Allantoin accumulation through overexpression of ureide permease1 improves rice growth under limited nitrogen conditions

Mark Christian Felipe R. Redillas1,*,‡, Seung Woon Bang†,‡, Dong-Keun Lee§, Youn Shic Kim†, Harin Jung‡, Pil Joong Chung§,†, Joo-Won Suh‡,* and Ju-Kon Kim*,†,‡

1Graduate School of International Agricultural Technology and Crop Biotechnology, Institute/GreenBio Science and Technology, Seoul National University, Pyeongchang, Korea
2Center for Nutraceutical and Pharmaceutical Materials, Division of Bioscience and Bioinformatics, Myongji University, Yongin, Gyeonggi, Korea

Summary
In legumes, nitrogen (N) can be stored as ureide allantoin and transported by ureide permease (UPS) from nodules to leaves where it is catabolized to release ammonium and assimilation to amino acids. In non-leguminous plants especially rice, information on its roles in N metabolism is scarce. Here, we show that OsUPS1 is localized in plasma membranes and are highly expressed in vascular tissues of rice. We further evaluated an activation tagging rice overexpressing OsUPS1 (OsUPS1OX) under several N regimes. Under normal field conditions, panicles from OsUPS1OX plants (14 days after flowering (DAF)) showed significant allantoin accumulation. Under hydroponic system at the vegetative stage, plants were exposed to N-starvation and measured the ammonium in roots after resupplying with ammonium sulphate. OsUPS1OX plants displayed higher ammonium uptake in roots compared to wild type (WT). When grown under low-N soil supplemented with different N-concentrations, OsUPS1OX exhibited better growth at 50% N showing higher chlorophyll, tiller number and at least 20% increase in shoot and root biomass relative to WT. To further confirm the effects of regulating the expression of OsUPS1, we evaluated whole-body-overexpressing plants driven by the GOS2 promoter (OsUPS1GOS2) as well as silencing plants (OsUPS1RNAi). We found significant accumulation of allantoin in leaves, stems and roots of OsUPS1GOS2 while in OsUPS1RNAi allantoin was significantly accumulated in roots. We propose that OsUPS1 is responsible for allantoin partitioning in rice and its overexpression can support plant growth through accumulation of allantoin in sink tissues which can be utilized when N is limiting.

Introduction
In crop production, nitrogen (N) supply and availability greatly influence the growth and yield of plants. To support the increasing demand in crops due to the rapid growth of world population, it is essential for crop researchers and breeders to come up with strategies to sustain production and meet demands. Improvement in crop management and agronomy coupled with conventional breeding and genetic engineering have been the major factors behind increased crop production (Han et al., 2015). However, for several decades synthetic N in the form of ammonium fertilizers showed to be the most practical way to increase crop production. In global cereal production alone, excessive use of synthetic N showed a dramatic increase from 11.6 Tg in 1961 to 104 Tg in 2006 (Mulvaney et al., 2009). Though high N input can increase yield, plants however do not take up these N completely and more than 50% are leached in soils resulting in the contamination of the environment (Gruber and Galloway, 2008).

 Nitrogen is transported in a plant vascular system in different forms depending on the plant species. Rice typically utilizes ammonia for assimilation to synthesize protein and other biochemical compounds once it enters the GS/GOGAT cycle (Xu et al., 2012). Other N forms such nitrate as well as asparagine and glutamine (amides) are also used for transport (Pate et al., 1980). Exogenous N is incorporated in plants in several steps starting from uptake followed by assimilation, translocation and recycling and remobilization at later stages each with its own enzymes and substrates. On the other hand, a number of N fixing plants, which are capable of utilizing atmospheric N through symbiotic relationship with rhizobia found within nodules of the roots, transport N in a different form.

Tropical nodulating legumes utilize the ureides, allantoin and allantoic acid, as the main transport form of organic N (Alamillo et al., 2010, Desimone et al., 2002; Pate et al., 1980; Pelissier et al., 2004). When symbiotic bacteria are present in nodules of N2-fixing plants, they produce ammonium which is then used for the synthesis of purines and uric acid. These uric acid are transported to neighbouring uninfected cells which are then used to synthesize allantoin in peroxisomes (Hanks et al., 1981). Allantoin and its first derivative allantoic acid play important roles in the assimilation and metabolism of N. In soybean, allantoin in cotyledons is transported to the shoot axis during germination (Duran and Todd, 2012) and concentration is highest in stems of green pods (Matsumoto et al., 1977). For example,
tropical and sub-tropical legumes such as Vigna unguiculata (cowpea), Glycine max (soybean) and Phaseolus vulgaris (French bean) dominantly transport ureides instead of amino acids under N-fixing conditions. Ureides also amount to 20 or 10 mM in the stem tissue or xylem (Layzell and Larue, 1982; Rainbird et al., 1984) and 94 mM in nodule exudates (Pelisser et al., 2004; Streeter, 1979). These ureides are loaded into the network of nodule-root xylem vessels and transported to aerial organs by the transpirational water current which is then enzymatically catabolized in series of reactions to yield ammonium (Baral et al., 2016). This ureide strategy is said to be more efficient than the amide strategy because of its lower carbon to N ratio, able to release four molecules of ammonium per allantoin, and utilized for plant survival under stressed conditions (Sagi et al., 1998; Todde et al., 2006; Watanabe et al., 2014). Allantoin level also responds to exogenous N status such that the concentration decreases under non-nodulating conditions where the amount of N present in soil is high (Amarante et al., 2006; Pate et al., 1980).

