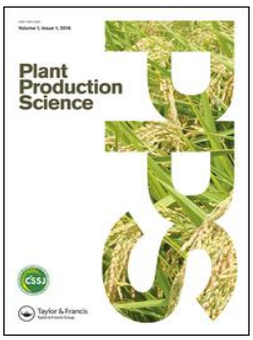

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# 【Short Report】Effects of Nitrogen on the Expression of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Small Subunit Multigene Family Members in Rice (*Oryza sativa* L.)

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【Short Report】

# Effects of Nitrogen on the Expression of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Small Subunit Multigene Family Members in Rice (*Oryza sativa* L.)

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**Abstract: Five ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit genes identified in the rice (*Oryza sativa*) genome are designated as *OsRBCS1*, *2*, *3*, *4* and *5*. *OsRBCS1* transcripts were not detectable. The mRNA levels of *OsRBCS2*, *OsRBCS3*, *OsRBCS4* and *OsRBCS5* in leaf blades were increased by $NH_4NO_3$ supply to nitrogen-starved plants, but the extent of increment varied with the genes. The mRNA levels of *OsRBCS2*, *OsRBCS3* and *OsRBCS4* in leaf blades were increased by $NH_4NO_3$ or glutamine supply to detached shoots. On the other hand, $NH_4NO_3$ and glutamine had little effect on the expression of *OsRBCS5* gene. The mRNA level of *OsRBCS4* was increased only by cytokinin supply to the detached shoots. The expression of the promoters of *OsRBCS2* and *OsRBCS3* was enhanced by $NH_4NO_3$ supply.**

**Key words: Cytokinin, Gene expression, Nitrogen, Promoter, Ribulose-1,5-bisphosphate carboxyase/oxygenase small subunit multigene family, Rice.**

Nitrogen (N) is an essential nutrient and a metabolic signal that controls gene expression in plants. Nitrate can serve as a signal of N that regulates gene expression (Forde, 2002; Scheible et al., 2004; Wang et al., 2004). For example, nitrate induces the expression of genes encoding proteins that include nitrate transporters, nitrate reductase, nitrite reductase, and enzymes in the pentose phosphate and glycolytic pathways. Nitrate is converted to nitrite and ammonium, and is ultimately incorporated into amino acids. Nitrite has been shown to act as a potent signal for global regulation of gene expression (Wang et al., 2007). Ammonium can induce expression of genes including those that encode phosphoenolpyruvate carboxylase (Sugiharto and Sugiyama, 1992) and an asparagin synthetase (Wong et al., 2004). Studies using methionine sulfoximine, a nonmetabolizable analog of glutamine (Gln) that irreversibly inhibits glutamine synthetase, have implied that the assimilated forms of N such as Gln or glutamate may also serve as signals that coordinate regulation of gene expression (Oliveira and Coruzzi, 1999; Rawat et al., 1999).

Cytokinin has been implicated in N responses as a pivotal substance communicating N availability from roots to shoots (Takei et al., 2002). Nitrate application increases cytokinin biosynthesis in roots, and cytokinins are transported to shoots (Rahayu et al., 2005; Sakakibara et al., 2006). The cytokinin signal is transmitted to target genes through His-Asp phosphorelay systems (Sakakibara et al., 2006). In this signal transduction pathway, response regulator family members function as nuclear transcription factors binding to target promoters that contain the (A/G)GGAT(T/C) sequence (Ferreira and Kieber, 2005).

A highly positive correlation between photosynthetic capacity and N content of leaves is widely recognized. There are also strong linear relationships between N content and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in higher plants (Evans, 1989). Rubisco is composed of eight small subunits (rbcS) encoded by a nuclear multigene family and eight large subunits encoded by a single gene in the multicopy chloroplast genome. In rice, *Oryza sativa,* the *rbcS* multigene family consists of 5 members designated as *OsRBCS1*, *2*, *3*, *4* and *5* (AK059909, AK121444, AK068555, AK070257 and AK099574, respectively; Suzuki et al., 2007). *OsRBCS1* mRNA levels in leaf blades were very limited

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**Abbreviations**: BA, benzyladenine; Gln, glutamine; LUC, luciferase; N, nitrogen; PCR, polymerase chain reaction; rbcS, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; sqRT-PCR, semi-quantitative reverse transcription-polymerase chain reaction.

