the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **81,** 367–376 doi: 10.1111/tpj.12736

TOND1 confers tolerance to nitrogen deficiency in rice

Yangjun Zhang[1], Lubin Tan[1], Zuofeng Zhu[1], Lixing Yuan[2], Daoxin Xie[3] and Chuanqing Sun[1,*]

[1]*State Key Laboratory of Plant Physiology and Biochemistry, National Center for Evaluation of Agricultural Wild Plants (Rice), Beijing Key Laboratory of Crop Genetic Improvement, Department of Plant Genetics and Breeding, China Agricultural University, Beijing 100193, China,*

[2]*Key Laboratory of Plant–Soil Interaction of Ministry of Education, Center for Resources, Environment and Food Security, College of Resources and Environmental Sciences, China Agricultural University, Beijing 100193, China, and*

[3]*Key Laboratory of Bioinformatics of Ministry of Education, School of Life Sciences, Tsinghua University, Beijing 100084, China*

Received 7 September 2014; revised 10 November 2014; accepted 27 November 2014; published online 2 December 2014.
*For correspondence (e-mail suncq@cau.edu.cn).

SUMMARY

Nitrogen (N), the most important mineral nutrient for plants, is critical to agricultural production systems. N deficiency severely affects rice growth and decreases rice yields. However, excessive use of N fertilizer has caused severe pollution to agricultural and ecological environments. The necessity of breeding of crops that require lower input of N fertilizer has been recognized. Here we identified a major quantitative trait locus on chromosome 12, *Tolerance Of Nitrogen Deficiency 1* (*TOND1*), that confers tolerance to N deficiency in the *indica* cultivar Teqing. Sequence verification of 75 *indica* and 75 *japonica* cultivars from 18 countries and regions demonstrated that only 27.3% of cultivars (41 *indica* cultivars) contain *TOND1*, whereas 72.7% of cultivars, including the remaining 34 *indica* cultivars and all 75 *japonica* cultivars, do not harbor the *TOND1* allele. Over-expression of *TOND1* increased the tolerance to N deficiency in the *TOND1*-deficient rice cultivars. The identification of *TOND1* provides a molecular basis for breeding rice varieties with improved grain yield despite decreased input of N fertilizers.

Keywords: *TOND1*, quantitative trait locus, tolerance of nitrogen deficiency, rice, N fertilizer, *Oryza sativa*.

INTRODUCTION

Nitrogen (N) is the most important macronutrient required for crop growth and development. To enhance crop productivity, the level of N fertilizer used on croplands has globally increased several fold in the last 60 years (Mulvaney *et al.*, 2009). N deficiency affects rice growth and development, producing yellow leaves, dwarf plants and lower grain yields (Borrell *et al.*, 1998, 2001; Lian *et al.*, 2005). As a result of utilization of high-yielding semi-dwarf rice varieties, the quantity of N fertilizer applied for rice production has rapidly increased worldwide. Applications of N fertilizer substantially increase rice yield, which has assisted in averting chronic food shortage. However, agricultural and ecological environments have been gradually polluted because of the excessive use of N fertilizer (Matson *et al.*, 2002; Robertson and Vitousek, 2009; Guo *et al.*, 2010). The necessity of creating crops that require lower input of N fertilizers while producing higher yields has been recognized in the call for a 'Second Green Revolution' (Zeigler and Mohanty, 2010; McAllister *et al.*, 2012).

Many quantitative trait loci (QTLs) associated with tolerance of N deficiency in rice have been identified using molecular methods of genetic analysis. Shan *et al.* (2005) detected 12 QTLs for N concentration/accumulation and N-use efficiency for biomass production in rice plants. Tong *et al.* (2006) identified 31 QTLs that control plant height, the number of panicles per plant, chlorophyll content, shoot dry weight and grain yield per plant, using a population of chromosome segment substitution lines at the late developmental stage under two N conditions. Cho *et al.* (2007) mapped 20 QTLs and 58 pairs of epistatic loci for the N concentration in grain, N concentration in straw, N content of shoots, harvest index, grain yield, straw yield and physiological N-use efficiency under both normal and low N conditions. Wang *et al.* (2009) identified several QTLs related to panicle number and grain yield in field experiments under normal fertilization and low-N treatments. Lian *et al.* (2005) found that very few QTLs were detected under both low and normal N conditions, and most of the QTLs for the relative measurements were different from QTLs for traits under the two N treatments.

Several genes controlling the absorption, transportation and utilization of N have been identified in rice. *OsNRT1*

encodes a constitutive component of the low-affinity nitrate uptake transporter (Lin *et al.*, 2000). Knockdown of *OsNAR2.1* simultaneously suppressed expression of *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.3a* (Yan *et al.*, 2011). Knockdown of *OsNRT2.3a* influenced long-distance nitrate transport from root to shoot under low nitrate supply (Tang *et al.*, 2012). The product of *OsNPF2.4* plays a key role in long-distance nitrate transport in rice (Xia *et al.*, 2015). The product of the *OsAMT1;1* gene affects ammonium uptake and content (Hoque *et al.*, 2006). *OsAMT2;2* is N-inducible, and its expression was detected in both roots and shoots (Li and Shi, 2006). Over-expression of rice NADH-dependent glutamate synthases encoded by *NADH-GOGAT* genes enhanced grain filling (Yamaya *et al.*, 2002), but knockout of *NADH-GOGAT1* in rice decreased overall biomass and panicle production, as well as yield (Tamura *et al.*, 2010). Dof1, the transcription factor proposed to affect N-use efficiency, influenced N assimilation under low N condition (Yanagisawa *et al.*, 2004). Recently, *qNGR9*, a major rice QTL for N-use efficiency, was cloned and found to be synonymous with the previously identified *DEP1 (DENSE AND ERECT PANICLE 1)* gene (Sun *et al.*, 2014).

Here we describe the isolation and detailed characterization of *Tolerance Of Nitrogen Deficiency 1* (*TOND1*), located on chromosome 12, which confers tolerance to N deficiency in the *indica* cultivar Teqing. We show that, among 75 *indica* and 75 *japonica* cultivars from 18 countries and regions, only 41 *indica* cultivars contain *TOND1*, whereas the remaining 34 *indica* cultivars and the 75 *japonica* cultivars do not harbor the *TOND1* allele. Over-expression of *TOND1* increased the tolerance to N deficiency in *TOND1*-deficient rice cultivars.

RESULTS AND DISCUSSION

Characterization of the N-deficiency intolerant line YIL105

To clone the gene responsible for N-deficiency tolerance, we used a set of introgression lines based on an accession of Yuanjiang common wild rice (YJCWR, *Oryza rufipogon*) as a donor and an elite *indica* cultivar Teqing (*Oryza sativa*) as the recipient (Tan *et al.*, 2007) to identify an introgression line YIL105 that displayed intolerance to N deficiency (Figure 1). YIL105 harbored three YJCWR chromosomal segments on the long arms of chromosomes 2, 9 and 12 (Figure S1). The growth, development and production of YIL105 were significantly inhibited under N-deficient conditions compared with N-sufficient conditions. Compared with Teqing, YIL105 exhibited reductions in plant dry weight (DW), N concentration (NC), total N amount per plant (TN), chlorophyll relative content (SPAD), plant height (PH), root length (RL) (Figures 1b and S2a), the number of panicles per plant (PN), grain number per plant (GN), grain yield per plant (GY) and the number of tillers (Figures 1c and S2b) under N-deficient conditions relative to N-sufficient conditions.

