Root aeration improves growth and nitrogen accumulation in rice seedlings under low nitrogen

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Abstract. In wetland soils, changes in oxygen (O2) level in the rhizosphere are believed to influence the behaviour of nutrients and their usage by plants. However, the effect of aeration on nitrogen (N) acquisition under different N supply conditions remains largely unknown. In this study, the rice cultivars Yangdao 6 (YD6, with higher root aerenchyma abundance) and Nongken 57 (NK57, with lower root aerenchyma abundance) were used to evaluate the effects of aeration on rice growth and N accumulation. Our results showed that the number of adventitious roots and the root surface area increased significantly, and ethylene production and aerenchyma formation decreased in both cultivars after external aeration (EA). Five N treatments, including no N (−N), 0.125 mM NH4NO3 (LN), 1.25 mM Ca(NO3)2 (NO3-N), 1.25 mM (NH4)2SO4 (NH4-N) and 1.25 mM NH4NO3 (N/N), were applied to YD6 and NK57 for 2 days under internal aeration or EA conditions. External aeration increased the root biomass in both cultivars and the shoot biomass in NK57 by 18–50 %. The total N concentrations in roots of YD6 grown under −N and LN and of NK57 grown under NO3-N were increased by EA. Expression of OsPAD4, one of four putative genes regulating aerenchyma formation, showed a similar pattern alongside changes in the ethylene level in the EA-treated rice irrespective of the N treatments. Furthermore, expression of the high-affinity nitrate transporter gene OsNRT2.1 was increased by EA under −N, LN and NO3-N conditions. Our data provide evidence of an interaction between O2 and the supply of N in ethylene production, aerenchyma formation and N nutrition through modification of the expression of OsPAD4 and OsNRT2.1.

Keywords: Aerenchyma formation; ethylene; gene expression; nitrogen; Oryza sativa; oxygen.

Introduction

Wetland plants commonly grow under alternating drought and flood conditions (Alberda 1953; Colmer 2003a; Qian et al. 2004; Nishiuchi et al. 2012). They can develop aerenchyma—gas-filled interconnected spaces or lacunae in the roots, stems and leaves—to adapt to low dissolved oxygen (O2) levels during waterlogging (Arenovski and Howes 1992; Colmer et al. 1998; Armstrong and Armstrong 2005; Steffens et al. 2011). Oxygen, ethylene and methane can be transported from the shoots to the roots via the aerenchyma and released into the rhizosphere (Armstrong 1979; Jackson and Armstrong 1999; Colmer 2003b; Teal and Kanwisher 1966; Yu et al. 1997). This leads to variable dissolved O2 levels at wetland plant root surfaces in paddy fields. Oxygen directly or indirectly affects nitrification dominated by nitrifying bacteria, or chemical oxidation...
for the conversion of NH$_4^+$ to NO$_3^-$ at the root surface, as well as the form of nitrogen (N) taken up by wetland plants (Kludze et al. 1993; Li et al. 2008). Oxygen levels and rate of nitrification in the paddy rhizosphere can also affect total N usage by wetland plants (Li et al. 2008; Xu et al. 2012a).

Aerenchyma form in the parenchyma as a result of programmed cell death (PCD) and extend from below the ground up through the stems and leaves (Drew et al. 2000). Formation of aerenchyma is affected by external ethylene but to different extents among different cultivars (Justin and Armstrong 1991; Kong et al. 2009). In plant roots, external ethylene can inhibit root elongation (Visser and Pierik 2007) and aerenchyma formation (Takahashi et al. 2015). In Arabidopsis, the formation of lysigenous aerenchyma as a result of PCD is dependent on the plant defence regulators AtLSD1, AtEDS1 and AtPAD4 (Mühlenbock et al. 2007). These genes influence PCD by operating upstream of ethylene production and reactive oxygen species in the roots (Mühlenbock et al. 2007). Expression of PAD4 was shown to be strongly associated with ethylene concentrations in Arabidopsis (Durrant and Dong 2004; Lei et al. 2014) and rice (Qiu et al. 2007; Ding et al. 2008). In rice, a gene related to disease resistance, OsBphi008a, was located downstream of the ethylene signalling pathway and was a positive indicator of ethylene levels (Yuan et al. 2004; Hu et al. 2011), and overexpression of OsPDCD5 induced PCD in transgenic lines (Su et al. 2006). In addition, OsLSD1.1, OsLSD2, OsPAD4 and OsEDS in rice, which are homologous to the Arabidopsis AtLSD1, AtPAD4 and AtEDS1 genes, respectively, may also play similar roles during aerenchyma formation via PCD in the roots.

Although ammonium (NH$_4^+$) is the primary form of available N in flooded fields, and rice prefers NH$_4^+$ over NO$_3^-$ (Wong et al. 1993), physiological studies have shown that lowland rice is exceptionally efficient at acquiring NO$_3^-$ through nitrification in the rhizosphere (Li et al. 2006; Duan et al. 2007). A mixed supply of NH$_4^+$ and NO$_3^-$ to both upland and lowland rice cultivars resulted in significant increases in the dry weight and grain yield compared with application of either NH$_4^+$ or NO$_3^-$ as the sole N source (Qian et al. 2004; Duan et al. 2007). In rice, a number of ammonium transporter genes (Sonoda et al. 2003; Zhao et al. 2014) and nitrate transporter genes (Feng et al. 2011; Xu et al. 2012b) have been functionally characterized. The ammonium transporter genes OsAMT1.1, OsAMT1.2 and OsAMT1.3 exhibited ammonium transport activity in yeast (Sonoda et al. 2003) and OsAMT1.1 contributed more than did the other two genes to N accumulation (Zhao et al. 2014). Knockdown of OsNAR2.1, which encodes a partner protein of the NRT2 transporter, decreased the total N accumulation by $\sim$63–66 % (Yan et al. 2011). OsNRT2.3a plays a key role in long-distance NO$_3^-$ transport from the roots to shoots in rice under low NO$_3^-$ conditions (Tang et al. 2012). Physiological studies on the relationship between N and the aerenchyma in rice have shown that the aerenchyma can influence N absorption and utilization (Yoshida and Ancajas 1973; Gebauer et al. 1996; Li et al. 2008). In addition, the different forms of N could also affect aerenchyma formation. The NO$_3^-$ treatment yielded a significantly greater increase in root porosity than did NH$_4^+$ treatment (Yang et al. 2012), and N deficiency induced aerenchyma formation in the roots of maize (Drew et al. 1989; He et al. 1994) and rice (He et al. 1992).

