Identification and Characterization of Proteins Involved in Rice Urea and Arginine Catabolism[1][W]

Feng-Qiu Cao, Andrea K. Werner, Kathleen Dahncke, Tina Romeis, Lai-Hua Liu, and Claus-Peter Witte*

Key Laboratory of Plant and Soil Interactions, College of Resources and Environmental Sciences, China Agricultural University, 100193 Beijing, China (F.-Q.C., L.-H.L.); and Department of Plant Biochemistry, Dahlem Centre of Plant Sciences, Freie Universität Berlin, 14195 Berlin, Germany (F.-Q.C., A.K.W., K.D., T.R., C.-P.W.)

Rice (*Oryza sativa*) production relies strongly on nitrogen (N) fertilization with urea, but the proteins involved in rice urea metabolism have not yet been characterized. Coding sequences for rice arginase, urease, and the urease accessory proteins D (UreD), F (UreF), and G (UreG) involved in urease activation were identified and cloned. The functionality of urease and the urease accessory proteins was demonstrated by complementing corresponding Arabidopsis (*Arabidopsis thaliana*) mutants and by multiple transient coexpression of the rice proteins in *Nicotiana benthamiana*. Secondary structure models of rice (plant) UreD and UreF proteins revealed a possible functional conservation to bacterial orthologs, especially for UreF. Using amino-terminally StrepII-tagged urease accessory proteins, an interaction between rice UreD and urease could be shown. Prokaryotic and eukaryotic urease activation complexes seem conserved despite limited protein sequence conservation for UreF and UreD. In plant metabolism, urea is generated by the arginase reaction. Rice arginase was transiently expressed as a carboxyl-terminally StrepII-tagged fusion protein in *N. benthamiana*, purified, and biochemically characterized (K_m = 67 mM, k_{cat} = 490 s^{-1}). The activity depended on the presence of manganese (K_d = 1.3 μM). In physiological experiments, urease and arginase activities were not influenced by the external N source, but sole urea nutrition imbalanced the plant amino acid profile, leading to the accumulation of asparagine and glutamine in the roots. Our data indicate that reduced plant performance with urea as N source is not a direct result of insufficient urea metabolism but may in part be caused by an imbalance of N distribution.

Nitrogen (N) availability often limits plant performance in natural ecosystems (Vitousek and Howarth, 1991), causing a selective pressure to optimize the use of N resources. This ecophysiological selection has even led to a reduction of the N content of plant proteins in comparison with animal orthologs (Elser et al., 2006). Because N is a limiting resource, plants do not only require efficient N uptake mechanisms but also possess enzymatic pathways for N remobilization.

Arg is the most important single metabolite for N storage in plant seeds. In a survey of 379 plant species, Arg N accounted on average for 17.3% of total seed N (Vanetten et al., 1967). In several rice (*Oryza sativa*) varieties, values ranging from 16.1% to 17.1% were measured (Mosse et al., 1988). To access the N stored in the guanidinium group of Arg, it must first be hydrolyzed by mitochondrial arginase to Orn and urea. Urea leaves the mitochondria and is hydrolyzed by urease in the cytosol, releasing ammonia, which is reassimilated into amino acids by the combined action of Gln synthetase and Glu synthase.

Urea not only originates from Arg breakdown but may also be taken up from the environment by urea transporters (Kojima et al., 2007; Wang et al., 2008). Therefore, urease is involved in N remobilization as well as in primary N assimilation. Plant ureases and arginases are housekeeping enzymes found in many if not all plant species (Witte and Medina-Escobar, 2001; Brownfield et al., 2008). Urease is a nickel metalloenzyme that in Arabidopsis (*Arabidopsis thaliana*) requires three urease accessory proteins (UAPs; AtUreD, AtUreF, and AtUreG) for activation (Witte et al., 2005a). Studies in bacteria demonstrated that UAPs form a complex with apo-urease and are required for posttranslational Lys carboxylation of apo-urease and the subsequent incorporation of two nickel ions into the active center. After activation, the UAPs dissociate from urease. The exact molecular function of each accessory protein in this process is not yet understood (Carter et al., 2009). Like urease, arginase is a metalloenzyme. It is best activated by manganese (Carvajal et al., 1996; Hwang et al., 2001), not requiring accessory proteins for activation.

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* Corresponding author; e-mail cpwitte@zedat.fu-berlin.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Claus-Peter Witte (cpwitte@zedat.fu-berlin.de).

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Urea plays an important role in agriculture because it is the most used N fertilizer worldwide (http://www.fertilizer.org/ifa), intensively employed in Asia for the cultivation of rice. Urea N partly reaches the plant as ammonium or nitrate because the fertilizer is already degraded in the environment by microbial ureases and may then be subject to nitrification. Alternatively, plants are capable of taking up urea from fertilization directly and assimilate its N (Kojima et al., 2007; Wang et al., 2008). Although rice is a major crop plant and rice production is heavily dependent on urea fertilization, the enzymes and the corresponding genes involved in rice urea metabolism have not yet been investigated. In this study, we identified the genes and cloned the corresponding cDNAs coding for rice arginase, urease, and the UAPs UreD, UreF, and UreG. The functionality of the corresponding proteins was demonstrated and biochemical parameters were determined. The general gene and protein structure of plant UreD and UreF were investigated and a direct interaction of rice UreD with apo-urease was discovered, leading to a refinement of the mechanistic view of plant urease activation. In physiological experiments, rice urease and arginase activities showed no significant response to different N-fertilizing regimes, while the amino acid composition in urea-grown plants was strongly imbalanced, indicating that urea N disturbs plant metabolism downstream of N assimilation.

RESULTS

Cloning and Functional Testing of Rice Urease and UAPs

Genes and coding sequences for rice *urease* and the UAPs *ureD*, *ureF*, and *ureG* were predicted using the rice genome annotation, EST data, the corresponding Arabidopsis sequences, and multiple protein alignments of the corresponding proteins from monocotyledons generated from The Institute for Genome Research EST assemblies (Childs et al., 2007). Total RNA from rice subspecies Indica cv Hunan late 2 (PI 503035; National Small Grains Collection) was used to clone all cDNAs. Minor sequence differences of urease (Supplemental Fig. S1) and UreD (Supplemental Fig. S2) cloned from the rice variety used in this study from the sequences in the published genomes were found to be genuine judged from the results of repeated cloning and sequencing.

For Arabidopsis, the functionality of urease and UAPs was demonstrated by simultaneous coexpression in *Escherichia coli* and detecting urease activity (Witte et al., 2005a). Although the corresponding rice proteins could also be (co)expressed in *E. coli*, functional urease was not generated (data not shown). As an alternative approach for functional testing, Arabidopsis mutants of *urease* and *UAP* genes were transformed with cDNA constructs of the corresponding rice genes. Urease activity quantification in leaf extracts of several T1 plants in comparison with the respective mutants showed that rice *urease*, *ureD*, and *ureG* complemented the mutants, while *ureF* did not. However, reverse transcription (RT)-PCR of *OsureF* transformants revealed that the transgene was expressed (Fig. 1). The Dissociator element insertion in the transformed *ureF* mutant (*ureF-1*) may lead to the expression of an almost complete AtUreF protein with only a short C-terminal truncation (Witte et al., 2005a). It is possible that OsUreF cannot replace this putative dominant-negative AtUreF mutant variant. As a third approach for functional testing of rice urease and UAPs, a transient expression system in *Nicotiana benthamiana* was used. Only when all four rice genes were coexpressed was urease activity in leaf extracts increased clearly and statistically different from controls (Fig. 2A). These data demonstrate that the cloned rice genes are functional and encode OsUrease, OsUreD, OsUreF and OsUreG. Slightly increased activity over controls was observed even when one of the rice UAPs was missing (Fig. 2A, compare lanes II–IV with lanes VI and VII). Although these differences were not statistically significant, it appeared that the rice urease activation process was partially complemented

