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# QTL and QTL × Environment Effects on Agronomic and Nitrogen Acquisition Traits in Rice

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## Abstract

**Agricultural environments deteriorate due to excess nitrogen application. Breeding for low nitrogen responsive genotypes can reduce soil nitrogen input. Rice genotypes respond variably to soil available nitrogen. The present study attempted quantification of genotype × nitrogen level interaction and mapping of quantitative trait loci (QTLs) associated with nitrogen use efficiency (NUE) and other associated agronomic traits. Twelve parameters were observed across a set of 82 double haploid (DH) lines derived from IR64/Azucena. Three nitrogen regimes namely, native (0 kg/ha; no nitrogen applied), optimum (100 kg/ha) and high (200 kg/ha) replicated thrice were the environments. The parents and DH lines were significantly varying for all traits under different nitrogen regimes. All traits except plant height recorded significant genotype × environment interaction. Individual plant yield was positively correlated with nitrogen use efficiency and nitrogen uptake. Sixteen QTLs were detected by composite interval mapping. Eleven QTLs showed significant QTL × environment interactions. On chromosome 3, seven QTLs were detected associated with nitrogen use, plant yield and associated traits. A QTL region between markers RZ678, RZ574 and RZ284 was associated with nitrogen use and yield. This chromosomal region was enriched with expressed gene sequences of known key nitrogen assimilation genes.**

**Key words:** doubled haploid population; nitrogen acquisition; quantitative trait loci; quantitative trait loci × environment interaction; rice.

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Rice (*Oryza sativa* L.) is the staple food crop for more than 50% of the world's population. To feed the fast increasing global population in the coming years, a considerable quantity of rice will be required (FAO 2002) and this increase in production will have to come from the receding extent of land, soil and water. This warrants the situation in which other production needs to be prudently used. Among the production inputs, nitrogen remains as the most naturally scarce primary input in irrigated rice ecosystems. For over a century now, rice farmers have become increasingly dependent on chemical nitrogenous fertilizers. However, the efficiency of fertilizer nitrogen use by rice is low due to substantial nitrogen losses from flooded soil (De Datta and Burresh 1989). Further, the excessive use of nitrogen fertilizers causes serious adverse ecological consequences (Conway and Pretty 1988). Socolow (1999) cautioned that eutrophication and loss of biodiversity in the ecosystem would be a very serious danger due to the over-application of nitrogen in agriculture.

The efficiency of the rice plant to use soil available nitrogen effectively is the prime factor to its overall yield potential. Efforts to improve this must be guided by a thorough understanding of the genetic processes that govern nitrogen use efficiency (NUE). Significant genotypic differences in NUE have been shown in

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rice, which can be better exploited in the rice improvement (Tirol-Padre et al. 1996). The limited understanding on the genetics of this complex trait remains as an impediment to breeders in genetic enhancement of rice for better NUE. Nevertheless, the genotypes are having varying responses to different nitrogen sources and levels, indicating the presence of strong genotype × environment (G × E) interaction.

Quantitative genetic analyses in recent times rely extensively on mapping of loci on molecular linkage maps to be used in marker assisted selection (MAS) programs. Hirel et al. (2001) mapped quantitative trait loci (QTLs) for several agronomic and physiological traits such as nitrate content, nitrate reductase (NR) and glutamine synthetase (GS) in maize. Rauh et al. (2002) mapped specific QTLs to varying nitrogen sources such as nitrate, ammonium, and ammonium nitrate in *Arabidopsis*. Similarly, Loudet et al. (2003) mapped 48 QTLs for traits such as shoot growth, total nitrogen, nitrate and free amino acid contents in *Arabidopsis*. In rice, Obara et al. (2004) found that the QTL region of chromosome 2 was linked to panicle number and panicle weight contained a regulatory gene (*GS1*) for glutamine synthetase activity, and selections based on this QTL region proved to be superior in tillering ability, panicle number and total panicle weight, particularly under low nitrogen. Lian et al. (2005) mapped QTLs for low nitrogen tolerance at the seedling stage involving a recombinant inbred lines (RIL) population in rice.

Based on the success of the above works, we attempted to locate the QTLs associated with nine traits of agronomic importance presumably related to nitrogen nutrition and three parameters that are directly associated with nitrogen uptake and nitrogen use efficiency in dihaploid (DH) lines of IR 64 × Azucena grown under three nitrogen regimes.

## Results

### Phenotypic variation and heritability

The mean performance of parents and DH lines under three nitrogen regimes is given in Table 1. IR64 recorded higher grain yield per plant in g (SPY) together with higher values of number of tillers (TLN), number of productive tillers (PTN) and spikelet fertility percentage (SFP) than Azucena under all three nitrogen levels. IR64 was dwarfed and early flowering compared to Azucena. It recorded higher values for percentage nitrogen content (NCP) in plant and nitrogen uptake in g (NUP). IR64 was more nitrogen responsive compared with Azucena. However, Azucena recorded higher NUE than IR64 at the native N level. The limitation of nitrogen availability had a systematic decreasing effect on all of the agronomic traits substantially in IR64 except for spikelets per primary panicle (SPP) and SFP. The mean values of DH lines were in between the parental values for all of the traits. However, a large number of transgressive segregants were observed for every trait. The broad sense heritability ($h^2$) values for all agronomic traits except plant height in cm (PHT) recorded moderate to low values (Table 2). All of the nitrogen parameters recorded a very low heritability. For NCP, the $h^2$ value ranged between 27% and 42% across three nitrogen levels, whereas it was 12%–56% for NUP and 49%–53% for NUE.

### Correlation analysis

The correlation analysis carried out across the twelve parameters, indicated that the traits NUP and NUE were positively associated with SPP, filled grains per panicle (FGP), SFP and SPY (Table 3). NCP showed positive correlations with NUP; while it had significant negative associations with days-to-flowering (DTF), PHT and panicle length in cm (PLG). NUP had positive association with PHT, TLN and PTN. Among agronomic traits, SPY showed significant positive correlation with PTN, SPP, FGP and SFP.

