Ammonia emission from rice leaves in relation to photorespiration and genotypic differences in glutamine synthetase activity

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INTRODUCTION

The exchange of gaseous NH3 between plants and the atmosphere depends on the gradient between substomatal cavities in the leaf and the atmosphere: NH3 emission takes place when the concentration of NH3 in the atmosphere is lower than that of NH3 in the substomatal cavities of leaves, while NH3 absorption occurs in the opposite case (Schjoerring et al., 2000). The NH3 concentration at which NH3 absorption balances NH3 loss, resulting in zero net flux of NH3, is the NH3 compensation point (Farquhar et al., 1980). Generally, the concentration of NH3 is higher within the leaf than the atmosphere. Thereby, NH3 flux occurs from the leaf to the atmosphere. Net emissions range from less than 10 to more than 70 kg NH3-N ha−1 per season, depending on the plant species, nitrogen status of the plant and soil, and climatic conditions. Such emissions may lead to a significant loss (up to 5 %) of the shoot’s N content (Schjoerring et al., 2000). Thus, the NH3 emission by crops may affect their productivity because N is essential for key physiological processes leading to dry-matter production.

There are several processes generating NH4+ in the leaf, including photosynthesis, nitrate/nitrite reduction, lignin biosynthesis and protein turnover (Leegood et al., 1995). In the photorespiratory cycle, NH4+ is released during the decarboxylation of glycine in mitochondria (Keys et al., 1978). Nitrate is converted to NH4+ by the sequential action of the cytosolic nitrate reductase and chloroplastic nitrite reductase (Lea and Ireland, 1999). In the lignin biosynthetic pathway, a significant amount of NH4+ is generated directly in the leaf apoplast (Nakashima et al., 1997). NH4+ is released from protein degradation and amino acid deamination in the cytosol (Olea et al., 2004). Photosynthetic NH4+ production may occur at rates up to ten times that of nitrate/nitrite reduction (Lea et al., 1992; Leegood et al., 1995). Among these processes, photosynthesis is probably the largest source of liberated NH4+. The NH4+ generated is in equilibrium with NH3, depending on pH in the compartment.

Glutamine synthetase (GS), a key enzyme in the GS–glutamate synthase cycle, plays a pivotal role in the recycling of NH4+ that is released during photosynthesis by generating glutamate from NH4+ and glutamine (Leegood et al., 1995).
Ammonia emission from leaves of rice cultivars

Kumagai et al.

There are two isoforms of GS in higher plants, cytosolic GS1 and chloroplastic GS2, with recycling of photorespiratory $\text{NH}_3$ depending on GS2 (Keys and Leegood, 2002). Despite the operation of this efficient $\text{NH}_3$ recycling system, part of the $\text{NH}_3$ is lost from the leaves into the atmosphere. Previous studies of the inhibition of GS by methionine sulfoximine (MSO) and of mutants with reduced GS activity in barley (Hordeum vulgare) unambiguously demonstrated the involvement of GS in $\text{NH}_3$ emission (Mattsson and Schjoerring, 1996; Mattsson et al., 1997). However, Husted et al. (2002) found that the antisense oilseed rape (Brassica napus) plants with reduced GS2 activity showed a similar $\text{NH}_3$ emission rate (AER) to that of wild plants. Thus, the extent and/or the mechanism of involvement of GS in $\text{NH}_3$ emission may differ between barely and oilseed rape plants. Further analyses with different species are required to understand the physiological mechanisms responsible for foliar $\text{NH}_3$ emission.

Rice (Oryza sativa), one of the most important cereal crops, loses significant amounts of volatile $\text{NH}_3$ from its leaves (da Silva and Stutte, 1980; Stutte and da Silva, 1981). We found that foliar applications of MSO and an inhibitor of photorespiration (pyrid-2-yl hydroxymethylene sulfonate) to rice plants dramatically increased and decreased, respectively, AER (Kumagai et al., 2009), suggesting the involvement of photorespiration in $\text{NH}_3$ emission and a pivotal role of GS in the recycling of $\text{NH}_3$ in rice plants, as in barley (Mattsson et al., 1998). In another study, we found that ‘Kasalath’, a cultivar with low GS activity, had a higher AER and higher $\text{NH}_3$ content in its leaves than ‘Akenohoshi’, a cultivar with high GS activity (Kumagai et al., 2011). These data suggest that rice cultivars differ in the AER of their leaves and that this difference may be explained, at least in part, by different GS activities.

Kamiji and Horie (1989) reported that AER was correlated with the transpiration rate ($Tr$) in flag leaves of rice plants during the ripening stage and proposed that the leaves release $\text{NH}_3$ along with water during transpiration. However, they did not consider the stomatal conductance to water vapour ($gs$). $\text{NH}_3$ emission should be evaluated based on the diffusive conductance as well as the transpiration flux (Massad et al., 2008). The possible involvements of $gs$ and $Tr$ in AER of rice leaves remain to be investigated.