Ureides are produced through purine degradation and oxidation in the purine metabolism pathway (Brychkova et al., 2008; Werner and Witte, 2011; Werner et al., 2010; Zrenner et al., 2006). Catabolism of allantoin involves the first key enzyme called allantoinase (ALN) which produces allantoate/allantoic acid (Werner et al., 2013) and when completely broken down to glyoxylate releases ammonium ions as by-products which is then utilized during remobilization or periods of high N demand (Muriz et al., 2001; Watanabe et al., 2014; Yang and Han, 2004). Transport of the ureides have been well described in legumes which are facilitated by ureide permease (UPS) proteins (Collier and Tegeder, 2012; Desimone et al., 2002; Lescano et al., 2016; Pelisser et al., 2004). Five UPS genes were reported in Arabidopsis (Desimone et al., 2002; Schmidt et al., 2004, 2006), one in French bean (Pelisser et al., 2004) and two in soybean (Collier and Tegeder, 2012).

Our study is the first to evaluate the effects of UPS overexpression on the allantoin content in cereal crops such as rice. We evaluated an activation tagging line where the rice ureide permease 1 gene (OsUPS1) was highly activated due to a T-DNA containing a tetramerized 35S enhancer sequence inserted at 1.1 kb upstream of the transcriptional start site of OsUPS1 resulting in allantoin accumulation. In addition, the overexpression of OsUPS1 gene resulted in the changes in the metabolic profile of panicles as such asparagine and glutamine during the grain filling stage and exhibited improved growth under suboptimal N conditions. Allantoin partitioning was also altered in overexpression plants driven by a GOS2 promoter (OsUPS1GOS2) as well as in silencing plants (OsUPS1RNAi). We demonstrate that OsUPS1 is beneficial to rice under N-limited conditions.

**Results**

**Characterization of OsUPS1 expression**

The metabolite allantoin is a potential N source in non-leguminous plants which can be utilized under N-limiting conditions, thus we characterized one of its transporters in rice. The rice ureide permease 1 (OsUPS1) is a homologue of those found in legumes such as soybean (GmUPS1) and French bean (PvUPS1) which are known transporters of ureides. To confirm the subcellular localization of OsUPS1, we linked the coding sequence of OsUPS1 without the stop codon to GFP. The cassette was driven by a 35S promoter and inserted into the pHBT vector (GenBank accession number EF090408) producing the plasmid 35S:OsUPS1-GFP. Plasmids were then transiently co-expressed with a plasma membrane marker (CD3-1007) (Nelson et al., 2007) bearing an mCherry protein in rice protoplasts. Another cassette without the OsUPS1 gene was used as control producing the plasmid 35S:GFP. Similar to other reported UPS genes, the GFP signal overlapped with the PM marker confirming the transmembrane localization of OsUPS1 (Figure 1a). In addition, in situ hybridization analysis on the stem section of 6-day-old rice seedlings revealed intense signal around the vascular tissues (Figure 1b) similar to what was observed in soybean (Collier and Tegeder, 2012). Phylogenetic analysis on UPS proteins showed that legumes form a separate clade with non-leguminous plants such as Arabidopsis and rice (Figure S1). Similar to other UPS proteins, OsUPS1 has the same configuration of transmembrane localization wherein 10 helical domains span across the membrane with a large central loop in the middle (Figure S2).

To characterize the endogenous expression of OsUPS1, we extracted total RNA from different tissues collected from different growth stages of rice. qRT-PCR showed that OsUPS1 was highly expressed in seedlings younger than 7 days and the expression significantly declined in leaves as the plants mature. The expression of OsUPS1 was significantly high in roots, stem and flowers compared to leaves (Figure 1c). To determine whether OsUPS1 is responsive to exogenous N status, 3-week-old rice plants (Oryza sativa var. Japonica) grown in Yoshida solution containing 2.8 mM N ((NH₄)₂SO₄) were exposed to N-starvation for 10 days by feeding with Yoshida solution minus (NH₄)₂SO₄. Plants were then resupplied with the same N-concentration starting on the 11th day. Results showed that transcript levels of OsUPS1 were quickly down-regulated within 24 h of N-starvation and almost no transcripts were detected starting on the 7th day (Figure 1d). When N was re-introduced, OsUPS1 expression increased within 12 h suggesting that OsUPS1 was closely associated with exogenous ammonium status.

**T-DNA insertion activates expression of OsUPS1**

To understand the role of OsUPS1 in rice, we acquired a T-DNA activation tagging line kindly provided by Prof. Gyuneung An of Kyung Hee University, Korea. The T-DNA contains a tetramerized 35S enhancer sequence at the left border of the pGA2772 vector (Figure 2a) (An et al., 2003). In silico BLAT analysis of the flanking sequence showed that the T-DNA was inserted upstream of OsUPS1 and the left border was oriented towards the OsUPS1 gene. To confirm the insertion site, genomic PCR using primers specific to the T-DNA right border and the plant genome confirmed the insertion of T-DNA 1.1 kb upstream of the transcriptional start site considered as the promoter region of OsUPS1 producing the activation tagging plants OsUPS1OX. In addition, we found two putative UPS genes located upstream (Os12 g5053300) and downstream (Os12 g502800) of OsUPS1 based on annotation and protein sequence similarity (Figure 2b).

