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# *Chrysanthemum* CmNAR2 interacts with CmNRT2 in the control of nitrate uptake

SUBJECT AREAS:

PLANT PHYSIOLOGY

PLANT MOLECULAR BIOLOGY

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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.

In soil, inorganic nitrogen (N) is available for plants as nitrate and ammonium<sup>1</sup>, and plants have evolved various means of coping with nitrogen deficiency<sup>2</sup>. Genes belonging to the nitrate transporter families (*NRT1*, *NRT2*) and the nitrate assimilation related family (*NAR2*) ensure the capacity of root cells to regulate the uptake of nitrate, through two high-affinity transport systems (HATS) and one low-affinity transport system (LATS) depending on nitrate concentrations<sup>3,4</sup>. The *Arabidopsis thaliana* (*A. thaliana*) gene *AtNPF6.3* has been shown to encode a dual-affinity nitrate transporter<sup>5</sup>. Certain elements of HATS are inducible (iHATS) while others are constitutively expressed<sup>6,7</sup>. Full activity of iHATS in the *A. thaliana* root requires the presence of both *AtNRT2.1* and *AtNRT2.2*<sup>8</sup>. Nevertheless, it was shown that the expression of high affinity nitrate transport in *Chlamydomonas* sp. requires the presence of *NRT2.1*, *NRT2.2* and *NAR2*<sup>9</sup>, an observation confirmed in experiments based on a *Xenopus* oocyte expression system<sup>10</sup>. In T-DNA insertional mutant *atnar2.1-1* and *atnar2.1-2*, constitutive HATS (cHATS) was reduced by 34% and 89%, while iHATS was reduced by 92% and 96% respectively<sup>11</sup>. In the *atnrt2.1-1* mutant, the HATS under either high or low nitrate growth concentrations was affected<sup>12</sup>. *AtNAR2.1* and *AtNRT2.1* interact with one another during the HATS process<sup>13</sup>, while the product of *AtNAR2* is involved in the constitutively induced high affinity influx<sup>14</sup>. Similarly in rice, *OsNAR2.1* interacts with *OsNRT2.1*, *2.2* and *2.3a*<sup>15</sup>. In *A. thaliana*, the *AtNRT2* transporters interact strongly with *AtNAR2.1* except for *AtNRT2.7*<sup>8</sup>.

*Chrysanthemum* (*Chrysanthemum morifolium*) is a commercially important ornamental species, for which a sufficient supply of nitrogenous fertilizer is required to promote vegetative growth<sup>16,17</sup>. However, excessive fertilizer application, especially when coupled with a low nitrate utilization rate, risks a significant wastage of nutrients, which can lead to problems of environmental pollution. Thus studying the HATS and LATS in *chrysanthemum* is necessary for cultivation. As yet, no genes involved in nitrate uptake have been isolated in *chrysanthemum*, nor have any interactions between nitrate transporters and nitrate assimilation related proteins been defined. Here, the isolation of *CmNRT2* and *CmNAR2* cDNA was described, and their inducibility by nitrate and ammonium was described. A series of assays was used to show that their gene products interact with one another. The behavior of transgenic *A. thaliana* confirmed that *CmNAR2* probably serves as a partner to *CmNRT2* in controlling nitrate uptake.

## 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 LCYAP, 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 *CmNAR2.1* promoter harbored 5 A(C/G)TCA motifs and 18 GAGABOX motifs, along with a single nitrogen-responsive EMHVCHORD motif (Fig. S6).

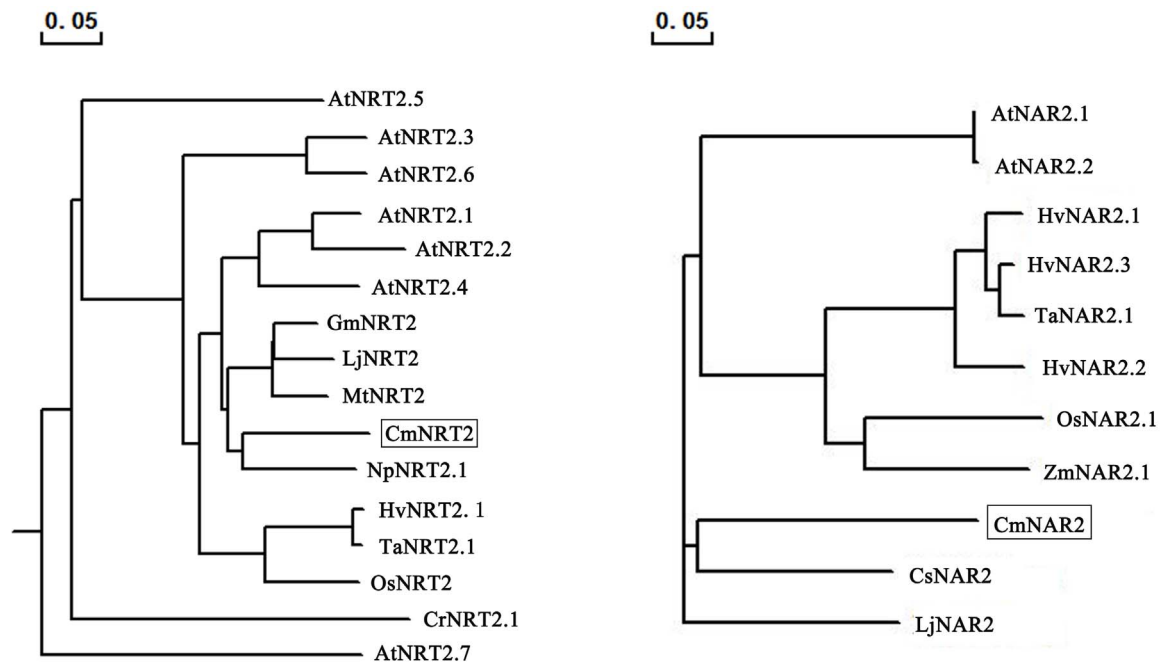
#### Transcript accumulation of *CmNRT2* and *CmNAR2* is inducible.

