

Nitrogen use efficiency (NUE) in rice links to NH_4^+ toxicity and futile NH_4^+ cycling in roots

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Abstract

Aims Rice is known as an ammonium (NH_4^+)-tolerant species. Nevertheless, rice can suffer NH_4^+ toxicity, and excessive use of nitrogen (N) fertilizer has raised NH_4^+ in many paddy soils to levels that reduce vegetative biomass and yield. Examining whether thresholds of NH_4^+ toxicity in rice are related to nitrogen-use efficiency (NUE) is the aim of this study.

Methods A high-NUE (Wuyunjing 23, W23) and a low-NUE (Guidan 4, GD) rice cultivar were cultivated hydroponically, and growth, root morphology, total N and NH_4^+ concentration, root oxygen consumption,

and transmembrane NH_4^+ fluxes in the root meristem and elongation zones were determined.

Results We show that W23 possesses greater capacity to resist NH_4^+ toxicity, while GD is more susceptible. We furthermore show that tissue NH_4^+ accumulation and futile NH_4^+ cycling across the root-cell plasma membrane, previously linked to inhibited plant development under elevated NH_4^+ , are more pronounced in GD. NH_4^+ efflux in the root elongation zone, measured by SIET, was nearly sevenfold greater in GD than in W23, and this was coupled to strongly stimulated root respiration. In both cultivars, root growth was affected more severely by high NH_4^+ than shoot growth. High NH_4^+ mainly inhibited the development of total root length and root area, while the formation of lateral roots was unaffected.

Conclusions It is concluded that the larger degree of seedling growth inhibition in low- vs. high-NUE rice genotypes is associated with significantly enhanced NH_4^+ cycling and tissue accumulation in the elongation zone of the root.

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Keywords Rice · Nitrogen-use efficiency · NH_4^+ toxicity · Free NH_4^+ concentration · NH_4^+ efflux · Root respiration

Introduction

Ammonium (NH_4^+), one of the two inorganic nitrogen sources used by plants (NH_4^+ and NO_3^-), is beneficial

for plant growth under many circumstances and, indeed, serves as a ubiquitous intermediate in plant metabolism (Glass et al. 1997). Its assimilation furthermore entails lower energy costs compared to NO_3^- (Mehrer and Mohr 1989). Additionally, studies have shown NH_4^+ can improve the capacity to tolerate water stress in rice in comparison with NO_3^- (Guo et al. 2007), and has been shown to act as an inducer of resistance against salinity conditions in other species (Fernandez-Crespo et al. 2012). Nevertheless, NH_4^+ frequently reaches levels in soils that affect plant growth negatively. These negative effects manifest in stunted root growth, yield depression, and chlorosis of leaves (Britto and Kronzucker 2002; Balkos et al. 2010; Li et al. 2011b). However, higher plants display widely differing responses to NH_4^+ nutrition (Marschner 1995) and, accordingly, can be divided into tolerant and sensitive species (Britto and Kronzucker 2002). Based on a series of comparative studies, more than 18 kinds of plants or plant species, including eight kinds of wild plants, have been classified as highly adapted to NH_4^+ as a nitrogen source (Britto and Kronzucker 2002; Rios-Gonzalez et al. 2002; Cruz et al. 2006; Dominguez-Valdivia et al. 2008; Omari et al. 2010). Further, more than 22 kinds of plants or plant species, eight of which wild, have been classified as sensitive to the NH_4^+ source (Britto and Kronzucker 2002; Cruz et al. 2006; Roosta et al. 2009). Rice is regarded as unique in its high degree of NH_4^+ tolerance (Wang et al. 1993a, b). Studies have suggested that NH_4^+ -tolerant plants generally possess higher glutamine synthetase (GS) activity and less accumulation of free NH_4^+ in plant tissues (Magalhaes and Huber 1991; Balkos et al. 2010). In addition, several important hypotheses have been proposed, such as carbon depletion in roots induced by NH_4^+ assimilation (Finnemann and Schjoerring 1999), NH_4^+ -induced pH reduction in the root zone (Chaillou et al. 1991), deficiencies of mineral cations (Siddiqi et al. 2002), impairments in the N-glycosylation of proteins (Barth et al. 2010) and futile and energy-costly NH_4^+ cycling at the plasma membrane of both root and shoot cells (Britto et al. 2001; Kronzucker et al. 2001; Britto and Kronzucker 2002; Szczerba et al. 2008a; Li et al. 2010). However, to date, no single mechanism has been able to fully elucidate NH_4^+ toxicity (Britto and Kronzucker 2002; Roosta and Schjoerring 2008). On the basis of the fact that all of the hypothesized mechanisms with regard to NH_4^+ toxicity are linked to the permeation of NH_4^+ (or perhaps NH_3) into the cell, useful clues can be obtained by

studying transmembrane NH_4^+ fluxes. Britto et al. (2001) studied NH_4^+ fluxes across the root plasma membranes of barley and rice by using a high-precision positron tracing technique and found, at elevated levels of NH_4^+ , a significantly larger NH_4^+ efflux, accounting for up to 80 % of primary influx, in barley cells, which carried a high energetic cost and was independent of N metabolism. Britto et al. (2001) furthermore suggested that rice, unlike barley, was resistant to the respiratory drain induced by futile NH_4^+ cycling.

Despite its reputation as an NH_4^+ -tolerant species, rice can be affected negatively by elevated NH_4^+ , particularly at low K^+ (Balkos et al. 2010), which, in turn, may be relieved by elevated K^+ , similar to conclusions reached in *Arabidopsis* (Li et al. 2010; Zou et al. 2012). Several studies have shown declines in K^+ -bearing clay minerals over extended cultivation periods in many rice-growing areas of China (Li et al. 2003). In fact, K^+ deficiency has been observed in about 70 % of rice paddies in southeastern China (Yang et al. 2005). Similar declines have been noted in other parts of Asia (Cassman et al. 1997). On the other hand, in recent years, excessive use of N fertilizers, irrational fertilization patterns, and deposition of atmospheric $\text{NH}_3/\text{NH}_4^+$ (Pearson and Stewart 1993; Li et al. 2011a) have resulted in the accumulation of excess NH_4^+ in many agricultural soils, and, consequently, soil-solution NH_4^+ concentrations have been reported in ranges of 2–20 mM, and some as high as 40 mM (Glass et al. 2002; Kronzucker et al. 2003).

