Comparative and functional analysis of NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 in rice and Medicago

Wei Liu

Thesis committee

Promotor
Prof. dr. T. Bisseling
Professor of Molecular Biology
Wageningen University

Co-Promotor
Dr. ir. R. Geurts
Associate professor, Laboratory of Molecular Biology
Wageningen University

Other members
Prof. dr. M.E. Schranz, Wageningen University
Prof. dr. W.-C. Yang, Chinese Academy of Sciences, Beijing, China
Dr. Y. Bai, Wageningen University
Dr. G.B. de Deijn, Wageningen University

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

Comparative and functional analysis of NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 in rice and Medicago

Wei Liu

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof.dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 26 August 2013
at 1:30 p.m. in the Aula.

Wei Liu

Comparative and functional analysis of NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 in rice and Medicago.

147 pages.

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English

ISBN 978-94-6173-636-9

List of contents

Chapter 1

From evolution of legume-rhizobium symbiosis

to

revolution of nitrogen fixing crops

Wei Liu

Department of Plant Science, Laboratory of Molecular Biology, Wageningen University, 6708 PB Wageningen, The Netherlands

According to reports of the United Nations, the global human population will consistently reach 9.1 billion by 2050 (FAO, 2009). Consequently, agricultural production should steadily increase to keep pace with food demands. In past decades, increase of crop production was achieved mainly by application of artificial fertilizers and to date, about half of the world's population is dependent on fertilizer-driven agriculture. Especially, production of ammonia through the industrial Haber-Bosch process is an invention with highest impact (Smil, 1999). However, downside of this success is the unprecedented perturbation of the nitrogen cycle, which is considered as the second most disturbing anthropogenic activity (Erisman et al., 2008; Rockstrom et al., 2009; Sutton et al., 2011). Production of artificial fertilizer is responsible for ~30% of the fossil fuel consumption in agriculture, thereby contributing to greenhouse effects (Wood and Cowie, 2004). Even more critical is the extreme low 'use efficiency' of fertilizers (Robertson et al., 2011). Most of the applied fixed nitrogen is not taken up by the plant, but runs off and causes eutrophication in terrestrial and aquatic systems (Snyder et al., 2009). In addition, it increases the activity of denitrifying bacteria causing release of the highly potent greenhouse gas nitrous oxide (Erisman et al., 2008). Taken together these environmental effects, in combination with the increasing costs of fertilizer, it is inevitable that future agriculture has to rely on less fertilizer inputs (Olivares et al., 2013).

Legume plants (Fabaceae) have overcome the problem of nitrogen limitation by establishing an endosymbiosis with nitrogen fixing rhizobium bacteria. Symbiotic diazotrophic rhizobia form a paraphyletic group of different genera of α and β-proteobacteria (Sawada et al., 2003). These bacteria have in common that they combine their ability to fix molecular dinitrogen, with a set of symbiotic genes that enable them to live in endosymbiosis with legumes. To accommodate rhizobium, novel root lateral organs are formed, so-called nodules. In nodules, rhizobium finds the optimal conditions to fix atmosphere nitrogen gas into ammonia, which is transported to the plant. In return, the legume plants reward the bacteria with carbohydrates and other nutrients. Transfer of the nitrogen fixing rhizobium symbiosis to non-legume crops in a biotech approach is seen as an alternative strategy to reduce artificial fertilizer input (Charpentier and Oldroyd, 2010; Beatty and Good, 2011; Sutherland et al., 2012). Activities in this direction gained new attention driven by the knowledge obtained with model legumes (see below). The research presented in this thesis can be seen in light of this activity. In this introductory chapter, I will present current knowledge

of nodule formation in legumes and discuss the possibility to transfer this trait to non-legume crops.

Root nodule formation in legume species

The Legume family found it origin shortly after the Cretaceous-Paleogene extinction, 60-65 million years ago, and rapidly diverged into 14 crown clades that to date represent over 18,000 species (Lavin et al., 2005) (Figure 1A). The vast majority of these species are able to establish a nitrogen fixing symbiosis with rhizobium, suggesting early evolution of this character in the legume family (Doyle, 2011). The relatively long period of evolution of the symbiosis in different crown clades resulted in significant variation of this trait; e.g. in nodule histology (Figure 1B to 1F). However, all nodule types have a large central tissue in common that hosts the bacteria intracellularly, generally as transient organelle-like structures, named symbiosomes. Symbiosomes contain one, or multiple, rhizobium bacteria that are surrounded by a plant derived membrane; the peribacteroid membrane (Roth and Stacey, 1989). A single nodule cell can be packed with hundreds of symbiosomes, so a legume nodule can host thousands of symbiotic rhizobia that provide ammonia to the host, and in return obtain carbohydrates and other nutrients. The exchange of nutrients is facilitated by the peribacteroid membrane, which functions as a symbiotic interface between plant and microbe. Some legumes in the *Caesalpinioid* and *Dalbergioids* tribes host rhizobia in thread-like structures; so-called fixation threads (Naisbitt et al., 1992; Lavin et al., 2001). Like symbiosomes, these threads are surrounded by a plant-derived membrane. In general fixation-threads are considered to be more primitive when compared to symbiosomes (Brewin, 1998; Sprent, 2007).

Root nodule formation is triggered by rhizobium secreted signal molecules; named Nodulation (Nod) factors. Nod factors are lipo-chitooligosaccharides (LCOs), consisting of a β-1, 4-linked chitin oligomeric backbone with an N-linked fatty acid at the non-reducing glucosamine residue (Lerouge et al., 1990). Further modifications can be present at the chitin backbone that in part determines the host specificity of the legume-rhizobium interaction (Roche et al., 1991). When a legume root perceives Nod factors from a compatible rhizobium, some differentiated cortical parenchyma cells become mitotically active, resulting in the formation of a nodule primordium (Yang et al., 1994; Timmers et al., 1999). Simultaneously to the formation of root nodule primordia, the bacteria form infection structures to reach the dividing cortical cells. Generally, root hair first curls around a Nod factor producing

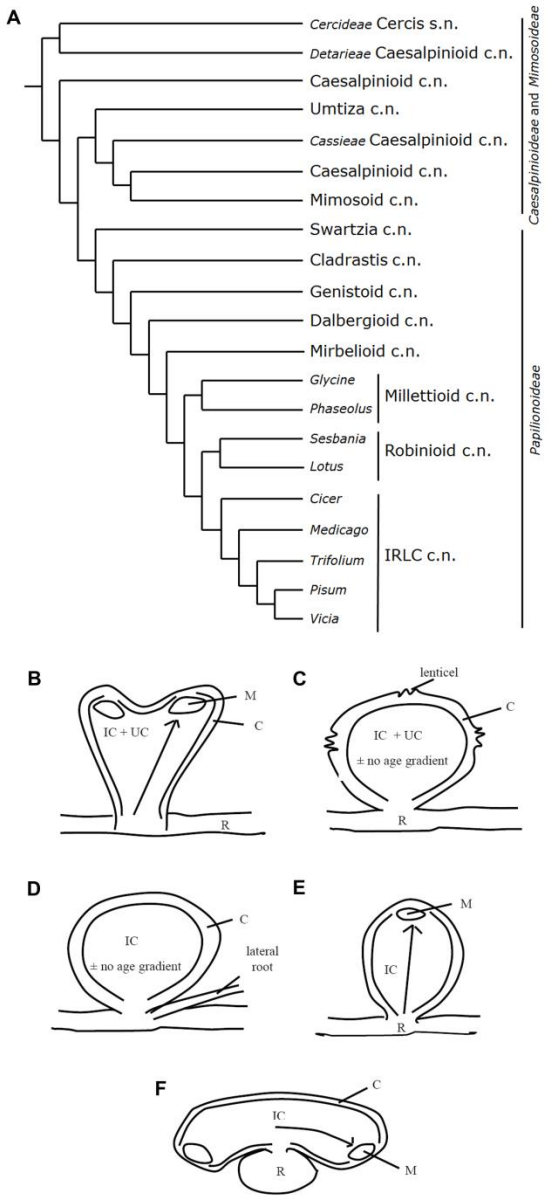


Figure 1. Legume phylogenetic tree and major types of nodules. Phylogenetic tree of legume is drawn based on Lavin et al. (2005) and Doyle (2011). All 14 crown nodes (c.n.) and one stem node (s.n.) are presented in **A**. IRLC: inverted-repeat-loss clade. Major types of nodule shape and structure are shown in **B-F** following Sprent et al. (2007). **B**: Indeterminate nodule as found in all three sub-families, with one or more apical meristem(s) (M), infected tissue with a mixture of infected (IC) and uninfected (UC) cells (the arrow indicates an age gradient with the head pointing to the youngest cells), nodule cortex (C), subtending root (R). **C**: Desmodioid nodule with lenticels on the outside and infected tissue of uniform age. **D**: Aeschynomenoid nodule. **E**: Indeterminate nodule, superficially like that of A, but with uniformly infected tissue in the center; this type may be common in tribe Genisteae. **F**: Lupinoid nodule.

rhizobium and entrap it in a closed pocket (Esseling et al., 2003; Jones et al., 2007). Then, the cell wall inside the pocket is partially degraded and a tube-like structure, called the infection thread, is formed upon invagination of the plasma membrane (Brewin, 2004). Infection threads grow towards the root cortical cells and this is accompanied by clonal propagation of the rhizobia. In this way the rhizobia reach the newly formed primordia where they are released from the infection thread as symbiosomes.

In some legumes belonging to several genera of the Dalbergioid and Genistoid crown clades, the rhizobium entry of legume root does not depend on root hair based infection thread formation (Chandler, 1978; Subba-Rao et al., 1995). In *Geniseae*, which includes *Lupinus*, infection threads are rarely present and the infections occur between epidermal cells close to the root hairs bases (González-Sama et al., 2004). In some legume species, e.g. *Trifolium repens* and *Lotus uliginosus*, besides to the infection through infection thread, under certain stress condition, the rhizobia can infect the plants through the breaks in the root epidermis or wounds formed by lateral roots outgrowth, therefore, named as crack entry (Sprent and James, 2007). In this case, intercellular or intracellular infection threads are formed in the outer cortical cells and direct the rhizobia to the growing nodule primordia. This more primitive infection mechanism is wide spread in more basal legume species.

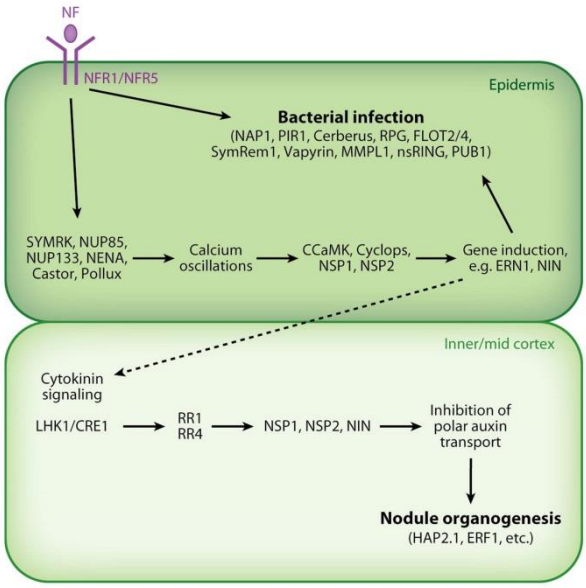
Legume model species to genetically dissect rhizobium LCO-based root nodulation

For two decades two legume species are used as model for genetic studies; namely *Lotus japonicus* (Lotus) and *Medicago truncatula* (Medicago) (Handberg and Stougaard, 1992; Cook, 1999). Both species represent crown clades that make different nodule types; Medicago (IRLC crown clade) has indeterminate nodules and Lotus (Robinioid crown clade) has determinate nodules (Figure 1A). These two main nodule types differ in the persistence of the meristem, which enables the nodule to grow (Figure 1B and 1C). Persistent meristem activity in an indeterminate nodule leads to a zonation of the nodule with subsequent developmental stages. Such nodule can be divided into 5 zones in the longitudinal direction from the tip to the base: the apical meristem zone I, where the cells are actively dividing; the infection zone II, where the infection threads reach and bacteria are released into the plant cells; the fixation zone III, where the bacteria differentiate and gain the ability to fix nitrogen, and therefore, is the main place for nitrogen fixation; the senescence zone IV, where the nitrogen fixation ceases and the cells enter degradation; and saprophytic zone V, where bacteria escape from

retained infection thread into the senesced plant cells (Vasse et al., 1990; Timmers et al., 2000). In contrast, the initial meristem of determinate nodules is not maintained and loses activity after some time, resulting in a round shaped morphology of the nodule. Indeterminate and determinate nodules also differ in ontogeny. Nodule primordia that give rise to indeterminate nodules always originate from root inner cortical cells, and can even involve root endodermis and pericycle cell layers. In contrast, primordia that will give rise to a determinate nodule are initiated in more outward positioned cortical cell layers. *De novo* primordium formation in roots is typically associated with a local auxin maximum. *In silico* modelling of auxin homeostasis revealed that a spatially controlled decrease of auxin efflux can result in an auxin maximum similar as observed in *in vivo* experiments using auxin reporter constructs (Deinum et al., 2012). The position of this local auxin maximum can be shifted (in or outward) by slight changes in the ratio of central to peripheral efflux activity, simulating the difference as observed between primordia of indeterminate and determinate nodule types.

Genetics in *Lotus japonicus* and *Medicago truncatula* identified a conserved set of symbiosis genes (Figure 2). Two types of LysM domain containing receptor kinases (named NFP and LYK3 in Medicago; NFR5 and NFR1 in Lotus) were identified to be essential for Nod factor recognition (Amor et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Smit et al., 2007). These plasma membrane localized receptor kinases function as a heterodimer transducing a signal into the cytosol (Madsen et al., 2011). Upon Nod factor perception a series of responses are induced in the root epidermal cells, starting with an influx of calcium ions followed by an efflux of chloride ions. These ion fluxes cause an alkalinisation of the cytoplasm and a depolarization of plasma membrane (Felle et al., 1998). Induction of these responses requires additional symbiotic genes that include the trans-membrane receptor-like kinase (named Symbiotic Receptor Kinase (SYMRK) in Lotus or DMI2 in Medicago) and specific cation ion channel proteins localized in the perinuclear membrane (CASTOR and POLLUX in Lotus or DMI1 in Medicago) (Endre et al., 2002; Imaizumi-Anraku et al., 2004; Bersoult et al., 2005; Limpens et al., 2005; Charpentier et al., 2008). Additionally, Nod factor induced calcium oscillations are facilitated by the nuclear pore complex compounds (NUP85 and NUP133 in Lotus) (Kanamori et al., 2006; Saito et al., 2007). The Ca^{2+} oscillations activate a nuclear localized protein complex of a Ca^{2+}/calmodulin dependent protein kinase (CCaMK/DMI3) and a facilitator protein (CYCLOPS in Lotus or IPD3 in Medicago) (Levy et al., 2004; Mitra et al., 2004; Yano et al., 2008; Horvath et al., 2011; Ovchinnikova et al.,

2011). CCaMK forms a key node in the symbiotic signalling network as dominant active forms of the CCaMK protein trigger spontaneous root nodule formation (Levy et al., 2004; Mitra et al., 2004; Gleason et al., 2006; Tirichine et al., 2006). To do so, several symbiotic transcription factors are essential, including two GRAS family transcriptional regulators NSP1 and NSP2 (Kalo et al., 2005; Smit et al., 2005), one ethylene response factor ERN1 (Andriankaja et al., 2007), *NODULE INCEPTION* (*NIN)* (Marsh et al., 2007) and one CCAAT-binding family transcription factor HAP2 (Combier et al., 2006).



(Oldroyd et al., 2011)

Figure 2. A schematic representing the Nod factor signalling involved in the nodule organogenesis and bacterial infection. Epidermal cells are capable of perceiving Nod factor (NF) through receptor-like kinases (NFR1/NFR5) that activate calcium spiking which require a suite of proteins (NUP85, NUP133, CASTOR, POLLUX). CCaMK perceives calcium spiking and functions with transcription factors (such as NSP1/2, NIN and ERN1) to activate gene expression. This signaling pathway has two outcomes: the initiation of bacterial infection at the epidermis and the promotion of cell division in the cortex which requires an unknown diffusible signal (dotted line). In the inner or mid-cortex, NF-induced cytokinin signaling involves the cytokinin receptor LHK1/CRE1, response regulators (RRs), and the transcription factors NSP1, NSP2, and NIN. A target for cytokinin signaling is the suppression of polar auxin transport that is sufficient to promote nodule organogenesis. (Oldroyd et al., 2011)

Nod factor signalling intertwines with the hormonal signalling pathways, especially cytokinin and auxin. It was found that the histidine receptor kinase (MtCRE1, LjLHK1), the cytokinin receptor, is essential for root nodule formation, and upon rhizobium Nod factor signalling several cytokinin response factors are transcriptionally activated (Gonzalez-Rizzo et

al., 2006; Tirichine et al., 2007; Op den Camp et al., 2011; Plet et al., 2011; Ariel et al., 2012). The precise position of cytokinin signalling in the symbiotic signalling network remains elusive, especially in relation to NSP1 and NSP2 (Op den Camp et al., 2011; Ariel et al., 2012). The importance of this hormone in symbiosis is further underlined by identification of a dominant positive allele, *snf2* in *Lotus japonicus*, that triggers spontaneous nodule formation (Tirichine et al., 2007). As cytokinin is known to be an inhibitor of the auxin efflux carriers of the PIN family (Moubayidin et al., 2009; Pernisová et al., 2009; Růžička et al., 2009; Marhavý et al., 2011), it may explain how a local auxin maximum in the root cortex is established in response to Nod factor signalling (Deinum et al., 2012; Suzaki et al., 2013).

Table 1. Nod factor signaling genes in legume and their non-legume homologs

Medicago truncatula	*Lotus japonicus*	*Populus trichocarpa*	*Vitis vinifera*	*Arabidopsis thaliana*	*Zea mays*	Rice (*Oryza sativa*)
LYK3	NFR1	XP_002301610.1 XP_002317145.1	CBI28844.3	-	NP_001146346.1 ACG29351.1	Os08g42580
NFP	NFR5	XP_002310198.1	CBI17584.3 XP_002280070.1	-	DAA44181.1 AFW89120.1	Os03g13080
DMI1	CASTOR POLLUX	XP_002331112.1 XP_002328774.1 XP_002330696.1 XP_002325606.1	XP_002263318.1 XP_002274786.1 CBI24699.3	At5g49960	DAA56623.1 AFW67006.1 AFW67008.1 ABD67493.1	Os03g62650 Os01g64980
DMI2	SYMRK	CAO99188.1 XP_002310339.1	XP_002272055.2	-	ACG43469.1 NP_001105860.1	Os07g38070
DMI3	CCaMK	XP_002315401.1	XP_002273342.1	-	NP_001105906.1	Os05g41090
IPD3	CYCLOPS	XP_002304379.1 XP_002326769.1	CBI37458.3 XP_002263862.2	-	ACR37710.1 AFW75287.1	Os06g02520
n.a.	NUP85	XP_002324219.1	XP_002283798.1	AT4G32910	NP_001168171.1	Os01g54240
n.a.	NUP133	XP_002301581.1	XP_002272021.1	AT2G05120	AFW89129.1 AFW89130.1	Os03g12450
NSP1	NSP1	XP_002299589.1 XP_002304134.1	XP_002273108.1	At3g13840	DAA45744.1 AFW88089.1	Os03g29480
NSP2	NSP2	XP_002302249.1 XP_002306621.1	XP_002284426.2 XP_002276350.1	At4g08250	DAA44514.1	Os03g15680

Non-legume homologs of Nod factor signaling genes are determined by the protein sequence similarity and previous publications. "n. a.": not available. "-": indicates no homolog in *Arabidopsis thaliana*.

Legume root nodule evolution

Rhizobium Nod factors are structurally very similar to LCOs produced by endomycorrhizal fungi (Maillet et al., 2011). In this way rhizobium co-opted the signalling network that plants use to establish an endomycorrhizal symbiosis. As the endomycorrhizal symbiosis is ancient (450 million years old) and widespread among land plants, orthologs of endomycorrhizal symbiosis signalling genes are present in most plants species (Table 1). This provides a genetic toolbox to which, in theory, all plants would allow convergent evolution of rhizobium symbiosis in lineages outside of legumes. This indeed has happened in the genus *Parasponia* of the *Cannabaceae*. *Parasponia* gained its symbiotic trait independent from legumes, as both lineages are only remotely related (Doyle, 2011). By demonstrating that the

Parasponia-rhizobium symbiosis is also founded on the same symbiosis signalling genes, evidence was provided that genetic constraints guided the convergent evolution of this trait (Op den Camp et al., 2011). However, *Parasponia* nodules are rather distinct from legumes nodules, as they contain a central vascular bundle that is surrounded with infected cells. Furthermore, in *Parasponia* nodules, rhizobium remains within fixation threads, similar as found in some basal legume species (Trinick and Galbraith, 1976; Trinick, 1979).

Parasponia nodules show strong resemblance with root nodules formed by the nitrogen fixing filamentous bacteria of the genus *Frankia* (Trinick, 1979; Lancelle and Torrey, 1985; Pawlowski and Sprent, 2008). This symbiosis can occur in eight lineages in the orders *Rosales*, *Cucurbitales* and *Fagales*. Reverse genetic studies on *Datisca glomerata* revealed that also the *Frankia* symbiosis is, at least in part, founded on the symbiosis signalling genes (Markmann et al., 2008). As the orders *Rosales*, *Cucurbitales* and *Fagales* are phylogenetically related to the legume order *Fabales*, it may imply that the genetic basis to evolve a nodular symbiosis evolved already 100 million years ago in a common ancestor that has given rise to these 4 orders (Soltis et al., 1995; Doyle, 2011; Geurts et al., 2012). In line with this, these orders form a clade that is named "the nitrogen fixation clade" (Figure 3).

As genetic approaches in model legumes species have identified a conserved set of symbiosis signalling genes, it may imply that these genes specifically evolved in legumes to support rhizobium symbiosis. However, this basically holds only for the LysM-type LCO receptors (MtLYK3/LjNFR1 and MtNFP/LjNFR5) that co-evolved with the Nod factor structures of the host rhizobia. All other symbiosis signalling genes are highly conserved in higher plants and legume knockout mutants can be trans-complemented with non-legume orthologs (Gleason et al., 2006; Godfroy et al., 2006; Chen et al., 2007; Yokota et al., 2010). These experiments are generally done using either a constitutive active promoter (e.g. CaMV35S) or the promoter of the corresponding legume orthologous gene. This implies that in evolution protein function remained conserved. As most of the symbiosis signalling genes are single copy, it implies that the primary function is integrated in rhizobium LCO signalling.

Some symbiosis signalling genes have been duplicated early in evolution of the legume lineage, and are maintained as paralogous gene pair in Medicago, Lotus and soybean; e.g. the LysM-type Nod factor receptors and the transcription factor ERN1 (Andriankaja et al., 2007;

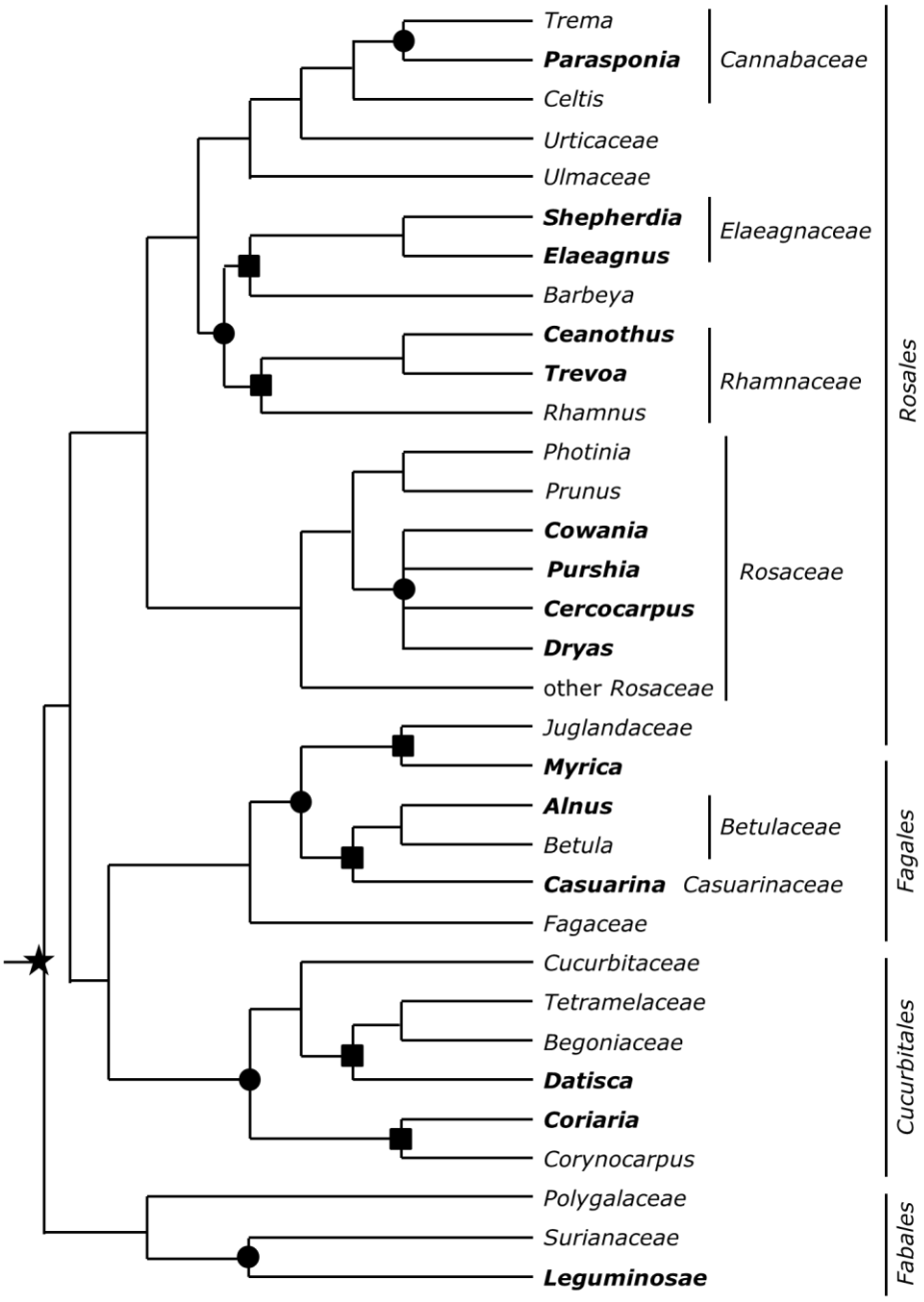


Figure 3. Phylogenetic tree indicates the distribution of the nitrogen fixation clade (NFC). Nodulating taxa are in bold type. Star indicates the origin of the predisposition for nodulation. Circles mark main origins of nodulation. Boxes mark possible additional origins of nodulation within these lineages. Modified from Doyle (2011).

Smit et al., 2007). These gene duplications are likely the result of a whole genome duplication event that has occurred in the subfamily, about 58 million years ago (Lavin et al., 2005; Schmutz et al., 2010). This provided an additional possibility to evolve new functions.

Transcriptome profiling studies in Medicago showed that a large fraction of the paralogous gene pairs displayed a diverged expression profile, including the paralogous pairs *MtNFP-MtLYR1* and *MtERN1-MtERN2*. Whereas *MtNFP* and *MtERN1* expression is induced upon rhizobium Nod factor signalling, their paralogous counterparts are highly expressed in mycorrhized roots (Young et al., 2011). This suggests that the paralogous genes sub-functionalized: one copy controls rhizobium symbiosis; the other maintains its function in mycorrhization. Subsequently, genes can neofunctionalize a process that likely has occurred for the Nod factor receptor MtNFP in Medicago and LjNFR5 in Lotus as both proteins gained specificity for Nod factors of their compatible bacterium.

Challenges for a revolution in nitrogen-fixing crops

Non-legumes can obtain limited fixed nitrogen from free-living nitrogen fixing bacteria that colonize their rhizosphere, such as *Azospirillum*, *Enterobacter*, *Klebsiella* and *Pseudomonas* (Hayat et al., 2010). However, the nitrogen fixing capacities of these associations is limited, and an order of magnitude less efficient than rhizobium. Additionally, lose associations in the rhizosphere are highly sensitive to environmental changes.

Since a century now scientists speculated whether it is possible to transfer the nitrogen fixing rhizobium symbiosis to non-legume crops (Roger and Watanabe, 1986; Ladha and Reddy, 2003; Soft and Wani, 2007). As *Parasponia* shows that such symbiotic relation indeed can be gained by a non-legume lineage, it suggests that such transfer by means of a biotech approach is feasible.

Three objectives have to be fulfilled to achieve a transfer of the rhizobium symbiosis to non-legume crops: (I) the formation of root nodules, (II) the intracellular infection of nodule cells by rhizobium, and (III) the efficient nitrogen fixation and transfer of ammonium to the plant. However, till now none of these objectives have been achieved. In theory, a strategy to achieve nodule organogenesis in non-legumes could be based on a transfer of legume symbiosis signalling genes; especially the LysM-type Nod factor receptors. Such strategy was tried by introducing 8 legume symbiosis signalling genes in their genomic context into several different non-legume species. However, this turned out to be insufficient to trigger rhizobium-specific responses (Untergasser et al., 2012). This demonstrates that other genetic underlie the rhizobium symbiosis trait. Unveiling these determinants will be a prerequisite to advance the objectives.

REFERENCES

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

Andriankaja, A., Boisson-Dernier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D.G., and de Carvalho-Niebel, F. (2007). AP2-ERF transcription factors mediate Nod factor dependent *MtENOD11* activation in root hairs via a novel cis-regulatory motif. Plant Cell **19,** 2866-2885.

Ariel, F., Brault-Hernandez, M., Laffont, C., Huault, E., Brault, M., Plet, J., Moison, M., Blanchet, S., Ichante, J.L., Chabaud, M., Carrere, S., Crespi, M., Chan, R.L., and Frugier, F. (2012). Two direct targets of cytokinin signaling regulate symbiotic nodulation in *Medicago truncatula*. Plant Cell **24,** 3838-3852.

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

Beatty, P.H., and Good, A.G. (2011). Plant science. Future prospects for cereals that fix nitrogen. Science **333,** 416-417.

Bersoult, A., Camut, S., Perhald, A., Kereszt, A., Kiss, G.B., and Cullimore, J.V. (2005). Expression of the *Medicago truncatula* DM12 gene suggests roles of the symbiotic nodulation receptor kinase in nodules and during early nodule development. Mol. Plant Microbe Interact. **18,** 869-876.

Brewin, N.J. (1998). Tissue and cell invasion by Rhizobium: the structure and development of infection threads and symbiosomes. In The rhizobiaceae (Springer), pp. 417-429.

Brewin, N.J. (2004). Plant cell wall remodelling in the Rhizobium–legume symbiosis. Crit. Rev. Plant Sci. **23,** 293-316.

Chandler, M.R. (1978). Some observations on infection of *Arachis hypogaea* L. by Rhizobium. J. Exp. Bot. **29,** 749-755.

Charpentier, M., and Oldroyd, G. (2010). How close are we to nitrogen-fixing cereals? Curr. Opin. Plant Biol. **13,** 556-564.

Charpentier, M., Bredemeier, R., Wanner, G., Takeda, N., Schleiff, E., and Parniske, M. (2008). *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. Plant Cell **20,** 3467-3479.

Chen, C., Gao, M., Liu, J., and Zhu, H. (2007). Fungal symbiosis in rice requires an ortholog of a legume common symbiosis gene encoding a Ca^{2+}/calmodulin-dependent protein kinase. Plant Physiol. **145,** 1619-1628.

Combier, J.P., Frugier, F., de Billy, F., Boualem, A., El-Yahyaoui, F., Moreau, S., Vernie, T., Ott, T., Gamas, P., Crespi, M., and Niebel, A. (2006). MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. Gene Dev. **20,** 3084-3088.

Cook, D.R. (1999). *Medicago truncatula*-a model in the making! Curr. Opin. Plant Biol. **2,** 301-304.

Deinum, E.E., Geurts, R., Bisseling, T., and Mulder, B.M. (2012). Modeling a cortical auxin maximum for nodulation: different signatures of potential strategies. Front. Plant Sci. **3,** 96.

Doyle, J.J. (2011). Phylogenetic perspectives on the origins of nodulation. Mol. Plant Microbe Interact. **24,** 1289-1295.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. Nature **417,** 962-966.

Erisman, J.W., Sutton, M.A., Galloway, J., Klimont, Z., and Winiwarter, W. (2008). How a century of ammonia synthesis changed the world. Nat. Geosci. **1,** 636-639.

Esseling, J.J., Lhuissier, F.G., and Emons, A.M. (2003). Nod factor-induced root hair curling: continuous polar growth towards the point of Nod factor application. Plant Physiol. **132,** 1982-1988.

FAO. (2009). How to Feed the World in 2050, UN, ed, pp. 1-35.

Felle, H.H., Kondorosi, É., Kondorosi, Á., and Schultze, M. (1998). The role of ion fluxes in Nod factor signalling in *Medicago sativa*. Plant J. **13,** 455-463.

Geurts, R., Lillo, A., and Bisseling, T. (2012). Exploiting an ancient signalling machinery to enjoy a nitrogen fixing symbiosis. Curr. Opin. Plant Biol.**15,** 438-443.

Gleason, C., Chaudhuri, S., Yang, T., Munoz, A., Poovaiah, B.W., and Oldroyd, G.E. (2006). Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature **441,** 1149-1152.

Godfroy, O., Debelle, F., Timmers, T., and Rosenberg, C. (2006). A rice calcium and calmodulin-dependent protein kinase restores nodulation to a legume mutant. Mol. Plant Microbe Interact. **19,** 495-501.

González-Sama, A., Lucas, M.M., De Felipe, M.R., and Pueyo, J.J. (2004). An unusual infection mechanism and nodule morphogenesis in white lupin (*Lupinus albus*). New Phytol. **163,** 371-380.

Gonzalez-Rizzo, S., Crespi, M., and Frugier, F. (2006). The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. Plant Cell **18,** 2680-2693.

Handberg, K., and Stougaard, J. (1992). *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. Plant J. **2,** 487-496.

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion. Ann. Microbiol. **60,** 579-598.

Horvath, B., Yeun, L.H., Domonkos, A., Halasz, G., Gobbato, E., Ayaydin, F., Miro, K., Hirsch, S., Sun, J., Tadege, M., Ratet, P., Mysore, K.S., Ane, J.M., Oldroyd, G.E., and Kalo, P. (2011). *Medicago truncatula* IPD3 is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses. Mol. Plant Microbe Interact. **24,** 1345-1358.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., and Vickers, K. (2004). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature **433,** 527-531.

Jones, K.M., Kobayashi, H., Davies, B.W., Taga, M.E., and Walker, G.C. (2007). How rhizobial symbionts invade plants: the Sinorhizobium-Medicago model. Nat. Rev. Microb. **5,** 619-633.

Kalo, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R.M., Hirsch, S., Jakab, J., Sims, S., Long, S.R., Rogers, J., Kiss, G.B., Downie, J.A., and Oldroyd, G.E. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science **308,** 1786-1789.

Kanamori, N., Madsen, L.H., Radutoiu, S., Frantescu, M., Quistgaard, E.M.H., Miwa, H., Downie, J.A., James, E.K., Felle, H.H., Haaning, L.L., Jensen, T.H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2006). A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proc. Natl. Acad. Sci. USA **103,** 359-364.

Ladha, J.K., and Reddy, P.M. (2003). Nitrogen fixation in rice systems: state of knowledge and future prospects. Plant Soil **252,** 151-167.

Lancelle, S.A., and Torrey, J.G. (1985). Early development of Rhizobium-induced root nodules of *Parasponia rigida*. II. Nodule morphogenesis and symbiotic development. Can. J. Bot. **63,** 25-35.

Lavin, M., Herendeen, P.S., and Wojciechowski, M.F. (2005). Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. Syst. Biol. **54,** 575-594.

Lavin, M., Pennington, R.T., Klitgaard, B.B., Sprent, J.I., de Lima, H.C., and Gasson, P.E. (2001). The dalbergioid legumes (Fabaceae): delimitation of a pantropical monophyletic clade. Am. J. Bot. **88,** 503-533.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C., and Denarie, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344,** 781-784.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C., and Debelle, F. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science **303,** 1361-1364.

Limpens, E., Mirabella, R., Fedorova, E., Franken, C., Franssen, H., Bisseling, T., and Geurts, R. (2005). Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by DMI2. Proc. Natl. Acad. Sci. USA **102,** 10375-10380.

Madsen, E.B., Antolin-Llovera, M., Grossmann, C., Ye, J., Vieweg, S., Broghammer, A., Krusell, L., Radutoiu, S., Jensen, O.N., Stougaard, J., and Parniske, M. (2011). Autophosphorylation is essential for the *in vivo* function of the *Lotus japonicus* Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J. **65,** 404-417.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E.A., Driguez, H., Becard, G., and Denarie, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469,** 58-63.

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., and Kleine-Vehn, J. (2011). Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. Dev. Cell **21,** 796-804.

Markmann, K., Giczey, G., and Parniske, M. (2008). Functional Adaptation of a Plant Receptor-Kinase Paved the Way for the Evolution of Intracellular Root Symbioses with Bacteria. PLoS Biol. **6,** e68.

Marsh, J.F., Rakocevic, A., Mitra, R.M., Brocard, L., Sun, J., Eschstruth, A., Long, S.R., Schultze, M., Ratet, P., and Oldroyd, G.E. (2007). *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. Plant Physiol. **144,** 324-335.

Mitra, R.M., Gleason, C.A., Edwards, A., Hadfield, J., Downie, J.A., Oldroyd, G.E., and Long, S.R. (2004). A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. USA **101,** 4701-4705.

Moubayidin, L., Di Mambro, R., and Sabatini, S. (2009). Cytokinin-auxin crosstalk. Trends Plant Sci. **14,** 557-562.

Naisbitt, T., James, E., and Sprent, J. (1992). The evolutionary significance of the legume genus Chamaecrista, as determined by nodule structure. New Phytol. **122,** 487-492.

Oldroyd, G.E., Murray, J.D., Poole, P.S., and Downie, J.A. (2011). The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Genet. **45,** 119-144.

Olivares, J., Bedmar, E.J., and Sanjuan, J. (2013). Biological nitrogen fixation in the context of global change. Mol. Plant Microbe Interact. **26,** 486-494.

Op den Camp, R.H., De Mita, S., Lillo, A., Cao, Q., Limpens, E., Bisseling, T., and Geurts, R. (2011). A phylogenetic strategy based on a legume-specific whole genome duplication yields symbiotic cytokinin type-A response regulators. Plant Physiol. **157,** 2013-2022.

Ovchinnikova, E., Journet, E.P., Chabaud, M., Cosson, V., Ratet, P., Duc, G., Fedorova, E., Liu, W., den Camp, R.O., Zhukov, V., Tikhonovich, I., Borisov, A., Bisseling, T., and Limpens, E. (2011). IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and Medicago *Spp*. Mol. Plant Microbe Interact. **24,** 1333-1344.

Pawlowski, K., and Sprent, J.I. (2008). Comparison between actinorhizal and legume symbiosis: nitrogen-fixing actinorhizal symbioses. In Nitrogen Fixation: Origins, Applications, and Research Progress, K. Pawlowski and W.E. Newton, eds (Springer Netherlands), pp. 261-288.

Pernisová, M., Klíma, P., Horák, J., Válková, M., Malbeck, J., Souček, P., Reichman, P., Hoyerová, K., Dubová, J., and Friml, J. (2009). Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc. Natl. Acad. Sci. USA **106,** 3609-3614.

Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., Crespi, M., and Frugier, F. (2011). MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. Plant J. **65,** 622-633.

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

Robertson, G.P., Hamilton, S.K., Del Grosso, S.J., and Parton, W.J. (2011). The biogeochemistry of bioenergy landscapes: carbon, nitrogen, and water considerations. Ecol. Appl. **21,** 1055-1067.

Roche, P., Debelle, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Denarie, J., and Prome, J.C. (1991). Molecular basis of symbiotic host specificity in Rhizobium meliloti: nodH and nodPQ genes encode the sulfation of lipo-oligosaccharide signals. Cell **67,** 1131-1143.

Rockstrom, J., Steffen, W., Noone, K., Persson, A., Chapin, F.S., Lambin, E.F., Lenton, T.M., Scheffer, M., Folke, C., Schellnhuber, H.J., Nykvist, B., de Wit, C.A., Hughes, T., van der Leeuw, S., Rodhe, H., Sorlin, S., Snyder, P.K., Costanza, R., Svedin, U., Falkenmark, M., Karlberg, L., Corell, R.W., Fabry, V.J., Hansen, J., Walker, B., Liverman, D., Richardson, K., Crutzen, P., and Foley, J.A. (2009). A safe operating space for humanity. Nature **461,** 472-475.

Roger, P.A., and Watanabe, I. (1986). Technologies for utilizing biological nitrogen fixation in wetland rice: potentialities, current usage, and limiting factors. In Nitrogen Economy of Flooded Rice Soils, S.K. Datta and W.H. Patrick, Jr., eds (Springer Netherlands), pp. 39-77.

Roth, L., and Stacey, G. (1989). Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. Eur. J. Cell Biol. **49,** 13.

Růžička, K., Šimášková, M., Duclercq, J., Petrášek, J., Zažímalová, E., Simon, S., Friml, J., Van Montagu, M.C., and Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc. Natl. Acad. Sci. USA **106,** 4284-4289.

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., and Umehara, Y. (2007). NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. Plant Cell **19,** 610-624.

Sawada, H., Kuykendall, L.D., and Young, J.M. (2003). Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J. Gen. Appl. Microbiol. **49,** 155-179.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.C., Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., and Jackson, S.A. (2010). Genome sequence of the palaeopolyploid soybean. Nature **463,** 178-183.

Smil, V. (1999). Nitrogen in crop production: An account of global flows. Global Biogeochem. Cy. **13,** 647-662.

Smit, P., Raedts, J., Portyanko, V., Debelle, F., Gough, C., Bisseling, T., and Geurts, R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science **308,** 1789-1791.

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

Snyder, C.S., Bruulsema, T.W., Jensen, T.L., and Fixen, P.E. (2009). Review of greenhouse gas emissions from crop production systems and fertilizer management effects. Agr. Ecosyst. Environ.**133,** 247-266.

Soft, P., and Wani, S. (2007). Prospects of nitrogen fixation in rice. Asian J. Plant Sci. **6,** 203-213.

Soltis, D.E., Soltis, P.S., Morgan, D.R., Swensen, S.M., Mullin, B.C., Dowd, J.M., and Martin, P.G. (1995). Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc. Natl. Acad. Sci. USA **92,** 2647-2651.

Sprent, J.I. (2007). Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. New Phytol. **174,** 11-25.

Sprent, J.I., and James, E.K. (2007). Legume evolution: where do nodules and mycorrhizas fit in? Plant Physiol. **144,** 575-581.

Subba-Rao, N.S., Mateos, P., Baker, D., Pankratz, H., Palma, J., Dazzo, F., and Sprent, J. (1995). The unique root-nodule symbiosis between Rhizobium and the aquatic legume, Neptunia natans (L. f.) Druce. Planta **196,** 311-320.

Sutherland, W.J., Aveling, R., Bennun, L., Chapman, E., Clout, M., Cote, I.M., Depledge, M.H., Dicks, L.V., Dobson, A.P., Fellman, L., Fleishman, E., Gibbons, D.W., Keim, B., Lickorish, F., Lindenmayer, D.B., Monk, K.A., Norris, K., Peck, L.S., Prior, S.V., Scharlemann, J.P., Spalding, M., and Watkinson, A.R. (2012). A horizon scan of global conservation issues for 2012. Trends Ecol. Evol. **27,** 12-18.

Sutton, M.A., Oenema, O., Erisman, J.W., Leip, A., van Grinsven, H., and Winiwarter, W. (2011). Too much of a good thing. Nature **472,** 159-161.

Suzaki, T., Ito, M., and Kawaguchi, M. (2013). Genetic basis of cytokinin and auxin functions during root nodule development. Front. Plant Sci. **4**.

Timmers, A.C., Auriac, M.C., and Truchet, G. (1999). Refined analysis of early symbiotic steps of the Rhizobium-Medicago interaction in relationship with microtubular cytoskeleton rearrangements. Development **126,** 3617-3628.

Timmers, A.C., Soupene, E., Auriac, M.C., de Billy, F., Vasse, J., Boistard, P., and Truchet, G. (2000). Saprophytic intracellular rhizobia in alfalfa nodules. Mol. Plant Microbe Interact. **13,** 1204-1213.

Tirichine, L., Sandal, N., Madsen, L.H., Radutoiu, S., Albrektsen, A.S., Sato, S., Asamizu, E., Tabata, S., and Stougaard, J. (2007). A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. Science **315,** 104-107.

Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L.H., Miwa, H., Nakagawa, T., Sandal, N., Albrektsen, A.S., Kawaguchi, M., Downie, A., Sato, S., Tabata, S., Kouchi, H., Parniske, M., Kawasaki, S., and Stougaard, J. (2006). Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. Nature **441,** 1153-1156.

Trinick, M.J. (1979). Structure of nitrogen-fixing nodules formed by Rhizobium on roots of *Parasponia andersonii* Planch. Can. J. Microbiol. **25,** 565-578.

Trinick, M.J., and Galbraith, J. (1976). Structure of root nodules formed by Rhizobium on the non-legume *Trema cannabina* var. *scabra*. Arch. Microbiol. **108,** 159-166.

