

QTL analysis of nitrogen content of plant shoot under two nitrogen conditions in rice (*Oryza sativa* L.)

Shi-kai Hu, Da-li Zeng, Yan Su, Zhen-yuan Shi, Wei-jun Ye, Guo-jun Dong, Li Zhu, Jiang Hu, Qian Qian*, Long-biao Guo*

State Key Lab for Rice Biology, China National Rice Research Institute, Hangzhou 310006, P. R. China

*Corresponding author: qianqian188@hotmail.com; guolongb@mail.hz.zj.cn

Abstract

As an essential macronutrient, nitrogen (N) is one of the most important factors which could limit agricultural productivity and plays an important role in rice yield formation. To investigate the genetic mechanism of N absorption and utilization efficiency and to improve N utilization efficiency in rice a QTL analysis was done. The main-effect quantitative trait loci (QTLs), epistatic QTLs and QTL-by-environment interactions (QEs) for nitrogen content (NC) of plant shoot at heading and mature stages in two N conditions were identified using a double haploid population derived from a cross between a *japonica* Chun Jiang06 (CJ06) and an *indica* Taichung Native 1 (TN1). A total of three NC QTLs were detected on chromosomes 5, 6 and 8 in two N conditions. The phenotypic variation explained by individual QTL ranged from 9.73 to 39.50%. Two pairs of digenic epistatic loci were associated with NC. The QTL qNc8-4 was detected in both high nitrogen (HN) and low nitrogen (LN) conditions, and explained phenotypic variation 18.35% and 39.5% in HN and LN conditions, respectively. The analysis revealed that the additive effect of qNc8-4 came from TN1. Then the qNc8-4 was considered as the main effect loci affecting NC of rice shoot. We suggest that QTL markers of qNc8-4, RM4085 and RM1111, expressing in LN conditions, may be used to improve the N absorption and utilization efficiency in low input rice varieties through marker assisted selection (MAS) breeding programs. It is the first time to use a genetic population like this to map the QTLs of NC of plant shoot in rice, and these results will supply references and research forecast for further study on practicable methods of N utilization, cloning the genes which affect NC and improving N utilization efficiency of varieties by MAS.

Keywords: Nitrogen; Double haploid population; Quantitative trait loci; Rice (*Oryza sativa* L.).

Abbreviations: CJ06_ *Japonica* cultivars ChunJiang06; DH_Double Haploid; HN_High Nitrogen; LN_Low Nitrogen;

MAS_Molecular Marker Assisted Selection; NC_Nitrogen Content; NCh_Nitrogen Content in heading stage; NCm_Nitrogen

Content in mature stage; ORF_Open Reading Frame; QEs_QTL-by-environment interactions; QTL_Quantitative Trait Loci; TN1_ *Indica* cultivars Taichung Native 1.

Introduction

Nitrogen (N) is central to living systems and is an essential component of agricultural cropping systems in modern crop management. Application of N is one of the major reasons that crop production has kept pace with human population growth. However, out of ~120 Tg year⁻¹ of N is used for food production globally, only ~10% is consumed by people (Gutiérrez, 2012; Robertson and Vitousek, 2009). Most of the unutilized N, between 50% and 75% of the N applied to the field, is not used by crops and hence is lost by leaching into the soil or is released to the atmosphere as nitrogen gases (Hirel et al., 2011). Moreover, the amount of mineral N fertilizers applies to agricultural crops increase by 7.4 fold, whereas the overall yield increase is only 2.4 fold over 40 years from 1966 to 2006 (Tilman et al., 2002). N plays a crucial role in the yield formation and the increasing and stabilizing production of rice (de Datta and Broadbent, 1990; Gutiérrez, 2012; Hirel et al., 2007; Tadahiko, 1997; Zhang et al., 2004). The latest reports indicated that the rice yield would remain stagnant along with the increasing N utilization. What has caused this phenomenon? Some studies indicated that the unreasonable utilization and the low efficiency of N fertilization utilization may be the main

reasons besides the differences of varieties (Borrell et al., 1998; Peng et al., 1996; Peng et al., 2006). So, it is necessary to cultivate varieties with high N efficiency, discover the genetic mechanism of N absorption and utilization efficiency and map the QTLs controlling NC in rice.

