Chrysanthemum CmNAR2 interacts with CmNRT2 in the control of nitrate uptake

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Nitrate transporters are an important component of plant growth and development. *Chrysanthemum morifolium* is an important ornamental species, for which a sufficient supply of nitrogenous fertilizer is required to maintain economic yields. In this study, the full-length cDNA of the nitrate transporter genes *CmNRT2* and *CmNAR2* were isolated. *CmNRT2* transcript accumulation was inducible by both nitrate and ammonium, but the latter ion down-regulated the transcript accumulation of *CmNAR2*. *CmNRT2* might be a plasma membrane localized protein, while *CmNAR2* was distributed throughout the cell. *CmNAR2* was shown to interact with *CmNRT2* by *in vitro* and *in vivo* assays. *Arabidopsis thaliana* plants heterologously expressing *CmNRT2* showed an increased rate of nitrate influx, while this trait was unaltered in plants expressing *CmNAR2*. Double transformants (*CmNRT2* plus *CmNAR2*) exhibited an enhanced rate of nitrate influx into the root. Our data indicated that the interaction of *CmNAR2* with *CmNRT2* contributed to the uptake of nitrate.

**Results**

The *CmNRT2* and *CmNAR2* coding and promoter sequences. The degenerate PCR amplified a 959 bp fragment of *CmNRT2* and a 300 bp fragment of *CmNAR2*. Extension from these amplicons to the full length cDNAs via RACE PCR identified that the two genes had an ORF of length, respectively, 1590 bp (DDBJ accession...
AB921547) and 597 bp (DDBJ accession AB921548), implying polypeptide products composed of 530 (molecular weight 57.6 kDa) and 199 (22.3 kDa) residues. The alignment of the CmNRT2 sequence with those of homologous NRT2 proteins showed levels of similarity ranging between 39.78% and 79.81% (Table S1), while that of CmNAR2 was between 39.13% and 54.50% (Table S2, Fig. S3). Only three conserved motifs LCYPAP, WRK and YGQ were included in CmNAR2 with its related NAR2 sequences (Fig. S4). On the other hand, a comparison with a set of full-length NRT2 and NAR2 proteins from other species clustered CmNRT2 with NpNRT2.1 and CmNAR2 with CsNAR2 in the evolutionary, respectively (Fig. 1). The 1413 bp (CmNRT2) and 1683 bp (CmNAR2) sequences upstream of the translation initiation site were obtained by TAIL-PCR and anchored PCR. By reference to the PLACE database, the CmNRT2 promoter included a number of nitrate-responsive cis elements including 5 A(C/G)TCA and 22 GAGABOX motifs (Fig. S5), while the CmNAR2 promoter harbored 5 A(C/G)TCA motifs and 18 GAGABOX motifs, along with a single nitrogen-responsive EMHVCHORD motif (Fig. S6).

Transcript accumulation of CmNRT2 and CmNAR2 is inducible. To gain the expression pattern of CmNRT2 and CmNAR2 under various external NO3− and NH4+ concentrations, qRT-PCR was carried out to analyze samples. In the presence of 0.5 mM nitrate following a period of nitrogen starvation, CmNRT2 transcript abundance increased gradually over the first 4 h, but decreased marginally thereafter (Fig. 2A). Its behavior was similar to the two higher nitrate concentrations (Fig. 2C, E). CmNAR2 transcript accumulation was also induced, peaking 6 h after the initial provision of each of the three different concentrations of nitrate, then declining gradually (Fig. 2B, D, F). CmNRT2 transcript abundance was substantially raised after a 4 h exposure to 5 mM ammonium, but that of CmNAR2 was decreased (Fig. 2H).

Subcellular localization of CmNRT2 and CmNAR2. To explore subcellular localization of CmNRT2 and CmNAR2, the transient assays of GFP fusion constructs transformed into onion epidermal cells by particle bombardment were used. As shown in Fig. 3, the transient expression of CmNRT2::GFP and CmNAR2::GFP fusions in onion epidermal cells suggested that CmNAR2 localized throughout the entire cell, whereas CmNRT2 might localize to the plasma membrane, however a conclusion can’t be drawn at present because of lack of co-localization with PM makers. In contrast, 35S-GFP alone resulted in diffused distribution of green fluorescence throughout the cell (Fig. 3).

