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ORIGINAL PAPER

Expression of the Legume-Specific Nod Factor Receptor Proteins Alters Developmental and Immune Responses in Rice

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Abstract

Legumes form symbiosis with rhizobia, which fix nitrogen for the benefit of host plant in return for carbon resources. Development of this unique symbiosis in legumes is triggered by rhizobia-secreted nodulation (Nod) factors (NFs). NFs, upon perception, activate Nod signaling cascade, leading to reprogramming of host cell (root) developmental networks to pave way for accommodating rhizobial symbionts. A long-cherished goal of legume-rhizobia symbiosis research is to extend this symbiotic nitrogen-fixing capacity to cereal plants such as rice. As a part of achieving this ultimate goal, in this work, initially we expressed legume-specific Nod factor receptor protein (NFRP) genes, *MtNFP*, *MtLYK3*, and *LjLNP*, in rice and assessed their impact on NF perception and consequently triggered biological responses in roots. RNA-seq analysis revealed that roots of both control and NFRP-expressing plants perceive NFs, but NFs elicited contrasting impacts on gene expression patterns in roots of these plants. In contrast to suppressive role of NFs on expression of several genes involved in innate immune response in roots of control plants, in NFRP-expressing plants, NFs triggered massive upregulation of a vast array of genes associated with signaling, defense response, and secondary metabolism networks in roots. Expression of NFRPs in rice also conferred root hairs the ability to respond to NFs in terms of exhibiting deformations, albeit at low levels. Together, results of the study demonstrated that rice plants have inherent ability to perceive NFs, but the expression of legume NFRPs rendered rice roots hypersensitive to NFs.

Keywords *Oryza sativa* · Legume Nod factor receptor proteins · Rice transformation · Nod factor perception · Symbiosis · Transcriptome

Key Message

• Extensive dissimilarities in gene expression patterns in control and NFRP-expressing rice plants in response to NFs suggest that the "putative NF receptors" of rice and the "NF receptors" of legumes transmit Nod signal differently, activating distinct signaling pathways to elicit disparate gene expression cascades in rice roots.

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Introduction

Rice (*Oryza sativa* L.) is one of the most widely cultivated cereals in the world in a range of climatic zones, and it is a staple food for nearly 50% of the human population. Nitrogen (N) is an essential nutrient for rice production and plays a crucial role in sustaining high yields. At current levels of N use efficiency, it is estimated that about 70% more N fertilizer will be required to increase rice production by 2050 to meet the food demands of growing human population (Africare and Oxfam 2010). Considering the existing practice, it is normal to envision that the increase in need for N to ensure crop production will be supplied by industrially produced synthetic N fertilizer. Excessive use of synthetic fertilizers not only entails high economic costs but also contributes to environmental degradation including climate change. To avoid these problems, an apt option to provide the N fertilizer requirement is via biological nitrogen fixation (BNF) (Ladha and Reddy 1995; Beatty and Good 2011). Thus, it is envisioned to construct a BNF system, such as the one similar to rhizobial symbiosis with legumes or through direct *nif* (nitrogen fixation) gene transfer, in rice plant itself to substantially minimize our dependence on fertilizer-N.

Legume plants form a symbiotic relationship with rhizobia, which fix atmospheric nitrogen and supply it to the host plant thereby effectively freeing the host legume from dependency on exogenous N supplies (Oldroyd 2013). Legumes, besides forming N_2-fixing root nodule symbiosis (RNS) with rhizobia, are also able to develop symbiotic associations with mycorrhizal fungi to form arbuscular mycorrhizae (AMS) to facilitate phosphate acquisition (Markmann and Parniske 2009). During the formation of rhizobial symbiosis, flavonoids secreted by legume roots activate production of rhizobial lipo-chitooligosaccharide (LCO) molecules known as Nod factors (NFs), which upon perceived by the root cell plasma membrane-anchored NF receptor kinases (MtLYK3/LjNFR1 and MtNFP/LjNFR5 in the model legumes *Medicago truncatula* or *Lotus japonicus*, respectively) trigger signaling cascade to initiate the plant processes necessary for the development of RNS (Oldroyd 2013). In *Lotus japonicus*, another unique receptor protein known as lectin nucleotide phosphohydrolase (LjLNP) has also been shown to participate in NF perception (Roberts et al. 1999). In legumes, acting downstream of the NF receptors are a conserved set of gene products that participate in the development of both RNS and the more widespread and ancient AMS (Oldroyd and Downie 2008; Markmann and Parniske 2009). Thus, the signaling pathway mediated by these genes that promote both RNS and AMS is termed as "common symbiosis pathway" (CSP) (Markmann and Parniske 2009). Although rice cannot develop symbiotic association with rhizobia, it can interact symbiotically with mycorrhizal fungi to form AMS (Gutjahr et al. 2008). It has been shown that CSP that mediate AMS formation in legumes is also conserved in rice and plays a similar role in promoting AMS development in rice roots (Banba et al. 2008; Chen et al. 2007, 2008, 2009; Gutjahr et al. 2008; Yano et al. 2008). Therefore, these findings imply that a part of the signal transduction pathway needed for RNS is conserved in rice, and this genetic network can form building blocks to add on additional genetic circuits needed for the development of rhizobial symbiosis in rice. At present, there is enhanced interest in ascertaining whether rhizobia would be able to form symbiosis with monocot like rice and carry out N_2 fixation (Reddy et al. 2013; Rogers and Oldroyd 2014).

In rice, homologues of the legume NF receptor kinases have been identified (i.e., OsCERK1 and OsNFR5, the rice homologues of the *Medicago truncatula* LYK3 and NFP, respectively) (Miyata et al. 2014, 2016; Zhang et al. 2015). Nevertheless, their extracellular domains (ECDs) are poorly conserved in comparison with the ECD regions that recognize NFs in the legume NF receptor kinases (the ECD of OsCERK1 is only 40% identical to the ECD of MtLYK3 at amino acid level, while the ECD of OsNFR5 exhibited 47% identity to the ECD of MtNFP), even though kinase domains (KDs) are reasonably well preserved (the KD of OsCERK1 is 74% identical to the KD of MtLYK3, while the KD of OsNFR5 exhibited 45% identity to the KD of MtNFP). Similarly, homologue of LjLNP, another NF binding receptor protein (a member of a distinct class of apyrases from legumes; Roberts et al. 1999) is also poorly conserved in rice. The closest homologue of the *Lotus japonicus* LNP in rice is an apyrase (AK066262), which showed only 48% identity at amino acid level with LjLNP; particularly, it is very lowly conserved at the N-terminal region. The absence of suitable NF receptors that are able to perceive and transmit rhizobial Nod signals may be one reason for the inability of rice roots to interact symbiotically with rhizobia. Thus, we hypothesized that if proper Nod factor receptor proteins (NFRPs) are expressed in rice plant, then, the roots may be able to perceive and transmit Nod signal appropriately to the downstream components of CSP to trigger symbiotic responses.

Hence, in this work, we have expressed in rice plants the legume NF receptor genes involved in perception and early Nod signal transduction, and assessed their ability in promoting symbiotic reactions and transcriptional changes in transgenic rice roots in response to NFs. Incorporation of genes involved in the perception of nodulation signals allows us to expand our knowledge on the ability of rice to interact symbiotically with rhizobia and establish the groundwork for assembling symbiotic nitrogen fixation in rice.

Materials and Methods

Construction of Plant Transformation Vector Carrying Multi-gene Expression Cassettes

The coding sequences of *MtNFP* (DQ496250.1) (Arrighi et al. 2006), *MtLYK3* (AY372406.1) (Limpens et al. 2003),

MtSYMREM1 (JQ061257.1) (Lefebvre et al. 2010), and *LjLNP* (AF156780.1) (Roberts et al. 1999) were amplified by RT-PCR using gene-specific primers (Supplementary Table S1) based on the mRNA sequences available in databases and cDNA prepared from total RNA derived from the roots of *Medicago truncatula* A17 or *Lotus japonicus* MG-20. *MtNFP* was amplified using the forward 5′-AGATCTATGTCTGCCTTCTT TCTTCCTTCTAG-3′ and reverse 5′-GGTACCTTAACGAG CTATTACAGAAGTAACAAC-3′, MtLYK3 with the forward 5′-GAGCTCATGAATCTCAAAAATGGATTAC-3′ and reverse 5′-ACTAGTTCATCTAGTTGACAACAGATTTATG-3′, MtSYMREM1 with the forward 5′-AGATCTGCTGTAAC CCTAGAGATACATTATGG-3′ and reverse 5′-CCCG GGCTAACTGAAAAACCTTAAACCGCTGA-3′, and *LjLNP* with the forward 5′-CTCGAGGCATTGGACTAAAG CCATGG-3′ and reverse 5′-CCCGGGCAGGCTTA AGCAGGTACTAGTA-3′ primer sets. The *OsRCg2* promoter, 1656-bp region upstream to the ATG codon of the *OsRCg2* gene (LOC_Os10g40430) (Xu et al. 1995), was isolated from genomic DNA of rice (*Oryza sativa*, cv. Murasaki R86) by PCR using promoter specific forward (5′-ACCGGTCTGCAGCT GATCTCAACAGT-3′) and reverse (5′CTCGAGGG ATGCAGCTAGCGAGCTAGTGAT-3′) primers (Supplementary Table S1). All PCR amplifications (see Supplementary Table S2 for PCR conditions) were performed using Advantage-2 polymerase mix (Takara Bio USA, Inc.). Restriction sites were included into the PCR primers to enable cloning of the amplified DNA products. All PCR-amplified promoter or gene products were initially cloned into pGEM-T Easy vector (pGEMTE; Promega, Madison, USA) and sequence-verified to confirm their fidelity, prior to assembling them into intermediate vectors for generating expression cassettes.

The plant transformation vector carrying the linked expression cassettes of the legume nodulation-specific *MtNFP*, *MyLYK3*, *MtSYMREM1*, and *LjLNP* genes was constructed utilizing the pSAT-RCS2-HPT modular vector system (Chung et al. 2005). Initially, intermediate vectors were modified as described below to make them amenable for receiving the PCR-amplified genes and promoters for assembling them into expression cassettes. Modification of pUbi.Tm1: A part of the polylinker/multicloning site (MCS: BamHI, XmaI, and KpnI) downstream of the maize ubiquitin promoter in the pUbi.tm1 vector (Wang and Waterhouse 2000) was replaced with a new synthetic polylinker containing BamHI, SpeI, XmaI, and KpnI sites (Supplementary Table S3) to generate pUbi-MCS-Tm1. Development of pWAct1-MCS-nosT: First, the rice Actin1 promoter from pWAct.GUS (M-B. Wang, CSIRO Plant Industry, Canberra, Australia, cited in Schünmann et al. 2003) was amplified using PrAct1-For (5′-AGCATACTCGAGGTCATTCATATG-3′) and PrAct1-Rev (5′-CTCCATGGGTCGACTCTACCTACAAAAAAGCT CCGCACG-3′) primers (Supplementary Table S1), and then, the PCR product was digested with XhoI and NcoI, and re-cloned into the correspondingly digested pWAct.GUS, replacing the original Act1 promoter; this cloning step permitted the insertion of a SalI site upstream of NcoI site at the 3′-end of the Act1 promoter in the pWAct.GUS. This newly modified vector was designated as pWActS-GUS. Subsequently, the GUS coding sequence in the pWActS-GUS was excised out by digesting with SalI and KpnI, and replaced with a similarly digested synthetic polylinker containing the MCS of SalI, XbaI, XmaI, SpeI, and KpnI (Supplementary Table S3), resulting in the plasmid pWAct1-MCS-nosT. Development of the promoterless pS3AWoP-masT, pS4AWoP-35ST, and pS6AWoP-agsT vectors: Mannopine synthase (mas), 35SCaMV, and RbcS promoters in the pSAT3A, pSAT4A, and pSAT6A (Chung et al. 2005), respectively, were excised out using AgeI and BglII and replaced with a synthetic AgeI/BglII adaptor (5′-CCGGTCTCCAGTGACATA-3′ and 5′-GATCTATGTCACTGGAGA-3′) (Supplementary Table S3), giving rise to pS3AWoP-masT, pS4AWoP-35ST, and pS6AWoP-agsT vectors.

