Elevated CO₂ Decreases the Photorespiratory NH₃ Production but Does not Decrease the NH₃ Compensation Point in Rice Leaves

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(Received October 6, 2013; Accepted June 17, 2014)

The exchange of gaseous NH₃ between the atmosphere and plants plays a pivotal role in controlling the global NH₃ cycle. Photorespiration generates NH₃ through oxygenation instead of carboxylation by the CO₂-fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The future increase in the atmospheric CO₂ concentration, [CO₂], is expected to reduce plant NH₃ production by suppressing RuBisCO oxygenation (Vₒ). We measured the net leaf NH₃ uptake rate (FNH₃) across NH₃ concentrations in the air (nₐ) ranging from 0.2 to 1.6 nmol mol⁻¹ at three [CO₂] values (190, 360 and 750 µmol mol⁻¹) using rice plants. We analyzed leaf NH₃ gas exchange using a custom-made whole-leaf chamber system, and determined the NH₃ compensation point (γ), a measure of potential NH₃ emission, as the x-intercept of the linear relationship of FNH₃ as a function of nₐ. Our γ values were lower than those reported for other plant species. γ did not decrease under elevated [CO₂], although leaf NH₄⁺ content decreased with decreasing Vₒ at higher [CO₂]. This was also the case for γ estimated from the pH and NH₄⁺ concentration of the leaf apoplastic solution (γ'). γ' of rice plants, grown at elevated [CO₂] for months in a free-air CO₂ enrichment facility, was also not decreased by elevated [CO₂]. These results suggest that suppression of RuBisCO oxygenation by elevated [CO₂] does not decrease potential leaf NH₃ emission in rice plants.

Keywords: Ammonia • Elevated CO₂ • NH₃ compensation point • Nitrogen metabolism • Photorespiration.

Abbreviations: γ, NH₃ compensation point determined by the gas exchange method; γ', NH₃ compensation point determined by the apoplastic method; [CO₂], CO₂ concentration; FACE, free-air CO₂ enrichment; FNH₃, leaf net NH₃ uptake rate on an area basis; nₐ, the NH₃ concentration in the air; [NH₃], NH₃ concentration; [NH₄⁺], NH₄⁺ concentration; [O₂], O₂ concentration; PFD, photosynthetic photon flux density; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; Vₒ, RuBisCO carboxylation rate on a leaf area basis; Vₚ, RuBisCO oxygenation rate on a leaf area basis.

Introduction

Intensive application of synthetic fertilizers at agricultural sites since the Green Revolution, which had been promoted from the 1940s to the 1970s, has resulted in a significant increase of global ammonia (NH₃) emission and subsequent NH₃ deposition (Tilman et al. 2002, Galloway et al. 2004). Excessive NH₃ deposition causes severe damage to forests and agricultural crops, probably due to ammonium (NH₄⁺) toxicity for most higher plant species, pH perturbation in the soil or a combination of the two (Pearson and Stewart 1993).

The exchange of gaseous NH₃ between plants and the atmosphere plays a pivotal role in controlling the global NH₃ cycle, and is a potential target for improving the nitrogen-use efficiency of plants (Weiland and Stutte 1985, Sutton et al. 1995, Schjoerring et al. 2000). Farquhar et al. (1980) demonstrated that the net NH₃ uptake rate by French bean on a leaf area basis (FNH₃) was linearly correlated with the NH₃ concentration in ambient air (nₐ) over a range from 5 to 50 nmol mol⁻¹, reaching a value of zero at some concentration of NH₃ (γ, the NH₃ compensation point). In general, γ varies among plant cultivars (Husted et al. 1996), among growth stages (Farquhar et al. 1979, Morgan and Parton 1989) and among soil nitrogen levels (Schjoerring et al. 1998). NH₃ is emitted from a leaf when nₐ is below γ, whereas atmospheric NH₃ is assimilated by a leaf when nₐ is above γ. Thus, γ can measure the potential of a plant to emit NH₃.

According to Farquhar et al. (1980), FNH₃ can be calculated as follows:

$$ F_{NH_3} = g_{NH_3}(n_a - \gamma) $$

(1)
where $g_{NH3}$ is the stomatal conductance for NH$_3$. $\gamma$ is considered to be equal to [NH$_3$] above the water film (the apoplastic water) in the cell wall of the mesophyll cells (Husted and Schjoerring 1995). $\gamma$ increases with increasing pH and [NH$_4^+$] in the leaf apoplast, and also with increasing leaf temperature (Farquhar et al. 1980, Husted and Schjoerring 1995).

Photorespiration takes place simultaneously with photosynthesis. The first step in photorepiration is the oxygenation of ribulose-1,5-bisphosphate instead of the carboxylation that occurs during photosynthesis by the CO$_2$-fixing enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The resultant products of the oxygenation are 3-phosphoglycerate, a metabolic intermediate in the Calvin cycle, and 2-phosphoglycolate, which is toxic to plants. When 2-phosphoglycolate is recycled back to 3-phosphoglycerate through the photorespiratory C2 cycle, half a molecule each of CO$_2$ and NH$_3$ is produced from the mitochondria. NH$_3$ is re-assimilated in the chloroplasts via the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle.

The Rubisco oxygenation rate ($V_o$) amounts to 40–50% of the carboxylation rate ($V_c$) at 25–30°C under ambient CO$_2$ and O$_2$ concentrations, with [CO$_2$] and [O$_2$] at 360 $\mu$mol mol$^{-1}$ and 21%, respectively (Sage 2013). The $V_o/V_c$ ratio decreases with increasing [CO$_2$] and increases with increasing [O$_2$] (Sage 2013). The NH$_3$ production rate from the mitochondria is considered to be half of $V_o$ (von Caemmerer 2000). The atmospheric [CO$_2$] has increased from 280 to 390 $\mu$mol mol$^{-1}$ during the past 200 years, and it is predicted to continue increasing despite various efforts to reduce CO$_2$ emission from fossil fuel combustion (Fisher et al. 2007). This suggests that $\gamma$ will decrease at elevated [CO$_2$] due to the suppression of photorepiration.

