Title

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

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Abstract

Aims

Excess biuret, a common impurity in urea fertilizers, is toxic to plants. Little is known about the mechanisms of biuret toxicity in plants. This study aimed to investigate the accumulation of biuret and the changes in metabolites in rice (*Oryza sativa*) plants under biuret toxicity.

Methods

A previous study had shown that transgenic rice plants overexpressing bacterial *biuret hydrolase* had improved biuret tolerance. Here, we grew wild-type and bacterial *biuret hydrolase*-overexpressing rice plants in hydroponics at different biuret levels. Concentrations of biuret and allantoin, a nitrogenous intermediate in the purine degradation pathway, in the plants were determined. The expression levels of genes related to purine degradation and ureide metabolisms were analyzed using wild-type plants. Additionally, we performed a metabolome analysis using rice suspension cells.

Results

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. The concentration of allantoin in shoots of wild-type plants 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 *allantoinase*, *allantoate amidohydrolase* and putative allantoin transporter genes. Furthermore, another nitrogenous compound citrulline, which is a non-protein amino acid, accumulated in rice suspension cells under biuret toxicity.

Conclusion

The accumulation of these compounds suggests that rice plants subjected to biuret toxicity need to reduce the concentration of surplus ammonium ions via synthesizing nitrogen-rich compounds.

Key words: allantoin, biuret, citrulline, nitrogen, 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, albeit more slowly than urea does (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 and Yamamoto, 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 caused by biuret as the impurity in urea fertilizers.

We previously found that rice plants overexpressing *biuret hydrolase* from a soil bacterium had an enhanced biuret tolerance (Ochiai et al., 2020). The experiment used ^{15}N-labeled biuret and showed 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 accumulation in plants causes injury. Plants grown with 0.3 mmol L^{-1} biuret showed stress symptoms and accumulated biuret in the shoots. The overexpression of *biuret hydrolase* led to biuret consumption and stress alleviation.

In addition, we hypothesized that biuret inhibits the metabolism of compounds with a similar structure, specifically ureide compounds, in a competitive manner. Among ureide, 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). Additionally, 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 examined whether biuret disrupted allantoin metabolism and found that allantoin accumulated in biuret-treated rice shoots. However, contrary to our expectations, biuret did not show competitive inhibition of the allantoin-degrading enzyme; therefore, we further investigated the mechanism of allantoin accumulation.

Furthermore, in addition to the analysis targeting allantoin metabolism, we took a comprehensive approach to examine metabolic processes affected by biuret toxicity. Concretely, we performed a metabolome analysis using rice suspension cells and showed a citrulline accumulation in cells 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 at the desired concentration 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, B3-9-1 and B2-3-3 (Ochiai et al., 2020). 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 were resuspended in 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, 5 µm, 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). The allantoin concentration in rice plants determined by HPLC was consistent with that measured by the colorimetric method (Supplemental Fig. S1).

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).

Determination of free amino acids

Free amino acids were extracted from freeze-dried plant powder with 80% ethanol at 80°C for 20 min. The concentration of amino acids in the extract was determined by the ninhydrin method (Moore and Stein, 1954) using leucine as a standard.

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 at final concentration of 0, 0.5, and 5 mmol L^{-1} was added to the reaction mixture together with the allantoin. The total volume of the reaction mixture was 0.5 mL. The reaction mixture was incubated at 30ºC 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 and 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. Experiments were repeated twice. 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*, 5´-TCTGACAAGAGTGGCACGAC-3´ and 5´- GACAGGCCCTTGTTCAATGT-3´for *allantoate amidohydrolase*, 5´-TATTCAGCCCTTTGCCTGAC-3´ and 5´-GGTGGTAGGGCTGATTTTGA-3´ for *ureidoglycine aminohydrolase*, 5´-TCGCATGTGGAAATTGATGT-3´ and 5´-ATAATAGCACGCCACGGTTC-3´ for *ureidoglycolate amidohydrolase*, 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 and 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 (see Supplemental Figure S2 for the typical symptoms). 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 as the biuret concentration in the culture solution increased (Fig. 1b). Consistent with our 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 bacterial *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. S2). 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 WT plants was the cause of biuret injury.

