The auxin influx carrier, OsAUX3, regulates rice root development and responses to aluminium stress

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Abstract
In rice, there are five members of the auxin carrier AUXIN1/LIKE AUX1 family; however, the biological functions of the other four members besides OsAUX1 remain unknown. Here, by using CRISPR/Cas9, we constructed two independent OsAUX3 knock-down lines, osaux3-1 and osaux3-2, in wild-type rice, Hwayoung (WT/HY) and Dongjin (WT/DJ). osaux3-1 and osaux3-2 have shorter primary roots (PRs), decreased lateral root (LR) density, and longer root hairs (RHs) compared with their WT. OsAUX3 expression in PRs, LRs, and RHs further supports that OsAUX3 plays a critical role in the regulation of root development. OsAUX3 locates at the plasma membrane and functions as an auxin influx carrier affecting acropetal auxin transport. OsAUX3 is up-regulated in the root apex under aluminium (Al) stress, and osaux3-2 is insensitive to Al treatments. Furthermore, 1-naphthylacetic acid accentuated the sensitivity of WT/DJ and osaux3-2 to respond to Al stress. Auxin concentrations, Al contents, and Al-induced reactive oxygen species-mediated damage in osaux3-2 under Al stress are lower than in WT, indicating that OsAUX3 is involved in Al-induced inhibition of root growth. This study uncovers a novel pathway alleviating Al-induced oxidative damage by inhibition of acropetal auxin transport and provides a new option for engineering Al-tolerant rice species.

KEYWORDS
heavy metal stresses, lateral root initiation, polar auxin transport, primary root elongation, root hair development

1 | INTRODUCTION

Auxin is a critical plant hormone that regulates every aspect of plant growth and development (Kepinski, 2007; Ljung, 2013; Teale, Paponov, & Palme, 2006). Auxin is produced particularly in shoot and root meristems and is transported over long distance in a non-polar fashion in the vasculature to other parts of the plant. A second mode involves a cell-to-cell or polar auxin transport (PAT) employing carriers in the plasma membrane (Kramer & Bennett, 2006). Auxin carriers include members of the AUXIN1/LIKE AUX1 (AUX1/LAX), PIN-FORMED, and ATP Binding Cassette B/P-glycoprotein families (Bennett et al., 1996; Cho, Lee, & Cho, 2007; Geisler et al., 2005; Kerr & Bennett, 2007; Murphy, Hoogner, Peer, & Taiz, 2002; Noh, Murphy, & Spalding, 2001; Petrásek et al., 2006; Sparup et al., 2008; Yang & Murphy, 2009). In recent years, members of the PIN-LIKES family were also reported to be involved in auxin transport and homeostasis (Barbez & Kleine-Vehn, 2013). PAT plays an important role in various aspects of plant growth and development, which is involved in
regulation of embryogenesis, organogenesis, vascular tissue formation, lateral root (LR) initiation, and tropic responses (Friml & Palme, 2002; Petrasek & Friml, 2009; Peret et al., 2012; Swarup & Bennett, 2003; Vieten, Sauer, Brewer, & Friml, 2007).

In Arabidopsis, the AUX1/LAX family consists of four highly conserved members, AUX1, LAX1, LAX2, and LAX3 (Peret et al., 2012). AUX1 and LAX3 influence roots development, whereas LAX2 regulates vascular patterning in cotyledons (Bennett et al., 1996; Bhosale et al., 2018; Marchant et al., 2002; Peret et al., 2012; Swarup et al., 2001; Swarup et al., 2008; Vandenbussche et al., 2010). In tomato (Solanum lycopersicum), five AUX/LAX (SILAX1 to 5) genes revealed heterogeneous expression patterns, with tissue and developmental-stage specificity (Pattison & Catala, 2012). In Medicago truncatula, MtLAX2, a paralogue of Arabidopsis AUX1, is required for nodule organogenesis (Roy et al., 2017). In Chinese cabbage (Brassica rapa L. ssp. pekinensis), it is suggested that BrLAX genes may be involved in PAT during leafy head development (Gao et al., 2017). In rice, there are five members of the AUX1/LAX family (Shen et al., 2010). OsAUX1 was reported to function in regulation of LR development (Zhao et al., 2015). Previously, we have shown that OsAUX1, besides negatively regulating primary root (PR) elongation, positively regulates root hair (RH) development and responds to Cd stress (Yu et al., 2015). The roles of the other rice AUX1/LAX member remain unknown.

