High water uptake ability was associated with root aerenchyma formation in rice: evidence from local ammonium supply under osmotic stress conditions

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Abstract

Root water uptake is strongly influenced by the morphology and anatomical structure of roots, which are regulated by nitrogen forms and environmental stimuli. To further illustrate the roles of different nitrogen forms on root water uptake under osmotic stress, a split-root system was supplied with different nitrogen forms and osmotic stress simulated by adding 10% (w/v) polyethylene glycol (PEG, 6000). The local effects of nitrogen form and osmotic stress on root morphology, anatomical structure, root lignin content, and water uptake rate were investigated. Under osmotic stress conditions, ammonium markedly promoted the formation and elongation of the lateral root, whereas a significant decrease in numbers of lateral roots was observed under local nitrate supply. Under nitrate supply in split-root systems, osmotic stress significantly promoted root cell death and more aerenchyma formation, as well as accelerated the lignification of the root. However, osmotic stress had no negative effect on the root anatomical structure under ammonium supply. The root water uptake rate was significantly higher in split-root supplied with ammonium than nitrate under osmotic stress conditions. In conclusion, the high water uptake ability in local ammonium supply was associated with the more lateral roots development and the lower cell death, aerenchyma formation and lignification under osmotic stress.

Key words: Nitrogen forms; Aerenchyma; Osmotic stress; Split-root systems; Water uptake

Abbreviations

PEG: polyethylene glycol (molecular weight 6,000 Da; PPFD: photosynthetic photon flux density; PBS: phosphate-buffered saline; RFW: root fresh weight; Pn: photosynthetic rate; g_s: stomatal conductance; C_i: intercellular CO_2 concentration

1. Introduction

Roots play a vital role in delivering water from the growing environment to shoot to keep normal metabolism in higher plants (Steudle and Peterson 1998). The ability of root water uptake is determined by its morphological, anatomical, biochemical and molecular characteristics, which are the results of interaction of genotype and its growing environment (Schiefelbein and Benfey 1991; Henry et al. 2012). Morphological, anatomical, biochemical and molecular characteristics of root have a strong plasticity (Robinson 1994), which is easy to be influenced by growing environment, such as nutrient deficiency (Linkohr et al. 2002; Gahoonia and Nielsen 2004; Insalud et al. 2006; Maniou et al. 2014; Abiko and Obara 2014), waterlogging (Kadempir et al. 2014; Marashi and Mojaddam 2014), drought (Cruz et al. 1992; Henry et al. 2012; Chimungu et al. 2015) and nitrogen forms (Gao et al. 2010; Yang et al. 2012; Ding et al. 2015).

Root hydraulic conductivity is an important parameter reflecting root water absorption capacity. The flux of water through the soil-plant-air continuum is driven by the differences in water potential and affected by hydraulic conductivity of roots, xylem and shoot (Frensch 1997; Knipfer and Fricke 2011). Water flux from root surface to shoot is composed of two steps; the first step is radial transport that water from the root surface to the root xylem; the second step is axial transport that water is delivered from root to shoot via xylem (Frensch 1997). Hydraulic resistances occur at the root and shoot limit the water transport through the plant (Landsberg and Fowkes 1978; Frensch 1997). The pressure of osmotic and hydrostatic are prevailing driving forces in xylem and shoot, and the hydraulic resistance in this process is weak, while hydraulic resistance is relatively great in root radial water transport, which is caused by complex, composite anatomical structure of roots (epidermis, exodermis, cortex, endodermis and stele) (Frensch 1997; Rieger and Litvin 1999; Miyamoto et al. 2001; Knipfer and Fricke 2011). In addition to Casparian band, the number of passage cells in the endodermis, the suberization of the endodermis or exodermis (Melchior and

Steudle 1993; Steudle 2000), root diameter, root cortical width and aerenchyma may also cause variability in root hydraulic conductivity (Melchior and Steudle 1993; Rieger and Litvin 1999; Yang et al. 2012).

Aerenchyma, the parenchyma tissue comprising a high proportion of intracellular space (Evans 2004), takes a central role in transporting oxygen from the aerial environment to roots (Videmšek et al. 2006). There are two types of root aerenchyma presenting in wetland plants, such as rice, including constitutive and inducible aerenchyma (Arikado et al., 1990). Constitutive aerenchyma, forming in aeration condition, is beneficial for rice growth (Jackson et al., 1985). However, the aerenchyma formed in upland crops (e.g. maize and wheat) is inducible, and it is beneficial for plants to acclimate abiotic stresses, including waterlogging (Abiko et al. 2012; Marashi and Mojaddam 2014), nutrient deprivation (Siyiannis et al. 2012; Maniou et al. 2014).

In whole root systems, we previously demonstrated that ammonium could increase the resistance of rice seedlings to osmotic stress as compared to nitrate, which closely related to the higher aquaporin activity, more lateral roots formation and lower aerenchyma formation in rice seedlings supplied with ammonium than those of rice seedlings supplied with nitrate under osmotic stress conditions, leading to a higher root water absorption ability, and enhanced the tolerance to osmotic stress (Li et al. 2009; Gao et al. 2010; Yang et al. 2012; Ding et al. 2015; Gao et al. 2017). In rice seedlings fed with nitrate, osmotic stress significantly promoted ethylene production and the formation of root cortical aerenchyma (Gao et al. 2017), and inhibiting root elongation and lateral root formation (Ding et al. 2015), resulting in the suppression of water uptake and rice growth. At the same time, in split-root systems, we also proved that local osmotic stress decreased photosynthetic characteristics and rice growth when rice seedlings supplied with nitrate (Chen et al., 2006, in Chinese). However, it still remains unclear that the effect of different nitrogen forms and osmotic stress on rice water uptake, root morphology and structure in the split-root

systems. Therefore, the four treatments (detailed description in the section of plant material and growth conditions) were designed in present study. Here with a split-root system, we investigated the effect of local nitrogen forms and osmotic stress on the rice plant growth, photosynthetic rate, root morphology and anatomical structure, and we also determined the root lignin content, water uptake. We analyzed the physiological mechanism of osmotic stress inhibited water uptake in rice seedlings when half of the roots were supplied with nitrate. Furthermore, the response of root morphology and anatomical structure to different nitrogen forms and osmotic stress was preliminarily explored.