Development of pS6A-ZmUbiP-MtNFP-Tm1, pS4A-OsAct1P-MtLYK3-nosT, pS3A-OsRCg2P-LjLNP-masT, and pS5A-MtSYMREM1-masT auxiliary vectors For developing pS6A-ZmUbiP-MtNFP-Tm1 intermediate vector, first, *MtNFP* was isolated by restricting the pGEMTE-MtNFP with BglII and KpnI and cloned into BamHI and KpnI digested pUbi-MCS-Tm1, resulting in the pUbi-MtNFP-Tm1 primary intermediate vector. Subsequently, ZmUbiP-MtNFP-Tm1 expression cassette was excised out from pUbi-MtNFP-Tm1 by digesting with HindIII and NotI and cloned into similarly digested pS6AWoP-agsT (by replacing agsT) to generate pS6A-ZmUbiP-MtNFP-Tm1 vector.

pS4A-OsAct1P-MtLYK3-nosT was generated by routing the *MtLYK3* coding sequence via pWAct1-MCS-nosT vector. This was achieved initially by isolating *MtLYK3* from pGEMTE-MtLYK3 by digesting with EcoICRI and SpeI, and cloning into SmaI-SpeI restricted pWAct1-MCS-nosT to develop pWAct1-MtLYK3-nosT. Subsequently, the Act1-MtLYK3-nosT expression cassette was isolated from pWAct1-MtLYK3-nosT by digesting with XhoI-NotI and inserted into analogously restricted S4AWoP-35ST (by replacing 35ST) giving rise to pS4A-Act1-MtLYK3-nosT.

For developing pS3A-OsRCg2P-LjLNP-masT vector, the AgeI-XhoI restricted *OsRCg2* promoter was obtained from pGEMTE-OsRCg2 and cloned into similarly digested pS3AWop-masT to produce pS3A-OsRCg2P-masT. Later, *LjLNP* was derived from pGEMTE-LjLNP by digesting with XhoI-XmaI and ligated into correspondingly digested pS3A-OsRCg2P-masT to generate pS3A-OsRCg2P-LjLNP-masT.

Finally, pS5A-MasP-MtSYMREM1-masT was developed by first cloning the XhoI-XmaI restricted *MtSYMREM1*, derived from pGEMTE-MtSYMREM1, under the

transcriptional control of MasP-masT in the similarly digested pSAT3A, giving rise to pS3A-MasP-MtSYMREM1-masT. Consequently, the expression cassette MasP-MtSYMREM1-masT was excised by digesting with AgeI-NotI from pS3A-MasP-MtSYMREM1-masT and moved into analogously digested pAUX3132 (equivalent to S5A devoid of any promoter/terminator) (Goderis et al. 2002) to generate pS5A-MasP-MtSYMREM1-masT.

Development of the binary plant transformation vector pPhL

To achieve this, the expression cassettes *ZmUbiP-MtNFP-Tm1*, *OsAct1P-MtLYK3-nosT*, *OsRCg2P-LjLNP-masT*, and *MasP-MtSYMREM1-masT* were released from the auxiliary vectors pS6A-ZmUbiP-MtNFP-Tm1, pS4A-OsAct1P-MtLYK3-nosT, pS3A-OsRCg2P-LjLNP-masT, and pS5A-MtSYMREM1-masT by digesting with PI-PspI, I-SceI, I-PpoI, and I-CeuI, respectively, and sequentially assembled in that order at the corresponding restriction sites in pRCS2-HPT binary vector (Fig. 1a) (Chung et al. 2005), thus finally generating a composite plant transformation vector carrying the linked expression cassettes of *PrOcs:HPT:ocsT* + *PrOsRCg2:LjLNP:masT* + *PrOsAct:MtLYK3:nosT* + *MasP:MtSYMREM:masT* + *PrZmUbi:MtNFP:Tm1* (Fig. 1b). This composite binary plant transformation vector was designated as pPhL.

Generation of Transgenic Plants and Confirmation of Transgene Presence

Mature seeds of rice (*Oryza sativa* ssp. *japonica*, cv. Murasaki R86) were surface-sterilized and used for callus induction according to Sreevidya et al. (2005). To obtain transgenic rice plants, embryonic calli were transformed with the composite plant transformation vector pPhL carrying four expression cassettes (see above) employing particle bombardment protocol (http://www.staceylab. missouri.edu/biolistic-transformation-of-rice) using GJ-1000 High-pressure Gas Gene Gun (Ningbo Scientz Biotechnology Co. Ltd., Ningbo, Zhejiang, China). Putatively transformed calli were selected for hygromycin resistance and regenerated according to Toki et al. (2006) with some modifications (phytohormone concentration in REIII medium was adjusted to NAA 0.1 mg/L and kinetin 2 mg/L).

Presence of transgenes in putative transgenic rice plants was confirmed by PCR using plant DNA extraction and amplification kit (Sigma-Aldrich, St. Louis, MO, USA) and the gene-specific primers for *MtNFP* (MtNFP 5′-F: 5′-GCCT TCTTTCTTCCTTCTAG-3′ and MtNFP 3′-R: 5′-GTAA CTTATCTGCAGTCTCG-3′; fragment size 0.864 kb), *MtLYK3* (MtLYK3 5′-F: 5′-GTGTGATGTAGCTTTAGCTT C-3′ and MtLYK3 3′-R: 5′-GATACGTAAACTCTGTCGAC TTTG-3′; 0.853-kb fragment), *MtSYMREM1* (MtRem-F: 5′-AGATCTGCTGTAACCCTAGAGATACATTATGG-3′ and MtREM-R: 5′-CCCGGGCTAACTGAAAAACCTTAAAC CGCTGA-3′; 0.7-kb fragment), and *LjLNP* (LjLNP-F: 5′-CTCGAGGCATTGGACTAAAGCCATGG-3′ and LjLNP-R: 5′-CCCGGGCAGGCTTAAGCAGGTACTAGTA-3′; 1.3-kb fragment) under PCR conditions described in the Supplementary Table S2. PCR-confirmed transgenic plants were hardened on vermiculite supplemented with Yoshida nutrient solution (Yoshida et al. 1976) and subsequently shifted to soil and maintained in the greenhouse. Wild type or the plants transformed with the empty vector pRCS2-HPT (Ortiz-Berrocal et al. 2017) served as controls.

Rice plants confirmed for the presence of all four legume transgenes were designated as PhL plants and the plants transformed with the empty vector pRCS2-HPT were termed as RMh plants (from now on, RMh plants will be called “vector control RMh plants”).

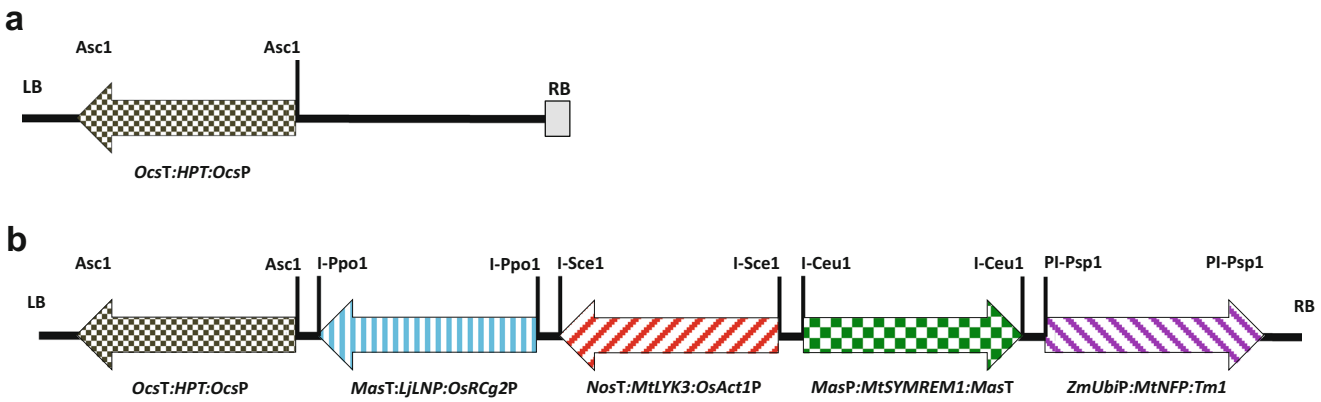


Fig. 1 Schematic representations of the **a** pRCS2-HPT vector and **b** pPhL transformation vector carrying expression cassettes of *M. truncatula* LysM domain-containing receptor-like kinase 3 (*MtLYK3*), *M. truncatula* SYMBIOTIC REMORIN 1 (*MtSYMREM1*), *M. truncatula* Nod factor perception (*MtNFP*), *L. japonicus* lectin nucleotide phosphohydrolase (LjLNP) and hygromicin phosphotransferase (*HPT*-selection marker) genes. *Ocs*P, octopine synthase promoter; *OsRCg2*P, rice protease inhibitor/seed storage/LTP family protein promoter; *OsAct1*P, rice actin 1 promoter; *Mas*P, mannopine synthase promoter; *ZmUbi*P, maize ubiquitin promoter; RB, right border; LB, left border. Diagram not drawn to the scale

Transgenic plants were also confirmed by Southern analysis as follows: Genomic DNA was isolated from young leaves according to Dellaporta et al. (1983), digested with HindIII (for vector control RMh plants) or BamHI (for PhL plants), separated on 0.8% agarose gel, transferred to Hybond-N^+ nylon membrane (Amersham Bioscience, Buckinghamshire, UK), and hybridized with digoxigenin-11-dUTP-labeled *HPT* (for RMh plants) or *NFP* (for PhL plants). Probe preparation, hybridization, stringent washes, blocking, and chemiluminiscent detection were performed following the instructions of the manufacturer (Roche Applied Science, Mannheim, Germany).

Preparation of Rhizobial Strains

The plasmid pDG77-FITA-DO was developed by inserting the hybrid *nodD* (FITA, flavonoid-independent transcription activator) gene derived from pMP604 (Spaink et al. 1989) into pDG77 (Gage 2002) carrying the reporter system DsRed. This plasmid was introduced into the rhizobial strains *Sinorhizobium meliloti* 1021 and *Rhizobium* NGR234-Ω-nodD2 by triparental mating, using *Escherichia coli* Hb101 harboring pDG77-FITA-DO plasmid as a donor and *E. coli* pRK600 as a helper strain. Transformed rhizobial strains were grown in TY medium (tryptone 6 g/L, yeast extract 3 g/L, $CaCl_2$ 0.38 g/L) supplemented with appropriate antibiotics (nalidixic acid 20 μg/mL plus tetracycline 12.5 μg/mL for *S. meliloti* pDG77-FITA-DO, and rifampicin 100 μg/mL plus kanamycin 50 μg/mL and tetracycline 12.5 μg/mL for *R.* NGR234-Ω-nodD2 pDG77-FITA-DO) in dark at 30 °C and 200 rpm for 24 h, and used for inoculating rice seedlings to evaluate root hair deformation and rhizobial colonization in roots.