Untergasser, A., Bijl, G.J.M., Liu, W., Bisseling, T., Schaart, J.G., and Geurts, R. (2012). One-step agrobacterium mediated transformation of eight genes essential for rhizobium symbiotic signaling using the novel binary vector system pHUGE. PLoS One **7,** e47885.

Vasse, J., de Billy, F., Camut, S., and Truchet, G. (1990). Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. **172,** 4295-4306.

Wood, S., and Cowie, A. (2004). A review of greenhouse gas emission factors for fertiliser production. In IEA bioenergy task, pp. 20.

Yang, W.-C., de Blank, C., Meskiene, I., Hirt, H., Bakker, J., van Kammen, A., Franssen, H., and Bisseling, T. (1994). Rhizobium nod factors reactivate the cell cycle during infection and nodule

primordium formation, but the cycle is only completed in primordium formation. Plant Cell **6,** 1415-1426.

Yano, K., Yoshida, S., Muller, 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.L., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., and Parniske, M. (2008). CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc. Natl. Acad. Sci. USA **105,** 20540-20545.

Yokota, K., Soyano, T., Kouchi, H., and Hayashi, M. (2010). Function of GRAS proteins in root nodule symbiosis is retained in homologs of a non-legume, rice. Plant Cell Physiol. **51,** 1436-1442.

Young, N.D., Debelle, F., Oldroyd, G.E.D., Geurts, R., Cannon, S.B., Udvardi, M.K., Benedito, V.A., Mayer, K.F.X., Gouzy, J., Schoof, H., Van de Peer, Y., Proost, S., Cook, D.R. et al. (2011). The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature **480,** 520-524.

Chapter 2

Exogenous fixed nitrogen affects rhizobium Nod factor signalling in a differential way

Wei Liu, Ton Bisseling and René Geurts

Department of Plant Science, Laboratory of Molecular Biology, Wageningen University, 6708 PB Wageningen, The Netherlands

ABSTRACT

At low fixed nitrogen levels, legumes can interact with N_2-fixing rhizobium bacteria. In such case a root nodule is formed to host rhizobia. In nodule cells the bacteria are maintained as organelle-like structures that are surrounded by a plant derived membrane. These structures are called symbiosomes, and in which differentiated rhizobia fix elemental nitrogen into ammonia that can be utilized by the plant. In return the plant feeds carbohydrates to the bacteria. Consequently, legumes have to control this symbiotic interaction to balance their energy flow. To achieve this, nodule formation is tightly linked to the nitrogen status of the plant. Here we used *Medicago truncatula* and investigated whether this block is mediated by down-tuning the perception and signalling of bacterial Nod factors. We show that there is differentially sensitivity of root epidermal cells to the presence of an exogenous nitrogen source correlating with the developmental stage they are in. Young mature root hairs that just stopped growing are less sensitive to exogenous applied 10 mM NH_4NO_3, when compared to younger or older root hairs. We found that young mature root hairs still respond to a relative low concentration of rhizobium Nod factors (10^{-10} M) by monitoring *MtENDO11* expression and root hair deformation. These responses are absent in other epidermal cells in presence of this nitrogen concentration. 10 mM NH_4NO_3 prohibits root nodule formation when rhizobium is present, suggesting that the symbiotic responses in a small developmental window are insufficient to achieve a symbiotic engagement. By ectopically expressing a dominant active allele of CCaMK we demonstrate that this block can be passed, and root nodules can be formed. This suggests that fixed nitrogen controlled suppression of nodulation occurs upstream of CCaMK.

INTRODUCTION

Fixed nitrogen is generally a growth-limiting factor for plants. Legumes can establish an endosymbiosis with nitrogen fixing rhizobium to overcome this problem. To do so, root nodules are formed to host the rhizobia intracellularly, as (transient) nitrogen fixing organelles. This intimate association provides the plant with an unlimited access to fixed nitrogen. However, to achieve this, the plant has to invest up to 20% of its photosynthetic assimilates in this symbiotic relationship (Minchin and Pate, 1973). Therefore, legumes have evolved control mechanisms that enable them to block root nodule formation when grown in soils that are sufficiently rich in fixed nitrogen. This suppression of symbiosis can occur at several levels; e.g. the formation of new nodules is blocked, and existing nodules are terminated (Carroll and Gresshoff, 1983; Streeter, 1985; Imsande, 1986; Streeter and Wong, 1988; Carroll and Mathews, 1990).

Rhizobium secretes lipo-chitooligosaccharides (named Nod factors) upon recognition of plant-derived molecules, which are often flavonoids (Peters et al., 1986). Nod factors are sufficient to trigger symbiotic responses in the root, including symbiotic gene expression, root hair growth responses and nodule primordium formation (Truchet et al., 1991). When a Nod factor producing rhizobium attaches to a young root hair, it can affect the growth direction of the hair by which the root hair curls around the bacterium. The closed environment within the pocket of the curled root hair enables the rhizobium to form a micro-colony and to initiate formation of intracellular infection threads. These threads grow towards the newly formed nodule primordia (Murray, 2011).

In the young root, the root hair containing epidermal cells can be divided into three zones based on the development stage: Zone I, growing root hairs; Zone II, growth terminating root hairs; and Zone III, mature root hairs (Heidstra et al., 1994). In case Nod factors are applied, mainly, root hairs in Zone II display root growth responses. These root hairs first swell and then form a new growth tip. The new outgrowth is in a random direction, giving these hairs a deformed appearance (Heidstra et al., 1994), so named as root hair deformation. Root hair deformation can be used to study early Nod factor induced signalling.

In past two decades, genes essential for Nod factor signalling have been identified through forward genetic screens in two model species; *Medicago truncatula* and *Lotus japonicus.* In brief, Nod factors are perceived by two distinct LysM-domain receptor-like-kinases (named NFP and LYK3 in *M. truncatula* and NFR5 and NFR1 in *L. japonicus*) that

might form a heterodimeric signalling complex (Amor et al., 2003; Radutoiu et al., 2003; Smit et al., 2007; Madsen et al., 2011). Besides these LysM-receptors, another plasma membrane localized receptor kinase is essential for Nod factor induced signalling. This receptor, named DMI2 in *M. truncatula* or SYMRK in *L. japonicus*, contains three LRR domains and one large domain of unknown function in its extracellular portion, as well as one kinase domain in the intracellular portion (Catoira et al., 2000; Endre et al., 2002). Downstream of these plasma membrane localized receptors, components have been identified that are essential to induce and interpret Ca^{2+} spiking in the nucleus. One of these genes, named DMI1 in *M. truncatula* or CASTOR and POLLUX in *L. japonicus*, encodes a presumed potassium channel that localizes in the perinuclear envelope (Catoira et al., 2000; Wais et al., 2000; Shaw and Long, 2003; Ane et al., 2004). Ca^{2+} spiking subsequently activates Ca^{2+}/Calmodulin dependent kinase (CCaMK) encoded by DMI3 in *M. truncatula*. This CCaMK forms a key node in the symbiotic signalling cascade, as a truncated form that lacks the auto-inhibition domain induces root nodule formation in absence of rhizobia (Catoira et al., 2000; Levy et al., 2004; Gleason et al., 2006). Knocking out any of these genes blocks basically all rhizobium Nod factor induced responses.

It has been known for a long time that the availability of an external nitrogen source can inhibit the rhizobium symbiosis (Gi öbel, 1926; Fred et al., 1932; Allison and Ludwig, 1934). Time-laps experiments in vetch (*Vicia sativa*) revealed that this inhibition occurs in a time frame of 1 to 2 days (Heidstra et al., 1994). Studies in *L. japonicus* revealed that high levels of exogenously available fixed nitrogen does not affect the expression of symbiotic signalling genes expression, suggesting that inhibition Nod factor signalling occurs by a different, yet unknown, mechanism (Barbulova et al., 2007). We conducted studies in *M. truncatula* and found that the sensitivity of root epidermal cells to NH_4NO_3 is depending on their developmental stage. Young mature root hairs are less sensitive to NH_4NO_3 and still respond to rhizobium Nod factors, whereas the responses are blocked in cells that either young or older in the age. With the help of a dominant active form CCaMK*, we found that spontaneous nodule formation is not affected by exogenous nitrogen source.

RESULTS

10 mM NH_4NO_3 suppresses Nod factor induced responses in the root epidermis differentially

In *M. truncatula*, Nod factor signalling can be monitored by the expression of *MtENOD11* in the root (Journet et al., 2001; Charron et al., 2004) as well as by root hair deformation. Therefore we addressed the question to what extend these responses are blocked when plants are grown in the presence 10 mM NH_4NO_3, an amount of fixed nitrogen that blocks rhizobium induced root nodule formation (Catoira et al., 2000). To answer this question, *M. truncatula* seedlings containing the *pMtENOD11::GUS* reporter gene were grown in Fåhraeus slides for 5 days (see Materials and methods). The liquid medium was changed every 24 hours (h), and we included 10 mM NH_4NO_3 for 0, 24 or 48 h, respectively, prior Nod factor application. Subsequently, 10^{-10} M Nod factors were supplied, the lowest concentration that is still sufficient to trigger maximal *MtENOD11* expression and root hair deformation under nitrogen-limiting conditions. *MtENOD11* expression, based on GUS staining, and root hair deformation were studied 3 h post Nod factor application. Roots that were grown in absence of NH_4NO_3, GUS staining was clearly visible in a broad zone ranging from just emerging root hairs up to the older hairs (Zone I, II and part of III) (Figure 1A). In the roots that were pre-treated with 10 mM NH_4NO_3 for 24 h or 48 h, GUS activity could also be observed, however the responding zone was smaller, and basically restricted to zone II. Also, the GUS staining intensity was markedly reduced, with the lowest intensity upon 48h NH_4NO_3 pre-treatment (Figure 1B and 1C). This indicates that Nod factor induced *MtENOD11* expression is suppressed by exogenously applied NH_4NO_3, though the different zones of epidermal cells display a differential sensitivity to this inhibition.

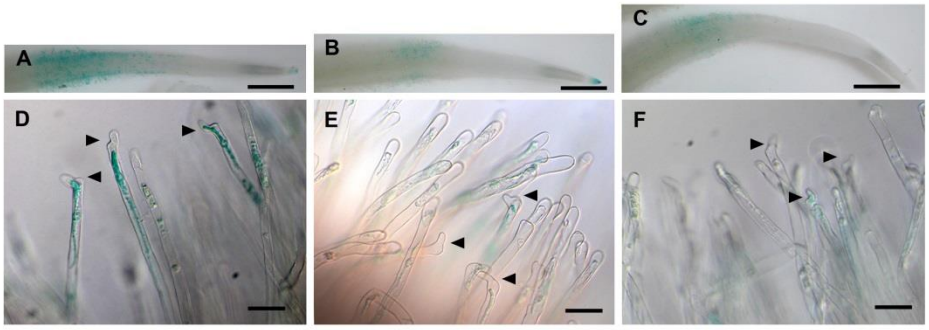


Figure 1. Ammonium nitrate affects Nod factor induced responses in zone I & III, but not in zone II of roots of *M. truncatula*. Roots from *M. truncatula MtENOD11::GUS* transgenic line are grown in 10 mM NH_4NO_3 0 h (A), 24 h (B) and 48 h (C), and then treated with *Sinorhizobium meliloti* Nod factors (10^{-10} M) for 3 hours. Afterwards, these roots are stained with *GUS* staining solution. Root hair deformation response of GUS stained roots grown in 10 mM NH_4NO_3 0 h (D), 24 h (E) and 48 h (F). Arrowheads indicate deformed root hairs. The scale bars are equal to 1 mm in A, B and C, and equal to 10 µm in D, E and F.

Although Nod factor induced *MtENOD11* expression is inhibited in zone I and III, it still can be triggered in zone II. Zone II cells are known for their root hair deformation responses in presence of Nod factors. We tested whether these root hairs maintain their responsiveness when grown in NH_4NO_3, which showed to be the case (Figure 1D to 1F). Taken together, these data demonstrate that Nod factor induced signalling in *M. truncatula* young mature epidermal root hair cells (zone II) are least sensitive for exogenously applied fixed nitrogen, when compared to the other developmental zones.

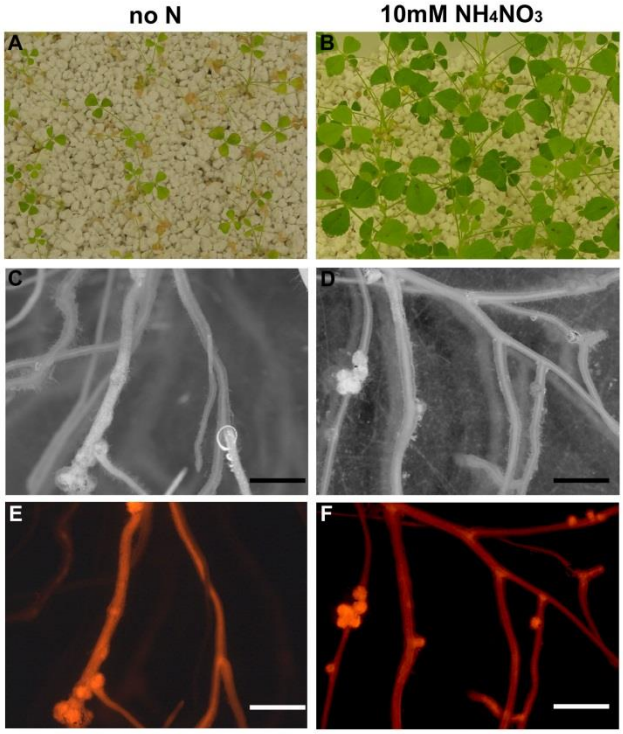


Figure 2. Spontaneous nodule formation induced by a dominant active form of MtCCaMK is independent of the nitrogen status of the plant. *M. truncatula* plants harboring transgenic roots expressing a dominant active form of *MtCCaMK* and *DsRED1* as selectable marker. Plants are grown for 4 weeks without fixed nitrogen (A, C, E) or in medium containing 10 mM NH_4NO_3 (B, D, F). Without nitrogen plants stay relative small and limited development, when compared to plants grown on medium containing fixed nitrogen (A, B). However, under both growth conditions spontaneous nodulation occurred (C-F). Transgenic roots are visualized by the red fluorescence under the stereomicroscope corresponding to the DsRED marker (E, F). Scale bars in C-F are equal to 5 mm.

Dominant active *CCaMK* induces nodule formation in presence of 10 mM NH_4NO_3

The occurrence of Nod factor signalling activation in a small developmental window of epidermal cells in presence of 10 mM NH_4NO_3 may suggests that occasionally root nodule

formation will also be triggered under such growth conditions. However, this is not the case (data not shown). We hypothesize that this is due to the level of signalling that is achieved under such conditions, as not only the spatial regulation of *MtENOD11* induction is affected, but also the expression level seems to be affected. To test this hypothesis, we ectopically expressed a dominant active allele of CCaMK (CCaMK*) and determined whether (spontaneous) root nodule formation can be triggered under these growth conditions.

A dominant active form of MtCCaMK (MtCCaMK*) was introduced into wild type *M. truncatula* plants using *A. rhizogenes* mediated hairy-root transformation. Two weeks post transformation, plants were scored for transgenic roots and transgenic compound plants were transferred to perlite containing either 0 or 10 mM NH_4NO_3. Four weeks post the transfer, a clear morphological difference was apparent between plants grown under different nitrogen regimes. Whereas plants grown at 10 mM NH_4NO_3 were green and had 3-4 fully developed compound leaves, the plants grown in absence of external nitrogen remain small and pale (Figure 2A and 2B). Next, the transgenic root systems were checked for spontaneous nodules (in absence of rhizobia). As expected, spontaneous nodulation occurred on roots of nitrogen-starved plants. All plants that carried transgenic roots formed 5-10 nodules per plant (Figure 2C and 2E). Interestingly, spontaneous nodule formation also occurred with same efficiency on transgenic plant roots grown under 10 mM NH_4NO_3 (Figure 2D and 2F). This finding demonstrates that suppression of Nod factor signalling by fixed nitrogen can be overcome by ectopic expression of MtCCaMK*.

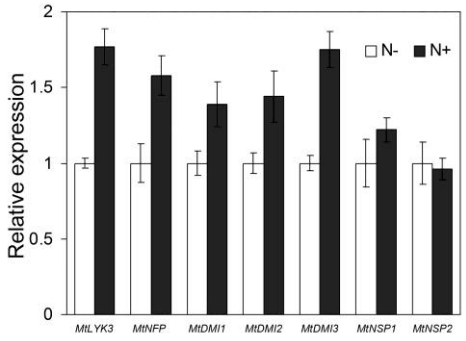


Figure 3. Expression of Nod factor signalling genes in *M. truncatula* roots is not affected by the nitrogen status of the plant. The relative expression levels of *MtLYK3*, *MtNFP*, *MtDMI1*, *MtDMI2*, *MtDMI3*, *MtNSP1* and *MtNSP2* detected by real time qRT-PCR in roots grown in medium without a fixed nitrogen source or medium containing 10 mM NH_4NO_3. The relative expression level of each gene in no fixed nitrogen condition is set 1. Data are means ±SD.

10 mM NH_4NO_3 does not affect the expression of Nod factor signalling genes

As we demonstrate that the plant nitrogen-status interferes with Nod factor signalling, we determined the expression level of Nod factor signalling genes. Wild type *M. truncatula* seedlings were grown on Fåhraeus medium containing either no fixed nitrogen or 10 mM NH_4NO_3 for 3 days, respectively. Total RNA was extracted from the Nod factor susceptible zone of 7-day-old seedlings and used as template in real time qRT-PCR experiments to compare the relative expression level of these Nod factor signalling genes, normalized against an ubiquitin gene. This revealed that the expression levels of *MtNFP*, *MtLYK3*, *MtDMI1*, *MtDMI2*, *MtCCaMK*(*MtDMI3*), *MtNSP1* and *MtNSP2* are not down-regulated in high nitrogen grown plants. Contrary, even a slight up-regulation could be observed for most of these genes (Figure 3). Taken together, this demonstrates that in *M. truncatula* Nod factor signalling is also not controlled on a transcriptional level of key genes in the signalling pathway.

DISCUSSION

Here we showed that in *M. truncatula* an exogenously applied nitrogen source suppresses Nod factor induced *MtENOD11* expression, but does so in a differential way. Whereas *MtENOD11* expression in young developing root hairs as well as old mature hairs is suppressed, *MtENOD11* can still be induced in the small zone of root hairs that are just mature. This coincides with the root zone of which root hairs are most responsive for Nod factor induced deformation.

As 10 mM NH_4NO_3 blocks root nodule formation in *M. truncatula*, we ask the question whether this block can be effectively overcome by a dominant active form of *CCaMK** allele. This revealed that MtCCaMK* activity is not affected by the nitrogen status of the plant, and may suggest that fixed nitrogen inhibits Nod factor signalling upstream of MtCCaMK, by a yet unknown mechanism (Figure 4). These results are in contrast to data obtained in *L. japonicus*. In *L. japonicus snf1* mutant, plants carrying a dominant active allele of LjCCaMK, caused by a point mutation, do not form spontaneous nodules when grown on 20 mM KNO_3 (Tirichine et al., 2006), which contains "the same" concentration of fixed nitrogen as we used in our study. The reason for this difference can be in five-fold: (I) there could be different mechanisms in sensitivity to fixed nitrogen between both species; (II) there is variation in activity of the mutant *CCaMK** alleles, domain deletion mutant *vs*. point mutation mutant; (III) due to copy number variation of the trans-gene in *M. truncatula*; (IV) a differential effect of NH_4^+ and NO_3^-; and (V) differential effects between *M. truncatula* and *L. japonicus*.

Regardless the reasons of the difference between *L. japonicus* and *M. truncatula*, our experiments demonstrate that a growth condition can be created that effectively blocks root nodule formation by rhizobium, but does not fully abolish Nod factor signalling.

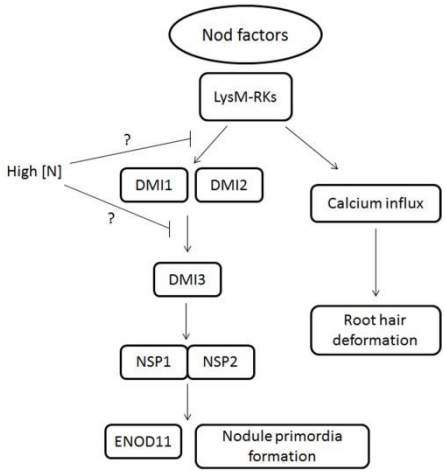


Figure 4. Schematic representation of putative fixed nitrogen inhibition sites in early Nod factor signalling in *M. truncatula*. Based on the results, (I) root hair deformation is not sensitive to the nitrogen status, (II) Expression of Nod factor response gene *ENOD11* is inhibited by the fixed nitrogen and (III) the auto-active form *CCaMK* can overcome the inhibition of high nitrogen concentration, indicating that the inhibition of Nod factor signalling by high nitrogen concentration should be located upstream of DMI3/CCaMK.

Studies in *L. japonicus* revealed different inhibitory effects of ammonia and nitrate on Nod factor induced root hair deformation (Barbulova et al., 2007). Whereas KNO_3 (10 mM) has no effect, root hair deformation is blocked upon application of NH_4NO_3 (10 mM). Similarly, 10 mM NH_4NO_3 blocks root hair deformation in Vetch (Heidstra et al., 1994). This suggests that the inhibitory effect on root hair deformation requires either a higher fixed nitrogen concentration, or NH_4^+ and NO_3^- have a differential effect on this response. In our studies on *M. truncatula* we used only NH_4NO_3, and did not observed an inhibition of root hair deformation in zone II. Possibly the difference in *M. truncatula* is due to the timing of NH_4NO_3 application, as we pre-treated the plant maximally for 48 h for a root hair deformation assay, comparable to the treatment in Vetch, but different to the *L. japonicus* plants which were grown for 6 days with NH_4NO_3. Likewise, studies in *M. truncatula* in which plants were treated for at least 7 days with 10 mM NH_4NO_3 resulted in a more severe block in the root hair deformation response (Catoira et al., 2000). This indicates that the period

of pre-treatment with fixed nitrogen affects the symbiotic read out, though can be bypassed by a dominant active *CCaMK** allele.

Nitrogen status controlled inhibition of rhizobium root nodule formation is well studied (Imsande, 1986; Streeter and Wong, 1988), however, studies on early Nod factor responses are limited. Here we studied these early inhibitory effects of the legume-rhizobium interaction using model legume species *M. truncatula*. Different to a previous report in *L. japonicus* (Barbulova et al., 2007), we mapped it upstream of CCaMK in Nod factor signalling. However, the mode of action of the N-status of the plant on symbiotic signalling remains unresolved.

MATERIALS AND METHODS

Plant materials and growth conditions

Medicago truncatula Jemalong A17 and an *pENOD11::GUS* reporter line (Journet et al., 2001) were used in this study. Plants were grown in a growth chamber at 20 ℃ with 16h/8h day/night regime. For seed germination, the seeds were pre-treated in 98% sulphuric acid for 7 minutes, and washed for 6 times with demi-water. Afterwards, the seeds were sterilized with commercial bleach for 7 minutes and washed with sterile demi-water for 6 times. The sterilized seeds are transferred on to Fåhraeus medium plates (1.5% agar) and placed at 4 ℃ for 24 hours to synchronize the germination. One-day-old seedlings are transferred to Fåhraeus medium plates for gene expression studies. For root hair deformation assay, one-day-old seedlings are transferred to Fåhraeus slides (Heidstra et al., 1994). The liquid Fåhraeus medium without NH_4NO_3 is changed every 24 h in the first two days. Then, liquid Fåhraeus medium with or without 10 mM NH_4NO_3 is applied and changed every 24 h dependent on the assay setup. Nod factors were isolated from *Sinorhizobium meliloti* Strain 2011 (pMH682), a Nod factor over-producing strain, as the previous described (Roche et al., 1991).

Histochemical staining of GUS gene expression pattern

pMtENOD11::GUS plant roots under different treatments were harvested and washed with phosphate buffer solution (PBS) (pH7.0) for 3 times. The roots were transferred into the GUS staining buffer (contains 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 10mM EDTA, 0.1% Triton X-100 and 1 mg/ml X-Gluc salt in 100mM phosphate buffer solution, pH 7.0) and vacuumed for 30 min. Then, the samples are incubated in 37 ℃ growth chamber for 6 hours under dark. The stained roots were washed with PBS for 3 times to terminate the reaction.

Real time qRT-PCR analysis

The root samples containing the Nod factor susceptible region are cut out and froze immediately in liquid nitrogen. Total RNA was isolated from these samples with RNeasy plant kit (QIAGEN) following the supplier's manual. Plant cDNA is synthesized using iScript cDNA synthesis kit (Bio-Rad) from 1μg total RNA. Real time qRT-PCR is set up in 20μl reaction system with 2x iQ SYBR Green Super-mix (Bio-Rad) and preceded with iQ5 Real time PCR detecting system according to the manufacturer's manuals. All primers used in this study were designed with Primer3Plus software (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) under qPCR settings (Untergasser et al., 2007). For data normalization, *M. truncatula* ubiquitin gene is used as internal control. All primers used in this assay are listed in Supplemental Table 1.

Constructs and plant transformation

For spontaneous nodule formation assay, a truncated MtCCaMK construct lacking auto-inhibition domain was made according to Gleason et al. (2006). A 933-bp fragment, coding 311 amino acids, of CCaMK was amplified from wild type root cDNA sample using primers cMtCCaMK-t and tMtCCaMK-b, and digested with *Sma I* and *Kpn I*. Then, this fragment was sub-cloned into pENTR-R2L3-ep35S-MCS-t35s in between an enhanced CaMV35S promoter and CaMV35S terminator. Afterwards, it was recombined into pHGW-RR-MGW-R4R3 by using LR Clonase II plus (Invitrogen). To transform this construct into *M. truncatula*, *Agrobacterium rhizogenes* strain MSU440 is used in hairy-root transformation. Transformation process follows the previous described method (Limpens et al., 2004). Two weeks after transformation, the compound plants were transferred into perlite and watered with Fåhraeus liquid medium with or without 10mM NH_4NO_3, respectively. The plant morphology and spontaneous nodule formation is checked and photographed at four weeks after the transfer.

Light and fluorescence microscopy analysis

The root hair deformation is detected and captured under the Leica microscope with 20× and 40× Nomarski interference contrast (NIC) optical lens following the manufacture's manual. Transgenic roots are visualized by the red fluorescence under Leica stereomicroscope corresponding to the DsRED marker. The photographs are taken under white light condition

without filter or under UV light condition combined with DsRed filter using Leica microscope software following the manual.

REFERENCES

Allison, F.E., and Ludwig, C.A. (1934). The cause of decreased nodule formation on legumes supplied with abundant combined nitrogen. Soil Sci. **37,** 431-444.

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

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarie, J., and Cook, D.R. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science **303,** 1364-1367.

Barbulova, A., Rogato, A., D'Apuzzo, E., Omrane, S., and Chiurazzi, M. (2007). Differential effects of combined N sources on early steps of the Nod factor-dependent transduction pathway in *Lotus japonicus*. Mol. Plant Microbe Interact. **20,** 994-1003.

Carroll, B.J., and Gresshoff, P.M. (1983). Nitrate inhibition of nodulation and nitrogen fixation in white clover. Z. Pflanzenphysiol. **110,** 77-88.

Carroll, B.J., and Mathews, A. (1990). Nitrate inhibition of nodulation in legumes. In Molecular biology of symbiotic nitrogen fixation. P.M. Gresshoff, ed (CRC Press), pp. 159-180.

Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Denarie, J. (2000). Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. Plant Cell **12,** 1647-1666.

Charron, D., Pingret, J.L., Chabaud, M., Journet, E.P., and Barker, D.G. (2004). Pharmacological evidence that multiple phospholipid signaling pathways link Rhizobium nodulation factor perception in *Medicago truncatula* root hairs to intracellular responses, including Ca^{2+} spiking and specific ENOD gene expression. Plant Physiol. **136,** 3582-3593.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. Nature **417,** 962-966.

Fred, E.B., Baldwin, I.L., and McCoy, E. (1932). Factors that influence nodule production. In Root nodule bacteria and leguminous plants (Madison, Winsconsin, USA: Univ. Wisconsin), pp. 192-209.

Gi öbel, C.G. (1926). The relation of the soil nitrogen to nodule development and fixation of nitrogen by certain legumes. (Bulletin: New Jersey Agricultural Experiment Stations: Rutgers University), pp. 1-125.

Gleason, C., Chaudhuri, S., Yang, T., Munoz, A., Poovaiah, B.W., and Oldroyd, G.E. (2006). Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature **441,** 1149-1152.

Heidstra, R., Geurts, R., Franssen, H., Spaink, H.P., Van Kammen, A., and Bisseling, T. (1994). Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiol. **105,** 787-797.

Imsande, J. (1986). Inhibition of nodule development in soybean by nitrate or reduced nitrogen. J. Exp. Bot. **37,** 348-355.

Journet, E.P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., Arnould, C., Morandi, D., Barker, D.G., and Gianinazzi-Pearson, V. (2001). *Medicago truncatula* ENOD11: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol. Plant Microbe Interact. **14,** 737-748.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C., and Debelle, F. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science **303,** 1361-1364.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T., and Geurts, R. (2004). RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. J. Exp. Bot. **55,** 983-992.

Madsen, E.B., Antolin-Llovera, M., Grossmann, C., Ye, J., Vieweg, S., Broghammer, A., Krusell, L., Radutoiu, S., Jensen, O.N., Stougaard, J., and Parniske, M. (2011). Autophosphorylation is essential for the *in vivo* function of the *Lotus japonicus* Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J. **65,** 404-417.

Minchin, F.R., and Pate, J.S. (1973). The carbon balance of a legume and the functional economy of its root nodules. J. Exp. Bot. **24,** 259-271.

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

Peters, N.K., Frost, J.W., and Long, S.R. (1986). A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233**, 977-980.

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

Roche, P., Debelle, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Denarie, J., and Prome, J.C. (1991). Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: nodH and nodPQ genes encode the sulfation of lipo-oligosaccharide signals. Cell **67,** 1131-1143.

Shaw, S.L., and Long, S.R. (2003). Nod factor elicits two separable calcium responses in *Medicago truncatula* root hair cells. Plant Physiol. **131,** 976-984.

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

Streeter, J., and Wong, P.P. (1988). Inhibition of legume nodule formation and N_2 fixation by nitrate. CRC Cr. Rev. Plant Sci. **7,** 1-23.

Streeter, J.G. (1985). Nitrate inhibition of legume nodule growth and activity : I. Long term studies with a continuous supply of nitrate. Plant Physiol. **77,** 321-324.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J.-C., and Denarie, J. (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351,** 670-673.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. **35,** W71-74.

Wais, R.J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R.V., Cook, D., Gough, C., Denarie, J., and Long, S.R. (2000). Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. Proc. Natl. Acad. Sci. USA **97,** 13407-13412.

SUPPLEMENTAL DATA

Supplemental Table 1. All primers used in this study are listed.

Purpose	Name	Sequence	Restriction enzyme site
qRT-PCR	MtNFP-F	GCTCTAAGTTTGGCTTCTTTGGCAGTA	
	MtNFP-R	TGGTTCAGATGATGGTTGGTTGA	
	MtLYK3-F	TGATTCTGTTCTCAAGATGGCTCAA	
	MtLYK3-R	CAATCTTCAGTTGGTGATGAAAGTGTC	
	MtDMI1-F	TTATTTGCGGAGGAGGGGAAC	
	MtDMI1-R	CAATCTCCTTTCTTGTACGACCCCTA	
	MtDMI2-F	GGAGCTTGGTTGAATGGGCTAA	
	MtDMI2-R	TGCTACTTCCACAACTCTCCACAATG	
	MtDMI3-F	TTGTTTGACAACAACCGTGATGG	
	MtDMI3-R	CATACATCTGGAAGCACAAACGAAGA	
	MtNSP1-F	GCGATTTCGCCACTGGATTC	
	MtNSP1-R	CAGCCTCGCCTTCCATCATT	
	MtNSP2-F	GGCCTAGAATTGCAGGCTCGT	
	MtNSP2-R	TTGCAAAGCTCACCGGAACTC	
	MtUBQ10-F	CCCTTCATCTTGTCCTTCGTCTG	
	MtUBQ10-R	CACCTCCAATGTAATGGTCTTTCC	
Construct	cMtCCaMK-t	GGG *CCCGGG* ATGGGATATGGAACAA GAAAAC	*Sma* I
	tMtCCaMK-b	GG *GGTACC* GGCTTTCTCACCTTTGACC	*Kpn* I

The restriction enzyme sites in the sequences are shown as *italic*.

Chapter 3

Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2

Wei Liu[1,2], Wouter Kohlen[3], Alessandra Lillo[1], Rik Op den Camp[1], Sergey Ivanov[1], Marijke Hartog[1], Erik Limpens[1], Muhammad Jamil[3], Cezary Smaczniak[4], Kerstin Kaufmann[4], Wei-Cai Yang[2], Guido J. E. J. Hooiveld[5], Tatsiana Charnikhova[3], Harro J. Bouwmeester[3,6], Ton Bisseling[1,7] and René Geurts[1]

1. Department of Plant Science, Laboratory of Molecular Biology, Wageningen University, 6708 PB Wageningen, The Netherlands
2. Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
3. Department of Plant Science, Laboratory of Plant Physiology, Wageningen University, 6708 PB Wageningen, The Netherlands
4. Plant Research International–Bioscience, Laboratory of Molecular Biology, 6708 PB Wageningen, The Netherlands
5. Division of Human Nutrition, Wageningen University, 6703 HD Wageningen, The Netherlands
6. Centre for BioSystems Genomics, 6708 PB Wageningen, The Netherlands
7. College of Science, King Saud University, Riyadh 11451, Saudi Arabia

The Plant Cell **23**, 3853-3865 (2011)

ABSTRACT

Legume GRAS (GAI, RGA, SCR)-type transcription factors NODULATION SIGNALING PATHWAY1 (NSP1) and NSP2 are essential for rhizobium Nod factor-induced nodulation. Both proteins are considered to be Nod factor response factors regulating gene expression after symbiotic signaling. However, legume NSP1 and NSP2 can be functionally replaced by non-legume orthologs, including rice (*Oryza sativa*) NSP1 and NSP2, indicating that both proteins are functionally conserved in higher plants. Here, we show that NSP1 and NSP2 are indispensable for strigolactone (SL) biosynthesis in the legume *Medicago truncatula* and in rice. Mutant *nsp1* plants do not produce SLs, whereas in *M. truncatula*, NSP2 is essential for conversion of orobanchol into didehydro-orobanchol, which is the main SL produced by this species. The disturbed SL biosynthesis in *nsp1nsp2* mutant backgrounds correlates with reduced expression of *DWARF27*, a gene essential for SL biosynthesis. Rice and *M. truncatula* represent distinct phylogenetic lineages that split approximately 150 million years ago. Therefore, we conclude that regulation of SL biosynthesis by NSP1 and NSP2 is an ancestral function conserved in higher plants. *NSP1* and *NSP2* are single-copy genes in legumes, which imply that both proteins fulfill dual regulatory functions to control downstream targets after rhizobium-induced signaling as well as SL biosynthesis in non-symbiotic conditions.

INTRODUCTION

Strigolactones (SLs) exuded by plant roots into the rhizosphere are well-known stimuli for symbiotic arbuscular mycorrhizal fungi of the order *Glomeromycota* (Akiyama et al., 2005). These *ex planta* signals are coopted by root-parasitic plants of the *Orobanchaceae* family and are essential to induce their germination (Cook et al., 1966; Bouwmeester et al., 2007). Recently, it was found that SLs, or their derivatives, also function as endogenous plant hormones controlling outgrowth of axillary shoot buds (Gomez-Roldan et al., 2008; Umehara et al., 2008). They do so in crosstalk with auxin, the most prominent plant hormone (Beveridge and Kyozuka, 2010; Xie et al., 2010; Domagalska and Leyser, 2011). Biosynthesis and subsequent secretion of SLs is highly adaptive upon availability of nutrients, mainly phosphate (Yoneyama et al., 2007; López-Ráez et al., 2008; Umehara et al., 2008; Lin et al., 2009). To understand this adaptive regulation of these novel hormones, it is important to unravel the molecular mechanisms of SL biosynthesis, transport, and signaling. Here, we show that the GRAS-type transcription factors NODULATION SIGNALING PATHWAY1 (NSP1) and NSP2, which in legumes (Fabaceae) are essential for rhizobium root nodule formation, are indispensable for SL biosynthesis under non-symbiotic conditions.

Legumes can establish an endosymbiosis with nitrogen-fixing rhizobium bacteria. To host rhizobium, a novel lateral root organ, the root nodule, is formed in response to specific lipo-chito-oligosaccharides secreted by the bacterium. These signals, named nodulation (Nod) factors, show strong resemblance to lipo-chitooligosaccharides produced by mycorrhizal fungi (Maillet et al., 2011). In legumes, rhizobium Nod factors can trigger cell divisions in the root cortex, resulting in the formation of a nodule primordium. Furthermore, Nod factors are essential for intracellular infection by rhizobium. The Nod factor signaling cascade has been genetically dissected (reviewed by Kouchi et al., 2010). These studies showed that several signaling components have been recruited from the network that is also essential for endomycorrhizal symbiosis. Genes that are essential for mycorrhizal as well as rhizobium Nod factor induced signaling form the so-called common symbiotic signaling pathway and comprise a plasma membrane receptor kinase (DMI2 in *Medicago truncatula* and SYMRK in *Lotus japonicus*); several components in the nuclear envelope, including a cation ion channel (*M. truncatula* DMI1 and *L. japonicus* CASTOR and POLLUX); subunits of nuclear pores (*L. japonicus* NUP85 and NUP133); and a nuclear localized calcium calmodulin-dependent kinase (CCaMK) (*M. truncatula* DMI3 and *L. japonicus* CCaMK) (reviewed by Kouchi et al.,

2010). Mycorrhizae and rhizobium induced signaling bifurcates downstream of CCaMK, possibly because of differences in the nature of the calcium signal (Kosuta et al., 2008).

Nod factor signaling also requires several transcription factors, among which are the GRAS-type proteins NSP1 and NSP2 (Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006). Both of these transcription factors are essential for nearly all rhizobium-induced symbiotic responses, including Nod factor-induced early nodulin gene expression, nodule organogenesis, and nodule functioning (Catoira et al., 2000; Oldroyd and Long, 2003; Mitra et al., 2004b; Heckmann et al., 2006). Biochemical studies indicate that NSP1 and NSP2 form a protein complex that binds to a specific DNA element present in the promoter of some early nodulin genes, such as *ENOD11* (Hirsch et al., 2009). This suggests that NSP1 and NSP2 can function as Nod factor responsive transcription factors upon heterodimerization (Smit et al., 2005; Hirsch et al., 2009). NSP1 and NSP2, which are essential for rhizobium Nod factor induced signaling, are positioned downstream of CCaMK. Although both transcription factors are not essential for mycorrhizal symbiosis, it was recently found that an NSP2 dependent signaling pathway facilitates mycorrhizal root colonization (Maillet et al., 2011). This suggests that NSP2 does not function exclusively in rhizobium Nod factor signaling, a hypothesis that is supported by the presence of orthologous NSP genes in non-legume plant species (Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006). Here, we aim to characterize this novel function because it will provide insight into how these GRAS-type transcription factors have been recruited during nodule evolution.

GRAS-type transcription factors can be grouped in at least eight different classes, which are largely conserved in higher plants (Tian et al., 2004; Lee et al., 2008). For example, potential orthologs of NSP1 (class III) and NSP2 (class VII) can be found in many higher plant species, including rice (*Oryza sativa*) (Os03g29480/OsNSP1 and Os03g15680/OsNSP2) and *Arabidopsis thaliana* (At3g13840 and At4g08250) (Kaló et al., 2005; Smit et al., 2005; Heckmann et al., 2006; Murakami et al., 2006). *Arabidopsis* is unable to establish mycorrhizal symbiosis, which suggests a more generic function for both transcription factors. Notably, NSP1 and NSP2 are functionally conserved in higher plants as demonstrated in trans-complementation studies of legume *nsp1* and *nsp2* knockout mutants with non-legume NSP1 and NSP2 homologs (Heckmann et al., 2006; Yokota et al., 2010). This supports the idea that NSP1 and NSP2 fulfil a conserved function in plant growth and development.

We studied the genetic network controlled by NSP1 and NSP2 under non-symbiotic conditions and provide evidence that both transcription factors are indispensable for SL biosynthesis in legumes as well as non-legumes. *M. truncatula nsp1* mutants, *nsp1nsp2* double knockout mutants, and rice *Osnsp1Osnsp2* double knockdown lines hardly produce SLs. By analyzing the root transcriptome in *M. truncatula*, we found that the effect on SL production correlates with a strongly reduced *DWARF27 (MtD27)* expression. Likewise, rice *Osnsp1Osnsp2* double RNA interference (RNAi) knockdown lines have reduced *DWARF27 (OsD27)* expression. This underlines a conserved function of NSP1 and NSP2 in regulating SL biosynthesis.

RESULTS

M. truncatula NSP1 and NSP2 regulate genes in the carotenoid biosynthetic pathway

To identify genes that are directly or indirectly activated by NSP1 and NSP2 under non-symbiotic conditions, we analyzed the transcriptome of *M. truncatula nsp1* and *nsp2* knockout mutants. NSP1 and NSP2 are expressed mainly in root and nodules (Benedito et al., 2008). Therefore, we conducted microarray studies on 7-d-old roots and compared the *nsp1-1* and *nsp2-2* mutants with the wild type *M. truncatula* (Jemalong A17) grown on minimal medium without a nitrogen source (1.7 mM Pi). Expression values were obtained from three independent biological replicates for each of the two mutants against the wild type (National Center for Biotechnology Information Gene Expression Omnibus accession no. GSE26548). Because NSP1 and NSP2 can act as heterodimers (Hirsch et al., 2009), we searched for genes with decreased expression (at least two-fold) in both mutants. In total, 42 probe sets (representing 39 genes) fulfil this criterion (Supplemental Table 1). The expression of these genes was subsequently studied by quantitative (q) RT-PCR in an independent experiment that now also included the *M. truncatula nsp1nsp2* double mutant. Reduced expression in roots of both *nsp* mutants could be confirmed for 16 genes. These genes were also down-regulated in the double mutant (Table 1).

To obtain insight in the biological function of the down-regulated genes, extended sequences were retrieved from the *M. truncatula* genome data (IMGAG genome annotation version MT3.5) and were compared with homologous genes in other plant species. Two genes seemed to be legume specific, whereas the remaining 14 genes have clear homologs in non-legume species (Table 1). Four of these encode a protein that catalyses a conversion in the carotenoid biosynthetic pathway (a carotenoid isomerase homolog) or pathways leading from

carotenoids to the plant hormones SL and abscisic acid (a MAX1 homolog, a 9-cis-epoxycarotenoid dioxygenase homologous to Arabidopsis NCED4 and an iron-containing protein

Table 1. Genes down-regulated in root tissue of *M. truncatula nsp1*, *nsp2*, and *nsp1nsp2*

		Fold Change Microarray		Fold Change qRT-PCR		
Gene	Gene ID	nsp1	nsp2	nsp1	nsp2	nsp1 nsp2
DWARF27	–	−3.2	−3.4	−6.1	−8.0	−7.4
Carotenoid isomerase	–	−2.1	−2.2	−2.2	−2.5	−3.5
MAX1	Medtr3g104560	−2.5	−2.3	−1.9	−2.0	−1.6
9-*cis*-epoxycarotenoid dioxygenase 4	–	−2.0	−3.0	−2.6	−2.4	−3.2
1-Cys peroxiredoxin	–	−3.6	−3.7	−2.3	−1.9	−9.2
Abnormal gametophytes	Medtr3g014420	−3.4	−2.8	−3.2	−2.5	−2.5
Ala–tRNA ligase	Medtr4g074670	−3.3	−3.4	−1.9	−1.7	−4.4
Allyl alcohol dehydrogenase	–	−2.1	−2.0	−18.2	−14.9	−23.8
C2H2-type Zinc finger protein 6	Medtr7g082260	−7.8	−10.1	−5.0	−5.4	−29.4
Cytokinin-specific binding protein	Medtr3g055120	−2.4	−2.5	−1.9	−1.7	−2.1
Dirigent-like protein	Medtr4g122110	−6.1	−5.4	−4.4	−3.5	−5.3
Man-6-phosphate isomerase	Medtr3g104560	−2.5	−2.3	−1,7	−1.8	−1.6
Nudix hydrolase	Medtr5g078020	−3.5	−4.5	−1.5	−3.4	−15.6
Seed maturation protein LEA 4	Medtr7g093160	−3.2	−3.0	−1.2	−1.2	−11.1
Legume-specific protein	Medtr5g061550	−7.7	−7.7	−1.9	−1.8	−10.5
Legume-specific protein	–	−4.6	−7.8	−1.5	−3.0	−2.5

Plants were grown in the absence of a nitrogen source. Gene expression was analyzed using microarray analysis and qRT-PCR. Gene ID is based on *M. truncatula* genome annotation (IMGAG MT3.5).

with high sequence similarity to rice DWARF27 (OsD27)) (Lin et al., 2009; Beveridge and Kyozuka, 2010; Xie et al., 2010). In particular, the expression of the *DWARF27* homologous gene was strongly affected (~90% reduced expression) in roots of both *nsp1* and *nsp2* mutants and in the double mutant (Figure 1A). Subsequent searches in the *M. truncatula* genome and EST resources revealed that *M. truncatula* has only a single DWARF27 homologous gene that was covered by two probes on the *M. truncatula* GeneChip (Supplemental Table 1). The encoded *M. truncatula* protein displays 54% identity and 70% similarity with rice D27 (Figure 2A). Subsequent phylogenetic analysis revealed that the *M. truncatula* protein and rice D27 belong to the same orthology group (Figure 2B; Supplemental Data Set 1). Common ancestry is further supported by a largely conserved exon-intron structure of both genes (Figure 2C). Based on these findings, we named the corresponding *M. truncatula* gene MtDWARF27 (MtD27), although it must be noted that we did not provide a formal proof for orthology because functional trans-complementation experiments of the rice *d27* knockout mutant have not been done.