To gain the expression pattern of *CmNRT2* and *CmNAR2* under various external  $\text{NO}_3^-$  and  $\text{NH}_4^+$  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 at 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

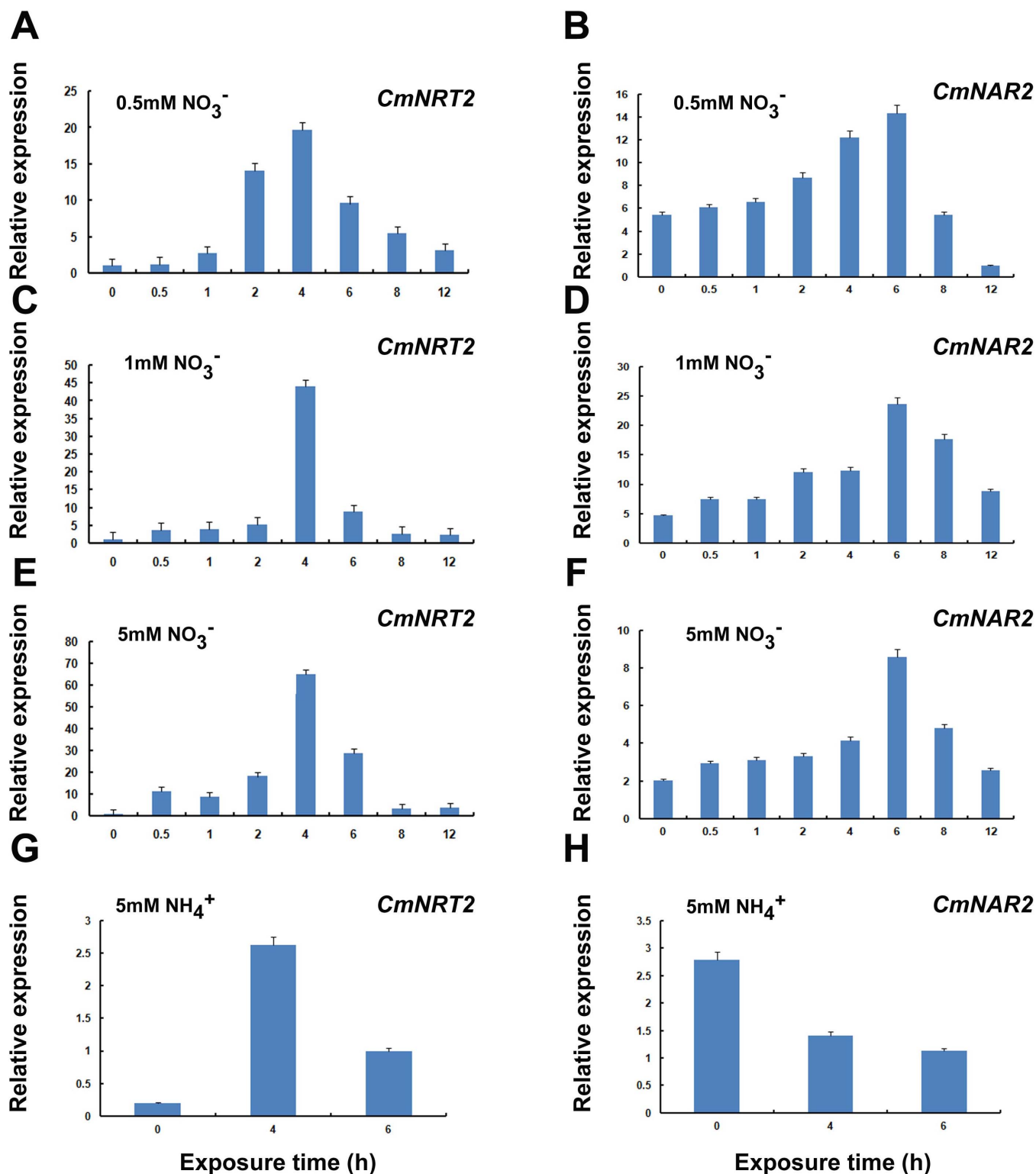
abundance was substantially raised after a 4 h exposure to 5 mM ammonium, but that of *CmNAR2* was decreased (Fig. 2H).