Map-based cloning of *TOND1*

Genetic linkage analysis using 311 F_2 individuals derived from the cross between YIL105 and Teqing showed that the N-deficiency tolerance, as indicated by the relative plant dry weight, was controlled by a major QTL, referred to as *TOND1*, which was located between simple sequence repeat markers RM235 and RM17 on the long arm of chromosome 12 (Figure 2a). Using a larger F_2 population of 3180 individuals, we further localized *TOND1* to within a 269 kb region between the M4 and RM17 markers (Figure 2b), in which 45 putative genes are predicted to occur according to the Rice Annotation Project Database (Yuan *et al.*, 2003) (Figure 2c).

Expression profiling using an Affymetrix whole-genome array and real-time quantitative PCR assays showed that, among the 45 predicted genes, only a single gene (*Os12g0630100*) in the *TOND1* mapping region exhibited a significant expression change. *Os12g0630100* expression in YIL105 was obviously lower than that in Teqing, and the relative expression of *Os12g0630100* under N-deficiency conditions was significantly decreased in YIL105 (Figure 2d).

To genetically examine whether *Os12g0630100* is responsible for N-deficiency tolerance, we generated an RNA interference (RNAi) construct that was designed to target the *Os12g0630100* gene of Teqing (Figure 2e,f). The results, shown in Figure 2, demonstrate that down-regulation of the *Os12g0630100* gene in Teqing leads to a decreased tolerance to N deficiency. The RNAi transgenic plants (Ri1 and Ri2) exhibited an obvious reduction in DW, NC, TN, SPAD, PH and RL, in comparison with that of the control plants, under N-deficient hydroponic conditions (Figures 2g and S3a). Furthermore, PN, GN and GY of the RNAi transgenic plants were lower than that of control plants under a variety of N-deficiency levels (Figure 2h). These results suggest that the *Os12g0630100* gene in Teqing corresponds to *TOND1*.

Subcellular localization and expression pattern of *TOND1*

The *TOND1* gene comprises 549 bp with a single exon (Figure S4), and encodes a thaumatin protein containing an N-terminal signal peptide and the thaumatin motif (Figure S5a). Using the full-length protein sequence of TOND1 as a query for BLASTp searches against the non-redundant protein database (http://www.ncbi.nlm.nih.gov/BLAST/), we identified 27 putative homologs in other crop species (Figure S5b). The sequences for thaumatin motif were highly conserved between TOND1 and its homologs in other crop plants (Figure S5c). A TOND1–GFP fusion protein localized to the plasma membrane (Figure 3a). Histochemical analysis of *japonica* cultivar Zhonghua 17, which is transgenic for the GUS reporter gene driven by the

