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Introduction of the ZmDof1 gene into rice enhances carbon and nitrogen assimilation under low-nitrogen conditions

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Summary

The excessive application of nitrogen fertilizer to maximize crop yields causes negative environmental effects such as pollution and ecological imbalance. To overcome this problem, researchers have attempted to improve the nitrogen assimilation capacity of crops. Maize Dof1 (ZmDof1) is a plant-specific transcription factor shown to promote nitrogen assimilation in *Arabidopsis thaliana* (Arabidopsis) even under nitrogen-deficient conditions. The present study examines the effect of the introduction of the *ZmDof1* gene on carbon and nitrogen assimilation in rice. ZmDof1 induced the expression of phospho*enol*pyruvate carboxylase (PEPC) genes in transgenic rice plants and transactivated the PEPC promoters in protoplast transient assays, showing similar effects in rice as in Arabidopsis. Transgenic rice expressing *ZmDof1* and grown in the presence of 360 μM (nitrogen-sufficient) or 90 μM (nitrogen-deficient) of nitrogen concentrations showed modulation of metabolite content and gene expression associated with the anaplerotic pathway for the TCA cycle, suggesting an increased carbon flow towards nitrogen assimilation. Furthermore, increases in carbon and nitrogen amounts per seedling were found in Dof1 rice grown under nitrogen-deficient conditions. Nitrogen deficiency also resulted in the predominant distribution of nitrogen to roots, accompanied by significant increases in root biomass and modification of the shoot-to-root ratio. Measurement of the CO_2 gas exchange rate showed a significant increase in the net photosynthesis rate in Dof1 rice under nitrogen-deficient conditions. Taken these together, the present study displayed that ZmDof1 expression in rice could induce gene expressions such as PEPC genes, modulate carbon and nitrogen metabolites, increase nitrogen assimilation and enhance growth under low-nitrogen conditions.

Introduction

Soaring global populations, the subsequent increase in food demands and current trends favouring bio-ethanol as an alternative energy source have led to a demand for increases in agricultural production. To meet these increased demands, current agricultural practices rely heavily on nitrogenous fertilizers in the form of ammonium or nitrate. However, the use of large amounts of nitrogen (N) fertilizer has resulted in negative environmental effects such as pollution and ecological imbalance (Miller and Cramer, 2004). One of the possible solutions to these environmental problems is the enhancement of N assimilation in crops.

Different strategies using biotechnology to optimize N assimilation have been developed in a variety of plants. Overexpression of the high affinity ammonium transporter *OsAMT1-1* in rice increased N uptake, but was also associated with a decrease in biomass (Kumar *et al.*, 2006) and growth impairment (Hoque *et al.*, 2006). Glutamine synthetase (GS), the key enzyme for primary N assimilation in plants, is also a popular target for the enhancement of N assimilation, but the effects of its overexpression have been inconsistent. Oliveira *et al.* (2002) showed that the overexpression of cytosolic GS1 in tobacco enhanced growth and leaf soluble protein, while overexpression of GS in the roots of *Lotus japonicus* resulted in decreased plant biomass (Limami *et al.*, 1999). Promising results were obtained recently through the expression of the barley amino transferase gene in the roots of canola (Good *et al.*, 2007) and rice (Shrawat *et al.*, 2008). In both studies, plant biomass, N content and yield were increased in the transformants.

Difficulties obtaining positive results for N assimilation could be attributed to the close relationship between N and carbon (C) metabolism, which implies that these two processes are tightly coregulated (Oliveira and Coruzzi, 1999; Stitt *et al.*, 2002). Therefore, the effects of alterations in the amount and/or activity of any single enzyme in the N metabolic pathway may be masked by concurrent mechanisms that are activated to maintain homoeostasis, including post-transcriptional, translational and/or feedback regulation. Engineering of biological mechanisms under the complex regulations, such as the N metabolic pathway, might be achieved more efficiently with the help of transcription factors, because they have the capacity to modulate simultaneously the expression of multiple genes. In fact, *Arabidopsis thaliana* (Arabidopsis) plants expressing maize Dof1 (ZmDof1), a plant-specific transcription factor, showed both improved N assimilation and increased plant growth under low-N conditions (Yanagisawa *et al.*, 2004). The effect of *ZmDof1* expression in Arabidopsis was proposed to be mediated by the modulation of the expression of genes involved in the production of the C skeleton for amino acid biosynthesis,

including the phospho*enol*pyruvate carboxylase (PEPC) genes (Yanagisawa *et al.*, 2004). The expression of the *ZmDof1* gene also altered the amino acid content, in particular that of glutamine (Gln), in Arabidopsis and potato plants. However, the effect of *ZmDof1* expression has not been tested in monocots to date.

Rice (*Oryza sativa* L.) is used as a model plant for the study of monocots, and it is an important crop because it is the main dietary staple for more than half of the world population. Approximately 80 million tons of chemical N fertilizer are used annually to sustain high yields of rice (Hoque *et al.*, 2006; FAO, 2008), suggesting that improving the N assimilation capability of rice is quite valuable.

The present study examined the effects of ZmDof1 expression on N assimilation in rice (cv. Nipponbare) and on the growth of rice plants under N-deficient conditions through the production and characterization of transgenic rice plants expressing the *ZmDof1* gene (Dof1 rice). The present results showed that *ZmDof1* expression modulates the concentration of C metabolites associated with the anaplerotic pathway for the TCA cycle and improves N assimilation in rice. This study also showed that the expression of *ZmDof1* in rice enhances growth under N-deficient conditions in association with an increase in the net photosynthesis rate (Pn) and a decrease in the shoot-to-root dry weight ratio (S/R ratio).

Results

Generation of transgenic rice plants expressing the *ZmDof1* gene

The maize ubiquitin promoter (*ZmUbi-1* promoter) was used to construct a binary vector for the expression of *ZmDof1* in rice (Figure 1a) because of its ability to direct a stronger constitutive downstream gene expression in monocots than the cauliflower mosaic virus 35S (CaMV35S) promoter (Christensen *et al.*, 1992). Agrobacterium-mediated transformation (Toki *et al.*, 2006) using the constructed vector generated 22 independent T_0 generations that contained the *ZmDof1* gene at a single locus or at multiple loci. Vector control rice plants (VC rice) were generated through the replacement of the *ZmDof1* gene with the *mGFP* gene in the expression vector. Analysis of the genomic DNA from T_1 generations of Dof1 rice by Southern hybridization analysis with selection marker (hygromycin resistance gene)- and Dof1-specific DNA probes showed the insertion of one to five copies of the *ZmDof1* gene into multiple loci across the genome (data not shown). However, similar to findings in Arabidopsis (Yanagisawa *et al.*, 2004), segregation of the selectable marker gene revealed that *ZmDof1* homozygotes could not be obtained from T_0, T_1 or T_2 generations of Dof1 rice plants harbouring the *ZmDof1* gene at a single locus and that heterozygous plants harbouring the *ZmDof1* gene at a single locus exhibited unstable inheritance of the gene (data not shown). Although these results reflect the difficulties in further characterizing the plants containing the *ZmDof1* gene at a single locus, a successful inheritance of the *ZmDof1* gene was obtained in transgenic lines harbouring the *ZmDof1* gene at multiple loci, and all analyses were therefore performed on T_3 progenies of these plants. Three lines (referred to as Dof1-64, Dof1-202 and Dof1-203) that were established from independent T_0 seedlings were selected for further analysis based on the stable yield of seeds and successful inheritance of the *ZmDof1* gene. These lines had two copies of the *ZmDof1* gene,

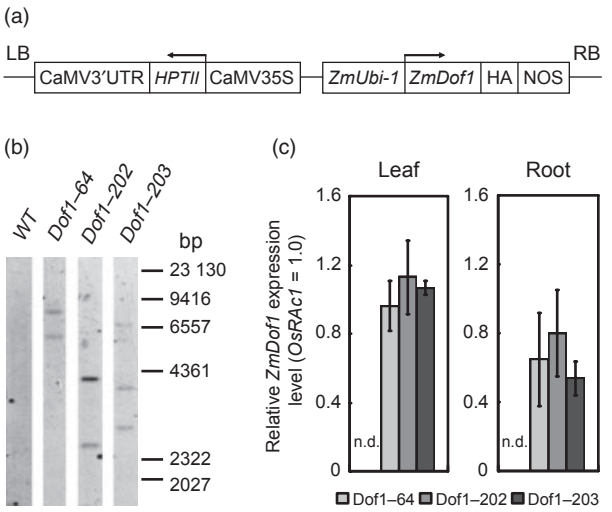

Figure 1 Generation of transgenic rice plants expressing *ZmDof1*. (a) The plasmid used for transformation. The *ZmUbi-1* promoter and the nopaline synthase terminator were used for the expression of *ZmDof1*, and the hemagglutinin (HA) epitope was used as a tag. The HA tag does not affect the activity of Dof1 (Yanagisawa *et al.*, 2004). The hygromycin resistance gene (*HPTII*) was used as a selectable marker and is shown between the left (LB) and right (RB) borders of the T-DNA. Transcription start sites and the direction of transcription are shown by arrows. (b) Genomic Southern blot analysis. Genomic DNA from wild-type (WT) and three Dof1 rice lines (Dof1-64, Dof1-202 and Dof1-203) was digested with *Hin*dIII and analysed with a Dof1-specific DNA probe. Because the probe has no *Hin*dIII site, a single band should be detected when the analysed plant has a single insertion of the transgene. (c) Quantitative RT-PCR analysis of *ZmDof1* transcripts in the leaves (left) and roots (right). RT was performed with RNA from the leaves of the T_3 generations of VC rice and three Dof1 rice lines. Values are expressed as the means ± SD of eight replicates, after normalization with actin (*OsRac1*). The letters 'n.d.' indicate 'not detected'.

as indicated by Southern hybridization analyses using both hygromycin- and Dof1-specific probes with three different restriction enzymes, *Hin*dIII, *Eco*RI and *Bgl*II. An example of Southern hybridization using the Dof1-probe with *Hin*dIII-digested genomic DNA is shown in Figure 1b. The selection of this working line did not allow the evaluation of the integrated gene copy numbers or the distinction between the homozygous and heterozygous status of *ZmDof1* at each locus. However, the system enabled the investigation of the effects of *ZmDof1* expression in individual plants that tested positive for the presence of the gene by reverse transcription (RT)-PCR. The results of quantitative RT-PCR (qRT-PCR) analysis are depicted in Figure 1c, which shows comparable levels of *ZmDof1* mRNA in three independent Dof1 rice lines.