Aluminium (Al) rhizotoxicity is a major environmental stress that reduces crop production through inhibiting root elongation (Foy, 1988; Delhaize & Ryan, 1995; Kochian, 1995; von Uexküll & Mutter, 1995). Under heavy metal stresses, plants are known to dynamically regulate the transcription of auxin-related genes to adjust the effective accumulation of auxin within the plant for their survival (Wang, Wang, Zhao, Yang, & Song, 2015). Several studies have demonstrated that Al-regulated inhibition of root growth may interact with auxin signalling. Auxin accumulation and distribution in roots was altered by the presence of Al (Kollmeier, Felle, & Horst, 2000; Yang et al., 2014; Yang et al., 2017; Zhu et al., 2013). Auxin negatively mediates Al distribution in plant cells and Al tolerance by regulating ALS1 expression (Zhu et al., 2013). Auxin is a key signalling molecule that triggers an increase of malic acid against Al toxicity in wheat (Liu et al., 2017). Further, in the root apex transition zone of Arabidopsis, Al induces a localized enhancement of auxin signalling to regulate auxin biosynthesis (Yang et al., 2014). In OsPIN2 overexpression lines, it was found that the auxin efflux carrier alleviates Al-induced cell rigidity in rice root apex (Wu, Shen, Yokawa, & Baluska, 2014). The expression of auxin transport-erigeroninlike proteins and auxin efflux carrier components is significantly higher in Al-stressed alfalfa roots than in the control, whereas the expression of an auxin conjugate hydrolase is significantly lower (Zhou, Yang, Ren, Huang, & An, 2014). However, the mechanism of Al-induced disruption of root PAT most likely resulting in inhibition of root growth remains unclear.

In this study, we decipher the roles of auxin influx carrier, OsAUX3, related to PR elongation, LR initiation, and RH development and in response to Al stress. OsAUX3 transcription was induced by Al treatment, and the osaux3 mutant was insensitive to Al stress, indicating that OsAUX3 functions as a negative regulator decreasing rice Al tolerance in roots. Knowledge of this molecular mechanism may contribute to the breeding of Al-tolerant crops.

## METHODS AND MATERIALS

### 2.1 Plant materials and growth conditions

Japonica wild-type rice Hwayoung (WT/HY) and Dongjin (WT/DJ), OsAUX3-related transgenic mutants, and OsAUX3 overexpression lines were planted in nutrient solution as previously described (Wang et al., 2014; Xu et al., 2014). Phytohormone treatment was performed with 1 μM of indole-3-acetic acid (IAA), or 0.01 μM of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.01 μM of 1-naphthalacetic acid (NAA) for 7 days. Al treatment was performed in a 0.5 mM CaCl2 solution (pH 4.5) with different concentration of AlCl3 as indicated in the figure legends.

### 2.2 Construction and identification of osaux3 mutants

The CRISPR/Cas9 system was used to establish OsAUX3 knock-down lines (Xie, Minkenberg, & Yang, 2015). WT/HY and WT/DJ were infected by the Agrobacterium strain EHA105 transformed with OsAUX3-pRGEB32 as previously described (Hiei, Ohta, Komari, & Kumashiro, 1994). The related editing site was found through DNA sequencing. The design of specific primers was based on editing sites to identify the original generation (T0) of transgenic rice. Seeds of self-fertilized T0 were harvested as transgenic line (T1). Homozygous osaux3 mutants were identified with Cas9 specific primers and OsAUX3 specific primers. Cas9 label was removed from the homozygous osaux3 mutants. Homozygous T2 seeds were used throughout this study. The primers used for plasmid construction are listed in Table S1.

### 2.3 Construction and transformation of binary vectors

The ORF of OsAUX3 (Os05g37470) was amplified from the full-length cDNA of WT/DJ using the primers listed in Table S1 and cloned into pCAMBIA1300-sGFP to create the 35S:OsAUX3-sGFP fusion construct. For constructing the ProOsAUX3:OsAUX3-sGFP and ProOsAUX3:GUS (pBII1013.3), 2.3 kb of the OsAUX3 promoter was used to replace the CaMV35S promoter. These vectors were introduced into Agrobacterium strain EHA105 using electroporation and transformed into WT/DJ using the callus infection method as described previously (Hiei et al., 1994).

### 2.4 Subcellular localization of OsAUX3

35S:OsAUX3-sGFP and ProOsAUX3:OsAUX3-sGFP fusion constructs were transiently expressed in tobacco epidermal cells by agrobacterium-mediated transformation as previously described (Qi et al., 2012). The two constructs were also polyethylene glycol–calcium transfected into rice protoplasts, which were prepared from stems of 10-day-old rice seedlings. Images were acquired using the two-photon microscope, Zeiss LSM710 (Carl Zeiss, Oberkochen, Germany).
2.5 | β-Galactosidase staining and analysis of β-galactosidase activity

The maximum auxin response reporter, DR5:GUS, was introduced into Agrobacterium strain EHA105 and was transformed into WT/HY and osaux3-1 mutants. β-Galactosidase (GUS) staining was performed as described previously (Jefferson, Kavanagh, & Bevan, 1987). Root tissues were vacuumed-infiltrated in staining solution for 20 min and incubated at 37°C. After staining, tissues were soaked in 70% ethanol to remove chlorophyll and surface dyes and observed by using Nikon AZ100 microscope (Nikon Corporation, http://www.nikoninstruments.com.cn/index.html). For quantification of GUS activity, 50 mg of root tissues were grounded in liquid nitrogen, resuspended in phosphate-buffered saline (PBS) solution (pH 7.4), and centrifuged at 4200 g for 10 min, and the supernatant was collected. GUS activity was measured by NanoQuant infinite M200 pro (www.eastwin.com.cn) spectrophotometrically at a wavelength of 450 nm using the Plant GUS ELISA Kit (www.bangyi-sh.com).