**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-VP16<sup>15</sup>. 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  $\beta$ -galactosidase activity assays (LacZ). Our data showed that *CmNAR2* interacted with *CmNRT2* (Fig. 4A, B). Confirmation was obtained from X- $\alpha$ -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



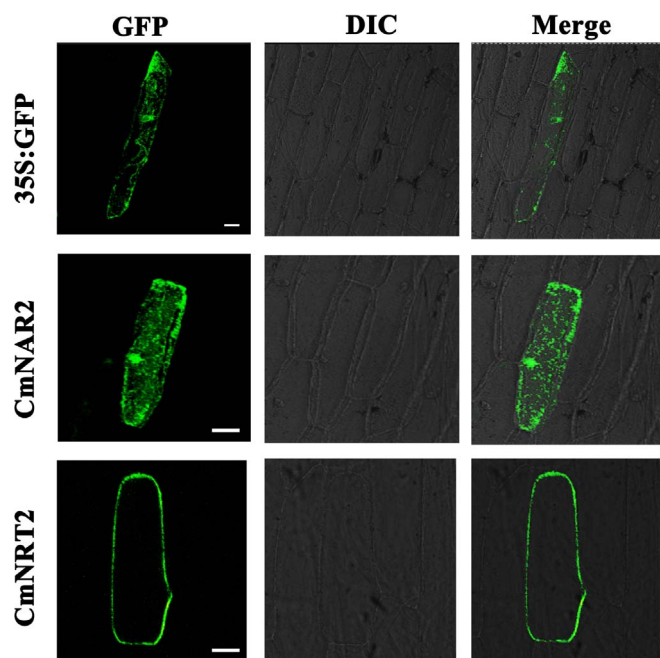
**Figure 1 | Phylogeny of NRT2 and NAR2 polypeptides.** Bootstrap percentages are given for each branch and the scale bar represents 0.05 substitutions per site. (A) The relationship of *CmNRT2* with related NRT2s. The genes encoding these products and their GenBank accession numbers are: *A. thaliana* *AtNRT2.1* (AAC64170), *AtNRT2.2* (AAC35884), *AtNRT2.3* (BAB10099), *AtNRT2.4* (BAB10098), *AtNRT2.5* (AAF78499), *AtNRT2.6* (CAB89321) and *AtNRT2.7* (CAB87624), *Nicotiana. plumbaginifolia* *NpNRT2.1* (CAA69387), *Lotus japonicus* *LjNRT2* (CAC35729), soybean *GmNRT2* (AAC09320), rice *OsNRT2* (BAA33382), barley *HvNRT2.1* (AAC49531), wheat *TaNRT2.1* (AAK19519), *Chlamydomonas reinhardtii* *CrNRT2.1* (CAA80925), *Medicago truncatula* *MtNRT2* (XP\_003596775). chrysanthemum *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* (BAI63584), rice *OsNAR2.1* (AP004023.2), wheat *TaNAR2.1* (AAV35210), maize *ZmNAR2.1* (NP\_001105929). chrysanthemum *CmNAR2* (DDBJ accession AB921548) is shown boxed.



**Figure 2** | The induced gene expression level of (A, C, E, G) *CmNRT2* and (B, D, F, H) *CmNAR2* in roots exposed, following a period of N starvation, to (A, B) 0.5 mM nitrate, (C, D) 1 mM nitrate, (E, F) 5 mM nitrate, (G, H) 5 mM ammonium, as assayed by qRT-PCR. Mean values and standard deviation calculated from triplicated assays.

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*-YFP<sup>N</sup>/*CmNAR2*-YFP<sup>C</sup>, but were not observed when coexpressed with *CmNRT2*-YFP<sup>N</sup>/YFP<sup>C</sup>, or YFP<sup>N</sup>/*CmNAR2*-YFP<sup>C</sup>, (Fig. 5).

**Nitrate uptake in *Xenopus* oocytes.** To assay the amount of nitrate accumulated inside the oocytes, a colorimetric method was used in the paper<sup>18</sup>. *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  $\text{NaNO}_3$  for 16 h, there was no significant difference in nitrate uptake between the single injection (*CmNRT2* or *CmNAR2*) and water injected



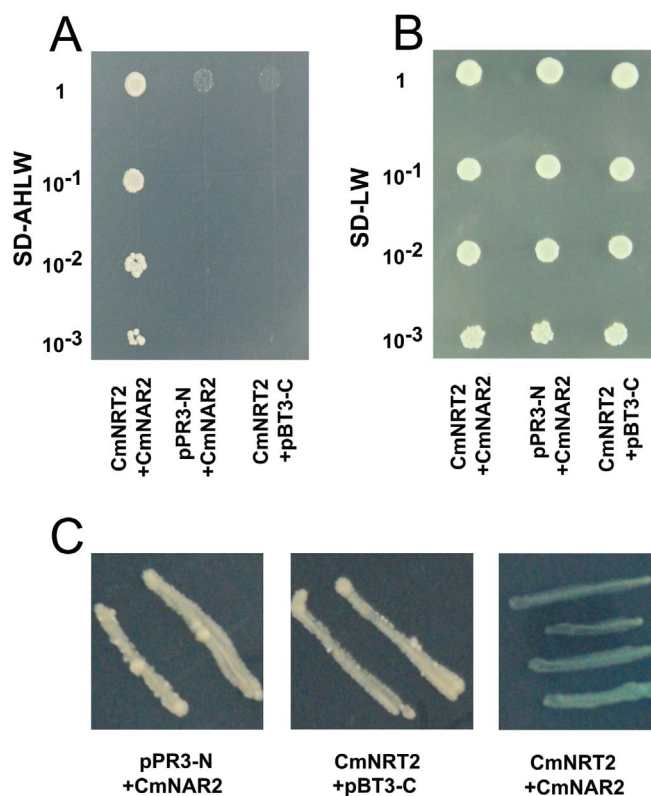
**Figure 3 | Subcellular localization of CmNRT2 and CmNAR2.** Inserted plasmid carry *GFP*, *CmNAR2::GFP*, *CmNRT2::GFP*. Images DIC taken under bright light to display the morphology of the cell, and images GFP under dark field to show GFP fluorescence; images Merge are formed by merging GFP and DIC,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