QTL mapping is the most available method towards understanding the molecular genetics mechanisms of complex quantitative traits behind phenotypic complexity (Agrama et al., 1999; Guo et al., 2004; Septiningsih et al., 2003; Shan et al., 2005; Tanksley et al., 1993; Wang et al., 1999; Yano and Sasaki, 1997; Zeng et al., 1994; Zhang et al., 2011). At present, there were a few studies for QTL researches of NC in rice, and the previous researches mainly focused on the QTL researches for NC of flag leaf in rice. Ishimaru et al. (2001) reported that there was one QTL on Chromosome 2, controlling NC of flag leaf in rice. Yang et al. (2005) chose IR24 (*indica*) and Asominori (*japonica*) and their chromosome segment substitution lines (CSSLs) as the material to detect NC of flag leaf at five different growth stages and detected seven QTLs, which located on chromosome 2, 3, 8 and 11, respectively. However, there was no report available about QTL analysis of NC of plant

shoot in rice. Moreover, Wei et al. (2008) found that N utilization efficiency was positive and significantly correlated with N accumulation during the heading and mature stages.

The correlation analysis between N utilization efficiency and the accumulation at different growth stages has suggested that there is a negative correlation during the periods from transplant to effective tiller critical leaf age and from effective tiller critical leaf age to jointing. There is also significant positive correlation during the periods from the jointing to heading and from heading to mature. Accordingly, the current study was carried out to identify QTLs for NC of plant shoot at the heading and mature stages in the controlled conditions using 116 doubled haploid (DH) lines derived from a *japonica* and *indica* cross, which will help us to understand the genetic mechanisms of the uptake and utilization mechanism of N fertilizer in rice, clone the related regulatory loci or genes, and obtain molecular markers for breeders in molecular marker assisted breeding.

Results

NC performance of the parents and DH lines

NC of plant shoot for the parents (CJ06 and TN1) and DH population were summarized in Table 1. There was significant difference between the parents, especially in HN conditions at the heading stage, while NC of CJ06 was similar to that of TN1 in LN conditions at the mature stage. In HN conditions, at the heading stage, NC of CJ06 was 2.14%, much higher than that of TN1 (1.69%), while NC of the DH lines ranged from 1.2% to 3.4%. While at the mature stage, NC of CJ06 was 1.32%, which was lower than that of TN1 (1.67%), and NC of the DH lines were declined, respectively, and ranged from 1.00% to 2.41%. In LN conditions, at the heading stage, NC of CJ06 was 1.85%, while TN1 was 1.36%. The highest and the lowest NC in DH lines were 2.21% and 0.79%. But at the mature stage, there was no significant difference between the parents (Table 1).

The statistical analysis between the two stages in HN and LN conditions discovered that the difference of NC among the DH lines was obvious, and NC had a continuous normal distribution (Fig. 1). In addition, some lines showed best parent heterosis and separated widely among the DH lines, which indicated that the target trait controlling NC was quantitative trait controlled by multiple genes in accordance with the QTL mapping requirements.

QTLs for NC of plant shoot

The main QTL effect of NC at the two stages in HN and LN treatment was shown in Table 2. In HN conditions, two putative QTLs (qNCh5-4 and qNCh8-4) were associated with NC at the heading stage, while only one was related to NC at mature stage (qNCm6-12). The detected QTL qNCh5-4 was mapped between the markers RM3170 and RM480 on chromosomes 5, which explained 10.85% of the phenotypic variation. The allele of QTL qNCh5-4 from CJ06 tended to increase NC. The qNCh8-4 was mapped between RM4085 and RM1111 on chromosomes 8, which explained 18.35% of the phenotypic variation. The qNCm6-12 was mapped between RM527 and RM3 on chromosomes 6, which explained 9.73% of the phenotypic variation (Fig. 2, Table 2).

In LN level, only one putative QTL (qNCh8-4) was detected at the heading stage, which explained 39.5% of the phenotypic variation, and the allele of QTL qNCh8-4 from

TN1 tended to increase NC. The same locus could be detected in the two different conditions, which located between RM4085 and RM1111, and the QTL in HN conditions explained the phenotypic variation higher than that in LN conditions. But we did not detect any effective QTL locus at the mature stage.

In order to confirm validation of the three detected NC QTLs, we compared with the reported QTLs/genes of yield-related traits (grains per plant, primary branches per panicle, 1000-grains weight, etc.). We found that the gene, *Ghd8/DTH8/Hd5*, was very closely to the region of detected QTL on chromosome 8(qNC8-4). Sequencing analysis showed that there was a little difference between CJ06 and TN1 in the position of *Ghd8/DTH8/Hd5* (Fig. 3). It showed qNc8-4 may be the same gene as *Ghd8/DTH8/Hd5*, but it needed further confirmation.