2. Materials and methods

2.1. Plant material and growth conditions

Rice seeds (*Oryza sativa* L., cv. 'Shanyou 63' hybrid *indica* China) were disinfected in 10% H_2O_2 (w/w) for 30 mins and then germinated in 2.0 mmol L^{-1} $CaSO_4$ solutions. The seedlings were transplanted into 7 L plastic buckets containing a quarter-strength mixture of ammonium (A) and nitrate (N) nutrient solution (A: N = 1:1, for composition, see below), when seedlings had developed an average of two visible leaves. Three days after transplantation, seedlings were supplied with half-strength nutrient solution for a further three days, and then cultured in full-strength nutrient solution for seven days. The uniform seedlings with eight roots, by removing extra roots (including the seminal roots) were selected for the split-root experiment. The seminal root is a root directly developed from the radicle of the seed, and there is only one seminal root under normal growth conditions. Therefore, we removed the seminal root when rice seedlings were transferred into split-root systems. At the same time, eight adventitious roots were remained per plant to ensure that the length of rice root was as consistent as possible when transplanting. Roots of the seedlings were carefully divided into two halves and transferred into split-root systems, supplied with ammonium and nitrate nutrition on either of both sides or each side, respectively. After another seven days, osmotic stress was simulated by adding polyethylene glycol

(PEG, molecular weight 6,000 Da) to a concentration of 10% (w/v) in the nutrient solution (–0.15 MPa) (Michel and Kaufmann 1973). Four treatments were included: A-N, half of the roots were supplied with ammonium and the other half with nitrate; A-AP, both sides of roots were supplied with ammonium and half of the roots under osmotic stress; N-NP, both sides of roots were supplied with nitrate and half of the roots under osmotic stress; AP-NP, half of the roots were supplied with ammonium and the other half with nitrate, both sides under osmotic stress.

Nutrient composition of hydroponic culture solution was as follows: macronutrients (mmol L^{-1}): 2.85 N as $(NH_4)_2SO_4$ or $Ca(NO_3)_2$, 0.32 P as KH_2PO_4, 1.02 K as K_2SO_4 and KH_2PO_4, 1.65 Mg as $MgSO_4$, 0.1 Si as $Na_2SiO_3 \cdot 9H_2O$; micronutrients (μmol L^{-1}): 35.8 Fe as Fe-EDTA, 9.1 Mn as $MnCl_2 \cdot 4H_2O$, 0.52 Mo as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 18.5 B as H_3BO_3, 0.15 Zn as $ZnSO_4 \cdot 7H_2O$, and 0.16 Cu as $CuSO_4 \cdot 5H_2O$. The Ca^{2+} content in ammonium solution was compensated by adding $CaCl_2$. Dicyandiamide, as nitrification inhibitor was added to each nutrient solution to prevent ammonium oxidation (Willey and Tang 2006; Xie et al. 2008). Nutrient solutions were changed every two days, and pH was adjusted to 5.50 ± 0.05 daily with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH. The temperature in the growth chamber was maintained at 30/18°C day/night. The light was supplied with SON-T AGRO 400 W bulbs, maintaining a light intensity at a minimum of 1000 μmol photons m^{-2} s^{-1} (photosynthetically active radiation) at the leaf level for 14 h photoperiods.

2.2. Gas exchange and chlorophyll content measurements

Gas exchange measurements were determined on fully expanded leaves by a portable photosynthesis system (LI-6400, Li-COR, Lincoln, NE, USA). The leaf temperature during the measurements was maintained at 28±1.0°C, with a photosynthetic photon flux density (PPFD) of 1500 μmol photons m^{-2} s^{-1}. The CO_2 concentration in the cuvette was maintained at 400 μmol CO_2 mol^{-1} with a CO_2 mixture, and the relative humidity was maintained at 40%. Data were recorded after equilibration to a steady-state (approximately 15 mins).

Following the gas exchange measurements, the chlorophyll content (SPAD) of the leaves was non-destructively quantified using a portable SPAD-502 chlorophyll meter (Konica Minolta Sensing, Inc., Japan).

2.3. Leaf area measurements

The leaf area was measured by the imprint gravimetric method, assuming that all leaves were homogenous. The leaf area (SLA) of the fully expanded leaf per gram was measured by imprint method, and the total leaf area was calculated as follows:

The total leaf area = SLA × dry weight of total leaf

Where the dry weight of total leaf was oven-dried at 105°C for 30 mins, and then at 70°C until constant weight, and then weighted using a balance with an accuracy of 1/ 10000.

2.4. Cell death and root morphology measurements

Cell death was estimated by trypan blue staining (Duan et al. 2010). The 7-8 cm long newly formed adventitious roots were immersed in trypan blue (Beijing Solarbio Science & Technology Co., Ltd. China) solution (10 mg mL^{-1}) for 15 mins at room temperature. Then, the samples were rinsed with 0.01 M phosphate buffer solution (PBS pH=7.4) until no floating color attached to the root surface. Root samples were observed by light microscopy (LG-PS2, Olympus, Japan). The dark color of roots stained with trypan blue was counted as cell death. Rice roots were immersed in water and gently separated with a toothpick to avoid injury before imaged. Root morphology (root length, root surface area, root volume and root tips) of each root was analyzed by a LA1600 scanner (Canadian Regent Win RHizo2003b).