To confirm that down-regulation of *MtD27* was the result of the knockout of NSP genes and not of a background mutation present in both mutants, we complemented the *Mtnsp1*

mutant with a *pMtNSP1::MtNSP1* construct and subsequently determined the *MtD27* expression in transgenic roots. This revealed that the expression of *MtD27* did indeed depend on the presence of a functional MtNSP1 protein (Figure 1B).

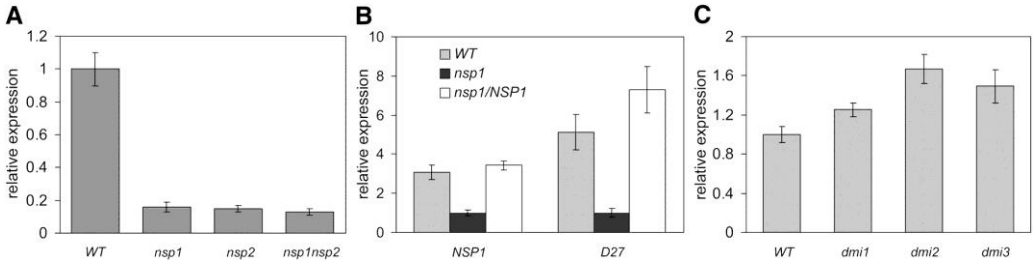


Figure 1. *M. truncatula D27* Expression is NSP1 and NSP2 dependent. (A) Relative expression level of *MtD27* in nitrogen-starved roots of *M. truncatula* wild-type (WT), *nsp1*, *nsp2*, and *nsp1nsp2* mutant plants as determined by qRT-PCR. (B) Expression of *D27* can be rescued in the roots of nsp1 mutant complemented with pMtNSP1:MtNSP1. (C) Expression of *D27* in nitrogen-starved roots of the *M. truncatula* common symbiotic signalling pathway mutants, *dmi1*, *dmi2*, and *dmi3*, determined by qRT-PCR. Data are means ± SD.

Next, we determined whether expression of *MtD27* under non-symbiotic conditions depends on components of the common symbiotic signaling pathway that is essential for rhizobium Nod factor signaling and mycorrhizal signaling. *MtD27* transcript levels were quantified in roots of *M. truncatula dmi1*, *dmi2*, and *dmi3* mutants. *MtD27* expression was not reduced in these mutants (Figure 1C). Therefore, we conclude that the regulation of *MtD27* expression in the absence of rhizobium or a mycorrhizal fungus does not depend on the common symbiotic signaling pathway but does depend on MtNSP1 and MtNSP2.

SL biosynthesis in *M. truncatula* requires NSP1 and NSP2

In rice, *OsD27* is essential for SL biosynthesis (Lin et al., 2009). Because the expression of *MtD27* in *M. truncatula* is nearly undetectable in roots of *nsp1*, *nsp2*, and *nsp1nsp2* mutants grown in the absence of a nitrogen source, we determined whether SL biosynthesis is affected in these mutants. To this end, we characterized the SLs produced by *M. truncatula*. It has been reported that the amount of SLs in root exudate strongly increases upon phosphate starvation (Yoneyama et al., 2007; López-Ráez et al., 2008; Umehara et al., 2008; Lin et al., 2009). Therefore, we conducted two experiments and grew plants in one-half-strength Hoagland medium under either low and normal nitrogen conditions (0.14 and 2.8 mM NH4NO3, respectively) or no and normal phosphate conditions (0.2 mM Pi). First, we determined the expression levels of *MtNSP1*, *MtNSP2*, and *MtD27* in root systems grown under these

conditions. Expression levels of *MtNSP1* and *MtNSP2* were not affected by the nutrient levels in the medium. By contrast, *MtD27* already displayed a strong (>30-fold) up-regulation after 1 d of phosphate starvation, whereas nitrogen starvation had little to no effect on *MtD27* expression (Figures 3A and 3B).

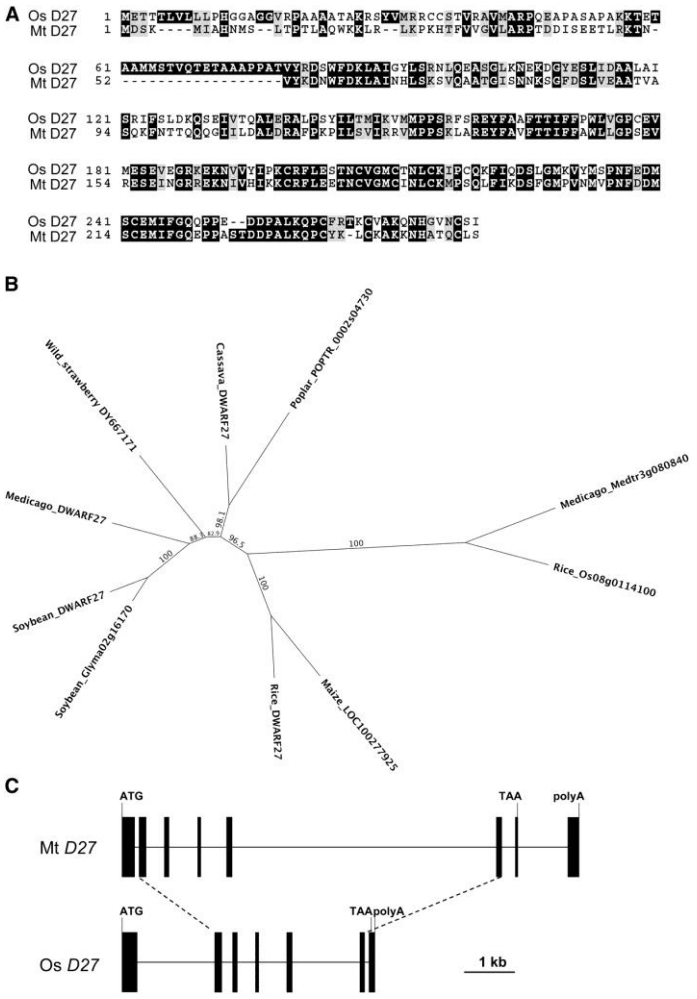


Figure 2. *M. truncatula D27* is orthologous to rice *D27*. (A) Protein alignment of *M. truncatula* D27 and rice D27 (70% homology/54% identity). (B) Maximum-likelihood phylogenetic analysis of rice D27 homologous proteins of *M. truncatula* (*M. truncatula*), soybean (*Glycine max*), poplar (*Populus trichocarpa*), cassava (*Manihot esculenta*), wild strawberry (*Fragaria vesca*), maize (*Zea mays*), and rice. Branch lengths are proportional to the number of amino acid substitutions per site. Branch support was obtained from 100 bootstrap repetitions. (C) Largely conserved exon-intron structure in *M. truncatula* and rice *D27*. The lengths of exons 2 to 6 are conserved between *M. truncatula D27* and rice *D27* (aligned with dashed lines). Black boxes, exons; line, introns. GenBank accession number JN629088.

Next, root exudates from phosphate starved and non-starved plants and from nitrogen starved and non-starved plants were collected and purified using C18 column chromatography. In all

root exudates, two peaks were detected that could correspond to SLs. The major peak had a retention time and transitions corresponding to didehydro-orobanchol (Supplemental Figure 1A). The nature of this SL was confirmed by co-injection and comparison of that of the tandem mass spectrometry (MS/MS) spectrum with the MS/MS spectrum obtained from one of the didehydro-orobanchol isomers found in tomato (*Solanum lycopersicum*) (López-Ráez et al., 2008) (Supplemental Figure 1B). The minor peak was identified as orobanchol based on the retention time and transitions (Supplemental Figure 1C), and this was confirmed by comparing this MS/MS spectrum with that of an orobanchol standard (Supplemental Figure 1D). Taken together, we conclude that *M. truncatula* produces two different SLs: didehydro-orobanchol and orobanchol. Although both SLs could be detected in the exudate of non-phosphate starved plants (with or without nitrogen), amounts increased ~10-fold upon phosphate starvation (Figures 3C and 3D). This indicates that, as with many other species, SL secretion, and likely biosynthesis, by *M. truncatula* roots is induced by phosphate limiting conditions, whereas the nitrogen status of the plant has no effect. This increase in SL secretion under phosphate starvation correlates with the transcriptional up-regulation of *MtD27*.

Subsequently, the *M. truncatula nsp1*, *nsp2*, and *nsp1nsp2* mutants as well as an independent set of the wild type plants were grown under phosphate starvation. Profiling of *MtD27* expression under these growth conditions revealed that *MtD27* expression was repressed in all three mutants. In *nsp1* and *nsp1nsp2* mutant plants, *MtD27* expression was nearly absent, whereas in roots of the *nsp2* mutant, *MtD27* expression was reduced to ~20% of wild type levels (Figure 3E). The finding that *MtD27* expression in phosphate-starved roots is dependent on MtNSP1 and MtNSP2 is in line with results obtained in the microarray study, although it also shows that, under phosphate limitation, transcriptional activation of *MtD27* can occur partly independent of MtNSP2. Analyses of root exudates by multiple reaction monitoring-liquid chromatography (MRM-LC)-MS/MS showed that no SLs are secreted by the *nsp1* mutant or the *nsp1nsp2* double mutant (Figure 3F). By contrast, in root exudates of the *nsp2* mutant, orobanchol accumulated in 10-fold higher levels than those observed in exudate of the wild type plants (Figure 3F). Interestingly, didehydro-orobanchol was absent in the root exudate of this mutant, suggesting that MtNSP2 controls a specific step in SL conversion. To rule out the possibility that NSP1 and MtNSP2 are merely affected in secretion of certain SLs and not in biosynthesis, we also investigated the SL content of root extracts. This revealed similar results as observed in root exudates (Supplemental Figure 2). Taken together, these

findings demonstrate that genes controlled by MtNSP1 and MtNSP2 are essential in the SL biosynthetic pathway and include *MtD27*.

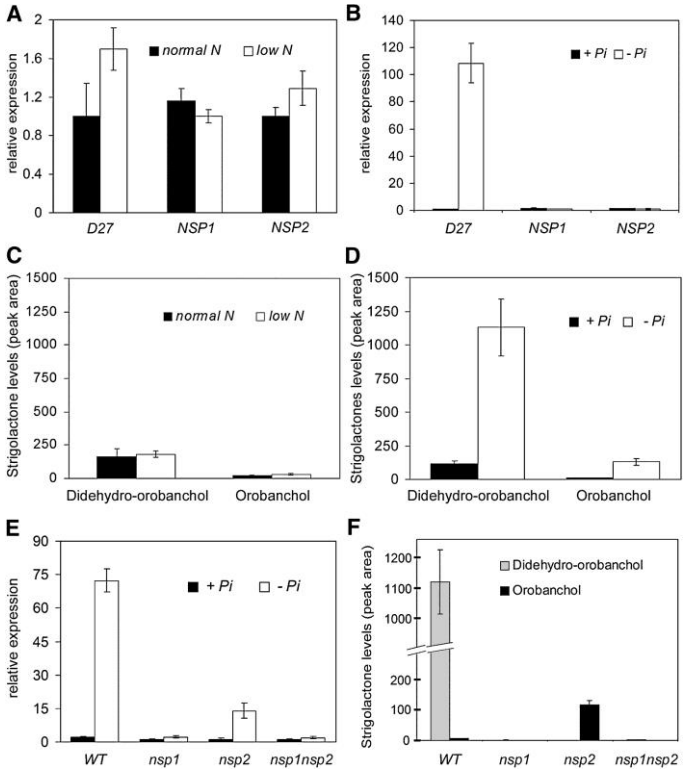


Figure 3. SL Biosynthesis Correlates with *M. truncatula D27* Expression. (A) Relative expression levels as detected by qRT-PCR of *D27*, *NSP1*, and *NSP2* in *M. truncatula* wild-type roots under low (0.14 mM for 24 h) and sufficient (2.8 mM) NH_4NO_3 growth conditions. Data are means ± SD. (B) Relative expression levels as detected by qRT-PCR of *D27*, *NSP1*, and *NSP2* in *M. truncatula* wild-type roots under no (for 24 h) and sufficient (0.2 mM Pi) phosphate growth conditions. Lowest expressed gene is set to 1. Data are means ± SD. (C) Levels of the SLs didehydro-orobanchol and orobanchol in *M. truncatula* wild-type root exudates as detected by MRM-LC-MS/MS under low (0.14 mM for 7 d) and sufficient (2.8 mM) NH_4NO_3 growth conditions. Data are means ± SE. (D) Levels of the SLs didehydro-orobanchol and orobanchol in *M. truncatula* wild-type root exudates as detected by MRM-LC-MS/MS under no (for 7 d) and sufficient (0.2 mM Pi) phosphate conditions. Data are means ± SE. (E) Relative expression levels as detected by qRT-PCR of *MtD27* in roots of *M. truncatula* wild-type (WT), *nsp1*, *nsp2*, and *nsp1nsp2* mutant plants under no (for 24 h) and sufficient (0.2 mM Pi) phosphate growth conditions. Data are means ± SD. (F) Analysis of the SLs didehydro-orobanchol and orobanchol in root exudates of phosphate-starved (0.14 mM for 7 d) *M. truncatula* wild-type, *nsp1*, *nsp2*, and *nsp1nsp2* mutant plants (n = 3). Data are means ± SE.

NSP1/NSP2 control SL biosynthesis in rice

Because MtNSP1 and MtNSP2 orthologous genes are present in non-legume species, we investigated whether the control of SL biosynthesis is a conserved function of these proteins. The role of DWARF27 in SL biosynthesis is characterized in rice (Lin et al., 2009; Wang and Li, 2011), and rice, as a monocot, is phylogenetically distinct from *M. truncatula*. Therefore, we decided to focus on this species. Real time qRT-PCR analysis on root RNA showed that transcripts of both NSP genes are present, although the level of *NSP2* expression was close to the detection limit (Figure 4A). This is in line with a previous study that reported that the expression levels of rice *NSP1* and *NSP2* are extremely low (Yokota et al., 2010).

To study whether OsNSP1 and OsNSP2 are essential for SL biosynthesis in rice, double RNAi knockdown lines were created by *Agrobacterium tumefaciens* mediated transformation. In total, 14 lines were obtained, and two of these showed severe knockdown (>90%) of *OsNSP1* in roots (Figure 4B). Because *OsNSP2* is expressed at a very low level, the knockdown level of this gene could not be determined reliably. The expression of *OsD27* in these knockdown lines is reduced >90% when compared with the wild type (Figure 4C), showing that in rice, as in *M. truncatula*, the expression of *OsD27* relies on the presence of OsNSP1 and possibly OsNSP2.

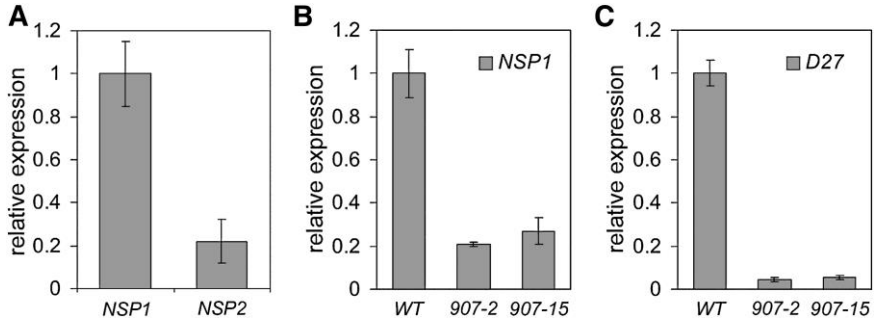


Figure 4. Expression analysis of *NSP1, NSP2,* and *D27* in rice determined by qRT-PCR. (A) *NSP1* and *NSP2* expression in roots of wild-type rice (ZH11). (B) Expression of *NSP1* in roots of the rice *nsp1nsp2* double knockdown lines 907-2 and 907-15. WT, wild type. (C) Expression of *D27* in roots of the rice *nsp1 nsp2* double knockdown lines 907-2 and 907-15. Data are means ± SD.

To determine whether down-regulation of rice *OsD27* expression correlates with reduced SL biosynthesis, root exudates of *nsp1nsp2* RNAi lines were analyzed using MRM-LC-MS/MS analysis. In rice, 2′-epi-5-deoxystrigol and orobanchol are the major SLs (Umehara et al., 2008), and both could be detected in root exudates of the wild type rice plants

(Figure 5A). In the *nsp1nsp2* RNAi lines, the amounts of 2′-epi-5-deoxystrigol and orobanchol in the root exudates were approximately five- and eight-fold lower, respectively, than in the wild-type plants (Figure 5A). This reduction was also visualized in a *Striga hermonthica* seed germination bioassay. Seeds of this parasitic plant are known to respond in a semi-quantitative way to SLs (Matusova et al., 2005). The germination-inducing activity of the rice *nsp1nsp2* RNAi root exudates was four to five-fold reduced compared with exudates from the wild-type plants (Figure 5B). Therefore, we conclude that NSP1 and possibly NSP2 are essential for SL biosynthesis in rice as well.

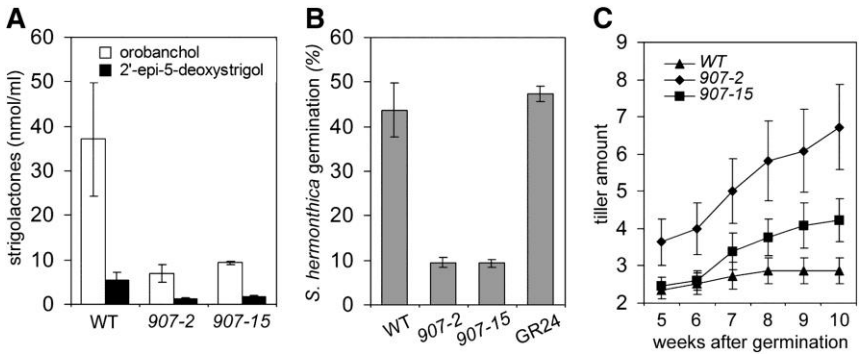


Figure 5. Tillering phenotype and SL concentrations in root exudates of wild type rice (zh11) and two independent rice *nsp1nsp2* knockdown lines. (A) Analysis of 2′-epi-5-deoxystrigol and orobanchol content in root exudates of phosphate-starved wild type (WT) rice (ZH11) and *nsp1nsp2* knockdown lines 907-2 and 907-15 (n = 3). (B) Germination of *S. hermonthica* seeds induced by root exudates of the wild type (ZH11) and *nsp1nsp2* knockdown lines 907-2 and 907-15 (n = 3). Data are means ± SE. (C) Quantification of tillering in rice. Tillers of wild-type rice (ZH11) and nsp1 nsp2 double knockdown lines (907-2 and 907-15) were quantified 5 to 10 weeks after planting (n = 15). Data are means ± SE.

NSP1/NSP2 control lateral shoot growth in rice, but not in *M. truncatula*

In rice, *Osd27* knockout mutation causes increased tillering in combination with reduced plant height (Lin et al., 2009). Because the absence of NSP1-NSP2 expression in rice and *M. truncatula* resulted in markedly reduced SL biosynthesis in root tissue, we tested whether these mutants plants also display a more branched shoot phenotype. In rice, the amount of tillers was quantified at different time points from 5 to 10 weeks after germination. The *nsp1nsp2* RNAi lines had an increased number of tillers when compared with the wild type rice (Figure 5C). However, these knockdown lines did not display obvious differences in plant height, as reported for the rice *d27* knockout mutant (Lin et al., 2009). Therefore, although the increased tillering is in line with the phenotype of the rice *d27* knockout mutant,

plant height seems less critical because residual levels of *OsD27* expression and SL biosynthesis seem to be sufficient to support normal shoot growth. Next, we studied shoot branching in the *M. truncatula nsp1nsp2* double mutant and compared it with the wild type. Wild type *M. truncatula* formed three to four branches at the first four knots and well-developed secondary branches during 10 weeks of plant growth. We did not find an increased amount of branches in the *nsp1nsp2* double mutant. Therefore, we conclude that severe reduction in SL concentration in roots of the *M. truncatula nsp1nsp2* mutant plants has no obvious effect on shoot architecture.

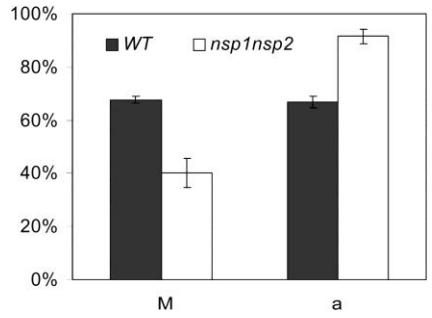


Figure 6. Mycorrhizal colonization of *M. truncatul*a wild type and *nsp1nsp2* double mutant. ($P < 0.05$). Data are means ± SE. a, presence of arbuscules in the mycorrhized root segments; M, intensity of mycorrhization in total root system.

M. truncatula nsp1nsp2 double mutant displays reduced mycorrhizal infection

Because NSP1 and NSP2 control biosynthesis of SLs, which are important *ex planta* stimuli for branching of mycorrhizal hyphae, it seems probable that these GRAS-type transcription factors also have a function in the interaction of plants with arbuscular mycorrhizae. A recent study in *M. truncatula* indeed revealed that the *nsp2* mutant displays a ~40% reduction in mycorrhizal colonization (Maillet et al., 2011). Our study revealed that the *nsp2* knockout mutant still produces orobanchol. We investigated the mycorrhizal phenotype of the *nsp1nsp2* double mutant that does not produce detectable amounts of SLs. Mutant and wild type plants were grown under low phosphate conditions and inoculated with *Rhizophagus irregularis* (formerly named *Glomus intraradices*). Six weeks after inoculation, the mycorrhizal status of the root systems was evaluated. It was found that *nsp1nsp2* can be mycorrhized effectively by *R. irregularis*. However, the fraction of the root system that was mycorrhized was significantly lower in the mutant than in the wild-type plants (40.1% versus 67.6%, respectively) (Figure 6). When quantifying the number of arbuscules within mycorrhized root sections, we noted that such segments of the *nsp1nsp2* mutant contained more arbuscules when compared with the wild type (91.4% versus 66.7%, respectively)

(Figure 6). These data suggest that absence of SLs affects the initial infection of *R. irregularis*, whereas intracellular arbuscule formation is not hampered.

DISCUSSION

Here, we show that the GRAS-type transcriptional regulators NSP1 and NSP2 regulate SL biosynthesis in *M. truncatula* and rice. These two species represent distinct phylogenetic lineages that split ~150 million years ago (Moore et al., 2007; Smith et al., 2010). Therefore, we conclude that the regulation of SL biosynthesis by NSP1 and NSP2 is an ancestral function conserved in higher plants. During evolution of legumes, these two transcriptional regulators have been recruited to play an essential role in rhizobium root nodule symbiosis. NSP1 and NSP2 are single copy genes in legumes, which imply that single proteins fulfill both functions in these species.

The absence of SL biosynthesis in *nsp1nsp2* mutant backgrounds correlates with a reduced expression of several genes that encode enzymes of the carotenoid and SL biosynthetic pathways, including *D27*. The precise biochemical function of the plastid-localized, iron-containing protein D27 remains to be elucidated, but it was shown to be essential for SL biosynthesis in rice (Lin et al., 2009; Wang and Li, 2011). Our study in *M. truncatula* revealed that the transcriptional regulation of the SL biosynthetic enzyme D27 is tightly regulated by the nutrient status of the plant. Phosphate starvation in particular triggers a dramatic up-regulation of *D27* transcription. Because *NSP1* and *NSP2* expression is not affected or is only very mildly affected by the nutrient status of the plant (Barbulova et al., 2007), we conclude that the activity of both transcription factors is controlled at the protein level, similar to their hypothesized symbiotic functioning in rhizobium Nod factor-induced signaling in legumes (Geurts et al., 2005).

We showed that *M. truncatula* produces two different SLs (orobanchol and didehydro-orobanchol), similar to its close relative red clover (*Trifolium pratense*) (Yokota et al., 1998; Xie et al., 2010). Intriguingly, *M. truncatula* SL biosynthesis is differentially regulated by NSP1 and NSP2. In the *nsp1* mutant background, no detectable amounts of SLs are produced, but the *nsp2* mutant specifically secretes orobanchol in concentrations higher than those found in the wild type plants (Figure 7). This shows that in *M. truncatula*, the markedly decreased expression level of *D27* in an *nsp2* mutant background is still sufficient to control orobanchol biosynthesis. Because the precise biochemical pathway of different SLs has not yet been elucidated, the finding that the *M. truncatula nsp2* mutant accumulates orobanchol rather than

didehydro-orobanchol, as found in the wild-type *M. truncatula* plants, may provide a tool to identify key enzymes in the biosynthesis of different SLs. It is assumed that 5-deoxystrigol is the first genuine SL and that all other SLs are derived from it (Matusova et al., 2005; Rani et al., 2008). Subsequently, didehydro-orobanchol is derived from orobanchol in a three-step process: a homoallylic hydroxylation of orobanchol to hydroxyorobanchol, an oxidation or dehydration to oxo-orobanchol, and a migration of the methyl group leading to didehydro-orobanchol (Matusova et al., 2005; Beveridge and Kyozuka, 2010; Xie et al., 2010). The enzymes involved in these steps might be under the transcriptional regulation of NSP2 (Figure 7). A preliminary analysis of *M. truncatula* genes that are differentially down-regulated in *nsp2* mutant roots but not in the wild type or the *nsp1* mutant revealed a subset of such genes (Supplemental Table 2) that included several candidates that encode enzymes that could be involved in this process. Functional analysis of these genes could provide access to key enzymes in SL conversion.

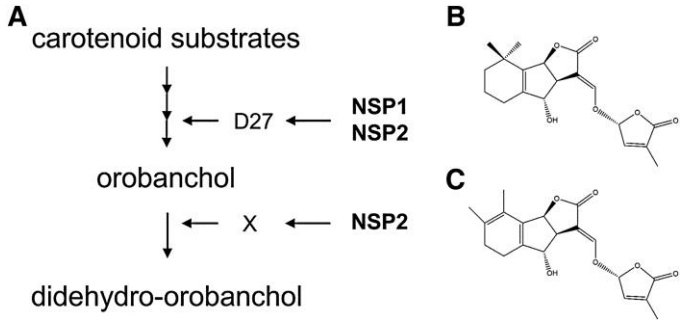


Figure 7. Model for SL biosynthesis and structures of the two main SLs in *M. truncatula*. (A) Schematic representation of the SL biosynthesis pathway in *M. truncatula* controlled by the GRAS-type transcription factors NSP1 and NSP2. Orobanchol is synthesized in a multistep process from carotenoid substrates and requires D27, which is under transcriptional control of NSP1 and NSP2. In this step, NSP2 is partly redundant. The conversion of orobanchol in didehydro-orobanchol requires unknown enzyme X, which is under direct or indirect control of NSP2. WT, wild type. (B) Structural formula of orobanchol. (C) Putative structure formula of didehydro-orobanchol.

Besides D27, two different carotenoid cleavage dioxygenase (CCD) enzymes are essential for SL biosynthesis, specifically CCD7 and CCD8. However, the precise relation between both CCDs and D27 remains to be elucidated (Xie et al., 2010). *M. truncatula* homologs of both CCD genes are represented on the GeneChip (*CCD7*/Medtr7g045370.1/ probe ID Mtr.32038.1.S1_at and *CCD8*/Medtr3g109610.1/ Mtr.1606.1.S1_at). Interestingly, the absence of functional NSP1 or NSP2 does not affect the expression of these *M. truncatula CCD7* and *CCD8* homologous genes. Assuming that both *CCD* genes are also essential for SL

biosynthesis in *M. truncatula*, this suggests that key enzymes essential for this biosynthetic pathway are controlled by different transcriptional networks.

In legumes, NSP1 and NSP2 are key components in the rhizobium Nod factor-induced signaling pathway, and a knockout mutation in either of these genes impairs gene induction by Nod factors, including *ENOD11* in *M. truncatula* (Catoira et al., 2000; Oldroyd and Long, 2003; Mitra et al., 2004a). *ENOD11* is a direct target of NSP1 because this transcription factor binds to the conserved *cis*-regulatory binding element AATTT (Hirsch et al., 2009). This motif is named the nodulation-responsive element (NRE), and NSP1 binds to this element in an NSP2-dependent manner. *ENOD11* has two NREs in the -500 bp promoter region. Interestingly, NRE-like motifs are abundantly present in putative promoter regions of rice and *M. truncatula D27* (Supplemental Figures 3A and 3B). In the *M. truncatula D27* promoter, nine NRE-like motifs can be found in about 1000 bp region: seven times in the forward orientation and two times in the reverse orientation, respectively (Supplemental Figure 3A). This raises the question of whether NSP1 also can bind directly to the *D27* promoter. Preliminary studies using *in vitro* electrophoretic mobility shift assays suggest that this is the case (Supplemental Figures 3C to 3E), indicating that *M. truncatula D27* indeed could be a direct target of NSP1. However, such studies need to be confirmed by complementary approaches.

The finding that *M. truncatula D27* expression is under (direct or indirect) control of NSP1/NSP2 leads to a puzzling difference in regulation when compared with functioning of both GRAS-type transcription factors in the Nod factor signaling pathway. In the latter case, NSP1 needs to be activated upon Nod factor signaling. By contrast, none of the components of this signaling pathway, not even the kinase CCaMK directly active upstream of NSP1 and NSP2, are required for *D27* expression. The finding that both NSPs can function independent of CCaMK is in line with the fact that some plant species that cannot establish a mycorrhizal symbiosis (e.g., *Arabidopsis*) have lost many of the components in the common symbiotic signaling pathway, including CCaMK, but have maintained NSP1 and NSP2 orthologous genes (Kaló et al., 2005; Smit et al., 2005; Zhu et al., 2006). The recruitment of a conserved transcription factor into the signaling pathway triggered by rhizobium Nod factors, as occurred in legumes, is therefore intriguing. It suggests that in the event of Nod factor signaling, the NSP-controlled transcriptional regulation is activated in a different manner. It remains to be elucidated how this difference is created at a molecular level. We hypothesize that this is achieved by modification of the NSP1 and NSP2 protein complexes upon CCaMK activity.

This might be a direct modification or may involve a different component that affects the binding affinity of the transcription factor complex. However, the occurrence of NRE-like *cis* regulatory elements in the *M. truncatula D27* promoter region suggests that both mechanisms could have parts in common.

SLs are stimuli for mycorrhizal hyphae, and SL biosynthesis knockout mutants in tomato (*ort1*) and pea (*Pisum sativum*; *ccd8*) display reductions in mycorrhizal root colonization (Gomez-Roldan et al., 2008; Koltai et al., 2010). The *M. truncatula nsp1nsp2* double mutant has a similar reduction in mycorrhizal root infection (Figure 6). We also investigated arbuscule formation and showed that the *nsp1nsp2* mutant efficiently forms well-developed arbuscules. This finding suggests that SLs are not essential for arbuscule formation. Therefore, we hypothesize that SLs stimulate root colonization exclusively *ex planta*, in line with this are the mycorrhizae symbiotic phenotypes of *nsp* mutants that can be fully explained by the role of both transcription factors in SL biosynthesis.

Plant mutants affected in SL biosynthesis or signaling are known for a distinctive shoot branching phenotype. However, in *M. truncatula nsp1* and *nsp2*, we did not observe such phenotype. This is in contrast with rice *nsp* knockdown lines. This suggests that *M. truncatula* is possibly not ideal for detecting shoot branching phenotypes, likely because it does not display strong apical dominance. Because shoot architecture can be influenced by environmental conditions, a more detailed study on bud outgrowth, stem length, and/or polar auxin transport may reveal weaker phenotypes in SL biosynthesis mutants in *M. truncatula*. Additionally, the D27 dependent shoot architecture in rice may be enhanced by the expression profile of the corresponding gene. In rice, *D27* is transcribed in shoot bases, culms, panicles, and axillary buds (Lin et al., 2009), whereas in *M. truncatula D27* is expressed exclusively in roots (Supplemental Figure 4) (Benedito et al., 2008).

During the past decade, genes encoding enzymes essential for SL biosynthesis have been elucidated. To our knowledge, we have identified the first two transcription factors, NSP1 and NSP2, which are key regulators of SL biosynthesis. SL biosynthesis is highly regulated by environmental and other conditions. Therefore, NSP1 and NSP2 will be important tools in future studies on the molecular mechanisms of how environmental sensing is translated into regulation of SL biosynthesis.

MATERIALS AND METHODS

Plant materials, growth conditions and transformation

Medicago truncatula was grown in a growth chamber at 20 ℃ and 16-h-day/8-h-night regime. Jemalong A17, *nsp1-1* (B85) (Catoira et al., 2000; Smit et al., 2005), and *nsp2-2* (0 to 4) (Oldroyd and Long, 2003; Kaló et al., 2005) were used as the wild type, *nsp1*, and *nsp2*, respectively. The *nsp1nsp2* double mutant was obtained by pollinating *nsp1-1* plants with *nsp2-2* pollen. *nsp1nsp2* homozygote plants were selected by PCR-based genotyping of F2 individuals.

M. truncatula plants used for gene expression analysis were grown vertically on Fåhraeus medium plates without nitrate (Fåhraeus, 1957). RNA was isolated from the Nod factor-susceptible zone of 7-d-old roots samples snap-frozen in liquid N_2. Total RNA was extracted using the E.Z.N.A. Plant RNA Kit (Omega Bio-Tek), combined with Qiagen RNase-Free DNase Set for on-column DNase treatment.

Agrobacterium rhizogenes-based root transformation of *M. truncatula* was conducted according to Limpens et al. (2004). Plants with transgenic roots were selected based on DsRED1 fluorescence.

Mycorrhization studies were conducted with fine sand and calcined clay mixtures, which contain spores of *Rhizophagus irregularis*. Six weeks after transplantation, the whole-root systems were harvested and evaluated according to Trouvelot et al. (1986).

Rice (*Oryza sativa* ssp *japonica* cv Zhonghua 11) was used as the wild-type rice. Rice plants were grown in a greenhouse at 28 ℃ in a 16-h-day/8-h-night regime. For tillering assays, rice plants were grown on fine sand with one-half-strength full-nutrient Hoagland solution (Hoagland and Arnon, 1950) and watered once a week. Rice transformations were conducted using *Agrobacterium tumefaciens* strain AGL1 according to Toki et al. (2006) and subsequent Hygromycin B selection.

Constructs and plasmids

For complementation of the *M. truncatula nsp1* mutant, we used the *pMtNSP1:MtNSP1* construct as generated by Smit et al. (2005).

Based on the principle that the RNAi functions through an ~20-nucleotide fragment, a chimeric RNAi construct was made by fusing two fragments from OsNSP1 (295 bp) and OsNSP2 (488 bp) in a single hairpin. First, fragments of OsNSP1 and OsNSP2 were PCR

amplified from rice genomic DNA with primer pairs OsNSP1-F/OsNSP-mr and OsNSP-mf/OsNSP2-R, respectively (Supplemental Table 3). Subsequently, both fragments were fused by overlap PCR with primer pair OsNSP1-F and OsNSP2-R (Supplemental Table 3). This chimeric fragment then was subcloned into pENTRL1L2, resulting in pENTRL1L2_OsNSP1-2i. Then, this construct was recombined into pHGWIWG2 (II)-RR-R1R2 to get the binary construct pHGWIWG2 (II)-RR-OsNSP1+2i.

Affymetrix GeneChip oligoarray hybridization, scanning, and quality control

Total RNA (5 μg) was labeled using the Affymetrix One-Cycle Target Labeling Assay kit (Affymetrix). Labeled RNA samples were hybridized on Affymetrix *M. truncatula* Genome arrays (product 900735) and washed, stained, and scanned on an Affymetrix GeneChip 3000 7G scanner. The detailed protocols that we used for array handling can be found in the GeneChip Expression Analysis Technical Manual, section 2, chapter 2 (Affymetrix; product 701028, revision 5). Packages from the Bioconductor project (Gentleman et al., 2004) were used to analyze the array data. Various advanced quality metrics, diagnostic plots, pseudoimages, and classification methods were used to determine the quality of the arrays before statistical analysis (Heber and Sick, 2006, Brettschneider et al. 2008). In brief, the library "affy" was used to determine for each array the average background signal, percentage present calls, scale factor, and RNA degradation plots essentially as described by Alvord et al. (2007). All arrays passed the guidelines recommended by Affymetrix, as described in the Affymetrix Microarray Suite Users Guide, Version 5.0. In addition, the library "AffyPLM" was used to fit probe-level linear models that provide parameter estimates for probes and arrays on a probe-by-probe basis (Bolstad et al., 2005). Two-dimensional pseudoimages of the arrays based on probe-level quantities, namely the weights and residuals computed by fitPLM, were inspected for stains, scratches, and other artifacts. Even if present, all artifacts covered less than 5% of the area of the array. Moreover, numerical quality was assessed based on two distributions computed at the probe set level, the normalized unscaled se, and relative log expression (RLE). No outlier arrays were observed for all these quality control parameters; that is, all arrays were reasonably centered around the median normalized unscaled se of 1, and boxplots for RLE had a small spread and were centered at an RLE of 0. Array data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE26548.

Expression estimates were obtained by GeneChip Robust Multiarray Averaging analysis, using the empirical Bayes approach to adjust for background (Wu et al., 2004) in the Bioconductor library "gcrma." Differentially expressed probe sets were identified using linear models, applying moderated *t* statistics that implemented empirical Bayes regularization of se values (library "limma"). The moderated Student's *t* test statistic has the same interpretation as an ordinary Student's *t* test statistic, except that these values have been moderated across genes (i.e., shrunk to a common value) using a Bayesian model (Smyth, 2004). To adjust for both the degree of independence of variance relative to the degree of identity and the relationship between variance and signal intensity, the moderated *t* statistic was extended by a Bayesian hierarchical model to define an intensity-based moderated *t* statistic (Sartor et al., 2006). Intensity-based moderated *t* statistics improve the efficiency of the Empirical Bayes moderated t statistics and thereby achieve greater power while correctly estimating the true proportion of false positives. P values were corrected for multiple testing using a false discovery rate method proposed by Storey and Tibshirani (2003). Probe sets that satisfied the criterion of false discovery rate < 5% (q value < 0.05) were considered to be significantly regulated.

Real time qRT-PCR analysis

Total RNA (1 μg) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the supplier's manual. Real-time PCR was set up in a 20-μL reaction system using the Eurogentec qPCR Core kit and the iQ5 Real-time PCR detecting system according to the manuals supplied by the manufacturers. Gene-specific primers were designed with Primer-3-Plus software (Untergasser et al., 2007) and are listed in Supplemental Table 3 online. For *M. truncatula*, ubiquitin (UBQ) and polypyrimidine tract binding protein were used as internal control, whereas for rice, UBQ was used (Lin et al., 2009).

Phylogenetic analysis

Maximum-likelihood phylogenetic analysis was performed on rice *D27* homologous proteins of *M. truncatula*, soybean (*Glycine max*), poplar (*Populus trichocarpa*), cassava (*Manihot esculenta*), wild strawberry (*Fragaria vesca*), maize (*Zea mays*), and rice. Protein sequences were aligned using Geneious Pro software package 5.4.6 (Biomatters) by applying Blosum62 matrix, a gap open penalty of 12, and a gap extension penalty of 3. Phylogenetic trees were reconstructed by maximum likelihood and 1000 bootstrap repetitions to evaluate

statistical support of the branches. Rice Os08g0114100 was used as an out-group because it is the closest paralog of rice *D27*.

Characterization and quantification of SLs

For SL analysis, 15 seven-day-old *M. truncatula* plants were transplanted (16 h of light, 23 °C, 60% relative humidity) into an X-stream 20 aeroponic system (Nutriculture) operating with 5 L of modified one-half-strength Hoagland solution (Hoagland and Arnon, 1950; López-Ráez et al., 2008). Four weeks after transplanting, phosphate starvation was introduced by replacing the nutrient solution with one-half-strength Hoagland solution without phosphate. Twenty-four hours before exudate collection, the nutrient solution was refreshed to remove all accumulated SLs. For SL analysis in rice, plants were grown as previously described by Jamil et al. (2010).

M. truncatula exudates were purified and concentrated as previously described by López-Ráez et al. (2010) with some modifications. Five liters of root exudate was loaded onto a pre-equilibrated C18 column (Grace Pure C18-Fast 5000 mg/20 mL) per sample. Subsequently, columns were washed with 50 mL of deionized water and 50 mL of 30% acetone/water. SLs were eluted with 50 mL of 60% acetone/water. Rice exudates were purified and concentrated as previously described by Jamil et al. (2010). All exudates were collected within 3 h and stored at −20°C before further use. SLs were extracted from *M. truncatula* root material as previously described for tomato (*Solanum lycopersicum*) by López-Ráez et al. (2010).

Analysis of SLs was performed by comparing retention times and mass transitions with those of available SL standards (sorgolactone, strigol, 2′-epistrigol, orobanchol, 2′-epiorobanchol, 5-deoxystrigol, 2′-epi-5-deoxystrigol, solanacol, and orobanchyl acetate, sorgomol, 7-oxoorobanchol, 7-oxoorobanchyl acetate) using ultra-performance LC coupled to MS/MS, as previously described by Kohlen et al. (2011). Didehydro-orobanchol MS/MS fragmentation spectra of *M. truncatula* and tomato root exudates were obtained as previously described López-Ráez et al. (2008).

Root exudates obtained from different rice lines were assessed for germination stimulatory activity by germination bioassays with *Striga hermonthica* as previously described by Jamil et al. (2010).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assay experiments were performed mainly as described previously (Kaufmann et al., 2005). The coding sequence of *M. truncatula NSP1* was PCR amplified from a pool of cDNA using the following primers with *Nco*I (forward) and *Cla*I (reverse) restriction sites (Supplemental Table 3). The amplified fragment was cloned into the pSPUTK, an in vitro transcription/translation vector (Stratagene). The protein was produced by in vitro transcription/translation with the TNT SP6 High-Yield Wheat Germ Protein Expression System following the manufacturer's instructions (Promega). Promoter fragments of *M. truncatula D27* and *ENOD11* were PCR amplified from *M. truncatula* genomic DNA and cloned via TA-cloning into the pGEM-T vector (Supplemental Table 3). Biotin-labeled pGEM-T–specific primers, directly flanking the cloning site, were used for generation of the DNA probes by PCR. The Mt NSP1 protein (2 μL of the in vitro reaction) was incubated with 40 fmol of biotin-labeled DNA probes in binding solution (1 mM EDTA, pH 8.0, 0.25 mg/mL BSA, 7 mM HEPES, pH 7.3, 0.7 mM DTT, 60 μg/mL salmon sperm DNA, 1.3 mM spermidine, 2.5% 3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate, and 8% glycerol) for 1 h on ice. DNA–protein complexes were analyzed on a 5% native PAGE (37.5:1 acrylamide:bisacrylamide). After electrophoresis, the gel was blotted to Amersham Hybond-N^+ membrane, and the signal was detected using the chemiluminescent nucleic acid detection module (Pierce Chemical Co.) in a Genius:BOX gel documentation system (Westburg).

ACCESSION NUMBERS

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: *M. truncatula nsp1* and *nsp2* microarray data, GSE26548; and *M. truncatula* D27, JN629088. The following genes were used in qRT-PCR experiments: *M. truncatula* UBQ, BT053109; *M. truncatula* polypyrimidine tract binding protein, CT963079; *M. truncatula* NSP1, AJ972478; *M. truncatula* NSP2, AJ832138; rice NSP1, AC135559; rice NSP2, AC135206; rice D27, FJ641055; and rice UBQ, AC103891.

ACKNOWLEDGMENTS

This research was funded by the Chinese Academy of Sciences (CAS) and the Royal Netherlands Academy of Arts and Sciences (KNAW) (CAS-KNAW Joint PhD Training Programme 06PhD12 to W.L., W.C.Y., T.B., and R.G.), the Dutch Science Organization NWO (VIDI 864.06.007 to R.G., Dutch Russian Research Cooperation 047.018.001 to S.I.,

E.L., R.G., and T.B., VICI 865.06.002 to H.J.B., and Equipment 834.08.001 to H.J.B.), EC Marie Curie Research Training Network Program MRTN-CT-2006-035546 NODPERCEPTION (to A.L. and R.G.), and Pakistan Higher Education Commission (to M.J.). H.J.B. is co-financed by the Centre for BioSystems Genomics, which is part of the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research.

REFERENCES

Akiyama K., Matsuzaki K., Hayashi H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature **435**: 824-827.

Alvord W.G., Roayaei J.A., Quiñones O.A., Schneider K.T. (2007). A microarray analysis for differential gene expression in the soybean genome using Bioconductor and R. Brief. Bioinform. **8**: 415-431.

Barbulova A., Rogato A., D'Apuzzo E., Omrane S., Chiurazzi M. (2007). Differential effects of combined N sources on early steps of the Nod factor–dependent transduction pathway in *Lotus japonicus*. Mol. Plant Mircobe Interact. **20**: 994-1003.

Beveridge C.A., Kyozuka J. (2010). New genes in the strigolactone-related shoot branching pathway. Curr. Opin. Plant Biol. **13**: 34-39.

Benedito V.A., et al. (2008). A gene expression atlas of the model legume *Medicago truncatula*. Plant J. **55**: 504-513.

