



# Isolation and characterization of the *Chrysanthemum* nitrate transporter CmNRT1

C.S. Gu, X.X. Zhang, S.M. Chen, T. Li, Y. Chen, J.F. Jiang and F.D. Chen

College of Horticulture, Nanjing Agricultural University, Nanjing, China

Corresponding author: F.D. Chen

E-mail: chenfd@njau.edu.cn

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**ABSTRACT.** In this study, the nitrate transporter gene *CmNRT1* was isolated from the chrysanthemum variety 'Nannongxuefeng'. The full-length cDNA contains an open reading frame of 1761 bp encoding 587 residues. Using qRT-PCR, we found that *CmNRT1* was induced by 10 mM NO<sub>3</sub><sup>-</sup> in roots and shoots. Two *Arabidopsis thaliana* transgenic plants expressing *CmNRT1* were selected for functional analyses. Root <sup>15</sup>N influx in wild-type and transgenic *A. thaliana* lines under 10 or 0.2 mM <sup>15</sup>NO<sub>3</sub> was tested. Our results indicate that *CmNRT1* encodes a constitutive component for a low-affinity transporter.

**Key words:** Chrysanthemum; Nitrogen; Nitrate transporter

## INTRODUCTION

Nitrogen, an essential macronutrient for plants (Covelo et al., 2008; Zhang et al., 2008), is a constituent of numerous important compounds including amino acids, proteins (enzymes), nucleic acids, chlorophyll, and several plant hormones (Britto and Kronzucker, 2004; Guo et al., 2008). Because of the important influence of nitrogen on plant growth, a number of studies have examined the basis of nitrogen use efficiency in cotton (Li et al., 2007), rice (Koutroubas and Ntanos, 2003) and maize (Presterl et al., 2002). Three nitrate transporter gene families *NRT1s*, *NRT2s*, and *NAR2s* have been identified that code for a high-affinity nitrate transport system and low-affinity nitrate transport system (Kotur et al., 2012). The first plant nitrate transporter discovered, CHL1, was identified in *Arabidopsis* (Tsay et al., 1993) and was shown to be a dual-affinity nitrate transporter involved in multiple phases of nitrate uptake (Liu et al., 1999). The functions of *AtNRT1* gene family members have since been gradually established. *AtNRT1.5* is responsible for xylem loading of nitrate (Lin et al., 2008); *AtNRT1.6* has a role in early embryo development (Almagro et al., 2008); *AtNRT1.7* is responsible for source-to-sink remobilization of nitrates (Fan et al., 2009); *AtNRT1.8* functions in nitrate removal from the xylem sap and mediates cadmium tolerance (Li et al., 2010); *AtNRT1.9* is important in phloem nitrate transport (Wang and Tsay, 2011), and *AtNRT1.11* and *AtNRT1.12* play a key role in redistributing xylem-borne nitrates to enhance plant growth (Hsu and Tsay, 2013). *NRT1* genes have also been identified in *Brassica napus* (Zhou et al., 1998; Faure-Rabasse et al., 2002), rice (Lin et al., 2000; Ma et al., 2011), and Chinese cabbage (Yang et al., 2012).

Chrysanthemums (*Chrysanthemum morifolium*) are one of the world's four most popular species for cut flowers. Owing to their high commercial value, large amounts of nitrogen fertilizer are applied to chrysanthemum cultivation to induce high flower production. However, a significant proportion of the applied nitrogen fertilizer is lost to the atmosphere or ground water due to the low nitrogen use efficiency of commercial chrysanthemum cultivars. This loss not only causes serious pollution to the environment but also adversely affects the yield and quality of cut chrysanthemum (Barbosa et al., 2000). The selection of chrysanthemum varieties that have high nitrogen acquisition and the identification of genes that code for nitrogen uptake transporters will therefore be important for improving chrysanthemum cultivation. In this study, we screened for chrysanthemum genotypes for high nitrogen acquisition. This screen identified a candidate nitrogen uptake transporter, *CmNRT1*, that was inducible by nitrates. Analysis of transgenic *Arabidopsis thaliana* suggested that *CmNRT1* encodes a constitutive component for a low-affinity transporter.