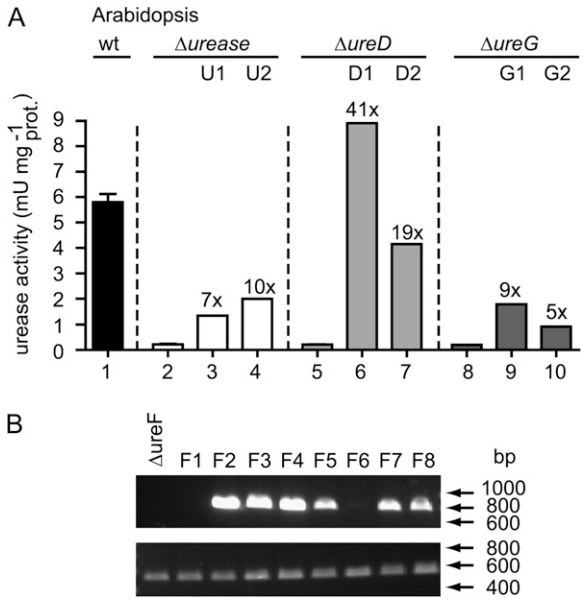

Figure 1. Complementation of Arabidopsis *urease* and *urease accessory protein* mutants with the corresponding cDNAs from rice. A, Quantification of urease activity from leaf extracts of Arabidopsis wild type (wt; lane 1), *urease*, *ureD*, and *ureG* mutants (lanes 2, 5, and 8), and two independent complementation lines for each mutant (lanes 3 and 4, 6 and 7, 9 and 10). The numerical values above the columns for the complementation lines indicate the fold increase of activity over background measured in the corresponding mutant. Wild-type activity was determined in three biological replicates, activity in the mutants in two replicates, and activity in the complementation lines only by a single measurement due to the limited amount of material. Error bars indicate SD. prot., Protein. B, RT-PCR amplification of rice *ureF* transcript (primers 1970 and 1697) and Arabidopsis *actin 2* (primers 1033 and 1034) in the Arabidopsis *ureF-1* mutant and several transgenic lines of rice *ureF* in this background.

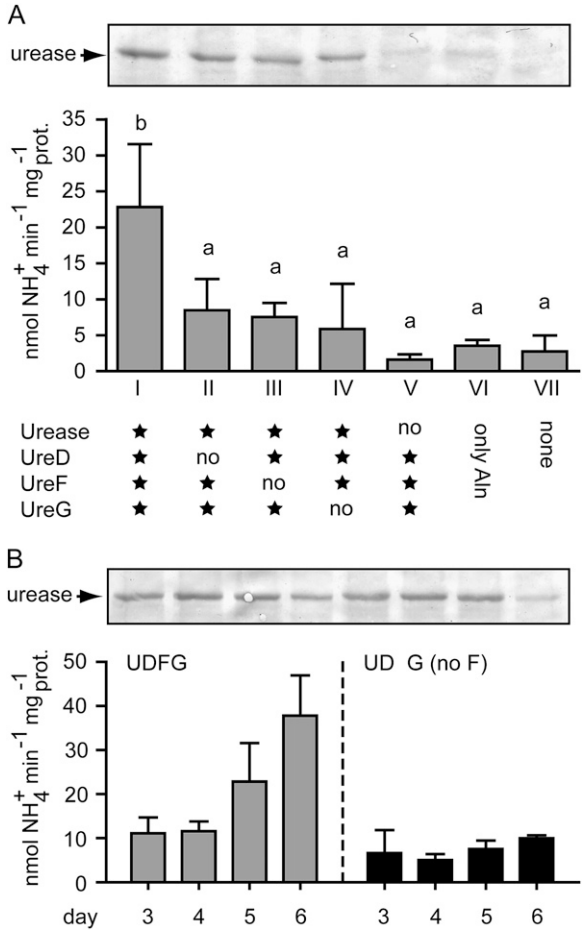

Figure 2. Functional test of rice urease and UAPs expressed in *N. benthamiana*. A, Western blot loaded with 40 μg of protein (prot.) per lane and probed with anti-urease antibody (top) and urease activity quantification (bottom) using leaf extracts from *N. benthamiana* after 5 d of transient coexpression of different combinations of OsUrease, OsUreD, OsUreF, OsUreG (lanes I–V) and Atallantoinase (lane VI) as a control. Uninfected leaves were used as an additional control (lane VII). Each urease activity was quantified using three independent leaves from different plants ($n = 3$). Error bars indicate SD. As indicated by lowercase letters, only activity I is different from all others with statistical significance ($P < 0.01$). B, Western blot and urease activity as in A from leaves coexpressing either OsUrease, OsUreD, OsUreF, OsUreG (gray columns) or OsUrease, OsUreD, OsUreG, but not OsUreF (black columns). Activities were assessed in a time course from 3 to 6 d after infiltration of the plants with *Agrobacterium*. Error bars indicate SD ($n = 3$ independent leaves).

by the corresponding proteins from *N. benthamiana*. Interestingly, urease protein expression preceded urease activation by several days, indicating that the activation process is a relatively slow process, at least in the heterologous expression system used (Fig. 2B).

Plant UreF and UreD Protein Structure and Interaction with Apo-Urease

In the current model of urease activation in bacteria, UreD binds stoichiometrically to apo-urease and facilitates the binding of UreF, which directly interacts with UreD, changing the conformation of the complex. Subsequently, UreG and UreE bind and urease is activated (Carter et al., 2009). Most bacterial ureases are trimers of heterotrimers where each heterotrimeric subunit (encoded by *ureA*, *ureB*, and *ureC*) carries one active site. Plant ureases are highly similar to bacterial ureases in sequence, but the three types of subunits are fused in a collinear fashion to a single polypeptide chain (*ureA*-linker-*ureB*-linker-*ureC*) that forms trimers or hexamers (Carter et al., 2009). Because of these similarities, the process of urease activation may be analogous in plants and bacteria. However, the amino acid sequences of plant and bacterial UreD and UreF are only marginally conserved, with identities of only about 20%, while ureG proteins are over 40% identical and ureases are over 50% identical (UreE is absent in plants; Witte et al., 2005a, 2005b; Supplemental Figs. S1–S4). We hypothesized that UreD and UreF proteins from plants and bacteria may be structurally conserved despite the low amino acid identity. Structural studies on these proteins are complicated by low native expression, instability, and the formation of insoluble agglomerates upon overexpression. Comparing a secondary structure model of rice UreF (Fig. 3A) based on an alignment of 19 plant UreF proteins (Supplemental Fig. S3) with (1) a partial UreF protein structure from *Helicobacter pylori* made recently available (Protein Data Bank accession no. 2WGL) and (2) a bacterial UreF structural model (Salomone-Stagni et al., 2007) revealed that plant and bacterial UreFs are α-helical proteins with similar overall structure (Fig. 3A). In contrast, comparing a secondary structural model of rice UreD based on an alignment of 16 plant UreD protein sequences (Supplemental Fig. S2) with a model of UreD from *Klebsiella aerogenes* based on an alignment of 100 bacterial UreD proteins indicated that plant and bacterial UreD proteins, although similar in part, also possess major differences in structure (Fig. 3B). The different structural architectures of plant and bacterial UreD may reflect the different requirements for interaction with the bacterial heterotrimeric urease or the plant homomeric urease. To test whether OsUreD directly interacts with rice urease, both proteins were coexpressed in *N. benthamiana* leaves. When N-terminally StrepII-tagged OsUreD was affinity purified from leaf extracts, OsUrease but also urease from *N. benthamiana* were copurified. This was not observed when purifying tagged OsUreG after coexpression with rice urease (Fig. 3C). Unfortunately, tagged OsUreF was not expressed in sufficient amounts for protein detection (RNA was present; data not shown). These data indicate that OsUreD directly interacts with rice urease while OsUreG does not. In bacteria, UreD interacts with urease most directly as well, while urease-UreF or urease-UreG complexes were not found (Carter et al., 2009). UreF was hypothesized to be a GTPase-activating protein, maybe acting on UreG (Salomone-Stagni et al., 2007), which resembles a small G protein. It appears that UreF and UreG