oocytes. However the co-injection of *CmNRT2* and *CmNAR2* had a substantial positive increase on nitrate uptake (Fig. 6).

**Root  $^{15}\text{N}$  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  $^{15}\text{N}$  influx. Through *Agrobacterium*-mediated transformation, four single-gene transformed 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 *CaMV* 35S::*GUS* (vector) *A. thaliana* plants (Fig. 7 A, B). After 5 min soaked in nutrient solution containing 0.2 mM  $^{15}\text{N-NO}_3^-$ , lines RT-3, RT-20, D1 and D2 showed a significantly enhanced rate of nitrate influx than wild-type and empty vector control transgenic lines (Fig. 8), indicating that the presence of *CmNRT2* was responsible for heightened nitrate uptake. The  $^{15}\text{N}$  influx in the double transgenic gene lines was much greater than in the single gene ones indicating that *CmNAR2* can interact with *CmNRT2* to promote root  $^{15}\text{N}$  influx. There was no significant difference in nitrate uptake performance between the *CmNAR2* transformants and the non-transformed control (Fig. 8). After 5 min culture in nutrient solution containing 6 mM  $^{15}\text{N-NO}_3^-$ , the data showed that no significant difference in root nitrate influx was found in transgenic and non-transgenic *A. thaliana* lines (Fig. S7).

## Discussion

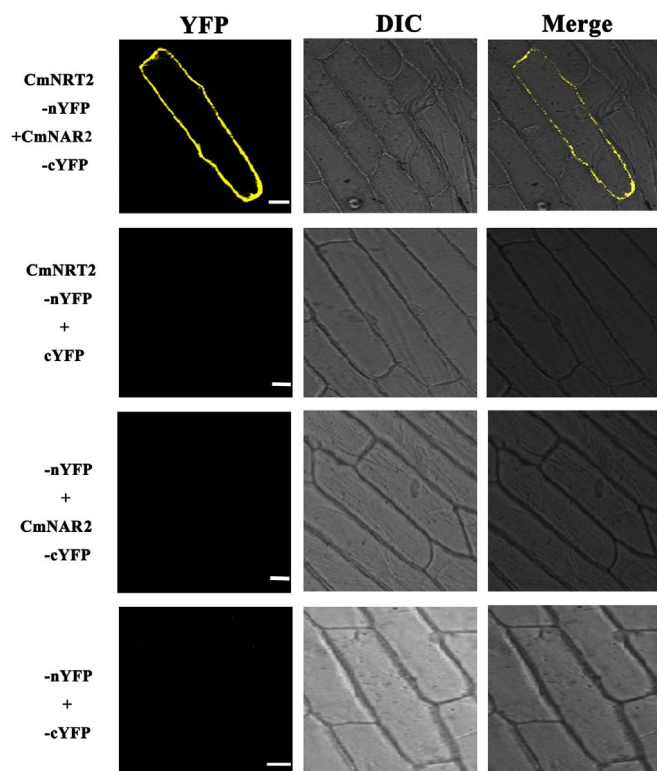
Genes encoding NRT2 or NAR2 proteins are present in many plant species<sup>2,10,19–21</sup>. According to degenerated primers, seven chrysanthemum NRT2 homolog *CmNRT2* and one NAR2 homolog *CmNAR2* were isolated by RT-PCR and RACE methods (data not shown). Here, we focus on the molecular characterization of one *CmNRT2* gene and *CmNAR2*. The *CmNRT2* sequence proved to be highly homologous with those of other NRT2 proteins, but con-



**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)  $\beta$ -galactosidase assay of the interaction between *CmNRT2* and *CmNAR2*. Yeast strain NMY51 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.