To determine the expression of OsUPS1 in the activation plants, total RNA from leaves of randomly selected 1-week-old homozygous T₃ OsUPS1OX plants was extracted and for qRT-PCR. Transcript levels of OsUPS1 were highly up-regulated in leaves of young plants (Figure 2c) confirming that the 35S enhancer sequence present in the T-DNA cassette was effective in activating the expression of endogenous OsUPS1 in OsUPS1OX plants. At the reproductive stage 14 days after flowering (14 DAF), we sacrificed three sister lines of OsUPS1OX plants as well as WT and collected the flag leaves, leaves (the leaves right below
the flag leaf) and panicles. From these tissues, we extracted total RNA qRT-PCR analysis. Relative to WT, OsUPS1 expression in leaf, flag leaf and panicle were all highly induced (Figure 2d). To determine whether the enhancer sequence affects the expression of nearby genes, qRT-PCR was performed on genes Os12 g0502800 (downstream) and Os12 g0503300 (upstream) (Figure 2e). Transcript levels of both genes were not activated indicating that the effect of the enhancer was limited to OsUPS1 and is a good material for further analysis, thus we further analysed these tissues for metabolite analysis.

Metabolites are altered in sink tissues of mature OsUPS1OX plants

To determine the effects of OsUPS1 overexpression on the endogenous allantoin content in OsUPS1OX, allantoin was measured in leaf, flag leaf and panicle tissues of 14 DAF plants. Following the overexpression of OsUPS1, the endogenous concentration of allantoin in leaves was higher than WT and more significantly in panicles (Figure 3a). Since the sampling time was done during active stage of nutrient remobilization, and since allantoin can serve as ammonium source when catabolized we further measured the ammonium and free amino acids. Ammonium levels in all tissues were relatively higher in OsUPS1OX than WT but was significantly higher in flag leaf (Figure 3b). The total free amino acid content in leaf tissues were relatively similar in both plants while in panicles, OsUPS1OX showed almost twice to those of WT (Figure 3c, Table S1). The amino acids aspartic acid (Asp), asparagine (Asn), glutamine (Gln) and glutamic acid (Glu), which are closely linked to N-assimilation, showed relatively similar concentration in leaf of OsUPS1OX and WT (Figure 3d–e). Since glutamine is required for the production of allantoin following ammonium assimilation, we further tested the OsUPS1OX plants response to ammonium starvation and repletion at the root level.

Ammonium level is elevated in roots of OsUPS1 overexpressing plants

In legumes, allantoin synthesis requires ammonium released during N-fixation while in non-leguminous plants, ammonium is imported in roots through the action of ammonium transporters. Since we observed elevated levels of allantoin in sink tissues of mature OsUPS1OX plants, we measured the ammonium in roots of plants...
that were normally fed N, or starved for 10 days and those that were resupplied following starvation. At normal N-feeding concentration of 1 mM N, ammonium content in OsUPS1\(^{\text{OX}}\) was twice to those of WT while both plants showed relative ammonium level after 10 days of N-starvation. During N-repletion, three N-concentrations were used, that is, 0.01, 0.1 and 1 mM N to determine whether OsUPS1\(^{\text{OX}}\) will show an advantage over WT in terms of ammonium uptake. At ammonium concentration of 0.01 mM, both OsUPS1\(^{\text{OX}}\) and WT plants were relatively similar in ammonium content though at 1 and 3 h after N-feeding OsUPS1\(^{\text{OX}}\) showed slightly elevated content than WT (Figure 4a). At 0.1 mM N, OsUPS1\(^{\text{OX}}\) showed higher ammonium content with a more pronounced accumulation peak at 3 h compared to WT (Figure 4b). When fed 1 mM N, OsUPS1\(^{\text{OX}}\) showed higher ammonium content than WT reaching a peak at 12 h before depleting back to a concentration close to the control plants suggesting that OsUPS1 expression affects the ammonium uptake of rice (Figure 4c). These changes in ammonium concentration also showed a more positive slope in OsUPS1\(^{\text{OX}}\) compared to WT under different N-concentrations (Figure 4d–f).

Purine synthesis pathway is induced in OsUPS1\(^{\text{OX}}\) plants

Since glutamine is used as a substrate for purines synthesis, we measured the transcript levels of enzymes involved in the purine de novo synthesis pathway (Stasolla et al., 2003) of OsUPS1\(^{\text{OX}}\) roots grown under normal conditions. We found that amido phosphoribosyltransferases 1, 2 and 3 (Atase1, 2, 3), GAR synthetase (GARS), GAR formyl transferase (GART), FGAM synthetase (FGAMS), AIR synthetase (AIRS), AIR carboxylase (AIRC), adenylosuccinate lyase (ASL), AICAR formyl transferase (ATIC), SAMP synthetase (ASS), IMP dehydrogenase (IMPDH) and GMP synthetase (GMPs) were all highly up-regulated in OsUPS1\(^{\text{OX}}\) plants compared to WT (Figure S3). These suggest that the purine synthesis pathway was induced in OsUPS1\(^{\text{OX}}\) plants. In addition, the transcript levels of the enzymes xanthine dehydrogenase (XDH) and uricase (uricase), which are involved in the conversion of purines to allantoin, did not differ to those of WT (Figure S4) suggesting that the turnover of purines to allantoin was not feedback inhibited, thus allowing allantoin to accumulate. We also found that the Ct values of these genes were similar to those of the internal control OsUbi1 suggesting that the endogenous expression of XDH and uricase was already very high. It appears then that in rice, the production of allantoin is not solely dependent on the expression of the two key upstream genes for allantoin synthesis but also the expression of the genes in the purine synthesis pathway such that the process leans towards the biosynthesis of allantoin when induced. Furthermore, we measured the transcripts of the enzymes in the allantoin degradation pathway starting from the Allantoinase (ALN), the key enzyme responsible for the start of allantoin degradation, Allantoate amidohydrolase (AAH), Ureidoglycine amino hydrolase (UGAH) and Ureidoglycolate amidohydrolase (UH) and found that the transcript levels were relatively similar to WT (Figure S5) indicating that the increase in purine and allantoin synthesis was not coupled with allantoin degradation, thereby allowing it to accumulate.
Vegetative growth of OsUPS1OX plants is improved under suboptimal N supply

