Sites of Action of Elevated CO₂ on Leaf Development in Rice: Discrimination between the Effects of Elevated CO₂ and Nitrogen Deficiency

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Elevated CO₂ concentrations (eCO₂) trigger various plant responses. Despite intensive studies of these responses, the underlying mechanisms remain obscure. In this work, we investigated when and how leaf physiology and anatomy are affected by eCO₂ in rice plants. We analyzed the most recently fully expanded leaves that developed successively after transfer of the plant to eCO₂. To discriminate between the effects of eCO₂ and those of nitrogen deficiency, we used three different levels of N application. We found that a decline in the soluble protein content (on a leaf area basis) at eCO₂ was only observed under N deficiency. The length and width of the leaf blade were reduced by both eCO₂ and N deficiency, whereas the blade thickness was increased by eCO₂ but was not affected by N deficiency. The change in length by eCO₂ became detectable in the secondly fully expanded leaf, and those in width and thickness in the thirdly fully expanded leaf, which were at the leaf developmental stages P₄ and P₃, respectively, at the onset of the eCO₂ treatment. The decreased blade length at eCO₂ was associated with a decrease in the epidermal cell number on the adaxial side and a reduction in cell length on the abaxial side. The increased thickness was ascribed mainly to enhanced development of vascular bundles and epidermal cell files. The increased blade length at eCO₂ was only observed under N deficiency. The length and width of the leaf blade were reduced by both eCO₂ and N deficiency, whereas the blade thickness was increased by eCO₂ but was not affected by N deficiency.

Introduction

Photosynthesis of C₃ plants, which account for about 90% of terrestrial plant species, is limited by the supply of CO₂, and elevation of the ambient CO₂ level is expected to enhance photosynthesis if other environmental conditions are optimal. Free-air CO₂ enrichment (FACE) experiments carried out using various C₃ plant species have shown that elevated CO₂ (eCO₂) stimulates leaf photosynthesis and photosynthetic carbon gain (Long et al. 2004, Leakey et al. 2009). In rice, for example, both biomass and yield are increased by about 20% in eCO₂ (Ainsworth 2008). However, the stimulation of photosynthesis and yield is much less than expected, due to plant acclimation responses to eCO₂ (Long et al. 2004).

Responses to eCO₂ observed in experiments using FACE fields, open-top chambers and closed growth chambers are essentially identical, though their extent often differs between the three types of experiments. Responses commonly observed in most plant species are a decline in the level of the CO₂ fixation enzyme of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and reduction in stomatal conductance (gₛ; Makino and Mae 1999, Ainsworth and Rogers 2007, Leakey et al. 2009). The former results in a decline of the initial slope of the response curve of net photosynthesis rate (A) to intercellular CO₂ concentration (Cᵢ) in a low Cᵢ range, which is called down-regulation of photosynthesis (Long et al. 2004). In accordance with the reduced gₛ, the stomatal density per leaf area is reduced in most cases (Woodward and Kelly 1995). Leaf size and anatomy are often altered by eCO₂, and increases in the total leaf area per plant, single-leaf area and leaf thickness have been reported for many species (Pritchard et al. 1999). These changes, however, do not always reflect acclimation responses to eCO₂, because features altered by eCO₂ are often affected by other growth conditions, for example nutrient availability (Stitt and Krapp 1999). Neither the Rubisco level nor the shape of the A–Cᵢ curve is significantly affected when nitrogen
(N) supply is sufficient (Sage 1994, Makino and Mae 1999, Seneweera et al. 2002). Leaf size and anatomy are also affected by N supply, but only a few studies have investigated the contribution of N conditions to the observed changes (Sims et al. 1998b).

Development of leaves is strongly affected by the environment experienced by mature leaves. Mature leaves of Arabidopsis thaliana exposed to eCO2 transmit a long-distance signal that controls stomatal development in expanding leaves (Lake et al. 2001). Phytohormones such as jasmonate and ethylene, reactive oxygen species and carbohydrates are candidates for the signal that controls stomatal development (Lake et al. 2001). The mechanisms of systemic regulation and the nature of the long-distance signal have not yet been clarified.