Effect of *ZmDof1* expression on PEPC gene expression in transgenic rice

To investigate the effect of *ZmDof1* expression in rice, VC and Dof1 rice plants were first evaluated under N-sufficient conditions. For the present study, we applied 360 μM of N as the sufficient N concentration based on the results of preliminary experiments (see Materials and Methods). Expression of the ZmDof1 gene in Arabidopsis resulted in enhanced PEPC gene expression and increased PEPC activity (Yanagisawa *et al.*,

2004). PEPC catalyzes the reaction between carbon dioxide and phospho*enol*pyruvate (PEP) to produce oxaloacetate (OAA) and inorganic phosphate and plays a central role in the anaplerotic provision of C skeletons for amino acid biosynthesis (Matsuoka *et al.*, 2001). Increased activity of PEPC can therefore be an important factor in N assimilation. To examine the effect of exogenously introduced *ZmDof1* on PEPC gene transcription in rice, qRT-PCR was performed to investigate the expression of three cytosolic PEPC genes, *OsPpc1-3*, in Dof1 rice. Because a novel gene encoding a chloroplastic PEPC named *OsPpc4* was recently found (Masumoto *et al.*, 2010), the expression of *OsPpc4* was additionally examined. All Dof1 rice lines had significantly higher expression of *OsPpc1* in leaves, compared to that in VC rice (Figure 2a). Furthermore, the Dof1-202 line showed a significant increase in the expression of all the cytosolic *OsPpc* genes examined (Figure 2b,c), consistent with the highest *ZmDof1* expression (Figure 1c). The expression levels of *OsPpc4* in the Dof 1 and VC rice plants were comparable (Figure 2d). These results suggest that ZmDof1 could play a role in the induction of PEPC gene expression in rice.

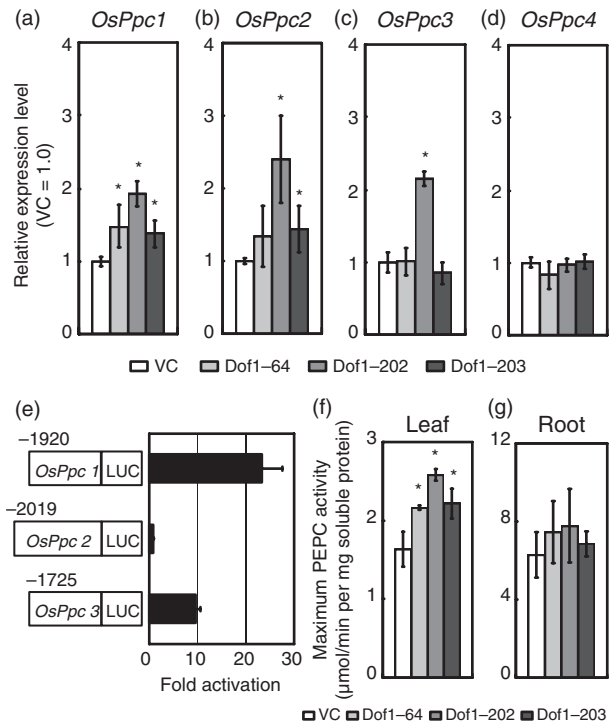

Figure 2 Effects of ZmDof1 expression on PEPC in rice. Quantitative RT-PCR analysis of transcripts of *OsPpc1* (a), *OsPpc2* (b), *OsPpc3* (c) and *OsPpc4* (d) using RNA obtained from the leaves of T_3 generations of VC rice and three Dof1 rice lines. Values are expressed as the means ± SD of at least four replicates after normalization with the expression of actin (*OsRac1*). Asterisks indicate statistically significant differences at $P < 0.05$ when compared with VC samples. (e) Transactivation of PEPC gene promoters by ZmDof1. Reporter constructs generated through the fusion of rice PEPC promoters (*Osppc1, Osppc2* and *Osppc3*) with the LUC reporter gene were cotransfected into maize protoplasts together with the *ZmDof1* expression vector or an empty vector and the UBI-GUS internal control. Relative LUC activity was calculated against GUS activity, and ZmDof1-dependent activation was determined by the ratio between the activity in protoplasts transfected with the *ZmDof1* expression vector and in those transfected with the empty vector. Values represent the means ± SE of at least four replicates. PEPC maximum activity in rice plants was measured in leaves (f) and roots (g) of individual plants. Values are expressed as the means ± SD of at least four replicates. Asterisks indicate statistically significant differences at $P < 0.05$, when compared with VC samples.

Effect of *ZmDof1* expression on PEPC activity in transgenic rice

To investigate whether the alterations in *OsPpc* gene expression in transgenic rice were direct effects of ZmDof1, transient assays were performed in maize protoplasts using an expression vector for *ZmDof1* and reporter constructs in which three rice PEPC gene promoters were fused to the luciferase (LUC) gene. Cotransfection of protoplasts with the expression vector and reporter constructs revealed a significant activation of the *OsPpc1* and *OsPpc3* promoters (Figure 2e). Contrary to the enhanced *OsPpc2* expression in the Dof1-202 line (Figure 2b), the transactivation of the *OsPpc2* promoter could not be observed (Figure 2e), suggesting that ZmDof1 might enhance *OsPpc2* expression by interacting with a region outside of the promoter sequence. These results showed that at least two PEPC genes in rice could also be targets of ZmDof1.

To further investigate whether the enhanced gene expression altered PEPC enzymatic activity in Dof1 rice, a kinetic analysis was carried out to determine the *Vmax* of PEPC in VC and Dof1 rice plants. In accordance with the observed induction of PEPC gene expression, PEPC enzymatic activity was 30%–60% higher in Dof1 rice leaves compared with VC rice, and the Dof1-202 line showed the highest PEPC activity (Figure 2f). In the roots, both the transcript levels and PEPC activity were comparable between VC and Dof1 rice (Supplementary Figure S1 and Figure 2g). These results revealed that ZmDof1 expression affected PEPC gene expression and PEPC activity in leaves and that activity changes were directly correlated with expression levels of *ZmDof1* in rice plants. These results also suggested that the effect of ZmDof1 on the modulation of C and N metabolism reported in Arabidopsis could also be present in rice.

Effect of *ZmDof1* expression on the anaplerotic pathway for the TCA cycle

The increase in PEPC activity could result in changes in the anaplerotic pathway for the TCA cycle and in amino acid biosynthesis in Dof1 rice. The previous work in Arabidopsis showed that in addition to changes in PEPC gene expression, Dof1 transgenic plants showed alterations in the expression of genes encoding pyruvate kinase (PK), which drives C into the glycolytic pathway (Yanagisawa *et al.*, 2004). Therefore, the expression of PK and other genes involved in the anaplerotic pathway for the TCA cycle were examined by qRT-PCR. Contrary to the results in Arabidopsis, the expression of PK gene was not changed in the leaves of Dof1 rice plants (Figure 3). However, all the Dof1 lines showed a significant increase in the expression of a gene encoding the NADP-malate dehydrogenase (*NADP-MDH*) and a cytosolic isocitrate dehydrogenase, *ICDH3*, in the leaves compared to VC rice (Figure 3). Similar to the results of *OsPpc* expression, the Dof1-202 line, characterized by the highest *ZmDof1* expression, also showed the most significant changes in the expression of *ICDH1* and *ICDH2* (Figure 3). These results suggested that the PEPC-mediated pathway rather than the PK-mediated glycolytic pathway, which regulates C flux into the TCA cycle, was activated in the Dof1 rice. Based on these

