LysM domains mediate lipochitin–oligosaccharide recognition and *Nfr* genes extend the symbiotic host range

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Legume–*Rhizobium* symbiosis is an example of selective cell recognition controlled by host/non-host determinants. Individual bacterial strains have a distinct host range enabling nodulation of a limited set of legume species and vice versa. We show here that expression of *Lotus japonicus* *Nfr1* and *Nfr5* Nod-factor receptor genes in *Medicago truncatula* and *L. filicaulis*, extends their host range to include bacterial strains, *Mesorhizobium loti* or DZL, normally infecting *L. japonicus*. As a result, the symbiotic program is induced, nodules develop and infection threads are formed. Using *L. japonicus* mutants and domain swaps between *L. japonicus* and *L. filicaulis* NFR1 and NFR5, we further demonstrate that LysM domains of the NFR1 and NFR5 receptors mediate perception of the bacterial Nod-factor signal and that recognition depends on the structure of the lipochitin–oligosaccharide Nod-factor. We show that a single amino-acid variation in the LysM2 domain of NFR5 changes recognition of the Nod-factor synthesized by the DZL strain and suggests a possible binding site for bacterial lipochitin–oligosaccharide signal molecules.

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Introduction

Multicellular organisms share common components and strategies that enable them to distinguish between self and nonself and to establish specific cell–cell interactions

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(Ausubel, 2005). We investigated such mechanisms in the plant nitrogen-fixing symbiosis. In this interaction, compatible bacteria (collectively called *Rhizobium*) recognize specific legume hosts and induce development of root nodules, which will host the bacteria and develop into symbiotic organs. The legume–*Rhizobium* symbiotic interaction is a sequential process where development of nodule primordia from dedifferentiated root cortical cells initiating a meristem, and a bacterial invasion process, run in parallel. In most legumes, the process starts with rhizobial attachment to plant root hair tips. Subsequent physiological and morphological responses result in root hair curling, entrapping bacteria in an infection pocket (Oldroyd *et al*, 2005; Miwa *et al*, 2006). Inwards growing infection threads are then formed by the plant and colonized by rhizobia. These infection threads act as rhizobial conduits from which the bacterial symbiont ultimately will be endocytosed into the plant nodule cells. In a fully compatible interaction, symbiosomes consisting of membrane-enclosed rhizobia differentiated into nitrogen-fixing bacteroids will form.

The developmental program leading to nodule organogenesis and infection is controlled by the legume through a suite of nodulin genes which are temporally and spatially regulated (Schultze and Kondorosi, 1998; Colebatch *et al*, 2004; Mergaert *et al*, 2006). In the model legume *Lotus japonicus*, perception of the rhizobial-synthesized lipochitin–oligosaccharide (Nod-factor) is required for initiation of the nodule developmental process through a signaling pathway encoded by seven genes, *SymRK*, *Castor*, *Pollux*, *Nup133*, *Nup85*, *CCaMK* and *Cyclops* (Kistner *et al*, 2005) and downstream of this pathway putative transcription factors encoded by *LjNin* (Schauser *et al*, 1999), *LjNsp1* and *LjNsp2* (Heckmann *et al*, 2006) are required for initiation of nodule organogenesis.

The ability to invade roots of leguminous plants and induce development of root nodules is shared by several bacterial species belonging to both the α - and the β -proteobacteria (Moulin *et al*, 2001). The relationship between the app. 18000 legume species and their bacterial microsymbionts is nevertheless selective and this specificity led to the definition of cross-inoculation groups used to describe the symbiotic diversity of legume–*Rhizobium* symbiosis and to classify bacterial species. Among narrow host range interactions, *Sinorhizobium meliloti* (*S. meliloti*) and alfalfa, together with other *Medics* like *Medicago truncatula*, belong to one cross-inoculation group, whereas *Mesorhizobium loti* (*M. loti*) and *Lotus* spp. like *L. japonicus*, belong to another. Reciprocal nodulation between these two groups does not occur. This catalog of cross-inoculation groups provides a practical overview of legume–*Rhizobium* relationships although broad host range strains like NGR234 (Pueppke and Broughton, 1999) and *Bradyrhizobium* sp strains lacking the common Nod-factor biosynthetic genes (Giraud *et al*, 2007) are difficult to accommodate.

A deeper understanding of the classical symbiotic interaction involving Nod-factor signaling emerged from characterization of the two-way signal exchange. Rhizobial NodD proteins mediate host recognition by interacting with specific flavonoids or isoflavonoids exuded from host roots (Mulligan and Long, 1985; Spaink *et al*, 1989). Flavonoid-activated NodD promotes transcription of bacterial *nod*-genes involved in synthesis and secretion of lipochitin-oligosaccharides, called Nod-factors, required for initiation of nodulation (Mulligan and Long, 1985; Lerouge *et al*, 1990; Spaink *et al*, 1991). The ability of NodD to recognize the type of flavonoid exuded by the plant is one of the primary steps determining host range and expression of a constitutively active *nodD* transcriptional activator can extend the bacterial host range, bypassing flavonoid activation (Cardenas *et al*, 1995). For the rhizobial signal, the length of the Nod-factor carbohydrate moiety, the size and degree of saturation of the acyl chain and substitutions of the reducing and nonreducing glucosamine residues (Supplementary Figure 1) are characteristics for each species and these structural features determine whether the bacteria are able to infect legume plants and which plants are infected (Lerouge *et al*, 1990; Spaink *et al*, 1991; D'Haese and Holsters, 2002 and references therein).

Analysis of loss-of-function mutants has previously shown that the *L. japonicus* *LjNfr1* and *LjNfr5* genes are required for the earliest cellular and physiological responses to *M. loti* and *M. loti* synthesized Nod-factor. Neither root hair deformation, Ca^{2+} spiking nor induction of nodulin gene expression was detected in these mutants (Madsen *et al*, 2003; Radutoiu *et al*, 2003; Miwa *et al*, 2006). The corresponding receptor kinase proteins, *LjNFR1* and *LjNFR5*, were predicted to have a topology where single pass transmembrane domains anchor LysM-containing extracellular domains and intracellular serine/threonine kinase domains (Madsen *et al*, 2003; Radutoiu *et al*, 2003). Combining this prediction with the genetic evidence, a receptor complex composed of *LjNFR1* and *LjNFR5* was proposed to initiate signal transduction in response to Nod-factor (Radutoiu *et al*, 2003).

