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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 accented 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; Swarup 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

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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* (*SlLAX1 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 & Mutert, 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 transporter-like 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.

2 | 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-naphthylacetic acid (NAA) for 7 days. Al treatment was performed in a 0.5-mM $CaCl_2$ solution (pH 4.5) with different concentration of $AlCl_3$ 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* (pBI101.3), 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-ethynyl-2′-deoxyuridine (EdU) of culture medium for 1–2 hr as described in EdU Flow Cytometry Assay Kits (http://www.ribobio.com/sitecn/Products.aspx?id=37) and observed under a LSM710 NLO microscope (Zeiss, http://www.zeiss.com/corporate/en_de/home.html).

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 ddH_2O, 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 ^{3}H-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 rice 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 $AlCl_3$ was excised, washed six times with 0.2-mM $CaCl_2$, 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 $CaCl_2$ and sliced into 100-μm section by using a vibratome (Leica VT1000 S). Slices were Morin stained for 5 min and washed twice with 0.2-mM $CaCl_2$, 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 H_2O_2 and CAT concentrations

Analysis of H_2O_2 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 two times with 0.2-mM $CaCl_2$. Root tips were then observed under a LSM710 NLO Zeiss microscope (Zeiss, http://www.zeiss.com/corporate/en_de/home.html). For measurement of H_2O_2 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 H_2O_2 and CAT concentration by NanoQuant infinite M200 pro (www.eastwin.com.cn) spectrophotometrically at a wavelength of 450 nm by using the Plant H_2O_2 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

