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Increased nitrogen-use efficiency in transgenic rice plants over-expressing a nitrogen-responsive early nodulin gene identified from rice expression profiling

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ABSTRACT

Development of genetic varieties with improved nitrogen-use efficiency (NUE) is essential for sustainable agriculture. In this study, we developed a growth system for rice wherein N was the growth-limiting factor, and identified N-responsive genes by a whole genome transcriptional profiling approach. Some genes were selected to test their functionality in NUE by a transgenic approach. One such example with positive effects on NUE is an early nodulin gene *OsENOD93-1*. This *OsENOD93-1* gene responded significantly to both N induction and N reduction. Transgenic rice plants over-expressing the *OsENOD93-1* gene had increased shoot dry biomass and seed yield. This *OsENOD93-1* gene was expressed at high levels in roots of wild-type (WT) plants, and its protein product was localized in mitochondria. Transgenic plants accumulated higher concentrations of total amino acids and total N in roots. A higher concentration of amino acids in xylem sap was detected in transgenic plants, especially under N stress. *In situ* hybridization revealed that *OsENOD93-1* is expressed in vascular bundles, as well as in epidermis and endodermis. This work demonstrates that transcriptional profiling, coupled with a transgenic validation approach, is an effective strategy for gene discovery. The knowledge gained from this study could be applied to other important crops.

Key-words: amino acids; nitrogen induction; nitrogen limitation; nitrogen reduction; transcriptional profiling.

INTRODUCTION

Within the next 50 years, increased human population and crop consumption per capita, together with environmental issues, will necessitate considerable increases in crop production to prevent serious societal conflict. Given current trends, an approximate doubling of yields worldwide will be required, and it will take a considerable effort to accomplish this (Rothstein 2007). Nitrogen (N), being an essential element for plant growth, is considered to be the main controlling factor for plant productivity after water deficiency (Lea & Morot-Gaudry 2001). The production of high-yielding crops is associated with the application of large quantities of N fertilizers. These inputs, which are currently estimated at 90 million metric tons worldwide, have become the major cost in crop production (Frink, Waggoner & Ausubel 1999). N incorporated into agricultural crops, however, rarely exceeds 40% of the applied N, indicating a serious inefficiency in N utilization (Raun & Johnson 1999; Glass 2003). The remaining N from fertilizer is lost to the atmosphere or leached into the groundwater and other freshwater bodies (Raun & Johnson 1999; Glass 2003), which is causing serious N pollution and becoming a threat to global ecosystems (Nosengo 2003; Giles 2005). Therefore, there is an urgent need to develop crops with improved N-use efficiency (NUE).

There are two main stages of N use in the plant life cycle. The first one is the vegetative stage, when N assimilation is most involved. The second one is the reproductive stage, when both N assimilation and remobilization are important (Hirel *et al.* 2007). NUE was defined as the yield of grain per unit of available N in the soil (Moll, Kamprath & Jackson 1982, 1987), although more definitions and evaluation methods have been developed over the years (Good, Shrawat & Muench 2004). Generally, NUE can be divided further into two parts: assimilation efficiency and utilization efficiency.

While improved agricultural practice is one way to increase NUE (Jing *et al.* 2009), there is increasing interest in understanding the genetics of NUE (Coque *et al.* 2008; Sylvester-Bradley & Kindred 2009). Attempts have been made to improve NUE in transgenic crops by ectopic regulation of key enzymes involved in N metabolism (Good *et al.* 2004, 2007; Shrawat *et al.* 2008). In addition, efforts have been directed towards the molecular basis of plant responses to N and the identification of N-responsive genes

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(Crawford & Forde 2002). Micro-array technology is a useful tool for analyzing genome-scale gene expression; a long list of N-responsive genes representing a wide range of processes has been generated for *Arabidopsis thaliana* (Wang *et al.* 2000, 2003; Palenchar *et al.* 2004; Price *et al.* 2004; Scheible *et al.* 2004; Bi *et al.* 2007; Gutierrez *et al.* 2007, 2008; Peng *et al.* 2007b; Wang, Xing & Crawford 2007; Gifford *et al.* 2008). There are also studies of N-responsive genes in tomato roots (Wang, Garvin & Kochian 2001), and of expression profiles for 10 422 genes in rice seedlings at an early stage of low-N stress (Lian *et al.* 2006). However, none of these N-responsive genes has been evaluated for NUE.

Rice is a staple food for almost half the world's population, and a model plant for monocot species research because of its small genome size and completion of the rice genome sequencing project (Feng *et al.* 2002; Sasaki *et al.* 2002; Matsumoto *et al.* 2005). Previously, we developed a hydroponic growth system for *A. thaliana* where N is the growth-limiting factor, and searched for N-responsive genes in *Arabidopsis* under chronic N stress, as well as transient transcriptional change after increases in short- or long-term N availability (Bi *et al.* 2007). Furthermore, we developed a defined N soil growth system for *Arabidopsis* (Bi *et al.* 2005), isolated the *NLA* gene involved in the adaptability of *Arabidopsis* to N limitation (Peng *et al.* 2007a) and did genome-wide analysis of N responses using this system (Peng *et al.* 2007b). In the present study, we developed a semihydroponic growth system for rice wherein N was the growth-limiting factor, identified N-responsive genes by a whole-genome transcriptional profiling experiment and evaluated some of these genes for their functionality in NUE by a transgenic approach. One such example is presented in this report.