Here, we investigate the role of Nod-factor receptor genes in the biodiversity of cross-inoculation groups, and examine their role in specifying the legume's ability to distinguish between different rhizobial strains. We show that legume Nod-factor receptors are important components of the two-way signal and recognition processes determining the host range in plant-bacterial symbiotic interactions. We have further examined the selectivity of the predicted extracellular LysM domains of *LjNFR1* and *LjNFR5* in relation to Nod-factor recognition and the ability of rhizobial strains to induce root nodule development. We used domain swaps, amino acid substitutions and modeling to demonstrate that the LysM2 domain of NFR5 is important for Nod-factor perception, and present a model for the binding site.

Results

To understand the basis for host/non-host relations, we aimed to identify components required for extending the host range of wild-type legumes. Two different host plants, *M. truncatula* (*Mt*) and *L. filicaulis* (*Lf*), were genetically transformed with *L. japonicus* (*Lj*) Nod-factor receptor genes, *LjNfr1* and *LjNfr5*, and inoculated with rhizobial strains nodulating *L. japonicus*, but unable to nodulate

M. truncatula and *L. filicaulis* wild-type plants. The range of bacterial strains normally able to induce a symbiotic developmental program in *M. truncatula* plants is limited to strains of *S. meliloti*, and *L. filicaulis* does not form nodules with the genetically modified *R. leguminosarum* biovar *viciae* DZL strain (Pacios-Bras *et al*, 2000, 2003), which, in contrast, induced fully infected although ineffective nodules on *L. japonicus* (Pacios-Bras, 2003 and this study). All the *LjNfr1* and *LjNfr5* gene constructs (Supplementary Table I) were transferred via *A. rhizogenes* into plant cells giving rise to transgenic roots (Stougaard *et al*, 1987; Hansen *et al*, 1989). Composite plants having both wild-type and transgenic roots were tested for their nodulation capacity. The rhizobial strains used to determine host range changes were *M. loti*, the symbiont of *Lotus* species and a modified *R. leguminosarum* bv. *viciae* DZL strain that contains a flavonoid-independent NodD activator, *NodZ* and *NoIL* genes (Supplementary Table II). This strain synthesizes Nod-factors resembling Nod-factor synthesized by *M. loti*. Rhizobium strains that are not natural symbionts of *M. truncatula*, *L. japonicus* or *L. filicaulis*, respectively, were genetically modified with a constitutive NodD activator. This modification ensured Nod-factor synthesis independent of host-secreted flavonoids (Spaink *et al*, 1989).

Host range extension in wild-type *M. truncatula*

Introduction of *L. japonicus* *LjNfr1* + *LjNfr5* into *M. truncatula* resulted in development of nodules on transgenic roots after inoculation with *M. loti* (Figure 1A and Supplementary Figure 2A and Supplementary Table III). Transgenic roots of composite plants (136/499 plants) formed on average 1.7 nodules per plant with *M. loti* (Table I). Inoculation of *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots with *S. meliloti* resulted in 5.8 nodules per transgenic root (43/56 plants). This demonstrated that both *M. loti* and *S. meliloti* were recognized by *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots (Figure 1A and B). Nodules were not observed on *M. loti*-inoculated transformed roots of *M. truncatula* carrying the empty vector or *LjNfr1* and *LjNfr5*, separately (Table I; Supplementary Figure 2B). Additionally, inoculation with *M. loti* *nodC::Tn5*, a modified strain unable to produce Nod-factors, did not result in nodule formation on *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots (Table I). These results demonstrated the involvement of both *LjNFR1* and *LjNFR5* in perception of *M. loti* Nod-factors and in the subsequent triggered nodule development in *M. truncatula*.

Nodulin gene expression in *M. loti* induced *M. truncatula* nodules

Plant genes known as nodulins are sequentially activated or upregulated after compatible rhizobial inoculation or Nod-factor application on host legume roots, and their expression serves as molecular markers for Nod-factor perception, nodule organogenesis and rhizobial infection (Schultze and Kondorosi, 1998; Stougaard, 2000). To confirm that *M. loti*-induced nodules on *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots indeed result from activation of the symbiotic developmental program, the expression of nodulin genes (*Enod11*, *Enod12*, *Enod2*, *N6* and *Nin*) was monitored by quantitative RT-PCR or promoter-GUS activation. *Enod11* and *Enod12* mark the early symbiotic events in root epidermis and cortex, whereas in nodules their expression was

Figure 1 Nodule development on *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots. (A) *M. loti*-induced nodule. (B) *S. meliloti*-induced nodule. (C) *Enod12*-GUS expression at different stages of *M. loti*-induced nodule during development. (D) *Enod12*-GUS expression in the *M. loti*-induced nodules (arrows), but not in the lateral roots (arrowheads). (E) *Enod2*-GUS expression in the *M. loti*-induced nodules (arrows) and lateral root primordia (arrowheads). (F) Section of *M. loti*-induced nodule. (G) Section of *S. meliloti*-induced and -infected nodule. (H) Methylene blue staining of cleared *M. loti*-induced nodule. Arrows indicate peripheral vascular bundles. (I) Methylene blue staining of cleared lateral root. Arrow indicate central root vascular bundles. (J) Cell divisions in the inner cortex initiate nodule primordia (arrow) upon *M. loti* inoculation. Scale bars = 100 µm.

Table I Nodulation frequencies of transformed *M. truncatula* roots

Construct	Rhizobia	Fraction of nodulated plants	Nodules per total plants
Vector	Uninoculated	0/98	0
	<i>M. loti</i>	0/295	0
	<i>M. loti</i>	0/20	0
	<i>nodC::Tn5</i>		
	DZL ^a	0/50	0
<i>LjNfr1</i>	<i>S. meliloti</i>	58/69	5.0
	Uninoculated	0/20	0
	<i>M. loti</i>	0/59	0
	DZL ^a	0/29	0
	<i>S. meliloti</i>	37/43	6.9
<i>LjNfr5</i>	Uninoculated	0/18	0
	<i>M. loti</i>	0/79	0
	DZL ^a	0/30	0
	<i>S. meliloti</i>	34/43	4.5
<i>LjNfr1</i> + <i>LjNfr5</i>	Uninoculated	0/111	0
	<i>M. loti</i>	136/499	1.7
	<i>M. loti</i>	0/51	0
	<i>nodC::Tn5</i>		
	DZL ^a	22/42	3.4
	<i>S. meliloti</i>	43/56	5.8

^a*R. leguminosarum* bv. viciae 5560 strain DZL.

