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REGULAR ARTICLE

Involvement of auxin in the regulation of ammonium tolerance in rice (*Oryza sativa* L.)

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Abstract

Background and aims Ammonium (NH_4^+) is an important nitrogen source and is widely used as a fertilizer in agricultural systems. However, excess NH_4^+ inhibits root growth, and, subsequently, vegetative shoot growth and yield. This study examines whether auxin is involved in differential NH_4^+ tolerance in rice (*Oryza sativa* L.), and how auxin is regulated under high-NH_4^+ conditions in rice.

Methods An NH_4^+-sensitive (Kasalath, Kas) and an NH_4^+-insensitive (Koshihikari, Kos) rive cultivar were cultured hydroponically with or without exogenous indole-3-acetic acid (IAA) and auxin biosynthesis inhibitors. Root growth, root area, tissue IAA content, and transcription of genes involved in auxin biosynthesis, conjugation and degradation were determined.

Results *p*DR5::GUS staining and auxin measurement show that high NH_4^+ can decrease free IAA content in roots. In addition, quantitative RT-PCR, pharmacology, and genetics analysis suggest that Kos possesses a higher capacity for auxin biosynthesis and a weaker capacity for auxin metabolism compared to Kas under high-NH_4^+ stress.

Conclusion We conclude that the NH_4^+-tolerant cultivar possesses a higher capacity to maintain auxin homeostasis under high-NH_4^+ stress, and that this advantage is incurred by promotion of auxin biosynthesis and a suppression of auxin metabolism.

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Keywords Ammonium toxicity · Auxin level · Nitrogen fertilizer · Rice · Root development

Abbreviations

DAO	Dioxygenase for Auxin Oxidation
GH3	Group II GRETCHEN HAGEN3 acyl amido synthetases

IAA	indole-3-acetic acid
IAGLU	INDOLE-3-ACETATE BETA-D-GLUCOSYLTRANSFERASE
IPyA	indole-3-pyruvate acid
Kyn	L-kynurenine
NH_4^+	Ammonium
OxIAA	2-oxoindole-3-acetic acid
TAR	TRYPTOPHAN AMINOTRANSFERASE RELATED
Tryptophan	Trp
YUC	YUCCA
Yucasin	5-(4-chlorophenyl)-4H-1, 2, 4-triazole-3-thiol

Introduction

Ammonium (NH_4^+) is an important inorganic nitrogen source used by plants, and is of special importance to rice cultivation in paddy soils (Tobin and Yamaya 2001). However, excessive NH_4^+ is toxic to plant growth and development, which manifests in the inhibition of root growth, yield depression, and chlorosis of leaves (Britto and Kronzucker 2002; Esteban et al. 2016a; Li et al. 2014). In agricultural fields, soil NH_4^+ concentrations can exceed 20 mM, particularly when concentrated fertilization practices or strip-dressing are adopted (Britto and Kronzucker 2002; Raven et al. 1993). Higher plants possess widely differing sensitivities to stress from excess NH_4^+ (Britto and Kronzucker 2002), and significant differences can also be found among cultivars of a given species. Rice is one of the most important food crops worldwide and is widely known as an NH_4^+-tolerant species (Balkos et al. 2010; Britto et al. 2001; Kronzucker et al. 2010). Our previous study examined differential NH_4^+ sensitivity among cultivars of rice, showing that differential tolerance was linked to futile cycling of NH_4^+ at the root surface (Chen et al. 2013), a phenomenon known to be associated with NH_4^+ toxicity in many species (Britto et al. 2001; Coskun et al. 2013). However, whether other factors are involved in the differential manifestation of NH_4^+ tolerance in rice remained unclear.

Auxin is an extremely important hormone involved in plant growth and development, affecting such processes as root elongation and development, apical dominance, flowering, senescence, and stress responses in general (Di et al. 2016a). The distribution and levels of the active form of auxin (the most abundant form is indole-3-acetic acid, IAA, which exists as the negatively charged species under most physiological conditions) are tightly controlled through the processes of transport and synthesis, and through inactivation or conversion to 2-oxoindole-3-acetic acid (oxIAA) (Korasick et al. 2013). Recently, progress has been made in characterizing the physiological and molecular processes of auxin under environmental stress in Arabidopsis and maize. Aluminum (Al) stress was shown to lead to the upregulation of the transcription of *AtTAA1* and *AtYUC*s and the accumulation of free IAA in the transition zone of roots, followed by the inhibition of root growth (Liu et al. 2016; Yang et al. 2014). Moreover, osmotic and salt stress were shown to inhibit lateral root growth via promotion of transcription of auxin-conjugating genes, *AtIAGLU* (also named *UGT75D1*), *AtUGT84B2,* and *AtGH3.1*, associated with reduced free IAA accumulation (Ding et al. 2015). High doses of nitrate can decrease free IAA and inhibit root growth in maize and Arabidopsis (Tian et al., 2008; Kiba et al., 2011). Furthermore, our previous study and other studies had suggested that HA can reduce free IAA in the roots of various species (Cao et al. 1993; Esteban et al. 2016b; Li et al. 2010; Liu et al. 2013; Song et al. 2013). Root growth inhibition as a consequence of NH_4^+ exposure of shoots has also been linked to IAA transport in Arabidopsis (Li et al. 2011). However, little is known about the role of auxin in the world's leading crop, rice, under HA stress (Tamura et al. 2010).

Rice also uses the indole-3-pyruvic acid (IPyA) pathway as its main auxin-biosynthesis pathway and contains four TARs and fourteen YUCCAs homologs (Wang et al. 2018). Inhibition of enzyme activity or disturbance of gene transcription of TARs or YUCs cause obvious auxin-deficient phenotypes and decreases in free IAA content (Fujino et al. 2008; Kakei et al. 2017; Qin et al. 2017; Yamamoto et al. 2007; Yoshikawa et al. 2014). Furthermore, rice contains thirteen GH3s and two IAGLUs, which can catalyze the conjugation of free IAA to amino compounds or sugar, and one DAO, which functions in the conversion of IAA to inactive oxIAA in rice (Staswick et al. 2005; Zhao et al. 2013). A previous study suggested that overexpression of *GH3.2* (in a Mudanjiang background) could enhance tolerance to cold stress while increasing sensitivity to drought (Du et al. 2012). By contrast, *GH3.13*-overexpressing rice (in a Nipponbare background) exhibited enhanced tolerance to drought stress

(Zhang et al. 2009). These results suggest that the functions of *OsGH3* members may differ among rice cultivars. Although many regulatory mechanisms of auxin level under environmental stress have been investigated in Arabidopsis, only few studies exist in rice. The regulatory mechanisms of auxin under HA and the role of auxin in differential NH_4^+ tolerance in rice remains almost completely unknown.

In this study, we screened two rice cultivars, an NH_4^+-sensitive cultivar, Kasalath (Kas), and an insensitive cultivar, Koshihikari (Kos), from an original collection of 25 cultivars. Molecular, genetic, pharmacological, and physiological analyses demonstrate that auxin is involved in the inhibition of root growth by HA in rice and that auxin plays a key role in differential NH_4^+ tolerance among rice cultivars.

Materials and methods

Plant materials

Twenty-five rice (*Oryza sativa*) cultivars were used in the screening for NH_4^+ tolerance (Supplementary Table 1), and two of these, Kas and Kos, were chosen as experimental materials. Os*dao* (Zhao et al. 2013) (Nipponbare, Nip) and Os*GH3.2ox* (Fu et al. 2011) (Mudanjiang, MDJ) were also used in this research.

