Effects of Elevated CO₂ Concentration on Photosynthetic Carbon Metabolism in Flag-Leaf Blades of Rice before and after Heading

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Abstract: We monitored the effects of elevated atmospheric CO₂ concentrations on the photosynthetic carbon metabolism in the flag leaves of rice plant (Oryza sativa L. cv. Akitakomachi) before and after heading. The plants were grown under ambient (350 ppm: control) or elevated (650 ppm) CO₂ conditions. Flag-leaf blades grown under high CO₂ accumulated more starch than control leaf blades before heading, but the level of starch declined to almost zero under both CO₂ concentrations as soon as the development of ears began. Before heading, the transcript level of sucrose-phosphate synthase (SPS) (EC 2.4.1.14), a key enzyme in the sucrose synthesis in flag-leaf blades was significantly higher under elevated CO₂ conditions than under elevated CO₂ (P<0.01). The difference in the expression of SPS decreased after heading, coinciding with a change in starch contents in both groups. These results showed that the effects of elevated CO₂ concentration on rice plants might vary with the growth stage of the leaf blades. We also discussed the influence of the changes in the carbohydrate metabolism of rice plants caused by elevated CO₂ concentration on yield.

Key words: Elevated CO₂ concentration, Rice, Starch, Sucrose-phosphate synthase, Yield

The concentration of CO₂ ([CO₂]) in the global atmosphere, currently 350 ppm, is increasing and is predicted to double by the end of this century. It is of great interest to know how this will influence photosynthetic CO₂ fixation, photoassimilative metabolism, and source-sink relationships in plants. The effects of elevated [CO₂] on photosynthetic response and carbon metabolism have been studied, but mainly in plants in the vegetative growth stage (Webber et al. 1994; Koch, 1996; Wolfe et al., 1998).

In rice plants (Oryza sativa L.), transport of photoassimilates changes before and after heading. Before heading, photoassimilates accumulate mainly in the form of starch, in the stems and (to a lesser extent) in the form of sucrose in the flag-leaf blades. After heading, the stored sugar is remobilized and transported to the ears, the new sink organs (Cook and Yoshida, 1972). The contribution of carbohydrate assimilated before heading to grain yield is in the range of 20~40% of grain yield (Cook and Yoshida, 1972; Murata and Matsushima, 1975; Ishi, 1993). In contrast to what is the studies on the vegetative growth stage, relatively little information has been reported on the carbon metabolism and allocation of photoassimilates under elevated [CO₂] before and after heading in rice plants.

Sucrose-phosphate synthase (SPS) is a regulatory enzyme involved in the partitioning of photoassimilates between sucrose and starch in leaves (Huber and Huber, 1996). The ratio of starch to sucrose content has been correlated with SPS activity in rice leaf blades, which accumulate mainly sucrose and little starch (Ono et al., 1999). In leaf blades grown at elevated [CO₂], starch and sucrose contents are about two-fold as high as in control plants (Nakano et al., 1997; Hussain et al., 1999) and the maximum activity and/or activation state of SPS is increased (Seneweera et al., 1995).

Rapid development of plant molecular techniques aided our understanding of the acclimatization of plants to elevated [CO₂] (Moore et al., 1999). The steady-state level of mRNA for the small subunit (SSU) of ribulose - 1,5 - bisphosphate carboxylase/oxygenase (rubisco) is reduced by elevated [CO₂] (Moore et al., 1999). For other photosynthetic proteins, the available information on the response to elevated [CO₂] is limited. Under high [CO₂], the transcripts for ADP glucose pyrophosphorylase show little change in Lycopersicon.
infrared CO$_2$ analyzer (ZFP9GD11, Fuji Electric Co., Tokyo, Japan), and a computer-controlled pure CO$_2$ injection system replaced CO$_2$ during the day and removed it at night. Air temperature and relative humidity were controlled by feedback control systems and measured with a temperature and humidity sensor (HN -Q500-1, Chino, Tokyo, Japan). The relative humidity was kept at 80% throughout the experiment. Three of the 6 Climatrons were used to house control plants ([CO$_2$] 350/430 ppm, daytime/night-time), and the other 3 were used for the “high-CO$_2$” plants ([CO$_2$] 650/730 ppm). Mean air temperature and solar radiation during the growth period were measured at Tsukuba (Hayashi et al., 1998).