MATERIALS AND METHODS

Plant growth conditions

For the micro-array experiments, peat moss and vermiculite (1:4; SunGro Horticulture Canada Ltd, Vancouver, BC, Canada) were used to grow wild-type (WT) *Oryza sativa japonica* cv. Donjin plants, adding nutrient solution once a week until harvest. The nutrient solution contained 4 mM $MgSO_4$, 5 mM KCl, 5 mM $CaCl_2$, 1 mM KH_2PO_4, 0.1 mM Fe–ethylenediaminetetraacetic acid (EDTA), 0.5 mM MES (pH 6.0), 9 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 46 μM NaB_4O_7 and 0.2 μM Na_2MoO_4. Ten millimolar nitrate (KNO_3) was used as the high-N condition, 5 mM nitrate as medium N and 1 mM nitrate as low N. The plants were grown in a growth room with 16 h light (~500 μmol m^{-2} s^{-1}) at 29 °C, and 8 h dark at 23 °C for 4 weeks. Shoots and roots were harvested separately, and three biological replicates were collected.

To test the transgenic plants, the same soil mixture as above was used, and the cultivar used for producing the transgenic events was 'Kaybonnet' rice (*O. sativa* L.), which also belongs to the *japonica* race. The 1 mM N solution was added once a week for 4 weeks when screening the lines at the vegetative stage. The 3 mM N solution was added once a week until harvest for screening at the reproductive stage. The plants were grown in a growth room with 16 h light (~500 μmol m^{-2} s^{-1}) at 29 °C, and 8 h dark at 23 °C for the first 4 weeks, then 1 week of short-day treatment (10 h light/14 h dark) to promote flowering, and then returned to the long-day regime until harvest.

Micro-array hybridization

Five micrograms of total RNA from each sample was used to synthesize double-stranded cDNAs. Labelled complementary RNA, synthesized from the cDNA, was hybridized to a custom-designed rice whole-genome GeneChip array (SYNG003a), which contains 50 188 probe sets representing 50 119 known and predicted rice genes (Zhu 2003). Among them, 39 201 had high homology to non-transposable element-related protein-coding sequences (Yuan *et al.* 2005). On average, each gene contains approximately 11 perfect match probes, selected from the 3′ end of the coding region (Zhu 2003). The hybridization signal of the arrays was acquired by the GeneChip scanner 3000 and quantified by MAS 5.0 (Affymetrix, Santa Clara, CA, USA). The probe set measurement was summarized as a value of weighted average of all probes in a set, subtracting the bottom 5% of average intensity of the entire array using a custom algorithm. The overall intensity of all probe sets of each array was further scaled to a target intensity of 100 to enable direct comparison.

Micro-array data analysis

Data analysis was conducted using GeneSpring (Agilent, Palo Alto, CA, USA). The data were normalized with a default setting of the program, followed by gene filtering which required that each gene must have either a 'P' or 'M' flag in the three replicate samples. The following eight comparisons were made among the samples (four each for shoots and roots): (1) high N versus medium N (10 mM nitrate to 5 mM nitrate); (2) high N versus low N (10 mM nitrate to 1 mM nitrate); (3) low N (1 mM nitrate) versus low to high N (2 h N induction); and (4) high N (10 mM nitrate) versus high to low N (2 h N reduction). In these comparisons, genes with at least twofold change were identified first, and then analysis of variance (ANOVA) was used to identify significance (Welch *t*-test with *P* value cut-off at 0.05).

Transgenic rice plants

The constructs for over-expressing NUE candidate genes were made using a ubiquitin promoter. *Agrobacterium*-mediated transformation was performed, and the T_1 transgenic seeds were harvested. Phosphomannose isomerase (PMI) tests were used for genotyping the selectable PMI marker (Negrotto *et al.* 2000; Reed *et al.* 2001; Wright *et al.* 2001).

Expression analysis by quantitative real-time PCR

Total RNA was isolated from plant tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). Primer sequences for *LOC_Os06g05010* were: 5′-CGAACCTTGC AGGGGTTAAG-3′ and 5′-GTTCAGGTGCGTTCTC GAAG-3′; and for internal control *LOC_Os10g36650* (actin2): 5′-TCTTACGGAGGCTCCACTTAAC-3′ and 5′-TCCACTAGCATAGAGGGAAAGC-3′; and real-time PCR was performed according to Kant *et al.* (2006). Relative quantification (RQ) values for each target gene were calculated by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001) using *ACTIN2* as an internal reference gene.

Biochemical assays

Frozen shoot or root tissue was used for several biochemical assays. Nitrate was extracted in 50 mM HEPES–KOH (pH 7.4), and analysed by a colorimetric assay according to Cataldo *et al.* (1975). Total soluble amino acids were extracted at room temperature successively with 80, 50 and 0% ethanol in 10 mM HEPES–KOH (pH 7.4), and the supernatants were pooled and assayed according to Rosen (1957). Total soluble protein was extracted with buffer containing 100 mM HEPES–KOH, pH 7.5, and 0.1% Triton X-100, and assayed using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The percent total nitrogen in dry tissues was measured by the micro-Dumas combustion analysis method using a Carlo Erba NA1500 C/N analyser (Carlo Erba Strumentazione, Milan, Italy). Relative anthocyanin concentration was analysed based on Neff & Chory (1998). Chlorophyll was extracted in 80% acetone, and assayed according to Arnon (1949).

Subcellular localization of ENOD93-1

The coding sequence of *OsENOD93-1* was amplified by PCR, and cloned into a C-terminal YFP reporter gene fusion gateway-compatible vector pEarleyGate 101 (Earley *et al.* 2006). The OsENOD93-1–YFP and control βATPase–RFP (GenBank accession number for βATPase P17614) constructs were biolistically bombarded into onion epidermal cells, and fluorescent images were acquired 20 h post-bombardment through epiflourescnce microscopy.

In situ RNA hybridization

For *in situ* analysis, samples were taken from 4-week-old WT rice roots, cut into 0.5–1.0 cm sections and fixed in 4% polyformaldehyde. The tissues were subsequently dehydrated and embedded in paraffin. The 8-μm-thick tissue sections were prepared using a microtome, and mounted on microscope slides coated with poly-Lysine (Biolabs, Ipswich, MA, USA). After deparaffination by treatment with xylene, the sections were treated for 30 min with proteinase K (10 μg mL^{-1}). *In situ* hybridization was performed as described previously (Komminoth 1992), using digoxigenin (DIG)-labelled antisense and sense RNA probes (Roche Diagnostics, Penzberg, Germany). Photographs were taken using a DMIR2 microscope (Leica) and a DC 500 camera (Leica, Wetzlar, Germany).

