

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

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- **Background and Aims** Rice (*Oryza sativa*) plants lose significant amounts of volatile NH₃ from their leaves, but it has not been shown that this is a consequence of photorespiration. Involvement of photorespiration in NH₃ emission and the role of glutamine synthetase (GS) on NH₃ recycling were investigated using two rice cultivars with different GS activities.
- **Methods** NH₃ emission (*AER*), and gross photosynthesis (*P_G*), transpiration (*Tr*) and stomatal conductance (*g_s*) were measured on leaves of ‘Akenohoshi’, a cultivar with high GS activity, and ‘Kasalath’, a cultivar with low GS activity, under different light intensities (200, 500 and 1000 μmol m⁻² s⁻¹), leaf temperatures (27.5, 32.5 and 37.5 °C) and atmospheric O₂ concentrations ([O₂]: 2, 21 and 40 %, corresponding to 20, 210 and 400 mmol mol⁻¹).
- **Key Results** An increase in [O₂] increased *AER* in the two cultivars, accompanied by a decrease in *P_G* due to enhanced photorespiration, but did not greatly influence *Tr* and *g_s*. There were significant positive correlations between *AER* and photorespiration in both cultivars. Increasing light intensity increased *AER*, *P_G*, *Tr* and *g_s* in both cultivars, whereas increasing leaf temperature increased *AER* and *Tr* but slightly decreased *P_G* and *g_s*. ‘Kasalath’ (low GS activity) showed higher *AER* than ‘Akenohoshi’ (high GS activity) at high light intensity, leaf temperature and [O₂].
- **Conclusions** Our results demonstrate that photorespiration is strongly involved in NH₃ emission by rice leaves and suggest that differences in *AER* between cultivars result from their different GS activities, which would result in different capacities for reassimilation of photorespiratory NH₃. The results also suggest that NH₃ emission in rice leaves is not directly controlled by transpiration and stomatal conductance.

Key words: Ammonia assimilation, ammonia emission, glutamine synthetase, nitrogen, *Oryza sativa*, photorespiration, rice cultivars.

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 photorespiration, 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). Photorespiratory 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, photorespiration 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 photorespiration by generating glutamate from NH₄⁺ and glutamine (Leegood *et al.*, 1995).

There are two isoforms of GS in higher plants, cytosolic GS1 and chloroplastic GS2, with recycling of photorespiratory NH_4^+ depending on GS2 (Keys and Leegood, 2002). Despite the operation of this efficient NH_4^+ recycling system, part of the 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 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 NH_3 emission rate (AER) to that of wild plants. Thus, the extent and/or the mechanism of involvement of GS in 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 NH_3 emission.

Rice (*Oryza sativa*), one of the most important cereal crops, loses significant amounts of volatile 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 hydroxymethane sulfonate) to rice plants dramatically increased and decreased, respectively, AER (Kumagai et al., 2009), suggesting the involvement of photorespiration in NH_3 emission and a pivotal role of GS in the recycling of 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 NH_4^+ 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 (T_r) in flag leaves of rice plants during the ripening stage and proposed that the leaves release NH_3 along with water during transpiration. However, they did not consider the stomatal conductance to water vapour (g_s). 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 g_s and T_r in AER of rice leaves remain to be investigated.

To clarify the roles of photorespiration in NH_3 emission and of GS in 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 ($[\text{O}_2]$) 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 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 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 spongy 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 $(\text{NH}_4)_2\text{SO}_4$ was used instead of NH_4NO_3 : 2.86 mM $(\text{NH}_4)_2\text{SO}_4$, 0.51 mM K_2SO_4 , 1.00 mM CaCl_2 , 1.67 mM MgSO_4 , 0.32 mM NaH_2PO_4 , 0.04 mM FeCl_2 , 9.09 μM MnCl_2 , 0.08 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 18.2 μM H_3BO_3 , 0.15 μM ZnSO_4 , 0.16 μM CuSO_4 and 3.57 mM Na_2SiO_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 NH_3 emission of the uppermost 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 °C 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 °C before determination of the NH_4^+ content and GS activity.

