Biuret toxicity induces accumulation of nitrogen-rich compounds in rice plants

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Abstract

Excess biuret, a common impurity in urea fertilizers, is toxic to plants. Little is known about the mechanisms of biuret toxicity in plants. A previous study had shown that transgenic rice (*Oryza sativa*) plants overexpressing bacterial *biuret hydrolase* improved biuret tolerance. Additionally, the *biuret hydrolase*-overexpressing plants showed a higher ^{15}N ratio than wild-type plants when the roots were fed ^{15}N-labeled biuret. Here, we determined biuret accumulation in rice seedlings by directly measuring the biuret. We found that the *biuret hydrolase*-overexpressing plants did not contain biuret, whereas wild-type plants accumulated biuret in shoots in the order of mmol L $^{-1}$ tissue water. We also found that the concentration of allantoin, a nitrogen-rich intermediate compound in the purine degradation pathway, in rice shoots under biuret toxicity was higher than those in control conditions. Inhibition of allantoinase activity by biuret was not detected, and allantoin accumulation appeared to be associated with changes in the expression of putative allantoin transporter genes. Furthermore, another nitrogenous compound citrulline, which is a nonprotein amino acid, accumulated in rice suspension cells under biuret toxicity. The accumulation of these two nitrogen-rich compounds suggests that rice plants subjected to biuret toxicity may need to reduce the generation of surplus ammonium ions.

Title

Biuret toxicity induces accumulation of nitrogen-rich compounds in rice plants

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-overexpressing plants showed a higher ^{15}N ratio than wild-type plants when the roots were fed ^{15}N-labeled biuret. Here, we determined biuret accumulation in rice seedlings by directly measuring the biuret. We found that the *biuret hydrolase* -overexpressing plants did not contain biuret, whereas wild-type plants accumulated biuret in shoots in the order of mmol L^{-1} tissue water. We also found that the concentration of allantoin, a nitrogen-rich intermediate compound in the purine degradation pathway, in rice shoots under biuret toxicity was higher than those in control conditions. Inhibition of allantoinase activity by biuret was not detected, and allantoin accumulation appeared to be associated with changes in the expression of putative allantoin transporter genes. Furthermore, another nitrogenous compound citrulline, which is a nonprotein amino acid, accumulated in rice suspension cells under biuret toxicity. The accumulation of these two nitrogen-rich compounds suggests that rice plants subjected to biuret toxicity may need to reduce the generation of surplus ammonium ions.

Key words: allantoin, biuret, citrulline, nitrogen, *Oryza sativa*, rice

Introduction

Biuret is a common impurity in urea fertilizers. It is a byproduct of the urea granulation process and is formed by the thermal condensation of two urea molecules. When urea fertilizers are added to arable lands, biuret, a contaminant in these fertilizers, is also applied. Biuret in the soil is decomposed by microorganisms and eventually produces ammonia and carbon dioxide, relatively slow but the same as urea's fate (Aukema et al., 2020; Cameron et al., 2011; Robinson et al., 2018). Biuret is not known to be toxic to animals; however, excess biuret can stunt plant growth and cause chlorosis of leaves (Mikkelsen, 1990). Consequently, the permissible biuret concentration in many countries is 1.2% for urea fertilizers.

Previous studies on biuret toxicity have shown that it inhibits protein synthesis in plants (Ogata T. & Yamamoto M., 1959; Webster et al., 1957). A recent experiment also found that biuret toxicity can alter the expression levels of many genes involved in environmental stress response (Ochiai et al., 2020). However, the mechanisms underlying biuret toxicity are still not well understood. Clarification of this mechanism may help prevent potential plant injury.

We previously found that rice plants that were overexpressing *biuret hydrolase* from a soil bacterium had an enhanced biuret tolerance (Ochiai et al., 2020). The experiment used ^{15}N-labeled biuret to show that *biuret hydrolase* overexpressing plants take up more biuret than wild-type plants. However, the form of ^{15}N in plants after uptake is not known. In this study, we examined the biuret accumulation, in wild-type and *biuret hydrolase* overexpressing rice plants using HPLC-UV, to understand how the biuret concentration in plants causes injury.