Nod Factor Isolation

For NF purification, we have employed NF overproducing strains of *S. meliloti* pDG77-FITA-DO and *R.* NGR234-Ω-nodD2 pDG77-FITA-DO following the protocol of Cárdenas et al. (1995) with some modifications. Briefly, first the rhizobial strains were grown in dark at 30 °C and 200 rpm for 48 h in 100 mL of TY medium supplemented with appropriate antibiotics. Later, these rhizobial cultures were inoculated into 3 L of B-medium without antibiotics and grown under the conditions described above for 48 h. Subsequently, NFs were extracted with 600 mL of water-saturated *n*-butanol, dried under vacuum and reconstituted with 20 mL of 60% aqueous acetonitrile with vigorous shaking. Purification was done first using octadecyl (C18) extraction columns (J.T. Baker, Deventer, Holland) and later by reverse-phase Waters HPLC system (Waters Associates Inc., Milford, MA). The HPLC separation was performed at a flow rate of 0.7 mL min^{-1}, with monitoring of eluents at 206 nm. Eluent fractions corresponding to a pre-verified peak (identified based on the ability to induce root hair deformation—see below) were pooled and dried (Lopez-Lara et al. 1995). Finally, NFs were dissolved in 0.1% CHAPS [(3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)], and concentration was calculated based on peak intensity in comparison with a reference NF of the LCO NodRlv-V (Spaink et al. 1991).

Biological activity of the isolated NFs was evaluated, using a concentration of 10^{-9} M, for their ability to induce root hair deformation on 3-day-old *Medicago sativa* seedlings germinated from surface sterilized seeds on N-deprived Summerfield medium (Summerfield et al. 1978). After confirming the efficacy of the isolated NFs on *M. sativa*, they were used for studies on rice.

Root Hair Deformation Assays in Rice Using Rhizobial Inoculation and NF Treatments

First, T_2 seeds from segregating transgenic PhL rice plants were surface-sterilized and germinated on Yoshida medium (Yoshida et al. 1976). After 8 days, seedlings were analyzed by PCR for the presence of transgenes, and the PCR-confirmed T_2 plants were transferred to tubes containing 30 mL of agarized N minus Yoshida medium and inoculated with 1 mL of rhizobial suspension (*Rhizobium* NGR234-Ω-nodD2 pDG77-FITA-DO or *S. meliloti p*DG77-FITA-DO; $OD_{600} = 0.2$). Eight days post-inoculation, seminal and crown roots were observed under light microscope and number of deformed root hairs per unit length root was accounted for at the differentiation/maturation zone.

For treatment with NFs, 8-day-old seedlings derived from the surface-sterilized T_2 seeds obtained from the segregating transgenic rice populations were PCR-confirmed for the presence of transgenes and transferred from the N-sufficient to N-deficient Yoshida liquid medium (the lower part of the tubes was covered with aluminum foil to avoid exposer of roots to light). Later, after 24-h incubation, the plants were supplemented with 10^{-9} M purified NFs of *S. meliloti* or *Rhizobium* NGR234 and observations were scored for root hair deformation after 1 day. Seedlings treated with 0.001% CHAPS served as control. The results obtained with the PhL transgenic plants were compared with the similarly treated vector control RMh plants.

Statistical Analysis

The percentages of root hair deformation in RMh and PhL plants subjected to treatments with the *Rhizobium* NGR234 and *S. meliloti* NFs and the rhizobial inoculation were evaluated separately with two-way analysis of variance (ANOVA), using type III sum of squares (R ver. 3.12014). Percentages of deformed root hairs were angular transformed to improve

normality and homogenize variances. In order to perform the analysis, a model simplification-based approach of Crawley (1993) was followed in which the maximal model (containing all factors and interactions) was initially fitted and then simplified by step-wise deletion tests, starting with the interaction and then the main effects. Terms not statistically significant were removed, and the treatment levels reduced in case of no statistical differences in comparisons between the simplified model and the full model. After model simplification, we used Tukey's honestly significant difference test to compare the means of each treatment category.

Gene Expression Analysis

RNA isolation, library preparation, and high-throughput sequencing were performed as previously reported (Ortiz-Berrocal et al. 2017).

Plant Cultivation and RNA Isolation Surface-disinfected T_2 seeds from PhL and vector control RMh lines were germinated in vitro, PCR-confirmed, and grown in 80-mL culture tubes containing liquid Yoshida medium for 2 months in sterile conditions in growth room at 28 °C and 16-h/8-h light/dark photoperiod; roots were protected from light by covering the lower portion of tube with aluminum foil. Later, the plants were taken out, roots were rinsed with sterile Yoshida medium lacking nitrogen, and the plants were shifted to N-free Yoshida medium for a day and then treated for 24 h with *S. meliloti* NFs (10^{-9} M). N-deprived Yoshida medium augmented with CHAPS served as control. Subsequently, roots derived from individual plants (three biological replicates for each treatment) were obtained and total RNA was prepared from 50 mg of liquid nitrogen–frozen roots of PhL and vector control RMh transgenic plants using Spectrum Plant Total RNA Kit (Sigma-Aldrich, USA). Purity of RNA samples was assessed using Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA).

RNA-seq Analysis Total RNA samples (≈ 20 μg) with values of OD260/280 between 2.13 and 2.16 and OD260/230 between 2.19 and 2.22 were sent to Beijing Genome Institute (BGI) Americas Laboratory (Tai Po, Hong Kong) for Illumina HiSeq 2000 high-throughput sequencing (quantification). RNA-seq was performed with RNA samples isolated from the root samples derived from three plants each of RMh and PhL plants subjected or not to NF treatment ((A) RMh untreated, (B) RMh treated with NFs, (C) PhL untreated, and (D) PhL treated with NFs). After sequencing, the quality of raw reads was assessed with FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed with Trim Galore v0.3.7 (https://www.bioinformatics.babraham.ac.uk/projects/trimgalore/). Subsequently, reads were aligned to the *O. sativa* CDS collection (Os-Nipponbare-Reference genome V 7.1; http://rice.plantbiology.msu.edu/) using Bowtie aligner (Langmead et al. 2009), with the ensuing parameters: bowtie -aS -X 800 –offrate 1 transcripts -1. Expression of transgenes was confirmed through aligning reads to the FASTA file of *MtLYK3*, *MtNFP*, *MtSYMREM1*, and *LjLNP* coding sequences with the aligner BWA version 0.7.12-r1039 (Li and Durbin 2009).

Differential expression analysis between samples was performed with the NOISeq version 2.6.0 using non-parametric Noiseq-sim method, which simulates technical replicates from a multinomial distribution to estimate differential expression probabilities, with the recommended q value of 0.9 (Tarazona et al. 2015). Noiseq-sim was used with the following parameters: size of simulated sample (pnr) = 0.2, number of simulated replicates (nss) = 5, allowed variability (v) = 0.02. Enrichment analysis of Gene Ontology (GO) terms was based on the TopGO v. 3.0.2. (Alexa and Rahnenführer 2016), and a P value of 0.05 was used to define the most significant GO terms. Differentially expressed genes (DEGs; $|\log2 \text{ ratio}| \geq 1.5$) in the root samples were compared in combinations of C vs A, B vs A, and D vs C. The presence and distribution of all up- and downregulated DEGs in different pathways were analyzed with the KEGG PATHWAY database (https://www.genome.jp/kegg/pathway.html) (Tanabe and Kanehisa 2012). Heatmap representations for the DEGs in the KEGG pathways were done using gplots package v2.14.2 in R software environment (https://www.r-project.org/).

RT-PCR for Evaluation of Transgene Expression RNA isolation and RT-PCR for evaluation of transgene expression in roots were performed according to Ortiz-Berrocal et al. (2017). Transgene expression was evaluated in 2-month-old PhL plants grown in Yoshida solution and then incubated in N-deprived Yoshida solution for 2 days prior to analysis. cDNA was prepared from 3 μg of freshly isolated, DNase-treated RNA using RevertAid™ First strand cDNA synthesis kit according to the manufacturer's instructions (Thermo Scientific, EU, Lithuania). Transgene expression was assessed by PCR using gene-specific primers (Supplementary Table S1).

Real-Time Quantitative RT-PCR Analysis

To validate the RNA-seq results, real-time quantitative reverse transcription-PCR (RT-qPCR) analysis of transcript abundance was performed with selected DEGs using three biological replicates with two technical replicates. Total RNA (2 μg) was used as template to synthesize cDNA using the RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Genomic DNA removal, cDNA synthesis, and quality verification for RT-qPCR were performed as reported (Hernández et al. 2007; Ramírez et al. 2013). Resulting

cDNAs were then diluted to 60 μL, and 0.066 μg (2 μL) of cDNA was used per RT-qPCR reaction. Reactions were performed in a 96-well format Real-Time PCR System and 7300 System Software (Applied Biosystems, Foster City, CA, USA) using SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) following the manufacturer's instructions. The primers used for RT-qPCR are as follows: nicotianamine synthase (LOC_Os03g19420.1), OsNS-F (CTGAGTGC GTGCATAGTAATCC) and OsNS-R (ACCTCTTG CTTTCTCAGCACC); expressed protein 1 (LOC_Os02g33070.1), OsEP1-F (TCGCCCAACCTGAT CCGCTG) and OsEP1-R (TTCTTGACTCCTTTCCCACT GTACAG); glutathione S-transferase GSTU6 (LOC_Os10g38340.1), OsGST-F (CCAGTTGAAGTTTG CTGAATCC) and OsGST-R (TAGAATAGCCACTT CCCAC); oxidoreductase, aldo/keto reductase family protein (LOC_Os04g27060.1), OsAKR-F (ACGTTGTGCAAGGT GACAG) and OsAKR-R (CACATGGCAAAAAC ACTGGAACA); major facilitator superfamily antiporter (LOC_Os11g04020.1), OsMFSA-F (TTGCGTTGACCTTC AAGCCG) and OsMFSA-R (GTCTATGCTTGCAT CCACGC); expressed protein 2 (LOC_Os08g05960.1), OsEP2-F (TGCTAGTGTTGCTAGGGAGG) and OsEP2-R (CAACAGGATAACGAGTACAGAGC); transketolase (LOC_Os07g09190.1), OsTK-F (GCACTTCAGCTCAG CTGAA) and OsTK-R (CAAGAAACACATACACATGG GC); B12D protein (LOC_Os07g41350.1), OsB12D-F (GTGACCGTCAAAACTGATCC) and OsB12D-R (TAAGTTCGCACGTATGCGAC). Relative expression was calculated using the comparative Ct method (Livak and Schmittgen 2001). The threshold cycle (Ct) value obtained in each reaction was normalized with the geometrical average of Ct values of expression levels of three reference genes, namely protein kinase 1 (LOC_Os06g48970.1), an expressed protein (LOC_Os07g02340.1), and a nucleic acid binding protein (LOC_Os06g11170.1) (Narsai et al. 2010). The Student *t* test was performed with a *P* value cutoff of 0.05.

Results

Vector Construction, Genetic Transformation, and Characterization of Transgenic Rice Plants

To make rice plant amenable to perceive and transmit Nod signal appropriately, we chose to express legume-specific NFRPs—two NF-perceiving LysM-Receptor-like Kinases (MtNFP and MtLYK3) together with their interacting protein MtSYMREM1, and a NF-recognizing lectin nucleotide phosphohydrolase (LjLNP)—in rice. *MtSYMREM1* was included because it was shown to be a scaffolding symbiotic receptor-binding protein that specifically interacts with the core set of symbiotic receptor kinases (MtNFP and MtLYK3) that are essential for Nod signal perception (Lefebvre et al. 2010). It is worthwhile to mention here that the closest homologue of MtSYMREM1 in rice is poorly conserved, with only 41% identity at amino acid level (LOC_Os04g45070). Hence, we chose to use the legume *MtSYMREM1* to replicate Nod factor receptor system (MtNFP and MtLYK3 together with their interacting protein MtSYMREM1) in rice similar to that in legumes. Lectin nucleotide phosphohydrolase (LjLNP) was included in the study because it possesses not only a NF binding domain but also a lectin binding domain. The lectin binding domain enables close interaction with lectins present on surface of rhizobia (Roberts et al. 1999) and aids in bringing about intimate association of bacteria with root epidermal cell surface including root hairs (Sreevidya et al. 2005). It has been suggested that the close association may facilitate accumulation of NFs secreted by rhizobia to reach critical concentrations at root surface to foster cellular reactions effectively (Goedhart et al. 2000).