Interaction between CmNRT2 and CmNAR2. To test whether CmNRT2 interacted with CmNAR2, the yeast two hybrid technique was used in our paper. CmNRT2 protein was fused to the mutated N-terminal half of ubiquitin (NubG), and the CmNAR2 protein was fused to the C-terminal half of ubiquitin (Cub) and the transcription factor LexA-VP1615. When NubG and Cub are brought together as fusions by CmNRT2 and CmNAR2 interacted, split-ubiquitin is restored. Then two types of reporter genes are activated by VP16 which allows growth tests on selective media (HIS3 and ADE2) and β-galactosidase activity assays (LacZ). Our datas showed that CmNAR2 interacted with CmNRT2 (Fig. 4A, B). Confirmation was obtained from X-α-Gal detection (Fig. 4C). No activity was detectable when either CmNAR2/pPR3-N or CmNRT2/pBt3-C were co-transformed. In addition, the BiFC technique was also used to characterize protein interactions in vivo. The pSAT4A-CmNAR2 vector contains a fusion of CmNAR2 at the C terminus of

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**Figure 1 | Phylogeny of NRT2 and NAR2 polypeptides.** Bootstrap percentages are given for each branch and the scale bar represents 0.05 substitutions per site. (A) The relationship of CmNRT2 with related NRT2s. The genes encoding these products and their GenBank accession numbers are: A. thaliana AtNRT2.1 (AAC64170), AtNRT2.2 (AAC55884), AtNRT2.3 (BAB10099), AtNRT2.4 (BAB10098), AtNRT2.5 (AAP78499), AtNRT2.6 (CA89321) and AtNRT2.7 (CA89826), Nicotiana plumbaginifolia NpNRT2.1 (CA669387), Lotus japonicus LjNRT2 (CAG35729), soybean GmNRT2 (AAC0320), rice OsNRT2 (BAA3382), barley HvNRT2.1 (AAC49531), wheat TaNRT2.1 (AAK19519), Chlamydomonas reinhardtii CrNRT2.1 (CA480925), Medicago truncatula MtNRT2 (XP_003596775), caryophyllaceum CmNRT2 (DDBJ accession AB921547) is shown boxed. (B) The relationship of CmNAR2 with its related NAR2 sequences. The genes encoding these products and their GenBank accession numbers are: A. thaliana AtNAR2.1 (NP_199831), AtNAR2.2 (CAC36942), Cucumis sativus CsNAR2.1 (ACV33078), barley HvNAR2.1, HvNAR2.2 and HvNAR2.3 (AAP31850, AAP31851, AAP31852), L. japonicus LjNAR2.1 (BAA65384), rice OsNAR2.1 (AP004023.2), wheat TaNAR2.1 (AAV35210), maize ZmNAR2.1 (NP_001105929), caryophyllaceum CmNAR2 (DDBJ accession AB921548) is shown boxed.
YFP, and the pSAT4A-CmNRT2 vector contains a fusion of CmNRT2 at the N terminus of YFP. The two recombinant DNA constructs were introduced into onion epidermal cells by particle bombardment. Signals were detected in the onion epidermal cell membrane when the cells were transformed with CmNRT2-YFPN/CmNAR2-YFPC, but were not observed when coexpressed with CmNRT2-YFPN/YFPC, or YFPN/CmNAR2-YFPC, (Fig. 5).

Nitrate uptake in Xenopus oocytes. To assay the amount of nitrate accumulated inside the oocytes, a colorimetric method was used in the paper18. CmNRT2 and CmNAR2 were subcloned in to expression vector pT7Ts and then used as template to synthesize mRNA. After incubation in MBS solution including 0.5 mM NaNO₃ for 16 h, there was no significant difference in nitrate uptake between the single injection (CmNRT2 or CmNAR2) and water injected.
be highly homologous with those of other NRT2 proteins, but con-
utrient solution containing 6 mM 15N-NO₃

Species2,10,19–21. According to degenerated primers, seven chrys-
genous solution containing 0.2 mM 15N-NO₃

soaked in nutrient solution containing 0.2 mM 15N-NO₃

A number of techniques have been developed to reveal gene inter-

4. The interaction between CmNRT2 and CmNAR2 based on the

A. thaliana

transgenic and non-transgenic

CmNRT2

CmNAR2

GFP

DIC

Merge

Figure 3 | Subcellular localization of CmNRT2 and CmNAR2. Inserted plasmid carry GFP, CmNAR2::GFP; CmNRT2::GFP. Images DIC taken under bright light to display the morphology of the cell, and images GFP under dark field to show GFP fluorescence; images Merge are formed by merging GFP and DIC, respectively. Bars: 50 μm.