In addition, we hypothesized that biuret inhibits the metabolism of compounds with a similar structure, specifically ureido compounds. As an ureido, we focused on allantoin because of its multiple roles and importance to plants. Allantoin, a compound composed of a hydantoin ring and ureido group, is an intermediate in the purine degradation pathway. It contains four nitrogen (N) atoms per molecule and contributes to N recycling in plants (Soltabayeva et al., 2018). Allantoin and allantoic acid are the dominant forms of assimilated-N transported from roots to shoots through the xylem in tropical leguminous plants (Schubert et al., 1986). Many plant species accumulate allantoin under abiotic stress such as salinity, drought, and heavy metal toxicity (Casartelli et al., 2019; Kaur et al., 2021; Lescano et al., 2016; Nourimand and Todd, 2016; Watanabe et al., 2013). The accumulated allantoin can enhance the abiotic stress tolerance of plants (Watanabe et al., 2013). We investigated the effect of biuret on the accumulation and metabolism of allantoin in rice seedlings.

Furthermore, we performed a metabolome analysis using rice suspension cells to examine metabolite changes under biuret toxicity.

Materials and methods

Plant materials and growth conditions

Seeds of *japonica* rice (*Oryza sativa*) cultivar Nipponbare were purchased from Nouken (Kyoto, Japan). Transgenic rice lines overexpressing bacterial *biuret hydrolase* were developed from Nipponbare (Ochiai et al., 2020), and the T_3 generation was used in this study. Seeds of rice were soaked in distilled water added with fungicide (Trifumin; Nippon Soda, Tokyo, Japan) for two days. Ten seeds were sown on a mesh (18 mesh, 23 x 34 mm) stretched on a plastic slide mount and floated on a culture solution. The culture solution contained 1 mmol $L^{-1}(NH_4)_2SO_4$, 0.5 mmol L^{-1} KCl, 0.25 mmol $L^{-1}KH_2PO_4$, 0.5 mmol $L^{-1}CaCl_2$, 0.5 mmol $L^{-1}MgCl_2$, and Arnon's micronutrient (cited by Hewitt, 1966). Iron was supplied at the rate of 5 mg Fe L^{-1} as Ethylenediamine-N,N,N',N'-tetraacetic acid iron(III) sodium salt. Biuret was added to the solution whenever necessary. The culture solution was prepared with tap water and not aerated. At most, six nets were floated in a 1-L plastic container. Plants were raised in a growth chamber (NS-280 FHW; Takayama Seisakusyo, Kyoto, Japan) under the following conditions: temperature, 30°C; photoperiod, 12 h; and light intensity, 350 μmol m^{-2} s^{-1}.

The rice Oc cell suspension culture line (Baba et al., 1986) was provided by RIKEN BRC, participating in the National BioResource Project of the MEXT/AMED, Japan. The cells were maintained as described by Ochiai et al. (2020).

Confirmation of transgene in *biuret hydrolase*overexpressing rice plants

DNA was extracted from the second leaves of individual T_3 plants of *biuret hydrolase* overexpressing lines. Second leaves were also excised from the wild-type plants to equalize the effects of leaf clipping. The transgene was detected by PCR using the primers 5´-ATGAAGACACTTTCCAGCGC-3´ and 5´-TGGCAAATGCCTCTCAAGG-3´ and Blend Taq polymerase (Toyobo, Osaka, Japan). Plants confirmed to possess *biuret hydrolase* were used in the analysis.

Determination of biuret and allantoin in rice seedlings

At harvest, the rice roots were rinsed for 3 mins, three times with 100 mL of distilled water. Several seedlings were combined into a single sample, blotted and dried with paper towels, separated into shoots and roots, weighed, and freeze-dried. After determining the dry weights, the samples were ground into a powder using a ball mill.

About 10 mg of the powdered sample was extracted with 250 μL of distilled water. After centrifugation, a 35 μL aliquot of the supernatant was mixed with 465 μL of acetonitrile and centrifuged again. A 20 μL aliquot of the supernatant was injected into the HPLC system (LC-10AS; UV detector: SPD-10A, Shimadzu, Kyoto, Japan) equipped with a hydrophilic interaction chromatography (HILIC) column (YMC-Triart Diol-HILIC, 5μL, 4.6 x 250 mm, YMC Co. Ltd., Kyoto, Japan). The isocratic eluent was a mixture of 930 mL of HPLC-grade acetonitrile and 70 mL of distilled water. In some experiments, we modified the eluent to a mixture of 940 mL of acetonitrile and 60 mL of distilled water to improve the separation. In this case, a 94:6 mixing ratio was used for sample preparation. Elution was performed at a flow rate of 0.5 mL min^{-1}, and the effluent was monitored at 190 nm. The colorimetric determination of allantoin was performed as described by Young and Conway (1942).