To understand the mechanisms underlying the responses to eCO2, it is necessary to know when and how eCO2 exerts its effects on leaf physiology and anatomy. Leaf development in rice had been intensively studied, and a series of morphological changes occurring from formation of a leaf primordium to full development of a leaf were described in detail (Kauffman 1959, Yamazaki 1963a, Hoshikawa 1989). Rice leaf development is classified into seven stages using the plastochron number (Pi) from P0 to P6 (Itoh et al. 2005). P0 represents a state of cells determined to form a new leaf primordium, and P6 is a mature leaf with fully expanded blade and sheath. When the uppermost leaf is fully developed (P0), five leaves at different developmental stages from a leaf primordium (P1) to a rapidly expanding leaf (P5) are present at the same time (Yamazaki 1963a, Hoshikawa 1989). In addition, rice leaves develop at a relatively constant rate (Hoshikawa 1989). Thus, the developmental stage affected by eCO2 can be determined by the analysis of successively developing leaves. In this study, we consecutively analyzed the uppermost leaves that had developed and fully expanded after plant transfer from aCO2 to eCO2 using rice plants (Oryza sativa L. ssp. japonica cv. Nipponbare). To discriminate between the effects of eCO2 and those of N deficiency, rice plants were grown under different N conditions. We found that a decline in soluble protein and Chl contents at eCO2 did not occur in the presence of sufficient N. By analyzing the effects of eCO2 on leaf size and anatomy under sufficient N supply, we could identify the developmental stages affected by eCO2.

### Results

#### Experimental design

We used three different N conditions by applying the amounts of N fertilizer corresponding to 1/3 (very low; VL), 2/3 (low; L) and 5/3 (excess; Ex) of the standard level. Under the Ex-N conditions, no signs of N deficiency, such as the death of lower leaves or bleaching of the upper leaf, were observed up to the plant age of 12 (expressed as the leaf number on the main culm). The CO2 treatments, aCO2 (300–400 p.p.m.) or eCO2 (approximately 1000 p.p.m.), were started when the seventh leaf blades were fully expanded, but those of the eighth leaves had not yet emerged from the sheaths of older leaves (plant age = 7.0; a decimal fraction represents the length of the next leaf blade emerged from the sheaths of older leaves, relative to the full length). The seventh leaf and each of the successive younger leaves were analyzed when the blade of the next leaf was expanded to two-thirds of its full length (leaf age = 0.6–0.7). At this leaf stage, the soluble protein content of the uppermost fully expanded leaf blades reached maximum (see Supplementary Fig. S1 for the seventh leaf). The widest region of each blade, usually at approximately one-third from the tip, was used for the analyses.

Essentially identical experiments were performed twice, in which the growth chambers for aCO2 and eCO2 treatments were exchanged [designated experiment (Exp.) 1 and 2]. Rice plants were grown until the plant age of 13 under three different N conditions.

#### Effects of eCO2 and applied N level on the uppermost fully expanded leaf blade

The emergence of leaves on the main culm was accelerated with high N supply, but it was not significantly affected by eCO2, whereas the number of tillers increased with increasing amounts of applied N, and was further increased by eCO2 (Exp. 1; Fig. 1). This is in line with previous reports for some other rice cultivars (Makino et al. 2000, Seneweera et al. 2002).

Leaf soluble protein and Chl contents (on a leaf area basis) increased with increasing N level (Fig. 2A, B; Supplementary Fig. S2A, B). Protein levels in the seventh leaf blades at VL-N were lower than those at higher N levels at both aCO2 and eCO2, indicating N deficiency in these blades at VL-N. eCO2 reduced the soluble protein earlier at lower N levels: the decline by eCO2 in protein level (by about 10%) was first observed in the seventh leaf blades at VL-N and the eighth leaf blades at L-N, but was only observed in the 12th blade at Ex-N (Fig. 2A). These results suggest that the decreased protein content at eCO2 was caused by N deficiency. eCO2 also decreased the Chl content in an N supply-dependent manner (Fig. 2B). The Chl content measured using a Chl meter (SPAD) was similar for aCO2 and eCO2 up to the 10th leaf, but became higher at eCO2 in successive leaves (Fig. 2C). The effect of eCO2 on protein and Chl contents also became undetectable in the 11th and
12th blades at VL-N and L-N, which may be due to an increase of blade thickness induced by eCO2, as described below.