Bolstad B., Collin F., Brettschneider J., Simpson K., Cope L., Irizarry R., Speed T.P. (2005). Quality assessment of Affymetrix GeneChip data. In Bioinformatics and computational biology solutions using r and bioconductor. Book Series: Statistics for Biology and Health, Gentleman R., Vincent J.C., Wolfgang H., Irizarry R.A., Dudoit S., eds (New York: Springer) pp. 33-47.

Bouwmeester H.J., Roux C., Lopez-Raez J.A., Bécard G. (2007). Rhizosphere communication of plants, parasitic plants and AM fungi. Trends Plant Sci. **12**: 224-230.

Brettschneider J., Collin F., Bolstad B.M., Speed T.P. (2008). Quality assessment for short oligonucleotide microarray data. Technometrics **50**: 241-264.

Catoira R., Galera C., de Billy F., Penmetsa R.V., Journet E.P., Maillet F., Rosenberg C., Cook D., Gough C., Dénarié J. (2000). Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. Plant Cell **12**: 1647-1666.

Cook C.E., Whichard L.P., Turner B., Wall M.E., Egley G.H. (1966). Germination of witchweed (*Striga lutea* Lour.): Isolation and properties of a potent stimulant. Science **154**: 1189-1190.

Domagalska M.A., Leyser O. (2011). Signal integration in the control of shoot branching. Nat. Rev. Mol. Cell Bio. **12**: 211-221.

Fåhraeus G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. **16**: 374-381.

Gentleman R.C., et al. (2004). Bioconductor: Open software development for computational biology and bioinformatics. Genome Biol. **5**: R80.

Geurts R., Fedorova E., Bisseling T. (2005). Nod factor signaling genes and their function in the early stages of Rhizobium infection. Curr. Opin. Plant Biol. **8**: 346-352.

Gomez-Roldan V., et al. (2008). Strigolactone inhibition of shoot branching. Nature **455**: 189-194.

Heber S., Sick B. (2006). Quality assessment of Affymetrix GeneChip data. OMICS **10**: 358-368.

Heckmann A.B., Lombardo F., Miwa H., Perry J.A., Bunnewell S., Parniske M., Wang T.L., Downie J.A. (2006). *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiol. **142**: 1739-1750.

Hirsch S., Kim J., Muñoz A., Heckmann A.B., Downie J.A., Oldroyd G.E. (2009). GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in *Medicago truncatula*. Plant Cell **21**: 545-557.

Hoagland A., Arnon D. (1950). The water-culture method for growing plants without soil. California Agricultural Experimental Station Circular, No. **347** (Berkeley, CA: University of California), pp. 1-32.

Jamil M., Charnikhova T., Verstappen F., Bouwmeester H. (2010). Carotenoid inhibitors reduce strigolactone production and *Striga hermonthica* infection in rice. Arch. Biochem. Biophys. **504**: 123-131.

Kaló P., et al. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science **308**: 1786-1789.

Kaufmann K., Anfang N., Saedler H., Theissen G. (2005). Mutant analysis, protein-protein interactions and subcellular localization of the Arabidopsis B sister (ABS) protein. Mol. Genet. Genomics **274**: 103-118.

Kohlen W., Charnikhova T., Liu Q., Bours R., Domagalska M.A., Beguerie S., Verstappen F., Leyser O., Bouwmeester H., Ruyter-Spira C. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host Arabidopsis. Plant Physiol. **155**: 974-987.

Koltai H., LekKala S.P., Bhattacharya C., Mayzlish-Gati E., Resnick N., Wininger S., Dor E., Yoneyama K., Yoneyama K., Hershenhorn J., Joel D.M., Kapulnik Y. (2010). A tomato strigolactone-impaired mutant displays aberrant shoot morphology and plant interactions. J. Exp. Bot. **61**: 1739-1749.

Kosuta S., Hazledine S., Sun J., Miwa H., Morris R.J., Downie J.A., Oldroyd G.E.D. (2008). Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. Proc. Natl. Acad. Sci. USA **105**: 9823-9828.

Kouchi H., Imaizumi-Anraku H., Hayashi M., Hakoyama T., Nakagawa T., Umehara Y., Suganuma N., Kawaguchi M. (2010). How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. Plant Cell Physiol. **51**: 1381-1397.

Lee M.H., et al. (2008). Large-scale analysis of the GRAS gene family in *Arabidopsis thaliana*. Plant Mol. Biol. **67**: 659-670.

Limpens E., RamOsJ., Franken C., Raz V., Compaan B., Franssen H., Bisseling T., Geurts R. (2004). RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. J. Exp. Bot. **55**: 983-992.

Lin H., Wang R., Qian Q., Yan M., Meng X., Fu Z., Yan C., Jiang B., Su Z., Li J., Wang Y. (2009). DWARF27, an iron-containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. Plant Cell **21**: 1512-1525.

López-Ráez J.A., Charnikhova T., Gómez-Roldán V., Matusova R., Kohlen W., De VOsR., Verstappen F., Puech-Pages V., Bécard G., Mulder P., Bouwmeester H. (2008). Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol. **178**: 863-874.

López-Ráez J.A., Kohlen W., Charnikhova T., Mulder P., Undas A.K., Sergeant M.J., Verstappen F., Bugg T.D.H., Thompson A.J., Ruyter-Spira C., Bouwmeester H. (2010). Does abscisic acid affect strigolactone biosynthesis? New Phytol. **187**: 343-354.

Maillet F., et al. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469**: 58-63.

Matusova R., Rani K., Verstappen F.W.A., Franssen M.C.R., Beale M.H., Bouwmeester H.J. (2005). The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. Plant Physiol. **139**: 920-934.

Mitra R.M., Gleason C.A., Edwards A., Hadfield J., Downie J.A., Oldroyd G.E.D., Long S.R. (2004a). A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. USA **101**: 4701-4705.

Mitra R.M., Shaw S.L., Long S.R. (2004b). Six non-nodulating plant mutants defective for Nod factor-induced transcriptional changes associated with the legume-rhizobia symbiosis. Proc. Natl. Acad. Sci. USA **101**: 10217-10222.

Moore M.J., Bell C.D., Soltis P.S., Soltis D.E. (2007). Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc. Natl. Acad. Sci. USA **104**: 19363-19368.

Murakami Y., Miwa H., Imaizumi-Anraku H., Kouchi H., Downie J.A., Kawaguchi M., Kawasaki S. (2006). Positional cloning identifies *Lotus japonicus* NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. DNA Res. **13**: 255-265.

Oldroyd G.E. and Long S.R. (2003). Identification and characterization of nodulation-signaling pathway 2, a gene of *Medicago truncatula* involved in Nod actor signaling. Plant Physiol. **131**: 1027-1032.

Rani K., Zwanenburg B., Sugimoto Y., Yoneyama K., Bouwmeester H.J. (2008). Biosynthetic considerations could assist the structure elucidation of host plant produced rhizosphere signalling compounds (strigolactones) for arbuscular mycorrhizal fungi and parasitic plants. Plant Physiol. Biochem. **46**: 617-626.

Sartor M.A., Tomlinson C.R., Wesselkamper S.C., Sivaganesan S., Leikauf G.D., Medvedovic M. (2006). Intensity-based hierarchical Bayes method improves testing for differentially expressed genes in microarray experiments. BMC Bioinformatics **7**: 538.

Smit P., Raedts J., Portyanko V., Debellé F., Gough C., Bisseling T., Geurts R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science **308**: 1789-1791.

Smith S.A., Beaulieu J.M., Donoghue M.J. (2010). An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants. Proc. Natl. Acad. Sci. USA **107**: 5897-5902.

Smyth G.K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. **3**: Article 3.

Storey J.D., Tibshirani R. (2003). Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. USA **100**: 9440-9445.

Tian C., Wan P., Sun S., Li J., Chen M. (2004). Genome-wide analysis of the GRAS gene family in rice and Arabidopsis. Plant Mol. Biol. **54**: 519-532.

Toki S., Hara N., Ono K., Onodera H., Tagiri A., Oka S., Tanaka H. (2006). Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. Plant J. **47**: 969-976.

Trouvelot A., Kough J., Gianinazzi-Pearson V. (1986). Measuring the rate of VA mycorrhization of root systems. In Physiological and genetical aspects of mycorrhizae, Gianinazzi-Pearson V., Gianinazzi S., eds (Paris: INRA Press), pp. 217-221.

Umehara M., Hanada A., Magome H., Takeda-Kamiya N., Yamaguchi S. (2010). Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. Plant Cell Physiol. **51**: 1118-1126.

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

Untergasser A., Nijveen H., Rao X., Bisseling T., Geurts R., Leunissen J.A.M. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. **35**: W71-W74.

Wang Y., Li J. (2011). Branching in rice. Curr. Opin. Plant Biol. 14: 94-99.

Wu Z., Irizarry R., Gentleman R., Martinez-Murillo F., Spencer F. (2004). A model-based background adjustment for oligonucleotide expression arrays. J. Am. Stat. Assoc. **99**: 909-917.

Xie X., Yoneyama K., Yoneyama K. (2010). The strigolactone story. Annu. Rev. Phytopathol. **48**: 93-117.

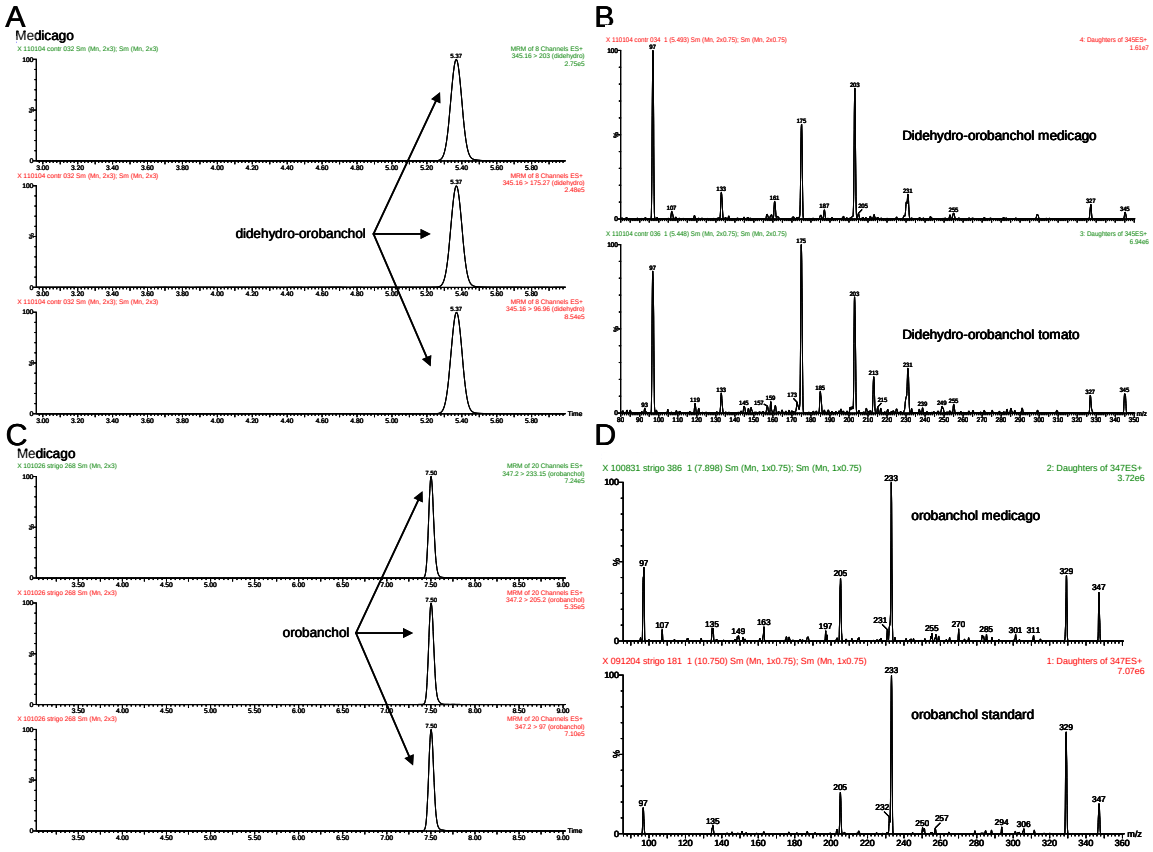
Yokota K., Soyano T., Kouchi H., Hayashi M. (2010). Function of GRAS proteins in root nodule symbiosis is retained in homologs of a non-legume, rice. Plant Cell Physiol. **51**: 1436-1442.

Yokota T., Sakai H., Okuno K., Yoneyama K., Takeuchi Y. (1998). Alectrol and orobanchol, germination stimulants for *Orobanche minor*, from its host red clover. Phytochemistry **49**: 1967-1973.

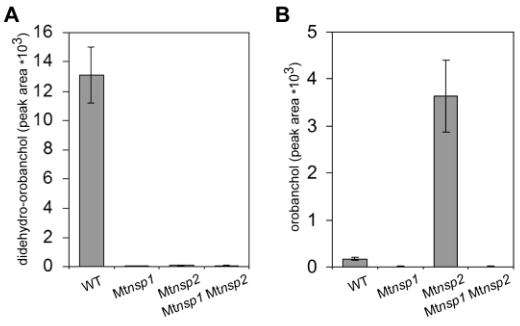
Yoneyama K., Yoneyama K., Takeuchi Y., Sekimoto H. (2007). Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. Planta **225**: 1031-1038.

Zhu H., Riely B.K., Burns N.J., Ané J.M. (2006). Tracing non-legume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. Genetics **172**: 2491-2499.

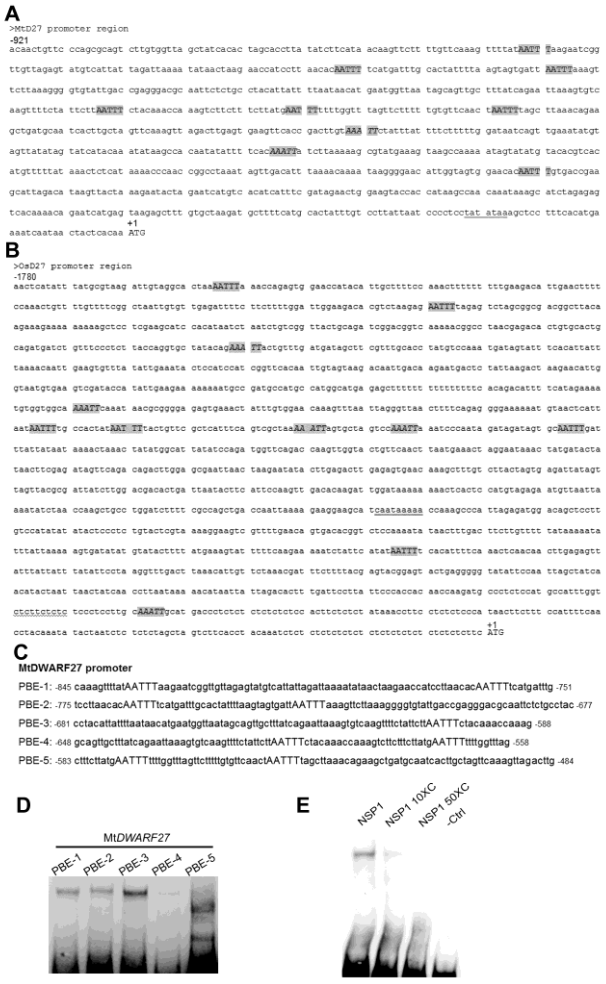
SUPPLEMENTAL DATA



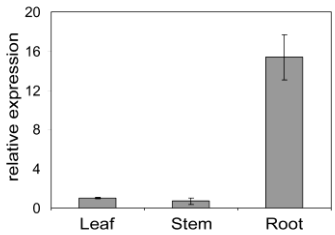
Supplemental Figure 1. **MRM-LC-MS/MS analysis of Medicago root exudates.** (**A**) Transitions 345 > 203, 345 > 175 and 345 > 96.8 for didehydro-orobanchol. (**B**) Full daughter ion scan MS/MS spectrum of didehydro-orobanchol in Medicago exudate and didehydro-orobanchol in tomato (*Solanum lycopersicum*). (**C**) Transitions 347 > 233, 347 > 205 and 347 > 96.8 for orobanchol. (**D**) Full daughter ion scan MS/MS spectrum of orobanchol in Medicago exudate and orobanchol standard.



Supplemental Figure 2. Amount of didehydro-orobanchol and orobanchol in *M. truncatula* root extracts of wild type, M*tnsp1*, M*tnsp2* and M*tnsp1*M*tnsp2* mutants (n=6). Data are means ±SE.



Supplemental Figure 3. Putative NRE-like *cis* regulatory binding elements for NSP1 in the promoter region of *M. truncatula* Mt*D27* and rice Os*D27*. (**A**) *M. truncatula* Mt*D27* promoter region (-920bp ~ +3bp). Nine putative MtNSP1 binding elements are marked in uppercase and shaded. Seven elements are present in the forward strand (marked in normal case) and 2 in the reverse strand (marked in Italic). (**B**) In the rice Os*D27* promoter (-1780bp ~ +3bp), 11 putative NSP1 binding elements are present (marked in uppercase letters and shaded). Six of these are localized in the forward chain (marked in normal case) and 5 are localized in the reverse chain (marked in Italic). Predicted transcription start sites (TSS) are underlined. (**C**) Putative binding element (PBE) probes used in Electrophoretic Mobility Shift Assays EMSA analysis originating from the Mt*D27* promoter regions. Given numbers mark position in promoter. The AATTT elements are shown in uppercase letters. (**D**) Five probes containing NRE-like elements (PBE-1 to PBE-5) originating from the Mt*D27* promoter region were used in an EMSA assay. (**E**) The competition assay was set up in PBE-3 by using unlabeled probes in 10× and 50×. Empty plasmid was used as negative control.



Supplemental Figure 4. **Expression analysis of Mt*D27* in Medicago shoot, stem and root tissue determined by qRT-PCR.**

Supplemental Table 1. *M. truncatula* probe sets representing genes down-regulated in root tissue of the Mt*nsp1* and Mt*nsp2* knockout mutant. Expression determined based on microarray analysis. FC: Fold Change.

	FC		Target Description	ID	Homolog(s) in *A. thaliana*
	nsp1	***nsp2***			
Mtr.32254.1.S1_at	-7.8	-10.1	C2H2 type Zinc finger protein 6	AW686309	At1g67030
Mtr.12833.1.S1_at	-7.7	-7.7	Unknown protein	TC146803	Legume specific
Mtr.9516.1.S1_at	-6.1	-5.4	Dirigent-like protein	TC158536	At1g22900 At5g42500
Mtr.25451.1.S1_at Mtr.10522.1.S1_s_at	-5.5 -3.3	-4.7 -3.4	Alanine-tRNA ligase	TC144165	At1g50200
Mtr.4797.1.S1_s_at Mtr.11343.1.S1_at	-5.1 -3.2	-6.2 -3.4	DWARF27	TC158596	At1g03055 At1g64680
Mtr.36679.1.S1_s_at	-4.7	-2.8	Unknown protein with homolog	TC158127	At5g66780
Mtr.34371.1.S1_at	-4.6	-7.8	Unknown protein	TC162100	Legume specific
Mtr.48470.1.S1_s_at	-3.7	-3.7	OB-fold Replication protein A1	NP7269740	Legume specific
Mtr.20927.1.S1_at	-3.6	-3.5	Calmodulin binding protein	TC143830	At3g13600
Mtr.11099.1.S1_at	-3.6	-3.7	1-cys peroxiredoxin	TC146236	At1g48130
Mtr.32982.1.S1_at	-3.5	-4.5	Nudix hydrolase	TC153996	At2g01670 At1g14860
Mtr.45041.1.S1_at	-3.4	-2.8	Abnormal gametophytes (AGM)	TC163978	At4g19950 At1g31130
Mtr.22664.1.S1_at	-3.3	-3.3	Avr9/Cf-9 rapidly elicited protein 146	TC168023	At1g52140 At4g29110
Mtr.2114.1.S1_at	-3.3	-3.7	Unknown protein	TC152884	Legume specific
Mtr.12358.1.S1_at	-3.2	-3.0	Seed maturation protein LEA 4	TC147896	At5g06760
Mtr.28502.1.S1_at	-3.0	-2.9	Gypsy-like retrotransposon protein	BG647160	At4g21420
Mtr.15641.1.S1_at	-2.9	-2.3	unknown protein	NP7273282	Legume specific

Mtr.11744.1.S1_at	-2.8	-2.5	(+)-abscisic acid 8'-hydroxylase	TC165592	At1g19630 At4g19230
Mtr.40725.1.S1_at	-2.5	-2.3	Mannose-6-phosphate isomerase	TC143057	At3g02570
Mtr.12616.1.S1_at	-2.5	-2.3	MAX1	TC143057	At2g26170
Mtr.42432.1.S1_at Mtr.12200.1.S1_at	-2.5 -2.1	-2.5 -2.2	Carotenoid isomerase	TC170207 TC165115	At1g06820
Mtr.38430.1.S1_at	-2.5	-2.6	CIPK4	TC165047	At3g23000 At4g14580
Mtr.17478.1.S1_at	-2.5	-2.4	26S proteasome subunit RPN5b	TC143513	At5g09900 At5g64760
Mtr.40755.1.S1_at	-2.4	-2.1	ERD7	TC160548	At2g17840 At3g51250
Mtr.33914.1.S1_at	-2.4	-2.3	Gibberellin 2-oxidase	BI273162	At1g78440 At1g30040
Mtr.42587.1.S1_at	-2.4	-2.5	Heat shock protein	TC153611	At5g03720 At5g16820
Mtr.17298.1.S1_at	-2.4	-2.5	NAD+ ADP-ribosyltransferase	NP7267637	At1g23550 At1g70440
Mtr.43078.1.S1_s_at	-2.4	-2.5	Cytokinin-specific binding protein	TC161067	-
Mtr.13426.1.S1_at	-2.3	-2.4	β-glucan-binding protein 4	TC145129	At5g15870 At1g18310
Mtr.28846.1.S1_at	-2.2	-2.7	ARR6	BQ124080	At5g62960
Mtr.50800.1.S1_at	-2.2	-2.3	2OG-Fe(II) oxygenase	TC144924	At1g06620 At2g30840
Mtr.38432.1.S1_at	-2.2	-2.0	Temperature stress-induced lipocalin	TC147968	At5g58070
Mtr.27101.1.S1_at	-2.1	-2.7	Unknown protein	AW559488	Legume specific
Mtr.37912.1.S1_at	-2.1	-2.0	Allyl alcohol dehydrogenase	TC159212	At5g16970
Mtr.7947.1.S1_at	-2.1	-2.8	Pherophorin-S precursor	BG447806	Legume specific
Mtr.30981.1.S1_at	-2.1	-2.7	Unknown protein	TC145768	Legume specific
Mtr.7136.1.S1_at	-2.0	-3.0	Nine-cis-epoxycarotenoid dioxygenase 4	CX549419	At4g19170
Mtr.7138.1.S1_at	-2.0	-2.4	Short-chain alcohol dehydrogenase	CX549464	At3g26770 At1g52340
Mtr.10681.1.S1_s_at	-2.0	-4.1	USP1-like protein	TC151489	At3g62550

Supplemental Table 2. *M. truncatula* probe sets representing genes down-regulated in root tissue of *Mtnsp2* mutant versus wild type and nsp1 mutant plants. Fold changes are determined based on microarray analysis.

Probe set ID	Fold Change nsp2-A17	Target Description
Mtr.43502.1.S1_at	-774.5	TC95479 /20S proteasome subunit alpha-5
Mtr.12315.1.S1_x_at	-434.0	TC94362 /Glutathione S-transferase GST 8
Mtr.13115.1.S1_at	-374.5	TC96958 /MLO-like protein 1
Mtr.12577.1.S1_s_at	-120.4	TC95279
Mtr.29613.1.S1_at	-113.8	TC98883
Mtr.38864.1.S1_at	-91.6	TC103695
Mtr.1122.1.S1_s_at	-72.4	F-box protein
Mtr.12577.1.S1_at	-70.6	TC95279
Mtr.42066.1.S1_at	-68.0	TC110677 /Phytase
Mtr.14020.1.S1_at	-45.7	TC99986 /Protein phosphatase
Mtr.29721.1.S1_at	-39.4	AL367580
Mtr.27518.1.S1_at	-36.8	BE203734 / Cell surface protein
Mtr.2291.1.S1_at	-35.2	BG584884 / P-type R2R3 Myb protein
Mtr.7710.1.S1_at	-32.5	AL367581
Mtr.38507.1.S1_at	-25.1	TC102945 /Ser/Thr protein phosphatase BSL1
Mtr.17523.1.S1_at	-17.5	Protein of unknown function
Mtr.13843.1.S1_at	-15.3	TC99351
Mtr.569.1.S1_s_at	-15.0	hypothetical protein
Mtr.38949.1.S1_x_at	-13.1	TC103888 /Albumin 2
Mtr.8470.1.S1_s_at	-12.8	TC100295 /Strictosidine-O-beta-D-glucosidase
Mtr.23596.1.S1_s_at	-11.8	AC146756
Mtr.7635.1.S1_at	-11.3	AJ548095 /Anther-specific protein
Mtr.42093.1.S1_at	-11.2	TC110744
Mtr.19517.1.S1_at	-10.9	Ribulose bisphosphate carboxylase
Mtr.13269.1.S1_at	-10.5	TC97491
Mtr.42093.1.S1_x_at	-10.4	TC110744
Mtr.2135.1.S1_s_at	-10.1	BF640276
Mtr.1666.1.S1_at	-9.9	AW694130
Mtr.43424.1.S1_at	-9.7	TC95281
Mtr.5739.1.S1_at	-9.2	BF646806 / LRR protein
Mtr.35945.1.S1_at	-9.0	TC99852 /UDP-glycose:flavonoid glycosyltransferase
Mtr.45231.1.S1_at	-8.8	TC99117 /Nodulin
Mtr.44995.1.S1_at	-7.9	TC98565
Mtr.7459.1.S1_at	-7.6	TC112501 /Resistance protein MG55
Mtr.38949.1.S1_at	-6.8	TC103888 /Albumin 2
Mtr.38494.1.S1_at	-6.7	TC102920
Mtr.33112.1.S1_at	-6.7	BF642307 /Patatin homolog
Mtr.38508.1.S1_at	-6.5	TC102946 /Serine/threonine protein phosphatase BSL1
Mtr.25451.1.S1_s_at	-6.3	alanine--tRNA ligase
Mtr.48470.1.S1_at	-6.2	AC119409
Mtr.41147.1.S1_at	-6.1	TC108815
Mtr.13904.1.S1_at	-6.0	TC99584/ 4-coumarate-CoA ligase
Mtr.1810.1.S1_at	-5.8	BE202654
Mtr.31788.1.S1_at	-5.8	AL380233 /F-box protein
Mtr.11820.1.S1_x_at	-5.7	TC111162 /UDP-glycosyltransferase
Mtr.48675.1.S1_s_at	-5.6	Purple acid phosphatase
Mtr.52163.1.S1_at	-5.3	hypothetical protein
Mtr.43310.1.S1_at	-5.3	TC95027 /blight resistance protein RGA1
Mtr.8426.1.S1_at	-5.0	TC100135 /Chlorophyll a/b binding protein

Mtr.48681.1.S1_at	-5.0	hypothetical protein
Mtr.40279.1.S1_at	-4.8	TC106943 /glutathione S-transferase
Mtr.10626.1.S1_at	-4.7	TC107414 /SRG1-like protein
Mtr.33562.1.S1_at	-4.5	BG453865 /type II keratin subunit protein
Mtr.10681.1.S1_at	-4.3	TC107596 /USP1-like protein
Mtr.13908.1.S1_at	-4.3	TC99593
Mtr.42339.1.S1_at	-4.2	TC111274
Mtr.35078.1.S1_at	-4.2	CX532386 /ATPase 2
Mtr.10604.1.S1_at	-4.2	TC107357 /Nitrate reductase
Mtr.8517.1.S1_at	-4.2	TC100462 /mannitol dehydrogenase
Mtr.45148.1.S1_at	-4.1	TC98922 /
Mtr.31557.1.S1_at	-4.1	AL370071
Mtr.11695.1.S1_at	-4.1	TC110748
Mtr.10450.1.S1_at	-4.0	TC106817
Mtr.25731.1.S1_at	-3.9	plastocyanin-like domain containing protein
Mtr.2334.1.S1_at	-3.9	BG586672
Mtr.21972.1.S1_at	-3.7	hypothetical protein
Mtr.13183.1.S1_at	-3.5	TC97194
Mtr.6718.1.S1_at	-3.5	CX519518
Mtr.47199.1.S1_at	-3.5	Calcineurin-like phosphoesterase-like
Mtr.35681.1.S1_at	-3.5	TC111048 /Metal-dependent amidase
Mtr.33530.1.S1_at	-3.5	BG453035
Mtr.47631.1.S1_s_at	-3.5	Transposase
Mtr.34638.1.S1_at	-3.5	BQ157221 /glutaredoxin-like protein
Mtr.42692.1.S1_at	-3.4	TC112168 /Glutaredoxin-like protein
Mtr.43831.1.S1_at	-3.4	TC96132 /F-box protein
Mtr.35757.1.S1_at	-3.4	TC112048 /Short chain alcohol dehydrogenase
Mtr.48064.1.S1_at	-3.3	hypothetical protein
Mtr.43048.1.S1_at	-3.3	TC94419 /Early nodulin 12A precursor
Mtr.31645.1.S1_at	-3.3	AL373660 /Deoxycytidine deaminase
Mtr.21112.1.S1_at	-3.3	Myb
Mtr.10545.1.S1_at	-3.3	TC107140
Mtr.30965.1.S1_at	-3.3	CX550363
Mtr.15275.1.S1_at	-3.2	lectin
Mtr.38954.1.S1_at	-3.2	TC103902 /Ferric-chelate reductase
Mtr.7244.1.S1_at	-3.2	TC104280
Mtr.37288.1.S1_at	-3.1	TC100298 /Beta-primeverosidase
Mtr.26939.1.S1_at	-3.1	AL379176
Mtr.12428.1.S1_at	-3.1	TC94767 /Anther-specific protein
Mtr.2631.1.S1_at	-3.1	BI311272 /Caffeic acid O-methyltransferase II
Mtr.34470.1.S1_s_at	-3.1	BQ148677 /Ca2+/H+ exchanger
Mtr.11820.1.S1_at	-3.1	TC111162 /UDP-glycosyltransferase
Mtr.11364.1.S1_at	-3.0	TC109714 /cytochrome P450
Mtr.38932.1.S1_at	-3.0	TC103858 /Malate synthase
Mtr.28845.1.S1_at	-3.0	BQ123925 /Cystinosin
Mtr.32209.1.S1_at	-3.0	AW684842
Mtr.41195.1.S1_at	-3.0	TC108916
Mtr.37084.1.S1_at	-2.9	TC112271
Mtr.5456.1.S1_at	-2.9	BE325502 /Valine--tRNA ligase
Mtr.37624.1.S1_s_at	-2.9	TC101083 /3-beta-hydroxysteroid dehydrogenase
Mtr.25691.1.S1_s_at	-2.9	TNP2-like transposon protein
Mtr.7052.1.S1_at	-2.9	CX537932 /Histone H1
Mtr.26276.1.S1_at	-2.9	1499.m00024 /CER1 protein
Mtr.41805.1.S1_at	-2.9	TC110159
Mtr.8612.1.S1_at	-2.9	TC100798
Mtr.4665.1.S1_at	-2.8	AL374081
Mtr.13947.1.S1_x_at	-2.8	TC99709 /EIX receptor 1

Mtr.28586.1.S1_at	-2.8	BI266323
Mtr.42446.1.S1_at	-2.8	TC111556 / Nitrate reductase
Mtr.40084.1.S1_at	-2.8	TC106463 /Sulfate transporter protein
Mtr.15010.1.S1_s_at	-2.7	Myb
Mtr.34799.1.S1_at	-2.7	CX524988
Mtr.17954.1.S1_s_at	-2.7	hypothetical protein
Mtr.39139.1.S1_at	-2.7	TC104268 /Pathogenesis-related protein 4A
Mtr.39091.1.S1_at	-2.7	TC104170 /Beta-glucan binding protein
Mtr.10175.1.S1_at	-2.6	TC105774 /Cytochrome P450
Mtr.9660.1.S1_at	-2.6	TC103997
Mtr.37623.1.S1_at	-2.6	TC101082 /3-beta-hydroxysteroid dehydrogenase
Mtr.7818.1.S1_at	-2.6	AW684444
Mtr.22108.1.S1_x_at	-2.6	EIX receptor 1
Mtr.44349.1.S1_at	-2.6	TC97188 /Zinc transporter
Mtr.13237.1.S1_at	-2.6	TC97378 /protein integral membrane protein
Mtr.39664.1.S1_at	-2.5	TC105397
Mtr.41148.1.S1_at	-2.5	TC108816 /Ca2+/H+ exchanger
Mtr.27598.1.S1_at	-2.5	BE239791 /WRKY transcription factor 33
Mtr.48991.1.S1_s_at	-2.5	hypothetical protein
Mtr.43078.1.S1_at	-2.5	TC94480 /Histone H1
Mtr.2200.1.S1_at	-2.5	BG455696
Mtr.37751.1.S1_at	-2.5	TC101337 /Isoflavone-7-O-methytransferase 9
Mtr.37989.1.S1_at	-2.5	TC101850
Mtr.47546.1.S1_at	-2.5	Albumin 1 precursor
Mtr.33270.1.S1_at	-2.5	BF648401 /cytochrome P450
Mtr.10187.1.S1_at	-2.5	TC105821 /LEXYL1
Mtr.31998.1.S1_at	-2.4	AL388370 /Epoxide hydrolase
Mtr.42265.1.S1_at	-2.4	TC111090 /Transmembrane protein kinase
Mtr.1405.1.S1_at	-2.4	AL370412
Mtr.11212.1.S1_s_at	-2.4	TC109255 /Isoflavonoid glucosyltransferase
Mtr.40085.1.S1_s_at	-2.4	TC106466 /18S ribosomal RNA gene
Mtr.1495.1.S1_at	-2.4	AW171775
Mtr.37578.1.S1_at	-2.4	TC100962
Mtr.20215.1.S1_at	-2.4	hypothetical protein
Mtr.10019.1.S1_at	-2.4	TC105283
Mtr.27663.1.S1_at	-2.4	BE940981
Mtr.3180.1.S1_at	-2.4	CX542025
Mtr.13527.1.S1_at	-2.4	TC98264 /Cytochrome b
Mtr.6640.1.S1_at	-2.4	BQ152527
Mtr.8550.1.S1_s_at	-2.4	TC100587 Leghemoglobin 29
Mtr.50032.1.S1_at	-2.4	Nucleoside phosphatase
Mtr.1532.1.S1_at	-2.4	AW267858
Mtr.13887.1.S1_at	-2.4	TC99521 /Tropinone reductase-I
Mtr.20804.1.S1_at	-2.3	serine/threonine protein kinase
Mtr.42525.1.S1_x_at	-2.3	TC111746
Mtr.27041.1.S1_at	-2.3	AW256402 /Salicylic acid-binding protein 2
Mtr.44643.1.S1_at	-2.3	TC97782
Mtr.35515.1.S1_at	-2.3	TC105090 / Leucoanthocyanidin dioxygenase-like
Mtr.44610.1.S1_at	-2.3	TC97710 /Valine--tRNA ligase
Mtr.43046.1.S1_at	-2.3	TC94416 / Early nodulin 12A precursor
Mtr.45331.1.S1_at	-2.3	TC99400
Mtr.15215.1.S1_at	-2.3	hypothetical protein
Mtr.34408.1.S1_s_at	-2.3	BQ147176
Mtr.38872.1.S1_at	-2.3	TC103704
Mtr.48196.1.S1_at	-2.3	F-box protein
Mtr.15199.1.S1_at	-2.3	hypothetical protein
Mtr.20427.1.S1_at	-2.3	Disease resistance protein

Mtr.27572.1.S1_at	-2.3	BE205155 /AtHVA22a
Mtr.41956.1.S1_at	-2.3	TC110457
Mtr.32765.1.S1_at	-2.3	BE321908 /Serine/threonine protein phosphatase BSL1
Mtr.35562.1.S1_at	-2.3	TC105695
Mtr.19822.1.S1_at	-2.2	hypothetical protein
Mtr.25950.1.S1_at	-2.2	ACC oxidase
Mtr.18528.1.S1_at	-2.2	Cyclin-like F-box
Mtr.44900.1.S1_at	-2.2	TC98362
Mtr.13654.1.S1_at	-2.2	TC98687 / Tyrosine aminotransferase
Mtr.8284.1.S1_s_at	-2.2	Leghemoglobin
Mtr.37708.1.S1_at	-2.2	TC101252 /Sulfate transporter
Mtr.3883.1.S1_at	-2.2	BM813684 /Beta-cyanoalanine synthase
Mtr.8783.1.S1_at	-2.2	TC101370 /RNA helicase
Mtr.28700.1.S1_at	-2.2	BI310751 /Pyrogallol hydroxytransferase small subunit
Mtr.7637.1.S1_at	-2.2	AJ548187 /Prostatic spermine-binding protein precursor
Mtr.29716.1.S1_at	-2.2	AL367132
Mtr.30879.1.S1_at	-2.2	CB893428 /Permease protein of ABC transporter
Mtr.46001.1.S1_s_at	-2.2	C2 calcium/lipid-binding
Mtr.34929.1.S1_at	-2.2	CX528280 /Zinc finger protein CONSTANS-LIKE 16
Mtr.22096.1.S1_at	-2.2	EIX receptor 1
Mtr.39963.1.S1_at	-2.2	TC106180
Mtr.7380.1.S1_at	-2.1	TC110898
Mtr.35316.1.S1_at	-2.1	CX542483 / Beta-xylosidase
Mtr.47087.1.S1_at	-2.1	Glucan 1,3-beta-glucosidase
Mtr.31558.1.S1_at	-2.1	AL370072
Mtr.13396.1.S1_at	-2.1	TC97887
Mtr.12729.1.S1_at	-2.1	TC95749 /Pr1-like protein
Mtr.49098.1.S1_at	-2.1	hypothetical protein
Mtr.11251.1.S1_at	-2.1	TC109352 /protein integral membrane protein
Mtr.41982.1.S1_at	-2.1	TC110501 /sulfate transporter ATST1
Mtr.40836.1.S1_at	-2.1	TC108150 /MYB-related transcription factor PHAN1
Mtr.13947.1.S1_at	-2.1	TC99709 /EIX receptor 1
Mtr.40342.1.S1_at	-2.1	TC107088
Mtr.9281.1.S1_at	-2.1	TC102903 /NOI protein
Mtr.7670.1.S1_at	-2.1	AJ846785 /Albumin 1 precursor
Mtr.13919.1.S1_s_at	-2.1	TC99619
Mtr.35701.1.S1_at	-2.1	TC111297 /N7 protein
Mtr.50231.1.S1_at	-2.1	P-type trefoil
Mtr.10130.1.S1_at	-2.1	TC105614
Mtr.33808.1.S1_at	-2.1	BI270332 / two-component response regulator
Mtr.12712.1.S1_at	-2.1	TC95685 /Alpha-expansin
Mtr.46868.1.S1_s_at	-2.1	Lipoxygenase
Mtr.2633.1.S1_s_at	-2.1	BI311333
Mtr.41817.1.S1_at	-2.0	TC110184 /sulfate transporter ATST1
Mtr.29083.1.S1_at	-2.0	CA917785
Mtr.32904.1.S1_s_at	-2.0	BF633401 / Phosphoglycerate kinase
Mtr.30266.1.S1_at	-2.0	BG580434
Mtr.5630.1.S1_at	-2.0	BF640372
Mtr.40866.1.S1_at	-2.0	TC108215 / Hydrolase-like protein
Mtr.39948.1.S1_at	-2.0	TC106137
Mtr.33147.1.S1_at	-2.0	BF644066
Mtr.37455.1.S1_at	-2.0	TC100688 /Glucosyltransferase-13
Mtr.23540.1.S1_at	-2.0	Nodule-specific protein Nlj70
Mtr.26318.1.S1_at	-2.0	hypothetical protein
Mtr.27985.1.S1_at	-2.0	BF641475
Mtr.3806.1.S1_at	-2.0	BG645951
Mtr.11200.1.S1_at	-2.0	TC109191 /Zinc finger protein

Supplemental Table 3. List of primers used in this study.