The involvement of photorepiration in leaf NH$_3$ emission has previously been investigated in several plant species by changing either [O$_2$] or [CO$_2$]. NH$_3$ emission (i.e. $-F_{NH3}$ at $n_2=0$) was enhanced at elevated [O$_2$] in soybean (Weiland and Stutte 1985), wheat (Morgan and Parton 1989) and two cultivars of rice (Kumagai et al. 2011), whereas no difference was observed in oilseed rape (Husted et al. 2002). Wang et al. (2013) recently demonstrated that $\gamma$ estimated from [NH$_4^+$] and the pH of the leaf apoplast solution ($\gamma'$) decreased when barley plants were grown at elevated [CO$_2$]. In these studies, however, the correlation between leaf NH$_3$ emission and $V_o$ was not quantitatively analyzed.

The major nitrogen source in waterlogged soils such as those of paddy fields is NH$_4^+$, whereas it is nitrate (NO$_3^-$) in upland (aerobic) field soils (Buresch et al. 2008). In the field, NH$_3$ emission from the aerial parts of wheat and barley plants peaked at around anthesis (Morgan and Parton 1989, Schjoerring et al. 1993). Quantification of the cumulated NH$_3$ emission from the canopy of barley, wheat, oilseed rape and pea crops over a complete growing season showed values ranging between 1 and 5 kg NH$_3$-N ha$^{-1}$ (Schjoerring and Mattsson 2001). In contrast, rice plants grown in paddy fields emit NH$_3$ only after the application of nitrogen fertilizer, but they absorb NH$_3$ from the atmosphere in other periods (Hayashi et al. 2008a, Hayashi et al. 2008b). The low NH$_3$ emission from rice leaves may result from the high atmospheric concentration of NH$_3$ emitted from the waterlogged soil of the paddy fields. However, the underlying mechanisms have not yet been clarified.

In this study, we constructed a whole-leaf chamber system to measure NH$_3$ gas exchange, based on the system developed in a previous study (Hayashi et al. 2008a) but designed to let us vary [CO$_2$] in the chamber and to measure both photosynthesis and transpiration of the lamina. Using this system, we found that the potential NH$_3$ emission, $\gamma'$, of rice leaves was very low, and that it was not decreased even if photorepiration was decreased by increased [CO$_2$].

### Results

#### The whole-leaf chamber system

In the original system developed for field experiments (Hayashi et al. 2008a), air was sucked from leaf chambers using a pump. In the system constructed in this study, air was pushed into the chambers to minimize the suction of NH$_3$ from air inside the growth cabinet where the rice plants were placed (Fig. 1). To analyze NH$_3$ gas exchange, each leaf chamber was connected to a filter for trapping NH$_3$ (#12 in Fig. 1). To analyze photosynthesis and transpiration, the chambers were connected to a CO$_2$/H$_2$O analyzer (#14). Laminae were exposed to three levels of [CO$_2$]: low (190 $\mu$mol mol$^{-1}$), ambient (360 $\mu$mol mol$^{-1}$) and high (750 $\mu$mol mol$^{-1}$) [CO$_2$]. The NH$_3$ concentration in the air entering the chambers was 0.4–1.6 nmol mol$^{-1}$ without the NH$_3$ absorber (#7 and #8) and 0.2–0.4 nmol mol$^{-1}$.
with it. Laminae of the uppermost fully expanded leaves at the vegetative stage were used for the analyses.

**Determination of \( V_o \) at three \([\text{CO}_2] \) concentrations**

To estimate the \( \text{NH}_3 \) production rate due to photorespiration, we determined \( V_o \) by simultaneous measurements of gas exchange and Chl fluorescence using a portable gas exchange system under conditions similar to those in the whole-leaf chamber system (Fig. 2, filled symbols). \( V_o \) and \( V_c \) were calculated from the net photosynthetic rate (\( P_n \)) and the PSII operating efficiency (\( \phi_{PSII} \)). The \( V_o \) obtained through this calculation decreased with increasing \([\text{CO}_2] \), whereas \( V_c \) increased (Fig. 2D), which are typical features observed in \( \text{C}_3 \) plants (Sage 2013). The \( V_o \) values were 15, 11 and 7 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at low, ambient and high \([\text{CO}_2] \), respectively. Hence, the leaf \( \text{NH}_3 \) production rate from the photorespiratory pathway (\( = V_o/2 \)) at the low \([\text{CO}_2] \) was more than twice the value at the high \([\text{CO}_2] \).

\( P_n \) and the transpiration rate (\( E \)) were also determined using the whole-leaf chamber system (Fig. 2A, B; open symbols). At low \([\text{CO}_2] \), the \( P_n \) values measured by the two systems were almost the same, whereas at higher \([\text{CO}_2] \), the value measured using the whole-leaf chamber system was larger than that measured with the portable system; however, none of these differences was statistically significant (Fig. 2A). The higher \( P_n \) at high \([\text{CO}_2] \) suggests that the actual \( V_o \) value in the whole-leaf chamber system may be lower than the value estimated using the portable system. In contrast, the \( E \) values were almost the same at every \([\text{CO}_2] \) between the two systems and they decreased with increasing \([\text{CO}_2] \) (Fig. 2B).