Allantoin accumulation in rice seedlings

The allantoin concentration in 7-day-old wild-type rice was higher in the roots than in the shoots (Fig. 1c). 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).

First, we examined whether biuret inhibited allantoin degradation enzyme, allantoinase (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 relating to the allantoin synthesis and degradation were then analyzed to investigate the mechanism of allantoin accumulation. 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-ureidoimidazoline. These steps are catalyzed by two enzymes, uric acid oxidase (UOX) and bifunctional allantoin synthase (ALNS). Allantoin is then hydrolyzed to allantoic acid by ALN. The downstream hydrolysis reactions resulting in allantoic acid to ureidoglycine, ureidoglycine to ureidoglycolate, and ureidoglycolate to hydroxyglycine are catalyzed by allantoate amidohydrolase (AAH), ureidoglycine aminohydrolase (UGlyAH), and ureidoglycolate amidohydrolase (UAH), respectively.

The expression level of genes in the allantoin synthetic pathway, *OsXDH* (Os03g0429800), *OsUO* (Os01g0865100), and *OsALNS* (Os03g0390700), was mostly 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 *OsXO* in the 7-day-old root and shoot (Fig. 3a-c). The expression level of *OsALN* (Os04g0680400) in 4 to 6-day old root decreased under the biuret treatments. On the other hand, the expression level in shoots did not differ between the treatments (Fig. 3d). The result of no difference in shoot *OsALN* expression was different from that of our preliminary study (Supplemental Fig. S3) but obtained by two independent trials (Fig. 3d). The expression level of *OsAAH* (Os06g0665500) in 5 and 6-day-old roots and 6, and 7-day-old shoots, and *OsUAH* (Os12g0597500) in 5-day-old roots showed significant decrease under the biuret treatment (Fig. 3, e and g). The expression level of *OsUGlyAH* (Os07g0495000) was not changed by the biuret treatment (Fig. 3f).

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. The biuret treatment significantly increased the expression level of *OsUPS1* in the shoots of 5-to 7-day-old seedlings (Fig. 3h). The expression levels of *OsUPS2* in the roots of 4 and 5-day-old seedlings were significantly lower in plants grown with biuret than in control plants (Fig. 3i). The expression level of *OsUPS3* was relatively low compared with the other two homologs and did not differ between the treatments (Fig. 3j).

Consistently, allantoin concentration in 8-day-old seedlings that were grown together with plants used for gene expression analysis was significantly higher in shoots under biuret treatment than in the control plants (data on three of six samples are shown in Fig. 6b).

Metabolite changes in rice suspension cells under biuret toxicity

Adding 0-1 mmol L^{-1} biuret to the culture medium reduced the growth of suspension-cultured rice cells in a concentration-dependent manner (Ochiai et al., 2020). Under 0.3 mmol L^{-1} biuret treatment, the cell fresh weight was significantly lower than control cells seven days after subculturing; however, the difference was not yet significant five days after subculturing (Ochiai et al., 2020).

We performed comprehensive metabolite analysis of suspension-cultured rice cells to investigate the changes in metabolome induced by 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 (Supplemental Data S1), 993 peaks consistently detected in replicates of at least one of the four sample groups were used for subsequent analysis.

Principal component analysis was performed to compare the metabolite 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 4 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 enhanced this decrease. These results suggest that most of the PC1 variability is explained by a growth component and for PC2 by an aging component.

We then compared the mean peak intensities between the control and biuret groups. Excluding one peak that was considered an artifact due to the assignment of different peak 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, a non-protein amino acid, accumulated in the biuret-treated rice suspension cells (Fig. 5).

Additionally, the intensity of the peak id 1202 showed a marked increase in biuret-treated cells (Fig. 5). There were two possible formulas, $C_{14}H_{20}N_6O_5S_1$ and $C_{21}H_{20}O_7$, for the observed m/z value of this peak. A peak, id 1240, was eluted at nearly the same retention time as 1202, with less intensity and 2.00 greater m/z value. Presence of the peak supported the former formula containing a sulfur with major stable isotopes, ^{32}S and ^{34}S. Therefore, it was shown that $C_{14}H_{20}N_6O_5S_1$, *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 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.