## MATERIAL AND METHODS

### Plant materials and growing conditions

The chrysanthemum varieties 'Nannongxuefeng', 'Nannongyuzhu', 'Nannongxiaoli', 'Nannonggongfen', 'Nannongxiaguang', 'Nannonghualian', 'Nannongshuangjiao', 'Nannongyudie', 'Nannongxunzhang', 'Nannongzixunzhang', 'Nannonghongxiu', 'Nannonghongfen', 'Nannongyupan', 'Nannongchifeng', 'Nannongyinshan', and 'Nannongjindie' were obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Cuttings were induced to form roots by immersion in 1/4 strength Murashige and Skoog medium for 2 weeks. Phenotypically uniform seedlings at the 8-leaf stage were transferred to a 8-L nutrient

solution (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. Two groups of five plants were treated with a low level of nitrogen (0.2 mM  $\text{NH}_4\text{NO}_3$ ) or a normal nitrogen level (4 mM  $\text{NH}_4\text{NO}_3$ ). The concentrations were selected based on previous reports on chrysanthemums (Jiang et al., 2008; Huang et al., 2010). The relative nitrogen content in chrysanthemums exposed to low nitrogen content compared to normal nitrogen content was used to estimate nitrogen uptake efficiency. Sixteen chrysanthemum varieties were grown for 21 days in sand culture to select those with the lowest and high nitrogen uptake. In the sand culture, the size of the quartz grains ranged from 0.45 to 1 mm. The quartz grains were first washed with distilled water, then with 2 M HCl for 24 h, washed again in distilled water, and sterilized at 170°C for 2 h in an oven (Liao et al., 2003). Each pot contained one plant.

### Determination of nitrogen content

At harvest, the seedlings were washed thoroughly with distilled water. To determine nitrogen contents, the plants were washed with 0.1 mM  $\text{CaSO}_4$  for 1 min, then placed at 105°C for 30 min to inactivate enzymes, and finally dried at 80°C. The dry weight was recorded. The concentration of nitrogen in the plants was determined by the Kjeldahl method (Shi et al., 2010). The experimental samples were digested with  $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$ , subjected to a distillation process, and then nitrogen levels were determined.

### Isolation and sequencing of *CmNRT1* full-length cDNA

For gene cloning, chrysanthemum seedlings were grown in IRR1 solution containing 4 mM  $\text{NH}_4\text{NO}_3$  for 4 weeks and then starved of nitrogen for 1 week. Total RNA was isolated from the root of nitrogen-starved seedlings given 5 mM  $\text{NO}_3^-$  (supplied as  $\text{KNO}_3$ ). Total RNA was extracted using the RNAiso reagent (TaKaRa) following the manufacturer instructions and treated with RNase-free DNaseI (TaKaRa). The concentration and the integrity of the RNA were assessed as described by Gu et al. (2011). The first cDNA strand was synthesized using Reverse Transcriptase M-MLV (RNase H<sup>-</sup>) (TaKaRa) according to the manufacturer instructions. A partial *CmNRT1* sequence was selected from chrysanthemum-expressed sequence tags (Chen et al., 2009). The remainder of the sequence was acquired using RACE-PCR as described by Gu et al. (2014a). The open reading frame (ORF) within the amplicons was identified using the ORF finder software ([www.ncbi.nlm.nih.gov](http://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.

### Real-time RT-PCR

Cuttings were grown in nutrient solution as above for 4 weeks and then starved of nitrogen for 1 week. Total RNA was isolated from the root tissue of plants supplied with either 0.5 or 10 mM  $\text{KNO}_3$  and sampled at 0, 0.5, 1, 2, 4, 6, 8, or 12 h. The transcription profiles of *CmNRT1* were obtained by real-time RT-PCR based on SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Perfect Real-Time) (TaKaRa). The PCR comprised an initial denaturation step of 95°C for 60 s, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 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 to generate the required denaturation curve. *CmpsaA* (AB548817) was used as the reference gene (Gu et al., 2011). Primer sequences are listed in Table 1. Each PCR was repeated as three biological replicates, and the relative changes in transcription were analyzed using the  $2^{-\Delta\Delta C_t}$  approach (Livak and Schmittgen, 2001).

