Biospectra : ISSN: 0973-7057, Vol. 20(1), March, 2025, pp. 209-218

An International Biannual Refereed Journal of Life Sciences

Plant Science

ISSN : 0973-7057

Bioengineering the nodulation pathway in rice from legume

Anjulata Singh*

TERI School of Advanced Studies & The Energy and Resources Institute, New Delhi, India.

Received : 25th January, 2025 ; Revised : 27th February, 2025

DOI:-https://doi.org/10.5281/zenodo.16613248

Abstract- For maximum productivity, rice, a staple food for a large percentage of the world's population, needs a lot of nitrogen input. The present dependence on artificial nitrogen fertilizers is a contributing factor to greenhouse gas emissions and environmental contamination. A sustainable substitute is rice that has been bioengineered to form a symbiotic relationship with nitrogen-fixing bacteria. By introducing important genes from legumes, this study tackles the problem of engineering the nodulation pathway in rice. Nodulation-specific transcription factor (TF) genes from *Medicago truncatula* were introduced into rice (*Oryza sativa*) as part of the study. This was accomplished by expressing nodulation-specific TFs genes using transformation techniques. Root hair deformation and colonization in response to Nod factors or rhizobial inoculation were used to assess the transgenic rice plants' capacity to initiate early symbiotic responses. The ability to start early symbiotic responses was shown by the modified rice plants that expressed genes for TFs specific to nodulation derived from legumes. The development of a nitrogen-fixing symbiosis depends on these reactions. Additionally, the study verified that transgenes were expressed in the engineered plants' roots. The development of nitrogen-fixing cereal crops, which could lessen the need for synthetic nitrogen fertilizers and encourage sustainable farming methods, is made possible in large part by this research. Further studies to improve the nitrogen-fixation process in rice and other cereals are made possible by the successful introduction and expression of nodulation genes in rice.

Keywords: Rice, nitrogen fixation, bioengineering, root hair deformation, *S. meliloti*

INTRODUCTION

Rice (*Oryza sativa*) is a staple crop that feeds nearly half of the global population. Increasing its productivity is vital to ensuring food security in the face of a growing population. Among various agronomic inputs, nitrogen (N) is the most critical nutrient for rice productivity-approximately 1 kg of nitrogen is required to produce 15-20 kg of rice grain. By 2050, global demand for rice is expected to necessitate a 70-80% increase in nitrogen input.[1] Currently, this demand is met primarily through the application of synthetic nitrogen fertilizers produced via the Haber-Bosch process, which relies heavily on non-renewable fossil fuels and contributes to environmental pollution and greenhouse gas emissions.[2,3] Alarmingly, 50-70% of applied nitrogen fertilizer is lost to the environment due to low nitrogen use efficiency in crops, leading to groundwater contamination, eutrophication, and nitrous oxide emissions.[4]

Biological nitrogen fixation (BNF), carried out by certain prokaryotes using the nitrogenase enzyme complex, offers a more sustainable alternative for nitrogen supply. This ATP-dependent enzymatic process converts atmospheric nitrogen (N_2) into ammonia (NH_3), which plants can assimilate. While free-living nitrogen-fixing organisms such as cyanobacteria and *Azolla* exist, their application in agriculture remains limited due to

*Corresponding author :
Phone : 9560115285
E-mail : anju.s0903@gmail.com

insufficient nitrogen contribution and economic constraints.[5-8]

Legumes, however, form efficient symbiotic associations with rhizobia, leading to the formation of root nodules wherein atmospheric nitrogen is fixed and transferred to the host plant. This root nodule symbiosis (RNS) represents one of the most advanced and effective BNF systems.[9,10] Initiation of this symbiosis is triggered by the secretion of flavonoids by legume roots under nitrogen-deficient conditions, which in turn induce the production of host-specific lipochitooligosaccharide Nod factors (NFs) in rhizobia.[11,12] Perception of NFs by plant receptors activates a cascade of symbiotic gene expression and developmental responses such as calcium spiking, root hair deformation, and cortical cell division, culminating in nodule formation.[13]

In *Medicago truncatula*, the LysM-type receptor kinases MtLYK3 and MtNFP mediate NF recognition,[14,15] initiating signal transduction through the common symbiotic pathway (CSP). The CSP includes MtDMI2 (a leucine-rich repeat receptor-like kinase), MtDMI1 (a cation channel), MtDMI3 (a calcium/calmodulin-dependent kinase), and IPD3 (a nuclear-localized coiled-coil protein).[16-19] Downstream, nodulation - specific transcription factors such as NSP1, NSP2, ERN1, and NIN regulate gene expression required for infection thread formation and nodule organogenesis.[20-23]

Notably, components of CSP are not only essential for RNS but also indispensable for the more ancient arbuscular mycorrhizal symbiosis (AMS) in both legumes and cereals. In rice, which naturally forms AMS with endomycorrhizal fungi to enhance phosphate uptake, orthologs of CSP genes-*OsCASTOR, OsPOLLUX, OsCCaMK,* and *OsCYCLOPS*-have been shown to be required for AM colonization.[24-27] Moreover, rice orthologs can complement CSP gene mutations in legumes, indicating a conserved evolutionary framework.[28,29]

However, rice lacks or has poorly conserved orthologs of key legume NF perception and transcriptional regulators, including *NFP, LYK3, NSP1, NSP2, ERN1*, and *NIN*.[30,31] As a result, rice is incapable of recognizing rhizobial NFs or initiating nodulation responses. To overcome this limitation, we hypothesize that the coordinated expression of legume-derived Nod factor receptors (*NFP* and *LYK3*) and transcription factors (*NSP1, NSP2, ERN1*, and *NIN*) in rice could enable the integration of Nod signal perception with the endogenous CSP, potentially triggering rhizobial responses in rice roots.