Free amino acids accumulation in rice seedlings

The accumulation of allantoin in intact plants and citrulline in cultured cells led us to consider the possibility that the reduced utilization of assimilated-N occurred before the accumulation of these N-containing compounds. Therefore, we measured free amino acids in rice seedlings. The free amino acids concentration in the roots of 8-day-old rice plants grown with 0.3 mmol biuret was slightly increased compared to the control, but this difference was not significant. The

shoot amino acids concentration was significantly increased in the biuret-treated sample (Fig. 6a). The shoot allantoin concentration in these plant samples was also significantly increased by the biuret treatment, consistent with other trials (Fig. 6b).

Discussion

In our previous study, we showed that rice plants overexpressing microbial *biuret hydrolase* were more tolerant to biuret than wild-type plants. Also, the ^{15}N-biuret uptake rate of the *biuret hydrolase*-overexpressing plants was greater than that of wild-type plants (Ochiai et al., 2020). In this study, we quantified biuret in plants and showed that biuret was not detected in *biuret hydrolase*-overexpressing plants grown with 0.3 mmol L^{-1} biuret (Fig. 1e). In contrast, wild-type rice plants accumulated significant amounts of biuret under the same condition (Fig. 1b). The results indicate that the *biuret hydrolase*-overexpressing rice plants hydrolyze most of the taken-up biuret. Thus, the accumulation of biuret in plants is the cause of biuret injury. Supposing 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 was estimated to be 0.5 and 1.8 mmol L^{-1}, respectively. Combined with minor injury in rice plants fed with 0.1 mmol L^{-1} biuret, a concentration of biuret in plants on the order of sub-millimolar is seemingly needed to cause injury. The severity of biuret toxicity in wild-type plants varied among trials, even when the biuret concentration in plants was nearly the same (Fig. 1, a and d), suggesting the presence of factors that enhance or alleviate biuret injury.

We hypothesized that biuret might specifically inhibit the metabolism of ureide compounds having structural similarity with biuret. Biuret in the culture solution increased allantoin concentration in rice shoots (Fig. 1, c and f), but our assumption was not supported as biuret did not inhibit ALN activity of the crude extract (Fig. 2).

Allantoin accumulation has been reported in plants under several environmental stresses (Casartelli et al., 2019; Kaur et al., 2021; Lescano et al., 2016; Nourimand and Todd, 2016; Watanabe et al., 2014). Allantoin alleviates reduced plant growth under environmental stress by activating abscisic acid metabolism (Watanabe et al., 2014) or by enhancing the activity of antioxidant enzymes (Nourimand and Todd, 2016). In plants under environmental stress, downregulation of the expression of *ALN* has been reported as a mechanism of allantoin accumulation (Casartelli et al., 2019; Irani and Todd, 2016; Lescano et al., 2016). The reduced expression of *OsALN* in roots and *OsAAH* in roots and shoots of the biuret-treated plants at some time points suggests that degradation suppression contributes to allantoin accumulation in biuret-injured rice plants (Fig. 3, d and e); however, the expression level of *OsALN* in rice shoots did not differ between the control and 0.3 mmol L^{-1} biuret-treated conditions (Fig. 3d). It is controversial whether biuret toxicity results in the suppression of *OsALN* because the expression of *OsALN* was significantly suppressed by biuret in one of the three independent trials performed in this study (Supplemental Fig. S3). We assume that biuret injury may have been enhanced by some unknown factors in the trial, resulting in the significant suppression of *OsALN* expression in shoots. Additionally, *OsALN* expression was lower in roots than in shoots (Fig. 3d), which is consistent with findings of greater allantoin concentration in roots than in shoots (Fig. 1, c and f, Fig. 6b), suggesting the contribution of *OsALN* expression regulation in controlling allantoin accumulation in the tissues. At the same time, however, our results show that the accumulation of allantoin in rice shoots under biuret toxicity can occur without significant suppression of *OsALN* in shoots.