Purpose	Primer Name	Sequence (5'-3')
qRT-PCR primers	Mtr.32254.1.S1_at-F	CATTTCCTCGCCAATGCTC
	Mtr.32254.1.S1_at-R	AAAAACTCCCGAACCTGCTG
	Mtr.43078.1.S1_s_at-F	TGCTCAGTCCAAGGATATCACC
	Mtr.43078.1.S1_s_at-R	CCCTCCATCCCCTTCAATTAC
	Mtr.9516.1.S1_at-F	GCCAATCCGTTGAATCTCAC
	Mtr.9516.1.S1_at-R	GACGAGGTTGGTTTGGTTTG
	Mtr.4797.1.S1_s_at-F	GAGATGATATTCGGCCAGGAAC
	Mtr.4797.1.S1_s_at-R	GCATGGTTTTTCTTAGCCTTGC
	Mtr.11343.1.S1_at-F	TCAAGCAGCAACAGGAATCAG
	Mtr.11343.1.S1_at-R	AAATTTCTGTGAAGCCACGGTAG
	Mtr.12616.1.S1_at-F	TTGGGTTTGGTTAGCCCTTG
	Mtr.12616.1.S1_at-R	CGCAGTTAGGGTCAAACCTTTC
	Mtr.42432.1.S1_at-F	AAAGCCGGAATTGTGAGCTG
	Mtr.42432.1.S1_at-R	AGCCCCTGACTCCCAAAATAG
	Mtr.40725.1.S1_at-F	TTGGGTTTGGTTAGCCCTTG
	Mtr.40725.1.S1_at-R	CGCAGTTAGGGTCAAACCTTTC
	Mtr.12833.1.S1_at-F	GCATCGATCAGATGTTGTTAGGG
	Mtr.12833.1.S1_at-R	TTCATCCATGTCGTGAAGACG
	Mtr.25451.1.S1_at-F	CCCCGAGCTAAAGCAAAATG
	Mtr.25451.1.S1_at-R	ATGGGACAACTCAAACGCTACC
	Mtr.34371.1.S1_at-F	CGAGGAAATTCTGGGAATAAGC
	Mtr.34371.1.S1_at-R	TTCCTTCCTTTCAGGCTTCC
	Mtr.11099.1.S1_at-F	GAACATGGATGAAGTGCTGAGG
	Mtr.11099.1.S1_at-R	TTCCAGTTAGCAGGGGTTGC
	Mtr.32982.1.S1_at-F	TTGCAGCGCTACAGAAAAGG
	Mtr.32982.1.S1_at-R	TTGCCCCTAAGGAAGTTTGG
	Mtr.45041.1.S1_at-F	TAACGAGGCCCCAAAGAATG
	Mtr.45041.1.S1_at-R	AATTGGACACAGGATCGTTCG
	Mtr.12358.1.S1_at-F	GGAGGAGCAAAGAAAACAGGAG
	Mtr.12358.1.S1_at-R	TCTCTTGAAATGTGGCCTTGG
	Mtr.37912.1.S1_at-F	AACCTCGAAAGGGTGAAACG
	Mtr.37912.1.S1_at-R	CAGCACAGCCAACAACATAGC
	Mtr.7136.1.S1_at-F	CTTTGCTCCTGTGGAAGAGTTG
	Mtr.7136.1.S1_at-R	CACCCTCTGGAAAATCATTCG
	Mtr.12200.1.S1_at-F	CACATGGGGGTTAAAGCAGAG
	Mtr.12200.1.S1_at-R	CCATATGGTTCCTCCAAACTGG
	qMtNSP1-F	GCGATTTCGCCACTGGATTC
	qMtNSP1-R	CAGCCTCGCCTTCCATCATT
	qMtD27-F	GAGATGATATTCGGCCAGGAAC
	qMtD27-R	GCATGGTTTTTCTTAGCCTTGC
	qMtUBQ-F	CCCTTCATCTTGTCCTTCGTCTG
	qMtUBQ-R	CACCTCCAATGTAATGGTCTTTCC
	qMtPTB-F	CGCCTTGTCAGCATTGATGTC
	qMtPTB-R	TGAACCAGTGCCTGGAATCCT
	qOsNSP1-F	GTGTCCTTCTGCTCGCTGTG

Purpose	Primer Name	Sequence (5'-3')
	qOsNSP1-R	CACGCCGTAGCGCTTAGTAAC
	qOsNSP2-F	TCAGCTGCTTCAACCACAGC
	qOsNSP2-R	TGTTGGGACCCGTCTCCTC
construct and genotyping primers	MtNSP1-t	CTTGTGCTGTAGCCATAAC
	MtNSP1-b	ACAGTAAGGCGAACAAGAG
	MtNSP2-5F	CAATGACCTCCACTTCTCTG
	MtNSP2-7R	TAAAAAGCCCTAACAACAGC
	OsNSP1-f	GGG *CTCGAG* GGCGCAGCAGGATGACA
	OsNSP-mf	GAGGCCGGCAACCTGTCGGTGCTACGCGT GGGGCGA
	OsNSP-mr	TCGCCCCACGCGTAGCACCGACAGGTTGC CGGCCTC
	OsNSP2-r	CGGGGTGGGAGCCGTCGA
EMSA primers	PBE-1F	CAAAGTTTTATAATTTAAGAATCGG
	PBE-1R	CAAATCATGAAAATTGTGTTAAG
	PBE-2F	TCCTTAACACAATTTTCATGATTTG
	PBE-2R	GTAGGCAGAGAATTGCGTC
	PBE-3F	CCTACATTATTTTAATAACATGAATG
	PBE-3R	CTTTGGTTTGTAGAAATTAAGAATAG
	PBE-4F	GCAGTTGCTTTATCAGAATTAAAG
	PBE-4R	CTAAACCAAAAAAATTCATAAGAAAG
	PBE-5F	CTTTCTTATGAATTTTTTTGGTTTAG
	PBE-5R	CAAGTCTAACTTTGAACTAG
	biotin-F	biotin*CATGGCCGCGGGATT
	biotin-R	biotin*GCGGCCGCACTAGTGATT
	cMtNSP1-*NcoI*-F	GGG *CCATGG* TGATGACTATGGAACCAAA TCCAAC
	cMtNSP1-*ClaI*-R	GGG *ATCGAT* CTACTCTGGTTGTTTATCCAG TTTCC

Supplemental Data Set 1. Sequenses used for hylogenetic analysis of D27 and its homologs

> Cassava_DWARF27
ATGGAAATACAATTTCTTTCACGAAACAGAAGCAATCTTCCATCAATACCTCTGCCGAGGCGTCTTCCCAAGCTTCA
TCGCTTGCCAGTTCTTGCAGTGCTTACTAGGCCGCCGGAGAAAATTACTGGAGTAAAAGAGAAGAATAGTTCAAAG
CCGGCAGATAAGGTAAGGGAACTCACGAGAAAGACTGTGTACAATGATAACTGGTTTGATAAACTAGCCATCAAC
CATCTTTCCAAGAGTGTTCAAGCTGCAACAGGGTTGAGAAACTGCAAGACTGGATATGAGAGCTTGGTGCAGGCAG
CAATGGATGCTTCACAAAAATTCAGCCTGATTCAACAACGTGAACTTGTGCTTCAAGCTCTTGATACAGCCTTCCCT
AGACTAATCCTTGACCTGGCAATAAAGGCAGTGATGCCCCAATCTCAATTTGCAAGAGAGTATTTTGCAGCTTTCA
CCACCTTGTTTTTCGTCTGGCTAATTGGGCCATGCGAGGTGCGGGAATCAGAGCTCAATGGAAGAAAAGAAAAGAA
TGTAGTCCATATAGAAAAGTGCAGGTTTTTAGAAGAGACTAATTGTGTTGGGATGTGTACTAATCTCTGTAAGGTTC
CTAGTCAAACCTTTATTAAGCACTCTCTGGGAATGCCAGTCAACATGGTTCCTAATTTTGATGATATGAGTTGCGAG
ATGATATTTGGTCAGGATCCTCCTATGCCAACTGAAGATCCAGCATTCAATCAACCATGCTATAAACTAT

>Maize_LOC100277925
ATGGAGGTCGCCGCCGCCACTTGTACGCTCCTTGCTGCCGTCGCCCTACCAGTTTCGTCCTCAGCAGCAGCAGCACG
CTCTGCGACGAAACAGAGCTACTCGAGGAGGAAGACGACGGCCGTGCGAGCCGTCATGGCGAGGCCGCATCAGGT
GCCGCCGCCTGCCACTGCCACCGCCACCGCCACCACGTACCGCGACAACTGGTTCGACAAGCTGGCCATCGGGTAC
CTGTCCCGGAACCTTCAAGAAGCTTCTGGGATGAAGAACGGAAAGGACGGCTACGAGGGCCTGATAGAGGCGGCC
CTGGCCATCTCCGCGCTGTTCAGGGTTGACCAGCAGTGGGACACTGTGGCCAGCGCTCTCCAACGGGCCTTCCCCA
GCTACATCCTCACCATGATCAAGGTGATGATGCCACCCTCTCGATTCTCGCGGGAGTATTTCGCCGCCTTCACCACG
GTGTTCTTCCCTTGGCTCGTCGGACCATGTGAGGTCAGGGAATCCCAAGTGGATGGAAGGGAAGAGAGGAACGTG
GTCTACATACCCAAATGCAGATTTCTGGAAAGCACCAACTGTGTCGGAATGTGCACGAACCTCTGCAAAATCCCAT
GCCAGAGGTTCATCCAGGATTCACTTGGCACGGCTGTCTACATGTCTCCCAATTTTGAGGACATGAGCTGCGAGAT
GATCTTTGGACAGCAACCACCTGAAGATGATCCAGCACTGAAGCAGCCCTGCTTCCGGACAAAATGCATCGCAAAG
CAGAATCATCAAGTGAATTGCTCTATT

>Medicago_DWARF27
ATGGATTCAAAGATGATTGCACATAACATGAGCCTAACACCAACTTTAGCTCAGTGGAAGAAGCTAAGACTTAAAC
CAAAACACACTTTTGTAGTTGGAGTTCTTGCAAGGCCTACAGATGATATTTCAGAAGAAACATTAAGAAAAACTAA
TGTATACAAAGATAATTGGTTTGACAAATTAGCTATAAACCATTTATCAAAAAGTGTTCAAGCAGCAACAGGAATC
AGTAACAATAAGAGTGGTTTTGATAGCCTTGTTGAGGCAGCTACCGTGGCTTCACAGAAATTTAATACTACTCAAC
AGCAAGGAATTATTCTAGATGCACTTGATAGAGCTTTTCCAAAGCCAATACTTTCAGTGATAAGGAGAGTAATGCC
ACCATCTAAATTAGCAAGGGAATATTTTGCCGTCTTCACCACTATATTCTTTGCTTGGTTACTCGGTCCCTCCGAGGT
AAGAGAGTCAGAGATCAACGGAAGAAGAGAAAAAAATATTGTCCACATTAAAAAATGCAGATTCTTAGAAGAAAC
AAATTGTGTTGGGATGTGTATTAATCTCTGCAAAATGCCATCTCAATTGTTCATAAAGGACTCGTTTGGTATGCCAG
TCAACATGGTCCCAAATTTTGATGACATGAGTTGTGAGATGATATTCGGCCAGGAACCCCCAGCATCAACTGATGA
TCCAGCACTCAAGCAACCCTGCTATAAACTTTGCAAGGCTAAGAAAAACCATGCAACTCAGTGTCTTAGT

>Medicago_Medtr3g080840
ATGGTAGTTCTTAGTTTTCAGGTTGTGCCTCAGCTAATAATAACTCCACCACAATATGCTCCAAGAGCAAGTGGTGC
TACCATTCGGTGTGGTATAGCTGAACCATCTGGTGAACCAGCTCCTTTAGGACAAAAGACACGTTACAATGACAGT
ATTTTTGAGAAGGTTTTCATGACTTTGTTTGCACGAAAAATGGAACCATTTGCAGAGCCTGTAATAGGGAATGCTA
AAAAGAAGAAGGAAAAGAAGGGTTTGTTGGATGTTTGGGAGTATGATTATGAGAGTTTTGTTGATGTGTCCAAAAG
AGTAATGTTGCGTCGGTCTCGTTTGCAGCAACAGCAAGTTGTTCGTGAGGTTCTTCTTTCTATGCTTCCTCCAGGTGC
GCCTGCTCAGTTTAGGAAATTATTTCCACCAACAAGATGGGCTGCAGAGTTTAATGCTGCATTGACAGTGCCTTTCT
TCCACTGGCTAGTTGGTCCTTCTGAGGTTATAGAAGTGGAGATAAATGGGGTGAAGCAAAAGAGTGGCGTCCATAT
AAAGAAATGCAGGTACCTGGAGAACAGTGGATGTGTTGGACAGTGTGTTAATATGTGCAAGATTCCTACCCAAGAT
TTTTTCACTAATGAATTTGGACTTCCATTAACAATGATTCCAAATTTTGAAGACATGAGTTGTGATATGGTTTATGG
CCAAACTCCGCCTTCATTTGAAGACGACCCAGTCTCCAAACAACCTTGCTATGCTGATATATGTCCTGTAGCGAATC
CAAACTCCAGTATATGTCCTAAGCTACAATCA

>Poplar_POPTR_0002s04730
ATGGATGCAAGATTTCTTTGTCAAACTAGAAGTCCTGTTCCATCCCTGCCGCGACAGAAGCGTGCTTGTAAGCTCAA
ACATCGCTCTCCTGTTCTTGCCGTTCTAACAAGAACACCAGATAATAATATGACTGGAGAGGAAAAGAAAGCTTCG
CATCCTACTGATATGTTAGCAGGACTTACGAAAAATACTACTGTCTATAACGATAGCTGGTTGGCTAAGCTAGCTAT
AAACTATCTATCGCAGCGATTTCAAGATGCAACAGGTTTAATCACGAAAACATGTGCAGGGATGAGAAACAGCAA
GAGAGATTATGAGAGCTTGACACAGACAGCTAGAGATACATGGCGGAAATTCAGTCCAACTCAACAACACGAGGT
TGTGCTTCAATCTCTTAATAGAGCCATCCCCGCAACGATATCGACCTTGGCAAGTTCGATGCATATTTTTCTTGTAA
AGATCATGCTACCACAGTGCACGTTTACAAGAGAATATTTTGCTGCCTTCACAACCTTGTTCTTTGTTTGGCTAGTC
GGACCCTGCGAGGTGCGGGAATCCGACTTCAATGGAAGAAAAGAAAAGAATGTGGTTCACATAAAAAAGTGCAGG
TTTTTAGAGGAGACAGACTGTATTGGGATGTGTACTAATCTATGCAAGGTTCCAAGTCAAACATTTATCAAGCACTC
CTTTGGAATGCCAGTTAACATGGTTCCAAATTTTGATGATATGAGCTGTGAGATGATATATGGCCAGGAACCTCCTG
CGATAACTGAAGATCCAGCATTCAAGCAGCCATGTTATAAACTATGTAATGATCCAAACCACCCTCACATAAGCAC
ATGCTCATGCATCCCTAAT

>Rice_DWARF27
ATGGAGACCACCACGCTTGTGCTGCTTCTTCCTCATGGCGGCGCCGGCGGCGTACGGCCGGCGGCAGCGGCAACGG
CGAAGCGAAGCTACGTGATGAGGAGGTGTTGCTCGACGGTGAGGGCGGTCATGGCGAGGCCGCAAGAGGCGCCGG
CGTCGGCGCCGGCCAAGAAGACGGAGACGGCGGCGATGATGTCGACGGTGCAGACGGAGACGGCGGCGGCGCCG
CCGGCGACGGTGTACCGGGACAGCTGGTTCGACAAGCTCGCCATTGGTTACCTGTCCAGGAACCTTCAAGAAGCTT
CTGGGCTAAAGAATGAAAAGGATGGCTACGAGAGCCTGATAGATGCCGCCCTAGCCATCTCAAGAATCTTCAGTCT
GGATAAACAAAGCGAGATTGTGACCCAAGCTCTTGAAAGAGCACTTCCAAGCTACATCCTCACAATGATCAAGGTG
ATGATGCCACCTTCAAGATTTTCCAGGGAGTACTTTGCTGCATTCACCACGATATTTTTTCCTTGGTTGGTTGGGCCG
TGTGAGGTTATGGAATCTGAAGTTGAAGGAAGGAAAGAGAAAAACGTGGTATATATCCCCAAATGCAGATTTCTG
GAAAGTACAAATTGTGTTGGTATGTGCACAAACCTTTGCAAGATTCCATGCCAGAAGTTCATCCAAGATTCACTTG
GCATGAAGGTCTACATGTCTCCCAATTTTGAAGACATGAGCTGTGAGATGATATTTGGACAGCAACCTCCTGAAGA
TGACCCTGCATTGAAGCAGCCATGCTTCCGGACAAAATGCGTCGCAAAGCAGAATCATGGTGTGAATTGCTCCATC

>Rice_Os08g0114100
ATGGCGGCGGCGGCGTTGCTGTTGGCGCCAGTGGCGGCGCCATGGTCGCGTGTACCGTCTGCAGGGAGGAGGGTTC
GGTGCGCGGCGACTGCGCCGGCGCCGATGGGGGAGAAGACGGAGTACCGGGACGGGCCGGTGGAGCGCGCCTTCA
TGGGGCTGTTCGCGCGCAAGATGGAGAAGTACGCCGTCGTCTCCAGCTCCGGCGGGAAGGGGAAGGAGAAGAAGA
AGGAGAAGAGCTCGAGGTCGGTGTGGGAGTGGGACTACGAGAGCTTCGTGGACGTGTCGCGGCGGGTGATGGTGG
GGCGGACGCGGGCGCAGCAGCAGGAGGCCGTCCGCGAGGTGCTCCTCTCCATGCTCCCCCCCGGCGCCCCCGAGC
AGTTCAAGAAGCTCTTCCCGCCGACGAGGTGGGCCTGCGAGTTCAACGCTGCCCTCACCGTCCCCTTCTTCCACTGG
CTCGTCGGCCCATCAGAGGTTGTGGAGGTTGAGGTCAATGGGGTGAAGCAGAAGAGTGGAGTGCTGATAAAGAAA
TGCAGGTACCTGGAGAACAGCGGCTGCGTCGGAATGTGCGTCAACATGTGCAAAATTCCGACGCAAAACTTCTTCA
CCAACGAGTTCGGACTCCCTCTCACCATGAATCCAAATTTTGAAGATATGAGCTGTGAAATGATTTACGGCCAAGT
GCCCCCACCCTTGGAAGAAGACCCAGCATCAAAACAGCCCTGCTATGCCAATCTATGTTCCATCTCAACACCCTCA
GCGCCAATTTGTCCCAAACTTCAGACT

>Soybean_DWARF27
ATGGAAGCAAAATTGGTTGCACAGTGCAAGAGTCCAACACTAAGTTTAGCTCACCGAAAACCAAAACACCCTTGTG
TGGTGGTTGGAGTGCTGGCAAGGCCTGTTGATGAGATTTCAGGAGAAGCAAGAAAAACAAATCATGTGTACAAAG
ATGGTTTGTTTGATCGCATAACCATAAACTATTTATCAAAATGCGTTCAAGAGGCAACAGGACTCAGAAATAATAA

GAGTGGGTATGAGAGCCTGGTAGATGCTGCTACCGTGGCTTCACAGAGATTTAGTCCTGTTGAACAACATCAACTT
GTCATTCAATCCCTTGACAGAGCCTTCCCCAAGCCAATGCTTTTATTGATAAGGAAACTGCTACCACCATCTAAATT
CGCAAGGAAATTGTTTGCCGTCTTCACCACTTTGTTCTTTGCTTGGTTAGTTGGGCCCTCTGAGGTGAGAGAATCGG
AGGTCGAGGGGAGAAGAGAAAGAAATGTAGTTCACATAAAAAAATGCAGGTTCTTAGAAGGGACAAATTGTGTAG
GAATGTGTATCAACCTTTGCAAACTCCCATCTCAATCGTTTATAAAGGATTCTCTGGGTATTTCAGTCAACATGGTC
CCTAATTTTGATGACATGAGTTGTGAGATGATATTTGGCCAGGATCCCCCAGAATCAACTGATGATCCAGCACTCA
AGCAGCCGTGTTTTAAACTATGGTTTCAGGTCAGCAAGGAAGAAACAAGAATGGCCCACCTTTCATATGCTGACAA
AGCTGAGACTCATCACCCATCGTTTTTATTAGGAAAACAGCTAAGTGGTCCAATGCACTTT

>Soybean_Glyma02g16170
ATGGAAGCAAAATTGGTTGCACAGTGCAATAGTCCAACACTAACTTTAGCTCACTGGAAACTAAAACAACCTTGTG
TGGTTGGAGTGCTGGCAAGGCCTGCTGATGACATTTCAGGAGAAGCAAGAAAAACTAATCATGTGTACAAAGATG
GCTTGTTTGATCGCATAGCCATAAACTATTTATCAAAATGCGTTCAAGAGGCAACAGGACTCAAAAATAGTAAGAG
TGGTTATGAGAGCCTGGTAGAGGCAGCTACCCTGGCTTCGCAGAGATTTAGTCCTATTGAACAGCATCAACTTGTC
ATTCAATCCCTTGACAGAGCCTTTCCCAAGCCAATGCTTTTACTGATAAGGACACTGCTACCACCCTCTAAATTCGC
AAGGAAATTGTTTGCCATCTTCACCACTTTGTTCTTTGCTTGGTTAGTCGGGCCCTCTGAGGTGAGAGAATCAGAGG
TGGAGGGGAGAAGAGAAAGAAATGTAGTCCACATAAAAAAGTGCAGGTTCTTAGAAGAGACAAATTGTGTAGGA
ATGTGTATCAATCTTTGCAAACTCCCATCTCAATCGTTTATAAAAGATTCTCTGGGTATGTCAGTCAACATGGTTCC
TAATTTTGATGACATGAGTTGTGAGATGATATTTGGCGAGGATCCCCCAGAATCAACTGATGATCCAGCACTGAAT
CAACCGTGTTTTAAACTATGCAAGGCAAAAAGAAGCCATGGAACCAATTGCCTCGTTATAAAT

>Wild_strawberry_Fv_DY667171
ATGTCTTCAAAACTTGCAAAACCAGTCCATTCTCAACTCTCTTCTTCTTCGATCTTCTTTCACCAACATCAAATGGAA
GCATCACATTTCTTACAAAGGAGCTTTGCTACAACTCTGCCTCACCACAGGCATGTGCATGCTCACAAGCTGAGGC
GCTCTTCGATAGTTGCAGTGCTGTCAACTCCGGCAGATGTTATAAACATAGAGGCGGATAAAAGCAGAATAAGACC
GGCAGATGGTGCAGCAGAAAAGAAAAGTGTCTATAATGATAACTGGTTTGATAAACTGGCCATTGATCATCTGTCT
CAAAATGTCCAAGCTGCATCAGGGTTGAGAAACAGAAAGAGTGGATTTGAGAGCCTGGTAGAGGCAGCTAGCGCT
GCGTCGCTAAACTTCAATCCAGAAAAACAAAGAGAAATAGTTGTTCAAGCTCTTGAGGGTGCATTTCCAAGGCCAA
TACTTTCCCTGATTAAGGCATTACTACCAGAATCTAAACTTGCAAGGGAATACTTCGCCGTCTTTACCACCATCTTC
TTCGCCTGGCTGGTCGGACCATGCGAGGTGAGAGAATCAGAGTTCAATGGAAGCAAAGAGAAAAATGTAGTCCAC
ATACCAAAATGCAGGTTCTTGGAGGAAACCAATTGTGTAGGGATGTGTATTAACATGTGTAAGATGCCATCTCAAA
GCTTTATAAAGGATACAATGGGAATGCCAGTCAACATGGTTCCAAATTTTGATGATATGAGCTGCCAGATGATATT
CGGGGAGGAGCCTCCAGAGCTACTTAATGATCCAGCACTCAAGCAGCCATGCTTCAAACTATGCAAAGTAAAACA
AAGGCACAATAACTGCTCCAGT

>Cassava_DWARF27
MEIQFLSRNRSNLPSIPLPRRLPKLHRLPVLAVLTRPPEKITGVKEKNSSKPADKVRELTRKTVYNDNWFDKLAINHLSKS
VQAATGLRNCKTGYESLVQAAMDASQKFSLIQQRELVLQALDTAFPRLILDLAIKAVMPQSQFAREYFAAFTTLFFVWLI
GPCEVRESELNGRKEKNVVHIEKCRFLEETNCVGMCTNLCKVPSQTFIKHSLGMPVNMVPNFDDMSCEMIFGQDPPMPT
EDPAFNQPCYKL

>Maize_LOC100277925
MEVAAATCTLLAAVALPVSSSAAAARSATKQSYSRRKTTAVRAVMARPHQVPPPATATATATTYRDNWFDKLAIGYLS
RNLQEASGMKNGKDGYEGLIEAALAISALFRVDQQWDTVASALQRAFPSYILTMIKVMMPPSRFSREYFAAFTTVFFPW
LVGPCEVRESQVDGREERNVVYIPKCRFLESTNCVGMCTNLCKIPCQRFIQDSLGTAVYMSPNFEDMSCEMIFGQQPPED
DPALKQPCFRTKCIAKQNHQVNCSI

>Medicago_DWARF27
MDSKMIAHNMSLTPTLAQWKKLRLKPKHTFVVGVLARPTDDISEETLRKTNVYKDNWFDKLAINHLSKSVQAATGISN
NKSGFDSLVEAATVASQKFNTTQQQGIILDALDRAFPKPILSVIRRVMPPSKLAREYFAVFTTIFFAWLLGPSEVRESEING
RREKNIVHIKKCRFLEETNCVGMCINLCKMPSQLFIKDSFGMPVNMVPNFDDMSCEMIFGQEPPASTDDPALKQPCYKL
CKAKKNHATQCLS

>Medicago_Medtr3g080840
MVVLSFQVVPQLIITPPQYAPRASGATIRCGIAEPSGEPAPLGQKTRYNDSIFEKVFMTLFARKMEPFAEPVIGNAKKKKE
KKGLLDVWEYDYESFVDVSKRVMLRRSRLQQQQVVREVLLSMLPPGAPAQFRKLFPPTRWAAEFNAALTVPFFHWLV
GPSEVIEVEINGVKQKSGVHIKKCRYLENSGCVGQCVNMCKIPTQDFFTNEFGLPLTMIPNFEDMSCDMVYGQTPPSFED
DPVSKQPCYADICPVANPNSSICPKLQS

>Poplar_POPTR_0002s04730
MDARFLCQTRSPVPSLPRQKRACKLKHRSPVLAVLTRTPDNNMTGEEKKASHPTDMLAGLTKNTTVYNDSWLAKLAIN
YLSQRFQDATGLITKTCAGMRNSKRDYESLTQTARDTWRKFSPTQQHEVVLQSLNRAIPATISTLASSMHIFLVKIMLPQ
CTFTREYFAAFTTLFFVWLVGPCEVRESDFNGRKEKNVVHIKKCRFLEETDCIGMCTNLCKVPSQTFIKHSFGMPVNMVP
NFDDMSCEMIYGQEPPAITEDPAFKQPCYKLCNDPNHPHISTCSCIPN

>Rice_DWARF27
METTTLVLLLPHGGAGGVRPAAAATAKRSYVMRRCCSTVRAVMARPQEAPASAPAKKTETAAMMSTVQTETAAAPPA
TVYRDSWFDKLAIGYLSRNLQEASGLKNEKDGYESLIDAALAISRIFSLDKQSEIVTQALERALPSYILTMIKVMMPPSRF
SREYFAAFTTIFFPWLVGPCEVMESEVEGRKEKNVVYIPKCRFLESTNCVGMCTNLCKIPCQKFIQDSLGMKVYMSPNFE
DMSCEMIFGQQPPEDDPALKQPCFRTKCVAKQNHGVNCSI

>Rice_Os08g0114100
MAAAALLLAPVAAPWSRVPSAGRRVRCAATAPAPMGEKTEYRDGPVERAFMGLFARKMEKYAVVSSSGGKGKEKKK
EKSSRSVWEWDYESFVDVSRRVMVGRTRAQQQEAVREVLLSMLPPGAPEQFKKLFPPTRWACEFNAALTVPFFHWLV
GPSEVVEVEVNGVKQKSGVLIKKCRYLENSGCVGMCVNMCKIPTQNFFTNEFGLPLTMNPNFEDMSCEMIYGQVPPPLE
EDPASKQPCYANLCSISTPSAPICPKLQT

>Soybean_DWARF27
MEAKLVAQCKSPTLSLAHRKPKHPCVVVGVLARPVDEISGEARKTNHVYKDGLFDRITINYLSKCVQEATGLRNNKSG
YESLVDAATVASQRFSPVEQHQLVIQSLDRAFPKPMLLLIRKLLPPSKFARKLFAVFTTLFFAWLVGPSEVRESEVEGRRE
RNVVHIKKCRFLEGTNCVGMCINLCKLPSQSFIKDSLGISVNMVPNFDDMSCEMIFGQDPPESTDDPALKQPCFKLWFQV
SKEETRMAHLSYADKAETHHPSFLLGKQLSGPMHF

>Soybean_Glyma02g16170
MEAKLVAQCNSPTLTLAHWKLKQPCVVGVLARPADDISGEARKTNHVYKDGLFDRIAINYLSKCVQEATGLKNSKSGY
ESLVEAATLASQRFSPIEQHQLVIQSLDRAFPKPMLLLIRTLLPPSKFARKLFAIFTTLFFAWLVGPSEVRESEVEGRRERN
VVHIKKCRFLEETNCVGMCINLCKLPSQSFIKDSLGMSVNMVPNFDDMSCEMIFGEDPPESTDDPALNQPCFKLCKAKRS
HGTNCLVIN

>Wild_strawberry_Fv_DY667171
MSSKLAKPVHSQLSSSSIFFHQHQMEASHFLQRSFATTLPHHRHVHAHKLRRSSIVAVLSTPADVINIEADKSRIRPADGA
AEKKSVYNDNWFDKLAIDHLSQNVQAASGLRNRKSGFESLVEAASAASLNFNPEKQREIVVQALEGAFPRPILSLIKALL
PESKLAREYFAVFTTIFFAWLVGPCEVRESEFNGSKEKNVVHIPKCRFLEETNCVGMCINMCKMPSQSFIKDTMGMPVN
MVPNFDDMSCQMIFGEEPPELLNDPALKQPCFKLCKVKQRHNNCS

Chapter 4

The strigolactone biosynthesis gene *DWARF27* is rhizobium Nod factor responsive in *Medicago truncatula*

Wei Liu[1,2], Tingting Xiao[1,2], Arjan van Zeijl[1,2], Erik Limpens[1], Ton Bisseling[1] and René Geurts[1]

1. Department of Plant Science, Laboratory of Molecular Biology, Wageningen University, 6708 PB Wageningen, The Netherlands
2. These authors contributed equally to this work.

ABSTRACT

Strigolactones are a class of plant hormones of which biosynthesis is activated in response to phosphate starvation. This involves at least 4 enzymes, including an iron-binding protein DWARF27 (D27). *D27* is under transcriptional regulation of the GRAS-type proteins, NSP1 and NSP2, both proteins that in legumes are also essential for rhizobium induced root nodule formation. Recently it was found that this symbiosis is less effective in pea (*Pisum sativum*) strigolactone biosynthesis mutants, suggesting a role of strigolactones in this interaction. We questioned whether *D27* is transcriptionally activated upon rhizobium Nod factor signalling in a NSP1 and NSP2 dependent manner. Studies in *Medicago truncatula* showed that this is indeed the case, whereas other strigolactone biosynthesis genes are not responsive. Analysis of *MtD27* expression using a promoter *GUS* reporter system revealed not only temporal, but also spatial regulation in response to rhizobium. Symbiotic *MtD27* expression is induced in the epidermis and all cell layers of the root cortex, but subsequently, is delineated to those cells that become susceptible for rhizobium infection. Knocking down *MtD27* expression by RNA interference causes a defect in nodule meristem maintenance and symbiosome development, the latter suggesting a role of strigolactones in intracellular accommodation of rhizobia.

INTRODUCTION

Legumes evolved the capacity to live in an intimate endosymbiosis with nitrogen fixing rhizobium bacteria. Specific root nodules are formed to host rhizobia intracellular. Such nodules provide optimal physiological conditions to the bacteria to fix atmospheric nitrogen gas into ammonia, which can be used by the plant. Two types of studies have revealed a link between this endosymbiosis and the phytohormone strigolactone. Strigolactone biosynthesis mutants in pea (*Pisum sativum*) display a reduced root nodule number (Foo and Davies, 2011; Foo et al., 2013). The second study focussed on the GRAS-type transcription regulators NSP1 and NSP2. These are originally identified as essential response factors in the rhizobium nodule symbiosis (Catoira et al., 2000; Oldroyd and Long, 2003; Kalo et al., 2005; Smit et al., 2005; Hirsch et al., 2009), but it was found that in the absence of rhizobia both transcriptional regulators are also needed for the expression of *DWARF27* (*D27*), encoding a key enzyme in strigolactone biosynthesis (Liu et al., 2011). So, (indirectly) NSP1 and NSP2 control strigolactone biosynthesis in the root. To determine whether NSP1 and NSP2 controlled strigolactone biosynthesis also occurs during nodule formation we focussed on the role of *D27* during nodulation.

Strigolactones are a class of terpenoids derived from β-carotene. They are produced mainly in the root of the plant and the biosynthesis of their basic structure involves at least 3 plastid localized enzymes; the iron-binding protein D27 and the carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8) (Alder et al., 2012). The subsequent activity of these 3 enzymes results in biosynthesis of carlactone, a precursor of strigolactones. The conversion of carlactone to strigolactones is not resolved yet, but is hypothesized to require a cytochrome P450 enzyme, possibly encoded by *MAX1* in *Arabidopsis thaliana* (Arabidopsis) (Booker et al., 2005). This biosynthetic pathway is under the control of a nutrient sensing mechanism and/or the nutrient status of the plant, and phosphate is most inductive (Yoneyama et al., 2007a; Yoneyama et al., 2012). Plants that either experience a sudden low phosphate concentration in their growth medium, or are continuously grown under phosphate-limited conditions, markedly increase *D27* expression and subsequent strigolactones production (Yoneyama et al., 2007a; Liu et al., 2011).

Substantial fractions of the strigolactones are actively exuded into the rhizosphere (Yoneyama et al., 2007b; Yoneyama et al., 2012). Studies in petunia (*Petunia hybrida*) revealed that this efflux requires a specific ATP-binding cassette (ABC) transporter

(Kretzschmar et al., 2012). Exuded strigolactones function as attractants for arbuscular mycorrhizal fungi (Akiyama et al., 2005). These obligatory biotrophic (symbiotic) fungi can sense strigolactones and respond with an increased hyphal branching, thereby, promoting colonization of plant roots (Akiyama et al., 2005; Besserer et al., 2006; Yoneyama et al., 2008). The endomycorrhizal symbiosis facilitates nutrient exchange between fungus and plant. Mycelium that remains in the soil markedly increases the plant root capacity to access nutrients, especially immobile phosphates. The plant receives these nutrients from the fungi at the expense of carbohydrates (Jennings, 1995). Although strigolactones are not essential for establishment of an endomycorrhizal symbiosis, they contribute significantly to increasing root infection levels (Kretzschmar et al., 2012).

Besides *ex planta* attractants for endomycorrhizal fungi, strigolactones also play a role as endogenous plant signals that intertwine with auxin and ethylene. Shoot localized strigolactones repress outgrowth of axillary buds thereby promoting apical dominance (Stirnberg et al., 2002; Gomez-Roldan et al., 2008). This is caused by dampening auxin transport through the main stem and affecting the subcellular localization of auxin efflux carriers, the PINs (Bennett et al., 2006; Ongaro and Leyser, 2008; Hayward et al., 2009; Leyser, 2011; Muller and Leyser, 2011). As a result the vasculature becomes a weak sink for auxin, preventing canalisation of auxin from the axillary buds into the vasculature (Leyser, 2011). Additionally, it was found that strigolactone analogs affect the outgrowth of lateral root primordia, but promote the growth of the primary roots (Ruyter-Spira et al., 2011). Also, strigolactones can promote root hair growth in an auxin and ethylene dependent manner. And the strigolactones induced ethylene biosynthesis is through transcriptional activation of an ACC synthase gene (Kapulnik et al., 2011).

Symbiotic diazotrophic rhizobia form a paraphyletic group that shares a set of symbiotic nodulation genes that are essential to establish a root nodule symbiosis. Most prominent genes are that encode proteins for biosynthesis of lipo-chitooligosaccharides (LCOs) that in structure are very similar to signal molecules secreted by endomycorrhizal fungi, as exemplified by the recent characterized LCOs of *Rhizophagus irregularis* (formerly named *Glomus intraradices*) (Maillet et al., 2011). In most legumes rhizobium LCOs (named Nod factors) are essential to trigger the formation of nodule primordia in the cortex of the root and to establish intracellular infection of the prokaryotic endosymbiont. This infection process starts in curled root hairs where a tube like structure is formed intracellularly, which guides the rhizobia to the newly formed nodule primordia. There, the rhizobia are released as organelle-like structures, named

symbiosomes, which remain surrounded by a plant-derived membrane. These symbiosomes act as nitrogen-fixing units that provide the ammonia to the plant cell in exchange of nutrients.

Molecular genetic studies in model legumes, like *Medicago truncatula*, revealed that rhizobium and endomycorrhizal fungi induced LCO-signalling share a common set of proteins to induce symbiotic responses. Upon perception of LCOs by specific LysM-type receptors, a common plasma membrane localized LRR-type receptor kinase (named MtDMI2 *in M. truncatula*) is required to transmit an unknown secondary messenger to the nucleus (Endre et al., 2002). In conjunction with a cation ion channel in the nuclear envelope (MtDMI1) specific Ca^{2+} signalling is induced that most likely is interpreted by a nuclear localized Ca^{2+}/calmodulin dependent protein kinase (MtCCaMK/MtDMI3) (Ane et al., 2004; Levy et al., 2004; Charpentier et al., 2013). Downstream of these common signalling proteins several transcription factors with differential functions in the either or both symbioses are active. Among these are the GRAS-type transcriptional regulators MtNSP1 and MtNSP2 (Kalo et al., 2005; Smit et al., 2005). Experiments in heterologous systems revealed that MtNSP2 can form heterodimer with MtNSP1 suggesting a regulatory link between both proteins (Hirsch et al., 2009). Both transcriptional regulators are essential for rhizobium symbiosis, but also promote mycorrhizal infection (Maillet et al., 2011).

In rice (*Oryza sativa*) and *M. truncatula* it was shown that under non-symbiotic growth conditions (in absence of rhizobium) NSP1 and NSP2 are essential for *D27* expression and strigolactone biosynthesis in response to phosphate starvation (Liu et al., 2011). As NSP1 and NSP2 are also indispensable for Nod factor induced responses we hypothesize that *MtD27* could be induced by Nod factors in an MtNSP1 and MtNSP2 dependent manner. We studied the transcriptional regulation of *MtD27* and other strigolactone biosynthesis genes in *M. truncatula* and found that only *MtD27* is Nod factor responsive. After the early activation in all the cortical cell layers, expression of *MtD27* becomes restricted to nodule primordia and subsequently to the nodule meristem and infection zone. As RNA interference of *MtD27* reveals a defect in symbiosome formation, we conclude that this gene, and likely strigolactones, plays a role in intracellular accommodation of rhizobia.

RESULTS

MtDWARF27 expression during rhizobium symbiosis

To obtain insight in the symbiotic function of *MtD27*, the spatiotemporal expression pattern is analysed in *M. truncatula*. For this, a ~1 kb fragment representing the 5' region upstream of the translational start site was cloned into a binary transformation vector in front of a β-glucuronidase (GUS) encoding sequence. This construct was used to create *M. truncatula* compound plants carrying transgenic roots. In non-inoculated plants, grown on BNM medium containing no nitrate, but relatively high phosphate concentration (500 µM PO_4^{3-}), *MtD27* expression was observed in the stele and pericycle cells (Figure 1A). Subsequently, transgenic plants were inoculated with *Sinorhizobium meliloti* strain 2011. Four days post inoculation (dpi) the *pMtD27::GUS* transgenic roots showed a patched GUS staining, which was associated with rhizobium root hair infections (Figure 1B). At 7 dpi, the expression in the root ceased, but GUS activity accumulated in nodule primordia (Figure 1C). Sectioning nodule primordia revealed expression of *pMtD27::GUS* in dividing pericycle cells (Figure 1D). This expression maintains in the developing nodule primordium (Figure 1E), but becomes delineated in newly formed nodule, where it is visible only in the meristem and distal infection zone (Figure 1F). This suggests a role of MtD27, and subsequently strigolactones, in rhizobium symbiosis.

***MtDWARF27* expression is Nod factor responsive**

As *MtD27* is expressed at early stages of the interaction we tested whether it is induced by rhizobium Nod factors. *M. truncatula* seedlings were grown in so-called Fåhraeus slides (Heidstra et al., 1994), which is a system optimized to study early responses induced by Nod factors in the root epidermis. *M. truncatula* roots of wild type plants were treated with *S. meliloti* Nod factors (~10^{-9} M) for 0, 1, 2 and 3 hours. Subsequently, total RNA was isolated from the Nod factor susceptible zone, a region of about 1 cm just above the root meristem. Real time qRT-PCR showed that *MtD27* is induced already at 2 hours post Nod factor application, and accumulates in a >20-fold increased level after 3 h (Figure 2C). This timing and induction level is comparable to that of *MtENOD11;* a gene frequently used as marker for Nod factor induced signalling in *M. truncatula* (Figure 2C) (Journet et al., 2001; Hirsch et al., 2009). We repeated the Nod factor inductive response, but now using transgenic plants expressing the *pMtD27::GUS* construct. These compound plants were grown *in vitro* on square agar plates and roots were treated with ~100 µl 10^{-9} M Nod factors solution. GUS staining was induced in epidermis and all cortical cell layers at 3 h (Figure 2A and 2B). Nod factor induced *MtENOD11* expression requires a signalling module downstream of Nod factor perception consisting of MtDMI1, MtDMI2 and MtDMI3/MtCCaMK (the common signalling

module) and the transcriptional regulators MtNSP1, MtNSP2 and MtERN1 (Catoira et al., 2000; Oldroyd and Long, 2003; Andriankaja et al., 2007). To investigate whether Nod factor induced *MtD27* expression is also dependent on this signalling module, we conducted real time qRT-PCR on Nod factor susceptible root zones of these mutant plants treated with *S. meliloti* Nod factors (10^{-9} M) for 3 hours and used *MtENOD11* as control. Firstly, we revealed that *MtD27* expression is not induced in the *Mtdmi1*, *Mtdmi2* and *Mtdmi3* mutant roots (Figure

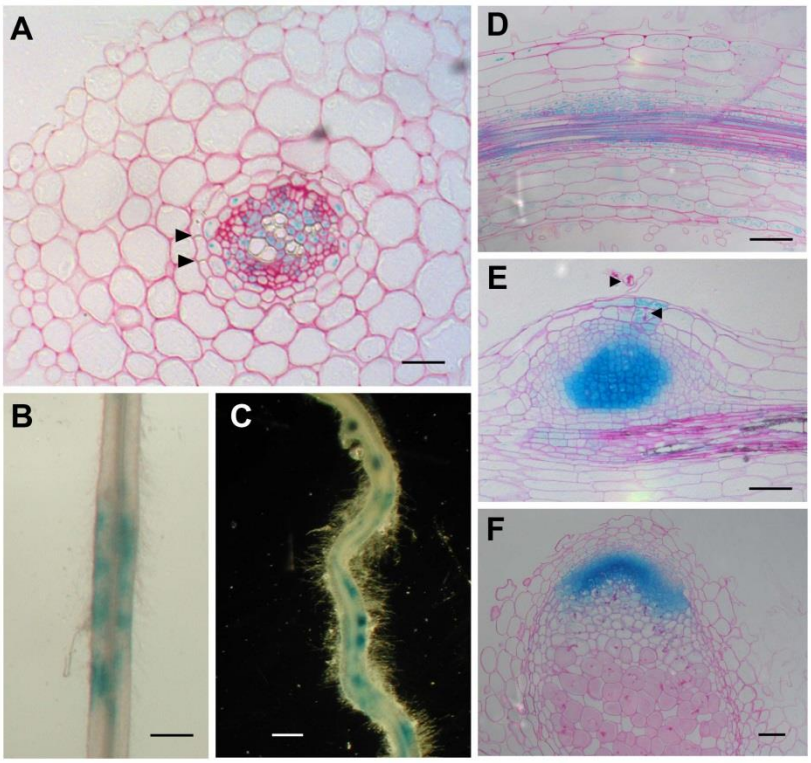


Figure 1. Spatial expression pattern of *MtD27* in *M. truncatula* roots and nodules. The *MtD27* expression pattern was analysed using a promoter GUS reporter system in transgenic *M. truncatula* roots. Under non-symbiotic condition, *MtD27* is mainly expressed in the stele. In a cross section of the root fragment GUS blue staining can be detected in precambium, pericycle cells, and in endodermis cells **(A)**. Arrowheads indicate Casparian strips, which mark the endodermal cell layer. Four days post inoculation (dpi) with *Sinorhizobium meliloti* strain 2011, *MtD27* expression delineates to some parts of the roots resulting in a patched pattern (**B**). *MtD27* expression is restricted to the nodule primordium 7 dpi (**C**). Longitudinal section of a young nodule primordium formed in the inner cortex 2 dpi (**D**) shows *MtD27* promoter is active in the dividing cells. *MtD27* promoter activity accumulates in the nodule primordium cells that are susceptible for rhizobium infection (**E**). The infection thread is indicated with arrowheads. At eighteen-day-old nodule *MtD27* is mainly expressed in nodule meristem and infecting zone (**F**). The scale bar is equal to 25 µm in A; 0.5mm in B and C and 50 µm in D, E and F.

2D). This indicates that the symbiotic *MtD27* induction requires the common signalling module genes. Previously, we had found that *MtD27* expression in non-inoculated roots is dependent on MtNSP1 and MtNSP2. We noticed that both GRAS-type proteins are also compulsory for Nod factor induction of *MtD27* (Figure 2E). Next, we monitored *MtD27* expression in the *Mtern1* mutant, in which Nod factor induced MtENOD11 expression is blocked (Figure 2G). For *MtD27* we found that the expression in non-inoculated roots is already about~70% lower when compared to wild type roots. This difference is maintained upon Nod factor induction (Figure 2F). Taken together, these results show that *MtD27* is a Nod factor responsive gene of which its symbiotic expression is dependent on the common signalling module and the transcription regulators MtNSP1, MtNSP2 and in part MtERN1.

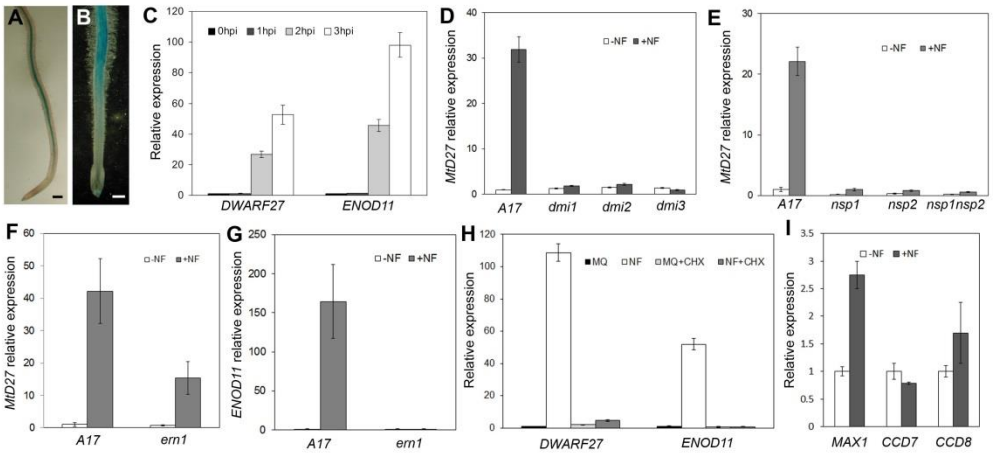


Figure 2. Symbiotic *MtD27* expression is under direct control of Nod factor signalling. Promoter GUS (**A-B**) and real time qRT-PCR studies (**C-I**) to investigate *S. meliloti* Nod factor (10^{-9} M) induced *MtD27* expression (**A-F, & H**) and compared to *MtENOD11* (**C, G-H**) and other strigolactone biosynthesis genes (**I**). Nod factor induced *MtD27::GUS* expression in the susceptible zone 3 hours post application. Under non-symbiotic conditions *MtD27::GUS* activity is mainly restricted to the stele (**A**), whereas upon Nod factor application the expression extends to all cell layers (**B**). Real time qRT-PCR shows *MtD27* is induced as early as 2 hours post Nod factor treatment, which correlates with the induction of the Nod factor response gene *MtENOD11* (**C**). *MtD27* expression is not induced (3 hours post Nod factor application) in the *Mtdmi1*, *Mtdmi2* and *Mtdmi3* (**D**), nor in the *Mtnsp1*, *Mtnsp2* or *Mtnsp1Mtnsp2* mutants (**E**), whereas in the *Mtern1* mutant *MtD27* induction is reduced by ~50% (**F**). This is different to *MtENOD11* of which the induction is completely abolished in *Mtern1* mutant roots (**G**). Nod factor mildly induced *MtD27* expression in presence of 50 µM cycloheximide (CHX) 3 hours post application. In comparison, *MtENOD11* is not induced in such condition (**H**). Other strigolactone biosynthesis genes *MtCCD7* and *MtCCD8* do not respond to Nod factor treatment, whereas *MtMAX1* is mildly induced (**I**). The scale bar is equal to 0.5 mm in **A** and **B**. In **C** to **I**, data are means ±SD.

Previously it was shown in *Vicia sativa* that the Nod factor inducibility of early nodulin genes is indirect, as an inhibition of protein synthesis by cycloheximide (CHX) blocks early nodulin genes expression (Vijn et al., 1995). As *MtD27* displays a comparable expression pattern as *MtENOD11*, we tested whether this gene is a direct target of Nod factor signalling. To this end, *M. truncatula* seedlings were grown in Fåhraeus slides for 3 days and pre-treated with 50 µM CHX for 30 minutes prior to Nod factor treatment (10^{-9} M, 3 h). The expression of *MtD27* and *MtENOD11* in the susceptible root zone was monitored by qRT-PCR. In contrast to *MtEOND11*, a slight 2-fold induction of *MtD27* could by observed. In comparison, in case proteins synthesis is not blocked, *MtD27* displayed over 100-fold induction upon Nod factor signalling (Figure 2H). This underlines that new protein synthesis is required for full induction of *MtD27* by Nod factors.

