Rice urea metabolism: Characterisation of proteins

Claus-Peter Witte
Freie Universität Berlin
Dahlem Centre of Plant Sciences
Department of Plant Biochemistry
Königin-Luise-Str. 12-16
14195 Berlin
Germany

Tel: +49/(0)30/83854787
Email: cpwitte@zedat.fu-berlin.de
Identification and characterisation of proteins involved in rice urea and arginine catabolism

Feng Qiu Cao¹,², Andrea K. Werner², Kathleen Dahncke², Tina Romeis², Lai Hua Liu¹, Claus-Peter Witte²

¹ China Agricultural University, College of Resources and Environmental Sciences, Key Laboratory of Plant and Soil Interactions, 100093 Beijing, China
² Freie Universität Berlin, Dahlem Centre of Plant Sciences, Department of Plant Biochemistry, Königin-Luise-Str. 12-16, 14195 Berlin, Germany
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Corresponding author:
Claus-Peter Witte
cpwitte@zedat.fu-berlin.de
ABSTRACT

Rice (Oryza sativa) production relies strongly on nitrogen (N) fertilisation with urea but proteins involved in rice urea metabolism have not yet been characterised. 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 (UAPs) was demonstrated by complementing corresponding Arabidopsis thaliana mutants and by multiple transient co-expression of the rice proteins in Nicotiana benthamiana. Secondary structure models of rice (plant) UreD and UreF proteins revealed a possible functional conservation to bacterial orthologues, especially for UreF. Using N-terminally StrepII-tagged UAPs, 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 C-terminally StrepII-tagged fusion protein in N. benthamiana, purified and biochemically characterised (K_M = 67 mM, k_cat = 490 s^{-1}). The activity depended on the presence of Mn (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 plants amino acid profile leading to 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.
INTRODUCTION
Nitrogen (N) availability often limits plant performance in natural ecosystems (Vitousek and Howarth, 1991) causing a selective pressure to optimise the use of N resources. This ecophysiological selection has even led to a reduction of the N content of plant proteins in comparison to animal orthologues (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 remobilisation.

Arginine is the most important single metabolite for nitrogen storage in plant seeds. In a survey of 379 plant species arginine N accounted on average for 17.3% of total seed nitrogen (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 nitrogen stored in the guanidinium group of arginine, it must first be hydrolysed by mitochondrial arginase to ornithine and urea. Urea leaves the mitochondria and is hydrolysed by urease in the cytosol releasing ammonia which is re-assimilated into amino acids by the combined action of glutamine synthetase and glutamate synthase.

Urea not only originates from arginine breakdown but may also be taken up from the environment by urea transporters (Kojima et al., 2007; Wang et al., 2008). Urease is therefore involved in N remobilisation 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 which in *Arabidopsis thaliana* requires three urease accessory proteins (*At*UreD, *At*UreF and *At*UreG) for activation (Witte et al., 2005a). Studies in bacteria demonstrated that urease accessory proteins (UAPs) form a complex with apo-urease and are required for posttranslational lysine carboxylation of apo-urease and the subsequent incorporation of two nickel ions into the active centre. 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.

Urea plays an important role in agriculture because it is the most used N fertiliser 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 fertiliser 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 fertilisation directly and assimilate its nitrogen (Kojima et al., 2007; Wang et al., 2008). Although rice is a major crop plant and rice
production is heavily dependent on urea fertilisation, 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 urease accessory proteins UreD, UreF and UreG. The functionality of the corresponding proteins was demonstrated and biochemical parameters determined. The general gene and protein structure of plant UreD and UreF urease accessory proteins were investigated and a direct interaction of rice UreD with apo-urease 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 fertilising 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 urease accessory proteins

