**RESEARCH ARTICLE**

**DEP1** is involved in regulating the carbon–nitrogen metabolic balance to affect grain yield and quality in rice (*Oriza sativa* L.)

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**Abstract**

The **DEP1** (*dense and erect panicle 1*) gene, which corresponds to the erect panicle architecture, shows a pleiotropic effect in increasing grain yield and nitrogen use efficiency (NUE) in rice. Nevertheless, it remains unclear whether the carbon–nitrogen metabolic balance changes as the dep1 allele enhances nitrogen uptake and assimilation. In this study, we generated transgenic Akitakomati plants by overexpressing dep1 and analyzed the carbon–nitrogen metabolic status, gene expression profiles, and grain yield and quality. Under either low or high nitrogen growth conditions, the carbon–nitrogen metabolic balance of dep1-overexpressed lines was broken in stem sheaths and leaves but not in grains; the dep1-overexpressed plants showed higher expressions of glutamine synthetase (GS) and glutamate synthase (GOGAT) genes than the wildtype, along with increased total nitrogen and soluble protein content in the straw at maturity. However, the ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) and phosphoenolpyruvate carboxylase (PEPC) genes were downregulated in dep1-overexpressed plants, leading to a decreased carbohydrate content and carbon/nitrogen ratio. Although the unbalanced carbon–nitrogen metabolism decreased the grain-filling rate, grain setting percentage, 1000 grain weight, and grain quality in dep1-overexpressed lines, it led to increased grain numbers per panicle and consequently increased grain yield. Our results suggest that an unbalanced carbon–nitrogen metabolic status is a major limiting factor for further improving grain yield and quality in erect panicle varieties.

**Introduction**

Nitrogen is an essential nutrient in the growth and development of plants [1]. A great deal of nitrogen fertilizer is applied to fields to maximize grain yield for its significant effect on crop productivity [2]. However, excessive nitrogen fertilizer application results in severe environmental pollution, particularly in aquatic ecosystems [3]. Thus, it is important to optimize the
use of nitrogen fertilizers to make agriculture more sustainable. One method of optimization is to increase nitrogen use efficiency (NUE) through genetic improvement, particularly in rice (*Oryza sativa* L.), which would increase grain yield with less nitrogen fertilizer [4].

Inorganic nitrogen is mainly absorbed by ammonium transporters (AMTs) in rice roots and is assimilated via glutamine synthetase (GS) within the plant; its product (glutamine) is digested into glutamate by the GS/GOGAT cycle or into asparagine by asparagine synthetase (As) [5]. In the past few decades, GS was the main focus in crop research due to its key role in controlling nitrogen assimilation [6]. Many previous studies have attempted to overexpress the GS gene in rice to obtain transgenic plants with higher NUE; however, GS-overexpressed plants exhibit poor plant growth and less grain yield [7–9]. Unbalanced carbon–nitrogen metabolism is the crucial reason for decreased grain yield in GS-overexpressed plants [8]. Metabolite profiles of a knockout mutant of rice *GS1;1* have revealed an imbalance in the levels of sugars, amino acids, and metabolites in the tricarboxylic acid cycle [10]. For these reasons, although the activity of GS is related to NUE, it is still difficult to increase rice yield by overexpressing it.

The synthesis of nitrogen-containing compounds, including various amino acids, proteins, or enzymes, requires the incorporation of ammonium into their carbon skeletons. The required energy and carbon skeletons for ammonium assimilation are provided from sucrose, glucose, organic acids, and other carbohydrates [11]. Maintaining an appropriate balance of carbon–nitrogen metabolites plays an important role in plant growth and development [12]. Many studies have indicated that numerous central metabolites involved in carbon and nitrogen metabolism can be altered in parallel rather than antagonistically if the nitrogen or carbon supply is changed [13–15]. In order to determine the carbon and nitrogen metabolism status of plant tissues, the carbon/nitrogen ratio is usually used as an empirical indicator [7–9].

Recently, a major quantitative trait locus for NUE in rice was cloned that is synonymous with the *DENSE AND ERECT PANICLES 1* (*DEP1*) gene [16]; the *DEP1* protein belongs to a γ subunit of the heterotrimeric G protein [17, 18], which not only plays an essential role in nitrogen signaling [16] but is also involved in carbon metabolism [19–21]. Rice plants carrying the gain-of-function *dep1* allele exhibit erect panicles, higher GS activity, and higher nitrogen uptake even under lower nitrogen growth conditions, and consequently, have increased NUE and grain yield [16]. Therefore, a feasible way to increase the grain yield is by introducing the *dep1* allele into varieties with curved panicles. However, it remains unknown whether the carbon–nitrogen metabolic balance and grain quality will change, while NUE and grain yield are improved in transgenic *dep1* plants.

Akitakomati, a *japonica* variety with good grain quality, carries the *DEP1* allele and exhibits curved panicles. A previous study found that the grain yield of Akitakomati is lower than that of some erect panicle varieties with the *dep1* allele [22]. In this study, we introduced *dep1* into Akitakomati by a binary vector, and then analyzed the carbon–nitrogen metabolic status, gene expression profiles, and grain yield and quality of the transgenic plants under low and high nitrogen growth conditions. The aim was to detect the effect of the *dep1* allele on the carbon–nitrogen metabolic balance, yield traits, and grain quality in curve panicle varieties, which may provide a theoretical basis for the improvement of rice varieties.