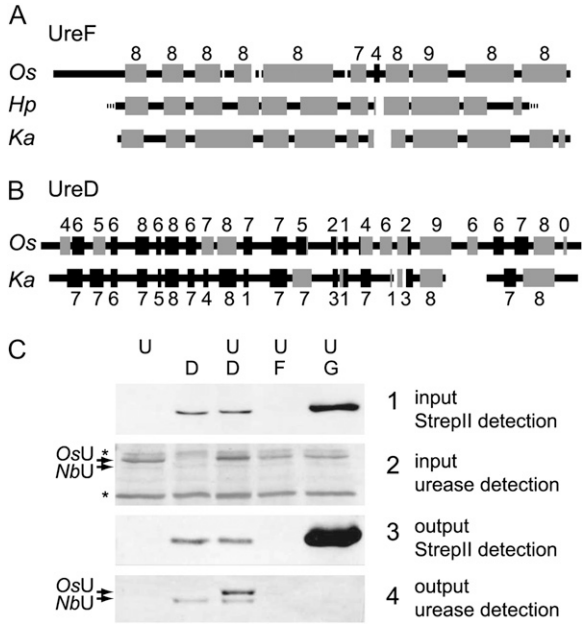

Figure 3. Structural comparison of plant and bacterial UreD and UreF proteins, and protein interactions of rice UAPs with urease. A, Schematic representation of secondary structure elements of UreF from rice (*Os*), from *H. pylori* (*Hp*), and from *K. aerogenes* (*Ka*). Proteins are drawn to scale; α-helices are displayed as gray boxes and β-elements as black boxes. The structure for the rice protein was predicted using the Jpred3 server with a multiple sequence alignment of 19 manually curated plant UreF proteins (Supplemental Fig. S3). The mean prediction confidence for each α- or β-element is given as a numerical value (scale 0–9, where 9 = highest confidence). The *H. pylori* partial UreF protein structure is based on experimental data but lacks the N and C termini of the protein. The *K. aerogenes* UreF protein structure was predicted by Salomone-Stagni et al. (2007). B, Schematic representation of secondary structure elements of UreD from rice (*Os*) and *K. aerogenes* (*Ka*). The structure for the rice protein was predicted using the Jpred3 server with a multiple sequence alignment of 16 manually curated plant UreD proteins (Supplemental Fig. S2). For the prediction of the *K. aerogenes* UreD structure, an alignment of 100 bacterial UreD sequences was used. C, Western blots of a copurification experiment. Rice urease (U) and N-terminally StrepII-tagged OsUAPs UreD, UreF, and UreG (D, F, and G) were coexpressed as indicated in leaves of *N. benthamiana* and affinity purified from extracts. Panels 1 and 3 show detection with StrepTactin alkaline phosphatase conjugate in crude extracts (1; input) and after affinity purification (3; output). Panels 2 and 4 show detection with anti-urease antibodies in crude extracts (2; input) and after purification (4; output). Stars label unspecific signals.

form the structurally conserved catalytic core for urease activation while UreD functions as an adapter protein for attachment of this core to the respective urease.

Plant UreF Expression Can Be Reduced by an Intron in the 5′ Leader Sequence

Bacterial UreD and UreF are expressed at very low levels, and overexpression hampers urease activation in bacteria (Lee et al., 1992; Park et al., 1994). It was suggested that differential splicing generating aberrant mRNA may reduce AtUreD expression in plants. Such a mechanism was not suggested for *AtureF* because it was found to be an intronless gene (Witte et al., 2005a). However, for *OsureF*, our gene analysis revealed that an intron in the noncoding 5′ leader sequence is predicted based on two ESTs (Fig. 4A). Splicing of this intron removes all AUG initiation codons upstream of the *OsureF* start codon from the primary transcript. We investigated whether such an intron is conserved in plant *ureF* genes and whether it is aberrantly spliced, contributing to low plant UreF expression. Using the Phytozome version 5.0 database of sequenced plant genomes (http://www.phytozome.net/), the structures of *ureF* genes from 16 higher plant species were investigated. Where EST or cDNA data were available for intron prediction (11 species), an intron in the 5′ leader was always found except for *ureF* from Arabidopsis. In almost every case, splicing of the transcript removes all AUG codons upstream of the start codon (Supplemental Table S1). Interestingly, the two *ureF* genes of soybean (*Glycine max*) are both spliced in the 5′ leader, but only for the paralogue on chromosome 2 (locus Gm02g44440) does splicing remove all nonstart AUG codons. The transcript of the other paralogue on chromosome 14 (locus Gm14g04380) retains an out-of-frame AUG codon upstream of the start codon and therefore may be nonfunctional due to inefficient translation. Based on our finding that plant *ureF* genes generally do contain an intron, we postulated that (1) *ureF* from Arabidopsis may also be spliced in the 5′ leader, removing several upstream nonstart AUG codons and (2) possibly the conserved 5′ leader intron of plant *ureF* genes

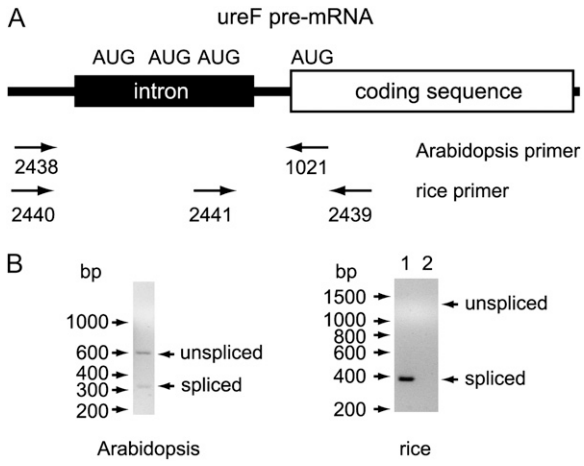

Figure 4. Structure of the transcript of plant *ureF* and splicing efficiency in rice and Arabidopsis. A, Schematic overview of the plant *ureF* transcript with the conserved intron in the 5′ leader sequence and primer positions used to investigate intron splicing in rice and Arabidopsis. B, RT-PCR products resolved on agarose gels. The left panel shows RT-PCR using Arabidopsis leaf RNA and primers 2438 and 1021, and the right panel shows RT-PCR using rice leaf RNA and primers 2440 and 2439 (lane 1) or 2441 and 2439 (lane 2).

is inefficiently spliced to limit UreF expression. For *AtureF*, both assumptions could be validated. An intron of 274 bp is spliced from the 5′ leader with low efficiency (Fig. 4B, left). For *OsureF*, splicing of the 5′ leader intron could be confirmed, but in contrast to the *AtureF* transcript, it was spliced efficiently (Fig. 4B, right). In conclusion, the *ureF* 5′ leader sequence may in some plant species play a functional role in reducing the amount of intact *ureF* mRNA by differential splicing (e.g. Arabidopsis) or translational inhibition (e.g. soybean).

Biochemical Characterization of Rice Urease and Arginase

For rice urease, a K_m of 0.53 ± 0.06 mM (confidence interval [ci], P = 95%; Fig. 5A) was determined using leaf extracts. This is in close agreement with the K_m value of 0.5 mM from ureases of *G. max* and *Zea mays* (Davies and Shih, 1984).