correlated with the presence of infection threads (Journet *et al*, 2001). *N6* expression was associated with plant cell preparation for rhizobial infection (Mathis *et al*, 1999). *Nin* regulates infection thread formation and nodule primordia initiation, whereas *Enod2* was induced at a later time point

upon infection, being expressed in the nodule parenchyma cells during development (Lauridsen *et al*, 1993; Vijn *et al*, 1995; Schauser *et al*, 1999; Marsh *et al*, 2007). *MtEnod11*, *MtN6* and *MtNin* were upregulated in nodules induced by *M. loti* on *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots (Figure 2). Confirming this, the promoter-GUS reporter analyses showed stage- and tissue-specific activation of *Enod12* and *Enod2* nodulin gene promoters in *M. loti*-inoculated *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots. The *Enod12* promoter was induced in root cortical cells where nodule primordia formed and showed a developmental activation pattern typical for indeterminate nodules (Figure 1C and D and Supplementary Figure 2C). The *Enod2* promoter was activated in the young nodule primordia and lateral root meristems (Figure 1E and Supplementary Figure 2D). Activation of genes involved specifically in the nodule developmental program and bacterial infection demonstrated that *M. loti* inoculation induced a symbiotic developmental program in *M. truncatula* roots carrying *LjNfr1* + *LjNfr5* genes.

Ontogeny and anatomy of *M. loti*-induced *M. truncatula* nodules

Nodules induced by *M. loti* on *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots show morphological and anatomical features similar to *S. meliloti*-induced nodules, but with no evidence for bacterial endocytosis into the plant cells as seen for the natural symbiont (Figure 1A, B, F and G). Staining of vascular tissues and microscopical analyses of semi-thin sections revealed branched peripheral vascular bundles connecting the nodules with root vasculature (Figure 1H),

Figure 2 Expression of *MtEnod11* (A), *MtNin* (B) and *MtN6* (C) nodulin genes in roots and nodules of transformed *M. truncatula*. Transcript levels of *MtEnod11*, *MtNin* and *MtN6* nodulin genes were determined by quantitative PCR. Induction of *MtEnod11*, *MtNin* and *MtN6* in nodules formed upon *S. meliloti* inoculation of transformed *M. truncatula* control roots (left, A. rhizogenes empty vector), and in nodules formed upon *M. loti* inoculation of LjNfr1 + Nfr5-transformed *M. truncatula* roots (right). Quantification of expression levels shows the induction of these nodulin genes in both *M. loti* and *S. meliloti* nodules compared to root tissue. Relative expression was determined as normalized ratios of the three nodulin genes and three housekeeping genes. Error bars represent the corresponding upper and lower 95% confidence intervals.

distinguishing them from lateral roots that have a central vasculature (Figure 1I). The *M. loti*-induced nodules originated from inner cortical cells (Figure 1J) and developed opposite protoxylem poles (Supplementary Figure 2D). These features are histological hallmarks of indeterminate *M. truncatula* root nodules induced by *S. meliloti* (Vasse *et al*, 1990; Figure 1B and G). Infection threads were observed using confocal microscopy after inoculation of LjNfr1 + LjNfr5-transformed *M. truncatula* roots with *M. loti* expressing an eGFP marker gene to visualize rhizobia. In contrast to the tubular appearance and root penetration of infection threads (Giovannelli *et al*, 2006) observed upon *S. meliloti* inoculation (3.9 infection threads/cm), most of the *M. loti*-induced infection threads were arrested inside the root hairs or ended in the epidermal cell layer with a swollen sac-like structure often observed when bacterial invasion is unsuccessful (Figure 3A and B). In few cases, some of these infection threads branched and progressed through the root cortical cell layers and reached the nodule primordia (Supplementary Figure 3A). However, no endocytosis of *M. loti* and therefore no symbiosome formation was observed (data not shown). On average, one *M. loti*-induced infection thread was counted per 3 cm of LjNfr1 + LjNfr5-transformed *M. truncatula* root and 10% of the analyzed nodules contained branched infection threads. The histological characteristics of nodules, and the presence of infection threads demonstrated that *M. loti* was recognized as a symbiont by *M. truncatula* roots carrying LjNfr1 + LjNfr5 genes. Hence, expression of LjNfr1 and LjNfr5 genes in *M. truncatula* confers a Nod-factor-dependent extension of host range.

Increased infection thread progression in DZL induced *M. truncatula* nodules

The LjNFR1- and LjNFR5-mediated nodulation of *M. truncatula* was further examined using an alternative microsymbiont, *R. leguminosarum* bv. *viciae* strain DZL, recognized by

L. japonicus (Pacios-Bras *et al*, 2000, 2003) but not by *M. truncatula* (Table I). Similar to our observation using *M. loti*, transfer of LjNfr1 + LjNfr5 extended the host range of *M. truncatula* to include the DZL strain and nodules developed on transgenic roots of 22/42 plants. Transgenic LjNfr1 + LjNfr5 *M. truncatula* roots inoculated with DZL developed on average more nodules (3.4) than those inoculated with *M. loti* (1.7) (Table I).

Confocal microscopy on LjNfr1 + LjNfr5-transformed *M. truncatula* roots inoculated with eGFP marked DZL bacteria (Figure 3D and E) revealed an invasion process similar to the early phases of *S. meliloti* invasion of wild-type *M. truncatula* (Figure 3B and C; Giovannelli *et al*, 2006). Most infection threads were tubular and arrested in root hairs or at the first epidermal cell layer. However, when infection threads were associated with nodule primordia, they progressed (Figure 3D), branched and invaded the nodule primordia (Figure 3E and F). Compared to *M. loti*, a larger number of infection threads were formed (1.6/cm), indicating a more effective interaction between DZL and the new host. Nevertheless, confocal, light and electron microscopy revealed that DZL infection threads end inside the nodules in expanded sac-like structures (Figure 3F-H and Supplementary Figure 3B). Most DZL bacteria colonized the nodule intercellular spaces (Figure 3H and Supplementary Figure 3B), and compared to *S. meliloti* inoculation (Figure 3I and Supplementary Figure 3C), no symbiosomes were observed.