**Materials and methods**

**Plant transformation**

Akitakomati carries the *DEP1* allele and exhibits curved panicles. In contrast, Liaojing5 with the *dep1* allele exhibits erect panicles. *DEP1* and *dep1* are a pair of alleles, and the erect panicle is dominant. In this variety, a 12-bp nucleotide sequence replaces a 637-bp region in the
middle of exon 5 at the DEP1 locus. [22–24]. The full coding sequence of dep1 (FJ039905) was isolated from the cDNA of the erect panicle variety Liaojing 5 (S1 Table) and was ligated into the binary vector pBWA(V)HS that uses the CaMV 35S promoter (Fig 1A). As the dep1 allele at the DEP1 locus shows a gain-of-function mutation [24], the binary vector carrying the dep1 allele was transformed into the curved panicle variety Akitakomati to obtain dep1-overexpressed plants (erect panicle mutants) via the Agrobacterium tumefaciens-mediated transformation method by Wuhan Biorun Biotechnology Company (www.biorun.net). Twenty-one transgenic plants were obtained from one independent transformation by using the vector pBWA(V)HS. The hygromycin resistance gene (HYG) was used as a selectable marker to identify positive transgenic plants by PCR (S1 Table), and copy numbers (S2 Table) were determined by qRT-PCR in the T0 generation [25]. Among the 21 transgenic plants, two positive transgenic plants with a single copy, TL35 and TL44, exhibiting erect panicle architecture and high expression of dep1 (Fig 1B–1D) were used to generate the T1 generation. Homozygous T1 generation plants (Fig 1E; S3 Table) were identified by qRT-PCR [26], and the seeds were selected and used to generate the T2 generation for subsequent study.

**Plant growth conditions**

The experiments were conducted in an isolated paddy field at the experimental farm of Shenyang Agricultural University, Shenyang (41.8˚N, 123.4˚E), China, in the summer of 2016. Seeds of dep1-overexpressed plants (TL35 and TL44) in the T2 generation and wildtype (Akitakomati) were sown in a seedling nursery on April 26, 2016 with one seedling being transplanted per hill on May 24, 2016. Seedlings were transplanted at 30 cm × 13 cm spacing. The
soil contained organic matter of 26.41 g kg\(^{-1}\), total nitrogen of 0.92 g kg\(^{-1}\), available nitrogen of 0.06 g kg\(^{-1}\), available phosphorus of 0.03 g kg\(^{-1}\), available potassium of 0.12 g kg\(^{-1}\), and pH of 5.87. Two nitrogen fertilizer treatments were used, including low nitrogen (LN, 0 kg ha\(^{-1}\) nitrogen, 60 kg ha\(^{-1}\) phosphorus, 100 kg ha\(^{-1}\) potassium) and high nitrogen (HN, 200 kg ha\(^{-1}\) nitrogen, 60 kg ha\(^{-1}\) phosphorus, 100 kg ha\(^{-1}\) potassium) conditions. These fertilizers were applied as a basal dressing using slow-releasing urea, P\(_2\)O\(_5\), and KCl. The field trials were performed in randomized complete blocks with three replications per line (TL35, TL44, and wild-type), and each plot area was 12 m\(^2\). Each replication was separated into two nitrogen treatments, and three lines were randomly arranged into each nitrogen treatment.

**Gene expression**

The dep1 allele was expressed in the root, leaf, culm, inflorescence meristem, and young inflorescence, and exhibited the highest expression in the inflorescence meristem at the stage of primary and secondary rachis branch formation [24]. We analyzed the effect of overexpressing the dep1 allele on the expressions of several genes involved carbon–nitrogen metabolism in leaves at the booting stage when the primary and secondary rachis branches were newly formed. The leaf materials of transgenic lines (TL35, TL44) and wildtype were sampled from three biological replications under the LN and HN conditions, frozen immediately in liquid nitrogen, and stored at ~80°C until use. Total RNA was extracted with TriZol reagent (Invitrogen, Germany) according to the manufacturer’s instructions. First strand cDNAs were synthesized from DNaseI-treated total RNA using a Primer Script RT reagent Kit with gDNA Eraser (Takara, Japan) following the manufacturer’s instructions. qRT-PCR was performed in an optical 96-well plate with a real-time PCR system (BIO-RAD). Each reaction contained 3.0 μl of first-strand cDNAs, 2 μl of 200 μM gene-specific primers, and 12.5 μl of 2×SYBR Green Master Mix reagent (Applied Biosystems) in a final volume of 25 μl. Amplification conditions were at 95°C for 3 min, followed by 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s. The specific primers of tested genes and the reference gene (ACTIN1) are listed in S1 Table. The qRT-PCR analysis was performed for each cDNA sample with four replications. Relative expression levels were calculated by \(2^{-\Delta\Delta CT} \) [27]. Normalized expression for TL35 or TL44 was calculated as \(\Delta ACT = (C_T, Target - C_T, actin)_{TL} - (C_T, Target - C_T, actin)_{wildtype}\). The results presented are the mean values of three biological replicates for each genotype.

**Chlorophyll content and gas exchange parameters**

As differences can usually be observed in phenotype after a change in gene expression levels, we measured the chlorophyll content and gas exchange parameters at the heading stage. One in every 30 plants of TL35, TL44, and wildtype grown under LN and HN conditions were selected to measure the chlorophyll content of flag leaves by a SPAD-502 plus leaf chlorophyll meter (Minolta Camera Co., Osaka, Japan) once every five days after the initial heading stage. At the full heading stage, one in every 12 plants of TL35, TL44, and wildtype grown under LN and HN conditions were selected to measure gas exchange parameters of flag leaves using a LI-6400 portable photosynthesis system (LI-COR, USA). The light intensity was set at 1,500 μmol m\(^{-2}\)s\(^{-1}\). The leaf temperature was kept at 25–30°C, along with a relative humidity of 60%–65%, a CO\(_2\) concentration of 380 μmol (CO\(_2\)) mol\(^{-1}\), and an air flow of 500 μmol s\(^{-1}\). All measurements were performed in the morning (9:00–11:30 am).