2.5. Root aerenchyma and porosity measurements

Root pieces (approximately 1-2 mm) were cut at distances of 3-4 cm from the root apex of 7-8 cm long newly formed adventitious roots using a razor blade. Then root pieces were vacuum infiltrated and fixed with 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH=7.0) for 3-5 h. After washing with phosphate buffer, root samples were subjected to dehydrated in a series of ethanol gradient and dried with a

vacuum freeze-dryer (ES-2030; Hitachi, Inc.). Dried root sections were glued onto specimen stubs and gold-coated with a vacuum ion sputter (ES-1010; Hitachi). Finally, root cross sections were observed and photographed by using a scanning electron microscope (3000N; Hitachi).

The determination of root porosity was according to a published method (Kludze et al. 1993; Kim et al. 1999; Wu et al. 2011; Yang et al. 2012). Adventitious roots were cut into 1-1.5 cm long segments using a razor blade and well mixed. Root segments (0.7-0.8 g) were separated from rice roots, and then the fresh weight of the root segments was determined before placing them into a 25-mL Pyrex pycnometer flask. The Pyrex pycnometer flask was filled with degassed water and was weighed before (W_1) and after (W_2) adding root segments. Root segments were then transferred to a scintillation vial, containing degassed water, and vacuum-infiltrated until no air bubbles were detected. The vacuum-infiltrated root segments were returned to the flask filled with degassed water and reweighted (W_3). Water temperature was measured after each weighing to correct the values. Root porosity was calculated as follows (Fan et al. 2003):

$$\text{Porosity } (\%) = (W_3 - W_2) \times 100 / (W_1 + \text{RFW} - W_2)$$

Where RFW represents root fresh weight.

2.6. Root lignin measurement

Root lignin contents were determined according to Vander et al. (1998) and Iiyama and Wallis (1990) with slight modification. Firstly, the cell wall was collected by grinding frozen rice roots in liquid nitrogen and followed by sequential extraction with methanol: water (1:1) twice, methanol: chloroform (2:1), acetone, ethanol and distilled water, centrifuged 10000 g 10 mins for each extraction. Samples were lyophilized for lignin content determination. Cell walls (5-15 mg) were digested with 2.5 ml AcBr (25%, v/v, acetyl bromide in acetic acid) and 0.1 ml 70% $HClO_4$ for lignin content measurement. After incubation at 70°C for 30 mins and immediately ice-cooled, the samples were transferred to a 50 mL volumetric flask containing a

mixture of 10 mL 2M NaOH, 12 mL acetic acid and 0.1 mL hydroxylamine hydrochloride. The solution was adjusted up to 50 mL with acetic acid, shaken up, and analysed at 280 nm after 1-2 h. The lignin content was estimated by a specific absorption coefficient value for graminaceous lignin of 20 g^{-1} L cm^{-1} (Iiyama and Wallis 1990).

2.7. Water uptake rate measurement

Measurement of water uptake rate was adjusted from a published method (Gao et al. 2010; Yang et al. 2012). The water uptake rate was determined by weighing the nutrient solution between 9:30 and 11:30 AM. The rice seedlings were transplanted from split-root systems to a 2L plastic bucket after blotting lightly with bibulous paper to remove excess nutrient solution and then sealed bucket tightly. The plastic bucket was filled with a nutrient solution, which was consistent with that in the split-root systems, and weighed (W_1) before transplantation of rice seedlings. After two hours later, the plastic bucket was weighed (W_2) again after moving rice seedlings away. Subsequently, the root was blotting lightly with bibulous paper to remove the excess nutrient solution, and then, the bibulous paper was weighed before (W_3) and after (W_4) blotting root. Water uptake rate was calculated as follows:

Water uptake rate (g g^{-1}FW h^{-1}) = $\{W_1–W_2–(W_4- W_3)\}$ / (RFW *2).

Where RFW represents root fresh weight.

2.8. Plant biomass

Rice seedlings were harvested and separated into root, stem and leaf fractions. All samples were oven-dried at 105°C for 30 mins, and then at 70°C until constant weight, and then weighted the shoot and root biomass, separately.

2.9. Statistical analyses

All data were analyzed by SigmaPlot 10.0 and the SPSS 18.0 statistical software package. One-way analysis of variance (ANOVA) among different treatments was performed using SPSS 18.0. Significant differences ($P < 0.05$) among treatments are indicated by different letters in the figures and tables.

3. Results

3.1. Effects of nitrogen forms and osmotic stress on shoot growth and leaf photosynthesis

Compared to A-N treatment, rice seedlings in treatment of AP-NP showed a suppression in the shoot biomass, plant height, tiller number, leaf area, photosynthetic rate (Pn), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), but there was no significant difference in the SPAD value and transpiration rate (E) between them (Table 1). Local osmotic stress significantly decreased the plant growth and gas exchange parameters (excluding C_i) when rice seedlings supplied with nitrate nutrition (treatment of N-NP), comparing with supplied with ammonium nutrition (treatment of A-AP) (Table 1).

Table 1

Effects of different nitrogen forms and osmotic stress on shoot growth and leaf photosynthesis of rice seedlings in split-root systems.