For this purpose, we generated the plant transformation vector pPhL carrying linked expression cassettes of *MtNFP*, *MtLYK3*, *LjLNP*, and *MtSYMREM1* (isolated from the legumes *Medicago truncatula* and *Lotus japonicus*) genes respectively driven by constitutive promoters *ZmUbi*P, *OsAct*P, *OsRCg2*P (from *Zea mays* and *Oryza sativa*), and *Mas*P (from *Agrobacterium tumefaciens*) (Fig. 1b), and used for genetic transformation of rice. Transformation of rice (*O. sativa* R86) was achieved by biolistic bombardment of the pPhL vector into rice calli. Plants transformed with the empty vector pRCS2-HPT (pRMh; Fig. 1a) (Ortiz-Berrocal et al. 2017) served as controls.

A total of 116 and 73 putative transgenic PhL and vector control RMh plants, respectively, were regenerated and analyzed by PCR amplification for the presence of transgenes. PCR analysis confirmed 63 PhL plants positive for all transgenes (*MtNFP*, *MtLYK3*, *LjLNP*, and *MtSYMREM1*) and 6 vector control RMh plants positive for *HPT* gene. Out of 63 PhL plants cultivated, only 3 plants turned out to be fertile and produced limited number of seeds (approximately 20 seeds in each T_0 PhL plants) compared with vector control RMh plants, which produced abundant number of seeds, about 80% filled seeds in each panicle (an average of 33 seeds per panicle, typically producing > 500 seeds per plant) (Table 1; Fig. 2). Pollen viability as tested by staining with KI or NBT was found to be less than 5% in T_0 PhL plants as compared with about 45% in vector control RMh plants (pollen viability in wild type plants was similar to RMh plants). In PhL plants, however, from T_2 generation onwards, seed production improved to about 40% of filled grains per panicle. Excepting for pollen viability and seed production, no other significant phenotypic differences were observed among flowers, leaves, tillers, or roots in PhL plants compared with wild type or vector

Table 1 Generation of transgenic rice plants. Transgenic rice (*Oryza sativa* L. cv. Murasaki R86) plants were obtained by biolistic transformation of embryogenic calli with the binary vectors pRMh or pPhL. Putative transformed calli were selected on medium containing hygromycin. Presence of transgenes in regenerated plants was confirmed by PCR and copy number was determined by Southern blot analysis

Transformation vector	Transgenes	No. of regenerated plants	No. of positive plants	No. of plants with seeds	Transgene copy number
RMh	*HPT*	73	6	6	2
PhL	*HPT* + *MtLYK3* + *MtNFP* + *MtSYMREM1* + *LjLNP*	116	63	3	6

control RMh plants. Nevertheless, in contrast to the vector control RMh plants, in old T_0 PhL plants, occasionally branched out tillers were developed from the main tillers (not shown).

Southern analysis revealed the presence of 6 copies of transgenes (with the same banding pattern) in all three fertile PhL plants suggesting that they all arose from a single transformation event (Supplementary Fig. S1). *HPT* gene copy number in all six vector control RMh plants was found to be 2 (Ortiz-Berrocal et al. 2017).

The RT-PCR analysis showed that in roots of PhL plants, relative to the expression level of the rice housekeeping elongation factor Tu gene (LOC_Os03g08020), *MtNFP*, *MtLYK3*, and *MtSYMREM*, transgene abundance was about 60–90% higher than that of *LjLNP* (Fig. 2g).

Response of Root Hairs to Rhizobial Inoculation and Nod Factor Treatment in Transgenic PhL Rice Plants

To assess whether the expression of NFRPs enables PhL plants receptive to rhizobial inoculation, PCR-confirmed T_2 transgenic plants (generated from surface sterilized seeds) grown in N-deprived Yoshida medium were challenged with *S. meliloti* or *Rhizobium* NGR234 and analyzed for their responsiveness in terms of exhibiting root hair deformation in roots. The former strain was selected because PhL plants express NF receptor-like kinases from *M. truncatula* that specifically recognize NFs from *S. meliloti* (Arrighi et al. 2006; Limpens et al. 2003), and the latter strain was chosen as it is highly promiscuous and capable of interacting symbiotically with a range of legume species that express a wide variety of NF receptor kinase proteins (Relić et al. 1993). As described in "Materials and Methods," both these strains were marked with *DsRED* (Gage 2002) to facilitate easy observation and carried *FITA* gene (Spaink et al. 1989) to aid production of NFs independently of inducer-flavonoids.

Microscopic examination revealed that the inoculated rhizobial cells were found growing in close proximity with outer surface of root hairs in both PhL and vector control RMh plants (Fig. 3a, c). On the other hand, colonization of rhizobia on root surface as well as at the sites lateral root emergence was found to be less dense in PhL plants as compared with vector control RMh plants (compare Fig. 3d with b). In case of the inoculated PhL plants, however, root hairs exhibited subtle but consistent deformations (such as curved and swollen tips, protuberances on root hairs) in root hairs localized in the differentiation/maturation zone of roots (Fig. 4e, f) as compared with the counterparts in the vector control RMh plants (Fig. 4b, c). In particular, root hairs in the PhL plants inoculated with *S. meliloti* showed relatively more conspicuous hook formation at their tips than the root hairs inoculated with *Rhizobium* NGR234 (compare Fig. 4e with f). Root hairs of uninoculated plants in both vector control RMh and PhL plants showed deformations rarely (Fig. 4a, d). The percentages of root hair deformation in transgenic plants subjected to inoculation treatments with *Rhizobium* NGR234 and *S. meliloti* were separately validated using two-way analysis of variance (ANOVA) (see "Materials and Methods"). In contrast to the microscopic observations, ANOVA indicated no significant differences in percentages root hair deformation in PhL and vector control RMh plants (Fig. 4g). The ANOVA full (maximal) model showed that both PhL and vector control RMh plants inoculated with rhizobia presented a significantly higher percentage of deformed root hairs compared with non-inoculated plants ($F_{(2,\ 25)} = 4.10$, $P = 0.0289$; Fig. 4g). Neither interaction among factors (type of rhizobial strain) nor transgenic plant lines (PhL and vector control RMh plants) were significant ($F_{(1,\ 25)} = 0.29$, $P = 0.5981$; $F_{(2,\ 25)} = 0.6525$, $P = 0.5294$; respectively). After the model simplification by elimination of non-significant terms, post hoc comparisons were performed between the means of each category of rhizobial inoculation levels. Both PhL and vector control RMh plants inoculated with rhizobia (*S. meliloti* 1021 and *Rhizobium* NGR234 strains) presented a significantly higher percentage of deformed root hairs compared with non-inoculated plants (Tukey's HSD; $P = 0.0335$ and $P = 0.0375$, respectively). No significant difference in percentage of deformed root hairs were observed in response to the rhizobial strain (Tukey's HSD; $P = 0.9987$; Fig. 4g).

To evaluate if root hair deformation was specifically modulated by NFs, we isolated NFs from both *S. meliloti* and *Rhizobium* NGR234 and tested their efficacy in inducing root hair deformation in transgenic rice roots (Fig. 5) and

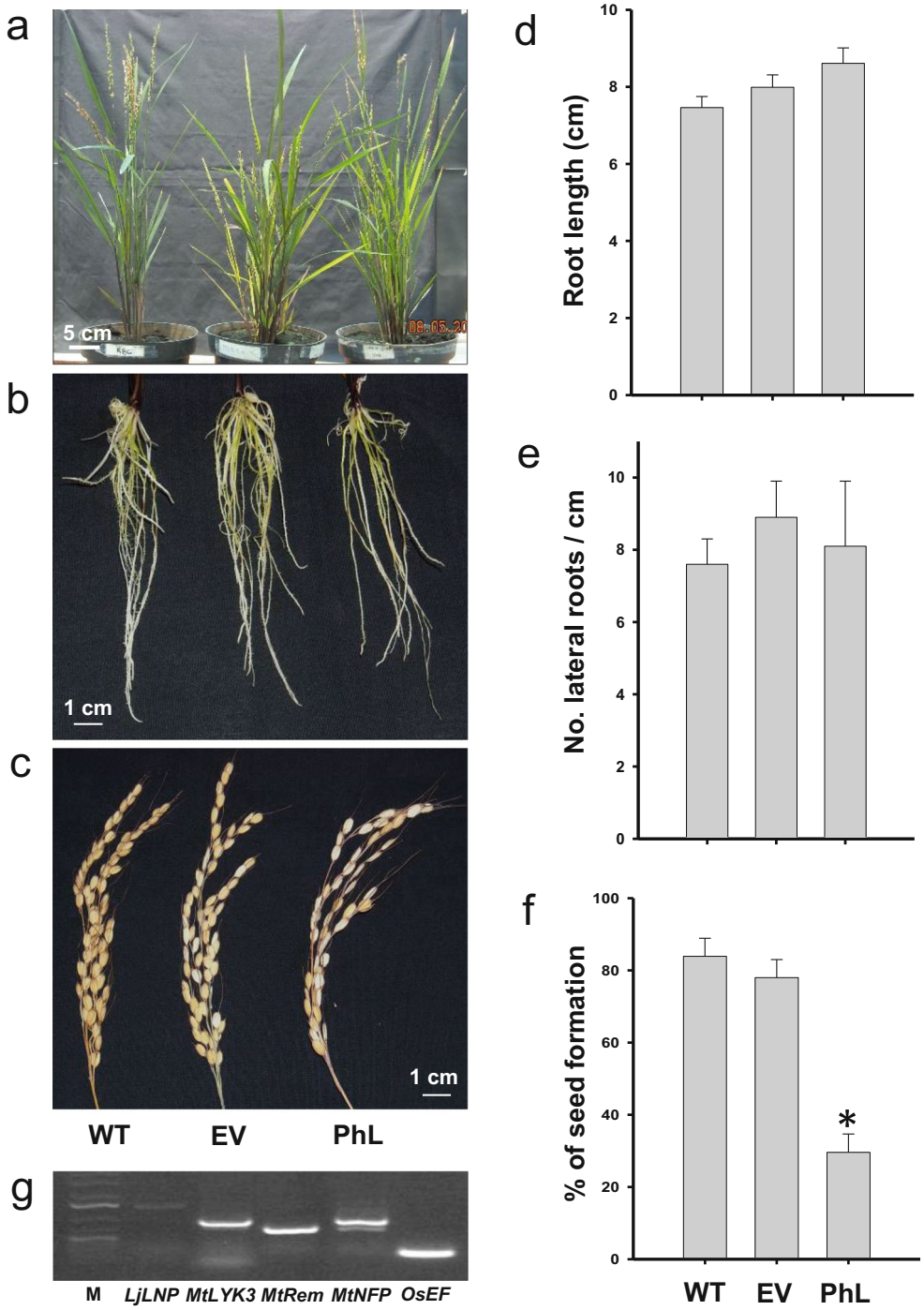


Fig. 2 Characterization of transformed rice plants. **a**, **b**, **c** Phenotype of PhL plants, roots, and panicles compared with wild type and vector control RMh plants. **d** Average root length of seven rice plants grown in Yoshida solution for 22 days post-germination (dpg). **e** Total number of lateral roots in differentiation/maturation zone of seminal roots of five plants 16 dpg in N-deprived Yoshida solution. **f** Percentage of matured seeds in ten panicles. Asterisk indicates percentage of filled seeds in PhL plants that is significantly lower than ($P = < 0.001$) in wild type and vector control plants. **g** Transgene expression analysis by RT-PCR in PhL roots. Expression of transgenes was compared with reference *O. sativa* elongation factor Tu (*OsEF*) gene expression

compared them with typical root hair deformations in *M. sativa* treated with the isolated NFs (10^{-9} M) for 24 h (Supplementary Fig. S2). Compared with vector control RMh plants, treatment of PhL plants with *Sm* or NGR NFs induced perceptible and consistent root hair deformations in the root hairs present in root differentiation/maturation zone similar to that induced by inoculation with rhizobial strains (compare Fig. 5b with e and e′ and c with f).