Genes and coding sequences for rice *urease* and the urease accessory proteins *ureD*, *ureF* and *ureG* were predicted using the rice genome annotation, expressed sequence tag (EST) data, the corresponding Arabidopsis sequences and multiple protein alignments of the corresponding proteins from monocotyledons generated from the TIGR EST assemblies (Childs et al., 2007). Total RNA from *Oryza sativa* ssp. Indica Hunan late 2 (PI 503035; National Small Grains Collection, Idaho, USA) 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 to 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 co-expression 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 (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 to the respective mutants showed that rice *urease*, *ureD* and *ureG* complemented the mutants while *ureF* did not. However, RT-PCR of *OsureF* transformants revealed that the transgene was expressed (Fig. 1). The Ds 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 co-expressed, urease activity in leaf extracts increased clearly and was 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 to IV with lanes VI and VII). Although these differences were not statistically significant, it appeared that the rice urease activation process was partially complemented 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) which form 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 and 2005b; Supplemental Figs. S1 to S4). We hypothesised 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 formation of insoluble agglomerates upon over-expression. Comparing a secondary structure model of rice UreF (Fig. 3A) based on an alignment of 19 plant UreF proteins (Supplemental Fig. S3) with (i) a partial UreF protein structure from Helicobacter pylori made recently available (PDB accession number:
2WGL) and (ii) 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 *K. aerogenes* based on an alignment of 100 bacterial UreD proteins indicated that plant and bacterial UreD proteins, although being 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 *Os*UreD directly interacts with rice urease both proteins were co-expressed in *N. benthamiana* leaves. When N-terminally StrepII-tagged *Os*UreD was affinity purified from leaf extracts, *Os*urease but also urease from *N. benthamiana* were co-purified. This was not observed when purifying tagged *Os*UreG after co-expression with rice urease (Fig. 3C). Unfortunately, tagged *Os*UreF was not expressed in sufficient amounts for protein detection (RNA was present, not shown). These data indicate that *Os*UreD directly interacts with rice urease while *Os*UreG does not. In bacteria, UreD interacts with urease most directly as well while urease-UreF or -UreG complexes were not found (Carter et al., 2009). UreF was hypothesised 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 form the structurally conserved catalytic core for urease activation while UreD functions as 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 level 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 *At*UreD expression in plants. Such a mechanism was not suggested for *AtureF* because it was found to be an intron-less gene (Witte et al., 2005a). However, for *OsureF* our gene analysis revealed that an intron in the non-coding 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 abnormally spliced contributing to low plant UreF expression. Using the Phytozome v. 5.0 database of sequenced plant genomes (http://www.phytozome.net/) the structure of *ureF* genes from 16 higher plant species was 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 thaliana. 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) splicing removes all non-start 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 may therefore be non-functional due to inefficient translation. Based on our finding that plant ureF genes generally do contain an intron, we postulated that (i) ureF from Arabidopsis may also be spliced in the 5’ leader removing several upstream non-start AUG codons and (ii) that possibly the conserved 5’ leader intron of plant ureF genes is inefficiently spliced to limit UreF expression. For AtureF both assumptions could be validated. An intron of 274 base pairs is spliced from the 5’ leader with low efficiency (Fig. 4B, left panel). 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 panel). 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 characterisation of rice urease and arginase

For rice urease a KM of $0.53 \pm 0.06 \text{ mM}$ (ci, $P = 95\%$, Fig. 5A) was determined using leaf extracts. This is in close agreement with the KM values 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 haemaglutinin (HA) and StrepII-tagged protein was highly purified from leaf extracts by StrepTactin affinity chromatography (Fig. 5B). A KM value of $67 \pm 9 \text{ mM}$ (ci, $P = 95\%$) and a turnover number of $490 \pm 19 \text{ s}^{-1}$ (ci, $P = 95\%$) were determined for the purified enzyme (Fig. 5C). Arginases of other plants possess similar KM constants: 83 mM were 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 Mn concentration of $1.3 \pm 0.6 \mu\text{M}$ (ci, $P = 95\%$). For the enzyme from Phaseolus vulgaris a $K_d$ for Mn of 0.47 $\mu\text{M}$ was determined but also Co, Ni and Cd activated the enzyme partially when used at 2 mM concentrations (Carvajal et al., 1996). The half-activation constant of arginase is in the same concentration range as found for other Mn-dependent enzymes.
indicating that full activation is reached at physiological concentrations of Mn (Werner et al., 2008). Other metals that are needed at high non-physiological 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 (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 arginine at this time point. Increases in arginase activity during germination have also been observed in several other species (Goldraij and Polacco, 1999; 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 (fw) to about 0.3 µmol g⁻¹ fw during germination, indicating that sufficient urease activity was present to metabolise the urea generated in the arginase reaction.