China has the second-largest area of rice cultivation and the highest rice production in the world, accounting for 19 % of the world's rice area and contributing 29 % of the world's rice production (FAO 2010), and rice is mainly cultivated in irrigated paddy fields, where anaerobic conditions prevail and inorganic nitrogen is maintained as NH_4^+ (Freney et al. 1985). In recent years, an N-fertilization rate averaging 300–350 kg N ha⁻¹ has been applied in many regions of China in a single rice season, with the goal of obtaining maximal grain yields (Wang et al. 2004). However, the high N-application rate has in fact decreased grain yields (Zhang et al. 2009; Sun et al. 2012; Qiao et al. 2012), and led to a drastic decline in N-utilization efficiency, with the additional consequence of increased N loss to the environment, polluting both the atmosphere and water systems (Zhu et al. 1997; Kondo et al. 2003). NUE, in an agronomic sense, can be defined as the ratio of grain yield to N supplied, namely, $\text{NUE} = \text{Gw}/\text{Ns}$, where Gw represents plant

grain yield and N_s represents plant-available N in the soil including soil-native N and N applied as fertilizer, and is composed of N-uptake efficiency and physiological N-use efficiency (De Macale and Velk 2004). Thus, high-NUE cultivars are designated as such when they display higher grain yield under identical nitrogen supply. With improvements in rice breeding technology, new rice cultivars have been produced that exhibit higher grain yield under identical N-application levels and possess stronger capacity for resistance to high application of N fertilizer in comparison with older cultivars which are usually referred to as relatively low-NUE cultivars. It is speculated that low-NUE rice cultivars suffer from more severe damage in development caused by high amount application of N fertilizer, primarily by excessive NH_4^+ , compared with high-NUE cultivars.

Here, we examine rice cultivars differing in NUE, with the emphasis on performance on high NH_4^+ reflective of excessive applications of N. We specifically explore the relationship between root NH_4^+ fluxes, N content, tissue NH_4^+ accumulation, root morphology, and the capacity for tolerance to high NH_4^+ . We hope our experiments will lay a foundation for research into the relationship of NUE and NH_4^+ tolerance.

Materials and methods

Plant material and treatments

Two rice (*Oryza sativa* L.) cultivars, Wuyunjing 23 (W23) and Guidan 4 (GD), were chosen as experimental materials. Our field experiment showed when 200 kg N ha^{-1} were applied, the grain yields of W23 and GD were 8.93 tha^{-1} and 5.09 tha^{-1} , respectively. Furthermore, when 270 kg N ha^{-1} were applied, W23 reached 9.28 tha^{-1} , compared to 4.27 tha^{-1} in GD. Based on these results, W23 was defined as a high-NUE cultivar, and GD as a low-NUE cultivar.

After germination on moist filter paper, rice seeds were transferred to a 2.0 mmol L^{-1} CaSO_4 solution for germination. After 3 days, seedlings were transferred to a 1/4-strength mixture of NO_3^- or NH_4^+ -containing nutrient solution, according to IRRI rice nutrient solution (Yoshida et al. 1972; for composition, see below). Three days after, seedlings were transferred to a half-strength mixture of NO_3^- or NH_4^+ -containing nutrient solution. Four days later, seedlings were supplied with

full-strength mixture of NO_3^- or NH_4^+ -containing nutrient solution for 1 week, and then seedlings were supplied with either “normal” N (2.86 mol m^{-3} NH_4^+ -N, provided as $(\text{NH}_4)_2\text{SO}_4$), namely CK treatment, or “high” N (15 mol m^{-3} NH_4^+ -N, provided as $(\text{NH}_4)_2\text{SO}_4$). In addition, varying NH_4^+ -N concentrations ($10, 20, 30 \text{ mol m}^{-3}$) were also supplied to examine the relationship between NH_4^+ fluxes and NH_4^+ tolerance of the two rice cultivars. All treatments had eight replicates, with completely randomized design. The placement of different treatments was randomized to avoid edge effects in the growth chamber.

Rice plants were cultivated in a growth chamber at 25°C with 70 % relative humidity and a 14-h light and 10-h dark cycle. The light intensity was set at $500 \mu\text{mol photon m}^{-2}\text{s}^{-1}$ at plant height. Except for N, composition of the nutrient solution was as follows: macronutrients (mol m^{-3}): 1.02 K as K_2SO_4 and KH_2PO_4 ; 0.32 P as KH_2PO_4 , 1.65 Mg as MgSO_4 , micronutrients (mmol m^{-3}): 35.8 Fe as Fe-EDTA; 9.10 Mn as MnSO_4 ; 0.15 Zn as ZnSO_4 ; 0.16 Cu as CuSO_4 ; 18.5 B as H_3BO_3 ; 0.52 Mo as $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$; 0.1 Si as Na_2SiO_4 . In NH_4^+ -containing nutrient solution, Ca^{2+} was supplied as CaCl_2 (1.43 mol m^{-3}), the pH of the nutrient solution was maintained at 5.50 ± 0.05 by adding 0.1 mol m^{-3} HCl or 0.1 mol m^{-3} NaOH daily, and the nitrification inhibitor DCD was added to each pot to prevent NH_4^+ oxidation. Nutrient solutions were exchanged every 2 days.

Biomass, total N accumulation and root morphology

15 days after treatments at normal and high N, rice plants were harvested and divided into above-ground parts and roots. All samples were kept in a drying oven at 105°C for 30 min, and then at 70°C until constant weight was achieved, at which point dry weight was recorded. Plant samples were digested with H_2SO_4 - H_2O_2 , and the concentration of N was determined using the Kjeldahl method. Root morphology including total root length, root volume, root surface area, average root diameter, and root tip number were analyzed using the root analysis instrument WinRhizo-LA1600 (Regent Instruments Inc., Quebec, Canada).

Tissue ammonium determination

Rice seedlings were harvested and desorbed in 10 mol m^{-3} CaSO_4 for 5 min, so as to remove extracellular

NH_4^+ . Roots and shoots were weighed separately and then transferred to polyethylene plastic vials with liquid N_2 for storage at -80°C . Approximately 0.5 g of root or shoot tissue was homogenized under liquid N_2 using a mortar and pestle, followed by addition of 6 ml formic acid (10 molm^{-3}) to extract NH_4^+ . Subsamples of the homogenate were centrifuged at 2°C for 10 min at 25,000 g, and filtered with $0.45\ \mu\text{m}$ nylon filter (Costar, Corning Inc., Lowell, MA, USA) and centrifuged at 5,000 g (2°C) for 5 min. Ammonium was analyzed by the *o*-phthalaldehyde (OPA) method using a high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA, equipped with a Phenomenex Gemini C18 analytical column $4.6\text{ mm}\times 150\text{ mm}$; particle size $5\ \mu\text{m}$). The analytical principle was based on detection of fluorescence upon reaction between the fluorochrome OPA and NH_4^+ as described by Husted et al. (2000).