Whereas *M. truncatula* control roots showed no root hair deformations upon inoculation with *M. loti* and DZL, the root hairs of LjNfr1-transformed *M. truncatula* roots showed a minor reorientation of root hair tip growth (Supplementary Figure 4A). The root hairs of LjNfr5-transformed *M. truncatula* roots curled and entrapped *M. loti* or DZL bacteria, without infection thread formation (Supplementary Figure 4B). However, the root hair response was not followed by the activation of *Enod12* gene promoter in *M. loti*-inoculated

Figure 3 Microscopic analyses of *Rhizobium* infection phenotypes on *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots. (A) Infection thread induced by *M. loti* stopped in the epidermal layer (arrow). (B) Infection thread induced by *S. meliloti* penetrates the root (C) *S. meliloti* infected nodule showing mainly endocytosed bacteria. (D) DZL induced infection thread penetrating a nodule primordium. (E) DZL-induced infection threads invaded the nodule tissue in a similar manner as *S. meliloti*. (F) DZL induced infection threads (arrow) penetrate the nodule. Inset: close-up of the nodule tissue with DZL-induced infection threads spreading in-between the cells, ending with a sac-like structure (arrow). (G) Sac-like structure (arrow) formed in the epidermal cell layer at the end of DZL-induced infection thread. (H) DZL bacteria colonizing the nodule intercellular space (arrow) is not endocytosed in the plant cells. (I) *S. meliloti* endocytosis (arrow) from infection thread into the nodule cell. Scale bars = 100 μ m in panels A–F, 2 μ m in panels G and H, and 1 μ m in I. Panels A–E are confocal images showing rhizobia marked with GFP (green), (G–I) are electron micrograph images. Notice bacteria (labeled with GFP) are contained in the infection threads induced by all three *Rhizobium* strains.

transformed roots (Supplementary Figure 4C) showing that activation of this nodulin gene expression requires the presence of both *LjNfr1* and *LjNfr5* (Figure 1C and D and Supplementary Figure 2C). Expression of both *LjNfr1* and *LjNfr5* genes in *M. truncatula* under the control of *L. japonicus* promoters therefore ensured *M. loti* and DZL recognition, infection thread formation and nodule organogenesis, but the resulting nodules arrest in late development before endocytosis and symbiosome formation.

Host range extension in *L. filicaulis*

In contrast to *L. japonicus*, the close relative *L. filicaulis* was not infected by the DZL strain (Pacios-Bras, 2003; Figures 4A and 5A, B, G–I). As a first approach to determine the basis of this difference in DZL strain specificity between *L. japonicus*

and *L. filicaulis*, we tested whether introduction of *LjNfr1* and *LjNfr5* genes into transgenic roots would enable *L. filicaulis* to develop nodules with DZL. At 7 weeks after inoculation, 6.5% of *L. filicaulis* plants transformed with *LjNfr1* + *LjNfr5* had developed nodules with DZL on the transformed roots (23/349 plants), whereas no nodules were observed on transgenic *L. filicaulis* roots (243 plants) transformed with empty vector (Figures 4A, B and 5C, F, J). *L. filicaulis* plants transformed with *LjNfr1* or *LjNfr5* separately formed nodules on 0.65% (1/154) and 1.7% (3/180) of the transgenic roots, respectively (Figures 4B and 5D, E). These results show that both genes contributed to the changed specificity. Control *L. filicaulis* roots transformed with *LjNfr1* + *LjNfr5* or empty vector (Figure 4C) were all nodulated by *M. loti* at comparable, normal efficiencies, showing that transgenic roots of

Figure 4 Receptor-mediated nodulation response of *L. japonicus* and *L. filicaulis*. (A) Nodulation efficiency of *A. rhizogenes* transformed wild-type plants inoculated with either DZL or *M. loti*. (B) Nodulation frequency of DZL inoculated *L. filicaulis* roots carrying the AR12 empty vector, *LjNfr1* or *LjNfr5* individually, or *LjNfr1* + *LjNfr5* together. (C) Nodulation frequency of *M. loti* inoculated *L. filicaulis* roots carrying *Nfr1* or *Nfr5* individually, *Nfr1* + *Nfr5* together or the AR12 empty vector. (D, E) Schematic outline of the chimeric receptor genes composed of segments encoding *L. filicaulis* LysM domains (gray) inserted into *L. japonicus* *Nfr1* and *Nfr5* genes. (F) Complementation efficiency in *Ljnfr1nfr5* double mutants transformed with either *L. japonicus* *LjNfr1* + *LjNfr5* wild-type genes or chimeric *FinG1* + *FinG5* genes and inoculated with either DZL or *M. loti*. (G) Complementation efficiency in *Ljnfr1* mutants transformed with either *LjNfr1* or chimeric *FinG1* gene and inoculated with either DZL or *M. loti*. (H) Complementation efficiency in *Ljnfr5* mutants transformed with either *LjNfr5* or chimeric *FinG5* gene and inoculated with either DZL or *M. loti*. The number of plants scored for nodulation is shown in parenthesis. Error bars represent the 95% confidence intervals.

L. filicaulis can be effectively nodulated and that concomitant transfer of *LjNfr1* and *LjNfr5* receptor genes does not perturb nodulation by the *M. loti* symbiont compatible with both *L. filicaulis* and *L. japonicus*. A reduction of the nodulation frequency of *M. loti* on *L. filicaulis* was observed after separate transfer of *LjNfr1* or *LjNfr5* possibly due to an imbalance between NFR1 and NFR5 receptors (Figure 4C). We conclude that *LjNFR1* and *LjNFR5* act in concert to confer perception of DZL Nod-factor molecules in *L. filicaulis*.

The role of LysM domains in Nod-factor recognition

To investigate whether the LysM domains may bind Nod-factor in a structure-dependent fashion, we took advantage of the ability of closely related *L. japonicus* and *L. filicaulis* hosts to recognize *M. loti* and distinguish DZL. Chimeric genes encoding composite NFR1 and NFR5 proteins were constructed. In these composite receptors, LysM-containing domains of *L. japonicus* (ecotype Gifu) *LjNFR1* and *LjNFR5* were exchanged with corresponding domains from

Figure 5 Nodulation phenotype of *L. filicaulis* and *L. japonicus* plants inoculated with *M. loti* and *R. leguminosarum* bv. *viciae* DZL. (A, B) Root segments showing the nodulation phenotype of *L. japonicus* (A) and *L. filicaulis* (B) when inoculated with *M. loti*. (C) Nodulation phenotype of *L. filicaulis* transformed with *LjNfr1* + *LjNfr5* inoculated with DZL. (D–F) Nodules formed by DZL on *L. filicaulis* transformed with *LjNfr1* (D), *LjNfr5* (E) and *LjNfr1* + *LjNfr5* (F). (G–J) Thin sections of *L. japonicus* (G, I) and *L. filicaulis* (H) or *L. filicaulis* transformed with *L. japonicus* *Nfr1* + *Nfr5* (J) infected by *M. loti* (G, H) or DZL (I, J). Sections were stained with toluidine blue.