Amino acid detection in xylem sap

Xylem sap was collected according to Shi *et al.* (2002). The sap was diluted with distilled water, and total amino acids were assayed according to Rosen (1957).

Statistical analysis

Data were statistically analysed by Fisher's protected LSD test using SAS statistical software (SAS Institute, Inc., Cary, NC, USA). The results shown are representative of two independent experiments, and within each experiment treatments were replicated three times, unless otherwise stated.

RESULTS

Developing defined nitrogen growth conditions for rice

A nitrogen growth condition was developed for rice (*O. sativa* L.) using a semihydroponic system (see Materials and methods), similar to that described previously for *Arabidopsis* (Bi *et al.* 2005, 2007; Peng *et al.* 2007a,b). Although rice, unlike other crops, utilizes mostly ammonium as an N source, some rice cultivars grow better using nitrate as an N source (Fan *et al.* 2005). 'Kaybonnet', the rice cultivar used to produce transgenic plants, was originally developed in Arkansas, USA (Wells *et al.* 1995), and can grow as well or slightly better on nitrate than on ammonium (Supporting Information Fig. S1). Therefore, nitrate was used as the N source for controlling the N-limiting conditions. Furthermore, nitrate was used for the previous *Arabidopsis* microarray studies (Bi *et al.* 2007), allowing for easier comparison of N responses between dicot and monocot plants.

WT rice plants at 4 weeks of age were evaluated for shoot biomass under different N conditions, with nitrate concentrations ranging from 0.1 to 20 mM. Under these N conditions, 10 mM nitrate was found to give maximal growth (data not shown). For subsequent experiments, 10 mM nitrate was used as the high-N condition; 5 mM nitrate as the medium-N condition, under which plant growth measured by shoot biomass was reduced to approximately 70% of that at 10 mM nitrate; and 1 mM nitrate as the low-N condition, under which plant growth measured by shoot biomass was reduced to approximately 30% of that at 10 mM nitrate (Supporting Information Fig. S2a). The reduction in root biomass under the two N-limiting conditions was in a similar range (Supporting Information Fig. S2b). Chlorophyll and anthocyanin concentrations in the leaves were similar under high- and medium-N conditions. Under the low-N condition, the plants were not obviously yellow or

purple, although the reduction of chlorophyll and the increase in anthocyanin were significant (Supporting Information Fig. S2c,d).

The free nitrate concentrations in the shoots and roots of plants grown under these three N conditions were determined. For shoots, medium N reduced the concentration to ~70% of that at 10 mM nitrate, whereas low N produced a small additional reduction (Supporting Information Fig. S3a). For roots, however, medium N reduced the nitrate concentration to about half, and low N reduced it to ~5% (Supporting Information Fig. S3b). Other indicators of the N status were also analysed. The concentration of total amino acids in the shoots was reduced to 77 and 35% under the medium- and low-N conditions, respectively, compared to the levels at 10 mM N (Supporting Information Fig. S3c); a similar reduction was observed in the roots (Supporting Information Fig. S3d). Total soluble protein, and total N in the shoots and roots were slightly lower under medium N, but significantly reduced under low N when compared to the high-N plants (Supporting Information Fig. S3e–h).

Identification of differentially expressed genes by expression profiling

The shoots and roots of 4-week-old WT rice plants grown under high N, medium N or low N were harvested for transcriptional profiling to compare baseline gene expression levels under different, but stable N conditions. In addition, at 2 h before the harvest, some plants grown under low N were transferred to high N to assess gene expression changes in response to nitrate resupply (referred to as N induction in this paper), and some plants grown under high N were transferred to low N to assess gene expression changes in response to nitrate removal (referred to as N reduction in this paper). All samples were taken in the middle of the day to minimize complications caused by diurnal changes in C and N metabolism. RNA was extracted and hybridized to a custom-designed rice GeneChip whole-genome array, and data were analysed to identify N-responsive genes (see Materials and methods).

Under the medium-N condition, plant growth, as measured by shoot or root biomass, was reduced by ~30% (Supporting Information Fig. S2a,b), and was accompanied by reductions in nitrate and amino acid concentrations, and small reductions in total protein and total N concentrations (Supporting Information Fig. S3a–h). The baseline expression levels of all genes in both shoots and roots were similar between plants grown under high- and medium-N conditions, indicating that plants adjusted well at the transcriptional level to the mildly N-limiting condition, although they stored less free nitrate and amino acids, and grew slower (Supporting Information Figs S2a,b & S3a–d). Under the low-N condition, plant growth was reduced by ~70% (Supporting Information Fig. S2a,b), and was accompanied by a ~40% reduction in nitrate concentration in shoots and a stunning ~95% reduction in nitrate concentration in roots (Supporting Information Fig. S3a,b), as well as significant reductions in amino acid, total protein and total N concentrations (Supporting Information Fig. S3c–h). A small number of differentially expressed genes were identified in the roots, including: down-regulated genes involved in carbon metabolism; photosynthesis; energy production; and different types of transporter genes for iron, zinc and ATPase; and a high proportion of up-regulated genes involved in nitrate assimilation such as nitrate and ammonium transporter genes, nitrate reductase and glutamine synthetase.