Plant growth conditions

Seeds were surface-sterilized with 3% H_2O_2 for 20 min, washed with sterile water, and then germinated in sterile water at 28 °C for two days. Germinated seeds were transferred into 1/2 modified Kimura's solution for pretreatment for two days; the solution composition was as follows: 0.5 mM $MgSO_4.7H_2O$, 0.36 mM $CaCl_2{\cdot}2H_2O$, 0.25 mM KCl, 0.2 mM NaH_2PO_4, 0.1 mM Fe-EDTA (Fe^{3+}), 50 μM H_3BO_3, 0.5 μM $Na_2MoO_4{\cdot}2H_2O$, 0.7 μM $ZnSO_4{\cdot}7H_2O$, 0.3 μM $CuSO_4{\cdot}5H_2O$, 9 μM $MnCl_2{\cdot}4H_2O$, 1 mM $NaNO_3$ and 1 mM NH_4Cl. Plants were grown in a growth chamber, with a 16 h / 8 h; 30 °C / 28 °C day/night cycle, a light intensity of 400 μmol m^{-2} s^{-1}, and a relative humidity of 65%. Following pretreatment, seedlings were transferred to normal modified Kimura's solution containing 1 mM NH_4Cl as control (NA) and 10 mM NH_4Cl as the HA treatment, for eight days. For other treatments, 1 mM KCl, 10 mM KCl, 1 mM KNO_3, 10 mM KNO_3, 2 nM indole-3-acetic acid (IAA), 10 μM L-kynurenine (Kyn) and 100 μM 5-(4-chlorophenyl)-4H-1, 2, 4-triazole-3-thiol (Yucasin) were added to the NA or HA medium.

Root morphology analysis

Total root length, total root area, and root tip number were analyzed using the root analysis instrument WinRhizo-LA1600 (Regent Instruments Inc., Quebec, Canada).

Histochemical GUS staining

For *p*DR5::GUS staining, the newly emerged roots of seedlings containing a *GUS* marker (Zhonghua 11, ZH11) (Li et al. 2016) grown in CK or HA medium were collected and then incubated in 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β -D-glucuronide) and 50 mM potassium phosphate buffer, pH 7.5, with 0.1% *v*/v Triton X-100 for GUS staining at 37 °C for 3 h before observation (Di et al. 2016b).

RNA isolation and qRT-PCR

RNA was extracted using Trizol agent (Sangon, http://www.sangon.com/). Reverse transcription was performed by HiScript 1st Strand cDNA Synthesis Kit (R111–01) (Vazyme Biotech co.,ltd). For synthesizing first-strand cDNA, 1 μg of total RNA was used. The cDNA was diluted 20 times for Real-Time PCR.

For quantitative RT-PCR (qRT-PCR), 20 μL amplification reaction volumes (10 μL ChamQ SYBR qPCR Master Mix (Q311–02) (Vazyme Biotech co., ltd), 0.8 μL of each primer (Supplementary Table S2), 1.6 μL cDNA and 6.4 μL ddH_2O) were used. The results of each primer pair were normalized relative to UBQ1.

All real-time qRT-PCR amplifications were performed in a Light Cycler® 480II (Roche). The following PCR program was used: An initial denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 10 s and 60 °C for 30 s. During the melting curve analysis, PCR reactions were carried out at 95 °C for 15 s, 60 °C for 60s and 95 °C for 15 s. Each experiment was repeated three times, and each reaction was performed in triplicate.

Quantification of IAA

For extraction of free IAA, whole roots were collected in three replicates (50 mg freeze-dried roots per sample).

Sample pretreatment was as described previously (Luo et al. 2017). Quantification was performed in an LC/MS/MS Triple Quad Mass Spectrometer (Applied Biosystems, USA), according to a method described previously (Luo et al. 2017).

Statistical analysis

Datasets were analyzed using Prism 6 software (GraphPad Software). Comparisons between two groups were made using Student's *t*- test. Values of *P* denote differences significant at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, respectively. Comparisons between multiple groups were made by one-way or two-way ANOVA tests, and $P < 0.05$ were considered significant. All values were presented as means ± SD.

Accession numbers

Listed in Supplementary Table S1.

Results

Cultivars differ strongly in NH_4^+ tolerance

To investigate the mechanism of NH_4^+ tolerance in rice, we first analyzed 25 rice cultivars grown in NA (normal ammonium, 1 mM NH_4^+) and HA (high ammonium, 10 mM NH_4^+) (Fig. S1, Table S1 and Table S3). Our results show that various cultivars possess different tolerance to HA, as also reported previously (Chen et al. 2013). The test cultivars exhibited different inhibition ratios in terms of stem height (50.1% - 98.1%), shoot dry weight (39.3% - 97.2%), root length (29.3%–59.9%), and root area (30.5% - 87.2%) under HA conditions (Figure S1 and Table S1). Since No. 14 (Kas) showed the highest sensitivity to NH_4^+ and No. 24 (Kos) showed the highest tolerance among the tested 25 cultivars, we chose Kas and Kos as the test materials for the following experiments. To determine whether the HA-induced inhibition of root elongation is due to HA, but not high salt or high nitrogen, we also examined the effect of high salt or nitrogen supply on root length and root area. The results show that both high salt (10 mM KCl) and high nitrogen (10 mM KNO_3) can slightly inhibit root growth; however, there were no obvious differences between Kas and Kos (Fig. S2). Taken together, these results suggest that Kas and Kos are appropriate materials to investigate differential NH_4^+ sensitivity among rice cultivars.

Furthermore, our observations show that the differences in inhibition of root length, root weight, and root area emerge by the fourth day following the onset of treatment with 10 mM NH_4^+ (Fig. 1a-c). With prolonged treatment time, these differences intensified (Fig. 1a-c). In addition, we also found that root tips, stem height, and shoot weight were less impacted by NH_4^+, especially in the NH_4^+-tolerant Kos (Fig. 1d and Fig. S3). Together, these results show that high NH_4^+ inhibits root growth predominantly in the early phases of HA treatment in rice, with strong differences among cultivars (Fig. 1a-d).

Cultivars differ in NH_4^+ tolerance in relation to auxin

Our previous work showed that NH_4^+ inhibition of root growth is linked to decreased free IAA content in the roots of Arabidopsis (Li et al. 2010). To investigate the mechanism of the root growth inhibition by HA in rice, we first observed *p*DR5::GUS staining under NA and HA conditions. As we show, NH_4^+ can decrease local staining and GUS-reported gene transcription in roots compared with NA, suggesting that NH_4^+-induced root growth inhibition is correlated with a reduction in free IAA in roots (Fig. 2a-b). To shed further light on the role of IAA involvement, we added low concentrations of IAA (2 nM) to HA media and measured total root length and area (Fig. 2c-d). The data show that low concentrations of IAA can rescue root growth under HA treatment in Kas, but not in Kos. Furthermore, we find that the function of IAA is dependent upon NH_4^+ concentration (Fig. 2c-d). To sum up, auxin plays an important role in NH_4^+-induced root growth inhibition, especially under moderate NH_4^+ stress.