Determination of total-N

The total-N in the plant samples was determined by the combustion method using an NC analyzer (Sumigraph NC-22F, Sumika Chemical Analysis Service, Osaka, Japan).

Inhibition assay for allantoinase activity

Allantoinase activity was assayed according to Duran and Todd (2012). Shoots of 9-day-old Nipponbare seedlings grown without biuret were weighed and homogenized with a five-fold volume of extraction buffer containing 50 mmol L^{-1} Tricine (pH 8.0) and 2 mmol L^{-1} $MnSO_4$. After centrifugation, the supernatant was used in the inhibition assay. The enzymatic reaction was initiated by the addition of allantoin as a substrate at a final concentration of 10 mmol L^{-1} to the supernatant. Biuret, 0, 0.5, and 5 mmol L^{-1} at final concentration, were added to the reaction mixture together with the allantoin. The mixture of 0.50 mL in

a total was incubated at 30 ºC for 30 min, and the reaction was stopped by adding 0.25 mL of 0.15 mol L^{-1}HCl. Allantoic acid in the reaction mixture was colorimetrically determined (Young & Conway, 1942). The allantoic acid content of the crude extract was also determined and subtracted.

Protein concentrations in the crude extracts were determined by the Bradford method using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan).

Gene expression analysis

Rice plants were grown with and without 0.3 mmol L^{-1}biuret supplementation in the culture solution. Four- to seven-day-old seedlings were harvested during the light period. Two to four plants were combined into a single sample. Total RNA was extracted from roots and shoots using the Plant Total RNA Extraction Miniprep System (Viogene, Taipei, Taiwan). First-strand cDNA was synthesized from total RNA using oligo dT primers and ReverTra Ace polymerase (Toyobo, Osaka, Japan). Quantitative real-time RT-PCR was performed using the TP850 Thermal Cycler Dice Real Time System Single (Takara Bio, Shiga, Japan) and THUNDERBIRD SYBR qPCR MIX (Toyobo, Osaka, Japan). Primer pairs used for target genes were 5´-CTGGAGCGTGCTATGTTTCA-3´ and 5´-AGCTTGTGGACCACCAAAAC-3´for *xanthine oxidase* , 5´-TCAGCTAAGGATGCCGAACC-3´ and 5´-ATGGTCCCGTGTGGTTCATC-3´ for*urate oxidase* , 5´-TAACGTCGCTCCTGGTTTCT-3´ and 5´-ACAGAGGGACATGGAAATGC-3´ for *allantoin synthase* , 5´-AACGCATACCCGATGTTCAG-3´ and 5´- TGTCTTTCATCGCACGTTGC-3´ for*allantoinase* , and 5´-TGAACCACAGCAACACCAAT-3´ and 5´-GCCACTTCAGGCTCTTGTTC-3´ for *ureide permease 1* , 5´-CTCTGCTGTACATGCCTCCA-3´ and 5´-TGTTTGGTTCCACACTTCCA-3´ for*ureide permease 2* , 5´-GAGAAGAGGCGATCCATCAA-3´ and 5´-CGAGAAGAGGGAGAAGCAGA-3 for *ureide permease 3* . The expression levels of the genes were normalized to the mean expression level of*Ubiquitin* (primer pairs: 5´-AGAAGGAGTCCACCCTCCACC-3´ and 5´-GCATCCAGCACAGTAAAACACG-3´; Yamaji & Ma, 2009) and *Actin 1*(primer pairs: 5´-ATCCTTGTATGCTAGCGGTCGA-3´ and 5´-ATCCAACCGGAGGATAGCATG-3´; Caldana et al., 2007).