**Table 1.** Sequences of PCR primers used in the study.

Primer	Sequence (5'-3')	Usage
Oligo (dT) primer	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT	3'-RACE <sup>a</sup>
CmNRT1GSP1	TGAATCACAGGTGGCAAAA	
CmNRT1GSP2	GGTAACGCTAACGCTGCTA	
dT-AP	GACTCGAGTCGACATCGA	
CmNRT1AGSP1	TTCTCCACGCCCGATA	5'-RACE
CmNRT1AGSP2	CAGCGTCTCCCAAGGTTG	
CmNRT1AGSP3	CCTCCTGTTCCGAGTGCG	
AAP	GGCCACGCGTCGACTAGTACGGGIIIGGGIIIGGGIIG	
AUAP	GGCCACGCGTCGACTAGTAC	
CmNRT1SP	TCTCCTTACTCTACATTTATTT	ORF amplification
CmNRT1AP	AACCTAACAAAATAACGG	
CmNRT1-P1	TCAGCAACAAAACCACCG	Promoter cloning
CmNRT1-P2	AGCCTTTACATGCCTCC	
CmNRT1-P3	GCTTGTCCAGCCGCCAGT	
CmNRT1-R1	CGGAGCTTCACTTGTCGG	
CmNRT1-R2	TGGGGCGTTTTCTGGGTCT	
CmNRT1-R3	CCGTGCGTTGCTCTGCTT	
qCmNRT1	ATGACGAAGAAATGACGGAG	qRT-PCR
qACmNRT1	AGAGGCTGCCATCAATACCC	
psaA-F	CCAATAACCACGACCGCTAA	
psaA-R	GGCACAGTCCTCCCAAGTAA	

RACE = rapid-amplification of cDNA ends.

## Transgene construction and *A. thaliana* transformation

The *CmNRT1* ORF was amplified using the primer pair CmNRTF/R (Table 1) and inserted into the *Bam*HI and *Sal*I sites of pCAMBIA1301-220 following digestion with these two enzymes. The construct was named pCAMBIA1301-220-*CmNRT1*. This construct was transformed into *A. tumefaciens* strain EHA105 using the freeze-thaw method (Liu et al., 2012). *A. thaliana* Col-0 (ecotype Columbia) was transformed via the floral dip method (Liu et al., 2012). T<sub>1</sub> seedlings were raised on agar containing 20 mg/L hygromycin and 25 mg/L ampicillin. Positive transformants were confirmed by histochemical evaluation of GUS expression in leaves (Remans et al., 2006), and by PCR using genomic DNA and the primer pair CmNRTF/R (Table 1). The qRT-PCR validation was based on *Arabidopsis* cDNA amplified by the primers CmNRTs/x (Table 1). The primers for the reference gene *AtUBQ* (NM\_116771.5) were AtUBQs/x (Table 1).

## Root <sup>15</sup>N influx

The influx of <sup>15</sup>NO<sub>3</sub><sup>-</sup> into seedlings of transgenic and non-transgenic lines was assayed as described previously (Ho et al., 2009). Briefly, 10-day-old wild-type and two *CmNRT1* transgenic lines were first exposed to pH 6.5 nitrate-free growth medium and then to pH 5.5 nitrate-free

medium for 3 h. The plants were washed and transferred to pH 5.5 nitrate medium containing 10 or 0.2 mM  $^{15}\text{NO}_3$  for 30 min and finally to 0.1 mM  $\text{CaSO}_4$  for 1 min. The influx of  $^{15}\text{N}$  was determined and calculated as described by Gu et al. (2014b). Dried root tissue was analyzed using the ANCA-MS system (PDZ Europa).