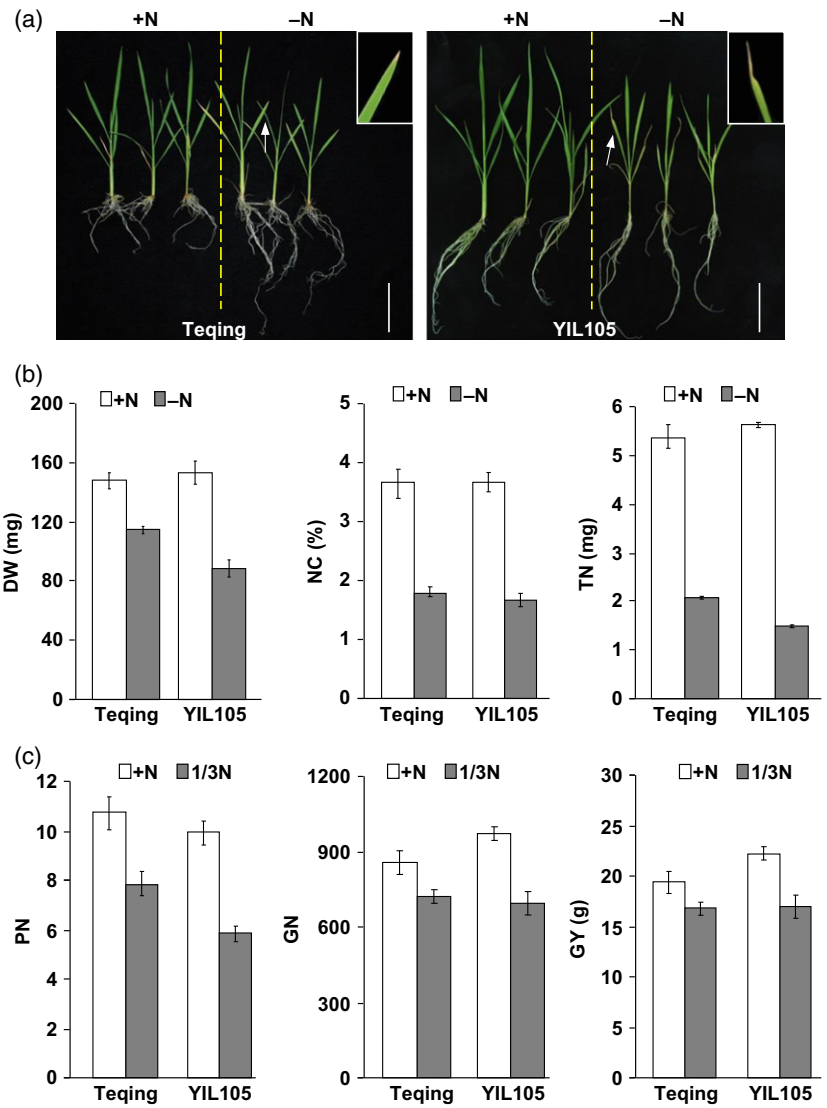


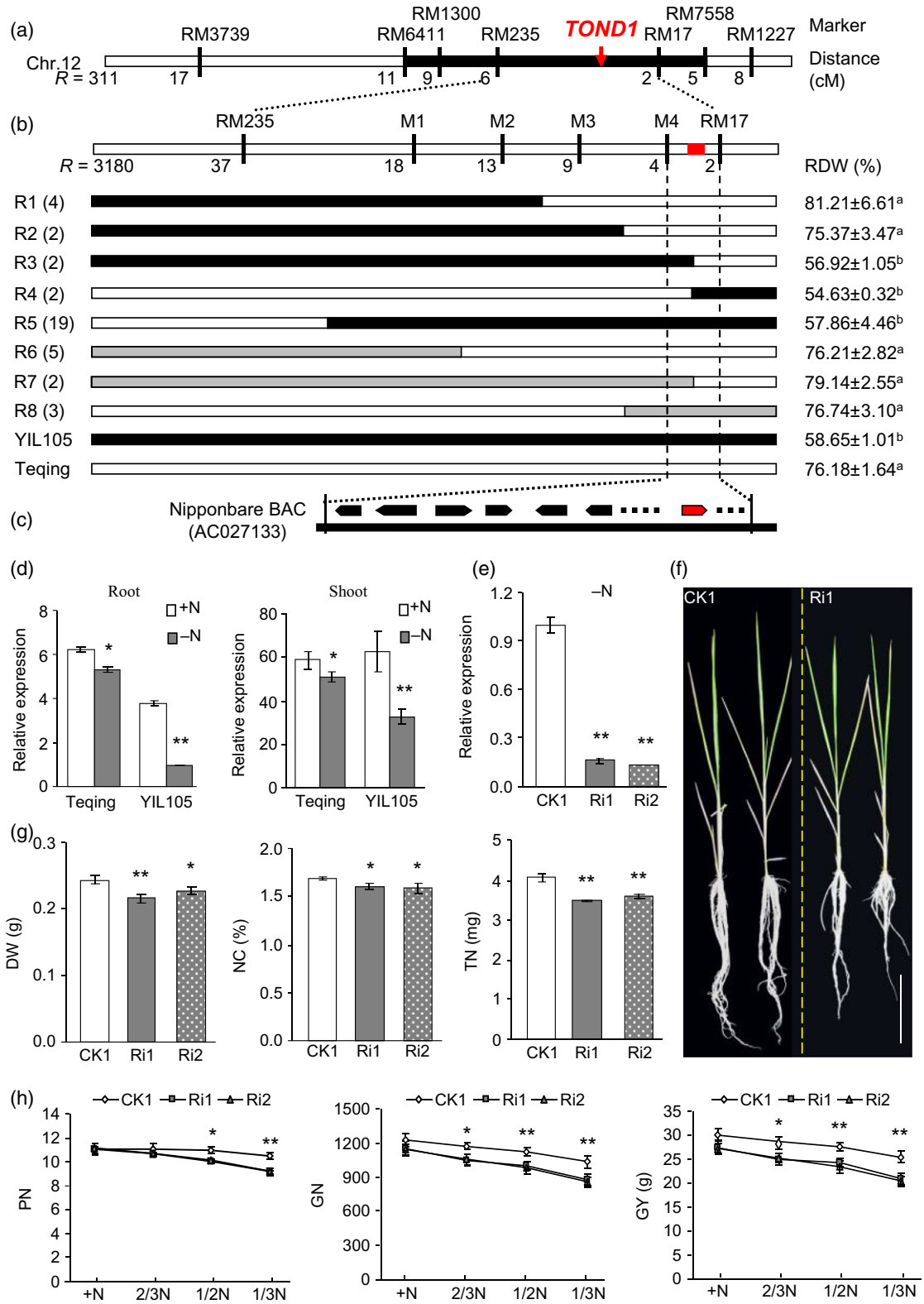
Figure 1. Phenotype of the rice *indica* cultivar Teqing (*Oryza sativa*) and the introgression line YIL105.
(a) Images of the recipient parent Teqing and YIL105 at 21 days after emergence of the third leaf under hydroponic conditions. +N, N-sufficient conditions; –N, N-deficient conditions. The arrows indicate the top third fully expanded leaf, and the leaf tip is magnified on the right. Scale bars = 10 cm.
(b) Comparison of plant dry weight (DW), N concentration (NC) and total N amount per plant (TN) between Teqing and YIL105 under hydroponic conditions.
(c) Comparison of panicle number per plant (PN), grain number per plant (GN) and grain yield per plant (GY) between Teqing and YIL105 under field conditions.
Values are means ± SD.

TOND1 2 kb regulatory region, indicated that *TOND1* is expressed in leaves at the seedling and heading stages, in spikelet hulls and anthers (Figure 3b–d), and particularly in the vascular bundles of leaf blades and pollen grains (Figure 3e,f). Real-time quantitative PCR also detected higher expression levels of *TOND1* in the leaf blades, leaf sheathes and spikelet hulls (Figure 3g).

Sequence comparison and functional site analysis of *TOND1*

Sequence comparison between Teqing and YIL105 revealed seven single nucleotide polymorphisms (SNP1–7) in the 2 kb regulatory region, and three SNPs (SNP8–10) in the coding region of *TOND1* (Figure 4a). Further sequence verification of these ten SNPs in 75 *indica* and 75 *japonica* cultivars from 18 countries and regions showed that only 41 *indica* cultivars, 27.3% of the tested cultivars, harbor a *TOND1* allele identical to Teqing, whereas 72.7% cultivars, including the remaining 34 *indica* and the 75 *japonica* cultivars, have sequences similar to the *tond1* allele of YIL105 (Table S1).

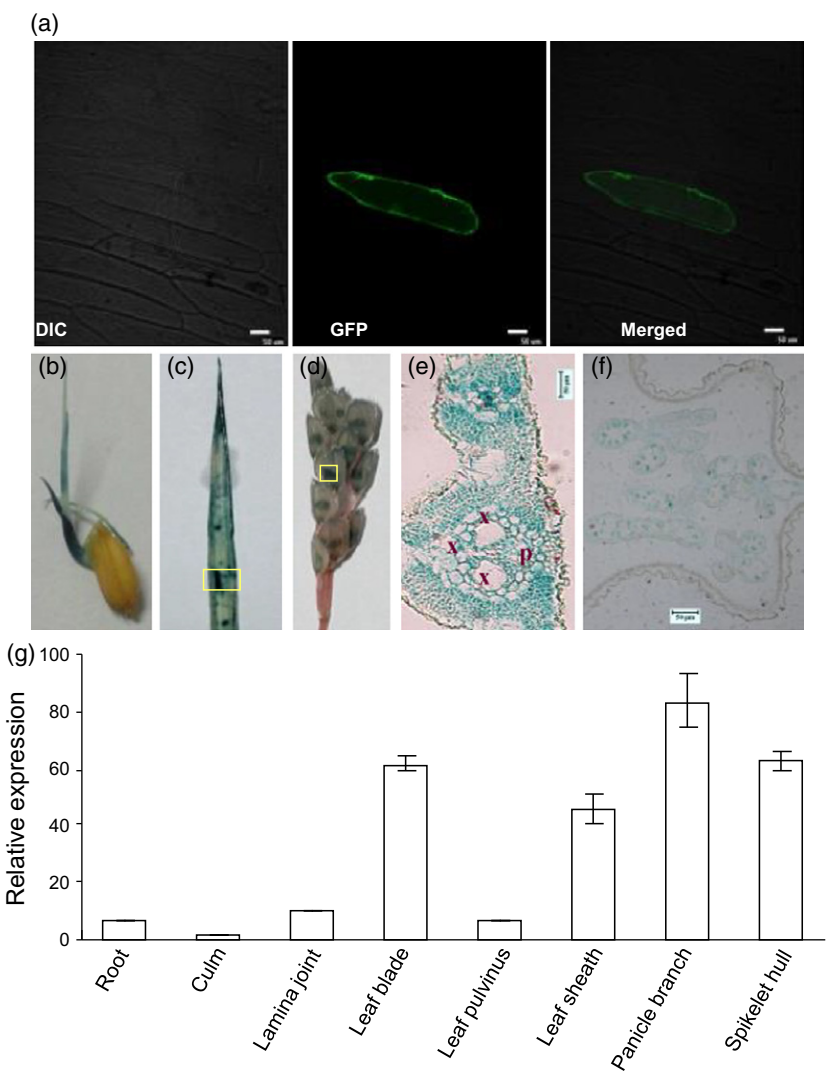
To determine whether rice cultivars harboring the *TOND1* allele exhibit stronger tolerance to N deficiency, 45 rice cultivars clustered in four haplotypes (H1–H4) were used to evaluate N-deficiency tolerance (Figure 4b and Table S2). We found that, similar to Teqing, H1 haplotypes (including 93-11), exhibited tolerance to N deficiency and showed higher *TOND1* expression, while other haplotypes (including Zhonghua 17 and Nipponbare) that are similar to YIL105 were intolerant to N deficiency and exhibited lower *tond1* expression (Figure 4d–h). A further test of association between N-deficiency tolerance and the ten SNPs identified between Teqing and YIL105 revealed that the strongest signal was present at SNP1, SNP4, SNP7,



