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Rice plant response to long term CO_2 enrichment: Gene expression profiling

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ABSTRACT

Effects of elevated CO_2 (68 Pa versus ambient 38 Pa) on gene expression were studied in rice leaves grown in soil medium with three different nitrogen conditions (0, 0.6 and 1.2 g N per 8-L pot) in CO_2 controlled chambers. Soluble protein contents were slightly decreased in leaves grown under elevated CO_2 regardless of N supplies, whereas the polypeptide profiles of soluble protein analyzed by 2DE using the same amount of protein were totally unchanged between ambient and elevated CO_2. In contrast, gene expressions examined by microarray analyses were significantly affected by elevated CO_2. Forty-six up-regulated genes (>1.5-fold) and 35 down-regulated genes (<0.68-fold) were identified and these included many signal transduction and transcription regulation related genes. By contrast, the expressions of most of the genes for primary metabolism were not significantly altered. Although changes were small, the expressions of genes for enzymes involved in CO_2 fixation (carbonic anhydrase, Rubisco, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase) were down-regulated, whereas that of genes encoding enzymes for RuBP regeneration (fructose bisphosphate phosphatase, fructose bisphosphate aldolase, sedoheptulose bisphosphate phosphatase and phosphoribulokinase) and starch synthesis (ADP-glucose pyrophosphorylase and starch synthase) were up-regulated under elevated CO_2. These results suggest that some sets of genes involved in primary metabolism pathway in the chloroplast are co-regulated by elevated CO_2.

1. Introduction

Global atmospheric CO_2 assume increase to 55 Pa by the end of this century [1]. This could expect to significant beneficial effects on plants by the enhancement of CO_2 assimilation. For this reason, the physiological and morphological impacts of elevated CO_2 have been extensively studied in various plant species [2,3]. Recently, FACE (free air CO_2 enrichment) facility has been developed all over the world and the effects of elevated CO_2 on plant growth are investigated at the managed field or natural ecosystem levels [2,4].

Photosynthesis is generally enhanced by elevated CO_2 and this also stimulates the growth and productivity in most C_3 plants [5]. However, prolonged exposure of elevated CO_2 causes down-regulation of photosynthesis typically showing reduction of Rubisco contents and light saturated CO_2 assimilation rate [5]. A high emphasis has been placed on the down-regulation because this significantly decreases the stimulation of final plant productivity by elevated CO_2 [6]. In addition to this down-regulation, plants also show some morphological changes under elevated CO_2. In general, plants became taller with larger stem diameter, increased branching and leaf number [5,7]. In rice, significant increase in biomass of leaf sheath was observed under elevated CO_2 where transiently accumulate fixed carbon as starch [8]. This could be an acclimation response to elevated CO_2 by changing the morphological characteristics in plants.

The response of plants to elevated CO_2 can relate to various signaling factors. An increase in the levels of soluble sugars in the cell often observed in the leaves under elevated CO_2, can influence the expression of sugar responsive genes and hexokinase 1 is suggested to be a sensor of soluble sugars [9]. In addition, the redox state in chloroplast can be an another signal of sugar responsive genes [10]. Enhancement of photosynthesis under elevated CO_2 may led to the decrease in Pi level in leaves [11] and PHR1 and SIZ1 can take part in the regulation of expression of Pi responsive genes [12,13]. Decrease in nitrogen content has been often observed in leaves under elevated CO_2 [5,6]. Dof transcription factors can regulate the genes related to nitrogen assimilation [14] and it may play some role in this phenomenon. In this manner, many signal

Abbreviations: FACE, free air CO_2 enrichment; 2DE, two-dimensional gel electrophoresis; *RbcS*, Rubisco small subunit gene; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate.

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transduction and transcription regulation related factors were expected to involve in CO_2 response and in addition, it is possible that these could interact with each other. However, the actual contributions of these signaling factors to CO_2 response in plants are still uncertain. In addition, these signaling factors including even most extensively characterized hexokinase have only reported in limited plant species [9–15]. These imply that there remain many questions left unanswered about the signaling mechanism of CO_2 response in plants.

In order to understand the molecular bases of CO_2 response, gene expression profiling using microarray were carried out in some plant species [16–20]. However, these profiles were diverse in response of genes related to metabolism and also selected signature genes. These suggest that response in gene expression to elevated CO_2 would significantly differ in plant species and seems difficult to find common features. In this study, we use rice, one of the most important crops for agriculture as a material and genome information and useful tools for molecular biology are well developed in this plant. Changes in gene expression due to exposure to elevated CO_2 were studied at protein level by two-dimensional gel electrophoresis (2DE) and transcript level by microarray including 44,000 oligo DNA.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice (*Oryza sativa* L. spp. Japonica cv. Nipponbare) plants were grown in naturally illuminated semi-closed growth chambers [21] at two different CO_2 levels (38 and 68 Pa as ambient and elevated CO_2, respectively). Three seedlings were transplanted in 8-L plastic pots filled with inceptisols (this soil originally contained approximately 2 mg g^{-1} N) with three different nitrogen supplies (0, 0.6 and 1.2 g per pot as low, medium and high N, respectively) applied as coated urea (LP-70, Chisso Asahi Fertilizer Co. Ltd., Tokyo, Japan). P_2O_5 and K_2O were applied 1.0 g per pot as basal dressing. The CO_2 treatment was started just after transplanting. Four chambers were used for the study, two being assigned to elevated CO_2 and the other two assigned to ambient CO_2. In each chamber, we also rotated the pots once per week to minimize any effects of microclimatic variation within the chambers. We also rotated the pots among the four chambers and then re-established the treatment conditions every 3 weeks during the treatment period so as to minimize the impact of any variations among the chambers. All the chambers were maintained at 32/22 °C (day/night) and 70% relative humidity. At 38 days after transplanting, the segments of about 2 cm for protein analysis and 20 cm for transcript analysis were harvested from the mid-section of the uppermost fully expanded 11th leaves (the flag leaf was 14th leaf in this study) at 11:00–12:00 and immediately frozen in liquid nitrogen. The samples were stored at −80 °C until use.