Biuret altered the expressions of *OsUPS1* and *OsUPS2* (Fig. 3, e and f). *OsUPS1* is a putative allantoin transporter gene. It is a homolog of the Arabidopsis *AtUPS1* which encodes an allantoin transporter (Desimone et al., 2002). In contrast to *AtUPS1*, which is up-regulated

379 under N deficiency (Desimone et al., 2002), *OsUPS1* expression is down-regulated under N
380 deficiency (Lee et al., 2018). OsUPS1 is supposedly related to the long-distance transport of
381 allantoin because it is localized to the plasma membrane and is expressed around vascular
382 tissues (Redillas et al., 2019). Allantoin accumulation in shoots can be caused by altered
383 translocation of allantoin between the roots and shoots. Since the expression of *OsUPS1* was
384 increased in shoots under biuret toxicity, an increase in the unloading of allantoin from xylem
385 vessels in shoots might have resulted in the observed increase in shoot allantoin concentration.
386 Alternatively, this may indicate increased allantoin transport within shoot cells or from shoots to
387 roots. The total-N concentration in the shoots of wild-type rice did not differ among biuret
388 treatments, suggesting that allantoin accumulation in biuret-injured rice plants was a
389 modification of the form of N within shoots. The shoot allantoin-N as a percentage of shoot
390 total-N was 0.45% and 0.42% in plants under 0 and 0.1 mmol L^{-1} biuret treatment, whereas it
391 increased to 0.71% in plants under 0.3 mmol L^{-1} biuret treatment. Furthermore, the expression
392 of *OsUPS1* was higher in roots where allantoin was more abundant than in shoots under control
393 conditions (Fig. 1c, Fig. 3h). In Arabidopsis roots, AtUPS5L localizes to endoplasmic reticulum
394 and trans-Golgi network/early endosome, in addition to the plasma membrane, and AtUPS5
395 may be involved in the vesicular export of allantoin under stress (Lescano et al., 2020a).
396 Although OsUPS1 has been shown to localize to the plasma membrane (Redillas et al., 2019), if
397 it is also localized to organelle membranes, increased allantoin accumulation may lead to more
398 active transport between organelles or vesicular export in rice shoots under biuret toxicity.
399 *OsUPS2* has not been characterized; thus, the significance of the change in its expression is
400 currently unclear. The differentially regulated expression of *OsUPS1* and *OsUPS2* in biuret-
401 injured rice plants showed that these two homologs play different roles in rice plants.

Metabolome analysis showed citrulline accumulation in the rice suspension cells treated with biuret (Fig. 5). Citrulline is a non-protein amino acid involved in the arginine synthesis pathway. It is synthesized from ornithine and carbamoyl phosphate. Similar to allantoin, citrulline is involved in plant stress responses. It has been reported that wild watermelon (*Citrullus lanatus*), native to deserts, and commercial watermelon plants substantially accumulate citrulline under drought stress (Kawasaki et al., 2000; Song et al., 2020). Arabidopsis plants under low CO_2 conditions also accumulate citrulline (Blume et al., 2019). Citrulline is considered to be a scavenger of hydroxyl radicals (Akashi et al., 2001) or NH_4^+ (Blume et al., 2019; Joshi and Fernie, 2017) generated under such environmental stresses.

In addition to their importance in the stress response, allantoin and citrulline are N-rich compounds. Allantoin and allantoic acid are the major forms of N transported from the roots to the shoots in legumes (Schubert et al., 1986). Allantoin accumulation in Arabidopsis plants is also induced by a low carbon to N ratio and NH_4^+-N in the culture medium (Lescano et al., 2020b). Similarly, citrulline contains three N per molecule and is considered an endogenous source of N in plants (Ludwig, 1993). The accumulation level of citrulline varies with N nutritional status in watermelon plants (Song et al., 2020) and is higher in NH_4^+-fed tobacco plants than in NO_3^--fed plants (Gupta et al., 2013). It is, of course, possible that intact rice and cultured cells responded differently to biuret, but it is also possible that the accumulation of these compounds was caused by an imbalance in N metabolism due to biuret injury.

Our results also revealed that the free amino acid concentration markedly increased in rice shoots under biuret toxicity (Fig. 6a). This result is consistent with previous studies showing that biuret inhibits the incorporation of amino acids into proteins (Ogata and Yamamoto, 1959; Webster et al., 1957) and other amino acids accumulate along with citrulline in watermelon plants under drought stress (Song et al., 2020).