servation of the *CmNAR2* sequence was less marked, although the LCYAP, WRK and YGQ motifs were still retained. The regulation of these genes is believed to occur at transcriptional levels<sup>22,23</sup>, and the motif content of the promoter sequences of the two genes was consistent with their both being involved in nitrate uptake and/or signaling. Like other nitrate-inducible genes such as *OsNRT2.1–2.4* in rice<sup>24</sup>, *TaNRT2.1–2.3* in wheat<sup>25</sup>, *AtNRT2.1* in *A. thaliana*<sup>26</sup> and *NpNRT2.1* in *Nicotiana plumbaginifolia*<sup>27</sup>, 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)<sup>26</sup>. The transcript accumulation of *CmNRT2* mirrored that of *AtNRT2.4* in showing a nitrate-inducible peak, which decayed with time. *CmNAR2* transcript accumulation followed a similar temporal profile, suggesting that these two chrysanthemum genes act in concert 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 levels<sup>23</sup>. Ammonium also has an inhibitory effect on the transcript accumulation of the *Dunaliella salina* gene *DsNRT2.1*<sup>28</sup>. 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 interactions, some involving *in vitro* assays, and others carried out *in*

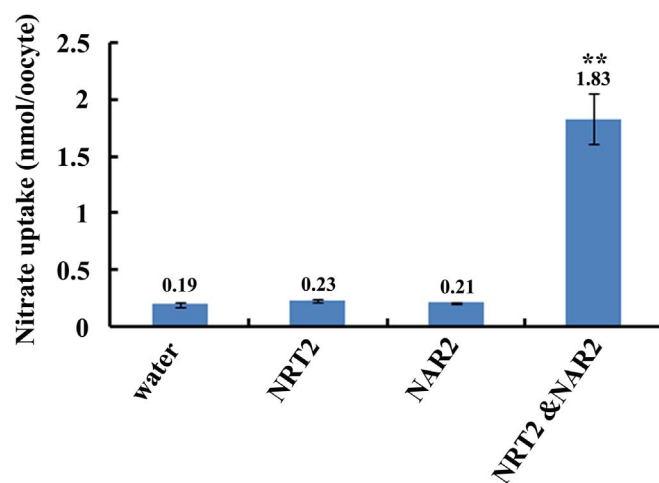


**Figure 5** | BiFC-based analysis of the CmNRT2 and CmNAR2 interaction in transiently transformed onion epidermal cells. YFP fluorescence, DIC and merged images are shown for each transformation combination. The construct pair of CmNRT2-nYFP and CmNAR2-cYFP fusions was used as positive control. The construct pairs of CmNRT2-nYFP and CmNAR2-cYFP with empty vector, respectively, were used as negative controls. Bars: 50  $\mu\text{m}$ .

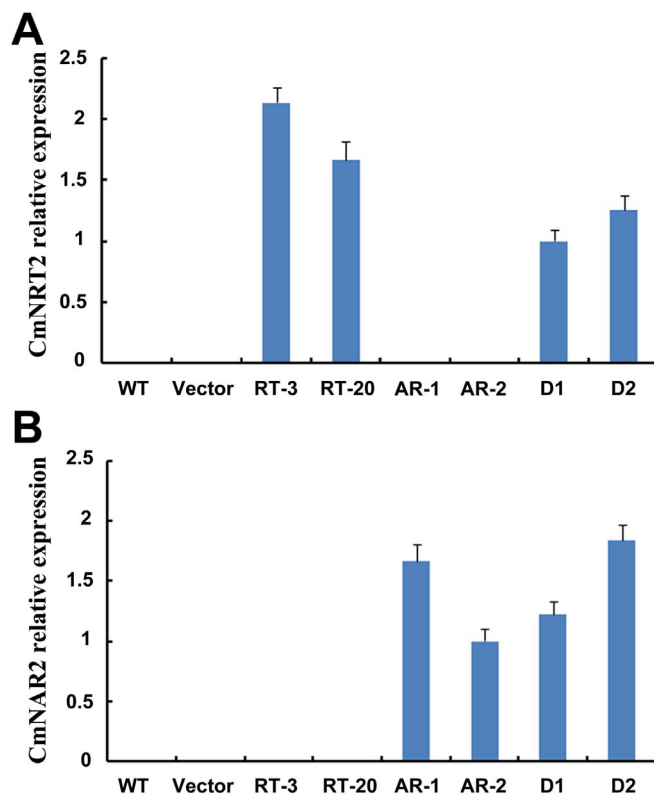
*in vivo*<sup>11,13,15,29</sup>. Here, use of the *in vitro* based yeast two-hybrid assay was able to establish that CmNAR2 interacts with CmNRT2 (Fig. 4), and this result was confirmed using the *in vivo* BiFC test (Fig. 5). The latter showed that the interaction took place in the plasma membrane, which 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<sup>29</sup>. 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)<sup>8</sup>. 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<sup>15</sup>. 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

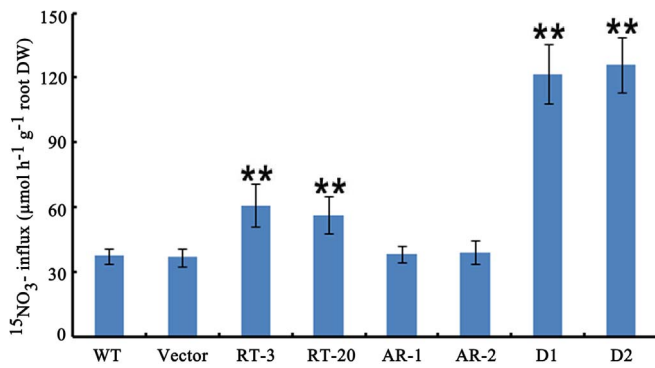
**Plant materials and growing conditions.** The chrysanthemum variety ‘Nannongxuefeng’ was obtained from the Chrysanthemum Germplasm Resource