Parameters	A-N	A-AP	N-NP	AP-NP
Shoot biomass (g DW plant^{-1})	4.19±0.23 b	4.82±0.17 a	2.35±0.66 d	3.08±0.35 c
Plant height (cm)	70.9±3.3 a	66.0±3.8 b	59.8±2.8 c	64.4±0.4 b
Tiller number	11.2±1.5 b	13.7±1.6 a	5.5±1.0 d	9.2±1.3 c
Leaf area (cm^2)	928±81 a	1034±92 a	444±127 c	633±42 b
SPAD value	41.3±0.7 a	42.0±0.7 a	35.0±1.7 b	41.7±0.4 a
Pn (μmol CO_2 m^{-2} s^{-1})	25.8±0.9 a	22.6±0.7 b	17.6±1.5 c	23.2±0.8 b
g_s (mol H_2O m^{-2} s^{-1})	0.49±0.05 a	0.34±0.02 b	0.27±0.05 c	0.33±0.03 b
C_i (μmol L^{-1})	297±3 a	263±6 b	263±6 b	266±6 b
E (mmol H_2O m^{-2} s^{-1})	6.31±0.32 ab	6.64±0.36 a	5.68±0.67 b	6.04±0.56 ab

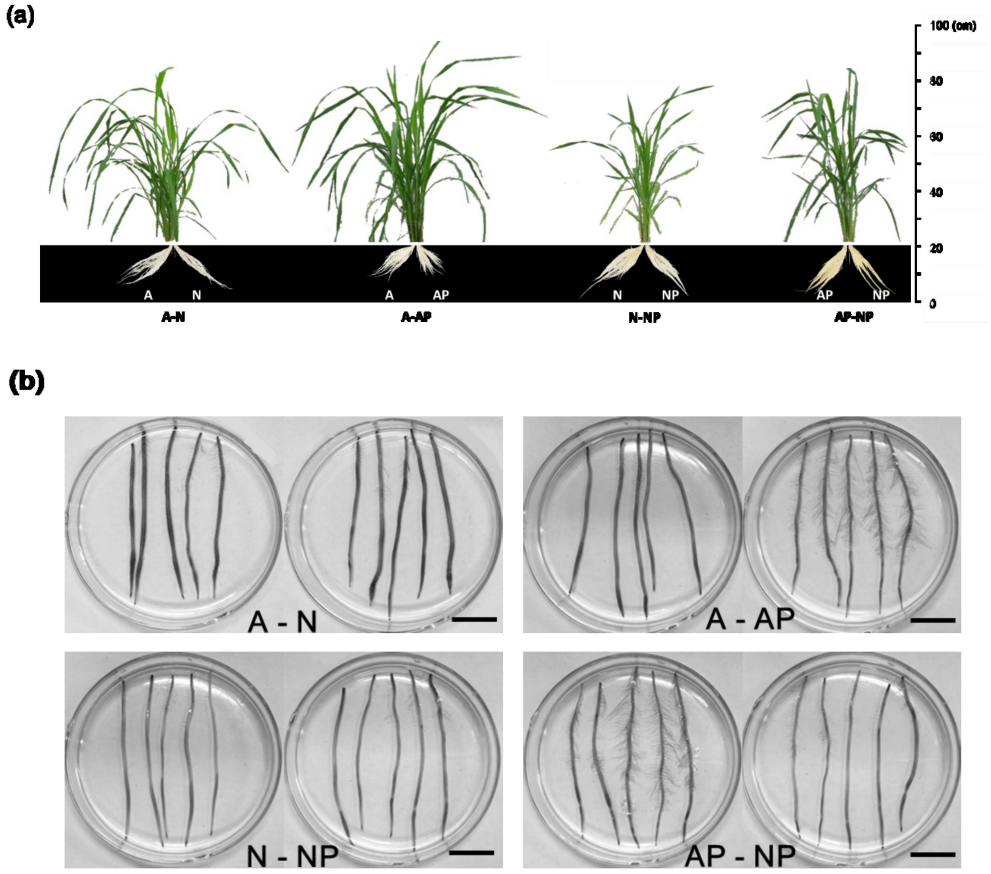
Net photosynthetic rate (Pn), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration rate (E). Rice seedlings were supplied with ammonium (NH_4^+, A) or nitrate (NO_3^-, N) in the absence or presence of osmotic stress [NH_4^+ + 10% (w/v) PEG 6000, AP; NO_3^-+ 10% (w/v) PEG 6000, NP]. Values represent the means ± SD, and different letters indicate significant differences among treatments at $P < 0.05$ significance level.

3.2. Effects of nitrogen forms and osmotic stress on root growth and water uptake in split-root systems

There was no significant difference in root biomass and root length between two sides of the A-N and AP-NP treatments (Fig. 1a and Table 2). Osmotic stress significantly restricted root growth compared with that in non-osmotic stress side, when rice seedlings supplied with nitrate alone. However, there was no difference in root biomass either root sides in the presence or absence of osmotic stress, when rice seedlings supplied with ammonium alone. Root surface area and volume of rice seedlings supplied with ammonium were significantly higher than nitrate under normal water conditions (Table 2), whereas more root tips were found in ammonium condition as compared with nitrate under osmotic stress conditions. Under normal water conditions, the root average diameter of rice seedlings supplied with nitrate was bigger than that supplied with ammonium. However, osmotic stress significantly caused root thinner in rice seedlings supplied with nitrate, while had no effect on that of rice seedlings supplied with ammonium.

Under normal water conditions, no significant difference in lateral root formation between rice seedlings supplied with ammonium and nitrate was observed. However, there was more lateral root formation of rice seedlings supplied with ammonium than that supplied with nitrate under osmotic stress conditions (Fig. 1b). Compared to the control side, local osmotic stress significantly promoted the formation of lateral root in rice seedlings supplied with ammonium, while only a few lateral roots were observed in rice seedlings supplied with nitrate.

Osmotic stress significantly inhibited root water uptake rate (except NP side in AP-NP treatment) in rice seedlings supplied with ammonium and nitrate on both sides or each side of split-root systems (Table 2), compared with that in rice seedlings absence of osmotic stress. Compared to the control side, local osmotic stress decreased the water uptake rate by 38% and 68% in rice seedlings supplied with ammonium and nitrate, respectively.