Additionally, the free amino acids measurement suggests that allantoin acts as a temporary pool of taken-up N in the roots, even in the control plants. Given that the amino acids in rice roots are predominantly glutamine (Ogasawara et al., 2021), there were approximately 200 μmol g^{-1} dw of amino acid-N and 80 μmol g^{-1} dw of allantoin-N in roots (Fig. 6). In shoots, on the other hand, N transported from roots as glutamine would be incorporated into proteins and would accumulate less as allantoin under control conditions. When biuret was supplied to the culture solution, protein synthesis was inhibited for direct or indirect reasons (Ogata and Yamamoto, 1959; Webster et al., 1957), increasing free amino acids and leading to an increase in allantoin. Since the de novo purine synthesis from glutamine is a complex process requiring energy input, and therefore, it is unclear whether accumulation of allantoin occurred via increasing de novo purine synthesis. Allantoin accumulation could also result from the degradation of existing purine bases. However, the accumulation of amino acids suggests a surplus of reduced N in rice plants under biuret toxicity. Glutamine and allantoin may trigger the downregulation of allantoin degradation, thereby inhibiting the generation of excess NH_4^+ and maintaining reduced N in a safer form.

In conclusion, we investigated changes in metabolites in rice plants under biuret toxicity to understand the mechanism of toxicity and found the accumulation of two N-rich compounds, allantoin in intact rice seedlings and citrulline in suspension-cultured cells. A surplus of amino acids appeared to occur prior to the accumulation of allantoin. Our results suggest that rice plants subjected to biuret toxicity need to maintain reduced N in secure forms and prevent the generation of NH_4^+. We are currently conducting experiments to investigate the effect of different N supply levels and sources on biuret injury in rice plants to clarify the significance of accumulating these N-rich compounds under biuret toxicity.

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575

Statements and Declarations

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Competing interests: The authors have no relevant financial or non-financial interests to disclose.

Author contributions: Kumiko Ochiai and Toru Matoh conceived and designed the research. Kumiko Ochiai and Yosuke Nomura performed experiments and analyzed the data. Asuka Uesugi generated *biuret hydrolase*-overexpressing rice lines. Kumiko Ochiai wrote the manuscript with input from other authors.

Data availability: the data supporting the findings of this study are available within the article and its supplementary materials.

Figure legend

Fig. 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). Different alphabets indicate significant difference in each organ ($p < 0.05$, Tukey's test, n = 3).

Fig. 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 30ºC 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).

Fig. 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. Data obtained from two independent trials,

each with three replicates, are combined and shown. The relative expression levels of *OsXO* (a), *OsUO* (b), *OsALNS* (c), *OsALN* (d), *OsAAH* (e), *OsUGlyAH* (f), *OsUAH* (g), *OsUPS1* (h), *OsUPS2* (i), and *OsUPS3* (j). 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 six samples. Asterisks indicate statistically significant difference between the treatments at the time point. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (n = 6, Welch's t-test). Numerical values above asterisks indicate log2 fold-change relative to the control plants.

Fig. 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.

Fig. 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.

Fig. 6 Free amino acids concentration (a) and allantoin concentration (b) in 8-day-old seedlings. Rice plants were hydroponically grown under the control condition and 0.3 mmol L^{-1} biuret toxicity. Five plants were combined for a single sample. Boxes indicate the mean of three samples,

642 and symbols indicate each sample. Asterisks indicate a statistically significant difference between

643 the treatments (Welch's t-test). $^{**}p < 0.01$; $^{***}p < 0.001$.