To clarify the roles of photorespiration in $\text{NH}_3$ emission and of GS in $\text{NH}_3$ recycling in rice plants, we investigated the response of AER in the leaves of two rice cultivars with different GS activities to light intensity, leaf temperature and $O_2$ concentration ($O_2$) with simultaneous measurements of photosynthetic gas exchanges to determine the relationships between AER and these processes. The measurement of AER under conditions with different photorespiratory rates clearly demonstrates that photorespiration is strongly involved in $\text{NH}_3$ emission by rice leaves. In addition, it is suggested that differences in AER between the two cultivars are due to different activities of GS involved in reassimilation of photorespiratory $\text{NH}_3$.

MATERIALS AND METHODS

Plant materials and cultivation

‘Akenohoshi’ is a japonica–indica cross, whereas ‘Kasalath’ is a traditional indica cultivar of rice (Oryza sativa L.). Imbibed seeds of the two cultivars were sown in nursery boxes in a glasshouse on 10 May 2009. After 21 d, young seedlings were transplanted into bottomless polyvinyl chloride cylinders (8 cm in diameter and 7 cm in height) sealed with spongious tissue (one seedling per cylinder). The cylinders were floated in 400-L water baths filled with the following solution recommended by Yoshida et al. (1972), with a slight modification, in which (NH$_4$)$_2$SO$_4$ was used instead of NH$_3$NO$_3$:

- 2.86 mm (NH$_4$)$_2$SO$_4$
- 0.51 mm K$_2$SO$_4$
- 1.00 mm CaCl$_2$
- 1.67 mm MgSO$_4$
- 0.32 mm NaH$_2$PO$_4$
- 0.04 mm FeCl$_2$
- 9.09 µm MnCl$_2$
- 0.08 µm (NH$_4$)$_6$Mo$_7$O$_{24}$
- 18.2 µm H$_3$BO$_3$
- 0.15 µm ZnSO$_4$
- 0.16 µm CuSO$_4$
- 3.57 mm Na$_2$SiO$_3$

The pH of the solution was adjusted every day to between 5.0 and 5.5 using HCl and NaOH. Each solution in the water baths was renewed at 2-week intervals. The seedlings were grown in the glasshouse under natural sunlight.

At 40–60 d after transplanting, we simultaneously measured photosynthetic gas exchange and $\text{NH}_3$ emission of the upper-most fully expanded leaves of the rice plants using the method described below. After the measurements, we sampled the leaves and measured their area. The leaves were then dried at 80 ºC for 3 d in an oven before measuring their N content. At mid-day, leaves from different plants were sampled, frozen in liquid $N_2$ and stored at −80 ºC before determination of the $\text{NH}_3$ content and GS activity.

Simultaneous measurements of photosynthesis, transpiration and $\text{NH}_3$ emission

Photosynthetic gas exchanges and $\text{NH}_3$ emission by the leaves were measured simultaneously using an open gas-exchange system based on an assimilation chamber (400 cm$^2$) equipped with a water jacket and a fan. The chamber was made of transparent acryl, ensuring low water, $CO_2$ and $NH_3$ adsorption. For all the measurements, we maintained [CO$_2$] and relative humidity of the entering air at 402 ± 5 µmol mol$^-1$ and 30 ± 5 %, respectively. Air flow through the chamber was adjusted to 2.0 L min$^-1$ throughout the measurements. Light was supplied to the chamber by a red–blue lighting system (LED-HLCN-P, Ollie Co., Ltd, Osaka, Japan). Leaf temperature was measured with T-type thermocouples and chamber temperature was maintained by the water jacket. Temperature-controlled water was circulated in the water jacket. Leaf temperature was always within ±1 ºC of the chamber temperature (data not shown). Four leaves per plant were inserted into the chamber for each measurement.

$[CO_2]$ and water vapour pressure in the reference and sample air were monitored with an infrared CO$_2$ and H$_2$O gas analysers (Li-6262, Li-COR, Lincoln, NB, USA). Net photosynthesis ($P_N$), dark respiration ($R_d$), $Tr$ and $gs$ were calculated according to the method of Long and Hallgren (1985). The gross photosynthesis ($P_G$) equalled the sum of $P_N$ and $R_d$. $NH_3$ in the air entering the chamber was removed by an upstream filter consisting of a three-stage cellulose filter (51A, Advantec, Tokyo, Japan). $NH_3$ emitted from leaves in the chamber was collected by passage through a downstream one-stage 51A filter impregnated with a mixed solution of 5 % (v/v) phosphoric acid (H$_3$PO$_4$) and 2 % (v/v) glycerol and then dried in $NH_3$-free air. $NH_3$ adsorbed by the
H₂PO₄-impregnated filter was extracted in 10 mL of de-ionized water. The concentration of extracted NH₃ was determined according to the indophenol blue method (Scheiner, 1976). AER was expressed as the amount of NH₃ emitted per unit leaf area per unit time (nmol m⁻² s⁻¹). The high efficiency (>95%) of NH₃ collection in this method was confirmed in our previous study (Kumagai et al., 2011).

