Regulation of ammonium acquisition and use in *Oryza longistaminata* ramets under nitrogen source heterogeneity

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Abstract

*Oryza longistaminata*, a wild rice, vegetatively reproduces and forms a networked clonal colony consisting of ramets connected by rhizomes. Although water, nutrients, and other molecules can be transferred between ramets via the rhizomes, inter-ramet communication in response to spatially heterogeneous nitrogen availability is not well understood. We studied the response of ramet pairs to heterogeneous nitrogen availability using a split hydroponic system that allowed each ramet root to be exposed to different conditions. Ammonium uptake was compensatorily enhanced in the sufficient-side root when roots of the ramet pairs were exposed to ammonium-sufficient and ammonium-deficient conditions. Comparative transcriptome analysis revealed that a gene regulatory network for effective ammonium assimilation and amino acid biosynthesis was activated in the sufficient-side roots. Allocation of absorbed nitrogen from the nitrogen-sufficient to the nitrogen-deficient ramets was rather limited. Nitrogen was preferentially used for newly growing axillary buds on the sufficient-side ramets. Biosynthesis of trans-zeatin (tZ), a cytokinin, was upregulated in response to the nitrogen supply, but tZ appeared not to target the compensatory regulation. Our results also implied that the *O. longistaminata* putative ortholog of rice (*Oryza sativa*) C-terminally encoded peptide1 plays a role as a nitrogen-deficient signal in inter-ramet communication, providing compensatory upregulation of nitrogen assimilatory genes. These results provide insights into the molecular basis for efficient growth strategies of asexually proliferating plants growing in areas where the distribution of ammonium ions is spatially heterogeneous.
Introduction

*Oryza longistaminata* is a wild rice species that preferentially grows in wetlands and vegetatively reproduces through rhizome growth that can be so vigorous as to occupy an area completely (Vaughan, 1994). Rhizome growth is characterized by the underground horizontal outgrowth of axillary buds to form new rhizomes that expand into new territory (Yoshida et al., 2016; Fan et al., 2017; Kyozuka, 2017; Bessho-Uehara et al., 2018; Toriba et al., 2020; Shibasaki et al., 2021). Rhizome tips developmentally transform into photosynthetic above-ground organs by growing into a new plantlet called a ramet. Continuous rhizome growth and subsequent transformation form a networked clonal colony. In addition to *O. longistaminata*, many other plant species vegetatively reproduce through rhizome growth, such as *Phyllostachys edulis* (Moso bamboo) and *Zozysa matrella* (Manila grass; Guo et al., 2021), and show vigorous fertility. Since ramets are connected via rhizomes, water, nutrients, and other molecules can be transferred between ramets (De Kroon et al., 1998). Therefore, the growth and metabolism of a ramet are not totally independent of the neighboring ramets but have some influence on each other via the rhizomes.

Nitrogen is one of the most limiting macronutrients for plants, so nitrogen acquisition and use efficiency significantly affect plant growth and development. In submerged and reductive soil conditions where wetland rice grows, plants mainly use ammonium ions as the inorganic nitrogen source (Yoshida, 1981). In rice (*Oryza sativa*), ammonium ions in the soil are taken up by ammonium transporters (AMTs; Sonoda et al., 2003; Suenaga et al., 2003; Yuan et al., 2007; Li et al., 2016; Jia and von Wirén, 2020; Lee et al., 2020a; Konishi and Ma, 2021), and then are initially assimilated into glutamine and glutamate by the glutamine synthetase (GS)/glutamate synthetase (GOGAT) cycle (Lea and Miflin, 1974). The cycle is mainly composed of cytosolic GS and plastidic NADH-GOGAT isoforms in the root (Ishiyama et al., 2004; Tabuchi et al., 2005, 2007; Funayama et al., 2013; Yamaya and Kusano, 2014; Ji et al., 2019; Lee et al., 2020b). Several amino acids are synthesized by amino-transfer reactions from glutamine and glutamate to organic acids derived from glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway. Previous studies using *O. sativa*, Arabidopsis (*Arabidopsis thaliana*), and tobacco (*Nicotiana tabacum*) revealed a wide and versatile regulatory network of gene expression in response to nitrogen (nitrate or ammonium) nutrition, including genes for the processes of uptake, assimilation, amino acid synthesis, carbon skeleton supply, and hormone signaling (Scheible et al., 1997, 2004; Wang et al., 2004; Sakakibara et al., 2006; Chandran et al., 2016; Yang et al., 2017; Sun et al., 2020).