Both the length and width of leaf blades were affected by both eCO2 and the applied N level (Fig. 3A, B; Supplementary Fig. S2D, E). At aCO2, both the length and width increased up to the 13th leaf at Ex-N, and up to the 10th leaf at VL-N and L-N, whereas at eCO2, they increased up to the ninth leaf, and then declined faster at VL-N and L-N than at Ex-N (Supplementary Fig. S2D, E), indicating that N deficiency was enhanced by eCO2. At Ex-N, the decline in length by eCO2 became detectable in the ninth leaf and that in width in the 10th leaf, and they became more pronounced in successive leaves (Fig. 3A, B). At Ex-N, the length of the 13th leaf blade was reduced by eCO2 by 24% and the width by 22%, resulting in an approximately 40% reduction in the leaf area.

The thickness of the leaf blade [defined as the mean thickness at the ridges of small vascular bundles (VBs)] was increased by eCO2 but was not affected by N supply (Fig. 3C; Supplementary Fig. S2F). The increase in thickness became detectable starting from the 10th leaf (Fig. 3C).

In the chamber swap experiment (Exp. 2), a marked decline in the leaf protein level with low N supply was first observed in the ninth leaf at both aCO2 and eCO2 (Supplementary Fig. S3A). In accordance with the delayed N deficiency in Exp. 2, the reduction in the blade length and width by low N supply was smaller in this experiment (Supplementary Fig. S3A) than in Exp. 1 (Supplementary Fig. S2D, E). In the presence of sufficient N supply (at Ex-N), the change in blade length induced by eCO2 became detectable in the ninth leaf and that in width in the 10th leaf (Supplementary Fig. S3B) in the same way as observed in Exp. 1.

The Rubisco contents of the ninth leaf blades of plants from Exp. 2 were compared (Supplementary Fig. S4). Among the six growth conditions, the Rubisco level per total soluble protein was not significantly affected, whereas the Rubisco content on a
leaf area basis was affected by low N supply and eCO2 in the same way as the soluble protein content. This confirms a previous observation that the Rubisco level in rice is determined by N availability but not by CO2 (Makino et al. 1997, Nakano et al. 1997).

Changes in the epidermal structure of the leaf blade

Epidermal structures on the adaxial and abaxial sides of the 12th leaves of Exp. 1 were analyzed using surface replicas (Fig. 4; see Supplementary Fig. S5). Low N supply reduced the blade length (Fig. 4A), but the epidermal cell length on both sides was not affected (Fig. 4B), suggesting a reduced epidermal cell number in the axial direction (Fig. 4C). eCO2 also reduced the blade length (Fig. 4A). The effects of eCO2 differed between the adaxial and abaxial surfaces. On the adaxial side, the cell number (but not the cell length) was reduced (Fig. 4B, C). In contrast, on the abaxial side, the cell length (but not the cell number) was significantly reduced by eCO2 (Fig. 4B, C). These results suggest that eCO2 and N deficiency reduce the blade length by different mechanisms. The length of a stomatal unit (a pair of guard and subsidiary cells) was reduced by N deficiency but was not affected by eCO2 (Fig. 4D). Next, we investigated the changes in parameters related to the blade width using leaf blades from Exp. 1. No significant change was observed in the epidermal cell width (data not shown). Although it is known that N deficiency reduces the number of large VBs (e.g., see Yamazaki 1963b), we observed only a marginal decrease in the number of large VBs at low N in
the 12th blade (6.86 at VL-N and 7.00 at L-N and Ex-N; means of eight plants). eCO₂ also did not affect the number of large VBs (not shown). However, both eCO₂ and N deficiency decreased the distance between large VBs (Fig. 5A) and the number of small VBs between two large VBs (Fig. 5B). Both eCO₂ and N deficiency also reduced the number of epidermal cell files between small VBs (Fig. 5C).

eCO₂ markedly reduces stomatal density (stoma number per leaf area) and the stomatal index (ratio of stomatal cells to total epidermal cells) in various plant species (Woodward and Kelly 1995). However, eCO₂ has an opposite effect in indica-type rice cultivars (Rowland-Bamford et al. 1990, Uprety et al. 2002). In the flag leaves of O. sativa L. cv. IR-30, an indica cultivar, the number of stomatal cell files per unit blade width was increased to a greater extent on the abaxial side than on the adaxial side (Rowland-Bamford et al. 1990). We confirmed these observations in a japonica cultivar, and found that the stomatal index in the lateral direction (ratio of stomatal cell files to total epidermal cell files) was increased at eCO₂ (Supplementary Fig. S6, top row). The increase was more noticeable on the abaxial than on the adaxial side, and resulted mainly from a selective decrease in the number of epidermal cell files other than stomatal cell files, because the number of stomatal cell files between small VBs was affected only slightly (Fig. 5D), whereas that of the total epidermal cell files was significantly decreased by eCO₂ (Fig. 5C). Stomatal density in the axial direction (stoma number per unit blade length) was not affected by eCO₂ (Supplementary Fig. S6, bottom row).