**Figure 6** | Nitrate uptake in *Xenopus* oocytes injected with the single gene assays or CmNRT2 and CmNAR2. Water injected oocytes were as a control. Oocytes were incubated in MBS with 0.5 mM  $\text{NaNO}_3$  for 16 h and washed four times with  $\text{NO}_3^-$  free MBS solution. Four oocytes were pooled for each sample. Values were average of  $n = 8 \pm \text{SD}$ . 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** | Detection of CmNRT2 and CmNAR2 in the T<sub>3</sub> 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  $2^{-\Delta\Delta\text{C}_T}$  (Livak and Schmittgen, 2001). Error bars represent SD of three biological replicates.



**Figure 8 | Influx of  $^{15}\text{N}$  into the root of transgenic and non-transgenic *A. thaliana*.** *A. thaliana* plants were grown for 42 d in hydroponics on 0.2 mM  $\text{NO}_3^-$ . Root  $^{15}\text{NO}_3^-$  was measured after 5 min labelling with complete nutrient solution containing 0.2 mM  $^{15}\text{NO}_3^-$ . 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. Values represent the mean  $\pm$  SE ( $n = 5$ ). Asterisks indicate means which differ significantly (by the t-test at  $P < 0.01$ ) from the wild type value.

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 seedlings at the 8 leaf stage were transferred into 8 L of 5 mM  $\text{NH}_4\text{NO}_3$ , 2.5 mM  $\text{K}_2\text{SO}_4$ , 1.5 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.33 mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 2.0 mM  $\text{CaCl}_2$ , 20  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 9  $\mu\text{M}$   $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.77  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.32  $\mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.39  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 20  $\mu\text{M}$   $\text{FeNaEDTA}$ , pH 6.5. To inhibit nitrification, 7  $\mu\text{M}$  dicyandiamide was added to the solution, which was refreshed each two days.

**Isolation and sequencing of full-length cDNA.** Batches of cuttings were grown in the above nutrient solution for four weeks and then were transferred into a nitrogen-free solution for one week. Total RNA was isolated from the roots 1 h after the addition of 5 mM  $\text{KNO}_3$ , 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 assessed following Gu et al.<sup>30</sup>. The first cDNA strand was synthesized using Reverse Transcriptase M-MLV (RNase H<sup>-</sup>) (TaKaRa, Japan) according to the manufacturer's instructions. Two pairs of degenerate primers *CmNRT2FD/RH* and *CmNAR2FD/RH* (sequences given in Table 1) were designed based on conserved regions of, respectively, *NRT2* (Fig. S1) and *NAR2* (Fig. S2) homologs from a range of plant species. The remainder of the sequence was acquired using RACE-PCR following Shan et al.<sup>31</sup>. 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.

**Isolation and sequence analysis of promoters.** Genomic DNA was isolated from leaves using the CTAB method<sup>32</sup> and diluted to 50 ng/ $\mu\text{L}$ . The *CmNAR2* and *CmNRT2* promoter sequences were isolated from this genomic DNA, via three rounds of PCR (primer sequences given in Table 1), where each round used the product of the previous one as template, applying both TAIL-PCR and anchored PCR<sup>33,34</sup>. Fragments 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 AC1, and those for the anchored PCR were R1, R2 and R3 in combination with AAP and AUAP. The resulting amplicons were sequenced. Analyses of the sequences upstream of each gene relied on RSTA (rsat.ulb.ac.be/rsat/) software, and the databases PLACE (dna.affrc.go.jp/PLACE/signalscan.html) and softberry (www.softberry.com/berry.phtml)<sup>35</sup>.

**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  $\text{KNO}_3$  after either 0, 0.5, 1, 2, 4, 6, 8 or 12 h, or of those supplied with 5 mM  $\text{NH}_4\text{Cl}$  after either 0, 4 or 6 h. The expression profiles of *CmNAR2* and *CmNRT2* were obtained by real time RT-PCR based on SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Perfect Real Time) (TAKARA, Japan). The PCR comprised an initial denaturation step of 95°C/60 s, followed by 40 cycles of 95°C/15 s, 55°C/30 s and 72°C/30 s. At the end of the PCR, the temperature was raised from 55 to 95°C at a rate of 0.5°C s<sup>-1</sup> to generate the required denaturation curve. The primer pair qCmNRT2-F/qCmNRT2-R and qCmNAR2-F/qCmNAR2-R were applied to amplify *CmNRT2* or *CmNAR2* fragment, respectively (Table 1). The reference gene used was a fragment of *CmpsaA* (AB548817)<sup>30</sup> with primer pair *psaA-*