Plants optimize their growth and development according to the amount of available nitrogen, and signaling molecules, including phytohormones and peptide hormones, play a key role in this regulation (Nishida and Suzuki, 2018; Ruffel, 2018; Sakakibara, 2021; Wheelon and Bennett, 2021). Cytokinin, especially trans-zeanin (tZ), promotes nitrogen-responsive shoot growth via modulating shoot meristem activity (Kiba et al., 2013; Davière and Achard, 2017; Kang et al., 2017; Osugi et al., 2017; Landrein et al., 2018). An abundant nitrate supply promotes de novo tZ biosynthesis via the upregulation of *ADENOSINE PHOSPHATE-ISOPENTENYLTRANSFERASE3* (IPT3) and *CYP73SA2* in Arabidopsis (Takei et al., 2004; Maeda et al., 2018; Naulin et al., 2019; Sakakibara, 2021). In *O. sativa*, a glutamine-related signal stimulates *OsIPT4* and *OsIPT5* expression that are involved in axillary bud outgrowth from shoots (Kamada-Nobusada et al., 2013; Ohashi et al., 2017). In *O. longistaminata*, the outgrowth of rhizome axillary buds in response to nitrogen nutrition is regulated by a similar process to that of the axillary shoot bud in *O. sativa*, despite differences in the physiological roles of these organs (Shibasaki et al., 2021). In contrast, strigolactone is involved in the nutrient-responsive suppression of shoot axillary bud outgrowth (Gomez-Roldan et al., 2008; Umehara et al., 2008; Minakuchi et al., 2010; Umehara et al., 2010). Strigolactone biosynthetic genes are known to be upregulated in the roots of *O. sativa* and maize (*Zea mays*) under nitrogen-deficient (Sun et al., 2014; Xu et al., 2015; Ravazzolo et al., 2019, 2021; Bellegarde and Sakakibara, 2021) and phosphorus-deficient (Umehara et al., 2010) conditions.

It is essential for plants to efficiently acquire nutrients for optimal growth and development even though mineral nutrients are heterogeneously distributed in the soil. Recent studies using a split-root system in Arabidopsis showed that when part of the root system is subjected to nitrogen deficiency, C-TERMINALLY ENCODED PEPTIDE1 (CEP1), synthesized in the deficient root, is translocated to shoots via the xylem (Ohyama et al., 2008; Tabata et al., 2014). The perception of CEP1 by the CEP1 RECEPTOR (CEPR) in shoots triggers the expression of CEP DOWNSTREAM (CEPD) proteins that are translocated to the root system via the phloem, thereby promoting compensatory nitrogen uptake by inducing the expression of high-affinity nitrate transporters, including *NITRATE TRANSPORTER2.1* in the nitrate-ample side (Tabata et al., 2014; Okamoto et al., 2016; Ruffel and Gojon, 2017; Ota et al., 2020; Ohkubo et al., 2017, 2021). In rhizomatous plants, ramets connected by rhizomes are regarded as one individual; however, it remains unknown whether each ramet responds to nitrogen availability independently or compensatively when growing in areas where nitrogen distribution is spatially heterogeneous.

In this study, we characterized the response of *O. longistaminata* ramet pairs, connected by a rhizome, to spatially heterogeneous nitrogen conditions and found a compensatory gene regulatory network for effective acquisition and assimilation of ammonium ions in the ample-side ramet root. Cytokinin biosynthesis was upregulated in response to ammonium supply, but this increase appears to be independent of the compensatory regulation. Our results also imply that an *O. longistaminata* ortholog of the *OsCEP1* gene plays a role in inter-ramet communication. This study provides
valuable hints for understanding the molecular basis of efficient growth strategies of vegetatively proliferating plants.

**Results**

**Compensatory promotion of ammonium uptake in response to a spatially heterogeneous ammonium supply in *O. longistaminata* ramet pairs**

To examine nitrogen-related inter-ramet communication via the rhizome, we established a split-hydroponic experimental system for *O. longistaminata* ramet pairs (Supplemental Figure S1). After preparing young ramet pairs of comparable growth stages that developed at adjacent rhizome nodes, we treated the roots of each ramet to different levels of nitrogen that developed at adjacent rhizome nodes, we treated the roots of each ramet to different levels of nitrogen at 24 h after the start of the + N, + N split, – N split, and – N treatments at a longer treatment period (48 h). As a result, the accumulation level of glutamine, asparagine, aspartate, alanine, glutamate, and arginine was significantly higher in the + N split roots than those in the + N treatment. Other amino acids except for arginine showed a similar tendency. These results are in line with the transcriptome changes in response to heterogeneous nitrogen availability.