Responses of P₉, Tr, gs and AER to light intensity (photosynthetic photon flux density, PPFD) were determined at a leaf temperature of 32.5 °C and [O₂] of 21% (i.e. ambient), with PPFD of 200, 500 and 1000 μmol m⁻² s⁻¹. Responses of P₉, Tr, gs and AER to temperature were determined at a PPFD of 500 μmol m⁻² s⁻¹ and [O₂] of 21%, with leaf temperatures of 27.5, 32.5 and 37.5 °C. Responses of P₉, Tr, gs and AER to [O₂] were also determined at a leaf temperature of 32.5 °C and a PPFD of 500 μmol m⁻² s⁻¹, with [O₂] of 2, 21 and 40%. Each measurement was repeated at least three times.

Estimation of photorespiration

Photorespiration (Rₚ) was estimated by subtracting the value of P₉ at 21 or 40% [O₂] from that at 2% [O₂] (Ye et al., 1994).

Determination of N and NH₄⁺ contents and GS activity in leaves

Dried leaves were powdered and the N content was determined using the semi-micro Kjeldahl procedure. NH₄⁺ content was measured according to Manderscheid et al. (2005). GS activity was measured according to O’Neal and Joy (1973).

Statistical analysis

Student’s t-test was applied to test the significance of differences between the data from ‘Akenohoshi’ and ‘Kasalath’. The tests were performed using version 3.1 of the SigmaStat software (Systat Software, Inc., Richmond, USA).

RESULTS

The N content in the leaves of ‘Akenohoshi’ was significantly higher than that of ‘Kasalath’, whereas the NH₄⁺ content in leaves of ‘Akenohoshi’ was significantly lower than that of ‘Kasalath’ (Table 1). GS activity in the leaves of ‘Kasalath’ was significantly smaller, only 57%, than that of ‘Akenohoshi’.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>N content (g m⁻²)</th>
<th>NH₄⁺ content (μmol g⁻¹)</th>
<th>GS activity (μmol g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Akenohoshi’</td>
<td>1.40 ± 0.02***</td>
<td>0.28 ± 0.01***</td>
<td>163.3 ± 19.2***</td>
</tr>
<tr>
<td>‘Kasalath’</td>
<td>0.92 ± 0.03</td>
<td>0.55 ± 0.03</td>
<td>92.4 ± 6.1</td>
</tr>
</tbody>
</table>

Values are given as the mean ± s.e. (n = 3). ***, Significant differences between the cultivars at P < 0.001.

An increase in PPFD from 200 to 1000 μmol m⁻² s⁻¹ increased AER in both cultivars (Fig. 1A). AER in ‘Kasalath’ was significantly higher than that in ‘Akenohoshi’ at 500 and 1000 μmol m⁻² s⁻¹ PPFD. P₉, Tr and gs of the two cultivars also increased with increasing PPFD (Fig. 1B–D), and the three parameters were significantly higher in ‘Akenohoshi’ than in ‘Kasalath’ at 1000 μmol m⁻² s⁻¹ PPFD.

An increase in leaf temperature from 27.5 to 37.5 °C also increased AER in both cultivars (Fig. 1E). AER in ‘Kasalath’ was significantly higher than that in ‘Akenohoshi’ at 32.5 and 37.5 °C. Tr of both cultivars also increased as leaf temperature increased (Fig. 1G). In contrast, the increase in leaf temperature caused slight decreases in P₉ and gs of both cultivars (Fig. 1F, H). No significant differences in P₉, Tr and gs were observed between the cultivars at any temperature.

An increase in [O₂] increased AER in both cultivars, but the increase was greater in ‘Kasalath’ than in ‘Akenohoshi’ (Fig. 1I), significantly so at 21 and 40% [O₂]. The increase in [O₂] caused a decrease in P₉ in both cultivars (Fig. 1J). Tr showed little response to the increase in [O₂] (Fig. 1K). The value of gs decreased slightly as [O₂] increased (Fig. 1L). There were no significant differences in P₉, Tr and gs between the cultivars.

There were high and significant positive correlations between Rₚ and AER for both ‘Kasalath’ and ‘Akenohoshi’ (Fig. 2; P < 0.001). However, the slope of the regression line was higher in ‘Kasalath’ than in ‘Akenohoshi’.