2.6 | EdU staining

One centimetre of root tips of 3-day-old seedlings was treated with 50-µM 5-ethyl-2'-deoxyuridine (EdU) of culture medium for 1–2 hr as described in EdU Flow Cytometry Assay Kits (http://www.ribobio.com/siten.cn/Products.aspx?id=37) and observed under a LSM710 NLO Zeiss microscope (Zeiss, http://www.zeiss.com/corporate/en_de/home.html). For quantification of GUS activity, 50 mg of root tissues were grounded in liquid nitrogen, resuspended in phosphate-buffered saline (PBS) solution (pH 7.4), and centrifuged at 4200 g for 10 min, and the supernatant was collected. GUS activity was measured by NanoQuant infinite M200 pro (www.eastwin.com.cn) spectrophotometrically at a wavelength of 450 nm using the Plant GUS ELISA Kit (www.bangyi-sh.com).

2.7 | TTC staining

PRs of 3- and 5-day-old seedlings were incubated in 0.4% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 3 hr, vacuum-treated for 20 min, washed three times with ddH2O, and dissociated in 10% hydrochloric acid for 10 min. Staining was observed under a Nikon SMZ 745T microscope (Nikon Corporation, http://www.nikoninstruments.com.cn/index.html) using 10% glycerin as transparent reagent.

2.8 | Measurement of IAA concentration and IAA transport

Free IAA concentrations in 1-cm sections of WT/HY and osaux3-1 mutant root tips were measured by NanoQuant infinite M200 pro (www.eastwin.com.cn) using the plant IAA ELISA Kit (www.bangyi-sh.com). For that, 50 mg of root samples were grounded in liquid nitrogen, resuspended in PBS solution (pH 7.4), and centrifuged at 4200 g for 10 min. The supernatant was used to measure IAA concentrations spectrophotometrically at 450 nm. For the analyses of polar 3H-IAA transport in rice roots, 1 cm of root tip was performed using a 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer, http://www.perkinelmer.com/) as described previously (Qi et al., 2008). IAA export from root protoplasts was performed as described in Yu et al. (2015).

2.9 | Al quantification and Morin staining

For a quantification of Al concentrations in root tips, 1 cm of root tips treated with 25-µM AlCl3 was excised, washed six times with 0.2-mM CaCl2, and incubated in 2-M HCl for 48 hr, and supernatants were then analysed by ICP-OES (Optical Emission Spectrometer, Optima8000, PerkinElmer). For Morin staining, 1-cm apical root cuts was washed for 5 min with 0.2-mM CaCl2 and sliced into 100-µm sections by using a vibratome (Leica VT1000 S). Slices were Morin stained for 5 min and washed twice with 0.2-mM CaCl2, and fluorescence intensity was observed under a LSM710 NLO Zeiss microscope (Zeiss, http://www.zeiss.com/corporate/en_de/home.html).

2.10 | Analyses of root H2O2 and CAT concentrations

Analysis of H2O2 accumulation in the root tips was performed by using 2',7'-dichlorofluorescin diacetate (H2DCF-DA). One-centimetre root tips were cut and incubated with 50-µM H2DCF-DA solution for 10 min under vacuum and washed twice with 0.2-mM CaCl2. Root tips were then observed under a LSM710 NLO Zeiss microscope (Zeiss, http://www.zeiss.com/corporate/en_de/home.html). For measurement of H2O2 and CAT concentration in root tips, 1-cm root tips of treated seedlings were grounded in liquid nitrogen, resuspended in PBS (pH 7.4), and centrifuged at 4200 g for 10 min. Collected supernatants were used to measure H2O2 and CAT concentration by NanoQuant infinite M200 pro (www.eastwin.com.cn) spectrophotometrically at a wavelength of 450 nm using the Plant H2O2 ELISA and Plant CAT ELISA Kits (www.bangyi-sh.com), respectively.

2.11 | RNA extraction and quantitative RT-PCR

Total RNA was extracted from tissues after various treatments using a commercial kit and according to the manufacturer’s instructions (Tiangen, Hangzhou, China http://www.tiangen.com/). Quantitative RT-PCR (qRT-PCR) was performed as described previously (Wang et al., 2010; Wang et al., 2014); OsACTIN (Os03g50885) and OsUBI (Os03g13170) were used as internal control for qRT-PCR. Primers used are listed in Table S2.