The NH_4^+-tolerant cultivar, Kos, possesses a high capacity for auxin biosynthesis and superior maintenance of auxin level under HA stress

To explore the regulatory mechanisms in the auxin-signaling chain, the transcription levels of auxin biosynthetic genes were investigated using qPCR. It is well known that IAA is mainly synthesized from tryptophan (Trp) and that it is involved in the activities of a variety of enzymes in Arabidopsis (Di et al. 2016a). Phylogenetic analysis showed that most genes involved in auxin biosynthesis have homologues in rice, including TARs, YUCCAs, NITs, and AMIs; however, only TARs and

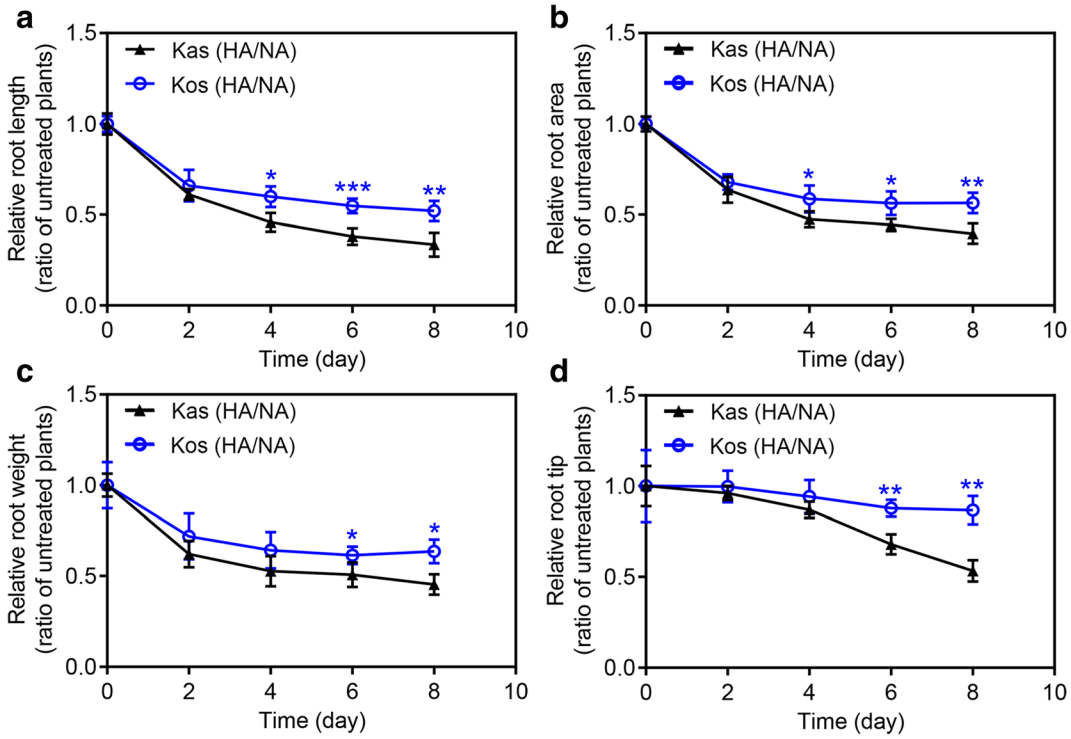

Fig. 1 Different cultivars possess different NH_4^+ sensitivity. a-d Relative root length (**a**), relative root area (**b**), relative root weight (**c**) and relative root tips (**d**) of Kas and Kos grown in NA (1 mM NH_4^+) and HA (10 mM NH_4^+) medium. All seedlings were transferred to pre-treatment medium (1/2 NA) two days, and then transferred to treatment medium (NA and HA) for another eight days. Error bars indicate ± SD. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (t-test). $n = 10$. In (**a**), 100% root length corresponds to 19.68 ± 1.01 cm $plant^{-1}$ for ten-day-old Kas and 23.53 ± 0.92 cm $plant^{-1}$ for ten-day-old Kos. In (**b**), 100% root area corresponds to 0.51 ± 0.018 cm^2 $plant^{-1}$ for ten-day-old Kas and 0.59 ± 0.021 cm^2 $plant^{-1}$ for ten-day-old Kos. In (**c**), 100% root weight corresponds to 4.12 ± 0.23 mg $plant^{-1}$ for ten-day-old Kas and 5.30 ± 0.61 mg $plant^{-1}$ for ten-day-old Kos. In (**d**), 100% root tip corresponds to 66.20 ± 6.58 for ten-day-old Kas and 105.60 ± 6.82 for ten-day-old Kos

YUCCAs have been clearly demonstrated in rice (Abu-Zaitoon 2014; Wang et al. 2018) (Fig. 3a). We then analyzed gene transcription levels in Kas and Kos under NA and HA conditions (Fig. 3b and c). The results show that TARs/YUCs are highly transcribed in the NH_4^+-tolerant cultivar, Kos, compared with the sensitive cultivar, Kas (Fig. 3b). In addition, our results show that HA inhibits the transcription of auxin-biosynthesis genes in both Kos and Kas, and to a much lesser extent in Kos (Fig. 3c). Overall, these data suggest that a higher capacity for auxin biosynthesis and maintenance of auxin level are critically tied to HA tolerance and that HA can downregulate the transcription of auxin-biosynthesis genes.

Inhibition of the enzyme activities of TARs and YUCs alters NH_4^+ tolerance in rice

Given the mediation of auxin biosynthesis through the TARs/YUCs pathway in rice and the transcription differences among cultivars that differ in NH_4^+ tolerance (Fujino et al. 2008; Kakei et al. 2017; Qin et al. 2017; Wang et al. 2018; Yamamoto et al. 2007; Yoshikawa et al. 2014) (Fig. 3), we then, to further clarify the role of auxin biosynthesis under HA stress, examined the effects of two chemical inhibitors, L-kynurenine (Kyn) and 5-(4-chlorophenyl)-4H-1, 2, 4-triazole-3-thiol (Yucasin), which are known to inhibit TAA1/TAR and YUC activity, respectively (He et al. 2011; Nishimura et al. 2014) (Fig. 4a). Our results show that the exogenous application of the auxin-biosynthesis inhibitors L-Kyn and Yucasin can decrease root growth in both Kas and Kos, reflected in both root length and root area (Fig. 4b-c). We also found that the NH_4^+-tolerant Kos exhibits a higher tolerance to inhibitors (Fig. 4b-c). Additionally, our results reveal that inhibitors can decrease NH_4^+ sensitivity in Kas, while increasing it in Kos (Fig. 4d, e). These data clearly demonstrate that the inhibition of auxin biosynthesis can alter the tolerance to HA.