### Genotype-by-environment interaction

The stratification of variance among genotypes, environments and G × E sources of variation varied appreciably from trait to trait (Table 2). The genotypic effect was large and significant for all traits except for the PTN and NUP, for which the environment effect was predominant. The environmental effect was significant for TLN and SPY also. For all other traits, the proportion of environment effect was negligible. Highly significant ($P < 0.01$) G × E effects were noticed for DTF, SPP, FGP, SFP, SPY, NCP, NUP and NUE. The proportion of G × E effect was the highest in NCP followed by genotype and environment effects. The G × E effect was significant ($P < 0.05$) for TLN, PTN and PLG. PHT was the only trait having non-significant G × E effect.

### Principal component analyses

Principal component analyses carried out independently for three nitrogen regimes, extracted four components cumulatively describing total variance of 75.20% (native nitrogen) to 81.09% (high nitrogen). In all cases, principal component 1 (PC1) and principal component 2 (PC2) together described more than 50% of the total variance. Component weights of individual factors, varied considerably between the nitrogen regimes, even shifting the direction of influence across regimes. For PC1, TLN and PTN under native levels showed high negative component weights, which shifted to low positive values in the optimum level and reverted back to negative values under high nitrogen regimes. Similar shifting of vector directions can be seen with NCP and NUP. NUE had positive component weights under native and normal nitrogen regimes and negative influence under high nitrogen (Table 4). The factor orientations also show

**Table 1.** Phenotypic mean values of parents and range for double haploid (DH) lines for various agronomic traits and nitrogen uptake parameters under different levels of nitrogen

| Trait | Mean | | | | | | Range | | |
|---|---|---|---|---|---|---|---|---|---|
| | IR64 | | | Azucena | | | DH lines | | |
| | Native | Normal | High | Native | Normal | High | Native | Normal | High |
| Days-to-flowering (DTF) | 105.33 | 118.33 | 96.33 | 118.33 | 128.00 | 126.00 | 88.70–129.70 | 89.00–126.00 | 95.00–134.70 |
| Plant height (PHT) (cm) | 58.67 | 61.00 | 62.67 | 99.83 | 105.67 | 107.00 | 45.70–127.70 | 46.30–124.70 | 47.00–131.30 |
| Number of tillers (TLN) | 6.33 | 7.00 | 10.67 | 2.00 | 3.00 | 4.00 | 2.00–9.30 | 3.30–9.30 | 3.30–13.30 |
| Number of productive tillers (PTN) | 6.33 | 7.00 | 10.67 | 1.67 | 2.67 | 4.00 | 2.00–7.00 | 2.70–9.30 | 3.30–12.70 |
| Panicle length (PLG) (cm) | 18.70 | 20.50 | 21.00 | 23.30 | 26.00 | 26.17 | 15.30–31.80 | 16.50–30.70 | 16.00–30.50 |
| Spikelets per primary panicle (SPP) | 80.44 | 173.40 | 149.84 | 175.76 | 186.87 | 143.69 | 22.00–185.00 | 20.30–188.31 | 18.12–198.00 |
| Filled grains per panicle (FGP) | 61.00 | 131.00 | 125.00 | 67.67 | 117.00 | 88.67 | 10.30–113.70 | 9.70–118.30 | 7.70–149.70 |
| Spikelet fertility percentage (SFP) | 75.83 | 73.55 | 83.42 | 38.50 | 62.61 | 61.71 | 8.00–83.20 | 7.10–78.00 | 8.30–90.30 |
| Grain yield per plant (SPY) (g) | 7.52 | 10.30 | 15.08 | 5.93 | 6.31 | 3.47 | 0.59–7.19 | 0.43–15.77 | 0.57–15.64 |
| Percent nitrogen content (NCP) | 1.14 | 1.09 | 1.16 | 1.07 | 0.82 | 0.80 | 0.58–1.25 | 0.67–1.25 | 0.63–1.19 |
| Nitrogen uptake (NUP) (g) | 0.17 | 0.19 | 0.34 | 0.14 | 0.16 | 0.18 | 0.05–0.21 | 0.08–0.30 | 0.12–0.38 |
| Nitrogen use efficiency (NUE) | 39.69 | 55.32 | 44.23 | 45.66 | 36.32 | 21.06 | 4.94–93.62 | 2.89–73.67 | 2.68–73.91 |

**Table 2.** Heritability and proportion of variances due to genotype (G), environment (E) and genotype × environment (G × E) for various agronomic traits and nitrogen uptake parameters under different levels of nitrogen

| Traits | Heritability ($h^2$) | | | Variance | | |
|---|---|---|---|---|---|---|
| | Native | Normal | High | Genotype (G) | Environment (E) | G × E |
| Days-to-flowering (DTF) | 0.65 | 0.74 | 0.57 | 76.32** | 0.00 | 23.68** |
| Plant height (PHT) (cm) | 0.85 | 0.92 | 0.87 | 93.30** | 1.22 | 5.48 |
| Number of tillers (TLN) | 0.24 | 0.32 | 0.39 | 44.10** | 36.24** | 19.66* |
| Number of productive tillers (PTN) | 0.23 | 0.30 | 0.42 | 38.82** | 45.19* | 15.99* |
| Panicle length (PLG) (cm) | 0.62 | 0.66 | 0.63 | 83.27** | 4.30 | 12.43* |
| Spikelets per primary panicle (SPP) | 0.34 | 0.28 | 0.43 | 63.84** | 4.10 | 32.06** |
| Filled grains per panicle (FGP) | 0.48 | 0.45 | 0.51 | 70.01** | 2.73 | 27.26** |
| Spikelet fertility percentage (SFP) | 0.53 | 0.48 | 0.60 | 75.83** | 0.15 | 24.02** |
| Grain yield per plant (SPY) (g) | 0.36 | 0.49 | 0.61 | 54.43** | 20.46** | 25.11** |
| Percent nitrogen content (NCP) | 0.42 | 0.27 | 0.27 | 41.29** | 3.44 | 55.25** |
| Nitrogen uptake (NUP) (g) | 0.56 | 0.12 | 0.29 | 25.09** | 47.31** | 27.60** |
| Nitrogen use efficiency (NUE) | 0.49 | 0.53 | 0.53 | 66.13** | 1.00 | 32.87** |