3 | RESULTS

3.1 | Knock-down mutants of OsAUX3 are insensitive to auxin, and OsAUX3 expression is induced by auxin in PR

In a previous study, we reported that OsAUX1 plays an important role in root development and in responses to Cd stress (Yu et al., 2015). To clarify the biological function of OsAUX3, the closest homology of OsAUX1 (Figures S1 and S2), we constructed two independent alleles of osaux3 mutants using CRISPR/Cas9 technology, and OsAUX3 overexpression lines in the WTs, HY and DJ (Figures S3 and S4). These homozygous T2 lines were tested under hydroponic culture condition for 7 days. Our
results show that the PR length of osaux3-1 or osaux3-2 mutants was 25–30% shorter than their corresponding WT, whereas the PR length of OsAUX3 overexpression lines, 35S:OsAUX3-3 and 35S:OsAUX3-6, was 35% longer than WT/DJ (Figure 1a,b). Importantly, the PR length of osaux3-complemented lines was close to their WT, indicating that the PR alteration in osaux3 or OsAUX3 overexpression lines was caused by altered OsAUX3 expression. The PR of the osaux3-2 mutant was not significantly reduced in comparison with WT/DJ in the presence of IAA or the synthetic auxins, 2,4-D or NAA, respectively (Figure 1c), suggesting that osaux3-2 is insensitive to auxin. Interestingly, OsAUX3 expression was highly induced by IAA, 2,4-D, and NAA, especially in the PR apex, as demonstrated by qRT-PCR and GUS staining (Figure 1d,e). This together suggests that OsAUX3 might participate in regulation of auxin-mediated PR development.

To deeper understand the cause of alteration of PR length in osaux3, EdU staining of the PR apex revealed reduced fluorescence intensities in osaux3-1 and osaux3-2 compared with WT/HY and WT/DJ, whereas fluorescence was higher in both OsAUX3 overexpression lines, 35S:OsAUX3-3 and 35S:OsAUX3-6, respectively. These results indicate that the cell division activity is dependent on OsAUX3, leading likely to a short-root phenotype in the mutants.

3.2 Deferred LR initiation and decreased expression in genes related to LR initiation and development in osaux3-2

To better understand OsAUX3 function during root development, the density of LRs in osaux3-2 and 35S:OsAUX3-3 was measured...
CDKs, cyclins and kinase Wee families (Guo et al., 2007). Moreover, it was shown that E2F transcription factors, CDK inhibitors, CDK subunit proteins, homologues of the retinoblastoma protein and the protein (CDK), E2F transcription factors, CDK inhibitors, CDK subunit protei ns, homologues of the retinoblastoma protein and the protein kinase Wee families (Guo et al., 2007). About 90 putative core cell cycle genes were reported to participate in cell division. They belong to cyclin-dependent kinases (CDK), E2F transcription factors, CDK inhibitors, CDK subunit proteins, homologues of the retinoblastoma protein and the protein kinase Wee families (Guo et al., 2007). Moreover, it was shown that cyclins and CDKs affect LR densities and that CYCDs regulate the G1-S transition to promote LR initiation (Nieuwland et al., 2009; Sanz et al. 2011). Especially, CYCD4;1 controls cell length in the pericycle of the basal meristem and affects the formation of LR (Nieuwland et al., 2009). Further, CDK is involved in LR induction in response to auxin by regulating the levels of interactor of CDK/Kip-Related Protein 2 (Verkest et al., 2005; Sanz et al., 2011). Our results show that the cell cycle-related genes OsCYCD4;1, OsCYCB2;2, OsCYCD4;3, OsCDKC;1, OsCDKC;3, and OsCKL:10 were dramatically reduced in osaux3-2 compared with WT/DJ but increased in 35S:OsAUX3-3, which suggests that OsAUX3 might also function in regulating LR initiation through mediating expressions of those cell cycle-related genes (Figure 2e).

3.3 Increased RH length in osaux3-2 and OsAUX3 expression in RH cell

In our previous research, we have shown that OsAUX1 expression is different in comparison with its paralogous gene, AtAUX1, which is not expressed in RHs but still able to regulate RH development (Jones et al., 2009; Yu et al., 2015). We wondered whether OsAUX3...
would be also involved in RH growth regulation and expressed in RHs, indicating that their molecular mechanisms would be conserved between rice and Arabidopsis. RH length in WT and osaux3-2 in 3-day-old seedlings were found to be increased by 60% compared with the WT (Figure 3a,b), which is different to the osaux1 mutant showing shorter RHs.

The expression pattern of OsAUX3 was investigated using transgenic rice expressing ProOsAUX3:OsAUX3-GFP (Figure 3c). The analysis revealed that OsAUX3 is expressed in each period RH including young RHs, developing RHs, and mature RHs. This is not the case for OsAUX1, which is not expressed in mature RHs, indicating a functional difference in regulating RH development not only in between monocot and dicot plants but also in close rice homologies.

3.4 | OsAUX3 contributes to an acropetal IAA translocation by functioning as an importer

Members of the AUX1/LAX family in Arabidopsis function as auxin influx carriers during auxin distribution. To deeper understand the biological function of OsAUX3, we further investigated its subcellular localization and its auxin transport capacity in osaux3 mutants. By using the 35S:OsAUX3-GFP constructs, OsAUX3 was co-localized with the plasma membrane marker, pm-rbCD3-1008, in tobacco and rice epidermal cells and protoplasts, respectively (Figure 4a), implying that OsAUX3 and AtAUX1 might share overlapping auxin transport functionalities. In agreement, both osaux3 alleles showed significantly enhanced IAA export from rice protoplasts prepared from osaux3-1 and osaux3-2 plants, in comparison with their corresponding WT (Figure 4b). Increased export caused by loss of function of a plasma membrane transporter can only be explained by an import directionality. In this scenario, a lack of reimport of effluxed radiolabeled IAA results in elevated net export compared with the WT. As a result, acropetal auxin transport in osaux3-1 and osaux3-2 roots is decreased drastically, in comparison with the WT (Figure 4c,d). These results suggest that OsAUX3 functions as an auxin importer involved in acropetal auxin transport.