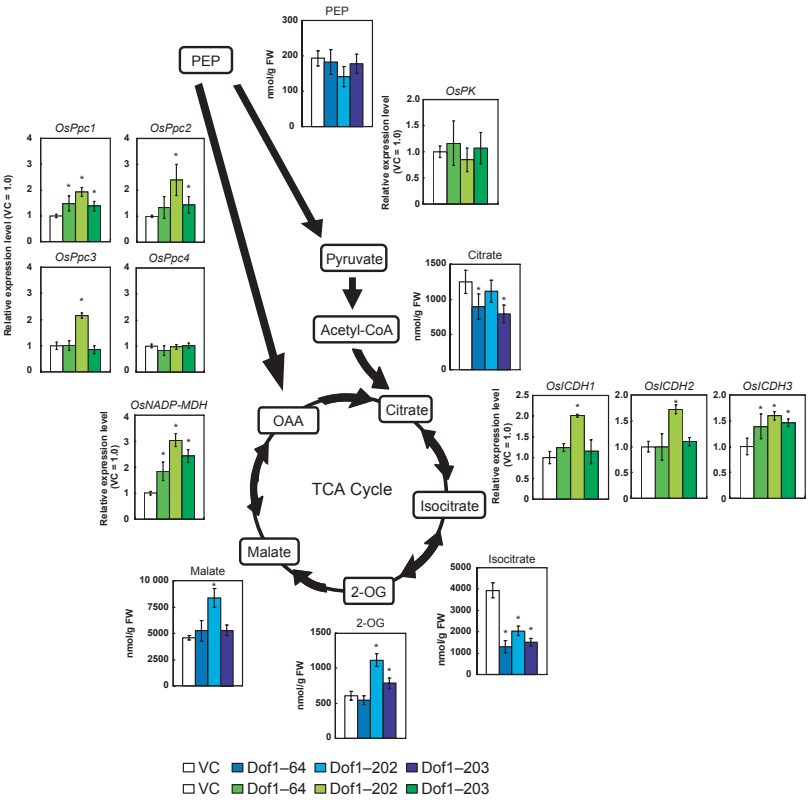

Figure 3 Effect of ZmDof1 on gene expression and the concentration of metabolites involved in the anaplerotic pathway for the TCA cycle. Quantitative RT-PCR analysis of transcripts of *OsPK*, *OsICDH1-3* and *OsNADP-MDH* was performed with RNA from leaves of T_3 generations of VC rice and three lines of Dof1 rice (green coloured figures). Values are expressed as the means ± SD of at least four replicates, after normalization with the expression of actin (*OsRac1*). Changes in leaf organic acid concentration induced by *ZmDof1* expression in rice are also displayed in this figure (blue coloured figures). Values represent the means ± SD of at least seven replicates. Asterisks indicate statistically significant differences at $P < 0.05$, when compared with VC samples.

results, the concentrations of related organic acids were measured by capillary electrophoresis coupled with mass-spectrometry (CE/MS). The results showed that malate, citrate and isocitrate concentrations were slightly altered in the leaves of Dof1 rice plants compared with VC rice plants, and the most significant change was the reduction in isocitrate concentration (Figure 3). Although an increase in the concentration of 2-oxoglutarate (2-OG) was not evident in transgenic lines, with the exception of the Dof1-202 line, these results further suggested that ZmDof1 expression affected the anaplerotic pathway for TCA cycle.

Effects of ZmDof1 expression on N assimilation, amino acid composition and related gene expression

Based on the increase in the provision of carbon metabolites resulting from the effect of ZmDof1 expression on the anaplerotic pathway, the effect of ZmDof1 on N assimilation was assessed by measuring the N concentration per gram of dry weight (DW) in various organs of Dof1 and VC rice. The N concentration in Dof1 rice was significantly higher than in VC rice in all organs examined (Figure 4), indicating that a larger amount of N was assimilated in ZmDof1 rice compared with VC rice. Assessment of the concentration of N-containing metabolites (free amino acids) per gram of fresh weight revealed differences in the free amino acid content of roots between the two types of rice (Table 1). The most significant change was an increase in the asparagine (Asn) concentration in the roots of Dof1 rice. Calculation of the amounts of Asn and Gln with respect to the total amino acid content reflected the prominent changes in the Asn concentration in Dof1 rice (Table 1), which differed from the results obtained in Arabidopsis.

The prior work in Arabidopsis showed that significant increases in glutamine and other amino acids caused by

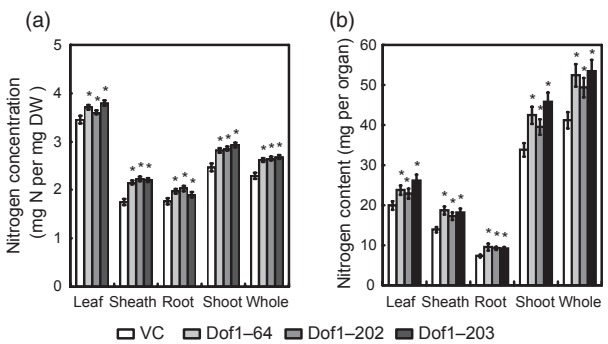

Figure 4 Nitrogen concentration and content of Dof1 rice grown under N-sufficient conditions. N concentration (a) and N content (b) of leaf blades, leaf sheaths, roots, shoots and whole rice plants grown with 360 μM of N. Values are expressed as the means ± SE of at least seven replicates. Asterisks indicate statistically significant differences at $P < 0.05$, when compared with VC samples.

ZmDof1 expression did not result in changes in the expression of the glutamine synthetase (*GS2*) and nitrate reductase genes (Yanagisawa *et al.*, 2004). The differences in the amino acid profile alterations caused by ZmDof1 expression between rice and Arabidopsis implied that ZmDof1 expression in rice might affect the expression of genes involved in amino acid biosynthesis, especially those associated with Asn. Asn biosynthesis in rice is catalyzed by Asn synthase (AS), and rice is reported to have only one gene encoding AS (D83378) (Nakano *et al.*, 2000). The expression of the AS gene was therefore analysed by qRT-PCR, but AS expression was not altered in the roots of Dof1 rice (Figure 5). Furthermore, the expression of other genes

Table 1 Amino acid concentrations (nmol/g FW) and their proportions to the total amino acid (in parentheses) of VC rice and Dof1 rice grown under N sufficient condition (360 μM of N)