Measurement of net NH_4^+ fluxes with the SIET system

Net fluxes of NH_4^+ were measured non-invasively using SIET (scanning ion-selective electrode technique, SIET system BIO-003A; Younger USA Science and Technology Corp.; Applicable Electronics Inc.; Science Wares Inc., Falmouth, MA, USA). The principle of this method and the instrument are detailed in Sun et al. (2009). Measurements were performed at room temperature ($24\text{--}26^\circ\text{C}$). After growth of rice seedlings in normal-N ($2.86\text{ molm}^{-3}\ \text{NH}_4^+\text{-N}$) and high-N ($15\text{ molm}^{-3}\ \text{NH}_4^+\text{-N}$) nutrient solution for 15 days, the roots of seedlings were equilibrated in measuring solution for 20–30 min. The equilibrated seedlings were then transferred to the measuring chamber, a small plastic dish (3-cm diameter) containing 2–3 mL of fresh measuring solution, and fixed for measurement. The microelectrode was vibrated in the measuring solution between two positions, $5\ \mu\text{m}$ and $35\ \mu\text{m}$ from the root surface, along an axis perpendicular to the root. The background was recorded by vibrating the electrode in measuring solution not containing roots. The glass microelectrodes with $2\text{--}4\ \mu\text{m}$ aperture were made and silanized by Xuyue Science and Technology Co., Ltd. $0.1\text{ molm}^{-3}\ \text{NH}_4^+$ as $(\text{NH}_4)_2\text{SO}_4$ was added as back-filling solution, followed by $20\ \mu\text{M}$ of commercially available ionophore cocktail for measuring NH_4^+ (NH_4^+ selective liquid ion-exchange cocktail #09879, Fluka Chemicals, Buchs, Switzerland) in front of the microelectrode. Prior to the flux

measurements, the ion-selective electrodes were calibrated using NH_4^+ concentrations of 0.05 and 0.5 molm^{-3} . The net fluxes of NH_4^+ at the meristem and elongation zone were measured individually. Each plant was measured once. The final flux values at each zone were the means of more than eight individual plants from each treatment. The measuring solution was composed of $1.02\text{ molm}^{-3}\ \text{K}$ as K_2SO_4 and KH_2PO_4 ; $0.32\text{ molm}^{-3}\ \text{P}$ as KH_2PO_4 , $1.65\text{ molm}^{-3}\ \text{Mg}$ as MgSO_4 , $1.43\text{ molm}^{-3}\ \text{Ca}$ as CaCl_2 and 0.5 g/L MES (pH5.5 adjusted with 1 M NaOH). All measurements of net NH_4^+ fluxes were carried out at Xuyue Science and Technology Co., Ltd (Beijing, China).

Root respiration

Excised roots of rice seedlings following 15-day treatment at normal and high N were used for root respiration measurements, using a Hansatech oxygen electrode and an Oxygraph control system (Hansatech Instruments, Norfolk, UK). Roots were cut into sections approximately 3 mm long while immersed in solution and using a razor blade, and were aged for a minimum of 3 h in aerated growth solution. About 0.3 g of root material was placed into 3 mL of growth solution, and the cuvette was sealed. The decline in O_2 concentration was monitored for 15 min, with the initial, linear decline used to calculate O_2 depletion rates (Balkos et al. 2010).

Statistical analysis

All data were statistically analyzed by means of the statistical software package SAS with LSD to identify differences. Significant differences ($P < 5\%$) between treatments are indicated by different letters.

Results

Total biomass accumulation and ratio of root to shoot

High NH_4^+ significantly inhibited total biomass accumulation in GD compared to growth on normal N level, by 27.3 %, while there was no statistically significant difference in W23 (Fig. 1a). Elevated NH_4^+ markedly suppressed the root:shoot ratio in both GD and W23, which indicated rice roots were