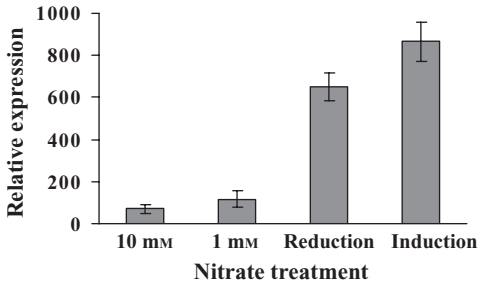
After 2 h N induction, the nitrate concentration in the shoot increased ~10% (Supporting Information Fig. S4a), and no significant difference in the expression levels was detected. In roots, however, nitrate levels increased ~90% ($P < 0.05$, Supporting Information Fig. S4b), and differentially expressed genes were identified. After 2 h N reduction, nitrate levels in the shoot decreased ~10% (Supporting Information Fig. S4c), and no significant difference in the expression levels was detected. Nitrate concentration in roots decreased ~20% ($P < 0.05$, Supporting Information Fig. S4d), and differentially expressed genes were identified. Some genes were significantly responsive to both N induction and N reduction, suggesting that they are sensitive to fluctuating N conditions. Notable functions of these genes are nitrogen and carbon metabolism; transportation of nitrate, ammonium, amino acid and growth hormones; signal transduction; and transcriptional regulation.

Generation and screening of transgenic rice plants over-expressing NUE candidate genes under N-limiting conditions

A small number of genes were selected from the significant gene lists as NUE candidate genes; some function in the transport of ammonium or nitrate, whereas others probably have a regulatory function (e.g. some transcription factor genes), and still others, such as an early nodulin gene *Os06g05010*, have a completely unknown function. While the change in expression for most of these genes was around two- to fourfold, the early nodulin gene was up-regulated over sevenfold, and responded to both N induction and N reduction (Fig. 1a). Full-length cDNA of all the selected genes was amplified and cloned into a binary vector. Transgenic rice plants were generated through *Agrobacterium*-mediated transformation to constitutively over-express these NUE genes. Five to ten independent events were generated for each construct. Around half of the events had a single chromosomal insertion site of the candidate gene as genotyping results revealed a 3:1 segregation ratio for these events (data not shown). When transcript levels were tested by semiquantitative RT-PCR, most events had higher levels of transgene expression (data not shown).

Two strategies were utilized to screen the transgenic plants. Firstly, plants were screened at a vegetative stage. This involved growing plants under the low-N condition for 4 weeks to monitor shoot and root biomass of transgenic lines and WT control plants. Secondly, plants were screened at the end of the reproductive stage. This involved growing

(a)



(b)

ATCTCATCTCATCTCGCTCAGCACTACTGATCCAACCAGTAGGCGATTCTCTCC
ACCTCCCAGCTACTTCACTTCAGGACTAGTTTCTTCTCAGCTCCATCGATCTTG
CTCGACA<u>ATGTCGACGACGGTGACCCGTGCTCACCTGGAGCAGAGGCTCGCCC
TCGCCAAGCGCTGCTCCCGGG</u>GTACGTACGCACGCACACTAAACCACCATGTT
TAATCTCGCTTTGTCTTGCATTCCATGGCGCTAATAACTGAATATATACAAACT
GATGTATGCTTCGTCGATCTGCAG<u>AGGCGAACCTTGCAGGGGTTAAGGCGGCT
GCTGTCGCGACCATCGCCTCTGCCGTTCCAACC</u>GTGAGTGTGCTCAGATGCTA
CTAGCTTCTTCTAATTCCTCCTTGTGTTGATTCTTCAGCAGAGAGTAGGTGTAC
TAACTTATTAGCTGCATATGTGTGTTGTTTCATCGATCAG<u>CTGGCGAGCGTGA
GGATGCTGCCATGGGCGAAGGCCAACATCAACCCGACGGGGCAGGCGCTGAT
CATCTGCACGGCGGCCGGGATGGCATACTTCGTCGCCGCCGACAAGAAGATCC
TCTCGCTGGCCAGGAGGCACTCCTTCGAGAACGCACCTGAACACCTCAAGAAC
ACCTCCTTCCAGGGCACCGGCCGCCCTCACCCTGCCTTCTTCAGGCCATGA</u>TC
AGCAAGCTAGTGCTGCTTGCAATCTGCTGCAGAAAGTAGCTAGTGTTGTGTAC
TTGGATGTAATATACTGTATGCTCCAGCTTGTATTAATAAACAATCTTAATTTG
ATGAACTCAAGAATGGATGCGGTGGTTCATGTGCACTGTAGTACATGTTTCTT
GTTCCTTGAACTCGAACGAATTTGAACTGCCTATACA

(c)

MSTTVTRAHLE<u>QRLALAKRCSREANLAGVKAAAVATIASAVPTLASVRML
PWAKANINPTGQALIICTAAGMAYFVAADKKILSLARRHS</u>FENAPEHLKN
TSFQGTGRPHPAFFRP

(d)

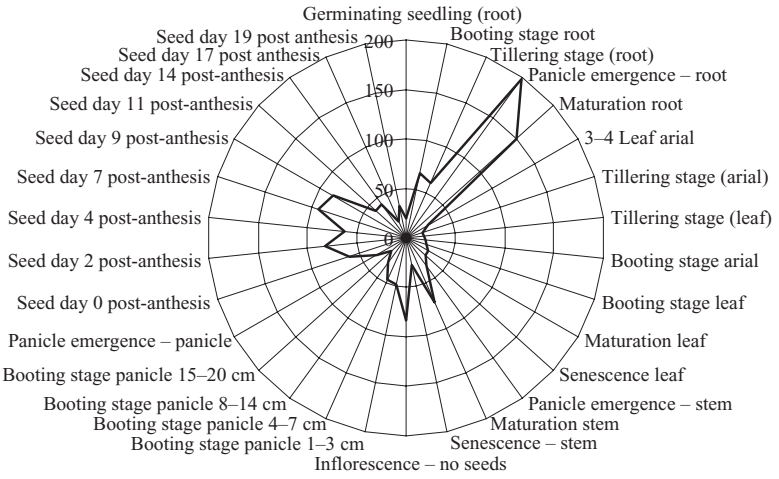


Figure 1. Expression and sequence analysis of *OsENOD93-1* gene. (a) Relative transcript abundance of *OsENOD93-1* in micro-array expression analysis. The plants were continuously grown at 10 or 1 mM nitrate for 28 d. Two hours before harvesting, the plants were switched from 1 to 10 mM (induction), and from 10 to 1 mM (reduction). (b) Genomic sequence and (c) predicted amino acid sequence of the *OsENOD93-1* gene. The underlined portions of the genomic sequences are exons. The ENOD93 domain is underlined in amino acid sequence. (d) Expression of the *OsENOD93-1* gene at different growth stages and plant parts in wild-type (WT) rice plants.