*$P = 0.05$; **$P = 0.01$.

that the position of IR64 was determined greatly by the direction encompassed by SPY, SFP, SPP, NUE and NUP, while position of Azucena was bracketed by PHT and PLG except in native nitrogen regime (Figure 1).

### Main-effect QTLs

Quantitative trait loci analysis revealed 16 main effect QTLs associated with the traits except NUP, distributed on chromosomes 1, 3, 5, 7, 8, 9 and 11 (Table 5). There were three QTLs identified each for PHT and PTN, two for NCP, and one each for DTF, TLN, PLG, FGP, SPP, SFP, SPY and NUE. The likelihood of odds (LOD) values for identified QTLs ranged from 2.37 to 12.43.

### Genetic effects of QTL and QTL-by-environment interaction

The QTL identified for PLG, FGP and SPP had non-significant additive effects within individual environments. The average additive genetic effect of putative QTL for DTF (*qDTF-3*) was 3.15 explaining 16% of the total phenotypic variation (Table 5). Of the three QTLs identified for PHT, *qPHT-1* and *qPHT-3* were contributed by IR64, while *qPHT-8* came from Azucena. These

**Table 3.** Pearson's correlation coefficients showing interrelations of agronomic traits and nitrogen uptake parameters across different levels of nitrogen

| Trait | DTF | PHT | TLN | PTN | PLG | SPP | FGP | SFP | SPY | NCP | NUP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PHT | 0.316** | | | | | | | | | | |
| TLN | −0.211 | −0.498** | | | | | | | | | |
| PTN | −0.218* | −0.476** | 0.965** | | | | | | | | |
| PLG | 0.146 | 0.762** | −0.474** | −0.438** | | | | | | | |
| SPP | 0.019 | 0.094 | −0.076 | 0.004 | 0.206 | | | | | | |
| FGP | −0.102 | 0.202 | −0.319** | −0.272* | 0.374** | 0.664** | | | | | |
| SFP | 0.110 | 0.007 | 0.132 | 0.208 | 0.004 | 0.767** | 0.062 | | | | |
| SPY | 0.062 | 0.030 | 0.192 | 0.297** | 0.112 | 0.822** | 0.334** | 0.826** | | | |
| NCP | −0.336** | −0.452** | 0.211 | 0.221* | −0.273* | 0.167 | 0.054 | 0.159 | 0.205 | | |
| NUP | 0.016 | 0.251* | 0.242* | 0.274* | 0.178 | 0.427** | 0.253* | 0.348** | 0.541** | 0.340** | |
| NUE | 0.041 | −0.072 | 0.050 | 0.151 | 0.081 | 0.718** | 0.296** | 0.744** | 0.851** | 0.040 | 0.099 |

*$P = 0.05$; **$P = 0.01$. DTF, days-to-flowering; FGP, filledgrains per panicle; NCP, percent nitrogen content; NUE, nitrogen use efficiency; NUP, nitrogen uptake (g); PHT, plant height (cm); PLG, panicle length (cm); PTN, number of productive tillers; SFP, spikelet fertility percentage; SPP, spikelets per primary panicle; SPY, grain yield per plant (g); TLN, number of tillers.

**Table 4.** Component weights of the extracted four principal components (PC1 to PC4) for twelve traits under three regimes of nitrogen

| Trait | Native N | | | | Normal N | | | | High N | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PC1 | PC2 | PC3 | PC4 | PC1 | PC2 | PC3 | PC4 | PC1 | PC2 | PC3 | PC4 |
| DTF | 0.164 | 0.001 | 0.357 | −0.405 | 0.095 | −0.217 | −0.192 | 0.022 | 0.026 | 0.103 | 0.455 | 0.351 |
| PHT | 0.378 | 0.118 | 0.477 | 0.061 | 0.126 | −0.430 | 0.238 | 0.303 | 0.098 | 0.446 | 0.124 | 0.419 |
| TLN | −0.449 | −0.179 | 0.154 | 0.060 | 0.021 | 0.491 | 0.201 | 0.315 | −0.293 | −0.386 | 0.047 | 0.331 |
| PTN | −0.424 | −0.238 | 0.131 | −0.036 | 0.079 | 0.486 | 0.230 | 0.293 | −0.313 | −0.382 | 0.050 | 0.282 |
| PLG | 0.332 | 0.023 | 0.446 | 0.289 | 0.177 | −0.402 | 0.248 | 0.146 | 0.112 | 0.463 | 0.044 | 0.280 |
| SPP | 0.241 | −0.429 | −0.086 | 0.159 | 0.461 | −0.059 | −0.066 | −0.177 | −0.370 | 0.322 | −0.016 | −0.178 |
| FGP | 0.130 | −0.181 | −0.186 | 0.716 | 0.283 | −0.233 | 0.219 | 0.033 | −0.148 | 0.364 | −0.267 | −0.240 |
| SFP | 0.178 | −0.358 | −0.020 | −0.385 | 0.425 | 0.138 | −0.238 | −0.211 | −0.404 | 0.088 | 0.200 | −0.042 |
| SPY | 0.160 | −0.505 | 0.027 | −0.115 | 0.490 | 0.134 | −0.030 | 0.065 | −0.479 | 0.112 | 0.072 | −0.019 |
| NCP | −0.284 | −0.249 | 0.076 | 0.047 | 0.026 | 0.131 | 0.296 | −0.773 | −0.126 | −0.018 | −0.647 | 0.046 |
| NUP | −0.211 | −0.325 | 0.509 | 0.149 | 0.253 | 0.066 | 0.628 | −0.022 | −0.275 | 0.127 | −0.382 | 0.497 |
| NUE | 0.284 | −0.359 | −0.313 | −0.142 | 0.399 | 0.115 | −0.400 | 0.149 | −0.387 | 0.094 | 0.300 | −0.305 |
| Eigenvalue | 3.335 | 2.982 | 1.513 | 1.195 | 3.456 | 3.026 | 1.572 | 1.209 | 3.825 | 2.804 | 1.777 | 1.325 |
| Cumulative variance (%) | 27.79 | 52.64 | 65.25 | 75.20 | 28.80 | 54.01 | 67.11 | 77.18 | 31.88 | 55.25 | 70.05 | 81.09 |