In contrast to the effects of eCO₂, N deficiency increased stomatal density and stomatal index in both the lateral and axial directions (Supplementary Fig. S6). These effects were observed on both the adaxial and abaxial sides in leaves that developed later.

**Changes in the internal structure of the leaf blade**

As described above, eCO₂ increased the leaf blade thickness (Fig. 3C). The effects of eCO₂ on structural components were investigated using fixed 13th blades from Exp. 2 (Fig. 6; Supplementary Fig. S7) or fresh tissue sections (data not shown); both approaches produced similar results. eCO₂ significantly increased the thickness of the mesophyll at VL-N, but only slightly at Ex-N (Fig. 6C). In contrast, eCO₂ markedly increased the thickness of the bundle sheath (BS) extension and BS on the adaxial side regardless of the applied N level (Fig. 6D, E); at Ex-N, BS extension increased by 31% and BS increased by 33%. The thickness of the sclerenchyma on the adaxial side was not affected by eCO₂ (Fig. 6F). The BS extension and sclerenchyma on the abaxial side were too thin to be measured precisely (Supplementary Fig. S7A, B). The sum of thicknesses of the two components increased at eCO₂ regardless of the applied N level (Fig. 6D, E); at Ex-N, BS extension increased by 31% and BS increased by 33%. The thickness of the sclerenchyma on the adaxial side was not affected by eCO₂ (Fig. 6F). The BS extension and sclerenchyma on the abaxial side were too thin to be measured precisely (Supplementary Fig. S7A, B). The sum of thicknesses of the two components increased at eCO₂ regardless of the applied N level (Fig. 6D, E); at Ex-N, BS extension increased by 31% and BS increased by 33%. The thickness of the sclerenchyma on the adaxial side was not affected by eCO₂ (Fig. 6F). The BS extension and sclerenchyma on the abaxial side were too thin to be measured precisely (Supplementary Fig. S7A, B). The sum of thicknesses of the two components increased at eCO₂ regardless of the applied N level (Fig. 6D, E); at Ex-N, BS extension increased by 31% and BS increased by 33%. The thickness of the sclerenchyma on the adaxial side was not affected by eCO₂ (Fig. 6F). The BS extension and sclerenchyma on the abaxial side were too thin to be measured precisely (Supplementary Fig. S7A, B). The sum of thicknesses of the two components increased at eCO₂ regardless of the applied N level (Fig. 6D, E); at Ex-N, BS extension increased by 31% and BS increased by 33%. The thickness of the sclerenchyma on the adaxial side was not affected by eCO₂ (Fig. 6F). The BS extension and sclerenchyma on the abaxial side were too thin to be measured precisely (Supplementary Fig. S7A, B). The sum of thicknesses of the two components increased at eCO₂ regardless of the applied N level (Fig. 6D, E); at Ex-N, BS extension increased by 31% and BS increased by 33%. The thickness of the sclerenchyma on the adaxial side was not affected by eCO₂ (Fig. 6F). The BS extension and sclerenchyma on the abaxial side were too thin to be measured precisely (Supplementary Fig. S7A, B).
Effects of eCO2 and N deficiency on leaf blade thickness and internal structure (Exp. 2). Cross-sections of leaf blades fixed in formalin/acetic acid/alcohol were stained with safranin and Fast Green FCF. (A) Measurements of thicknesses of internal structures. M, mesophyll; SVB, small VB; (i) sclerenchyma; (ii) bundle sheath (BS) extension; (iii) BS. (B–G) Thicknesses of the internal structures of the 13th leaf blades. The fixation shrank sclerenchymatous cells, including motor cells, so that the leaf thickness was reduced by about 10%. (B) Leaf blade thickness at ridges of small VBs. (C) Mesophyll thickness. (D–F) Thicknesses of the BS extension (D), BS (E) and sclerenchyma (F) on the adaxial side. (G) Sum of thicknesses of the sclerenchyma and BS extension on the abaxial side. Data represent means ± SD of four plants. *P < 0.05, **P < 0.01 between aCO2 and eCO2; ¹P < 0.05, ²P < 0.01 between VL-N and Ex-N by Student’s t-test.