Simultaneous measurements of photosynthesis, transpiration and NH_3 emission

Photosynthetic gas exchanges and NH_3 emission by the leaves were measured simultaneously using an open gas-exchange system based on an assimilation chamber (400 cm³) 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 $[\text{CO}_2]$ and relative humidity of the entering air at $402 \pm 5 \mu\text{mol mol}^{-1}$ and $30 \pm 5\%$, 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 °C of the chamber temperature (data not shown). Four leaves per plant were inserted into the chamber for each measurement.

$[\text{CO}_2]$ and water vapour pressure in the reference and sample air were monitored with an infrared CO_2 and H_2O gas analyser (Li-6262, Li-COR, Lincoln, NB, USA). Net photosynthesis (P_N), dark respiration (R_d), T_r and g_s 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_3PO_4) and 2 % (v/v) glycerol and then dried in NH_3 -free air. NH_3 adsorbed by the

H₃PO₄-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 *P_G*, *Tr*, *g_S* 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 *P_G*, *Tr*, *g_S* 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 *P_G*, *Tr*, *g_S* 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 (*R_P*) was estimated by subtracting the value of *P_G* at 21 or 40% [O₂] from that at 2% [O₂] (Yeo 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 Sigmasat 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 1. Nitrogen (N) content, NH₄⁺ content and glutamine synthetase (GS) activity in the two rice cultivars.

Cultivar	N content (g m ⁻²)	NH ₄ ⁺ content (μmol g ⁻¹)	GS activity (μmol g ⁻¹ h ⁻¹)
'Akenohoshi'	1.40 ± 0.02***	0.28 ± 0.01***	163.3 ± 19.2***
'Kasalath'	0.92 ± 0.03	0.55 ± 0.03	92.4 ± 6.1

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. *P_G*, *Tr* and *g_S* 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 *P_G* and *g_S* of both cultivars (Fig. 1F, H). No significant differences in *P_G*, *Tr* and *g_S* 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 *P_G* in both cultivars (Fig. 1J). *Tr* showed little response to the increase in [O₂] (Fig. 1K). The value of *g_S* decreased slightly as [O₂] increased (Fig. 1L). There were no significant differences in *P_G*, *Tr* and *g_S* between the cultivars.

There were high and significant positive correlations between *R_P* 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, *P_G* decreased because of enhanced *R_P* (Fig. 1J), which would be caused by accelerated oxygenation activity relative to the carboxylation activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Leegood et al., 1995). In both cultivars, high positive correlations were found between *R_P* 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 *P_G* both increased (Fig. 1A, B). Under these circumstances, *R_P* would also increase, keeping the ratio of *R_P* to *P_G* constant (Leegood et al., 1995). When leaf temperature increased, *AER* also increased, whereas *P_G* decreased slightly (Fig. 1E, F). This