To know the impact of transcriptome changes on the accumulation level of amino acids, we analyzed the amino acid concentrations in roots from the + N, + N split, – N split, and – N treatments at a longer treatment period (48 h). As a result, the accumulation level of glutamine, asparagine, aspartate, alanine, glutamate, and arginine was significantly higher in the + N split roots than in the – N split roots (Figure 3C). Among these amino acids, aspartate and alanine levels in roots from the + N split treatment were significantly higher than those in the + N treatment. Other amino acids except for arginine showed a similar tendency. These results are in line with the transcriptome changes in response to heterogeneous nitrogen availability.

On the other hand, 1,848 and 2,087 genes were upregulated in the – N and – N split treatments compared to the + N condition, respectively (Supplemental Tables S5 and S5). Overall, the expression of 9,935 genes overlapped between the two conditions (Supplemental Figure S2). One hundred and forty-five genes were upregulated in the + N treatment compared to the + N split treatment (Supplemental Table S6), and 15 genes overlapped with the – N upregulated genes (Supplemental Figure S2).
Allocation of absorbed nitrogen between ramets via the rhizome when nitrogen availability is spatially heterogeneous

To examine the allocation of absorbed nitrogen in ramet pairs when nitrogen availability is spatially different, 15NH4Cl was fed to + N and + N split roots with the same conditions as shown in Figure 1B, and the roots were grown for another 7 d with a nonlabeled nitrogen source. In this experimental condition, 15N was detected in both the shoots and roots of the systemic + N and –N split ramets that had not been fed with 15NH4Cl (Figure 4A), indicating that absorbed nitrogen is allocated to neighboring ramets via the rhizome. The percentage distribution of 15N was higher in the shoots than in the roots. Interestingly, the allocation of absorbed nitrogen from the + N split to the –N split ramets was less than that from the + N to the + N ramets.

Growth response of ramets in spatially heterogeneous nitrogen conditions

To know the long-term effects of nitrogen split treatment on the growth of ramets, we exposed ramet pairs to different nitrogen conditions for 5 weeks, and growth parameters for each ramet were monitored (Figure 4, B–E). Plant height, the number of fully developed leaves, chlorophyll content, and the number of growing axillary buds were significantly higher in the + N and + N split ramets than in the –N split and –N ramets. The chlorophyll content was lower in the –N split and –N ramet shoots, but the decrease was significantly alleviated in the –N split ramets compared to the –N ramets (Figure 4D), suggesting that the allocated nitrogen was used to retain photosynthetic function. In contrast, the change in the number of growing axillary buds was almost zero for the –N and –N split ramets, whereas the axillary bud number increased significantly in the + N split and + N ramets (Figure 4E). In particular, the axillary bud number was significantly higher in the + N split ramets than in the + N ramets. These results suggest that when neighboring ramets are exposed to different levels of nitrogen availability, the allocation of absorbed nitrogen from the N-sufficient ramet to the deficient ramet is rather limited and, nitrogen is preferentially allocated for newly growing axillary buds.
Response of cytokinin and strigolactone biosynthetic genes in response to a heterogeneous nitrogen supply

To gain insight into the growth promotion of axillary buds in the +N split ramets, we focused on cytokinin and strigolactone, two phytohormone families that promote and inhibit axillary bud outgrowth, respectively. We analyzed the expression levels of cytokinin and strigolactone biosynthetic genes by RT-qPCR using the roots of ramet pairs that had been split-treated for 7 d. The expression levels of cytokinin biosynthetic genes, OlIPT4, OlIPT5, OlCYP735A3, and OlCYP735A4 were higher in the roots of the +N and +N split ramets than in those of the –N and –N split ramets (Figure 5A), and there was no difference between the +N and +N split conditions.

We also analyzed the concentration of cytokinins in the roots. The level of tZ-type cytokinins, including the riboside and ribotide precursors, was higher in the +N and +N split roots compared to the –N split and –N roots, whereas the levels of N6-(Δ2-isopentenyl)adenine (iP)-type cytokinins were somewhat lower (Figure 5B; Supplemental Table S7). The tZ-type cytokinin content in the +N and +N split roots was comparable, suggesting that de novo tZ-type cytokinin biosynthesis is enhanced in response to the ammonium supply but is not under compensatory regulation.