**\( \text{NH}_3 \) gas exchange and leaf apoplast properties**

Using the whole-leaf chamber system, we measured \( F_{NH_3} \) at the three \([\text{CO}_2] \) values. To vary \( n_\text{a} \), we allowed the \([\text{CO}_2] \)-controlled gas to flow into the leaf chambers with or without the \( \text{NH}_3 \) absorber. At every \([\text{CO}_2] \), \( F_{NH_3} \) showed a strong and statistically significant linear correlation with \( n_\text{a} \) (\( R^2 = 0.7–0.9, P < 0.05; \) Fig. 3A), as was reported previously (Farquhar et al. 1980). The \( x \)-intercept (\( \gamma \)), the \( y \)-intercept or the slope (\( g_{NH_3} \)) of the regression lines did not differ significantly among the three \([\text{CO}_2] \) levels (Fig. 3B–D), indicating that the potential leaf \( \text{NH}_3 \) emission did not decrease at elevated \([\text{CO}_2] \), irrespective of the remarkable decline in \( V_o \) at high \([\text{CO}_2] \).

To confirm these results, we also estimated the \( \text{NH}_3 \) compensation point using the apoplast method (\( \gamma' \)) from the relationship between [\( \text{NH}_4^+ \)] and the pH of the leaf apoplast solution, using Equation (9), provided by Farquhar et al. (1980). In our experiment, the leaf [\( \text{NH}_4^+ \)] content on an area basis was also determined. The leaf [\( \text{NH}_4^+ \)] content decreased significantly with increasing \([\text{CO}_2] \) (Fig. 4A), and there was a positive relationship between leaf [\( \text{NH}_4^+ \)] content and \( V_o \) determined using the portable gas exchange system (Fig. 4B). In contrast, neither [\( \text{NH}_4^+ \)] nor the pH of the leaf apoplast differed significantly among the three \([\text{CO}_2] \) values (Fig. 4C, D). As a result, \( \gamma' \) was not significantly affected by [\( \text{CO}_2 \)] (Fig. 4E), as was observed in the \( \text{NH}_3 \) gas exchange experiments (Fig. 3B). However, the absolute values obtained using the apoplast method were higher than those determined using the gas exchange method.

**Changes in the \( \text{NH}_3 \) compensation point during growth at elevated \([\text{CO}_2] \)**

To investigate the effects of the growth at elevated \([\text{CO}_2] \) on the \( \text{NH}_3 \) emission potential, we investigated leaf apoplastic properties using rice plants grown at free-air \( \text{CO}_2 \) enrichment (FACE) facilities. In these experiments, rice plants at the mid reproductive stage and later were analyzed.

The [\( \text{NH}_4^+ \)] of the apoplastic solution at the mid reproductive stage (July 22) was significantly lower than those at the full-heading (August 9) and mid grain-filling stage (August 23) in both the control and the FACE plots (Fig. 5A; Table 1). The apoplastic pH was highest at the full-heading stage (August 9) in the control plot, whereas it was lowest at the mid grain-filling stage (August 23) in the FACE plot (Fig. 5B; Table 1). \( \gamma' \), estimated from the apoplastic [\( \text{NH}_4^+ \)] and apoplastic pH, was lowest at the mid reproductive stage, increased dramatically at the full-heading stage and then decreased at the mid grain-filling stage in both plots (Fig. 5C; Table 1). The marked increase in \( \gamma' \) at the full-heading stage resulted from the increases in both the apoplastic [\( \text{NH}_4^+ \)] and pH in the control plot, whereas it was ascribed to the increase in the apoplastic [\( \text{NH}_4^+ \)] in the FACE plot (Table 1).
Elevated $[\text{CO}_2]$ significantly increased $g_0$ (by 54%) compared with the control only at the mid reproductive stage (Fig. 5C). The increase in $g_0$ by elevated $[\text{CO}_2]$ was ascribed to the increase in the apoplastic pH (Fig. 5B).

**Discussion**

**Low NH$_3$ compensation points in rice leaves**

A previous report for oilseed rape demonstrated that the NH$_3$ compensation points determined using the NH$_3$ gas exchange method ($\gamma$) and the apoplast method ($\gamma'$) were comparable (Husted and Schjoerring 1995). In some grass species, it was reported that $g_0$ was much lower than $g$, though the underlying mechanisms remained unsolved (Mattsson and Schjoerring 2002). In contrast, $g_0$ was always much higher than $g$ in rice leaves: for example, the values were 1 nmol mol$^{-1}$ for $g'$ vs. 0.4 nmol mol$^{-1}$ for $g$ at low $[\text{CO}_2]$ (Figs. 3B, 4E). It is plausible that the higher $g'$ value measured by the apoplast method resulted from contamination of the apoplastic solution by xylem sap. If the apoplastic pH was assumed to be 6.3 (Fig. 4D), the $[\text{NH}_4^+]$ of the leaf apoplast estimated from the $g$ value using Equation (9) would be 14–21 $\mu$M, which is much lower than the level in the extracted apoplastic solution (40–60 $\mu$M; Fig. 4C). Rather, $[\text{NH}_4^+]$ of the extracted apoplastic solution was close to that of the xylem sap exudates from the rice stumps (80–100 $\mu$M; unpublished data). It is thus likely that the apoplastic $[\text{NH}_4^+]$ could be overestimated in rice plants using our method. The apoplastic $[\text{NH}_4^+]$ in oilseed rape was around 1 mM (Husted and Schjoerring 1995), which is 15–20 times the value for rice in our study; thus, the values of oilseed rape might not have been affected by contamination from the xylem sap.