QEs and digenic epistasis for NC of plant shoot

Compared with the main effects, the genotype-by-environment interactions may not be the major factor controlling NC, while the interaction effects had certain impact on NC, which made the genetic expression of NC different. At the heading stage, the environmental interactions were detected for two main-effect QTLs in HN conditions and the total QEs explained 34.3% of the phenotypic variation. The environmental interactions were detected for two main-effect QTLs in LN conditions, and the total QEs explained 29.48% of the phenotypic variation, which was 4.82% lower than that in HN conditions (Table 3). At the mature stage, two pairs of the epistatic QTLs in HN conditions were detected, and the total epistatic effects explained 51.04% of phenotypic variation. The environmental interactions were detected for two pairs of the epistatic QTLs in LN conditions, and the total epistatic effects explained 50.02% of the phenotypic variation, which was the same as that in HN conditions.

Discussion

The annual consumption of N fertilizer in China is 25 million tons. The decline of N application has become more and more serious; yield increasing of crop was stopped or even reduced under higher N fertilizer level (Shen et al., 2003). The effective ways to solve the problem are to discover new germplasm with higher N efficiency and to map new QTLs controlling NC and develop functional molecular marker for MAS. In this study, NC in the DH population and the parental cultivars (CJ06 and TN1) were investigated at the heading and mature stages in HN and LN conditions. The frequency distributions of the DH population for NC in different N conditions and growth stages were not bimodal (Fig. 1), suggesting that NC of plant shoot was quantitatively inherited traits controlled by multiple genes in rice. Yang et al. (2005) found that NC of the parents, IR24 and Asominori, displayed dynamic change at five different stages (Yang et al., 2005). Based on the data analysis, we found that NC of CJ06 was higher than that of TN1 at the heading stage, while NC of TN1 was higher than that of CJ06 at mature stage. This indicates that NC performed dynamic change at two different stages and showed similar results as Yang et al. (2005). The reason could be that different QTLs influenced the performance of NC of the parents at different stages, or the interaction among the QTLs caused the diverse performance of NC of the parents. That was a very interesting phenomenon and needed further researches.

Table 1. NC of the parents and their DH population in HN and LN conditions at heading stage and maturing stage.

Stage	Condition	Parents (Mean \pm SD)			DH population			
		CJ06	TN1	P value	Mean \pm SD	Range	Skewness	Kurtosis
Heading stage	HN	2.14 \pm 0.12%	1.69 \pm 0.13%	<0.0001	2.13 \pm 0.48%	1.20-3.40%	0.23	-0.66
	LN	1.85 \pm 0.09%	1.36 \pm 0.11%	<0.0001	1.39 \pm 0.31%	0.79-2.21%	0.27	-0.73
Mature stage	HN	1.32 \pm 0.09%	1.67 \pm 0.16%	<0.0001	1.50 \pm 0.27%	1.00-2.41%	0.69	0.57
	LN	1.04 \pm 0.07%	1.23 \pm 0.12%		1.04 \pm 0.17%	0.64-1.53%	0.60	-0.20