Although the physiological effects of N treatments on aerenchyma formation have been investigated in plants, limited information is available on the underlying molecular mechanisms. In previous research, we characterized two rice cultivars with high (Yangdao 6, YD6) and low (Nongken 57, NK57) N use efficiency (NUE), exhibiting different ammonia-oxidizing bacteria and nitrification activities in the root rhizosphere (Li et al. 2008). We reported the effects of root aeration on root aerenchyma formation, N accumulation and the expression of aerenchyma-formation-related and N transporter genes in both cultivars under diverse N conditions.

![Figure 1. The dissolved O$_2$ level in the solutions under IA and EA conditions. Measurements were continuous for 8 days in all experiments. IA, internal aeration (filled circles); EA, external aeration (open circles). The period between the two dashed lines indicates the time of N treatment. Significant differences relative to the dissolved O$_2$ level at 0 h are indicated by asterisks ($P < 0.05$, two-way ANOVA).](image)

| Source          | df | Mean$^2$ | F     | P     |
|-----------------|----|----------|-------|-------|
| Aeration        | 1  | 302.419  | 3121.742 | <0.0001 |
| Duration        | 11 | 0.920    | 9.500 | <0.0001 |
| Aeration × duration | 11 | 5.314    | 54.856 | <0.0001 |
| Error           | 96 |          |       |       |

Table 1. Results from a two-way ANOVA evaluating the influence of aeration and duration on dissolved O$_2$ content.
Figure 2. Rice growth, aerenchyma formation in root and ethylene production under IA and EA conditions. (A and B) Shoot and root phenotypes in seedlings of the YD6 (A) and NK57 (B) rice cultivars germinated and grown hydroponically in water for 5 days with IA or EA treatments. (C and D) Quantification of phenotypes. Numbers of adventitious roots (C) and root surface areas (D) were determined. (E and F) Partial aerenchyma formation visualized in sections of YD6 and NK57 roots. Resin-embedded sections obtained 1.5 cm (E) and 2.5 cm (F) from the root tips of YD6 and NK57 seedlings subjected to IA and EA treatments. (G and H) Quantification of aerenchyma formation in sections obtained 1.5 cm (G) and 2.5 cm (H) from the root tips, using Image J software. (I) Ethylene production in YD6 and NK57. Values represent the means ± SE (error bars) of three replicates. Significant differences are indicated by different letters (P < 0.05, two-way ANOVA).
after internal aeration (IA) and external aeration (EA) treatments. These results provided evidence for the relationship between aerenchyma formation and N nutrition at the gene expression level in rice.

Methods

Aeration treatments

Seeds of the YD6 and NK57 rice cultivars were surface sterilized with 30 % (v : v) NaClO (5.2 % available chlorine) for 30 min and then rinsed thoroughly five or six times with water. The sterilized seeds were distributed uniformly on plastic mesh grates with 1-mm² holes and placed in darkness for 2.5 days. The germinated seeds were divided equally into two groups for IA and EA treatments. Plastic grates containing germinated seeds for each group were placed on plastic buckets 30 cm in height and 25 cm in diameter. For the EA group, air was pumped continuously into the water in the bucket using an air pump. For seedlings in the IA group, air was not pumped into the water. The dissolved O₂ level with IA and EA treatments was detected with a portable dissolved O₂ detector (JPB-607, Leici Company, Shanghai, China) and recorded continuously during the 8 days of all experiments. Both groups of rice plants were placed in a growth chamber with a 16-h/8-h light/dark cycle and 30 °C/26 °C day/night temperature cycle. Relative humidity was maintained at 60 %. After 5 days of IA or EA treatment, the numbers of adventitious roots were counted and total root lengths and root surface areas were determined (scanned and analysed) using the WinRhizoV4.0b image analysis software (Regent Instruments, Canada). Root tips were collected for aerenchyma measurements and total root systems grown under the two aeration treatments were harvested 2 h into the light phase of the daily light cycle for analysis of OsPAD4 (AK243523), OsLSD1.1 (AK111759), OsBphi008a (NM_001048814), OsPDCD5 (AY749430), OsNRT2.1 (AB008519), OsNAR2.1 (AP004023), OsNRT2.3 (AK109776), OsAMT1.1 (AF289477), OsAMT1.2 (AF289479) and OsAMT1.3 (AF289478) expression using quantitative real-time polymerase chain reaction (PCR). The shoots and roots of rice seedlings were separately collected for measuring biomass and total N. The samples were desiccated in a forced-air oven at 70 °C for ~72 h to a constant weight, after which their dry weight was measured. The measurement of total N contents was performed according to Cai et al. (2012).

Determination and quantification of aerenchyma formation

After IA and EA treatments, roots with lengths of 5–8 cm were selected for aerenchyma measurements. Root tips were collected for aerenchyma measurements and total root systems grown under the two aeration treatments were harvested 2 h into the light phase of the daily light cycle for analysis of OsPAD4 (AK243523), OsLSD1.1 (AK111759), OsBphi008a (NM_001048814), OsPDCD5 (AY749430), OsNRT2.1 (AB008519), OsNAR2.1 (AP004023), OsNRT2.3 (AK109776), OsAMT1.1 (AF289477), OsAMT1.2 (AF289479) and OsAMT1.3 (AF289478) expression using quantitative real-time polymerase chain reaction (PCR). The shoots and roots of rice seedlings were separately collected for measuring biomass and total N. The samples were desiccated in a forced-air oven at 70 °C for ~72 h to a constant weight, after which their dry weight was measured. The measurement of total N contents was performed according to Cai et al. (2012).