In this study, we aimed to engineer rice plants to express these nodulation-specific genes from *Medicago truncatula* and evaluate their ability to activate early symbiotic responses such as calcium spiking, root hair deformation, and infection thread formation in response to Nod factors or rhizobial inoculation. This work represents a step toward the long-term goal of developing nitrogen-fixing cereal crops and reducing reliance on synthetic nitrogen fertilizers.

MATERIALS & METHODS

Callus induction, Growth conditions, and media

Mature rice seeds (*Oryza sativa* ssp. *japonica*, cv. Murasaki R86) were dehusked and surface sterilized with 70% (v/v) ethanol for 1 min followed by treatment with 4% sodium hypochlorite for 15 min with gentle shaking and washed five-six times with sterile autoclave water. The seeds were then left in sterile water to imbibe overnight at room temperature in the dark.[32]

Sterilized seed surfaces were placed on the N6D media, which is a callus induction agar medium that contains, N6D medium, 3% sucrose, and the plant hormone 2,4-Dichlorophenoxyacetic acid (2,4-D) 2 mg/L at a pH of 5.8. Ten to twelve seeds were added to each N6D agar plate. After sealing the plates with the sealer, they were left in a dark, plant-maintained chamber at 28 ± 2°C. After two weeks, calli extracted from the scutella area were moved to a fresh N6D medium and maintained in the same environment for two weeks. Before being bombarded, calli measuring 1-3 mm in diameter were placed in osmotic agar media (OM), which contained N6D media, 3% sucrose, 3% sorbitol, and 3% mannitol, as well as the plant hormones 2,4-Dichlorophenoxyacetic acid (2,4-D) 2 mg/L, pH 5.8, and the solidifying agent phyta gel 0.4%, for 4 hours before transformation.[33]

Tungsten Particle Preparation and plasmid Coating

Preparation of Tungsten particle stock: 60 mg of tungsten particles (Bio-Rad) were added to a 1.5-mL microfuge tube along with 1 mL of molecular grade 100% ethanol. The tube was vortexed for 2 min and centrifuged at 10,000 rpm for 1 min. The supernatant was discarded and tungsten particles were resuspended in 1 mL of sterile water and centrifuged at 10000 rpm for 1 min. The supernatant was discarded and tungsten particles were

resuspended in 1 mL of sterile water and stored at 4°C until further use for plasmid coating (http://staceylab.missouri.edu/biolistic-transformation-of-rice/).

Plasmid (pOmega, generous gift from Dr. P.M. Reddy) coating of tungsten particles: 50 µL of reconstituted tungsten particles (for two bombardment shots) were transferred to a 1.5 mL microcentrifuge tube, followed by the addition of 5 ?g of plasmid, 50 µL of 2.5-M $CaCl_2$, and 20 µL of 0.1M spermidine. Centrifuge at 10,000 rpm for 10 sec after finger tapping for 3 min and incubating the mixture at room temperature (RT) for 10 min. The pellet was cleaned with 250 µL of 100% ethanol after the supernatant was removed. It was then finger-tapped for 2 min and centrifuged for 1 min at 13,000 rpm. After removing the supernatant, the plasmid-coated pellet was reconstituted in 60 µL of 100% ethanol, and the tungsten particles coated with pJ5hL were placed on ice till particle bombardment (http://staceylab.missouri.edu/biolistic-transformation-of-rice/).

Biolistic Transformation of calli, Selection, and Regeneration

Calli incubated in OM are used for bombardment. Bio-Rad PDS-1000/He biolistic gun, settings used for the transformation (using the Mono Adaptor setup of the Bio-Rad PDS 1000/He particle gun) were as follows: Helium pressure at the tank regulator: 1300-1400 psi, Rupture discs: 1100 psi (Bio-Rad), macrocarrier (flying disc) assembly: level one from the top, Petri dish holder: level two from the bottom and vacuum (at the time of the shot): 27-28 Hg.[34]

Bombarded calli were incubated in the dark for 24 h at 28 ± 2 °C. Later, calli were transferred to Selection agar media (SM), which contained N6D media, 3% sucrose, as well as the plant hormones 2,4-Dichlorophenoxyacetic acid (2,4-D) 2 mg/L, pH 5.8, and the solidifying agent phyta gel 0.4%, along with antibiotic meropenem 25 mg/L and hygromycin 50 mg/L and kept in the dark at 28 ± 2 °C. After a fortnight calli were transferred to a fresh SM media agar plate with hygromycin (100 mg/L) and meropenem (25 mg/L) incubated in the dark at 28 ± 2 °C in a plant chamber. Subsequently, calli were transferred to regeneration media (RM), which contain N6D media, 3% sucrose, 3% sorbitol, plant hormones 1-Naphthylacetic acid 0.5 mg/L and BAP 3 mg/L, pH 5.8, and the solidifying agent phyta gel 0.4%, along with antibiotic meropenem 25 mg/L and hygromycin 50 mg/L and subculturing was done every fortnight at 28 ± 2 °C in 16 h photoperiod. The plantlets that produced roots were transferred to pots containing soilriteTM (Gardenesia, India) and kept in a plant chamber for 10-15 days for hardening. Afterwards, the regenerated putative Omega plants were transferred to a transgenic greenhouse and maintained at 28 ± 2 °C. We developed vector control (VC) transgenic rice plants that only had the *HPT* gene with the *OcsP* promoter. J5hL plants were developed by using pJ5hL plant transformation vector.[35]

Molecular characterization of transgenic plants

The CTAB technique was used to recover DNA from purported transgenic rice Omega leaves.[36] PCR amplification with gene-specific primers verified the existence of suspected transgenes Table 1.