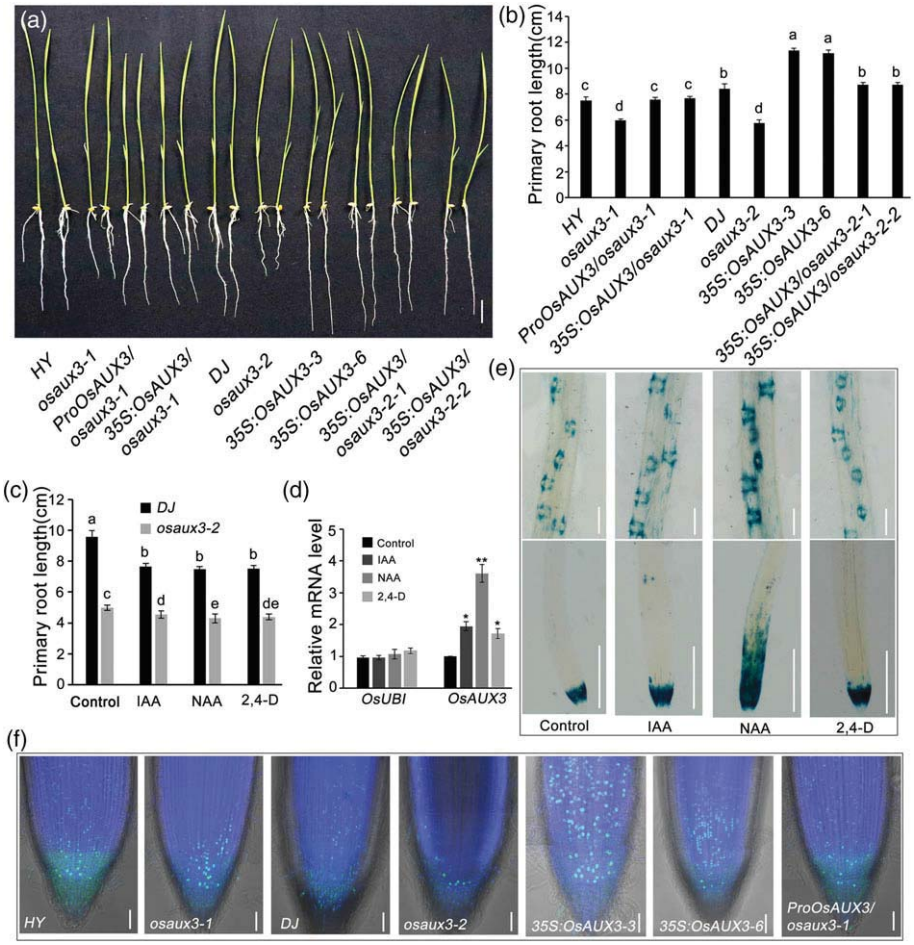

FIGURE 1 Phenotypic characterizing of *OsAUX3* gain and loss-of-function phenotypes. (a) Phenotypes of wild-type rice Hwayoung (WT/HY), *osaux3-1*, its complemented transgenic lines, *ProOsAUX3/osaux3-1* and *35S:OsAUX3/osaux3-1*; wild-type rice Dongjin (WT/DJ), *osaux3-2*, *OsAUX3* overexpression lines, *35S:OsAUX3-3* and *35S:OsAUX3-6*, and complemented transgenic lines, *35S:OsAUX3-2-1* and *35S:OsAUX3-2-2* for 7-day-old seedling, from left to right. Bar = 2 cm. (b) Quantification of primary root (PR) length of mentioned lines in (a). Significant difference to WT are indicated by letters, $n = 3$ with 10 seedlings each (Duncan's test, $P < 0.05$). (c) Quantification of PR length of WT/DJ and *osaux3-2* 7 days (days after germination) under grown on solvent, 1 μM of indole-3-acetic acid (IAA), 0.01 μM of 1-naphthylacetic acid (NAA), or 0.01-μM 2,4-dichlorophenoxyacetic acid (2,4-D) treatments. Significant difference to WT are indicated by letters, $n = 3$ with 10 seedlings each (Duncan's test, $P < 0.05$). (d) OsAUX3 expression in PR apex of WT/DJ for 3-day-old seedling under normal condition, 10 μM of IAA, 0.1 μM of NAA, and 0.1 μM of 2,4-D treatments. qRT-PCR experiments were analysed using three independent biological replicas. *OsACTIN* and *OsUBI* were used as internal controls. Asterisks indicate significant differences compared with solvent control (**$P < 0.01$; *$P < 0.05$; *t* test). (e) GUS staining of PRs of *ProOsAUX3:GUS* lines for 3-day-old seedling under normal condition, 10 μM of IAA, 0.1 μM of NAA, and 0.1 μM of 2,4-D treatments. In each treatment, 10 seedlings were used. Bar = 500 μm. (f) Ethynyl-2′-deoxyuridine staining for PR apex in WT, *osaux3* mutants, OsAUX3 overexpression lines and complemented transgenic lines for 3-day-old seedlings. In each treatment, 10 seedlings were used. Bar = 50 μm

(Figure 2a,b). LR initiation in *osaux3-2* was delayed in 3-day-old seedlings, and the density of LRs in *osaux3-2* was significantly decreased in 5- or 7-day-old seedlings. To get further insight into the basic reason for the reduced density of LRs in *osaux3-2*, LR primordia were observed by TTC staining (Figure 2c). In 3- and 5-day-old PRs, the number of LR primordia was significantly decreased in *osaux3-2* but increased in *35S:OsAUX3-3* (Figure 2c). These results indicate that knock-down of *OsAUX3* influences LR initiation. Both histochemical GUS staining in LRs of *ProOsAUX3:GUS* and fluorescence quantification in LRs of *ProOsAUX3:OsAUX3-GFP* showed that *OsAUX3* is expressed in LR primordia and extended LRs, further suggesting that OsAUX3 function is also involved in regulating LR initiation (Figure 2d).