3.5 | Al stress induces OsAUX3 expression in the rice root, and NAA increases the inhibition of PR growth under Al stress

Al toxicity inhibiting root elongation is a major limiting factor for rice growth. It was found that Al-regulated inhibition of root growth is regulated by auxin biosynthesis and signalling. In order to uncover if OsAUX3-mediated auxin transport is involved in responses to Al stress, we first quantified OsAUX3 expression in 3-day-old WT/DJ seedling under control conditions and Al treatment by qRT-PCR and GUS staining in the PR of ProOsAUX3:GUS plants (Yang et al., 2014). The results show that OsAUX3 expression was significantly increased by AlCl3 treatment for 3 hr (Figure 5a,b), suggesting that OsAUX3 might function in responses to Al stress.

To better understand the molecular mechanisms of OsAUX3 responses to Al stress, the morphology of WT/DJ, osaux3-2, and 35S:OsAUX3-3 in 7-day-old seedlings was observed under various concentrations of Al treatment. It was found that PR growth of WT/DJ, osaux3-2, and 35S:OsAUX3-3 was inhibited to a different degree by increasing Al concentrations (Figure 5c,d). However, compared with WT/DJ, PR growth in osaux3-2 was insensitive to Al stress, suggesting that decreased acropetal auxin transport in osaux3-2 might affect the sensitivity to Al stress. Interestingly, the inhibition of PR elongation by Al treatment was enhanced by addition of the synthetic auxin, NAA (Figure 5e,f). Similarly, root growth inhibition under different concentrations of Al stress for 24 hr was also significantly alleviated in osaux3-2 compared with the WT/DJ (Figure 5g), and after 24 hr of NAA exposure, the inhibition of PR elongation under Al stress

FIGURE 3 Morphology of root hair (RH) in wild-type rice Dongjin (WT/DJ) and osaux3 mutant and OsAUX3 expression in RH. (a) Comparative analysis of RH phenotype in WT/DJ and osaux3-2 for 3-day-old seedlings. Bar = 1 mm. (b) Statistical analysis of RH length of WT/DJ and osaux3-2 for 3-day-old seedlings. Ten biological replicas were measured for this test. Asterisks indicate significant differences compared with WT/DJ (**P < 0.01; t test). (c) OsAUX3 expression in the RH. Fluorescence microscopy of RH of 3-day-old ProOsAUX3:OsAUX3-GFP seedlings. Left, GFP channel (green represents GFP signals; yellow represents auto-fluorescence of roots); right, bright-field images. Upper to lower panels show mature RHs (length ≈800 μm), developing RHs (length ≈250 μm) and young RHs (length ≈100 μm), respectively. Bar = 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]
was enhanced (Figure 5h). According to a previous study, decreased auxin synthesis results in Al insensitivity in Arabidopsis (Yang et al., 2014). These findings together with our experiments imply that the sensitivity to Al stress might depend on local changes in auxin concentrations caused by reduced auxin transport in osaux3-2.

### 3.6 Reduced auxin concentration in osaux3-2 might confer Al stress insensitivity to osaux3-2

To confirm the effect of auxin on Al sensitivity, we further analysed the auxin distribution in WT and osaux3-1 lines using the auxin response marker, DR5:GUS, transformed into WT/HY and osaux3-1 (Figure 6a). DR5:GUS staining and DR5-GUS activity were significantly reduced in osaux3-1 compared with WT/HY (Figure 6b). In agreement, also, the content of auxin in the root tip of osaux3-2 mutant was reduced compared with the WT control (Figure 6c). Interestingly, the auxin concentration in both WT/DJ, osaux3-2, and 35S:OsAUX3-3 was increased under Al stress; however, the increase of auxin in osaux3-2 was less pronounced than in WT/DJ, suggesting that an inhibition of acropetal auxin translocation in osaux3-2 (Figure 4d) might be responsible for this event. These results with those from Figure 5 suggest that reduced auxin levels might be the primary cause for a reduced root sensitivity of osaux3-2 in response to Al.

Furthermore, we wondered whether decreased Al sensitivity also affected the Al content of osaux3-2 PR tips. Al contents of WT/DJ, osaux3-2, and 35S:OsAUX3-3 PR tips of 3-day-old seedlings exposed to 25 μM AlCl₃ for 3 hr were analysed by ICP and Morin fluorescence staining (Figure 6d,e). ICP analyses revealed that Al contents in osaux3-2 PR apex were reduced by 60% but found to be increased by 25% in 35S:OsAUX3-3 compared with the WT/DJ, respectively. Further, the distribution and accumulation of Al in the PR was visualized using the fluorochrome Morin (Del et al., 1990; Mile et al., 2015). Root sections of PRs indicate that Morin fluorescence intensities were significantly lower in osaux3-2 but higher in 35S:OsAUX3-3 compared with the WT/DJ. Both experiments demonstrate that Al accumulation in the PR apex of osaux3-2 is reduced.