## RESULTS

### Screening chrysanthemum genotypes for nitrogen acquisition abilities

As shown in Table 2, the relative nitrogen contents of ‘Nannongxiaoli’, ‘Nannongxuefeng’, and ‘Nannongyuzhu’ were 0.757, 0.840, and 0.788, while those in ‘Nannonghualian’, ‘Nannonggongfen’, and ‘Nannongxiaguang’ were 0.645, 0.568, and 0.616, respectively. The lowest and highest relative nitrogen contents were in ‘Nannonggongfen’ and ‘Nannongxuefeng’, respectively.

**Table 2.** Relative nitrogen content of 16 cut chrysanthemum cultivars.

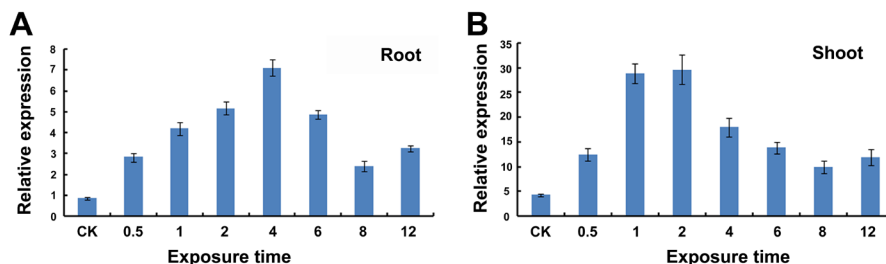
Cultivar	Relative nitrogen content*
Nannongxuefeng	0.840 ± 0.060 <sup>a</sup>
Nannongyuzhu	0.788 ± 0.025 <sup>b</sup>
Nannongxiaoli	0.757 ± 0.026 <sup>bc</sup>
Nannonggongfen	0.568 ± 0.017 <sup>f</sup>
Nannongxiaguang	0.616 ± 0.023 <sup>h</sup>
Nannonghualian	0.645 ± 0.013 <sup>h</sup>
Nannongshuangjiao	0.689 ± 0.012 <sup>g</sup>
Nannongyudie	0.692 ± 0.014 <sup>g</sup>
Nannongxunzhang	0.752 ± 0.018 <sup>bc</sup>
Nannongzixunzhang	0.723 ± 0.012 <sup>def</sup>
Nannonghongxiu	0.712 ± 0.016 <sup>efg</sup>
Nannonghongfeng	0.762 ± 0.016 <sup>bc</sup>
Nannongyupan	0.759 ± 0.020 <sup>bc</sup>
Nannongchifeng	0.759 ± 0.020 <sup>cd</sup>
Nannongyinshan	0.758 ± 0.020 <sup>cd</sup>
Nannongjindie	0.743 ± 0.017 <sup>cde</sup>

\*Results with the same letter are not significantly different.

### *CmNRT1* and its predicted product

The *CmNRT1* gene was isolated by RT-PCR and RACE methods using information from chrysanthemum EST libraries (Figure 1A). The full-length *CmNRT1* cDNA consisted of a 1761-bp ORF encoding 587 amino acids. The predicted gene product is a protein of molecular mass 64.90 kDa and a pI of 9.14. Its amino acid sequence shared high similarity with AtNRT1.1, BdNRT1.1, BnNRT1.2, CsNRT1.1, GmNRT1.1, NpNRT1.1, PpNRT1, and VvNRT1.1 (Figure 1B). GmNRT1.1 and CmNRT1 proteins were closest in the phylogenetic tree (Figure 1C).

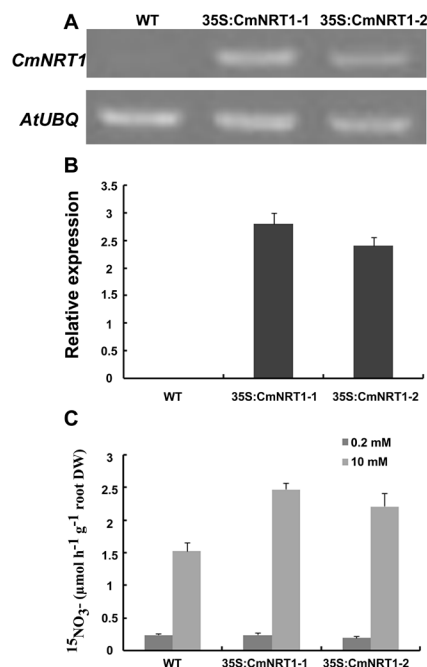




**Figure 2.** Induced transcription of *CmNRT1* in roots and shoots exposed to 10 mM nitrate following a period of N starvation. **A.** Roots. **B.** Shoots. Mean values and standard deviations calculated from triplicated assays.