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297Fig. 1. Effects of nitrogen forms and osmotic stress on the growth of rice seedlings in split-root

298systems. (a) Whole plant, (b) lateral root, bars=2cm. Rice seedlings were supplied with

299ammonium (NH_4^+, A) or nitrate (NO_3^-, N) in the absence or presence of osmotic stress [NH_4^+ +

30010% (w/v) PEG 6000, AP; NO_3^-+ 10% (w/v) PEG 6000, NP].

301

302Table 2

303Effects of different nitrogen forms and osmotic stress on root growth and water uptake of rice

304seedlings in the split-root systems.

Parameters	A-N		A-AP		N-NP		AP-NP	
	A	N	A	AP	N	NP	AP	NP
Root biomass (g DW plant^{-1})	0.54±0.02 bcd	0.46±0.08 cde	0.60±0.02 b	0.55±0.06 bcd	0.80±0.07 a	0.37±0.10 e	0.50±0.05 bcd	0.43±0.05 de
Root length (cm)	1055±152 bc	1041±164 bc	991±126 bc	835±101 c	1403±136 a	1435±128 a	1271±218 ab	1044±179 bc
Root surface area (cm^2)	501±27 b	425±33 c	420±43 cd	341±60 e	630±28 a	556±20 b	359±30 de	366±6 cde
Root volume (cm^3)	19.13±1.80 a	13.87±0.15 b	12.5±0.82 bc	11.12±2.53 bc	20.36±2.03 a	18.77±0.74 a	12.13±0.65 bc	10.35±1.44 c
Number of root tips	849±235 bc	984±337 bc	890±62 bc	762±119 bc	1457±269 a	1174±317 ab	1437±161 a	729±46 c

Root average diameter (mm)	1.21±0.05 cd	1.31±0.04 ab	1.22±0.07 cd	1.27±0.03 bc	1.36±0.01 a	1.14±0.02 de	1.23±0.05 bc	1.07±0.08 e
Root water uptake (g g^{-1}FW h^{-1})	1.49±0.40 ab	1.39±0.17 abc	1.58±0.16 a	0.99±0.18 d	1.42±0.06 abc	0.45±0.17 e	1.03±0.20 cd	1.13±0.19 bcd

Rice seedlings were supplied with ammonium (NH_4^+, A) or nitrate (NO_3^-, N) in the absence or presence of osmotic stress [NH_4^+ + 10% (w/v) PEG 6000, AP; NO_3^-+ 10% (w/v) PEG 6000, NP]. Values represent the means ± SD, and different letters indicate significant differences among treatments at $P < 0.05$ significance level.

3.3. Effects of nitrogen forms and osmotic stress on root cell death, aerenchyma formation and lignin content in split-root systems

No significant difference in the root cell death (the dark color of root tips stained with trypan blue) and aerenchyma formation was observed between rice seedlings supplied with ammonium and nitrate under non-osmotic stress conditions (Fig. 2, 3). However, osmotic stress significantly accelerated root cell death and increased aerenchyma formation in rice seedlings, when local root supplied with nitrate but not ammonium nutrition (Fig. 2, 3). Local osmotic stress significantly promoted root cell death and aerenchyma formation in rice seedlings fed with nitrate nutrition, but had no negative effects on root cell death and aerenchyma formation when rice seedlings supplied with ammonium (Fig. 2, 3).

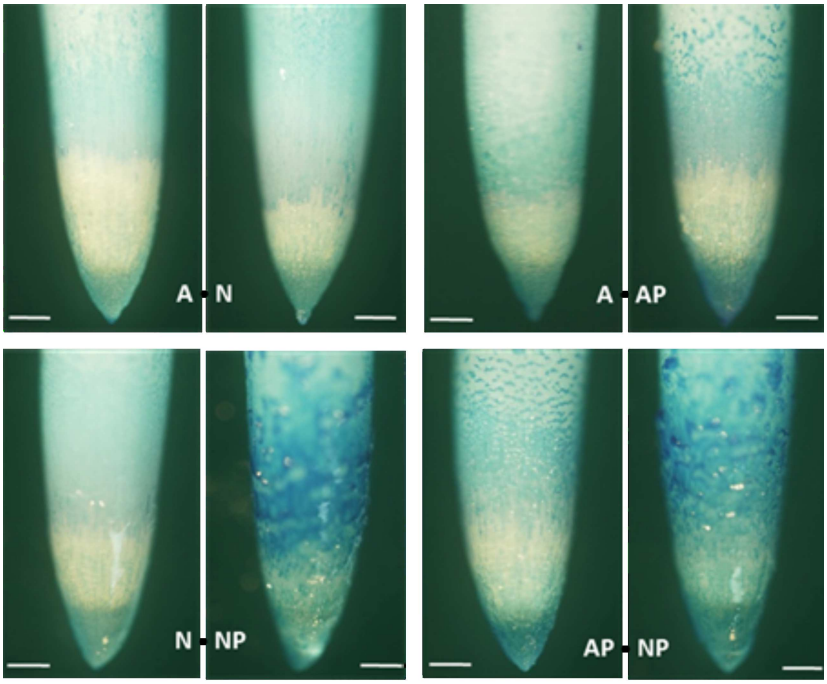

Fig. 2. Effects of nitrogen forms and osmotic stress on root cell death of rice seedlings in split-root systems. Rice seedlings were supplied with ammonium (NH_4^+, A) or nitrate (NO_3^-, N) in the absence or presence of osmotic stress [NH_4^+ + 10% (w/v) PEG 6000, AP; NO_3^-+ 10% (w/v) PEG 6000, NP]. The dark color of root tips region stained with trypan blue was estimated as cell death. Bars = 500 μm.