SNP8 and SNP9 ($P = 4.43 \times 10^{-19}$; Figure 4b,c). These results suggest that rice cultivars harboring the favorable *TOND1* allele exhibit tolerance to N deficiency, and that five SNPs (SNP1, SNP4, SNP7, SNP8 and SNP9) lead to the increase in N-deficiency tolerance in cultivars harboring *TOND1*.

Figure 2. Map-based cloning of *TOND1* in rice.
(a) The target gene was mapped between the simple sequence repeat markers RM235 and RM17 on the long arm of chromosome 12 by linkage analysis using 311 F_2 individuals created by crossing Teqing (TQ) and YIL105. *R* is the number of recombinants.
(b) *TOND1* was fine-mapped between markers M4 and RM17 using recombinants. The number of lines in each recombinant family are shown on the left. The phenotypic differences for each recombinant family compared with Teqing (TQ) and YIL105 are shown on the right. RDW (relative plant dry weight) is the mean value of plant dry weight for each line under N-deficient conditions relative to N-sufficient conditions.
(c) Predicted gene contained in the mapping region according to the Nipponbare genome.
(d) Real-time quantitative PCR for the relative expression of *Os12g0630100* in roots and shoots of Teqing (TQ) and YIL105 after 2 h of hydroponic growth under N-deficient and N-sufficient conditions.
(e) Expression of the *Os12g0630100* gene in the control (CK1) and RNAi transgenic plants (Ri1 and Ri2) by real-time quantitative PCR after 21 days of N-deficient hydroponic growth.
(f) Photos of the control (CK1) and RNAi transgenic plants (Ri1) taken after 21 days of N-deficient hydroponic growth. Scale bar = 10 cm.
(g) Comparison of DW, NC and TN between the control and RNAi transgenic plants under N-deficient hydroponic conditions.
(h) Comparison of PN, GN and GY between the control and RNAi transgenic plants under various N-deficiency field conditions.
Values are means ± SD. Asterisks indicate statistically significant differences compared with +N conditions or CK1 plants, as appropriate (*$P < 0.05$; **$P < 0.01$).

Figure 3. Subcellular localization and expression pattern of the *TOND1* gene in rice.
(a) Subcellular localization of *TOND1*. The *TOND1–GFP* fusion gene was expressed transiently in onion epidermal cells. DIC, a differential interference contrast image; GFP, the same cells showing TOND1-GFP green fluorescence in the plasma membrane; Merged, the merged image. Scale bars = 50 μm.
(b–f) Tissue localization of *TOND1*. Expression of *TOND1* was detected in a young leaf at the seedling stage (b), a leaf at the heading stage (c) and anthers (d) by GUS staining. (e) Magnification of cross section from the yellow box in (c). x, xylem; p, phloem. (f) Magnification of cross section from the yellow box in (d). Scale bars = 50 μm.
(g) Real-time quantitative PCR indicating the relative expression of *TOND1*.



Over-expression of *TOND1* enhances tolerance to N deficiency

As 72.7% of cultivars did not harbor the favorable *TOND1* gene, we investigated whether *TOND1* is able to enhance tolerance to N deficiency in *TOND1*-deficient rice. As shown in Figure 5, over-expression of the *TOND1* gene enhanced tolerance of N deficiency in YIL105. *TOND1* over-expressing transgenic plants (Ox1 and Ox2) in the YIL105 background exhibited an obvious increase in DW, NC, TN,

