The auxin transporter, OsAUX1, is involved in primary root and root hair elongation and in Cd stress responses in rice (Oryza sativa L.)

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SUMMARY
Auxin and cadmium (Cd) stress play critical roles during root development. There are only a few reports on the mechanisms by which Cd stress influences auxin homeostasis and affects primary root (PR) and lateral root (LR) development, and almost nothing is known about how auxin and Cd interfere with root hair (RH) development. Here, we characterize rice osaux1 mutants that have a longer PR and shorter RHs in hydroponic culture, and that are more sensitive to Cd stress compared to wild-type (Dongjin). OsAUX1 expression in root hair cells is different from that of its paralogous gene, AtAUX1, which is expressed in non-hair cells. However, OsAUX1, like AtAUX1, localizes at the plasma membrane and appears to function as an auxin transporter. Decreased auxin distribution and contents in the osaux1 mutant result in reduction of OsCyCB1;1 expression and shortened PRs, LRs and RHs under Cd stress, but may be rescued by treatment with the membrane-permeable auxin 1-naphthalene acetic acid. Treatment with the auxin transport inhibitors 1-naphthoxyacetic acid and N-1-naphthylphthalamic acid increased the Cd sensitivity of WT rice. Cd contents in the osaux1 mutant were not altered, but reactive oxygen species-mediated damage was enhanced, further increasing the sensitivity of the osaux1 mutant to Cd stress. Taken together, our results indicate that OsAUX1 plays an important role in root development and in responses to Cd stress.

Keywords: auxin, Cd stress, OsAUX1, Primary root, rice (Oryza sativa L.), root hair.

INTRODUCTION
Auxin moves within the plant body through non-polar auxin and polar auxin transport, the latter involving carrier-mediated transport (Kramer and Bennett, 2006). Polar auxin transport plays an important role in various aspects of plant growth and development (Friml and Palme, 2002; Swarup and Bennett, 2003). Transporters of auxin include the influx transporters of the AUXIN1/LIKE AUX1 (AUX1/LAX) family (Bennett et al., 1996; Kerr & Bennett 2007; Swarup et al., 2008), and efflux transporters encoded by members of the PIN-FORMED (PIN) and ATP binding cassette B/P-glycoprotein (ABC/PGP) families (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2005; Petrasek et al., 2006; Cho et al., 2007; Yang and Murphy, 2009). In recent years, PIN-LIKE proteins have also been reported to be involved in auxin transport (Barbez et al., 2012). Polar auxin transport in plants creates local auxin maxima that form the basis for plant development and differentiation, regulation of embryogenesis, organogenesis, vascular tissue formation, lateral root initiation and tropic responses (Vieten et al., 2007; Petrasek and Friml, 2009; Peret et al., 2012).

In Arabidopsis, the AUX1/LAX family consists of four highly conserved members, AUX1, LAX1, LAX2 and LAX3,
which have auxin uptake functions (Peret et al., 2012). AUX1 mediates the transport of indole-3-acetic acid (IAA) in Arabidopsis (Bennett et al., 1996). AUX1, which is asymmetrically localized at the plasma membrane of root protophloem cells, is proposed to promote the acropetal post-phloem movement of auxin to the root apex (Swarup et al., 2001). AUX1 and LAX3 influence lateral root development (Marchant et al., 2002; Swarup et al., 2008), while LAX2 regulates vascular patterning in cotyledons (Peret et al., 2012). In rice, the AUX1/LAX family has five members, which are homologous to AUX1 of Arabidopsis (Shen et al., 2010). OsAUX1 that was recently reported to function in the regulation of lateral root development and gravitropism (Zhao et al., 2015), but the function of other OsAUX members is not yet known.

The role of auxin in plant growth and development has been investigated in detail, but only a few studies have examined a possible role of auxin in regulating plant tolerance to abiotic stress, especially that caused by heavy metals. Cd is a major environmental pollutant and causes inhibition of plant growth and alteration of metabolism, leading to a decline in crop productivity. Further, it results in severe human health hazards through food chain contamination (Srivastava et al., 2014). Interestingly, alterations in IAA levels appear to be related to the activity of IAA oxidases in seedlings of pea (Pisum sativum) under Cd stress (Chaouki and El-Ferjani, 2005). Further, Cd stress may interfere with the metabolism of auxin, which triggers an increase in the activity of Gretchen Hagen3 (GH3) enzymes in poplar (Populus × canescens) (Ellobeid et al., 2012). Cadmium interferes with the maintenance of auxin homeostasis in Arabidopsis seedlings (Hu et al., 2013). However, the molecular mechanisms underlying the effects of Cd stress on auxin transport and homeostasis in rice are still unclear.