2.2. Extraction of leaf soluble protein and 2DE

Total leaf soluble proteins were extracted as described previously [22]. Samples were homogenized using a chilled mortar and pestle in an extraction buffer containing 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 5% polyvinylpolypyrrolidone and 10% (w/v) glycerol, with a small amount of sea sand. After total maceration, the homogenate was centrifuged at 15,000 × *g* for 5 min and resultant supernatant was collected as a total leaf soluble protein extract. 2DE was carried out according to the method of Tsuchida et al. [23]. The total soluble proteins of 30 μg were mixed with 4% CHAPS, 7 M urea, 2 M thiourea, 50 mM dithiothreitol, 0.002% bromophenol blue and 2% IPG buffer (GE Healthcare, Piscataway, NJ, USA). The proteins were first separated by IEF using Immobiline DryStrip (p*I* 4–7) and Multiphor II Electrophoresis Units (GE Healthcare), then by 12% SDS-PAGE, and stained with silver.

The soluble protein concentration was measured according to Bradford [24] using bovine serum albumin as a standard.

2.3. RNA extraction and microarray analysis

Total RNAs were isolated from leaves of four different plants grown in different pots for each treatment using the RNeasy plant mini kit (Qiagen, Carlsberg, CA, USA). Two sets of mixed total RNAs of two different plants (400 ng) were used for analysis. According to the manufacturer's instruction, the RNAs were labeled by Cyanine-3 or Cyanine-5 dye with the Low RNA Input Linear-Amplification/Labeling Kit (Agilent Technologies, Santa Clara, CA, USA) and the labeled cRNAs were purified using the RNeasy plant mini kit. After purification, Cyanine-3 and Cyanine-5 labeled cRNAs were hybridized with 44k Rice Oligo Microarray slides (Agilent Technologies). After hybridization, the signals were detected by DNA microarray scanner (Agilent Technologies), and spot intensities were digitalized using Feature Extraction software (Agilent Technologies). Difference in the expression of transcripts between ambient and elevated CO_2 were compared at each N levels. To separate the effects of nitrogen supply to CO_2 partial pressure, the comparison of low and medium N was also carried out. In total, four comparative analyses were done with two biological replicates and two technical replicates by dye swap.

Statistical data mining was performed to identify genes differentially expressed between treatments. Variances were normalized between dye swap samples, and then genes with Cyanine-5 signal value greater than 100 were selected. The mean and standard deviation were calculated for each gene and the data were adopted for *Z* transformation to calculate *Z* scores to estimate significant difference ($P < 0.05$) for each gene expression.

2.4. Semi-quantitative RT-PCR

RT-PCR was performed essentially as described by Fukayama et al. [25] using gene specific primers listed in Table 1. The 1st strand cDNA was synthesized from 5 μg of total RNA with oligo (dT)18 as the primer. AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) was first activated at 95 °C for 9 min, and PCR was carried out for 20–25 cycles of 15 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, followed by a final extension step for 7 min at 72 °C. Expression of the rice actin gene (RAc1, AB047313) was examined as an internal control.

Table 1
Primers used for RT-PCR.

Primer name	Primer sequence
PK-F	5′-TCACCGGGATGTGAAGACTA-3′
PK-R	5′-ATGAACGATGCACCATGGGC-3′
TPR-F	5′-CCGTTCATGGGGGCAGCTCA-3′
TPR-R	5′-GGCTTATTTATTTCACAAGCGT-3′
CBL-PK-F	5′-CCGAACCCGATGAAGAGGAT-3′
CBL-PK-R	5′-ATGATCCAGGGCGGCAATTC-3′
SynaC-F	5′-TGGACGTCGTGAACAACGGA-3′
SynaC-R	5′-GCGCCCAAAATGGATCACGA-3′
RbcS-F	5′-GCTTCGGCAACGTCAGCAAT-3′
RbcS-R	5′-CACACGAAACAAGGTGGGAG-3′
FBPase-F	5′-AGGACGTGTTACAGCCTGGA-3′
FBPase-R	5′-CACGCCTTGATGGTGCCAGA-3′
AGPase-F	5′-GTCCTGCATTTCTGAAGGCG-3′
AGPase-R	5′-CCGATGCCCCATCACATATT-3′
RAc1-F	5′-GCAACTGGGATGATATGGAGAA-3′
RAc1-R	5′-CCTCCAATCCAGACACTGTA-3′