### Root $^{15}\text{N}$ influx in transgenic *A. thaliana* lines

Overexpression of *NRT1* in wild-type plants has been shown to be an effective approach for studying *NRT1* function (Ma et al., 2011; Yang et al., 2012). To investigate the function of *CmNRT1*, we generated *A. thaliana* transgenic plants expressing *CmNRT1*. Based on PCR and RT-PCR assays (Figure 3A and B), two *CmNRT1* transgenic plants were identified and designated as 35S:*CmNRT1*-1 and 35S:*CmNRT1*-2. The nitrate uptake of wild-type plants and the two *CmNRT1* transgenic lines was compared after treatment with 0.2 or 10 mM  $^{15}\text{NO}_3$  for 30 min. We found that *CmNRT1* promoted  $^{15}\text{NO}_3$  uptake at 10 mM  $^{15}\text{NO}_3$  but not at 0.2 mM  $^{15}\text{NO}_3$ . This result suggested that *CmNRT1* encodes a constitutive component of a low-affinity transporter.



**Figure 3.** Influx of  $^{15}\text{N}$  into the roots of transgenic and non-transgenic *Arabidopsis thaliana* plants. **A. B. C.** Nitrate uptake of wild-type plants and transgenic *A. thaliana* using 10 or 0.2 mM  $^{15}\text{NO}_3$  for 30 min. Values are reported as means  $\pm$  SE (N = 3).

## DISCUSSION

Nitrogen is one of the most important mineral nutrients for plant development (Presterl et al., 2002). Under conditions of constant nitrogen levels, the relative ability of plants to acquire nitrogen is a crucial factor in achieving higher N efficiency (Moll et al., 1982). Here, we examined variation in nitrogen acquisition among sixteen chrysanthemum varieties. The relative nitrogen content of all genotypes was greater than 1, indicating that the nitrogen content under normal nitrogen levels (4 mM) was higher than under low nitrogen levels (0.2 mM) (Table 2). The chrysanthemum variety 'Nannongxuefeng' showed the highest nitrogen acquisition of the tested varieties; therefore, it potentially carries excellent nitrogen-related genes. Here, we isolated the *CmNRT1* homologue from this variety.

The sequence of *CmNRT1* shows strong conservation with those of other *NRT1* genes (Figure 2B). Genes in the *NRT1* family can be divided into constitutive and nitrate-inducible types (Crisuolo et al., 2012). *CmNRT1* is a nitrate-inducible gene like *BnNRT1* and *BcNRT1* (Faure-Rabasse et al., 2002; Yang et al., 2012). In contrast, rice *OsNRT1* (Lin et al., 2000), tomato *LeNRT1-1* (Lauter et al., 1996) and *Arabidopsis AtNRT1:2* (Huang et al., 1999) are constitutive genes, which show relatively stable expression before and after nitrate induction. Similar levels of *CmNRT1* expression were detected in roots and shoots. However, in other species, *NRT1* genes show widely variable levels of expression in different tissues. For example, *OsNRT1* is a root-specific gene with little or no expression in the shoot (Lin et al., 2000), whereas *OsNRT1.2* is only expressed in roots (Ma et al., 2011). In *Arabidopsis*, *AtNRT1.8* is preferentially expressed in vascular tissues (Li et al., 2010), while *AtNRT1.9* is predominantly expressed in roots (Wang and Tsay, 2011), and *AtNRT1.11* and *AtNRT1.12* are mainly expressed in larger expanded leaves (Hsu and Tsay, 2013).