Previously, auxin-induced LR initiation by the regulation of cell cycle-related gene expression was reported (Guo, Song, Wang, & Zhang, 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*, *OsCYCU4;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

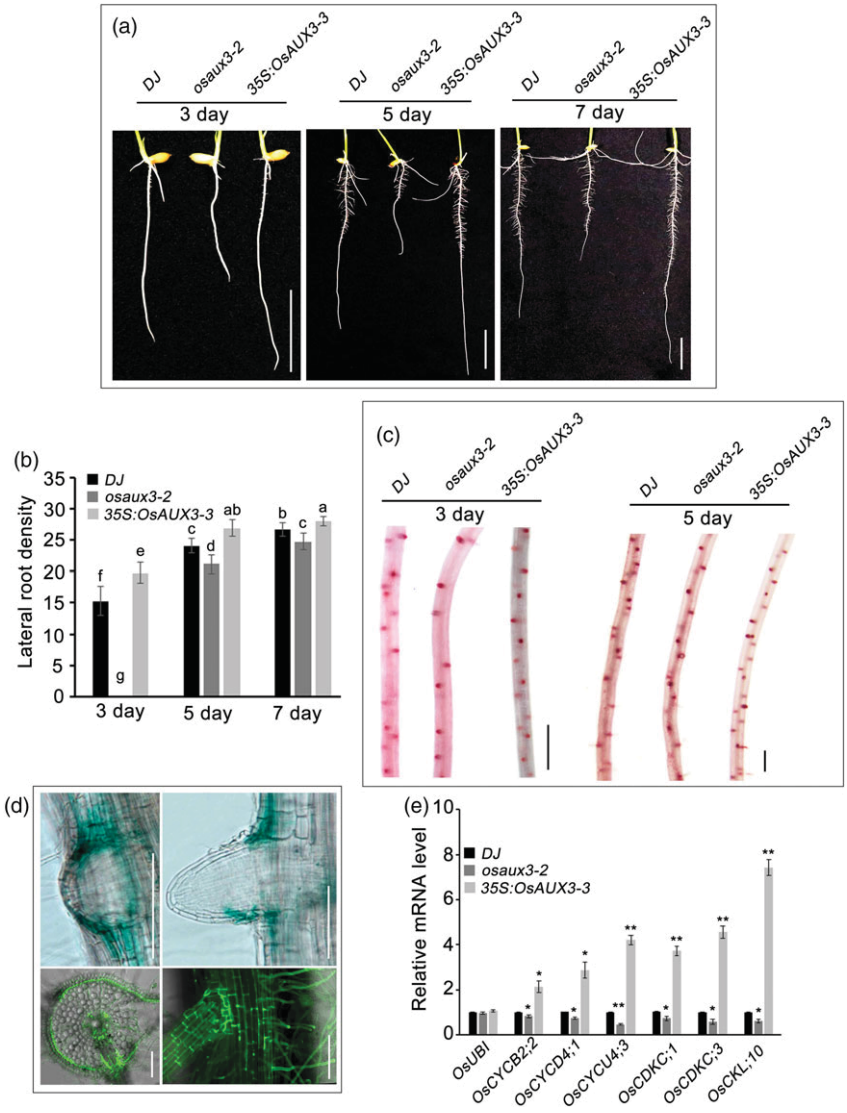

FIGURE 2 Analysis of OsAUX3 in lateral root (LR) development. (a) LR growth of wild-type rice Dongjin (WT/DJ), *osaux3-2*, and 35S:OsAUX3-3 seedlings at 3, 5, and 7 days. Bar = 2 cm. (b) Quantification of LR density in WT/DJ, *osaux3-2*, and *35S:OsAUX3-3*, respectively. Ten biological replicas were measured. Columns with different letters indicate significant differences to WT (Duncan's test, $P < 0.05$). (c) 2,3,5-triphenyltetrazolium chloride staining in primary root of WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* seedlings. Left, 0.5-cm samples without LR were taken from the root-stem base in the above three seedlings of 3-day-old of primary root; right, 1-cm samples without LR were taken from 4 cm of root-stem base in WT/DJ, 3 cm of stem base in *osaux3-2*, and 5 cm of root-stem base in *35S:OsAUX3-3*. Bar = 1 mm. (d) Expression pattern of OsAUX3 in LR. GUS staining in LR of 5-day-old *ProOsAUX3:GUS* and fluorescence imaging of LR of 5-day-old *ProOsAUX3:OsAUX3-GFP* seedlings. Upper panels from left to right show lateral root primordia and initiated LR, respectively, whereas lower panels from left to right indicate the transverse section and longitudinal section of the mature LR, respectively. Bar = 100 μm. (e) Relative mRNA levels of genes related to LR initiation in WT/DJ, *osaux3-2* and *35S:OsAUX3-3*. qRT-PCR experiments were analysed using three independent biological replicas. *OsACTIN* and *OsUBI* were used as internal controls. Asterisks indicate significant differences compared with WT/DJ, respectively (**$P < 0.01$; *$P < 0.05$; *t* test) [Colour figure can be viewed at wileyonlinelibrary.com]