DTF, days-to-flowering; FGP, filledgrains per panicle; NCP, percent nitrogen content; NUE, nitrogen use efficiency; NUP, nitrogen uptake (g); PHT, plant height (cm); PLG, panicle length (cm); PTN, number of productive tillers; SFP, spikelet fertility percentage; SPP, spikelets per primary panicle; SPY, grain yield per plant (g); TLN, number of tillers.

QTLs together explained 13.1% of the total phenotypic variation. There were three QTLs associated with PTN, of which two were located on chromosome 3 (*qPTN-3-1* and *qPTN-3-2*) and one on chromosome 9 (*qPTN-9*). Together, they accounted for 13.7% of the total phenotypic variation and were contributed by Azucena. For FGP, a QTL was located on chromosome 7, flanked by RG477 and PGMS0.7 (*qFGP-7*) having a contribution of 3.2%. The QTLs for TLN (*qTLN-1*) and SPP (*qSPP-1*) were found located on the same marker interval of chromosome 1 flanked by RG246 and K5. *qTLN-1* had 12.8% contribution with an average additive effect of –0.444, and had allelic influence from Azucena, while *qSPP-1* had an average additive effect of 2.81, with allelic favor of IR64. Two QTLs, *qPLG-11* and *qSFP-11* shared the same marker interval (RG247–RG103) on chromosome 11, with the QTL peaks located at 100 cM and 80 cM, respectively. The QTLs for SPY, NCP and NUE, *qSPY-3*, *qNCP-3-1* and *qNUE-3*, shared contiguous marker intervals on chromosome 3, involving markers, RG191, RZ678, RZ574 and RZ 284, all having considerable influence on their respective traits. Similarly the QTLs, *qDTF3*, *qPTN-3-2* and

**Table 5.** Effect of detected main effect quantitative trait loci (QTL) and QTL × environment interaction for three N regimes by composite interval mapping

| Trait | Chr. | Marker interval | QTL | Position of QTL peak (cM) | LOD | $R^2$ | QTL additive effect (a) | | | | MS (QTL × E) | General variance explained |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Native | Normal | High | Average | | |
| DTF | 3 | RZ448 – RZ519 | *qDTF-3* | 300 | 2.37 | 15.70 | 2.82* | 4.06** | 2.58* | 3.15** | 44.96* | 16.60 |
| PHT | 1 | RG690–RZ730 | *qPHT-1* | 198 | 12.43 | 50.30 | 5.06** | 6.26** | 6.18** | 5.84** | 14.69* | 13.10 |
| | 5 | RZ390–RG313 | *qPHT-5* | 16 | 4.64 | 24.00 | | 4.38* | | | 8.05* | |
| | 8 | RZ66–AC5 | *qPHT-8* | 142 | 5.76 | 27.70 | | −4.32* | −4.67* | −4.07* | 26.19* | |
| TLN | 1 | RG246–K5 | *qTLN-1* | 28 | 4.65 | 24.30 | −0.32* | −0.44** | −0.58** | −0.44** | 1.54* | 12.80 |
| PTN | 3 | RG348–RZ329 | *qPTN-3-1* | 28 | 4.37 | 22.00 | | | | | | 13.70 |
| | 3 | RZ519–Pgi1 | *qPTN-3-2* | 320 | 4.57 | 22.70 | | | | | | |
| | 9 | RZ12–RG667 | *qPTN-9* | 112 | 9.06 | 40.20 | −0.28* | −0.41* | −0.50* | −0.40** | | |
| PLG | 11 | RG247–RG103 | *qPLG-11* | 100 | 4.61 | 22.80 | | | | | 1.71* | 1.50 |
| FGP | 7 | RG477–PGMS0.7 | *qFGP-7* | 80 | 3.99 | 20.30 | | | | | 169.17* | 3.200 |
| SPP | 1 | RG246–K5 | *qSPP-1* | 46 | 4.19 | 22.20 | | | | | 809.92* | 0.000 |
| SFP | 11 | RG247–RG103 | *qSFP-11* | 80 | 4.71 | 23.30 | −9.82** | | −7.47* | −7.58** | 110.75* | 8.50 |
| SPY | 3 | RZ678–RZ574 | *qSPY-3* | 122 | 5.05 | 24.70 | 0.43* | 0.82* | 0.95* | 0.73* | 4.79* | 10.30 |
| NCP | 3 | RG191–RZ678 | *qNCP-3-1* | 58 | 6.12 | 29.100 | | −0.05* | −0.03* | −0.03* | | 48.20 |
| | 3 | Pgi1– CDO87 | *qNCP-3-2* | 372 | 4.67 | 23.600 | −0.04* | | | −0.03** | | |
| NUE | 3 | RZ574 – RZ284 | *qNUE-3* | 124 | 5.46 | 26.40 | | 5.66* | 3.64* | 4.11** | 19.12* | 11.00 |

*$P = 0.05$; **$P = 0.01$. DTF, days-to-flowering; FGP, filledgrains per panicle; LOD, likelihood of odds; NCP, percent nitrogen content; NUE, nitrogen use efficiency; NUP, nitrogen uptake (g); PHT, plant height (cm); PLG, panicle length (cm); PTN, number of productive tillers; SFP, spikelet fertility percentage; SPP, spikelets per primary panicle; SPY, grain yield per plant (g); TLN, number of tillers.