Metabolome analysis in rice suspension cells

Biuret treatment was initiated by subculturing 2 mL of seven-day-old cell suspension into 80 mL of the medium supplemented with or without 0.3 mmol L^{-1} biuret. Then, cells were harvested three and five days after subculturing. Cells were collected by suction filtration, rinsed with distilled water, frozen with liquid nitrogen, and stored at -80 °C until analysis. Samples were prepared in duplicate.

Metabolomic analysis was done by the Kazusa DNA Research Institute, Kisarazu, Chiba, Japan. Briefly, rice cells were extracted with methanol. A 5-μL aliquot of the extract was analyzed using liquid chromatography-mass spectrometry (LC-MS). LC-MS analysis was conducted on an Agilent 1200 series LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a TSK-GEL ODS-100V column (5μm, 3 × 50 mm; Tosoh, Tokyo, Japan) and connected to an LTQ ORBITRAP XL mass spectrometer (Thermo Fisher Scientific, Waltham, MS, US). The gradient mobile phase consisted of 0.1% formic acid in water (solution A), and acetonitrile (solution B). MS detection was performed using positive-ion mode electrospray ionization. The data were converted into text files using the MSGet software (http://www.kazusa.or.jp/komics/software/MSGet) and then organized using the PowerGet software (Sakurai et al., 2014).

Peaks detected in both the replicates of at least one of the four sample groups were used for subsequent analysis. Statistical analyses were performed using the R software (R Core Team, Vienna, Austria). The peak intensities were log2 transformed and then normalized to the median of each sample, and the missing values were replaced by half the value of the minimum peak intensity. The Welch's t-test was used to test the differences between the means of the two groups. The annotation information provided by Kazusa DNA Research Institute and a web program Metaboanalyst 5.0 (Pang et al., 2021) was used to identify candidate compounds for the peaks.

Results

Biuret accumulation in rice seedlings

First, biuret concentration was determined in 7-day-old rice (Nipponbare) seedlings grown with 0, 0.1, and 0.3 mmol L^{-1} biuret supplemented in the culture solution. Visual observation showed chlorosis on the leaves of plants under 0.3 mmol L^{-1} biuret treatment. The root and shoot dry weights were not significantly different among treatments. However, shoot dry weights tended to decrease with an increasing biuret concentration in the culture solution (Fig. 1a). Biuret concentration in roots and shoots increased with its concentration increase in the culture solution (Fig. 1b). Consistent with the previous ^{15}N-labeled biuret uptake experiment (Ochiai et al., 2020), the biuret concentration in the shoots was higher than that in the roots, suggesting that biuret accumulated in the shoots with the transpirational volume flow.

The biuret concentration was also determined for 9-day-old transgenic rice lines overexpressing bacterium *biuret hydrolase* under the control of a modified CaMV35S promoter. The dry weight of the wild-type plants was significantly reduced by 0.3 mmol L^{-1}biuret in the culture solution (Fig. 1d). In contrast, biuret injury was alleviated in the *biuret hydrolase* overexpressing lines grown in the same container (Fig. 1d, Supplemental Fig. S1). Biuret was not detected in *biuret hydrolase* overexpressing plants grown with 0.3 mmol L^{-1} biuret (Fig. 1f). The results indicated that excess biuret in plants was the cause of biuret injury.

Allantoin accumulation in rice seedlings

The allantoin concentration in rice plants determined by HPLC was consistent with that measured by the colorimetric method (Supplementary Fig. S2). The allantoin concentration in 7-day-old wild-type rice was higher in the roots than in the shoots. However, the allantoin concentration in roots did not differ among treatments. On the other hand, the shoot allantoin concentration was significantly higher in plants under 0.3 mmol L^{-1} biuret treatment than in control and 0.1 mmol L^{-1} biuret-treated plants. The shoot total N concentrations were 51.7, 49.8, and 49.4 mg g^{-1} dw for plants grown with 0, 0.1, and 0.3 mmol L^{-1} biuret, and there was no significant difference among treatments ($p < 0.05$, Tukey's test).

The shoot allantoin concentration of the 9-day-old *biuret hydrolase* overexpressing plants under 0.3 mmol L^{-1}biuret toxicity was not different from that of the control (Fig. 1f). This indicated that there was no increase in allantoin accumulation in the absence of injury. The root allantoin concentration of biuret-treated plants was lower than that of the control in the wild-type and line B-2-3-3 plants (Fig. 1f).