In this study, we report the roles of the auxin transporter, OsAUX1, in regulating primary root (PR) and root hair (RH) development, and in rice tolerance to oxidative stress caused by Cd treatment. We found that three independent osaux1 loss-of-function mutants were significantly more sensitive to Cd stress than WT. In osaux1 mutants, Cd increased the levels of the superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and thiobarbituric acid reactive substances (TBARS) compared with WT, suggesting a role for OsAUX1 in auxin transport during plant tolerance mechanisms to Cd stress via reactive oxygen species-mediated signaling.

RESULTS

Characterization of OsAUX1 loss- and gain-of-function rice lines

To understand the biological functions of OsAUX1 in rice, three T-DNA insertion mutants of OsAUX1 (osaux1-1 (3A-51110), osaux1-2 (1A-20543) and osaux1-3 (3A-01770)) were isolated, and over-expression lines of OsAUX1 (OsAUX1-O1, -O2 and -O3) were generated (Figure 1). T-DNA insertions were identified in the 3rd exon, 5th intron and 6th exon of the OsAUX1 gene by sequencing and comparison with the SIGnAL database at http://signal.salk.edu/cgi-bin/RiceGE (Figure 1a). Homozygous loss-of-function mutants of OsAUX1 were selected by DNA level and RNA level identifying (Figure 1b,c and Table S1). In the over-expression lines of OsAUX1, OsAUX1 expression was found to be increased eight- to tenfold (Figure 1d).

The PRs of the three osaux1 lines were all 30% longer than those of the wild-type (WT) (Dongjin, DJ) grown under hydroponic culture conditions for 7 days, while PRs of OsAUX1 over-expression lines were 30% shorter than those of the WT (Figure 1e). PRs of osaux1 mutants were insensitive to IAA and the synthetic auxin and herbicide 2,4-dichlorophenyl oxy acetic acid (2,4-D), but over-expression lines were hyper-sensitive to them. Interestingly, PRs of the three osaux1 mutants as well as the OsAUX1 over-expressing lines were sensitive to 1-naphthalene acetic acid (NAA), which diffuses into cells and thus rescues osaux1 mutants (Figure 1e).

A phenotypic analysis revealed that the mean lengths of the RHs of osaux1 were one-third those of WT (Figure 1f and Figure S1). Interestingly, the shorter RHs in osaux1 mutants were not rescued by IAA and 2,4-D treatments but were not rescued by NAA treatment. These results indicate that mutation of OsAUX1 disturbs IAA and 2,4-D transport, which depend on auxin transporters, but does not influence NAA influx, which is AUX1/LAX-independent.

OsAUX1 expression in root hair cells and OsAUX1 localization at the cell membrane

In the dicot Arabidopsis, AUX1 was reported not to be expressed in root hairs but to regulate root hair development (Jones et al., 2009). Based on the experiments described above, loss of OsAUX1 function had an effect on rice RH elongation and thus also showed a defect in RH development. Therefore, we wished to determine whether the molecular mechanisms of AUX1 regulation of RH development are the same in rice and Arabidopsis. The expression pattern of OsAUX1 was investigated using transgenic rice expressing ProOsAUX1:OsAUX1-sGFP and ProOsAUX1:GUS (Figure 2 and Figure S1). Using these constructs, OsAUX1 was constitutively expressed in each tissue (Figure S2) as described by Zhao et al. (2015). Expression of ProOsAUX1:OsAUX1-sGFP was detected during root hair initiation and when RHs were ~250 μm long, but was not found in mature RHs that were more than 800 μm long (Figure 2a). In 3-day-old seedlings, ProOsAUX1:GUS staining was observed in all RHs, including those close to the root cap and the proximal zone. However, in 5-day-old seedlings,
GUS staining was only detected in the distal zone close to root cap but not in the proximal zone (Figure 2b), i.e. OsAUX1 was not expressed in mature RHs. These results indicate that OsAUX1 has a specific spatio-temporal expression in RHs. Moreover, they confirm that OsAUX1 expression in rice root hair cells is different from that of

![Figure 1](image1.png)