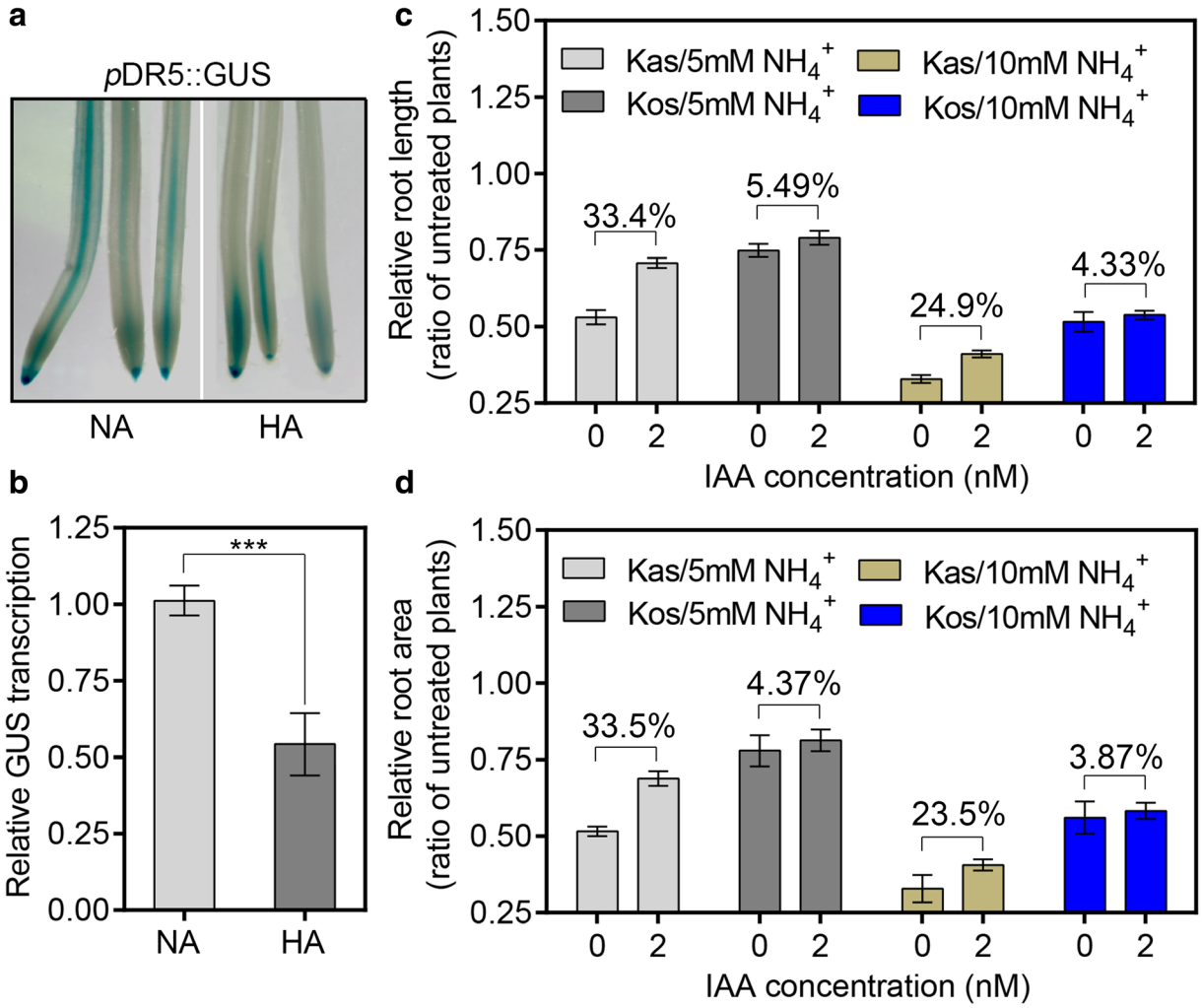

Fig. 2 Exogenous IAA can strengthen NH_4^+ tolerance in the sensitive cultivar Kas. **a** GUS staining of ZH11/*p*DR5::GUS. **b** Relative transcription level of *GUS* genes. Data are means of three biological replicates. Error bars indicate ± SD. $^{***}P < 0.001$ (t-test). **c**-**d** Relative root length (**c**) and root area (**d**) of Kas and Kos grown in NA and NH_4^+-containing medium with or without 2 nM IAA. For treatment, 10 pre-treatment seedlings were transferred to NA and NH_4^+-containing medium with or without 2 nM IAA and grown for eight days before measurement. Shown are mean values ± SD with n = 10. In (**c**), 100% corresponds to 20.80 ± 1.22 cm plant^{-1} for Kas and 21.42 ± 2.23 cm plant^{-1} for Kos. In (**d**), 100% corresponds to 0.49 ± 0.034 cm^2 plant^{-1} for Kas and 0.54 ± 0.051 cm^2 plant^{-1} for Kos

Auxin conjugation mediating free IAA reduction plays a positive role in the tolerance to HA stress in rice

Given that the distribution and levels of free IAA are tightly controlled by transport, synthesis, and inactivation (Korasick et al. 2013), in order to assess the potential contributions of auxin inactivation, including conjugation and degradation, in the regulation of root growth under HA stress, we first analyzed the transcriptional regulation of auxin-conjugating genes under HA. Our results show that the transcription of most auxin-conjugating genes is decreased under NH_4^+ stress (Fig. 5) and suggest that auxin conjugation plays a positive role under HA. Additionally, most auxin-conjugating genes exhibit similar, or identical, down-regulation under HA in both Kas and Kos, except for *GH3.2*, *GH3.3*, *GH3.5,* and *GH3.12*, revealing that auxin conjugation may have a positive function in the tolerance to HA.

Since Kas- and Kos-background genetic mutants are not available, we used OsGH3.2 overexpression lines (*GH3.2ox*, against a Mudanjiang background) to clarify

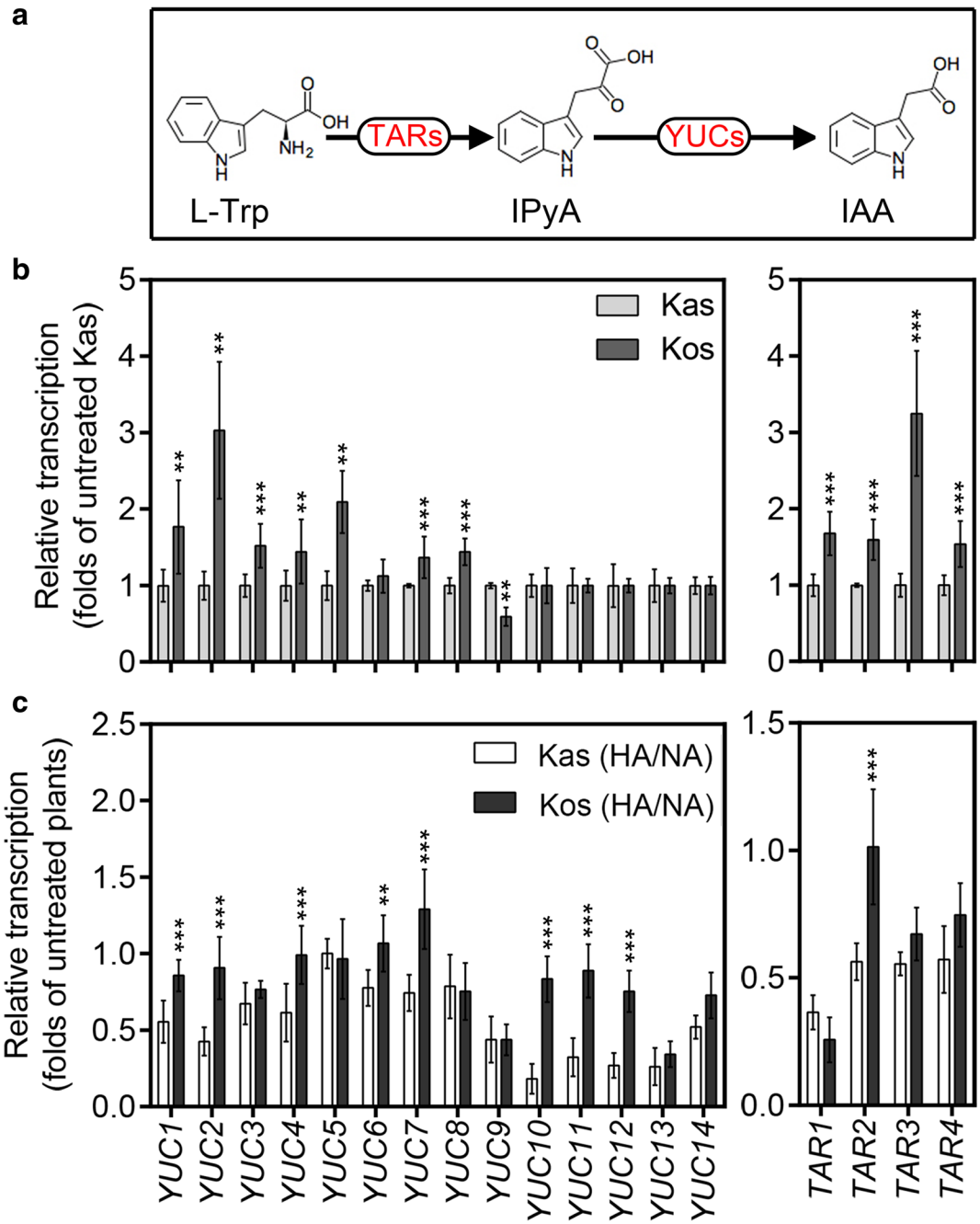

Fig. 3 Kos possesses higher capacity for auxin biosynthesis and auxin level regulation under HA stress. a The main auxin biosynthesis pathway in rice. **b** Transcription analysis of auxin biosynthesis genes in Kas and Kos. **c** The regulation of auxin biosynthesis genes transcription in Kas and Kos under HA. Data are means of three biological replicates. Error bars indicate ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ (t-test)