(Suzuki et al., 2009). The expression of *OsRBCS2, 3, 4* and *5* genes was enhanced similarly with increasing nitrogen supply (Suzuki et al., 2007). However, no sequence identity was found in the promoter regions of the *rbcS* multigene family members, and very limited data is available on the expression of the *rbcS* multigene family members in response to N supply and N signals that regulate the gene expression. Cytokinin stimulated accumulation of *rbcS* mRNAs in *Cucurbita pepo* (Lerbs et al., 1984) and *Lemma gibba* (Flores and Tobin, 1988), but the effect of cytokinin on the expression of the *rbcS* multigene family members in rice is not known.

Our ultimate goal is to understand the molecular mechanisms underlying N regulation of the expression of Rubisco genes in rice. In this study, we investigated the expression of the rice *rbcS* multigene family members in response to N supply, and signal molecules to regulate the gene expression. Furthermore, we determined the effect of N on the transcriptional rate of the *rbcS* promoters by analyzing transient expression.

## Materials and Methods

### 1. Plant materials

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were sterilized in 1.0% (v/v) NaClO for 1 hr and thoroughly rinsed five times in sterile water. Seeds were grown hydroponically in distilled water for a week in a controlled growth chamber with a day/night photoperiod of 12/12 hr at 250 $\mu$mol $m^{-2}$ $s^{-1}$ and humidity of 70% at 25ºC. Seedlings were transferred to nutrient solution (250 $\mu$M $NaH_2PO_4$, 75 $\mu$M $K_2SO_4$, 75 $\mu$M $CaCl_2$, 150 $\mu$M $MgCl_2$, 11.25 $\mu$M iron(III) monosodium-ethylenediaminetetraacetate, 12.5 $\mu$M $H_3BO_4$, 2.25 $\mu$M $MnSO_4$, 75 nM $CuSO_4$, 175 nM $ZnSO_4$, 250 nM $Na_2MoO_4$) (Mae and Ohira, 1981). Its pH was adjusted to 5.5 with 0.1 M HCl. After 8 d, the seedlings were transferred to the fresh nutrient solution or the nutrient solution containing 0.5 mM $NH_4NO_3$, and the solutions were renewed every day. After 3 d, third leaves were collected and frozen in liquid N immediately, and then stored at −80ºC until analysis.

### 2. Treatment of shoots

Shoots were cut from plants grown hydroponically using the nutrient solution without N sources for 18 d, and were dipped at their cut ends in the nutrient solution without N sources, or including 1 mM $NH_4NO_3$, 12 mM Gln or 10 $\mu$M benzyladenine (BA) for 4.5 hr. Then, the third leaf blades were frozen in liquid N and stored at −80ºC

### 3. Semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR)

Total RNA was isolated by the method described by Nagata and Saitou (2009) with minor modifications. To remove any DNA contamination, we treated the total RNA with DNase I (TaKaRa Bio Inc., Shiga, Japan). First-strand cDNA was synthesized from 0.9 $\mu$g total RNA, using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Bio Inc.) with random hexamer, according to the manufacturer's instructions. sqRT-PCR was performed with 1/5 of the first-strand reaction mix. Sequences of the primer for PCR were 5'-TGCATGCAGGTGTGGCCGATTGA-3' (forward) and 5'-GACGACGAAGGACATGCACTG-3' (reverse) for *OsRBCS1*, 5'-TGCATGCAGGTGTGGCCGATTGA-3' (forward) and 5'-CCAACAACATATAGTCGTAGCAGATACC-3' (reverse) for *OsRBCS2*, 5'-TGCATGCAGGTGTGGCCGATTGA-3' (forward) and 5'-GGCCATTGAGATGGTACATA ACCAAACT-3' (reverse) for *OsRBCS3*, 5'-TGCA TGCAGGTGTGGCCGATTGA-3' (forward) and 5'-AACAACGGCAGGTCGGAGCG-3' (reverse) for *OsRBCS4*, and 5'-TGCATGCAGGTGTGGCCGATTGA-3' (forward) and 5'-GGCCATTGAGATGGTACATAACCAAACT-3' (reverse) for *OsRBCS5*. The PCR condition was 94ºC for 30 s, 65ºC for 30 s, and 72ºC for 1 min 30 s. The reaction was repeated for 23−31 cycles to obtain an appropriate amount of DNA. The cycle numbers were determined to avoid the saturation of DNA amplification. The transcript of *18S rRNA* gene amplified was detected as an internal control (Nagata and Saitou, 2009). PCR products were separated on a 1.5% agarose gel, followed by 1 hr staining with ethidium bromide. The image of the stained gel was recorded with a gel image analyzer (Molecular Imager FX; Bio-Rad Laboratories, CA, USA). The image density of each stained PCR product was analyzed using the software provided with the analyzer (Quantity One software; Bio-Rad Laboratories). The amplified products were cloned and fully sequenced to confirm gene-specific amplification.