Nitrogen treatments

Rice seedlings of identical height cultivated under IA and EA conditions for 5 days were selected for N treatments for a further 2 days. Seedlings were divided into five groups for treatment under different N conditions. The seedlings were grown in tanks containing 10 L of nutrient solution. The five N treatments were listed as no N (−N), 0.125 mM NH₄NO₃ (LN), 1.25 mM Ca(NO₃)₂ (NO₃-N), 2.5 mM KNO₃ (KNO₃-N) and 5 mM Ca(NO₃)₂ (Ca(NO₃)₂-N).

Table 2. Results from a two-way ANOVA evaluating the influence of aeration on adventitious root number, root surface area, aerenchyma formation and ethylene production of different rice seedlings.

| Source | df | Mean² | F     | P     |
|--------|----|-------|-------|-------|
| (A) Adventitious root number | | | | |
| Cultivar | 1 | 14.450 | 34.000 | < 0.0001 |
| Aeration | 1 | 26.450 | 62.235 | < 0.0001 |
| Cultivar × aeration | 1 | 1.250 | 2.941 | 0.106 |
| Error | 16 | | | |
| (B) Root surface area | | | | |
| Cultivar | 1 | 0.302 | 0.580 | 0.454 |
| Aeration | 1 | 48.245 | 92.773 | < 0.0001 |
| Cultivar × aeration | 1 | 0.333 | 0.641 | 0.432 |
| Error | 16 | | | |
| (C) Aerenchyma formation (1.5 cm) | | | | |
| Cultivar | 1 | 12.592 | 8.885 | 0.018 |
| Aeration | 1 | 816.049 | 575.829 | < 0.0001 |
| Cultivar × aeration | 1 | 1.682 | 1.187 | 0.308 |
| Error | 8 | | | |
| (D) Aerenchyma formation (2.5 cm) | | | | |
| Cultivar | 1 | 356.430 | 167.600 | < 0.0001 |
| Aeration | 1 | 125.453 | 58.991 | < 0.0001 |
| Cultivar × aeration | 1 | 11.213 | 5.273 | 0.051 |
| Error | 8 | | | |
| (E) Ethylene production | | | | |
| Cultivar | 1 | 0.038 | 27.862 | 0.001 |
| Aeration | 1 | 0.191 | 140.355 | < 0.0001 |
| Cultivar × aeration | 1 | 0.001 | 0.674 | 0.435 |
| Error | 8 | | | |
1.25 mM (NH₄)₂SO₄ (NH₄-N) and 1.25 mM NH₄NO₃ (N/N). To inhibit nitrification, 7 μM dicyandiamide (DCD-C₂H₄N₄) was added to the nutrient solutions. Whole roots were collected after 0.5, 2, 6, 12, 24 and 48 h of growth in the nutrient solutions to analyse gene expression. Shoots and roots of rice seedlings were, respectively, collected 48 h later to measure biomass and total N concentration. Plant samples were desiccated in a forced-air oven at 70 °C for ~72 h to a constant weight, after which their dry weight was measured. The measurement of total N contents was performed according to Cai et al. (2012).

Quantification of ethylene production from rice

The ethylene levels of five seedlings of the two rice varieties were determined after aeration and N treatments. Seedlings were transferred into 10 mL gas chromatography (GC) vials (B7990-6A, National Scientific Company, Rockwood, TN, USA). We then added 4 mL of the same N solution as used for the N treatments into IA group vials or waterman filter paper soaked with same N solutions into EA group vials and collected ethylene released from the seedlings for 12 h. The 6 mL total air in these vials was introduced into 50 mL syringes and subjected to GC analysis (GC7890, Agilent Company, Palo Alto, CA, USA) as described previously (Xu et al. 2008). Three replicates were used for the data pool.

Quantitative real-time PCR

Total RNA was prepared from the roots of YD6 and NK57 seedlings using TRIzol reagent (Invitrogen; http://www.invitrogen.com). For quantitative PCR (qPCR) analysis, total RNAs were treated with DNaseI and reverse transcribed using SuperScript II (Invitrogen) to produce cDNA. Triplicate quantitative assays were performed on each cDNA dilution using SYBR Green Master Mix and an ABI 7000 sequence detection system according to the manufacturer’s protocol (Applied Biosystems, http://www.takara.com). Gene-specific primers were designed using the Primer Express software (Applied Biosystems). The relative quantification method was used to evaluate the quantitative variation between replicates. Amplification of OsRAc1 (Actin) was used as an internal standard to normalize all expression data. All primers used for qPCR are listed in Supporting Information—Table S1.

| Treatment | Dry weight (g) | | | Total N (mg g⁻¹) | | | |
|-----------|----------------|-----------------|-----------------|---------------------|-----------------|-----------------|-----------------|
|           | Shoot          | Root            | Shoot          | Root            | Shoot | Root | Shoot | Root |
|           | YD6            | NK57            | YD6            | NK57            | YD6   | NK57 | YD6   | NK57 |
|           | IA              | EA              | IA              | EA              | IA    | EA   | IA    | EA   |
| −N        | 3.4b            | 3.9b            | 3.8b            | 4.5a            | 1.8c  | 2.2b | 1.9c  | 2.5a |
| LN        | 3.6b            | 4.1b            | 4.0b            | 4.9a            | 1.6b  | 2.3a | 1.8b  | 2.6a |
| NO₃-N     | 4.1b            | 4.4b            | 4.1b            | 5.1a            | 1.9b  | 2.7a | 2.0a  | 2.7a |
| N/N       | 3.7c            | 4.2b            | 4.3b            | 5.0a            | 1.7b  | 2.3a | 2.1b  | 2.6a |
| NH₄-N     | 3.4c            | 4.2b            | 3.6c            | 5.4a            | 1.4b  | 2.3a | 1.4b  | 2.7a |