Effects of eCO2 on the leaf gas exchange and growth

To investigate the effects of eCO2 on leaf physiology and biochemistry in more detail, the ninth leaves from the third experiment (Exp. 3) were compared. eCO2 decreased the soluble protein content in the ninth leaf by approximately 16% at VL-N, but only slightly at Ex-N (Supplementary Fig. S7A). Accordingly, the effects of eCO2 on gas exchange characteristics were observed only at VL-N (Fig. 7). eCO2 reduced the carboxylation efficiency (the initial slope of the A–Ci response curve) at VL-N, and affected the shape of the A–Ci curve (Fig. 7A): A reached a maximum at a Ci of approximately 700–1,000 µmol mol⁻¹ at eCO2 and of 500 µmol mol⁻¹ at aCO2. eCO2 also reduced the gs at all Ci tested at VL-N, whereas a decline in gs at eCO2 was observed only at a Ci < 250 µmol mol⁻¹ under Ex-N (Fig. 7B). These features are typical for rice plants grown at eCO2 (Makino and Mae 1999, Seneweera et al. 2011).
Table 1 Summary of effects of N deficiency and eCO2 on leaf blade in rice

| Observed changes at eCO2 | Effects of N deficiency | eCO2 |
|--------------------------|-------------------------|------|
| Leaf blade constituent   |                         |      |
| Decline in protein content<sup>a</sup> | + | - | - |
| Decline in Rubisco content<sup>a</sup> | + | - | - |
| Decline in Chl content<sup>a</sup> | + | - | - |
| Leaf blade size          |                         |      |
| Decrease in length       | + | + | From ninth leaf (P<sub>3</sub>)<sup>b</sup> |
| Decrease in width        | + | + | From 10th leaf (P<sub>3</sub>)<sup>b</sup> |
| Increase in thickness    | - | + | From 10th leaf (P<sub>3</sub>)<sup>b</sup> |

<sup>a</sup> On a leaf area basis.  
<sup>b</sup> Leaf developmental stage at the onset of the eCO2 treatment.

Even at the plant age of around 10 (after exposure to eCO2 for 10–15 d) marked changes in plant growth were observed: the shoot dry weight and tiller number per plant were increased (Supplementary Fig. S8B).

**Discussion**

In this study, we were able to discriminate successfully between the effects of eCO2 and those of N deficiency. We found that the decline in leaf soluble protein and Chl contents in rice plants grown at eCO2 is caused by N deficiency, the decreased length and width of the leaf blade by both eCO2 and N deficiency, and the increased blade thickness by eCO2 only (Table 1).

**Mechanisms underlying the decline in protein content at eCO2**

Previous studies of hydroponically grown rice have demonstrated that Rubisco content (on a leaf area basis) is determined solely by N availability, giving the same regression line in plots against total leaf N at both aCO2 and eCO2 (Makino et al. 1997, Nakano et al. 1997). Using rice plants grown in soil, we confirmed that there is no significant decline in leaf soluble protein at eCO2 in the presence of sufficient N (Fig. 2A). Because the level of Rubisco per total soluble protein was not significantly affected (Supplementary Fig. S4A), a specific decline in Rubisco content by eCO2 is unlikely. In accordance with the decreased protein content, the carboxylation efficiency of Rubisco was lower in plants grown at eCO2 than in those grown at aCO2 under VL-N (Fig. 7A).