644

Supplemental Materials

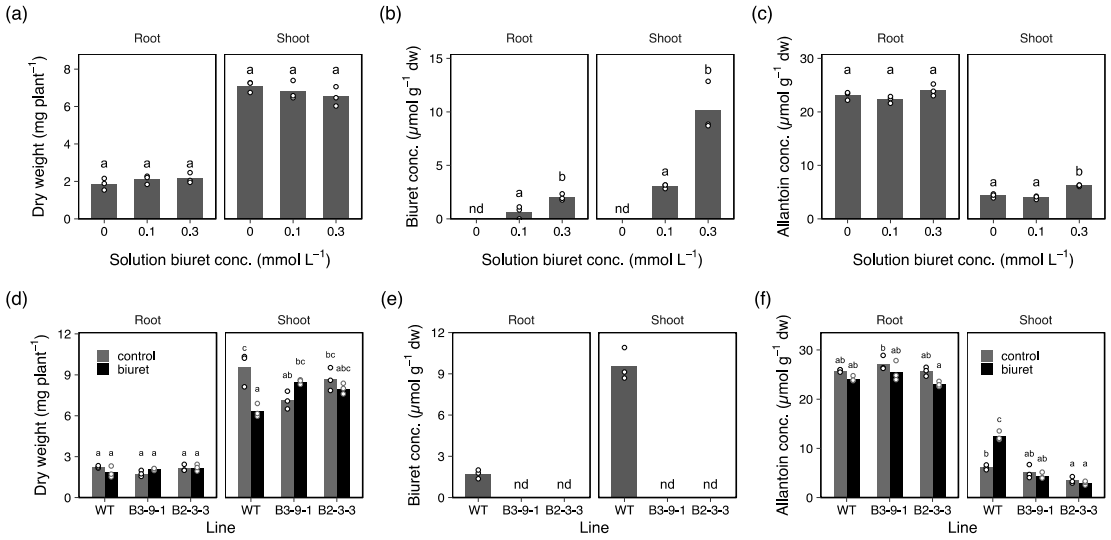
Supplemental Fig. S1 Allantoin concentration in 9-day-old rice shoots measured by colorimetric and HPLC-UV. Plants were grown hydroponically under the three biuret treatments. Control: plants did not receive biuret; NB: plants were grown without biuret for three days after sowing, and with 0.3 mmol L^{-1} 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 Fig. S2 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 Fig. S3 Relative expression levels of (a) *OsALN* and (b) *OsUO* in 3 to 9-day-old rice shoots in the preliminary experiment. 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 from those shown in Figure 3. 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). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Numerical values above asterisks indicate log2 fold-change relative to the control plants.

Supplemental Data S1 Peak intensities in Metabolome analysis.



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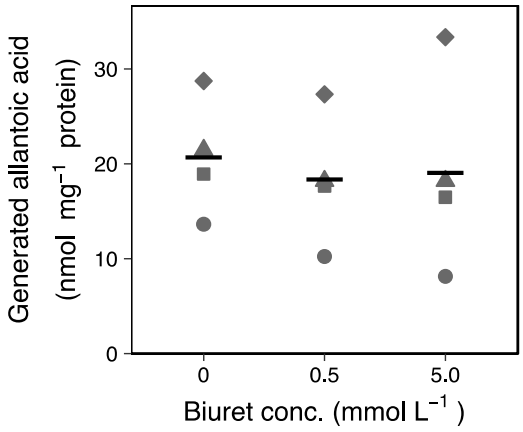