### 3.7 Oxidative damage in osaux3-2 is reduced under Al stress

Accumulation of reactive oxygen species (ROS) under Al stress has been widely reported (Yamamoto, Kobayashi, Devi, Rikiishi, & Matsumoto, 2002), and the concentration of H₂O₂ is often used as an indicator of oxidative damage. Root tips are susceptible to

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**FIGURE 4** Subcellular localization of OsAUX3 and auxin transport in wild-type (WT) and osaux3 mutants. (a) 35S:OsAUX3-GFP fusion construct transiently expressed in tobacco and rice. Co-transformation of 35S:OsAUX3-GFP with plasma membrane marker pm-rbCD3-1008. Left to right: green fluorescence of OsAUX3-GFP, bright-field images, red fluorescence of the protoplast membrane marker pm-rb CD3-1008, and merged microscope images. Bar = 50 μm. (b) Indole-3-acetic acid (IAA) export from osaux3-2 and osaux3-1 and from corresponding WT protoplasts, WT rice Dongjin (WT/DJ) and Hwayoung WT/HY, respectively, after 20 min. Significant differences (unpaired t test with Welch’s correction, \( P < 0.05 \)) to WT controls are indicated by an asterisk (mean ± SE; \( n \geq 3 \)). Analysis of acropetal IAA transport in primary root of 3-day-old (c) WT/DJ and osaux3-2. (d) WT/HY and osaux3-1 seedling. These experiments were performed using five independent biological replicas. Asterisk indicate significant differences (\( ^* P < 0.05; ^{\dagger} t \) test) [Colour figure can be viewed at wileyonlinelibrary.com]
oxidative damage under Al stress; hence, we used the fluorescence of H2DCF‐DA as a read‐out for the accumulation of H2O2 in roots. The results revealed no significant difference between WT/DJ, osaux3‐2, and 35S:OsAUX3 PRs in the absence of Al, whereas fluorescence was increased in WT/DJ and 35S:OsAUX3 under 25‐μM Al stress; however, there was no significant increase in osaux3‐2 (Figure 7a). Interestingly, the fluorescence increased in 35S:OsAUX3‐3 under Al stress was much more drastic than in WT/DJ. These results are consistent with a quantitative analysis of H2O2 concentration (Figure 7b), indicating that Al treatment increases the accumulation of H2O2 in the root apex of WT/DJ, osaux3‐2 and 35S:OsAUX3‐3. However, the lower Al accumulation in osaux3‐2 compared with the WT/DJ can reduce oxidative damage to PR apex in the presence of Al. On the other hand, expression of hydrogen peroxidase (CAT) in osaux3‐2 was significantly higher than that of WT/DJ.
under Al stress. Bar = 50 μm. (b) Quantification of ProDR5:GUS activity in the lines shown in (a). Five biological replicates were analysed in this test. Asterisks indicate significant differences compared with WT/DJ, respectively (*P < 0.05; t test). (c) Measurement of auxin concentrations in PR apex (1 cm) of 3-day-old WT/DJ, osaux3-2, and 35S:OsAUX3-3 seedlings under control and 25–μM AlCl3 treatments. Five biological replicates were analysed in this test. Asterisks indicate significant differences compared with WT/DJ, respectively (*P < 0.05; t test). (d) Al content of PR apex (1 cm) of 3-day-old WT/DJ, osaux3-2, and 35S:OsAUX3-3 seedlings under Al stress. Five biological replicates were analysed in this test. Asterisks indicate significant differences compared with WT/DJ, respectively (*P < 0.05; t test). (e) Morin staining of transverse section of PR apex under Al stress (Figure 7c).

and 35S:OsAUX3-3, suggesting that more H2O2 was reduced to H2O and O2 under Al stress (Figure 7c).

3.8 Al-resistant genes are up-regulated in osaux3-2

In rice, some genes have been reported to be involved in resistance to Al stress. Al RESISTANCE TRANSCRIPTION FACTOR1 (ART1), a transcription factor, encodes for a putative C2H2 zinc finger protein, which is involved in Al tolerance by regulating multiple Al-tolerant genes (Yamaji et al., 2009). The ART1-regulated downstream genes, Nramp Al transporter1 (OsNrat1), rice ALUMINIUM SENSITIVE1 (OsALS1), and MAGNESIUM TRANSPORTER1 (OsMGT1), have been reported (Tsutsui, Yamaji, & Feng, 2011). Nrat1 is localized to the plasma membranes of root tip cells and transports trivalent Al ions for Al detoxification (Xia, Yamaji, Kasai, & Ma, 2010). OsALS1 is localized to the tonoplast, which is required for detoxification of Al in rice through sequestration of Al into vacuoles (Huang, Yamaji, Chen, & Ma, 2012). OsMGT1, a putative rice Mg transporter, is able to alleviate Al toxicity through up-regulation of Mg concentrations under Al stress by displacing or competing with Al from binding sites of different cellular components (Chen, Yamaji, Motoyama, Nagamura, & Ma, 2012).