Table 3. Dry weights and total N of rice seedlings with different N supplies under IA and EA conditions. Seedlings were sampled under IA and EA conditions, with −N, LN, NO₃-N, NH₄-N and N/N added. Values represent the means ± SE (error bars). Letters associated with means correspond to the results of multiple comparison tests.
Statistical analysis

All statistical evaluations were conducted using IBM SPSS ver. 13 software. To determine whether dissolved O$_2$ in response to EA was changed during 192 h, a two-way analysis of variance (ANOVA) with aeration and time course as two main effects was used. One of the aims of the experiment was to investigate the nature of the aeration responses in rice plant. We measured adventitious root number, root surface area, aerenchyma formation, ethylene production and the expression of aerenchyma-formation- and N-related genes by aeration treatment of each cultivar individually. These data except gene expression were compared with the differences between cultivars under aeration treatment with two-way repeated-measures ANOVA. For the gene expression of each cultivar, one-way ANOVA was used to test significance of data difference. In order to study the interaction between aeration and N treatment on rice response, we used a full model factorial experiment ANOVA to test for main and interactive effects of cultivar, aeration and N treatments on rice dry weight, total N concentration and ethylene production. Furthermore, we investigated the influence of aeration during treatment time course on aerenchyma-formation- and N-usage-related gene expression in two rice varieties under LN and NO$_3$-N conditions. The significant differences of gene expression over aeration time under different N conditions were determined by the analysis of covariance (ANCOVA).

Table 4. Results from a full model factorial experiment ANOVA of dry weight, total N and ethylene production in rice seedlings with cultivar, aeration and N supplied conditions as factors.

| Source                   | df | Mean$^2$  | F     | P      |
|--------------------------|----|-----------|-------|--------|
| (A) Dry weight           |    |           |       |        |
| Cultivar                 | 1  | 8.817     | 93.208| <0.0001|
| Aeration                 | 1  | 34.051    | 359.975| <0.0001|
| N                        | 4  | 1.109     | 11.724| <0.0001|
| Cultivar × aeration      | 1  | 1.350     | 14.272| 0.001  |
| Cultivar × N             | 4  | 0.193     | 2.044 | 0.107  |
| Aeration × N             | 4  | 0.832     | 8.799 | <0.0001|
| Cultivar × aeration × N  | 4  | 0.172     | 1.815 | 0.145  |
| Error                    | 80 |           |       |        |
| (B) Total N              |    |           |       |        |
| Cultivar                 | 1  | 29.506    | 18.568| <0.0001|
| Aeration                 | 1  | 19.184    | 12.072| 0.001  |
| N                        | 4  | 32.510    | 20.458| <0.0001|
| Cultivar × aeration      | 1  | 1.576     | 0.991 | 0.325  |
| Cultivar × N             | 4  | 49.839    | 31.363| <0.0001|
| Aeration × N             | 4  | 17.035    | 10.720| <0.0001|
| Cultivar × aeration × N  | 3  | 37.267    | 23.451| <0.0001|
| Error                    | 80 |           |       |        |
| (C) Ethylene production  |    |           |       |        |
| Cultivar                 | 1  | 0.121     | 37.204| <0.0001|
| Aeration                 | 1  | 0.590     | 182.018| <0.0001|
| N                        | 4  | 0.041     | 12.761| <0.0001|
| Cultivar × aeration      | 1  | 0.010     | 3.048 | 0.088  |
| Cultivar × N             | 4  | 0.002     | 0.713 | 0.588  |
| Aeration × N             | 4  | 0.039     | 11.974| <0.0001|
| Cultivar × aeration × N  | 4  | 0.001     | 0.164 | 0.955  |
| Error                    | 40 |           |       |        |
Results

External aeration treatment increased root growth, and altered root aerenchyma formation and ethylene production under different N conditions

To examine the effect of the dissolved O$_2$ level on root growth, we quantified changes in the dissolved O$_2$ level of the rice-growing solutions after EA. Under IA conditions, the dissolved O$_2$ decreased from 4.3 to 3.8 mg L$^{-1}$ by 48 h and stabilized at 3.6 mg L$^{-1}$ after 120 h of growth (Fig. 1). Under EA conditions, the dissolved O$_2$ increased from 4.8 to 6.8 mg L$^{-1}$ by 48 h and reached 7.8 mg L$^{-1}$ by 120 h (Fig. 1). Both IA and EA significantly affected the dissolved O$_2$ in the solution (Table 1).

The N treatments were started after 120 h when the dissolved O$_2$ levels were stable. EA improved rice growth under different N supply conditions (Fig. 2A and B). Under conditions lacking N, both cultivars showed significant increases in the number of adventitious roots and root surface area (Fig. 2C and D) in response to EA treatment. While the number of adventitious roots showed significant higher in NK57 than YD6 at the same aeration condition (Fig. 2C and Table 2A), and the surface area had no significant difference (Fig. 2D and Table 2B) between cultivars. The shoot dry weight increased significantly in NK57, but not in YD6, after EA treatment (Table 3). In the treatments using different N supplies, EA treatment increased the shoot biomass by 15–40 % in NK57 and the root biomass by 20–50 % in both cultivars (Table 3). The results from a full model factorial experiment ANOVA of dry weight (Table 2) showed significant differences between cultivars, between aeration treatments and among N treatments (Table 4A). Furthermore, significant interactions were noted between cultivars and aeration, between aeration and N treatments, and between all three factors according to plant dry weight (Table 4A).

To detect the effects of aeration on aerenchyma formation, root segments were sectioned at 1–1.5 and 2–2.5 cm from the root tip. Transverse root sections showed that aerenchyma was more developed in YD6 than in NK57, and that it was less well developed under EA conditions in both cultivars (Fig. 2E and F; see Supporting Information—Fig. S1A and B). This effect was particularly noticeable in the sections obtained 1.5 cm from the root tip, in which aerenchyma formation was reduced from ~16 to 0 % by EA in both cultivars (Fig. 2G; see Supporting Information—Fig. S1C). In the sections obtained 2.5 cm from the root tip, aerenchyma formation was decreased in NK57 roots by ~9 % (Fig. 2H; see Supporting Information—Fig. S1D). Aerenchyma formation was significantly different between cultivars and between aeration treatments but not between cultivar and aeration treatment (Table 2C and D).