*qNCP-3-2* shared contiguous marker intervals on chromosome 3 bracketed by markers RZ448 and CDO 87.

Quantitative trait loci-by-nitrogen level interaction was generally low among the main effect QTLs identified barring a QTL for SPP. High level of interaction was shown by this QTL (*qSPP-1*) having high MS(QTL × E) value (809.92) and very low independent contribution toward the general variance. The additive effects of this QTL were insignificant among the nitrogen regimes. There were also differences among the additive effects of the QTLs under different nitrogen regimes. The QTLs for PTN and NCP did not show significant QTL × environment interaction.

## Discussion

Breeding crop plants for better NUE is one of the primary objectives in modern agriculture, considering the importance of imparting low nitrogen tolerance to mitigate the adverse effects of nitrogen saturation and also to decrease fertilizer usage, which bloats the cost of cultivation. This depends on the identification of heritable physiological or biochemical traits, causally related to grain yield. In the present study, a total of nine agronomic parameters associated with nitrogen nutrition and three parameters quantifying NUE were studied. The genetic loci associated with each of the parameters were mapped on a molecular linkage map of IR64/Azucena.

Considerable levels of genotype and genotype × environment variation were observed among the DH lines and parents for all of the traits studied under different nitrogen regimes. Responses of genotypes to the soil as well as applied nitrogen not only depend on the applied form (Rauh et al. 2002) but also on the levels (Gallais and Hirel 2004). The SPP and FGP of IR64 observed in the native regime are smaller than that of normal and high nitrogen regimes. This had possibly occurred due to low nitrogen susceptibility of the IR64 at low (native) levels, which might have considerably reduced the number of filled grains in panicles other than primary. Primary panicle had more filled spikelets because the development of which might have used more of mobilizing nitrogen within the plant system. In Azucena, FGP was lesser in low N conditions, but it produced more SPP, probably as an adaptive mechanism for low nitrogen. The mean performance of DH lines indicated that there were not many differences in DTF, PHT and PLG across the nitrogen levels, whereas there was an increasing trend in TLN, PTN, FGP, SPY and NUP with increasing nitrogen level. The genetic control of these traits is governed by quantitative genes, which may be dominant, additive and/or epistatic. They may have an array of suppressers and modifiers that are under disparate regulatory influences and modify the cumulative expression of the ultimate trait. They are thus differentially expressed under

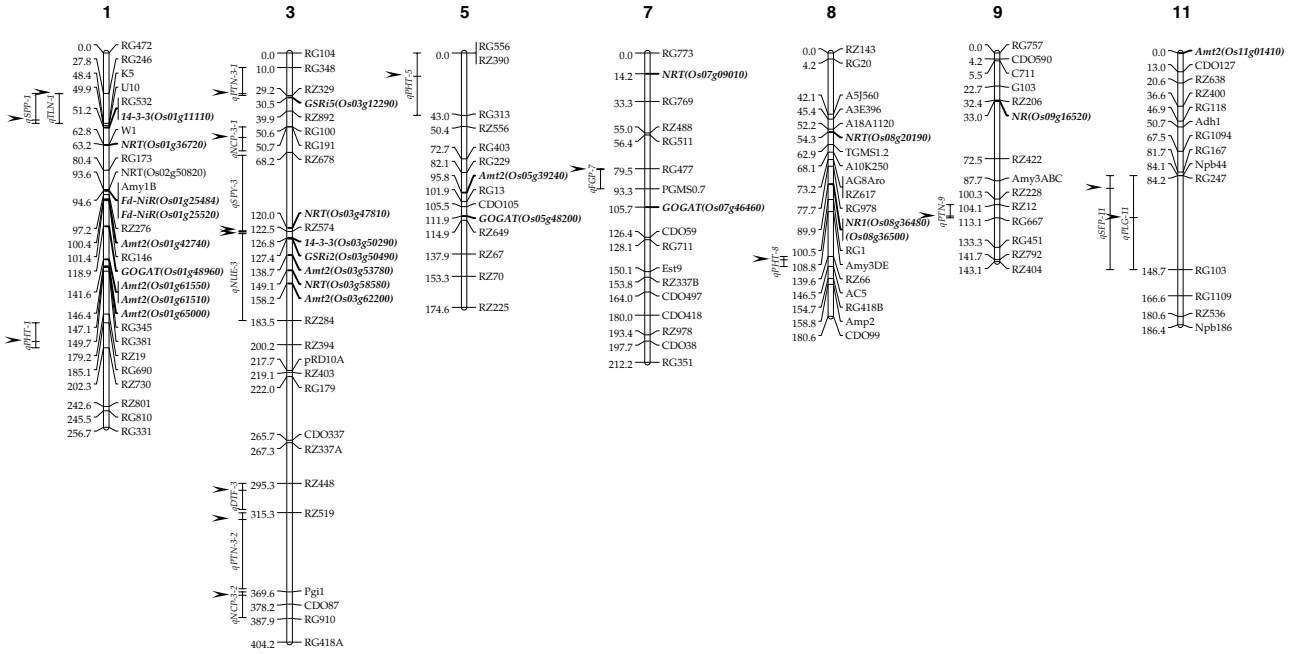


**Figure 1.** Molecular linkage map showing quantitative trait loci (QTL) positive linkage groups of IR64/Azucena double haploid (DH) populations, over which the putative QTL positions and the putative candidate gene (highlighted in bold and italics) locations have been superimposed.