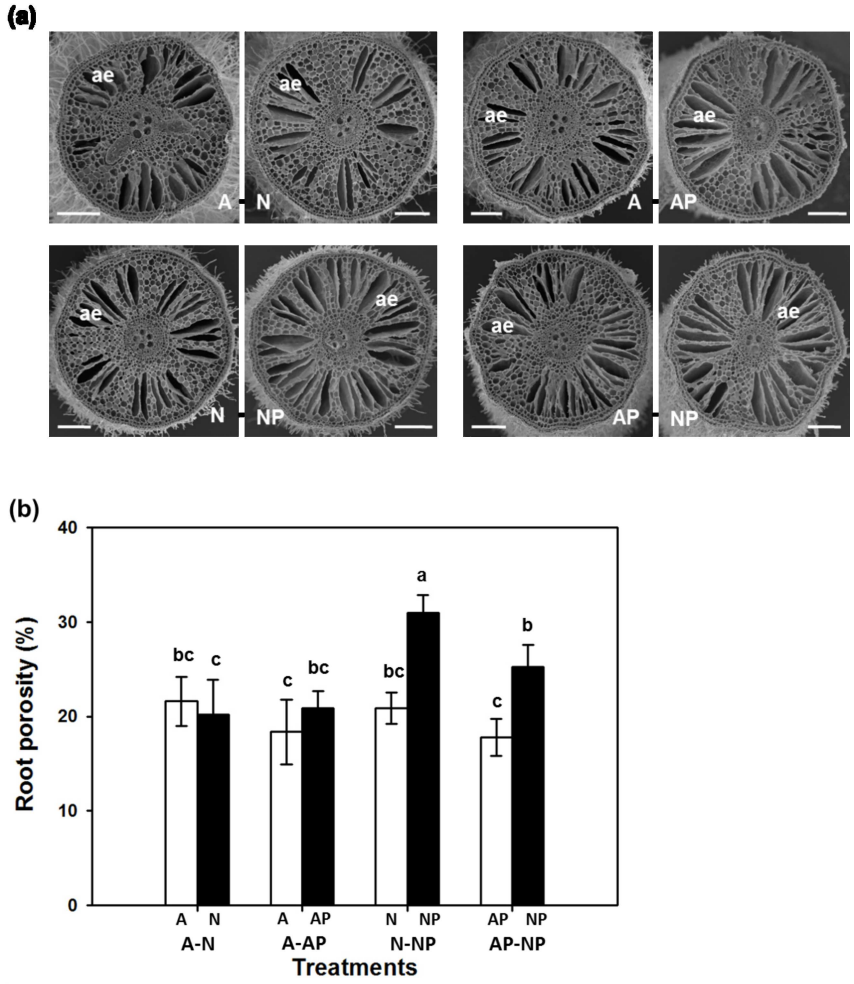

Fig. 3. Effects of nitrogen forms and osmotic stress on root aerenchyma formation of rice seedlings in split-root systems. (a) Scanning electron micrographs of transverse sections were cut at distances of 3-4 cm from root apex of 7–8 cm long newly formed adventitious roots of rice seedlings in split-root systems. ae, aerenchyma. Bars = 200 μm. (b) Root porosity of rice seedlings in split-root systems. Rice seedlings were supplied with ammonium (NH_4^+, A) or nitrate (NO_3^-, N) in the absence or presence of osmotic stress [NH_4^+ + 10% (w/v) PEG 6000, AP; NO_3^-+ 10% (w/v) PEG 6000, NP]. Values represent the means ± SD, and different letters indicate significant differences among treatments at $P < 0.05$ significance level.

Compared to A-N treatment, osmotic stress significantly increased root lignin content in local root supplied with nitrate, whereas did not affect on other local root supplied with ammonium in treatment of AP-NP (Fig. 4). When rice seedlings

supplied with nitrate alone, the root lignin content in local root with osmotic stress was higher than that under normal water conditions. However, no significant difference in root lignin content was observed in rice seedlings supplied with ammonium in the presence or absence of osmotic stress (Fig. 4).

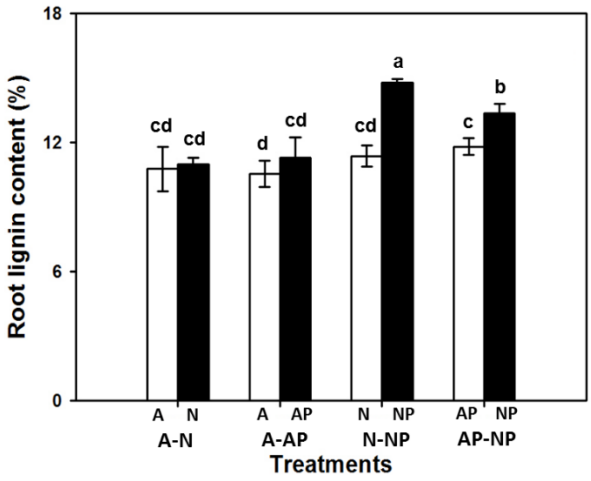

Fig. 4. Effects of nitrogen forms and osmotic stress on root lignin of rice seedlings in split-root systems. Rice seedlings were supplied with ammonium (NH_4^+, A) or nitrate (NO_3^-, N) in the absence or presence of osmotic stress [NH_4^+ + 10% (w/v) PEG 6000, AP; NO_3^-+ 10% (w/v) PEG 6000, NP]. Values represent the means ± SD, and different letters indicate significant differences among treatments at $P < 0.05$ significance level.

A significantly negative correlation was found between root porosity and root water uptake in local root supplied with nitrate in the absence or presence of osmotic stress across all the treatments. However, there was no correlation between them in the local root supplied with ammonium in the absence or presence of osmotic stress (Fig. 5).