Discussion

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transcriptional levels22,23, and the motif content of the promoter sequences of the two genes was con-

sistent with their both being involved in nitrate uptake and/or sig-

naling. Like other nitrate-inducible genes such as OsNRT2.1–2.4 in

rice24, TaNRT2.1–2.3 in wheat25, AtNRT2.1 in A. thaliana26 and

NpNRT2.1 in Nicotiana plumbaginifolia27, the expression level of

CmNRT2 in the root was strongly affected by the nitrogen status of

the growing medium (Fig. 2). The members of the AtNRT2 gene family are variously nitrate-inducible (AtNRT2.1, 2.2 and 2.4), nitrate-repressible (2.5) and constitutively expressed (2.3, 2.6 and 2.7)28. The transcript accumulation of CmNRT2 mirrored that of

AtNRT2.4 in showing a nitrate-inducible peak, which decayed with

time. CmNRT2 transcript accumulation followed a similar temporal

profile, suggesting that these two chrysanthemum genes act in con-

cert during nitrate uptake (Fig. 2). The transcript accumulation of

CmNRT2, but not that of CmNAR2, was inducible by ammonium. In

rice, it has been shown that the transcript accumulation in the root of

OsNAR2.1 is suppressed by the presence of 5 mM ammonium, while

that of various OsNRT2 genes (2.1, 2.2, 2.3a and 2.3b) is inhibited by

much lower levels29. Ammonium also has an inhibitory effect on the

transcript accumulation of the Dunaliella salina gene DoNRT2.128.

Thus, the induction/inhibition of NRT2 transcript accumulation by

nitrate or ammonium is quite gene and species dependent.

A number of techniques have been developed to reveal gene inter-

actions, some involving in vitro assays, and others carried out in

oocytes. However the co-injection of CmNRT2 and CmNAR2 had a

substantial positive increase on nitrate uptake (Fig. 6).

Root 15N influx in transgenic A. thaliana lines. To confirm the

function by in planta, we used different transgenic construction

expressed in A. thaliana to measure root 15N influx. Through

Agrobacterium-mediated transformation, four single-gene trans-

formed lines (RT-3 and RT-20 carrying p1301-220-CmNRT2, and

AR-1 and AR-2 carrying p1301-220-CmNAR2) and two dual-gene

transformed lines (D1 and D2, carrying p1301-220-CmNAR2-

CmNRT2) were arbitrarily chosen for study. Based on qRT-PCR

assay, target genes CmNRT2 and CmNAR2 were expressed in

transgenic A. thaliana lines but not wild-type (WT) and

wild-type (WT) and

transgenic and non-transgenic

A. thaliana

lines (Fig. S7).

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A. thaliana

transgenic

assay, target genes

CmNRT2

CmNRT2

1301-220-CmNAR2-

transformed lines (D1 and D2, carrying p1301-220-CmNRT2

and

CmNAR2

were isolated by RT-PCR and RACE methods (data not

shown). Here, we focus on the molecular characterization of one

CmNRT2 gene and CmNAR2. The CmNRT2 sequence proved to

be highly homologous with those of other NRT2 proteins, but con-

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25S:GFP

CmNAR2

Figure 4| The interaction between CmNRT2 and CmNAR2 based on the

DUAL membrane pairwise interaction assay. Cells grown on media

lacking (A) Ade, His, Leu and Trp, (B) Leu and Trp. (C) β-galactosidase assay of the interaction between CmNRT2 and CmNAR2. Yeast strain