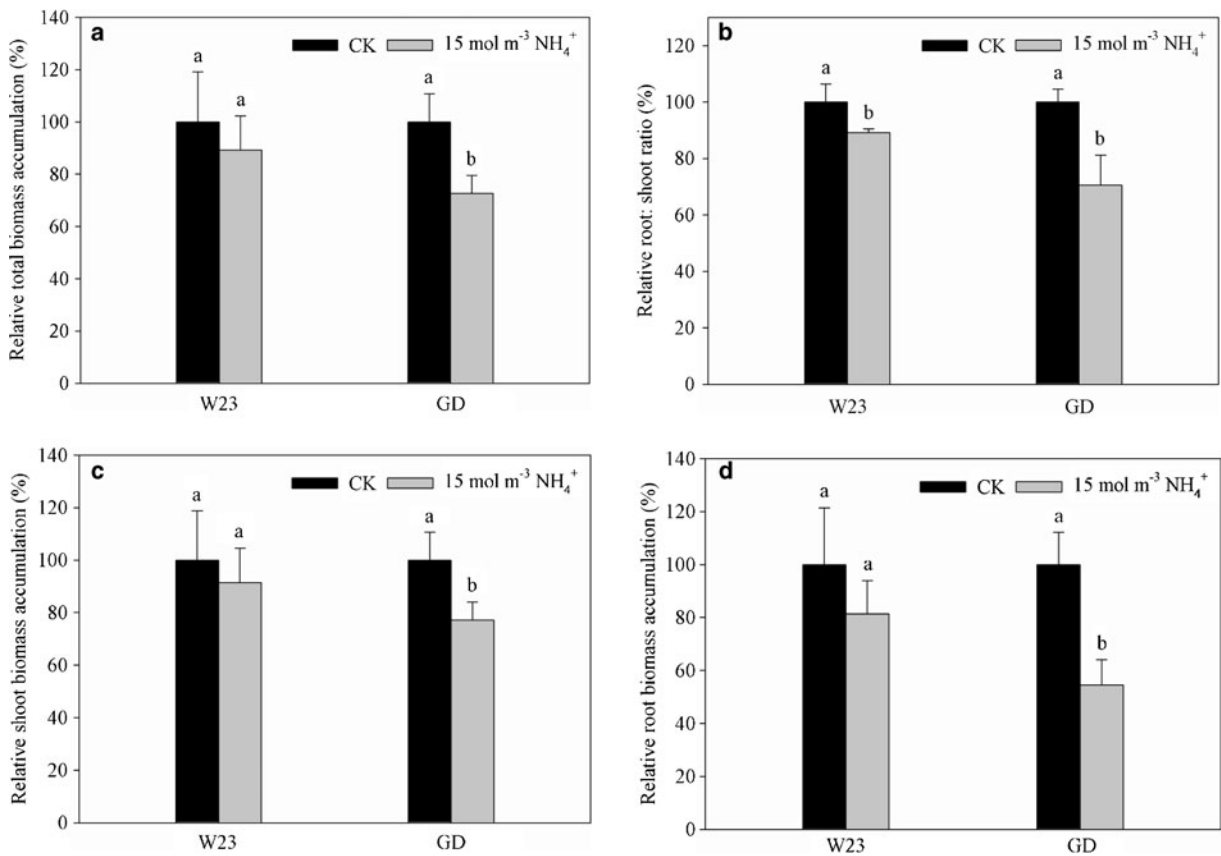


Fig. 1 Percentage inhibition of total biomass (a), root-to-shoot ratio (b), shoot (c) and root (d) biomass in Wuyunjing 23 (W23) and Guidan 4 (GD) supplied with normal N (CK) and high N (15 mol m⁻³ NH₄⁺) levels. In (a), (b), (c), and (d), growth on CK was considered as 100 %. In (a), 100 % corresponds to 0.51±0.10 g plant⁻¹ for W23 and 1.55±0.17 g plant⁻¹ for GD. In (b), 100 %

corresponds to 0.26±0.02 for W23 and 0.25±0.01 for GD. In (c), 100 % corresponds to 0.40±0.08 g plant⁻¹ for W23 and 1.25±0.13 g plant⁻¹ for GD. In (d), 100 % corresponds to 0.11±0.02 g plant⁻¹ for W23 and 0.31±0.04 g plant⁻¹ for GD. Bars show standard deviations and different letters indicate significant differences between normal N and high N levels in each rice cultivar (*P*<0.05)

more affected by elevated NH₄⁺ than shoots. The suppression of the ratio was more substantial (29.5 %) in GD than in W23 (<10.8 %) (Fig. 1b). Figure 1c, d further show that high NH₄⁺ suppressed root growth more than shoot growth, with decreases of 45.5 % and 18.6 % measured in roots of GD and W23, respectively, and only 22.8 % and 8.6 % in shoots.

Root morphology

As shown in Table 1, under high NH₄⁺, total root length and root surface area in GD were inhibited compared with growth on normal N. By contrast, no significant differences were observed in W23. In addition, high NH₄⁺ had little effect on root volume, average root diameter, or root tip number, showing

that high NH₄⁺ does not affect the formation of lateral roots in rice.

Total N accumulation and N concentration

N accumulation paralleled biomass accumulation in both cultivars, with smaller N accumulation observed in both root and shoot of GD in the high NH₄⁺ treatment compared to normal N treatments. Moreover, a larger extent of suppression in N accumulation was observed in roots than shoots in GD, by 41.1 % and 28.8 %, respectively. However, no obvious N-accumulation differences were found in either organ of W23 (Fig. 2a, b). In addition, no differences in N content were found, when expressed as a percentage, in either roots or shoots of W23 and GD (Fig. 2c, d).

Table 1 Root morphology of W23 and GD supplied with normal N (CK) and high N ($15 \text{ mol m}^{-3} \text{ NH}_4^+$)

Rice cultivar	N (NH_4^+) level	Total length (cm)	Volume (cm^3)	Surface area (cm^2)	Average diameter (cm)	Tip number
W23	CK	229±35.8 a	2.37±0.08 a	82.5±7.62 a	1.16±0.07 a	170±14.6 a
	15 mol m^{-3}	220±19.2 a	2.59±0.31 a	84.3±3.63 a	1.23±0.12 a	172±49.9 a
GD	CK	362±44.8 a	2.75±0.26 a	112±8.73 a	0.98±0.06 a	185±30.6 a
	15 mol m^{-3}	282±13.9 b	2.68±0.31 a	94.0±3.92 b	1.08±0.07 a	189±15.0 a

Data are means ± SD of three replicates; different letters indicate significant differences between normal N and high N levels in each rice cultivar ($P < 0.05$)

Tissue NH_4^+ concentration

When supplied with high NH_4^+ , markedly higher NH_4^+ concentrations were observed in both shoot and root of W23 and GD in comparison with normal-N treatments. Similarly increased percentages

were observed in shoots of both W23 and GD, 40 % and 41 %, respectively (Fig. 3a). However, dramatically larger NH_4^+ concentrations were detected in roots of GD when supplied with high NH_4^+ in comparison with those of W23: 255 % for GD, and only 83 % for W23 (Fig. 3b).