Rice arginase was cloned from the subspecies Indica Hunan late 2 by RT-PCR into the binary expression vector pXCS-HAStrep (Witte et al., 2004). After transient expression in *N. benthamiana*, the C-terminally hemagglutinin (HA) and StrepII-tagged protein was highly purified from leaf extracts by StrepTactin affinity chromatography (Fig. 5B). A K_m value of 67 ± 9 mM (ci, P = 95%) and a turnover number of 490 ± 19 s^{-1}(ci, P = 95%) were determined for the purified enzyme (Fig. 5C). Arginases of other plants possess similar K_m constants: 83 mM was determined for enzymes from ginseng (*Panax ginseng*; Hwang et al., 2001) and soybean (Kang and Cho, 1990), while turnover numbers were not reported. Manganese activated the rice enzyme (Fig. 5D). Half-activation was achieved with a manganese concentration of 1.3 ± 0.6 μM (ci, P = 95%). For the enzyme from *Phaseolus vulgaris*, a K_d for manganese of 0.47 μM was determined, but also cobalt, nickel, and cadmium activated the enzyme partially when used at 2 mM concentration (Carvajal et al., 1996). The half-activation constant of arginase is in the same concentration range as found for other manganese-dependent enzymes, indicating that full activation is reached at physiological concentrations of manganese (Werner et al., 2008). Other metals that are needed at high nonphysiological concentrations in vitro probably do not contribute to activation in vivo.

Urease and Arginase Activities during Germination

During germination, the total activity of urease in the whole seedling remained unchanged (Fig. 6). Urease activity decline in the seed was compensated by newly formed urease in the emerging shoot and root (data not shown). In contrast, seedling arginase activity rose during germination, especially at the onset of root and shoot emergence (day 2), indicating an increased turnover of Arg at this time point. Increases in arginase activity during germination have also been observed in several other species (Goldraij and Polacco, 1999;

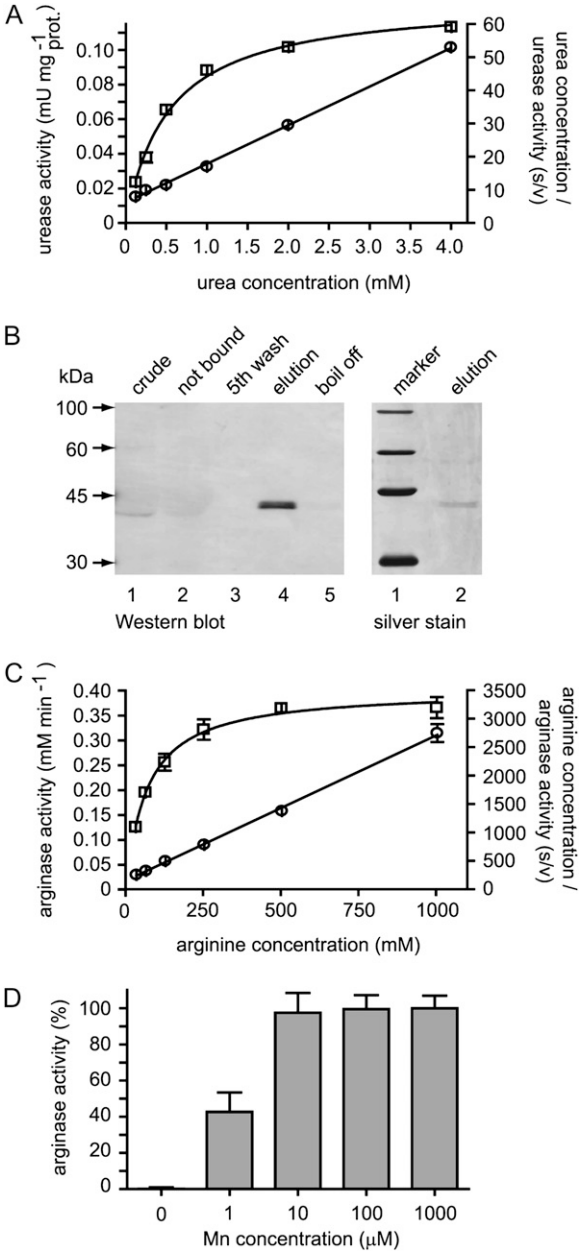

Figure 5. Characterization of rice urease and arginase. A, Michaelis-Menten graph (left axis, squares; r^2 = 0.990) and corresponding Hanes plot (right axis, circles; r^2 = 0.998) for rice urease. Error bars indicate SD (n = 3). prot., Protein. B, Western blot (left panel) documenting the StrepII tag-mediated affinity purification of rice arginase from leaf extracts of *N. benthamiana* after transient expression. Lane 1, Extract of soluble proteins; lane 2, proteins not bound after incubation with StrepTactin affinity matrix; lane 3, protein in the fifth wash supernatant; lane 4, pool of eluted protein; lane 5, affinity matrix boiled in SDS loading buffer after elution. Silver gel (right panel) to visualize the purity of rice arginase. Lane 1, Marker proteins; lane 2, affinity-eluted arginase (10 μL loaded). C, Michaelis-Menten graph (left axis, squares; r^2 = 0.977) and corresponding Hanes plot (right axis, circles; r^2 = 0.995) for purified rice arginase. Activity with reference to volume assay mixture is displayed. Error bars indicate SD (n = 4). D, Activation of purified arginase by manganese. Error bars indicate SD (n = 3).

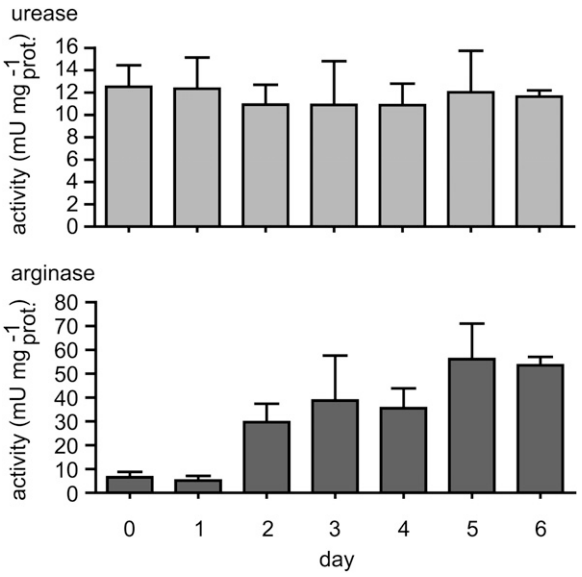

Figure 6. Changes of urease and arginase activity during rice germination. Urease activity (top) and arginase activity (bottom) are shown relative to total protein (prot.) in a time course taken during germination of subspecies Hwayoung from day 0 (dry seed) to day 6. Error bars indicate SD ($n = 3$).

Todd et al., 2001; Flores et al., 2008). Despite the increase in arginase activity, the urea concentration was always close to the detection limit and rose from about 0.1 μmol g^{-1} fresh weight to about 0.3 μmol g^{-1} fresh weight during germination, indicating that sufficient urease activity was present to metabolize the urea generated in the arginase reaction.

Growth on Urea Versus Ammonium Nitrate

Rice plants (subspecies Indica cv Hunan late 2) were grown from seed for 10 d under sterile conditions either on urea (5 mM) or ammonium nitrate (AN; 5 mM) to assess urease and arginase activities and several parameters of nutritional status using these alternative N sources (Fig. 7). An additional set of plants grown on 5 mM urea and limiting AN (0.25 mM) was also included to test whether the presence of a small amount of AN would improve urea usage. Controls either contained only limiting AN (0.25 mM) or no N.