**Figure 1.** Establishment of osaux1 and OsAUX1 over-expression lines. (a) T-DNA insertion sites in the osaux1-1, osaux1-2 and osaux1-3 mutants. Black boxes represent exons, white boxes represent untranslated regions, and black lines represent introns. Inverted triangles indicate T-DNA insertion sites. (b) PCR analysis of T-DNA insertion sites in osaux1-1, osaux1-2 and osaux1-2. Upper bands indicate the OsAUX1 gene fragment, while lower bands indicate T-DNA insertion fragments. (c) RT-PCR analysis of OsAUX1 expression. Upper bands indicate the abundance OsAUX1 (32 cycles), while lower bands show the abundance of the internal control OsACTIN (26 cycles). (d) Quantitative RT-PCR analysis of OsAUX1 expression. Relative mRNA levels of OsAUX1 in WT and three OsAUX1 over-expression lines, OsAUX1-O1, -O2 and -O2. OsACTIN (Os03g50855) was used as an internal control. Three independent biological repeats were performed. Asterisks indicate statistically significant differences compared with WT (***P < 0.01; Student’s t test) (e) Phenotypic characterization of WT and OsAUX1 mutants under control and various auxin treatments. From left to right: seedlings of WT, osaux1-1 osaux1-2, osaux1-3, OsAUX1-O1, OsAUX1-O2, OsAUX1-O3 under control, 1 μl IAA, 0.1 μl 2,4-D and 0.1 μl NAA treatments for 7 days. Scale bars = 2 cm. (f) Root hair morphology of WT and osaux1-1 under control, 1 μl IAA, 0.1 μl 2,4-D and 0.1 μl NAA treatments for 3 days. Scale bars = 1 mm.

![Figure 2](image2.png)

**Figure 2.** OsAUX1 expression in RH cells and subcellular localization of OsAUX1. (a-c) OsAUX1 expression in developing RH cells. ProOsAUX1:OsAUX1-sGFP was transformed into rice WT, and ten positive transgenic lines were imaged by laser scanning fluorescence microscopy. Left, GFP channel (green represents GFP signals; yellow represents auto-fluorescence of rice roots). Right, bright-field images. Scale bar = 100 μm. The images shows ProOsAUX1:OsAUX1-sGFP expression in mature RHs (a) (length 800 μm, blue arrows), developing RHs (b) (length 250 μm, red arrows), and cells initiating RHs (c) (white arrows). Red boxes indicate non-root hair cells. (d) OsAUX1 expression in RHs of a 3-day-old seedling (left) and a 5-day-old seedling (right). ProOsAUX1:GUS was transformed into rice WT, and ten positive transgenic lines were observed. Upper panels indicate the proximal zones of root hairs; lower panels indicate the distal zones of root hairs. Scale bars = 100 μm. (e) An OsAUX1-sGFP fusion construct was transiently expressed in tobacco and rice protoplasts either under the control of the CaMV 35 promoter (1st and 3rd rows) or under the control of its own promoter (2nd and 4th rows). Left to right: green fluorescence of OsAUX1-sGFP, bright-field images, red fluorescence of the protoplast membrane marker pm-rb CD3-1008, and merged microscope images. Scale bars = 10 μm.
its paralogous gene, AtAUX1, suggesting functional differences in regulating RH development between monocot and dicot plants.

To better understand the biological function of OsAUX1, we first investigated its subcellular localization. OsAUX1 was localized by transient expression of 35S:OsAUX1:sGFP and ProOsAUX1:OsAUX1-sGFP. Co-localization with the plasma membrane marker pm-rb CD3-1008 (Nelson et al., 2007) in leaf epidermal cells of Nicotiana benthamiana and rice protoplasts (Figure 2c) indicates a plasma membrane location as found for AtAUX1, implying that OsAUX1 and AtAUX1 share transport functionality on the plasma membrane.

**Cadmium stress induces OsAUX1 expression in the PRs, LRs and RHs of rice**

In recent years, scientists have started paying close attention to the relationship between auxin signaling and stress phenomena caused by heavy metals, such as cadmium (Strader and Bartel, 2009; Zhao et al., 2012; Hu et al., 2013; Tamas et al., 2014). To investigate the in vivo function of OsAUX1 in relation to Cd stress, we first tested OsAUX1 expression under CdCl2 treatment using quantitative RT-PCR and ProOsAUX1:GUS promoter analyses. Quantitative RT-PCR showed that OsAUX1 expression, and that of its homologs OsAUX2, OsAUX3, OsAUX4 and OsAUX5, increased two- to fivefold by treatment with CdCl2 for 12 h (Figure 3a). Analyses of ProOsAUX1:GUS and ProOsAUX1:OsAUX1:sGFP rice plants further demonstrated that OsAUX1 expression was distinctly induced in PRs, lateral roots (LRs) and RHs by 50 μM CdCl2 treatment (Figure 3b–e). These results suggest that OsAUX1 functions in Cd stress responses.