Irrespective of differences in the absolute values, the NH$_3$ compensation points determined by the two methods were not significantly affected by $[\text{CO}_2]$ in rice leaves at the vegetative stage (Figs. 3B, 4E). In addition, the $\gamma$ values for the rice leaves in the present study (0.4–0.6 nmol mol$^{-1}$) were much lower than those reported for other C$_3$ plant species at the vegetative stage: 2.5 and 5.5 nmol mol$^{-1}$ in French bean (at 26 and 33$^\circ$C, respectively; Farquhar et al. 1980), about 5 nmol mol$^{-1}$ in oilseed rape (at 25$^\circ$C; Husted and Schjoerring 1995) and 4–6 nmol mol$^{-1}$ in barley (at 20$^\circ$C; Husted et al. 1996). Although these and other $\gamma$ values reported so far were all determined as mean values of the whole aerial parts of plants, they could represent the values for the youngest fully developed leaves, because $\gamma'$ determined by the apoplast method was not greatly affected by the leaf age in oilseed rape as long as nitrogen supply was sufficient (Husted and Schjoerring 1995). A $\gamma$ value < 1 (0.44 nmol mol$^{-1}$ at 25$^\circ$C)
Fig. 4 The effects of the air CO₂ concentration on the apoplast properties of rice leaves (cv. ‘Nipponbare’). Laminae were set in the whole-leaf chamber system without the NH₃ absorber and treated with three different [CO₂] under illumination for 100–120 min in the same way as in the experiments in Fig. 3. (A) Leaf NH₄⁺ content on an area basis. (B) The relationship between the leaf NH₄⁺ content on an area basis and Vₑ determined using the portable gas exchange system. The Vₑ values are the same as those in Fig. 2D. (C) Apoplast [NH₄⁺]. (D) Apoplast pH. (E) The NH₃ compensation point (γ') at 30°C determined from the [NH₄⁺] and the pH of the apoplast solution using Equation (9). Data represent means ± SE of three laminae for the leaf [NH₄⁺] and three or four independent experiments for the apoplast properties. Values labeled with different letters in (A) indicate significant differences (one-way ANOVA, P < 0.05). No significant difference was detected among the values for the three [CO₂] in (C–E).

has been observed only under nitrogen limitation in oilseed rape (Husted and Schjoerring 1996). The low γ values of rice leaves in this study did not result from nitrogen limitation, because total soluble protein content on a leaf area basis was high enough, approximately 6.8 g m⁻² (see Tsutsumi et al. 2014).

These differences in γ between rice and other plant species may be explained by the difference in the major nitrogen source used: NH₄⁺ in waterlogged soils vs. NO₃⁻ in upland field soils (Buresh et al. 2008). Plant species used in the previous studies were grown with NO₃⁻ as the major nitrogen source in most cases: as has been confirmed in the literature, French bean plants were grown with 5.5 mM NO₃⁻ and 0.5 mM NH₄⁺ (Farquhar et al. 1980) and barley plants with 0.2–5.0 mM NO₃⁻ (Husted et al. 1996, and references therein). In addition, unlike rice plants, they are upland field crops which thrive better with NO₃⁻ than with NH₄⁺. In general, NO₃⁻ absorbed by roots is translocated to the shoot and then reduced to NH₄⁺ by sequential reactions by nitrate and nitrite reductases in the leaf mesophyll cells. In tomato and oilseed rape plants grown with NO₃⁻ as the sole nitrogen source, high levels of NH₄⁺ accumulated in the xylem sap, the apoplast solution and the leaf tissue water, increasing with increasing NO₃⁻ supply (Husted et al. 2000), and the apoplastic [NH₄⁺] could reach 2 mM (Husted and Schjoerring 1996). In contrast, the leaf apoplastic [NH₄⁺] of rice plants in this study did not exceed 70 μM (Figs. 4, 5). It is thus likely that high γ values in the previous studies resulted from high levels of NH₄⁺ produced from NO₃⁻ in the shoot.

The location of chloroplasts, the site of NH₄⁺ assimilation, inside the mesophyll cell may also play a role in the low NH₃ compensation points of rice leaves. In rice mesophyll cells, chloroplasts and their stroma-filled protuberances (stromules) occupy almost all the cell periphery (>95%), and hence the mitochondria are located inside the continuous layer of chloroplasts/stromules (Sage and Sage 2009). Such an organelle localization can reduce the diffusive efflux of NH₄⁺ produced through the photorespiratory cycle in the mitochondria to the outside of a cell, as demonstrated for photorespired CO₂ (Busch et al. 2013). The occupation by chloroplasts of the cell periphery would also be advantageous to assimilate NH₄⁺ in the mesophyll apoplast.

It was shown that γ increased after anthesis until the mid grain-filling stage in barley (Husted et al. 1996), and this increase was ascribed to the generation of NH₃ on protein degradation during senescence of leaves (Husted et al. 1996, Schjoerring et al. 1998). This was also the case in rice plants in the FACE experiments, and γ' was highest at the full-heading stage among the three stages tested (Fig. 5). The γ' value of rice leaves at this stage (10 nmol mol⁻¹) is comparable with reported values: 5 nmol mol⁻¹ in barley (at 20°C, mid grain-filling stage; Husted et al. 1996) and 20 nmol mol⁻¹ in wheat (at 25°C, early grain-filling stage; Morgan and Parton 1989).