Although strigolactone species in O. longistaminata have not been well characterized yet, we analyzed orthologs of O. sativa D27, D17, and D10 (OID27, OID17, and OID10, respectively) encoding enzymes involved in the production of carlactone, an intermediate of strigolactone biosynthesis (Alder et al., 2012). The expression level of OID10 was significantly higher in –N ramet roots than in other conditions, although OID27 and OID17 expression levels were essentially similar and slightly lower, respectively (Figure 5C). This result implies that de novo strigolactone biosynthesis might be upregulated in nitrogen-deficient roots.

Possible involvement of a CEP1-type peptide in inter-ramet nitrogen deficiency signaling

In Arabidopsis, CEP1 plays a key role in the systemic regulation of nitrate acquisition in response to heterogeneous nitrogen conditions as a root-to-shoot signaling molecule (Tabata et al., 2014; Okamoto et al., 2016). To investigate whether a CEP1-type peptide is involved in the observed inter-ramet communication, we analyzed the response of O. longistaminata CEP gene orthologs to spatially heterogeneous nitrogen availability. In O. sativa, 15 genes encode CEPs (Sui et al., 2016). We searched for the orthologs in O. longistaminata and found 15 sequences corresponding to each of the O. sativa genes (Supplemental Table S8), and eight of the genes (OICEP5, OICEP6.1, OICEP9, OICEP10, OICEP11, OICEP12, OICEP14, and OICEP15) were annotated in our RNAseq data. However, expression of these eight genes was not significantly different in –N roots compared to +N roots. Next, we focused on OICEP1 and analyzed its expression level by RT-qPCR because the peptide sequences encoded by OsCEP1 (also OICEP1) belong to the same group (group I) as Arabidopsis CEP1 based on the structural features (Delay et al., 2013; Sui et al., 2016). In the RT-qPCR analysis, the expression level of OICEP1 in –N roots was significantly higher than in the +N and +N split roots (Figure 6A).

To gain further insight into the involvement of OICEP1 gene products in nitrogen-related ramet-to-ramet signaling, we examined the effect of an exogenous application of OICEP1 peptides on the expression of OIAMT1;2, OIAMT1;3, and OIG51;2 genes. In addition to OsCEP1, the coding sequence of the OICEP1 gene contains multiple CEP sequences containing proline residues. Thus, we synthesized peptides
OICEP1a–OICEP1d (see "Materials and methods") and used a mixture. We treated ramet roots with the peptide mixture under nitrogen-deficient conditions to repress the endogenous OICEP1 expression. When we applied OICEP1 peptides to the roots of one side of the ramets, the accumulation level of transcripts for OIAMT1;2, OIAMT1;3, and OIGS1;2 increased in both the local side and systemic side of the ramet roots in 6 h. The transcript level significance was maintained in the systemic side of the root even at 24 h after treatment (Figure 6B), suggesting that CEP1-related signaling is possibly involved in the systemic regulation of the genes via the rhizomes.

We further explored homologs of the CEPR and CEPD genes in O. longistaminata. Since no previous studies of CEPR and CEPD have been conducted in O. sativa and O. longistaminata, we identified Ol12G001732 as a homolog of Arabidopsis CEPR1 (57.2% sequence identity) in the PLAZA database (Van Bel et al., 2018) and 10 CC-type glutaredoxin genes (GRX; Garg et al., 2010) as homologs of Arabidopsis CEPD1 (37.6%–58.3% sequence identity; Supplemental Figures S3 and S4; Supplemental Table S8). Our RT-qPCR analysis showed that the expression of Ol12G001732 was highest in the +N split shoot (Supplemental Figure S5A). Upregulation of Ol12G001732 was also observed in the CEP1 peptide treatment experiment (Supplemental Figure S6), suggesting that the CEPR homolog might play a role in ramet-to-ramet nitrogen signaling in O. longistaminata. On the other hand, there was no significant difference in the expression of the GRXs in the spatially heterogeneous nitrogen condition except for OlGRX15, but the upregulation of OlGRX15 expression was not +N split specific (Supplemental Figure S5B).

**Discussion**

In this study, we demonstrated inter-ramet communication occurs in O. longistaminata via rhizomes for a systemic response to spatially heterogeneous nitrogen availability. When ramet pairs of O. longistaminata connected by a rhizome were exposed to different ammonium ion conditions, a series of gene networks that allow complementary absorption and assimilation of ammonium was activated in the root of the ramet in the nitrogen-sufficient condition. This network also included genes capable of supplying the carbon skeletons for amino acid synthesis, such as those involved in glycolysis. The expression level of these genes in the nitrogen-sufficient side of the heterogeneous condition was higher than in the nitrogen homogeneously sufficient condition, suggesting that the nitrogen-deficient side ramet conveyed some kind of nitrogen deficiency signal to the
sufficient side ramet via the rhizome to trigger the systemic response. In our transcriptome analysis, a large part of the compensatorily upregulated genes overlapped with ammonium-responsive genes (295 genes/416 genes, Figure 2B), indicating that the expression of the compensatory genes is upregulated by the input of a systemic nitrogen-deficient signal from the adjacent ramet. It is not clear at present whether a derepression or a more facilitative regulatory event underlies the process.