Table 1: Primers used in the PCR confirmation

Gene	Primer name	Sequence
For amplification of transgene in rice plants		
MtNFP	Mt12-5'-F Mt12-3'-R	GCCTTCTTTCTTCCTTCTAG GTAACTTATCTGCAGTCTCG
MtLYK	LY3 5'-F LY3-Rev2	GTGTGATGTAGCTTTAGCTTC GATACGTAAACTCTGTCGACTTTG
MtDMI2	DMI2-5'-F DMI2-3'-R	CAACTAAAGGGTTTGAGAGC GAAAGTGGAGAAACACAGAG
MtSYMREM1	MtREM-BgF MtREM-XmR	AGATCTGCTGTAACCCTAGAGATACATTATGG CCCGGGCTAACTGAAAAACCTTAAACCGCTGA
LjLNP	LjLNP-F2 LjLNP-R2	TTGCGCTATGGAAGAGAAGC GGACGAATTTTGGCATTGGG
Cameleon	Cam-xhoF Camel-2-RQ	TTCTCGAGATGGTGAGCAAGGGCGAGGAGCTGTTCAC TGTCCAAGCGACCTCATAACG
HPT	HPT-FP HPT-RP	AGCTGCGCCGATGGTTTCTACAA ATCGCCTCGCTCCAGTCAATG
MtNSP1	MtNSP1XhF MtNSP1KpR	CCGCTCGAGATGACTATGGAACCAAATCC CGGGGTACCTACTCTGGTTGTTTATCCAG
MtNSP2	MtNSP2BgF MtNSP2KpR	GGAAGATCTATGGATTTGATGGACATGGATG CGGGGTACCTATAAATCAGAATCTGAAGAAG
MtERN1	MtERN-XmaF MtERN-XbaR	TAATCCCGGGATGGAAATTCAATTTCAGCAACC CTGTTCTAGATTAACAGAACAAGGAGCAC
MtNIN	MtNIN-F2 MtNIN-R2	CCTACCTCCAAGACCAACTG ACTGCTGCTGCTGCTGTTG
MtENOD40	E40-Sac-F E40-Xma-R	GAGCTCACTACCTTTCTATGTGGAG CCCGGGAGAAGAAAAGGAACATGAATTAATG
GUS	5'GUS 3'GUS	GGTGGGAAAGCGCGTTACAAG GTTTACGCGTTGCTTCCGCCA

Germination of transgenic rice seedlings

Transgenic rice seeds were dehusked, surface sterilized, and planted in Petri plates on MS agar medium.[37] They were then left to germinate in a culture chamber that was kept at 28 ± 2 °C and with a 16:8 h light:dark photoperiod. The arrangement of the petri plates was such that the roots developed vertically on the surface of the agar.

Semi-quantitative PCR analysis

Following the manufacturer's instructions, total RNA was isolated from Omega transgenic rice roots using the HiPurA® Plant and Fungal RNA miniprep Purification Kit (Himedia, India). A Oubit 3 fluorometer (Thermo

Scientific, USA) was used to measure the concentration of total RNA, and gel electrophoresis was used to verify its purity. In accordance with the manufacturer's instructions, 1µg of mRNA extracted from roots was used to create cDNA using the Revert Aid H Minus first strand cDNA synthesis kit (Thermo Scientific, USA). Using gene-specific primers, reverse-transcribed cDNA was utilized to analyse the expression of *MtNSP1, MtNSP2, MtERN1,* and *MtNIN* Table 2. To illustrate the relative changes in distinct gene expression levels across different transgenic plants, the constitutively expressed housekeeping gene elongation factor (*EFTu*) was used as a reference internal control. 2% agarose gels were used to separate the PCR products.

Table 2: Gene-specific primers for Semiquantitative PCR

For RT-PCR analysis of transgene expression in rice plants		
MtNSP1	MtNSP1-F2 MtNSP1-RQ	CAACATTGTTGCTGCTAGTACTAC AGACTTAGTTCATCTTCGGC
MtNSP2	MtNSP2-FQ MtNSP2-RQ	AACGCATGAAGTTGGTGATG TATCACTCGAGCTAAGTCAC
MtERN1	MtERN-FQ MtERN-RQ	CTCGAACCAACTTCATCACTC TACTTGGAGCAGAAGCAACAG
MtNIN	MtNIN-FQ MtNIN-R2	GCACACATCATCTCACACACG ACTGCTGCTGCTGCTGTTG
OsEFTu	OsEF Tu-F OsEF Tu-R	GTCATTGGCCACGTCGACTC CGTGCAGTAGTACTTGGTGG

Development and confirmation of Hybrid transgenic rice

J5hlOm plants were developed by crossing-pollinating emasculated flowers of the J5hl plant[35] (recipient) with pollen derived from the Omega plant (donor) employing a conventional plant breeding method. The seeds obtained from J5hLOm plants as a result of crosses were germinated, and F_1 hybrid seeds were confirmed by PCR using gene-specific primers of J5hL and Omega plants Table 1.

Rhizobium Inoculum Preparation

The GFP-tagged rhizobial strain *S. meliloti* was cultured on TY agar medium at 30° C in dark. A single colony was inoculated to 50 mL of liquid medium containing the antibiotics nalidixic acid (20 mg/L) and tetracycline (12.5 mg/L) and kept at 160 rpm on a shaker and incubated at 30°C. Centrifugation at 3000 x g for 10 min at room temperature was used to pellet down the bacterial cells after 8-10 hours. After being cleaned, the pallet was resuspended in Fahraeus[38] medium without nitrogen, which had been adjusted to an OD_{650} of 0.25.[2]

Colonization of *S. meliloti* on transgenic rice root

Both transgenic hybrid (J5hLOm) and wild type (WT) rice seeds were dehusked, surface sterilized, and planted in Petri plates on MS agar medium. The seeds were then allowed to germinate in a culture room with a 16-hour photoperiod and a temperature of 28 ± 2 °C. The arrangement of the Petri plates was such that the roots developed vertically on the surface of the agar. Three-day-old J5hLOm and WT rice seedlings were aseptically moved into 25 x 150 mm culture tubes with 20 mL of liquid nitrogen-free Fahraeus media and 3µM luteolin. The plants were then incubated for two days in the culture room. The seedlings were regrown for 30 days after being aseptically inoculated with the inoculum on the third day.