Factors affecting the NH₃ compensation point in leaves exposed to elevated [CO₂]

We found that the suppression of photorespiration by elevated [CO₂] did not decrease the NH₃ compensation point in rice
plants at the vegetative stage (Figs. 3, 4). This result contrasts with a previous observation in rice plants that the leaf NH₃ emission rate, measured at an air NH₃ concentration of zero, was significantly correlated with the photorespiratory activity, and was higher at higher \([\text{O}_2]\) (Kumagai et al. 2011). Although \(V_o\) was not determined in this previous study, it might not differ greatly from that in this study, because the net photosynthetic rate decreased by half when \([\text{O}_2]\) increased from 2% to 40% (Kumagai et al. 2011), whereas it increased by 30% at low \([\text{CO}_2]\) (Kumagai et al. 2011), which is similar to the change observed above that of NH₄⁺ (around 0.5 mM) in ambient \([\text{CO}_2]\) and it decreased to \(<1\) nmol mol⁻¹ at the elevated \([\text{CO}_2]\). Wang et al. (2013) reported that the decrease in \(\gamma'\) resulted from a decrease in the apoplastic \([\text{NH}_4^+]\). Doubling atmospheric \([\text{CO}_2]\) increased the net photosynthetic rate by 24–37% in barley leaves (Wang et al. 2013), as observed in rice leaves (Fig. 2A). Therefore, it seems unlikely that the difference in the \([\text{CO}_2]\) response of the NH₃ compensation point between barley and rice is due to a difference in \(V_o\). The transpiration rate decreased by about 30% at elevated \([\text{CO}_2]\) in barley, which is similar to our results for rice plants (Fig. 2B).

The inconsistent results between barley and rice plants may also result from the differences in the nitrogen source used. Wang et al. grew barley plants hydroponically with 2 mM NO₃⁻ or 1 mM NH₄NO₃. Irrespective of the nitrogen source used, the level of NO₃⁻ of the shoot (50–80 mM) was greatly above that of NH₄⁺ (around 0.5 mM) in ambient \([\text{CO}_2]\) and it decreased by 30–40% at elevated \([\text{CO}_2]\) due to the suppressed transpiration (Wang et al. 2013). In contrast, the shoot NH₄⁺ level significantly decreased at elevated \([\text{CO}_2]\) (by 30%) only with 2 mM NO₃⁻ but not with 1 mM NH₄NO₃ (Wang et al. 2013). The latter observation contrasts with our result that the leaf NH₄⁺ content was well correlated with \(V_o\) in rice leaves (Fig. 4B). In barley, the activity of nitrate reductase was significantly decreased (by 45–60%) at elevated \([\text{CO}_2]\) (Wang et al. 2013). Therefore, the reduced apoplastic \([\text{NH}_4^+]\) and the decline of \(\gamma'\) at elevated \([\text{CO}_2]\) observed by Wang et al. is ascribable to the reduced transport of NO₃⁻ from roots and the suppressed reduction of NO₃⁻ in the shoot by elevated \([\text{CO}_2]\), rather than the suppression of photorespiratory NH₄⁺ production.

Table 1 Results of Tukey’s post-hoc test for data from rice plants (cv. ‘Koshihikari’) grown at the FACE experimental site

<table>
<thead>
<tr>
<th>Date</th>
<th>July 22</th>
<th>August 9</th>
<th>August 23</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>Apoplast [NH₄⁺]</td>
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<td>Apoplast pH</td>
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<td>a</td>
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<td>(\gamma')</td>
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<td>Elevated [CO₂]</td>
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<td>Apoplast [NH₄⁺]</td>
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The inconsistent results between barley and rice plants may also result from the differences in the nitrogen source used. Wang et al. grew barley plants hydroponically with 2 mM NO₃⁻ or 1 mM NH₄NO₃. Irrespective of the nitrogen source used, the level of NO₃⁻ of the shoot (50–80 mM) was greatly above that of NH₄⁺ (around 0.5 mM) in ambient \([\text{CO}_2]\) and it decreased by 30–40% at elevated \([\text{CO}_2]\) due to the suppressed transpiration (Wang et al. 2013). In contrast, the shoot NH₄⁺ level significantly decreased at elevated \([\text{CO}_2]\) (by 30%) only with 2 mM NO₃⁻ but not with 1 mM NH₄NO₃ (Wang et al. 2013). The latter observation contrasts with our result that the leaf NH₄⁺ content was well correlated with \(V_o\) in rice leaves (Fig. 4B). In barley, the activity of nitrate reductase was significantly decreased (by 45–60%) at elevated \([\text{CO}_2]\) (Wang et al. 2013). Therefore, the reduced apoplastic \([\text{NH}_4^+]\) and the decline of \(\gamma'\) at elevated \([\text{CO}_2]\) observed by Wang et al. is ascribable to the reduced transport of NO₃⁻ from roots and the suppressed reduction of NO₃⁻ in the shoot by elevated \([\text{CO}_2]\), rather than the suppression of photorespiratory NH₄⁺ production.

\(\gamma'\) decreased by 70–80% when plants were exposed to more than twice the ambient atmospheric \([\text{CO}_2]\) (i.e. to 800 μmol mol⁻¹) for 3 weeks (Wang et al. 2013): \(\gamma'\) values were 2–3 nmol mol⁻¹ in ambient \([\text{CO}_2]\) and they decreased to \(<1\) nmol mol⁻¹ at the elevated \([\text{CO}_2]\). Wang et al. (2013) reported that the decrease in \(\gamma'\) resulted from a decrease in the apoplastic \([\text{NH}_4^+]\). Doubling atmospheric \([\text{CO}_2]\) increased the net photosynthetic rate by 24–37% in barley leaves (Wang et al. 2013), as observed in rice leaves (Fig. 2A). Therefore, it seems unlikely that the difference in the \([\text{CO}_2]\) response of the NH₃ compensation point between barley and rice is due to a difference in \(V_o\). The transpiration rate decreased by about 30% at elevated \([\text{CO}_2]\) in barley, which is similar to our results for rice plants (Fig. 2B).