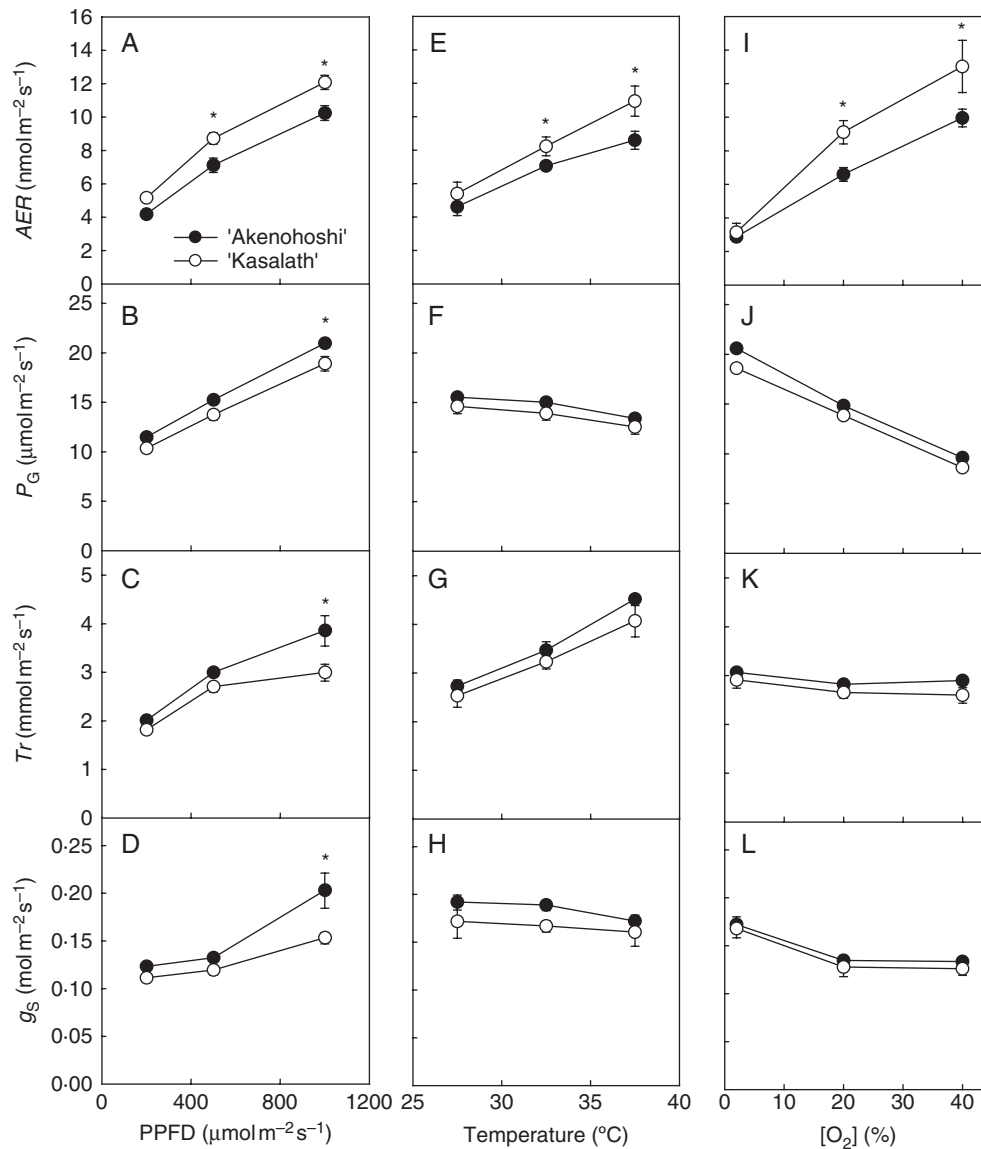


FIG. 1. Effects of increasing (A–D) light intensity (PPFD), (E–H) leaf temperature and (I–L) [O₂] on the NH₃ emission (*AER*), gross photosynthesis (*P_G*), transpiration (*Tr*) and stomatal conductance to water vapour (*g_s*) in two rice cultivars. Values are given as the means ± s.e. (*n* = 3). *Significant difference between the cultivars at *P* < 0.05.

decrease in *P_G* would be due partly to the enhanced *R_p* caused by a decline in CO₂ solubility in water compared with that of O₂ at higher temperatures (Jordan and Ogren, 1984; Brooks and Farquhar, 1985). However, other factors may also be involved in NH₃ 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 NH₄⁺ (Lawlor, 1979). Temperature influences foliar NH₃ emission by affecting the thermodynamic equilibrium between the aqueous NH₃ in the apoplast and the gaseous NH₃ 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% [O₂], *AER* derived from photorespiration at

ambient [O₂] (21%) was estimated by subtracting *AER* measured at 2% [O₂]. 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 NH₄⁺-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 NH₃ emission from rice leaves might be greater than that in other species.