**osaux1 mutants are sensitive to Cd stress but such sensitivity is alleviated by a low concentration of NAA**

To understand the molecular mechanism of OsAUX1 responses to Cd stress, phenotypes of WT and osaux1 mutants were observed after treatment with Cd (50 μM CdCl2) and/or auxin (10 nM NAA) for 7 days. We found that the length of PRs in WT was inhibited by 20% by CdCl2 treatment, while that in osaux1 was inhibited by 50% (Figure 4a,c). The number of LRs in WT decreased by 35%, while that in osaux1-1 decreased by 70% (Figure 4b,c) in response to CdCl2 treatment. The lengths of RHs in osaux1-1 were much shorter than in WT under CdCl2 treatment (Figure 4b,c) and the RH length of osaux1-1 was 45% decreased, while those of WT decreased by only 25%. Interestingly, the repression of root growth and development caused by Cd stress was retarded by simultaneous NAA application as indicated by PR length and LR density. Further, RH growth in the osaux1-1 mutant under Cd+NAA treatment was significantly increased compared to Cd treatment alone (Figure 4). Root growth responses to Cd+IAA treatment show similar trends to Cd+NAA treatment (Figure S3). These results imply that the sensitivity to Cd stress may be dependent on local changes in auxin concentrations caused by reduced auxin transport in osaux1-1.

**osaux1-1 shows reduced auxin contents and distribution under control and IAA treatments and under Cd stress**

To confirm that altered auxin contents do indeed have an effect on the sensitivity to Cd stress in rice roots, the auxin reporter DR5-GUS was transformed into WT and osaux1-1. IAA induced DR5 expression in WT and osaux1-1, while
CdCl₂ reduced it compared to controls (Figure 5a). However, DR5-GUS activity in osaux1-1 was always lower than in WT under control, IAA and Cd treatments (Figure 5a,b). In agreement, auxin contents in osaux1-1 were also reduced compared to WT under the conditions described above (Figure 5c).

In order to investigate whether reduced auxin contents were caused by defects in polar auxin transport, we examined acropetal and basipetal transport of [³H]-IAA under control and Cd stress conditions (Figure 5d). Acropetal transport of [³H]-IAA in osaux1-1 was significantly lower than in WT both under control and Cd stress conditions. In contrast, basipetal transport of [³H]-IAA in osaux1-1 was higher than in WT under control conditions, but was strongly decreased under Cd stress. These results coincide with phenotypes for osaux1-1 under Cd stress, and suggest that reduced polar auxin transport in osaux1-1 is the primary cause for decreased auxin contents. Our data also indicate that Cd treatments may negatively regulate polar auxin transport.

On the other hand, when 7-day-old WT seedlings were treated with 50 µM CdCl₂ for 5 days, the PR length was 15% shorter than for plants treated simultaneously with the auxin transport inhibitors 1-naphthoxyacetic acid (NOA) and N-1-naphthylphthalamic acid (NPA) (Figure 5e), indicating that both transport inhibitors significantly reduced the tolerance of WT rice plants to Cd stress. Taken together, these results indicate that decreased auxin contents and distribution are responsible for increased sensitivity toward Cd stress, suggesting that auxin plays an important role as a signaling molecular in response to Cd stress.

OsCyCB1;1 expression in osaux1-1 is decreased under control and IAA treatments and Cd stress

Cell-cycle regulation plays a crucial role in crop plants during Cd stress, as exemplified by the finding that cell cycle-related genes in soybean are induced by Cd treatment (Swarup and Bennett, 2003). To study further how OsAUX1 affects Cd stress, i.e. the relationship among

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auxin, cell-cycle regulation and Cd stress, we transferred the reporter ProCyCB1;1:GUS into WT and osaux1-1. ProCyCB1;1:GUS staining in WT and osaux1-1 was increased by IAA, NAA and Cd treatments compared to control conditions (Figure 6a). However, the staining intensity in osaux1-1 under the above conditions was significantly lower than in WT, suggesting that this is caused by decreased auxin contents in osaux1-1 (Figure 6b). Quantitative RT-PCR analysis of OsCyCB1;1 expression verified the reporter gene data (Figure 6c). These results suggest that OsAUX1 affects the ability of rice plants to respond to Cd stress via OsCyCB1;1. Reduced OsCyCB1;1 expression in osaux1-1 appears to further decrease cell division, and leads to shorter PRs compared to WT.