Arrowheads show positions of QTL peaks. The candidate gene locations were based on associated rice genetic marker information generated *in silico* (The Institute for Genomic Research (TIGR) Rice db, Gramene v22.0).

different environments, resulting in genotype × environment interaction. Segregation of traits among the DH lines that was transgressing over the parental values was signifying the action of complimentary, supplementary, and other epistatic genes, besides overdominance to some extent (Rieseberg et al. 1999). Presence of transgressive segregation and low heritability implied that these traits were under the control of an aggregate of genes causing the variation.

Principal component analysis is a powerful procedure in which multi-dimensional datasets are reduced to lower dimensions, in such a way that lower order components describe the maximum variance without affecting characteristics of the datasets (Abdi 2003). The factor component loadings that define the principal components linearly showed variation in both magnitude and direction across the nitrogen regimes, showing clear genotype × environment interaction for all of the traits. Rotation of factor component and scatter plots is seen across the nitrogen regimes (Figure 2). Congregation of factors based on the vector directions are seen between TLN and PTN, followed by PHT, DTF and PLG under all nitrogen regimes. SPY was in a group with SFP and NUE under normal and high nitrogen regimes, while a deviation was seen in native nitrogen. Similar deviations are seen with NCP and NUP also. This suggested that the nitrogen fertilization did not affect the association among agronomic traits, but influenced the factors related to nitrogen assimilation to a great extent. Positional orientation of IR64 and Azucena under normal and high nitrogen regimes was greatly influenced by similar factors. There was deviation in the position of these genotypes under native (low) nitrogen conditions, especially in the case of Azucena, which moved towards nitrogen assimilation factors. On the other hand, IR 64 was found moving away from NUE. This indicates that Azucena was less affected by low nitrogen situations than IR 64 and could adapt better under nitrogen-starved situations.

Some quantitative traits like nutrient assimilation efficiency are more complex than others. Macronutrients such as nitrogen are essentially required for plant growth and function (Mattson et al. 1991), as it forms the basic building blocks of amino acids and proteins. Moreover nitrogen is assimilated in the organic form. For assimilation, nitrogen needs to be absorbed exclusively from soil, though it is present in free form in the atmosphere. Conversion of atmospheric nitrogen into an inorganic form and its incorporation into soil is a complicated process, which does not take place easily in nature. Thus nitrogen becomes the most limited primary nutrient in nature. The inorganic nitrogen in the soil is available to plant either in nitrate or ammoniacal forms. Nitrate nitrogen is the most common form available to plants. On absorption, nitrate is converted into an ammoniacal form through an intermediary form of nitrite. The ammonia is then incorporated into organic

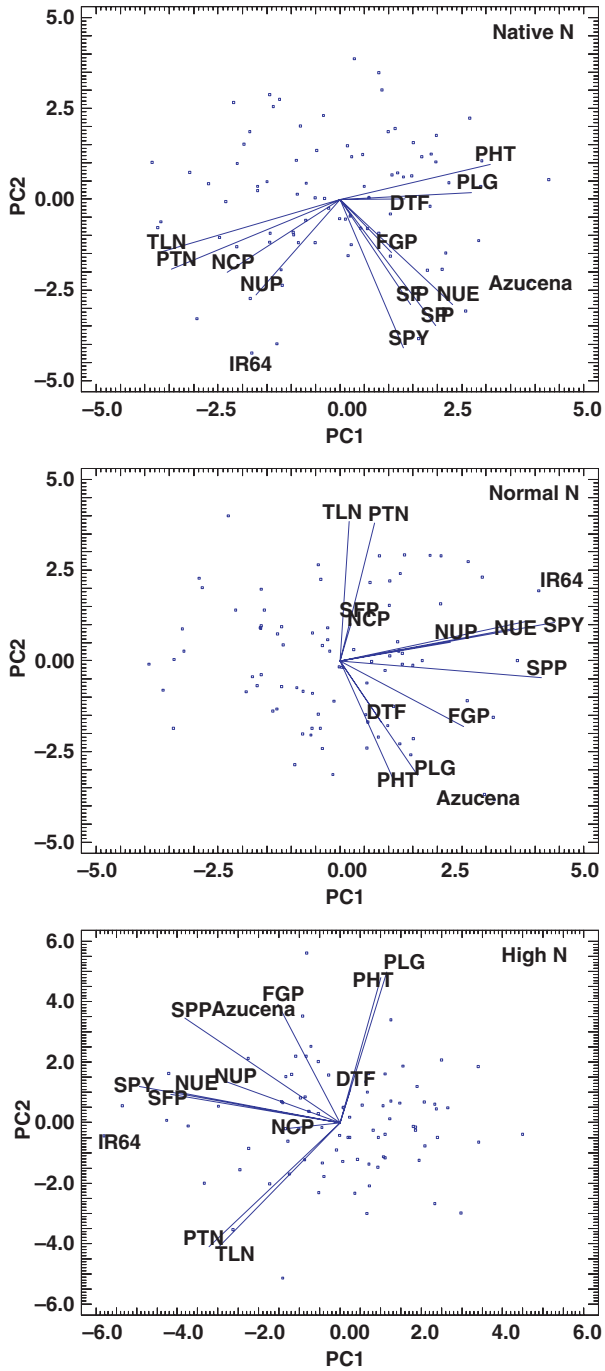


**Figure 2.** Biplots showing scatter of double haploid (DH) lines and components under three nitrogen regimes by principal component analyses.