plants under the moderate-N condition (limiting but still generating a reasonable seed yield) until the end of the reproductive stage to assess tiller and panicle number, shoot biomass and seed yield.

The initial T_1 genetic and phenotypic analysis involved screening five transgenic events per construct and ~16 plants per event to give a reasonable number of transgenic and non-transgenic plants for each event. PMI tests were used for genotyping to detect the selectable PMI marker (see Materials and methods). Data were averaged in order to compare PMI-positive plants with PMI-negative and WT control plants.

Table 1. Yield and yield attributes in transgenic rice plants over-expressing OsENOD93-1 and wild type (WT) at different nitrogen levels

	Total tillers	Shoot DW (g)	Spikes	Spikelets	Seed yield (g)
3 mM Nitrate					
OX-1	5.5 ± 0.25	5.1 ± 0.34	3.2 ± 0.13	203 ± 13	3.8 ± 0.23
OX-2	5.4 ± 0.24	4.9 ± 0.28	3.1 ± 0.14	197 ± 10	3.7 ± 0.17
WT	5.2 ± 0.29	4.4 ± 0.23*	2.7 ± 0.13*	173 ± 10*	3.1 ± 0.21*
10 mM Nitrate					
OX-1	7.0 ± 0.33	10 ± 0.51	4.8 ± 0.22	465 ± 24	8.7 ± 0.52
OX-2	6.9 ± 0.22	9.8 ± 0.56	4.9 ± 0.26	475 ± 25	8.8 ± 0.46
WT	6.7 ± 0.31	8.9 ± 0.61	4.1 ± 0.26*	395 ± 18*	7.7 ± 0.46*

The data are mean ± SE ($n = 10$ plants). Data of two separate T_3 lines derived from two independent T_2 events are presented.
*Values of WT which are significantly different ($P < 0.05$) from values of over-expresser (OX) lines.
DW, dry weight.

Transgenic rice over-expressing an early nodulin gene had increased shoot dry biomass and seed yield

Comparisons of PMI-positive and PMI-negative plants in the T_1 generation of *Os06g05010* over-expressors revealed that all five events produced higher seed yield (data not shown). Four T_2 lines derived from two independent events (genotyping gave a 3:1 segregation ratio for these two events) were then tested. At the end of the reproductive stage, although the tiller number was similar, the transgenic plants had 10–20% higher shoot dry biomass, number of spikes and spikelets and seed yield than WT control plants (data not shown). To further confirm the result, two T_3 progenies were tested again, grown not only under the defined N-limiting (3 mM nitrate) condition, but also under the high-N (10 mM nitrate) conditions. The same trend was observed from these T_3 plants, with the transgenic plants having similar tiller number, but a significant 10–20% increase in number of spikes and spikelets, and seed yield under both limiting and high-N conditions, as well as a significantly higher shoot dry biomass than WT plants under limiting N (Table 1). Growth of the transgenic and WT plants was also compared on slow-release fertilizer which contained ~50% ammonium. The results of yield and yield attributes from slow-release fertilizer (Supporting Information Table S1) showed similar trends as the plants grown on nitrate (Table 1). The consistent results from the three generations grown on different N sources and different N conditions convinced us that this early nodulin gene could have a positive effect on NUE, and therefore more detailed characterization of this gene was conducted.

Characterization of the early nodulin gene

There is no defined nomenclature for the early nodulin genes. This *Os06g05010* gene and five other genes showing sequence similarity in rice containing the ENOD93 domain have been designated as early nodulin 93 genes (TIGR rice genome annotation database). Thus, we designate this gene here as *OsENOD93-1*. It consists of three exons and two introns, and encodes a protein of 116 amino acids (Fig. 1b,c). Our previous expression profiling of different tissues at various growth stages (Zhu *et al.* 2003) showed that the *OsENOD93-1* gene is expressed at high levels in roots, especially at the panicle emergence stage (Fig. 1d). In the rice genome, there are five more *ENOD93* genes located in the same chromosomal region, with similar molecular weight and pI, all predicted to contain two transmembrane domains, and all but *Os06g04940* expected to be localized in mitochondria (Table 2). To examine the subcellular localization of the OsENOD93-1 protein, OsENOD93-1–YFP was biolistically bombarded into onion epidermal cells. Twenty hours post-bombardment, the protein sorted to punctuate structures throughout the cell, and this co-localized well with a known mitochondrial marker protein βATPase–RFP (Fig. 2), confirming that OsENOD93-1 is a mitochondrial membrane protein.

To understand the cell-specific expression of the *OsENOD93-1* gene, its transcript abundance in roots of WT plants was analysed via *in situ* hybridization. The expression was detected clearly in vascular bundles, as well as in epidermis and endodermis (Fig. 3), suggesting a potential role in the transport of compounds from root to shoot.