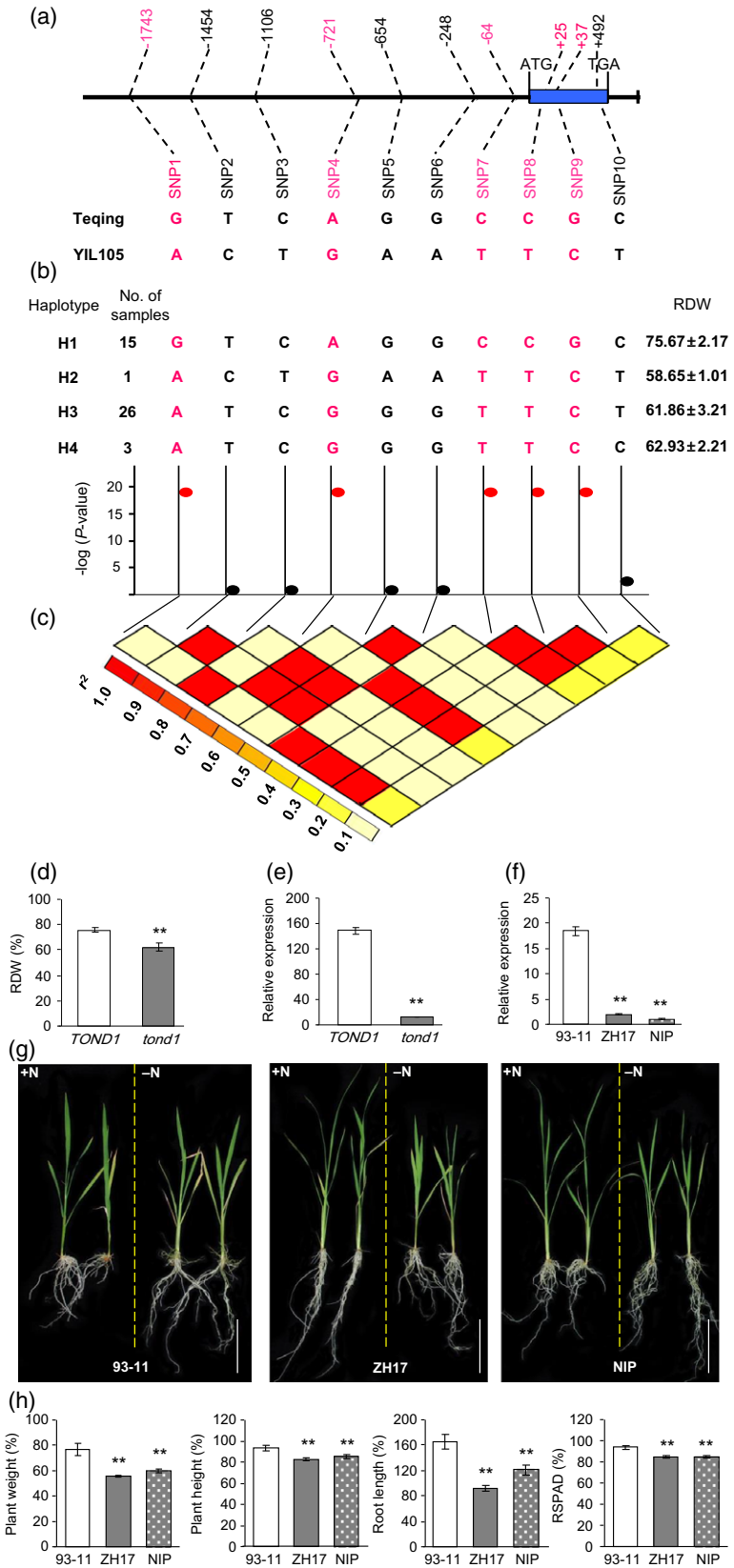


Figure 4. Sequence comparison between Teqing and YIL105, and functional sites analysis of *TOND1*.
(a) Sequence comparison of the 2 kb upstream regulatory region and coding region between Teqing and YIL105.
(b) Association testing of 10 variants in the regulatory region and coding region of *TOND1*. The black dots represent five variations; the red dots represent five proposed functional variant sites.
(c) A triangle matrix of pairwise linkage disequilibrium calculated for 45 samples.
(d) Comparison of mean values of relative plant dry weight (RDW) between varieties containing the *TOND1* allele and varieties containing the *tond1* allele after 21 days of hydroponic growth under N-deficient conditions relative to N-sufficient conditions.
(e) Comparison of mean values of relative expression of *TOND1* between varieties containing the *TOND1* allele and varieties containing the *tond1* allele.
(f) Relative expression of *TOND1* in 93-11, ZH17 and NIP.
(g) Photographs of 93-11, ZH17 and NIP taken after 21 days of hydroponic growth under N-deficient and N-sufficient conditions. Scale bar = 10 cm.
(h) Comparison of relative plant dry weight, relative plant height, relative root length and relative SPAD between 93-11, ZH17 and NIP under N-deficient conditions relative to N-sufficient conditions.
ZH17, Zhonghua 17; NIP, Nipponbare. Values are means ± SD. Asterisks indicate statistically significant differences compared with *TOND1* or 93-11, as appropriate (**$P < 0.01$).

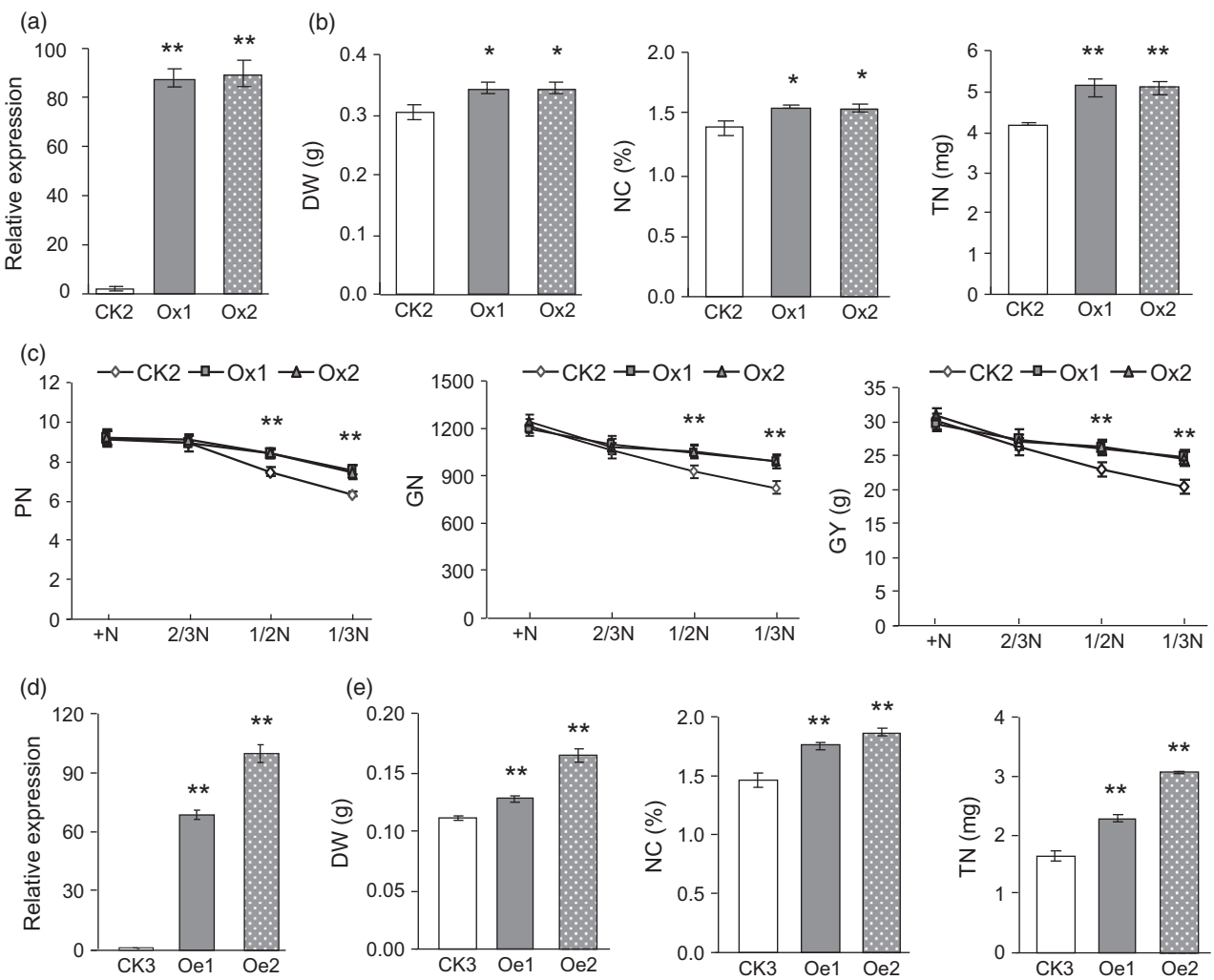


Figure 5. The phenotype of over-expressing transgenic rice plants.
(a) Expression of *TOND1* in over-expressing transgenic plants (Ox1 and Ox2) in the YIL105 background was assessed by real-time quantitative PCR.
(b) Comparison of DW, NC and TN between control (CK2) and over-expressing transgenic plants (Ox1 and Ox2) after 21 days of hydroponic growth under N-deficient conditions.
(c) Comparison of PN, GN and GY between control (CK2) and over-expressing transgenic plants under various N-deficiency field conditions.
(d) Expression of *TOND1* in over-expressing transgenic plants (Oe) in the *japonica* cultivar Zhonghua 17 background was assessed by real-time quantitative PCR.
(e) Comparison of DW, NC and TN between control (CK3) and over-expressing transgenic plants (Oe1 and Oe2) after 21 days of hydroponic growth under N-deficient conditions.
Values are means ± SD. Asterisks indicate statistically significant differences compared with +N conditions or control plants, as appropriate (*$P < 0.05$; **$P < 0.01$).