### 4. Construction of vectors for the biolystic method

To make reporter vectors, a *Renilla LUCIFERASE* (*LUC*) gene (*hRluc*) of the phRG-B vector (Promega, WI, USA) was ligated to the *Bam*HI/*Sac*I site of pBI221 (Clontech, CA, USA), replacing the *β-GLUCURONIDASE* gene, and generating *pCAMV35S-hRluc*. The 5' flanking regions of the *OsRBCS2* and *OsRBCS3* genes were amplified from genomic DNA of leaves by polymerase chain reaction (PCR). Sequences of the primer for PCR were 5'-CCCAAG CTTGGGTAGTAGAGATGCAACTTTACC-3' (forward) and 5'-CGGGATCCCGAGTACTTCTTGAGATGCACTGC-3' (reverse) for the *OsRBCS2* promoter, and 5'-CCCAAGC TTGGGAGGCGGTTCGTCCGTCAGC-3' (forward) and 5'-CGGGATCCCGTAGAGTATGTCCAGTAGCAGTG-3' (reverse) for the *OsRBCS3* promoter. Each forward and reverse primer was incorporated a *Hin*dIII site and *Bam*HI site (underlined). The PCR products were cut with *Hin*dIII and *Bam*HI, inserted each into the *Hin*dIII/*Bam*HI site of *pCAMV35S-hRluc*, replacing the cauliflower mosaic virus 35S promoter. The firefly *LUC* gene (*luc*$^+$) of the pGL3 basic vector (Promega) was inserted into the *Bam*HI/*Sac*I site of pBI221, replacing the *β-GLUCURONIDASE* gene.

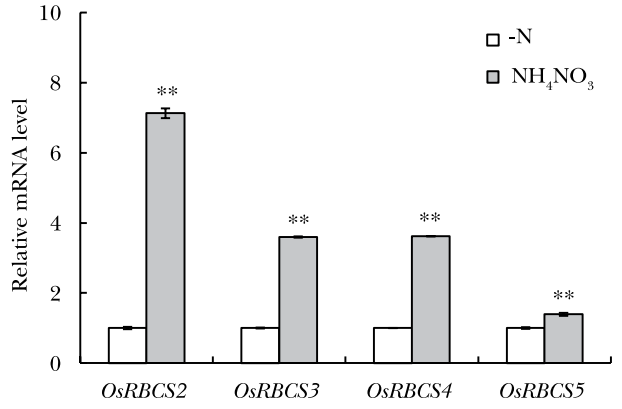


Fig. 1. Effect of $NH_4NO_3$ on the accumulation of mRNA for each member of the *rbcS* multigene family in leaf blades of rice. Rice seeds were precultured hydroponically in distilled water for 7 d and then in nutrient solution without N sources for another 8 d. Plants were transferred to the nutrient solution containing either no N or 0.5 mM $NH_4NO_3$ as a sole N source. After 3 d of the treatment, total RNA was isolated from the third leaf blades. The mRNA levels were normalized to 18S rRNA levels, and results were compared with the control value (without N sources) in each case, which was arbitrarily assigned 1. Bars show means ± SD (n = 3). ** indicates statistical significance at the 1% level by Student's *t*-test.

The resulting vector was used as an internal control.

**5. Transient gene expression**

DNA-coated microparticles were prepared by the $CaCl_2$/spermidine method as described by Sanford et al. (1993). An 80 ng aliquot of the reporter vector and 500 ng of the internal control vector were mixed with 375 $\mu$g of gold particles (1.0 Micron Gold; Bio-Rad Laboratories) in the presence of 2.5 M $CaCl_2$ and 0.1 M spermidine. The third leaf blades were bombarded from the reverse side with vector-coated gold particles (375 $\mu$g per bombardment) using a biolistic gun device (PDS-1000/He; Bio-Rad Laboratories), with the stopping screen positioned 3 cm below the rupture discs, the target leaf blades positioned 5 cm below the stopping screen, and a helium pressure of 7.6 MPa. After 20 hr of incubation in the growth conditions, bombarded leaf blades were homogenized with a pestle in an ice-cold mortar, and was measured *Renilla* and firefly LUC activities with the Dual-Luciferase® reporter assay system (Promega) according to the manufacturer's instructions, using a luminometer (GloMax™ 20/20n; Promega) to access chemiluminescence. The *Renilla* LUC activity values were normalized to firefly LUC activity values.