In response to NF treatment, percentage of deformed root hairs was significantly higher in PhL plants compared with vector control RMh plants (Tukey's HSD; $P < 0.0001$), and both PhL and vector control RMh plants treated with NFs showed significantly higher root hair deformation than the plants treated with solvent (control) alone (Tukey's HSD; PhL + NF vs vector control RMh, $P < 0.0001$; PhL + NF vs PhL, $P < 0.0001$; RMh + NF vs PhL, $P < 0.001$; RMh + NF vs vector control RMh, $P < 0.001$) (Fig. 5g). ANOVA indicated that treatment of PhL and vector control RMh plants with NFs from both strains increased the number of deformed root hairs, but more significantly in PhL plants compared with untreated

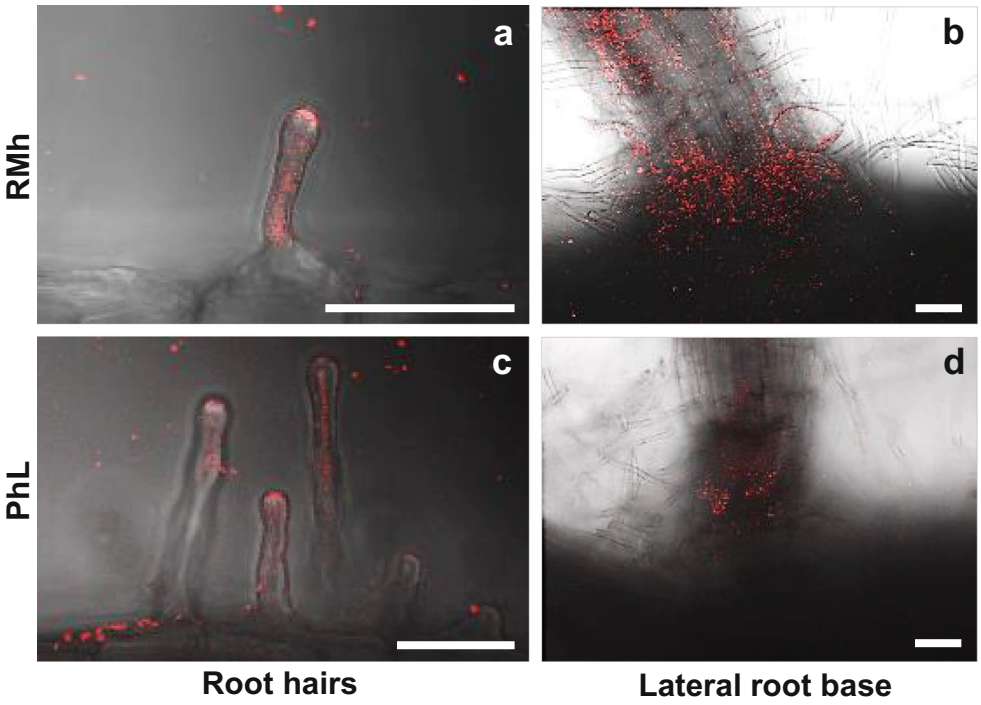
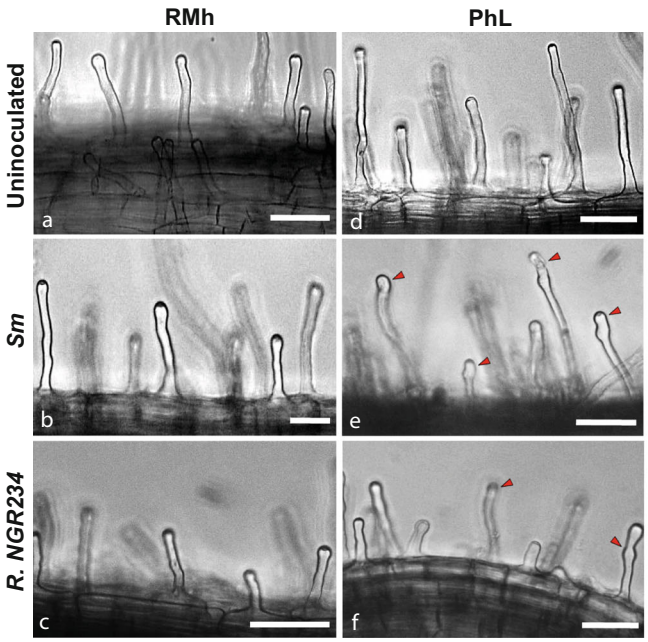


Fig. 3 Rhizobial colonization in rice roots of **a**, **b** vector control RMh plants and **c**, **d** PhL plants inoculated with *Rhizobium* NGR234 marked with DsRED and FITA. Confocal photomicrographs were taken 8 days post-inoculation. Bars, 50 μm

plants (Fig. 5g). ANOVA full model confirmed that the interaction was significant ($F_{(2,\ 24)} = 6.76$, $P = 0.0047$).

Display of root hair deformation in PhL plants in response to NF treatment indicates that the expression of NFRPs confers rice plants the ability perceive NFs more effectively.

Percentage of root hair deformation in NF-treated PhL plants is higher than the ones inoculated with rhizobia. Reason for such variations in levels of root hair deformations is difficult to decipher in the absence quantitative data pertaining to the amount of NFs secreted by rhizobial cells



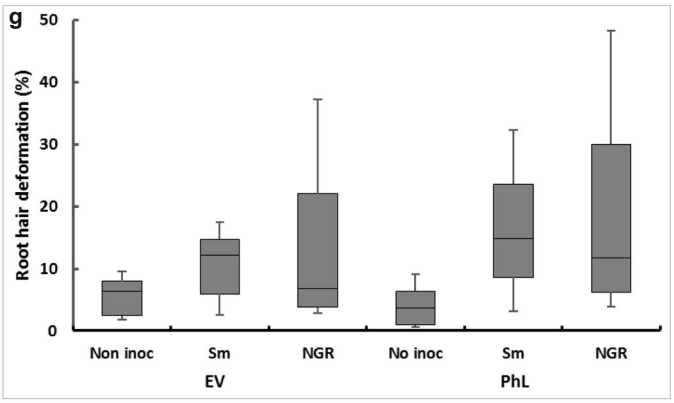


Fig. 4 Response of root hairs in **a**–**c** vector control RMh plants and **d**–**f** PhL plants to rhizobial inoculation, 8 dpi. Roots were **a**, **d** uninoculated (control) or **b**–**f** inoculated with rhizobial strains marked with DsRED and FITA. **b**, **e** *S. meliloti* or **c**, **f** *R.* NGR234. Photomicrographs were taken under compound microscope equipped with bright field optics. Deformations in root hairs are highlighted by arrowheads. Bar = 50 μm. **g** Box plots of percentages of root hairs showing deformation were accounted from differentiation/maturation zone of seminal root in vector control RMh and PhL plants inoculated with rhizobial strains. Percentages of deformed root hairs represent average values from 5 plants per treatment

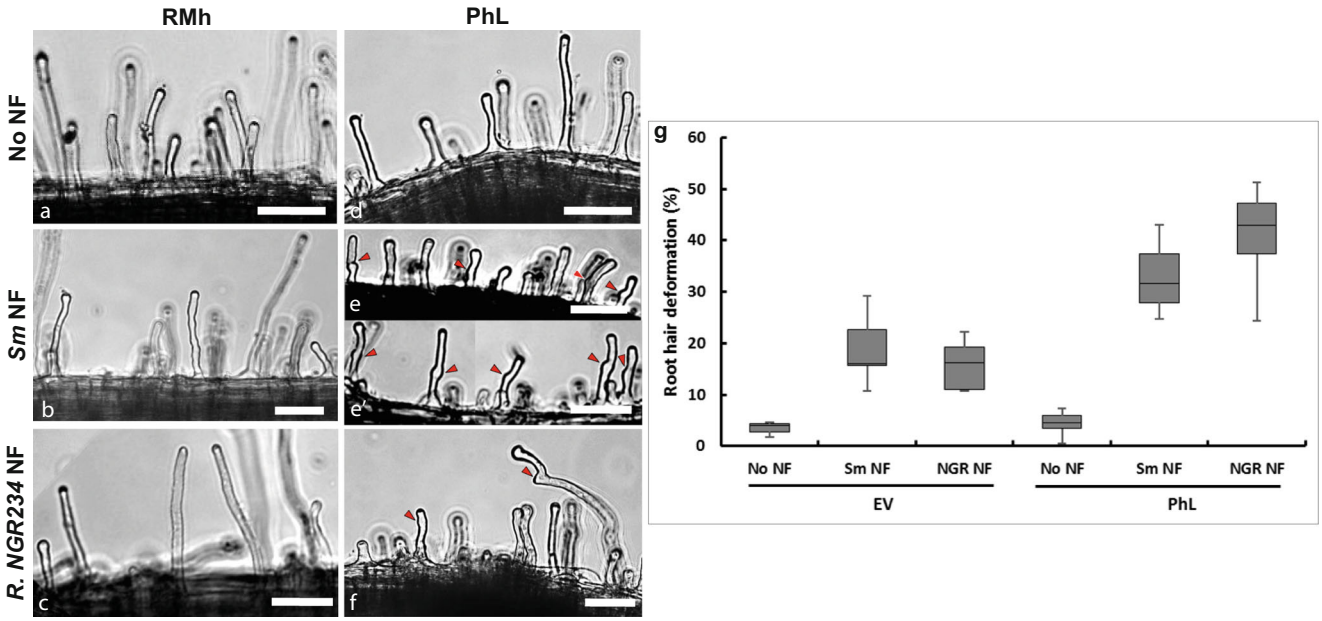


Fig. 5 Response of root hairs in **a**–**c** vector control RMh and **d**–**f** PhL plants to 24-h Nod factor treatment. Roots were treated with **a**, **d** CHAPS 0.001% (no NFs) or **b**–**f** with Nod factors (~ 10^{-9})M of **b**, **e** *S. meliloti* or **c**, **f** *R.* NGR234. Photomicrographs were taken under compound microscope equipped with bright field optics. Deformations in root hairs are highlighted by arrowheads. Bar = 50 μm. **g** Box plots of percentages of root hairs showing deformation were accounted from differentiation/maturation zone of seminal root in vector control RMh and PhL plants treated with Nod factors. Percentages of deformed root hairs represent average values from 5 plants per treatment

in rice root environment. It is likely that the concentration of NFs secreted by rhizobia may be relatively lower than the amount of pure NFs supplied in the medium, hence resulting in lower levels of root hair deformations. However, in the absence of quantitative data on endogenous Nod factor production levels, it is difficult to make a definitive conclusion in this regard.

Transcriptional Response of Rice Roots Due to Expression of NFRPs and Nod Factor Treatment

In order to further explore the influence of NFs on root biology of PhL plants expressing NFRPs, RNA-seq of roots of PhL and RMh plants treated with 10^{-9} M *S. meliloti* NFs or with 0.001% CHAPS for 24 h was performed (see "Materials and Methods" for details). The root RNA samples sent for transcriptome sequencing were derived from (a) vector control RMh plants without NF treatment, (b) vector control RMh treated with *S. meliloti* NFs, (c) PhL without NF treatment, and (d) PhL treated with *S. meliloti* NFs.

About 24 million reads were generated from polyadenylated RNA libraries for each treatment; of these, around 80% aligned to single positions in rice genome, and 5% aligned at multiple positions. Over 65% of alignments were complete, and between 8 and 18% were accepted with less than 2 bases unpaired. Scatter plots presented in Supplementary Fig. S3 illustrate relative levels of expression of 37,309 genes in roots of vector control RMh and NFRP-expressing PhL plants treated with or without Nod factors. From these, differentially expressed genes (DEGs) were selected according to FDR ≤ 0.001 and $|\log2\text{ratio}| \geq 1.5$. A detailed list of the DEGs obtained from comparisons between root samples of PhL and vector control RMh plants treated with or without NFs is provided in Supplementary Table S4. Noiseq-sim algorithm ($q = 0.9$) to analyze differential expression of genes between RNA-seq samples confirmed approximately 80% of DEGs selected previously.