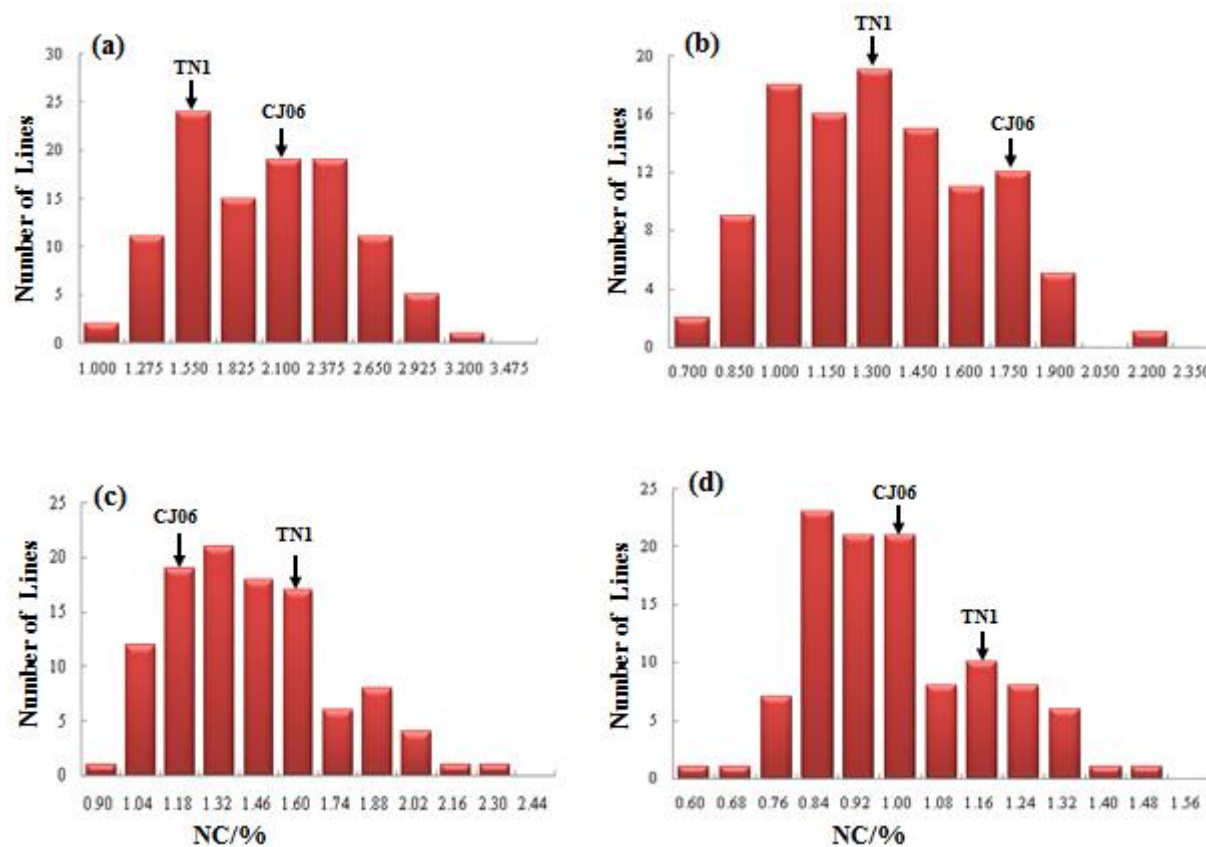


Fig 1. Distribution of NC in the DH population in HN and LN conditions. (a) In HN conditions at heading stage. (b) In LN conditions at heading stage. (c) In HN conditions at mature stage. (d) In LN conditions at mature stage.

Novel QTLs controlling NC of plant shoot

To the best of our knowledge, there is still no report on NC QTLs of plant shoots. On the other hand, in this study we, for the first time, used the genetic population to map the QTLs of NC of plant shoot in rice. Ishimaru et al. (2001) found that there was only one QTL on chromosome 2, controlling the NC of the flag leaf. Yang et al. (2005) detected 7 QTLs by analyzing NC of flag leaf at different five stages in the chromosome segment substitution lines (CSSLs), and none of which were as same as the QTLs we detected here. We have detected 3 QTLs controlling NC of plant shoot at two different stages in HN/LN conditions. In HN conditions, two QTLs, qNCh8-4 and qNCh5-4, were detected at the heading stage, while only one QTL qNCm6-12 was associated with NC at the mature stage. In LN conditions, only one QTL qNCh8-4 was related to NC at heading stage, but none was detected at the mature stage. Based on the results, the locus qNC8-4 could be detected at the heading stage in HN and LN conditions, suggesting that the locus may not be correlated with N fertilization supply. For the phenotypic variation, the locus showed significant difference in two conditions, qNCh8-4 in LN conditions explained 39.5% of the phenotypic variation, which was 21.15% higher than that of qNCh8-4 in HN conditions. Above all, we concluded that LN conditions might better induce the expression of the qNCh8-4, or qNCh8-4 and ensure the normal N fertilization supply in rice. Meanwhile, qNCh8-4 may act as the major QTL controlling NC in rice.