Urease and arginase activities were similar irrespective of the supplied N source. Higher specific activities (with reference to total protein) on medium with limiting N were not due to an increase in absolute activity but to decreased total protein contents (Fig. 7A). These data demonstrate that urease and arginase activities are not significantly altered when urea is the sole N source, despite their central role in plant urea metabolism. In experiments where 8-d-old plants grown on AN were placed on urea medium and urease and arginase activities were monitored for the following 3 d, similar results were obtained (Supplemental Fig. S5). Also, carbon starvation induced by placing the plants in the dark had no significant influence on these activities.

Shoot fresh weight was generally increased by ample N nutrition (AN, U, U+), while root fresh weight was reduced only by AN, indicating that exclusively AN was perceived as a rich N source (Fig. 7A; Supplemental Fig. S6). In accordance, total N and total protein content were increased in plants receiving AN compared with plants grown on urea (U, U+). Clear differences between AN and urea nutrition were observed in total amino acid N content and amino acid profiles. Plants grown on urea accumulated amino acid N in the root (Fig. 7A) in the form of Gln, Asn, and Arg (Fig. 7B; Supplemental Table S2). In the shoot, the concentration of most amino acids was reduced by urea nutrition in comparison with AN nutrition, with the exception of Asn, Asp, Glu, and Tyr. This reduction was less pronounced when urea was supplemented with limiting amounts of AN (U+). In accordance, U+ shoots (compared with U) contained more total amino acid N, matching the content of AN shoots (Fig. 7A). These data indicate that a proportion of the urea N was trapped in the root mainly in the primary amino acids of N assimilation and translocation, Gln and Asn (Lea et al., 2007). It appears that the translocation of N into the shoot and the distribution of N into other amino acids were insufficient with urea nutrition. The addition of a limiting amount of AN led to a slightly improved usage of urea, probably by improving N translocation and distribution. Irrespective of N source, the urea content remained close to the detection limit, ranging from 0.2 to 0.5 μmol g^{-1} fresh weight in shoot and root, indicating that urease activity is not limiting urea N conversions even when urea is the sole N source.

DISCUSSION

Plants possess urease to gain access to urea N from Arg breakdown and direct urea uptake. Other sources of urea in plant metabolism, like the degradation of ureides from purine catabolism, are controversial, and in a recent description of the complete ureide degradation pathway in Arabidopsis it was demonstrated that this plant can degrade ureides without a urea intermediate (Werner et al., 2010). Internal urea metabolism seems disconnected from external availability of urea, because neither the size of the internal urea pool nor urease or arginase activity is altered if urea is the sole N source. In contrast, rice arginase activity rises during germination, as observed in several other plants, because stored Arg needs to be mobilized. For example, in soybean, arginase activity is low during embryo development when Arg is deposited, rising steeply during germination when Arg is turned over. This separates the reactions of the urea cycle in time (Goldraij and Polacco, 1999). Plant arginases possess particularly high K_m constants (greater than 50 mM),

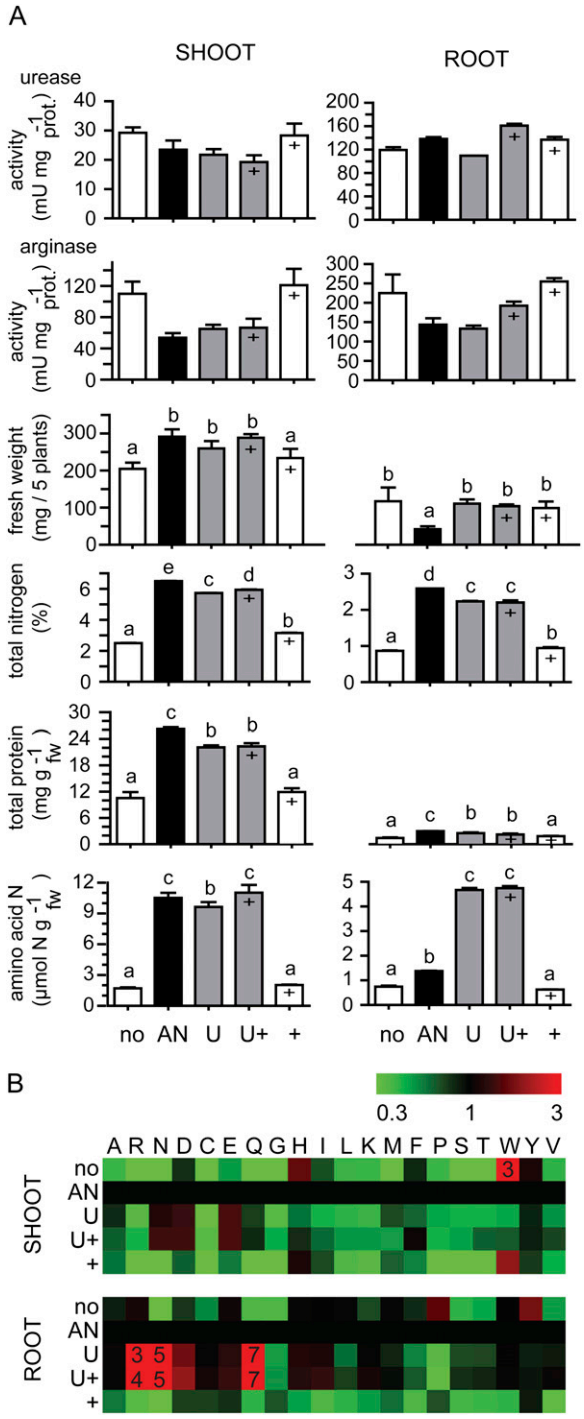

Figure 7. Analysis of rice plants grown under different N regimes. A, Urease activity, arginase activity, fresh weight (fw), total N, total protein (prot.), and amino acid N in shoot and root of plants grown without N (no), with 5 mM AN (AN), with 5 mM urea (U), with 5 mM urea and 0.25 mM AN (U+), and with limiting 0.25 mM AN (+) as sole N source. Error bars indicate SD ($n = 3$). Different letters indicate significant differences at $P < 0.05$. B, Heat map of relative amino acid quantification in shoot and root. Concentration changes are presented relative to concentrations in the AN-fertilized plants (set = 1). Increases at or above 3-fold are additionally labeled by indicating the fold increase in the corresponding square. Decreases below 3-fold cannot be distinguished in this graph. Absolute values are given in Supplemental Table S2.

indicating low affinity to Arg. In the presence of low amounts of arginase, therefore, Arg may be relatively stable, as observed by Goldraij and Polacco (1999). Beyond serving as N reserve, Arg is also required for polyamine biosynthesis and has been linked to nitric oxide biosynthesis in plants, while arginase appears to play a role in polyamine and nitric oxide homeostasis (Flores et al., 2008). A single arginase gene is present in rice (this study) and loblolly pine (*Pinus taeda*; Todd et al., 2001). Some plants (Arabidopsis, *Solanum lycopersicum*) possess two arginase genes, one being expressed after jasmonic acid or stress treatment (Chen et al., 2004; Brownfield et al., 2008), indicating that (some) plant arginases may exert functions beyond Arg catabolism for N mobilization.