A small part of the nitrogen absorbed in the ramet roots in the heterogeneous nitrogen-sufficient condition (+N split) was distributed to the adjacent nitrogen-deficient ramets (−N split), contributing to the maintenance of the chlorophyll content. However, no evidence was obtained to suggest that the distributed nitrogen was used for growth of the nitrogen-deficient side ramet. In a previous study in blue sedge (Carex flacca) examining water and nitrogen transfer between ramets via the rhizome, the direction of nitrogen nutrient transport depended on the direction of water transport (De Kroon et al., 1998). In rice cultivars, the nitrogen-sufficient condition elevates the leaf transpiration rate compared to the limited condition (Xiong et al., 2015). Thus, it is likely that the translocation of nitrogen from the sufficient side to the deficient side against the water flow is limited between rhizome-connected ramets experiencing different nitrogen conditions. Our results suggest that most compensatorily acquired nitrogen is locally used for growth on the sufficient side. Given that an O. longistaminata cluster is a single clonal colony, this use of nitrogen might be a strategy to ensure the colony’s survival in limited and heterogeneous nitrogen conditions.

Notably, genes for the biosynthesis of tZ-type cytokinins, IPTs and CYP735As, were upregulated by the ammonium supply. Still, the expression levels and accumulation of tZ-type cytokinins (tZ and its precursors) were comparable between the +N and +N split treatments in our experiments (Figure 5B). Therefore, we hypothesize that cytokinin synthesis is similarly upregulated in response to both homogeneous and heterogeneous nitrogen conditions. Ammonium acquisition and amino acid synthesis, however, are compensatorily enhanced in the nitrogen-rich ramet roots under heterogeneous conditions, resulting in a greater supply of nitrogen assimilates for axillary bud outgrowth. Both the cytokinin signal and the supply of building blocks could contribute to the preferential axillary shoot growth.

In our analysis, iP and its precursors were in low abundance in the +N and +N split roots, mainly due to the decreased levels of its ribotide precursor (iPRPs; Figure 5B). At present, we do not have a clear explanation for the opposite trend despite the upregulation of its biosynthetic genes, OiIPTs. The iP ribotide precursor might be over consumed by CYP735As to produce tZ-type species.

Previous studies in O. sativa and O. longistaminata indicated that NADH-GOGAT1 is under the same control as IPT4 in the local nitrogen response and that glutamine-related signaling is involved in the regulation (Kamada-Nobusada et al., 2013; Ohashi et al., 2017; Shibasaki et al., 2021). OiNADH-GOGAT2, an isogene, is compensatorily upregulated in our experimental conditions, whereas OiNADH-GOGAT1 had a similar expression pattern to OiIPT4 (Supplemental Figure S7). In O. sativa, NADH-GOGAT2 is mainly expressed in mature leaves and plays a role in providing glutamate for the GS1;1 reaction in vascular tissues for nitrogen remobilization and recycling (Tabuchi et al., 2005, 2007; Yamaya and Kusano, 2014). Our RNAseq data show that OiGS1;1 is upregulated in nitrogen-deficient conditions (Supplemental Tables S4 and S5) in a manner distinct from OiNADH-GOGAT2. Thus, the physiological role of the cytosolic GS isoforms and NADH-GOGAT isoforms might be different between O. sativa and O. longistaminata.

Systemic regulation of nitrate acquisition in response to heterogeneous nitrogen nutrient conditions by CEP1–CEP–CEPD has been identified in Arabidopsis (Tabata et al., 2014; Okamoto et al., 2016; Ohkubo et al., 2017, 2021; Ruffel and Gojon, 2017; Ota et al., 2020). In our analysis, the O. longistaminata ortholog of OsCEP1 was markedly induced by nitrogen deprivation, and exogenous application of the synthetic CEP1 peptides increased the expression level of OiAMT1;2.
OlAMT1;3, and OlGS1;2 on the systemic side as well as the local side root. In addition, Ol12G001732, the closest ortholog of Arabidopsis CEPR1, was upregulated in systemic side shoots in response to a spatially heterogeneous ammonium supply (Supplemental Figure S5A) and also in systemic and local side shoots treated with CEP1 peptides (Supplemental Figure S6). This finding implies that CEP plays a role in inter-ramet communication as a nitrogen-deficient signaling molecule via the rhizome. Upregulation of Ol12G001732 expression might sensitize the root-derived CEP1-signal. In contrast, there were no GRX genes whose expression increased only in +N split shoots in our experimental conditions (Supplemental Figure S5B). At present, it is unclear whether the whole set of CEP–CPER–CEPD module function in Oryza species. The systemic response mechanism responding to heterogeneous nitrogen conditions in monocots, including rice, is still largely unexplored. Characterizing the whole system at the molecular level will require identifying and characterizing the CEP receptor and associated downstream factors in Oryza species.