	VC			Dof1-64			Dof1-202			Dof1-203		
Leaf												
Lys	247.24 ±	103.01	(1.81% ± 0.80)	463.38 ±	295.70	(2.16% ± 0.70)	182.43 ±	55.28	(1.42% ± 0.39)	208.17 ±	80.25	(1.61% ± 0.45)
Arg	532.51 ±	188.50	(3.71% ± 0.89)	785.10 ±	201.10*	(4.16% ± 0.97)	371.51 ±	175.15	(2.81% ± 1.05)	404.30 ±	213.10	(2.93% ± 0.91)
His	89.14 ±	54.15	(0.65% ± 0.39)	341.06 ±	430.09	(1.36% ± 0.96)	118.14 ±	58.31	(0.96% ± 0.57)	115.70 ±	47.12	(0.89% ± 0.26)*
GABA	197.38 ±	99.66	(1.42% ± 0.72)	224.76 ±	124.22	(1.21% ± 0.72)	352.15 ±	294.35	(2.50% ± 1.40)	260.78 ±	115.20	(1.93% ± 0.48)
Gly	2167.07 ±	1281.39	(14.67% ± 5.70)	2503.16 ±	1115.55	(12.30% ± 2.27)	757.64 ±	195.09*	(5.87% ± 1.02)*	1717.61 ±	739.60	(12.98% ± 3.38)
Ala	1816.45 ±	513.83	(12.80% ± 2.58)	2101.78 ±	749.25	(10.78% ± 1.95)	1909.97 ±	366.77	(14.88% ± 1.68)	1535.45 ±	561.43	(11.61% ± 2.49)
Ser	1933.16 ±	547.32	(13.54% ± 2.19)	3811.14 ±	2611.90	(17.59% ± 3.56)*	1922.80 ±	526.75	(14.92% ± 2.96)	1854.08 ±	550.07	(14.30% ± 2.04)
Val	247.92 ±	59.07	(1.77% ± 0.39)	542.36 ±	319.87*	(2.54% ± 0.59)*	250.43 ±	55.75	(2.00% ± 0.61)	272.63 ±	48.43	(2.17% ± 0.41)
Ile	98.07 ±	50.27	(0.70% ± 0.34)	271.04 ±	225.89	(1.20% ± 0.49)*	101.28 ±	29.82	(0.82% ± 0.33)	124.73 ±	39.95	(0.98% ± 0.25)
Leu	112.52 ±	35.25	(0.82% ± 0.31)	222.85 ±	192.32	(0.98% ± 0.37)	109.00 ±	50.94	(0.89% ± 0.51)	134.01 ±	63.19	(1.05% ± 0.41)
Asn	480.35 ±	308.73	(3.20% ± 1.23)	243.85 ±	146.56	(1.02% ± 0.80)*	257.64 ±	153.28	(1.86% ± 0.85)*	376.02 ±	180.13	(2.83% ± 1.24)
Thr	552.13 ±	104.13	(3.96% ± 0.77)	818.43 ±	347.00	(4.07% ± 0.55)	571.91 ±	71.55	(4.53% ± 0.79)	595.70 ±	112.48	(4.70% ± 0.67)
Gln	2367.02 ±	998.24	(16.35% ± 3.33)	3271.97 ±	1459.47	(16.39% ± 5.32)	2735.74 ±	1894.07	(19.16% ± 8.15)	2146.46 ±	1037.00	(16.01% ± 3.35)
Pro	159.79 ±	31.19	(1.16% ± 0.29)	268.19 ±	203.38	(1.23% ± 0.33)	170.30 ±	25.78	(1.34% ± 0.24)	178.15 ±	48.49	(1.41% ± 0.34)
Glu	2664.96 ±	540.73	(19.29% ± 4.63)	3368.99 ±	1132.74	(17.46% ± 4.15)	2588.48 ±	419.44	(20.68% ± 4.86)	2523.08 ±	601.41	(19.94% ± 4.23)
Phe	125.94 ±	48.19	(0.92% ± 0.35)	292.63 ±	189.84	(1.34% ± 0.33)	144.49 ±	36.62	(1.16% ± 0.39)	171.40 ±	48.03	(1.35% ± 0.32)*
Tyr	83.53 ±	26.44	(0.60% ± 0.17)	212.71 ±	228.56	(0.88% ± 0.47)	94.42 ±	13.57	(0.75% ± 0.15)	92.23 ±	27.87	(0.73% ± 0.19)
Asp	358.93 ±	115.89	(2.63% ± 1.02)	708.07 ±	437.14*	(3.33% ± 0.68)	427.78 ±	63.50	(3.47% ± 1.09)	328.61 ±	135.76	(2.57% ± 0.84)
Total	14234.11 ±	3405.57		20420.99 ±	9069.92		13066.10 ±	3436.87		13039.11 ±	3714.45	
Root												
Lys	44.01 ±	20.86	(1.13% ± 0.43)	59.61 ±	17.69	(0.89% ± 0.20)	52.37 ±	25.61	(0.97% ± 0.68)	47.41 ±	27.21	(1.10% ± 0.51)
Arg	55.27 ±	27.77	(1.47% ± 0.55)	95.61 ±	26.83	(1.32% ± 0.24)	64.93 ±	33.10	(1.21% ± 0.68)	55.89 ±	19.07	(1.26% ± 0.22)
His	47.84 ±	16.55	(1.28% ± 0.24)	79.76 ±	24.49	(1.14% ± 0.12)	66.70 ±	21.14	(1.33% ± 0.50)	57.30 ±	21.04	(1.23% ± 0.35)
GABA	275.96 ±	84.98	(7.46% ± 2.00)	378.51 ±	146.22	(5.74% ± 2.60)	279.60 ±	53.14	(5.7% ± 1.55)	238.82 ±	49.73	(5.17% ± 1.44)*
Gly	91.40 ±	20.07	(2.39% ± 0.26)	105.22 ±	42.45	(1.53% ± 0.55)*	83.64 ±	24.23	(1.73% ± 0.53)*	121.94 ±	95.16	(2.65% ± 1.87)
Ala	246.00 ±	58.49	(6.85% ± 1.14)	464.22 ±	122.02*	(6.77% ± 2.07)	343.46 ±	59.11*	(7.06% ± 0.88)	320.24 ±	50.56*	(6.78% ± 1.02)
Ser	300.26 ±	78.64	(8.04% ± 1.14)	440.25 ±	115.09*	(6.34% ± 0.56)*	366.94 ±	87.60	(7.34% ± 0.78)	391.81 ±	148.07	(8.42% ± 2.57)
Val	61.02 ±	24.43	(1.58% ± 0.40)	105.15 ±	40.05*	(1.46% ± 0.35)	63.60 ±	32.00	(1.17% ± 0.32)	68.96 ±	30.51	(1.51% ± 0.48)
Ile	51.76 ±	14.46	(1.35% ± 0.18)	66.19 ±	14.02	(1.01% ± 0.24)*	49.65 ±	10.10	(0.96% ± 0.16)*	54.65 ±	19.26	(1.19% ± 0.32)
Leu	56.72 ±	13.01	(1.47% ± 0.28)	54.12 ±	10.64	(0.80% ± 0.18)*	44.35 ±	7.85*	(0.91% ± 0.23)*	49.77 ±	22.83	(1.08% ± 0.43)
Asn	853.24 ±	322.36	(23.22% ± 5.22)	2569.13 ±	1144.99*	(36.83% ± 5.40)*	1574.60 ±	666.06*	(28.72% ± 5.85)	1246.67 ±	344.97*	(26.45% ± 4.03)
Thr	43.89 ±	19.04	(1.17% ± 0.31)	94.79 ±	38.44*	(1.35% ± 0.22)	60.16 ±	24.73	(1.12% ± 0.17)	72.06 ±	35.57	(1.54% ± 0.60)
Gln	805.83 ±	189.63	(21.25% ± 2.20)	1363.15 ±	494.97*	(19.53% ± 2.05)	1123.04 ±	485.74	(22.88% ± 4.56)	1143.20 ±	261.19*	(23.85% ± 4.59)
Pro	49.55 ±	19.36	(1.26% ± 0.41)	46.57 ±	8.20	(0.70% ± 0.13)*	37.70 ±	4.72	(0.75% ± 0.10)	41.41 ±	19.75	(0.89% ± 0.35)
Glu	391.81 ±	56.25	(10.91% ± 2.24)	553.05 ±	141.50*	(8.46% ± 2.23)*	551.19 ±	96.97	(11.32% ± 2.96)	471.33 ±	82.61*	(9.91% ± 2.14)
Phe	19.19 ±	7.98	(0.53% ± 0.16)	28.77 ±	8.85*	(0.40% ± 0.13)	18.32 ±	5.12	(0.37% ± 0.13)	23.29 ±	18.53	(0.51% ± 0.36)
Tyr	38.00 ±	11.31	(1.00% ± 0.17)	43.77 ±	10.96	(0.62% ± 0.07)*	31.36 ±	5.05	(0.65% ± 0.16)*	40.47 ±	15.47	(0.87% ± 0.28)
Asp	315.92 ±	44.87	(8.45% ± 2.21)	359.49 ±	103.62	(5.35% ± 1.00)*	307.47 ±	86.19	(6.60% ± 2.39)	301.79 ±	69.35	(6.42% ± 2.03)
Total	3716.67 ±	763.70		6894.57 ±	2112.25*		5084.24 ±	1277.29*		4708.20 ±	751.97*	

Concentrations of each amino acid in leaf and root of rice plants grown under 360 μM of N treatment were measured with individual plants. Values are the means ± SD of at least eight replicates.

*Statistically significant differences at $P < 0.05$, when compared with VC samples.

known to play central roles in N metabolism, such as GS and glutamate synthase (GOGAT), was not altered in the roots of Dof1 rice (Figure 5).

Effect of ZmDof1 expression on biomass and C assimilation

Increased N assimilation (Figure 4) suggested a potential enhancement of biomass production in Dof1 rice because N amounts and biomass are known to be correlated. The DW of different organs of Dof1 and VC rice was measured to investigate the effect of ZmDof1 on biomass. Although significant differences in DW and C content between Dof1 and VC rice were not evident in any of the organs examined (Figure 6a,b), a tendency towards an increase in DW and C content in Dof1 rice was observed (Figure 6a,b).

Effect of ZmDof1 expression under nitrogen-deficient conditions

The present results showing increased N assimilation in Dof1 rice, together with the findings previously reported for Arabidopsis (Yanagisawa *et al.*, 2004), led to the speculation that the growth of Dof1 rice might be better than that of control rice under N-deficient conditions. For the present study, we applied 90 μM of N as the deficient N concentration based on the

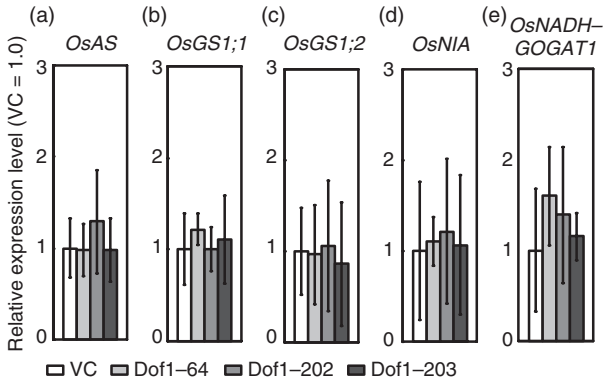

Figure 5 Effect of ZmDof1 on the expression of genes associated with N assimilation. Quantitative RT-PCR analysis of transcripts of *OsAS* (a), *OsGS1;1* (b), *OsGS1;2* (c), *OsNIA* (d), and *OsNADH-GOGAT1* (e) was performed with RNA from roots of T_3 generations of VC rice and three Dof1 rice lines. Values represent the means ± SD of at least four replicates, after normalization with the expression of actin (*OsRac1*).

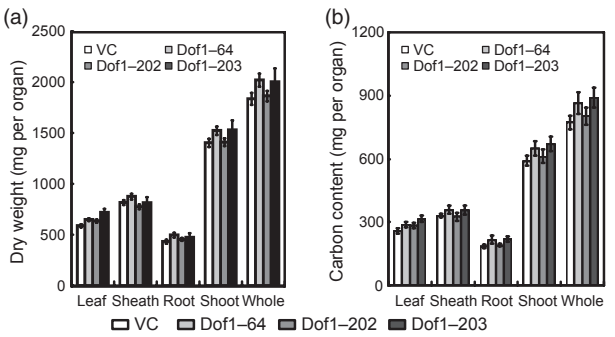

Figure 6 Growth of Dof1 rice under N-sufficient conditions. Dry weight (a) and C content (b) of leaf blades, leaf sheaths, roots, shoots and whole rice plants grown with 360 μM of N were measured in individual plants. Values are expressed as the means ± SE of at least seven replicates. Asterisks indicate statistically significant differences at $P < 0.05$, when compared with VC samples.