the role of auxin conjugation in response to HA (Fig. 6a-b). Our results show that transgenic *GH3.2ox* has shorter root growth compared with the wild type (Fig. 6a). In addition, *GH3.2ox* also displayed higher sensitivity to HA (Fig. 6c-d). To sum up, our results suggest that maintenance of auxin level plays an important role in NH_4^+ tolerance among cultivars, and auxin conjugation plays a positive role in this process.

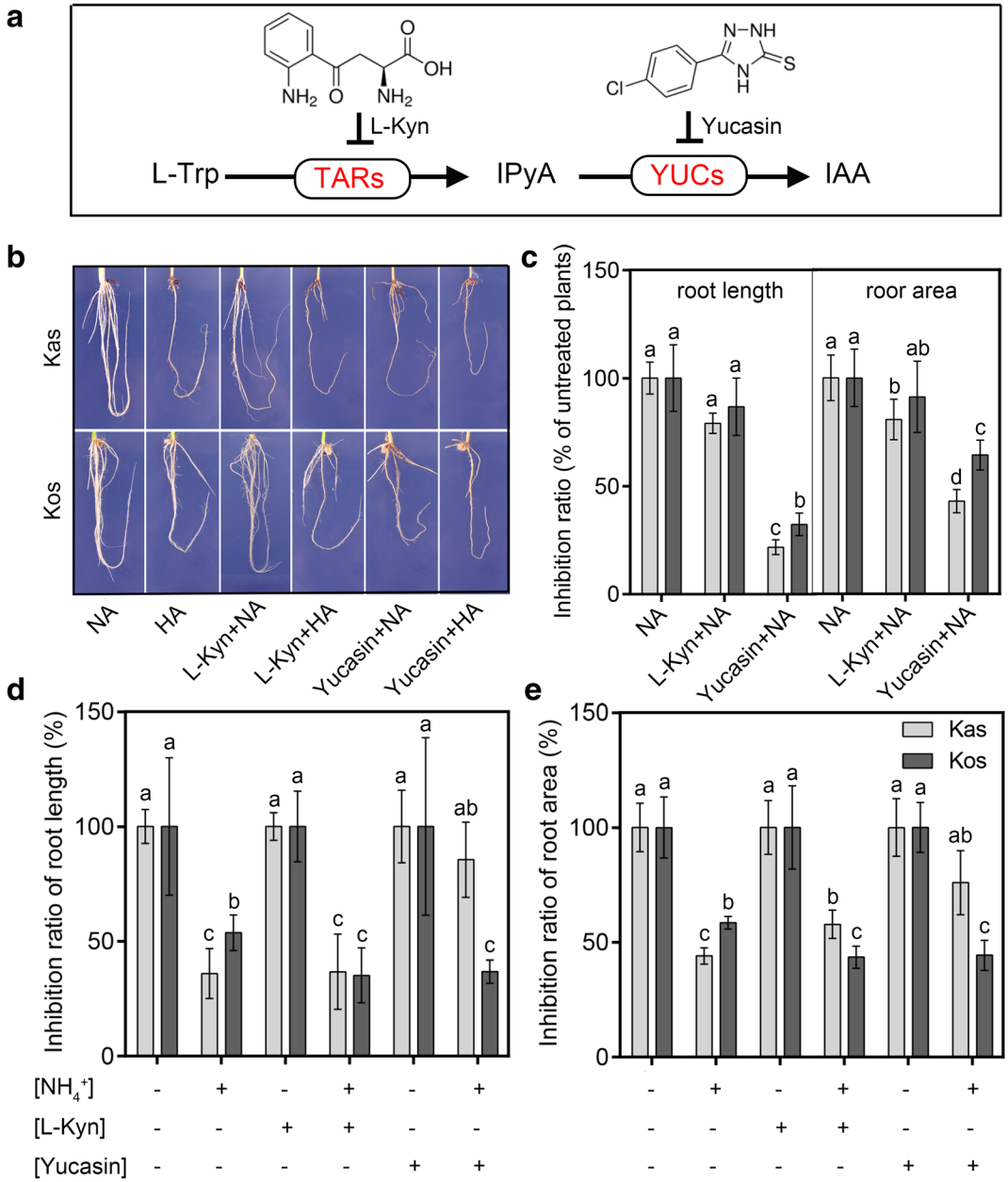

Fig. 4 Inhibition of TARs and YUCs alter the sensitivity to HA. a The location of inhibitor function for auxin biosynthesis; (**b**) Phenotypes of Kas and Kos grown in medium with different inhibitors; (**c**) Relative root length and root area grown in medium with different inhibitors; (**d-e**) Different inhibition ratios of root length (**d**) and root area (**e**) grown in medium with different inhibitors. Five-day-old seedlings were transferred to treated medium for another eight days (n = 10). Data are analyzed by two-way ANOVA following Duncan's test. Error bars with different letters represent a statistical difference ($P < 0.05$, Duncan's test). In (**c**), 100% root length corresponds to 23.86 ± 5.73 cm plant^{-1} for Kas and 25.91 ± 8.04 cm plant^{-1} for Kos, 100% root area corresponds to 0.41 ± 0.095 cm^2 plant^{-1} for Kas and 0.48 ± 0.15 cm^2 plant^{-1} for Kos. In (**d**), 100% corresponds to 10.20 ± 4.41 cm plant^{-1} for Kas and 15.26 ± 2.19 cm plant^{-1} for Kos. In (**e**), 100% corresponds to 0.20 ± 0.037 cm^2 plant^{-1} for Kas and 0.26 ± 0.059 cm^2 plant^{-1} for Kos