**Cd contents are not altered in osaux1**

P_{16}^-type heavy-metal ATPases (HMAs) are transmembrane metal-transporting proteins that play a key role in metal homeostasis (Lee et al., 2007). OsHMA3 in yeast transports Cd into the ER and thus increases Cd sensitivity (Ueno et al., 2010). Expression of three OsHMA genes, OsHMA5, OsHMA6 and OsHMA9, was induced by Cd stress, and these were therefore suggested to play roles in heavy-metal detoxification (Lee et al., 2007). We tested the expression of the above genes in WT and osaux1-1 under Cd treatment for the first 3 days after germination. All four genes (but especially OsHMA3) were significantly up-regulated in osaux1-1 compared to the WT under Cd stress.
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DISCUSSION

Auxin signaling has been reported to be involved in activation of Cd-induced morphogenic defense responses in root tips of barley (Hordeum vulgare) (Tamas et al., 2014). Auxin also alleviates Cd toxicity in wheat (Triticum aestivum) and Arabidopsis by enhancing antioxidant defense activities (Agami and Mohamed, 2013; Zhu et al., 2013). Auxin transport pathways may therefore be required for Cd-modulated LR development in Arabidopsis (Hu et al., 2013). However, the relationship between auxin transport and responses to Cd stress in rice is less well investigated. Here, we reveal that the auxin transporter OsAUX1 is implicated in responses to Cd stress.

OsAUX1 negatively regulates PR length but positively regulates RH development

In Arabidopsis, AUX1 functions in regulating LR and RH development (Swarup et al., 2008; Jones et al., 2009). In a recent paper, OsAUX1 was reported to function in regulating LR development as in Table S2 (Zhao et al., 2015). Here we show that three independent osaux1 mutants produce elongated primary roots under normal growth conditions and shorter RHs compared to WT (Figure 1e,f), indicating that OsAUX1 is also involved in PR and RH development. Loss of OsAUX1 function led to increases in PR length, suggesting that OsAUX1 negatively regulates PR length but positively regulates RH development. OsAUX1 is critical for RH development because the shorter RHs in osaux1 mutants were not rescued by treatment with IAA and 2,4-D, whose influx requires auxin importers, but were rescued...
by treatment with NAA, which may enter the cell by free diffusion, thus bypassing importers.

OsAUX1 regulates RH development differently from AUX1 in Arabidopsis

The root epidermis of Arabidopsis includes two cell types: those that form root hairs and those that do not. In Arabidopsis, AUX1 was localized in non-hair epidermal cells, suggesting that auxin transport through non-hair cells sustains root hair development (Jones et al., 2009). AUX1 expression in non-hair cells demonstrates that AUX1 affects the auxin content of both hair and non-hair cells. However, in rice, OsAUX1 was detected in root hair cells but not non-hair cells (Figure 2a), indicating that OsAUX1-mediated RH development is not the same as in Arabidopsis. Arabidopsis has a type 3 striped pattern of root hairs (Kwak and Schiefelbein, 2007; Horn et al., 2009), while rice may show the type 2 pattern, which depends on

Figure 7. Expression of OsHMA genes, localization of Cd and accumulation of Cd in roots of Cd-treated rice plants.

(a) Relative mRNA levels of OsHMA3, OsHMA5, OsHMA6 and OsHMA9 in roots of WT and osaux1-1 after treatment with 50 μM CdCl₂ for 0, 1 or 3 days. OsACTIN was used as an internal control. Quantitative RT-PCR experiments comprised three independent biological repeats. Letters indicate significant differences between means, determined using Duncan’s multiple range mean comparisons (5% α).

(b) Root Cd contents of WT, osaux1-1, -2 and -2. Rice plants were treated with 50 μM CdCl₂ for 5 days. Five biological repeats were used.