Root hair deformation assay

Wild rice seed (R86), vector control (VC), and J5hLOm transgenic hybrid rice plants were dehusked and surfaced sterilized. For germination in the culture room, surface-sterilized seeds were placed on an MS basal agar media plate. After six days, the roots' tips were immediately treated with 10^{-9} M pure *Sm*NF (which was a gift from Fabienne Maillet, LIPM, CNRS, France). Deformation of the root hair was seen at a fixed length of 2 cm, 0.5 cm from the tip. The observation was recorded at 6 dpi, 12 dpi, and 24 dpi.

Statistical analysis

All of the study's data points were statistically analyzed using OriginPro 2024b. For all metrics, we assess significant differences between the transgenic and WT plants using the student t-test. Unless otherwise noted, each experiment was conducted three times.

RESULTS

Development and Analysis of Genetically Modified Rice Plants

To generate transgenic plants, pOmega, we transformed four-week-old *Oryza sativa* (R86) calli by biolistic bombardment, and Omega transgenic rice plants were produced (Fig.1).

The development of J5hL plants and gene expression has been published in our previous paper.[35] The hygromycin selected calli regenerated into transgenic rice plants Omega was confirmed by PCR using gene-specific primers (Fig. 2A). Phenotypically there was no difference in transformed plants and untransformed plants (WT). The expression of the transgenes was confirmed from the roots of J5hL and Omega plants through semi-quantitative RT-PCR using gene-specific primers Fig. 2B. No expression of the gene was detected in WT (R86).

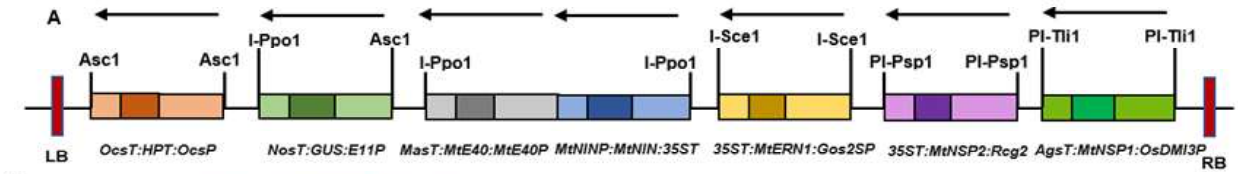

Fig.1 (A) pOmega plant transformation vector houbouring transcription factor genes; (B) Different stages of regenerating calli transformed with pOmega vector; (a) callus in callus induction media; (b) regeneration media and (c) rice plantlets in rooting medium; (d) transgenic plant in greenhouse. Bar scale represents (a-c) 1.75 cm, and (d) 8 cm.

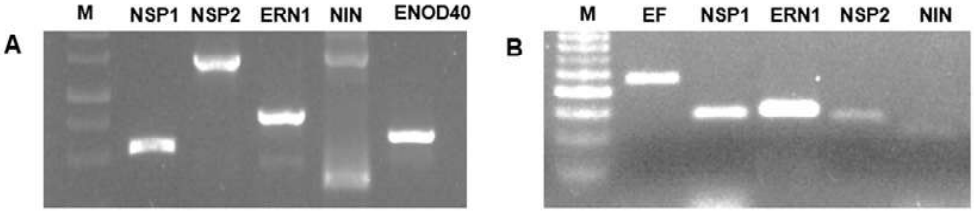

Fig. 2 (A) PCR confirmation of nodulation transcription factors genes in Omega plants (*NSP1, NSP2, ERN1, NIN* and *ENOD40*) genes, M- marker (1 kb ladder); (B) Semi-quantitative RT-PCR of transgenes in Omega plant. The PCR product was analyzed on 2% agarose. M- marker (50 bp).

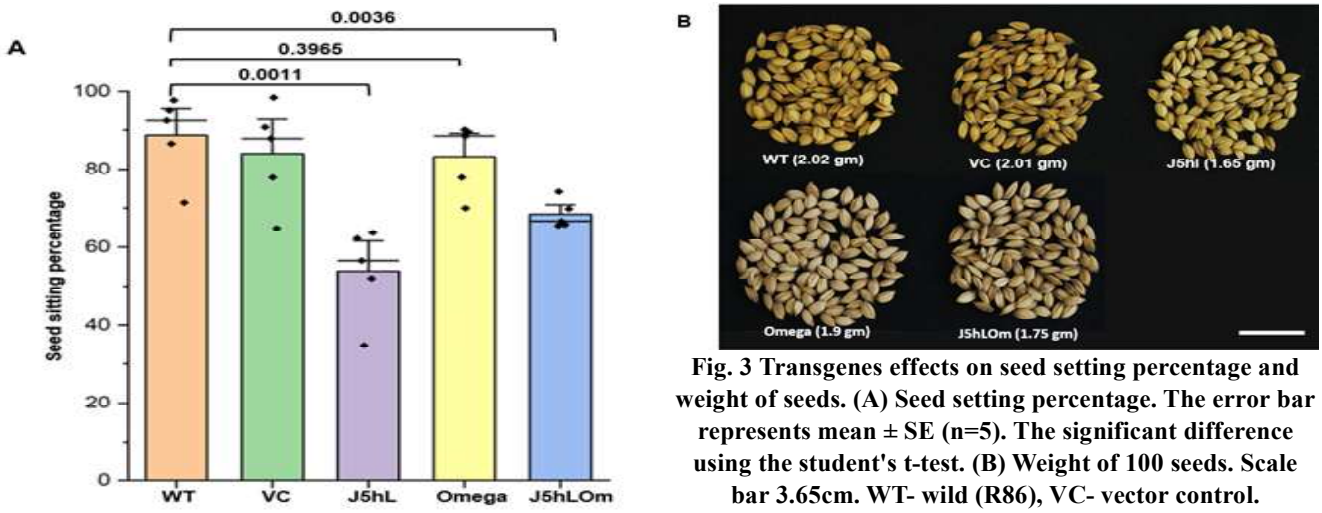

Fig. 3 Transgenes effects on seed setting percentage and weight of seeds. (A) Seed setting percentage. The error bar represents mean ± SE (n=5). The significant difference using the student's t-test. (B) Weight of 100 seeds. Scale bar 3.65cm. WT- wild (R86), VC- vector control.