## Results and Discussion

Following N starvation, rice seedlings grown hydroponically for 15 d were transferred to nutrient solutions containing either no N or 0.5 mM $NH_4NO_3$ as a sole N source. After 3 d of the treatment, total RNA was extracted from the third leaf blades, and the mRNA levels of each member of the *rbcS* multigene family were determined by sqRT-PCR. *OsRBCS1* transcripts were not detectable (data not shown). This result was consistent with that of Suzuki et al. (2007). The levels of *OsRBCS2*, *OsRBCS3*, *OsRBCS4* and *OsRBCS5* mRNAs increased in response to $NH_4NO_3$ supply (Fig.1). The mRNA level of *OsRBCS2* increased 7-fold, and the incremental ratio was higher than that of any other *rbcS* multigene family member. The incremental ratio of *OsRBCS5* was 1.4. These results indicate that the expression of the *rbcS* multigene family members is differently regulated by N in rice.

Suzuki et al. (2007) reported that *OsRBCS1* was only slightly expressed, and the expression of *OsRBCS2*, *OsRBCS3*, *OsRBCS4* and *OsRBCS5* was enhanced to the same relative degree with increasing N supply. In addition, the amino acid sequences except the transit peptide, deduced from rice four *rbcS* genes except for *OsRBCS1*, are 100% identical. Suzuki et al. (2007) hypothesized that adaptation of Rubisco to changes in N availability does not occur through the specific expression of five members of the rice *rbcS* multigene family. However, according to the data presented in Fig. 1, the incremental degree of the gene expression by N supply differed markedly with the member of the *rbcS* multigene family, and no sequence identity was found in the promoter regions of the *rbcS* multigene family members. We used fully expanded leaves for experiments, while Suzuki et al. (2007) used leaves which had emerged from the sheaths about 50%. These findings suggest the importance of the leaf developmental stage in understanding the response of the expression of the *rbcS* multigene family members to N supply.

Nitrate and N metabolites derived from nitrate reduction and assimilation pathways act as signaling molecules to regulate global gene expression in plants (Vidal and Gutiérrez, 2008). Nitrate application increases cytokinin biosynthesis in roots, and cytokinins are transported to shoots and function as a signal that regulates a wide variety of genes (Rahayu et al., 2005; Sakakibara et al., 2006). Therefore, we examined the effects of $NH_4NO_3$, Gln and cytokinin on the expression of the *rbcS* multigene family members. After keeping the detached shoots in the nutrient solution containing 1 mM $NH_4NO_3$, 12 mM Gln or 10 $\mu$M BA, a synthetic cytokinin, for 4.5 hr, we determined the mRNA levels of the *rbcS* multigene family members in the third leaf blades. $NH_4NO_3$ induced the expression of *OsRBCS2*, *OsRBCS3* and *OsRBCS4*, whereas the expression of *OsRBCS5* was unchanged (Fig. 2). The expression of *OsRBCS2*, *OsRBCS3* and *OsRBCS4* was enhanced by Gln, but that of *OsRBCS5* was not enhanced significantly. Although the relative amount of *OsRBCS4* mRNAs was increased by BA, the expression of the *rbcS* multigene family members other than *OsRBCS4* was not altered. These results suggest that Gln and/or its metabolite(s) can