The decline in the protein content in the seventh leaf at eCO2 under VL-N in Exp. 1 (Fig. 2A) was unexpected because the exposure time of this leaf to eCO2 (from plant age 7.0 to 7.7) was short (1.5–2 d). It is plausible that eCO2 suppresses synthesis and accumulation of soluble protein in the leaf blade in this period, during which the leaf protein content increased to reach a maximum at the plant age of 7.7 (Supplementary Fig. S1). The suppression of protein synthesis by eCO2 is not unlikely if the mechanisms of N assimilation in rice are taken into consideration. Rice plants assimilate ammonium (the major N source in waterlogged soil) in the roots, and the assimilation products (free amino acids) are transported to the shoot in the transpiration stream (Kiyomiya et al. 2001, Funayama et al. 2013). Decreases in g<sub>s</sub> and transpiration rate are common responses of plants to both short- and long-term exposure to eCO2 (Makino and Mae 1999, Long et al. 2004); in fact, we observed decreases in these parameters upon transfer of plants from aCO2 to eCO2 (Supplementary Fig. S9). In wheat and Arabidopsis, when the major nitrogen source is nitrate, eCO2 suppresses N assimilation in the leaves through suppression of photorespiration (Bloom et al. 2010). We suggest that, at least in rice, the suppressed transpiration plays a greater role in the decreases in leaf protein and total N as compared with plant species that preferentially use nitrate as the N source.

**eCO2 and N deficiency affect leaf structure in different ways**

eCO2 increases total leaf area in most species, and also single-leaf area in many species (Pritchard et al. 1999). In rice, eCO2 does not significantly affect total leaf area but reduces single-leaf area (Makino et al. 2000, Shimon et al. 2010). In this study, we found that both the length and width of leaf blades were reduced by eCO2 and N deficiency. The mechanisms of the reduction appear to be similar for the blade width (Fig. 5) but different for the blade length (Fig. 4). N deficiency reduced the number of epidermal cells over the entire blade length on both the adaxial and abaxial side, suggesting that it suppressed division of epidermal cells. eCO2 also reduced the cell number on the adaxial side, yet on the abaxial side it reduced the cell length, but not the cell number, indicating that cell elongation was suppressed. Thus, eCO2 decreased the blade length by influencing both cell division and cell elongation.

The increased blade thickness at the ridges of small VBs was only caused by eCO2. Detailed analyses of leaf internal structures showed that eCO2 and N deficiency affected internal components differently (Fig. 6; Supplementary Fig. S7). eCO2 thickened BS extensions on both the adaxial and abaxial sides, and also the adaxial BS, which together largely contributed to the increased blade thickness. Although we did not thoroughly analyze the ridges of large VBs, thickness at these ridges may be increased by eCO2 in similar ways: we observed that BS extensions and BS on the adaxial side of these ridges were also thickened at eCO2 with no change in the size of large VBs at VL-N (not shown). In contrast to the effects of eCO2, N deficiency resulted in shorter BS extensions on the adaxial side and the absence of BS extensions on the abaxial side, and in thicker sclerenchyma layers. The mesophyll thickness was also increased by eCO2 at VL-N and, albeit to a lesser extent, at
Ex-N (Fig. 6C). The increased thickness of the leaf blade and mesophyll in eCO2 has been reported in monocots (wheat, Masle 2000) and dicots (soybean, Sims et al. 1998b; poplar, Miyazawa et al. 2011) (for a review, see Pritchard et al. 1999).

The stomatal parameters were also affected by eCO2 and N deficiency in different ways (Supplementary Fig. 56). eCO2 only increased the stomatal index in the lateral direction, and the increase was noticeable on the abaxial side (Supplementary Fig. 56, top row). Despite the increased stomatal index by eCO2, the g, measured at the growth CO2 concentration was reduced to about two-thirds by eCO2 (Fig. 7B). N deficiency reduced the g, measured at the growth CO2 concentration only slightly. It is likely that rice plants down-regulate photosynthetic carbon assimilation by controlling the stomatal opening but not the stomatal density when carbon supply is in excess at eCO2.

Sites of action of eCO2 on rice leaf development

The change in blade length due to eCO2 became detectable in the ninth leaf and those in width and thickness in the 10th leaf in the presence of sufficient N. The ninth and 10th leaves were at the P3 and P4 developmental stages, respectively, at the beginning of the eCO2 treatment. At P3, the boundary between the blade and sheath is established, and various internal tissues and epidermal cells start to differentiate (Itoh et al. 2005). The P3 leaves are small, approximately 8 mm in height (Yamazaki 1963a). P4 is defined as a stage of rapid elongation of the leaf blade (Itoh et al. 2005). The P3 and P4 leaves are enclosed by older leaves, which makes the direct action of eCO2 on these leaves unlikely.