**Growth on urea versus ammonium nitrate**

Rice plants (ssp. Indica, Hunan) were grown from seed for 10 days under sterile conditions either on urea (5 mM) or ammonium nitrate (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 ammonium nitrate (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 nitrogen. Urease and arginase activities were similar irrespective of the supplied N source. Higher specific activities (referred to total protein) on media with limiting N were not due to an increase in absolute activity but to decreased total protein contents (Fig. 7A). The 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-day-old plants grown on AN were placed on urea media and urease and arginase activities were monitored for the following three days, similar results were obtained (Supplemental Fig. S5). Also carbon starvation induced by placing the plants into the dark had no significant influence on these activities. Shoot fresh weight was generally increased by ample nitrogen 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 to plants grown on urea (U and 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 to 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 to 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, glutamine and asparagine (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 fw 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 arginine 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 nor arginase activity are altered if urea is the sole N source. In contrast, rice arginase activity rises during germination as observed in several other plants, because stored arginine needs to be mobilised. For example in soybean, arginase activity is low during embryo development when arginine is deposited, rising steeply during germination when arginine 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 (> 50 mM) indicating low affinity to arginine. In the presence of low amounts of arginase, arginine may therefore be relatively
stable as observed by Goldraij and Polacco (1999). Beyond serving as nitrogen reserve, arginine is also required for polyamine biosynthesis and has been linked to NO biosynthesis in plants while arginase appears to play a role in polyamine and NO 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 arginine catabolism for N mobilisation.

Four proteins are needed in Arabidopsis to generate ureolytic activity (Witte et al., 2005a) and orthologous proteins are required in rice (Fig. 1 and 2). Corresponding genes / proteins are also found in other plants (Supplemental Figures S1 to 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 (Fig. 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 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 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 (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 non-start 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 sole N source, a significant increase in internal urea concentration was not observed in rice (this study and Gerendas et
al., 1998) and Arabidopsis (Merigout et al., 2008) while in *Brassica napus* a moderate rise of urea concentration was measured (Gerendas and Sattelmacher, 1999). The Michaelis constant 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 fertilisation 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. 5), potato (Witte et al., 2002) and several other plants (Gerendas et al., 1999).

Although urea is in general rapidly metabolised 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 to 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 to 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 to nitrate and often a mixed ammonium nitrate fertilisation is best (Britto and Kronzucker, 2002). While there is probably more than one reason why plants underperform on reduced N sources (discussed in Britto and Kronzucker, 2002) limiting root to shoot translocation of N in the absence of nitrate is one important aspect. Using 13N-labelled compounds it was demonstrated that nitrate when co-supplied 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 15N-labelled urea, Merigout et al. (2008) showed that Gln labelling 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 labelled N source offered in the pulse. A reduced N assimilation rate in plants supplied with urea may explain why protein nitrogen and total nitrogen content are generally lower than in AN-grown plants (Fig. 7A). Roots but also shoots contained sufficient glutamate and glutamine 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 2). 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 2). Despite a lower total protein and N content 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 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 fertiliser for rice, the molecular details of its usage by plants are not well investigated. Nitrogen 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