SPAD, PH and RL in comparison with control plants under N-deficient hydroponic conditions (Figures 5a,b and S3b). Furthermore, compared with control plants, PN, GN and GY of the over-expressing transgenic plants were significantly increased by approximately 17.3, 19.8 and 20.2%, respectively, under certain N-deficiency conditions (1/3N) (Figure 5c).

We further over-expressed *TOND1* in the commercial *japonica* cultivar Zhonghua 17, and found that the transgenic plants (Oe1 and Oe2), with higher expression levels of *TOND1*, exhibited tolerance of N deficiency, as indicated by increases in DW, NC, TN, SPAD, PH and RL under N-deficient hydroponic conditions (Figures 5d,e and S3c, d). These results suggest that over-expression of *TOND1* enhances tolerance of N deficiency in *TOND1*-deficient rice cultivars.

In this study, we identified *TOND1*, a major QTL in rice controlling tolerance of N deficiency. Increased expression of the *TOND1* gene led to a remarkable increase in the N-deficiency tolerance and grain yield of rice plants grown under N-deficient conditions. Previous studies showed that N-deficiency tolerance is associated with the *GS*/*GOGAT* cycle (Cai *et al.*, 2009; Tamura *et al.*, 2010). We found that expression levels of the *OsGS2*, *OsNADH-GOGAT1* and *OsNADH-GOGAT2* genes, which encode the essential enzymes in the *GS*/*GOGAT* cycle for N assimilation (Tabuchi *et al.*, 2005, 2007; Cai *et al.*, 2009, 2010; Tamura *et al.*, 2010, 2011), were significantly decreased in *TOND1* RNAi transgenic plants and increased in *TOND1* over-expressing transgenic plants, compared with control plants under N-deficiency conditions (Figure S6a–f). It would be interesting to verify whether the *TOND1* gene controls N-deficiency tolerance via modulation of the *GS*/*GOGAT* cycle.

China is the world's largest consumer of N fertilizers. The annual N fertilizer consumption in China has accounted for 30% of the annual global consumption of N fertilizer since the 1980s (Peng *et al.*, 2006). In recent years, the rate of N application and annual consumption of N fertilizers for rice production in China reached 180 kg ha^{-1} and 5.29 million tonnes, respectively (Peng *et al.*, 2006). However, only 30–45% of N fertilizer is efficiently used by rice plants (Ladha *et al.*, 1998; Zhu, 2000; Peng *et al.*, 2006), with the majority being released into the environment as non-point source pollution. As 72.7% of the cultivars analyzed do not harbor the *TOND1* allele (Table S1), introgression of *TOND1* into these *TOND1*-deficient rice cultivars may significantly decrease the use of N fertilizers, reduce the cost of rice production, alleviate pollution, and protect the environment. It is speculated that the *TOND1* gene has broad utilization prospects in rice breeding programs, and will play a vital role in the 'Second Green Revolution'.

EXPERIMENTAL PROCEDURES

Plant materials

The 150 varieties of cultivated rice, including 75 *indica* and 75 *japonica* varieties, used for the sequence comparison analysis are listed in Table S1, and the plant materials used for the association mapping are listed in Table S2.

Primers

The primers used in this study are listed in Table S3.

Phenotypic evaluation

All materials for phenotypic evaluation under hydroponic conditions were grown in nutrient solution under either N-deficient conditions (−N, 0.24 mM NH_4NO_3) or N-sufficient conditions (+N, 1.44 mM NH_4NO_3) (Yoshida *et al.*, 1976). Phenotypic evaluation was performed after emergence of the third leaf. The solution was matched with deionized water and refreshed every 2 days. After hydroponic growth for 21 days, the plants were sampled for phenotypic evaluation.

The second generation of transgenic plants (T_2) and control plants were planted in the field at the Experimental Station of China Agricultural University, Shangzhuang Road, Haidian District, Beijing, China. All materials were sown on 5 May 2013, and transplanted on 13 June 2013. The field trial followed a randomized block design with four N levels (+N, with 150 kg ha^{-1} N fertilizer; 2/3N, with 100 kg ha^{-1} N fertilizer; 1/2N, with 75 kg ha^{-1} N fertilizer; 1/3N, with 50 kg ha^{-1} N fertilizer). Under the four N conditions, P and K fertilizers were both applied at 100 kg ha^{-1}. Total P and K and 60% of N fertilizers were applied by top-dressing before transplantation, and the remaining 40% of N fertilizers was applied at the tillering stage. Twenty-one plants were planted for each transgenic line and control line, and the middle five plants were used for phenotypic evaluation. All materials were randomly arranged with three replications. Seedlings were transplanted into the field with a spacing of 13.3 cm between plants and 26.6 cm between rows. Field management followed normal agronomic procedures during the whole growth period (Shen *et al.*, 2007).

We surveyed plant height (PH), root length (RL), chlorophyll relative content (SPAD), dry weight (DW), N concentration (NC) and total N amount per plant (TN) under hydroponic conditions, and the number of tillers, the number of panicles number (PN), grain number per plant (GN) and grain yield per plant (GY) under field conditions.

Relative plant dry weight, relative plant height, relative root length and relative SPAD, between N-deficient and N-sufficient hydroponic conditions were calculated using the equation:

$$R(\%) = D/S \times 100\%,$$

where R is the relative value, and D and S are the values under N-deficient and N-sufficient conditions, respectively.

To evaluate the tolerance of each F_2 individual, 15 plants of the corresponding F_3 family were used for phenotyping under N-deficient and N-sufficient conditions.

QTL detection

QTL analysis for low N tolerance was performed using genotypic data and phenotypic data obtained by the single-marker regression method using Map Manager QTXb17 (Manly *et al.*, 2001). A probability of 0.01 was used as the threshold for single-point analysis. The proportion of the observed phenotype variance

explained by a particular QTL was estimated by the coefficient of determination (R^2) from the corresponding linear model analysis.