Four proteins are needed in Arabidopsis to generate ureolytic activity (Witte et al., 2005a), and orthologous proteins are required in rice (Figs. 1 and 2). Corresponding genes/proteins are also found in other plants (Supplemental Figs. S1–S4; Freyermuth et al., 2000; Witte et al., 2001; Bacanamwo et al., 2002). Plant urease activation complexes seem structurally conserved, because components from different plants can functionally replace each other (Figs. 1 and 2) and can also interact (Fig. 3C). Rice urease can even be activated by the complete set of Arabidopsis UAPs (Fig. 1A). There are also similarities between plant and bacterial urease activation systems, because UreG (from potato [*Solanum tuberosum*]) but not UreD and UreF (from Arabidopsis) complemented a urease operon of *K. aerogenes* lacking the gene of the respective UAP (Witte et al., 2001; Bacanamwo et al., 2002). However, UreF is conserved and UreD is partially conserved in secondary structure between plants and bacteria, despite very limited sequence similarity. As in bacteria (Carter et al., 2009), UreD directly interacts with (apo-)urease (Fig. 3). UreD and UreF from rice and Arabidopsis (data not shown) are unstable proteins (Fig. 3C). A similar observation has been made for UreF and UreD from *K. aerogenes* (Lee et al., 1992). This intrinsic instability of UreF and UreD but also differential splicing of the corresponding transcripts (Fig. 4; Witte et al., 2005a) and nonstart initiation codons in the 5′ leader sequence of some *ureF* mRNAs likely contribute to a low native expression of these proteins. Limited expression may be biologically required to ensure that UreF and UreD dissociate from urease after activation to release the active enzyme and that the putative GTPase-activating protein UreF (Salomone-Stagni et al., 2007) does not trigger UreG activity in the absence of urease. Consistently, increasing the expression of both proteins in *K. aerogenes* has a negative impact on urease activation in vivo (Lee et al., 1992; Park et al., 1994).

Urease activity in rice but also in other plants is more than sufficient to cope with metabolically produced urea. Even when urea was used as the sole N source, a significant increase in internal urea concentration was not observed in rice (Gerendas et al., 1998; this study) and Arabidopsis (Merigout et al., 2008),

while in *Brassica napus*, a moderate rise of urea concentration was measured (Gerendas and Sattelmacher, 1999). The K_m of urease (about 0.5 mM; Fig. 5A) exceeds urea concentrations in plant tissues by several orders of magnitude (assuming even distribution, urea concentrations are in the low nanomolar range). Consequently, increases of internal urea concentration directly after animal excretion or urea fertilization will result in a linear proportional rise of urea hydrolysis velocity. Because of this excess activity, an active regulation of urease is not required. Consistently, total urease activity is not influenced by the external N source (even if it is urea) in rice (Supplemental Fig. S5), potato (Witte et al., 2002), and several other plants (Gerendas et al., 1999).

Although urea is in general rapidly metabolized by plants when supplied with sufficient nickel for urease activation (Gerendas et al., 1999), it has been frequently observed that plants using urea as sole N source show reduced growth and signs of N starvation compared with plants supplied with mineral N (Gerendas et al., 1998; Gerendas and Sattelmacher, 1999; Tan et al., 2000; Merigout et al., 2008). However, rice plants receiving urea had only slightly reduced total N available compared with plants grown on AN (Fig. 7A), indicating that urea uptake was sufficient to supply N to the plant, at least under our experimental conditions (5 mM urea). Negative growth effects were also observed with ammonium nutrition compared with nitrate, and often a mixed AN fertilization is best (Britto and Kronzucker, 2002). While there is probably more than one reason why plants underperform on reduced N sources (for discussion, see Britto and Kronzucker, 2002), limiting root-to-shoot translocation of N in the absence of nitrate is one important aspect. Using ^{13}N-labeled compounds, it was demonstrated that nitrate when cosupplied to ammonium strongly enhanced root-to-shoot translocation of N in rice (Kronzucker et al., 1999). Consistently, high levels of amino acids used for N translocation (especially Gln and Asn) accumulate in roots of urea-grown plants (Fig. 7B; Gerendas et al., 1998; Gerendas and Sattelmacher, 1999; Merigout et al., 2008). Plants supplied with AN may also directly translocate nitrate to the shoot for reduction and amino acid biosynthesis, thereby avoiding massive amide amino acid biosynthesis in the root and consequently requiring less root-to-shoot translocation of assimilated N. In pulse-chase experiments feeding Arabidopsis roots with ^{15}N-labeled urea, Merigout et al. (2008) showed that Gln labeling is comparatively weak in plants that had been grown on urea, indicating a low ammonium assimilation rate. The reduced assimilation may be caused by a negative feedback of the assimilation products (Gln and Asn) that are not efficiently translocated. Consistently, Merigout et al. (2008) obtained indications of reduced root-to-shoot transport of label in urea-grown plants, irrespective of the type of labeled N source offered in the pulse. A reduced N assimilation rate in plants supplied with urea may explain why protein N and total N contents are generally lower than in AN-grown plants (Fig. 7A). Roots but also shoots contained sufficient Glu and Gln as amino group donors to sustain amino acid biosynthesis. Nonetheless, the concentrations of many amino acids were significantly lower in rice plants supplied with urea, especially in the shoot (Fig. 7B; Supplemental Table S2). Although the reason for this observation is unclear, it may indicate a limited availability of carbon skeletons for amino acid biosynthesis. Carbon limitation has been associated with the expression of Asn synthetase genes in several plants (Lea et al., 2007). Consistently, increased Asn concentrations were observed in root and shoot of our plants when supplied with urea (Supplemental Table S2). Despite lower total protein and N contents in rice plants supplied with urea, biomass was similar irrespective of N source as long as sufficient N was supplied (Fig. 7A). Older plants generally show a negative biomass response when supplied with urea (Gerendas et al., 1998; Gerendas and Sattelmacher, 1999; Tan et al., 2000; Merigout et al., 2008). Because we used relatively young plants that initially grew from the nutrient reserves of the seed, biomass was not significantly affected at this early growth stage.

In summary, it appears that growth reduction generally observed with ample urea as the sole N source is rather caused by inefficient N distribution than by insufficient urea uptake or urea hydrolysis. However, it is possible that uptake may become limiting at low urea concentrations when the influx relies only on high-affinity urea transporters.

Despite the great agricultural importance of urea as N fertilizer for rice, the molecular details of its usage by plants are not well investigated. N use efficiencies are only about 40% in rice, leading to great economic losses and environmental pollution. To improve this situation, biotechnological plant modifications may be a possible tool. This work contributes to the knowledge base for such approaches.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Rice (*Oryza sativa* subsp. Indica cv Hunan late 2 [PI503035] and subsp. Japonica cv Nipponbare [PI514663]) seeds were received from the National Small Grains Collection; seeds of subspecies Japonica cv Hwayoung were received from Postech University, Korea. Rice seeds were germinated for 2 d in distilled water at 28°C in an incubator. Plants were grown on a peat clay soil (Floragard Floraton 1) in 16-cm pots (height, 17 cm) in a greenhouse (14 h of light, 150 μmol m^{-2} s^{-1}, 24°C day, 18°C night). For sterile culture of plants, Indica seeds were dehusked, surface sterilized with 2% hypochloride solution for 15 min, rinsed, and germinated for 2 d in sterile water. Glass jars (14 cm height, 8 cm diameter) filled with 50 mL of growth medium solidified by 0.4% (w/v) plant agar (Duchefa) were used to grow six seeds each (greenhouse, 14 h of light, 24°C day, 18°C night). The basic growth medium contained 0.17 mM Na_2HPO_4, 0.27 mM K_2SO_4, 0.47 mM $CaCl_2$, 0.37 mM $MgSO_4$, 45 μM Fe-EDTA, 0.16 μM $CuSO_4$, 0.15 μM $ZnSO_4$, 0.10 μM Na_2MoO_4, 15 μM H_3BO_4, 4.6 μM $MnSO_4$, 1 μM $NiSO_4$, and 0.05% (w/v) MES, pH 5.7. N was supplied as 5 mM NH_4NO_3 (AN), 5 mM urea (U), 5 mM urea and 0.25 mM NH_4NO_3 (U+), and 0.25 mM NH_4NO_3 (+). Urea was not autoclaved but added from a sterile stock solution. Plants were harvested after 10 d, shoots and roots were weighed in

pools of five, and 100 mg of each pool was extracted (see below). The remaining material was frozen, and samples were prepared for total N content measurement and amino acid analysis. *Nicotiana benthamiana* was grown in a greenhouse at 22°C during the day and 18°C at night with 14 h of light. Three days before infiltration of *Agrobacterium tumefaciens*, 6 mL of $NiCl_2$ solution (1 mM) was added to the soil, except for plants used in copurification experiments.