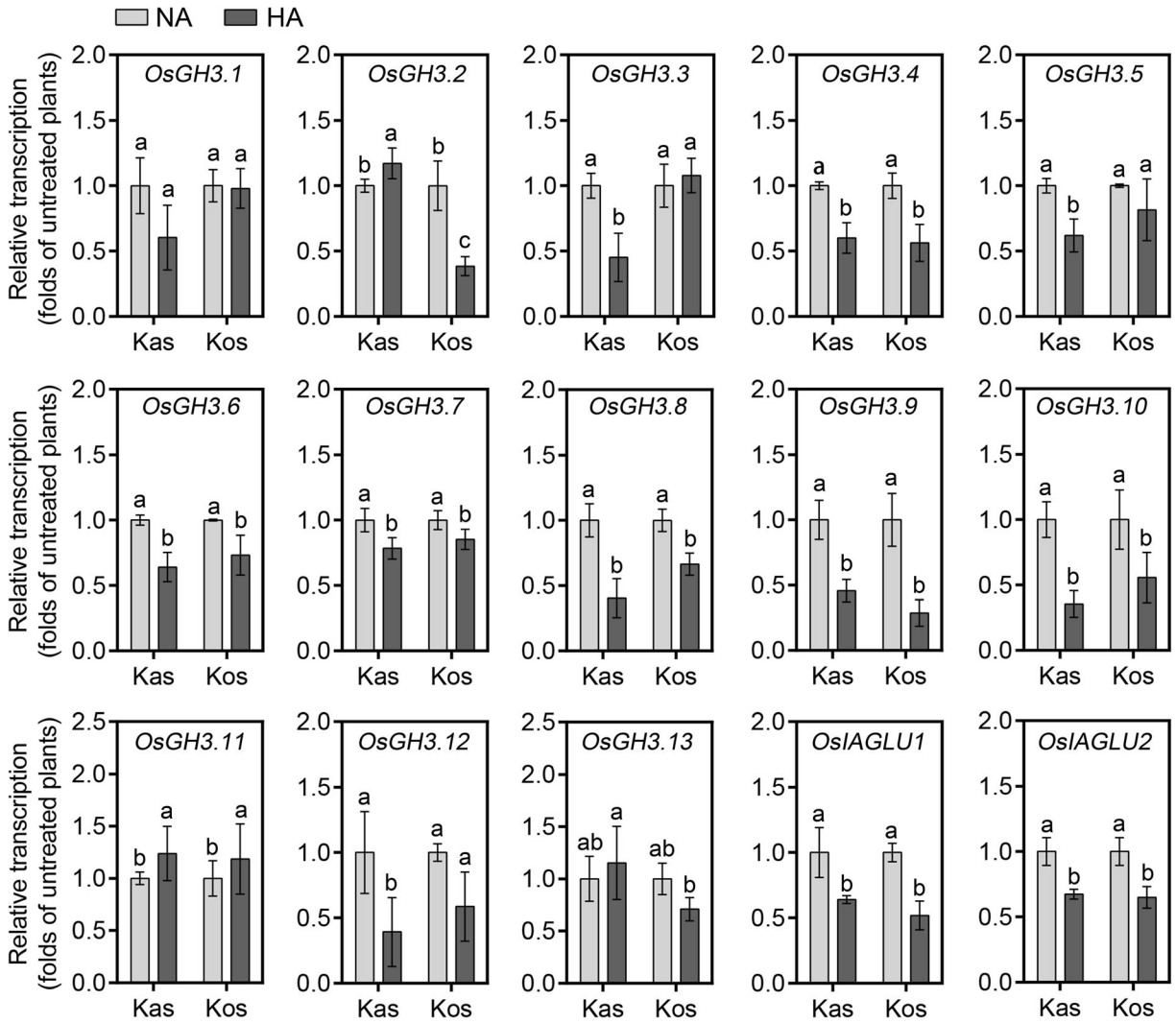

Fig. 5 Most auxin-conjugating genes are downregulated by HA. Five-day-old seedlings were transferred to treated medium for another eight days, and then roots are collected for RNA extraction. Three biological repeats per treatment. Data were analyzed by two-way ANOVA following Duncan's test. Error bars with different letters represent a statistical difference ($P < 0.05$, Duncan's test)

Auxin degradation mediated by DAO also functions in NH_4^+ tolerance in rice

Dioxygenase for Auxin Oxidation (DAO) converts the active form of IAA into oxIAA (Zhao et al. 2013) (Fig. 7a). To investigate the role of auxin degradation under HA conditions, we analyzed the transcriptional regulation of *DAO* in Kas and Kos (Fig. 7b). The results reveal that HA induces the transcription of *DAO* in Kas, but not in Kos (Fig. 7b), suggesting that the NH_4^+-insensitive Kos cultivar possesses a superior ability to maintain auxin level under stress (Fig. 7a-b). Since Kas- and Kos-background *Osdao* mutants are not available, we used Os*dao* in the Nip background to clarify the roles of OsDAO under HA conditions. Firstly, we analyzed the free IAA content in *Osdao* and its own background, and the results show that the mutation of *OsDAO* results in a slight increase in free IAA accumulation in roots under NA, and a slight decrease under HA (82.2 vs 88.3%) (Fig. 7c). Then, we observed and analyzed the phenotypes of the auxin degradation mutant *Osdao* (Fig. 7d-e). Consistent with our deduction, disturbance of auxin degradation slightly increased root growth (Fig. 7d-e). However, the minor difference in the inhibition ratio of *Osdao* and its background suggests that the function of DAO is limited under HA

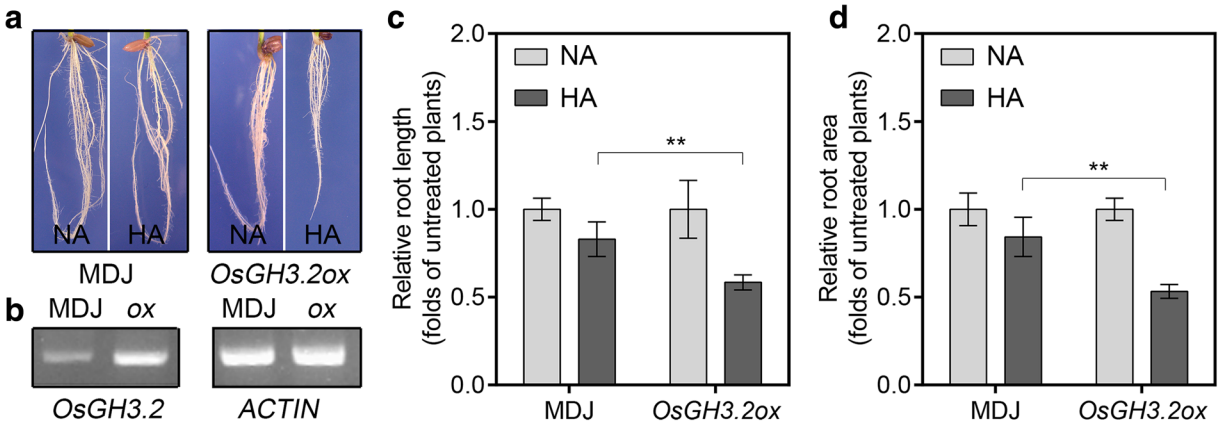

Fig. 6 Overexpression of *GH3.2* increases NH_4^+ sensitivity in rice. a Phenotypes of *OsGH3.2*ox in NA and HA medium. Five-day-old seedlings were transferred to new treatment medium for another eight days, and then phenotypes were observed; (**b**) Transcription level analysis by RT-PCR; (**c-d**) The total root length (**c**) and root area (**d**) of transgenic plants and their background. Five-day-old seedlings were transferred to treatment medium and grown for eight days prior to measurement. Shown are mean values ± SD with n = 10. $^{**}P < 0.01$ (t-test). In (**c**), 100% corresponds to 122.59 ± 6.62 cm plant^{-1} for MDJ and 87.50 ± 11.75 cm plant^{-1} for *OsGH3.2*ox. In (**d**), 100% corresponds to 1.96 ± 0.17 cm^2 plant^{-1} for MDJ and 1.47 ± 0.076 cm^2 plant^{-1} for *OsGH3.2*ox

conditions (Fig. 7c-d). Taken together, our results suggest that auxin degradation participates in the inhibition of root growth under HA by reducing the free IAA content, but that this function is restricted.