Strigolactone biosynthesis requires 4 genes. Besides *D27* these are *CCD7*, *CCD8* and a potential *MAX1* homolog (Alder et al., 2012). Previously we found that a *MAX1* homolog displays a lower expression in roots of the *M. truncatula Mtnsp1* and *Mtnsp2* mutants (Liu et al., 2011). We tested whether besides *MtD27*, these other strigolactone biosynthesis genes are also Nod factor responsive. Real time qRT-PCR revealed that *MtCCD7* and *MtCCD8* are not responsive to Nod factors, whereas *MtMAX1* shows 3-fold up-regulation (Figure 2I), which might indicate that *MtD27* expression is the main rate limiting step in strigolactone biosynthesis.

Phosphate deprivation induced *MtDWARF27* expression is independent of the common symbiotic signalling cascade

A (sudden) decrease of the phosphate concentration in the medium also elevates *MtD27* expression in *M. truncatula* roots (Liu et al., 2011). We aimed to determine whether the Nod factor signalling mechanism is required to induce *MtD27* expression in response to a low phosphate status. First it was determined to what extend the spatial *MtD27* expression in *M. truncatula* roots is affected by different phosphate regimes. Transgenic *M. truncatula* plants carrying the *MtD27* promoter GUS reporter construct were grown in perlite for 2 weeks at high phosphate (200 µM PO_4^{3-}), and subsequently they were transferred to no phosphate (0 µM PO_4^{3-}) medium. Plant roots were stained histochemically for GUS activity at 5 days after the transfer and compared to control roots. In all plants GUS staining could be observed in the stele of the roots, as well as in the root apical meristems (Figure 3A and 3B). Phosphate starved roots displayed a much more intense staining than control roots, which was most clear

in the root apical meristem (Figure 3C and 3D). However, the spatial expression pattern did not change in response to a different phosphate regime.

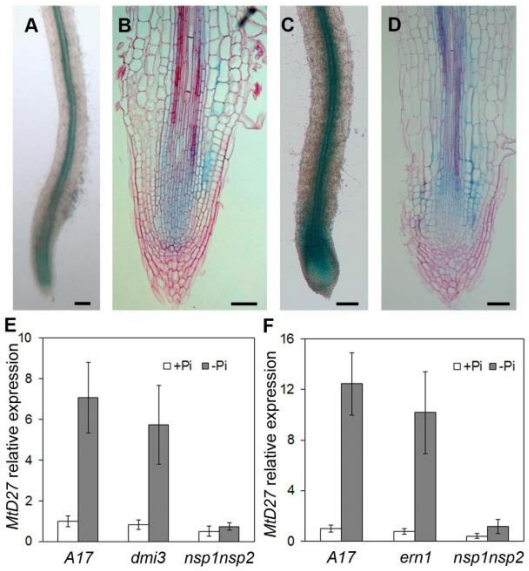


Figure 3. Spatial expression pattern of *MtD27* upon phosphate starvation. Promoter GUS (**A-D**) and real time qRT-PCR studies (**E**) investigate *MtD27* expression upon phosphate starvation. *M. truncatula* plants with transgenic roots containing pMtD27::GUS grown in full nutrient condition (200 µM Pi). *MtD27* is mainly expressed in the vascular bundle and the root meristem (**A-B**) . At 3 days of phosphate starvation (0 µM Pi), *MtD27* expression is enhanced, but spatially not changed (**C-D**). Quantification of *MtD27* expression in *Mtdmi3*, *Mtern1* and *Mtnsp1Mtnsp2* mutant background are analysed by qRT-PCR upon 3 days phosphate starvation. Only MtNSP1 and MtNSP2 are required for the phosphate-starvation response (**E-F**). The scale bar is equal to 250 µm in **A** and **C**, and equal to 100 µm in **B** and **D**. Data in **E** and **F** are means ±SD.

Previous studies revealed that phosphate deprivation induced *MtD27* expression is dependent on the symbiotic GRAS-type transcription regulators MtNSP1 and MtNSP2 (Liu et al., 2011). We investigated whether other symbiotic genes are also essential for this response. For this we selected *Mtdmi3* and *Mtern1*. MtDMI3/MtCCaMK is a key element in the Nod factor signalling pathway acting just upstream of MtNSP1 and MtNSP2, whereas ERF-type transcription factor MtERN1, in conjunction with MtNSP1 and MtNSP2, is essential for Nod factor induced *MtENOD11* expression. *M. truncatula Mtern1* and *Mtdmi3* knockout mutants were grown in an aeroponic system containing medium with high phosphate (200 µM PO_4^{3-}) and subsequently transferred to the medium containing no phosphate. To determine the expression of *MtD27* real-time qRT-PCR was conducted on RNA isolated from the meristematic zone of the root. This study reveals that in both mutants induction of *MtD27* in response to phosphate deprivation is similar to the induction found in roots of wild type plants

(Figure 3E and 3F). This indicates that the phosphate response of *MtD27* expression is independent of the MtDMI3/MtCCaMK Nod factor signalling cascade and the symbiotic transcription factor MtERN1.

Knocking down *MtDWARF27* expression causes a symbiotic phenotype

Strigolactone biosynthesis mutants *ccd7* and *ccd8* in pea display a decreased nodulation efficiency with about 50% (Foo and Davies, 2011; Foo et al., 2013). We conducted *MtD27* RNA knockdown studies (RNAi) using 35S promoter in *M. truncatula* roots. This results a similar reduction in nodule number as described for pea *ccd7* and *ccd8* knockout mutants, though with substantial variation between individual plants (Supplemental Table 1). The *MtD27* expression levels in the knockdown roots are reduced over 80% when compared to control roots (Figure 4A). Nodules formed on the *MtD27* RNAi roots remain relatively small, and the number of nodules was reduced to about 40% of wild type levels (Figure 4B). However, due to the high variation of the transgenic roots, this reduction is not statistically significant based on student *t-test* (p=0.139).

To characterize the *MtD27* RNAi nodules, we sectioned and analysed 13 nodules of 4 independent knockdown plants. This showed that 12 nodules had an aberrant phenotype, whereas only one nodule displayed a wild type phenotype. Three mutant nodules contained cells in the basal region that were well infected. However, several symbiosomes in these cells displayed an aberrant phenotype, containing only a small, not elongated, rhizobium bacterium, whereas the surrounding symbiosome membrane increased in size. Furthermore, these infected nodule cells did not contain a central vacuole, which is normally present in those cells in wild type nodules (Figure 4C and 4D). In other small nodules the basal infected cells already showed severe senescence and so it cannot be concluded whether also in these cells vacuole and symbiosome formation were affected in a similar manner. In all cases small mutant nodules have no or only a very small meristem. The absence of the meristem correlates with an endodermis that completely surrounds the central tissue (Figure 4D).

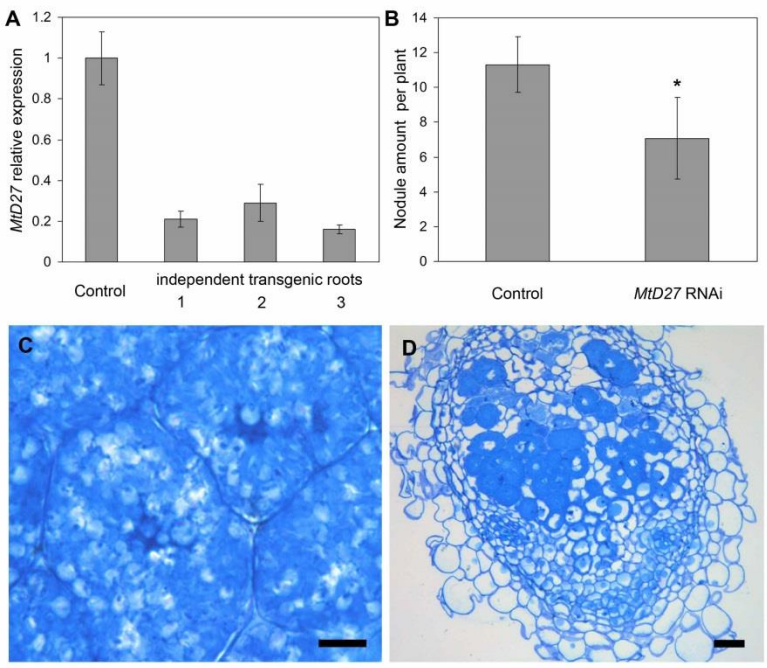


Figure 4. *MtD27* knockdown affects nodulation efficiency and symbiosome differentiation. Knockdown levels of *MtD27* in *M. truncatula* RNAi roots are detected by real time qRT-PCR in three independent transgenic roots (**A**). The average amount of nodules formed on transgenic *MtD27* RNAi roots compared to wild type plants at 18 days post inoculation (**B**). Longitudinal section of *MtD27* RNAi nodules stained with toluidine blue O (**C**-**D**). Infected cells contain large symbiosomes that harbour only a small bacterium (**C**). These nodules remain small and lack a meristem and infection zone (**D**). Data in **A** are means ±SD. Data in **B** are means ±SE. Star in **A** indicates p=0.139. The scale bar is equal to 10 µm in **C** and 50 µm in **D**, respectively.

DISCUSSION

Several lines of evidence indicate that strigolactones play a role in the rhizobium legume symbiosis (Soto et al., 2010; Foo and Davies, 2011; Foo et al., 2013). Here, we showed that *MtD27* is the symbiosis responsive gene in the strigolactone biosynthesis pathway in *M. truncatula*. The expression of *MtD27* is strongly elevated by rhizobium Nod factor induced signalling and sustains in nodule cells that are promiscuous for rhizobium infection.

A role of *MtD27* in intracellular infection was revealed by knockdown experiments. The small nodules formed on *MtD27* RNAi roots are affected in symbiosome development and display early root nodule senescence. Some nodule cells contain symbiosomes with an enlarged peribacteroid space, and bacteriods remain small. This phenotype coincides with the absence of a central vacuole in these cells. Infected cells in the central zone of a *M. truncatula*

nodule normally maintain a vacuole, although relatively small in size when compared to uninfected cells. The phenotype of enlarged peribacteroid membrane in combination with absence of a central vacuole is as similar as observed in the *M. truncatula Mtdnf2* knockout mutant, though less severe. *MtDNF2* encodes a phosphatidylinositol phospholipase C-like protein (Bourcy et al., 2013). The precise functioning of this protein remains elusive, but based on the phenotypic resemblance to the *MtD27* knockdown phenotype we suggest a mechanistic link between MtDNF2 and strigolactones.

The enlarged symbiosomes and/or premature senescence observed in *MtD27* RNAi nodules occur in cells in which this gene would be no longer transcriptionally active. The primary phenotype caused by the absence of *MtD27* expression could therefore occur earlier, namely in the nodule primordium and/or those cells just formed by the nodule meristem. In both cases these cells are promiscuous for rhizobium infection and can experience intracellular hosting of symbiosomes. Therefore we hypothesize that MtD27, and subsequently strigolactones, play a role during the early stage of intracellular infection.

MtD27 is Nod factor responsive, and its transcriptional activation is under control of the Nod factor signalling network, which includes MtDMI1, MtDMI2, MtDMI3/MtCCaMK, MtNSP1 and MtNSP2. *MtD27* expression is elevated within 2 hours post Nod factor application, by which it is among the earliest responsive genes. However, blocking protein synthesis with CHX largely diminishes induction of *MtD27* expression. This suggests that *de novo* protein synthesis is required to reach an *MtD27* expression maximum. Preliminary promoter binding studies using electrophoretic mobility shift assays (EMSA) revealed binding of MtNSP1 to the *MtD27* promoter, suggesting that *MtD27* is a primary target of this GRAS-type protein (Liu et al., 2011). Likewise, such binding was found for the *MtENOD11* promoter (Hirsch et al., 2009). However, in that case a second transcription factor, namely MtERN1, is essential to achieve Nod factor induced expression (Fig. 2G) (Cerri et al., 2012). We studied *MtD27* expression in *M. truncatula Mtern1* knockout mutant, which revealed that this transcription factor is not essential, though contributes for about 50-70% to the Nod factor inductivity of *MtD27*. This may hint to a similar regulatory mechanism for *MtD27* as identified for *MtENOD11* (Hirsch et al., 2009), with this difference that the functioning of MtERN1 is partially redundant. Alternatively, MtERN1 acts in an auto-activation loop that is essential to achieve maximal symbiotic *MtD27* expression.

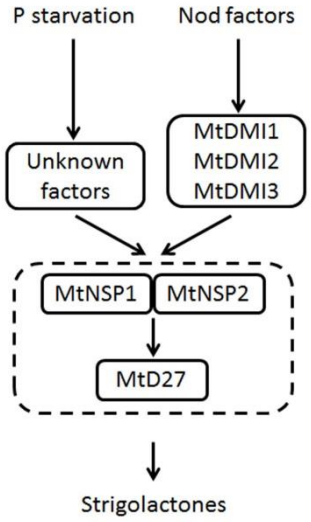


Figure 5. A model for MtNSP1-MtNSP2 controlled *MtD27* expression. A schematic model represents the regulation network of strigolactone biosynthesis in phosphate starvation and nodulation. *MtD27* expression and possibly strigolactone biosynthesis can be activated by phosphate starvation through unknown factors, as well as by nod factors through common symbiotic signalling cascade, MtDMI1, MtDMI2 and MtDMI3. In both cases, the activation requires a module component containing two GRAS-type transcription factors, MtNSP1 and MtNSP2, and an iron-containing protein MtD27.

MtD27 is also known for its responsiveness to phosphate starvation stress, a response conserved in *M. truncatula* and which is dependent on NSP1 and NSP2 (Liu et al., 2011). We studied the spatial regulation of this response using the *pMtD27::GUS* construct and found that the expression pattern remains unchanged in the stele and apical root meristem of the root upon phosphate starvation. The transcriptional activation of *MtD27* response to the phosphate status in the environment coincides with an increased exudation of strigolactones (Yoneyama et al., 2007b; Yoneyama et al., 2007a; Lopez-Raez et al., 2008). Generally, it is anticipated that this response is contributing to the attraction of endomycorrhizal fungi, which enhance phosphate acquisition from the environment (Yoneyama et al., 2008; Kretzschmar et al., 2012). We tested whether the *MtD27* expression response to phosphate starvation is dependent on the common signalling pathway as well as whether this response is (partially) dependent on MtERN1. Neither signalling components were involved in the phosphate starvation induced *MtD27* expression. Studies in Arabidopsis revealed that phosphate itself can function as a signal to report its availability. Such a phosphate regulatory response network would depend on NSP1 and NSP2, but acts independent from the other Nod factor signalling genes (Figure 5).

Since long there is an ambition to transfer the rhizobium symbiosis to other plant species, an objective that has not yet been achieved, but receives new attention with the unravelling of the symbiotic signalling pathway in legumes (Charpentier and Oldroyd, 2010; Untergasser et al., 2012). Strigolactone biosynthesis is largely conserved in higher plants. The finding of *MtD27* as the strigolactone biosynthesis gene that is responsive to rhizobium Nod factor signalling makes it to an excellent marker gene to study LCO signalling in non-legumes.

MATERIALS AND METHODS

Plant materials and growth conditions

Medicago truncatula Jemalong A17, *dmi1-1* (C71), *dmi2-1* (TR25), *dmi3-1* (TRV25), *nsp1-1* (B85), *nsp2-2* (0-4), *nsp1nsp2* (Liu et al., 2011) and *ern1* (*bit1-1*) were used in this study. Plants were grown in a growth chamber at 20 ℃ with 16h/8h day/night regime. For seed germination, seeds were pre-treated in 98% sulphuric acid for 7 minutes, and washed 6 times with demi-water. Afterwards, the seeds were sterilized with commercial bleach for 7 minutes and rinsed 6 times with sterile demi-water. The sterilized seeds were transferred on to Fåhraeus medium plates (1.5% agar) and left in cold room for 24 hours to synchronize germination. Then, the seeds plates were transferred into the growth chamber and left upside down for one day to initiate the germination. One-day-old seedlings were transferred to Fåhraeus slides for gene expression studies.

For phosphate starvation experiment, one-week-old seedlings were transferred into aeroponic system containing half strength Hoagland solution. Phosphate starvation was applied to 4-week-old plants by replacing the nutrient solution with half-strength Hoagland solution without phosphate.

Constructs and plant transformation

For *MtD27* promoter GUS reporter assay, a 921-bp fragment upstream the start codon of *MtD27* gene was amplified from *M. truncatula* Jemalong A17 genomic DNA sample using primer pair pMtD27-F and pMtD27-R. This fragment was recombined into pDONR-L4R1 with BP Clonase II (Invitrogen) according to the supplier's manual. The correct subcloned plasmid was recombined into pHGW-RR-MGW-R4R3 by using LR Clonase II plus (Invitrogen).

For *MtD27* RNAi construct, a 268-bp fragment in coding region was selected and PCR amplified from a *M. truncatula* Jemalong A17 root cDNA sample using the primer pairs MtD27i-F and MtD27i-R. Subsequently, this fragment is subcloned into a pENTR D-TOPO plasmid (Invitrogen) according to the supplier's manual. This clone was recombined into pKGWIWG2 (II)-RR-R1R2 to get the binary construct pKGWIWG2 (II)-RR-p35S-MtD27 RNAi. For an RNAi control construct, pENTR-CHEAP was recombined into pKGWIWG2 (II)-RR-R1R2 to get the binary plasmid pKGWIWG2 (II)-RR-p35S-RNAi-control.

For hairy root transformation in *M. truncatula*, *Agrobacterium rhizogenes* strain MSU440 was used following the previous described method (Limpens et al., 2004). Transgenic roots co-expressing DsRed marker were selected using Leica stereomicroscope. For the Nod factor inoculation experiment, compound plants were transferred to BNM solid medium containing 1 µM AVG. For the inoculation assay, two weeks after transformation the compound plants were transferred into perlite and watered with Fåhraeus liquid medium without nitrogen. One week after transfer, plants were inoculated with *Sinorhizobium meliloti* strain 2011 (OD600: 0.05-0.1). For the phosphate starvation experiment, the transgenic compound plants were transferred into perlite and watered with half strength Hoagland solution for one week. Afterward, plants were washed 3 times with demi-water to get rid of the nutrient salts and then watered with half strength Hoagland solution with or without phosphate, respectively.

Histochemical staining and microtome section

pMtD27::GUS transgenic roots and nodules samples under different treatments were rinsed 3 times with phosphate buffer solution (PBS) (pH7.0). The samples were transferred into the GUS staining buffer (contains 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 10 mM EDTA, 0.1% Triton X-100 and 1 mg/ml X-Gluc salt in 100 mM phosphate buffer solution, pH 7.0) and vacuumed for 30 min. Then, the samples were incubated in 37℃ growth chamber for 3 hours in the dark. The stained roots were rinsed with PBS (pH7.2) 3 times to stop the reaction.

For historesin imbedding, roots and nodule samples were fixed with 5% glutaraldehyde PBS (pH7.2) solution overnight. After fixation, the samples were rinsed with PBS (pH 7.2) 3 times and dehydrated through ethanol gradients (20%, 40%, 60%, 80% and 100%) . Afterwards, the samples were embedded with Technovit 7100 embedding kit (Fisher Scientific) following the supplier's protocol. Embedded GUS stained samples were sectioned to 4 µm thick with a Leica microtome and stained with 0.1% Ruthenium Red for one minute. Embedded nodule samples for structure detections were sectioned to 3 µm thick and stained with 0.05% Toluidine Blue O for 30 seconds. Images were obtained by Leica microscope and imaging software following the manual.

Real time qRT-PCR analysis

The samples were frozen immediately in liquid nitrogen. Total RNA were isolated from the frozen samples using the RNeasy plant kit (QIAGEN) following the supplier's manual.

1μg total RNA was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad). Real time qRT-PCR was set up in 20 μl reaction system with 2× iQ SYBR Green Super-mix (Bio-Rad) and proceeded with iQ5 Real time PCR detecting system according to the manufacturer's manuals. All primers used in this study were designed with Primer3Plus software (http://www. primer3plus.com/cgi-bin/dev/primer3plus.cgi) under qPCR settings (Untergasser et al., 2007). For data normalization, *M. truncatula* ubiquitin gene was used as internal control. All primers used in this assay are listed in Supplemental Table 2.

REFERENCES

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature **435,** 824-827.

Alder, A., Jamil, M., Marzorati, M., Bruno, M., Vermathen, M., Bigler, P., Ghisla, S., Bouwmeester, H., Beyer, P., and Al-Babili, S. (2012). The path from beta-carotene to carlactone, a strigolactone-like plant hormone. Science **335,** 1348-1351.

Andriankaja, A., Boisson-Dernier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D.G., and de Carvalho-Niebel, F. (2007). AP2-ERF transcription factors mediate Nod factor dependent *MtENOD11* activation in root hairs via a novel cis-regulatory motif. Plant Cell **19,** 2866-2885.

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarie, J., and Cook, D.R. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science **303,** 1364-1367.

Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C., and Leyser, O. (2006). The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. Curr. Biol. **16,** 553-563.

Besserer, A., Puech-Pages, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J.C., Roux, C., Becard, G., and Sejalon-Delmas, N. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biol. **4,** e226.

Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., Turnbull, C., Srinivasan, M., Goddard, P., and Leyser, O. (2005). MAX1 encodes a cytochrome P450 family member that acts downstream of MAX3/4 to produce a carotenoid-derived branch-inhibiting hormone. Dev. Cell **8,** 443-449.

Bourcy, M., Brocard, L., Pislariu, C.I., Cosson, V., Mergaert, P., Tadege, M., Mysore, K.S., Udvardi, M.K., Gourion, B., and Ratet, P. (2013). *Medicago truncatula* DNF2 is a PI-PLC-XD-containing protein required for bacteroid persistence and prevention of nodule early senescence and defense-like reactions. New Phytol. **197,** 1250-1261.

Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Denarie, J. (2000). Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. Plant Cell **12,** 1647-1666.

Cerri, M.R., Frances, L., Laloum, T., Auriac, M.C., Niebel, A., Oldroyd, G.E., Barker, D.G., Fournier, J., and de Carvalho-Niebel, F. (2012). *Medicago truncatula* ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and expression dynamics throughout rhizobial infection. Plant Physiol. **160**, 2155–2172.

Charpentier, M., and Oldroyd, G. (2010). How close are we to nitrogen-fixing cereals? Curr. Opin. Plant Biol. **13,** 556-564.

Charpentier, M., Vaz Martins, T., Granqvist, E., Oldroyd, G., and Morris, R. (2013). The role of DMI1 in establishing Ca^{2+} oscillations in legume symbioses. Plant Signal Behav. **8,** e22894.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. Nature **417,** 962-966.

Foo, E., and Davies, N.W. (2011). Strigolactones promote nodulation in pea. Planta **234,** 1073-1081.

Foo, E., Yoneyama, K., Hugill, C.J., Quittenden, L.J., and Reid, J.B. (2013). Strigolactones and the regulation of pea symbioses in response to nitrate and phosphate deficiency. Mol. Plant **6,** 76-87.

Gomez-Roldan, V., Fermas, S., Brewer, P.B., Puech-Pages, V., Dun, E.A., Pillot, J.P., Letisse, F., Matusova, R., Danoun, S., Portais, J.C., Bouwmeester, H., Becard, G., Beveridge, C.A., Rameau, C., and Rochange, S.F. (2008). Strigolactone inhibition of shoot branching. Nature **455,** 189-194.

Hayward, A., Stirnberg, P., Beveridge, C., and Leyser, O. (2009). Interactions between auxin and strigolactone in shoot branching control. Plant Physiol. **151,** 400-412.

Heidstra, R., Geurts, R., Franssen, H., Spaink, H.P., Van Kammen, A., and Bisseling, T. (1994). Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. Plant Physiol. **105,** 787-797.

Hirsch, S., Kim, J., Munoz, A., Heckmann, A.B., Downie, J.A., and Oldroyd, G.E. (2009). GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in *Medicago truncatula*. Plant Cell **21,** 545-557.

Jennings, D.H. (1995). The physiology of fungal nutrition. (Cambridge: Cambridge University Press).

Journet, E.P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., Arnould, C., Morandi, D., Barker, D.G., and Gianinazzi-Pearson, V. (2001). *Medicago truncatula ENOD11*: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol. Plant Microbe Interact. **14,** 737-748.

Kalo, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R.M., Hirsch, S., Jakab, J., Sims, S., Long, S.R., Rogers, J., Kiss, G.B., Downie, J.A., and Oldroyd, G.E. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science **308,** 1786-1789.

Kapulnik, Y., Resnick, N., Mayzlish-Gati, E., Kaplan, Y., Wininger, S., Hershenhorn, J., and Koltai, H. (2011). Strigolactones interact with ethylene and auxin in regulating root-hair elongation in Arabidopsis. J. Exp. Biol. **62,** 2915-2924.

Kretzschmar, T., Kohlen, W., Sasse, J., Borghi, L., Schlegel, M., Bachelier, J.B., Reinhardt, D., Bours, R., Bouwmeester, H.J., and Martinoia, E. (2012). A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature **483,** 341-344.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C., and Debelle, F. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science **303,** 1361-1364.

Leyser, O. (2011). Auxin, Self-Organisation, and the Colonial Nature of Plants. Curr. Biol. **21,** R331-R337.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T., and Geurts, R. (2004). RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. J. Exp. Bot. **55,** 983-992.

Liu, W., Kohlen, W., Lillo, A., Op den Camp, R., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C., Kaufmann, K., Yang, W.C., Hooiveld, G.J., Charnikhova, T., Bouwmeester, H.J., Bisseling, T., and Geurts, R. (2011). Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. Plant Cell **23,** 3853-3865.

Lopez-Raez, J.A., Charnikhova, T., Gomez-Roldan, V., Matusova, R., Kohlen, W., De Vos, R., Verstappen, F., Puech-Pages, V., Becard, G., Mulder, P., and Bouwmeester, H. (2008). Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol. **178,** 863-874.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E.A., Driguez, H., Becard, G., and Denarie, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469,** 58-63.

Muller, D., and Leyser, O. (2011). Auxin, cytokinin and the control of shoot branching. Ann. Bot. **107,** 1203-1212.

Oldroyd, G.E., and Long, S.R. (2003). Identification and characterization of nodulation signaling pathway 2, a gene of *Medicago truncatula* involved in Nod factor signaling. Plant Physiol. **131,** 1027-1032.

Ongaro, V., and Leyser, O. (2008). Hormonal control of shoot branching. J. Exp. Bot. **59,** 67-74.

Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter, N., Cardoso, C., Lopez-Raez, J.A., Matusova, R., Bours, R., Verstappen, F., and Bouwmeester, H. (2011). Physiological effects of the synthetic strigolactone analog GR24 on root system

architecture in Arabidopsis: another belowground role for strigolactones? Plant Physiol. **155,** 721-734.

Smit, P., Raedts, J., Portyanko, V., Debelle, F., Gough, C., Bisseling, T., and Geurts, R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science **308,** 1789-1791.

Soto, M.J., Fernández-Aparicio, M., Castellanos-Morales, V., García-Garrido, J.M., Ocampo, J.A., Delgado, M.J., and Vierheilig, H. (2010). First indications for the involvement of strigolactones on nodule formation in alfalfa (*Medicago sativa*). Soil Biol. Biochem. **42,** 383-385.

Stirnberg, P., van De Sande, K., and Leyser, H.M. (2002). MAX1 and MAX2 control shoot lateral branching in Arabidopsis. Development **129,** 1131-1141.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Rec. **35,** W71-74.

Untergasser, A., Bijl, G.J.M., Liu, W., Bisseling, T., Schaart, J.G., and Geurts, R. (2012). One-step Agrobacterium mediated transformation of eight genes essential for Rhizobium symbiotic signaling using the novel binary vector system pHUGE. PLoS One **7,** e47885.

Vijn, I., Martinez-Abarca, F., Yang, W.-C., das Neves, L., van Brussel, A., van Kammen, A., and Bisseling, T. (1995). Early nodulin gene expression during Nod factor-induced processes in *Vicia sativa*. Plant J. **8,** 111-119.

Yoneyama, K., Yoneyama, K., Takeuchi, Y., and Sekimoto, H. (2007a). Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. Planta **225,** 1031-1038.

Yoneyama, K., Xie, X., Kusumoto, D., Sekimoto, H., Sugimoto, Y., Takeuchi, Y., and Yoneyama, K. (2007b). Nitrogen deficiency as well as phosphorus deficiency in sorghum promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasites. Planta **227,** 125-132.

Yoneyama, K., Xie, X., Sekimoto, H., Takeuchi, Y., Ogasawara, S., Akiyama, K., Hayashi, H., and Yoneyama, K. (2008). Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. New Phytol. **179,** 484-494.

Yoneyama, K., Xie, X., Kim, H.I., Kisugi, T., Nomura, T., Sekimoto, H., Yokota, T., and Yoneyama, K. (2012). How do nitrogen and phosphorus deficiencies affect strigolactone production and exudation? Planta **235,** 1197-1207.

SUPPLEMENTAL DATA

Supplemental Table 1: The nodule numbers counting in the *MtD27* RNAi transgenic plants.

Transgenic plant	Amount of nodules in control plant	Amount of nodules in *MtD27* RNAi
1	8	5
2	20	8
3	6	10
4	14	5
5	16	7
6	9	4
7	20	5
8	9	2
9	14	35
10	6	4
11	17	1
12	8	6
Average	12.25	7.67

Supplemental Table 2: All primers used in this study are listed.

Purpose	Name	Sequence
qRT-PCR	qMtENOD11-F	ACTTCCTAAACCGCCGCAC
	qMtENOD11-R	TGGCGGTTTCTTCTGTGGT
	qMtD27-F	GAGATGATATTCGGCCAGGAAC
	qMtD27-R	GCATGGTTTTTCTTAGCCTTGC
	MtUBQ10-F	CCCTTCATCTTGTCCTTCGTCTG
	MtUBQ10-R	CACCTCCAATGTAATGGTCTTTCC
Constructs	MtD27i-F	*CACC* GGTCCCTCCGAGGTAAG
	MtD27i-R	GCAGGGTTGCTTGAGTGC
	pMtD27-F	GGGG *ACAACTTTGTATAGAAAAGTTG* AACAACTGTTCCCAGCGC
	pMtD27-R	GGGG *ACTGCTTTTTTGTACAAACTTGG* TTGTGAGTAGTTATTGATTTTCATGTGA

The recombination sites in the sequences are shown in *italic*.

Chapter 5

Dominant active OsCCaMK can induce spontaneous nodule formation in *Medicago truncatula*, but not in rice

Wei Liu, Ton Bisseling and René Geurts

Department of Plant Science, Laboratory of Molecular Biology, Wageningen University, 6708 PB Wageningen, The Netherlands

ABSTRACT

CCaMK is an essential gene in the common symbiosis signalling required for arbuscular endomycorrhizae (AM) and rhizobium root nodule formation in legumes. The encoded protein is highly conserved among land plants that are able to establish an AM symbiosis. In legumes CCaMK forms a central hub in the symbiotic signalling network, and dominant positive alleles (*CCaMK**) can spontaneously trigger root nodule formation. This response is dependent on the occurrence of the symbiotic transcriptional regulators NSP1 and NSP2 that control expression of symbiotic responsive genes; e.g. *DWARF27* (*D27*). Previous study showed that rice *OsCCaMK* can functionally complement the *Medicago truncatula ccamk* knockout mutant, including rhizobium induced root nodule formation. We raised the questions whether the CCaMK-NSP-D27 signalling module is functionally conserved in non-legume plants. Here we show that dominant active allele of *OsCCaMK** induces spontaneous nodule formation in *M. truncatula*, but not in rice. Additionally, we found that dominant active CCaMK* triggers the expression of *D27* in *M. truncatula*, but not in rice. Based on these data we conclude that CCaMK is functionally conserved in higher plants, but the signalling connection between this protein and NSP1-NSP2 to trigger rhizobium symbiosis related responses is non-functional in rice.

INTRODUCTION

To date, a common approach to improve crop yield is using more industrial fertilizer, particularly fixed nitrogen. This implies that growth of global human population will require more nitrogen fertilizer to achieve the expected crop yields (Smil, 1999). Legume crops, e.g. soybean, can bypass this limitation and obtain fixed nitrogen by the interaction with rhizobium bacteria. Through the establishment of an endosymbiosis in specific root nodules, rhizobia can fix atmospheric nitrogen gas into ammonia, which can be utilized by the legume host. Due to this, legume crops accumulate high amounts of proteins in leaves and seeds independent of the availability of nitrogen nutrients in the soil. The advantage of having a nitrogen fixing symbiosis with rhizobium was recognized and used in legume crop production from about a century ago (Bashan, 1998), and made researchers speculate whether a transfer of this intimate interaction to cereal crops would be possible (Khush et al., 1992; Charpentier and Oldroyd, 2010; Untergasser et al., 2012). Here we present a first approach in rice (*Oryza sativa*) where we introduced a dominant active allele of *CCaMK**, a key regulatory gene of root nodule formation, into rice.

Root nodule organogenesis and subsequent intracellular infection are key steps in the rhizobium symbiosis (Oldroyd and Downie, 2008). Plants exude flavonoids and isoflavonoids to attract rhizobia (Peters et al., 1986). When perceived by the rhizobia, the bacteria will activate genes that are responsible for the biosynthesis and secretion of lipo-chitooligosaccharides (LCOs)-type signal molecules, called Nod factors (Peters et al., 1986; Lerouge et al., 1990). Nod factor recognition by the host plant is required for both rhizobial infection and nodule organogenesis (Truchet et al., 1991). The infection starts with the root hair curling by which rhizobium is entrapped in a cavity. Subsequently, the bacteria divide and form a colony whereas a tunnel-like structure is formed that starts in the curl. This infection thread directs the rhizobia to the root cortex (Gage, 2004). In addition to the infection process, cortical cells in the root re-enter the cell cycle and form a nodule primordium (Yang et al., 1994). Once an infection thread reaches a nodule primordium, the bacteria are released in the primordial cells and become surrounded by a plant-derived membrane. These rhizobia are now called bacteroids and together with its plant membrane envelope form a transient organelle, named symbiosome. These symbiosomes differentiate in nitrogen fixation organelles (Brewin, 2004).

Forward genetics studies in the model legumes *Medicago truncatula* and *Lotus japonicus* uncovered a set of genes that is required for rhizobium Nod factor signalling (Catoira et al., 2000). These genes encode 3 plasma membrane receptors (two LysM-type and one LRR-type receptor-like kinases) (Endre et al., 2002; Amor et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006; Smit et al., 2007), two cation channels and two subunits of the nuclear pore complex localized in the perinuclear membrane (Ane et al., 2004; Imaizumi-Anraku et al., 2004; Kanamori et al., 2006; Saito et al., 2007; Charpentier et al., 2008), one nuclear localized Calcium Calmodulin dependent protein Kinase (CCaMK) and several transcriptional activators (Levy et al., 2004; Mitra and Long, 2004; Kalo et al., 2005; Smit et al., 2005; Andriankaja et al., 2007). Analysis of genomes of non-legume plants revealed the widespread occurrence of putative orthologs of these genes; e.g. rice (*Oryza sativa*) contains putative orthologs of all these Nod factor signalling genes (Markmann and Parniske, 2009) (Table 1). Trans-complementation studies have shown that OsCCaMK can rescue nodule formation in the *M. truncatula ccamk*/*dmi3* knockout mutant (Godfroy et al., 2006; Chen et al., 2007). Similar studies have been done for two GRAS-type transcription factors, NSP1 and NSP2, that are thought to function downstream of CCaMK (Catoira et al., 2000; Kalo et al., 2005; Smit et al., 2005). NSP1 and NSP2 are required for expression of several Nod factor early response genes, including *ENOD11*, *D27* and *NIN* (Catoira et al., 2000; Kalo et al., 2005; Marsh et al., 2007). Rice OsNSP1 and OsNSP2 can trans-complement the *L. japonicus Ljnsp1* and *Ljnsp2* knockout mutants, respectively (Yokota et al., 2010). These studies suggest that the evolution of the rhizobium nodule symbiosis did not require changed properties of these common signaling components.

Phenotypic analyses of the symbiotic mutants in legumes and in rice revealed that several of the Nod factor signalling genes are also required for the AM endosymbiosis with obligatory biotrophic fungi of the *Glomeromycota* order (Parniske, 2008). This interaction leads to the formation of arbuscular feeding structures in the inner cortex of the plant root where nutrient exchange takes place. The fungus provides the plant with minerals, especially phosphates that it can access with its extracellular mycelium. It receives carbohydrates in return (Parniske, 2008). The conserved set of genes essential for both symbioses includes the LRR-type plasma membrane receptor kinase SYMRK (MtDMI2 in *M. truncatula*) (Catoira et al., 2000; Endre et al., 2002; Bersoult et al., 2005; Limpens et al., 2005), perinuclear cation channels CASTOR & POLLUX (only a single gene in *M. truncatula* named MtDMI1) (Catoira et al., 2000; Ane et al., 2004; Imaizumi-Anraku et al., 2004; Charpentier et al., 2008)

and a nuclear localized CCaMK (named MtDMI3 in *M. truncatula*) (Catoira et al., 2000; Levy et al., 2004; Mitra et al., 2004). Studies in legumes revealed that these genes act genetically as a linear pathway. As this module is essential for 2 symbioses it is named common symbiotic signalling pathway or common SYM genes (Kistner and Parniske, 2002).

Table 1. List of Nod factor signaling genes and their homologs in rice.

Protein description	*M. truncatula*	*L. japonicus*	Rice (*Oryza sativa*)
LysM domain protein kinase	LYK3	NFR1	Os08g42580
LysM domain protein kinase	NFP	NFR5	Os03g13080
Ion channel protein	DMI1	CASTOR, POLLUX	Os03g62650, Os01g64980
LRR receptor kinase	DMI2	SYMRK	Os07g38070
Ca^{2+}/Calmodulin kinase	DMI3	CCaMK	Os05g41090
Nuclear protein	IPD3	CYCLOPS	Os06g02520
Nuclear pore protein	*n. a.*	NUP85	Os01g54240
Nuclear pore protein	*n. a.*	NUP133	Os03g12450
GRAS-type transcription factor	NSP1	NSP1	Os03g29480
GRAS-type transcription factor	NSP2	NSP2	Os03g15680

Nod factor signaling genes in *M. truncatula* and *L. japonicus* are listed. Meanwhile, their homologs in rice (*Oryza sativa*) are determined by the protein sequence similarity and previous published results. *n. a.* means not available.

Rhizobium Nod factors are perceived by 2 specific LysM-type receptor kinases that, in heterologous systems, can form a heterodimer complex (Madsen et al., 2011). It is hypothesised that such receptor complex activates the common signalling pathway, though the mechanism by which this occurs remains elusive. Recent studies revealed that the endomycorrhizal reference species *Rhizophagus irregularis* (formerly named *Glomus intraradices*) produces also a LCOs-type signalling molecule. These so-called Myc factors are very similar to rhizobium Nod factors, suggesting that they are perceived by a similar type of LysM-receptors (Maillet et al., 2011; Op den Camp et al., 2011). Arbuscular Mycorrhizae are wide spread among land plants and evolved about 450 million years ago. The presence of common symbiotic signalling genes in many plant species supports the theory that rhizobium Nod factor signalling in legumes was recruited from AM symbiosis (Parniske, 2008). In legumes the family of LysM-type receptor kinases markedly expanded when compared to non-legumes (Arrighi et al., 2006). This most likely provided the redundancy to evolve receptors that specifically recognise rhizobium Nod factors, thereby creating specificity in the interaction.

When Nod factor signalling is transmitted from plasma membrane to nuclear envelope, a calcium oscillation, also named Ca^{2+} spiking, is established. CCaMK, a key regulator in rhizobium symbiosis and AM symbiosis, is activated by this Ca^{2+} spiking (ref). In legumes a loss-of-function of CCaMK abolishes most of the rhizobium-induced responses including cortical cell divisions, rhizobium infection and induction of Nod factor responsive genes (Catoira et al., 2000; Levy et al., 2004). The protein consists of an N-terminal kinase domain and an auto-inhibitory domain that contains a Calmodulin (CaM) binding domain and three EF-hands (Levy et al., 2004). CaM and EF-hands are known to bind Ca^{2+}. The kinase domain is inhibited when the EF-hands have not bound Ca^{2+}. Ca^{2+} binding most likely triggers a conformational change, by which the kinase domain is not anymore inhibited by the auto-regulatory domain. Subsequently, auto-phosphorylation at a conserved threonine residue activates the kinase domain (Singh and Parniske, 2012). This is supported by a dominant active allele identified in *Lotus japonicus*, named *Ljsnf1*, which hosts a missense mutation at T265I. This mutation causes activation of the kinase resulting in nodule formation in absence of rhizobium (Tirichine et al., 2006). Likewise, deletion of the complete C-terminal regulatory domain can also induce nodule formation in *M. truncatula* in the absence of rhizobium (Gleason et al., 2006).

Full-length rice (*Oryza sativa*) *OsCCaMK* can restore nodule formation in a *M. truncatula Mtccamk/dmi3* mutant (Godfroy et al., 2006; Chen et al., 2007), indicating that the 2 genes are functionally conserved. As mentioned above, similar studies have been conducted for *OsNSP1* and *OsNSP2* (Yokota et al., 2010), which are thought to act immediately downstream of CCaMK. As the function of these proteins is conserved in monocot and dicot species, despite over 100 million years of independent evolution, strong purifying selection mechanisms seems to act on the underlying genes. The conserved function of CCaMK, together with the occurrence of dominant alleles, provides a good possibility to study the function of this gene in non-legumes. We studied a dominant active form of OsCCaMK and provide evidence that spontaneous nodule formation and activation of the symbiotic response gene *D27* can be induced in *M. truncatula*, but not in rice. This implies that the signalling connection between central regulator *CCaMK* and *D27* is lacking in rice.

RESULTS

A dominant active form of rice OsCCaMK induces spontaneous nodule formation in *Medicago truncatula*

Root nodule formation can be triggered by a dominant active form of CCaMK in *M. truncatula*. CCaMK is functionally conserved in legumes and non-legume species. We raised the question whether a non-legume CCaMK can also trigger spontaneous nodule formation in legumes, and/or in non-legume crops. We chose rice OsCCaMK to answer this question, because rice OsCCaMK can complement a *ccamk* mutant of *M. truncatula*.

We tested whether a dominant active form of rice CCaMK (OsCCaMK*) is sufficient to trigger spontaneous nodule formation in *M. truncatula*. To do so, a C-terminal deletion construct of OsCCaMK (1-303 aa), comparable to MtCCaMK (1-311 aa) in Gleason et al. (2006), was prepared (Figure 1A). This *OsCCaMK** driven by an enhanced CaMV35S promoter was transformed into a *M. truncatula ccamk* mutant through *Agrobacterium rhizogenes* mediated hairy-root transformation. Plants were co-transformed with *DsRed* by which transgenic roots can be distinguished from non-transgenic roots. In transgenic roots (DsRed fluorescence), root nodules were formed with an average of 5.1 ± 1.2 (mean $\pm$s.e.) in the absence of rhizobia. These nodules were only formed on transgenic roots (Figure 1B and 1C). When this mutant was transformed with MtCCaMK (1-311 aa), 6.8 ± 1.0 nodules were formed per root. This shows that CCaMK kinase domain is functionally conserved in monocots and dicot plants, and is sufficient to induce nodule formation in *M. truncatula*.

Cytological analyses showed that the nodules induced by *pCaMV35S::OsCCaMK** were well developed and display a similar tissue layout as rhizobium infected (wild type) nodules (Figure 2C). The surrounding peripheral tissues cover a central tissue, composed of two cell types: big sized cells containing enlarged nuclei, and normally sized cells containing starch granules (Figure 2E). The big cells are histologically similar to infected cells (albeit without rhizobia) as present in root nodules, whereas the small cells resemble uninfected cells in nodules. The big cells have an enlarged nucleus, which suggest that these cells went through rounds of endoreduplication, similar as seen in rhizobium infected nodule cells.

OsCCaMK promoter driven OsCCaMK* can also induce spontaneous nodule formation in *Medicago truncatula*

*pCaMV35S::OsCCaMK** can induce spontaneous nodule formation in *M. truncatula*, indicating that the kinase domain of legume CCaMK, has not evolved a new function to induce root nodule formation. We wondered whether the promoter of rice *OsCCaMK* also has all regulatory sequences to induce root nodule formation in *M. truncatula*. To study this, a rice *OsCCaMK* promoter driven *OsCCaMK** (*pOsCCaMK::OsCCaMK**) construct was

introduced into *M. truncatula ccamk* mutant. On transgenic roots, spontaneous nodules were visible with an average of 7.8 ±1.0 (mean ±s.e.) per root (Figure 2A and 2B).

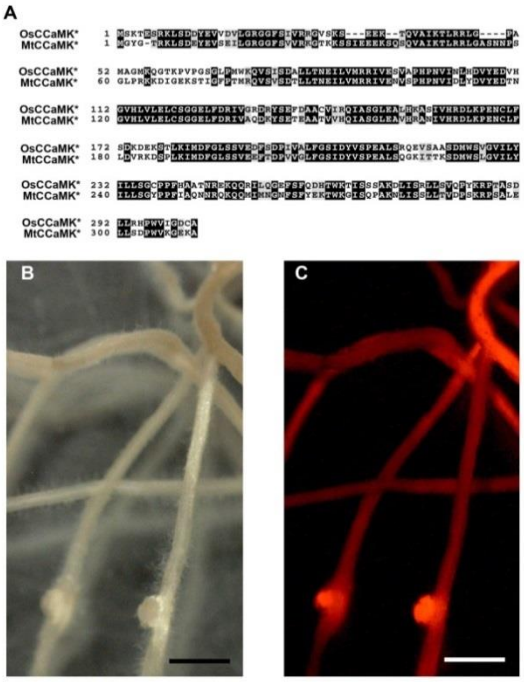


Figure 1. Spontaneous nodule formation induced by expression of dominant active form of OsCCaMK driven by the CaMV35S promoter. Protein sequence alignment of MtCCaMK and OsCCaMK kinase domain shows that they share 67% identity and 79% homology (A). The spontaneous nodules induced by *pCaMV35S::OsCCaMK** are detected with a stereomicroscope under white light field (B) and under UV light combined with a red filter to identify transformed roots (C). The scale bar is equal to 2 mm in B and C.