To determine whether the increased ammonium uptake and elevated allantoin concentration of OsUPS1OX plants support plant growth under suboptimal N conditions, we compared its growth with WT under low-N soil at the vegetative stage. Uniform seedlings of 7-day-old OsUPS1OX and WT plants pre-germinated in MS media were transplanted in a 4 L pot containing equal weight of low-N soil. Plants were then allowed to grow inside a glass house. All pots were rotated twice a week to avoid positional effects. In general, shoot and root phenotype of plants receiving higher N showed better vigour compared to those receiving less (Figure 5 a–b). OsUPS1OX was significantly taller than WT under both 100 and 50% N regime (Figure 5c). Chlorophyll content, represented by the SPAD values, was significantly higher in OsUPS1OX compared to WT (Figure 5d). In addition, it has been reported that tiller number of rice can give an indication of N availability such that soils with sufficient N result in higher plant tiller number compared to those receiving less (Wada et al., 1986). Here, we found that below the 100% N regime, OsUPS1OX consistently showed higher number of tillers compared to WT (Figure 5e). In addition, the shoot and root biomass of OsUPS1OX at 50% N was 26% and 20% higher than WT, respectively (Figure 5f). Collectively, these suggest that OsUPS1OX plants were well adapted in soils having suboptimal N content compared to WT.

Allantoin partitioning is reflected in constitutive whole-body overexpression and down-regulation of OsUPS1

To further confirm whether the regulation of OsUPS1 expression alters the allantoin partitioning in rice, we generated another overexpression lines using the whole-body overexpression GOS2 promoter (OsUPS1GOS2 1, 2, 13) as well as silencing lines for down-regulation (OsUPS1RNAi 10, 20, 33). Transcript levels of OsUPS1 in three independent homozygous plants showed significant up- and down-regulation of OsUPS1 in OsUPS1GOS2 and OsUPS1RNAi plants, respectively (Figure 6a). Quantification of allantoin in grains of OsUPS1GOS2 plants showed significant

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accumulation similar to what was observed in panicles of OsUPS1\textsuperscript{OX} activation lines (Figure 3a) while silencing lines showed significantly lower allantoin content compared to WT (Figure 6b). In addition, tissues from vegetative leaves, stem and roots of OsUPS1\textsuperscript{GOS2} lines showed significant increase in allantoin content (Figure 6c). Leaves of OsUPS1\textsuperscript{GOS2} plants exhibited the highest increase relative to WT while OsUPS1\textsuperscript{RNAi} showed the lowest allantoin content. Interestingly, the roots of OsUPS1\textsuperscript{RNAi} showed elevated allantoin relative to WT as a result of the down-regulation of OsUPS1 confirming that regulation of OsUPS1 expression does alter the partitioning of allantoin in rice. To confirm whether allantoin concentration is indeed catabolized during periods of N-starvation content was observed in OsUPS1\textsuperscript{GOS2} and OsUPS1\textsuperscript{RNAi} lines the ammonium content in roots when resupplied with N following 10 days of N-starvation (Figure 6e). Under normal N-feeding of 1 mM, OsUPS1\textsuperscript{GOS2} plants showed elevated ammonium content while OsUPS1\textsuperscript{RNAi} showed lower amount relative to WT. When exposed to complete removal of N source for 10 days, all plants showed similar ammonium level. During N-repletion with 1 mM N, OsUPS1\textsuperscript{GOS2} plants showed a faster accumulation of ammonium similar to what was observed in OsUPS1\textsuperscript{OX} activation plants (Figure 4) which also exhibited a peak during 12 h of N-feeding and subsequently reduced on the 24th h. On the other hand, though OsUPS1\textsuperscript{RNAi} showed similar pattern, the ammonium content did not significantly differ with those of WT.

Since there was an increase in ammonium uptake in OsUPS1\textsuperscript{GOS2}, we further measured the amino acid contents in both roots and leaves of plants. We found a higher free amino acid content in the young leaves of OsUPS1\textsuperscript{GOS2} compared to those of OsUPS1\textsuperscript{RNAi} and WT (Figure S6). Similar to the panicles of OsUPS1\textsuperscript{OX}, the increase in the free amino acid content was also due to the asparagine and glutamine fractions. Though there was an increase of glutamine in the sink tissues aboveground, the roots however showed no accumulation of glutamine which...
suggests that the glutamine pool present in roots was maintained possibly to a homeostatic level. This was probably achieved by readily utilizing glutamine as substrate for purine synthesis while simultaneously transporting some fractions to the shoot as observed in OsUPS1 overexpressing plants but not in WT and OsUPS1RNAi plants, thus avoiding accumulation in roots (Figure S7).

To determine whether the yield was affected in OsUPS1GOS2 and OsUPS1RNAI plants, we scored the grain filling rate under both normally fed (100%) and under N-limited conditions (50%, 20% and 0% N) Figure S8. We found that among the four N-feeding regimes, it was at the 20% feeding that OsUPS1GOS2 started to show a higher filling rate than OsUPS1RNAI and WT. In addition, one line from OsUPS1GOS2 exhibited a highly significant increase in filling rate at 20% N. It was however at 0% N-feeding that two out of the three OsUPS1GOS2 lines showed a very significant difference between OsUPS1RNAI and WT lines. These observations were even better than what we have observed in OsUSP1OX plants which showed growth advantage when N was lowered to 50% N. These results suggest that the increase in internal allantoin concentration due to OsUPS1 overexpression results in plants that are better adapted in N-limited growth conditions. Collectively, the overexpression of OsUPS1 either through gene activation or through the use of an overexpression promoter, can alter the allantoin partitioning and concentration in sink tissues, ammonium uptake in roots, as well as the glutamine fractions in sink tissues resulting in enhanced growth under limited N availability.