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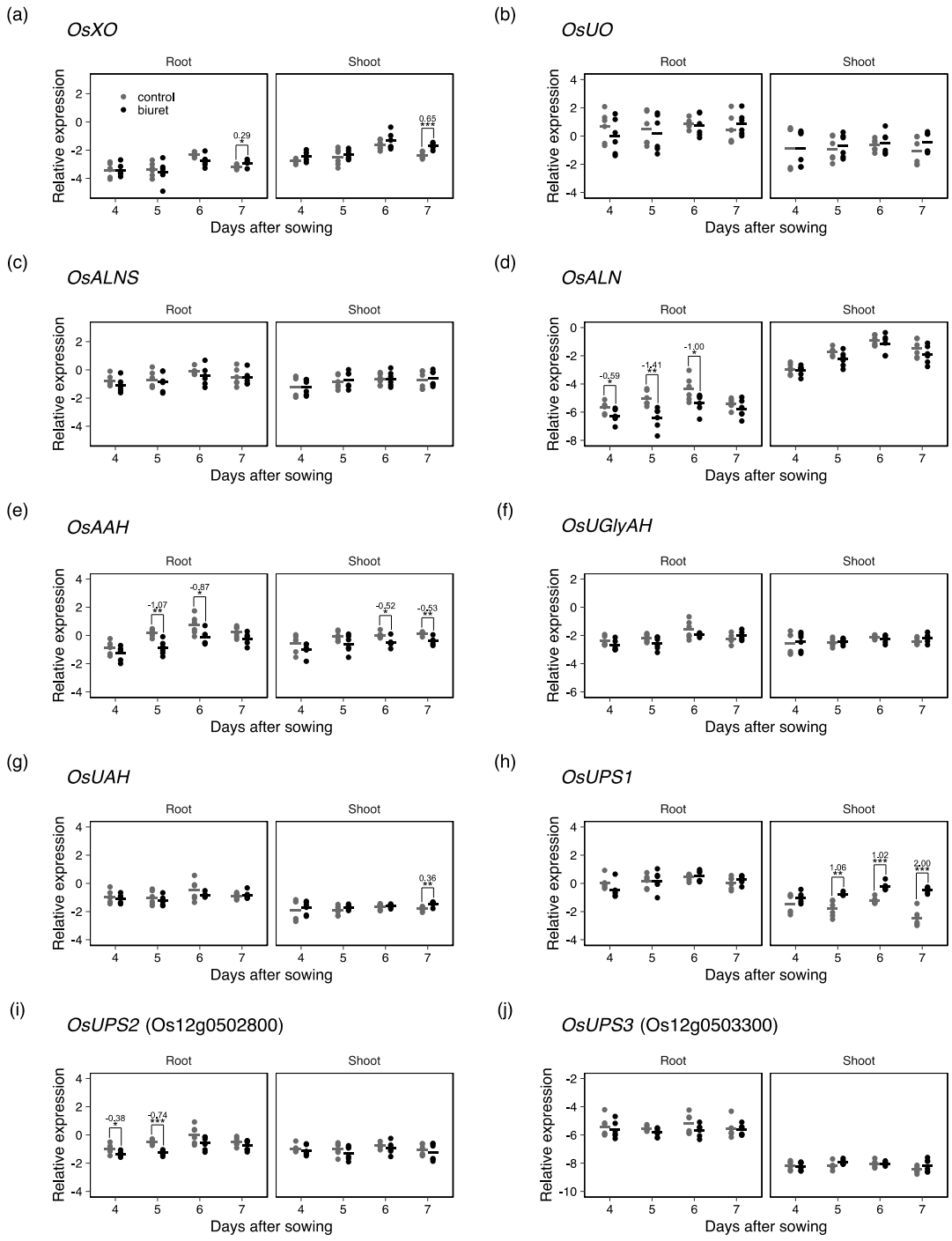

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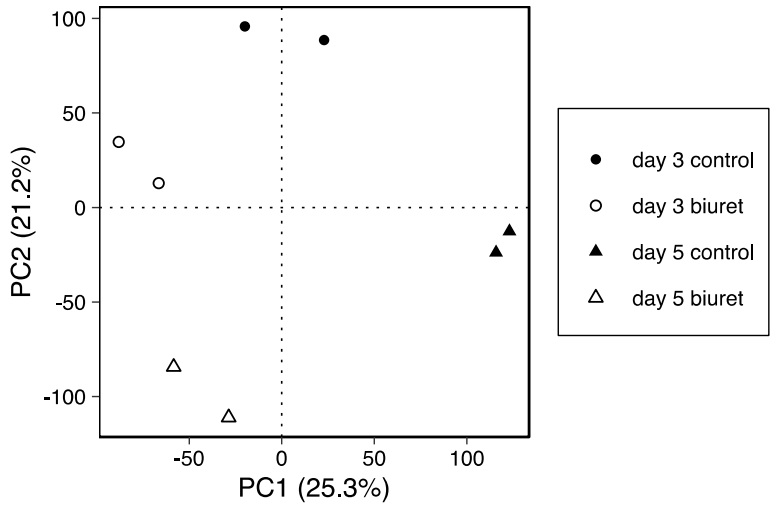