CHL1 is a dual-affinity nitrate transporter in *Arabidopsis* and is involved in multiple phases of nitrate uptake (Liu et al., 1999; Ho et al., 2009). Phosphorylated and dephosphorylated CHL1 show low and high levels of response, respectively (Ho et al., 2009). However, the *Arabidopsis* homolog, *AtNRT1:2*, is a low-affinity nitrate transporter (Huang et al., 1999; Liu et al., 1999). CHL1 homologous proteins in other plants, such as *LeNrt1.1* of tomato, *OsNRT1* of rice and *BcNRT1* of Chinese cabbage, are also low-affinity transporters (Lauter et al., 1996; Lin et al., 2000; Yang et al., 2012). Only low-affinity nitrate uptake activity was observed in *OsNRT1*-injected oocytes and *BcNRT1* can restore *chl1-5* nitrate uptake at 10 mM but not at 0.2 mM nitrate (Lin et al., 2000; Yan et al., 2011). Nitrate uptake in *A. thaliana* heterologously expressing *CmNRT1* (Figure 3) showed that *CmNRT1* has a similar function to *OsNRT1* and *BcNRT1*.

## Conflicts of interest

The authors declare no conflict of interest.

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## REFERENCES

- Almagro A, Lin SH and Tsay YF (2008). Characterization of the *Arabidopsis* nitrate transporter NRT1.6 reveals a role of nitrate in early embryo development. *Plant Cell* 20: 3289-3299. <http://dx.doi.org/10.1105/tpc.107.056788>
- Barbosa JG, Kampf AN, Prieto MHE, Koller OC, et al. (2000). Chrysanthemum cultivation in expanded clay. I. Effect of the nitrogen-phosphorus-potassium ratio in the nutrient solution. *J. Plant Nutr.* 23: 1327-1336. <http://dx.doi.org/10.1080/01904160009382103>
- Britto D and Kronzucker H (2004). Biotechnology of nitrogen acquisition in rice-implications for food security. In: Nitrogen Acquisition and Assimilation in Higher Plants (Amancio S and Stulen I, eds.). Kluwer Academic Publishers, The Netherlands, 261-281.
- Chen S, Miao H, Chen F, Jiang B, et al. (2009). Analysis of expressed sequence tags (ESTs) collected from the inflorescence of chrysanthemum. *Plant Mol. Biol. Rep.* 27: 503-510. <http://dx.doi.org/10.1007/s11105-009-0103-6>
- Covelo F, Rodriguez A and Gallardo A (2008). Spatial pattern and scale of leaf N and P resorption efficiency and proficiency in a *Quercus robur* population. *Plant Soil* 311: 109-119. <http://dx.doi.org/10.1007/s11104-008-9662-9>
- Crisuolo G, Valkov VT, Parlati A, Alves LM, et al. (2012). Molecular characterization of the *Lotus japonicus* NRT1(PTR) and NRT2 families. *Plant Cell Environ.* 35: 1567-1581. <http://dx.doi.org/10.1111/j.1365-3040.2012.02510.x>
- Fan SC, Lin CS, Hsu PK, Lin SH, et al. (2009). The *Arabidopsis* nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. *Plant Cell* 21: 2750-2761. <http://dx.doi.org/10.1105/tpc.109.067603>
- Faure-Rabasse S, Le Deunff E, Lainé P, Macduff JH, et al. (2002). Effects of nitrate pulses on *BnNRT1* and *BnNRT2* genes: mRNA levels and nitrate influx rates in relation to the duration of N deprivation in *Brassica napus* L. *J. Exp. Bot.* 53: 1711-1721. <http://dx.doi.org/10.1093/jxb/erf023>