Discussion

In this study, we evaluated the activation tagging plants overexpressing the rice ureide permease1 (OsUPS1OX) and showing significant allantoin accumulation in panicles of 14 DAF plants compared to WT (Figure 3a). Though present in rice, the role of
allantoin as a N source in rice is not established yet but has been reported to be involved in important biological processes such as stress tolerance (Casartelli et al., 2018; Degenkolbe et al., 2013; Nam et al., 2015) and as a growth enhancer (Wang et al., 2012). Allantoin is a stable nitrogen-rich heterocyclic compound that is a product of purine metabolism and a form N storage in ureide-metabolizing plants. The synthesis of allantoin in legumes requires ammonium, either acquired from soil or from N-fixation, which is assimilated into amino acids and ultimately incorporated into amide amino acids (asparagine or glutamine) or ureides (allantoin) for export to leaves (Buchanan et al., 2000). When catabolized, allantoin can produce four molar equivalents of ammonium (Zrenner et al., 2006) which is re-assimilated to produce amino acids.

Here, we found that OsUPS1 expression was regulated by the external N status such that OsUPS1 was down-regulated both in roots and leaves during complete removal of N source and rapidly
increased when N was resupplied (Figure 1e). A possible explanation is that under normal N conditions, OsUPS1 loads ureides into the xylem for transport to the aerial parts through the transpirational stream similar to what was described by Baral et al. (2016). This can be supported by the expression levels of OsUPS1 being high in roots and stems of plants under sufficient N (Figure 1d), indicative of transport from roots to shoots. However, in absence of N OsUPS1 was down-regulated possibly to limit the transport of allantoin to shoot allowing its breakdown to provide ammonium ions in roots. We hypothesize that due to overexpression of OsUPS1 there was a persistent transport of allantoin away from the source roots and towards the sink shoot tissues leading to accumulation in shoots. Thus, overexpression of OsUPS1 could provide N in plants by increasing the loading and transport of allantoin to sink tissues where it can be utilized later when the available N in soil becomes limited. In peas, allantoin is transported via the xylem to the leaves where it is either stored or metabolized and loaded into the phloem for transport to the growing parts of the plants (Atkins et al., 1982). Subcellular localization showed that OsUPS1 is located in plasma membranes and expressed in vascular tissues including the surrounding companion cells (Figure 1a–b). In French beans, PvUPS1 is expressed in phloem for long distance transport throughout the whole plant (Pelissier and Tegeder, 2007) and in soybean, both GmUPS1-1 and GmUPS1-2 are localized in plasma membranes and expressed in nodule cortex and vascular endodermis (Collier and Tegeder, 2012). These suggest that ureides are transported to locations where N is required either to roots or to shoots. The accumulation of allantoin in panicles of 14 DAF plants suggests roles of allantoin during N remobilization for grain filling. We further confirmed this in grains, leaves, stems and roots of OsUPS1 ox/ox overexpression plants indicating higher loading to sink tissues (Figure 6b–c). Similarly, when the common bean UPS1 transporter was expressed in cortex and endodermis cells of soybean nodules the delivery of N to shoot was significantly increased improving shoot N nutrition and seed development in legumes (Carter and Tegeder, 2016). In contrast, our OsUPS1RNAi silencing lines showed lower allantoin content in similar tissues except in roots where allantoin showed accumulation further indicating that OsUPS1 is required for loading of allantoin to vascular tissues. In soybean, the repressed expression of GmUPS1 through RNA interference showed accumulation of allantoin and allantoic acid in nodules, a decrease of the ureides in roots and xylem sap and resulted in N deficiency symptoms in leaves (Collier and Tegeder, 2012). We however did not find any N-deficient symptoms in OsUPS1 ROE plants even though the allantoin in shoot was reduced.

In rice, the concentration of allantoin is highest in stem during stem elongation period or panicle initiation to booting stages and decreases dramatically during maturity (Wang et al., 2010). This coincide with the reported transport pattern of allantoin where during senescence they are remobilized from vegetative tissues to seeds (Aveline et al., 1995; Diaz-Leal et al., 2012; Thomas and Schrader, 1981) and utilized during germination where it is transported from cotyledons to shoot axis (Duran and Todd, 2012; Quiles et al., 2009).