**Grain-filling rate**

Three plants each from TL35, TL44, and the wildtype grown under LN and HN conditions were selected to measure the dry weight of the superior (the top first and last two grains of the
upper three primary branches, and the top first grain of the second branch in the upper three primary branches) and inferior grains (the top third and fourth grains of the bottom three primary branches, and the last two grains of the second branch in the bottom three primary branches) every five days after the heading stage. Approximately 100 grains per plant were selected for measurement. Richards’s growth equations as described by Yang et al. [28] were used to simulate the grain-filling process and calculate the grain-filling rate: 

\[ W = \frac{A}{1 + Be^{-kt}}^{1/N}, \]

where \( W \) is the grain weight (g 100 grains \(^{-1}\)), \( A \) is the final grain weight, \( t \) is the days after heading, and \( B, k, \) and \( N \) are the parameters determined by regression analysis. Grain-filling duration was taken when \( W \) was from 10% \((t_1)\) to 90% \((t_2)\) of \( A \). The mean grain-filling rate during the active filling period was calculated from \( t_1 \) to \( t_2 \).

Grain yield and quality

At the maturity stage, the above-ground portions of 60 plants of TL35, TL44, and wildtype were harvested from each plot. After counting panicle numbers and measuring plant height, ten average-sized panicles were taken from each plot to observe the panicle length and the numbers of primary and secondary branches. Then, the panicles were hand-threshed and placed in water. Filled grains, sunk in water, were separated from the unfilled grains. To determine dry weight, the filled and unfilled grains were then oven-dried at 80˚C for two days. The number of grains per panicle and grain setting percentage were calculated using the above data. The stem sheaths, leaves, and remaining grains of plants were used to determine the biomass, actual yield, and harvest index.

Ten fully filled seeds from each plot were used to measure the grain length (GL), grain width (GW), and grain thickness (GT) using a Vernier caliper. Rough rice for each plot was de-husked and milled to measure the milling quality using a miller according to the National Standards GB/T17891-1999. One hundred milled head rice grains were used to measure the chalkiness grain rate and chalk size. The viscosity of the cooked rice grain was determined using a Rapid Visco Analyzer (RVA-4, Newport Scientific, Sydney, Australia) to obtain profile characteristics according to Standard Method AACC61-02 as recommended by the American Association of Cereal Chemists.

Carbon and nitrogen metabolites

At the maturity stage, the stem sheaths, leaves, and grains of TL35, TL44, and wildtype grown under LN and HN conditions were used to measure the carbon and nitrogen metabolites. Three samples from stem sheath, leaf, and grain materials from three biological replications were oven-dried at 85˚C for 48 h and ground. Samples of ~0.6 g DW (dry weight) were used to measure the total carbon and nitrogen content by a C/N analyzer (Elementar, Vario MAX CN, Germany) according to the manufacturer’s instructions, with L-glutamic acid as a standard. The NUE was calculated with the grain yield divided by the nitrogen accumulation in whole plants.

Dry samples of stem sheaths, leaves, and grain materials from three biological replications were used to measure soluble protein content [29]. Samples of ~0.5 g DW were homogenized by an extraction buffer [10 mM Trizma (pH 7.5), 10 mM MgSO\(_4\), 5 mM sodium glutamate, 1 mM dithiothreitol, 0.05% (v/v) Triton X-100, and 10% (v/v) glycerol]. Then the homogenates were centrifuged at 2,000 rpm at 4˚C for 30 min. The supernatant was used to measure the soluble protein content by Coomassie Brilliant Blue G-250 reagent (Sigma) with four replications for each sample.

Dry samples of stem sheaths, leaves, and grain materials from three biological replications were used to measure soluble carbohydrate content. Samples of ~0.2 g DW were homogenized
by 6 ml of 80% ethanol at 80°C for 30 min. The homogenates were centrifuged at 2,000 rpm at 4°C for 30 min, and the supernatant was collected three times, filtered by activated carbon, and diluted in 80% ethanol to 50 ml. The extract was used to measure the sucrose content by the resorcinol-photometric method and the soluble sugar content by the anthrone-photometric method [30]. In addition, the oven-dried residue was homogenized by 2 ml distilled water at 100°C for 20 min, and 2 ml of 9.2 mM perchloric acid was added. The homogenates were centrifuged in 2,000 rpm at 4°C for 30 min, and the supernatant was collected in duplicate. The extract was used to measure the starch content by the anthrone-photometric method [30] with four replications for each sample.

**Statistical analysis**

Data analysis was conducted for each trait by analysis of variance (ANOVA) in a general linear model by SPSS 19.0 (SPSS, Inc., Chicago, USA). The treatments with different amounts of nitrogen fertilizer and genotypes were considered as fixed effects. Probability values of less than 0.05 were considered to be significant. Means from three replicates were subjected to the Duncan’s multiple range tests at the $P < 0.05$ level.

**Results**

**The **dep1**-overexpressed plants exhibited erect panicles**

Using the **A. tumefaciens**-mediated method, the pBWA(V)HS vector carrying the **dep1** allele and 35S promoter (Fig 1A) was transformed into the curved panicle variety Akitakomati. Two positive transgenic plants, TL35 and TL44, were identified in the T₀ generation by the selectable marker **HYG** (Fig 1B). Although the transgenic plants TL35 and TL44 contained the wild-type allele (**DEP1**), they exhibited erect panicle architecture (Fig 1C), which was mainly attributed to the higher expression level of the transformed **dep1** allele in leaves, which is a gain-of-function (Fig 1D). Homozygous plants were obtained in the T₁ generation from the single-copy transgenic plants TL35 and TL44 (Fig 1E) and were used to generate the T₂ generation.