3. Determination of carbohydrate and chlorophyll contents and canopy net-photosynthesis

Samples of approximately 30 mg dry weight were powdered in liquid nitrogen with a mortar and pestle and extracted twice with 80% (v/v) ethanol at 80°C. After centrifugation at 2000×g for 5 min, the supernatants were collected, dried under vacuum, and used for the enzymatic determination of sucrose and glucose contents (Bergmeyer and Bernt, 1974). For the determination of the starch content, the pellets from centrifugation were boiled in distilled water for 2 h and digested with amyloglucosidase for 15 min at 55°C. The resultant glucose content was determined enzymatically as described above. All enzymes used in these procedures were obtained from Boehringer Mannheim GmbH (Mannheim, Germany).

For determination of the chlorophyll content in flag-leaf blades, samples of approximately 30 mg dry weight were soaked in 1 mL of 96% (v/v) ethanol until they became colorless. The chlorophyll content was measured by the method of Wintermans and De Mots (1965). The canopy net-photosynthesis was determined by calculating the amount of supplied CO$_2$ needed to maintain [CO$_2$] in the Climatrons.

4. RNA extraction and Northern blot analysis

For Northern blot analysis, total RNA was isolated as described by Wadsworth et al. (1988), with an extraction buffer containing 2 mM aurin tricarboxylic acid as a potent ribonuclease inhibitor. Total RNA, 20 µg per lane, was separated on a 1.15% (w/v) agarose gel containing formaldehyde, transferred to a nylon membrane (Hybond–N, Amersham Life Science, Buckinghamshire, England), and hybridized with $^{32}$P-labelled cDNA probes. The hybridization membrane was washed twice with 2×SSC (150 mM NaCl, 15 mM sodium citrate) plus 0.1% (w/v) sodium dodecyl sulfate (SDS) at room temperature for 5 min and with 0.2×SSC plus 0.1% (w/v) SDS at 55°C for 1 h, and then exposed to X-ray film. Hybridizing bands detected on the film were quantified with a high-resolution scanner (PowerLook 2000, UMAX Data Systems Inc., Taipei, ROC) and an image analysis software system (ZERO – Dscan,
Table 1. Effects of elevated CO\textsubscript{2} on the dry matter productivity (g DW plant\textsuperscript{-1}) and grain yield (g DW ear\textsuperscript{-1}) of rice plants grown in the Climatrons.

| Parameter       | 55 DAT (Middle vegetative stage) | 118 DAT (End of reproductive stage) |
|-----------------|----------------------------------|--------------------------------------|
|                 | Ambient                          | High CO\textsubscript{2}               | Ambient                          | High CO\textsubscript{2}               |
| Root            | 4.0±0.5                          | 3.7±0.3                               | 3.6±0.3                          | 4.1±0.2                               |
| Stems           | 11.9±0.6                         | 14.1±0.3**                            | 18.0±0.6                         | 21.3±1.0*                             |
| Leaf blade      | 5.7±0.4                          | 6.2±0.1                               | 3.3±0.1                          | 3.1±0.2                               |
| Ear             | 1.6±0.1                          | 1.5±0.1                               | 21.3±0.9                         | 23.4±1.0                              |

***, * mean significant difference at 0.01 and 0.05 levels, respectively.

Fig. 1. Time course of change in ear weight of rice plants grown under ambient or elevated CO\textsubscript{2} conditions (350 or 650 ppm). All of the ears examined came from main stems. Means are indicated by circles for control plants and triangles for high-CO\textsubscript{2}-grown plants. Standard errors (n=3) are indicated by vertical bars. Arrow indicates day of heading.

Scanalytics Inc., Virginia, USA). The probe was removed by washing the hybridisation membrane was washed with 2\times SSPE (150 mM NaCl, 10 mM Na\textsubscript{2}PO\textsubscript{4}, 1 mM EDTA) containing 90% (w/v) formamide and 10% (w/v) SDS at 80°C for 3 h. The data for three independent replications were averaged and presented as relative values. The 25S rRNA probe was used to check for equal loading on the gels, and the expression levels of the genes of interest were standardized to the 25S expression levels. Complementary-DNA fragments corresponding to the genes of the following enzymes were used to probe the Northern blots: rice CA (EC 4.2.1.1, Suzuki and Burnell (unpublished), DDBJ/EMBL/GenBank database accession number U08404, 194–849 bases), rice SSU (EC 4.1.1.39, D00644, full length (Matsuoka et al., 1988), rice cFBP (EC 3.1.3.11, AB007193, full length), and rice SPS (EC 2.4.1.14, Ohsugi [unpublished], corresponding to exons 6–10 of the gene published for this enzyme (Sakamoto et al., 1995; D45890), and rice 25S rRNA (M11585, 1474–2317 bases (Sugiura et al., 1985). The cDNA fragments of CA and 25S rRNA were amplified by reverse transcription-polymerase chain reaction from total RNA extracted from young green seedlings of rice, by using an RNA PCR Kit (ver. 2.1, Takara Biomedicals, Shiga, Japan).