Since catabolism of allantoin releases ammonium, these are then utilized for assimilation and synthesis of amino acids (Werner and Witte, 2011). We found significantly high fractions of asparagine and glutamine in panicles of OsUPS1 ox/ox compared to WT. The concentration of these two amino acids provides information on the N status in plants since they represent the primary amino acids derived from ammonium (Frunzillo et al., 2014). Soluble asparagine accumulates under a plentiful supply of reduced N (Lea et al., 2007) however, stress-induced accumulation of asparagine is also possible (Lea and Miflin, 2010). Since both OsUPS1 ox/ox and WT controls were grown in the same cultivating season and paddy field, effects of these stress-induced factors are minimal, if there is any. It is therefore likely that the altered free amino acid profile in the OsUPS1 ox/ox was a result of a high supply of reduced N due to the higher ammonium uptake of OsUPS1 overexpressing plants as shown in the plants response to N-repletion after being starved for 10 days (Figure 4). We also found that the expression of OsNRT2.3, a transporter known to be repressed by elevated ammonium content (Feng et al., 2011; Yan et al., 2011), was down-regulated in leaves of OsUPS1 ox/ox (Figure S9), which was consistent with high level of ammonium in OsUPS1 ox/ox leaves (Figure 3b). The accumulation of glutamine in panicles could also be a result of high ammonium availability in OsUPS1 ox/ox compared to WT. In addition, it is well established that both ammonium and glutamine are required for the synthesis of allantoin. For instance, in nodulated plants, bacteroids produce ammonium in nodules that are then transported to the cytosol where assimilation to glutamine occurs. Glutamine is then either transported to the shoot or utilized in the purine de novo synthesis pathway in plastids (Shep and Ireland, 1985) or mitochondria (Atkins and Storer, 1997). In other studies, it was reported that changes in ureide concentration in legumes are reflected in the concentration of glutamine and the response to the metabolites is related (amarante et al., 2006). Similar to legumes, both overexpression lines OsUPS1 ox/ox and OsUPS1 LOX showed higher ammonium content in roots of N-replete plants following a 10-day N-starvation. There was also a significant increase in the transcript levels of the enzymes found in the purine de novo synthesis pathway suggesting an increased turnover of glutamine to purines. A recent study by Colote et al. (2016) actually showed that in P. vulgaris, PvPRAT3 is responsible for the de novo synthesis of purines which in turn is utilized for ureide synthesis. This suggests that changes in the purine synthesis pathway can also affect the downstream ureide metabolism pathway. Generally, plants do not just accumulate purines but readily interconvert them to different biomolecules and one of these is allantoin. When allantoin is produced, it is transported to sink tissues such as the leaves where it can be stored or catabolized to supply N. Thus, the cycle of allantoin production in roots and catabolism in shoot is more pronounced in OsUPS1 ox/ox than in WT. These suggest that the overexpression of OsUPS1 can alter the N status of plants through enhanced production and translocation of allantoin.

Varying the exogenous N levels also influenced the overall vitality of plants as manifested when OsUPS1 ox/ox and WT plants were grown in soil with suboptimal N-concentrations. We observed that OsUPS1 ox/ox seemed to show an advantage over WT by exhibiting overall vigour, taller plant height, higher tiller number and biomass as well as higher SPAD values in soil having 50% N (Figure 5) indicating that overexpression of a ureide transporter can bring benefits to plants under N-limited conditions. (Tegeder, 2014) has reported that altering the import of amino acids or ureides into the collection phloem could improve biomass of plants at the vegetative stage. Indeed, we found that overexpression of OsUPS1 resulted in higher plant biomass at 50% N-feeding. In addition, the roots of OsUPS1 ox/ox plants fed 100% N showed reduced length compared to WT which is a general response of plants fed sufficient ammonium supply (Chen...
et al., 2013). Similarly, Hoque et al. (2006) reported that transgenic plants overexpressing OsAMT1-1 showed increased biomass only during lower ammonium feeding and suggested that the increased ammonium uptake was balanced with ammonium assimilation. The same can be claimed in our results where reducing the N application showed to be more beneficial to OsUPS1OX plants than at higher N.

In summary, we showed that OsUPS1 overexpression resulted in allantoin accumulation especially in sink tissues such as in grains of mature plants or in leaves and stems of young plants. We also showed that the increase in allantoin production resulted in an elevated ammonium uptake and N-assimilation to glutamine. Under limited N conditions, plants overexpressing OsUPS1 showed growth advantage over WT due to the availability of allantoin as an additional N source. Thus, we hypothesize that OsUPS1 is responsible for exporting allantoin out of the source cells across the plasma membrane and loading into the xylem for transport through the transpirational stream. In addition, the persistent export of allantoin out of the source roots promotes the synthesis of allantoin resulting in elevated allantoin concentration in plants overexpressing OsUPS1. We therefore propose a model for the improved growth of OsUPS1 overexpressing plants under limited N conditions (Figure 7). To produce higher ureide (allantoin) in rice plants that generally adopts the amide strategy for N metabolism, plants must increase the ammonium uptake for assimilation of N (ammonium) to glutamine. Glutamine then proceeds to two fates—first is the immediate transport to sink tissues where it can accumulate either in leaves of young plants or in grains of mature plants or second, serves as the substrate for purine synthesis leading to the synthesis of allantoin. Therefore, the persistent transport of allantoin in roots to shoots promoted the OsUPS1 overexpressing plants to uptake more ammonium leading to increased glutamine fractions, activation of purine synthesis pathway, and increased production of allantoin which in turn conferred growth advantage to the plants grown under limited N conditions.

Experimental procedures

Plant material, genotyping and rice transformation

T-DNA insertion line (PFG_2D-4064.L) was provided by Prof. Gynheung An of Kyunghee University and amplified on a rice paddy field. In this study, T3 seeds were planted on a rice paddy field at Kyungpook National University, Gunwi (128:34E/36:15N), Korea. Wild type (WT) Oryza sativa L. ssp. japonica cv. Dongjin and OsUPS1OX seedlings were transplanted into pots 25 days after sowing. Fertilizer was applied at 70N/40P/70K kg/ha after transplanting. For ammonium uptake assay, T4 plants were propagated in a rice paddy field at Kyungpook National University, Gunwi (128:34E/36:15N), Korea. T3 plants were used for further analysis.