NOIseq-validated deep sequencing data was utilized to analyze transcriptional reprograming of rice roots both due to the constitutive expression of legume NFRPs and the NF treatment. Transcriptome changes induced in roots due to expression of NFRPs were delimited by comparing the RNA-seq data obtained from PhL plants with the transcript data derived from vector control RMh plants (C/A). Similarly, effect of NFs on transcriptional reprogramming in roots of vector control RMh plants as well as PhL plants was evaluated by comparing transcriptome data derived from NF-treated plants vs mock-treated plants (i.e., comparisons between the samples B vs A and D vs C were made to delineate transcriptional changes induced in roots of vector control RMh and PhL plants, respectively, in response to NF treatment).

RNA-seq analysis revealed that the constitutive expression of legume-specific NFRPs in rice plants (PhL plants) induced downregulation of 398 genes and upregulation of 99 genes in roots as compared with vector control RMh plants (DEGs in "C" compared with "A" in Fig. 6; Supplementary Table S4). NF treatment, however, completely altered the gene

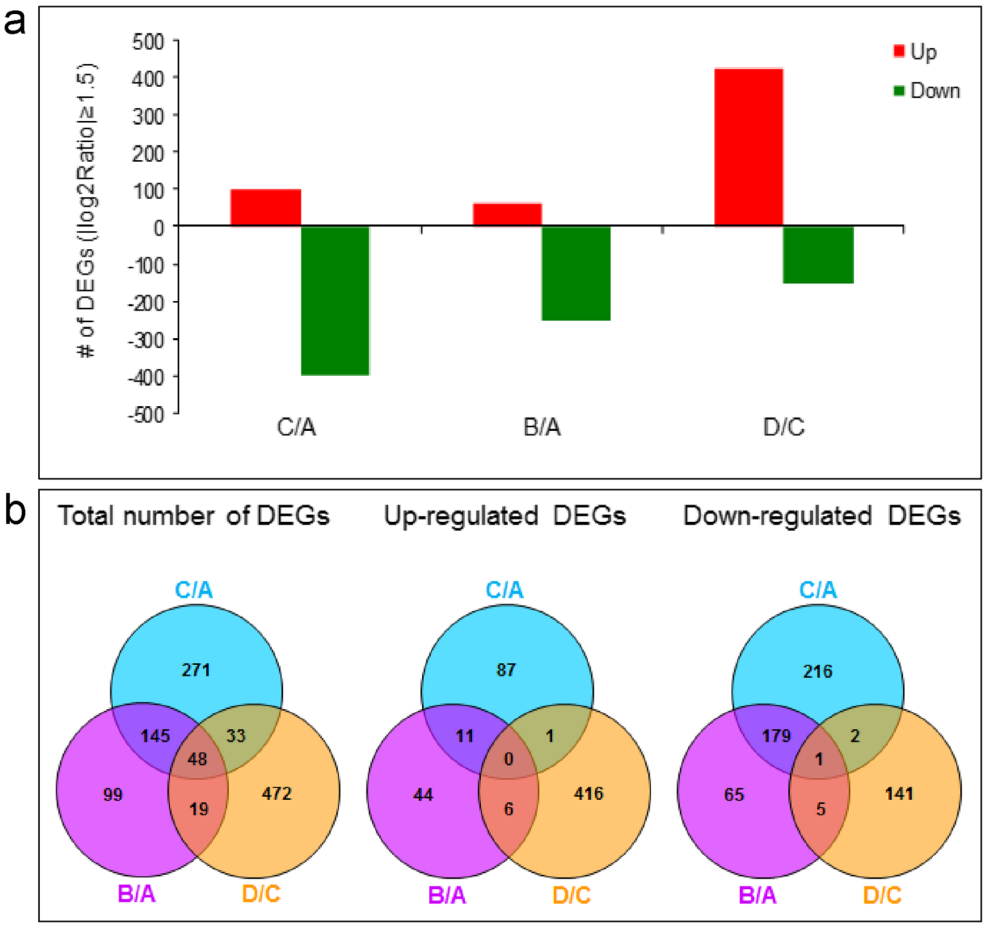


Fig. 6 RNA-seq analysis in Nod factor–treated rice roots. **a** Up- and downregulated genes (|log2ratio|≥ 1.5) in roots of vector control RMh and Nod factor receptor protein expressing PhL rice plants treated with or without NFs. **b** Interactive Venn diagrams representing the overlap in number of differentially expressed genes in roots of vector control RMh and PhL rice plants in response to Nod factor treatment. Differentially expressed genes in the roots of PhL vs vector control RMh plants (C/A), Nod factor–treated RMh plants vs untreated RMh plants (B/A) and Nod factor–treated PhL plants vs untreated PhL plants (D/C)

expression profiles in roots of PhL plants—with NF treatment a large number of genes were upregulated (423) compared with fewer downregulated genes (149) ("D" vs "C"). In contrast, in vector control RMh roots, NF treatment induced downregulation of more genes (250) as compared with upregulated genes (62) ("B" vs "A") (Fig. 6; Supplementary Table S4).

Transcriptome analysis showed that in comparison to RMh roots, the expression of NFRPs in PhL roots (compare "C" vs "A") induced downregulation of several genes coding for transcription factors (5 up/24 down), kinases (5 up/22 down), and calcium binding proteins (1 up/5 down) associated with signaling and biological processes (Supplementary Figs. S4 and S6-S9a), and those related to plant-pathogen interaction, stress/defense response, secondary metabolites, and phytohormone biosynthetic pathways (Figs. 7 and 8, Supplementary Figs. S4-S16). In contrast, NF treatment massively impacted gene expression in PhL roots, resulting in upregulation of expression of several genes encoding transcription factors (19 up/4 down), kinases (41 up/7 down), and calcium binding proteins (8 up/1 down) mainly associated with various biological and signal transduction pathways (Fig. 8; Supplementary Figs. S4 and S6-S9a) including those involved in plant hormone synthesis and signaling (Supplementary Figs. S15 and S16), in comparison with similarly treated vector control RMh plants. In addition, NF treatment of PhL plants also activated in roots various genetic networks/genes associated with defense response and secondary metabolites production—specifically, the genes involved in pathways related to plant-pathogen interaction (Fig. 7, Supplementary Fig. S5); phenylpropanoid biosynthesis (Supplementary Figs. S10 and S11); isoflavonoid and flavonoid biosynthesis (Supplementary Figs. S10, S12, and S13); diterpenoid biosynthesis (Supplementary Figs. S10, and S14a); glutathione metabolism (Supplementary Figs. S10 and S14d); and production of hydrolases and chitinases (Supplementary Figs. S6 and S9c), stilbenoid, diarylheptanoid and gingerol biosynthesis, cutin, suberine and wax biosynthesis (Supplementary Figs. S10 and S14b, c), and various transporters (Supplementary Fig. S4) in roots.

Transcriptional profiles of NFRP-expressing roots indicated that the induction of several genes associated with hormone biosynthetic pathways (except gibberellins) are suppressed relative to the expression in vector control RMh plants (Supplementary Figs. S15 and S16). But intriguingly, NF treatment of the same PhL plants highly upregulated the expression of hormone biosynthetic pathway genes including those associated with gibberellin, ethylene, brassinosteroids,

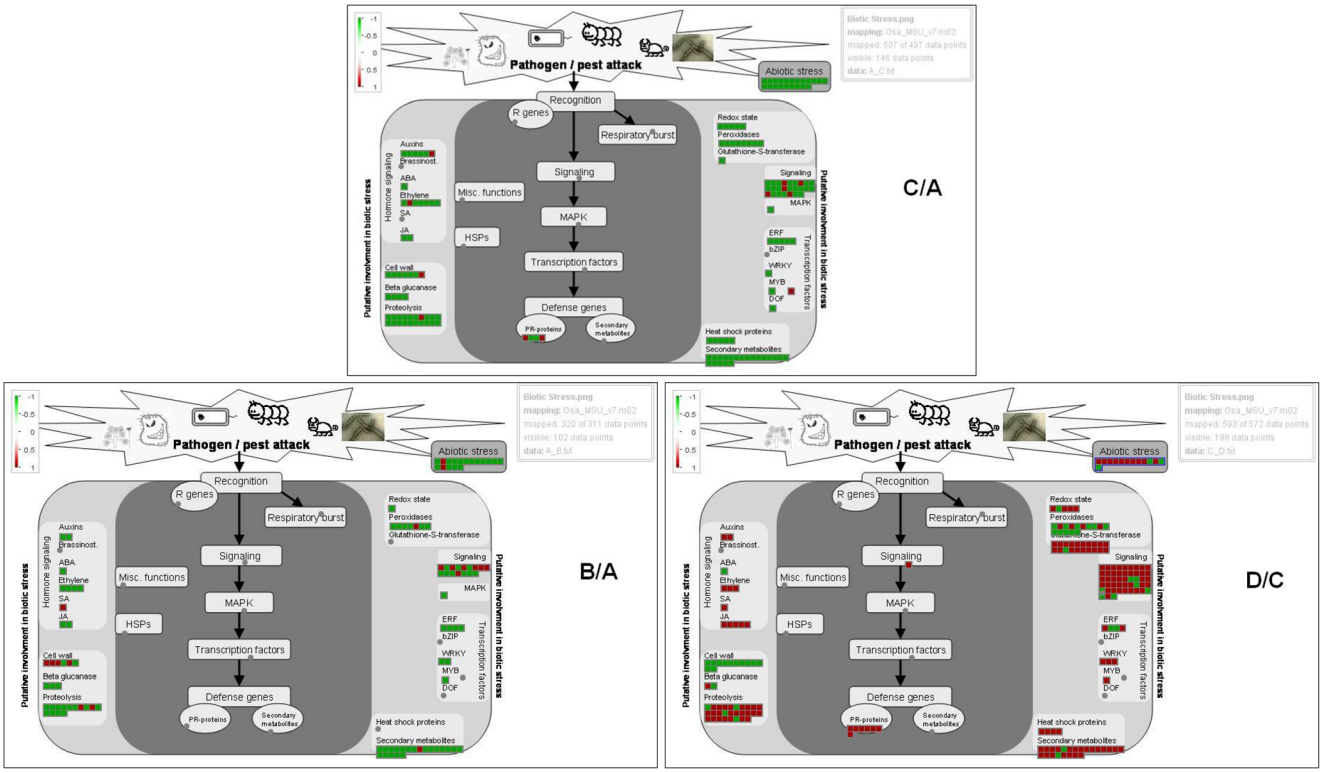


Fig. 7 MapMan diagram representing the differentially expressed genes related to biotic stress in roots of vector control RMh and PhL rice plants treated with or without Nod factors. PhL vs RMh plants (C/A), Nod factor–treated RMh plants vs untreated RMh plants (B/A), and Nod factor–treated PhL plants vs untreated PhL plants (D/C). Red: upregulated and green: downregulated

salicylic acid, jasmonic acid, and auxin biosynthesis, but excluding the genes involved in ABA and cytokinin biosynthesis. In contrast, in roots of vector control RMh plants, Nod factor treatment suppressed the induction of most hormone biosynthetic pathway genes.