Increased amino acids in the roots and the xylem sap of transgenic plants under N deficiency

The N status of shoots and roots of the transgenic and control plants growing under different N conditions was measured. Nitrate concentrations were similar in transgenic and WT plants (Supporting Information Fig. S5a–d). However, the roots of transgenic plants over-expressing *OsENOD93-1* had higher concentrations of total amino acids and total N at 30 d after sowing (DAS) than WT plants; this difference was accentuated as N stress increased, with transgenic plants grown under 1 mM nitrate showing significantly higher levels than the corresponding WT plants (Fig. 4a,c). At 60 DAS, the transgenic plants over-expressing *OsENOD93-1* had significant higher concentrations of total amino acids under both high-N and

Table 2. Predicted characteristics and structural domains in rice ENOD93 domain containing proteins

Gene	Protein similarity	Amino acids	Molecular mass	pI	Localization[a]	ENOD93 domain[b]	TMH
Os06g05010	ENOD93	116	12 424	10.95	Mitochondria	12....90	2
Os06g04990	ENOD93	115	12 307	10.95	Mitochondria	11....89	2
Os06g05020	ENOD93	115	12 277	10.95	Mitochondria	11....89	2
Os06g04950	ENOD93	115	12 233	10.29	Mitochondria	11....89	2
Os06g05000	ENOD93	115	12 167	10.35	Mitochondria	11....89	2
Os06g04940	ENOD93	139	14 897	9.98	Chloroplast	3....113	2

[a]PSORT (Horton *et al.* 2007)/TargetP1.1 (Emanuelsson *et al.* 2000).
[b]Pfam 22.0 (Finn *et al.* 2006).
TMH, transmembrane helices (TMHMM 2.0) (Krogh *et al.* 2001).

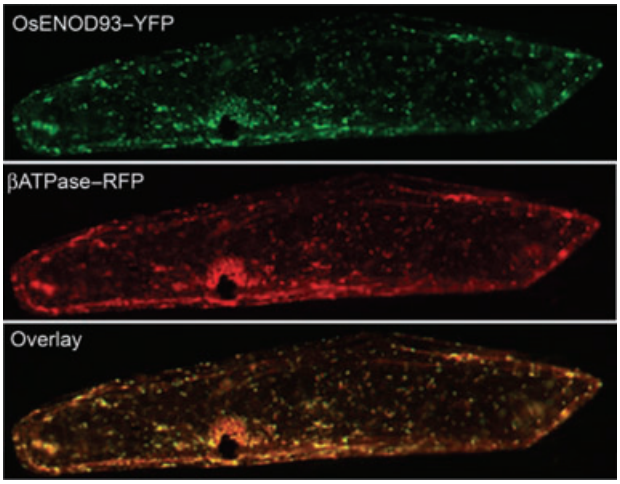


Figure 2. The subcellular localization of transiently expressed OsENOD93-1–YFP protein. OsENOD93-1–YFP and βATPase–RFP were co-transformed biolistically in onion epidermal cells, and images observed by epifluorescence microscopy. The subcellular localization of OsENOD93-1–YFP fusion protein (upper panel), βATPase–RFP fusion protein (middle panel) and overlay of two images (lower panel).

N-limiting conditions (Fig. 4b), and a moderate increase in total N (Fig. 4d). Amino acid concentrations in the shoots were similar in transgenic and WT plants (Supporting Information Fig. S6a,b), but total amino acid content per plant was significantly higher in the transgenic plants because of the higher biomass (Supporting Information Fig. S6c,d). Expression of *OsENOD93-1* under 1 mM nitrate condition was examined at 30 and 60 DAS by real-time PCR. The result confirmed the root-specific expression in WT plants, and the over-expression in the transgenic plants (Table 3). The expression of *OsENOD93-1* was slightly higher at a later developmental stage (60 versus 30 DAS) (Supporting Information Table S2), and its expression in the transgenic plants was constitutive, although the expression levels varied in some tissues (Supporting Information Table S2).

Because the expression of *OsENOD93-1* was detected strongly in the vascular bundles (Fig. 3), and the accumulation of amino acids was higher in the transgenic roots (Fig. 4), we determined the concentrations of amino acids in xylem sap from 4-week-old WT and transgenic plants growing under different N conditions. The transgenic plants over-expressing *OsENOD93-1* had higher total amino acids in the xylem sap than WT plants, especially when N supply

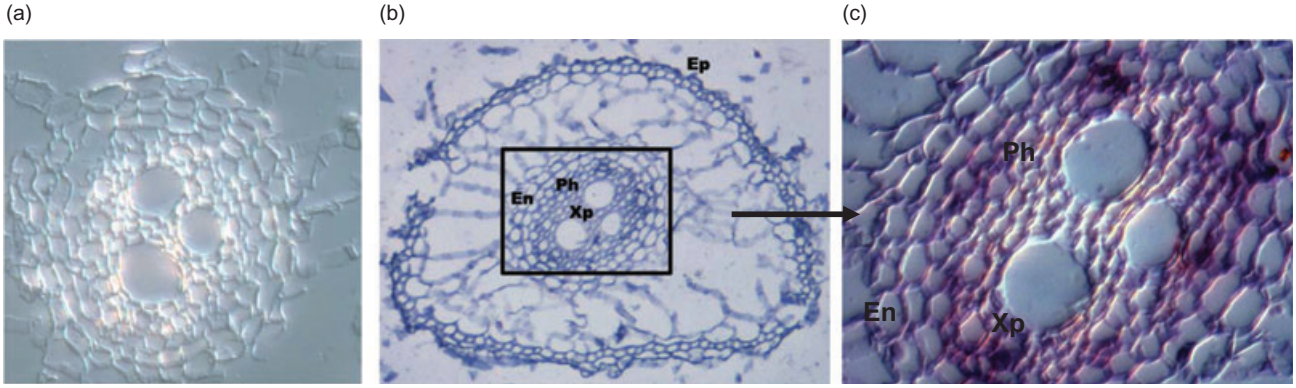


Figure 3. *In situ* localization of *OsENOD93-1* transcripts in rice roots. Sections of root from 4-week-old wild-type (WT) plants with a sense probe (a), an antisense probe (b) and an enlarged part of b (c). Ep, epidermis; En, endodermis; Xp, xylem; Ph, phloem.