DISCUSSION

Our results clearly indicated that an increase in [O₂] enhanced AER by both rice cultivars (Fig. 1I). Simultaneously, P₉ decreased because of enhanced Rₚ (Fig. 1J), which would be caused by accelerated oxygenation activity relative to the carboxylation activity of ribulose-1,5-bisphosphatase carboxylase/oxygenase (Leegood et al., 1995). In both cultivars, high positive correlations were found between Rₚ and AER (Fig. 2). These results show that photorespiration is strongly involved in NH₃ emission from rice leaves. Similar responses of AER to [O₂] were observed in soybean (Glycine max; Weiland and Stutte, 1985) and spring wheat (Triticum aestivum; Morgan and Parton, 1989). In oilseed rape, however, AER did not change despite a 300% increase in the ratio of O₂ to CO₂ (Husted et al., 2002). These data suggest that the extent of involvement of the photorespiratory process in foliar NH₃ emission differs among species. Recently, it was reported that GS2 was localized in mitochondria of Arabidopsis thaliana leaves (Taira et al., 2004). It may be possible that mitochondrial GS2 efficiently assimilates NH₄⁺ generated during photorespiration (Linka and Weber, 2005). Oilseed rape belongs to the family Brassicaceae together with Arabidopsis. Although GS2 has not been reported in mitochondria of species other than Arabidopsis so far, these contradictory results may be due to the specific difference in the intracellular localization of GS.

When PPFD increased, AER and P₉ both increased (Fig. 1A, B). Under these circumstances, Rₚ would also increase, keeping the ratio of Rₚ to P₉ constant (Leegood et al., 1995). When leaf temperature increased, AER also increased, whereas P₉ decreased slightly (Fig. 1E, F). This
decrease in $P_G$ would be due partly to the enhanced $R_P$ caused by a decline in CO$_2$ solubility in water compared with that of O$_2$ at higher temperatures (Jordan and Ogren, 1984; Brooks and Farquhar, 1985). However, other factors may also be involved in NH$_3$ emission, because $AER$ increased more rapidly in response to increasing leaf temperature than the corresponding decrease in $P_G$. In plants grown at high temperature, protein degradation is accelerated and accompanied by the release of NH$_4^+$ (Lawlor, 1979). Temperature influences foliar NH$_3$ emission by affecting the thermodynamic equilibrium between the aqueous NH$_3$ in the apoplast and the gaseous NH$_3$ in the substomatal cavity (Massad et al., 2008). Thus, such non-photorespiratory factors may also affect the temperature-dependent increase in $AER$.

As it is thought that photorespiration does not substantially occur at 2% [O$_2$], $AER$ derived from photorespiration at ambient [O$_2$] (21%) was estimated by subtracting $AER$ measured at 2% [O$_2$]. The values were 3.71 ± 0.14 and 5.96 ± 0.21 nmol m$^{-2}$ s$^{-1}$ in ‘Akenohoshi’ and ‘Kasalath’, respectively. These values accounted for 57 and 67% of the total $AER$ derived from all the processes, including NH$_4^+$-generating processes other than photorespiration. Morgan and Parton (1989) reported corresponding values of 15–50% in spring wheat. Based on data by Weiland and Stutte (1985), the value is approx. 35% for soybean. Thus, the extent of involvement of photorespiration in NH$_3$ emission from rice leaves might be greater than that in other species.

Husted and Schjoerring (1996) reported that in oilseed rape plants NH$_3$ emission increased linearly with $g_S$ as light intensity increased. We also found that $AER$, $Tr$ and $g_S$ increased with increases in PPFD. However, the patterns of increase differed somewhat among the three parameters (Fig. 1A, C, D).

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Fig. 1. Effects of increasing (A–D) light intensity (PPFD), (E–H) leaf temperature and (I–L) [O$_2$] on the NH$_3$ emission ($AER$), gross photosynthesis ($P_G$), transpiration ($Tr$) and stomatal conductance to water vapour ($g_S$) in two rice cultivars. Values are given as the means ± s.e. ($n$ = 3). *Significant difference between the cultivars at $P < 0.05$. 

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1384 Kumagai et al. — Ammonia emission from leaves of rice cultivars

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concept of the \( \text{NH}_3 \) compensation point (Farquhar et al., 1980), our measurements of \( \text{AER} \), which were carried out by use of \( \text{NH}_3 \)-free air, would lead to an overestimation relative to naturally occurring conditions in which the atmosphere inevitably contains some \( \text{NH}_3 \). Nevertheless, it seems likely that \( \text{NH}_3 \) emission will have a considerable influence on the \( \text{N} \) economy of rice plants, and suggests that reduced \( \text{NH}_3 \) emission should become a target trait in future rice breeding programmes.

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**LITERATURE CITED**