Mapping of reads of the expressed transgenes from roots of PhL plants treated with or without NFs was performed to determine the number of reads aligning with the sequences of the transgenes *LjLNP*, *MtLYK3*, *MtNFP*, and *MtSYMREM* used for generating transgenic rice plants. As expected, none of the transgene reads aligned with sequences of transcripts derived from vector control RMh plants transformed with empty vector. On the other hand, 559, 1437, 8182, and 2024 reads respectively aligned with *LjLNP*, *MtLYK3*, *MtNFP*, and *MtSYMREM* transgenes used for transformation of PhL plants (treated with solvent alone), with coverage of 57, 91, 100, and 100% (Supplementary Table S5). In case of the PhL plants treated with NFs, 752, 1706, 12291, and 3335 reads aligned with the transgenes *LjLNP*, *MtLYK3*, *MtNFP*, and *MtSYMREM* with a coverage of 68, 94, 100, and 100%, respectively (Supplementary Table S5). These results are in agreement with the levels of transgene expression evaluated by RT-PCR, in the roots of rice plants carrying *LjLNP*, *MtLYK3*, *MtNFP*, and *MtSYMREM* (Fig. 2g).

It is relevant to mention here that the expression levels of the rice homologues of the legume Nod signal pathway genes, namely *OsLYK3* (LOC_Os08g42580), *OsNFP* (LOC_Os03g13080), *OsSYMRK* (LOC_Os07g38070), *OsPOLLUX* (LOC_Os01g64980), *OsCASTOR* (LOC_Os03g62650), *OsNUP133* (03g12450) *OsNUP85* (LOC_Os01g54240), *OsCCaMK* (LOC_Os05g41090), *OsCYCLOPS* (LOC_Os06g02520), *OsNSP1* (LOC_Os03g29480), *OsNSP2* (LOC_Os03g15680), and *OsERN1* (LOC_Os07g10410), were found to be consistently low in all four RNA-seq samples (A, B, C, and D). Neither the expression of legume NFRPs nor NF treatment altered the expression levels of any of these genes in roots of rice plants (Supplementary Table S6).

On the other hand, with regard to the homologues of downstream early nodulin genes, NF treatment led to upregulation of nodulin2-like gene (Os04g59020) and suppressed the expression of homologues of four *ENOD93* and two *SWEET* genes in roots of the vector control RMh plants (Supplementary Table S7). Expression of legume NFRPs in rice (PhL plants), however, resulted in downregulation of homologues of 10 early nodulin-like genes, including six homologues of *ENOD93*, in roots, but NF treatment caused suppression of only one nodulin-like gene in roots of these PhL plants (Supplementary Table S7).

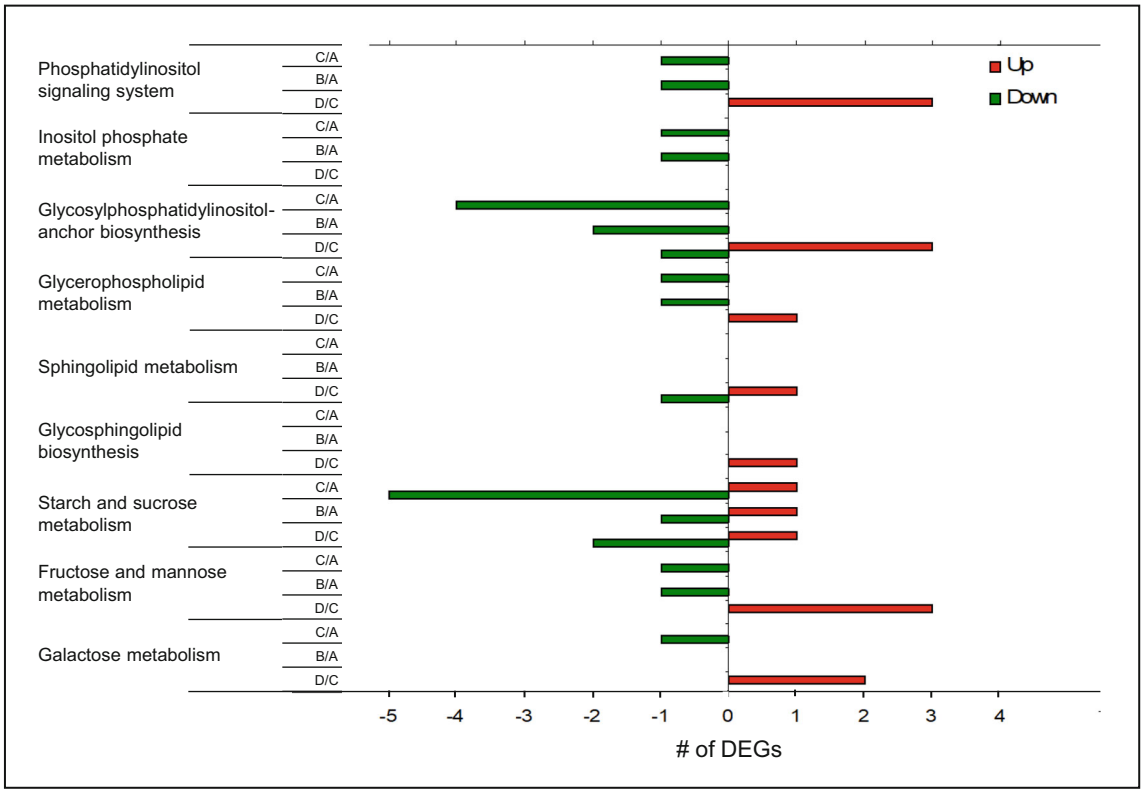


Fig. 8 KEGG analysis of differentially expressed signaling pathway genes (|log2ratio|≥ 1.5) in roots of RMh and PhL rice plants treated with and without Nod factors. (C/A) PhL vs RMh plants, (B/A) Nod factor–treated RMh plants vs untreated RMh plants and (D/C) Nod factor–treated PhL plants vs untreated PhL plants

Validation of RNA-seq Data by RT-qPCR Analysis

The RNA-seq results were validated by the RT-qPCR analysis of eight upregulated genes in roots of PhL plants treated with NFs, namely nicotianamine synthase (LOC_Os03g19420), expressed protein 1 (LOC_Os02g33070), glutathione S-transferase GSTU6 (LOC_Os10g38340), oxidoreductase, aldo/keto reductase family protein (LOC_Os04g27060), major facilitator superfamily antiporter (LOC_Os11g04020), expressed protein 2 (LOC_Os08g05960), transketolase (LOC_Os07g09190), and B12D protein (LOC_Os07g41350). Results obtained with RT-qPCR were found to be in agreement with RNA-seq data (Fig. 9).

Discussion

Rice Plants Have Inherent Ability to Perceive Rhizobial Nod Factors

NFs secreted by rhizobial cells play a pivotal role in promoting root infection and symbiotic nodule formation in legumes (Dénarié et al. 1996; Long 1996). Besides their role in the development of nitrogen-fixing symbiosis, NFs were also shown to have a beneficial influence on plant growth and development, such as promoting cell division, embryogenesis, seed germination, shoot biomass, root growth, lateral root branching, and alleviation of abiotic and biotic stresses in a wide range of legume as well as non-legume plants including monocots like maize, rice, and barley (De Jong et al. 1993; Rohrig et al. 1995; Khan et al. 2002; Souleimanov et al. 2002; Prithiviraj et al. 2003; Atti et al. 2005; Duzan et al. 2005; Olah et al. 2005; Jose et al. 2007; Miransari and Smith 2009; Miransari et al. 2006; Khan et al. 2011; Maillet et al. 2011; Kidaj et al. 2012; Schwinghamer et al. 2015; Sun et al. 2015; Tanaka et al. 2015). Recently, Liang et al. (2013) showed that *Arabidopsis thaliana*, which is neither mycorrhizal nor nodulation competent, is also capable of perceiving NFs.

NFs were found to induce gene expression in monocot plants such as rice. Reddy et al. (1998) using transgenic rice carrying *MtENOD12-GUS* reporter gene showed that NFs are able to induce the activity of *MtENOD12* promoter, indicating that rice has inherent ability to perceive NFs. Liang et al. (2013) found that NFs strongly suppress microbe-associated molecular pattern (MAMP)–triggered immune responses in *Arabidopsis* roots by means of a mechanism that results in diminished levels of pattern-recognition receptors in root plasma membrane. In the present study, transcriptome analysis of roots from vector control RMh plants revealed that NF

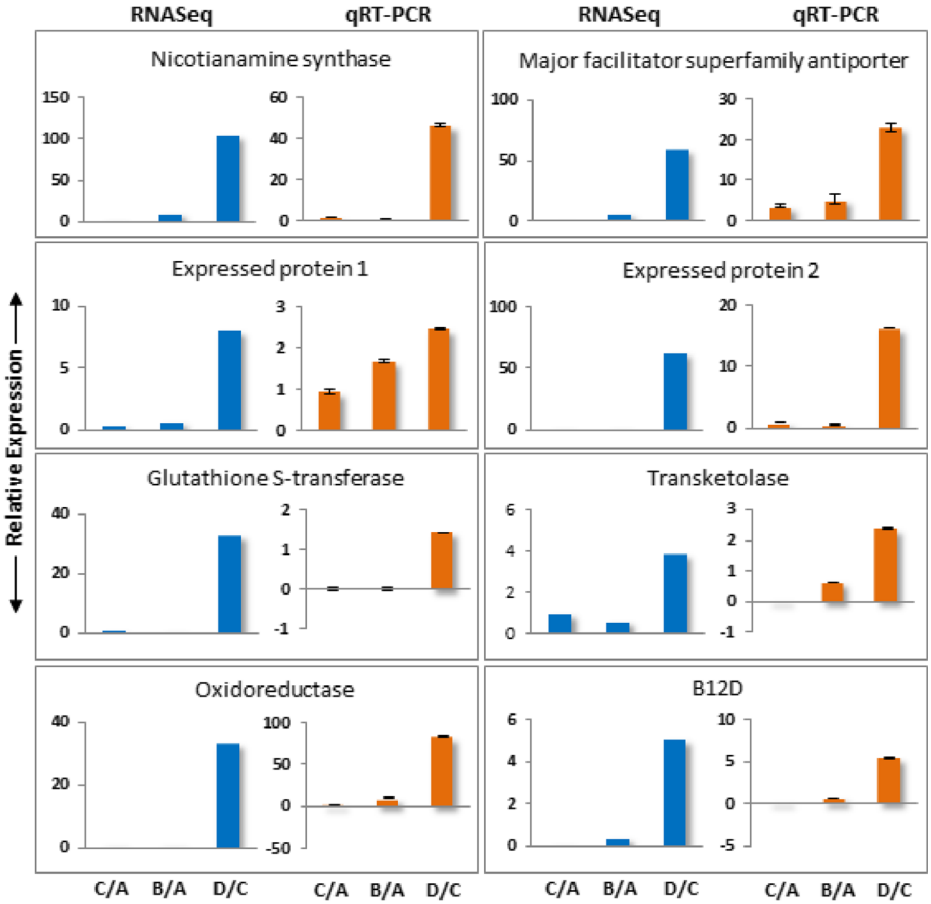


Fig. 9 Confirmation of expression profiles of candidate genes by RT-qPCR. In each box, the panel on the left presents the fold change expression values obtained from RPKM data of RNA-seq analysis, and the right panel represents the relative mRNA expression data from RT-qPCR. The relative expression values in RT-qPCR have been calculated keeping reference gene expression value as 1. The error bars represent the standard error between three biological replicates of each sample. The gene names are given on the top of each graph. Total RNA of root samples obtained from (A) vector control RMh plants; (B) vector control RMh plants treated with Nod factors; (C) PhL transgenic plants expressing NFRP genes; and (D) PhL transgenic plants treated with Nod factors. Accession numbers of the rice genes—nicotianamine synthase (LOC_Os03g19420.2), major facilitator superfamily antiporter (LOC_Os11g04020.1), expressed protein 1 (LOC_Os02g33070.1), expressed protein 2 (LOC_Os08g05960.1), glutathione S-transferase (LOC_Os10g38340.1), Transketolase (LOC_Os07g09190.1), oxidoreductase (LOC_Os04g27060.1), B12D (LOC_Os07g41350.1)

treatment induced changes in expression patterns of many genes. Among these, a significant number of genes associated with defense/immunity/stress responses (Fig. 7; Supplementary Figs. S5) and synthesis of secondary metabolites (Supplementary Figs. S6-S14) were downregulated, including genes encoding hydrolases, chitinases, and peroxidases (Supplementary Figs. S6 and S9c, d). The results pertaining to NF-mediated downregulation of defense/stress-related genes are in conformity with the results obtained with other monocot plants such as C_4 maize and *Setaria* (Tanaka et al. 2015). Altogether, these findings demonstrate that non-legume plants including rice not only have inherent ability to recognize NFs but also exhibit transcriptional response to NFs. It is not known whether downregulation of defense-related genetic programs is beneficial for accommodating symbiosis. *Arabidopsis* does not form symbiosis with either mycorrhizal fungi or rhizobia (Remy et al. 1994), but is able to recognize NFs (Liang et al. 2013). Similar to maize, *Setaria* (Tanaka et al. 2015) and rice (present study), NFs suppress MAMP-triggered immune responses in *Arabidopsis* (Liang et al. 2013). Based on these results, Liang et al. (2013) suggested that suppression of MAMP-triggered immunity by NFs represents a LCO recognition mechanism, which is not associated with processes related to symbiosis.