- Gu C, Chen S, Liu Z, Shan H, et al. (2011). Reference gene selection for quantitative real-time PCR in Chrysanthemum subjected to biotic and abiotic stress. *Mol. Biotechnol.* 49: 192-197. <http://dx.doi.org/10.1007/s12033-011-9394-6>
- Gu CS, Liu LQ, Zhao YH, Deng YM, et al. (2014a). Overexpression of *Iris. lactea* var. *chinensis* metallothionein IIMT2a enhances cadmium tolerance in *Arabidopsis thaliana*. *Ecotoxicol. Environ. Saf.* 105: 22-28. <http://dx.doi.org/10.1016/j.ecoenv.2014.04.002>
- Gu C, Zhang X, Jiang J, Guan Z, et al. (2014b). *Chrysanthemum* CmNAR2 interacts with CmNRT2 in the control of nitrate uptake. *Sci. Rep.-UK* 4: 5833
- Guo R, Li X, Christie P, Chen Q, et al. (2008). Influence of root zone nitrogen management and a summer catch crop on cucumber yield and soil mineral nitrogen dynamics in intensive production systems. *Plant Soil* 313: 55-70. <http://dx.doi.org/10.1007/s11104-008-9679-0>
- Ho CH, Lin SH, Hu HC and Tsay YF (2009). CHL1 functions as a nitrate sensor in plants. *Cell* 138: 1184-1194. <http://dx.doi.org/10.1016/j.cell.2009.07.004>
- Hsu PK and Tsay YF (2013). Two phloem nitrate transporters, NRT1.11 and NRT1.12, are important for redistributing xylem-borne nitrate to enhance plant growth. *Plant Physiol.* 163: 844-856. <http://dx.doi.org/10.1104/pp.113.226563>
- Huang CB, Fang WM, Yang Y, Chen F, et al. (2010). Effects of different nitrogen levels and forms on the appearance quality and photosynthesis characters of potted chrysanthemum with small inflorescences. *Acta Agr. Zhejiangensis* 1: 45-50.
- Huang NC, Liu KH, Lo HJ and Tsay YF (1999). Cloning and functional characterization of an *Arabidopsis* nitrate transporter gene that encodes a constitutive component of low-affinity uptake. *Plant Cell* 11: 1381-1392. <http://dx.doi.org/10.1105/tpc.11.8.1381>
- Jiang BB, Fang WM, Chen FD and Gu JJ (2008). Effects of N, P and K ratio on the growth and development of cut chrysanthemum 'Jinba'. *J. Zhejiang Forestry College* 6: 692-697.
- Kotur Z, Mackenzie N, Ramesh S, Tyerman SD, et al. (2012). Nitrate transport capacity of the *Arabidopsis thaliana* NRT2 family members and their interactions with AtNAR2.1. *New Phytol.* 194: 724-731. <http://dx.doi.org/10.1111/j.1469-8137.2012.04094.x>
- Koutroubas S and Nitanos D (2003). Genotypic differences for grain yield and nitrogen utilization in Indica and Japonica rice under Mediterranean conditions. *Field Crops Res.* 83: 251-260. [http://dx.doi.org/10.1016/S0378-4290\(03\)00067-4](http://dx.doi.org/10.1016/S0378-4290(03)00067-4)
- Lauter F-R, Ninnemann O, Bucher M, Riesmeier JW, et al. (1996). Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. *Proc. Natl. Acad. Sci. USA* 93: 8139-8144. <http://dx.doi.org/10.1073/pnas.93.15.8139>