L. filicaulis *LfNFR1* and *LfNFR5*, respectively. The chimeric genes were called *Filicaulis in Gifu* or *FinG1* and *FinG5*, respectively (Figure 4D and E). To diminish possible interference from endogenously expressed NFRs (Figure 4B and C), we exploited the non-nodulating *LjNfr1*, *LjNfr5* and *LjNfr1nfr5* double receptor mutants available in *L. japonicus* for *in planta* complementation. *A. rhizogenes*-induced

transgenic roots were inoculated with DZL or the normal *Lotus* symbiont *M. loti*, which provided a positive test for the function of chimeric constructs. Parallel experiments were performed in which mutants were transformed with *LjNfr1* and/or *LjNfr5* genes (Figure 4F–H).

Both *FinG1* + *FinG5* and *LjNfr1* + *LjNfr5* constructs complemented double mutants effectively after inoculation with *M. loti* (Figure 4F), demonstrating that sufficient active proteins were synthesized from both *FinG1* and *FinG5*. Complementation of *LjNfr1nfr5* mutants with *FinG1* + *FinG5* resulted in a low nodulation efficiency with DZL, amounting to 18% of the efficiency obtained with *LjNfr1* + *LjNfr5* genes (Figure 4F). To evaluate the cause of this significant change in specificity, *LjNfr1* and *LjNfr5* single mutants were transformed with *FinG1* and *FinG5*, separately. The complementation efficiency of *FinG1* in *LjNfr1* mutants inoculated with DZL was comparable to the efficiency of *LjNfr1* (Figure 4G). On the other hand, *FinG5* complementation efficiency in *LjNfr5* mutants inoculated with DZL was only 22% of the efficiency obtained with *LjNfr5* (Figure 4H). This corresponds to the low efficiency obtained with *FinG1* + *FinG5* in *LjNfr1nfr5* double mutants inoculated with DZL. Both *FinG1* and *FinG5* complemented as efficiently as *LjNfr1* and *LjNfr5* genes after inoculation with *M. loti* (Figure 4F–H). Taken together with results from transfer of *L. japonicus* *LjNfr1* and *LjNfr5* genes into *L. filicaulis*, these results clearly demonstrated that strain and Nod-factor specificity was provided by NFRs and that specificity could be changed by supplying variants of primarily NFR5 or NFR1 plus NFR5. Furthermore, domain swaps between *L. japonicus* and *L. filicaulis* proteins point at the LysM-containing domain of NFR5 as a major determinant in recognition.

Hybrid Nod-factor receptors

To examine the role of LysM modules in discrimination of Nod-factor, the amino-acid composition of *LfNFR1* and *LfNFR5* domains were compared to *LjNFR1* and *LjNFR5* proteins. Only four and three amino acids differ between the *L. japonicus* and *L. filicaulis* domains exchanged in the experiments described above (Figure 4D and E). *L. japonicus* NFR1 has Ile57, Ile171, Gln192 and Tyr213, whereas *L. filicaulis* NFR1 has Val, Leu, Arg and Asp, respectively. Ile/Val57 is located in the first, less conserved LysM domain, Ile/Leu171 and Gln/Arg192 are located in the third LysM and Tyr/Asp213 is positioned between the third LysM and the transmembrane domain. *L. japonicus* NFR5 has Lys30, Leu118 and Glu230, whereas *L. filicaulis* has Gln, Lys and Lys, respectively. Lys/Gln30 is located between the signal peptide and the first LysM domain, Leu/Lys118 is located in the second LysM domain, whereas Glu/Lys230 is in between the third LysM domain and the transmembrane domain.

For functional analysis, we constructed hybrid receptors combining the amino-acid variation by modification of *FinG1* and *FinG5*. Coding capacities were changed by site-directed mutagenesis such that *L. filicaulis* amino acids were individually replaced with corresponding amino acids from *L. japonicus*. Three hybrid genes were obtained for both *Nfr1* and *Nfr5*, and tested in transgenic roots of *LjNfr1* or *LjNfr5* mutant plants, respectively (Figure 6A and E). All six hybrid receptors were functional and complemented *LjNfr1* and *LjNfr5* mutants inoculated with *M. loti* effectively (Figure 6C and G). As no improvements or detrimental effects were

detected, we infer that different combinations of amino-acid variations between *L. japonicus* and *L. filicaulis* receptors in these hybrids did not significantly perturb domain structures or diminish/enhance NFR1 and NFR5 activity. Substitution of Gln30 with Lys, and Lys230 with Glu, both amino acids located outside of NFR5 LysM domains, did not appreciably alter DZL complementation efficiency compared to plants transformed with *FinG5* (Figure 6A and B). The third hybrid, where a basic hydrophilic Lys118 in the second LysM domain was replaced by a hydrophobic leucine residue, completely

restored complementation efficiency with DZL to *LjNfr5* levels (Figure 6A and B). The three NFR1 *L. filicaulis* to *L. japonicus* Val57 to Ile, Leu171 to Ile and Arg192 to Gln amino-acid replacements were tested without detecting any change in nodulation phenotype with DZL or *M. loti* (Figure 6E–G). We conclude that a Leucine/Lysine difference in amino-acid residue 118 of the second LysM module of NFR5 was largely responsible for the differential response of *L. japonicus* and *L. filicaulis* towards Nod-factor produced by DZL.

Figure 6 Nodulation of *Lotus japonicus* *LjNfr1* and *LjNfr5* mutants transformed with hybrid receptor genes. (A, E) Schematic outline showing domains exchanged between *L. japonicus* (white) and *L. filicaulis* (gray) in domain swap constructs. Positions of amino-acid differences between *L. japonicus* and *L. filicaulis* that were analyzed in hybrid receptor constructs are indicated relative to LysM domains. The less conserved LysM1 of *LjNfr1* is shaded light gray. (B–D) Complementation efficiency in *LjNfr5* mutants transformed with either *LjNfr5* *L. japonicus* wild-type receptor gene, chimeric *FinG5* gene, hybrid receptors where individual *L. filicaulis* amino acids were changed to the corresponding *L. japonicus* amino acid or the *LjNfr5*Leu118Ala gene. (F, G) Complementation efficiency in *LjNfr1* mutants transformed with either *LjNfr1*, chimeric *FinG1* gene or hybrid receptors where individual *L. filicaulis* amino acids were changed to corresponding *L. japonicus* amino acid. Roots were inoculated with either DZL (B, F) or *M. loti* (C, G). The number of plants scored for nodulation is shown in parenthesis.