Husted and Schjoerring (1996) reported that in oilseed rape plants NH₃ 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).

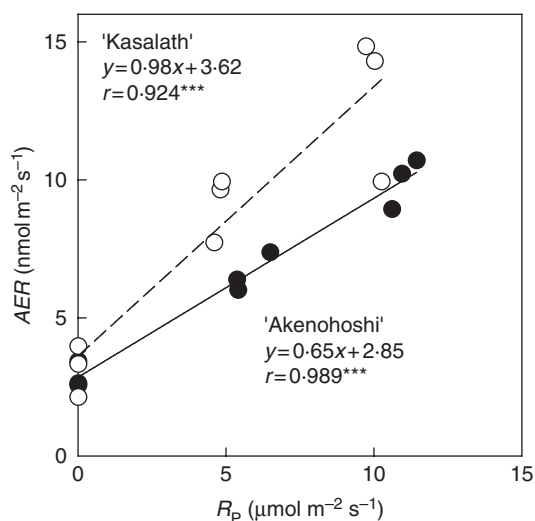


FIG. 2. Relationships between the photorespiration (R_p) and NH_3 emission (AER) for the two rice cultivars. ***Significant correlation at $P < 0.001$.

The increase in temperature increased AER and T_r (Fig. 1E, G) but decreased g_s slightly (Fig. 1H). Furthermore, the increase in $[\text{O}_2]$ increased AER (Fig. 1I), but did not greatly influence T_r (Fig. 1K) and decreased g_s slightly (Fig. 1L). These data suggest that AER in rice leaves is not directly controlled by the transpiration flux and g_s . Stutte and Weiland (1978) showed that the rates of NH_3 emission in several species were more closely correlated with temperature than with T_r . Husted and Schjoerring (1996) reported that g_s was not the only factor responsible for the increase in AER caused by increasing temperature.

Our study demonstrated that 'Kasalath' emits NH_3 at a higher rate than 'Akenohoshi' from their leaves under conditions of enhanced photorespiration. The amount of NH_4^+ in leaf tissues was higher in 'Kasalath' than in 'Akenohoshi', whereas GS activity showed the opposite trend (Kumagai et al., 2011; Table 1). Thus, the observed cultivar differences in AER may be due to differences in GS activity; 'Akenohoshi', with high GS activity, was able to reassimilate more of the NH_3 released by photorespiration than 'Kasalath', with low GS activity. Obara et al. (2000) reported that 'Kasalath' (an *indica* cultivar) has lower GS2 activity in its leaves than *japonica* and *javanica* cultivars of rice. The GS activity in leaves may therefore be one of the factors that determine differences in AER among rice cultivars.

Our study showed that 'Akenohoshi', with a higher leaf N content than 'Kasalath', loses less NH_3 from its leaves than 'Kasalath', as we found in our previous study (Kumagai et al., 2011). Based on the data in Fig. 1A, we estimated the amounts of N loss through foliar NH_3 emission during the life span of the two cultivars on the assumption of a 40-d leaf life span, an 8-h daylength with $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ mean daily PPFD (sufficiently high to support photorespiration), and a mean daily temperature of 32.5°C . The amounts of N loss per unit leaf area would be 0.165 and 0.195 g m^{-2} in 'Akenohoshi' and 'Kasalath', respectively. These N losses account for 12 and 21% of the leaf N contents in 'Akenohoshi' and 'Kasalath', respectively. According to the

concept of the NH_3 compensation point (Farquhar et al., 1980), our measurements of AER, which were carried out by use of NH_3 -free air, would lead to an overestimation relative to naturally occurring conditions in which the atmosphere inevitably contains some NH_3 . Nevertheless, it seems likely that NH_3 emission will have a considerable influence on the N economy of rice plants, and suggests that reduced NH_3 emission should become a target trait in future rice breeding programmes.

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