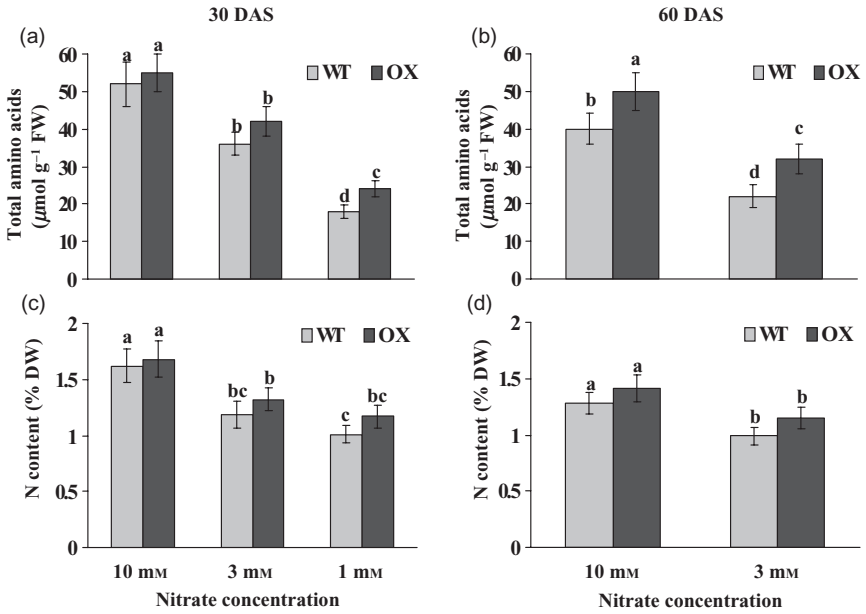


Figure 4. Effect of different nitrate levels on total amino acids (a,b) and nitrogen (c,d) concentrations in roots of wild-type (WT) rice and *OsENOD93-1* over-expression plants. The plants were grown continuously at 10, 3 or 1 mM nitrate. Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test). FW, fresh weight; DW, dry weight; DAS, days after sowing.

was limited (Fig. 5), suggesting that OsENOD93-1 could play a role in transporting amino acids from roots to shoot.

DISCUSSION

Defined N growth system for rice transcriptional profiling

Expression profiling is a useful tool to understand the molecular basis of plant responses to N. A defined N growth system is very important in order to discover the genes that are truly N responsive. We developed growth systems for *Arabidopsis* to delineate the responses at the transcriptional level when plants are grown under chronic N stress to identify genes most affected by different degrees of N limitation (Bi *et al.* 2007; Peng *et al.* 2007b). Similarly, an N growth system for rice was developed and used here to study global response under different N conditions. For rice, the N stress conditions were milder than used for *Arabidopsis*, both roots and shoots were sampled and N reduction as well as N induction was examined.

Table 3. Relative expression level of the *OsENOD93-1* gene in leaves and roots of wild-type (WT) and OX-OsENOD93-1 plants at low nitrogen level (1 mM)

	30 DAS		60 DAS	
	Leaf	Root	Leaf	Root
WT	1	50	1.6	105
OX	2050	1245	3674	2648

Relative transcript levels were determined by real-time PCR according to Livak & Schmittgen (2001) using *ACT2* as an internal control. The gene expression at each nitrate treatment was normalized to the WT shoot grown with 1 mM nitrate 30 DAS.
DAS, days after sowing; OX, over-expressor *Os*ENOD93-1.

In our previous *Arabidopsis* study, mild chronic N stress triggered only a small set of genes to be significantly different at the transcriptional level. When the N stress became severe, plant transcriptional responses were much more pronounced (Bi *et al.* 2007). A similar outcome was seen for rice: there was no significant difference for any gene between high- and medium-N conditions, and differentially expressed genes were identified when N supply became more limited.

In rice, shoots and roots responded very differently to N limitation as differentially expressed genes were identified only in root samples. The nitrate concentration in root and shoot was very different when N was limited. While there was a ~40% reduction of free nitrate in the shoots under the low-N condition, there was scarcely any free nitrate left in

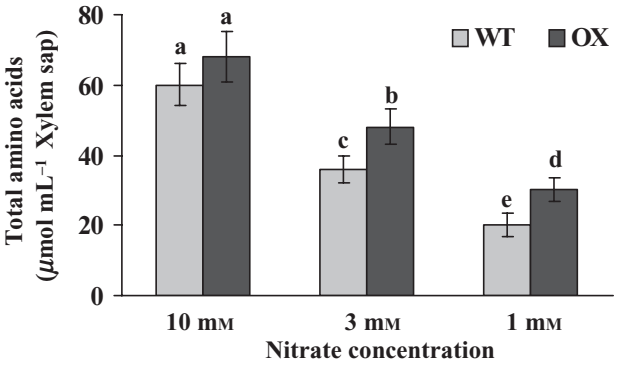


Figure 5. Effect of different nitrate levels on total amino acids in xylem sap of wild-type (WT) and OsENOD93-1 over-expression rice plants. Experimental conditions are as described in Fig. 4. Xylem sap was collected from 4-week-old plants. Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test).

the roots under that condition. For plants undergoing transient changes in N availability, differentially expressed genes were identified in roots, with the nitrate level being changed significantly in response to either N induction or N reduction. Therefore, it appears that the difference in nitrate concentration must reach a threshold before transcription is influenced. Recently, Lian *et al.* (2006) reported expression profiles for 10 422 genes at an early stage of low-N stress in rice seedling. They germinated and grew rice hydroponically with normal nutrient conditions until the emergence of the fourth leaf, then transferred the seedlings to a low-N solution, and harvested shoot and roots separately at 20, 60 and 120 min after the treatment. Like our findings, they only found transcriptional changes in the root and did not detect any significant gene expression changes in shoots at any of the three time-points after N supply was reduced.