Figure 7 Model of the *L. japonicus* NFR5 LysM2 domain and structure of Nod-factors produced by *R. leguminosarum* bv. *viciae* DZL and *M. loti*. (A) Surface representation of the model colored according to residue type: gray is hydrophobic, yellow is polar, red is acidic and blue is basic. The proposed Nod-factor binding site is highlighted and residues along this cleft with a plausible role in binding are labeled along with the important L118. (B, C) Differences in substitution of Nod-factor secreted by *R. leguminosarum* bv. *viciae* DZL (B) and *M. loti* (C). Numbering of Nod factor substitutions according to D’Haeze and Holsters (2002).

The importance of Leu118 for recognition of the DZL Nod-factor was further examined by changing the coding capacity of *LjNfr5* by site-directed mutagenesis. Leu118 was replaced by an alanine residue and the complementation

efficiency of this gene construct (*LjNfr5*Leu118Ala) was compared to the efficiency of the wild-type gene construct in an *LjNfr5* mutant. When inoculated with *M. loti* there was no detectable difference in complementation efficiencies between the *LjNfr5*Leu118Ala gene and the wild-type *LjNfr5* gene showing that Ala-substituted NFR5 was active. In contrast, a significantly reduced complementation efficiency of less than 15% was obtained with DZL (Figure 6D). This confirms the results obtained using FinG5 hybrids and provides independent evidence for the role of Leu118 in determining DZL Nod-factor specificity.

Homology model of the NFR5 LysM2 domain

A LysM domain structure from *Escherichia coli* membrane-bound MltD was solved by NMR (Bateman and Bycroft, 2000). We used this structural information to make a homology model of *LjNFR5* LysM2 (Figure 7A and Supplementary data) and identified a possible Nod-factor-binding cleft containing several aromatic residues (Y114, F124, Y130 and F159) and a conserved Asp (D120). The variable L/K118 is positioned at the entry/exit of the suggested binding cleft and would therefore be in a favorable position for interacting with substituents at the reducing or nonreducing end of Nod-factor known to determine *in vivo* specificity (D’Haeze and Holsters, 2002). The engineered DZL strain decorates pentameric Nod-factors with an acetylated fucosyl at C6 of the reducing sugar moiety (R6), an acetyl at C6 (R5) and an 18:1 or 18:4 acyl chain at C2 (R1) of the nonreducing sugar (Pacios-Bras, 2003; Figure 7B). *M. loti* synthesizes pentameric Nod-factors with an acetylated fucosyl at C6 (R6) of the reducing sugar, a hydrogen at C6 (R5), a carbamoyl at C4 (R4) and a *N*-methylated 18:1 acyl chain at C2 (R1 and R2) of the nonreducing sugar (López-Lara *et al*, 1995; Figure 7C). The structural differences between the *M. loti* and DZL Nod-factors are thus substitutions at C2, C4 and C6 positions of the nonreducing moiety. Taken together with our model, this predicts an orientation of Nod-factor with the nonreducing end at the L/K118 entry/exit of the *LjNFR5* LysM2 groove. We propose that the differences in these decorations at the nonreducing end influence affinity of the *M. loti* and DZL Nod-factors at the NFR5 LysM2 domain and that this affinity is also influenced by the amino acid present in position 118 of the LysM2. Supporting this notion, a change in strain specificity determined by the saturation of the acyl chain at C2 and the acetate at C6 of the nonreducing end has previously been reported (Spaink *et al*, 1991; Ardourel *et al*, 1994). It is now important to resolve the role of the binding site suggested here and the Nod-factor docking site suggested for the *M. truncatula* NFP LysM domain (Arrighi *et al*, 2006).

Discussion

The results presented show that *LjNFR1* and *LjNFR5* receptors act in concert as host determinants, transforming the non-hosts *M. truncatula* and *L. filicaulis* into hosts able to recognize and be infected by the *L. japonicus* symbionts *M. loti* or DZL and DZL, respectively. Recognition of these normally noncompatible bacteria triggered root cell dedifferentiation, redifferentiation and initiation of the developmental program leading to *de novo* nodule organogenesis. This extended NFR-mediated signal cascade depended on both Nod-factor presence and structure as shown with the

M. loti nodC mutant in *M. truncatula* and DZL in *L. japonicus* mutants complemented with chimeric and hybrid receptors. We have further demonstrated the crucial role of the putative extracellular LysM-containing domains of the NFR1 and NFR5 receptor kinases that were responsible for perceiving the rhizobial signal in the epidermal root hairs (Radutoiu *et al*, 2003; Miwa *et al*, 2006). Specific recognition of rhizobial bacteria by NFR1 and NFR5 receptors relied on these LysM domains and the LysM2 domain of NFR5 had a major function in discrimination of *M. loti* and DZL Nod-factors. A single amino acid, Leu118 of the NFR5 LysM2 domain, was found to be largely responsible for the specificity. Interestingly, LysM2 of NFR5 is the most diverged LysM domain among NFR1 and NFR5 homologs found in other plant species (Madsen *et al*, 2003) and during evolution of plant symbiosis, this flexibility may have allowed specificity in Nod-factor perception to emerge. The functional role of the other LysM domains present in NFR1 and NFR5 was not revealed by our studies. The presence of three LysM domains in the *LjNFR5*, *MtNFP* and two conserved and one more variable LysM in the *LjNFR1*, *MtLYK3*, *MtLYK4* receptors characterized so far (Limpens *et al*, 2003; Madsen *et al*, 2003; Radutoiu *et al*, 2003; Arrighi *et al*, 2006), suggests a mechanism involving more than one LysM domain in Nod-factor perception. The non-nodulation phenotype caused by amino-acid substitutions in the LysM1 domains of the *MtNFP* and *MtLYK3* proteins supports this notion (Arrighi *et al*, 2006; Smit *et al*, 2007). Furthermore, we report here the requirement for both the *LjNfr1* and *LjNfr5* genes for nodulation of *L. filicaulis* by the DZL strain and for changing the non-host *M. truncatula* into a host for the *Lotus* microsymbionts *M. loti* and DZL.