NM51 carried each pairs of bait and prey plasmid (pBT3-C and pPR3-N

are the control vectors with no cloned cDNA). The construct pair of

CmNRT2 and CmNAR2 fusions was used as positive control. The

construct pairs of CmNRT2 and CmNAR2 with empty vector, respectively,

were used as negative controls.
In vivo-based yeast two-hybrid assay was able to establish that CmNAR2 interacts with CmNRT2 (Fig. 4), and this result was confirmed using the in vivo BiFC test (Fig. 5). The latter showed that the interaction took place in the plasma membrane, which again suggested a possible plasma membrane localization of CmNRT2 (Fig. 3). AtNRT2.1 localizes mostly to the plasma membrane of root cortical and epidermal cells, and the BiFC assay has shown that an intact AtNRT2.1/AtNAR2.1 complex is formed within the A. thaliana plasma membrane. CmNAR2 localized throughout the cell (Fig. 3). The natural hypothesis is therefore that CmNRT2 requires the presence of CmNAR2 to form a transporter unit. The effect on the rate of nitrate influx of constitutively expressing both chrysanthemum sequences as transgenes in A. thaliana simultaneously was much more positive than when either CmNAR2 or CmNRT2 on their own were expressed, underlining the importance of the CmNAR2/CmNRT2 interaction. In A. thaliana, AtNAR2.1 has been identified as chaperonin proteins, and AtNRT2.1–2.6 all require the presence of AtNAR2.1 to be effective (this does not apply to AtNRT2.7). In rice, the alteration in the influx of nitrate observed in an osnar2.1 knockdown mutant was taken to show that both HATS and LATS were impaired. However, LATS was not affected using the different transgenic constructions in our results, which may be that CmNRT2 or CmNAR2 was responsible in HATS and CmNRT2 interacted with CmNAR2 at the protein level in HATS. Our work has further helped to confirm the proposed complementary roles of NRT2 and NAR2 proteins.

Methods

Plant materials and growing conditions. The chrysanthemum variety ‘Nannongxuefeng’ was obtained from the Chrysanthemum Germplasm Resource

![Figure 5](image-url) BiFC-based analysis of the CmNRT2 and CmNAR2 interaction in transiently transformed onion epidermal cells. YFP fluorescence, DIC and merged images are shown for each transformation combination. The construct pair of CmNRT2-nYFP and CmNAR2-cYFP fusions was used as positive control. The construct pairs of CmNRT2-nYFP and CmNAR2-cYFP with empty vector, respectively, were used as negative controls. Bars: 50 μm.

![Figure 6](image-url) Nitrate uptake in Xenopus oocytes injected with the single gene assays of CmNRT2 and CmNAR2. Water injected oocytes were as a control. Oocytes were incubated in MBS with 0.5 mM NaNO3 for 16 h and washed four times with NO3−-free MBS solution. Four oocytes were pooled for each sample. Values were average of n = 8 ± SD. Asterisks signify that co-injection of NRT2 and NAR2 produced significantly higher nitrate uptake (by the t-test at P < 0.01) than others.

![Figure 7](image-url) Detection of CmNRT2 and CmNAR2 in the T3 generation of transgenic A. thaliana lines. (A): The CmNRT2 expression based on qRT-PCR. (B): The CmNAR2 expression based on qRT-PCR. WT: wild type A. thaliana; Vector: empty vector control transgenic line; RT-3 and RT-20: CmNRT2 transgenics; AR-1 and AR-2: CmNAR2 transgenics; D1, D2: CmNRT2/CmNAR2 transgenics; AtUBQ: A. thaliana reference gene. Transcript abundance was normalized against the expression of the constitutively expressed AtUBQ. The data efficiency of each reaction was 2^ΔΔCT (Livak and Schmittgen, 2001). Error bars represent SD of three biological replicates.
Preserving Centre, Nanjing Agricultural University, China. Cuttings were induced to form roots by a two week exposure to a liquid culture 1/4 MS. Phenotypically uniform transgenic A. thaliana.

**Isolation and sequencing of full-length cDNA.** Batches of cuttings were grown in nutrient solution for four weeks and then transferred into a nitrogen-free solution for one week. Total RNA was isolated from the roots 1 h after the addition of 5 mM KNO₃, using the RNAiso reagent (TaKaRa, Japan) following the manufacturer’s instructions and treating with RNase-free DNaseI (TaKaRa, Japan) to remove any DNA contamination. The concentration and the integrity of the resulting RNA were measured according to Shan et al.31. The open reading frame (ORF) within the amplicons was identified using ORF finder software (www.ncbi.nlm.nih.gov), and its deduced peptide sequence was subjected to a BLASTp search to identify homologs. The phylogeny of the sequences was derived using the DNAman software package. The remainder of the sequence was acquired using RACE-PCR following Shan et al. The interactions between CmNAR2 and CmNRT2 were tested using a DUAL membrane pairwise interaction kit (www.nature.com/scientificreports).

**Real-time RT-PCR.** Cuttings were grown in nutrient solution as above for four weeks and then starved of nitrogen for one week. Total RNA was isolated from the root tissue of plants supplied with either 0.5 mM, 1 mM or 5 mM KNO₃ after either 0, 0.5, 1, 2, 4, 6, 8 or 12 h, or of those supplied with 5 mM NH₄Cl after either 0 or 4 h. The expression profiles of CmNAR2 and CmNRT2 upstream of the translation initiation codon were isolated using the latter procedure, while the former used these sequences as the template. The TAIL-PCR primer pairs were P1, P2 and P3 in combination with AAP primers, while the former used these sequences as the template. The TAIL-PCR primer pairs were P1, P2 and P3 in combination with AAP primers, while the former used these sequences as the template.