Microarray analysis

Total RNA was extracted using Trizol reagent (Invitrogen, http://www.invitrogen.com) and purified using an RNeasy kit (Qiagen, http://www.qiagen.com). The methods for cDNA synthesis, cRNA synthesis, cRNA fragmentation, microarray hybridization, washing and staining are described in the GeneChip® Expression Analysis Technical Manual (http://www.affymetrix.com/). The arrays were scanned using a GeneChip 3000 scanner located in the Bioinformatics Center at China Agricultural University.

Vector construction and transformation

The *TOND1* RNAi construct was generated by insertion of a hairpin sequence containing two 487 bp cDNA inverted repeat fragments targeting the *TOND1* sequence of Teqing into pJL1460 (Yu *et al.*, 2007), under the control of the ubiquitin promoter. We got 10 independent RNAi transgenic lines, and phenotypically evaluated two of the RNAi lines, Ri1 and Ri2. The construct p*35S::TOND1-GFP* containing *TOND1* fused to GFP under the control of the CaMV 35S promoter was transformed into onion epidermal cells by particle bombardment. The p*TOND1::GUS* construct was generated by cloning a 2 kb regulatory region harboring the Teqing *TOND1* promoter into the vector pCAMBIA1381 (Yu *et al.*, 2007). The over-expression construct harboring only the coding sequence of *TOND1* cDNA, a 549 bp fragment including the whole coding region, was inserted into the vector pCAMBIA1301 under the control of the ubiquitin promoter (Yu *et al.*, 2007). This was subsequently transformed into YIL105 and Zhonghua 17, and nine and 16 independent transgenic lines were harvested, respectively. Ox1, Ox2, Oe1 and Oe2 were used for phenotypic evaluation.

Real-time quantitative PCR

Real-time quantitative PCR was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, http://www.appliedbiosystems.com). cDNA corresponding to 5 ng of total RNA was used as the template, and was amplified using primers *TOND1*-qRTF and *TOND1*-qRTR and SYBR Green Master Mix (Applied Biosystems). We normalized the levels of *TOND1* transcripts using endogenous ubiquitin transcripts amplified with primers UBIQRT-2F and UBIQRT-2R. Each set of experiments was repeated three times, and the relative quantification method (Zhang *et al.*, 2006) was used to evaluate quantitative variation.

Subcellular and tissue localization

p*35S::TOND1-GFP* was bombarded into onion epidermal cells using a helium biolistic device (Bio-Rad PDS-1000) (Bio-Rad, http://www.bio-rad.com). We examined the bombarded tissues using a Nikon C1 Si confocal laser-scanning microscope (Nikon, http://www.nikon.com). GUS staining of tissues from plants harboring the p*TOND1*::GUS construct was performed as previously described (Scarpella *et al.*, 2003).

Association mapping

Association testing was performed by Fisher's exact test using a simple linear model with the R program (Lin *et al.*, 2012).

ACKNOWLEDGEMENTS

This research was supported by the National Natural Science Foundation of China (grant number 30725023), the China National High-tech Research and Development ('863') Program (grant number 2012AA10A301), the Special Fund for Agro-Scientific Research in the Public Interest (grant number 201003021), and self-regulated projects of the State Key Laboratory of Plant Physiology and Biochemistry.

SUPPORTING INFORMATION

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

Figure S1. Genotype of introgression line YIL105.

Figure S2. Phenotype comparison between Teqing and YIL105.

Figure S3. Phenotypic comparison between transgenic plants and their corresponding controls.

Figure S4. Gene sequence of *TOND1* in Teqing.

Figure S5. Phylogenetic analysis of TOND1.

Figure S6. Relative expression of *OsGS2*, *OsNADH-GOGAT1* and *OsNADH-GOGAT2* genes.

Table S1. Materials used in the sequence analysis of *TOND1*.

Table S2. Materials used in the association analysis of *TOND1*.

Table S3. Primers used in this study.

REFERENCES

Borrell, A.K., Garside, A.L., Fukai, S. and Reid, D.J. (1998) Season, nitrogen rate, and plant type affect nitrogen uptake and nitrogen use efficiency in rice. *Aust. J. Agric. Res.* **49**, 829–843.

Borrell, A., Hammer, G. and Van Oosterom, E. (2001) Stay-green: a consequence of the balance between supply and demand for nitrogen during grain filling. *Ann. Appl. Biol.* **138**, 91–95.

Cai, H.M., Zhou, Y., Xiao, J.H., Li, X.H., Zhang, Q.F. and Lian, X.M. (2009) Overexpressed *glutamine synthetase* gene modifies nitrogen metabolism and abiotic stress responses in rice. *Plant Cell Rep.* **28**, 527–537.

Cai, H.M., Xiao, J.H., Zhang, Q.F. and Lian, X.M. (2010) Co-suppressed *glutamine synthetase*2 gene modifies nitrogen metabolism and plant growth in rice. *Chin. Sci. Bull.* **55**, 823–833.

Cho, Y.I., Jiang, W.Z., Chin, J.H., Piao, Z.Z., Cho, Y.G., McCouch, S.R. and Koh, H.J. (2007) Identification of QTLs associated with physiological nitrogen use efficiency in rice. *Mol. Cells*, **23**, 72–79.

Guo, J.H., Liu, X.J., Zhang, Y., Shen, J.L., Han, W.X., Zhang, W.F., Christle, P., Goulding, K.W.T., Vitousek, P.M. and Zhang, F.S. (2010) Significant acidification in major Chinese croplands. *Science*, **327**, 1008–1010.

Hoque, M.S., Masle, J., Udvardi, M.K., Ryan, P.R. and Upadhyaya, N.M. (2006) Over-expression of the rice *OsAMT1-1* gene increases ammonium uptake and content, but impairs growth and development of plants under high ammonium nutrition. *Funct. Plant Biol.* **33**, 153–163.

Ladha, J.K., Kirk, G.J.D., Bennett, J., Reddy, C.K., Reddy, P.M. and Singh, U. (1998) Opportunities for increased nitrogen-use efficiency from improved lowland rice germplasm. *Field. Crop. Res.* **56**, 41–71.

Li, S.M. and Shi, W.M. (2006) Quantitative characterization of nitrogen regulation of *OsAMT1;1*, *OsAMT1;2*, and *OsAMT2;2* expression in rice seedlings. *Russ. J. Plant Physiol.* **53**, 837–843.

Lian, X.M., Xing, Y.Z., Yan, H., Xu, C.G., Li, X.H. and Zhang, Q.F. (2005) QTLs for low nitrogen tolerance at seedling stage identified using a recombinant inbred line population derived from an elite rice hybrid. *Theor. Appl. Genet.* **112**, 85–96.

Lin, C.M., Koh, S., Stacey, G., Yu, S.M., Lin, T.Y. and Tsay, Y.F. (2000) Cloning and functional characterization of a constitutively expressed nitrate transporter gene, *OsNTR1*, from rice. *Plant Physiol.* **122**, 379–388.

Lin, Z.W., Li, X.R., Shannon, L.M. *et al.* (2012) Parallel domestication of the *shattering1* genes in cereals. *Nat. Genet.* **44**, 270–275.