- Li JY, Fu YL, Pike SM, Bao J, et al. (2010). The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. *Plant Cell* 22: 1633-1646. <http://dx.doi.org/10.1105/tpc.110.075242>
- Li XN, Sheng JD, Hou J and Xing X (2007). Screening for nitrogen efficiency in different cotton varieties at seeding stage. *J. Xinjiang Agr. Univ.* 3: 010.
- Liao JP, Lin XG, Cao ZH, Shi YQ, et al. (2003). Interactions between arbuscular mycorrhizae and heavy metals under sand culture experiment. *Chemosphere* 50: 847-853. [http://dx.doi.org/10.1016/S0045-6535\(02\)00229-1](http://dx.doi.org/10.1016/S0045-6535(02)00229-1)
- Lin CM, Koh S, Stacey G, Yu SM, et al. (2000). Cloning and functional characterization of a constitutively expressed nitrate transporter gene, *OsNRT1*, from rice. *Plant Physiol.* 122: 379-388. <http://dx.doi.org/10.1104/pp.122.2.379>
- Lin SH, Kuo HF, Canivenc G, Lin CS, et al. (2008). Mutation of the *Arabidopsis* NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. *Plant Cell* 20: 2514-2528. <http://dx.doi.org/10.1105/tpc.108.060244>
- Liu KH, Huang CY and Tsay YF (1999). CHL1 is a dual-affinity nitrate transporter of *Arabidopsis* involved in multiple phases of nitrate uptake. *Plant Cell* 11: 865-874. <http://dx.doi.org/10.1105/tpc.11.5.865>
- Liu Z, Gu C, Chen F, Yang D, et al. (2012). Heterologous expression of a *Nelumbo nucifera* phytochelatin synthase gene enhances cadmium tolerance in *Arabidopsis thaliana*. *Appl. Biochem. Biotechnol.* 166: 722-734. <http://dx.doi.org/10.1007/s12010-011-9461-2>
- Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  Method. *Methods* 25: 402-408. <http://dx.doi.org/10.1006/meth.2001.1262>
- Ma C, Fan XR and Xu GH (2011). Responses of rice plants of Wuyunjing 7 to nitrate as affected by over-expression of *OsNRT1.2*. *Chin. J. Rice Sci.* 4: 349-356.
- Moll R, Kamprath E and Jackson W (1982). Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization. *Agron. J.* 74: 562-564. <http://dx.doi.org/10.2134/agronj1982.00021962007400030037x>
- Presterl T, Groh S, Landbeck M, Seitz G, et al. (2002). Nitrogen uptake and utilization efficiency of European maize hybrids developed under conditions of low and high nitrogen input. *Plant Breed.* 121: 480-486. <http://dx.doi.org/10.1046/j.1439-0523.2002.00770.x>
- Remans T, Nacry P, Pervent M, Girin T, et al. (2006). A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in *Arabidopsis*. *Plant Physiol.* 140: 909-921. <http://dx.doi.org/10.1104/pp.105.075721>
- Shi WM, Xu WF, Li SM, Zhao XQ, et al. (2010). Responses of two rice cultivars differing in seedling-stage nitrogen use efficiency to growth under low-nitrogen conditions. *Plant Soil* 326: 291-302. <http://dx.doi.org/10.1007/s11104-009-0007-0>
- Tsay YF, Schroeder JI, Feldmann KA and Crawford NM (1993). The herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. *Cell* 72: 705-713. [http://dx.doi.org/10.1016/0092-8674\(93\)90399-B](http://dx.doi.org/10.1016/0092-8674(93)90399-B)
- Wang YY and Tsay YF (2011). *Arabidopsis* nitrate transporter NRT1.9 is important in phloem nitrate transport. *Plant Cell* 23: 1945-1957. <http://dx.doi.org/10.1105/tpc.111.083618>
- Yan M, Fan X, Feng H, Miller AJ, et al. (2011). Rice *OsNAR2.1* interacts with *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.3a* nitrate transporters to provide uptake over high and low concentration ranges. *Plant Cell Environ.* 34: 1360-1372. <http://dx.doi.org/10.1111/j.1365-3040.2011.02335.x>
- Yang X, Sun F, Xiong A, Wang F, et al. (2012). *BcNRT1*, a plasma membrane-localized nitrate transporter from non-heading Chinese cabbage. *Mol. Biol. Rep.* 39: 7997-8006. <http://dx.doi.org/10.1007/s11033-012-1646-4>
- Zhang L, Spiertz JHJ, Zhang S, Li B, et al. (2008). Nitrogen economy in relay intercropping systems of wheat and cotton. *Plant Soil* 303: 55-68. <http://dx.doi.org/10.1007/s11104-007-9442-y>
- Zhou JJ, Theodoulou FL, Muldin I, Ingemarsson B, et al. (1998). Cloning and functional characterization of a *Brassica napus* transporter that is able to transport nitrate and histidine. *J. Biol. Chem.* 273: 12017-12023. <http://dx.doi.org/10.1074/jbc.273.20.12017>