Leaf development in the lateral direction. The midrib and large VBs start to differentiate in P3, and extend to the entire blade length in P5 (Kaufman 1959, Yamazaki 1963a). Small VBs and epidermal cells, including stomatal cells, start to form basipetally from the tip in P3. Because eCO2 reduced the numbers of small VBs and epidermal cell files between large VBs (Fig. 5), but not the numbers of large VBs, we suggest that eCO2 suppresses differentiation of small VBs and epidermal cells in P3, resulting in the decreased blade width.

Leaf development in the axial direction. The leaf blade rapidly elongates in P3, and this elongation has been attributed to the activity of the intercalary meristem in the basal region of the blade (Kaufman 1959). The decreased blade length under N deficiency resulted from the decreased number of epidermal cells on both the adaxial and abaxial side. Therefore, the decreased blade length can be explained simply by suppression of cell division in the intercalary meristem in P3. A decrease in the blade length by shading occurs in P4 (Yamazaki 1964). N deficiency and shading both suppress assimilation of inorganic nitrogen and carbon. The decreased length probably results from a limited supply of these assimilates and energy required for cell division in the intercalary meristem. It is more difficult to interpret the effect of eCO2, because it probably affects both cell division and elongation.

Leaf blade thickness. At eCO2, ridges of small VBs started thickening in P3. In rice seedlings, eCO2 increased the midrib thickness in the P3 leaf primordium (Jitla et al. 1997); midrib ridges and large VBs also became thicker in wheat exposed to eCO2 (Masle 2000). Although we did not analyze these large ridges in detail, they might be affected in a manner similar to those of small VBs. eCO2 enlarged BS extensions and expanded the adaxial BS (Fig. 6). The enlarged BS extensions, however, are probably not the cause but rather the result of the increased thickness, because they differentiate after the formation of VBs and sclerenchyma (Kaufman 1959). Elevated CO2 might affect the entire formation and maturation of the vasculature.

As discussed above, the mechanisms involved in the reduction in the blade width may be relatively simple. In contrast, phenomena associated with the decreased length and the increased thickness are complex, and we could not identify the process affected by eCO2. The blade length and thickness may be determined (i) by several independent consecutive processes differently affected by eCO2, or (ii) by central regulators (if any) of these processes affected by eCO2. If a long-distance signal is involved, it should persist or be generated for some time, because larger changes in the blade size were observed in the leaves that developed later. This hypothesis is plausible, because the DROOPING LEAF (DL) gene, a regulator of midrib formation, is highly expressed from P1 to P3 and is down-regulated in P4 (Yamaguchi et al. 2004). The P3 and P4 leaves in rice plants may not be influenced by external environmental factors such as the atmospheric CO2 concentration, humidity, and the intensity and quality of light, in contrast to most dicot species in which developing leaves in buds can sense the light environment for regulation of chloroplast differentiation (Yano and Terashima 2001). An approach and information provided by this study would be useful for identifying the long-distance signal and understanding the effects of eCO2 on leaf development in rice.

Materials and Methods

Plant materials and growth conditions

Rice plants (Oryza sativa L. ssp. japonica cv. Nipponbare) were germinated and sown in a commercial soil mixture (Masumoto et al. 2010). They were grown in growth chambers on a day/night cycle (14–14.5 h day period) under illumination from metal halide lamps at a photosynthetic photon flux density (PPFD) of 300–600 μmol m−2 s−1. After the fourth leaves were fully expanded, four seedlings were transplanted into a 1/5,000-are Wagner pot containing non-fertilized granular soil (3.2 liters per pot; 7 mg N kg−1 as NH4+ and 10 mg N kg−1 as NO3−; Kanuma Sangyou), supplemented with coated urea (LP-70, Chisso) to the N amounts of 0.1 g per plant (VL-N), 0.2 g per plant (L-N) and 0.5 g per plant (Ex-N) (the standard N level is 0.3 g per plant). The soil was also supplemented with a compound fertilizer containing P2O5 (0.45 g per plant) and K2O (0.35 g per plant) as basal dressing. The plants were grown
under waterlogged conditions and ambient air in two identical growth chambers (inside dimensions: width 1,294 mm × depth 900 mm × height 1,800 mm; TGL-1, Especmic) on a 28°C day/23°C night cycle (14 h day period) at a relative humidity of 70–80%. At least four pots were used for each growth condition.