**Physiological traits in **dep1**-overexpressed lines**

The chlorophyll content and gas exchange parameters were compared between transgenic lines and the wildtype after the initial heading stage (Fig 2). The chlorophyll content of flag leaves reached the maximum at 15 and 10 d after the initial heading date under LN (Fig 2A) and HN (Fig 2B) conditions, respectively, and then began to decrease gradually. During this period, the chlorophyll content of transgenic lines (TL35 and TL44) carrying the **dep1** allele declined slowly compared with the wildtype. However, transgenic lines (TL35 and TL44) did not exhibit a significant increase in photosynthetic rate, stomatal conductance, or transpiration rate compared with the wildtype under LN and HN conditions at the full heading stage (Fig 2C–2E).

Compared with the wildtype, transgenic lines (TL35 and TL44) exhibited higher total nitrogen and soluble protein content in stem sheaths and leaves under LN and HN conditions at the maturity stage (Table 1). In contrast, these transgenic lines had lower total carbon, starch, sucrose, and soluble sugar content in stem sheaths and leaves, which led to a decrease in the carbon/nitrogen ratio in these tissues under LN and HN conditions. There was no significant difference in most carbon–nitrogen metabolites in grains between transgenic lines and the wildtype.
Nitrogen absorption and carbohydrate assimilation of these lines were also analyzed at the maturity stage (S4 Table). Compared with the wildtype, transgenic lines (TL35 and TL44) had higher nitrogen accumulation in whole plants under LN and HN conditions, whereas most of the carbohydrate accumulation of these transgenic lines was decreased in stem sheaths and leaves but increased in grains.

Gene expression patterns in dep1-overexpressed lines

Key genes in the carbon–nitrogen metabolic pathway were detected at the booting stage; these genes had different expression patterns between transgenic lines TL35 and TL44 and the wildtype plants (Fig 3 and S1 Fig). Under LN conditions, compared with the wildtype, the expression levels of GS1;1, GS1;2, NADH-GOGAT1, NADH-GOGAT2, AS, and PEPC1 were significantly increased in the transgenic lines TL35 and TL44, whereas the expressions of NR1, NR2, Fd-GOGAT, GDH1 RUBISCO, PEPC2, PEPC3, PEPC4, PEPC6, and PEPC7 were significantly decreased (Fig 3B). Under HN conditions, compared with the wildtype, the expression...
levels of GS1;1, GS1;2, NADH-GOGAT1, and AS were significantly increased in the transgenic lines TL35 and TL44, whereas those of NR1, NR2, NiR, NADH-GOGAT2, GDH1, RUBISCO, PEPC1, PEPC2, PEPC3, PEPC6, and PEPC7 were significantly decreased (Fig 3C).

### Grain yield and quality in transgenic dep1 lines

The *dep1*-overexpressed lines (TL35 and TL44) exhibited erect panicle types along with a shorter plant height and panicle length, and had increased primary and secondary panicle branches and grain density (Table 2). Under LN conditions, transgenic lines (TL35 and TL44) showed increased grain yields of 27.07% and 34.53% compared to the wildtype, which was mainly attributed to the higher number of panicles per plant, the number of grains per panicle, and biomass. Under HN conditions, the grain yields of transgenic lines (TL35 and TL44) were 14.82% and 13.08% higher than that of the wildtype due to the raised grain numbers per panicle and harvest index. These transgenic lines also exhibited higher NUE than the wildtype under LN and HN conditions. However, the grain setting percentage and 1000 grain weight were significantly decreased in transgenic lines (TL35 and TL44) under either LN or HN conditions. There was a difference in grain weight accumulation after the heading stage between transgenic line (TL35 or TL44) and the wildtype (Fig 4). Grain-filling dynamic analysis for transgenic lines TL35 and TL44 showed smaller initial filling power ($R_0$), maximum grain-filling rate ($GR_{\text{max}}$), mean grain-filling rate ($GR_{\text{mean}}$), and longer time reaching the maximum

#### Table 1. Carbon and nitrogen metabolites at maturity stage in the wildtype (WT) and transgenic lines (TL35 and TL44) under low nitrogen (LN) and high nitrogen (HN) conditions.