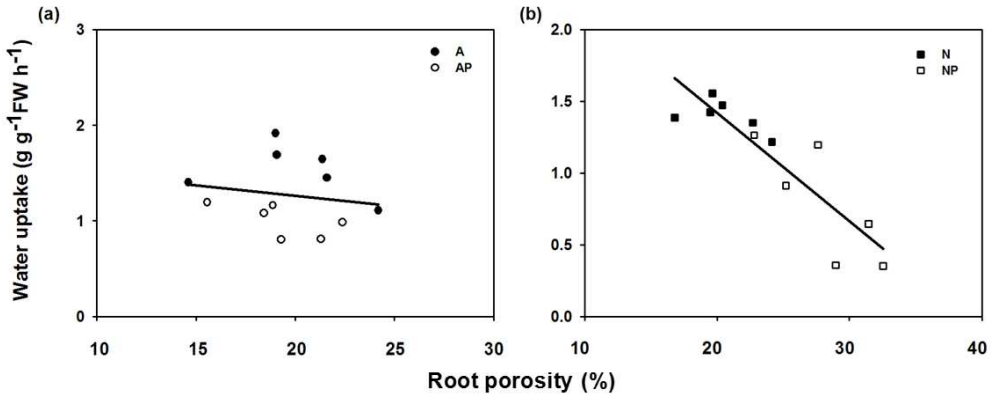

Fig. 5. Relationships between root porosity and root water uptake under ammonium (a) and nitrate (b) treatments in the split-root systems. Lines represent linear regressions, and the regression equations are (a) y = -0.022 x + 1.700, R = 0.169, $P > 0.05$; (b) y = -0.076 x + 2.935, R = 0.884, $P < 0.01$.

4. Discussion

Our previous studies have demonstrated that osmotic stress apparently inhibited the rice biomass, photosynthetic rate and water uptake rate in rice seedlings supplied with nitrate, while had no effects on rice seedlings supplied with ammonium. Furthermore, we have proved that the higher aquaporin activity, more lateral roots formation and lower aerenchyma formation in rice seedlings supplied with ammonium could lead to a higher root water absorption ability, and enhance the tolerance to osmotic stress, comparing with rice seedlings supplied with nitrate (Li et al. 2009; Gao et al. 2010; Yang et al. 2012; Ding et al. 2015; Gao et al. 2017). The previous studies were based on the whole root systems; however, the effects of different nitrogen forms and osmotic stress on rice seedling growth, root morphology and structure in split-root systems are still poorly understood. Similar results were found in the split-root systems and whole-root systems, osmotic stress significantly decreased the rice biomass, photosynthetic rate and water uptake rate in rice seedlings when the local root was supplied with nitrate, while does not affect that of rice seedlings in local root were was supplied with ammonium(Table 1, 2). In present split-root systems, we preliminarily explored the response mechanism of root

morphology and anatomical structure to different nitrogen forms and osmotic stress.

Root morphology and anatomical structure are closely related to the ability of root water uptake (Schiefelbein and Benfey 1991; Henry et al. 2012). The lateral root is an essential component of the root, which plays an important role in the absorption of water and nutrient. The development of lateral roots is strongly influenced by the local environments, i.e., drought, nitrogen forms and nutrients (nitrogen, phosphorus or potassium) availability (Sattelmacher and Thoms 1995; Johnson et al. 1996; Zhang et al. 2009; Lima et al. 2010; Ding et al. 2015). It was demonstrated that locally nitrate supply stimulated lateral root production and elongation (Granato et al. 1989; Zhang et al. 1999), whereas, high nitrate concentration had a strong inhibit on lateral root development (Zhang et al. 1999). On the other hand, Lima et al. (2010) reported that ammonium stimulated lateral root via a higher number of first-and higher-order lateral roots. In our studies, local osmotic stress significantly promotes the lateral root formation and elongation in rice seedlings supplied with ammonium (AP sides of A-AP and AP-NP treatments), but only a little lateral root formation, when rice seedlings supplied with nitrate (NP sides of N-NP and AP-NP treatments) (Fig. 1b, Table 2). Osmotic stress significantly promoted ethylene production in rice seedlings fed with nitrate, comparing with ammonium (Ding et al. 2015; Gao et al. 2017). It has been demonstrated ethylene accumulation positively controls auxin biosynthesis, and thereby inhibiting root elongation (Xu et al. 2013; Ding et al. 2015) and lateral root formation (Negi et al., 2008). Therefore, under osmotic stress conditions, lateral root formation and elongation of rice seedlings supplied with ammonium were beneficial to maintain a higher ability of water uptake (Wasson et al. 2012), thus increased drought resistance. According to ways of plant response to drought stress, drought resistance mechanisms have been identified three adaptation types: drought escape, drought avoidance and drought resistance (Price et al., 2002). In N-NP plant, the biomass of N root side was significantly greater than that of NP root side, and the root average diameter of NP root side was thinner than that of N root side. We speculated

that the response of rice supplied with nitrate to osmotic stress might be a drought avoidance mechanism. When rice seedlings supplied with nitrate, osmotic stress promoted the transportation of photosynthetic products to the half root in the absence of osmotic stress, stimulating the root growth, and then improving the absorption of water. The different response of root morphology and anatomical structure to nitrogen forms and osmotic stress might be due to the distinct resistance mechanisms of ammonium and nitrate.