Ammonium uptake assay

For ammonium uptake, T4 OsUPS1OX and WT plants were grown in a 10 L tank containing Yoshida solution (Yoshida et al., 1976) for 3 weeks. The solution was changed every 3 days to ensure consistent supply of nutrients. On the 4th week, plant roots were washed with running water and plants were transferred to a new tank containing Yoshida solution without N and allowed to grow for 10 more days. After 10 days of N-starvation, ammonium sulphate corresponding to three N-concentrations (0.01, 0.1 and 1 mM) was introduced. Before sampling, roots were washed with water containing 1 mM CaSO4 for 1 min to remove any ammonium present on the surface of roots. For confirmation using different overexpression system, three independent homozygous T3 OsUPS1GOS2 (1, 2, 13) and OsUPS1RNAi (10, 20, 32) were separated by a GUS sequence of the p700-GOS2-RNAi vector (Lee et al., 2016) through Gateway system (Invitrogen, Carlsbad, CA). Plasmids were introduced into Agrobacterium tumefaciens LBA4404 through triparental mating and embryonic calli from rice seeds (Oryza sativa L. ssp. japonica cv. Dongjin) were transformed as previously described (Jang et al., 1999). Copy number were determined in T0 plants through Taq-Man PCR as described by (Bang et al., 2015) and single-copy plants were selected and propagated in a rice paddy field at Kyungpook National University, Gunwi (128:34E/36:15N), Korea. T3 plants were used for further analysis.

Figure 7 Schematic representation of allantoin partitioning in OsUPS1-overexpressing plants. OsUPS1-overexpressing plants OsUPS1GOS2 and OsUPS1GOS2 both showed higher allantoin content in their sink tissues compared to WT and OsUPS1RNAi. This can be attributed to the proposed exporter function of Ureide permease1 in roots. The persistent loading of allantoin into the vascular tissues resulted in the increased tendency of plants to synthesize more allantoin in roots. As a result, plants increased its ammonium uptake, thus elevating the glutamine fractions which follows to fates-first, transported to shoot where it can accumulate in sink tissues and second, used as substrate for purine and allantoin synthesis.
OsUPS1 overexpression improves rice growth under low N 1299

33) as well as WT plants were subjected to the same stress as the OsUPS1ox plants. Samples were ground in liquid nitrogen and kept in a –80 °C freezer until use.

Metabolite analysis

Amino acid quantification was sent to National Instrumentation Center for Environmental Management (NICEM) in Seoul National University, Korea. Analysis was done using HPLC Ultimate 3000 equipped with column VD Spher 100 C18-E (4.6 mm × 150 mm, 3.5 μmVDS, Optiliab, Germany) and FL detector 1200 FLD (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer’s manual. For allantoin quantification, samples were analysed based on differential chemical hydrolysis of ureides and measured using a spectrophotometer (NanoQuant, Infinite M200, Switzerland) at 535 nm wavelength as described by (Vogels and Van der Drift, 1970). Concentration was measured according to the standard curves for allantoin (Sigma-Aldrich, Korea), allantoin acid (TRC, Canada) and glyoxylate standards (Sigma-Aldrich, Korea).

For ammonium analysis, the Berthelot reaction was followed with some modifications. Preweighed ground samples were first extracted with 2% sulpho-salisylic acid and passed through a syringe filter (ADVANTEC, Toyo Roshi Kaisha LTD., Japan). The reagents were Reagent A (0.33 mM sodium phenolate in 2 N NaOH with pH adjusted to pH 13), Reagent B (0.01% sodium nitroprussiate in water) and Reagent C (26 mM sodium hydrochlorite). Ten μL of each extract was reacted with 50 μL of each of the reagents in a sequential manner in triplicates. The reaction was performed in a 96 well plate (SPL Life Sciences, Korea) for 1 h and read through a spectrophotometer (NanoQuant, Infinite M200, Switzerland) at 635 nm wavelength. Results were then compared through a standard curve using ammonium sulphate (Sigma-Aldrich, http://www.sigmaaldrich.com) as standard compound. For glutamine analysis, the glutamine analysis kit from (USBiological Life Sciences, Marblehead, MA, USA) was performed according the manufacturer’s instructions.

Subcellular localization and microscopy

Leaf sheaths from 50 etiolated rice seedlings were cut into 1- to 2- mm pieces using a fresh sharp razor blade on a glass plate. The leaf sheath pieces were quickly transferred to 10 mL digestion buffer solution containing 5 mM KC1, 5 mM D-glucose 2 mM MES, pH 5.7) was done and the pooled collected protoplasts. The suspension was passed through 70 μm and 40 μm nylon mesh. The pooled suspension was centrifuged at 320 g for 8 min and washed three times with WS solution. The pellet was resuspended in MMg solution (4 mM MES, pH 5.7, 0.6 mM mannitol, and 15 mM MgCl2). Protoplasts were quantified by microscopy using a haemocytometer before measurement.

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Subcellular localization and microscopy

Leaf sheaths from 50 etiolated rice seedlings were cut into 1- to 2- mm pieces using a fresh sharp razor blade on a glass plate. The leaf sheath pieces were quickly transferred to 10 mL digestion solution containing 0.6 mM mannitol, 0.6 mM 4-morpholinoeethanesulfonic acid (MES), 1.5% cellulase RS (Yakult, Japan), 0.75% macerozyme (Yakult, Japan), 1 mM CaCl2, 0.1% Bovine Serum Albumin (BSA), 5 mM beta-mercaptoethanol, pH 5.7. Samples were incubated for 4–5 h in dark with gentle shaking (40–50 rpm) at 28 °C and subjected to vacuum infiltration every hour. After incubation, the digestion solution containing the protoplasts was collected in 50 mL tube. Subsequent washing using 10 mL (twice) of WS solution (154 mM NaCl, 125 mM CaCl2, 5 mM KC1, 5 mM D-glucose 2 mM MES, pH 5.7) was done and pooled the collected protoplasts. The suspension was passed through 70 μm and 40 μm nylon mesh. The pooled suspension was centrifuged at 320 g for 8 min and washed three times with WS solution. The pellet was resuspended in MMg solution (4 mM MES, pH 5.7, 0.6 mM mannitol, and 15 mM MgCl2). Protoplasts were quantified by microscopy using a haemocytometer before transformation. The 35S:OsUPS1-GFP plasmid were co-transformed with mCherry protein marker specific for plasma membrane (CD3-1008) (Nielsen et al., 2007) to protoplasts using PEG-mediated transformation. After 12 h incubation at 28 °C, the protoplasts were harvested by centrifugation at 300 g for 2 min and viewed using Leica SP8 STED laser scanning confocal microscope (Leica). Images were processed using Leica LAS AF Lite Software. GFP was excited at 488 nm and the emitted light was detected between 512 and 560 nm. RFP was excited at 555 and emission was detected between 584 and 610 nm.