Materials and methods

Plant materials and growth conditions

The perennial wild rice species O. longistaminata (IRGC10404) was hydroponically grown under natural light conditions in a greenhouse with a nutrient solution described by Kamachi et al. (1991), except that the solution contained 1-mM NH₄Cl as the sole nitrogen source and was renewed once every 3 or 4 d. Pairs of young ramets grown on the proximal nodes of rhizomes were excised and used in experiments. The growth stage of all ramet pairs was similar; each ramet had two to three fully developed leaves (Supplemental Figure S1).

Split treatment

The roots of each ramet pair, connected by rhizomes, were incubated in two separate pots (Supplemental Figure S1). Nutrient solution of 1 L was used for each ramet. For the +N split treatment, one of the ramet pairs was treated with the nutrient solution containing 2.5-mM NH₄Cl (+N split), and the other was treated with 0-mM NH₄Cl (–N split). For
comparison, both ramet pairs were treated with 2.5-mM NH₄Cl (+N) or 0-mM NH₄Cl (–N). Treatments for the growth analysis, and cytokinin and strigolactone response analysis were conducted in a greenhouse. All other treatments were conducted in a growth cabinet (LPH-411PFQDT-SPC; NK System, Osaka, Japan) with the following environmental conditions: 16-h (30°C) light/8-h (30°C) dark at 400 mol photons m⁻² s⁻¹.

15NH₄ tracer experiments
The ramet pairs were initially incubated with water for 3 d, followed by a 24-h split treatment with stable isotope-free nitrogen nutrient solutions (2.5-mM NH₄Cl for the +N ramet and the +N split ramet, and 0-mM NH₄Cl for the –N ramet). Roots of the ramets were soaked in 800 mL of 1 mM CaSO₄ for 1 min to remove the nitrogen treatment solution. The solution for the +N-treated side was then replaced with a solution containing 2.5-mM 15NH₄Cl (15N 9 atom%; Shoko Science Co., Ltd., Yokohama, Japan), and 15NH₄⁺ was allowed to be absorbed for 5 min. The roots of ramet pairs were then soaked in 600 mL of 1-mM CaSO₄ for 1 min to wash out 15N and then treated again with the stable isotope-free +N or –N hydroponic solutions for 22 h to measure the ammonium absorption activity and for 7 d to measure the absorbed nitrogen distribution with daily changing of the culture solution in a growth cabinet (LPH-411PFQDT-SPC, NK System) with 16 h (30°C) light/8 h (30°C) dark at 400-μmol photons m⁻² s⁻¹.

The above-ground tissues, rhizomes, and roots were separately harvested and dried in an oven at 70°C for at least 5 d. All dried tissues were weighed and ground into fine powders. The 15N and total nitrogen contents were analyzed by Shoko Science Co. with an elemental analyzer–isotope ratio mass spectrometer (Flash2000-DELTA plus Advantage ConFlo III System; Thermo Fisher Scientific, Waltham, MA, USA).

The absorption activity of ammonium was calculated as follows. The increase in 15N (μmol) in each sample (Δ15Ni) was calculated from Equation (1). W, dry weight (DW) of the sample (gDW); N, the total nitrogen concentration of the sample (%); 15Ns, the 15N concentration of the sample; and 15N₀, the naturally occurring 15N concentration (%) of the sample.

\[
\Delta^{15}N_i = W_i \times 10^6 \times \frac{N_i}{100} \times \frac{15N_i - 15N_0}{100} \times \frac{1}{15} \tag{1}
\]

The Δ15N of each sample (above-ground tissues and roots) were summed to obtain the increase of 15Ni on each root. The absorption activity of ammonium (μmol gDW⁻¹ h⁻¹) in roots under +N or +N split conditions was calculated using Equation (2). A, the absorption
activity of ammonium; $W_r$, the DW of the root that absorbed $^{15}$N.