The development and growth of lateral roots lead to an increase in absorbing surface area, promoting water uptake, while root anatomical structure, such as Casparian bands, the number of passage cells in the endodermis, lignin deposition in sclerenchyma and stellar cells, suberization of the endodermis or exodermis and root cortical width, are closely related to root hydraulic conductivity, (Melchior and Steudle 1993; Rieger and Litvin 1999; Steudle 2000; Schreiber 2010; Ranathunge and Schreiber 2011),which plays a crucial role in the ability of water uptake (Mu et al. 2006). Yang et al. (2012) explained that the reduction of water uptake rate caused by nitrate in osmotic stress rice seedlings was due to the larger aerenchyma formation in the root cortex, which could decrease root hydraulic conductivity and further impeded the water radial transports in the cylinder. In split-root systems, we also found that osmotic stress increased aerenchyma formation and root porosity supplied with local nitrate, but no promoting effect with ammonium was observed in the treatment of AP-NP. Moreover, local osmotic stress significantly promoted the aerenchyma formation and increased root porosity in root of N-NP treatment, but not in the treatment of A-AP (Fig. 3). Furthermore, when rice seedlings supplied with nitrate, significant negative correlation was observed between root porosity and water uptake rate, whereas, this negative correlation was not found in ammonium (Fig. 5). Therefore, the excess aerenchyma formed in roots of rice seedlings supplied with local nitrate under osmotic stress conditions markedly restrict water uptake and reduce the drought resistance, resulting in lower biomass accumulation, comparing with rice seedlings

locally supplied with ammonium. However, aerenchyma formation was beneficial for upland plants adapting to the suboptimal growing environment (Fan et al., 2003). For example, root cortical aerenchyma formation in maize enhanced drought tolerance by reducing root respiration, resulting to increase greater root length in deeper soil layers for absorbing water from drying soil (Zhu et al. 2010){Zhu, 2010 #2396}{Zhu, 2010 #2396}. The other studies showed that the aerenchyma formation in roots of wheat enhanced the ability to waterlogging tolerance (Marashil & Mojaddam, 2014; Xu et al., 2013), which transports the oxygen from aerobic shoots to anaerobic roots (Armstrong, 1971). Therefore, we hypothesize that root cortical aerenchyma formation is a double-edged sword. The exceeded aerenchyma formation in root of rice seedlings supplied with local nitrate caused by osmotic stress causes disadvantageous effect on rice growth, but root cortical aerenchyma formation enhance the drought tolerance for maize, which was beneficial to maize growth, and enhanced the ability to waterlogging tolerance for wheat, which transports the oxygen from aerobic shoots to anaerobic roots. The adaptation to the local environment and differences in species, rice is a wetland plant, but maize and wheat are upland plants, resulting in double effects of root cortical aerenchyma formation on adversity tolerance ability.

The aerenchyma formation is considered as a specific status of programmed cell death (Jackson and Armstrong 1999; Evans 2004; Joshi and Kumar 2012). Under normal water condition, there were no visible cell death, which was assessed by trypan blue stained, were observed in both supplied with local ammonium and nitrate; while the cell death in roots of nitrate-supplied rice seedlings significantly increased, comparing with ammonium-supplied rice seedlings under osmotic stress conditions (Fig. 2). At the same time, we also found that local osmotic stress significantly promotes the cell death in root of NP in N-NP treatment, but not in AP of A-AP treatment (Fig. 2). As a result, the effects of different nitrogen forms and osmotic stress on root cell death were consistent with those of aerenchyma formation in whole

root (Gao et al. 2017) and split-root systems (Fig. 2, Fig. 3), indicating that osmotic stress induces root cell death and cause more aerenchyma formation in the rice seedlings supplied with nitrate. Meanwhile, in whole root systems, we have demonstrated that water stress increased additional aerenchyma formation in rice seedlings fed with nitrate rather than ammonium (Yang et al. 2012; Gao at al. 2017). Furthermore, compared to control treatment, water stress increased ethylene production in water-stressed roots under nitrate nutrition, in association with faster ethylene synthesis and regulated by the related genes (*ACS*, *L.S.D.*, *EDS* and *PAD*), promoting the formation of root cortical aerenchyma. However, no significant differences in these factors, causing additional aerenchyma formation, when rice seedlings fed with ammonium without water stress or subjected to water stress conditions (Gao at al. 2017).

Besides, the deposition of lignin in outer cortical layers is considered to be necessary for aerenchyma formation (Purnobasuki and Suzuki 2004). Extensive amounts of aerenchyma were observed in the root cortex of maize (Abiko et al. 2012) and wheat (Xu et al. 2013), which was associated with a heavy lignin deposition in the outer cortical layers of root. In present studies, compared to A-N treatment, osmotic stress markedly increases the lignin content in the root of rice seedlings supplied with local nitrate, whereas it does not affect that of rice seedlings supplied with local ammonium (Fig. 4). Meanwhile, compared to normal water conditions, local osmotic stress promotes the lignin content in the root of NP in N-NP treatment, but not in AP of A-AP treatment (Fig. 4). More aerenchyma was formed in the root, which is unanimous with higher lignification of the root in rice seedlings local fed with nitrate (Fig. 3, Fig. 4). Osmotic stress significantly accelerated the lignification of root relative to plants grown in well-watered conditions (Cruz et al. 1992; Lee et al. 2007). Furthermore, in the whole root systems, osmotic stress significantly increased aerenchyma formation in root of rice seedlings supplied with nitrate (Yang et al. 2012; Gao et al. 2017), which were associated with deeper color of cross-section peripheral

and middle column of root stained by Safranine (Fig. S1), which could estimate root lignification (Duan et al. 2010), and higher lignin content (Fig. S2). Therefore, we conferred that lignin is deposited in root cortical cells and related to aerenchyma formation.

5. Conclusion

Under osmotic stress conditions, more lateral root development and the lower cell death, aerenchyma formation and lignification in local ammonium supply resulted in a higher water uptake rate. This implicates that root development depends on local nitrogen supply, which contributes to the resistance of rice to osmotic stress.

Authors' contributions

CM Gao conceived, designed and performed the experiments, and was a major contributor in writing the manuscript. M Wang analyzed and interpreted the data, and was another major contributor in writing the manuscript. L Ding, YP Chen and ZF Lu performed the experiments. J Hu, SW Guo conceived and designed the experiments, and revised the manuscript. All authors read and approved the final manuscript.

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Declaration of interest

The authors declare that they have no competing interests.

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