The inconsistent results between barley and rice plants may also result from the differences in the nitrogen source used. Wang et al. grew barley plants hydroponically with 2 mM NO₃⁻ or 1 mM NH₄NO₃. Irrespective of the nitrogen source used, the level of NO₃⁻ of the shoot (50–80 mM) was greatly above that of NH₄⁺ (around 0.5 mM) in ambient \([\text{CO}_2]\) and it decreased by 30–40% at elevated \([\text{CO}_2]\) due to the suppressed transpiration (Wang et al. 2013). In contrast, the shoot NH₄⁺ level significantly decreased at elevated \([\text{CO}_2]\) (by 30%) only with 2 mM NO₃⁻ but not with 1 mM NH₄NO₃ (Wang et al. 2013). The latter observation contrasts with our result that the leaf NH₄⁺ content was well correlated with \(V_o\) in rice leaves (Fig. 4B). In barley, the activity of nitrate reductase was significantly decreased (by 45–60%) at elevated \([\text{CO}_2]\) (Wang et al. 2013). Therefore, the reduced apoplastic \([\text{NH}_4^+]\) and the decline of \(\gamma'\) at elevated \([\text{CO}_2]\) observed by Wang et al. is ascribable to the reduced transport of NO₃⁻ from roots and the suppressed reduction of NO₃⁻ in the shoot by elevated \([\text{CO}_2]\), rather than the suppression of photorespiratory NH₄⁺ production.
Taken together, contradictory responses of the NH₃ compensation point among various plant species cannot be ascribed to differences in Vₑ per se, but could instead be derived from the amount and form of inorganic nitrogen available for leaf NH₄⁺ assimilation. Most upland plant species such as oilseed rape, wheat and barley preferentially use NO₃⁻ as the nitrogen source, whereas NH₄⁺ is the major inorganic nitrogen form in paddy fields, and rice plants preferentially use NH₄⁺. The absence of elevated [CO₂] effects on leaf NH₃ exchange in this study (Figs. 3, 4) might be a specific feature of rice plants grown in waterlogged soil.

To what extent does the lamina NH₃ uptake account for the root NH₃ uptake in rice?

In contrast to wheat and barley (Morgan and Parton 1989, Schjoerring et al. 1993), rice plants in paddy fields emit NH₃ from their leaves only after the application of nitrogen fertilizers, but they usually absorb NH₃ from the atmosphere (Hayashi et al. 2008a, Hayashi et al. 2008b). We estimated the total lamina NH₃ uptake rate using the data in Fig. 3A and the nₑ values that have been observed in paddy fields (Hayashi et al. 2008a, Hayashi et al. 2013) as well as from the total root NH₄⁺ uptake using data reported previously (Wang et al. 1993, Britto et al. 2001). The estimation indicated that the lamina NH₃ uptake rate accounted for 3–7% of the root NH₄⁺ uptake rate on a whole-plant basis (Supplementary Table S1). It is suggested that the lamina NH₃ uptake provides a small but significant contribution to the nitrogen budget of rice plants.

Materials and Methods

Plant materials and growth conditions

For our experiments using the portable gas exchange system and the whole-leaf chamber system, we grew rice plants (Oryza sativa L. cv. 'Nippobare') under natural light conditions with supplemental light illumination from metal halide lamps in an air-conditioned room (27/22°C and 60/60% relative humidity for 16/8 h of the day/night period). Seeds imbibed tap water at 28°C for 3 d. Germinated seeds were grown in a commercial soil mixture (Granular soil K; Kumiai Chemicals) in a small container for 10 d in the glasshouse. Seedlings were then transplanted into Wagner pots (1/10,000- or 1/5,000-a pots; 113 or 159 mm in internal diameter and 1.4 or 3.8 liters in volume) containing granular soils (7 and 10 mg N kg⁻¹ soil as NH₄⁺ and NO₃⁻, respectively; Kanuma Sand), which had been supplemented with compound fertilizer (N: P₂O₅: K₂O = 1: 1: 1 by weight; 0.16g each per plant), with ammonium sulfate as the nitrogen source. Potted plants were grown in the glasshouse under waterlogged conditions. Laminae of the uppermost fully expanded leaves (the eighth leaves on the main culm) were used for all analyses. The content of soluble protein, extracted and quantified as described previously (Tsutsumi et al. 2014), was 68 ± 0.5 g m⁻² (mean ± SD; n = 4) on a leaf area basis.

For the FACE experiments, we grew the rice cultivar ‘Koshihikari’ in a FACE facility located at Tsukubamirai City, Ibaraki Prefecture, Japan (35°58′N, 139°60′E, 10 m above sea level; for details, see Hasegawa et al. 2013). Seedlings were transplanted into the plots on May 23, 2012. Both the control and the FACE plots received an equal amount of nitrogen (8 g m⁻²) prior to transplanting; this was provided as 2 g N m⁻² as urea and 6 g N m⁻² as slow-release coated urea.Sunlit upright leaves were harvested at the mid reproductive stage (July 22). Flag leaves were harvested at the full-heading stage (August 9) and the mid grain-filling stage (August 23). Both the plots were kept waterlogged until August 25. CO₂ fertilization was begun on May 30. Average daytime CO₂ concentrations from May 30 to August 23 (at the end of the experiments) were 386 and 567 μmol mol⁻¹ in the control and FACE plots, respectively.

Contents of soluble protein and nitrogen of flag leaves from late July to early September were reported previously (Chen et al. 2014).