Impact of Expression of Legume NFRPs on NF Perception in Rice Roots

In the present study, we have found that the expression of legume NFRPs profoundly impacts transcriptional responses in rice roots. The RNA-seq analysis revealed that in NFRP-expressing rice roots, the expression of diverse genes was

downregulated including genes related to microbe-associated molecular pattern (MAMP)–triggered immunity and other defense responses as well as those involved in biosynthesis of secondary metabolites (Fig. 7, Supplementary Figs. S5-S14) and phytohormones (Supplementary Figs. S15 and S16).

However, upon treatment with NFs, the NFRP-expressing PhL plants exhibited profoundly different gene expression patterns in contrast to untreated PhL plants. In general, many genes participating in diverse biological processes were highly upregulated in NF-treated roots. GO and KEGG analysis of DEGs revealed that highly enriched categories of genes belonging to biological processes are particularly related to response to stress (including abiotic, biotic, and defense responses), metabolic processes (including primary and secondary metabolic processes together with pathways for generation of primary precursor metabolites and secondary metabolites), transcriptional regulation, hormonal stimulus, and transport (Fig. 7; Supplementary Figs. S4-S16).

Transient co-expression of MtNFP and MtLYK3 was found to induce cell death in tobacco leaves (Pietraszewska-Bogiel et al. 2013). The induction of cell death due to co-production of MtNFP and MtLYK3 was found to be related to defense-like response, as indicated by compromised membrane permeability and accumulation of phenolic compounds. Likewise, transient co-expression of orthologous LysM-RLKs, LjNFR5, and LjNFR1from *L. japonicus* also produced similar damaging effects in tobacco leaves as well as in epidermal cell peels of *Allium ampeloprasum* (Madsen et al. 2011). However, results of the present study showed that co-expression of MtNFP and MtLYK3 in rice did not produce such a detrimental effect.

In legumes, addition of NFs to root hairs causes a surge of ROS production within 30 s, and within subsequent few minutes prompt extrusion of ions across cell plasma membrane, resulting in alkalization of medium (Cárdenas et al. 2008; Radutoiu et al. 2003). ROS production and alkalization are also early plant defense responses in MAMP recognition (Zipfel et al. 2006). If such physiological responses are left unchecked by cellular regulatory mechanisms, they have a potential to cause cell death (Madsen et al. 2011). In legume symbiosis, upon NF perception, the LysM-RLKs (MtLYK3-MtNFR or LjNFR1-LjNFR5) also trigger burst of ROS in root hairs and extracellular alkalization, but they are only transiently exhibited. It is possible that these effects are promptly suppressed by symbiosis-specific cellular regulatory components, thus averting cell damage/death. Hence, it was hypothesized that due to the absence of symbiosis-specific regulatory components in tobacco, the LysM-RLKs-mediated signaling are interpreted differently leading to activation of defense response and ultimate cell death (Madsen et al. 2011; Pietraszewska-Bogiel et al. 2013).

In rice, we co-expressed NF receptor-like kinases, MtNFP and MtLYK3, along with their interacting protein, MtSYMREM1, and another NF receptor LjLNP. Unlike in tobacco, co-expression of MtNFP and MtLYK3 did not cause any detrimental effect in rice. It is not known whether the lack of such a negative effect in rice is due to co-expression of MtNFP and MtLYK3 along with other symbiosis-related components, MtSYMREM1 and LjLNP, or whether rice has inherent mechanism to interpret the LysM-RLKs-mediated signaling differently to avert such cell damage/death. Incidentally, co-expression of these NFRPs resulted in the downregulation of expression of several defense-related genes in rice. Upon treatment with NFs, diverse defense responsive genes were upregulated in the rice roots expressing the legume NFRPs. Even such an upregulation of defense response genes did not cause any visible damage in rice plants, excepting some loss in pollen viability. In legume-*Rhizobium* symbiosis, a number of genes involved in the defense response against pathogen are abundantly induced during initial stages of rhizobial infection, but their expression was quickly repressed during subsequent phases of nodule development (Kouchi et al. 2004).

Expression of Legume-Specific NFRPs Hypersensitizes Rice Roots and Primes Root Hairs to Respond to NFs

In legumes, NF treatment induces root hair deformation. This morphological change occurs due to reactivation of polar growth in root hairs resulting in branching, curling, etc. (Larrainzar et al. 2015). Root hair curl (the shepherd's crook) creates a niche for entrapment of rhizobia to enable bacterial cells to initiate infection thread formation. Earlier studies with more than 50 rice varieties including some wild species of rice demonstrated that unlike in legumes, rice lacks the ability to respond to rhizobial NFs in terms of exhibiting any morphological changes in root hairs (PM Reddy and JK Ladha, unpublished). Present study showed that the expression of legume NFRPs enables rice root hairs to efficiently respond to NFs (Fig. 5). Elicitation of root hair deformation in NFRP-expressing PhL plants in response to NFs indicates that the legume NFRPs are able to function normally in rice cellular milieu to enable perception and transmission of Nod signal appropriately to trigger phenotypic changes in root hairs.

Influence of Legume NFRP Expression on Colonization of Rice Roots by Rhizobia

Results of the current study showed that colonization of rhizobia on root surface as well as at the sites of lateral root emergence was lower in rice plants expressing legume NFRPs as compared with vector control RMh plants (compare Fig. 3d with b). It may be relevant to mention here that *Rhizobium* NGR234 strain used in these studies carried hybrid *nodD* (flavonoid-independent transcriptional activator, FITA) gene, which is capable of inducing NF production independently of

flavonoids (Spaink et al. 1989). Our study with the RNA-seq analysis of PhL roots clearly evidenced that the constitutive expression of legume NFRPs make rice roots hypersensitive to NFs, prompting massive upregulation of diverse genes associated with defense response. Thus, it is tempting to speculate that reduction in density of rhizobial colonization in the roots of NFRP-expressing PhL plants may be a direct consequence of NF-activated plant defense, which likely hinders bacterial growth. However, it should be emphasized here that in case of legume roots, when inoculated with rhizobia, expression of several defense-related genes in roots are transiently upregulated, but at later stages, their expression subsides, paving way for bacterial infection and development of symbiosis (Gourion et al. 2015; Larrainzar et al. 2015). It will be interesting to study if such a phenomenon occurs in PhL plants (rice plants expressing NFRPs) as well when inoculated with rhizobia.

To the best of our knowledge, no information is available on dynamics of gene expression in legumes exposed to purified NFs/LCOs for prolonged durations (beyond 48 h; Wang et al. 2012). In soybean, LCO (NodBj-V (C18:1, MeFuc) foliar spray promoted expression of various stress resistance genes, especially those involved in defense, cold, oxidative stress, and phosphate deficiency (Lindsay 2007; Wang et al. 2012). In current study with PhL rice plants expressing NFRPs, 24-h NF treatment resulted in drastic upregulation of defense-related genes in roots. In order to verify if NF effect on upregulation of defense genes is transitory, as is the case with legume roots inoculated with rhizobia (in legumes, defense responses gradually wane off beyond 48 h after rhizobial inoculation, e.g., Cook et al. 1995), gene expression studies need to be performed with longer incubation periods with NFs. However, it will be difficult to undertake such studies as NFs are gradually degraded and their concentration tapers off due to the activity of hydrolases/chitinases in root environment. Under such circumstances, it will be challenging to interpret if apparent waning of defense-related gene expression (if any) is due to lowering of concentration of NFs as a consequence of degradation, or is it really because of inherent plasticity of plants to re-calibrate gene expression to original levels after initial surge in gene expression.

Expression of NFRPs Modulates Hormone Biosynthetic Pathways in PhL Rice Roots

Phytohormones such as salicylic acid, jasmonic acid, ethylene, abscisic acid, and brassinosteroids have been implicated in playing positive role in conferring immunity in rice, while auxins and gibberellins were shown to function as negative regulators (Yang et al. 2013).

Analysis of transcriptional profiles of NFRP-expressing rice roots indicated that several genes associated with hormone biosynthetic pathways (except gibberellins) are downregulated relative to the expression in vector control RMh plants (Supplementary Figs. S15 and S16). But, intriguingly, NF treatment of the same NFRP-expressing plants highly upregulated the expression of brassinosteroid, jasmonic acid, salicylic acid, and ethylene biosynthetic pathway genes including those associated with auxin and gibberellin biosynthesis. Future studies can only answer if NF-activated biosynthesis of hormones in turn helps regulate immunity-related genetic circuits in rice plants.

Rice Native Receptors and the Legume NF Receptors Activate Divergent Nod Signaling Cascades in Rice Roots

Both vector control RMh plants and PhL plants expressing the legume NFRPs perceive NFs, but set off vastly divergent gene expression patterns. Upon perception of NFs, expression of several defense/stress response genes was suppressed/downregulated in vector control RMh plants, while in PhL plants expressing the legume NFRPs, expression of entirely a divergent set of defense/stress response genes was activated/upregulated. These results suggest that the "native receptors" of rice and the "NF receptors" of legumes interpret Nod signal differently and activate distinct signaling cascades leading to disparate gene expression patterns in rice roots.

Conclusion

A long-cherished goal of legume-rhizobia symbiosis research is to extend this symbiotic nitrogen-fixing capacity to cereal plants such as rice to render them independent of synthetic N fertilizers. As a part of achieving this ultimate goal, in this work, initially, we expressed three legume-specific nodulation (Nod) factor receptor protein genes in rice and assessed their impact on Nod factor (NF) perception and consequently triggered biological responses in roots. In the current study, we report for the first time that the expression of legume Nod factor receptor proteins rendered rice roots hypersensitive to rhizobial NFs and conferred root hairs the ability to respond to NFs in terms of exhibiting deformations, albeit at lower levels, reminiscent of legume initial symbiotic reactions. Moreover, the study demonstrated that the legume-specific NF receptors transmit Nod signal differently, activating distinct signaling pathways in rice roots. This study forms a foundation for adding on additional genetic circuits needed for the development of rhizobial symbiosis in rice.

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Author Contributions AA-M performed experiments and wrote manuscript. LL carried out bioinformatic analysis. MO-B, MR, SS, and SD-R contributed in primer designing and quantitative RT-PCR experiments. LM, MLV-H, and SS-K performed Southern blot analyses. NM performed statistical analysis. GH critically reviewed the article and provided inputs. PMR conceptualized and planed the project and vector construction and contributed to manuscript writing.

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Data Availability All data generated from this investigation are included in the main text of the manuscript and in the Supplementary Files. The complete RNA-seq data have been deposited in NCBI's Bioproject under identifiers PRJNA545468, GSE131964 (https://www.ncbi.nlm.nih.gov/bioproject/?term=GSE131964).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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