The positions of both the parents IR 64 and Azucena are shown. DTF, days-to-flowering; FGP, filledgrains per panicle; NCP, percent nitrogen content; NUE, nitrogen use efficiency; NUP, nitrogen uptake (g); PHT, plant height (cm); PLG, panicle length (cm); PTN, number of productive tillers; SFP, spikelet fertility percentage; SPP, spikelets per primary panicle; SPY, grain yield per plant (g); TLN, number of tillers.

form. On the other hand, a limited number of plants are capable of absorbing nitrogen in the ammoniacal form and rice is one among them (Wang et al. 1993; Kronzucker et al. 1999). However, absorption of nitrate and ammonia by roots depends on many transport systems, namely, high affinity transport systems (HATS) and low affinity transport systems (LATS). Members of nitrate transporters and ammonium transporters belonging to both these systems are reported (Glass et al. 2002). Among these genes, NRT 1 and NRT 2 families are very crucial in nitrate uptake in plants (Crawford and Glass 1998). Furthermore, the nitrate pathway is primarily regulated by enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) and glutamate synthase (GOGAT) (Meyer and Stitt 2001). In addition, many genes encoding for enzymes like glutamate dehydrogenase, aspargene synthase, urease may also be involved (Gerendas et al. 1998; Glevarec et al. 2004; Lillo 2004). Microarray analyses had revealed, as many as 115 genes upregulated in tomato roots (Wang et al. 2001) and 1 176 genes either being up or downregulated in *Arabidopsis* roots after nitrate induction, based on the mRNA hybridization (Wang et al. 2003).

The prime upshot of this study is the establishment of main effect QTLs, and their interactions to the varying nitrogen regimes, for describing the phenotypic performance of the DH population. One prominent QTL was identified with 12.43 LOD and an $R^2$ value of 50.3 for PHT in the present study. This QTL, *qPHT1* was located on chromosome 1 (RG690–RZ730) and found expressed in all of the nitrogen levels. This can be considered as a stable QTL. Fang and Wu (2001) found a stable QTL flanked between RZ730–RZ801, corresponding to the locus of the semi-dwarfing gene, *sd-1*, which was tightly linked to the marker RZ730 on chromosome 1 in IR64/Azucena DH line population (Yu 1991; Cho et al. 1994; Huang et al. 1996). The other two QTLs for PHT were not detected under native nitrogen, but in normal and high nitrogen regimes. Similar detection of QTLs for plant height that interacted with nitrogen levels has been reported (Fang and Wu 2001). It was seen here that chromosome 3 harbored seven QTLs out of 16 identified. Among this, the QTL for SPY was detected in all of the nitrogen regimes, while its nearest interval addressing NUE was expressed only under nitrogen-fertilized regimes. This suggested that this location might be sensitive to added nitrogen. Fang and Wu (2001) identified a plant height affecting QTL associated closely with marker RZ284, sensitive to low nitrogen levels under nutrient solution culture and soil culture. Abundance of QTLs affecting nitrogen-related traits on chromosome 3 has also been reported recently by Laza et al. (2006). These QTLs were found to be associated with nitrogen uptake, shoot nitrogen content, plant height, filled grains per panicle, panicle number, and above ground dry weight. In this study, no QTL was identified for NUP, which recorded the highest proportion of environment effect followed by genotype × environment effect.

It is striking to note that 11 out of 17 QTLs detected showed significant ($P < 0.05$) interaction with nitrogen levels. This indicates that significant influences are on for agronomic and nitrogen assimilation rates, at varying levels of nitrogen. Studies on quantitative genetic control of nitrogen assimilation across species show complex patterns of regulatory mechanisms. Evidence suggests that HATS and LATS genes in higher plants are more root-specific, and nitrate inducible and are repressed by nitrogen metabolites (Daniel-Vedele et al. 1998). Also, it is likely that both the transporter systems are active during the early stages of crop growth, but HATS continue to function even in the later stages. Thus HATS probably plays a major role in improving grain protein content and the number of fully developed grains (Abrol et al. 1999). Besides, key enzymes in the nitrogen assimilation pathway show complex regulatory mechanisms. Particularly, nitrate reductase is very sensitive to stress and shows low affinity to nitrate, and is unstable *in vitro*. Higher plants also harbor multiple conserved sequences of the *Nia* gene encoding for nitrate reductase, and evidence suggests that plants do not benefit from excess activity of this enzyme. However, it may be worth mentioning here that overexpression of the enzymes of ammonia assimilation increases the total protein accumulation under nitrogen deprivation and showed higher carbon dioxide assimilation (Chichkova et al. 2001). This may further support the studies of Hirel et al. (2001), wherein the QTLs were detected for physiological traits such as nitrate content, nitrate reductase, and glutamine synthetase activities in maize. Yamaya et al. (2002) have reported putative QTLs associated with the contents of glutamine synthetase, which is probably involved in the export of nitrogen from senescing organs.

It is known that nitrogen content in plants is predominantly affected by the Rubisco content, which strongly affects photosynthesis. About 50% of the total soluble protein and 25% of the total nitrogen are in Rubisco protein in rice leaves (Makino 2003). Ishimaru et al. (2001) detected QTL for Rubisco and soluble protein on chromosome 8 in rice. An additional QTL for Rubisco was also identified on chromosome 9. To have the better results, the physiological parameters should be measured by rapid nitrate tissue tests (Diphenylamine-, Merckoquant-, Reflectoquant-method) and chlorophyll meter-tests to evaluate the nitrogen-nutritional status of individual genotype across environments. The rapid nitrate tests were already used in wheat, barley, rape, sugar beet and maize in field experiments at sites differing in total nitrogen content of the soil and in mineralization rate of nitrogen.