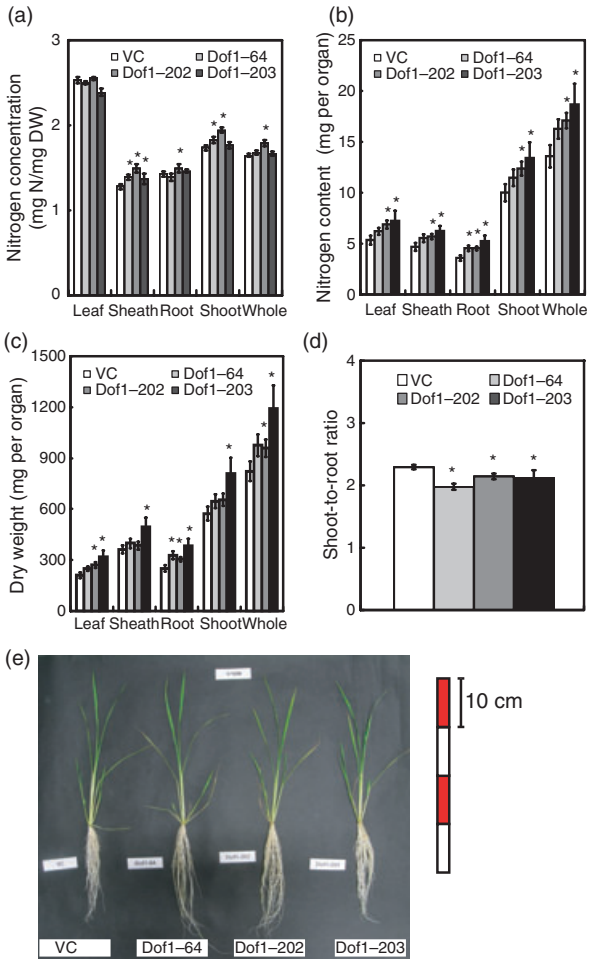

Figure 7 N concentration, N content, biomass, shoot-to-root ratios and plant phenotypes of Dof1 rice grown under N-deficient conditions. N concentration (a), N content (b), biomass (c) of rice plants grown under 90 μM of N were measured. (d) The shoot-to-root ratios of rice plants were calculated in individual plants based on their DW. Values represent the means ± SE of at least seven replicates. Asterisks indicate statistically significant differences at $P < 0.05$, when compared with VC samples. (e) Photographs of the VC and Dof1 rice plants. The plants were grown hydroponically for 2 weeks under only ion-exchanged water followed by 6 weeks under 90 μM of N. The pH of the 90 μM of N nutrient solution was adjusted to 5.5 daily and exchanged every 2 days.

results of preliminary experiments (see Materials and Methods) and examined the effect of ZmDof1.

As expected, N concentration (mg N/mg DW) and N content (g N/g Organ) were higher in Dof1 rice compared with VC rice plants (Figure 7a,b), but the differences were less significant than those observed in plants grown in a solution containing 360 μM of N (Figure 4). Although alterations in amino acid concentrations were mild, significant increases in Asn concentration were noted in the roots of all Dof1 plants grown under nitrogen-deficient conditions, as was the case under the 360 μM N condition (Table 2). These results indicated that ZmDof1 expression also enhanced N assimilation in rice grown under nitrogen-deficient conditions. Differences in biomass were more evident between Dof1 and VC rice plants, when they were grown using 90 μM N rather than 360 μM of N, and these differences were more significant in the roots than in the shoots (Figure 7c). In roots, 20%–50% increases were observed, whereas 10%–40% increases were detected in shoots. The different DW increase ratios between roots and shoots resulted in a significant reduction in the S/R ratio (Figure 7d). A representative figure is shown in Figure 7e.

Effect of ZmDof1 expression on the net photosynthesis rate under nitrogen-deficient conditions

The increases in biomass implied an increase in C assimilation, which can only be achieved through the enhancement of Pn. To validate this hypothesis, the light response curves of net photosynthesis were drawn for both VC and Dof1 rice by measuring the Pn under multiple irradiances. The values obtained under the 90 μM of N condition revealed that although the maximum Pn under 2000 μmol/m^2/s photosynthetic photon flux density (PPFD) was not statistically different between VC and Dof1 rice, Dof1 rice had a higher Pn, especially below 1000 μmol/m^2/s PPFD (Figure 8a and Table 3). Further analyses showed that the stomatal conductance at the different irradi-

Table 2 Amino acid concentrations (nmol/g FW) and their proportions to the total amino acid (in parentheses) of VC rice and Dof1 rice grown under N deficient condition (90 μM of N)

	VC	Dof1-64	Dof1-202	Dof1-203
Leaf				
Lys	111.02 ± 21.77 (2.63% ± 0.52)	60.16 ± 7.35* (1.34% ± 0.30)*	46.98 ± 7.76* (0.69% ± 0.16)*	47.84 ± 20.29 (1.06% ± 0.37)*
Arg	17.24 ± 4.63 (0.41% ± 0.11)	16.03 ± 4.47 (0.34% ± 0.04)	23.31 ± 8.50 (0.33% ± 0.09)	15.95 ± 6.55 (0.35% ± 0.10)
His	56.03 ± 8.88 (1.32% ± 0.22)	61.06 ± 19.21 (1.30% ± 0.19)	86.97 ± 31.02* (1.23% ± 0.28)	45.76 ± 11.36 (1.03% ± 0.21)*
GABA	39.78 ± 13.59 (0.93% ± 0.26)	61.95 ± 27.62 (1.40% ± 0.71)	77.47 ± 46.76* (1.10% ± 0.58)	82.79 ± 51.68 (1.95% ± 1.33)
Gly	296.56 ± 61.34 (7.03% ± 1.61)	294.35 ± 109.26 (6.22% ± 1.46)	382.7 ± 98.25 (5.53% ± 1.26)	315.50 ± 73.65 (7.15% ± 1.63)
Ala	467.8 ± 142.55 (10.96% ± 3.02)	497.15 ± 42.37 (11.09% ± 2.47)	682.66 ± 183.84* (9.82% ± 2.22)	525.58 ± 131.45 (11.89% ± 2.92)
Ser	621.34 ± 69.65 (14.63% ± 0.94)	808.6 ± 294.89 (16.90% ± 2.56)*	1291.57 ± 257.74* (18.65% ± 3.56)*	751.55 ± 21.05 (16.77% ± 3.67)
Val	115.10 ± 21.51 (2.71% ± 0.48)	129.17 ± 73.04 (2.61% ± 0.77)	177.55 ± 82.95 (2.50% ± 0.76)	87.84 ± 22.72 (1.95% ± 0.28)*
Ile	39.23 ± 11.57 (0.93% ± 0.28)	53.93 ± 60.26 (1.00% ± 0.81)	82.62 ± 79.62 (1.10% ± 0.77)	24.29 ± 8.68 (0.54% ± 0.11)*
Leu	27.45 ± 8.24 (0.65% ± 0.20)	34.91 ± 32.60 (0.68% ± 0.42)	54.91 ± 43.63 (0.75% ± 0.43)	21.34 ± 4.35 (0.48% ± 0.05)*
Asn	32.67 ± 20.10 (0.79% ± 0.55)	26.79 ± 12.53 (0.56% ± 0.19)	32.69 ± 18.78 (0.49% ± 0.32)	40.72 ± 30.13 (0.87% ± 0.55)
Thr	89.58 ± 19.51 (2.10% ± 0.33)	103.27 ± 19.19 (2.23% ± 0.20)	132.10 ± 15.62* (1.91% ± 0.18)	90.89 ± 21.24 (2.03% ± 0.21)
Gln	594.28 ± 46.5 (14.10% ± 1.78)	666.30 ± 211.04 (14.07% ± 1.14)	1566.18 ± 498.29* (22.06% ± 3.28)*	759.29 ± 154.88 (17.13% ± 2.83)*
Pro	97.26 ± 12.66 (2.30% ± 0.27)	61.51 ± 10.91* (1.34% ± 0.21)*	107.43 ± 19.76 (1.56% ± 0.33)*	63.49 ± 10.13 (1.44% ± 0.24)*
Glu	1247.50 ± 161.44 (29.33% ± 2.38)	1374.10 ± 295.62 (29.56% ± 3.13)	1738.84 ± 285.31* (25.10% ± 3.47)*	1214.59 ± 232.05 (27.20% ± 1.51)
Phe	83.81 ± 31.32 (1.98% ± 0.74)	68.88 ± 44.57 (1.39% ± 0.53)	91.54 ± 35.62 (1.29% ± 0.38)*	38.50 ± 9.87 (0.87% ± 0.21)*
Tyr	46.38 ± 6.60 (1.09% ± 0.13)	46.94 ± 17.44 (0.99% ± 0.16)	70.16 ± 21.46* (1.00% ± 0.17)	39.58 ± 9.12 (0.89% ± 0.11)*
Asp	260.49 ± 51.59 (6.13% ± 1.08)	325.80 ± 80.35 (6.97% ± 0.99)	343.50 ± 78.22* (4.90% ± 0.64)*	289.00 ± 94.64 (6.41% ± 1.39)
Total	4243.51 ± 328.64	4690.90 ± 1160.71	6989.19 ± 1231.71*	4454.52 ± 713.71
Root				
Lys	74.00 ± 51.57 (2.41% ± 1.03)	60.91 ± 21.94 (2.08% ± 0.69)	54.50 ± 6.19 (1.90% ± 0.46)	59.35 ± 12.46 (1.91% ± 0.70)
Arg	79.52 ± 72.78 (2.41% ± 1.78)	48.62 ± 11.14 (1.68% ± 0.72)	43.02 ± 21.74 (1.51% ± 0.48)	51.93 ± 13.13 (1.58% ± 0.19)
His	73.01 ± 34.54 (2.40% ± 0.69)	88.22 ± 31.87 (2.90% ± 0.91)	68.86 ± 12.59 (2.32% ± 0.43)	77.51 ± 22.79 (2.36% ± 0.55)
GABA	244.61 ± 82.33 (9.54% ± 3.76)	225.33 ± 72.14 (7.15% ± 1.79)	258.67 ± 46.21 (8.57% ± 2.02)	311.66 ± 197.10 (8.96% ± 2.93)
Gly	110.61 ± 81.10 (3.32% ± 1.97)	118.04 ± 58.19 (4.00% ± 1.73)	57.15 ± 13.30 (1.79% ± 0.23)	73.07 ± 15.21 (2.33% ± 0.82)
Ala	198.22 ± 68.07 (6.78% ± 0.91)	195.24 ± 54.04 (6.32% ± 0.82)	197.10 ± 47.62 (6.29% ± 0.76)	215.14 ± 55.99 (6.56% ± 1.22)
Ser	336.00 ± 138.13 (11.03% ± 2.47)	336.92 ± 101.13 (10.94% ± 2.65)	301.89 ± 71.05 (9.48% ± 0.98)	303.55 ± 82.27 (9.25% ± 1.59)
Val	70.41 ± 40.75 (2.33% ± 0.75)	82.18 ± 30.19 (2.61% ± 0.44)	72.05 ± 23.58 (2.25% ± 0.36)	84.26 ± 34.23 (2.49% ± 0.54)
Ile	31.29 ± 21.57 (1.02% ± 0.42)	31.71 ± 11.43 (1.06% ± 0.30)	30.14 ± 8.27 (0.99% ± 0.20)	33.84 ± 11.00 (1.02% ± 0.22)
Leu	48.11 ± 46.12 (1.52% ± 0.98)	39.37 ± 14.25 (1.33% ± 0.41)	32.19 ± 6.42 (1.04% ± 0.17)	39.13 ± 12.24 (1.18% ± 0.31)
Asn	256.51 ± 111.83 (8.72% ± 3.47)	497.48 ± 42.92* (14.43% ± 6.78)*	523.57 ± 274.97* (16.36% ± 6.14)*	670.64 ± 53.76* (17.52% ± 8.47)*
Thr	63.08 ± 27.00 (2.13% ± 0.48)	71.71 ± 23.51 (2.32% ± 0.58)	62.59 ± 16.84 (1.98% ± 0.26)	67.54 ± 18.31 (2.03% ± 0.22)
Gln	440.78 ± 81.79 (15.82% ± 2.82)	443.47 ± 201.02 (13.68% ± 2.31)	592.69 ± 233.93 (17.49% ± 4.56)	531.10 ± 192.20 (15.98% ± 3.76)
Pro	37.07 ± 25.47 (1.16% ± 0.52)	29.13 ± 11.22 (0.99% ± 0.33)	32.98 ± 12.71 (1.00% ± 0.32)	29.44 ± 7.58 (0.92% ± 0.26)
Glu	447.63 ± 63.96 (16.01% ± 2.41)	530.19 ± 64.25* (16.53% ± 4.32)	491.38 ± 78.21 (15.82% ± 1.76)	480.52 ± 154.14 (14.91% ± 4.19)
Phe	25.49 ± 20.88 (0.81% ± 0.43)	23.91 ± 12.34 (0.79% ± 0.35)	18.84 ± 8.67 (0.63% ± 0.24)	18.98 ± 5.67 (0.58% ± 0.18)
Tyr	44.68 ± 18.47 (1.53% ± 0.37)	41.23 ± 12.54 (1.30% ± 0.30)	31.06 ± 7.51 (1.01% ± 0.20)*	37.90 ± 7.87 (1.17% ± 0.23)
Asp	288.58 ± 47.55 (10.41% ± 1.50)	294.69 ± 58.05 (9.24% ± 2.40)	277.19 ± 42.58 (8.79% ± 1.61)	269.87 ± 152.72 (7.93% ± 2.21)*
Total	2829.51 ± 605.47	3508.01 ± 1215.50	3314.52 ± 770.88	3404.84 ± 1236.32