Ethylene production in NK57 was higher than that in YD6 after IA treatment, but no difference was observed after EA (Fig. 2I). Under EA conditions, ethylene production in both cultivars was significantly lower than that under IA treatments without N added (Fig. 2I and Table 2E). Under different N supplied conditions, ethylene production showed significant differences between cultivars, aeration conditions and N treatments. There was also a significant interaction between aeration and N treatments (Fig. 3 and Table 4C). Furthermore, under IA conditions, LN and NO$_3$-N enhanced ethylene production in both rice cultivars compared with N/N and NH$_4$-N treatments (Fig. 3).

External aeration treatment increased total N concentration in the roots under N-limited or nitrate-only conditions

Since root morphology and aerenchyma formation were both affected by EA treatment, and EA could affect N transport and acquisition in plants (Hu et al. 2014; Saengwilai et al. 2014), we further explored the effects of aeration on total N accumulation under varying N supply conditions. No significant differences were observed in the shoot N concentration in either cultivar in response to EA treatment (Table 3). In the roots of YD6 grown without N or in a LN solution, and in those of NK57 grown in a NO$_3$-N solution, total N concentration increased significantly after EA treatment (Table 3). However, the total N concentration in the roots of both rice cultivars did not change significantly when grown under ammonium only or a mixed supply of nitrate and ammonium (Table 3). And the total N concentration showed no significant difference between cultivars.
but there was a significant difference for aeration or N treatment (Table 4B).

**External aeration treatment affected the expression of aerenchyma-formation-related genes in roots, depending on the rice cultivar and N supply**

Since root aerenchyma formation was strongly affected by aeration treatment (Fig. 2), we determined the effects of aeration on the expression of aerenchyma-formation-related genes in roots. External aeration decreased the expression of the *OsBphi008a* and *OsPDCD5* genes by 12–43 % (Fig. 4A and B) and of *OsPAD4* by ~60 % in both cultivars compared with 1A treatment (Fig. 4C). Expression levels of *OsLSD1.1* (Fig. 4D), *OsLSD2* (Fig. 4E) and *OsEDS* (Fig. 4F) changed significantly after EA treatment in YD6 (downregulated by 58, 48 and 32 %, respectively). The expression of these genes in NK57 was not significantly different between the 1A and EA treatments, except for *OsEDS*, which was upregulated (Fig. 4F). Nitrogen deficiency and NO$_3$-N enhanced the porosity of, and aerenchyma formation in, rice roots (Wang *et al.* 2005; Abiko and Obara 2014). Therefore, a detailed time-course analysis of the expression was performed for aerenchyma-formation-related genes under different supplies of LN and NO$_3$-N. The expression of *OsPAD4* and *OsLSD1.1* in YD6 peaked at 2 h under LN conditions (Fig. 5A and C) and at 0.5 h under NO$_3$-N (Fig. 5B and D) conditions after 1A treatment compared with EA treatment. After 48 h of aeration,
the expression of OsPAD4 was not affected in either rice variety compared with IA treatment under LN conditions (Fig. 5A and Table 5A). However, the expression of OsLSD1.1 during 48 h of aeration showed the opposite trend in both rice varieties (Fig. 5C). OsLSD1.1 expression was suppressed in YD6, but induced in NK57, by EA under LN conditions (Fig. 5C and Table 5B). Interestingly, the expression patterns of OsPAD4 and OsLSD1.1 under NO3-N supply differed from those under LN supply; expression levels of both OsPAD4 (Fig. 5B) and OsLSD1.1 (Fig. 5D) were significantly suppressed during aeration in both rice varieties (Table 5C and D). We also evaluated the expression of OsPAD4 and OsLSD1.1 under N/N and NH4-N conditions [see Supporting Information—Fig. S2]. The results showed that OsPAD4 expression was significantly downregulated in YD6 but significantly upregulated in NK57 by EA treatment with N/N [see Supporting Information—Fig. S2A] and NH4-N [see Supporting Information—Fig. S2B] supply.

External aeration treatment regulated the expression of several N transporter genes in the roots under different N conditions

Since EA increased the total N concentration in rice roots under limited N or NO3-N supply conditions (Table 3), we explored the effects of the N treatments on the expression of N transporter genes in rice seedlings grown with IA and EA treatments. The expression levels of the N-usage-related genes (OsNRT2.1, OsNAR2.1, OsNRT2.3; OsAMT1.1, OsAMT1.2 and OsAMT1.3) were determined under −N supply. OsNRT2.1 expression was increased by ~30 % after EA treatment in both cultivars (Fig. 6A). OsNAR2.1 (Fig. 6B) and OsNRT2.3 (Fig. 6C) expression levels changed significantly after EA treatment in YD6 only. In YD6, OsNAR2.1 (Fig. 6B) was downregulated by 31 %, but OsNRT2.3 was upregulated (Fig. 6C). Interestingly, the expression of OsNAR2.1 and OsNRT2.3 in NK57 did not differ significantly between IA and EA treatments (Fig. 6B and C). The expression of OsAMT1.1 (Fig. 6D) and OsAMT1.3 (Fig. 6F), ammonium transporter genes, was significantly reduced by ~20 and 75 % in YD6, respectively, while OsAMT1.2 (Fig. 6E) expression was enhanced 2-fold in NK57 after EA treatment. It was also noted that expression of OsNRT2.1, OsNAR2.1 and OsAMT1.3 in YD6 and NK57 with or without EA changed over time in LN and NO3-N nutrient solutions (Fig. 7 and Table 6). Under LN conditions after 48 h of aeration, the expression levels of OsNRT2.1 (Fig. 7A and Table 6A) and OsAMT1.3 (Fig. 7E and Table 6B) increased in YD6; however, only OsAMT1.3 was significantly upregulated in NK57 (Fig. 7E). Under NO3-N treatment, the expression of OsNRT2.1 in YD6 was markedly downregulated.
Table 5. Results from an ANCOVA evaluating the influence of aeration and duration on aerenchyma-formation-related gene expression in different rice varieties under LN and NO₃-N conditions.