Photosynthesis measurements using a portable gas exchange system

We measured gas exchange and Chl fluorescence using a portable CO₂/H₂O gas exchange and Chl fluorescence analysis system equipped with a red/blue light-emitting diode (LED) and a CO₂ injection system (LI-6400-40; Li-Cor). Rates of Rubisco carboxylation (Vc) and oxygenation (Vo) per unit leaf area were calculated using the following equations based on the NAADPH consumption rate (von Caemmerer 2000).

\[ V_c = \left[ \frac{1}{2} + 4(P_n + R_d) \right] / 6 \] (2)

and

\[ V_o = \left[ 4 - (P_n + R_d) \right] / 6 \] (3)

where Ψₑ, Pₙ, and Rₙ are the electron transport rate, net photosynthetic rate and day respiration rate, respectively. j was calculated as:

\[ j = \frac{\alpha \cdot 1 - \phi_{PSI}}{\phi_{PSII}} \] (4)

where α, j, and φ₂ are the fraction of the absorbed irradiance that reaches PSII, the incident photosynthetic photon flux density (PPFD) and the operating efficiency of PSII, respectively. The α value was set at 0.46 (a value for rice; Makino et al. 2002). The eighth leaf blades (8–9 mm in width) were not large enough to occupy the leaf chamber fully (2 cm²). Therefore, we placed two leaves in the chamber, without overlap. We assumed that Rₙ equaled the dark respiration rate. The leaf to air vapor pressure deficit and the leaf temperature were 1.2–1.5 kPa (at 58–65% relative humidity) and 29–30°C, respectively. Based on the equation in the LI-6400 manual, the boundary layer conductance for water vapor in air, for one leaf surface, was 4.65 mol m⁻² s⁻¹.

Gas exchange measurements using a custom-made whole-leaf chamber system with NH₃ traps

The whole-leaf chamber system. To measure NH₃ gas exchange by rice leaves at a range of CO₂ concentrations, we constructed a whole-leaf chamber system (Fig. 1), based on the system developed for field measurements by Hayashi et al. (2008a). In this system, NH₃ in the air from each leaf chamber was trapped in cellulose filter paper impregnated with phosphoric acid.

Each lamina was held in a transparent cylindrical chamber (40 × 3.6 cm in length × diameter; Fig. 1, #10) with silicon plugs at both ends. The plug at the bottom end was slit to allow insertion of the lamina. Air was drawn from the growth cabinet using a pump (#2; CD-15TFA, Enomoto Micro Pump) through a polystyrenecoated filter (a 1 mm in height, #1; pore size = 1 μm). After CO₂ was absorbed by passing through a 0.4 liter column containing soda lime (#4), the air was supplied with the desired concentration of CO₂ from a CO₂ cylinder (#5, 99.999%) using a mass-flow meter (#6; MC-3000L; Lintec). When [NH₃] in air entering the leaf chambers was reduced, the [CO₂]-controlled air was passed through an NH₃ absorber consisting of an acrylic column (1 liter in volume; #7) and a filter (#8). The column contained 1 kg of 3 mm diameter glass beads, which had been spread with 2% (v/v) phosphoric acid and 5% (v/v) glycerol and dried for 4 d at 60°C. In the filter, a sheet of cellulose filter paper (45 mm in diameter: No. 51A; Advantec), which had been impregnated with the phosphoric acid/glycerol solution and dried as above, was set in a filter holder (NI-L; NI-LU Products).

To analyze NH₃ gas exchange by a lamina, each leaf chamber was connected to a mass-flow meter (#11; 3810DS; Kofloc), and NH₃ in air from the chamber was trapped with an NH₃-absorbing filter (#12; a sheet of cellulose filter paper (30 mm in diameter; No. 51A; Advantec) which had been treated with the phosphoric acid/glycerol solution, as described above). To analyze CO₂ and H₂O gas exchange by a lamina, each leaf chamber was connected to a switching device that allowed analysis of the gas from one lamina at a time (#13; FC15S-6C; Meiwafosis), and CO₂ and water vapor concentrations in air from the chambers were determined using an infrared gas analyzer (#14; LI-840; Li-Cor).

The leaf chambers (#10) and NH₃-absorbing filters (#12) were installed in a growth cabinet, in which air temperature and relative humidity were controlled at 27°C/22°C and 65%/60% for the day/night cycle, respectively. The growth
cabinet light (from metal halide lamps) was turned on at 05:00 h and turned off at 19:00 h. The flow rate of the [CO$_2$]-controlled air entering each leaf chamber was adjusted to between 3.1 and 3.3 min$^{-1}$ by rotameters #3 and #9 (maximum flow rates were 30 and 5 l min$^{-1}$, respectively).

**Calculation of the leaf NH$_3$ uptake rate.** The leaf NH$_3$ uptake rate on a leaf area basis ($F_{NH_3}$) was calculated according to the methods of Hayashi et al. (2008a) and von Caemmerer and Farquhar (1981), as follows:

\[
F_{NH_3} = (n_o - n_a) \cdot u_o / LA - n_o \cdot E
\]

where $n_o$ and $n_a$ are the mole fractions of NH$_3$ in the air leaving the control (without plants) and sample (containing plants) chambers, respectively, $u_o$, LA and $E$ are the average flow rate of air entering the chamber measured with rotameters #9, the lamina area, and the respiration rate per unit leaf area, respectively. $n_o$ was determined as:

\[
n_o = N/(u_o \cdot t)
\]

where $N$, $u_o$, and $t$ are the amount of NH$_3$ trapped in the filter (#12), the flow rate measured with a mass-flow meter (#11) and the sampling period, respectively. $n_a$ was determined in the same way.

**Estimation of photosynthetic and transpiration rates.** We calculated the photosynthetic rate ($P_o$) and the transpiration rate ($E$) per unit leaf area according to the equations provided by von Caemmerer and Farquhar (1981):

\[
P_o = (c_o - c_e) \cdot u_o / LA - c_e \cdot E
\]

\[
E = (w_o - w_e) \cdot u_o / (1 - w_o) \cdot LA
\]

where $c_o$ and $c_e$ are the mole fractions of CO$_2$ in air leaving from the control and sample chambers, respectively. $w_o$ and $w_e$ are the mole fractions of water vapor in air leaving from the sample and control chambers, respectively.