$$A = \frac{\Delta^{15}N_w}{X} \times 1 \times \frac{60}{W_r} \times 5 \quad (2)$$

The increases in $^{15}$N in each part of the ramet pair ($\Delta^{15}N_s$) were summed to obtain the increase of $^{15}$N in the whole ramet pair ($\Delta^{15}N_w$), and the percentage (%) of $^{15}$N distributed in each part of the ramet pair was calculated using Equation (3). $P$, the percentage of $^{15}$N distributed in each part.

$$P = \frac{\Delta^{15}N_x}{\Delta^{15}N_s} \times 100 \quad (3)$$

RT-qPCR analysis

Total RNA was extracted from frozen and ground tissues using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with the RNase-Free-DNase Set (Qiagen) according to the supplier’s protocols. One microgram of total RNA was used to synthesize cDNA using the ReverTra Ace qPCR Master Mix (TOYOBO, Osaka, Japan) according to the supplier’s protocol. Twenty-nanogram cDNA was used for each RT-qPCR reaction with the KAPA SYBR FAST qPCR Master Mix (2X) (KAPA Biosystems, Wilmington, MA, USA) and a real-time PCR system (Applied Biosystems QuantStudio 3).

Expression levels were estimated using the relative quantification method (Livak and Schmittgen, 2001) with Ol/TBC, a homolog of TBC1 domain family member 22A (Maksup et al., 2013), the internal standard for normalization. Gene locus IDs and the specific primers used for amplification are listed in Supplemental Table S9.

Determination of free amino acids

Free amino acids were extracted as described by Konishi et al. (2014). Derivatization of amino acids was carried out using the AccQ-Tag Ultra Derivatization Kit (Waters Corp., Milford, MA, USA). The resulting AccQ-Tag-labeled derivatives were separated and quantified using an high-performance liquid chromatography (HPLC) System (Alliance 2695 HPLC system/2475, Waters Corp.) with the AccQTag Column (3.9 × 150 mm; Waters Corp.) as described in the instruction manual.

RNA-Seq analysis

Libraries were prepared with the TruSeq Standard mRNA Library Prep Kit (Illumina, San Diego, CA, USA) using 1 µg of total RNA. Sequence analysis of 40-M reads was performed with a NovaSeq 6000 Sequencing System (Illumina). Library preparation and sequencing were conducted by Macrogen Japan, Inc. (Tokyo, Japan). The sequencing reads were mapped to the O. longistaminata genome obtained from the O. longistaminata Information Resource (http://olinfres.nigac.jp/; Reuscher et al., 2018) using HISAT2 (Kim et al., 2019), followed by featureCounts (Liao et al., 2014) for counting reads and edgeR (Robinson et al., 2009) for differential expression analysis. Low expression genes were removed using filterByExpr function of edgeR with the default setting. We obtained the presumed function and MAPMAN BIN code for each gene and the presumed corresponding gene ID of O. sativa from Supplemental data 2 of Reuscher et al. (2018). GO analysis was performed using the corresponding O. sativa gene ID list with the analysis tool agrigo (http://systemsbiology.cau.edu.cn/agriGOv2/) (Du et al., 2010; Tian et al., 2017).

Growth analysis

Ramet pairs at a similar growth stage were first grown hydroponically in water for 4 d and subsequently exposed to nitrogen split conditions for 5 weeks in a greenhouse using the hydroponic culture solution with (+ N split, 2.5 mM NH$_4$Cl) or without (−N split, 0 mM NH$_4$Cl) nitrogen. The hydroponic solution was refreshed every 3–4 d. Growth changes were analyzed at 7-d intervals by measuring plant height, the number of fully expanded leaves, the number of axillary buds growing more than 1 cm, and the chlorophyll content. The chlorophyll content (SPAD value) was measured with a SPAD-502 Plus Chlorophyll Meter (Konica Minolta, Tokyo, Japan) at the tip, middle, and basal parts of leaves, and the average was taken as the SPAD value. For the controls, both ramets of each pair were fed 2.5 mM NH$_4$Cl (+N) or 0 mM NH$_4$Cl (−N)-containing hydroponic solution for 5 weeks.

Phytohormone quantification

Cytokinins were extracted and semi-purified from about 100-mg fresh weight (FW) of root tissues as described previously (Kojima et al., 2009). Cytokinins were quantified using an ultra-performance liquid chromatography (UPLC)-tandem quadrupole mass spectrometer (ACQUITY UPLC System/XEVO-TQXS; Waters Corp.) with an octadecylsilyl column (ACQUITY UPLC HSS T3, 1.8 µm, 2.1 mm × 100 mm, Waters Corp.; Kojima et al., 2009).