In the purine degradation pathway, the primary substrate xanthine is oxidized to uric acid by xanthine dehydrogenase (XDH). Uric acid is then oxidized to allantoin via 5-hydroxyisourate and 2-oxo-4-hydroxy-4-carboxy-5-ureido-imidazoline. These steps are catalyzed by two enzymes, uric acid oxidase (UOX) and bifunctional allantoin synthase (ALNS). Allantoin is then hydrolyzed to allantoic acid by allantoinase (ALN).

First, we examined whether biuret inhibited ALN activity. In the assay using crude extracts prepared from shoots of rice seedlings grown without biuret and 5 mmol L^{-1} allantoin as the substrate, biuret up to 5 mmol L^{-1} did not inhibit the allantoic acid-producing activity of ALN (Fig. 2).

The expression levels of genes in the purine degradation pathway were then analyzed. The relative expression level of *OsXDH*(Os03g0429800), *OsUO* (Os01g0865100), *OsALNS*(Os03g0390700), and *OsALN* (Os04g0680400) was not changed by the 0.3 mmol L^{-1} biuret treatment in 4 to 7-day-old seedlings. However, there was a significant increase in the expression level of *OsUO* in the 5-day-old seedlings (Fig. 3a-d).

Additionally, the expression levels of the putative allantoin transporter gene *OsUPS1* (*ureide permease 1* , Os12g0503000) and the two homologous genes *OsUPS2* (Os12g0502800) and*OsUPS3* (Os12g0503300) were examined. *OsUPS1* is a homolog of *Arabidopsis AtUPS1* , which codes the gene of an allantoin transporter (Desimone et al., 2002). The biuret treatment significantly reduced the expression level of *OsUPS1* in the shoots of 5-to 7-day-old seedlings (Fig. 3e). The expression levels of *OsUPS2* in the roots of 5 and 7-day-old seedlings and in shoots of 7-day-old seedlings were significantly higher in plants grown with biuret than in control plants.

Allantoin concentration in 8-day-old seedlings that were grown with plants used for gene expression analysis was significantly higher in shoots under biuret treatment than in the control plants (Fig. 3f).

Metabolite changes in rice suspension cells under biuret toxicity

We performed metabolome analysis using suspension-cultured rice cells to comprehensively investigate the changes in metabolites under biuret toxicity. Metabolites of rice suspension cells at two-time points, day 3 (d3) and day 5 (d5) after subculturing, for two treatments, biuret treatment supplied 0.3 mmol L^{-1} biuret and a control treatment without biuret, were analyzed using LC-MS technique. Of the 3,566 peaks detected (Supplementary Data S1), 993 peaks detected in replicates of at least one of the four sample groups were used for subsequent analysis.

Principal component analysis (PCA) was performed to compare the metabolic profiles of rice suspension cells. The first principal component (PC1) accounted for 25.3% of the total variance, the second (PC2) for 21.2%, and the third (PC3) for 15.9%. Figure 1 shows the score plots for PC1 and PC2. These two components clearly separated the four sample groups (Fig. 4). Increasing the culture period increased the PC1 scores, and the biuret group had a smaller PC1 score than the control group. Meanwhile, increasing the culture period decreased the PC2 scores, and the biuret treatment accelerated this decrease. PC1 may be a growth component and PC2 an aging component.

We then compared the mean peak intensities between the control and biuret groups. After excluding one peak that was considered an artifact due to the assignment of different ids to the same compound in different samples, 38 peaks showed significantly different intensities between the two groups (Fig. 5).

Only two of these peaks, id 310 and id 326, matched the standard compounds. They were identified as citrulline and citrulline-related compounds, indicating that citrulline accumulated in the biuret-treated rice suspension cells (Fig. 5). Citrulline is a non-proteinaceous amino acid with an ureido group, and thus, it was shown that biuret excess caused accumulation of a N-rich compound in cultured cells.

Additionally, the intensity of the peak id 1202 showed a marked increase in the number of biuret-treated cells (Fig. 5). Two possible compounds responsible for this peak were $C_{14}H_{20}N_6O_5S_1$and $C_{21}H_{20}O_7$. Peak id 1240, having nearly the same retention time and m/z value of 2.00 greater, indicated that peak 1202 was a sulfur-containing compound. Therefore, it was shown that S -adenosyl homocysteine (SAH) was highly accumulated in biuret-treated cells.