| Cultivar | Source          | df | Mean² | F      | P   |
|----------|-----------------|----|-------|--------|-----|
| (A) OsPAD4 expression under LN conditions |                 |    |       |        |     |
| YD6      | Duration        | 1  | 0.024 | 2.543  | 0.119|
|          | Aeration        | 1  | 0.051 | 5.285  | 0.027|
|          | Error           | 39 |       |        |     |
| NK57     | Duration        | 1  | 0.153 | 3.223  | 0.080|
|          | Aeration        | 1  | 0.142 | 2.983  | 0.092|
|          | Error           | 39 |       |        |     |
| (B) OsLSD1.1 expression under LN conditions |                 |    |       |        |     |
| YD6      | Duration        | 1  | 0.010 | 2.094  | 0.156|
|          | Aeration        | 1  | 0.020 | 4.153  | 0.048|
|          | Error           | 39 |       |        |     |
| NK57     | Duration        | 1  | 0.006 | 2.060  | 0.159|
|          | Aeration        | 1  | 0.004 | 1.158  | 0.288|
|          | Error           | 39 |       |        |     |
| (C) OsPAD4 expression under NO₃-N conditions |                 |    |       |        |     |
| YD6      | Duration        | 1  | <0.0001 | 0.385  | 0.538|
|          | Aeration        | 1  | <0.0001 | 0.264  | 0.610|
|          | Error           | 39 |       |        |     |
| NK57     | Duration        | 1  | 0.003 | 1.369  | 0.249|
|          | Aeration        | 1  | 0.001 | 0.233  | 0.632|
|          | Error           | 39 |       |        |     |
| (D) OsLSD1.1 expression under NO₃-N conditions |                 |    |       |        |     |
| YD6      | Duration        | 1  | 0.001 | 0.594  | 0.446|
|          | Aeration        | 1  | 0.001 | 0.544  | 0.465|
|          | Error           | 39 |       |        |     |
| NK57     | Duration        | 1  | <0.0001 | 0.066  | 0.798|
|          | Aeration        | 1  | 0.001 | 0.161  | 0.690|
|          | Error           | 39 |       |        |     |

by EA compared with IA (Fig. 7B and Table 6D). In NK57, the expression of OsNRT2.1 after 48 h of aeration was increased by 40 % (Fig. 7B), while OsNAR2.1 expression was not significantly different after 48 h of aeration under LN or NO₃-N conditions (Fig. 7C and D and Table 6C and E).

Discussion

External aeration treatment improved rice growth

Field oxygenation levels can affect rice growth and generally most rice cultivars show greater root biomass in deep-water or drained soils in comparison with conventional waterlogged growth conditions (Colmer et al. 2006; Parlanti et al. 2011). Oxygenation of hydroponic solutions used for rice growth increased adventitious root length, the absorption area and total biomass (Xu et al. 2012a). However, several reports have also shown that flooding treatments decrease adventitious root emergence and elongation (Visser and Pierik 2007; Dawood et al. 2014), and this was reviewed recently by Voesenek and Bailey-Serres (2015). In this study, we evaluated whether EA of the roots could improve rice plant growth and whether the response to O₂ differed among rice cultivars (YD6 and NK57) with different aerenchyma formation. The number of adventitious roots, root surface area and root dry weight were increased by EA in YD6 and NK57 five days after germination (Fig. 2 and Table 3). The interaction between aeration and N on adventitious roots and biomass (Tables 2 and 4) indicated that O₂ effects on rice plant growth depended on the N situation. Therefore, we deduced that the effect of O₂ on adventitious root development of rice may depend on the different N conditions, since previous studies (Visser and Pierik 2007; Dawood et al. 2014) have focussed mainly on the effect of flooding on rice plants in potting soil conditions but not under specific N treatments. Although the effects of aeration on rice growth differed under different N conditions, EA treatment increased root dry mass more in the cultivar with less-developed aerenchyma (NK57) than in the cultivar with more-developed aerenchyma (YD6) (Table 3). However, the 48-h N treatments did not result in any significant differences in plant growth or N contents with different N treatments. This may be because the 48-h N treatment was too short to show any significant effects on rice growth.

External aeration treatment decreased ethylene production and affected aerenchyma formation by regulating related gene expression

Root aerenchyma formation was increased by water flooding and inhibited by aeration or in drained soils (Colmer et al. 2006; Parlanti et al. 2011). Our results showed that aerenchyma formation and ethylene production in rice roots were reduced by EA treatment (Figs 2 and 3), and this is consistent with the findings from above. External aeration significantly inhibited ethylene production in two rice cultivars and decreased the formation of the root aerenchyma (Figs 2 and 3). The effect of ethylene on root aerenchyma formation was documented previously in rice (Nishiuchi et al. 2012). However, we found a significant interaction between aeration and N supply on rice ethylene production (Fig. 3 and Table 4). Under nitrate and –N conditions, the effects of IA and EA on rice ethylene production were much larger than those under ammonium or mixed N supply conditions (Fig. 3). This indicated that the ethylene signal in response to the external N supply appeared before the signal related to the biomass or N
content (Fig. 3 and Tables 2 and 4). We also confirmed previous data showing that aerenchyma formation in different cultivars responded to external ethylene treatments (Justin and Armstrong 1991; Kong et al. 2009) using Tukey’s test, which showed a significant difference between cultivars (Fig. 3 and Table 4). This indicated that the external ethylene treatment would be similar to the internal ethylene production, even under different N supply conditions. Surprisingly, the effect of aeration on ethylene production did not depend on the rice cultivar since the interaction between aeration and cultivar was not significant (Table 4). However, there was a significant interaction between aeration and N treatment, indicating that the external N effect on ethylene synthesis depends on the aeration conditions (Table 4). There was no interaction between cultivar and N treatment or between aeration and cultivar on ethylene production (Table 4).