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 $AlCl_3$ 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

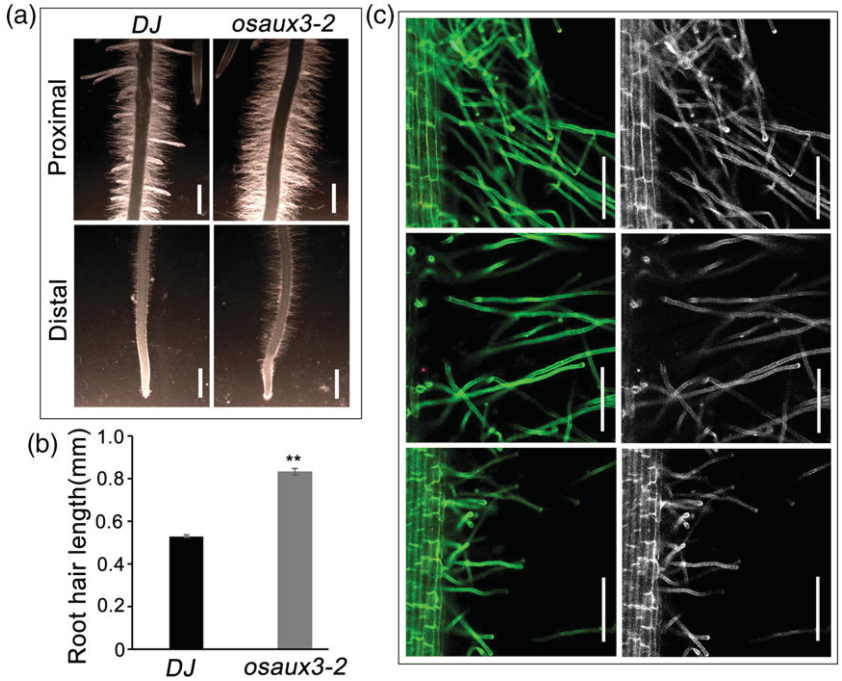

FIGURE 3 Morphology of root hair (RH) in wild-type rice Dongjin (WT/DJ) and *osaux3* mutant and OsAUX3 expression in RH. (a) Comparative analysis of RH phenotype in WT/DJ and *osaux3-2* for 3-day-old seedlings. Bar = 1 mm. (b) Statistical analysis of RH length of WT/DJ and *osaux3-2* for 3-day-old seedlings. 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]