**Table 1** | Primer sequences used in this study

| Primer | Sequence (5’–3’) |
|--------|------------------|
| CmNRT2 FD | CGCCGGCTGGGgngnaoytggg |
| CmNRT2 RH | TCGGACCGCGGACGGTGCggcagyyaeat |
| CmNAR2 FD | GAACTCGTGGTGAAGGCTTCAGG |
| CmNAR2 RH | GGGGGCGGCTTGGCggtrcngmncot |
| AC1 | ACGATGAGCCTCCAGAG |
| CmNRT2-P1 | AAATCTCCGAAAGCCGGAGGC |
| CmNRT2-P2 | CCGGATGAGGCGAAGATGGGA |
| CmNRT2-P3 | TCCGAGTCCCAAGGTAAGTGG |
| CmNAR2-P1 | CAAAAATCATCAAATACGCC |
| CmNAR2-P2 | AGAAGGCTACTGGCAGGGA |
| CmNAR2-P3 | CGCCATAGTGAAGAAATTAC |
| CmNRT2-R1 | CGACCCAAACCCACGCAAACTGAAAC |
| CmNAR2-R2 | ACCAACAAATGGCGTGCAATCCAGGA |
| CmNAR2-R3 | GCAACAAATTTTGCCGCAAGAAGG |
| qCmNAR2-R2 | ACCACTCGTGGAGCGGGAAT |
| qCmNAR2-R3 | ATGCTCGGAATAGACCCAATCT |
| qCmNAR2-R4 | AATTGTCAAGATGGCATAA |
| psaA | CCAAATACCCAGCAGCCTAA |
| psaA-R | GGCAACAGTTTCCTCCAAAGTAA |
| prP3-N-2-JS | ATAAACAAAGCGCATTACGCGCATCTGAGGG |
| prP3-N-2-JX | ACTGGATTGGCCCGGACGGCCCACCATGGT |
| psbT3-C-NAR2-JS | ATATCAAAGGCCCATACGGCAGGAATATACAT |
| psbT3-C-NAR2-JX | AATGGGATGGCCGGCGGCCCACCATGGT |
| NRT2-Sali-103 | GCGTGCAGATCCGTGAGTGGTAAGG |
| NRT2-Nol-103 | GAGCGGCGCGAAGAATGTGTTGGGGTG |
| NAR2-BamHI-103 | GGCGATCTCGGGGTGAACTTCTTGCT |
| NAR2-Nol-103 | GAGCGGCGCGAAGAATGTGTTGGGGTG |
| GUS-BamHI-103 | GCGGATCTCAGTTGGCCTGTGAGAACCC |
| GUS-Xhol-103 | CGGCTGCTGAGTGTGTTGCTGTGTTCG |
| CmNRT2-Bi-S | CGGAACTCTGCTGCTGCTGAGAACCC |
| CmNRT2-Bi-X | CGGAATCTAGGCTGCTGAGAACCC |
| CmNAR2-Bi-S | CGGAGATCTAGGCTGCTGAGAACCC |
| CmNAR2-Bi-X | CGGAAATCTAGGCTGCTGAGAACCC |
| CmNRT2-qF | ACTGGATCGTGCCCGGGGTCG |
| CmNRT2-qR | GAAAGTCAGAAAGATGCTATG |
| NAR2-qF | ATGTCGATTTTACATCGTG |
| NAR2-qR | ACTTTATAGCTGTTG|
| AtUBQs | AGGACAAAGGAGTGATCCCA |
| AtUBQx | GAGGACAAAGGAGTGATCCCA |