Arabidopsis (*Arabidopsis thaliana*) plants were grown at 20°C with a 14-h light period (150 μmol m^{-2} s^{-1}) in a greenhouse. The following mutants were used: *ure-1*, *ureD-1*, *ureF-1*, and *ureG-1* (Witte et al., 2005a). Three days before urease assays, plants were supplemented with 1 mL of a 1 mM $NiCl_2$ solution.

Cloning and RT-PCR

Two similar binary vectors allowing the 35S promoter-driven expression of N-terminally StrepII-tagged proteins were generated: pXNS1pat-Strep and pXNS2pat-Strep, which only differ by encoding either an *Nde*I or an *Nco*I recognition sequence in the multiple cloning site, respectively. Corresponding vectors without StrepII tag sequence were also generated: pXS1pat and pXS2pat. All vectors carry a glufosinate (Basta) resistance for selection in planta. Primers 1860 and 1861 were cloned into pamPAT-MCS (accession no. AY436765) using the *Xho*I and *Eco*RI restriction sites, generating pXNS1pat-Strep. Similarly, primers 1862 and 1863 were used to generate pXNS2pat-Strep. With primers 1775 and 1776 or 1777 and 1778, pXNS1pat-Strep or pXNS2pat-Strep was converted to pXS1pat or pXS2pat using *Xho*I and *Nde*I or *Xho*I and *Nco*I, respectively. RNA from rice leaves was prepared using TRI reagent (Sigma) and treated with DNaseI (Fermentas) following the manufacturer's instructions. Moloney murine leukemia virus reverse transcriptase (Promega) and a poly-T primer were employed for RT using 1.0 μg of total RNA. cDNAs were amplified by PCR and cloned into pCR-Blunt (Invitrogen). Two consecutive amplifications (and clonings) were performed for all cDNAs, either because sequences surrounding the initiation codon were problematic for primer design (low complexity), requiring nested PCR, or because changes in cloning strategy required the use of different restriction sites than initially planned. Primers are found in Supplemental Table S3. *Osurease* was first amplified with primers 2537 and 2538, cloned, reamplified with primers 1694 and 1695, and cloned into pCR-Blunt (Invitrogen). After sequencing, the clone was first cut with *Xba*I and then with *Eco*RI and brought into pXCS-HAStrep (Witte et al., 2004) via three-point ligation. The cloned cDNA contains a stop codon; therefore, the protein is expressed without tag from this vector. *OsureD* was first amplified with primers 1705 and 1683, cloned, reamplified with primers 1723 and 1701, and cloned into pCR-blunt. The insert was released with *Nco*I and *Bam*HI and inserted into pXS2pat and pXNS2pat-Strep, expressing an untagged and a N-terminally StrepII-tagged protein, respectively. Similarly, *OsureF* and *OsureG* were cloned into pXS2pat and pXNS2pat-Strep using primers 2539/2540 and 1970/1697 and cloning via *Nde*I and *Xba*I for *ureF* and using primers 2541/2542 and 169/1699 and cloning via *Nco*I and *Bam*HI after partial digestion for *ureG*. *Osarginase* was amplified with primers 2265/2266 and 2267/2268 in two nested PCRs and cloned into pCR-Blunt. After sequencing, the cDNA was cloned via *Eco*RI and *Sma*I into pXCS-HAStrep, expressing a C-terminally HA and StrepII-tagged protein. Constructs encoding untagged proteins were used for Arabidopsis mutant complementation. Plants were transformed by flower dipping and selected by spraying transformants 1 week after germination with glufosinate (Basta). The same constructs were also used to assess urease and UAP function in *N. benthamiana*. Constructs encoding tagged proteins were used for copurifying urease with UAPs after transient expression in *N. benthamiana* and for the purification of Osarginase. To study *ureF* splicing, cDNA was prepared from Arabidopsis (ecotype Columbia 0) and rice subspecies Japonica Nipponbare as described above. *AtureF* was amplified with primers 2438 and 1021, *OsureF* was amplified either with primers 2440 and 2439 or 2441 and 2439. Control reactions omitting the reverse transcriptase were negative in all cases.

Transient Expression in *N. benthamiana*

Transient expression in *N. benthamiana* was performed as described by Witte et al. (2004). For functional tests, OsUrease (U), OsUreD (D), OsUreF (F), and OsUreG (G) were coexpressed in different combinations (UDFG, UFG, UDG, UDF, and DFG). The optical densities at 600 nm (OD_{600}) of *Agrobacterium* in the mixture were adjusted to 0.2 for those encoding U and G and to 0.05 for those encoding D and F on the respective constructs. To ensure even bacterial load for each infiltration, the OD_{600} of each mixture was brought to 0.5 with *Agrobacterium* carrying a construct encoding Atallantoinase-HAStrep (Werner et al., 2008), unrelated to the process of urease activation. Finally, *Agrobacterium* carrying a silencing inhibitor construct (Witte et al., 2004) was added, bringing the total OD_{600} to 0.6. For copurification experiments, N-terminally StrepII-tagged variants of OsUreD, OsUreF, or OsUreG were coexpressed with untagged OsUrease (each at a final OD_{600} of 0.2). In controls, OsUrease or N-terminally tagged OsUreD was expressed alone but mixed with *Agrobacterium* carrying a promoter-GUS construct (unrelated to the process of urease activation) to reach the same bacterial concentration in all treatments. Finally, *Agrobacterium* carrying a silencing inhibitor construct (Witte et al., 2004) was added, bringing the total OD_{600} to 0.5.

Preparation of Extracts

All extracts were prepared using an extraction buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and 10 mM dithiothreitol (DTT). Fresh Arabidopsis and *N. benthamiana* leaves were extracted using a 1:2 ratio of fresh weight to buffer and centrifuged (15 min at 4°C and 20,000*g*). Supernatants were desalted with spin columns as described by Witte and Medina-Escobar (2001) equilibrated in 10 mM HEPES, pH 7.5 (slurry density, 0.16 g mL^{-1}). To determine native urease and arginase activities in rice, as well as ammonia, urea, and total protein contents, fresh rice leaves or seeds (100 mg) were extracted in 1 mL of extraction buffer using six steel beads (4 mm diameter) in a mill (Retsch; MM 301) for 4 min at a frequency of 25 s^{-1}. After centrifugation (15 min, 20,000*g*, 4°C), 600 μL of supernatant was frozen for the determination of ammonia and urea contents and 100 μL of supernatant was desalted (spin columns) for activity measurements. For the determination of the K_m value of rice urease, leaves were extracted in a mortar using a 1:4 ratio of fresh weight to buffer and centrifuged (20 min, 20,000*g*, 4°C). Supernatants were desalted with High Trap G25 columns (GE Healthcare) equilibrated in half-strength extraction buffer without 4-(2-aminoethyl)benzenesulfonyl fluoride and DTT. Protein concentrations in the desalted plant extracts were determined with a commercial Bradford reagent (Bio-Rad) and bovine serum albumin as the standard.

Affinity Purification

For copurification experiments of rice urease with StrepII-tagged UAPs, proteins were purified as described by Werner et al. (2008) but using HEPES buffer at pH 7.5 throughout and only a single elution step with 60 μL of elution buffer. Affinity purification of C-terminally StrepII-tagged rice arginase was performed according to Werner et al. (2008) but using the extraction buffer described above with the addition of 100 μg mL^{-1} avidin. The wash buffer contained 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, and 2 mM DTT. For elution, 10 mM biotin was added to the wash buffer. After elution, polyethylene glycol 4000 was added, reaching a final concentration of 5%, to stabilize the enzymatic activity for freezing. For analysis of the activation by manganese, arginase was purified, omitting EDTA in the last two wash steps and in the elution buffer. Purified enzyme was quantified using the Nano-Orange kit (Invitrogen).