QTLs of NC may be related directly to other agronomy traits

Nitrogen is the component of organic N compounds which is necessary for the plant growth and development. The rice leaves are large and dark green, and the chlorophyll content is high when the supply of N is excessive. The rice yield declines when N level is insufficient or excessive. This condition affects the leaf chlorophyll synthesis and causes some symptoms such as yellow leaves or early senescence, resulting to a yield reduction. We analyzed the dynamic variation of chlorophyll content at different growth stages with the same mapping population. We found that there was one QTL controlling the chlorophyll content in the interval RM4085-RM1111, and that may affect the chlorophyll synthesis. Consequently, we concluded that there was a certain relationship between NC and the chlorophyll content (Huang et al., 2008; Wang et al., 2002; Yang et al., 2003). The locus qNCh8-4 may affect NC and the chlorophyll content, then influence the plant photosynthetic efficiency, even the biomass synthesis and yield formation. We compared the map position of the detected QTLs of NC with the reported QTLs/genes of yield-related traits (grains per plant, primary branches per panicle, 1000-grains weight, etc.). We found that *Ghd8/DTH8/Hd5*, which had first been cloned by Wei et al. (2010) and Yan et al. (2010), played pleiotropic roles in regulating grain productivity, plant height and heading date in rice. It was very closely to the region of the detected QTL on chromosome 8(qNc8-4) (Yano et al., 1997; Lin et al., 2003; Wei et al., 2010; Yan et al., 2010). We suggested that qNc8-4 may be the same gene with the gene, *Ghd8/DTH8/Hd5*, based on the sequencing results. However, it needed further investigation. Because N fertilizer is very important in rice yield formation and improvement, especially in super hybrid rice in China, the QTL of NC may influence the yield-related traits (heading date, 1000-grains weight, etc.) simultaneously by direct reflect on

performance of yield-related traits. In addition, the same QTL may locate on the region with little difference in the chromosome in different mapping populations and genetic linkage maps.

There were no reported QTLs/genes matched or closed to the region of the other two QTLs, qNch5-4 and qNcm6-12, which meant that those two QTLs were the new loci controlling NC.

Epistatic effects between NC and main-effect QTLs

The influence of the epistatic effects on NC also varied considerably among different stages. The total effects of the epistatic QTLs were even higher than those of the main-effect QTLs. At the heading stage, no digenic epistatic QTL was detected either in HN or LN conditions. But the effects of epistasis were most prominent at the mature stage, which were detected in two pairs of loci, none of which involved in the main-effect QTLs (Table 3). Based on all the results above, we could conclude that although the major QTLs were frequently the most important determinants of NC, but interactions between minor effect QTLs (or even those that do not have detectable effects by single locus analysis) may also have significant effects on NC. Moreover, the effects of both major and minor genes were also sometimes subject to environmental modifications, which can cause dramatic differences in the phenotypic effects of the QTLs (Fan et al., 2005; Senapathy et al., 2008).

It was essential for improving rice cultivars and increasing N utilization efficiency to clarify the molecular mechanism of differential expression in two N levels, by which we can use marginal land for rice production and protect the environment. We characterized the genetic components including main-effect quantitative trait loci (QTLs), epistatic QTLs and QTL-by-environment interactions (QEs), which were involved in controlling NC in the two different conditions and at two stages. The results varied considerably when the same QTL was detected in the two different N conditions.

Materials and methods

Plant materials

A DH population consisted of 116 DH lines was used in the present experiment. This population was developed through another culture of the F₁ hybrid between an *indica* rice variety Taichung Native 1 (TN1) and a *japonica* rice variety Chun Jiang 06 (CJ06).

Seedling preparation and fertilizer treatment

Uniform seeds of all DH lines and the parents were soaked in distilled water in the dark at 30°C for 2 days from May 18th to 20th, and germinated in distilled water at 35°C for 12 h. The germinated seeds then sown in the paddy field on May 21th in the experimental farm of China National Rice Research Institute, Hangzhou with necessary irrigation. After 25 days, rice seedlings with the similar size were selected and transplanted to the randomized blocks. Two treatments of the parents and each DH line were applied with three replications: HN and LN conditions and each DH line was planted to three rows, with six plants in each row. The seedlings were transplanted to the paddy yields with the same N fertilization level, supplemented with 75 kg ha⁻¹ and 225 kg ha⁻¹ in LN and HN conditions during the full growth period, respectively.

Table 2. QTLs of NC in the DH population of CJ06/TN1 in HN and LN conditions.