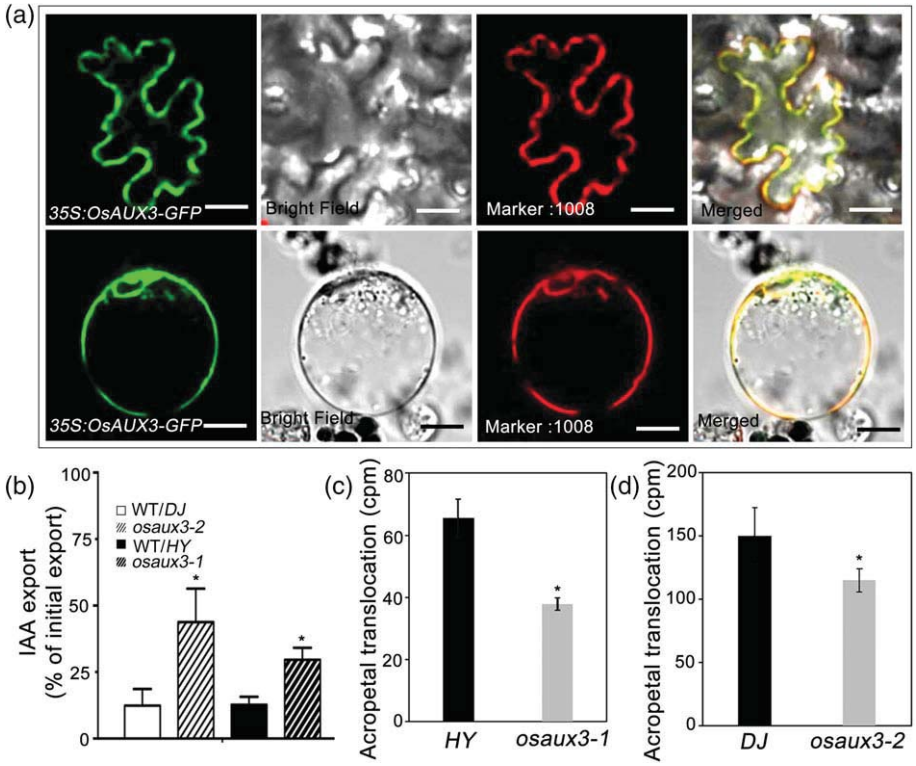

FIGURE 4 Subcellular localization of OsAUX3 and auxin transport in wild-type (WT) and *osaux3* mutants. (a) 35S:OsAUX3-GFP fusion construct transiently expressed in tobacco and rice. Co-transformation of 35S:OsAUX3-GFP with plasma membrane marker pm-rbCD3-1008. Left to right: green fluorescence of OsAUX3-GFP, 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 (*$P < 0.05$; *t* test) [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 H_2O_2 is often used as an indicator of oxidative damage. Root tips are susceptible to