Concentrations of each amino acid in leaf and root of rice plants grown under 90 μM of N treatment were measured with individual plants. Values are the means ± SD of at least eight replicates.

*Statistically significant differences at $P < 0.05$, when compared with VC samples.

ances was increased in Dof1 rice plants (Figure 8b), while no differences were detected on CO_2 concentration in the intercellular space (Ci) between VC rice and Dof1 rice plants (Figure 8c). A detailed investigation revealed that the initial slopes of Pn light response curves were much steeper in Dof1 rice compared with VC rice (Table 3), suggesting a higher light-use efficiency in Dof1 rice. Additional experiments investigating initial slopes of CO_2 response curves under 1500 μmol/m^2/s PPFD, an index for the maximum carboxylation efficiency, also displayed no differences between VC rice and Dof1 rice plants (Supplemental Figure S2), which is in agreement with the finding that the content and activity of RubisCO did not differ between VC rice and Dof1 rice plants (Supplemental Figure S3a,b). Taken together, these results showed that differences in Pn could have resulted in an increase in the biomass of Dof1 rice under N-deficient conditions.

Discussion

ZmDof1 gained attention as a potential transcription factor to improve N assimilation based on results obtained in Arabidopsis and potato (Yanagisawa *et al.*, 2004), but the effect of ZmDof1

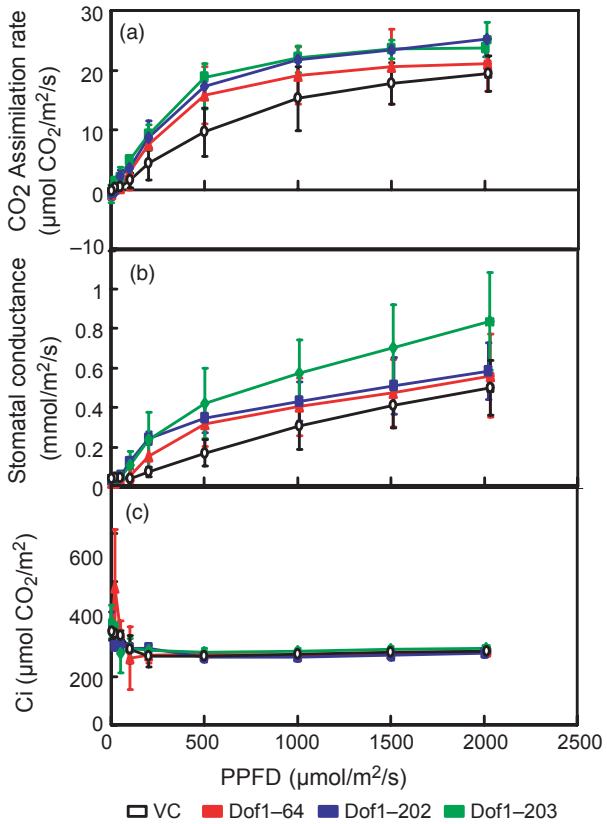

Figure 8 Light response curves and Ci response curves of Dof1 rice grown under nitrogen-deficient conditions. Light response curves of Pn (a), stomatal conductance (b) and Ci (c) of Dof1 rice plants grown under multiple irradiances. Values are expressed as the means ± SD of at least four replicates. Refer to Table 3 for the statistical differences obtained by comparison with VC samples.

expression in monocots has not been investigated. In the present study, *ZmDof1* was introduced into rice, which is one of the main staple crops in the world, and its effect was investigated in the transgenic rice plants.

Consistent with the results obtained with ZmDof1-expressing transgenic Arabidopsis, the increase in the expression of and transactivation of rice PEPC genes (Figure 2a–c,e), as well as higher PEPC activity (Figure 2f), was found in the leaves of Dof1 rice. It was also similar that Dof1 rice lines homozygous for the *ZmDof1* gene could not be established, although the reason for this phenomenon is unclear. Most significantly, N uptake was increased in plants grown in both sufficient (360 μM) and deficient (90 μM) N concentrations (Figures 4a,b and 7a,b), and growth enhancement and an increase in biomass were observed in Dof1 rice plants grown under 90 μM of N (Figure 7c). These results indicated that ZmDof1 was capable of inducing both C and N assimilation in rice as well as in Arabidopsis. Furthermore, we showed that the effects of *ZmDof1* expression included the promotion of root development under conditions of N deficiency.

ZmDof1 expression and PEPC

Prior studies have reported that PEPC is one of the potential direct targets of ZmDof1 regulation (Yanagisawa and Sheen, 1998; Yanagisawa *et al.*, 2004). The present qRT-PCR results showed that *OsPpc1* expression was increased in all Dof1 transgenic rice plants (Figure 2a). The Dof1-202 line showed an additional enhanced expression of other cytosolic *OsPpc* genes (Figure 2a–c). Transient expression assays using rice PEPC promoters further supported the direct regulation of the expression of *OsPpc1* and *OsPpc3* by ZmDof1 (Figure 2e). Contrary to the changes in the expression level of *OsPpc2* in the Dof1-202 and Dof1-203 lines (Figure 2b), transactivation of the *OsPpc2* promoter by ZmDof1 was not observed in the present study (Figure 2e). A possible explanation for this finding is that the *OsPpc2* promoter region used might not contain the ZmDof1 binding region. This assumption is supported by recent works showing that binding sites of transcription factors sometimes exist downstream of the coding regions in Arabidopsis (Zheng *et al.*, 2009; Kaufmann *et al.*, 2010).