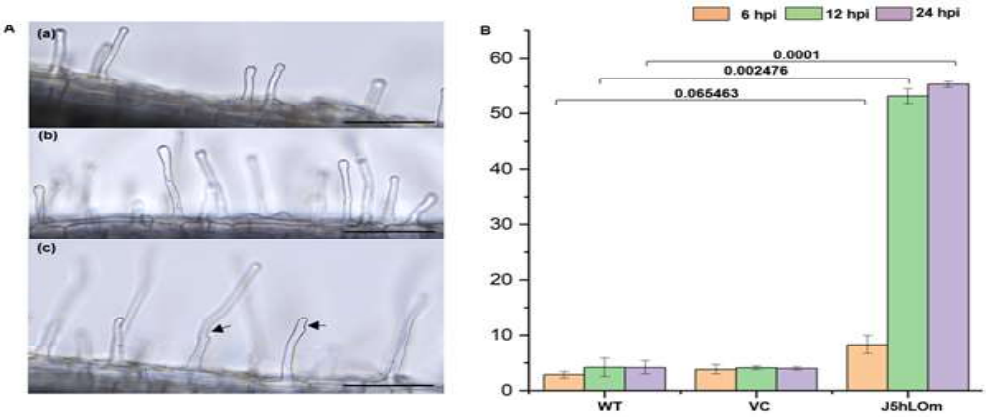

Fig. 4 (A) Representative picture of the response of root hairs when treated with *Sm*NF. The arrow indicates the distorted roots. (a, c, d) root hairs response of WT, VC, and J5hLOm. Scale bars are 50µm; (B) percentage of deformed root hairs treated with *Sm*NF. The whisker marks mean ± 1.5 SE. The student's t-test was highly significant. n=3.

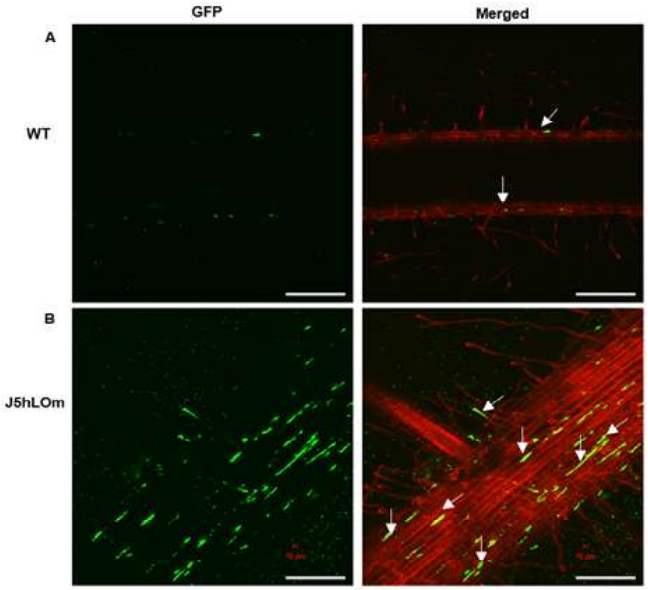

Fig. 5 Photomicrographs showing colonization of rhizobial cells on the surface of rice root of WT and J5hL inoculated with GFP tagged *S. meliloti* 30 dpi. (A) confocal image of WT rice root; (B) J5hLOm root. Green colour indicates the *S. meliloti* tagged with GFP. The red colour of the root is due to propidium iodide (PI) staining. Micrographs were taken using a confocal scanning microscope (Zeiss LSM 710). Scale bars is 10 µm.

We chose to use the conventional method of crossing the transgenic plants to obtain 10 genes in a single plant because, although numerous studies over the past few decades have shown how to develop transgenic rice using particle bombardment, these studies only produced plants with a few genes. Homozygous transgenic J5hL and omega plants were crossed to produce J5hLOm hybrid plants with the *MtNFP, MtLYK3, MtDMI2, MtSYMREM1, LjLNP, MtNSP1, MtNSP2, MtERN1, MtNIN,* and *ENOD40* genes. Using gene-specific primers, PCR was used to confirm J5hLOm plants. Although the hybrid plants J5hLOm and J5hL and Omega plants shared morphological similarities with WT plants in terms of vegetative growth patterns and plant architecture, their seed setting rates differed from WT. Omega plants had seed settings that were just 6% lower than WT, but J5hL plants had seed settings that were about 39% lower than WT. Additionally, the seed sitting percentage of the hybrid J5hLOm is 22% lower than that of WT plants, falling between that of J5hL and Omega plants (Fig. 3A). In comparison to WT plants, seed weight dropped by roughly 18.3% in J5hL plants, 5.9 % in Omega plants and 13.36 % in J5hLOm plants (Fig. 3B).

Aberrant root hair responses to NF in transgenic rice

Following the addition of *Sm*NF (10-9 M) to the root of WT, VC, and J5hLOm, we observed that root hair deformations were 8% at 6 hpi, but sharply increased to 50% by 12 hpi and 54% by 24 hpi (Fig. 4A). However, even after 24 hours post-inoculation, less than 10% of the root hairs in WT and VC plants showed deformations after being inoculated with *Sm*NF. In J5hLOm plants, we saw deformed root hair tips, disorganized root hair stalk development, and primitive branching, but in WT and VC, we only observed swelling on the tip (Fig. 4B).