Treatment	QTL	Marker interval	Genetic distance (cM)	P-value	Additive effect(A)	H ² (A)(%)
HN	qNCh8-4	RM4085-RM1111	45.4-62.7	<0.0001	-0.2150	18.35
	qNCh5-4	RM3170-RM480	49.6-59.5	<0.0001	0.1159	10.85
LN	qNCm6-12	RM527-RM3	68.5-78.1	<0.0001	0.9743	9.73
	qNCh8-4	RM4085-RM1111	50.4-68.5	<0.0001	-0.2068	39.50

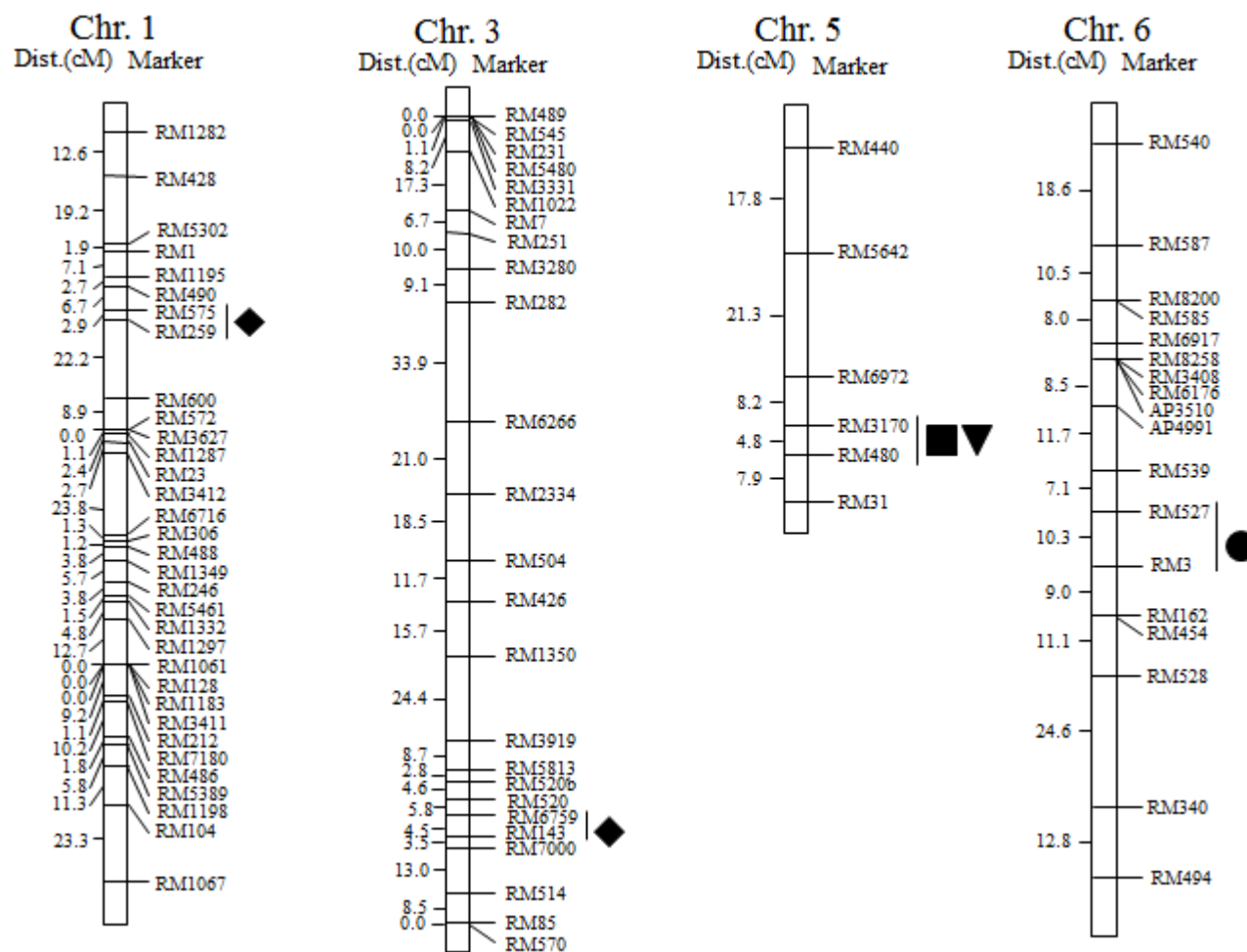


Fig 2

Continue fig 2.