SAH is a byproduct of methylation reactions using$S$ -adenosylmethionine as a methyl donor and is a potential competitive inhibitor of methylation reactions. SAH is associated with DNA hypomethylation (Huang et al., 2019; Rocha et al., 2005). Thus, the accumulation of SAH observed here could be related to the upregulation of many stress-related genes in rice suspension cells under biuret toxicity (Ochiai et al., 2020).

No peaks that could be attributed to allantoin were detected in this analysis, and no differences between the treatment groups were observed.

Discussion

No biuret was detected in *biuret hydrolase* overexpressing lines grown with 0.3 mmol L^{-1} biuret, whereas significant amounts of biuret accumulated in control plants (Fig 1). Combined with the results of previous ^{15}N-labelled biuret uptake experiments (Ochiai et al., 2020), *biuret hydrolase*overexpressing rice plants took up biuret but decomposed most of it in plants. On the other hand, wild-type rice seemingly accumulated biuret without decomposing it. The alleviation of biuret injury in the biuret hydrolase overexpressing lines (Fig 1c, Supplemental Fig. S1) indicates that the excess accumulation of biuret in plants was the cause of injury. Under a uniform distribution of biuret in tissue water, the shoot biuret concentration in wild-type rice seedlings grown with 0.1 and 0.3 mmol L^{-1} biuret were found to be 0.5 and 1.8 mmol L^{-1}, respectively. This suggests that a relatively high biuret concentration in plants is needed to cause injury, and nonspecific effects of biuret such as hydrogen bonding with polar residues are the cause of injury. The severity of biuret toxicity in wild-type plants, however, differed among trials, even when the biuret concentration in plants was nearly the same (Fig. 1), suggesting the presence of factors that enhance or alleviate biuret injury. To investigate the metabolic steps that are specifically inhibited, we plan to search for binding partners using affinity.

Biuret-injured rice shoots accumulated higher concentrations of allantoin than control plants (Fig. 1c, 1f). This was contrary to our assumption that biuret might inhibit the metabolism of ureide compounds, since biuret in our study did not inhibit ALN activity (Fig. 2). The existing knowledge about factors that affect the allantoin concentration in non-leguminous plants includes growing substrate (Wang et al., 2007), N sufficiency status or C/N ratio (Casartelli et al., 2019; Lee et al., 2018; Lescano et al., 2020), the form of N in the N source (Lescano et al., 2020), and environmental stress (Casartelli et al., 2019; Kaur et al., 2021; Lescano et al., 2016; Nourimand and Todd, 2016; Watanabe et al., 2013). In our study, there were no differences in the conditions around the roots other than the biuret concentration, and there were no differences in the total-N concentration in the shoots, indicating that the allantoin accumulation observed here occurred due to changes in the form of the assimilated N within rice seedlings.

Downregulation of the expression of *ALN* is often reported in plants under environmental stress, as a mechanism of allantoin accumulation (Casartelli et al., 2019; Lescano et al., 2016). In contrast, an increase in the expression level of *OsALN* following allantoin accumulation was observed in the shoots of N-deficient rice plants (Lee et al., 2018). In our study, the expression level of*OsALN* in rice seedlings under the control condition and those grown with 0.3 mmol L^{-1} biuret was not different; nevertheless, the allantoin concentration was higher in the biuret-treated plants (Fig. 3d). However, in another independent experiment performed by us, the expression of the *OsALN* was suppressed by biuret (Fig. S3). It is, therefore, difficult to generalize the relationship between allantoin accumulation and*ALN* expression. It most probably is influenced by the severity of the stress.

Additionally, the expression levels of the genes involved in allantoin synthesis were essentially unchanged by biuret toxicity (Fig. 3a–d). Therefore, one possible mechanism for allantoin accumulation under biuret toxicity could be the increased amount of degraded purine base. Another explanation could be the change in the distribution of allantoin within the plant by controlling transport activity. Biuret, most likely, induced suppression of the putative allantoin transporter gene*OsUPS1* in the shoots (Fig. 3e). *OsUPS1* is supposedly related to the long-distance transport of allantoin because it is localized to plasma membrane and expressed around vascular tissues (Redillas et al., 2019). Additionally, the expression of *OsUPS2*had also increased in roots and shoots at several time points because of biuret (Fig. 3f). Given that there are no previous studies on the function of *OsUPS2* , the significance of this increase is unknown at this time.