To clarify how OsAUX3 responds to Al stress, we further analysed expression levels of these genes related to Al tolerance in WT/DJ, osaux3-2, and 35S:OsAUX3-3 under Al stress by qRT-PCR (Figure 7d). The results reveal that the expression of these genes in the osaux3-2 was higher than in WT/DJ and that their expression was dramatically up-regulated in osaux3-2 after Al treatment, whereas we found no significant increase in the 35S:OsAUX3-3 compared with the WT/DJ. This suggests that significant up-regulation of these Al-resistant genes in osaux3-2 confers insensitivity to Al in osaux3-2.

4 DISCUSSION

In the recent years, a participation of IAA in Al resistance of plants has frequently been reported. Inversely, Al has also been shown to affect root growth by modifying the levels of auxin (Ponce, Barlow, Feldman, & Cassab, 2005); however, this study only presented indirect evidence on the involvement of polar transportation of auxin in Al-induced inhibition of root growth. The underlying molecular mechanisms of the effects of Al stress on auxin transport in rice are still unclear. Here, we uncover OsAUX3 as an auxin influx carrier, functioning in the regulation of root and its implication in responses to Al stress.

4.1 Unlike OsAUX1, OsAUX3 positively regulates PR growth

In our previous report, OsAUX1 was shown to negatively regulate PR elongation (Yu et al., 2015). We therefore wondered if the role of OsAUX3 in PR development is similar to OsAUX1. According to sequence analysis of amino acids, a special domain (Figure S2c, with red box) in OsAUX3 is different to other OsAUX family members, including OsAUX1. Therefore, we used the CRISPR-Cas9 method to remove this special domain for constructing both osaux3 mutants. As shown in Figure 1, both osaux3 mutants revealed a short-root phenotype different to osaux1 (Yu et al., 2015). Therefore, this special domain in OsAUX3 might play an important role in PR development. In this study, we find short PR traits in osaux3 mutants and longer PR in OsAUX3 overexpression lines that are opposite to the phenotypes of OsAUX1-related mutants and overexpression lines, respectively (Figure 1). Knock-down of OsAUX3 led to decreases in PR...
length, which suggests that OsAUX3 is also implicated in PR growth but positively regulates PR elongation. The expression of OsAUX3 in PR was induced by auxin, and shorter roots of osaux3-2 were insensitive to auxin, which is a significant auxin defective phenotype. EdU fluorescence staining showed a decrease of meristem cell activity in the PR of osaux3-2, indicating that inhibition of meristem cell division in PRs leads to reduced PR elongation. In agreement, the auxin distribution and concentration in osaux3 was significantly decreased (Figure 6a-c), which demonstrates that OsAUX3 positively regulates PR growth by maintaining the auxin distribution in PRs to support meristem cell division required for normal PR growth.

4.2 OsAUX3 controls LR initiation in analogy to AtAUX1, AtLAX3, and OsAUX1

LR formation includes two phases: initiation and emergence (Bhalerao et al., 2002; Laskowski, Williams, Nusbaum, & Sussex, 1995; Marchant et al., 2002). It was reported that AtAUX1 participates in the control of LR initiation and thus causes a reduction of LR number (Marchant et al., 1999; Marchant et al., 2002; Swarup et al., 2008). In Arabidopsis, the AUX1 and LIKE-AUXIN3 (LAX3) auxin influx carriers are required for auxin signalling activating LBD16 and LBD18 (lateral organ boundaries [LBD]) to control LR development (Lee, Cho, & Kim, 2015). In rice, the osaux1 mutant has a reduced number of LR primordia (Zhao et al., 2015). In osaux3-2, all these features including delayed LR initiation, decreased LR primordia, reduced LR density, and decreased expression of genes related to LR initiation suggest that OsAUX3 plays an important role during LR initiation, which would be consistent with a conserved function to its homologues/analogues, AtAUX1, AtLAX3, or OsAUX1 (Figure 2).

4.3 OsAUX3 regulates RH development different than AtAUX1 but similar to OsAUX1

In Arabidopsis, the root epidermis consists of two cell types: one is called an RH cell forming RHs, and the other one is a non-hair cell, which does not form RHs. AtAUX1 is localized in non-hair epidermal cells, whereas OsAUX1 is expressed in young RH cells (Jones et al., 2019; Laskowski et al., 1995; Marchant et al., 2002; Swarup et al., 2008). In rice, OsAUX3 has been shown to regulate RH development differently than AtAUX1 but similar to OsAUX1 (Marchant et al., 2002; Swarup et al., 2008). This suggests a conserved role for auxin influx carriers in controlling cell fate during RH development, which may be due to their ability to regulate auxin distribution and concentration in RH cells.
OsAUX3 plays an important role in regulating the auxin content of osaux3 growth inhibition under Al stress was also significantly alleviated in tents was less pronounced than in WT/DJ (Figure 6c). Hence, root the root apex of rice. However, in