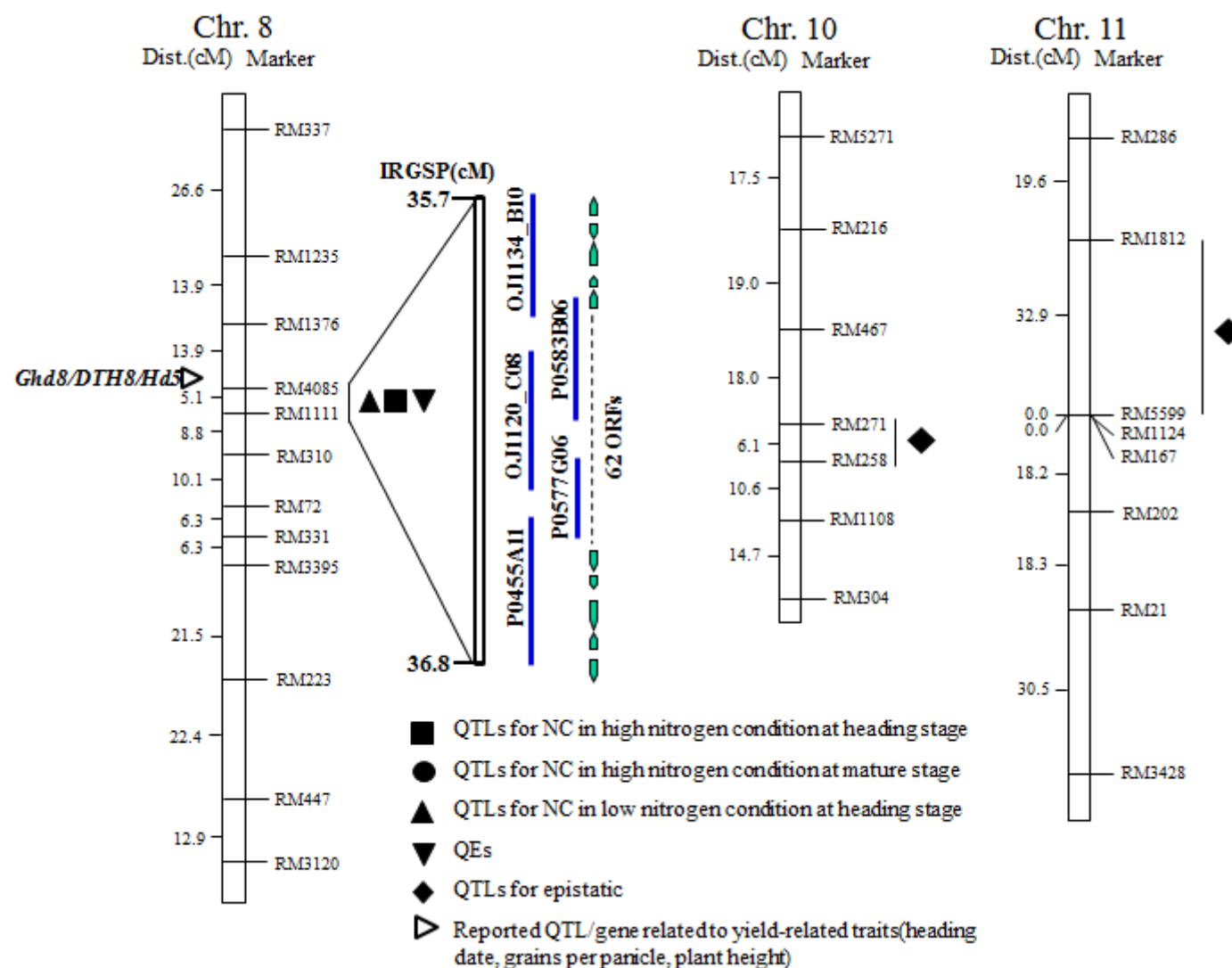
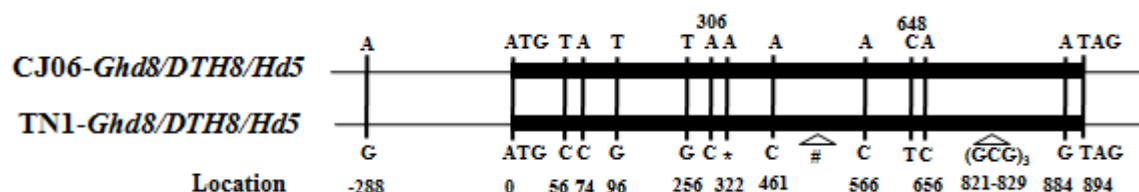