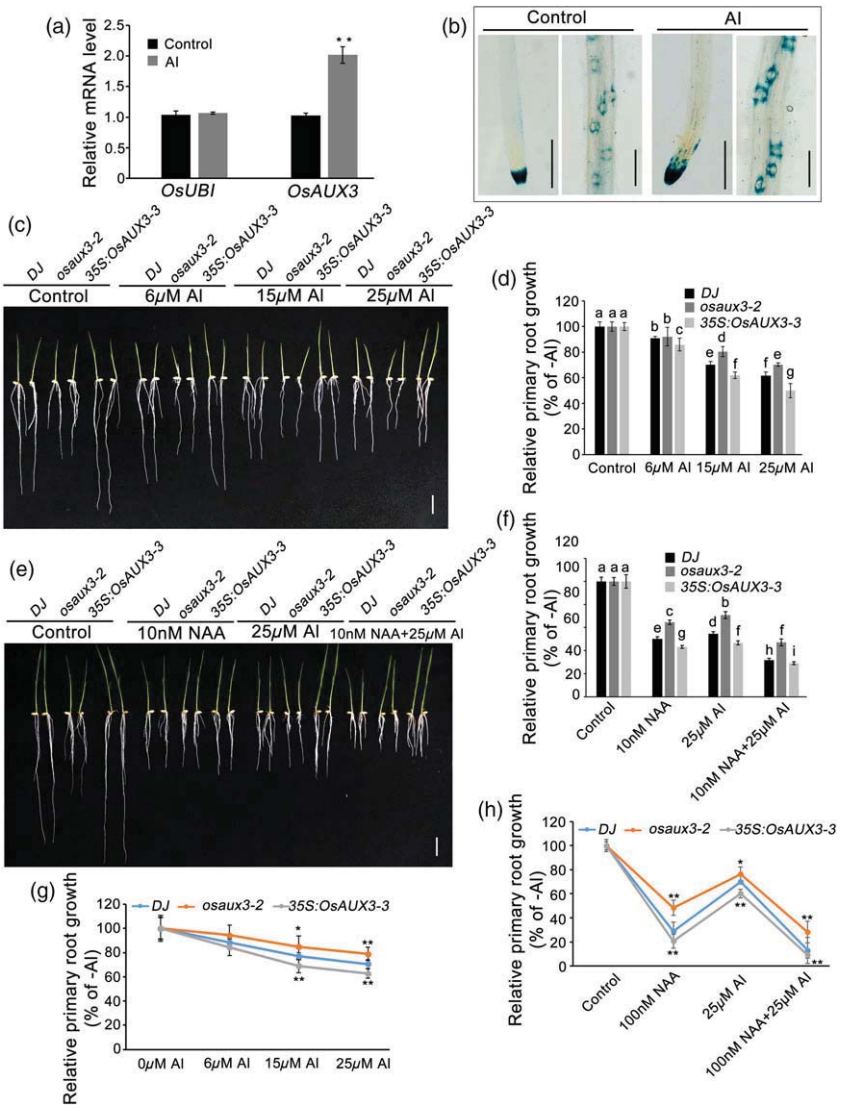

FIGURE 5 OsAUX3 expression level and primary root (PR) growth in wild-type rice Dongjin WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under aluminium (Al) treatment. (a) Relative mRNA level of OsAUX3 in the PR apex of 3-day-old WT/DJ seedling under control and Al treatment. qRT-PCR experiments were analysed using three independent biological replicas. *OsACTIN* and *OsUBI* were used as internal controls. Asterisks indicate significant differences (**P < 0.01; t test). (b) β-Galactosidase staining in the PR of 3-day-old *ProOsAUX3:GUS* seedlings with and without Al treatment. For each treatment (n = 3), 10 seedlings were used for this test. Bar = 500 μm. (c) Phenotypic characterization of WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under 0-, 6-, 15-, or 25-μM $AlCl_3$ treatment for 7 days. Bar = 2 cm. (d) Analysis of relative PR growth of WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under 0-, 6-, 15-, or 25-μM $AlCl_3$ treatment for 7 days. Ten seedlings of each line were used here, n = 3. Columns with different letters indicate significant differences (Duncan's test, P < 0.05). (e) Phenotypic characterization of WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under 0, 10-nM 1-naphthylacetic acid (NAA), 25-μM $AlCl_3$, or 10-nM NAA + 25-μM $AlCl_3$ treatments for 7 days. Bar = 2 cm. (f) Analysis of relative PR growth of WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under 0, 10-nM NAA, 25-μM $AlCl_3$, or 10-nM NAA + 25-μM $AlCl_3$ treatments for 7 days. Ten seedlings of each line were used here, n = 3. Columns with different letters indicate significant differences (Duncan's test, P < 0.05). (g) Analysis of relative PR growth in WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under 0-, 6-, 15-, or 25-μM $AlCl_3$ treatment for 24 hr. Ten seedlings of each line were used here, n = 3. Asterisks indicate significant differences compared with WT/DJ, respectively (**P < 0.01; *P < 0.05; t test). (H) Analysis of relative PR growth of WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* under 0, 100-nM NAA, 25-μM $AlCl_3$, or 100-nM NAA + 25-μM $AlCl_3$ treatments for 24 hr. Ten seedlings of each line were used here, n = 3. Asterisks indicate significant differences compared with WT/DJ, respectively (**P < 0.01; *P < 0.05; 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 H_2O_2 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 H_2O_2 concentration (Figure 7b), indicating that Al treatment increases the accumulation of H_2O_2 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