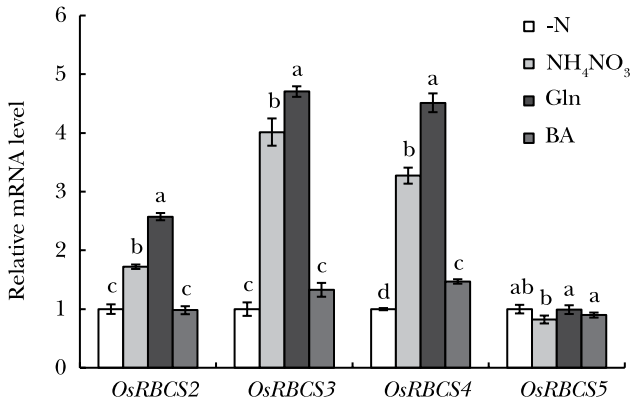


Fig. 2. Effect of $NH_4NO_3$, Gln and BA on the expression of the *rbcS* multigene family members in detached shoots of rice. Shoots were cut from N-starved plants, and were dipped at their cut ends in the nutrient solution without N sources, or including 1 mM $NH_4NO_3$, 12 mM Gln or 10 $\mu$M BA for 4.5 hr. Total RNA was isolated from the third leaf blades. The mRNA levels were normalized to 18S rRNA levels, and results were compared with the control value (without N sources) in each case, which was arbitrarily assigned 1. Bars show means ± SD (n = 3). The data were statistically analyzed with Tukey's multiple-range test for differences between means. Columns with the same letters are not significantly different ($p < 0.05$).

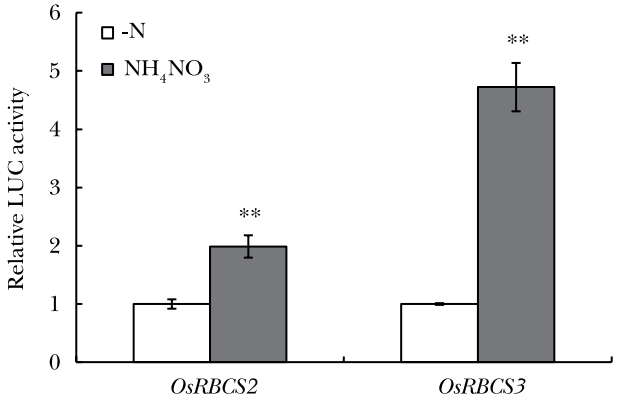


Fig. 3. Effect of $NH_4NO_3$ on the expression of the promoters of the *OsRBCS2* and *OsRBCS3*. *Renilla LUC* reporter genes were obtained by fusion to the *Renilla LUC* gene of the promoter of *OsRBCS2* or *OsRBCS3*. The third leaf blades of plants grown using the nutrient solution without N sources, or with 0.5 mM $NH_4NO_3$ for 3 d, were bombarded with the *Renilla LUC* reporter genes and internal control vectors containing the firefly *LUC* gene. After 20 hr of incubation, *Renilla* and firefly LUC activities were measured. *Renilla* LUC activity was normalized relative to the firefly LUC activity, and results were compared with the control value (without N sources) in each case, which was arbitrarily assigned 1. Bars show means ± SD (n = 3). ** indicates statistical significance at the 1% level by Student's *t*-test.

be the signal molecules for N to regulate the expression of *OsRBCS2*, *OsRBCS3* and *OsRBCS4*.

The expression of the *rbcS* multigene family members was enhanced by $NH_4NO_3$ supply, but the degree of enhancement shown in Fig. 1 was not consistent with that in Fig. 2. This suggests that the response time of the gene expression for N supply differs with the *rbcS* multigene family member since the treatment time was 3 d and 4.5 hr in Fig. 1 and 2, respectively.

The expression of *OsRBCS4* was enhanced by BA (Fig. 2). The (A/G)GGAT(T/C) sequences, which are recognized by response regulator family members in the cytokinin signal transduction pathway (Ferreira and Kieber, 2005), was present at the −98 nucleotide position of the *OsRBCS4* promoter. These observations imply that cytokinin regulates the *OsRBCS4* promoter.

The incremental ratio of the levels of *OsRBCS2* and *OsRBCS3* mRNAs in response to $NH_4NO_3$ supply was high (Figs. 1, 2). We bombarded with either the *OsRBCS2* or *OsRBCS3* promoter fused to the *Renilla LUC* reporter gene and the internal control vector containing the firefly *LUC* gene in the third leaf blades. Analysis of the transient expression of these genes showed that $NH_4NO_3$ enhanced the expression of the reporter gene driven by either promoter (Fig. 3). These results implied that the promoters of *OsRBCS2* and *OsRBCS3* have regulatory element(s) involved in N induction.

In conclusion, the expression of each member of the *rbcS* multigene family is regulated independently by N in rice, and signal molecules for N to regulate the gene expression vary with the *rbcS* multigene family member. Furthermore, the promoters of *OsRBCS2* and *OsRBCS3* can regulate N-inducible expression. Further studies are needed to identify the cis- and trans-regulatory elements involved in N induction in these promoters.

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