5. Statistical analysis
The data shown are mean values±SE. Data were analyzed by ANOVA. Differences between the mean values were analyzed by using the least significant difference (P=0.05) with Duncan’s multiple range test.

Results
1. Growth, chlorophyll content, and grain yield
We compared the dry weights of separated parts of the control and high-CO\textsubscript{2}-grown plants at 55 DAT (i.e. during the middle vegetative stage) and 118 DAT (at the end of the reproductive stage) (Table 1). At both 55 and 118 DAT, the dry weights of the stems were significantly greater in the high-CO\textsubscript{2}-grown plants than in the control plants. The other plant parts (i.e., root and leaf blade) were unaffected by elevated [CO\textsubscript{2}]

Table 1 (continued)

2. Carbohydrate contents of flag-leaf blades before and after heading
During the period just after the onset of ear emergence (Fig. 1), considerable amounts of starch, sucrose and glucose accumulated transiently in flag-leaf blades of both rice plants grown under elevated CO\textsubscript{2} concentration (high-CO\textsubscript{2}-grown leaves) and flag-leaf blades of control plants (control leaves) (Figs. 2A, B, C). Before ear development began, the levels of starch and glucose in the flag-leaf blades declined to almost zero, but the
of SPS in high-CO₂-grown leaf blades were significantly higher than those in control leaf blades (P < 0.01). Therefore, we hypothesize that the presence of a high starch content may induce the higher expression of CA to facilitate the diffusion of CO₂ from the intercellular air space to rubisco in chloroplasts. CA catalyzes the interconversion of CO₂ and HCO₃⁻ and facilitates the diffusion of CO₂ and HCO₃⁻ into the chloroplasts.

3. Levels of transcripts for enzymes involved in photosynthetic carbon metabolism in flag-leaf blades before and after heading

Using the same leaf-blade samples that were used to measure carbohydrate content, we investigated changes in the steady-state levels of mRNAs for several enzymes related to photosynthetic carbon metabolism: carbonic anhydrase (CA), rubisco, and two regulatory enzymes of sucrose synthesis, cytosolic fructose-1,6-bisphosphatase (cFBP) and SPS. In the control leaf blades, levels of mRNA for CA and SSU were changed with a time course similar to that of starch and glucose (Figs. 2, 3A, B), although there was no difference between high-CO₂-grown and control leaf blades except for SSU at 65 DAT. Before heading (at 55 and 65 DAT), the transcript levels of SPS in high-CO₂-grown leaf blades were significantly higher than those in control leaf blades (P < 0.01). The level of cFBP mRNA in the flag-leaf blades of rice plants grown under high CO₂ tended to be higher than those of the control plants.

Discussion

The flag-leaf blade functions as a temporary sink organ before heading (Gock and Yoshida, 1972). To our knowledge, this is the first report describing the effects of elevated [CO₂] on carbon allocation and the expression of genes in rice flag-leaf blades before and after heading. The carbohydrate content and the expression of SPS changed with the growth stage of flag-leaf blades in both the high [CO₂] and control groups; this change was especially apparent around the time of heading. At 65 DAT (just before the appearance of ears), the starch content of high-CO₂-grown leaves was significantly higher than that of control leaves (Fig. 2). These results suggest that the high-CO₂-grown leaves may function as stronger sinks than the control leaves. Therefore, a significant difference in starch content was observed just before heading, when the ability to accumulate starch in stems reaches a maximum. Monitoring of the starch contents in stems under elevated [CO₂] would show whether this hypothesis is correct.