The QTLs mapped can be regarded as intermediaries in the genetic analyses, between statistically defined loci and Mendelian genes (Paran and Zamir 2003). This multiple trait QTL mapping method will certainly improve the efficiency of QTL mapping as indicated by (Jiang and Zeng 1995). Of a total of 26 expressed candidate gene sequences associated with enzymes involved in the nitrogen metabolism in rice distributed over some of the identified QTL positions in the present study can be compared to the loci of few expressed candidate genes involved in nitrogen metabolism in rice. We attempted to place these candidate genes by *in silico* methods on rice chromosomes 1, 3, 5, 7, 8, 9 and 11 (Figure 1). It was interesting to find that co-localization of genes putatively expressing glutamine synthetase, glutamate synthase, nitrate transporter, ammonium transporter and 14-3-3-like protein along with the detected QTLs for nitrogen use efficiency and plant yield. Furthermore, high level of QTL effects and QTL × environment interactions observed in this study implies manifold regulation of nitrogen response genes in rice. This could be facilitated additionally by QTLs distributed outside the vicinity of candidate gene loci. This corroborates the need to generate precise information about the QTL mapped for nitrogen response in rice using explicit phenotypic parameters and accurate phenotyping, which will positively lead to the establishment of functionality to the genes of nitrogen metabolism by combining various approaches such as QTL mapping, candidate genes mapping and *in silico* biology.

## Materials and Methods

### Plant materials and environments

A subset of 82 double haploid rice (*Oryza sativa* L.) lines drawn out of 135 lines obtained from the International Rice Research Institute, the Philippines was used in this study. These lines were generated through *in vitro* anther culture of $F_1$ between IR64, an *indica* variety adapted to irrigated conditions and Azucena, a traditional upland *japonica* variety from the Philippines (Guiderdoni et al. 1992).

Sowing of the selected lines along with their parents was carried out in seedling boxes. Twenty-day-old seedlings of each line were planted in pots measuring 25 × 30 cm filled with approximately 4.5 kg of soil (12.74% moisture). The soil was a nitrogen-deficient clay loam falling under the group Typic Haplustalf with an ammoniacal N (KCl extractable) concentration of 37.07 μg/g of soil and available N ($KMnO_4$-N) concentration of 277 kg/ha. A single plant was maintained in each pot from seedling to maturity. A total of nine pots were maintained for each line split into three sets corresponding to different nitrogen levels (native, optimum and high) with three replications per level.

Three levels of nitrogen, namely, native (low), optimum and high were maintained. Native level was not supplied with any nitrogen fertilization (0 kg/ha); optimum level was applied with 1 630 mg of nitrogen fertilizer (750 mg N) per pot in the form of urea, which is the normal recommended level for rice (100 kg/ha) and high level was added with 3 260 mg of urea per pot (1 500 mg N) that was two times more than the normal dose (200 kg/ha). Nitrogen was applied in four split doses: 25% as

basal and 25% each at tillering, panicle initiation and heading stages. Other major nutrients, phosphorus and potassium were applied to all of the pots in the form of superphosphate and muriate of potash at the rate of 2 345 mg (375 mg $P_2O_5$) and 625 mg (375 mg $K_2O$) per pot, respectively. All fertilizers were applied in the form of aqueous solution.

### Agronomic traits

Nine traits of agronomic importance that are presumably affected by nitrogen nutrition, namely, days-to-flowering (DTF), plant height (PHT), total number of tillers including non-productive ones (TLN), number of productive tillers alone (PTN), panicle length (PLG), spikelets per primary panicle (SPP), filled grains per primary panicle (FGP), spikelet fertility in percentage (SFP) and grain yield (SPY) were measured on a single plant basis from all three replications across different nitrogen levels. At maturity, the plants were cut just above ground level. The grains were threshed manually and sun-dried. Filled and unfilled grains were separated manually. Grain yield at 14% moisture level was recorded.

### Nitrogen analysis

The dry weight of filled grains, unfilled grains, straw and rachis were determined after oven drying to a constant weight at 70 °C. The oven-dried unfilled grains, straw, and rachis were pooled and ground in a cyclone mill and the grains were ground separately to obtain fine powdered samples. Three grams of composite sample was prepared by taking a portion of the straw sample (straw + unfilled grain + rachis) and the grain sample proportionate to their weight and stored in tightly sealed vials. A sub-sample of 0.5 g was used to determine total nitrogen content by the Microkjeldhal method (Humphries 1956). The biomass of filled grains, unfilled grains, rachis and straw were added and expressed as plant dry weight (g/plant) on oven-dry weight basis (70 °C). Three parameters, namely, percent nitrogen content in plant (percentage of nitrogen in the pooled sample of straw and grain, NCP), total nitrogen uptake (plant dry weight (g/plant) × percent nitrogen content in plant, NUP) and nitrogen use efficiency (grain yield (g/plant)/total nitrogen uptake, NUE) were computed.

### Data analysis

The data on individual traits under varying nitrogen regimes were subjected to analysis of variance, association analysis, principal component analysis and QTL analyses. The phenotypic and genotypic variance and heritability of the traits were computed. The frequency distribution of the traits in the mapping population was also determined.

The molecular marker data developed by Huang et al. (1997) for IR64/Azucena mapping population with 175 markers including 135 restriction fragment length polymorphism (RFLP) markers and 40 isozyme and/or Random Amplified Polymorphic DNA (RAPD) markers was used to identify putative QTL for various traits involved in the study. The mean phenotypic values from three replications for all 12 parameters under three different nitrogen regimes were subjected to composite interval mapping using multi-environment analysis option of PLABQTL v1.2 (Utz and Melchinger 2003). To get an unbiased estimate of LOD threshold for each trait, 1 000 permutations were run using a permutation test (Churchill and Doerge 1994). The detected QTLs were named as per McCouch et al. (1997), with a "q" prefixed to the trait name followed by the chromosome number separated by a hyphen (-). If more than one QTL was located on the same chromosome for the same trait, the QTLs were serially numbered and prefixed by another hyphen (-).

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