HA decreases free IAA content in the roots of Kas and Kos

To further elucidate the function of HA in auxin level, we measured free IAA content in the roots of Kas and Kos (Table 1). The results show that HA can decrease free IAA in the roots of both cultivars (Table 1). Interestingly, we also found that Kos contains higher levels of free IAA than Kas under NA, suggesting that Kos possesses higher biosynthesis capacity (Table 1). In addition, Kos also exhibited a smaller decrease in free IAA in comparison with Kas after NH_4^+ treatment, revealing that Kos maintains auxin level more competently than Kas (Table 1). Overall, these data show that HA affects auxin level and offers novel insight into the differential sensitivities to NH_4^+ seen among cultivars.

Discussion

In this study, we show that two rice cultivars, Kas and Kos, possess differential tolerance to HA and that their auxin level responds differently. We also demonstrate that the NH_4^+-tolerant Kos has a stronger capacity for auxin biosynthesis compared to the NH_4^+-sensitive Kas. Moreover, auxin conjugation, mediated by GH3s or IAGLUs, and auxin oxidation, catalyzed by DAO, also makes a small contribution to this differentiation in NH_4^+ tolerance. In addition, our results suggest that NH_4^+ decreases free IAA content mainly by inhibiting IAA biosynthesis but not IAA conjugation or oxidation.

Maintenance of auxin level is critical to NH_4^+ tolerance in rice cultivars

Excessive NH_4^+ affects plant growth negatively, especially roots, which act as the first contact with the toxicant under most conditions, and as an NH_4^+ sensor (Dominguez-Valdivia et al. 2008; Li et al. 2010). However, higher plants display widely varying responses to NH_4^+ and, accordingly, can be divided into tolerant and sensitive species (Britto and Kronzucker 2002). Although rice is known to be exceptionally tolerant to NH_4^+, a trait shared with species such as late-successional trees of the Northern hemisphere (Kronzucker et al. 1997) but that is rare among cereals, different rice cultivars nevertheless possess differential sensitivities (Chen et al. 2013; Wang et al. 1993a; Wang et al. 1993b). It is important to understand the foundation for differential sensitivities to NH_4^+ among rice cultivars, as tolerance traits may eventually be transferred to other cereals, which carries great agronomic

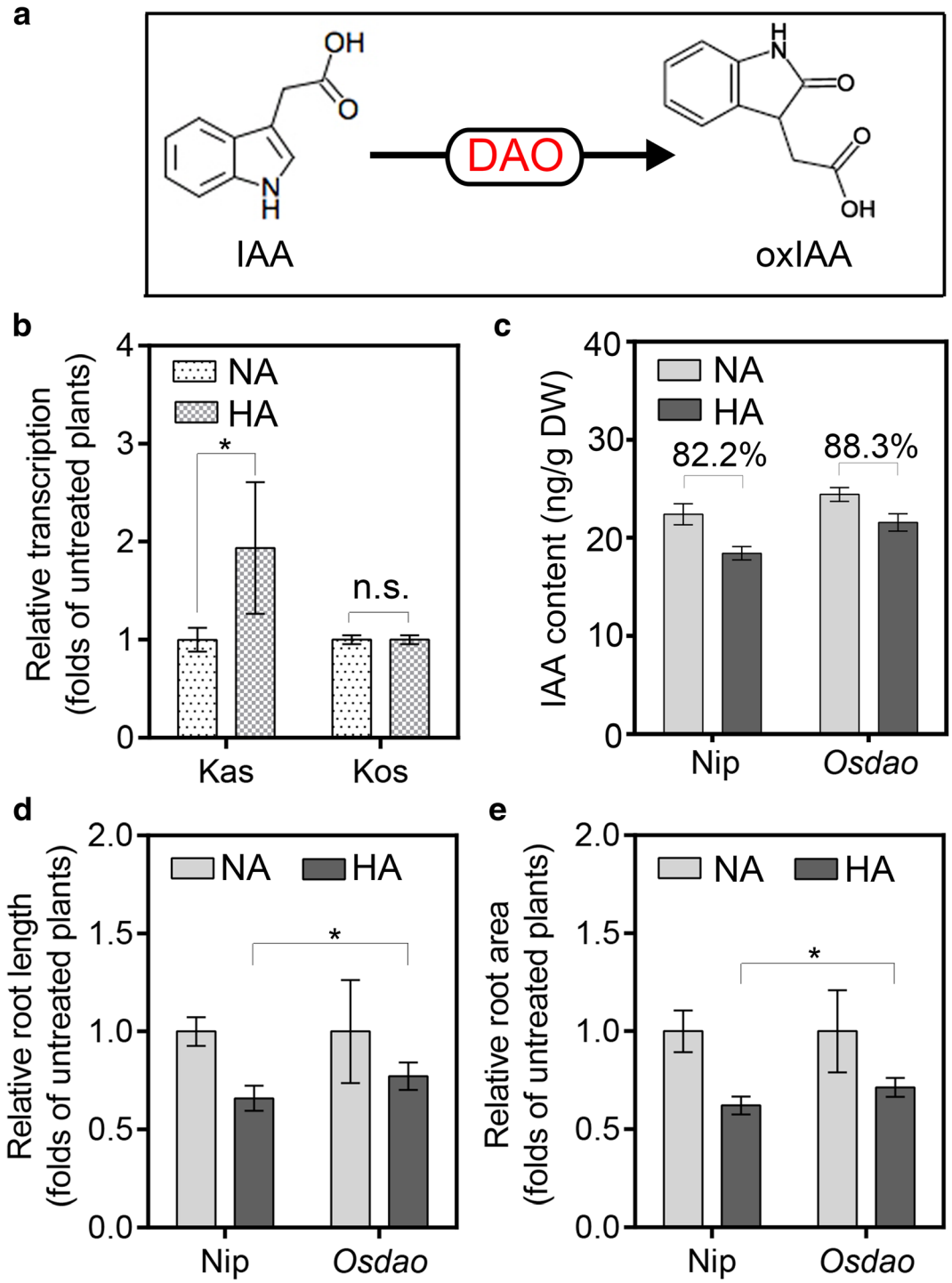

Fig. 7 OsDAO plays a negative role in NH_4^+ sensitivity between cultivars. a OsDAO catalyzes the conversion of IAA to oxIAA in vivo; (**b**) HA up-regulates *OsDAO* transcription in NH_4^+-sensitive Kas, but not in NH_4^+-tolerant Kos. Three biological repeats per treatment. Error bars indicate ± SD. $^*P < 0.05$ (t-test). n.s. indicates not significantly different; (**c**) Free IAA contents in Nip and *Osdao* under NA and HA conditions; (**d-e**) Total root length (**d**) and root area (**e**) of Nip and *Osdao1*. Five-day-old seedlings were transferred to treatment medium and grown for eight days prior to measurement. Shown are mean values ± SD with $n = 15$. Error bars indicate ± SD. $^*P < 0.05$ (t-test). In (**d**), 100% corresponds to 86.14 ± 5.61 cm plant^{-1} for Nip and 88.99 ± 21.99 cm plant^{-1} for *Osdao*. In (**e**), 100% corresponds to 1.30 ± 0.12 cm^2 plant^{-1} for Nip and 1.53 ± 0.30 cm^2 plant^{-1} for *Osdao*

importance (Zhao et al. 2018). Previous studies have suggested that NH_4^+-tolerant plants possess higher glutamine synthetase activity and accumulate less free NH_4^+ (Balkos et al. 2010; Magalhaes and Huber 1991). Our previous research also showed that futile cycling of the NH_4^+ ion at the root surface is more pronounced in NH_4^+-sensitive cultivars (Chen et al. 2013), a phenomenon also observed in tree species (Kronzucker et al. 2003). In addition, several hypotheses have been proposed to explain the different sensitivities to NH_4^+ among plants: NH_4^+ assimilation induces carbon depletion, free NH_4^+ accumulation in tissues, bringing about deficiencies in other cations (Finnemann and Schjoerring 1999; Groot et al. 2003; Siddiqi et al. 2002), acidification of the root zone (Chaillou et al. 1991), and impairments in the N-glycosylation of proteins (Barth et al. 2010). Our recent studies in Arabidopsis furthermore reveal that NH_4^+ can decrease the content of free IAA in root tissue. Whether auxin plays a role in differential tolerance among cultivars in rice is still unclear.