Colonization of rhizobium

To check the invasion process and the colonization pattern during rice-rhizobial interaction, we visualized 30-day-old infected roots under a confocal scanning microscope. Before visualising, the tissue was washed and stained with propidium iodide (10 µM). The image indicates that *S. meliloti* in GFP tagged was seen colonizing on the surface of J5hLOm plants, but on WT colonization was almost negligible compared to J5hLOm roots.

DISCUSSION

Our study demonstrates the feasibility of introducing and expressing legume-derived nodulation signalline genes in rice to initiate early symbiotic responses. The hybrid transgenic plants expressing Nod factor receptor genes and nodulation specific TF genes from *Medicago truncatula* showed the ability to initiate early symbiotic responses, which are essential for the establishment of a nitrogen-fixing symbiosis. These findings suggest that the introduction of legume Nod factor receptor genes and nodulation specific TF genes can partially reconstitute the signalling pathway required for rhizobial interaction in rice. While rice possesses orthologs of certain genes involved in the Common Symbiosis Pathway, it lacks key receptors and transcriptional regulators necessary for recognizing rhizobial Nod factors and initiating a complete nodulation. The introduction of *Medicago truncatula* genes encoding Nod factor receptors and transcription factors (*NSP1, NSP2, ERN1,* and *NIN*) could bridge this gap, enabling rice to perceive rhizobial signals and activate downstream symbiotic responses. The introduction of the *IFS* gene from soybean into rice also conferred the ability to produce flavonoids that induce nod gene expression in rhizobia.[39] This suggests that engineering rice to produce signalling molecules that attract and activate rhizobia could be a viable strategy for promoting nitrogen fixation. The

ability of engineered rice roots to respond to Nod factors indicates that at least a portion of the signal transduction machinery important for legume nodulation exists in rice.[40] This suggests that the existing cellular components in rice can be co-opted to establish a novel symbiotic interaction. Root hair deformation is an early characteristic of root response to rhizobium and NF in legumes. Different types of root hair deformation can occur in legumes, like swelling, distortion, and branching, after inoculation of rhizobium or NF.[41,42] Previously, it has been shown that rice root hair is deformed after inoculation with rhizobium.[43] But later Reddy *et al.* (1997)[2] results demonstrated that aberrant lateral root growth was visible rather than root hair deformation in rice, after 2 weeks of inoculation with different rhizobium strains. In our study, hybrid transgenic rice harbouring NF receptor genes and nodulation TF genes showed similar root hair deformation to legumes, deformed root hair tips, disorganized root hair stalk development, and primitive branching. Previously, Altúzar-Molina *et al.* (2020)[44] showed colonization in transgenic rice having NF receptor genes without *DIM2*, which was very low. While our results showed higher colonization in both J5hL plants and J5hLOm plants, which harbour NFs receptor genes along with *DMI2* gene. Although this study demonstrates the potential for engineering nitrogen fixation in rice, further research is needed to optimize the process. Future efforts should focus on:

- Enhancing the expression levels and stability of the introduced nodulation genes.
- Investigating the spatial and temporal regulation of gene expression to mimic the natural nodulation process in legumes.
- Exploring the use of alternative promoters to drive gene expression in specific root tissues.
- Identifying and overcoming potential bottlenecks in the downstream signaling pathway.
- Evaluating the effectiveness of different rhizobial strains in establishing symbiotic interactions with the engineered rice plants.

Engineering nitrogen fixation in rice represents a promising approach to reduce the reliance on synthetic nitrogen fertilizers, which contribute to environmental pollution and greenhouse gas emissions. By creating cereal crops that can fix their own nitrogen, we can promote sustainable agricultural practices and enhance food security for a growing global population.

ACKNOWLEDGEMENTS

We gratefully acknowledge Fabienne Maillet (LIPM, CNRS, France) for providing us with isolated *S. meliloti* NF. In memoriam to Dr. Pallavolu Maheshwara Reddy, who was responsible for this study. The authors would also like to acknowledge the support and guidance of the Director TERI, New Delhi, India

FUNDING

This research was supported by grants from DBT (BT/IN/UK-VNC/41/DLN/2015-2016), India,

AUTHORSHIP CONTRIBUTION STATEMENT

ALS conceptualized the work, performed and was involved in all the experiments, prepared the manuscript and finalized the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCE

1. **Africare, Oxfam, WWF. 2010.** More rice for the people. More water for the plante. System of Rice Intensification (SRI). 40
2. **Reddy P.M., Ladha J.K., So R.B., Hernandez R.J., Ramos M.C., Angeles O.R., Dazzo F.B., Bruijin Frans J. 1997.** Rhizobial communication with rice roots: Induction of phenotypic changes, mode of invasion and extent of colonization. *Plant Soil.* **194:**81–98. https://doi.org/10.1007/978-94-011-5744-5_9
3. **Ladha J.K., Reddy P.M. 2003.** Nitrogen fixation in rice systems: State of knowledge and future prospects. *Plant Soil*. **252:**151–167. https://doi.org/10.1023/A:1024175307238
4. **Erisman J.W., Sutton M.A., Galloway J., Klimont Z., Winiwarter W. 2008.** How a century of ammonia synthesis changed the world. *Nat Geosci.* **1:**636–639. https://doi.org/10.1038/ngeo325
5. **Ladha J.K., Reddy P.M. 1995.** Extension of nitrogen fixation to rice - Necessity and possibilities. *Geo Journal* **35:**363–372. https://doi.org/10.1007/BF00989144

6. **Santi C., Bogusz D., Franche C. 2013.** Biological nitrogen fixation in non-legume plants. *Ann Bot.* **111:** 743–767. https://doi.org/10.1093/aob/mct048

7. **Soumare A., Diedhiou A.G., Thuita M., Hafidi M. 2020.** Exploiting Biological Nitrogen Fixation/ : A Route. *Plants* 1–22