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

Primer	Sequence (5'-3')
<i>CmNRT2</i> FD	CGCCGGCTGGgnaayatggg
<i>CmNRT2</i> RH	TCGGACCGGCAGTTCtngcraaytt
<i>CmNAR2</i> FD	GAACGTCAAGGTGAAGCTGtgytaygncc
<i>CmNAR2</i> RH	GGGGCGTCTGGCrrtangnacyt
AC1	ACGATGGACTCCAGAG
<i>CmNRT2</i> -P1	AAATACTTCCAGAAACCGAGGC
<i>CmNRT2</i> -P2	CCGGATGATGGGGACAAGTGA
<i>CmNRT2</i> -P3	TTCGGAGTCCACAGGTAAGTCG
<i>CmNAR2</i> -P1	CAAAACCATCAAATACGC
<i>CmNAR2</i> -P2	AGAGGCATGACGGAAAG
<i>CmNAR2</i> -P3	CGGCAATGATGAAAATAGC
<i>CmNRT2</i> -R1	CGACCCAACCCAGCCACCCAAGTCAAAC
<i>CmNRT2</i> -R2	AACACCCATTATGACCCGGTCAACAAAAGT
<i>CmNRT2</i> -R3	ACCAATAACTGCTTCATATCTTTGAACCGACA
<i>CmNAR2</i> -R1	CCGTGATATCAATAAGCTACAAAGACCAT
<i>CmNAR2</i> -R2	ACCAACAATTGGCGGTCACTTAACGGA
<i>CmNAR2</i> -R3	GTC AAGGGACCAAACCCGATACGAGTCA
qCmNRT2-F	ACCACTGCTGAACGGGAAAT
qCmNRT2-R	ATGGCTGGAATAGAACCTCA
qCmNAR2-F	AATGGCTGAAAACCTCAAGAA
qCmNAR2-R	AATCATGCAATGGCATAAAC
<i>psaA</i> -F	CCAATAACCACGACCCTAA
<i>psaA</i> -R	GGCACAGTCTCCCAAGTAA
pPR3-N-2-JS	ATTAACAA GGCCATTACGGCCATGCTGAGG TTGAAGGT
pPR3-N-2-JX	AACTGATT GGCCGAGGCGCCAACATGGTTG GGGGTG
pBT3-C-NAR2-JS	ATTAACAAGGCCATTACGGCCAAAAATGAAATC CATTTTGCTC
pBT3-C-NAR2-JX	AACTGATT GGCCGAGGCGGCCCC GCTTTTCGACTGC
<i>NRT2</i> -SalI-103	GCGTCGAC ATGCCTGAGGTTGAAGGT
<i>NRT2</i> -NotI-103	GAGCGGCCGCGA AACATGGTTGGGGGTG
<i>NAR2</i> -BamHI-103	CGGGATCCGGATGAAATCCATTTTGCTC
<i>NAR2</i> -NotI-103	GAGCGGCCGCGGACTTTTCTGACTGTC
<i>GUS</i> -BamHI-103	CGGGATCCATGGTCCGCTCTAGAAACCC
<i>GUS</i> -XhoI-103	CCGCTCGAGTATTGTTTGCCTCCCTGCTGC
<i>CmNRT2</i> -Bi-S	CGGAATTCATGCCTGAGGTTGAAGGT
<i>CmNRT2</i> -Bi-X	CGGGATCCCAACATGTTGGGGGTG
<i>CmNAR2</i> -Bi-S	CGGAATTCATGAAATCCATTTTGCTC
<i>CmNAR2</i> -Bi-X	CGGGATCCCGCTTTTTCGACTGTC
<i>CmNRT2</i> -qF	TCCTGATGCCCGTGTGTTGTTGAG
<i>CmNRT2</i> -qR	GAAGTACGAAGATCCATGTCGGA
<i>NAR2</i> -qF	ATGTGCTATTTTCATCATTTGCCG
<i>NAR2</i> -qR	CTTCTTATCATTTGTGGTTTGCC
AiUBQs	AGGACAAAGAGGGATCCCA
AiUBQx	CAGACCAAGACCAAGTGAA

F/*psaA*-R (Table 1). Each PCR was repeated as three biological replicates, and relative changes in transcript accumulation were analysed using the 2<sup>- $\Delta\Delta\text{C}_T$</sup>  approach<sup>35</sup>.

**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 pBI121, *CmNAR2* and *CmNRT2* ORFs (lacking their stop codon) were amplified employing primer pairs *GUS*-*Bam*HI-103/*GUS*-*Xho*I-103, *NAR2*-*Bam*HI-103/*NAR2*-*Not*I-103 and *NRT2*-*Sal*I-103/*NRT2*-*Not*I-103 (Table 1), then they were sub-cloned into pMD19-T (TAKARA, Japan) and confirmed by DNA sequencing. The pMD19-T constructs and the pENTR1M1A dual selection vector (Invitrogen) were cut by *Bam*HI I/*Xho*I (*GUS*), *Bam*HI I/*Not*I (*CmNAR2*) and *Sal*I/*Not*I (*CmNRT2*), ligated using T4 DNA ligase (TAKARA, Japan), and finally introduced into the pEarleyGate 103 plasmid using an LR Clonase<sup>™</sup> II enzyme mix (Invitrogen) to produce pEarleyGate 103-GFP, pEarleyGate 103-*CmNAR2/CmNRT2* vector respectively<sup>36</sup>. The transgenes were transiently expressed in onion epidermal cells using a He-driven PDS-1000 particle accelerator (Bio-Rad). pEarleyGate 103-GFP was used as a control. After bombardment, the onion peels were kept for 24 h in the dark on Murashige and Skoog (1962) medium plates. 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