In the present work, we first analyzed NH_4^+ tolerance in 25 rice cultivars (Fig. S1 and Table S1). The results show that there is a large difference in tolerance to NH_4^+ among rice cultivars (Fig. S1). We then selected two cultivars at the extremes of the tolerance spectrum, Kas and Kos, to examine in detail. Our results show different inhibition ratios in root length, root area, and root fresh weight following treatment with 10 mM NH_4^+ for four days, and show that root tips display differentiation from the sixth day onward (Fig. 1). To investigate the relationship between NH_4^+ and auxin in rice roots, we then monitored *p*DR5::GUS staining and analyzed the transcription level of the *GUS* gene under HA, and the results show that both staining and gene transcription decrease under HA (Fig. 2a-b). Interestingly, exogenous IAA applied in the medium with 10 mM NH_4^+ can strengthen NH_4^+ tolerance of Kas (Fig. 2c-d). Overall, our results suggest that HA decreases free IAA in rice roots, and that auxin plays a role in the differential tolerance between Kas and Kos.

To clearly elucidate the function of auxin in differential NH_4^+ tolerance between Kas and Kos, we analyzed transcription of auxin biosynthesis genes under NA and following HA treatment. The data reveal that most of the auxin biosynthesis genes involved in the IPyA pathway are transcribed at higher levels in Kos compared with Kas (Fig. 3a-b). In addition, Kos exhibits higher tolerance than Kas to the inhibitors L-Kyn and Yucasin, which inhibit the enzyme activities of TARs and YUCs, respectively (Fig. 4b-c). Considering that other proposed auxin biosynthesis genes, such as NITs, and AMIs, also exhibited higher transcription in Kos, we conclude that superior capacity for auxin biosynthesis is critical to the degree of NH_4^+ tolerance among the cultivars (Fig. S4).

We further analyzed the relative transcription of auxin biosynthesis genes under HA in Kas and Kos (Fig. 3c). The results show that, compared with Kas, transcription of auxin biosynthesis genes in Kos is significantly less impacted under HA (Fig. 3c; Fig. S5), suggesting that improved maintenance of auxin level plays an important role in differential NH_4^+ tolerance between Kas and Kos. In addition to auxin biosynthesis, auxin conjugation and auxin degradation also contribute to auxin level in plants (Korasick et al. 2013). To examine the degree of involvement of conjugation and degradation, we also analyzed alterations in transcription of *GH3*s, *IAGLU*s, and *DAO*, which are reported to be responsible for the catalysis of auxin conjugation and oxidation, respectively (Du et al. 2012; Staswick et al. 2005; Zhang et al. 2009; Zhao et al. 2013). The data show that the transcription of most auxin-conjugating genes is reduced to a similar extent in both cultivars under HA, except for *GH3.2*, *GH3.3*, *GH3.5,* and *GH3.12* (Fig. 5). Given that *OsGH3.2*ox transgenic plants exhibit enhanced sensitivity to NH_4^+, we conclude that auxin conjugation may play a positive role in response to HA stress. Analysis of the transcriptional regulation of *DAO* under HA reveals that NH_4^+ can upregulate *DAO* transcription in Kas but not in Kos (Fig. 6a-b). Given the small change in tolerance to HA in the *Osdao* mutant and mildly IAA decrease in *Osdao* roots, and the low transcriptional level of *DAO* in roots (Fig. 7) (Zhao et al. 2013), we deduce that auxin degra-

Table 1 Free IAA contents in Kas and Kos under NA and HA conditions

Cultivars	Free IAA (ng/g DW)		Inhibition ratio #
	NA	HA	
Kas	18.08 ± 0.49 b	13.05 ± 0.34 c	0.28 ± 0.027
Kos	21.43 ± 0.19 a	18.43 ± 0.71 b	0.14 ± 0.009

#Inhibition ratio indicates: [IAA cont.(NA)- IAA cont.(HA)]/ IAA cont.(NA). Data are analyzed by two-way ANOVA following Duncan's test. Error bars with different letters represent a statistical difference ($P < 0.05$, Duncan's test)

dation meditated by DAO plays a limited role in the differential NH_4^+ tolerance. Altogether, our results show that a greater capacity to maintain auxin level in roots plays an important role in the regulation of NH_4^+ tolerance between Kas and Kos. Previous work suggested that auxin level in roots is controlled by auxin biosynthesis, conjugation, degradation, and transport, and whether the disturbance of auxin distribution is also involved in the response to HA warrants future investigation. Moreover, with increasing NH_4^+ concentrations, the rescue ability of exogenous IAA disappears (Fig. 2c-d). These results suggest that auxin is insufficient to protect against cell death induced by HA under prolonged NH_4^+ stress (Qin et al., 2008).

HA decreases free IAA content mainly through inhibition of auxin biosynthesis

Auxin level is controlled by auxin biosynthesis, conjugation and degradation (Korasick et al. 2013). Our recent study suggests that HA mainly stimulates auxin conjugation, leading to reduced free IAA contents in the roots of Arabidopsis (unpublished data). However, whether auxin plays such a role under HA in the NH_4^+-tolerant species rice, and which processes are involved, is unknown. Our study presented here provides four lines of evidence to indicate that HA can decrease free IAA content in the roots of rice mainly through inhibition of auxin biosynthesis, but does not involve auxin conjugation or degradation. First, *p*DR5::GUS staining and transcription decreased in the roots under HA treatment, which was accompanied by a reduced free IAA content in the roots under HA treatment in both cultivars (Fig. 2 a-b and Table 1). Second, both Kas and Kos have inhibited transcription of auxin biosynthesis genes, though the extent of down-regulation is different (Fig. 3). Additionally, after treatment with the inhibitors L-Kyn and Yucasin, Kas and Kos exhibited altered responses to HA treatment (Fig. 4). Third, most auxin-conjugating genes, including *GH3s* and *IAGLUs*, also displayed down-regulation under HA treatment, suggesting the regulation of auxin conjugation may be a rescue mechanism in rice under HA (Fig. 5). Fourth, the transcription of *DAO* was up-regulated in Kas, but not in Kos (Fig. 7b). In addition, DAO was mainly expressed in anther, and less in roots, and a knock-out of *DAO* only slightly influenced tolerance to HA. Taken together, these data reveal that auxin degradation plays a restricted role in the regulation of auxin level under HA.

The elucidation of the mechanisms of inhibition of roots under HA stress is critical to our efforts to develop superior screens for NH_4^+ tolerance in crops, and learning what mechanisms underpin tolerance in rice is of particular importance, given rice's unique standing among cereals as an NH_4^+-tolerant crop (Britto and Kronzucker 2002). The systematic analysis of the capacity for maintenance of auxin level between two major cultivars, Kas and Kos, provides an important new clue towards developing HA tolerant cultivars.

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