Selection of NUE candidate genes and screening of transgenic rice plants

Some of the N-responsive rice genes identified from this study were selected as NUE candidate genes, and a transgenic validation approach was adapted directly to test the functionality of these selected genes. A ubiquitin (Ubi) promoter (Christensen & Quail 1996) was used to drive the constitutive expression of these NUE genes in transgenic plants. With limited resources and limited information on the function of some NUE candidate genes, the Ubi promoter was chosen to examine the phenotypic changes that might result. Once the functions of the candidate genes are characterized in more details, specific promoters can be used in order to maintain any positive effects while avoiding negative pleiotropic effects.

It is important to establish an effective strategy to screen transgenic plants. In our case, two strategies were performed, one at the vegetative stage and the other at the end of the reproductive stage, because transgenic plants with improved NUE at an early vegetative stage do not necessarily translate into higher final biomass or seed yield. Other strategies such as determining N uptake rate and/or biochemical changes may reveal more information on the function of some unknown genes.

Transgenic rice over-expressing *OsENOD93-1* showed improved NUE

An early nodulin gene (*OsENOD93-1*) was one of the NUE candidate genes selected because of its strong response to both N induction and N reduction. Transgenic plants over-expressing this *OsENOD93-1* gene had higher seed yield and yield-contributing factors than WT plants. In leguminous plants, the early nodulin (*ENOD*) genes are expressed at early stages of nodule development and may mediate rhizobial infection and/or nodule organogenesis (Oldroyd & Downie 2008). Furthermore, differential roles of *ENOD* genes have been postulated. As an example, *ENOD40* has been shown to be expressed during the early stage of nodule initiation (Kouchi & Hata 1993; Yang *et al.* 1993), but its protein product may be involved in cortical cell division (Charon *et al.* 1997), differentiation of vascular bundles (Kouchi *et al.* 1999) or photosynthate transport (Kouchi & Hata 1993; Yang *et al.* 1993). A number of homologs of *ENOD* genes have been identified in non-leguminous plants (Trevaskis *et al.* 1997; Reddy, Kouchi & Ladha 1998), but most are of unknown function. An early nodulin-like protein accumulates in the sieve element plasma membrane (SEPM) of *Arabidopsis* (Khan *et al.* 2007). Some sucrose transporters localized in the SEPM are involved in sucrose loading, recovery during transport and unloading (Kuhn *et al.* 2003). In addition, other transporters including those for amino acids are also localized in the SEPM (Hirner *et al.* 1998). In our case, the *OsENOD93-1* gene was expressed at high levels in roots in WT plants, and its protein product was localized in mitochondria. *In situ* hybridization showed that *OsENOD93-1* was expressed in vascular bundles, as well as in epidermis and endodermis. Over-expression of this *OsENOD93-1* gene promoted the accumulation of amino acids in roots and in xylem sap. How this contributed to the increase in final seed yield and in yield-contributing traits in the transgenic plants is yet to be determined. A recent study highlighted the importance of mitochondria in the control of N–C balance and amino acid homeostasis (Weigelt *et al.* 2008). The role of this mitochondrial *OsENOD93-1* gene is still under investigation. A homology search against the *OsENOD93-1* gene in rice indicates that there is a family of at least six genes possessing the ENOD93 domain, with five of them predicted to be localized in mitochondria (Table 2). Further characterization of these genes will hopefully delineate their function.

The *OsENOD93-1* gene would not have been selected for testing in transgenic rice plants if it had not been identified through its transcriptional response to N levels. Thus, transcriptional profiling is an effective approach for the discovery of genes that may one day contribute to improved crop genetics for this important trait.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Growth of wild-type (WT) rice plants under different N regimes. The plants were continuously grown at 3 and 10 mM ammonium, and 3 and 10 mM nitrate; picture was taken when plants were 28 days old.

Figure S2. Effect of different nitrate levels on dry weight, chlorophyll concentration and anthocyanin concentration in shoot and roots of wild-type (WT) rice. The plants were continuously grown at three different nitrogen levels: 10 mM nitrate, high N; 5 mM nitrate, medium N and 1 mM nitrate, low N, for 28 d. Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test).

Figure S3. Effect of different nitrate levels on the concentrations of nitrate, total amino acid, total soluble protein and N in shoot and roots of wild-type (WT) rice plants.

Experimental conditions are as described in Supporting Information Fig. S2. Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test). FW, fresh weight; DW, dry weight.

Figure S4. Effect of different nitrate treatments on nitrate concentrations in shoot and roots of wild-type (WT) rice plants. The plants were continuously grown at 10 or 1 mM nitrate for 28 d. Two hours before harvesting, the plants were switched from 1 to 10 mM (induction), and from 10 to 1 mM (reduction). Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test).

Figure S5. Effect of different nitrate levels on nitrate concentrations and contents in shoot and roots of 4-week-old wild-type (WT) and transgenic rice plants. The plants were continuously grown at three different nitrogen levels: 10, 3 and 1 mM nitrate, for 28 d. Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test). FW, fresh weight; DAS, days after sowing.

Figure S6. Effect of different nitrate levels on amino acid concentrations or contents in shoots of 4-week-old wild-type (WT) and transgenic rice plants. Experimental conditions are as described in Supporting Information Fig. S5. Data are mean ± SD ($n = 3$–5). Bars with different letters indicate significant difference at $P < 0.05$ (Fisher's protected least significant difference test). FW, fresh weight; DAS, days after sowing.

Table S1. Yield and yield attributes in transgenic over-expressing OsENOD93 and wild-type (WT) rice plants grown with slow-release fertilizer containing both ammonium and nitrate nitrogen.

Table S2. Relative expression level of the *OsENOD93-1* gene in plant parts of wild-type (WT) and transgenic plants.