Nitrogen deficiency and NO$_3$-N treatments, compared with sufficient ammonium supply, enhance the porosity and formation of the aerenchyma in roots (Drew et al. 1989; He et al. 1994; Wang et al. 2005; Abiko and Obara 2014). Recently, Yang et al. (2012) reported that different sources of N nutrition affect aerenchyma formation in rice roots. However, the regulation of gene expression during aerenchyma formation in rice is unknown. In Arabidopsis, the AtPAD4, AtLSD1 and AtEDS1 genes play a role in ethylene synthesis and aerenchyma formation (Mühlenbock et al. 2007). AtLS1 is a negative regulator of PCD and plays a role in lysigenous aerenchyma formation. AtPAD4 and AtEDS positively regulated the induction and amount of lysigenous aerenchyma formation, thereby counteracting the inhibitory action of AtLS1 (Mühlenbock et al. 2007). Based on these reports and our ethylene data, we explored the regulation of gene expression in rice roots by examining the expression of relevant genes, such as OsBphi008a, OsPDCD5, OsPAD4 and OsLSD1.1.

In the aeration treatment experiment, aerenchyma formation was decreased in YD6 and NK57 roots (Fig. 2), and the PCD-related genes, OsBphi008a and OsPDCD5, were downregulated in roots (Fig. 4A and B). The expression pattern of OsBphi008a, which plays a positive role in ethylene synthesis (Hu et al. 2011), and OsPDCD5, which is involved in PCD (Drew et al. 2000), also exhibited positive regulation during rice aerenchyma formation, similar to the regulation pattern of ethylene and PCD in aerenchyma formation of Arabidopsis (Mühlenbock et al. 2007). Our results showed that OsPAD4 expression was downregulated; the amount of root aerenchyma decreased in both rice cultivars, and OsLSD1.1 expression was downregulated only in YD6 by EA (Fig. 4C and D). Therefore, we hypothesized that OsPAD4 positively controlled aerenchyma formation in rice roots based on ethylene content, and that OsLSD1.1 may be located upstream of OsPAD4 in rice. In Arabidopsis, aeration and waterlogged treatments were performed
under NO₃-N conditions, and the expression of AtPAD4 increased under waterlogging conditions (Mühlenbock et al. 2007). However, we compared the effect of aeration on OsPAD4 under different N treatments, in which EA altered the expression of OsPAD4 in a NO₃-N solution (Fig. 5B) but not under low N conditions (Fig. 5A), a mixture of N or in a NH₄-N solution [see Supporting Information—Fig. S2]. The gene expression data showed that the ethylene-synthesis-related genes OsPAD4 and OsLSD1.1 peaked within 6 h of N treatment (Fig. 5). This indicates that the regulation of OsPAD4 by EA was dependent on the external N supply and occurred during the very early stages of N treatment. Furthermore, the regulatory mechanism of aerenchyma formation during aeration in rice may differ from that in Arabidopsis.

External aeration treatment increased rice N accumulation by increasing expression of the N transporter gene

Simultaneous supply of ammonium and nitrate improves plant growth, both in hydroponics and in soil (Xu et al. 1992; Qian et al. 2004; Ruan et al. 2007; Song et al. 2011). Most previous studies have reached this conclusion without considering the effect of O₂ in the soil when applying different forms of N. In this study, it was shown that adding O₂ through EA of the roots improved rice plant growth (Fig. 2) and N accumulation (Table 3), and that the N accumulation response to O₂ differed among rice cultivars with different NUE. The results showed that total N concentration increased significantly by EA in YD6 roots without any supply of N (Table 3). Under

Figure 7. Real-time quantitative RT-PCR analysis of N-usage-related gene expression in the roots of rice seedlings grown in LN and NO₃-N nutrient solutions with IA and EA treatments. (A and B) Time course of OsNRT2.1 expression under LN (A) and NO₃-N (B) conditions. (C and D) Time course of OsNAR2.1 expression under LN (C) and NO₃-N (D) conditions. (E) Time course of OsAMT1.3 expression under LN conditions. Seedlings grown with or without aeration were transferred to a LN or NO₃-N nutrient solution, and the roots were collected for gene expression analysis at 0, 0.5, 2, 6, 12, 24 and 48 h. Values represent the means ± SE (error bars) of three replicates.
Table 6. Results from an ANCOVA evaluating the influence of aeration and duration on N-usage-related gene expression in different rice varieties under LN and NO3-N conditions.

| Cultivar | Source | df | Mean² | F     | P     |
|----------|--------|----|-------|-------|-------|
|          | (A) OsNRT2.1 expression under LN conditions | | | | |
| YD6      | Duration | 1  | 13.176 | 27.213 | <0.0001 |
|          | Aeration  | 1  | 0.042  | 0.088  | 0.769  |
|          | Error     | 39 |        |        |        |
| NK57     | Duration  | 1  | 16.935 | 17.086 | <0.0001 |
|          | Aeration  | 1  | 3.363  | 3.392  | 0.073  |
|          | Error     | 39 |        |        |        |
|          | (B) OsAMT1.3 expression under LN conditions | | | | |
| YD6      | Duration  | 1  | 60.377 | 19.198 | <0.0001 |
|          | Aeration  | 1  | 1.495  | 0.475  | 0.495  |
|          | Error     | 39 |        |        |        |
| NK57     | Duration  | 1  | 68.054 | 5.189  | 0.028  |
|          | Aeration  | 1  | 0.702  | 0.054  | 0.818  |
|          | Error     | 39 |        |        |        |
|          | (C) OsNAR2.1 expression under LN conditions | | | | |
| YD6      | Duration  | 1  | 26.407 | 27.197 | <0.0001 |
|          | Aeration  | 1  | 0.276  | 0.284  | 0.597  |
|          | Error     | 39 |        |        |        |
| NK57     | Duration  | 1  | 208.806 | 8.585  | 0.006  |
|          | Aeration  | 1  | 88.635 | 3.644  | 0.064  |
|          | Error     | 39 |        |        |        |
|          | (D) OsNRT2.1 expression under NO3-N conditions | | | | |
| YD6      | Duration  | 1  | 37.915 | 13.750 | 0.001  |
|          | Aeration  | 1  | 6.627  | 2.403  | 0.129  |
|          | Error     | 39 |        |        |        |
| NK57     | Duration  | 1  | 17.972 | 15.068 | <0.0001 |
|          | Aeration  | 1  | 0.260  | 0.218  | 0.643  |
|          | Error     | 39 |        |        |        |
|          | (E) OsNAR2.1 expression under NO3-N conditions | | | | |
| YD6      | Duration  | 1  | 21.900 | 28.819 | <0.0001 |
|          | Aeration  | 1  | 2.602  | 3.424  | 0.072  |
|          | Error     | 39 |        |        |        |
| NK57     | Duration  | 1  | 118.552 | 45.685 | <0.0001 |
|          | Aeration  | 1  | 0.048  | 0.018  | 0.893  |
|          | Error     | 39 |        |        |        |