(c) Confocal microscopy imaging of Cd in primary roots and lateral roots of WT and osaux1-1 after 50 μM CdCl₂ treatment for 5 days. From left to right: staining using the Cd-specific probe Leadmium™ Green AM, propidium iodide staining, bright-field images and merged images. Scale bars = 100 μm.

(d) Effect of Cd stress on H₂O₂ and O₂⁻, lipid peroxidation (TBARS) and cell death. Accumulation of O₂⁻, H₂O₂ and TBARS was measured in WT and osaux1-1 rice exposed to 50 μM CdCl₂ for 0, 1, 3 and 5 days. Cell death was also assessed for of WT and osaux1-1 exposed to 50 μM CdCl₂ for 0, 1, 3 and 5 days. Samples were analyzed by Evans blue staining. Letters indicate significant differences between means, determined using Duncan’s multiple range mean comparisons (5% α).
asymmetrical cell divisions (Clowes, 2000). Our data on OsAUX1 localization further support the idea that rice RHs belong to type 2. 

AUX1 functions as an auxin influx carrier that is thought to transport charged IAA into the cytoplasm in Arabidopsis (Dharmasiri et al., 2006; Carrier et al., 2008; Peret et al., 2012; Robert and Friml, 2009). OsAUX1 in rice is localized to plasma membranes (Figure 2c), as for AUX1 in Arabidopsis. However, whether OsAUX1 functions as an importer or exporter remains to be confirmed.

Reduced auxin content increases the sensitivity to Cd stress in an action involving OsCyCB1;1

In recent years, evidence has accumulated showing that auxin and Cd stress responses are interconnected. Our data show that OsAUX1 expression in rice roots is induced by Cd stress (Figure 3), indicating that the in vivo function of OsAUX1 may be related to Cd stress responses. A detailed study of the three osaux1 mutants revealed that their PRs, LRs and RHs are more sensitive to Cd compared to WT, but that the inhibitory effect of Cd was alleviated by a low concentration of NAA (Figure 4). Exogenous NAA treatment improves Cd tolerance in osaux1 mutants, suggesting that local auxin gradients provided by OsAUX1 are essential for Cd tolerance in rice. Auxin content and distribution were reduced in osaux1-1 under control, IAA treatment and Cd stress, while application of the auxin transport inhibitors NOA and NPA increased the Cd sensitivity in WT (Figure 5). Taken together, this series of molecular, morphological and biochemical analyses strongly demonstrates that a reduction of auxin content in rice plants increases their sensitivity to Cd stress.

Previous reports had already suggested that auxin signaling and cell-cycle regulation function to regulate plant root development in Arabidopsis, especially under unfavorable conditions (De Smet et al., 2010; Potters et al., 2007; Perez Torres et al., 2009). Our study shows that OsAUX1 also affects OsCyCB1;1 expression under IAA and Cd treatments (Figure 6), suggesting that OsAUX1 and OsCyCB1;1 may play a role during auxin responses, cell-cycle regulation and Cd stress. However, the genes related to cell-cycle regulation include a large family of 44 cyclins, which are known to be functionally redundant and conserved in rice (Guo et al., 2007). Hence, whether other cell-cycle genes are involved in OsAUX1-mediated responses to Cd stress needs to be addressed in further research.

Increased oxidative damage in osaux1 results in increased Cd sensitivity

Cd, which usually enters humans through the food chain via plants or cigarette smoke, is highly toxic to most organisms. Hence, inhibiting Cd transport or fixing Cd within the plant root may be a useful strategy for decreasing the accumulation of Cd in humans (Ueno et al., 2010). For example, OsHMA3 controls Cd over-accumulation in rice through transport of Cd into vacuoles, partially limiting the root-to-shoot Cd translocation in rice plants (Miyadate et al., 2011; Tezuka et al., 2010). On the other hand, Cd stress disturbs auxin homeostasis by affecting its distribution and metabolism in Arabidopsis (Hu et al., 2013). In our study, we show that OsHMA3 and its homologs are significantly up-regulated in osaux1-1 under Cd stress compared to WT (Figure 7d), suggesting that OsAUX1 may negatively regulate expression of OsHMA3 and it homologs, explaining why osaux1-1 is more sensitive to Cd stress. However, we did not detect a difference in Cd content within roots of WT and osaux1-1, indicating that the regulatory mechanism mediated by OsAUX1 in response to Cd stress is intricate.