8. **Bloch S.E., Ryu M.H., Ozaydin B., Broglie R. 2020.** Harnessing atmospheric nitrogen for cereal crop production. *Curr Opin Biotechnol.* **62:**181–188. https://doi.org/10.1016/J.COPBIO.2019.09.024

9. **Nap J.P., Bisseling T. 1990.** Developmental biology of a plant-prokaryote symbiosis: The legume root nodule. *Science* (80-) **250:**948–954. https://doi.org/10.1126/science.250.4983.948

10. **Downie J.A. 2014.** Legume nodulation. *Curr Biol.* **24:** R184–R190.https://doi.org/10.1016/j.cub.2014.01.028

11. **Perret X., Staehelin C., Broughton W. J. 2000.** Molecular Basis of Symbiotic Promiscuity. *Microbiol Mol Biol Rev.* **64:**180–201. https://doi.org/10.1128/mmbr.64.1.180-201.2000

12. **Reddy P.M., Anaya Martha Rendon, Khandual S. 2007.** Flavonoids as Signaling Molecules and Regulators of Root Nodule Development. *Dyn Soil, Dyn Plant.* **1:**83–94

13. **Kouchi H., Imaizumi-Anraku H., Hayashi M., Hakoyama T., Nakagawa T., Umehara Y., Suganuma N., Kawaguchi M. 2010.** How many peas in a pod? Legume genes responsible for mutualistic symbioses underground. *Plant Cell Physiol.* **51:**1381–1397. https://doi.org/10.1093/pcp/pcq 107

14. **Limpens E., Franken C., Smit P., Willemse J., Bisselling T., Geurts R. 2003.** LysM Domain Receptor Kinases Regulating Rhizobial Nod Factor-Induced Infection. *Science* (80-) **302:**630–633. https://doi.org/10.1126/science.1090074

15. **Arrighi J-FFX, Barre A., Ben Amor B., Bersoult A., Campos Soriano L., Mirabella R., Carvalho-Niebel F., Journet E., Gherardi M., Huguet T., Geurts R., Denarie J., Rouge P., Gough C. 2006.** The *Medicago truncatula* lysine motif-receptor-like kinase gene family includes NFP and new nodule-expressed genes. *Plant Physiol.* **142:**265–279. https://doi.org/10.1104/pp.106.084657

16. **Endre G., Kereszt A., Kevei Z., Mihacea S., Kalo P., Kiss G.B. 2002.** A receptor kinase gene regulating symbiotic nodule development. *Nature*. **417:**962–966. https://doi.org/10.1038/nature00842

17. **Ané J.M., Kiss G.B., Riely B.K., Penmetsa R.V., Oldroyd G.E.D., Ayax C., Levy J., Debelle F., Baek J.M., Kalo P., Rosenberg C., Roe B.A., Long S.R., Denarrie J., Cook D.R. 2004.** *Medicago truncatula* DMI1 Required for Bacterial and Fungal Symbioses in Legumes. *Science* (80-) **303(5662):**1364–1367. https://doi.org/10.1126/science.1092986

18. **Lévy J., Bres C., Geurts R., Chalhoub B., Kulikova O., Duc G., Journet E.P., Ane J.M., Lauber E., Bisseling T., Denarie J., Rosenberg C., Debelle F. 2004.** A Putative Ca^{2+} and Calmodulin-Dependent Protein Kinase Required for Bacterial and Fungal Symbioses. *Science* (80-) **303(5662):**1361–1364. https://doi.org/10.1126/science.1093038

19. **Chen C., Ané J.M., Zhu H. 2008.** OsIPD3, an ortholog of the *Medicago truncatula* DMI3 interacting protein IPD3, is required for mycorrhizal symbiosis in rice. *New Phytol.* **180:**311–315. https://doi.org/10.1111/j.1469-8137.2008.02612.x

20. **Kaló P., Gleason C., Edwards A., Marsh J., Mitra R.M., Hisch S., Jakab J., Sims S., Long S.R., Rogers J., Kiss G.B., Downie J.A., Oldroyd G.E.D. 2005.** Nodulation signaling in legumes requires NSP2, a member of the GRAS family of transcriptional regulators. *Science*. **308:**1786–1789. https://doi.org/10.1126/science.1110951

21. **Smit P., Raedts J., Portyanko V., Debella F., Gough C., Bisseling T., Geurts R. 2005.** NSP1 of the GRAS protein family is essential for rhizobial nod factor-induced transcription. Science. **308:**1789–1791. https://doi.org/10.1126/science.1111025

22. **Marsh J.F., Rakocevic A., Mitra R.M., Brocard L., Sun J., Eschstruth A., Long S.R., Schultze M., Ratel P., Oldroyd G.E.D. 2007.** *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol.* **144:**324–**335. https://doi.org/10.1104/pp.106.093021**

23. **Middleton P.H., Jakab J., Penmetsa R.V., Starker C.G., Doll J., Kalo P., Prabhu R., Marsh J.F., Mitra**

R.M., Kereszt A., Dudas B., Vandenbosch K., Long S.R., Cook D.R., Kiss G.B., Oldroyd G.E.D. 2007. An ERF transcription factor in *Medicago truncatula* that is essential for nod factor signal transduction. *Plant Cell*. **19:**1221–1234. https://doi.org/10.1105/tpc.106.048264

24. **Chen C., Gao M., Liu J., Zhu H. 2007.** Fungal symbiosis in rice requires an ortholog of a legume common symbiosis gene encoding a Ca^{2+}/calmodulin-dependent protein kinase. *Plant Physiol.* **145:**1619-1628. https://doi.org/10.1104/pp.107.109876

25. **Banba M., Gutjahr C., Miyao A., Hirochika H., Paszkowski U., Kouchi H., Imaizumi-Anraku H. 2008.** Divergence of evolutionary ways among common sym genes: CASTOR and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway. *Plant Cell Physiol.* **49:**1659–1671. https://doi.org/10.1093/pcp/pcn153