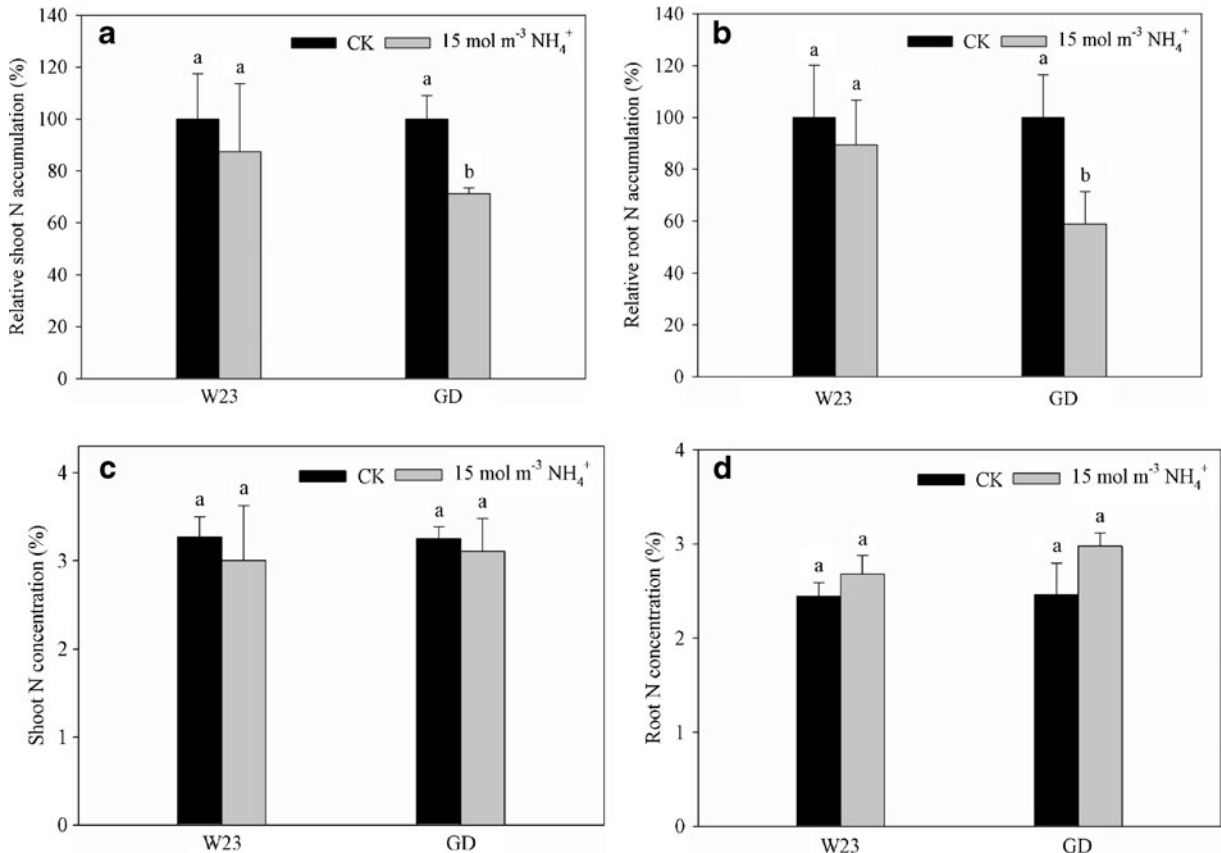


Fig. 2 Percentage inhibition of shoot-N (a) and root N accumulation (b), as affected by high NH_4^+ , shoot N concentration (c) and root N concentration (d) of W23 and GD supplied with normal N (CK) and high N ($15 \text{ mol m}^{-3} \text{ NH}_4^+$) levels. In (a), (b), N accumulation on CK was considered as 100 %. In (a), 100 % corresponds to $13.0 \pm 2.27 \text{ mg plant}^{-1}$ for W23 and $40.5 \pm$

$3.64 \text{ mg plant}^{-1}$ for GD. In (b), 100 % corresponds to $2.57 \pm 0.52 \text{ mg plant}^{-1}$ for W23 and $9.38 \pm 1.54 \text{ mg plant}^{-1}$ for GD. Bars show standard deviations and different letters indicate significant differences between normal N and high N levels in each rice cultivar ($P < 0.05$)

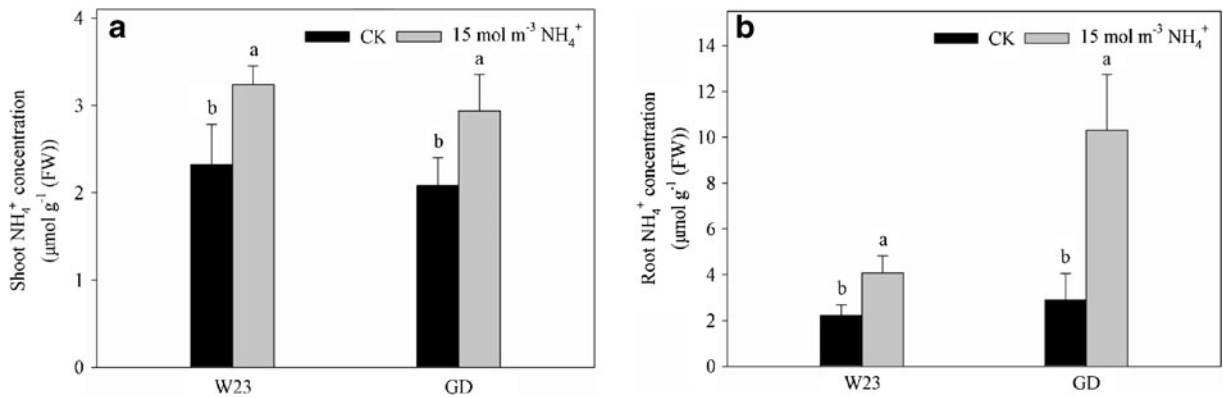


Fig. 3 Shoot NH₄⁺ concentration (a) and root NH₄⁺ concentration (b) of W23 and GD supplied with normal N (CK) and high N (15 mol m⁻³ NH₄⁺) levels. Bars show standard deviations and

different letters indicate significant differences between normal N and high N levels in each rice cultivar ($P < 0.05$)

Net NH₄⁺ fluxes at the meristem and elongation zones of rice roots

Net NH₄⁺ fluxes at the surfaces of meristem and elongation zones of W23 and GD, assayed by SIET, showed NH₄⁺ influx primarily in the meristem zones in both W23 and GD when supplied with normal N and high NH₄⁺, whereas high NH₄⁺ stimulated NH₄⁺ efflux in the elongation zones of both cultivars (Fig. 4a, b, c, d). Greater NH₄⁺ influx was observed in the meristem zone of W23 compared with GD, and high NH₄⁺ did not change the NH₄⁺ flux direction in the meristematic zones in either rice cultivar (Fig. 4a, b, e). NH₄⁺ efflux was detected in the elongation zones of both W23 and GD when supplied with high NH₄⁺, but there was a significantly larger NH₄⁺ efflux in GD, some 670 % higher than W23 (Fig. 4c, d, f).

Root respiration

High NH₄⁺ stimulated O₂ consumption in roots to differing extents in W23 and GD when compared with normal-N treatments. No significant differences were observed in W23. However, in GD, high NH₄⁺ markedly increased root O₂ consumption (Fig. 5).

The relationship between net NH₄⁺ fluxes and NH₄⁺ tolerance of the two rice cultivars

As shown in Fig. 6a, total biomass accumulation was decreased with increasing NH₄⁺ concentration from CK (2.86 mol m⁻³ NH₄⁺) to 30 mol m⁻³ NH₄⁺. However,

larger degrees of growth suppression were detected in GD with increasing NH₄⁺ concentration in comparison with W23. Meanwhile, a larger degree of gradually increased NH₄⁺ effluxes in the elongation zone was observed in GD with increasing NH₄⁺ concentration. In addition, NH₄⁺ effluxes in the meristematic zone were also detected in GD when NH₄⁺ concentrations were raised to 20 and 30 mol m⁻³ (Fig. 6b, c). This indicates that NH₄⁺ efflux at the root surface is linked to the capacity for NH₄⁺ tolerance in rice.