The accumulation of reactive oxygen species under Cd stress is a common phenomenon, which causes oxidative damage in plants. O$_{2}^{-}$ is the initial reactive oxygen species produced in response to environmental stresses, such as toxic heavy metals, triggering formation of more reactive oxygen species such as OH$^{-}$ and O$_{2}^{\bullet-}$ (Halliwell, 2006). H$_{2}$O$_{2}$ is usually employed as an oxidative stress biomarker and is an important signaling molecule, depending on its cellular concentration. A certain concentration of H$_{2}$O$_{2}$ appears to be necessary to activate H$_{2}$O$_{2}$-dependent signaling pathways, stimulating the expression of H$_{2}$O$_{2}$-responsive genes (Michelet et al., 2013). However, the link between H$_{2}$O$_{2}$ and the auxin signaling pathway in Cd-stressed rice roots is poorly understood. It has been reported that H$_{2}$O$_{2}$ may act as a signal influencing auxin signaling and regulating growth of the root system in plants under Cd stress (Zhao et al., 2012). Upon Cd exposure for 1 day, the H$_{2}$O$_{2}$ concentration was highly increased in WT, and similar results were found in aux1 of Arabidopsis under arsenite treatment (Krisnamurthy and Rathinasabapathi, 2013). Over-production of O$_{2}^{-}$ and H$_{2}$O$_{2}$ is the cause of oxidative stress, and the concentration of O$_{2}^{-}$ and H$_{2}$O$_{2}$ was higher in osaux1 than in WT under Cd treatment. Cd-induced lipid peroxidation and damage to membrane stability are well-known consequences of O$_{2}^{-}$ and H$_{2}$O$_{2}$ generation. Our results indicated that lipid peroxidation and cell integrity were not altered differently in osaux1 compared to the WT under control conditions. However, osaux1-1 revealed a high degree of oxidative damage under Cd treatment for 5 days, implying that OsAUX1 has a positive impact during the response to Cd stress.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Seeds of WT/Dongjin, osaux1-1, osaux1-2 and osaux1-3, and OsAUX1 over-expression lines were germinated and planted in rice nutrient solution as previously described (Wang et al., 2014;
Xu et al. (2014). Phytohormone treatment was performed using 1 μM IAA, 0.1 μM 2,4-D or 0.1 μM NAA; the duration of treatments is indicated in the figure legends.

**Identification of osaux1 mutants**

T-DNA insertion sites in three independent mutants of OsAUX1 [osaux1-1 (3A-51110), osaux1-2 (1A-20543) and osaux1-2 (3A-01770)], were determined by comparison with the SIGnal database at http://signal.salk.edu/cgi-bin/RiceGE. Insertions were confirmed by PCR using OsAUX1-specific and T-DNA border primers (2715LB or Ngus-RB, see Figure 1b and Table S3). OsAUX1 expression in the three osaux1 mutants was analyzed by RT-PCR using the primers shown in Table S3.

**RNA extraction and quantitative RT-PCR**

Total RNA was isolated using a plant RNA extract kit according to the manufacturer’s instructions (Tiangen, http://www.tiangen.com/). Reverse transcriptase-PCR was performed as described previously (Wang et al., 2010, 2014). The primers used are listed in Table S3.

**Construction and transformation of binary vectors**

For the 3SS:OsAUX1 construct, the ORF of OsAUX1 was amplified from cDNA of Dongjin (DJ) using the primers listed in Table S3, and then cloned into the binary vector pCAMBIA1300 (http://www.cambia.org/). For the 35S:OsAUX1:sGFP construct, the ORF of OsAUX1 (without terminator) was cloned into binary vector pCAMBIA1300 containing a 3SS:sGFP cassette. For the ProOsAUX1:OsAUX1-sGFP construct, the CaMV 3SS promoter was replaced by the native OsAUX1 promoter (ProOsAUX1). For the ProOsAUX1:GUS construct, 2 kb upstream of the ATG of OsAUX1 gene were amplified from genomic DNA of WT, and cloned into binary vector pBI101.3 (Kang et al., 2013). All vectors were introduced into Agrobacterium strain EHA105 and transformed into WT rice as described previously (Hei et al., 1994).

**Subcellular localization of OsAUX1**

3SS:OsAUX1:sGFP and ProOsAUX1:OsAUX1: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 prepared from stems of 10-day-old rice seedlings (Yoo et al., 2007). Images were acquired using an LSM710 microscope (Zeiss, http://www.zeiss.com/corporate/en/de/home.html).