Several studies have reported the detrimental effect of increased PEPC activity on Pn or biomass. Overproduction of C_4-PEPC in rice resulted in reduced photosynthesis rates because of increased respiration rates under light conditions (Fukayama *et al.*, 2003). In other studies, the overproduction of

Table 3 Net photosynthesis rate (μmol/m^2/s) under multiple irradiance and initial slope of VC rice and Dof1 rice grown under N deficient condition (90 μM of N).

	VC	Dof1-64	Dof1-202	Dof1-203
Pn				
0 PPFD	−0.24 ± 0.98	−0.69 ± 0.32	−0.56 ± 1.25	−0.78 ± 1.52
20 PPFD	0.20 ± 0.40	0.15 ± 0.80	1.28 ± 0.25*	−0.41 ± 0.30
50 PPFD	0.40 ± 0.49	0.30 ± 0.77	1.97 ± 1.25	2.22 ± 1.54
100 PPFD	1.52 ± 1.22	2.83 ± 2.86	4.80 ± 0.38*	3.54 ± 0.63*
200 PPFD	4.36 ± 2.73	7.58 ± 1.12	9.22 ± 2.26*	8.68 ± 2.20*
500 PPFD	9.63 ± 4.02	15.78 ± 4.80*	18.83 ± 0.40*	17.23 ± 3.82*
1000 PPFD	15.27 ± 5.38	19.10 ± 4.76	22.07 ± 0.61*	21.80 ± 2.18*
1500 PPFD	17.77 ± 3.50	20.55 ± 6.29	23.60 ± 0.53*	23.47 ± 1.62*
2000 PPFD	19.50 ± 2.97	21.00 ± 4.50	23.70 ± 1.51	25.13 ± 2.84*
Initial Slope	0.017 (R^2 = 0.96)	0.034 (R^2 = 0.93)	0.050 (R^2 = 0.97)	0.046 (R^2 = 0.93)

Net photosynthesis rate of upper-most expanded leaf grown under 90 μM of N were measured with individual plants. Initial slope was determined by the best-fit linear line between 0 to 100 PPFD (μmol/m^2/s). R^2 in parentheses indicates coefficient of determination. Values are the means ± SD of at least eight replicates.
*Statistically significant differences at $P < 0.05$, when compared with VC samples.

PEPC resulted in severely stunted growth (Chen *et al.*, 2004; Rademacher *et al.*, 2002). The negative effects of too strongly increased PEPC activity might explain the slightly lower biomass of the Dof1-202 line that showed the highest PEPC gene expression (Figures 2a–c and 7c) and maximum PEPC activity (Figure 2f). However, possible differences in the expression of the introduced ZmDof1 among Dof1 rice lines because of positional effects cannot be ruled out, despite the use of the constitutive *ZmUbi-1* promoter.

Expression of ZmDof1 and other genes

2-oxoglutarate is an intermediate between C and N metabolisms, and its amount is reported to affect N uptake and assimilation (Yuan *et al.*, 2007). One of the key enzymes of 2-OG biosynthesis is isocitrate dehydrogenase, and it exists in two forms depending on the coenzyme used for the biosynthesis of 2-OG: isocitrate dehydrogenase using NAD^+ (IDH) and isocitrate dehydrogenase using $NADP^+$ (ICDH). In the present study, the expressions of IDH and ICDH were measured by qRT-PCR, and only ICDH expression was altered in Dof1 rice (Figure 3). The correlation between ZmDof1 expression and ICDH expression suggests that the observed increase in ICDH gene expression was caused by *ZmDof1*. Although, similar to the induction of the PEPC genes, the enhancement of the transcription of the ICDH gene was significant but mild (usually less than twofold of VC), alterations in metabolite concentrations suggested that modulations of gene expression can be used to evaluate the effect of ZmDof1. Transient expression assays using ICDH promoters similar to the transient expression of the PEPC genes in the present study (Figure 2e) or chromatin immunoprecipitation experiments would reveal if ICDH is another target of ZmDof1 and should be investigated in a future study.

Another effect of ZmDof1 expression was the enhancement of the NADP-MDH expression in Dof1 rice (Figure 3). MDH catalyzes the reversible reaction between OAA and malate, which strongly favours malate synthesis. OAA synthesized by PEPC is likely converted into malate by NADP-MDH and possibly transported to mitochondria, where the TCA cycle is taken place. The present results indicate that the expression of ZmDof1 in rice might have accelerated carbon flow from PEP towards 2-OG anaplerotically by altering multiple genes involved in the pathway and through positive feedback activation by the substrates. It is currently unknown that ZmDof1 directly regulated the NADP-MDH expression in the Dof1 rice.

Effects of ZmDof1 expression on N metabolism

In Arabidopsis, *ZmDof1* expression induced a remarkable increase in glutamine content, which caused an increase in the free amino acid content (Yanagisawa *et al.*, 2004). Furthermore, a recent report showing the role of a member of the Dof transcription factor family in the expression of GS in pine (Rueda-Lopez *et al.*, 2008) suggested the possibility that a Dof transcription factor might directly promote glutamine synthesis. Although we found the promotion of N assimilation in Dof1 rice, *ZmDof1* expression did not have a significant effect on the concentration of amino acids in rice, with the exception of the Asn concentration in roots (Tables 1 and 2). The present results therefore imply that the effects of *ZmDof1* expression on N metabolism may differ according to plant species, in accordance with the unique N metabolism characteristics in different plants.

Although the introduction of *ZmDof1* into rice significantly changed the concentration of Asn, the expression of the rice Asn synthase gene (Nakano *et al.*, 2000) in the roots of transgenic rice was comparable to that of control plants (Figure 5). The mechanism mediating the effect of *ZmDof1* expression on Asn biosynthesis therefore remains to be elucidated. Prior studies have shown that plants adapted to nutrient deficiency, including N deficiency, exhibit a steady state of transcripts and metabolome, in particular those involved in primary metabolism, after reaching equilibrium (Yokota-Hirai *et al.*, 2004). In the present study, rice plants were provided with constant N for 6 weeks, suggesting that a steady state was reached, which might have masked the changes in Asn expression. Therefore, a detailed analysis including transcriptome analysis, metabolite profiling and time course experiments might help elucidate the mechanisms mediating the effect of ZmDof1 on the direct and/or indirect modulation of C and N assimilation in rice.

ZmDof1 expression and growth under N deficiency in rice

Nitrogen deficiency suppresses biomass production because of the significant partitioning of N (approximately 30% in rice) to RubisCO (Makino *et al.*, 1983) and chlorophyll, both of which influence the rates of photosynthesis. A correlation between Rubisco content and the rate of photosynthesis was reported (Makino *et al.*, 1987). The increases in biomass in the present study observed under N-deficient conditions suggested an elevated Pn in Dof1 rice because net photosynthesis is reported to account for at least 90% of biomass production (Zelitch, 1982). Light response curves of Pn showed that the observed increases in biomass in plants grown under the 90 μM N condition were caused by increased Pn. Further investigation revealed that increases in stomatal conductance, but not changes in RubisCO content or activity, were possibly responsible for the enhanced Pn (Figure 8 and Supplementary Figure S3).

In addition to the enhanced Pn, a decrease in the S/R ratio in Dof1 rice was found to be an important phenotype of the Dof1 rice in the present study. A decrease in the S/R ratio is a common plant response to N-limiting conditions aimed at increasing the N uptake capability (Hutchings and John, 2003). The significant reduction in the S/R ratio of Dof1 compared to VC rice plants occurred in plants grown in 90 μM of N (Figure 7d) and could reflect a similar adaptive response to low-N availability. Importantly, the observed reduction in the S/R ratio in Dof1 rice occurred without a concurrent reduction in shoot biomass (Figures 6a and 7c). This morphological change therefore indicates the capacity of Dof1 rice to efficiently adapt to low-N conditions. At this stage, it is uncertain whether this phenotype was induced by the ZmDof1 expression directly or through changes in metabolite contents. However, this morphological change could nevertheless have contributed to the enhancement in N uptake in Dof1 rice plants.

Experimental procedures

Generation of transgenic rice plants

A plasmid for plant transformation was constructed by modifying the binary vector pCambia 1302 (Cambia, Canberra, Australia), replacing the CaMV35S promoter and the gene for mGFP with the *ZmUbi-1* promoter from pAHC27 and *ZmDof1* cDNA (Yanagisawa *et al.*, 2004), respectively. The constructed plasmid was introduced into rice (cv: Nipponbare) by *Agrobacterium*

(EHA105)-mediated transformation (Toki *et al.*, 2006) with a slight modification of the method, using Claforan (Sanofi Aventis, Paris, France) instead of carbenicillin. VC rice plants were also generated with pCambia 1302 by replacing the CaMV35S promoter with the *ZmUbi-1* promoter. Re-generated rice plants were grown under controlled light and temperature conditions in a greenhouse (Day/Night cycle: 14/10 h; 27 °C in daytime, 22 °C at night).

