: 1.0
Nov. 2002	Diploma examination; average: 1.0
Oct. 2003-Oct. 2004	PhD (The Sainsbury Laboratory, John Innes Centre, Norwich, United Kingdom; Prof. Dr. Martin Parniske)
Oct. 2004-July 2008	PhD continued (Ludwig-Maximilians-Universität München, Genetics; Prof. Dr. Martin Parniske)
Aug. 2008-present	Post-doctoral scientist (Centre for carbohydrate recognition and signalling (CARB), University of Aarhus, Aarhus, Denmark; Prof. Dr. Jens Stougaard)

INTERNATIONAL CONFERENCES

July 24-27, 2004	6th European Nitrogen Fixation Conference (Toulouse, France; joint poster presentation)
July 22-26, 2006	7th European Nitrogen Fixation Conference (Aarhus, Denmark; poster presentation)
Jan. 21-26, 2007	Joint 15th International Congress on Nitogen Fixation & 12th International Conference of the African Association for Biological Nitrogen Fixation (Cape Town, South Africa; invited oral presentation)
Aug. 30-Sept. 03, 2008	8th European Nitrogen Fixation Conference (Ghent, Belgium; poster presentation)

AWARDS AND GRANTS

Scholarship	Scholarship of the German National Merit Foundation (Studienstiftung des deutschen Volkes) (Jan. 2000- Jan. 2003)
Course Studentship	Spanish language course, funded by the German National Merit Foundation (April 2003)
Poster Award	1st poster prize at the 7th European Nitrogen Fixation Conference (Aarhus, Denmark)
Research Grants	Grants by the BFHZ (Bayerisch-Französisches Hochschulzentrum) (2004- 2006) and PROCOPE/DAAD (Deutscher Akademischer Austausch Dienst) (2006-2007) supporting collaborative projects with a French research team

PUBLICATIONS

Markmann, K. and Parniske, M. (2009).
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Perry, J., Welham, T., Brachmann, A., Charpentier, M., **Markmann, K**., Wang, T. and Parniske, M.
Mining the symbiotic component of the *Lotus japonicus* genome using classical genetics and thematic TILLING. Manuscript in preparation.