**Conditions and procedures for gas exchange measurements.** We measured air temperatures inside the leaf chambers using thermocouples, and found a range of 30–33°C. The relative humidity was 60–70%. PFD measured above the chambers was 600 mol m$^{-2}$ s$^{-1}$ (LI-189; Li-Cor). $u_o$ (the flow rate measured with mass-flow meter #11) and $u_e$ (the average flow rate entering the chamber, measured with rotameter #9) ranged from 0.07 to 0.1 m s$^{-1}$ and from 0.10 to 0.12 mol min$^{-1}$, respectively. $u_o$ values were estimated from the flow rate and air temperature inside the chambers. The wind speed inside the leaf chambers was calculated to be 0.1 m s$^{-1}$. The boundary layer conductance to water vapor in air for one leaf surface, calculated from the wind speed and the average lamina length inside the chambers (Nobel 1999), was 0.2 mol m$^{-2}$ s$^{-1}$.

NH$_3$-free air was flushed into the entire system at least 12 h prior to our experiments. Rice plants were transferred from the glasshouse to the growth cabinet, and the CO$_2$ treatments of the leaves were started 2 h into the light period (around 07:00 h). CO$_2$ and water vapor concentrations in the air leaving each leaf chamber were monitored for 5 min at around 09:00 h. Then, NH$_3$ gas exchange was measured as follows. First, NH$_3$ in air entering the chambers ($n_o$) was reduced to <0.4 nmol mol$^{-1}$ using the NH$_3$ absorber (#7 and #8), and NH$_4^+$ in air from each chamber was trapped by the trapping filter (#12) for 100–120 min. Next, the NH$_3$ absorber was removed from the system to increase $n_o$ and NH$_4^+$ from each chamber was trapped as described above. Because of the low leaf NH$_3$ gas exchange rates in rice, a relatively long period (>60 min) was required to trap NH$_4^+$ sufficient for precise quantification of NH$_4^+$. All gas exchange measurements were completed by 14:30 h. Leaf samples were harvested immediately after the measurements.

**Leaf sampling and measurement of leaf area.** The middle portion of the lamina (15 cm in length) was harvested to obtain the leaf apoplastic solution. When we determined the leaf NH$_4^+$ content, a small segment (3 cm in length) was excised from the mid section of the harvested lamina and immediately frozen in liquid N$_2$. The area of the segment was calculated from its length and width measured with a pair of callipers based on the assumption that its shape was a trapezoid. We measured the area of the residual parts of the lamina which had been treated inside the chambers using an area meter (AAM-9; Hayashi-Denko), and used the sum of these areas as LA.

**Extraction of the apoplastic solution**

We extracted the apoplastic solution from the lamina by means of vacuum infiltration, according to the methods described by Husted and Schjoerring (1995) and Nouchi et al. (2012). Leaves were infiltrated with an isotonic sorbitol solution containing 0.01% (v/v) Tween-20. We determined the concentrations of sorbitol (550 and 400 mM for the chamber and FACE experiments, respectively) based on the osmotic pressure of sap squeezed from laminae harvested during the daytime, which we measured using an osmometer (Vapro Osmometer 5520; Wescor).

After the infiltration, the leaf surface was blotted dry with tissue paper (5-200; Kimwipes), then the leaf was cut into segments (1.5–2 cm in length) and placed into centrifugal filter units (Nanosep: 0.45 μm, Pall Corporation). After centrifugation at 2,000 x g for 30 s at room temperature, the resultant filtrates were taken as the apoplastic solution. We obtained 50–70 μl of the apoplastic solution from 2–3 laminae. After we measured the pH with a semi-micro pH meter (InLab Surface; Mettler Toledo), we determined the NH$_4^+$ content of the apoplastic solution. The NH$_4^+$ compensation point ($\gamma'$) was calculated from the [NH$_4^+$]apo and the pH of the apoplastic solution using the equation provided by Farquhar et al. (1986):

\[
\gamma' = R \cdot T \cdot (T - 0.00052 - 0.207 T/77) [\text{NH}_4^+][\text{H}^+]_{apo}
\]

where R is the gas constant (= 0.08311 bar K$^{-1}$ mol$^{-1}$), T, [NH$_4^+$]apo and [H$^+$]apo are the temperature, the apoplastic NH$_4^+$ concentration and the apoplastic proton concentration, respectively. T was set at 30°C.

**Determination of NH$_4^+$**

NH$_3$ trapped in the filter paper of the NH$_3$-absorbing filter was dissolved into 1.0 ml of distilled water and the NH$_4^+$ content was then determined. Leaf samples were powdered in liquid N$_2$ and their NH$_4^+$ was extracted using 20 mM formic acid according to the methods of Husted et al. (2000). NH$_4^+$ was determined fluorometrically after reaction with o-phthalaldehyde using HPLC (474 Scanning Fluorescence Detector; Waters).

**Statistical analyses**

Statistical analyses were performed using the R statistical software (R Foundation for Statistical Computing, software version 3.0.1, www.r-project.org). All tests were performed at a significance level of P < 0.05.

**Supplementary data**

**Supplementary data are available at PCP online.**

**Funding**

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [Grants-in Aid for Scientific Research on Innovative Areas (22114516 and 24114712 to M.M.)]; the Ministry of Agriculture, Forestry, and Fisheries of Japan [Genomics for Agricultural Innovation, grant No. GPN0006 to M.M.].

**Acknowledgments**

We thank Drs. Mitsutoshi Kitao and Kenichi Yazaki, Forestry and Forest Products Research Institute, Tsukuba, Japan, for
allowing us to use their portable gas exchange analyzer and their osmometer. We also thank Dr. Eiichi Minami, National Institute of Agrobiological Sciences, for allowing us to use the HPLC. We thank the journal’s three anonymous reviewers for their valuable comments on our manuscript.

Disclosures

The authors have no conflicts of interest to declare.

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