Peptide synthesis

OsCEP1a (DVRHypTNPGSHypGIGH), OsCEP1b (DVRHypTNHypGSHypGIGH), OsCEP1c (GVRHypTNPGSHypGIGH), and OsCEP1d (GVRHypTNHypGSHypGIGH) were synthesized on a CS 136X synthesizer (CS Bio, Menlo Park, CA, USA) using Fmoc solid phase peptide synthesis chemistry. Hydroxyproline (Hyp) was introduced with Fmoc- Hyp(Boc)-OH purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). The obtained crude peptides were purified by reverse-phase HPLC on Jasco, Inc. (Tokyo, Japan) preparative instruments with a Jupiter C18 column (5 µm, 300 Å pore size, 21.2-mm internal diameter × 250 mm) (Phenomenex, Torrance, CA, USA), and lyophilized to yield pure peptides.

Treatment with synthesized CEP1 peptides

Ramet pairs at a similar growth stage were first grown in hydroponic solution for 3 d in a growth chamber (LPH-411PFQDT-SPC, NK System). Some of the root tips (about 3 cm) from both ramets were removed with a razor blade.
to facilitate uptake of the exogenously supplied CEP1 peptides. Water on the root surface was wiped off with a Kim Towel (Nippon Paper Cressia, Tokyo, Japan). The root from one side of the ramet was immediately submerged into 100 mL of an ammonium solution containing CEP peptides (+ CEPs local: 2.5-mM NH4Cl, 30 μM of each CEP1a–d). Simultaneously, the root on the other side was submerged in the ammonium solution (systemic: 2.5-mM NH4Cl) for the indicated period. For the mock control, the roots of each ramet pair were treated with 2.5-mM NH4Cl.

Phylogenetic analysis
Alignment and phylogenetic reconstructions of Arabidopsis CEPDs and O. sativa glutaredoxin family proteins, GRX and GRL. Were performed using the function “build” of ETE3 version 3.1.1 (Huerta-Cepas et al., 2016) as implemented on the GenomeNet (https://www.genome.jp/tools/ete/).

Statistical analysis
Graphs represent the means ± standard error (SE) of biologically independent experiments using independently prepared ramet pairs. The statistical significance was assayed using means a two-tailed Student’s t test or Tukey’s honestly significant difference test (P < 0.05). The choice of test and the replicate numbers are provided in the corresponding figure legend.

Accession numbers
RNA-seq data were deposited to the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE182486. CEP1, At1g47485; CEPR1, At5g49660. Other locus IDs of genes (TAIR and RAP-DB) analyzed in this study are shown in Supplemental Figure S4 and Supplemental Tables S8 and S9.

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. The split hydroponic experimental system for O. longistaminata ramet pairs.

Supplemental Figure S2. A Venn diagram showing the overlap among genes upregulated in the –N treatment compared to the + N treatment, those upregulated in the –N split treatment compared to + N treatment, and those upregulated in the + N treatment compared to + N split treatment.

Supplemental Figure S3. Phylogenetic analysis of Arabidopsis CEPDs (AtCEPD1, AtCEPD2, AtCEDL1, and AtCEPDL2) and O. sativa glutaredoxin family proteins, GRX and GRL.

Supplemental Figure S4. An alignment of the amino acid sequences used for the phylogenetic analysis in Supplemental Figure S4.

Supplemental Figure S5. Expression pattern of OI12G001732 and OIGRXs.

Supplemental Figure S6. Expression of OI12G001732 in ramet shoots in response to exogenously supplied CEP1 peptides.

Supplemental Figure S7. Transcript abundance of OINADH-GOGAT1 in the roots of ramet pairs after a 24-h split treatment as measured by RT-qPCR.

Supplemental Table S1. Genes upregulated in the + N split treatment compared to the + N treatment.

Supplemental Table S2. Genes upregulated in the + N treatment compared to the –N treatment.

Supplemental Table S3. Genes upregulated in the + N treatment compared to the –N split treatment.

Supplemental Table S4. Genes upregulated in the –N treatment compared to the + N treatment.

Supplemental Table S5. Genes upregulated in the –N split treatment compared to the + N treatment.

Supplemental Table S6. Genes upregulated in the + N treatment compared to the + N split treatment.

Supplemental Table S7. Cytokinin concentrations in the roots of + N, + N split, –N split, and –N treated ramets.

Supplemental Table S8. Gene correspondence between O. sativa and O. longistaminata.

Supplemental Table S9. Primers used for RT-qPCR analysis.

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