| Trait                  | Tissue           | LN              | HN              |
|------------------------|------------------|-----------------|-----------------|
|                        | WT               | L35             | L44             |
|                        |                  | WT              | L35             | L44             |
| Total nitrogen content (%) | Stem sheath | 0.39e           | 0.48d           | 0.49d           | 0.63c           | 0.81a           | 0.69b           |
|                        | Leaf             | 1.02e           | 1.30c           | 1.15d           | 1.50b           | 1.63a           | 1.66a           |
|                        | Grain            | 1.03b           | 1.07b           | 1.06b           | 1.16a           | 1.18a           | 1.18a           |
| Total carbon content (%) | Stem sheath | 37.15a          | 36.46b          | 35.81c          | 37.40a          | 36.14b          | 36.31b          |
|                        | Leaf             | 38.09a          | 37.36b          | 37.52b          | 38.89a          | 37.66b          | 36.62c          |
|                        | Grain            | 38.22d          | 38.57d          | 39.52b          | 39.04c          | 39.17b          | 40.33a          |
| Carbon/nitrogen ratio   | Stem sheath | 94.84a          | 76.65b          | 73.29b          | 59.78c          | 44.80e          | 52.62d          |
|                        | Leaf             | 37.54a          | 28.65c          | 32.70b          | 26.00d          | 23.07e          | 22.08e          |
|                        | Grain            | 37.18a          | 36.09ab         | 37.34a          | 33.55c          | 33.22c          | 34.43bc         |
| Soluble protein content (mg g\(^{-1}\) DW) | Stem sheath | 15.93d          | 18.02bc         | 17.87c          | 17.40bc         | 19.07a          | 18.85ab         |
|                        | Leaf             | 13.45bc         | 12.80c          | 13.77ab         | 13.20bc         | 14.00a          | 13.33ab         |
|                        | Grain            | 13.19a          | 13.11a          | 13.42a          | 12.55a          | 13.24a          | 14.01a          |
| Starch content (mg g\(^{-1}\) DW) | Stem sheath | 212.66a         | 180.30b         | 171.07b         | 145.67c         | 111.01d         | 106.88d         |
|                        | Leaf             | 125.79a         | 103.71bc        | 98.51cd         | 113.28b         | 98.25cd         | 88.94d          |
|                        | Grain            | 272.13b         | 274.77b         | 273.43b         | 300.41a         | 307.60a         | 304.21a         |
| Sucrose content (mg g\(^{-1}\) DW) | Stem sheath | 94.01b          | 62.98cd         | 57.97d          | 133.64a         | 86.62b          | 73.13c          |
|                        | Leaf             | 56.08a          | 31.81c          | 26.82c          | 41.71b          | 28.74c          | 26.82c          |
|                        | Grain            | 44.33bc         | 50.45b          | 57.29a          | 38.86cd         | 36.08d          | 46.24b          |
| Soluble sugar content (mg g\(^{-1}\) DW) | Stem sheath | 190.77b         | 137.68c         | 132.71c         | 242.74a         | 118.87d         | 104.47e         |
|                        | Leaf             | 90.67a          | 46.68c          | 35.18d          | 70.03b          | 30.58e          | 22.70f          |
|                        | Grain            | 57.08b          | 60.14a          | 64.34b          | 45.20c          | 47.13c          | 47.19c          |

Data are shown as the means estimated from three plots (each plot contained 20 randomly mixed plant materials) per line per fertilizer. Different letters following the mean values indicate significant differences at $P < 0.05$ by Duncan’s multiple range tests.

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filling rate ($T_{max}$) and active filling period (D) for both superior and inferior grains, compared with those of wildtype under both LN and HN conditions (S5 Table).

We further determined the grain appearance, milling, and cooking quality in transgenic lines (TL35 and TL44) and the wildtype (Table 3). Under LN and HN conditions, only transgenic line TL44 showed decreased grain length, grain length/width ratio, and head rice rate compared with the wildtype. However, some RVA profile parameters, including the peak viscosity, cool paste viscosity, breakdown value, and consistence value were decreased in transgenic lines (TL35 and TL44), whereas the setback viscosity and peak time were increased compared with wildtype, particularly under HN conditions.

**Discussion**

Rice plants carrying the dominant DEP1 allele (dep1) have higher expression levels of *GS1;1* and GS activity, exhibiting nitrogen-insensitive vegetative growth coupled with increased nitrogen uptake and assimilation [16]. However, many studies have found that the balance of carbon–nitrogen metabolism can be broken if the nitrogen metabolism activity is increased in GS-overexpressed plants [8–10]. In this study, the carbon/nitrogen ratio of *dep1*-overexpressed lines was lower in stem sheaths and leaves than that of the wildtype under either LN or HN conditions, which was not only attributed to the increased total nitrogen content but also...
decreased total carbon content. Similar results in previous studies have also shown that more carbohydrate or nitrogen accumulating in plants automatically results in lower concentrations of other components [31–34]. However, the carbon/nitrogen ratio is sometimes considered to be a poor indicator of the carbon and nitrogen metabolism status of plant tissues [34]. Thus, to provide evidence of unbalanced carbon−nitrogen metabolism in dep1-overexpressed lines, we showed an increase in soluble protein content and decreases in starch, sucrose, and soluble sugar content in the straw of these lines. The gene expression patterns involved in carbon-nitrogen metabolism were further analyzed. The dep1-overexpressed lines had higher expressions of GS and GOGAT genes, and thus showed higher nitrogen metabolic activity than the wildtype under either LN or HN conditions. Meanwhile, the genes involved in carbon metabolism, such as RUBISCO and PEPC, were suppressed, which caused unbalanced carbon−nitrogen metabolism in stem sheaths and leaves under both LN and HN conditions.

Previous studies have shown that unbalanced carbon−nitrogen metabolic status can result in some negative effects, such as poor plant growth, inferior photosynthetic capacity, lower nitrogen transfer ability, and decreased yield [8–10, 35, 36], but the dep1 allele can lead to an increased number of grains per panicle, and consequently, increase grain yield [24, 37]. Moreover, the dep1 allele makes panicles dense and erect, which causes improvements in the population structure, light-interception capacity, and ecological environment [38, 39]. In this study, overexpressed dep1 led to greater nitrogen absorption and NUE, and the dep1-overexpressed lines (TL35 and TL44) exhibited higher numbers of panicles per plant, grain numbers per panicle, biomass, and grain yield under LN conditions, and had higher grain numbers per panicle, harvest index, and grain yield under HN conditions. Meanwhile, despite the unbalanced carbon−nitrogen metabolism in stem sheaths and leaves, the dep1-overexpressed lines also had higher carbon accumulation in grains and whole plants, leading to higher grain yield than the wildtype under LN and HN conditions.