Recognition of the normally noncompatible bacteria *M. loti* and DZL in *M. truncatula* induced infection thread formation, but endocytosis was not observed. Compared to *M. loti*, the engineered *R. leguminosarum* bv. *viciae*, strain DZL, induced an increased number of nodules and an infection process that progressed further through the cortical cells and invaded the nodule space of *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots. This difference may be due to the particular structural similarity of the DZL Nod-factor to the *S. meliloti* Nod-factor that differs only in the type of acyl chain and the acetyl-fucose decoration (Supplementary Figure 1). After the epidermal recognition ensured by *L. japonicus* receptors, perception of the DZL Nod-factor may function more effectively with endogenous *M. truncatula* signaling components than the *M. loti* Nod-factor. In *M. truncatula*, 17 genes encoding LysM receptor-like kinases similar to the *LjNFR5* or *LjNFR1* receptor proteins have been identified, 8 of them being expressed in roots and nodules (Arrighi *et al*, 2006) and the possibility that one or more of these putative receptors could promote infection thread progression and cortical invasion process of DZL, cannot be excluded. The *MtLYK3* protein suggested to interact with the nonreducing end of *S. meliloti* Nod-factor and to promote infection thread progression in *M. truncatula* could be such an example (Smit *et al*, 2007). In addition to Nod-factor, specific surface carbohydrates are required for rhizobia to become efficient symbionts (Pellock *et al*, 2000; Laus *et al*, 2004; Mathis *et al*, 2005) and this may account for differences observed between *M. loti* and DZL. Like the DZL strain on transgenic *M. truncatula*, several lipopolysaccharide-deficient (*lps*) mutants of

S. meliloti induce infection threads penetrating the core nodule tissue and a Fix⁻ phenotype (Campbell *et al*, 2003), but in contrast to the DZL strain, the *lps* mutants are usually endocytosed (Niehaus *et al*, 1998). The early arrest of infection thread growth observed in *M. loti*-inoculated transgenic *M. truncatula* resembles the phenotype reported for exopolysaccharide-deficient (*exo*-) *S. meliloti* mutants in wild-type alfalfa (Cheng and Walker, 1998). On the plant side, lectins were shown to be involved in host range presumably by recognizing bacterial exopolysaccharides (Diaz *et al*, 1989; van Rhijn *et al*, 2001). Transfer of the soybean *Le1* gene into *L. corniculatus* resulted in a *Bradyrhizobium japonicum*-induced nodulation phenotype reminiscent of *LjNfr1* + *LjNfr5*-transformed *M. truncatula* roots inoculated with *M. loti* (van Rhijn *et al*, 1998). Our results are consistent with the notion that increased binding of *B. japonicum* Nod-factor, which is similar but not identical to the *M. loti* Nod-factor, to pass the threshold required for perception by *L. corniculatus* (van Rhijn *et al*, 1998).

Our results provide circumstantial evidence that NFR1 and NFR5 are components of the same receptor complex or alternatively that balanced signaling through two independent NFR1 and NFR5 receptors is crucial. First, inefficiency of a receptor complex composed of NFRs from the two *Lotus* species in perception of DZL Nod-factor may explain the difference in nodulation efficiency of DZL and *M. loti* in *LjNfr1* + *LjNfr5*-transformed *L. filicaulis* (Figure 4B and C). Secondly, an imbalance between NFR1 and NFR5 may cause the reduced nodulation efficiency observed in *M. loti*-inoculated *L. filicaulis* plants transformed with *LjNfr1* and *LjNfr5*, separately (Figure 4C). The observed reduced complementation efficiency of *LjNfr1* mutants transformed with *LjNfr1* (Figure 4G) further implies that Nod-factor perception is particularly sensitive towards NFR1 expression levels or NFR1-mediated signaling. Our results demonstrate a higher nodulation frequency with the DZL strain in *M. truncatula* transformed with *LjNfr1* and *LjNfr5* than observed in similarly transformed *L. filicaulis* plants. This difference may suggest a higher level of (ineffective) complex formation between endogenous *L. filicaulis* receptors and *LjNFR1* and *LjNFR5* receptors expressed from transgenes in *L. filicaulis* than in the more distantly related *M. truncatula*. Although complex formation between NFR1 and NFR5 components would explain these results, a final verification of the Nod-factor receptor composition and its interacting partners await a detailed biochemical analysis. At the amino-acid sequence level there is certainly sufficient variation to account for differences in protein-protein interactions. NFR5 proteins of *L. japonicus* and *L. filicaulis* are 99% identical (591 out of 595 amino acids) compared to the 76% amino-acid identities between the corresponding *L. japonicus* NFR5 and *M. truncatula* NFP proteins (Madsen *et al*, 2003).

Adding further complexity, involvement of a third component is likely to account for the incomplete change in DZL specificity observed in the *L. japonicus* genetic background when complementing *LjNfr1nfr5* double mutants with *FinG1* + *FinG5*. The 18% nodulation efficiency in *L. japonicus* is significantly different from the absence of DZL nodulation seen in the *L. filicaulis* background. At present, the identity and function of this third component differing between *L. filicaulis* and *L. japonicus* is undescribed, but both a direct

and an indirect involvement in Nod-factor recognition appears possible. A Nod-factor binding protein presenting the Nod-factor ligand at the receptor binding sites would serve a direct function. The high-affinity Nod-factor-binding sites identified in *Medicago* may represent such a function (Gressent *et al*, 1999). A more effective signal transduction component enabling nodulation of *L. japonicus* at a Nod-factor signaling level that was below the threshold in *L. filicaulis* would be an example of an indirect function. Similar differences in Nod-factor perception between closely related legume host plants have previously been observed between *M. truncatula* and alfalfa (*Medicago sativa*). These two *Medicago* species differ in their sensitivity towards unsulfated Nod-factor produced by *S. meliloti nodH* mutants and secondary components or threshold values were suggested to account for the observed difference (Wais *et al*, 2002).