When the seventh leaves were fully expanded (plant age = 7.0–7.1), the CO2 concentration inside one chamber was raised to approximately 1,000 p.p.m. (eCO2). High-purity CO2 gas (99.999%) was used to minimize possible contamination by ethylene in the CO2 gas cylinder (Morison and Gifford 1984). For aCO2 treatments, the CO2 concentration inside the growth chamber was either not regulated or adjusted to approximately 400 p.p.m. CO2 concentrations inside the chambers were monitored with portable CO2 sensors (SenseAir, SenseAir AB), and chamber CO2 settings were adjusted to attain the desired CO2 concentrations at the plant height. PPFD from metal halide lamps, measured at the plant height with a quantum sensor (Li-189, Li-Cor), was 600–700 μmol m−2 s−1 (just after transplanting) and 800–1,000 μmol m−2 s−1 (at the plant age 10 and later). Pot positions were changed twice a week during the CO2 treatments to avoid positional effects on plant growth.

Exps. 1 and 2 were carried out under essentially identical conditions except the CO2 concentration inside the growth chamber was either not regulated or adjusted to approximately 400 p.p.m. CO2 concentrations inside the chambers were monitored with portable CO2 sensors (SenseAir, SenseAir AB), and chamber CO2 settings were adjusted to attain the desired CO2 concentrations at the plant height. PPFD from metal halide lamps, measured at the plant height with a quantum sensor (Li-189, Li-Cor), was 600–700 μmol m−2 s−1 (just after transplanting) and 800–1,000 μmol m−2 s−1 (at the plant age 10 and later). Pot positions were changed twice a week during the CO2 treatments to avoid positional effects on plant growth.

Protein and Chl determination. Blade segments were ground as described previously (Tsuchida et al. 2001) except that phenylmethylsulfonyl fluoride and insoluble polyvinylpolypyrrolidone were not included in the grinding medium. A portion of the homogenate was used for Chl extraction, and the remaining homogenate was centrifuged at 15,000 × g for 10 min. The supernatant was used as total soluble protein. Protein determination and SDS–PAGE were carried out as described previously (Tsuchida et al. 2001). Chl was extracted with 80% acetone and determined according to Porra et al. (1989).

Analyses of leaf anatomy. A region between the first and the second large VBs from the midrib was analyzed, as the blade thickness and parameters related to stoma in this region were almost equal to the mean values for the entire blade width. The means of data for both sides of the midrib were taken as the data for the single blade.

To determine the blade thickness, segments were embedded in 5% (w/v) agar and sliced with a microslicer (Dosaka EM). Cross-sections (approximately 40 μm thick) were observed under a light microscope (AX70, Olympus) equipped with a digital camera. Thicknesses at all ridges of small VBs were determined and averaged. To analyze the epidermal structure, surface replicas were made by pouring transparent nail varnish into the molds made of dental putty described above. After drying, the replicas were peeled from the molds. Four photomicrographs of the objective region (1.77 mm long in the axial direction) were taken with the focus on different areas. Because surfaces adjacent to the large veins could not be replicated precisely due to the tall ridges at large VBs, a mid-portion of the objective region containing at least three small VBs was analyzed to determine the number of epidermal cell files and the epidermal cell length. To determine the cell length in the axial direction, the number of cells in each cell file was counted, and the file length was divided by the cell number. To investigate the internal structure, segments were fixed with formalin/acetic acid/alcohol, dehydrated with an ethanol/acetone series and embedded in paraffin (Tsutsumi et al. 2006). Cross-sections (10 μm thick) were mounted on slides, stained with safranin and Fast Green FCF, and observed under the light microscope.

Gas exchange measurements
Gas exchange was measured with an open gas exchange system (LI-6400, Li-Cor). The leaf to air vapor pressure difference was controlled using a dew point generator (LI-610, Li-Cor). Illumination was obtained from light-emitting diodes (peak wavelengths of 470 and 665 nm, Li-Cor). Gas exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981). When gas exchange was continuously monitored inside the growth chamber, ambient air inside the growth chamber was allowed to flow into the leaf chamber of LI-6400 through a plastic container (2.4 liters) at a rate of 500 μmol s−1. The light intensity on the leaf surface was
automatically regulated to match that inside the growth chamber.

**Supplementary data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

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