Manly, K.F., Cudmore, J.R.H. and Meer, J.M. (2001) Map Manager QTX, cross-platform software for genetic mapping. *Mamm. Genome*, **12**, 9309–9332.

Matson, P., Loshe, K.A. and Hall, S.J. (2002) The globalization of nitrogen deposition: consequences for terrestrial ecosystems. *Ambio*, **31**, 113–119.

McAllister, C.H., Beatty, P.H. and Good, A.G. (2012) Engineering nitrogen use efficient crop plants: the current status. *Plant Biotechnol. J.* **10**, 1011–1025.

Mulvaney, R.L., Khan, S.A. and Ellsworth, T.R. (2009) Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. *J. Environ. Qual.* **38**, 2295–2314.

Peng, S.B., Buresh, R.J., Huang, J.L., Yang, J.C., Zou, Y.B., Zhong, X.H., Wang, G.H. and Zhang, F.S. (2006) Strategies for overcoming low agronomic nitrogen use efficiency in irrigated rice systems in China. *Field. Crop. Res.* **96**, 37–47.

Robertson, G.P. and Vitousek, P. (2009) Nitrogen in agriculture: balancing the cost of an essential resource. *Annu. Rev. Environ. Resour.* **34**, 97–125.

Scarpella, E., Rueb, S. and Meijer, A.H. (2003) The *RADICLELESS1* gene is a required for vascular pattern formation in rice. *Development*, **130**, 645–658.

Shan, Y.H., Wang, Y.L. and Pan, X.B. (2005) Mapping of QTLs for nitrogen use efficiency and related traits in rice (*Oryza sativa* L.). *Agric. Sci. China*, **4**, 721–727.

Shen, B., Zhuang, J.Y., Zhang, K.Q., Dai, W.M., Lu, Y., Fu, L.Q., Ding, J.M. and Zheng, K.L. (2007) QTL mapping of chlorophyll content in rice. *Agric. Sci. China*, **6**, 17–24.

Sun, H.Y., Qian, Q., Wu, K. *et al.* (2014) Heterotrimeric G proteins regulate nitrogen-use efficiency in rice. *Nat. Genet.* **46**, 652–656.

Tabuchi, M., Sugiyama, K., Ishiyama, K., Inoue, E., Sato, T., Takahashi, H. and Yamaya, T. (2005) Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase1;1. *Plant J.* **42**, 641–651.

Tabuchi, M., Abiko, T. and Yamaya, T. (2007) Assimilation of ammonium ions and reutilization of nitrogen in rice (*Oryza sativa* L.). *J. Exp. Bot.* **58**, 2319–2327.

Tamura, W., Hidaka, Y., Tabuchi, M., Kojima, S., Hayakawa, T., Sato, T., Obara, M., Kojima, M., Sakakibara, H. and Yamaya, T. (2010) Reverse genetics approach to characterize a function of NADH-glutamate synthase1 in rice plants. *Amino Acids*, **39**, 1003–1012.

Tamura, W., Kojima, S., Toyokawa, A., Watanabe, H., Tabuchi, M., Hayakawa, T. and Yamaya, T. (2011) Disruption of a novel NADH-glutamate synthase2 gene caused marked reduction in spikelet number of rice. *Front. Plant Sci.* **2**, 57.

Tan, L.B., Liu, F.X., Xue, W., Wang, G.J., Ye, S., Zhu, Z.F., Fu, Y.C., Wang, X.K. and Sun, C.Q. (2007) Development of *Oryza rufipogon* and *Oryza sativa* introgression lines and assessment for yield-related quantitative trait loci. *J. Integr. Plant Biol.* **49**, 871–884.

Tang, Z., Fan, X.R., Li, Q., Feng, H.M., Miller, A.J., Shen, Q.R. and Xu, G.H. (2012) Knock down of a rice stellar nitrate transporter alters long distance translocation but not root influx. *Plant Physiol.* **160**, 2052–2063.

Tong, H.H., Mei, H.W., Yu, X.Q., Xu, X.Y., Li, M.S., Zhang, S.Q. and Luo, L.J. (2006) Identification of related QTL at late developmental stage in rice (*Oryza sativa* L.) under two nitrogen levels. *Acta Genet. Sin.* **33**, 458–467.

Wang, Y., Sun, Y.J., Chen, D.Y. and Yu, S.B. (2009) Analysis of quantitative trait loci in response to nitrogen and phosphorus deficiency in rice using chromosomal segment substitution lines. *Acta Genet. Sin.* **35**, 580–587.

Xia, X.D., Fan, X.R., Wei, J., Feng, H.M., Qu, H.Y., Xie, D., Miller, A.J. and Xu, G.H. (2015) Rice nitrate transporter OsNPF2.4 functions in low affinity acquisition and long distance transport. *J. Exp. Bot.* **66**, 317–331.

Yamaya, T., Obara, M., Nakajima, H., Sasaki, S., Hayakawa, T. and Sato, T. (2002) Genetic manipulation and quantitative-trait loci mapping for nitrogen recycling in rice. *J. Exp. Bot.* **53**, 917–925.

Yan, M., Fan, X.R., Feng, H.M., Miller, A.J., Shen, Q.R. and Xu, G.H. (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.

Yanagisawa, S., Akiyama, A., Kisaka, H., Uchimiya, H. and Miwa, T. (2004) Metabolic engineering with Dof1 transcription factor in plants: improved nitrogen assimilation and growth under low-nitrogen conditions. *Proc. Natl Acad. Sci. USA*, **101**, 7833–7838.

Yoshida, S., Forno, D.A., Cock, J.H. and Gomez, K.A. (1976) *Laboratory Manual for Physiological Studies of Rice*, 3rd edn. Manila, The Philippines: The International Rice Research Institute.

Yu, B.S., Lin, Z.W., Li, H.X. *et al.* (2007) *TAC1*, a major quantitative trait locus controlling tiller angle in rice. *Plant J.* **52**, 891–898.

Yuan, Q.P., Ouyang, S., Liu, J., Suh, B., Cheung, F., Sultana, R., Lee, D., Quackenbush, J. and Buell, C.R. (2003) The TIGR rice genome annotation resource: annotating the rice genome and creating resources for plant biologists. *Nucleic Acids Res.* **31**, 229–233.

Zeigler, R.S. and Mohanty, S. (2010) Support for international agricultural research: current status and future challenges. *New Biotechnol.* **27**, 565–572.

Zhang, X., Zhou, S.X., Fu, Y.C., Su, Z., Wang, X.K. and Sun, C.Q. (2006) Identification of a drought tolerant introgression line derived from Dongxiang common wild rice (*O. rufipogon* Griff.). *Plant Mol. Biol.* **62**, 247–259.

Zhu, Z.L. (2000) Loss of fertilizer N from plant-soil system and the strategies and techniques for its reduction. *Soil Environ. Sci.* **9**, 1–6 (in Chinese with English abstract).