(Dualsystems Biotech, Schlieren, Switzerland). Full-length of *CmNAR2* and *CmNRT2* cDNAs were amplified using PrimeSTAR<sup>®</sup> HS DNA polymerase (TAKARA, Japan) with the primer pairs pBT3-C-NAR2-JS/pBT3-C-NAR2-JX and pPR3-N-2-JS/pPR3-N-2-JX (Table 1), then cloned into pBT3-C (LEU2, KanR) and pPR3-N (TRP1, AmpR) expression vectors, respectively, and co-transformed into yeast strain NMY51 (*MA Ta his3 trp1 leu2 ade2 LYS2::HIS3 ura3::lacZ ade2::ADE2 GAL4*) using a DS yeast transformation kit (Dualsystems Biotech). Transformed colonies were selected in SD-LW medium to which had been added 5 mM 3-amino-1,2,4-triazole (3-AT), and allowed to grow out for the selection of positive transformants<sup>15</sup>. Several independent positive transformants were selected and grown in SD-LW liquid medium at 30°C overnight. When the OD<sub>546</sub> of the cultures reached 1.0 they were serially diluted (10×, 100× and 1,000×). A 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<sup>37</sup> 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*-cEYFP-N1. Similarly, the *CmNRT2* ORF was amplified using the primer pair *CmNRT2*-Bi-S/X (Table 1) to construct pSAT4A-*CmNRT2*-nEYFP-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 YFP.

**Functional characterization in *Xenopus* oocytes.** Oocyte preparation and cRNA injection were carried out as described<sup>18,38</sup>. Oocytes injected with cRNA or water were incubated in NO<sub>3</sub><sup>-</sup> MBS solution consisting of 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 0.71 mM CaCl<sub>2</sub>, 0.82 mM MgSO<sub>4</sub> and 15 mM HEPES of pH 7.4 for 48 h. To monitor NO<sub>3</sub><sup>-</sup>, injected oocytes were incubated in MBS solution supplied with 0.5 mM NaNO<sub>3</sub> at 18°C for 16 h<sup>18</sup>. Then, they were washed with NO<sub>3</sub><sup>-</sup> free MBS solution. The supernatants were collected using methods as described by Feng et al.<sup>18</sup>. A 25 ng aliquot of cRNA was injected for each of the single gene assays (*CmNRT2*) and 25 ng of each of *CmNRT2* and *CmNAR2* for the two gene assay<sup>8</sup>. 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*)/F/R (Table 1) and inserted into the *Bam*HI and *Sac*I sites of pCambia1301 following digestion with these two enzymes to generate 35S::*CmNRT2* and 35S::*CmNAR2* constructs. The constructs were named p1301-220-*CmNAR2* and p1301-220-*CmNRT2*. *CmNRT2* was amplified using the primer pair *CmNRT2* BS/BX (Table 1) and the amplicon cloned into the *Bgl*II/*Bst*EII site of p1301-220-*CmNAR2* replacing the *GUS* sequence to form p1301-220-*CmNAR2*-*CmNRT2*. This construct was transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method<sup>39</sup>. *A. thaliana* Col-0 was transformed via the floral dip method<sup>40</sup>. T<sub>1</sub> seedlings were raised on agar containing 20 mg/L hygromycin and 25 mg/L ampicillin. Positive transformants were validated by PCR. Specific primers of quantitative RT-PCR for *CmNRT2* and *CmNAR2* were *CmNRT2*-qF/*CmNRT2*-qR for *CmNRT2* and *NAR2*-qF/*NAR2*-qR for *CmNAR2* (Table 1). A portion of the *A. thaliana* *AtUBQ* gene (NM\_116771.5), amplified by primer pair *AtUBQs*/*AtUBQx*, was used as the reference (Table 1).

**Root <sup>15</sup>N influx.** The influx of <sup>15</sup>N into seedlings of transgenic and non-transgenic lines was assayed as described previously<sup>12</sup>. All lines were in the similar developmental stage for root <sup>15</sup>N influx testing. Plants were supplied with nutrient media as follows: 0.1 mM KNO<sub>3</sub>, 0.05 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 2.45 mM K<sub>2</sub>SO<sub>4</sub>, 2.15 mM CaCl<sub>2</sub> and 0.7 mM CaCl<sub>2</sub>. Nutrient solutions were used at half-strength during the first 2 weeks and changed every 2 days<sup>12</sup>. After 42 days culture, plants were first exposed to 0.1 mM CaSO<sub>4</sub> for 1 min and then to a complete nutrient solution containing 0.2 mM <sup>15</sup>N-NO<sub>3</sub><sup>-</sup> for 5 min and finally to 0.1 mM CaSO<sub>4</sub> for 1 min. Homogenized root tissue was dried by baking overnight at 80°C, and analyzed using the ANCA-MS system (PDZ Europa). The influx of <sup>15</sup>N was calculated from the <sup>15</sup>N concentrations of the roots.

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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

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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