Some previous studies have reported that the dep1 allele can decrease the grain-filling percentage and the 1000 grains weight [23, 37, 40]. DEP1, like GS3 that encodes a γ subunit of the heterotrimeric G protein, regulates grain size and shape by protein−protein interactions [41,

Table 2. Agronomic traits at maturity stage in the wildtype (WT) and transgenic lines (TL35 and TL44) under low nitrogen (LN) and high nitrogen (HN) conditions.

| Trait                                | LN          | HN          |
|--------------------------------------|-------------|-------------|
|                                      | WT          | TL35        | TL44        | WT          | TL35        | TL44        |
| Plant height (cm)                    | 85.40c      | 77.02d      | 77.02d      | 108.86a     | 91.48b      | 91.48b      |
| No. of panicles per plant           | 6.86c       | 9.23b       | 9.51b       | 13.50a      | 13.79a      | 13.87a      |
| Panicle length (cm)                 | 17.15b      | 15.05d      | 15.06d      | 18.04a      | 16.37c      | 16.70bc     |
| No. of primary panicle branches     | 8.00c       | 10.00b      | 10.00b      | 10.00b      | 11.00a      | 11.00a      |
| No. of secondary panicle branches   | 10.50d      | 13.47c      | 13.57c      | 14.57b      | 19.4a       | 20.17a      |
| Grain density (g·cm$^{-3}$)         | 4.21d       | 6.01b       | 6.04b       | 5.34c       | 7.11a       | 7.16a       |
| Number of grains per panicle        | 72.13d      | 90.33c      | 90.73c      | 96.10b      | 116.23a     | 119.5a      |
| Grain setting percentage (%)        | 96.84a      | 92.81b      | 93.22b      | 92.36b      | 88.21c      | 87.75c      |
| 1000-grains weight (g)              | 26.02ab     | 24.87c      | 24.66c      | 26.49a      | 25.38bc     | 25.22bc     |
| Yield (t·ha$^{-1}$)                 | 3.62d       | 4.60c       | 4.87c       | 7.49b       | 8.60a       | 8.47a       |
| Biomass (t·ha$^{-1}$)               | 6.29c       | 7.54b       | 7.84b       | 13.60a      | 13.67a      | 13.68a      |
| Harvest index                       | 0.58ab      | 0.61a       | 0.62a       | 0.55b       | 0.63a       | 0.62a       |
| NUE                                  | 61.69b      | 70.19a      | 70.65a      | 57.76b      | 58.35b      | 61.38b      |

Data are shown as the means estimated from three plots (each plot comprised 60 plants) per line per fertilizer. Different letters following the mean values indicate significant differences at $P < 0.05$ by Duncan’s multiple range tests.

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Overexpression of the \textit{DEP1} allele leads to large grains, whereas that of the \textit{dep1} allele results in small grains [41]. \textit{DEP1} interacts with \textit{RGB1} to promote grain growth, while \textit{GS3} regulates grain size by repressing the function of \textit{DEP1} [41]. This study showed that sink capacity improved by increasing the grain number per panicle in the \textit{dep1}-overexpressed lines under LN or HN conditions, but the source was insufficient due to a poor photosynthetic rate, resulting in a decrease of the grain-filling rate, grain-setting percentage, grain length, and 1000 grains weight. Moreover, there was a decrease in peak viscosity, cool paste viscosity, breakdown value, and consistence value, and an increase in setback viscosity and peak time in grains of \textit{dep1}-overexpressed lines under LN or HN conditions. The viscosity of cooked rice is negatively correlated with setback viscosity and breakdown value [43], and the amylose content of cooked rice is positively correlated with setback viscosity [44]. Rice varieties with good eating quality (such as Akitakomati used in this study) usually have higher peak viscosity and breakdown values, but smaller setback viscosity compared to common varieties [45]. This study suggests that unbalanced carbon–nitrogen metabolism resulted in decreased grain quality in \textit{dep1}-overexpressed lines.

Fig 4. Accumulations of grain weight after heading stage in the wildtype (WT) and transgenic lines (TL35 and TL44) under low nitrogen (A and B) and high nitrogen (C and D) conditions. Data shown as mean ± SD (n = 3 plants).

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In conclusion, metabolic and gene expression profile analysis showed that the carbon--nitrogen metabolic status was unbalanced in stem sheaths and leaves but not in grains of dep1-overexpressed lines. This status did not result in decreased grain yield, though it reduced the grain-filling rate, grain setting percentage, 1000 grain weight, and grain quality. These results can explain the reasons for poor grain quality in most of erect panicle varieties. They also suggest that some other genes related to grain size or grain quality should be aggregated with the dep1 allele to improve grain yield and grain quality together for super rice breeding.

Supporting information

S1 Table. Primer sequences used in this study.
(DOCX)

S2 Table. Copy number of exogenous gene in transgenic T0 plants identified by quantitative real-time PCR.
(DOCX)

S3 Table. Zygosity analysis of exogenous gene in transgenic T1 plants identified by quantitative real-time PCR.
(DOCX)

S4 Table. Carbon and nitrogen accumulation at maturity stage in the wildtype (WT) and transgenic lines (TL35 and TL44) under low nitrogen (LN) and high nitrogen (HN) conditions.
(DOCX)
S5 Table. Parameters of grain filling in the wildtype (WT) and transgenic lines (TL35 and TL44) under low nitrogen (LN) and high nitrogen (HN) conditions.

S1 Fig. Fold change corresponding to the ratio of the gene expression level in transgenic lines TL35 (A) and TL44 (B) relative to the wildtype plants.