Discussion

Inhibited development of rice plants and root morphology under high NH₄⁺ in relation to NUE

Excessive NH₄⁺ is known to inhibit the growth of most crop species (Roosta and Schjoerring 2008). In particular, stunted root growth is a principal symptom of the ion's toxicity (Gerendas et al. 1997; Britto and Kronzucker 2002; Balkos et al. 2010; Roosta and Schjoerring 2008). In our study, a larger degree of inhibition of rice development, in particular in roots, was observed in the lower-NUE cultivar GD compared with the higher-NUE W23 (Fig. 1a, b, c, d), indicating that lower NUE in the two cultivars examined is associated with a lower threshold for NH₄⁺ toxicity. More severe inhibition of rice root than shoot growth was observed in both W23 and GD (Fig. 1c, d), producing reduced root:shoot ratios in both cultivars when NH₄⁺ was elevated (Fig. 1b). This agrees with observations of others, who related the effect to a competition for carbon skeletons between root growth

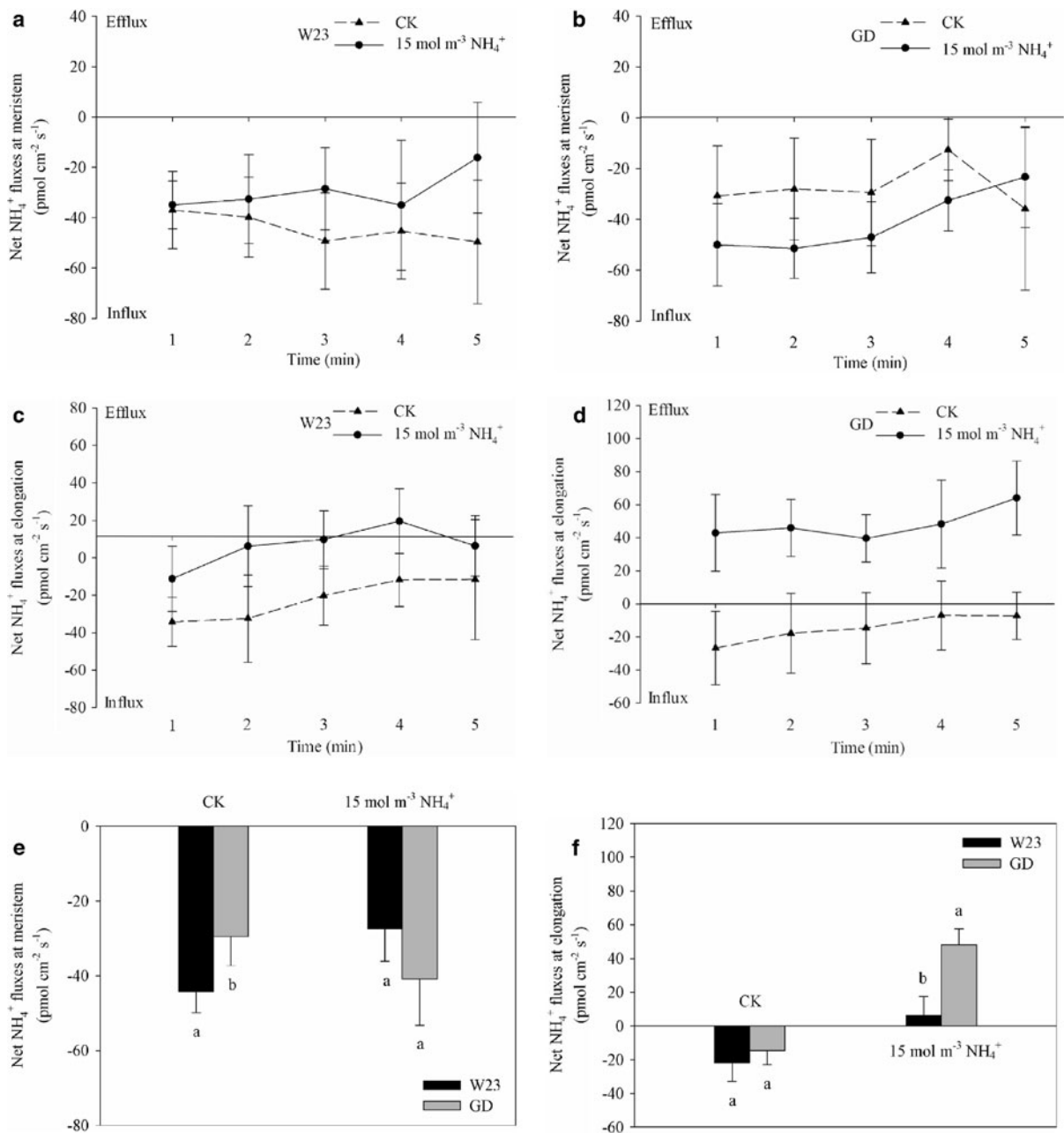


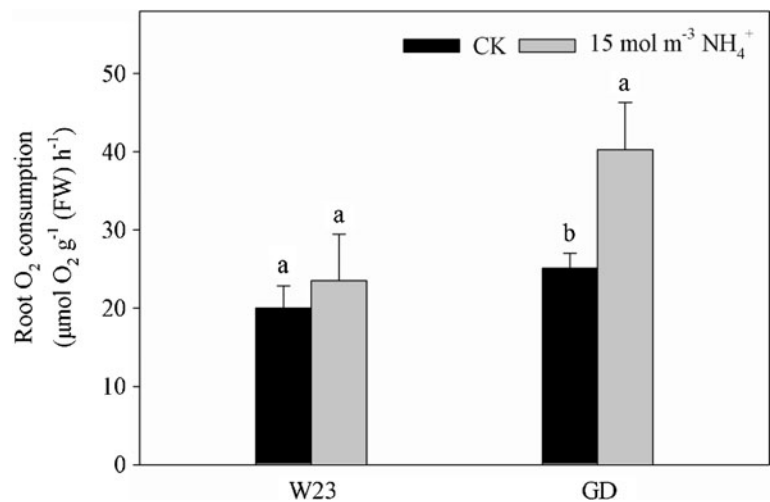
Fig. 4 Net plasma-membrane NH_4^+ fluxes in W23 and GD supplied with normal N (CK) and high N ($15 \text{ mol m}^{-3} \text{ NH}_4^+$) levels in the meristematic zones (a), (b), and the elongation zones (c) (d), and mean values of NH_4^+ fluxes from (a), (b)

and (c), (d), respectively. Each point represents the mean \pm SD of eight individual plants, and different letters indicate significant differences between normal N and high N levels in each rice cultivar ($P < 0.05$)

and NH_4^+ assimilation in NH_4^+ -fed plants (Roosta and Schjoerring 2008). While carbohydrates were not determined in the present study, we measured enhanced NH_4^+ efflux (Fig. 4c, d, f) and respiratory oxygen consumption in roots (Fig. 5), which may have

contributed to the reduced root:shoot ratio (see later discussion). Root architecture plays an essential role in the efficiency of nutrient and water acquisition from the soil (Lynch 1995), and, therefore, root morphologies of GD and W23 were further analyzed to assess