*Oryza sativa* ssp. *Indica* Hunan late 2 seeds (PI 503035) and *Japonica* Nipponbare (PI514663) were received from the National Small Grains Collection, USA; *Oryza sativa* ssp. *Japonica* Hwayoung seeds were received from Postech University, Korea. Rice seeds were...
germinated for 2 days 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 light, 150 μmol m⁻² s⁻¹, 24°C day, 18°C night). For sterile culture of plants, Indica seeds were dehusked, surface sterilised with 2% hypochlorite solution for 15 min, rinsed and germinated for two days in sterile water. Glass jars (14 cm height, 8 cm diameter) filled with 50 ml growth medium solidified by 0.4% (w/v) plant agar (Duchefa) were used to grow six seeds each (greenhouse, 14 h light, 24°C day, 18°C night). The basic growth medium contained 0.17 mM Na₂HPO₄, 0.27 mM K₂SO₄, 0.47 mM CaCl₂, 0.37 mM MgSO₄, 45 μM Fe-EDTA, 0.16 μM CuSO₄, 0.15 μM ZnSO₄, 0.10 μM Na₃MoO₄, 15 μM H₃BO₃, 4.6 μM MnSO₄, 1 μM NiSO₄, 0.05% (w/v) MES, pH 5.7. Nitrogen sources were supplied as 5 mM NH₄NO₃ (AN), 5 mM urea (U), 5 mM urea and 0.25 mM NH₄NO₃ (U+), and 0.25 mM NH₄NO₃ (+). Urea was not autoclaved but added from a sterile stock solution. Plants were harvested after 10 days, shoot and roots weighed in pools of five and 100 mg of each pool extracted (see below). The remaining material was frozen and samples prepared for total nitrogen content measurement and amino acid analysis. *Nicotiana benthamiana* was grown in a greenhouse at 22°C during day time and 18°C at night with 14 h light. Three days before infiltration of Agrobacteria, 6 ml NiCl₂ solution (1 mM) were added to the soil, except for plants used in co-purification experiments. *Arabidopsis thaliana* were grown at 20°C, 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 NiCl₂ 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 *NdeI* or an *NcoI* 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 number: AY436765) using the *XhoI* and *EcoRI* 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 were converted to pXS1pat and pXS2pat using *XhoI* and *NdeI* or *XhoI* and *NcoI*, respectively. RNA from rice leaves was
prepared using TRI reagent (Sigma) and treated with DNaseI (Fermentas) following the manufacturers’ instructions. Moloney murine leukemia virus reverse transcriptase (Promega) and a poly-T primer were employed for reverse transcription using 1.0 μg 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 and re-amplified with primers 1694 and 1695 and cloned into pCR-Blunt (Invitrogen). After sequencing, the clone was first cut with XbaI and then with EcoRI and brought into pXCS-HASrept (Witte et al., 2004) via three point ligation. The cloned cDNA contains a stop codon; the protein is therefore expressed without tag from this vector. OsureD was first amplified with primers 1705 and 1683, cloned, re-amplified with primers 1723 and 1701 and cloned into pCR-blunt. The insert was released with NcoI and BamHI 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 NdeI and XbaI for ureF and primers 2541, 2542 and 1698, 1699 and cloning via NcoI and BamHI after partial digest for ureG. Osarginase was amplified with primers 2265, 2266 and 2267, 2268 in two nested PCR reactions and cloned into pCR-Blunt. After sequencing, the cDNA was cloned via EcoRI and SmaI into pXCS-HASrept 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 one 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 co-purifying urease with UAPs after transient expression in N. benthamiana and for the purification of Osarginase. To study ureF splicing, cDNA was prepared from Arabidopsis (Col 0) and rice ssp. 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, *Os*Urease (U), *Os*UreD (D), *Os*UreF (F) and *Os*UreG (G) were co-expressed in different combinations (UDFG, UFG, UDG, UDF and DFG). The optical densities at 600 nm (OD\textsubscript{600}) of Agrobacteria 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\textsubscript{600} of each mixture was brought to 0.5 with Agrobacteria carrying a construct encoding *At*allantoinase-HAStrep (Werner et al., 2008) unrelated to the process of urease activation. Finally Agrobacteria carrying a silencing inhibitor construct (Witte et al., 2004) were added bringing the total OD\textsubscript{600} to 0.6. For co-purification experiments, N-terminally StrepII-tagged variants of *Os*UreD or *Os*UreF or *Os*UreG were co-expressed with untagged *Os*urease (each at final OD\textsubscript{600} of 0.2). In controls, *Os*Urease or N-terminally tagged *Os*UreD were expressed alone but mixed with Agrobacteria carrying a promoter-GUS construct (unrelated to the process of urease activation) to reach the same bacterial concentration in all treatments. Finally Agrobacteria carrying a silencing inhibitor construct (Witte et al., 2004) were added bringing the total OD\textsubscript{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 (AEBSF) and 10 mM dithiothreitol (DTT). Fresh Arabidopsis and *N. benthamiana* leaves were extracted using a 1 to 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 in Witte and Medina-Escobar (2001) equilibrated in 10 mM HEPES, pH 7.5 (slurry density: 0.16 g ml\textsuperscript{-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 extraction buffer using 6 steal beads (4 mm diameter) in a mill (Retsch, MM 301) for 4 min at a frequency of 25 sec\textsuperscript{-1}. After centrifugation (15 min, 20,000g, 4°C) 600 µl of supernatants were frozen for the determination of ammonia and urea contents and 100 µl of supernatants were desalted (spin columns) for activity measurements. For the determination of the K\textsubscript{M} value of rice urease, leaves were extracted in a mortar using a 1 to 4 ratio of fresh weight to buffer and centrifuged (20 min, 20,000g, 4°C). Supernatants were desalted with High Trap G25 columns (GE Healthcare) equilibrated in half-strength extraction buffer without AEBSF and DTT. Protein concentrations in the desalted plant extracts were determined with a commercial Bradford reagent (Biorad) and bovine serum albumin as standard.
**Affinity purification**