26. **Gutjahr C., Banba M., Croset V., An K., Miyao A., An G., Hirochika H., Imaizumi-Anraku H., Paszkowski U. 2008.** Arbuscular Mycorrhiza-Specific Signaling in Rice Transcends the Common Symbiosis Signaling Pathway. Plant Cell 20:2989–3005. https://doi.org/10.1105/tpc.108.062414

27. **Yano K., Yoshida S., Muller J., Singh S., Banba M., Vickers K., Markmann K., White C., Schuller B., Sato S., Asamizu E., Tabata S., Murooka Y., Perry J., Wang T.L., Kawaguchi M., Imaizumi-Anraku H., Hayashi M., Parniske M. 2008.** CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc Natl Acad Sci U S A 105:20540–20545. https://doi.org/10.1073/pnas.0806858105

28. **Godfroy O., Debellé F., Timmers T., Rosenberg C. 2006.** A rice calcium- and calmodulin-dependent protein kinase restores nodulation to a legume mutant. *Mol Plant-Microbe Interact.* **19:**495–501. https://doi.org/10.1094/MPMI-19-0495

29. **Markmann K., Giczey G., Parniske M. 2008.** Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. *PLoS Biol.* **6:**0497–0506. https://doi.org/10.1371/journal.pbio.0060068

30. **Miyata K., Hayafune M., Kobae Y., Kaku H., Nishizawa Y., Masuda Y., Shibuya N., Nakagawa T. 2016.** Evaluation of the role of the LysM receptor-like kinase, OsNFR5/OsRLK2 for AM symbiosis in rice. *Plant Cell Physiol*. **57:**2283–2290. https://doi.org/10.1093/pcp/pcw144

31. **Miyata K., Kozaki T., Kouzai Y., Ozawa K., Ishii K., Asamizu E., Okabe Y., Umehara Y., Miyamoto A., Kobae Y., Akiyama K., Kaku H., Nishizawa Y., Shibuya N., Nakagawa T. 2014.** The bifunctional plant receptor, OsCERK1, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice. *Plant Cell Physiol.* **55:**1864–1872. https://doi.org/10.1093/pcp/pcu129

32. **Sreevidya V.S., Hernandez-Oane R.J., So R.B., Sullia S.B., Stacey G., Ladha J.K., Reddy P.M. 2005.** Expression of the legume symbiotic lectin genes psl and gs52 promotes rhizobial colonization of roots in rice. *Plant Sci.* **169:**726–736. https://doi.org/10.1016/j.plantsci.2005.05.024

33. **Toki S., Hara N., Ono K., Onodera H., Tagiri A., Oka S., Tanaka H. 2006.** Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. *Plant J.* **47:**969–976. https://doi.org/10.1111/j.1365-313X.2006.02836.x

34. **Tanwar N., Rookes J.E., Cahill D.M., Lenka S.K. 2023.** Carotenoid Pathway Engineering in Tobacco Chloroplast Using a Synthetic Operon. *Mol Biotechnol.* **65:**1923–1934. https://doi.org/10.1007/s12033-023-00693-3

35. **Singh A., Altúzar-Molina A., Kaur G., Sidhu G.K., Mandal P.K., Reddy P.M. 2025.** Activation of Ca2+ Oscillations in Rice (*Oryza sativa*) Root Hairs Expressing the Legume-Specific Nod Factor Receptor Kinases. *Mtnfp and Mtlyk.* **3:**1–25

36. **Doyle J.J., Doyle J.L. 1990.** A rapid total DNA preparation procedure for fresh plant tissue. *Focus (Madison).* **12:**13–15

37. **Murashige T., Skoog F. 1962.** A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol Plant*. **15:**473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

38. **Fahraeus G. 1957.** The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J. Gen. Microbiol.* **16:**374–381. https://doi.org/10.1099/00221287-16-2-374

39. **Sreevidya V.S., Srinivasa Rao C., Sullia S.B., Ladha J.K., Reddy P.M. 2006.** Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. *Int Journal of Experimental Botany.* 1957–1969

40. **Reddy P.M., Ladha J.K., Ramos M.C., Maillet F., Hernandez R.J., Torrizo L.B., Oliva N.P., Datta S.K., Datta K. 1998.** Rhizobial lipochitooligo saccharide nodulation factors activate expression of the legume early nodulin gene ENOD12 in rice. *Plant J.* **14:**693–702. https://doi.org/10.1046/j.1365-313x.1998.00170.x

41. **Heidstra R., Geurts R., Franssen H., Spaink H.P., Kammen A.V., Bisseling T. 1994.** Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. *Plant Physiol.* **105:**787–797. https://doi.org/10.1104/pp.105.3.787

42. **Catoira R., Galera C., De Billy F., Penmetsa R.V., Journet E.P., Maillet F., Rosenberg C., Cook D., Gough C., Denarie J. 2000.** Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. *Plant Cell.* **12:**1647–1665. https://doi.org/10.1105/tpc.12.9.1647

43. **Plazinski J., Innes R.W., Rolfe B.G. 1985.** Expression of Rhizobium trifolii early nodulation genes on maize and rice plants. *J. Bacteriol*. **163:**812–815. https://doi.org/10.1128/jb.163.2.812-815.1985

44. **Altúzar-Molina A., Lozano L., Ortíz-Berrocal M., Ramire M., Martinez L., Velazquez-Hernandez M.L., Dhar-Ray S., Silvente S., Mariano N., Shishkova S., Hernandez G., Reddy P.M. 2020.** Expression of the Legume-Specific Nod Factor Receptor Proteins Alters Developmental and Immune Responses in Rice. *Plant Mol Biol Report.* **38:**262–281. https://doi.org/10.1007/s11105-019-01188-9

***