F/psaA-R (Table 1). Each PCR was repeated as three biological replicates, and relative changes in transcript accumulation were analysed using the 2^(-ΔΔCt) approach35. Subcellular localization of CmNAR2 and CmNRT2. Plasmids for transient expression were generated using the Invitrogen Gateway system. The GUS sequence (without the stop codon) from pB721 (CmNAR2) and CmNRT2 ORFs (lacking their stop codons) were amplified employing primers CmNRT2-F/psaA and CmNRT2-R and cloned into pEarleyGate 103 (GUS), BamHI/NotI (GUS), BamHI/NotI (CmNAR2) and Sal I/NotI (CmNAR2) (Table 1), then they were sub-cloned into pMD19-T (TAKARA, Japan), and finally introduced into the pEarleyGate 103 plasmid using LR Clonase™ II enzyme mix (Invitrogen) to produce pEarleyGate 103-GFP, pEarleyGate 103-CmNRT2/CmNRT2 vector respectively35. The transgenes were transiently expressed in onion epidermal cells using a He-driven PDS-1000 particle accelerator (Bio-Rad). Each transgene was bombarded on leaf segments of Allium cepa L. at 4400 psi. Confocal laser microscopy was used to monitor the expression of GFP. Protein–protein interaction assays. The interactions between CmNAR2 and CmNRT2 were tested using a DUAL membrane pairwise interaction kit.
and SD-AHLW solid media, respectively, and incubated at 30°C overnight. When the OD600 of the cultures reached 1.0 they were serially diluted (10^3, 10^2 and 10^1) and 5 µl aliquot of each dilution was spotted on to SD-LW and SD-AHLW solid media, respectively, and incubated at 30°C for 3 d. Positive clones were selected and assayed for β-galactosidase activity.

**BifC assay.** The BifC assay was used to identify the interaction between CmNAR2 and CmNRT2. The CmNAR2 ORF, excluding its termination codon, was amplified with the primer pair CmNAR2-Bi-S/X (Table 1) and introduced into the pSAT4A vector to give the construct pSAT4A-CmNAR2-eYFP-N1. Similarly, the CmNRT2 ORF was amplified using the primer pair CmNRT2-Bi-S/X (Table 1) to construct pSAT4A-CmNRT2-eYFP-N1. The two constructs were mixed with 1:1 gold particles (Bio-Rad) and transformed as described above for the transient expression study. Confocal laser microscopy was used to monitor the expression of eYFP.

**Functional characterization in Xenopus oocytes.** Oocyte preparation and cRNA injection were carried out as described previously. Oocytes injected with cRNA or water were incubated in NO3⁻-MBS solution consisting of 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.71 mM CaCl2, 0.82 mM MgSO4, and 15 mM HEPES of pH 7.4 for 48 h. To minuter NO3⁻-injected oocytes were incubated in MBS solution supplied with 0.5 mM NaNO3 at 18°C for 16 h. Then, they were washed with NO3⁻ free MBS solution. The supernatants were collected using methods as described by Feng et al. 2012. A 25 ng aliquot of cRNA was injected for each of the single gene constructs (CmNRT2) and 25 ng of each of CmNAR2 and CmNRT2 for the two gene assay. Nitrate uptake assays were performed as set out in the nitrate/nitrite colorimetric assay kit (Cayman Chemical Company).

**Transgene construction and A. thaliana transformation.** The CmNAR2 and CmNRT2 ORFs were amplified using the primer pair CmNAR (CmNRT2)/FR (Table 1) and inserted into the BanHI and SacI sites of pCAMBIA1301 following digestion with these two enzymes to generate 35S:CmNAR and 35S:CmNRT2 constructs. The constructs were named p1301-220-CmNAR2 and p1301-220-CmNRT2. CmNRT2 was amplified using the primer pair CmNAR2 BS/BX (Table 1) to construct 35S::CmNRT2. The ORF was amplified using the primer pair CmNRT2 BS/BX (Table 1) to construct 35S::CmNRT2. The constructs were named p35S::CmNRT2 and CmNRT2. The CmNRT2 gene was amplified using the primer pair CmNRT2 BS/BX (Table 1) to construct CmNRT2. The two constructs were mixed with 1:1 gold particles (Bio-Rad) and transformed as described above for the transient expression study. Confocal laser microscopy was used to monitor the expression of eYFP.

**Root ¹⁵N influx.** The influx of ¹⁵N into seedlings of transgenic and non-transgenic plants was assayed as described previously. All lines were in the similar developmental stage. The influx of ¹⁵N was calculated from the ¹⁵N concentrations of the seedlings. The influx of ¹⁵N into seedlings of transgenic and non-transgenic lines was assayed as described previously. All lines were in the similar developmental stage. The influx of ¹⁵N was calculated from the ¹⁵N concentrations of the seedlings.
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Author contributions

C.G., S.C. and F.C. conceived the study and designed the experiments. C.G., X.Z., J.J., Z.G. and S.Z. performed the experiments and C.G., W.F. and Y.L. analysed the data with suggestions by S.C. and F.C. and C.G. wrote the manuscript. All authors read and approved the final manuscript.

Additional information

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