Activity Assays

Urease activity was quantified essentially as described by Witte and Medina-Escobar (2001). In brief, desalted supernatants were incubated at 50°C for 3 min. To start the reaction, urea was added to a final concentration of 25 mM. In a time course (usually up to 30 min), samples of 10 μL were diluted in 190 μL of water followed by the addition by 50 μL of phenol reagent and 100 μL of hypochloride reagent for colorimetric ammonia detection. Ammonia standards were prepared in the complete chemical background of the assay, including the protein extract to account for possible interference with the color development. For the determination of urease K_m, urea concentrations of 0.125, 0.25, 0.5, 1, 2, and 4 mM were employed. To start the reaction, 125 μL of desalted leaf extract was mixed with 125 μL of 2-fold-concentrated urea solutions in 20 mM HEPES (pH 7.5). The assay was performed at 50°C. In a time course of 0, 3, 6, and 9 min, 20-μL samples were added to 280 μL of water followed by 50 μL of phenol reagent and 100 μL of hypochloride reagent for colorimetric detection of ammonia. All samples were taken in triplicate.

For the determination of arginase activity in rice extracts, the following reaction mixture was prepared: 25 μL of Arg (500 mM; pH 9.0), 12.5 μL of water, 2.5 μL of phenylphosphorodiamidate (2 mM; a urease inhibitor), and

0.5 μL of $MnCl_2$ (100 mM). After preincubation of this reaction mixture for 3 min at 30°C, the reaction was started by the addition of 10 μL of extract. After 0 and 30 min, samples of 10 μL were added to 100 μL of water and frozen in liquid N for the later determination of urea (see below). In preliminary experiments, a constant rate of urea production was observed for at least 40 min under these assay conditions. A long preincubation of the enzyme preparation with manganese before starting the assay, frequently employed by others, had no influence on activity in our hands. For activity measurements of affinity-purified rice arginase, an enzyme preparation containing 5 μL of purified enzyme (5.4 ng μL^{-1}), 2 mM DTT, and 4 mM $MnCl_2$ in a total of 25 μL was first placed on ice for 6 min and then at 30°C for 3 min. To start the reaction, the enzyme preparation was mixed with a solution of 25 μL of Arg (pH 9.0; also incubated for 3 min at 30°C). Samples of 10 μL were taken directly after adding the substrate and, after 6 min, diluted in 100 μL of water and frozen in liquid N. The kinetic constants were determined at final Arg concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 mM in four repeated measurements, respectively. For all arginase measurements, urea standards were prepared in the exact chemical background of the assay to account for interference. For each Arg concentration, a separate urea standard was required. EDTA-free protein was used in assays containing distinct manganese concentrations (0, 1, 10, 100, and 1,000 μM) and 250 mM Arg as substrate. Activities were measured in triplicate and expressed as urea production rate. The detection of urea was based on the method described by Kyllingsbaek (1975). The frozen samples were thawed in 300 μL of color reagent and incubated for 30 min at 80°C, cooled for 2 min on ice, and measured at 527 nm in a plate photometer.

SDS-Gel Electrophoresis, Western-Blot Analysis, and Protein Staining

SDS-gel electrophoresis, western blotting for the detection of StrepII-tagged proteins, and silver staining were performed according to Witte et al. (2004). StrepTactin alkaline phosphatase conjugate was diluted 1:4,000 for arginase detection and 1:1,000 for the detection of OsUAPs. Plant ureases were detected using a polyclonal rabbit anti-jackbean urease antibody (Rockland 100–4182; dilution, 1:1,000) and a mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma A2306; dilution, 1:10,000).

Metabolite Analysis

For total N determinations, 2.7 to 2.9 mg of freeze-dried plant material was analyzed in an elemental analyzer (EuroEA 3000) in three replicates per sample. For the determination of the ammonia and urea contents, 600 μL of 1:10 extracts (see above) was further purified by the addition of 300 μL of chloroform, 15 min of incubation at 4°C (rotation wheel), and phase separation (12,000*g*, 10 min, 4°C). The aqueous phase (500 μL) was split into 2 × 250 μL for ammonia and urea determination, respectively. To quantify the urea content, the urea was converted to ammonia by adding 100 units of jackbean urease (Sigma type IX, U4002) and incubated for 20 min at 30°C. Ammonia was purified with strong cation-exchange columns and quantified as described by Witte et al. (2002). For colorimetric ammonia detection, 100 μL of the column efflux was used. Standards were treated identically to samples to account for losses and interference.

For amino acid analysis, extracts were prepared as described by Geiger et al. (1998). Bulk frozen samples were ground in liquid N using a mortar. For shoot material, frozen aliquots of 30 mg were passed into 1.5-mL microcentrifuge tubes, and 50 μL of an internal standard solution (norvaline and sarcosine, 0.5 mM each) for absolute amino acid quantifications was added. Four sequential extractions with 112.5-μL ethanol/water solutions buffered in 10 mM HEPES-KOH, pH 7.0, were performed at 70°C for 4 min, and all extracts were pooled. Each extraction was followed by a 5-min centrifugation step (14,000*g*, room temperature). The four extraction solutions contained 80% ethanol for the first two extractions, then 50%, and finally no ethanol. For root extracts, aliquots of 15 mg were extracted and all volumes were halved. To reduce sample concentration, shoot samples were diluted 1:1 in extraction buffer without ethanol, whereas root samples were used nondiluted. Three independent extracts were analyzed for each shoot sample and two extracts for each root sample. The quantification of amino acids was performed after automated precolumn derivatization with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate on an Agilent 1200 SL HPLC system according to the Agilent application note 5990-4547EN. The samples (1 μL of derivatized extract) were separated on a ZORBAX Eclipse Plus C18 (3.0 × 150, 3.5 μm) column at 40°C using a binary mobile phase gradient of mobile phase A (10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and 0.5 mM NaN_3) and mobile phase B (acetonitrile:methanol:water, 45:45:10, v/v). A diode array detector and a fluorescence detector were employed for detection. For quantification, an amino acid standard from Sigma Aldrich (AAS18) additionally supplemented with Asp, Glu, Trp, citrulline, and Orn was used at four concentrations (9, 22.5, 90, and 225 μM). Norvaline and sarcosine (40 μM each) were also included as internal standards. Calibration curves were generated using Agilent ChemStation, and recalibrations were performed after every eight samples.

Statistical Analyses

For comparison of multiple groups, one-way ANOVA followed by Tukey's posttest was performed using the GraphPad Prism 4 statistics software.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: pXNS1pat-Strep (HM439359), pXNS2pat-Strep (HM439360), pXS1pat (HM439361), pXS2pat (HM439362), *Osurease* coding sequence (HM369060), *OsureD* coding sequence (HM369057), *OsureF* coding sequence (HM369058), *OsureG* coding sequence (HM369059), *Osarginase* (HM369061), and spliced fragment of *AtureF* transcript (HM369062).

Supplemental Data

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

Supplemental Figure S1. Multiple protein sequence alignment of ureases.

Supplemental Figure S2. Multiple protein sequence alignment and secondary structure model of UreD.

Supplemental Figure S3. Multiple protein sequence alignment and secondary structure model of UreF.

Supplemental Figure S4. Multiple protein sequence alignment of UreG.

Supplemental Figure S5. Urease and arginase activity responses to N nutrition and darkness.

Supplemental Figure S6. Phenotypes of rice plants grown under different N regimes.

Supplemental Table S1. Analysis of initiation codons in plant *ureF* gene leader sequences.

Supplemental Table S2. Amino acid quantification in plants grown on different N sources.

Supplemental Table S3. Primers used in this study.

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