Rice accumulates more sucrose than starch in flag-leaf blades (Ono et al., 1999), but high accumulation of starch has been reported in high-CO₂-grown rice leaf blades (Nakano et al., 1997). Nomura et al. (1995) observed that huge accumulated starch grains deformed the thylakoids and grana of chloroplasts in rice leaves under elevated [CO₂] with sufficient phosphorus nutrition. It is likely that such a large amount of accumulated starch would affect photosynthesis and/or carbon metabolism of rice leaves, even at a transcriptional level. Enhancement of the expression of CA mRNA by elevated [CO₂] has been reported in leaves of Arabidopsis (Raines et al., 1992). In our study, the expression of CA changed with the growth stage, and, interestingly, the changes in the expression of CA coincided with the starch content in both high-CO₂-grown and control flag-leaf blades (Fig. 3A). Starch that has accumulated in the steady-state levels of mRNAs for several enzymes related to photosynthetic carbon metabolism: carbonic anhydrase (CA), rubisco, and two regulatory enzymes of sucrose synthesis, cytosolic fructose-1,6-bisphosphatase (cFBP) and SPS. In the control leaf blades, levels of mRNA for CA and SSU were changed with a time course similar to that of starch and glucose (Figs. 2, 3A, B), although there was no difference between high-CO₂-grown and control leaf blades except for SSU at 65 DAT. Before heading (at 55 and 65 DAT), the transcript levels of SPS in high-CO₂-grown leaf blades were significantly higher than those in control leaf blades (P < 0.01). The
Fig. 3. Expression levels of enzymes involved in photosynthetic sucrose metabolism in flag leaf blades before and after heading. All of the leaf blades examined came from main stems. Two series of samples, I from an ambient-CO₂ chamber and I from a high-CO₂ chamber, were loaded on the same gel and transferred to the same membrane. Levels of mRNA for CA (A), SSU (B), cFBP (C), and SPS (D) are expressed as relative values on the basis of the expression levels in the control plants on the day the sampling started (55 DAT). Means are indicated by circles for control plants and triangles for high-CO₂-grown plants. Standard errors (n=3) are indicated by vertical bars. Arrows indicate day of heading. * means significant differences at \( P < 0.05 \) by Duncan's multiple range test.
to relieve the restricted CO₂ diffusion. Our finding of a high level of expression of CA in high-CO₂-grown leaf blades containing a large amount of starch supports this hypothesis. Many papers have described a reduction in SSU levels under elevated [CO₂] in leaves at early growth stage (Moore et al., 1999). In our study, we detected an increase in SSU levels under elevated [CO₂] before heading (Fig. 3B). We used flag leaves at just before heading date, and the different result may be explained by this difference in materials. The effect of elevated [CO₂] on SSU expression in leaves should be examined further at different growth stages.

At 55 and 65 DAT before heading, the amount of SPS mRNA in high-CO₂-grown leaves was significantly higher than in control leaves (Fig. 3D). There have been several reports describing the increased SPS activity in rice leaf blades under elevated [CO₂] (Hussain et al., 1993; Seneweera et al., 1995). However, it is not clear exactly how increased SPS activity is caused by an elevation in [CO₂], since SPS activity is regulated at various steps (Huber and Huber, 1996). It is also not clear whether the transcriptional level of SPS gene reflects the activity of the enzyme, although our data suggested that an increase in SPS activity through an elevation in [CO₂] might be indeed caused at a transcriptional level. There was no difference in the transcriptional level of SPS after heading (Fig. 3D). Therefore, elevated [CO₂] had different effects on SPS mRNA levels before and after heading. The up-regulation of SPS before heading might be related to the accumulation of starch, which could have an oppressive effect on the chloroplasts.

The enhancement effect of elevated [CO₂] on photosynthetic ability by rice canopy was investigated (data not shown), confirming the result by Rowland-Bamford et al. (1991). There was no difference in the sucrose contents of the high-CO₂-grown and the control leaf blades before heading. These results suggest that an elevated [CO₂] increases the amount of carbon supplied to the stems, significantly increasing their dry weights (Table 1). In rice plants, starch stored prior to heading contributes to 20% to 40% in the grain yield (Cook and Yoshida, 1972; Murata and Matsushima, 1975; Ishii, 1993). However, there was no difference in grain yield in high-CO₂-grown plants and control plants (Table 1), although ear weight tended to increase faster in the former plants than in the controls (Fig. 1). The sink capacity of the grains is determined by genetic factors and/or by the level of nitrogen applied as fertilizer (Kumura, 1993). The effects of CO₂ enrichment (560 to 700 ppm [CO₂]) on the grain yield of rice have been shown to vary from 0% to 30% (Barker and Allen, 1993; Kim et al., 1996; Moya et al., 1998; Ziska et al., 1997). These differences may be attributed to the varietal difference in capacity. At the end of the reproductive stage (118 DAT) the dry weights of stems had been significantly increased by CO₂ enrichment (Table 1).

These results suggest that some of the stored starch might be left in the stems, because the grain sink capacity is limited. Further study on carbon flow with 13C is needed to clarify this point.

Our results showed that the effect of elevated [CO₂] on rice plants varied with the growth stage (e.g. before or after heading). This point has not been considered in our study on the effect of elevated [CO₂]. Further studies on the effect of elevated [CO₂] at various growth stages are necessary.

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