low N conditions, the total N concentration increased in YD6 roots (Table 3), while in NK57 roots, NO3-N conditions with EA increased the total N concentration (Table 3). Tukey’s test showed a significant interaction among the aeration conditions, cultivars and N treatment, which indicated that the external effect of N on different cultivar biomass and total N content depended on the aeration situation (Table 4).

The difference in N transporter gene expression between the two cultivars could explain the different N responses to EA treatments. The total N concentration in the roots of OsNAR2.1 RNAi mutant plants was 63–66 % of that in wild-type roots grown in 0.2 mM N under IA conditions (Yan et al. 2011). In the OsNAR2.1 RNAi mutant, the expression levels of OsNRT2.1 and OsNRT2.3 decreased markedly (Yan et al. 2011). In the aeration experiment, the expression of OsNRT2.1 and OsNRT2.3 increased significantly in YD6 roots after 5 days of EA treatment; however, only OsNRT2.1 expression was increased by EA treatment in NK57 roots (Fig. 6A–C). The gene expression data confirmed that the activities of enzymes involved in root N metabolism were enhanced by EA, and that the effect of EA on root N metabolism might be genotype specific (Xu et al. 2013). Under LN conditions, the expression levels of OsNRT2.1 and OsAMT1.3 were upregulated in YD6; however, expression of only OsAMT1.3 was increased by EA in NK57 (Fig. 7). This difference in expression levels of OsNRT2.1 (Fig. 7A) and OsAMT1.3 (Fig. 7E) between the two cultivars may have caused the changes in N concentration in rice to EA under LN conditions (Table 3). However, under NO3-N supply conditions, the expression of a primary high-affinity transporter gene in rice roots (Araki and Hasegawa 2006), OsNRT2.1, was increased by EA in NK57 (Fig. 7B) but decreased in YD6 (Fig. 7B). The increase in OsNRT2.1 expression may explain the increase in N concentration in NK57 in the presence of NO3-N condition with EA treatment. We conclude that the increased rice growth and total N acquisition by EA may be associated with the abundance of root endogenous aerenchyma and be dependent on the forms of N supplied.

Conclusions
Following the aeration experiment, rice roots showed the different abundance of aerenchyma and growth pattern in YD6 and NK57 cultivars. The ethylene production from whole rice plant was also altered by aeration treatment in both cultivars. Combining aeration and external N treatments, we found a strong interaction effect between aeration and N supplies on rice growth and total N concentration in plant. Furthermore, the increase of rice growth and total N acquisition can be explained by altering expression of the OsPAD4 and OsNRT2.1 genes in aeration under LN and NO3-N supplies.

As in waterlogged conditions, the dissolved O2 was low and main N form was ammonium (Kludze et al. 1993; Wang et al. 1993). In contrast, in upland soil, the dissolved O2 was high and main N form was nitrate N (Kludze et al. 1993; Li et al. 2008). Apparently, the gene variation in
responding to EA under different N conditions suggested that aeration and external N interaction were complex for plant adaptation to environmental change from waterlogged conditions into upland soil conditions. The EA could affect the formation of aerenchyma which in turn affected the growth of roots and shoots, and thus total N acquisition by altering expression of the OsPAD4 and OsNRT2.1 genes.

Accession Numbers
Sequence data from this article can be found in the Rice Genome Initiative/GenBank data libraries under accession numbers listed in table [see Supporting Information—Table S1].

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Contributions by the Authors
J.Z. and J.L. conducted all the experiments. Z.X. was involved in ethylene measurement. X.F. designed the experiments and wrote the manuscript. Q.Z. was involved in soluble O2 measurement. Q.S. and G.X. were involved in editing the manuscript.

Conflict of Interest Statement
None declared.

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Supporting Information
The following additional information is available in the online version of this article –

Table S1. Primers and accession numbers used for quantitative real-time PCR.

Figure S1. Effects of aeration on aerenchyma formation of 7-day-old seedlings. Transverse section of root visualized in resin-embedded sections of YD6 (A) and NK57 (B) roots. Resin-embedded sections obtained 1.5 and 2.5 cm from the root tips of YD6 and NK57 seedlings subjected to IA and EA treatments. Quantification of aerenchyma formation in sections obtained 1.5 cm (C) and 2.5 cm (D) from the root tips, using Image J software. Values represent the means ± SE (error bars) of three replicates. Significant differences are indicated by different letters (P < 0.05, two-way ANOVA).

Figure S2. Real-time quantitative RT-PCR analysis of OsPAD4 and OsLSD1.1 expression in roots of rice seedlings grown in N/N and NH4-N nutrient solution. Time course of OsPAD4 expression in N/N (A) and NH4-N (B) nutrient solution. Time course of OsLSD1.1 expression in N/N (C) and NH4-N (D) nutrient solution. Seedlings grown with or without aeration were transferred to a LN nutrient solution and roots were collected for gene expression analysis at 0, 0.5, 2, 6, 12, 24 and 48 h. IA, internal aeration; EA, external aeration. Values represent the means ± SE (error bars) of three replicates.

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