No peaks corresponding to allantoin were detected in the metabolome analysis performed using rice suspension cells, irrespective of the treatment. However, another nitrogenous compound, citrulline, significantly increased in the biuret-treated cells (Fig. 5). Citruline is a non-proteinous amino acid abundant in Cucurbitaceae plants (reference) and accumulates substantially in wild watermelons (*Citrullus lanatus*), native to deserts, under drought stress (Kawasaki et al., 2000). Its accumulation have also been observed in*Arabidopsis thaliana* under low CO_2 (Blume et al., 2019). Citrulline is considered a scavenger of hydroxyl radicals (Akashi et al., 2001) or NH_4^+ (Blume et al., 2019; Joshi and Fernie, 2017). Regarding the latter, Blume et al. (2019) suggested that photorespiration is the origin of NH_4^+ reassimilated as citrulline. However, NH_4^+ produced by protein degradation or from NH_4^+ providing N source may also have contributed to the assimilation of citrulline, since the rice suspension-cultured cells were grown in the dark.

To conclude, we demonstrated the accumulation of two N-rich compounds, allantoin in intact rice seedlings, and citrulline in suspension-cultured cells under biuret toxicity. Although these compounds may be involved as functional molecules in the response to biuret excess, rice plants might have to modulate N compounds to assimilate or reduce surplus NH_4^+ ions produced in cells because of environmental stress. We are currently conducting experiments to investigate the effect of different N supply levels and N sources on biuret injury in rice plants to clarify the significance of accumulating these N-rich compounds under biuret toxicity.

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Author contributions: KO and TM conceived and designed the research. KO and YN performed ex-

periments and analyzed the data. AU generated *biuret hydrolase* overexpressing rice lines. KO wrote the manuscript with input from other authors.

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Figure legend

Figure 1 Effects of biuret on dry weight (a, d), biuret concentration (b, e), and allantoin concentration (c, f) in roots and shoots of 7-day-old wild-type rice plants (a–c) and 9-day-old*biuret hydrolase* overexpressing rice lines (d–f). Bars and circles represent mean and each sample, respectively. nd means not detected. (a–c) Wild-type plants were grown with 0, 0,1, and 0.3 mmol L^{-1} biuret supplemented in the culture solution. Ten seedlings were combined for a single sample. Different alphabets indicate significant difference among treatments in each organ ($p< 0.05$, Tukey's test, n = 3). (d–f) Wild-type and two independent transgenic lines (B3-9-1 and B2-3-3) were grown with or without 0.3 mmol L^{-1} biuret in the culture solution. Four to six plants were combined into one sample. Gray and black bars represent control and biuret-treated plants, respectively. Asterisks indicate significant difference between the treatment (*$p< 0.05$; **$p < 0.01$, Welch's t-test, n = 3).

Figure 2 Inhibitory effect of biuret for allantoinase activity. Crude extracts were prepared from shoots of 9-day-old rice seedlings hydroponically grown without biuret. Extracts were incubated at 30^{o}C with 10 mmol L^{-1} allantoin, 50 mmol L^{-1} Tricine-NaOH (pH8.0), 2mmol $L^{-1}MnSO_4$, and the desired concentration of biuret for 30min. The amount of allantoic acid produced from allantoin was colorimetorically determined. Same shape symbols indicate a same crude extract. Crossbars represent the mean value. The means were not significantly different among treatments at 5% level (One-way ANOVA with blocking, n = 4).