Plant materials and growth

T_3 generation seeds obtained from three independent T_0 generations of Dof1 rice (ZmDof1-64, ZmDof1-202 and ZmDof1-203 lines) and VC rice were sterilized in 1% (v/v) NaClO for 20 min and then submerged in ion-exchanged water containing hygromycin (50 μg/mL) at 14 °C for a week to induce and synchronize germination. Sixteen seedlings of each transgenic line showing hygromycin resistance were further grown hydroponically with ion-exchanged water in a greenhouse (Day/Night cycle: 14/10 h; 27 °C in daytime, 22 °C at night) for 2 weeks. For N-sufficient treatment, the Yoshida nutrient solution ingredients were diluted to one-quarter-strength, resulting in a final concentration of 360 μM N. For N-deficient treatment, the Yoshida nutrient solution ingredients were also diluted to one-quarter-strength with the exception of N concentration, which was further reduced to 1/16, resulting in a final concentration of 90 μM N. Two-week-old seedlings were transferred to a corresponding diluted Yoshida nutrient solution. The pH of the nutrient solution was adjusted to 5.5 daily, and the solution was exchanged every 2 days. After 6 weeks of nitrogen supplementation, plants from each line were sampled between 10:00 and 11:00 am. The samples for measurements of metabolite content were divided into shoots and roots and then immediately dipped into liquid nitrogen and kept at −80 °C until use. The samples for biomass were divided into leaf blades, leaf sheaths and roots. Integration of T-DNA into the genome of analysed T_3 seedlings was confirmed by independent PCR using Dof1-specific primers and genomic DNA from individual plants.

'Sufficient' and 'deficient' N concentrations were determined by preliminary experiments, where growth rates of wild-type (WT) rice plants were compared with multiple N concentrations. In details, WT rice plants were first grown for 2 weeks with ion-exchanged water to completely deplete stored nutrients within seeds. Uniform seedlings were then transferred to modified Yoshida nutrient solution that did not include citrate (Yoshida *et al.*, 1976) with six different N concentrations: 1.44, 720, 360, 180, 90 and 45 μM of N. WT rice plants grown under 1.44, 720 and 360 μM of N showed no growth difference during 60 days of cultivation, when the pH of the nutrient solution was adjusted to 5.5 daily, and the nutrient solution was exchanged every 2 days. Whereas WT rice plants showed reduced growth, when N concentrations were further decreased. Furthermore, WT plants could not survive beyond 60-day cultivation when N concentration was 45 μM. We therefore decided to use 360 μM and 90 μM of N as sufficient and deficient N conditions, respectively.

Southern hybridization analysis

Genomic DNA was extracted from the leaves of transgenic and wild-type plants by the CTAB method (Murray and Thompson, 1980). Genomic DNA (10 μg) was digested with *Hin*dIII and electrophoresed on a 0.6% agarose gel before transfer to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). Dof1-specific DNA probes were generated by PCR amplification using the primer sets 5′-AGCGAGATCACCACGGAGACTG-3′ and 5′-CCTCGGGAGGTTGAGGAAGAT-3′. Prehybridization and hybridization as well as washing and detection were carried out according to the manufacturer's instructions for the AlkPhos Direct Labelling and Detection System (GE Healthcare, Buchinghamshire, UK). To detect hybridization signals, a KODAK BioMax XAR Film (Carestream Health, Paris, France) was exposed for 4 h. The Southern hybridization analyses were repeated using *Bgl*II or *Eco*RI for further confirmation (data not shown).

RNA extraction, cDNA synthesis and quantitative RT-PCR analysis

Total RNA was extracted from the fully expanded, upper-most leaves of 8-week-old seedlings using an RNA Plant Mini kit (QIAGEN K.K. Japan, Tokyo, Japan), according to the manufacturer's instructions. After elimination of DNA with TURBO DNase (Applied Biosystems Japan, Tokyo, Japan), total RNA was quantified by spectrophotometric analysis. First-strand cDNA synthesis was performed with 500 ng of total RNA and oligo$(dT)_{20}$ primers using Thermoscript (Invitrogen Japan, Tokyo, Japan) in a 20-μL reaction mixture. A volume of 0.5 μL of the reaction mixture was used for each quantitative RT-PCR. The conditions used for quantitative RT-PCR with a real-time PCR machine (ABI7300 model; Applied Biosystems Japan) and SYBR Green (Invitrogen) were 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of 95 °C for 15 s and 57 °C for 30 s. The amount of product was quantified using a standard curve after normalization with transcripts from an actin gene (*OsRac1*). Primer sets used for quantitative RT-PCR are listed in Supplementary Table S1.

Transactivation assay of the rice PEPC gene promoters in maize leaf protoplasts

The isolation of maize leaf protoplasts, followed by the cotransfection of effector and reporter constructs into protoplasts and a reporter enzyme assay, was performed according to previous reports (Sheen, 1990; Yanagisawa and Sheen, 1998). The effector constructs used were 35SC4PPDK-Dof1 for Dof1 expression and 35SC4PPDK-none as a negative control (Yanagisawa and Sheen, 1998) and reporter constructs that were generated by the replacement of the promoter sequence in the EBS reporter construct (Yanagisawa *et al.*, 2003) with rice PEPC gene promoters. Detailed information on the rice PEPC gene promoters (*OsPpc1* and *OsPpc2*) was obtained from previous work (Sanchez and Cejudo, 2003). An additional PEPC gene in japonica rice was identified in the present database search, and this gene is referred to as *Osppc3* for convenience. The promoters for *OsPpc1* (AK065029), *OsPpc2* (AK101274) and *OsPpc3* (AK073703) were obtained by PCR using sets of specific primers as follows: 5′-cccaagcTTAGTGGAACCAGAGAAAAGGAG-3′ and 5′-catgccatggCTCCCTCTCTCTCGCTTACAGCT-3′ for *OsPpc1*, 5′-cccaagcTTCTAGCGTGGATACGAATAGAC-3′ and 5′-catgccatggCTCCCCGAAAGCCGACTCCTTTT-3′ for *OsPpc2* and 5′-cccaagcTTGCTATGGCCTATGGTTTC-3′ and 5′-catgccatggCCCCCCCAAAACCACCCGATTCAA-3′ for *OsPpc3*, where small letters indicate intentionally added restriction enzyme sequences for cloning. After verification, the cloned DNA fragments were used as the PEPC gene promoters. Because the plasmid UBI-GUS

(Yanagisawa and Sheen, 1998) was used as an internal control in the cotransfection experiment, relative LUC activity was normalized against GUS activity.

Measurement of DW, and C and N contents

Shoots and roots of 8-week-old seedlings were dried for at least 72 h in an oven at 70 °C to measure their DW. The DW of the shoots was considered as the sum of the DWs of leaf blades and leaf sheaths. Total DW was calculated as the sum of the DW of leaf blades, leaf sheaths and roots. The samples were also measured on their N and C contents with an NC analyzer (vario MAX CN model; Elementar, Hanau, Germany) according to the manufacturer's instructions.

Measurement of amino acid content

Frozen upper-most expanded leaf blades and roots (50 mg) were finely ground using a Multi-Beads Shocker (Yasui-Kikai Co., Osaka, Japan), followed by the extraction of metabolites using the method described by Sato *et al.* (2004) and Wakayama *et al.* (2010). Briefly, 250 μL of 100% methanol was added to the powdered sample material, followed by 250 μL of water containing 400 μM methionine sulfone as an internal standard. The extract was filtered through a 5-kDa ultrafilter (Millipore, Bedford, MA) with centrifugation at 12 000 ***g*** for 40 min at 4 °C. Filtrates were analysed using a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) and the quadruple mass spectrometer Finnigan TSQ Quantum Discovery Max (Thermo Fisher Scientific, San Jose, CA).

Measurement of CO_2 assimilation and photosynthetic components

Pn (μmol CO_2/m^2/s), stomatal conductance (mmol/m^2/s^1) and intercellular CO_2 concentration (μmol CO_2/mol) were measured in the attached upper-most, fully expanded leaves using Li6400 with a fluorometer attachment (LI-COR, Lincoln, NE). All measurements were performed at 27 °C, 65% relative humidity, and the flow rate was constant at 500 μmol/s. To obtain a light response curve, the growth chamber CO_2 concentration was maintained at 380 μmol/mol, and Pn was measured at the following PPFDs: 2000, 1500, 1000, 500, 300, 200, 100, 50, 20 and 0 μmol/m^2/s. The initial slopes of light response curves were drawn with the values obtained from 100, 50, 20 and 0 μmol/m^2/s PPFD. To obtain a Ci-curve, the PPFD was maintained at 1500 μmol/m^2/s, and Pn was measured at the following CO_2 concentration: 2000, 1500, 1000, 500, 300, 200, 100, 50 and 0 μmol/mol. The initial slopes were drawn with the values obtained from 200, 100, 50 and 0 μmol/mol CO_2.

Assessment of PEPC activity, RubisCO content and activity

Frozen second upper-most, fully expanded leaf blades were homogenized using a chilled mortar and pestle on ice with 1 ml of extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 2 mM EDTA (pH 7.0), 10 mM $MgCl_2$, 5 mM DTT, 10% (v/v) glycerol and 2% (w/v) polyvinylpolypyrrolidone. The homogenates were centrifuged at 13 200 ***g*** for 10 min at 4 °C, and the supernatants were used for the assessment of PEPC activity, RuBisCO content and its activity according to the methods described by Jiao *et al.* (2005), Kanbe *et al.* (2009) and Du *et al.* (1996), respectively.

Statistical analysis

Data were analysed using the Student's *t*-test (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL). Significant differences were determined based on $P < 0.05$.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Effects of ZmDof1 expression on PEPC in roots of rice.
Figure S2 Effects of ZmDof1 on maximum carboxylation efficiency under nitrogen deficient conditions.
Figure S3 Effects of ZmDof1 on RubisCO in rice.
Table S1 Lists of primer sets used for qRT-PCR analyses.

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