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References

1. Olson R, Kurtz LT. Crop nitrogen requirements, utilization, and fertilization. Nitrogen Agri. Soils. 1982; 567–604.
2. Good AG, Shrawat AK, Muench DG. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci. 2004; 9: 597–605. https://doi.org/10.1016/j.tplants.2004.03.008 PMID: 15564127
3. Guo J, Liu X, Zhang Y, Shen J, Han W, Zhang W, et al. Significant acidification in major Chinese croplands. Science. 2010; 327: 1008–1010. https://doi.org/10.1126/science.1182570 PMID: 20150447
4. Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gauflchin L, Suzuki A. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of Botany. 2010; 106:1141–1157. https://doi.org/10.1093/aob/mcq028 PMID: 20298346
5. Xu G, Fan X, Miller AJ. Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol. 2012 63:153–182. https://doi.org/10.1146/annurev-arplant-042811-105332 PMID: 22224450
6. Edwards JW, Walker EL, Coruzzi GM. Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. Proc Natl Acad Sci USA. 1990; 87:3459–3463. PMID: 1970638
7. Cai H, Zhou Y, Xiao X, Li X, Zhang Z, Lian X. Overexpressed glutamine synthetase gene modifies nitrogen metabolism and abiotic stress responses in rice. Plant Cell Rep. 2009; 28:527–537. https://doi.org/10.1007/s00299-008-0665-5 PMID: 19123004
8. Bao A, Zhao Z, Ding G, Shi L, Xu F, Cai H. Accumulated expression level of cytosolic glutamine synthetase 1 gene (OsGS1;1 or OsGS1;2) alters plant development and the carbon-nitrogen metabolic status in rice. PLoS One. 2014; 9:e95581. https://doi.org/10.1371/journal.pone.0095581 PMID: 24743556
9. Bao A, Liang Z, Zhao Z, Cai H. Overexpressing of OsAMT1-3, a high affinity ammonium transporter gene, modifies rice growth and carbon-nitrogen metabolic status. Int J Mol Sci. 2015; 16:9037–9063. https://doi.org/10.3390/ijms16059037 PMID: 25915023

10. Kusano M, Tabuchi M, Fukushima A, Funayama K, Diaz C, Kobayashi M, et al. Metabolomics data reveal a crucial role of cytosolic glutamine synthetase 1 in coordinating metabolic balance in rice. Plant J. 2011; 66(3):456–466. https://doi.org/10.1111/j.1365-313X.2011.04506.x PMID: 21255162

11. Zheng Z. Carbon and nitrogen nutrient balance signaling in plants. Plant Signal Behav. 2009; 4:584–591. https://doi.org/10.4161/psb.4.7.8540 PMID: 19820356

12. Coruzzi GM, Zhou L. Carbon and nitrogen sensing and signaling in plants: Emerging “matrix effects”. Curr Opin Plant Biol. 2001; 4:247–253. PMID: 11312136

13. Geiger M, Haake V, Ludewig F, Sonnewald U. Stitt M. The nitrate and ammonium supply have a major influence on the response of photosynthesis, carbon metabolism, nitrogen metabolism and growth to elevated carbon dioxide in tobacco. Plant Cell Environ. 1999; 22:1177–1199.

14. Matt P, Schurr U, Krapp A. Stitt M. Growth of tobacco in short day conditions leads to high starch, low sugars, altered diurnal changes of the NIA transcript and low nitrate reductase activity, and an inhibition of amino acid synthesis. Planta. 1998; 207:27–41. https://doi.org/10.1007/s004250050452 PMID: 9951717

15. Yang W, Peng S, Dionisio-Sese ML, Laza RC, Visperas RM. Grain filling duration, a crucial determinant of genotypic variation of grain yield in field-grown tropical irrigated rice. Field Crops Res. 2008; 105:221–227.

16. Sun H, Qian Q, Wu K, Luo J, Wang S, Zhang C, et al. Heterotrimetric G-proteins regulate nitrogen-use efficiency in rice. Nat Genet. 2014; 46:652–656. https://doi.org/10.1038/ng.2958 PMID: 24777451

17. Chakravorty D, Trustov Y, Zhang W, Acharya BR, Sheahan MB, McCurdy DW, et al. An atypical heterotrimeric G-protein γ-subunit is involved in guard cell K+ channel regulation and morphological development in Arabidopsis thaliana. Plant J. 2011; 67:840–851. https://doi.org/10.1111/j.1365-313X.2011.04638.x PMID: 21575088

18. Ashikari M, Wu J, Yano M, Sasaki T, Yoshimura A. Rice gibberellin-insensitive dwarf mutant gene Dwarf 1 encodes the α-subunit of GTP-binding protein. Proc Natl Acad Sci USA. 1999; 96:10284–10289. PMID: 10468600

19. Johnston CA, Taylor JP, Gao Y, Kimple AJ, Grigston JC, Chen JG, et al. GTPase acceleration as the rate-limiting step in Arabidopsis G protein-coupled sugar signaling. Proc Natl Acad Sci USA. 2007; 104:17317–17322. https://doi.org/10.1073/pnas.0704751104 PMID: 17951432

20. Urano D, Phan N, Jones JC, Yang J, Huang J, Grigston J, et al. Endocytosis of the seven-transmembrane RGST protein activates G-protein-coupled signalling in Arabidopsis. Nat Cell Biol. 2012; 14:1079–1088.