Figure 3 Relative expression of genes related to purine degradation and ureide metabolisms in roots and shoots of 4 to 7-day old rice seedlings. Rice plants were hydroponically grown under the control condition and 0.3 mmol L^{-1} biuret toxicity. (a-h) Relative expression levels of *OsXO* (a), *OsUO* (b),*OsALNS* (c), *OsALN* (d), *OsUPS1* (e), *OsUPS2*(f), and *OsUPS3* (g). The expression levels were normalized to the expression of *Ubiquitin* and *Actin1* and expressed in log2 scale. Gray and black symbols indicate control and biuret treated samples, respectively. Crossbars indicate means of the three samples. (h) Allantoin concentration in 8-day-old seedlings. Ten plants were combined for a single sample. Boxes indicate mean of three samples, and symbols indicate each sample. Asterisks indicate statistically significant difference between the treatments at the time point (Welch's t-test). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 4 Principal component analysis of metabolomics profile of control and biuret-treated rice suspension cells. Rice cells were transferred into a medium without biuret or with 0.3 mmol L^{-1} biuret and harvested 3 and 5 days after transfer. Closed symbols indicate control cells, and open symbols indicate biuret-treated cells. Circles indicate day 3 samples, and triangles indicate day 5 samples.

Figure 5 Normalized peak intensities of differentially accumulated metabolites between control and biuret treated rice suspension cells. Peaks with significantly different intensity between the control-group and biuret-

group are shown in the list ($p< 0.05$, Welch's t-test, n = 4). RT column indicate retention time in second. In the formula column, the formula is shown when the formula is uniquely determined from the m/z value, blank when there are multiple possible candidates, and unknown when there are no candidates. D3C: day 3 control cell sample; D3B: day 3 biuret-treated cell sample; D5C: day 5 control cell sample; D5B: day 5 biuret-treated cell sample.

Supplemental Materials

Supplemental Figure S1 Pictures of 9-day-old wild-type rice seedlings and two *biuret hydrolase* overexpressing lines. From left to right: wild type and overexpressing lines B3-9-1 and B2-3-3. Upper: seedlings grown in the control culture solution. Lower: seedlings grown in the culture solution supplemented with 0.3 mmol L^{-1} biuret. Bars show 10 cm.

Supplemental Figure S2 Allantoin concentration in 9-day-old rice shoots measured by colorimetric and HPLC-UV. Plants were grown hydroponically under the three biuret treatments. Control: plants did not receive biuret; NB: plants were grown without biuret for three days after sowing, and with 0.3 mmol L^{-1} biuret supplemented in the culture solution from the fourth day; BN: plants were grown with 0.3 mmol L^{-1} biuret for 6 days after sowing and transferred to new culture solution without biuret on the seventh day. Fresh shoots of 9-day-old seedlings were ground under liquid N_2 and extracted with 10-fold volume of distilled water. After centrifugation, the supernatant was used for allantoin determination. Gray boxes indicate mean allantoin concentration determined colorimetrically and black boxes indicate that determined by the HPLC method. Symbols indicate each sample. The statistical significance of the differences between the methods was determined through paired t-test (n = 2). ns: not significant.

Supplemental Figure S3 Relative expression levels of (a)*OsALN* and (b) *OsUO* in rice seedlings. Rice plants were hydroponically grown with or without 0.3 mmol L^{-1}biuret supplied in the culture solution. Measurements were made using plants grown in an independent trial, as shown in Figure 5. The relative expression levels were normalized to those of *Ubiquitin* and*Actin1* and expressed on a log2 scale. Gray and black squares indicate control and biuret-treated plants, respectively; data represent the mean ± SD (n = 4). Asterisks indicate statistically significant differences between treatments at the time point (Welch's t-test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p< 0.001$.

Supplemental Data S1 Peak intensities in Metabolome analysis.

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Figure 1 Effects of biuret on dry weight (a, d), biuret concentration (b, e), and allantoin concentration (c, f) in roots and shoots of 7-day-old wild-type rice plants (a–c) and 9-day-old*biuret hydrolase* overexpressing rice lines (d–f). Bars and circles represent mean and each sample, respectively. nd means not detected. (a–c) Wild-type plants were grown with 0, 0,1, and 0.3 mmol L^{-1} biuret supplemented in the culture solution. Ten seedlings were combined for a single sample. Different alphabets indicate significant difference among treatments in each organ ($p< 0.05$, Tukey's test, n = 3). (d–f) Wild-type and two independent transgenic lines (B3-9-1 and B2-3-3) were grown with or without 0.3 mmol L^{-1} biuret in the culture solution. Four to six plants were combined into one sample. Gray and black bars represent control and biuret-treated plants, respectively. Asterisks indicate significant difference between the treatment ($^{*}p< 0.05$; $^{**}p < 0.01$, Welch's t-test, n = 3).

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