Cytological analyses showed that these nodules were less developed when compared to nodules triggered by *OsCCaMK** under control of the CaMV35S promoter. *pOsCCaMK::OsCCaMK** induced nodules contain only normal sized cells in the central zone with small nuclei. So enlarged cells as detected in *pCaMV35S::OsCCaMK** nodules or wild type nodule are lacking (Figure 2D). Starch granules are present in the central located cells (Figure 2F). This indicates that regulation of *CCaMK* expression directly affects the developmental program of root nodules, including endoreduplication of cells in the nodule central zone.

OsCCaMK* is not sufficient to induce spontaneous nodule formation in rice

As *OsCCaMK** can induce spontaneous nodule formation in *M. truncatula*, we studied whether *OsCCaMK** is able to induce spontaneous nodule formation in rice. To prove this, transgenic rice lines with *OsCCaMK** driven by either a CaMV35S promoter or the *OsCCaMK* native promoter were generated through *Agrobacterium tumefaciens* mediated stable transformation. Four independent transgenic lines were obtained for each construct. In T_1 generation seedlings, transgenic *OsCCaMK** expression demonstrated in roots with real time qRT-PCR. Transgenic rice plants were grown in fine sand and watered with Hoagland's solution with a low concentration of fixed nitrogen. The root systems are checked in four transgenic lines, including fifteen plants of each line, three weeks after germination. However, no nodules were detected on the roots. Longitudinal section of the roots did not reveal any cortical cell division induced by *OsCCaMK**.

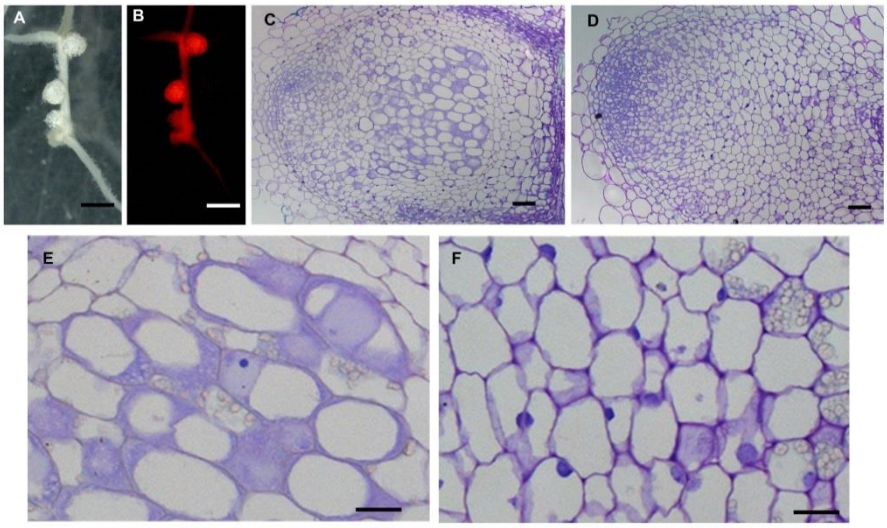


Figure 2. Spontaneous nodules induced by OsCCaMK* in *M. truncatula*. The spontaneous nodule induced by *pOsCCaMK::OsCCaMK** is identified with a stereomicroscope under white light field (A) and under UV light combined with a red filter to identify transgenic roots (B). Longitudinal section of nodule induced by *pCaMV35S::OsCCaMK** shows enlarged cells containing a big nucleus (C and E). In contrast, in nodules induced by *pOsCCaMK::OsCCaMK** these enlarged cells are not present in the central tissue (D and F). The scale bar is equal to 2 mm in A and B; 50 μm in C and D, and 20 μm in E and F.

DWARF27 is activated in CCaMK* transgenic roots in *Medicago truncatula*, but not in rice

M. truncatula expressing *MtCCaMK** can induce the Nod factor response gene *MtENOD11* in the absence of rhizobium (Gleason et al., 2006). This expression is MtNSP1

and MtNSP2 dependent. Unfortunately an *ENOD11* ortholog of rice is not known. To study whether *OsCCaMK** can activate a Nod factor response gene that occurs in *M. truncatula* as well as rice, we searched for an alternative response gene. Similar to *MtENOD11*, *MtD27* is also activated through Nod factor signalling and requires MtCCaMK, MtNSP1 and MtNSP2. We studied in *M. truncatula* whether *MtCCaMK** can induce *MtD27* expression. The expression level of *MtD27* was determined with real time qRT-PCR in *M. truncatula MtCCaMK** transgenic roots and control roots. This shows that *MtD27* has a >9-fold up-regulation in *MtCCaMK** roots (Figure 3A). So, similar to the induction of *MtENOD11*, MtCCaMK* can activate *MtD27* expression in absence of rhizobium. Since rice has a *D27* ortholog this gene can be used to monitor the activation by Nod factor signalling in both species.

We studied whether *OsD27* is activated in *OsCCaMK** transgenic rice. To study this, *OsD27* expression level was determined in roots of *pOsCCaMK::OsCCaMK**, *pCaMV35S::OsCCaMK** transgenic rice lines and wild type. Two independent transgenic lines (for each construct) were studied and in all cases *OsD27* expression was not affected by *OsCCaMK** (Figure 3B). This shows that *OsCCaMK** is not sufficient to elevate *OsD27* expression in rice.

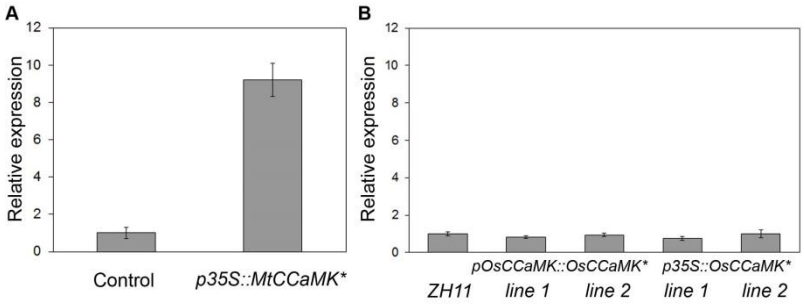


Figure 3. *DWARF27* expression is studied in CCaMK* transformed *M. truncatula* and rice, respectively. *MtD27* expression, studied by real time qRT-PCR, is induced by MtCCaMK***** in *M. truncatula* roots (A). However, *OsD27* is not activated by OsCCaMK* in rice (B). Neither the construct driven by native OsCCaMK promoter nor byCaMV35S promoter results an increased *OsD27* expression in two independent transgenic lines. Data are means ±SD.

DISCUSSION

CCaMK is a highly conserved protein and indispensable for endomycorrhizal symbiosis in higher plants (Catoira et al., 2000; Weidmann et al., 2004; Chen et al., 2007). Additionally,

CCaMK is co-opted by rhizobium to induce root nodule formation in legumes (Levy et al., 2004; Mitra et al., 2004). This evolutionary event most likely did not require specific adaptation of the protein sequence; as OsCCaMK of rice can rescue root nodule formation in a *M. truncatula ccamk* knockout mutant and a dominant active form induces nodule formation (Godfroy et al., 2006; Chen et al., 2007). We asked the question to what extend the downstream pathway of CCaMK is conserved in legumes and non-legumes and tested whether an artificially constructed dominant active *OsCCaMK** allele lacking the auto-inhibitory domain can trigger rhizobium associated symbiotic responses in rice. This showed not to be the case. Hence, we conclude that in rice the signalling connection of OsCCaMK is insufficient to trigger nodule formation.

We tested the functionality of the *OsCCaMK** in *M. truncatula*. To do so, we used two constructs that differ in promoter to drive *OsCCaMK** expression; namely the constitutively active CaMV35S and the native rice OsCCaMK promoter. In both cases spontaneous nodules can be triggered, suggesting that essential *cis*-regulatory elements required for root nodule development were already present in an ancestral *CCaMK* gene that predates the monocot-dicot split, which occurred ~150 million years ago (Moore et al., 2007; Smith et al., 2010). Nevertheless, slight differences in nodule phenotype could be observed between *pOsCCaMK::OsCCaMK** and *pCaMV35S::OsCCaMK** induced nodules in *M. truncatula*. We noted that nuclei of cells in the central tissue of *pOsCCaMK::OsCCaMK** induced nodules remain relatively small compared to *pCaMV35S::OsCCaMK** induced nodules. Similar enlarged cells with enlarged nuclei occur in MtCCaMK* (Gleason et al., 2006). Enlarged nuclei hint to endoreduplication, a process that is common for infected cells that host rhizobia (Kondorosi et al., 2000). Reduced endoreduplication, e.g. by knocking down the expression of MtCCS52A, a key regulator in this process, hampers nodule differentiation in *M. truncatula*, indicating the importance of this process in nodule development (Vinardell et al., 2003). Our data indicate that endoreduplication is under control of rhizobium Nod factor signalling and induced by CCaMK. As CCaMK expression driven by the native rice promoter is insufficient to trigger endoreduplication, it suggests that the rice promoter lacks some *cis*-regulatory elements.

As in rice lines expressing *OsCCaMK** nodule-like structures are not formed, we raised the question whether Nod factor responsive genes could be activated in rice. In legumes early nodulin genes, like *MtENOD11* in *M. truncatula*, are used as symbiotic markers. *MtENOD11* expression is triggered within a few hours in the root epidermis, and remains active in the

newly induced nodule primordium (Journet et al., 2001). However, *MtENOD11*-like genes are unique for legumes. Recently, we identified *MtDWARF27* (*MtD27*) as Nod factor responsive gene in *M. truncatula*, of which the symbiotic expression pattern largely overlaps with *MtENOD11* (Chapter 4 in this thesis). *MtD27* encodes an enzyme in the strigolactone biosynthesis pathway and is conserved in most plant species; including rice (Lin et al., 2009). *D27* expression in roots is dependent on the GRAS-type transcriptional regulators NSP1 and NSP2 in *M. truncatula* as well as rice (Liu et al., 2011). Symbiotic *MtD27* expression is dependent on a functional Nod factor signalling cascade including MtNSP1 and MtNSP2 that act downstream of MtCCaMK (Chapter 4 in this thesis). Here we used *D27* as symbiotic response marker and show that in *M. truncatula MtD27* expression is elevated in roots expressing MtCCaMK*. However, in transgenic rice lines containing *OsCCaMK**, *OsD27* expression is not affected. Rice contains putative orthologs of all Nod factor signalling genes, including OsNSP1 and OsNSP2, indicating that additional factors are lacking to achieve a signalling connection between OsCCaMK activity and *OsD27* expression. Alternatively, *OsD27* lacks *cis*-regulatory elements that are specific for rhizobium Nod factor induced expression.

With the current knowledge of nodule formation in legumes, a transfer of this symbiosis to non-legumes is no longer an untouchable question (Charpentier and Oldroyd, 2010). However, a previous study showed that transgenic non-legume plants hosting a cascade of eight genes essential for Nod factor signalling are unable to induce root nodule formation (Untergasser et al., 2012). Together with our results, this clearly demonstrates that a transfer of symbiosis is not straightforward. Our data suggests that the failure to trigger symbiotic responses in Untergasser et al. (2012) could be explained by the absence of a signalling connection between CCaMK and two important transcription factors NSP1 and NSP2 to their downstream targets. Uncovering the genes responsible to this connection in legumes is indispensable to achieve nitrogen fixation in non-legume crops.

MATERIALS AND METHODS

Plant materials and growth conditions

Medicago truncatula Jemalong A17 and *ccamk* (*dmi3-1*/TRV25) were used in this study. Plants were grown in a growth chamber at 20℃ with 16h/8h day/night regime. For seed germination, the seeds were pre-treated in 98% sulphuric acid for 7 minutes, and washed for 6 times with demi-water. Afterwards, the seeds were sterilized with commercial bleach for 7

minutes and washed with sterile demi-water for 6 times. The sterilized seeds were transferred to Fåhraeus medium plates (1.5% agar) and left in cold room for 24 hours to synchronize germination. Then, the seeds plates were transferred into the growth chamber and left upside down for one day to initiate the germination. One-day-old seedlings were transferred to Fåhraeus medium for initiating gene expression studies. Seven-day-old seedlings are used for hairy-root transformation.

Rice (*Oryza sativa* ssp *japonica cv* Zhonghua11) was used in this study as wild type. Plants were grown in a greenhouse at 28 ℃ with 16-hour-day/8-hour-night regime. For seed germination, the seeds were sterilized with commercial bleach for 30 minutes and washed with sterile demi-water for 6 times. Then, seeds were transferred to CHU (N6) medium plates and grown vertically in 28 ℃ growth chamber for sampling of real time qRT-PCR analysis. For root phenotype experiments, 5-day-old seedlings are transferred into pots containing fine sand and watered with half-strength Hoagland's solution (Hoagland and Arnon, 1950).

Constructs and plant transformations

For spontaneous nodule formation assay, a rice OsCCaMK* construct (1-303 aa) correlated to MtCCaMK* (1-311 aa) was made by amplified a 909 bp fragment from rice root cDNA sample with primer pair cOsCCaMK-F and trOsCCaMK-R and digested with *Sma* I and *Kpn* I. Then, this fragment was sub-cloned into pENTR-R2L3-ep35S-MCS-t35s in between an enhanced CaMV35S promoter and CaMV35S terminator after digestion. Correct sub-clone was recombined into pHGW-RR-MGW by using LR Clonase II plus (Invitrogen) and got *pCaMV35S::OsCCaMK**. For *pOsCCaMK::OsCCaMK* construct, 1007 bp fragment representing OsCCaMK promoter was amplified from rice genomic DNA and subcloned into pENTR-R2L3-MCS-t35s to get pENTR-R2L3-pOsCCaMK-MCS-t35s. And the following cloning process was same to *pCaMV35S::OsCCaMK* construct. Primer sequences are listed in Supplemental Table 1.

To transform these constructs into *M. truncatula*, *Agrobacterium rhizogenes* strain MSU440 was used in hairy-root transformation. Transformation process follows the previous described method (Limpens et al., 2004). Two weeks after transformation the compound plants were transferred into perlite and watered with Fåhraeus liquid medium without nitrogen supply. The plant morphology and spontaneous nodule formation were checked and photographed at three weeks after the transfer. Transgenic roots were visualized by the red fluorescence under Leica stereomicroscope corresponding to the DsRED marker. The

photographs were taken under white light condition without filter or under UV light condition combined with DsRed filter using Leica microscope software following the manual.

Rice transformations were conducted using *Agrobacterium tumefaciens* strain AGL1 according to Toki et al. (2006) (Toki et al., 2006) and hygromycin B was used for transgenic plant selection. The phenotype was analysed in T_1 generation seedlings.

Real time qRT-PCR analysis

The root samples were frozen immediately in liquid nitrogen after sampling. Total RNA was isolated from these samples with RNeasy plant kit (QIAGEN) following the supplier's manual. First strand cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) from 1 µg total RNA. Real time qRT-PCR was set up in 20 µl reaction system with 2× iQ SYBR Green Super-mix (Bio-Rad) and proceeded with iQ5 real time PCR detecting system according to the manufacturer's manuals. All primers used in this study were designed with Primer3Plus software (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) under qPCR settings (Untergasser et al., 2007). For data normalization, in *M. truncatula* ubiquitin gene was used as an internal control, whereas OsUBQ is used in rice. All primers used in this assay are listed in Supplemental Table 1.

REFERENCES

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

Andriankaja, A., Boisson-Dernier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D.G., and de Carvalho-Niebel, F. (2007). AP2-ERF transcription factors mediate Nod factor dependent *MtENOD11* activation in root hairs via a novel *cis*-regulatory motif. Plant Cell **19,** 2866-2885.

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarie, J., and Cook, D.R. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science **303,** 1364-1367.

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

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol. Adv. **16,** 729-770.

Bersoult, A., Camut, S., Perhald, A., Kereszt, A., Kiss, G.B., and Cullimore, J.V. (2005). Expression of the *Medicago truncatula* DM12 gene suggests roles of the symbiotic nodulation receptor kinase in nodules and during early nodule development. Mol. Plant Microbe Interact. **18,** 869-876.

Brewin, N.J. (2004). Plant cell wall remodelling in the Rhizobium-legume symbiosis. Crit. Rev. Plant Sci. **23,** 293-316.

Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Denarie, J. (2000). Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. Plant Cell **12,** 1647-1666.

Charpentier, M., and Oldroyd, G. (2010). How close are we to nitrogen-fixing cereals? Curr. Opin. Plant Biol. **13,** 556-564.

Charpentier, M., Bredemeier, R., Wanner, G., Takeda, N., Schleiff, E., and Parniske, M. (2008). Lotus japonicus CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. Plant Cell **20,** 3467-3479.

Chen, C., Gao, M., Liu, J., and Zhu, H. (2007). Fungal symbiosis in rice requires an ortholog of a legume common symbiosis gene encoding a Ca^{2+}/calmodulin-dependent protein kinase. Plant Physiol. **145,** 1619-1628.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. Nature **417,** 962-966.

Gage, D.J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. **68,** 280-300.

Gleason, C., Chaudhuri, S., Yang, T., Munoz, A., Poovaiah, B.W., and Oldroyd, G.E. (2006). Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition. Nature **441,** 1149-1152.

Godfroy, O., Debelle, F., Timmers, T., and Rosenberg, C. (2006). A rice calcium and calmodulin dependent protein kinase restores nodulation to a legume mutant. Mol. Plant Microbe Interact. **19,** 495-501.

Hoagland, D.R., and Arnon, D.I. (1950). The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular. No. 347 (Berkeley, CA: University of California), pp. 1-32.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., and Vickers, K. (2004). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature **433,** 527-531.

Journet, E.P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., Arnould, C., Morandi, D., Barker, D.G., and Gianinazzi-Pearson, V. (2001). *Medicago truncatula*

ENOD11: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol. Plant Microbe Interact. **14,** 737-748.

Kalo, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R.M., Hirsch, S., Jakab, J., Sims, S., Long, S.R., Rogers, J., Kiss, G.B., Downie, J.A., and Oldroyd, G.E. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science **308,** 1786-1789.

Kanamori, N., Madsen, L.H., Radutoiu, S., Frantescu, M., Quistgaard, E.M.H., Miwa, H., Downie, J.A., James, E.K., Felle, H.H., Haaning, L.L., Jensen, T.H., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. (2006). A nucleoporin is required for induction of Ca^{2+} spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proc. Natl. Acad. Sci. USA **103,** 359-364.

Khush, G.S., Bennett, J., and Institute, I.R.R. (1992). Nodulation and nitrogen fixation in rice: potential and prospects. (Manila, Philippine: International Rice Research Institute), pp. 1-136.

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

Kondorosi, E., Roudier, F., and Gendreau, E. (2000). Plant cell-size control: growing by ploidy? Curr. Opin. Plant Biol. **3,** 488-492.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C., and Denarie, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344,** 781-784.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C., and Debelle, F. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science **303,** 1361-1364.

Limpens, E., Mirabella, R., Fedorova, E., Franken, C., Franssen, H., Bisseling, T., and Geurts, R. (2005). Formation of organelle-like N2-fixing symbiosomes in legume root nodules is controlled by DMI2. Proc. Natl. Acad. Sci. USA **102,** 10375-10380.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T., and Geurts, R. (2004). RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. J. Exp. Bot. **55,** 983-992.

Lin, H., Wang, R., Qian, Q., Yan, M., Meng, X., Fu, Z., Yan, C., Jiang, B., Su, Z., Li, J., and Wang, Y. (2009). DWARF27, an iron-containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. Plant Cell **21,** 1512-1525.

Liu, W., Kohlen, W., Lillo, A., Op den Camp, R., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C., Kaufmann, K., Yang, W.C., Hooiveld, G.J., Charnikhova, T., Bouwmeester, H.J., Bisseling, T., and Geurts, R. (2011). Strigolactone biosynthesis in *Medicago truncatula*

and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. Plant Cell **23,** 3853-3865.

Madsen, E.B., Antolin-Llovera, M., Grossmann, C., Ye, J., Vieweg, S., Broghammer, A., Krusell, L., Radutoiu, S., Jensen, O.N., Stougaard, J., and Parniske, M. (2011). Autophosphorylation is essential for the in vivo function of the *Lotus japonicus* Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J. **65,** 404-417.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E.A., Driguez, H., Becard, G., and Denarie, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469,** 58-63.

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

Marsh, J.F., Rakocevic, A., Mitra, R.M., Brocard, L., Sun, J., Eschstruth, A., Long, S.R., Schultze, M., Ratet, P., and Oldroyd, G.E. (2007). *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. Plant Physiol. **144,** 324-335.

Mitra, R.M., and Long, S.R. (2004). Plant and bacterial symbiotic mutants define three transcriptionally distinct stages in the development of the *Medicago truncatula*/*Sinorhizobium meliloti* symbiosis. Plant Physiol. **134,** 595-604.

Mitra, R.M., Gleason, C.A., Edwards, A., Hadfield, J., Downie, J.A., Oldroyd, G.E., and Long, S.R. (2004). A $Ca^{2+/}$calmodulin-dependent protein kinase required for symbiotic nodule development: gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. USA **101,** 4701-4705.

Moore, M.J., Bell, C.D., Soltis, P.S., and Soltis, D.E. (2007). Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc. Natl. Acad. Sci. USA **104,** 19363-19368.

Oldroyd, G.E.D., and Downie, J.A. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu. Rev. Plant Biol. **59,** 519-546.

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

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

Peters, N.K., Frost, J.W., and Long, S.R. (1986). A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science **233,** 977-980.

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

Saito, K., Yoshikawa, M., Yano, K., Miwa, H., Uchida, H., Asamizu, E., Sato, S., Tabata, S., Imaizumi-Anraku, H., and Umehara, Y. (2007). NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. Plant Cell **19,** 610-624.

Singh, S., and Parniske, M. (2012). Activation of calcium- and calmodulin-dependent protein kinase (CCaMK), the central regulator of plant root endosymbiosis. Curr.Opin. Plant Biol. **15,** 444-453.

Smil, V. (1999). Nitrogen in crop production: an account of global flows. Global Biogeochem. Cy. **13,** 647-662.

Smit, P., Raedts, J., Portyanko, V., Debelle, F., Gough, C., Bisseling, T., and Geurts, R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science **308,** 1789-1791.

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

Smith, S.A., Beaulieu, J.M., and Donoghue, M.J. (2010). An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants. Proc. Natl. Acad. Sci. USA **107,** 5897-5902.

Tirichine, L., Imaizumi-Anraku, H., Yoshida, S., Murakami, Y., Madsen, L.H., Miwa, H., Nakagawa, T., Sandal, N., Albrektsen, A.S., Kawaguchi, M., Downie, A., Sato, S., Tabata, S., Kouchi, H., Parniske, M., Kawasaki, S., and Stougaard, J. (2006). Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. Nature **441,** 1153-1156.

Toki, S., Hara, N., Ono, K., Onodera, H., Tagiri, A., Oka, S., and Tanaka, H. (2006). Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. Plant J. **47,** 969-976.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J.-C., and Denarie, J. (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351,** 670-673.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J.A. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. **35,** W71-74.

Untergasser, A., Bijl, G.J.M., Liu, W., Bisseling, T., Schaart, J.G., and Geurts, R. (2012). One-step agrobacterium mediated transformation of eight genes essential for rhizobium symbiotic signaling using the novel binary vector system pHUGE. PLoS One **7,** e47885.

Vinardell, J.M., Fedorova, E., Cebolla, A., Kevei, Z., Horvath, G., Kelemen, Z., Tarayre, S., Roudier, F., Mergaert, P., Kondorosi, A., and Kondorosi, E. (2003). Endoreduplication

mediated by the anaphase-promoting complex activator CCS52A is required for symbiotic cell differentiation in *Medicago truncatula* nodules. Plant Cell **15,** 2093-2105.

Weidmann, S., Sanchez, L., Descombin, J., Chatagnier, O., Gianinazzi, S., and Gianinazzi-Pearson, V. (2004). Fungal elicitation of signal transduction-related plant genes precedes mycorrhiza establishment and requires the DMI3 gene in *Medicago truncatula*. Mol. Plant Microbe Interact. **17,** 1385-1393.

Yang, W.-C., de Blank, C., Meskiene, I., Hirt, H., Bakker, J., van Kammen, A., Franssen, H., and Bisseling, T. (1994). Rhizobium Nod factors reactivate the cell cycle during infection and nodule primordium formation, but the cycle is only completed in primordium formation. Plant Cell **6,** 1415-1426.

Yokota, K., Soyano, T., Kouchi, H., and Hayashi, M. (2010). Function of GRAS proteins in root nodule symbiosis is retained in homologs of a non-legume, rice. Plant Cell Physiol. **51,** 1436-1442.

SUPPLEMENTAL DATA

Supplemental Table 1. All primers used in this study are listed.

Purpose	Primer name	Primer sequence	Restrict enzyme
qRT-PCR	qMtD27-F	GAGATGATATTCGGCCAGGAAC	
	qMtD27-R	GCATGGTTTTTCTTAGCCTTGC	
	MtUBQ10-F	CCCTTCATCTTGTCCTTCGTCTG	
	MtUBQ10-R	CACCTCCAATGTAATGGTCTTTCC	
	qOsUbq-F	GCCCAAGAAGAAGATCAAGAAC	
	qOsUbq-R	AGATAACAACGGAAGCATAAAAGTC	
	qOsD27-F	TCTGGGCTAAAGAATGAAAAGGA	
	qOsD27-R	AGAGCTTGGGTCACAATCTCG	
Constructs	cOsCCaMK-F	GGG *CCCGGG* ATGTCCAAGACTGAGAGCAGAA	*SmaI*
	trOsCCaMK-R	GGG *GGTACC* GGCGCAGTCTCCAATCAC	*KpnI*
	pOsCCaMK-F	GG *GGCGCGCC* GCTTTGGAGATTCGGCATAGT	*AscI*
	pOsCCaMK-R	GGG *CCCGGG* TATGGACTTCGACTGCCTGCT	*SmaI*

The restriction enzyme sites in the sequences are shown in *italic*.

Concluding remarks

Legume nitrogen fixation

Most legume species can form root nodules upon interaction with rhizobia, a paraphyletic group proteobacteria that can reduce molecular nitrogen into ammonia (Sawada et al., 2003; Sprent, 2007). Rhizobia have in common that they combine the nitrogen fixing capacity with a set of nodulation (*nod, nol* & *noe*) genes that provide the possibility to live in symbiosis with legume plants (Downie, 1998). Core *nod* genes encode enzymes for biosynthesis of lipo-chitooligosaccharides (LCOs) signal molecules, named Nod factors, that in structure strongly resemble LCOs of arbuscular endomycorrhizal (AM) fungi (Lerouge et al., 1990; Maillet et al., 2011). AM fungi are obligate biotrophs that can establish a very successful endosymbiotic interaction with plant roots, as it is ancient (450 million years), but maintained by the vast majority of plant species (Remy et al., 1994; Parniske, 2008). In legumes LCO producing rhizobia can activate a signalling cascade that includes the mycorrhizal symbiosis signalling pathway (Delaux et al., 2013). This leads to the formation of root nodules, which became infected by rhizobia (Geurts et al., 2012). Inside the nodule, rhizobia are accommodated intracellular as transient organelles that meet optimal conditions to covert molecular dinitrogen into ammonia (Roth and Stacey, 1989).

Although rhizobium nodule symbiosis is primary occurring in the legume family (Fabaceae; ~20,000 species), symbiosis signalling genes are conserved in most non-legume species, including monocots, which diverged from legumes ~150 million years ago (Moore et al., 2007; Delaux et al., 2013). Therefore, understanding the molecular and cellular functioning of these symbiosis signalling genes is extremely helpful in underpinning the evolutionary trajectory that has led to the symbiotic capacities of legumes. Also, this knowledge will be instrumental in a future transfer of the rhizobium nitrogen fixing symbiosis to non-legume crop species. In this thesis, I focused mainly on comparative and functional analysis of two GRAS-type transcriptional regulators; NSP1 and NSP2. I studied the functioning of these genes under non-symbiotic condition in the legume model *Medicago truncatula* and the monocot species rice (*Oryza sativa*).

Knockout mutations in either NSP1 or NSP2 in *M. truncatula*, *Lotus japonicus* or pea (*Pisum sativum*) completely block Rhizobium LCO induced root nodule formation (Catoira et al., 2000; Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006). The last common

ancestor of these species lived about 50 million years ago, a period shortly after emergence of the legume Papilionoid subfamily (Lavin et al., 2005). This indicates that in this subfamily both genes are essential for rhizobium root nodule formation. Interestingly, *L. japonicus* and *M. truncatula nsp1* and *nsp2* knockout mutants can be functionally trans-complemented with gene orthologs of rice (Yokota et al., 2010). As rice is unable to establish a nodular symbiosis with rhizobium, it suggests that both genes commit another function in this species. In my thesis I focussed on this function and showed that NSP1 and NSP2 control the expression of the carotenoid isomerase DWARF27 (D27), an enzyme that is essential for strigolactone biosynthesis (Chapter 3 & 4). Additionally I aimed to engineer the NSP1-NSP2 expression module in rice in such that symbiotic responses can be triggered by ectopic expression of a dominant active form of an upstream acting Calcium Calmodulin Kinase (CCaMK). This showed to be an unsuccessful strategy (Chapter 5). In this last Concluding Chapter, I will discuss the evolutionary trajectory of symbiosis signalling genes to position results presented in this thesis in a broader perspective.

Evolution of symbiosis signalling genes

To get insight in the molecular mechanisms that control rhizobium Nod factor induced root nodule formation extensive molecular genetic studies have been conducted on *L. japonicus* and *M. truncatula*. These studies uncovered basically the same set of genes that form a small genetic network (Figure 1). These core symbiotic genes are essential for Nod factor perception, subsequent signalling to the nucleus and modulation of transcriptional activity. Some of these genes have close paralogs suggesting that they experienced a recent duplication event. Gene duplication generally provides a new degree of freedom to evolve *de novo* functions, and it can be envisioned that this was an important mechanism in the evolutionary trajectory towards rhizobium symbiosis. A mechanism of gene duplication acted on the genes that gave rise to the LysM-type Nod factor receptors as well as some key symbiotic transcription factors; e.g. ERN1 of the ERF family (Young et al., 2011; Cerri et al., 2012). Other symbiotic genes remained single copy, thereby restricting evolutionary freedom to gain novel functions.

Nod factors are perceived by 2 LysM-type receptor kinases that are distinct in sequence, but physically can interact to form a heterodimeric complex (Madsen et al., 2011; Broghammer et al., 2012). One of these receptor kinases, named LjNFR1 in *L. japonicus* and MtLYK3 in *M. truncatula,* is part of a larger gene family that is the result of at least two

rounds of ancestral legume duplications followed by lineage specific duplication events (Lavin et al., 2005; Streng et al., 2011; De Mita et al., unpublished data). The latter may have resulted in some gene redundancy and further functionalization. For example, *M. truncatula* has 2 close homologs of *MtLYK3*, whereas *L. japonicus* has only one gene (LjNFR1) in this orthology group. This may explain the difference in phenotype in between *Ljnfr1* and *Mtlyk3* knockout mutations. Whereas in the *L. japonicus* mutant Nod factor induced responses are completely abolished, the *Mtlyk3* knockout mutant still responds and is specifically blocked in infection stage (Smit et al., 2007). As LjNFR1/MtLYK3 is a close homolog of the Chitin Elicitor Receptor kinase (CERK) of *Arabidopsis thaliana*, it is tempting to speculate that triggering innate immunity is the ancestral function of this receptor. Functional comparison of the kinase domains of LjNFR1 and AtCERK1 pinpointed 2 domains, the so-called YAQ motif and the kinase activation loop (AL), that are determinants in response specificity (Nakagawa et al., 2011). However, the variation in YAQ and AL motifs as found in AtCERK1 are not present in other non-legume CERK1 homologs (De Mita et al., unpublished data). Therefore, these motifs are more likely the result of specific evolution in the *Brassicaceae* or *Arabidopsis* lineage, rather than representing an ancestral stage of the protein. As rhizobium Nod factors are highly similar to Myc factor LCOs of endomycorrhizal fungi, I argue that it is more probable that non-legume *NFR1/LYK3*-like genes function in Myc factor recognition. Upon the duplication events early in legume evolution, the NFR1/LYK3 lineage could gain specificity for rhizobium Nod factors and subsequently co-evolve with the Nod factor structural features produced by its host rhizobia.

A similar evolutionary trajectory occurred for the second LysM type receptor kinase; LjNFR5 and MtNFP. Phylogenetic reconstruction revealed that putative orthologous genes of *MtNFP* are present in many non-legume species (Zhu et al., 2006; Streng et al., 2011), with the exception of, for example, species of the *Arabidopsis* genus. Reversed genetic studies in *Parasponia*, the only non-legume able to establish a nitrogen fixing symbiosis with rhizobium, revealed that NFP fulfills a function in two symbiosis; rhizobium as well as endomycorrhizae (Op den Camp et al., 2011). In legumes, the *LjNFR5-MtNFP* gene is duplicated, which is likely the result of a whole genome duplication (WGD) that occurred early in the Papilionoid family, but after the evolution of the rhizobial symbiosis (Cannon et al., 2010; Young et al., 2011). Transcriptome profiling studies in *M. truncatula* revealed that the *MtNFP* paralogous gene *MtLYR1* is induced specifically during mycorrhization, whereas *MtNFP* is essential for Myc factor induced gene expression (Gomez et al., 2009; Young et al., 2011; Czaja et al.,

2012). Taken together, these results suggest that the gene ancestral to *MtNFP* acted as a Myc factor receptor that was co-opted for Nod factor perception when the rhizobial symbiosis evolved in Fabaceae. When this ancestral gene was duplicated upon whole-genome duplication, the two functions were separated by sub-functionalization; *MtLYR1* remained as a Myc factor receptor gene and *MtNFP* became a Nod factor receptor gene.

Besides Nod factor LysM-type receptors, also a symbiotic ethylene response factor type transcription factor, *ERN1*, is affected by the Papilionoid-specific WGD (Young et al., 2011). In *M. truncatula* this has led to 3 paralogous copies, *MtERN1*, *MtERN2* and *MtERN3*, that are able to bind the Nod factor responsive *cis*-regulatory element (so-called NF-box) and subsequently activate gene expression; e.g. *MtENOD11*. (Andriankaja et al., 2007; Middleton et al., 2007). This suggests that ERN-type transcription factors are part of the Nod factor signal transduction pathway activating symbiotic gene expression. *Mtern1* knockout mutants are blocked in rhizobium infection and nodule formation (Middleton et al., 2007). However, as *Mtern1* knockout mutants are not completely blocked in symbiotic responses, it suggests functional redundancy with *MtERN2*. This was proven in trans-complementation studies in where the *M. truncatula Mtern1* mutant could be complemented with *MtERN2* driven by the *MtERN1* promoter (Cerri et al., 2012).

Interestingly, the remaining symbiotic signalling genes, encoding key proteins in the connective network between the LysM-type Nod factor receptors and symbiosis induced gene expression, are single copy in *M. truncatula* and *L. japonicus*, suggesting that the paralogous gene copies have been lost after the ancestral WGD event. These symbiosis signalling genes encode a plasma membrane localized LRR-type receptor kinase (LjSYMRK, MtDMI2), perinuclear localized potassium channel subunits CASTOR and POLLUX (MtDMI1), and a nuclear localized Calcium Calmodulin Kinase (CCaMK) and its interacting protein (LjCYCLOPS, MtIPD3), and 2 GRAS type transcriptional regulators NSP1 and NSP2. All these genes have been identified in genetic screens for rhizobium symbiosis mutants, but additional phenotyping revealed that most of these mutants are affected in endomycorrhizal symbiosis as well (Parniske, 2008; Maillet et al., 2011). As these symbiosis genes remained single copy, four possibly evolutionary scenarios may have enable them to gain a symbiotic function: (1) regulatory changes; (2) non-synonymous mutations in the coding region; (3) a combination of scenario 1 and 2; or (4) no adaptive changes. To date, these evolutionary trajectories were not investigated systematically for all the symbiotic signalling genes, though some studies on individual genes provide insights in the evolutionary trajectories.

Knockout mutations in *symrk* restrict root infection by rhizobium or AM fungi, and block rhizobium induced root nodule organogenesis (Catoira et al., 2000; Endre et al., 2002; Stracke et al., 2002). This suggests a critical role for SYMRK in the root epidermis, where initial bacterial entry occurs, and a secondary signalling pathway is activated to initiate root nodule organogenesis. Detailed studies on the *M. truncatula symrk* (*dmi2*) knockout mutant suggests a role in stabilizing the actin cytoskeleton in tip growing root hairs, a phenotype that is also apparent under non-symbiotic conditions (Esseling et al., 2004). However, the precise functioning of SYMRK remains elusive. The extracellular domain of symbiotic receptor kinase SYMRK consists of 3 domains; an N-terminal extracellular region (NEC), a highly conserved GDPC motif and a Leucine Rich Repeat (LRR) region (Endre et al., 2002; Stracke et al., 2002; Markmann et al., 2008; Kosuta et al., 2011). Of these, especially the NEC domain can be highly variable in orthologous genes. For example, in rice the NEC domain of OsSYMRK is extremely short. Nevertheless, OsSYMRK can functionally trans-complement a *L. japonicus Ljsymrk* knockout mutant (Markmann et al., 2008). This indicates that functioning of SYMRK is highly conserved and protein evolution was not essential towards functioning in rhizobium symbiosis.

SYMRK interact with 3-hydroxyl 3-methylglutaryl coenzyme A reductase 1 (HMGR1), which is required for nodule development (Kevei et al., 2007). This suggests that HMGR1 may play a role in transducing signals from the plasma membrane to the nuclear envelope. There, two nuclear potassium channels, CASTOR and POLLUX, are instrumental for Nod factor induced oscillations in the nuclear Ca^{2+} concentration (Ane et al., 2002; Ane et al., 2004; Imaizumi-Anraku et al., 2004; Peiter et al., 2007; Riely et al., 2007; Charpentier et al., 2008; Kosuta et al., 2008; Capoen et al., 2011). Putative orthologs of both genes can be found in most plant species and mutant analysis in rice underlines the ancient functioning of these genes in AM symbiosis (Zhu et al., 2006; Banba et al., 2008; Gutjahr et al., 2008; Chen et al., 2009; Wang et al., 2010). Trans-complementation studies of *M. truncatula dmi1* mutants with rice OsPOLLUX showed that the nodulation, but not rhizobia infections, can be restored (Chen, et al., 2009). Though, since also Arabidopsis contains a putative ortholog of POLLUX that is abundantly expressed in roots (Ane et al., 2004), and a single POLLUX gene, in absence of a CASTOR homolog, can commit the full symbiotic function (Venkateshwaran et al., 2012), I hypothesize that CASTOR and POLLUX did not experience specific evolutionary changes towards a function in rhizobium symbiosis.

All above described components in the Nod factor signalling cascade are essential for symbiotic nuclear Ca^{2+} oscillation responses as triggered by AM fungi and rhizobia. This secondary messenger signal is most probable recognized by the CCaMK-IPD3/CYCLOPS protein complex, in legumes as well as non-legume species (Levy et al., 2004; Mitra et al., 2004; Godfroy et al., 2006; Gutjahr et al., 2008; Yano et al., 2008; Wang et al., 2010; Horvath et al., 2011). Though how legumes discriminate between both symbioses remains largely unknown. Trans-complementation experiments using non-legume CCaMK and IPD3/CYCLOPS orthologs demonstrated that no specific evolution occurred on protein level (Banba et al., 2008; Gutjahr et al., 2008; Wang et al., 2010). However, in case of CCaMK specific regulatory evolution has shaped the expression of CCaMK in legumes. By comparing CCaMK putative promoter regions, Bijl and Geurts identified a conserved element in legume CCaMK genes that is essential for bacterial release from infection threads in nodule cells (Bijl and Geurts, unpublished data). This element may have been instrumental for symbiosome formation in legumes. However, several legume species maintain rhizobia in thread-like infection structures, so-called fixation threads (Sprent, 2007), suggesting that this function of CCaMK may be a later invention in legume evolution.

CCaMK-IPD3/CYCLOPS activate symbiotic gene expression, involving distinct transcriptional activators. Among others these include ERN1, ERN2 and the GRAS-type proteins NSP1 and NSP2. NSP1 and NSP2 are highly conserved across higher plants, and legume mutants can be functionally trans-complemented with non-legume orthologous genes; e.g. rice (Yokota et al., 2010). However, experimental validation of promoter evolution has not been conducted, so it remains unclear whether in legumes both genes are shaped by adaptations of *cis*-regulatory elements. As in legumes NSP1 and NSP2 are constitutively expressed in root tissue, both proteins have been positioned as Nod factor response factors that primarily control expression of Nod factor responsive genes (Smit et al., 2005; Hirsch et al., 2009). However, the mechanism of action of NSP1 and NSP2 in symbiosis is more complex. First, it remains unclear how both proteins become activated. Genetic studies suggest that this is committed by CCaMK, but no direct experimental evidence for this has been found. Second, studies in *L. japonicus* indicate that the epistatic relation of NSP1-NSP2 with other Nod factor signalling genes is complex. NSP1 and NSP2 are not only positioned directly downstream of CCaMK, but are also essential for cytokinin mediated root nodule formation; a developmental signalling network that acts downstream of Nod factor signalling. This places

NSP1 and NSP2 functioning in multiple positions in the signalling network (Madsen et al., 2010) (Figure 1).

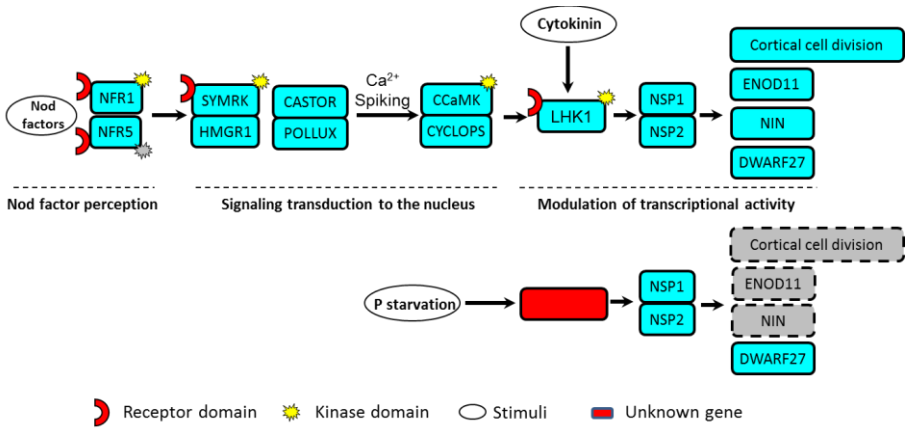


Figure 1. A schematic model of the behaviors of NSP1 and NSP2 responding to Nod factors, cytokinin and phosphate starvation. NSP1 and NSP2 activation can be modulated by three different stimuli: Nod factors, cytokinin and phosphate starvation. In rhizobium symbiosis, NSP1 and NSP2 are activated through Nod factor signaling pathway, which includes two Nod factor receptors NFR1 and NFR5 in the plasma membrane, several proteins (SYMRK, HMGR1, CASTOR and POLLUX) responsible for transduction the signal into the nucleus, a nuclear localized CCaMK and its associated protein CYCLOPS, and cytokinin receptor LHK1. Cytokinin can stimulate legume root cortical cell division and, in some species, the root nodule formation. Cytokinin induced nodule acts downstream of common Nod factor signaling and requires cytokinin receptor LHK1 and NSP1-NSP2 are required. In both cases, NSP1 and NSP2 activated downstream Nod factor responsive genes (*ENOD11*, *NIN* and *DWARF27*) expression and cortical cell divisions (Top model). NSP1 and NSP2 can also be activated under the non-symbiotic condition by phosphate starvation. Different to previous discussed two cases, Nod factor signaling genes and cytokinin receptors are not required for their function, and only *DWARF27* is activated. This implies that unknown cofactor(s) and/ or signaling cascade is required to specify this activation (bottom model).

I hypothesize that activation of NSP1-NSP2 is not committed upon direct interaction with CCaMK. For this I have 3 arguments: (I) I showed that in *M. truncatula* MtNSP1-MtNSP2 dependent *MtD27* expression triggered by phosphate starvation occurs in a CCaMK independent manner. This indicates that an alternative mode for NSP1-NSP2 activation can occur (Chapter 3); (II) This finding is supported by the absence of *CCaMK* in *Brassicaceae* species that harbour NSP1 and NSP2 orthologous genes, suggesting that both proteins can be activated by a different mechanism; (III) I found that in rice a functional CCaMK-NSP2-NSP2 signalling module that activates *D27* expression cannot be created simply by introducing a

dominant active *CCaMK** allele. This indicates that a signalling connection between CCaMK and NSP1-NSP2 is lacking in rice.

Taken together, it seems likely that NSP1-NSP2 can be activated by alternative mechanism, other that direct interaction with CCaMK. Here, I present a speculative model based on current knowledge of GRAS family DELLA proteins.