Fig 2. Locations of main effect QTLs for NC of plant shoot in the DH population of CJ06/TN1. Individual QTLs are designated with the different shapes. Square indicates QTLs for NC in HN conditions at heading stage, round shape indicates the QTLs for NC in HN conditions at mature stage. The triangle indicates the QTLs for NC in LN condition at heading stage. The inverted triangle indicates QEs. The rhombus indicates the QTLs for epistatic. The hollow triangle indicates reported QTLs/genes related to yield-related traits (heading date, grains per panicle, plant height, etc). Five bacterial artificial chromosome (BAC) clones and 62 open reading frames (ORFs) locate in the region between the markers RM4085 and RM1111 by searching against the IRGSP (<http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgsp-status.cgi>) and the Rice GAAS (<http://ricegaas.dna.affrc.go.jp/>) databases. The distances between markers (cM) are listed left of each chromosome figure.

Table 3. Environmental interactions for NC in the DH population of CJ06/TN1.

Environment	QTL _i	QTL _j	ae _i	h ² ae _i	ae _j	h ² ae _j	aae _{ij}	h ² aae _{ij}
HN	qNCh5-4	qNCh8-4	0.0444	12.45%	-0.0631	21.85%		
	qNCm1-7	qNCm3-19					0.0457	23.02%
	qNCm10-4	qNCm11-2					0.0256	28.02%
LN	qNCh5-4	qNCh8-4	-0.0084	10.85%	-0.0481	18.63%		
	qNCm1-7	qNCm3-19					-0.0448	22.87%
	qNCm10-4	qNCm11-2					-0.0264	27.33%

ae_i, ae_j and aae_{ij} are effects of the environmental interaction of locus i, j and epistasis, respectively. h²ae_i, h²ae_j and h²aae_{ij} are the percentages of the phenotypic variations explained by ae_i, ae_j and aae_{ij}, respectively.

**Fig 3.** Sequence analysis of the structure of *Ghd8/DTH8/Hd5* between CJ06 and TN1.

*Indicates the single-base deletion of the structure of *Ghd8/DTH8/Hd5* in TN1 compare with CJ06,

#represents the sequence “CGGCGGCGCCGCCGCGCGCGCCACGGCGAGGGC

GGTTGCTGCGGCGGCGGCGG”, triangle indicates the sequence deletion of the structure of *Ghd8/DTH8/Hd5* in TN1.

NC Measurement

At the heading and mature stages of each line of DH population, the plants were harvested by cutting the stems from each replication. Then plants were placed into the DHG series heating and drying oven to de-enzyme at 105°C for 2 h, then in 80°C for 72 h, after which the materials were ground to flour and 0.1 ± 0.005 g of the flour were weighted to measure NC of each treatment by the Kjeldahl method (Lang, 1958), in which there were three steps: digestion, distillation and titration. Each line of the DH population and the parents were measured three times and averaged.

Statistical analysis and QTL mapping

The molecular marker data for the DH population consisted of 227 polymorphic loci including simple sequence repeat (SSR) and sequence-tagged site (STS) markers. The markers covered all 12 chromosomes, among which 178 markers were uniformly distributed in the 12 chromosomes used in the linkage map construction. The 1,674.8 cM linkage map covering nearly the whole rice genome was used for QTL (define QTL) analysis. This data set was used for mapping the genes for quantity traits of the DH population data. The average of NC for each line of DH population was used for QTL analysis. QTLs and epistatic effects as well as QEs in the DH population were mapped by the software QTLNetwork version 2.0 (Yang and Zhu., 2005) based on the mixed linear model (Wang et al., 1999). The QTLs were determined with a threshold of P<0.05 for claiming putative QTLs. The genetic parameters, additive effects, and accounted variation of each QTL were also estimated. The relative contribution of a genetic component was calculated as the proportion of phenotypic variation explained by that component in the selected model (McCouch et al., 1997).

Conclusion

It was concluded that there were three QTLs, qNc5-4, qNc6-12 and qNc8-4. The qNc8-4 was considered as the main effect loci affecting NC of plant shoot in rice. The markers of QTL qNc8-4, RM4085 and RM1111, expressing in LN conditions, may be used as the functional markers to

improve N absorption and utilization efficiency in low-in-put rice varieties through marker assisted selection (MAS) breeding. It was the first time to use the genetic population to map the QTLs of NC of plant shoot in rice. The results in this study laid a solid foundation for QTL cloning, and also had significant implication that it was the most effective way to solve or relieve the contradiction of high N input but low agricultural output to cultivate high N efficiency rice varieties.

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