**GUS staining and analysis of GUS activity**

GUS staining of WT and osaux1 seedlings transformed with ProOsAUX1:GUS, DR5:GUS (Umasov et al., 1997) and ProOsCyCB1;1:GUS was performed as described previously (Jefferson et al., 1987). After staining, the tissues were soaked in 70% ethanol to remove chlorophyll and surface dyes. Quantification of GUS activity was performed as described by Jefferson et al. (1987) by monitoring cleavage of the GUS substrate 4-methylumbelliferyl-β-D-glucuronide (Sigma, http://www.sigmaaldrich.com/china-mainland.html). GUS-stained roots were imaged using a Nikon 16 Eclipse 80i microscope (Nikon Corporation, http://www.nikon-instruments.com.cn/index.html).

**Analysis of IAA concentrations and polar auxin transport**

For measurement of IAA concentrations, 20 mg of roots of 7-day-old WT and the osaux1-1 mutant were washed several times with deionized water, and samples were ground into a fine powder under liquid nitrogen. The extraction of IAA was performed as described by Wang et al. (2014). IAA purification and quantification were performed as described by Ljung et al. (2005) using a FOCUS GC-DSQII gas chromatograph (Thermo Fisher Scientific, http://www.thermofisher.com/en/home.html). Analyses of polar H1-IAA transport in rice roots (1 cm segments from the tip) was performed using a 1450 MicroBeta TriLux liquid scintillation counter (Perkin-Elmer, http://www.perkinelmer.com/) as described previously (Qi et al., 2008).

**Cd localization and quantification**

The distribution of Cd in WT and osaux1-1 after 50 μM CdCl2 treatment for 5 days was visualized using the Cd-specific probe Lead-ium™ Green AM (Invitrogen, http://www.lifetechnologies.com/cn/zh/home/brands/invitrogen.html) according to the manufacturer’s instructions. Twenty PRs of WT or osaux1-1 were immersed in the dye solution at room temperature for 3 h in the dark, and then washed with 0.85% NaCl. Roots were stained with propidium iodide for 10 min, washed with 0.85% NaCl for three times at room temperature, and micrographs were obtained by confocal microscopy.

For measurement of Cd concentrations, harvested roots (after treatment with or without 50 μM CdCl2 treatment for 5 days) were washed first in distilled water and then in 0.01 mM EDTA solution, and dried at 80°C until the materials reached constant weights. Then 0.1 g of each sample was digested with HNO3/H2O2 at 110°C for 0.5 h using a microwave digestor (Anton Paar, http://www.anton-paar.com/cn/cn/). The samples were then dissolved in deionized water to 25 ml of constant volume for analysis of Cd content using an OPTIMA 8000DV inductively coupled plasma optical emission spectrometer (Perkin Elmer). Five biological replicates were performed for each sample in all experiments.

**Quantification of H2O2, O2, and TBARS and cell death detection**

Root H2O2 levels were measured as described by Jana and Choudhuri (1981). Root samples (0.1 g) were homogenized in 6 ml phosphate buffer (50 mmol/l, pH 6.5). The absorbance at 410 nm was read using a spectrophotometer (UV-2550, Shimadzu, http://www.shimadzu.com.cn/). Superoxide anion radicals were measured by the hydroxylamine oxidation method (Elstner and Heupel, 1976). The absorbance was measured at 520 nm using the above-mentioned spectrophotometer. Lipid peroxidation was quantified on the basis of TBARS, which represent malondialdehyde and other end products of lipid peroxidation, as described by Hodges et al. (1999). The distal 2 cm of the root tips were excised from 40 roots and stained in 0.025% w/v Evans blue solution for 20 min at room temperature, and then washed with double distilled H2O to remove excessive dye. Root tips were treated with 50% methanol and 1% SDS for 1 h at 50°C, and the absorbance at 600 nm was quantified as described by Baker and Mock (1994).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Root hair length in WT and osaux1-1 under various treatments.
Figure S2. Expression pattern of OsAUX1.
Figure S3. Phenotype of WT and osaux1 under Cd and IAA co-treatment.
Table S1. PR lengths of 7-day-old seedlings of WT, osaux1 mutants and OsAUX1 over-expression lines under various treatments.
Table S2. LR number of 7-day-old seedling of WT, osaux1 mutants and OsAUX1 over-expression lines under various treatments.
Table S3. Primers used in this study.

REFERENCES


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