4.4 OsAUX3 is an auxin influx carrier playing an important role in acropetal auxin transport

AtAUX1 is an auxin influx carrier functioning in auxin uptake (Carrier et al., 2008; Dharmasiri et al., 2006; Peret et al., 2012; Robert & Friml, 2009; Zazimalova, Murphy, Yang,Hoyerova, & Hosek, 2010). LAX3 has been shown to create cell-specific auxin sinks (Swarup et al., 2008; Vandenbussche et al., 2010). Like AUX1 or LAX3 in Arabidopsis and OsAUX1 in rice, also, OsAUX3 is localized to plasma membrane (Figure 4a). Further, our study revealed reduced IAA export from osaux3-1 and osaux3-2 protoplasts, indicating that OsAUX3 functions also as auxin influx carrier (Figure 4b). Acropetal auxin transport of osaux3-1 and osaux3-2 roots was found to be decreased in comparison with their WTs (Figure 4c,d), suggesting that OsAUX3 functions in acropetal transport of auxin. Decreased acropetal auxin transport results in a reduced auxin distribution and concentration in the PR apex of osaux3 (Figure 6a–c).

4.5 OsAUX3 is induced in the root apex under Al stress and is involved in Al-induced inhibition of root growth

Several studies have demonstrated that Al may interact with auxin-signalling pathways, leading to alterations of auxin accumulation and distribution in roots (Doncheva, Amenos, Poschenrieder, & Barcelo, 2005; Kollmeier et al., 2000). In response to Al stress, auxin signalling in the root transition zone is enhanced, which results in auxin-regulated root growth inhibition through altering auxin response factors in Arabidopsis (Yang et al., 2014). In barley, auxin enhances Al-induced root growth inhibition in response to toxic Al stress (Bai et al., 2017). In wheat, MAPK-mediated auxin signal transduction pathways triggers an increase of malic acid against Al toxicity (Liu et al., 2017).

Rice is one of the most Al-tolerant species. Our results demonstrate that the presence of Al promotes the accumulation of auxin in the root apex of rice. However, in osaux3-2, the increase of auxin contents was less pronounced than in WT/DJ (Figure 6c). Hence, root growth inhibition under Al stress was also significantly alleviated in osaux3-2 compared with WT/DJ (Figure 5c–h), uncovering that OsAUX3 plays an important role in regulating the auxin content of the root apex in the presence of Al. On the other hand, auxin negatively regulates Al tolerance through altering ALS1 expression and Al distribution within Arabidopsis cells (Zhu et al., 2013). Al suppresses root growth due to an abnormal accumulation of auxin and cytokinin in Arabidopsis (Daspute et al., 2017). In agreement, our experiments show that Al accumulation in the PR apex of osaux3-2 was reduced compared with WT/DJ (Figure 6d,e). This confirms that auxin negatively regulates Al tolerance in rice and that this process involves OsAUX3-mediated auxin transport.

4.6 Reduced oxidative damage in the PR of osaux3-2 under Al stress is regulated through enhanced expression of Al-tolerance-related genes

Auxin was suggested to regulate ROS level and to direct the role of ROS in oxidative stress (Iglesias, Terrile, Bartoli, D’ippoitolo, & Casalengue, 2010; Krishnamurthy & Rathinasabapathi, 2013b). Auxin plays an important role in responses to oxidative stress and effects the distribution of Al in cells (Krishnamurthy & Rathinasabapathi, 2013a; Zhu et al., 2013). Phytohormones and ROS activate various transcriptional responses, including the expression of genes related to increased Al tolerance under Al treatment (Daspute et al., 2017). Our results show that loss of OsAUX3-mediated acropetal auxin transport causing decreased Al distribution in root cells resulted in distinctly alleviated Al-induced oxidative cellular damage in the rice root apex of osaux3-2 (Figures 4 and 7). We uncover a novel pathway that employs inhibition of auxin acropetal transport to alleviate Al-induced oxidative cellular damage. Our findings offer a novel path for engineering Al-tolerance rice species by altering the expression of the auxin influx carrier OsAUX3.

Previous studies identified some Al-tolerance-related genes in rice, including OsART1, OsNrat1, OsALS1, and OsMGT1, whose expression was induced by Al (Chen et al., 2012; Huang et al., 2012; Xia et al., 2010; Yamaji et al., 2009). Our results further show that expression of these Al-tolerance-related genes in osaux3-2 was more up-regulated than in WT/DJ upon Al treatment. As previously reported, OsNrat1 and OsALS1 play important role in detoxifying Al through transportation and sequestration of Al into vacuoles (Huang et al., 2012; Xia et al., 2010). The up-regulation of these Al-tolerance-related genes might result in the reduced oxidative damage to the PR apex in osaux3-2 in the presence of Al.

Taken together, our results indicate that decreased auxin transport in osaux3 mutants is responsible for its reduced sensitivity towards Al stress, underlining that auxin plays an important role as a signalling molecular in response to Al stress.

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REFERENCES


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.