Fig. 5 Root O₂ consumption of W23 and GD supplied with normal N (CK) and high N (15 mol m⁻³ NH₄⁺) levels. Bars show standard deviations and *different letters* indicate significant differences between normal N and high N levels in each rice cultivar ($P < 0.05$)



root changes induced by elevated NH₄⁺ and found that both total root length and root surface area were more severely inhibited in GD than in W23 (Table 1). As reported by Li et al. (2010) in *Arabidopsis*, the distance from the root apex to the first root hair was reduced markedly under high NH₄⁺, the latter study further demonstrated that cell elongation rather than cell division was affected when the root tip was in direct contact with NH₄⁺, indicating that root tip may function as a sensor in the perception of NH₄⁺ stress. Based on these findings, the suppressed total root length in GD is attributed to inhibited cell elongation in the root elongation zone where, as in the previous study (Li et al. 2010), markedly larger NH₄⁺ efflux was also detected (Table 1 and Fig. 4d, f). In addition, the number of the root tip itself was not affected by high NH₄⁺ (Table 1), indicating that the “sensor system” for perceiving NH₄⁺ stress remains intact under NH₄⁺ stress in rice. Also interesting was that the formation of lateral roots was not obviously affected by high NH₄⁺, another conclusion that agrees with the earlier study by Li et al. (2010), who found high NH₄⁺ did not inhibit the formation of lateral roots in *Arabidopsis*, a relatively NH₄⁺-sensitive species. Fundamentally, therefore, there appears to be little difference in the manner in which NH₄⁺ affects the root architecture in NH₄⁺-tolerant and NH₄⁺-sensitive species, and species as well as cultivar differences are more a matter of threshold and degree than fundamental mechanistic difference.

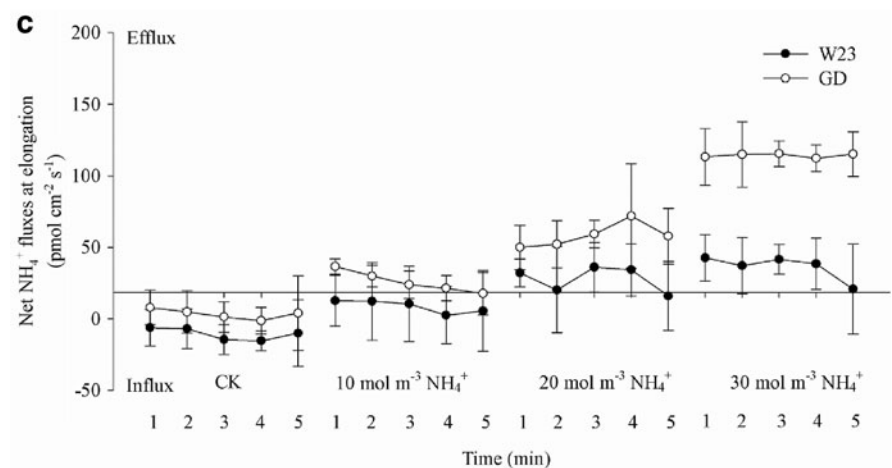
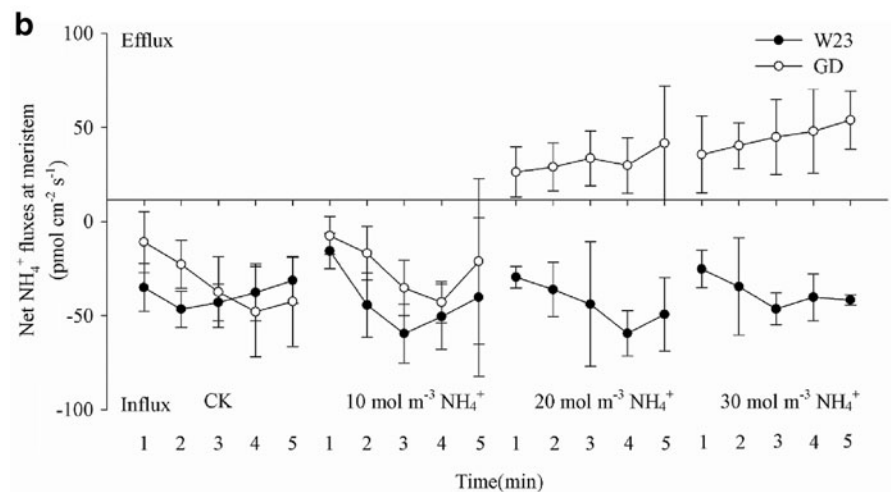
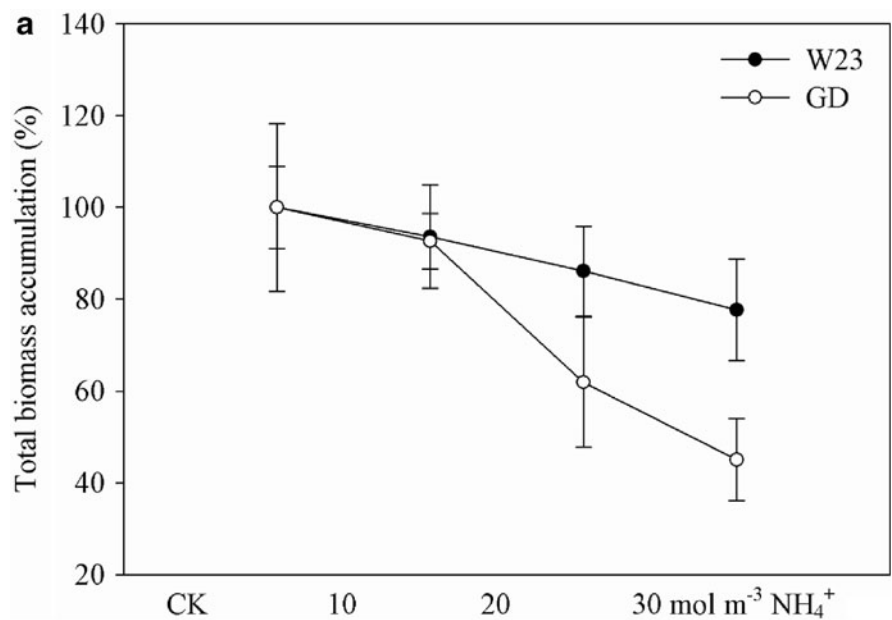
In our study, high NH₄⁺ also suppressed N accumulation in rice, reflecting the trend in biomass (Fig. 2a, b). As per De Macale and Velk (2004), pertinent to the seedling (vegetative) stage, physiological N-use efficiency is

