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The role of the kinase OXI1 in cadmium- and copper-induced molecular responses in *Arabidopsis thaliana*

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ABSTRACT

The hypothesis that mitogen-activated protein kinase (MAPK) signalling is important in plant defences against metal stress has become accepted in recent years. To test the role of oxidative signal-inducible kinase (OXI1) in metal-induced oxidative signalling, the responses of *oxi1* knockout lines to environmentally realistic cadmium (Cd) and copper (Cu) concentrations were compared with those of wild-type plants. A relationship between OXI1 and the activation of lipoxygenases and other initiators of oxylipin production was observed under these stress conditions, suggesting that lipoxygenase-1 may be a downstream component of OXI1 signalling. Metal-specific differences in OXI1 action were observed. For example, OXI1 was required for the up-regulation of antioxidative defences such as catalase in leaves and Fe-superoxide dismutase in roots, following exposure to Cu, processes that may involve the MEKK1-MKK2-WRKY25 cascade. Moreover, the induction of Cu/Zn superoxide dismutases in Cu-exposed leaves was regulated by OXI1 in a manner that involves fluctuations in the expression of miRNA398. These observations contrast markedly with the responses to Cd exposure, which also involves OXI1-independent pathways but rather involves changes in components mediating intracellular communication.

Key-words: heavy metals; oxidative stress; signalling.

INTRODUCTION

A central role for mitogen-activated protein kinases (MAPKs) in the cellular defence against metal toxicity was originally revealed in studies on yeast cells and animals (Koga *et al*. 2000). Subsequent research also reported a strong activation of several MAPK components under both cadmium (Cd) and copper (Cu) toxicity in plants (Yeh, Hung & Huang 2003; Jonak, Nakagami & Hirt 2004; Yeh, Chien & Huang 2007; Liu *et al*. 2010; Opdenakker *et al*. 2012). In spite of different experimental set-ups and test species, all these experiments concluded distinctly regulated MAPK activation because of the different chemical properties of Cd and Cu.

Reactive oxygen species (ROS) are hypothesized to be key modulators of the MAPK (in)activation (Mittler 2002; Mishra, Tuteja & Tuteja 2006). A major role for NADPH oxidases in the Cd-induced ROS production and corresponding MAPK induction is often suggested (Rodriguez-Serrano *et al*. 2006; Yeh *et al*. 2007; Remans *et al*. 2010; Cuypers *et al*. 2012). In addition, the functional state of mitochondria modulates MAPK activities under Cd stress (Yeh *et al*. 2007; Keunen *et al*. 2011). A direct Cu-induced ROS production, due to the redox-active properties of this element, is a possible underlying mechanism of the activation of the MAPK pathway under Cu stress (Desikan *et al*. 2001; Rentel *et al*. 2004).

Oxidative signal-inducible kinase (OXI1) is known to play a central role in ROS sensing during cellular processes such as root hair growth (Anthony *et al*. 2004) and stress defence (Rentel *et al*. 2004). Both gene expression and activity of OXI1 are strongly induced by hydrogen peroxide (H_2O_2) (Rentel *et al*. 2004), which emphasizes the importance of OXI1 in connecting oxidative burst signals and downstream responses. Moreover, this kinase also operates as an intermediate between redox and lipid signalling, via phosphatidic acid (PA) in a phosphoinositide-dependent kinase (PDK1)-related manner (Anthony *et al*. 2004). Downstream targets of OXI1 include MPK3 and MPK6, because H_2O_2-triggered activation of these kinases was reduced in *oxi1* mutants (Rentel *et al*. 2004).

In the present study, the involvement of MAPK signalling and its correlation with the cellular redox status during metal stress was investigated in roots and leaves of *Arabidopsis thaliana*. Because of the strongly increased *OXI1* transcript levels during Cd and Cu exposure (Opdenakker *et al*. 2012), we hypothesize a central signalling function for this enzyme in both cases. Therefore, both redox and signalling signatures (Nakagami, Pitzschke & Hirt 2005; Sunkar, Kapoor & Zhu 2006; Yang, Xue & An 2007; Dugas & Bartel 2008) were compared between wild-type plants and mutants lacking OXI1 (*oxi1*, Rentel *et al*. 2004) to reveal any metal-specific involvement of OXI1 in previously unravelled oxidative stress-related parameters (Cuypers *et al*. 2011).