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				Normalized intensity							
ID	RT (sec)	m/z	Formula	D3C1	D3C2	D5C1	D5C2	D3B1	D3B2	D5B1	D5B2
47	37	102.0913	C5H11N1O1					3	3	3	4
239	54	170.0576	C4H12N1O4P1					-2	-1	-1	-1
310	55	176.1029	C6H13N3O3	3	3	3	3	5	5	5	5
326	56	159.0763	C6H10N2O3	1	2	1	1	2	3	3	3
342	57	120.0655	C4H9N1O3	2	3	3	1	3	4	3	4
372	58	138.0549	C7H7N1O2	3	4	3	4	3	1	3	2
432	58	268.0848	C9H17N1O6S1					-1	-1	0	0
448	59	385.1288						2	3	3	0
811	80	206.0480	C7H11N1O4S1					1	2	2	3
826	82	236.0585	C11H10N3O1Cl1					-3	0	1	1
864	83	238.0742	C8H15N1O5S1					-2	0	0	1
1100	99	284.1337	C10H21N1O8					0	1	1	1
1117	102	376.1283						0	2	1	2
1127	103	247.1286	C10H18N2O5					0	0	0	-2
1161	106	163.0599	C6H10O5	0	1	2	0		0		
1202	110	385.1287					0	5	7	6	6
1240	114	387.1245						0	2	1	2
1247	114	136.0616	C5H5N5					-3	1	-1	0
1248	114	193.5696	Unknown Peak					1	4	2	4
1251	114	170.0651	Unknown Peak					2	4	2	4
1354	138	296.1160	C14H18N3O2Cl1	-1				0	-1	0	-1
1719	263	529.1268						2	1	1	3
1722	264	190.1072	C8H15N1O4	2	2	3	2	3	3	3	4
1805	278	285.0901						0	1	1	2
1867	292	149.1171	C7H16O3					0	-1	0	0
1954	309	304.1389	C13H21N1O7	1	1	2	2	1	0	1	0
2067	322	229.1545	C11H20N2O3	0				2	-1	2	2
2219	339	352.1389	C17H21N1O7	0	0	1	1				0
2222	339	445.0944						2	3	4	4
2340	360	587.2546		-1				2	1	1	-1
2444	386	293.1164						1	2	1	1
2470	394	159.1014	C8H14O3					0	1	1	2
2476	395	335.1334		2	3	3	3	3	4	5	5
2477	395	370.1706		4	3	4	3	4	5	5	5
2669	487	141.1273	C9H16O1					0	0	1	1
2837	645	1118.7230		-4				-3	-3	-2	-2
3022	806	522.3552	C26H52N1O7P1	-1				0	0	1	-2
3183	965	487.3603	Unknown Peak	-1	-1	-1	-1				-2

Fig. 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.

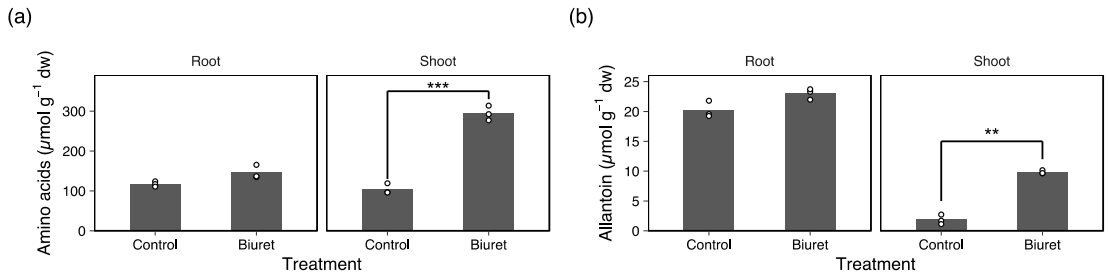

Fig. 6 Free amino acids concentration (a) and allantoin concentration (b) in 8-day-old seedlings. Rice plants were hydroponically grown under the control condition and 0.3 mmol L^{-1} biuret toxicity. Five plants were combined for a single sample. Boxes indicate the mean of three samples, and symbols indicate each sample. Asterisks indicate a statistically significant difference between the treatments (Welch's t-test). $^{**}p < 0.01$; $^{***}p < 0.001$.