defined as biomass accumulation relative to N accumulation (Shi et al. 2010). In our work, a significantly larger decrease in N accumulation was observed in GD when supplied with high NH₄⁺ relative to W23, which indicated a significantly more reduced N-uptake efficiency in the low-NUE GD when supplied with high NH₄⁺ in the seedling stage (Fig. 2a, b). However, high NH₄⁺ had little effect on physiological N-use efficiency in both W23 and GD, as no significant differences in N content (the ratio of N accumulation to biomass) were found (Fig. 2c, d). Therefore, N-uptake efficiency, rather than physiological N-use efficiency, declined in GD under high NH₄⁺ condition compared to W23 in the seedling stage.

Relationship between free NH₄⁺ content in roots and NUE under high NH₄⁺

Ammonium influx by rice roots has been shown to exhibit a biphasic dependence on external NH₄⁺ concentration (Wang et al. 1993a, b). At low NH₄⁺ concentrations, NH₄⁺ influx is mediated by a saturable HATS (high-affinity transport system), and at higher, including toxic, concentrations of NH₄⁺, it is mediated by a LATS (low-affinity transport system) (Wang et al. 1993b; Kronzucker et al. 2000; von Wiren et al. 2000; Britto and Kronzucker 2006). Moreover, the activity of LATS is apparently not downregulated, indeed might even be upregulated, and could cause higher fluxes with increased nitrogen status of the plant (Wang et al. 1993b; Rawat et al. 1999; Cerezo et al. 2001), and consequent NH₄⁺ hyperaccumulation in tissues, considered a hallmark symptom of NH₄⁺

Fig. 6 Percentage inhibition of total biomass accumulation (**a**) as affected by a series of high NH_4^+ concentrations, and net plasma-membrane NH_4^+ fluxes in W23 and GD in the meristematic zone (**b**) and elongation zone (**c**) of roots, supplied with increasing NH_4^+ concentration (2.86 (CK), 10, 20, 30 mol m^{-3} $\text{NH}_4^+\text{-N}$). In (**a**), growth on CK was considered as 100 %. In (**a**), 100 % corresponds to 0.57 ± 0.05 g plant^{-1} for W23 and 1.55 ± 0.28 g plant^{-1} for GD. Bars show standard deviations



toxicity (Britto et al. 2001; Szczerba et al. 2008b; Balkos et al. 2010; Li et al. 2012). In our study, high NH_4^+ stimulated free NH_4^+ accumulation in both shoot and root of W23 and GD (Fig. 3a, b). However, a dramatically larger free NH_4^+ content was detected in the root tissue of GD under high NH_4^+ compared with W23, an increase, compared to normal-N control, of 83 % in W23, and of 255 % in GD (Fig. 3b). Britto et al. (2001) suggested rice could maintain lower symplastic concentrations of NH_4^+ than barley (known to be susceptible to NH_4^+ toxicity) under elevated NH_4^+ , in part because it is capable of shifting the trans-plasmamembrane electrical potential ($\Delta\psi$) to more positive values with increasing NH_4^+ , a response not seen in barley, which, like *Arabidopsis* (Hirsch et al. 1998), fails to down-regulate $\Delta\psi$, which has the important biophysical consequence of lowering NH_4^+ influx through root cation channels and the ceiling for NH_4^+ accumulation in the rice cytosol. In addition, Wang et al. (1994) furthermore found the decline of membrane polarization with increasing NH_4^+ in rice was not followed by a restoration of that polarization in the steady state, possibly reflecting rice's relatively higher NH_4^+ tolerance. Although this was not measured here and will be the focus of a future study, the relatively lower free root NH_4^+ content detected in W23 compared with GD (Fig. 3b) may well be related to a stronger capacity of adjusting $\Delta\psi$ across the plasma membrane in W23.

Relationship between NH_4^+ efflux in the elongation zone and root respiration

In the toxic range of NH_4^+ supply, NH_4^+ acquisition is mediated by a high-capacity, energetically passive, low-affinity transport system (LATS), and low-affinity NH_4^+ influx is accompanied by a nearly equal magnitude of NH_4^+ efflux, constituting a futile cycle of NH_4^+ ion across the plasma membrane (Britto and Kronzucker 2001, 2002, 2006). The substantial efflux of NH_4^+ under high NH_4^+ conditions has been demonstrated to be associated with high respiratory activity, in particular in NH_4^+ -sensitive species (Britto et al. 2001; Britto and Kronzucker 2001; Kronzucker et al. 2001; Britto and Kronzucker 2006). In our study, a significantly larger NH_4^+ efflux was observed in the elongation zone in GD under high NH_4^+ supply compared with W23 (Fig. 4c, d, f). Furthermore, markedly stronger O_2 consumption was detected in GD than in W23 (Fig. 5), and

this was in turn associated with suppressed growth, supporting the predictions from earlier models. Li et al. (2010) also observed significant NH_4^+ efflux in the elongation zone of *Arabidopsis*, which was coincident with the inhibition of root elongation. It is clear that significantly different thresholds exist within rice in response to NH_4^+ in terms of NH_4^+ entry, the degree of futile cycling, the increase in root respiration, and the suppression of growth, and these thresholds relate to NUE. Whether NUE and NH_4^+ toxicity relate to each other in this manner more generally will be examined in the future using large accessions of rice germplasms.

Conclusion

It is concluded that two rice cultivars with different NUE experience differential tolerance to high NH_4^+ in the seedling stage. In our study, growth development of relatively low-NUE GD was suppressed by high NH_4^+ to a larger extent than the higher-NUE W23 in the seedling stage, especially so for root growth. Total root length and root area were particularly affected. Furthermore, dramatically higher free NH_4^+ was detected in roots of GD than W23, which was related to relatively poor regulation of NH_4^+ influx at the plasma membrane under high NH_4^+ . Larger NH_4^+ efflux in the elongation zone of the root was observed in GD under high NH_4^+ . This was accompanied by significantly higher root respiration in comparison with W23. In conclusion, the relatively high-NUE W23 showed superior tolerance to high NH_4^+ compared with the relatively low-NUE GD in the seedling stage, and this was at least partially related to enhanced respiration and futile transmembrane cycling of the NH_4^+ ion in the sensitive genotype.

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