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MATERIAL AND METHODS

Plant material and treatment

A. thaliana oxi1 knockout mutants (Rentel *et al.* 2004) and wild-type plants, both ecotype Wassilewskija, were grown in a hydroponic set-up, as described previously (Smeets *et al.* 2008b). Three-week-old seedlings were exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ via the roots. The Cd concentrations are similar to those commonly found in pore water of contaminated soils (Krznaric *et al.* 2009). After 24 h of exposure, the entire aerial part as well as the root system were separated and snap frozen in liquid nitrogen prior to biological measurements. One sample, mentioned as one biological replicate, contained at least one individual plant, depending on the required sample weight. The number of biological replicates for each measurement (within an experiment) was sampled out of more than one pot, to correct for coincidental variation. The number of biological replicates is specified in the corresponding table and/or figure legends. In order to verify the results, parameters were repeatedly measured in different experiments. All samples were analysed within 2 months.

Element analysis

Roots and leaves were dried at 80 °C and digested in HNO_3 (70–71%) using a heat block. Metal concentrations in the extracts were determined by inductively coupled plasma–atomic emission spectrometry. Blanks (only HNO_3) and a certified reference sample [NIST Spinach (1570a)] were analysed for control purposes. Roots were washed twice for 10 min at 4 °C with 10 mM $Pb(NO_3)_2$ and deionized water to exchange surface-bound elements (Cuypers, Vangronsveld & Clijsters 2000). Leaves were washed twice with deionized water.

H_2O_2 measurements

H_2O_2 levels were determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies, Carslbad, CA, USA) in roots and leaves. This is a one-step fluorimetric method that uses 10-acetyl-3,7-dihydroxyphenoxazine to detect H_2O_2. A triplicate of 20 mg root or leaf samples was processed for each exposure concentration and measured together with a H_2O_2 standard curve. The fluorescence emission spectrum (590 nm) was determined at an excitation wavelength of 530 nm using a Photon Technology International QuantaMaster Model QM-6/2005 spectrofluorometer (Photon Technology International, Birmingham, NJ, USA) equipped with FeliX32 software (Photon Technology International) and BryteBox interface (Photon Technology International).

Determination of lipid peroxidation

Thiobarbituric acid (TBA)-reactive compounds in roots and leaves were measured spectrophotometrically to estimate the amount of lipid peroxidation. Plant tissue (200 mg) was homogenized with 3 mL 0.1% trichloroacetic acid (TCA) buffer, and 4 mL 0.5% TBA was added to the extract. After the extract was heated at 95 °C for 30 min, immediately cooled in an ice-bath and centrifuged for 10 min at 4 °C and 20.000 *g*, the absorbance of the supernatant was measured at 532 nm and corrected for unspecific absorbance at 600 nm.

Gene expression analysis

Frozen tissue (approximately 100 mg) was ground thoroughly under frozen conditions using two stainless steel beads (2 mm diameter) in each sample in the Retsch Mixer Mill MM2000 (Retsch, Haan, Germany). RNA and miRNAs were extracted from the disrupted tissue using the miRVANA total RNA isolation procedure (Life Technologies). The concentration of the (mi)RNA was determined spectrophotometrically at 260 nm (NanoDrop Technologies, Wilmington, DE, USA). The (mi)RNA purity was checked based on 260/280 and 260/230 ratios, which were all above 1.8. (mi)RNA integrity was checked and approved based on Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) patterns.

The RNA sample was briefly incubated in gDNA wipe-out buffer at 42 °C for 2 min to effectively remove contaminating gDNA. First-strand cDNA synthesis was primed with a combination of oligo(dT) primers and random hexamers according to the manufacturer's instructions using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Equal amounts of starting material (total RNA) were used (1 μg). Quantitative PCR was performed with the ABI Prism 7000 (Life Technologies), SYBR green chemistry. Primers were designed and optimized using Primer Express (Life Technologies) and the genes with primer sequences are represented in Table 1. PCR amplifications were performed in a total volume of 20 μL, containing 4 μL cDNA sample (10X diluted), 10 μL Power SYBR green Master Mix (Life Technologies), 0.6 μL primers (10 μM) and 5.4 μL RNase-free H_2O. All samples were tested in duplicate for the reference genes as well as for the genes of interest.

Specificity of the primers was checked *in silico* using TAIR BLAST (http://www.arabidopsis.org) and after qPCR by verifying peaks on the melting curve. The amplification efficiencies of all primer sets were investigated by a twofold serial dilution over six dilution points and were in between 85 and 115% ($r^2 > 0.98$). Gene expression data were calculated relative to multiple reference genes: *actin2 (ACT2)*, *At2g28390*, *At4g26410, At5g15710* and *At5g80290*, as described previously by Remans *et al.* (2008) (Vandesompele *et al.* 2002; Czechowski *et al.* 2005).