For co-purification 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 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, poly (ethylene glycol) 4000 was added reaching a final concentration of 5% to stabilise the enzymatic activity for freezing. For the 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 NanoOrange 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 water followed by the addition by 50 µl phenol reagent and 100 µl 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 interferences with the colour 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 desalted leaf extract were mixed with 125 µl two-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 water followed by 50 µl phenol reagent and 100 µl 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 arginine (500 mM, pH 9.0), 12.5 µl H₂O, 2.5 µl phenylphosphorodiamidate (2 mM, a urease inhibitor) and 0.5 µl MnCl₂ (100 mM). After pre-incubation of this reaction mixture for 3 min at 30°C, the reaction was started by the addition of 10 µl extract. After 0 and 30 min, samples of 10 µl were added to 100 µl of water and frozen in liquid nitrogen 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 pre-incubation 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 purified enzyme (5.4 ng µl\(^{-1}\)) and 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 arginine (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 nitrogen. The kinetic constants were determined at final arginine 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 interferences. For each arginine 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 arginine 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 colour 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 stainings**

SDS-gel electrophoresis, Western blot 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 were analysed in an elemental analyser (EuroEA 3000) in three replicates per sample. For the determination of the ammonia and urea contents, 600 µl 1:10 extracts (see above) were further purified by addition of 300 µl chloroform, 15 min incubation at 4°C (rotation wheel) and phase separation (12,000g, 10 min, 4°C). The aqueous phase (500 µl) was split into 2 x 250 µl for ammonia and urea determination, respectively. To quantify the urea content, the urea was
converted to ammonia by adding one hundred 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 in Witte et al. (2002). For colorimetric ammonia detection, 100 µl of the column efflux were used. Standards were treated identical to samples to account for losses and interferences.