21. Fu Y, Lim S, Urano D, Tunc-Ozdemir M, Phan NG, Elston TC, et al. Reciprocal encoding of signal intensity and duration in a glucose-sensing circuit. Cell. 2014; 156:1084–1095. https://doi.org/10.1016/j.cell.2014.01.013 PMID: 24581502

22. Zhao M, Sun J, Xiao Z, Cheng F, Xu H, Tang L, et al. Variations in DENSE AND ERECT PANICLE 1 (DEP1) contribute to the diversity of the panicle trait in high-yielding japonica rice varieties in northern China. Breeding Sci. 2016; 66, 599–605.

23. Zhou Y, Zhu J, Li Z, Yi C, Liu J, Zhang H, et al. Deletion in a quantitative trait gene qPE9-1 associated with panicle erectness improves plant architecture during rice domestication. Genetics. 2009; 183:315–325. https://doi.org/10.1534/genetics.109.102681 PMID: 19546322

24. Huang X, Qian Q, Liu Z, Sun H, He S, Luo D, et al. Natural variation at the DEP1 locus enhances grain yield in rice. Nat Genet. 2009; 41:494–497. https://doi.org/10.1038/ng.352 PMID: 19305410

25. Weng H, Pan A, Yang L, Zhang C, Liu Z, Zhang D. Estimating number of transgene copies in transgenic rapeseed by real-time PCR assay with HMG I/Y as an endogenous reference gene. Plant Mol Bio Rep. 2004; 22:289–300.

26. German MA, Kandel-Kfir M, Swarzberg D, Matsevitz T, Granot D. A rapid method for the analysis of zygosity in transgenic plants. Plant Sci. 2003; 164:183–187.

27. Livak KJ, Schmittgen, TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001; 25:402–408. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

28. Yang W, Peng S, Dionisio-Sese ML, Laza RC, Visperas RM. Grain filling duration, a crucial determinant of genotypic variation of grain yield in field-grown tropical irrigated rice. Field Crops Res. 2008; 105:221–227.

29. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. PMID: 942051
30. Li H. Modern plant physiology. Beijing: Higher Education Press; 2002.
31. Poorter H, Berkel V, Baxter R, den Hertog J, Dijkstra P, Gifford RM, et al. The effect of elevated carbon dioxide on the chemical composition and construction costs of leaves of 27, C3 species. Plant Cell Environ. 1997; 20:472–482.
32. Ferrario-Mery S, Thibaud MC, Betsche T, Valadier MH, Foyer CH. Modulation of carbon and nitrogen metabolism, and of nitrate reductase, in untransformed Nicotiana plumbaginifolia during CO₂ enrichment of plants grown in pots and hydroponic culture. Planta. 1997; 202:510–521.
33. Tissue DT, Thomas RB, Strain BR. Atmospheric CO₂ enrichment increases growth and photosynthesis of Pinus taeda, a 4 year experiment in the field. Plant Cell Environ. 1997; 20:1123–1134.
34. Stitt M, Krapp A. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ. 1999; 22:583–621.
35. Bao A, Zhao Z, Ding G, Shi L, Xu F, Cai H. The stable level of glutamine synthetase 2 plays an important role in rice growth and in carbon-nitrogen metabolic balance. Int J Mol Sci. 2015; 16:12713–12736. https://doi.org/10.3390/ijms160612713 PMID: 26053400
36. Chen J, Zhang Y, Tan Y, Xu G, Fan X. The effects of OeNRT2.1 over-expression on plant growth and nitrogen use efficiency in rice Nipponbare (Oryza sativa L. ssp. japonica) Mol Plant Breeding. 2016; 14:1–9.
37. Wang J, Tetsuya N, Chen S, Chen W, Saito H, Tsuliyama T, et al. Identification and characterization of the erect-panicle gene EP conferring high grain yield in rice (Oryza sativa L.). Theor Appl Genet. 2009; 119: 85–91. https://doi.org/10.1007/s00122-009-1019-0 PMID: 19407986
38. Xu X, Chen Y, Zhou H, Zhang L, Yang S. Physiological and ecological characteristics of rice with erect panicle and prospects of their utilization. Chinese Sci Bulletin. 1996; 19:1642–1648.
39. Xu Z, Lin H, Ma D, Wang J, Xu H, Zhao M, et al. Research and application of the panicle type improved theory and technology in northern japonica rice. J Shenyang Agric Univ. 2012; 6:650–659.
40. Yi X, Zhang Z, Zeng S, Tian C, Peng J, Li M, et al. Introgresion of qPE9-1 allele, conferring the panicle erectness, leads to the decrease of grain size per plant in japonica rice (Oryza sativa L.). J Genet Genomics. 2011; 38:217–223. https://doi.org/10.1016/j.jgg.2011.03.011 PMID: 21621743
41. Sun S, Wang L, Mao H, Shao L, Li X, Xiao J, et al. A G-protein pathway determines grain size in rice. Nat Commun. 2018; 9:851. https://doi.org/10.1038/s41467-018-03141-y PMID: 29487318
42. Liu Q, Han R, Wu K, Zhang J, Ye Y, Wang S, et al. G-protein betagamma subunits determine grain size through interaction with MADS-domain transcription factors in rice. Nat Commun. 2018; 9:852. https://doi.org/10.1038/s41467-018-03047-9 PMID: 29487282
43. Shu Q, Wu D, Xia Y, Gao M, Mc Clung A. Relationship between RVA profile character and eating quality in Oryza sativa L. Sci Agric Sin. 1998; 31:25–29.
44. Juliano BO, Bautista GM, Lugay JC, Reyes AC. Studies on the physicochemical properties of rice. J Agric Food Chem. 1964; 12:131–138.
45. Jin Z, Qiu T, Sun Y, Jin B. Study on the varietal variation of the cooking and eating quality properties of rice grain in Heilongjiang. Heilongjiang Agric Sci. 2000; 1:1–4.