The GRAS family DELLA proteins

GRAS family proteins form an ancient, but conserved group of plant-specific transcriptional regulators that fulfil essential functions in various signal transduction pathways controlling development. The GRAS family can be divided into ~13 classes (Bolle, 2004; Tian et al., 2004; Zhang et al., 2012) that share a conserved C- terminal GRAS domain with five distinct motifs (leucine heptad repeat I (LHR I), VHIID, leucine heptad repeat II (LHR II), PFYRE and SAW). This GRAS domain is essential for homo and hetero dimerization, and other protein-protein interactions. Sequence profile searches, structural comparisons and phylogenetic analysis indicate that the GRAS domain emerged first in bacteria, were it can be found in Rossmann fold methyltransferases that bind small organic molecules (Zhang et al., 2012). Plants could have gained the GRAS domain by horizontal gene transfer of a single bacterial gene that gave rise to the current GRAS family in plants. It is speculated that the GRAS domain of some plant proteins still retained the capacity to bind small organic molecules; e.g. plant hormones.

Most plant GRAS proteins are nuclear-localized and share some structural domain similarities with animal-specific DNA binding STAT proteins, although it is debated whether this is correct (Richards et al., 2000; Hirsch and Oldroyd, 2009). Nevertheless, it generally lets to the assumption that plant GRAS proteins act as canonical transcription factors. However, direct support for this assumption is limited, with most convincing data coming from studies on *M. truncatula* NSP1 for which it was shown to bind directly to an AT-rich element of the promoters of *MtENOD11*, *MtERN1* and *MtD27* (Hirsch et al., 2009; Liu et al., 2011). Studies on other GRAS proteins, however, provided convincing evidence that these proteins can also regulate gene expression in an indirect way, by forming protein complexes. Of these, studies on the DELLA sub-class are most comprehensive.

DELLAs have been identified as mediators of multiple environmental signals. Genetic analyses in Arabidopsis and rice identified several DELLAs (e.g. GAI, RGA, RGL1 and

RGL2) that repress gibberellic acid (GA) signalling. These proteins have two motifs in common in their N-terminal region (DELLA and VHYNP), which enable them to interact with GA receptors. DELLA proteins can also interact with various transcription factors that maintain an inactive state upon binding; e.g. PHYTOCHROME-INTERACTING FACTORs (PIFs), ALCATRAZ, MYC2 and *BRAZINAZOL RESISTANT 1* (BZR1) (Davière et al., 2008; Feng et al., 2008; Arnaud et al., 2010; Gallego-Bartolomé et al., 2010; Gallego-Bartolome et al., 2012; Hong et al., 2012). DELLAs are actively degraded upon GA binding by the GA receptor, resulting in the release of associated transcription factors, enabling them to bind to regulatory sequences of their target genes. In this way DELLAs form a key component in transcription modules thereby controlling a diverse range of transcriptional networks.

I postulate that the mode of action of DELLA proteins could be more generic for plant specific GRAS-type proteins, including NSP1 and NSP2. Crystallography of the DELLA protein AtGAI revealed that its N-terminal part contains a so-called intrinsically disordered region (IDR) (Murase et al., 2008). IDRs enable a protein to fold differently in order to recognize and bind different partners. IDRs lack a secondary and/or tertiary structure in absence of a binding partner, yet can commit cellular responses upon protein binding (Uversky, 2010). Studies of the GRAS family indicate that the N-domains of all GRAS proteins contain IDR motifs, including NSP1 and NSP2 (Sun et al., 2011; Sun et al., 2012). This may provide GRAS proteins with a degree of binding plasticity and may explain their functional versatility.

In case of NSP1-NSP2, it would mean that secondary signals of unknown nature, generated by CCaMK-IPD3/CYCLOPS, cytokinin signalling and/or phosphate starvation, activate the NSP1-NSP2 transcriptional module. In analogy to DELLAs this could require interaction with receptor-like molecules. Alternatively, the secondary signals bind directly to NSP1-NSP2. This hypothesis finds some support by the structural similarities of binding motifs of methyltransferases that bind small organic molecules and the GRAS domains of plant GRAS proteins (Zhang et al., 2012). Upon activation, NSP1 could act directly as a canonical transcription factor, a mode of action that is supported by binding studies of NSP1 to specific promoter elements of the *MtENOD11*, a Nod factor responsive gene (Hirsch et al., 2009). In line with DELLAs, indirect transcriptional activation could also occur via associated transcription factors. The latter is supported by studies presented in Chapter 4, where I show that NSP1-NSP2 mediated activation, either by phosphate starvation or by Nod factor signalling, results in a differential transcriptional read out. Whereas *MtENOD11* and *MtD27* are induced upon Nod factor signalling, phosphate starvation only activates *MtD27* in an

NSP1-NSP2 dependent manner. Identification of NSP1-NPS2 interacting transcription factors that are essential for *MtD27* expression could support this model.

perspectives

Unravelling the molecular mechanisms of rhizobium Nod factor signalling will be instrumental for a future transfer of this symbiosis to other plant species. It will provide insight in the evolutionary trajectory of the genes involved, and can elucidate functioning of orthologous genes in non-legume species. The research presented in my thesis provided insight in the complex functioning of two GRAS-type transcriptional regulators, NSP1 and NSP2. It uncovered the role of both genes in strigolactone biosynthesis in response to phosphate starvation, and the integration of this signalling module in rhizobium symbiosis. Simultaneously, it provided insight in the complex regulation of the NSP1-NSP2 transcription module. Future research will uncover the precise molecular functioning of both GRAS proteins and provide insight the evolutionary trajectory of the underlying genes.

REFERENCES

Andriankaja, A., Boisson-Dernier, A., Frances, L., Sauviac, L., Jauneau, A., Barker, D.G., and de Carvalho-Niebel, F. (2007). AP2-ERF transcription factors mediate Nod factor dependent *MtENOD11* activation in root hairs via a novel *cis*-regulatory motif. Plant Cell **19,** 2866-2885.

Ane, J.M., Levy, J., Thoquet, P., Kulikova, O., de Billy, F., Penmetsa, V., Kim, D.J., Debelle, F., Rosenberg, C., Cook, D.R., Bisseling, T., Huguet, T., and Denarie, J. (2002). Genetic and cytogenetic mapping of DMI1, DMI2, and DMI3 genes of *Medicago truncatula* involved in Nod factor transduction, nodulation, and mycorrhization. Mol. Plant Microbe Interact. **15,** 1108-1118.

Ane, J.M., Kiss, G.B., Riely, B.K., Penmetsa, R.V., Oldroyd, G.E., Ayax, C., Levy, J., Debelle, F., Baek, J.M., Kalo, P., Rosenberg, C., Roe, B.A., Long, S.R., Denarie, J., and Cook, D.R. (2004). *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes. Science **303,** 1364-1367.

Arnaud, N., Girin, T., Sorefan, K., Fuentes, S., Wood, T.A., Lawrenson, T., Sablowski, R., and Østergaard, L. (2010). Gibberellins control fruit patterning in *Arabidopsis thaliana*. Genes Dev. **24,** 2127-2132.

Banba, M., Gutjahr, C., Miyao, A., Hirochika, H., Paszkowski, U., Kouchi, H., and Imaizumi-Anraku, H. (2008). Divergence of evolutionary ways among common sym genes: CASTOR and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway. Plant Cell Physiol. **49,** 1659-1671.

Bolle, C. (2004). The role of GRAS proteins in plant signal transduction and development. Planta **218,** 683-692.

Broghammer, A., Krusell, L., Blaise, M., Sauer, J., Sullivan, J.T., Maolanon, N., Vinther, M., Lorentzen, A., Madsen, E.B., Jensen, K.J., Roepstorff, P., Thirup, S., Ronson, C.W., Thygesen, M.B., and Stougaard, J. (2012). Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. Proc. Natl. Acad. Sci. USA **109,** 13859-13864.

Cannon, S.B., Ilut, D., Farmer, A.D., Maki, S.L., May, G.D., Singer, S.R., and Doyle, J.J. (2010). Polyploidy did not predate the evolution of nodulation in all legumes. PLoS One **5,** e11630.

Capoen, W., Sun, J., Wysham, D., Otegui, M.S., Venkateshwaran, M., Hirsch, S., Miwa, H., Downie, J.A., Morris, R.J., Ane, J.M., and Oldroyd, G.E. (2011). Nuclear membranes control symbiotic calcium signaling of legumes. Proc. Natl. Acad. Sci. USA **108,** 14348-14353.

Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E.P., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Denarie, J. (2000). Four genes of *Medicago truncatula* controlling components of a nod factor transduction pathway. Plant Cell **12,** 1647-1666.

Cerri, M.R., Frances, L., Laloum, T., Auriac, M.C., Niebel, A., Oldroyd, G.E., Barker, D.G., Fournier, J., and de Carvalho-Niebel, F. (2012). *Medicago truncatula* ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and expression dynamics throughout rhizobial infection. Plant Physiol. **160,** 2155–2172.

Charpentier, M., Bredemeier, R., Wanner, G., Takeda, N., Schleiff, E., and Parniske, M. (2008). *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. Plant Cell **20,** 3467-3479.

Chen, C., Fan, C., Gao, M., and Zhu, H. (2009). Antiquity and function of CASTOR and POLLUX, the twin ion channel-encoding genes key to the evolution of root symbioses in plants. Plant Physiol. **149,** 306-317.

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

Davière, J.-M., de Lucas, M., and Prat, S. (2008). Transcriptional factor interaction: a central step in DELLA function. Curr. Opin. Genet. Dev. **18,** 295-303.

Delaux, P.-M., Ségalon-Delmas, N., Bécard, G., and Ané, J.-M. (2013). Evolution of the plant-microbe symbiotic 'toolkit'. Trends Plant Sci. doi: 10.1016/j.tplants.2013.01.008.

Downie, J.A. (1998). Functions of rhizobial nodulation genes. In The Rhizobiaceae (Springer), pp. 387-402.

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P., and Kiss, G.B. (2002). A receptor kinase gene regulating symbiotic nodule development. Nature **417,** 962-966.

Esseling, J.J., Lhuissier, F.G., and Emons, A.M. (2004). A nonsymbiotic root hair tip growth phenotype in NORK-mutated legumes: implications for nodulation factor-induced signaling and formation of a multifaceted root hair pocket for bacteria. Plant Cell **16,** 933-944.

Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J.M., and Kircher, S. (2008). Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. Nature **451,** 475-479.

Gallego-Bartolomé, J., Minguet, E.G., Marín, J.A., Prat, S., Blázquez, M.A., and Alabadí, D. (2010). Transcriptional diversification and functional conservation between DELLA proteins in Arabidopsis. Mol. Biol. Evol. **27,** 1247-1256.

Gallego-Bartolome, J., Minguet, E.G., Grau-Enguix, F., Abbas, M., Locascio, A., Thomas, S.G., Alabadi, D., and Blazquez, M.A. (2012). Molecular mechanism for the interaction between gibberellin and brassinosteroid signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. USA **109,** 13446-13451.

Geurts, R., Lillo, A., and Bisseling, T. (2012). Exploiting an ancient signalling machinery to enjoy a nitrogen fixing symbiosis. Curr. Opin. Plant Biol. **15,** 438-443.

Godfroy, O., Debelle, F., Timmers, T., and Rosenberg, C. (2006). A rice calcium- and calmodulin-dependent protein kinase restores nodulation to a legume mutant. Mol. Plant Microbe Interact. **19,** 495-501.

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

Gutjahr, C., Banba, M., Croset, V., An, K., Miyao, A., An, G., Hirochika, H., Imaizumi-Anraku, H., and Paszkowski, U. (2008). Arbuscular mycorrhiza–specific signaling in rice transcends the common symbiosis signaling pathway. Plant Cell **20,** 2989-3005.

Heckmann, A.B., Lombardo, F., Miwa, H., Perry, J.A., Bunnewell, S., Parniske, M., Wang, T.L., and Downie, J.A. (2006). *Lotus japonicus* nodulation requires two GRAS domain regulators, one of which is functionally conserved in a non-legume. Plant Physiol. **142,** 1739-1750.

Hirsch, S., and Oldroyd, G.E. (2009). GRAS-domain transcription factors that regulate plant development. Plant Signal Behav. **4,** 698-700.

Hirsch, S., Kim, J., Munoz, A., Heckmann, A.B., Downie, J.A., and Oldroyd, G.E. (2009). GRAS proteins form a DNA binding complex to induce gene expression during nodulation signaling in *Medicago truncatula*. Plant Cell **21,** 545-557.

Hong, G.J., Xue, X.Y., Mao, Y.B., Wang, L.J., and Chen, X.Y. (2012). Arabidopsis MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. Plant Cell **24,** 2635-2648.

Horvath, B., Yeun, L.H., Domonkos, A., Halasz, G., Gobbato, E., Ayaydin, F., Miro, K., Hirsch, S., Sun, J., Tadege, M., Ratet, P., Mysore, K.S., Ane, J.M., Oldroyd, G.E., and Kalo, P. (2011). *Medicago truncatula* IPD3 is a member of the common symbiotic signaling pathway required for rhizobial and mycorrhizal symbioses. Mol. Plant Microbe Interact. **24,** 1345-1358.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., and Vickers, K. (2004). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature **433,** 527-531.

Kalo, P., Gleason, C., Edwards, A., Marsh, J., Mitra, R.M., Hirsch, S., Jakab, J., Sims, S., Long, S.R., Rogers, J., Kiss, G.B., Downie, J.A., and Oldroyd, G.E. (2005). Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. Science **308,** 1786-1789.

Kevei, Z., Lougnon, G., Mergaert, P., Horvath, G.V., Kereszt, A., Jayaraman, D., Zaman, N., Marcel, F., Regulski, K., Kiss, G.B., Kondorosi, A., Endre, G., Kondorosi, E., and Ane, J.M. (2007). 3-hydroxy-3-methylglutaryl coenzyme a reductase 1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. Plant Cell **19,** 3974-3989.

Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R.J., Downie, J.A., and Oldroyd, G.E. (2008). Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. Proc. Natl. Acad. Sci. USA **105,** 9823-9828.

Kosuta, S., Held, M., Hossain, M.S., Morieri, G., MacGillivary, A., Johansen, C., Antol ń-Llovera, M., Parniske, M., Oldroyd, G.E., and Downie, A.J. (2011). *Lotus japonicus* symRK-14 uncouples the cortical and epidermal symbiotic program. Plant J. **67,** 929-940.

Lavin, M., Herendeen, P.S., and Wojciechowski, M.F. (2005). Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. Syst. Biol. **54,** 575-594.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C., and Denarie, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344,** 781-784.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., Denarie, J., Rosenberg, C., and Debelle, F. (2004). A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science **303,** 1361-1364.

Liu, W., Kohlen, W., Lillo, A., Op den Camp, R., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C., Kaufmann, K., Yang, W.C., Hooiveld, G.J., Charnikhova, T., Bouwmeester, H.J., Bisseling, T., and Geurts, R. (2011). Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. Plant Cell **23,** 3853-3865.

Madsen, E.B., Antolin-Llovera, M., Grossmann, C., Ye, J., Vieweg, S., Broghammer, A., Krusell, L., Radutoiu, S., Jensen, O.N., Stougaard, J., and Parniske, M. (2011). Autophosphorylation is essential for the in vivo function of the *Lotus japonicus* Nod factor receptor 1 and receptor-mediated signalling in cooperation with Nod factor receptor 5. Plant J. **65,** 404-417.

Madsen, L.H., Tirichine, L., Jurkiewicz, A., Sullivan, J.T., Heckmann, A.B., Bek, A.S., Ronson, C.W., James, E.K., and Stougaard, J. (2010). The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. Nat. Commun.**1,** 10.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E.A., Driguez, H., Becard, G., and Denarie, J. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469,** 58-63.

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

Middleton, P.H., Jakab, J., Penmetsa, R.V., Starker, C.G., Doll, J., Kalo, P., Prabhu, R., Marsh, J.F., Mitra, R.M., Kereszt, A., Dudas, B., VandenBosch, K., Long, S.R., Cook, D.R., Kiss, G.B., and Oldroyd, G.E. (2007). An ERF transcription factor in *Medicago truncatula* that is essential for Nod factor signal transduction. Plant Cell **19,** 1221-1234.

Mitra, R.M., Gleason, C.A., Edwards, A., Hadfield, J., Downie, J.A., Oldroyd, G.E., and Long, S.R. (2004). A Ca^{2+}/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. USA **101,** 4701-4705.

Moore, M.J., Bell, C.D., Soltis, P.S., and Soltis, D.E. (2007). Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc. Natl. Acad. Sci. USA **104,** 19363-19368.

Murase, K., Hirano, Y., Sun, T.P., and Hakoshima, T. (2008). Gibberellin-induced DELLA recognition by the gibberellin receptor GID1. Nature **456,** 459-463.

Nakagawa, T., Kaku, H., Shimoda, Y., Sugiyama, A., Shimamura, M., Takanashi, K., Yazaki, K., Aoki, T., Shibuya, N., and Kouchi, H. (2011). From defense to symbiosis: limited alterations in the kinase domain of LysM receptor-like kinases are crucial for evolution of legume-Rhizobium symbiosis. Plant J. **65,** 169-180.

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

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

Peiter, E., Sun, J., Heckmann, A.B., Venkateshwaran, M., Riely, B.K., Otegui, M.S., Edwards, A., Freshour, G., Hahn, M.G., Cook, D.R., Sanders, D., Oldroyd, G.E., Downie, J.A., and Ane, J.M. (2007). The *Medicago truncatula* DMI1 protein modulates cytosolic calcium signaling. Plant Physiol. **145,** 192-203.

Remy, W., Taylor, T.N., Hass, H., and Kerp, H. (1994). Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc. Natl. Acad. Sci. USA **91,** 11841-11843.

Richards, D.E., Peng, J., and Harberd, N.P. (2000). Plant GRAS and metazoan STATs: one family? BioEssays **22,** 573-577.

Riely, B.K., Lougnon, G., Ane, J.M., and Cook, D.R. (2007). The symbiotic ion channel homolog DMI1 is localized in the nuclear membrane of *Medicago truncatula* roots. Plant J. **49,** 208-216.

Roth, L., and Stacey, G. (1989). Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. Eur. J. Cell Biol. **49,** 13.

Sawada, H., Kuykendall, L.D., and Young, J.M. (2003). Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J. Gen. Appl. Microbiol. **49,** 155-179.

Smit, P., Raedts, J., Portyanko, V., Debelle, F., Gough, C., Bisseling, T., and Geurts, R. (2005). NSP1 of the GRAS protein family is essential for rhizobial Nod factor-induced transcription. Science **308,** 1789-1791.

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

Sprent, J.I. (2007). Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. New Phytol. **174,** 11-25.

Stracke, S., Kistner, C., Yoshida, S., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., and Szczyglowski, K. (2002). A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature **417,** 959-962.

Streng, A., op den Camp, R., Bisseling, T., and Geurts, R. (2011). Evolutionary origin of rhizobium Nod factor signaling. Plant Signal Behav. **6,** 1510-1514.

Sun, X., Jones, W.T., and Rikkerink, E.H. (2012). GRAS proteins: the versatile roles of intrinsically disordered proteins in plant signalling. Biochem. J. **442,** 1-12.

Sun, X., Xue, B., Jones, W.T., Rikkerink, E., Dunker, A.K., and Uversky, V.N. (2011). A functionally required unfoldome from the plant kingdom: intrinsically disordered N-terminal domains of GRAS proteins are involved in molecular recognition during plant development. Plant Mol. Biol. **77,** 205-223.

Tian, C., Wan, P., Sun, S., Li, J., and Chen, M. (2004). Genome-wide analysis of the GRAS gene family in rice and Arabidopsis. Plant Mol. Biol. **54,** 519-532.

Uversky, V.N. (2010). The mysterious unfoldome: structureless, underappreciated, yet vital part of any given proteome. J. Biomed. Biotech. **2010,** 568068.

Venkateshwaran, M., Cosme, A., Han, L., Banba, M., Satyshur, K.A., Schleiff, E., Parniske, M., Imaizumi-Anraku, H., and Ané, J.-M. (2012). The recent evolution of a symbiotic ion channel in the legume family altered ion conductance and improved functionality in calcium signaling. Plant Cell **24,** 2528-2545.

Wang, B., Yeun, L.H., Xue, J.Y., Liu, Y., Ane, J.M., and Qiu, Y.L. (2010). Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. New Phytol. **186,** 514-525.

Yano, K., Yoshida, S., Muller, 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.L., Kawaguchi, M., Imaizumi-Anraku, H., Hayashi, M., and Parniske, M. (2008). CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc. Natl. Acad. Sci. USA **105,** 20540-20545.

Yokota, K., Soyano, T., Kouchi, H., and Hayashi, M. (2010). Function of GRAS proteins in root nodule symbiosis is retained in homologs of a non-legume, rice. Plant Cell Physiol. **51,** 1436-1442.

Young, N.D., Debelle, F., Oldroyd, G.E.D., Geurts, R., Cannon, S.B., Udvardi, M.K., Benedito, V.A., Mayer, K.F.X., Gouzy, J., Schoof, H., Van de Peer, Y., Proost, S., Cook, D.R., Meyers, B.C., Spannagl, M., Cheung, F., De Mita, S., Krishnakumar, V., et al.(2011). The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature **480,** 520-524.

Zhang, D., Iyer, L.M., and Aravind, L. (2012). Bacterial GRAS domain proteins throw new light on gibberellic acid response mechanisms. Bioinformatics **28,** 2407-2411.

Zhu, H., Riely, B.K., Burns, N.J., and Ane, J.M. (2006). Tracing nonlegume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. Genetics **172,** 2491-2499.

Summary

Legume species can interact with rhizobium and form new lateral organs, called root nodules. Rhizobium bacteria are hosted in this organ and fix elemental nitrogen into ammonia. In return, the plant provides a carbon source to feed the bacteria. This co-dependence is known as nitrogen fixing symbiosis. To balance the energy flow, legume plants control the symbiosis based on their nitrogen status. Only when fixed nitrogen is limited the plant will enter this symbiotic state. In the past two decades, genetic analysis in model legume species *Medicago truncatula* and *Lotus japonicus*, uncovered genes essential to establish this symbiotic interaction. Among these are genes encoding a small signalling network that recognizes rhizobium secreted signal molecules named Nod factors. This network subsequently sets in motion root nodule formation. Mutating these symbiosis signalling genes abolishes most of the rhizobium induced symbiotic responses, whereas dominant active alleles can trigger spontaneous root nodule formation in absence of rhizobium. This underlines the importance of the symbiotic signalling network in root nodule formation.

In Chapter 2, I investigated the transcriptional regulation of symbiosis signalling genes in response to an exogenous nitrogen source and the subsequent effect on the interaction with rhizobium. It was discovered that ammonium nitrate affects symbiotic responses differentially in root epidermal cells. Whereas symbiotic gene expression is repressed in young growing hairs and mature hairs, it is still induced in a small zone of root hairs that just stopped growing. These hairs also display symbiotic growth responses in root hairs upon rhizobium Nod factor signalling. Additionally, I found that nitrate inhibition of root nodule formation could be overcome by a dominant-positive allele of the symbiosis signalling gene *CCaMK*. This indicates that the nitrogen status of plants affects the Nod factor signalling pathway upstream of the nuclear localized CCaMK protein. This mechanism likely acts post-transcriptionally, as no changes in expression of symbiosis signalling genes could be monitored in response to nitrate.

Although a nitrogen fixing symbiosis with rhizobium is basically unique for legumes, putative orthologs of symbiosis signalling genes can be found in many non-legume species, including monocots such as rice (*Oryza sativa*). This indicates that the symbiosis signalling genes predate the birth of the legume family and implies that the original function might been kept in non-legumes. Support for this hypothesis was found already in previous studies that

showed that some genes are also essential for the widespread and very ancient symbiosis of higher plants and endomycorrhizal fungi. In Chapter 3, I focussed on two GRAS-type transcriptional regulators, NSP1and NSP2, that are essential only for rhizobium root nodule formation, whereas *nsp1* and *nsp2* knockout mutants can affectively mycorrhized. Uncovering the non-symbiotic function NSP1 and NSP2 helps to understand the evolutionary origin of the rhizobium symbiosis and can be instrumental for a future transfer of rhizobium symbiosis to other (non-legume) crops. I used *M. truncatula* and the non-legume rice to uncover the non-symbiotic function of NSP1 and NSP2. By conducting comparative transcriptome and metabolome profiling of *M. truncatula nsp1*, *nsp2* mutant and wild type roots it was discovered that both transcription factors control the strigolactone biosynthetic pathway through transcriptional regulation of *DWARF27*. This regulation is dependent on the phosphate status of the plant. *DWARF27* is a conserved gene in higher plants. By studying rice I could demonstrate that that phosphate deprivation induced *DWARF27* expression also in this species is NSP1-NSP2 dependent. Based on these results, I concluded that regulation of strigolactone biosynthesis in response to phosphate starvation is an ancestral function of NSP1 and NSP2. As strigolactones act as *ex planta* stimuli for root mycorrhization I conclude that also NSP1 and NSP2 have co-opted from the ancient endomycorrhizae symbiosis.

In Chapter 4, I questioned whether NSP1-NSP2 dependent *DWARF27* expression has been co-opted in rhizobium symbiosis. By using qRT-PCR and a *GUS* reporter construct, I discovered that *DWARF27* is a Nod factor responsive gene of which its spatial expression pattern is tightly regulated. This regulation is dependent on the symbiosis signalling pathway. This in contrast to *DWARF27* induced expression in response to phosphate starvation, which requires NSP1 and NSP2, but is independent of other symbiosis signalling genes. These results indicate that in legumes the NSP1-NSP2-DWARF27 regulation module gained a Nod factor signalling dependent regulatory element of unknown nature.

In Chapter 5, I further investigated regulation of the NSP1-NSP2-DWARF27 signalling module. By using a *CCaMK* dominant positive allele it was demonstrated that *DWARF27* expression in *M. truncatula* could be activated in a CCaMK independent way, whereas this showed not to be the case for rice. This implies that the signalling connection between CCaMK and NSP1-NSP2, which is essential for root nodule formation, is non-functional in rice.

In Chapter 6, I place the data described in this thesis in a broader perspective.

Samenvatting

Vlinderbloemige planten kunnen een stikstofbindende symbiose aangaan met rhizobium-bacteriën, wat resulteert in de vorming van wortelknolletjes. De rhizobium-bacteriën die in deze knolletjes zitten, zijn in staat moleculaire stikstof - aanwezig in de atmosfeer - om te zetten in ammonia. Dit komt ten gunste van de plant. In ruil hiervoor worden de bacteriën voorzien van carbohydraten, met als resultaat een wederzijdse profijtelijke interactie: een symbiose. Vlinderbloemige planten houden altijd controle over de symbiotische relatie met de rhizobium-bacteriën, en alleen bij gebrek aan gebonden stikstof zullen wortelknolletjes worden gevormd en/of in stand worden gehouden.

Door gebruik te maken van vlinderbloemige modelplanten, zoals *Medicago truncatula* (Medicago) en *Lotus japonicus,* zijn symbiotische genen geïdentificeerd. Een deel van deze genen vormt een klein genetisch netwerk dat essentieel is voor de herkenning van een door rhizobium uitgescheiden signaalmolecuul, de Nod-factor. Herkenning van Nod-factoren door de plant leidt tot de vorming van wortelknolletjes. Het belang van de symbiotische genen komt het best tot uiting in de analyse van mutanten. Het uitschakelen (muteren) van symbiotische genen blokkeert het vermogen van de plant om wortelknolletjes te vormen, terwijl dominant-actieve mutaties de vorming van knolletjes induceert in afwezigheid van rhizobium (of Nod-factoren).

In Hoofdstuk 2 onderzoek ik het effect van het toevoegen van gebonden stikstof (ammoniumnitraat) op door Nod-factoren geïnduceerde responsen en de expressie van symbiotische genen in Medicago. Een van de eerste responsen die door Nod-factoren worden geïnduceerd is de expressie van het gen *MtENOD11*. Het toevoegen van ammoniumnitraat remt deze respons, maar niet gelijkmatig in alle epidermiscellen. Epidermiscellen met wortelharen die net gestopt zijn met groeien zijn het minst gevoelig voor ammoniumnitraat, en reageren nog steeds op Nod-factoren. Daarnaast heb ik ontdekt dat de door ammoniumnitraat opgeworpen blokkade *overruled* kan worden door een dominant-actieve vorm van het symbiotische gen *CCaMK*. Introductie van dit gen leidt tot vorming van spontane knollen - zonder rhizobium - en deze respons is onafhankelijk van de stikstofstatus van de plant.

Hoewel de stikstofbindende rhizobiumsymbiose bijna exclusief bij vlinderbloemige planten voorkomt, zijn (homologen van) de symbiotische genen wijdverspreid. Dit geeft aan dat deze genen waarschijnlijk (veel) ouder zijn dan de vlinderbloemige planten zelf, en dus ook een andere functie hebben vervuld, of misschien nog steeds vervullen. Inzicht in deze

oude functie was reeds eerder verkregen, toen bleek dat een aantal symbiotische mutanten niet alleen geblokkeerd zijn in rhizobiumsymbiose maar ook in de symbiose met mycorrhizae-schimmels. Deze symbiose is wijdverbreid in het plantenrijk en mogelijk zeer oud.

In Hoofdstuk 3 focus ik op de functie van twee symbiotische transcriptieregulatoren van de GRAS-familie: NSP1 en NSP2. Het uitschakelen van een van beide transcriptieregulatoren in Medicago blokkeert de rhizobiumsymbiose volledig, maar heeft nauwelijks effect op de symbiose met mycorrhizae-schimmels. Om inzicht te krijgen in de niet-symbiotische functie heb ik het *transcriptome* en *metabolome* in de wortel van gemuteerde *nsp1* en *nsp2* Medicago-planten vergeleken met niet gemuteerde planten. Hierdoor heb ik ontdekt dat beide transcriptieregulatoren de expressie reguleren van het gen *DWARF27,* en dat deze regulatie afhankelijk is van de fosfaatstatus van de plant. Het *DWARF27*-gen codeert voor een enzym dat een essentiële stap in de biosynthese van strigolactonen reguleert. Door vergelijkbare studies bij rijst (*Oryza sativa*) te doen kon ik aantonen dat NSP1 en NSP2 ook in deze plant de expressie van *DWARF27* reguleren. Op basis van deze vindingen concludeer ik dat de oude functie van NSP1-NSP2 het reguleren van de strigolacton-biosynthese is. Een respons die optreedt als reactie op fosfaatgebrek. Strigolactonen in planten hebben een dubbele functie: ze werken als endogeen plantenhormoon, maar worden uitgescheiden door de wortel om mycorrhizae-schimmels aan te trekken. Dit laatste suggereert dat NSP1 en NSP2 toch nodig zijn voor de wijdverspreide mycorrhizae-symbiose.

In Hoofdstuk 4 stel ik de vraag of *DWARF27* ook een rol speelt in de rhizobiumsymbiose. Dat blijkt zo te zijn. Door middel van qRT-PCR en het gebruik van een promoter-GUS reporter construct heb ik aangetoond dat *DWARF27* een Nod-factor *responsive* gen is, waarvan de expressie strikt wordt gereguleerd gedurende de knolvorming. Nod-factor geïnduceerde *DWARF27*-expressie vereist een functioneel symbiose signaleringsnetwerk. Dit in tegenstelling tot de door fosfaatstress geïnduceerde *DWARF27*-expressie. Aangezien beide afhankelijk zijn van NSP1-NSP2, kom ik tot de conclusie dat vlinderbloemigen een nieuwe regulatie van de NSP1-NSP2-DWARF27 signaleringsmodule hebben verworven.

In Hoofdstuk 5 onderzoek ik of ik de NSP1-NSP2-DWARF27 signaleringsmodule in rijst kan activeren met een dominant-actieve vorm van CCaMK. Dit bleek niet mogelijk, wat aangeeft dat de signaleringsconnectie tussen CCaMK en NSP1-NSP2 niet functioneel is in rijst.

Het laatste hoofdstuk beschrijft een korte samenvatting van mijn resultaten, en daarin plaats ik deze in een breder perspectief.

Acknowledgements

I believe that, many years later, most parts of this thesis will be left in the history, covered with dust, and even erased from my own mind. But this part will be with me all the way. All your helps and all your encouragements support me to date.

I would like to thank Prof. Weicai Yang for giving me the chance to join the CAS-KNAW joint PHD program and providing me the opportunity to join such an active group. Grateful acknowledgement gives to my promoter Prof. Ton Bisseling for accepting me to be a PhD student in his lab. I enjoyed all the discussions with you, and I'm still struggling with your simple but difficult question, "why". I would like to give many thanks to my supervisor Dr. Rene Geurts for giving me an opportunity to work in his group. Thanks for your encouragement and support in these years, and for your suggestions and revises during the writing of this thesis. Your self-confidence always supports me to uncover the unknown.

I would like to thank all my colleagues in Molbi. Together with you all, I enjoyed every moment in Molbi. I miss all the days we shared.

I would like to thank Maria Augustijn and Marie-Jose van Iersel for preparing all the documents for my stay. Without your supports, I cannot imagine how I can stay in The Netherlands legally and safely. I would like to give my thanks to Jan Verver for ordering and organizing the enzymes and chemicals, Jan Hontelez for keeping all the equipment and wastes in correct position with strict rules, Marijke Hartog for ordering my primers, sequencing my constructs, organizing the greenhouse and communicating with the gardeners, and Carolien Franken for keeping the lab clean and clear which letting me be shame to make it dirty again. All of your hard work guaranteed my experiments go smoothly.

I fell grateful to Joan Wellink for organizing the lab journals every week. When you come to me, I know I'm in your list already. Many thanks to Boudewijn van Veen for keeping my computer perfect and maintaining the microscopes. But you know I have to visit some Chinese websites and I still cannot read the whole manual of the confocal microscope although you suggested me several times. Many many thanks to Henk Franssen for sharing ideas, experiments, and also the rapping. I'm admiring your optimism. And that is why I believed you merely took a long vacation that year. I would like to thank Olga Kulikova and Ludmila Mlynarova for sharing me the knowledge of Arabidopsis and the discussion of my project. My heart feels grateful to Elena Fedorova. Your supporting and encouraging are

always there for me. Thanks to Erik Limpens for sharing his knowledge of symbiosis. I miss all the discussions with all of you.

I would like to thank Arend Streng for showing me the lab on my first day in the lab, Gerben Bijl for teaching me the principle of qPCR and solving the software and hardware problems of my computer, Silvester de Nooijer for explaining the principle of sailing to me, Andreas Untergasser for showing me your Primer-3-Plus program and teaching me the Gateway system. I also would like to thank Stefan Schilderink for inviting me to join the dinner in the first week I arrived at Wageningen. That is the first Dutch dinner I joined. I miss all the chats with you, experiments, life, future and your mountain bike. Many thanks to Rik op den Camp. I'm missing your warm-hearted helps and admiring your plenty of fast arising ideas. Thanks to Stéphane De Mita to teaching me bioinformatics knowledge. Thanks to Alessandra Lillo for the days we shared the office. Many thanks to Evgenia Ovchinnikova for showing me the knowledge of pea and explaining me the principle of mapping. I also would like to thank Natalya Savelyeva for providing me the pea mutants and sharing her data. My special thanks to my best friend and my best comrade, Sergey Ivanov. I'm missing those weekends and holidays that we shared in the lab. Of course, most time, you were working alone. And I also miss all the chats with you: science, family, career, life, and everything. Thanks to Eva Deinum for showing me the principle of modeling. I also would like to thank Rik Huisman, Sjef Moling, Vid Karmarkar, Adam Folta, Arjan van Zeijl, Maryam Seifi Kalhor, Trupti Sharma, Aleksandr Gavrin, and Timofey Nemankin for sharing me all the moments in Molbi.

I would like to thank all collaborators and people in WUR, Prof. Harro Bouwmeester, Prof. Sacco de Vries, Prof. Dolf Weijers, Wouter Kohlen, Muhammad Jamil, Carolien Ruyter-spira, Tatsiana Charnikhova, Cezary Smaczniak, Kerstin Kaufmann, Guido Hooiveld and Kim Boutilier. Thanks for you showing me the science from different angles.

I also would like to give my special thanks to all my Chinese friends in The Netherlands. Cheng Xi, Cheng Xu, Dong Lemeng, Fan Huajie, Gao Wei, Gao Dongli, He Hanzi, He Jun, Hu Yuming, Hua Chenlei, Zhang Lisha, Huang Xingfeng, Tang Xiaomin, Ji Xianwen, Lang Chunting, Li Huchen, Li Hui, Li Jimeng, Lin Ke, Zhang Ningwen, Liu Junwei, Liu Qing, Yang Ting, Luo Hong, Ma Lisong, Xu Fang, Peng Ke, Qi Weicong, Chen Xi, Qin Wei, Song Chunxu, Song Wei, Sun Zhongkui, Sun Xiaoxue, Sun Pulv, Tang Jifeng, Wan Jinbo, Ting Hieng Ming, Lin Ya-Fen, Ren Guiling, Wang Bo, Wang Guodong, Wang Yiding, Xiao Dong,

Xiao Tingting, Xie Songlin, Zhang Yanxia, Zhang Ying, Zhang Zhao, Zhao Chunzhao, Zheng Zheng, Zhu Feng and Zhu Suxian. Thanks to Bai Yuling, Wang Wenbin, Huang Sanwen, Li Guangcun, Li Junming and Zhao Jianjun.

感谢大家在这些年里对我们全家的照应。忘不了和樊华杰一起炖猪手的日子；忘不了到达荷兰后的第一顿米饭是在唐季凤万金波家吃到；忘不了第一次在国外的中秋节是在黄兴峰唐晓敏家吃的包子，领略了赵建军老师实在是厉害的面食手艺；忘不了李慧饱含氢气喊出的我的名字；忘不了和宋炜一起在刘庆杨婷家灌香肠时的情景和那缭绕的肉香；忘不了罗红家那两只恋家的鹦鹉；忘不了陈希的龙猫，戚维聪的架子鼓以及他们自制的美味糕点；忘不了凝文怀着宝宝挺着肚子坐在灶台前为我们摊的鸡蛋饼和那美味的烤鸭；忘不了张钊那满墙的古董相机、那面条机压制出的烩面；忘不了在陈贤明林雅芬家吃到的各种台湾和马来西亚的美食；忘不了张莹包的口口香的陕西饺子；忘不了写论文时在婷婷家连续蹭饭的日子；忘不了万金波的工具永远是最好的；忘不了郑铮那需要健胃消食片才能缓解的大盘鸡永远是最美的；忘不了我们共同在那块小菜园不断拔草和播种的日子；忘不了我们共同在一起讨论实验的日子；忘不了每天午饭我们海阔天空的时光；忘不了我们一起玩驴牌的日子；忘不了那点点滴滴汇聚在一起的快乐时光。。。

“饮其流者怀其源，学其成时念吾师”。非常感谢李喜文先生和李迎化老师十多年来在生活和工作上对我们的支持和鼓励。虽然李喜文先生已经仙逝，但他的音容笑貌永远和我们在一起。

感谢我的妻子李娜在这些年来的支持和鼓励，很抱歉这些年我不断失眠给你带来的痛苦，愿你每天都能睡个好觉。感谢你这些年来在学业上与我并肩作战，每次和你讨论实验都感觉受益匪浅，都有着无尽的期待和前进的力量。

感谢父母对我无限的关怀，支持和鼓励。非常抱歉这么些年来一直没能陪伴在你们的身边，在你们生病的时候也没有陪在床前，而让你们承受着空巢的痛苦。感谢我的妹妹对我工作生活的支持，关心和挂念。感谢我的岳父母对我们生活学习的支持、理解和关怀。

Curriculum vitae

Wei Liu (刘巍) was born in Jilin City, Jilin Province, China on August 7, 1976. From 1982 to 1995, he enjoyed his childhood and finished the primary and middle school studies in his hometown. From 1995 to 1999, he studied biotechnology in Department of Life Science, North-East Normal University, China for his B. Sc. degree. After graduation, he worked in Jilin Eurasia Agriculture Co., Ltd. as a technician. From 2002, he joined Wei-Cai Yang's lab in the Institute of Genetics and Developmental Biology (IGDB), Chinese Academy of Sciences (CAS) as a graduate student under a master and doctoral combined program. In 2006, he was selected in the Chinese Academy of Sciences - Royal Netherlands Academy of Arts and Sciences (CAS-KNAW) joint PhD program. From 2007 to 2011, he joined Rene Geurts' group in the Laboratory of Molecular Biology, Wageningen University as a sandwich PhD fellow. From February 2012 to date, he joined Wei-Cai Yang's lab in IGDB, CAS as a guest researcher and will be a postdoc researcher after receiving the doctorate. His current projects mainly focus on the proteomics analysis of the root nodule symbiosomes in soybean, as well as the molecular mechanisms of *Parasponia-Rhizobium* symbiosis, which is funded by the National Natural Science Foundation of China- Netherlands Organization for Scientific Research (NSFC-NWO) joint project.

List of publications

Liu, W., Kohlen, W., Lillo, A., Op den Camp, R., Ivanov, S., Hartog, M., Limpens, E., Jamil, M., Smaczniak, C., Kaufmann, K., Yang, W.-C., Hooiveld, G.J., Charnikhova, T., Bouwmeester, H.J., Bisseling, T., and Geurts, R. (2011). Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. **Plant Cell 23**: 3853-3865.

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

Ovchinnikova, E., Journet, E.P., Chabaud, M., Cosson, V., Ratet, P., Duc, G., Fedorova, E., **Liu, W.,** Op den Camp, R., Zhukov, V., Tikhonovich, I., Borisov, A., Bisseling, T., and Limpens, E. (2011). IPD3 controls the formation of nitrogen-fixing symbiosomes in pea and *Medicago Spp*. **Mol. Plant Microbe Interact. 24**: 1333-1344.

Untergasser, A., Bijl, G.J.M., **Liu, W.**, Bisseling, T., Schaart, J.G., and Geurts, R. (2012). One-step Agrobacterium mediated transformation of eight genes essential for rhizobium symbiotic signaling using the novel binary vector system pHUGE. **PLoS One 7**: e47885.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Wei Liu**
Date: **26 August 2013**
Group: **Molecular Biology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
nod factor signalling genes in rice (*Oryza sativa*)	Nov 02, 2007
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1,5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day (2007), Wageningen University	Sep 13, 2007
EPS PhD student day (2009), Leiden University	Feb 26,2009
► **EPS theme symposia**	
EPS theme 1 'Developmental biology of plants' Wageningen University	Oct 11, 2007
EPS theme 1 'Developmental biology of plants', Leiden University	Jan 30, 2009
EPS theme 1 'Developmental biology of plants', Wageningen University	Jan 28, 2010
EPS theme 1 'Developmental biology of plants', Leiden University	Jan 20, 2011
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2007
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
Flying Seminars (4X)	Sep 2007- Jun 2008
seminars 2007 (2X)	2007
seminars 2008 (7X)	2008
seminars 2009 (9X)	2009
seminars 2010 (16X)	2010
seminars 2011 (1X)	2011
Workshop: "Systems Biology for Plant Design"	Jul 08-11, 2009
Plant Sciences Seminars series	Sep 2009- Feb 2011
EPS symposium “Ecology and Experimental Plant Sciences 2”	Sep 22, 2009
Symposium ‘The more you see: spectroscopy in molecular Biophysics’	Sep 24, 2009
Symposium Dr. Pim Zabel 'Art meets Science'	Oct 16, 2009
Symposium "Photosynthesis: from femto to Peta and from nano to Global"	Nov 05, 2009
CBSG2012 Seminars "BGI Genomics in China" (H. Yang, S. Huang and R. Luo)	Apr 01, 2010
Systems Biology Day	Jun 16, 2010
Plant physiology Seminars (Koichi Yoneyama, Takahito Nomura and Kaori Yoneyama)	Jun 25, 2010
Mini symposium: How to write a world-class paper	Oct 26, 2010
2nd EPS Cellular Signaling Symposium (Amsterdam)	Nov 04, 2010
Symposium on advances in Life Sciences Technology	Nov 25, 2010
Seminar KEYS 2011 (Jim Haseloff and Drew Endy)	Sep 13, 2011
Symposium ‘Plant Breeding in the Genomics Era’	Nov 25, 2011
► **Seminar plus**	
► **International symposia and congresses**	
8th European Nitrogen Fixation Conference	Aug 30-Sep 03, 2008
11th International Symposium on Nitrogen Fixation with Non-Legumes	Sep 03-04, 2008
► **Presentations**	
2010 EPS theme 1 'Developmental biology of plants' (oral presentation)	Jan 20, 2011
4th international PHD school on plant development (oral presentation)	Oct 05-07, 2011
EPS Autumn School 'Host-Microbe Interactomics' (poster)	Nov 01-03, 2011
► **IAB interview**	Dec 05, 2008
► **Excursions**	
Subtotal Scientific Exposure	*18,3 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Systems Biology: ~Omics Data Analysis	Dec 08-11, 2008
Utrecht summerschool "environmental signaling"	Aug 24-26, 2009
The Legume-Rhizobium Symbiosis: from Molecules to Farmers’ Fields	Oct 18-22, 2010
4th international PHD school on plant development	Oct 05-07, 2011

	Introduction to R for statistical analysis	Oct 24-25, 2011
	EPS Autumn School 'Host-Microbe Interactomics'	Nov 01-03, 2011
►	**Journal club**	
	Literature discussion in Laboratorium of Molecular Biology	2007-2011
►	**Individual research training**	
	Using EMSA to investigate DNA-protein interaction (Gerco Angenent's Lab, PRI, WUR)	Feb 15-Mar 21, 2011
	Subtotal In-Depth Studies	*11,9 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Time Planning and Project Management	Mar 18, Apr 01, Apr. 29, 2008
	BCF career event	May 26, 2011
	Communication with the Media and the General Public (COM-MGP)	Nov 09 Dec 10, 2011
	EPS Career Day (ExPectationS 2011)	Nov18, 2011
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3,1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*