For amino acid analysis extracts were prepared as described by Geiger et al. (1998). Bulk frozen samples were ground in liquid nitrogen using a mortar. For shoot material, frozen aliquots of 30 mg were passed into 1.5 ml micro-centrifuge tubes and 50 µl of an internal standard solution (norvaline and sarcosine, 0.5 mM each) for absolute amino acid quantifications 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 pooled. Each extraction was followed by a 5 min centrifugation step (14,000g, 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 non-diluted. Three independent extracts were analysed for each shoot sample and two extracts for each root sample. The quantification of amino acids was performed after automated pre-column derivatisation with o-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) on an Agilent 1200 SL HPLC system according to the Agilent application note 5990-4547EN. The samples (1 µl derivatised extract) were separated on a ZORBAX Eclipse Plus C18 (3.0 x 150, 3.5 µm) column at 40°C using a binary mobile phase gradient of mobile phase A (10 mM Na2HPO4, 10 mM Na2B4O7, pH 8.2, 0.5 mM NaN3) and mobile phase B (acetonitrile: methanol: water / 45:45:10 by volume). A diode array detector (DAD) and a fluorescence detector (FLD) were employed for detection. For quantification an amino acid standard from Sigma Aldrich (AAS18) additionally supplemented with Asp, Glu, Trp, citrulline, ornithine was used at four concentrations (9 µM, 22.5 µM, 90 µM 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 Turkey’s post test was performed using the GraphPad Prism 4 statistics software.
GenBank accession numbers
pXNS1pat-Strep (HM439359), pXNS2pat-Strep (HM439360), pXS1pat (HM439361),
pXS2pat (HM439362), Osurease cds (HM369060), OsureD cds (HM369057), OsureF cds
(HM369058), OsureG cds (HM369059), Osarginase (HM369061) spliced fragment of
AtureF transcript (HM369062).

SUPPLEMENTAL MATERIAL

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 response to N nutrition and darkness
Supplemental Figure S6. Phenotype 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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FIGURE LEGENDS

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, 8) and two independent complementation lines for each mutant (lanes 3, 4; 6, 7; 9, 10). The numerical values above the columns of 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 limited amount of material. Error bars are sd. 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 urease accessory proteins expressed in N. benthamiana. A, Western blot loaded with 40 µg protein per lane and probed with anti-urease antibody (upper panel) and urease activity quantification (lower panel) using leaf extracts from N. benthamiana after five days of transient co-expression of different combinations of Osurease, OsUreD, OsUreF, OsUreG (I to V) and Atallantoinase (VI) as control. Uninfected leaves were used as additional control (VII). Each urease activity was quantified using three independent leaves from different plants (n = 3). Error bars are sd. As indicated by lower case 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 co-expressing either OsUrease, OsUreD, OsUreF, OsUreG (gray columns) or OsUrease, OsUreD, OsUreG
but not OsUreF (black columns). Activities were assessed in a time course from three to six days after infiltration of the plants with Agrobacterium. Error bars are sd (n = 3, independent leaves).

**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 *Helicobacter pylori* (Hp) and from *Klebsiella aerogenes* (Ka). Proteins are drawn to scale, α-helices are displayed as gray boxes, 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 numerical value (scale 0 to 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 co-purification experiment. Rice urease (U) and N-terminally StrepII-tagged OsUAPs UreD, UreF and UreG (D, F, G) were co-expressed as indicated in leaves of *N. benthamiana* and affinity purified from extracts. Panels 1 and 3, detection with StrepTactin alkaline phosphatase conjugate in crude extracts (1, input) and after affinity purification (3, output). Panels 2 and 4, detection with anti-urease antibodies in crude extracts (2, input) and after purification (4, output). Stars label unspecific signals.

**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 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. Left panel, using Arabidopsis leaf RNA and primers 2438 and 1021. Right panel, using rice leaf RNA and primers 2440 and 2439 (lane 1) or 2441 and 2439 (lane 2).
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Figure 6. Changes of urease and arginase activity during rice germination. Urease activity (upper panel) and arginase activity (lower panel) relative to total protein in a time course taken during germination of ssp. Hwayoung from day 0 (dry seed) to day 6. Error bars are sd (n = 3).

Figure 7. Analysis of rice plants grown under different N regimes. A, Urease activity, arginase activity, fresh weight, total nitrogen, total protein and amino acid nitrogen in shoot and root of plants grown without nitrogen (no), with 5 mM ammonium nitrate (AN), with 5 mM urea (U), with 5 mM urea and 0.25 mM ammonium nitrate (U+) and with limiting 0.25 mM ammonium nitrate (+) as sole N source. Error bars are 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 fertilised plants (set = 1). Increases at or above 3-fold are additionally labelled by indicating the fold-increase in the corresponding square. Decreases below 3-fold can not be distinguished in this graph. Absolute values are given in Supplemental Table S2.
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