The *in planta* assays used in this study measure NFR-mediated Nod-factor perception in its biological context and in addition to the factors mentioned above the results may also be influenced by the variable amounts of Nod-factor secreted by rhizobia and Nod-factor degradation by host chitinases or hydrolases (Ovtsyna *et al*, 2000). To promote a better understanding of the quantitative and structural requirement for Nod-factor function and ligand-binding site interactions we have modeled the NFR5 LysM2 structure. Based on the *E. coli* MltD LysM structure (Bateman and Bycroft, 2000), modeling of the NFR5 LysM2 domain predicts a cleft for Nod-factor binding and positions the L/K118 amino acid participating in the *in vivo* recognition of Nod-factor structure at the entry or exit of the groove. A similar homology model of NFR5 LysM2 based on the crystal structure of the *B. subtilis* ykuD protein supports our binding site prediction (Supplementary Figure 5). The most likely interpretation is a structure-dependent binding of Nod-factor at the LysM2 domain and this is in accordance with studies in both bacteria and plants. LysM domains from bacterial autolysin bind peptidoglycans and more recently the high-affinity chitin-binding protein CEBiP from rice, involved in perception of chitin oligosaccharide elicitors, was shown to carry two LysM domains (Kaku *et al*, 2006). Further characterization of these putative binding sites is a challenge for the future and *in vitro* biochemical investigations of the binding affinities and kinetics as well as high-resolution structures of the LysM domains will be required for a detailed understanding of Nod-factor receptor function during nodule organogenesis.

Materials and methods

Plant material, hairy root transformation and nodulation tests

The *Ljnr1* and *Ljnr5* loci are in *L. japonicus* Gifu ecotype background (Madsen *et al*, 2003; Radutoiu *et al*, 2003). Homozygosity for mutant alleles was confirmed by sequencing of PCR fragments spanning the point of mutation. Transgenic hairy roots on *Lotus* and *Medicago truncatula* plants (cultivar J5), were generated using the *Agrobacterium rhizogenes* strain AR12 or AR1193 (Stougaard *et al*, 1987; Hansen *et al*, 1989). For nodulation tests in Magenta containers (Sigma), seedlings with emerging hairy roots were planted in a 3:1 mixture of Leca (Optiroc):Vermiculite and supplemented with 70–80 ml of 1/4 strength B&D medium (Radutoiu *et al*, 2003). Nodulation and gene expression were assayed after 40–42 days of growth at 21/16°C and 16/8 h day/night regime. *Medicago* experiments: four composite plants were transferred to one Magenta box supplemented with 1 or 0.1 mM KNO₃; each plant was inoculated with 700 µl of *Rhizobium* inoculum, OD₆₀₀ = 0.01–0.02. *Lotus* experiments: after planting in Magenta

boxes supplemented with 1 mM KNO₃, 2.8 ml inoculum (OD₆₀₀ ~ 0.01–0.02) of NZP2235 or DZL was added per Magenta containing seven seedlings; to verify the presence and identity of DZL bacteria in nodules of *L. filicaulis* transformed with *L. japonicus Nfr1+Nfr5*, nodules were surface sterilized in hypochlorite (0.6–1.4% active chlorine) for 10 min, washed three times for 10 min in sterile water, crushed in sterile water and plated on YMB medium supplemented with appropriate antibiotics. An aliquot of the 3rd wash was plated as a sterilization control.

Rhizobial strains

M. loti NZP2235 and *S. meliloti* 2011 or 1021 (Supplementary Table II) were grown to high density in liquid YMB supplemented with tetracycline 2 µg/ml for *S. meliloti* at 28°C with shaking for 2 days before use. *R. leguminosarum* DZL was grown at 28°C on solid YMB supplemented with rifampicine 20 µg/ml, spectinomycin 100 µg/ml, tetracycline 2 µg/ml for 2 days and then transferred at high density to liquid YMB without selection and grown for 3–4 h at 28°C with shaking before use.

Microscopic analyses

Enod2-GUS and *Enod12-GUS* activity in transgenic roots was assayed according to Kosugi *et al* (1990). Whole-root and -nodule analyses were made by staining with 0.01% aqueous solution of methylene blue, followed by clearing with 2.4% sodium hypochlorite for 3 min and visualization under stereomicroscope. Sectioning of roots and nodules was carried out as described previously (van Spronsen *et al*, 2001). For infection thread screening, fresh, 1 cm long segments of transformed roots were observed using a Zeiss fluorescence microscope with FITC filters. Hand-cut sections of nodules were optionally stained with propidium iodide (0.1% (v/v), Fluka) in PEM buffer (PIPES 0.05 M, EGTA 1 mM, MgCl₂ 0.5 mM, pH 6.9) with 0.01% (v/v) Triton X-100, for 30 min at room temperature. Images were collected with the Zeiss LSM 510 Meta confocal microscope (488 nm excitation, 505–530 bandpass filter for GFP emission and 585–620 nm bandpass filter for propidium iodide emission). The number of root segments analyzed for presence of infection threads is presented in Supplementary Table IV. Electron microscopy was according to James and Sprent (1999).

Expression analyses

Total RNA was isolated using Trizol (Sigma-Aldrich) and RNA was treated with RQ1-DNase (Promega). Transcript levels were determined by quantitative real-time RT-PCR (Radutoiu *et al*, 2003). All cDNA samples were tested for contaminating DNA using PCR primers specific for the *LYK3* gene promoter. Primers for transcript amplification are listed in Supplementary Table V. For each sample, normalized relative ratios of nodulin genes and three independent housekeeping genes (*Protein phosphatase2A*, *TIP41* and *Tubulin beta-chain*; Czechowski *et al*, 2005) were calculated using the Relative Quantification Software (Roche). The geometric mean of relative expression ratios for three biological, and three technical repetitions and corresponding upper and lower 95% confidence intervals were calculated (Vandesompele *et al*, 2002).

Construction of chimeric Nfr receptor genes

The *FinG1* and *FinG5* genes were based on the *Nfr1* and *Nfr5* complementation construct (Madsen *et al*, 2003; Radutoiu *et al*, 2003), which were modified using standard cloning techniques and transferred into the pIV10 integration vector (AM235368). The constructs were integrated into *A. rhizogenes* strain AR12 (Hansen *et al*, 1989). *FinG1* was constructed as follows: a DNA fragment from position 4090 to position 4993 of the *Nfr1* gene (AJ575246/AJ575247) was substituted by the corresponding fragment from *L. filicaulis*. *FinG5* was constructed as follows: a DNA fragment from position 1267 to position 2102 of *Nfr5* (AJ575254) was substituted by the corresponding fragment from *L. filicaulis* produced by PCR.

Construction of hybrid Nfr receptor genes

The GeneEditor™ *in vitro* Site-Directed Mutagenesis System (Promega) or a PCR-based method (Ke and Madison, 1997) were used for site-directed mutagenesis using *FinG1* and *FinG5* genes as starting constructs. Polymorphic amino acids between *L. filicaulis* and *L. japonicus* were replaced individually by changing the coding nucleotide triplets. All constructs and the transformed roots were checked by sequencing for the presence of the characteristic codon.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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