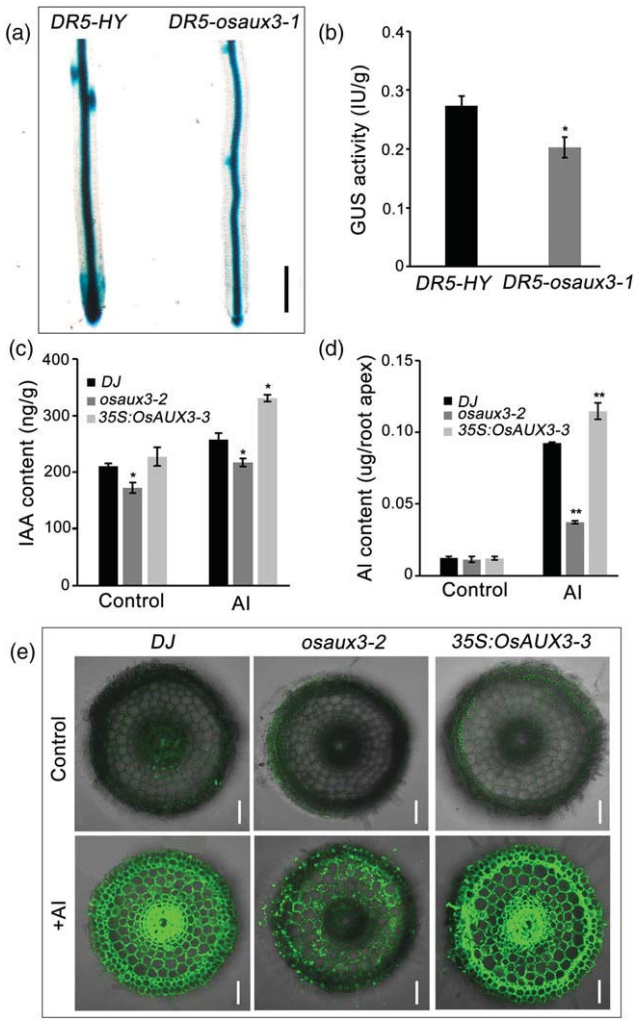

FIGURE 6 Auxin content and aluminium (Al) content in wild-type rice Dongjin (WT/DJ), *osaux3-2*, and *35S:OsAUX3-3* under Al stress. (a) β-Galactosidase (GUS) staining in primary root (PR) of *ProDR5:GUS* and *osaux3-1* seedlings for 3-day-old. Ten biological replicas were analysed. Bar = 250 μm. (b) Quantification of *ProDR5:GUS* activity in the lines shown in (a). Five biological replicas were analysed in this test. Asterisks indicate significant differences to *DR5-HY* (*$P < 0.05$; *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 $AlCl_3$ treatments. Five biological replicas 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 replicas were analysed in this test. Asterisks indicate significant differences compared with WT/DJ, respectively (**$P < 0.01$; *t* test). (e) Morin staining of transverse section of PR apex (1 cm) of 3-day-old WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* seedlings under Al stress. Bar = 50 μm. IAA: indole-3-acetic acid [Colour figure can be viewed at wileyonlinelibrary.com]

and *35S:OsAUX3-3*, suggesting that more H_2O_2 was reduced to H_2O and O_2 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 7 d). 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