Phenotypic and agronomic trait analysis under suboptimal N supply

A total of 40 uniformly grown seedlings for each OsUPS1ox and WT plants were transplanted in a 4-L pot filled with equal weight (3.5 kg) of low-N soil containing 0.023% (w/w) total nitrogen (TN) which was four times lower than ordinary nursery soil (0.101% (w/w) TN). Soil was excavated from a hill (2 metres deep) in Yongin City, Korea (127:12E/37:7N). Ten plants were used for each N level of 100, 50, 20 and 0% N. The concentration of 100% N is equal to 0.215 g of urea supplied per pot. During the tillering stage, 30% of the initial N was supplied and another 20% during the booting stage. All pots were supplemented once with 0.48 and 0.19 g of phosphorus and potassium, respectively (Farm Hannong Co., Ltd, Korea). Images were taken using Sony Alpha α5000 camera (SONY CORP., Japan). Chlorophyll was measured using SPAD (KONICA MINOLTA INC., Japan). For the filling rate of OsUPS1GOS2 and OsUPS1p2OS, similar procedures were done except that the plants were grown in a rain-off shelter at Kyungpook National University, Gunwi (128:34E/36:15N), Korea.

Quantitative real-time PCR analysis

RNaseay plant mini-kit (QIAGEN) was used to isolate total RNA from all the tissues mentioned in this study. For cDNA synthesis, 2000 ng of total RNA was used as initial template and the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) was used for cDNA synthesis. Real-time PCR analysis was performed using the Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA). Reactions were performed at 98 °C for 10 min, followed by 40 cycles of 98 °C for 20 s, 60 °C for 40 s, 72 °C for 20 s in a 20 μL volume mix containing 1 μl EvaGreenTM Mix (SolGenet, Deajeon, Korea), 0.25 μM primers and 20 ng cDNA. Primers were designed using the Primer-BLAST designing tool (www.ncbi.nlm.nih.gov) and listed in Table S2. To ensure measurement of only the required PCR product at a specific melting point, a melting curve analysis was performed at 55–95 °C at a heating rate of 0.1 °C/s according to the procedure of (Park et al. 2010). OsUbi1 gene (AK121590) was used as an internal standard. Relative gene expression was calculated through the 2−ΔΔCT method by Livak and Schmittgen (2001). Values are the means ± SD (standard deviation) of three biological samples and three experimental repeats.

In situ hybridization

In situ hybridization experiments were performed as previously described (Lee et al., 2003), with minor modifications. Briefly, wild type rice plants Oriza sativa L. ssp. japonica cv. Ilmi were grown on MS media for 6 days and stem sections were fixed in a solution containing 50% ethanol, 5% acetic acid and 3.7% formaldehyde. They were then embedded in paraplast (Sigma, St Louis,MO) and 8 μm cross-sectional cuts were made using a microtome. Digoxigenin (DIG)-labelled OsUPS1 antisense and sense probes were generated through in vitro transcription from the 5’UTR to the coding region (1.4 kb) with DIG-labelled UTP (Roche, Mannheim, Germany) using the SP6 RNA polymerase and the T7 RNA polymerase, respectively (Roche, Mannheim, Germany). Paraplast were removed and the sections were then dried
on a slide warmer and incubated in a humidified box containing one of the probes (0.8 µg/slide) for 12 h. After hybridization, sections were washed and colour-reacted in a solution containing blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and levamisole for 36 h in a dark humidified box and mounted with Permoun® (Fisher Scientific, Fair Lawn, NJ). Photographs were taken under a bright field illumination using a microscope (ZEISS AXIO Imager.A2, Germany) equipped with a camera (ZEISS Axiocam 506 Color, Germany).

Acknowledgements

We thank Prof. Gynheung An of Kyung Hee University for kindly providing us with seeds of OsUP71 activation tagging lines. This research was supported by the Rural Development Administration under the Next-Generation BioGreen 21 Program (Project No. PJ013666 to J.-K.K.) and by the Basic Science Research Program through the National Research Foundation of Korea, Ministry of Education (NRF-2017R1A2B4007457 to J.-K.K.).

Conflicts of interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic analysis of UPS proteins from leguminous and non-leguminous plants.

Figure S2 Topology of UPS from different plant species.

Figure S3 Transcript levels of enzymes in the purine synthesis pathway.

Figure S4 Transcript levels of enzymes in the allantoin synthesis pathway.

Figure S5 Transcript levels of enzymes in the allantoin degradation pathway.

Figure S6 Free amino acid contents in OsUPS1GOSS and OsUPS1RNA plants.

Figure S7 Glutamine concentration in roots of OsUPS1OX plants after re-supplying N.

Figure S8 Filling rate of OsUPS1GOSS and OsUPS1RNA plants grown under different N-concentrations.

Figure S9 Expression of OsNRT2.3 in leaf tissues of 14 DAF OsUPS1OX plants.

Figure S10 Vectors used for rice transformation with overexpression and silence cassettes.

Table S1 Free amino acid content in leaf, flag leaf and panicles of 21 DAF plants.

Table S2 List of oligomers used in this study.