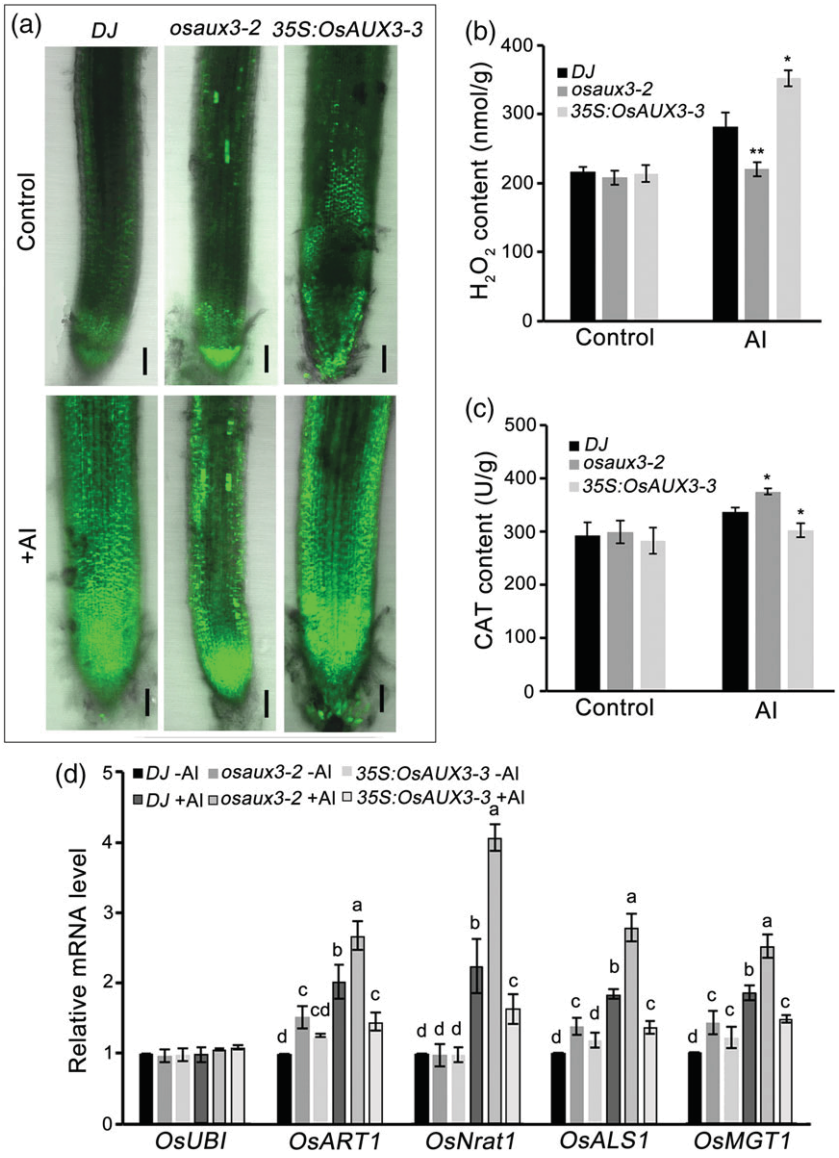

FIGURE 7 H_2O_2 accumulation under aluminium (Al) stress in wild-type rice Dongjin (WT/DJ), *osaux3-2*, and *35S:OsAUX3-3* and expression level of Al-resistant genes in primary root (PR) apex. (a) 2′,7′-Dichlorofluorescin diacetate staining (green) of PR apex (1 cm) in 3-day-old WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* seedlings after Al treatment. Bar = 100 μm. (b) H_2O_2 content of PR apex (1 cm) in 3-day-old WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* seedlings after Al stress. The experiment was analysed using five independent biological replicas. Asterisks indicate significant differences compared with WT/DJ, respectively (**P < 0.01; *P < 0.05; t test). (c) Catalase content of PR apex (1 cm) in 3-day-old WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* seedlings after Al treatment. The experiment was analysed using five independent biological replicas. Asterisks indicate significant differences compared with WT/DJ, respectively (*P < 0.05; t test). (d) Relative mRNA level of Al-resistant genes in PR apex of 3-day-old WT/DJ, *osaux3-2*, and *35S:OsAUX3-3* seedling after Al treatment. *OsACTIN* and *OsUBI* were used as internal control. Columns with different letters indicate significant differences to control (Duncan's test, P < 0.05) [Colour figure can be viewed at wileyonlinelibrary.com]

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 domain [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 feature 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.,

2009; Yu et al., 2015). This means that AtAUX1 sustains RH development through regulating auxin transport in non-hair cells, whereas OsAUX1 directly regulates RH, at least in young RH cells. Here, we show that OsAUX3 is expressed in either young or mature RH cells (Figure 3c), indicating that regulation of OsAUX3-mediated RH development is similar to OsAUX1 but different to AtAUX1 and that OsAUX3 might have a stronger biological function in regulating mature RH development than OsAUX1. Previous reports revealed that Arabidopsis belongs to the striped pattern (type 3) of RHs, whereas rice was suggested to be type 2, which depends on asymmetrical cell divisions (Clowes, 2000; Kwak & Schiefelbein, 2007; Horn et al., 2009). In this study, the expression position of OsAUX3 further supports that rice RHs belong to type 2.

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

AtAUX1 is an 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'Ippolito, & Casalongue, 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 Os*ART1*, *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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