The miRNA samples were processed using the miRNA reverse transcription kit and the TaqMan MicroRNA Assays from Applied Biosystems (Life Technologies). miRNA expression was normalized to the reference miRNA172, after checking its stability.

Statistical analyses

The datasets were analysed using linear mixed-effects models (Verbeke and Molenberghs 2000). Transformations were applied when necessary to approximate normality. Both

Table 1. Primer sequences and amplicon information

Gene (accession nr)	Forward primer	Reverse primer	Amplicon length	Intron-spanning (Y/N)
LOX1 (At1g55020)	TTGGCTAAGGCTTTTGTCGG	GTGGCAATCACAAACGGTTC	101	Y
LOX2 (At3g45140)	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC	102	Y
RBOHC (At5g51060)	TCACCAGAGACTGGCACAATAAA	GATGCTCGACCTGAATGCTC	101	Y
RBOHD (At5g47910)	TATGCATCGGAGAGGCTGCT	TAGAGACAACACGTTCCCGGG	96	Y
RBOHE (At1g19230)	GTGATGCAAGATCAACCCTGA	GCCTTGCAAAATGTGTTCTCA	105	Y
RBOHF (At1g64060)	GGTGTCATGAACGAAGTTGCA	AATGAGAGCAGAACGAGCATCA	99	Y
CSD1 (At1g08830)	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC	102	Y
CSD2 (At2g28190)	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC	101	Y
FSD1 (At4g25100)	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC	101	Y
CAT1 (At1g20630)	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA	103	Y
APX1 (At1g07890)	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA	101	Y
APX2 (At1g07890)	GAGATGTGTTTGGTCGGATGG	CTCGAATCCTGAACGCTCC	101	Y
GST2 (At4g02520)	ATCACCAGTTCGACCCAGTG	CTCCTCTTCTGCAACAACGG	101	N
ANP1 (At1g09000)	AAGAGAGGACACTGCTCGTGG	TTGCGTCTGTTGCTCTTGAAG	101	N
ANP2 (At1g54960)	GGTGACTGGAAAAGCTCCTTG	TTGTCAGGGATTGGAGGATG	96	Y
MEKK1 (At4g08500)	TGAGATATCGTGGCACAGCC	CCCGAAGCTGGTATCTTTGG	105	Y
MKK2 (At4g29810)	GGATCCAAACAGTCGAAGCTC	TGCATCTGTGAAGTAGGACGC	103	Y
MPK3 (At3g45640)	GACGTTTGACCCCAACAGAA	TGGCTTTTGACAGATTGGCTC	103	Y
MPK4 (At4g01370)	ACATGTCGGCTGGTGCAGT	AATATGGGTGGCACAACGC	96	Y
MPK6 (At2g43790)	TAAGTTCCCGACAGTGCATCC	GATGGGCCAATGCGTCTAA	101	Y
CDPK1 (At1g18890)	CAAAGCTGGGCTTCAGAAGG	AAACCCATTTCCATCGACATC	91	Y
SOS3 (At5g24270)	CGCTTCTTCACGAATCCGA	TCGTTTTTGCGGTCTGCTT	91	Y
SOS2 (At5g35410)	ATTATCTTCGATCAAGGCCGG	GTTTCACCAGCAGCCTTTCTT	102	Y
WRKY22 (At4g01250)	AAACCCATCAAAGGTTCACCA	GGGTCGGATCTATTTCGCTC	101	Y
WRKY25 (At2g30250)	GAAAGATCCGCAGCAGACG	TCCCAATAATTTCACGAGCG	101	N
WRKY29 (At4g23550)	CATGGGCGTGGCGTAAATA	TTGTTTTCTTGCCAAACACCC	104	Y
ACT2 (At3G18780)	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT	68	N
At2g28390	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC	61	Y
At4g26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC	81	Y
At5g08290	TTACTGTTTCGGTTGTTCTCCATTT	CACTGAATCATGTTCGAAGCAAGT	61	N
OXI1 (At3g25250)	CGATTATTGTCCGGGACAGA	CTAATACAAGCTCCGCCGC	104	Y

ACT, actine; ANP, NPK1-related protein kinase; APX, ascorbate peroxidase; CAT, catalase; CDPK, calcium-dependent protein kinase; CSD, copper zinc superoxide dismutase; FSD, iron superoxide dismutase; GST, glutathione-S-transferase; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; MEKK, MAPkinase kinase kinase; MKK, MAPkinase kinase; RBOH, respiratory burst oxidase homolog; SOS, salt overly sensitive protein (CBL-interacting protein kinase); WRKY, DNA-binding protein (transcription factor).

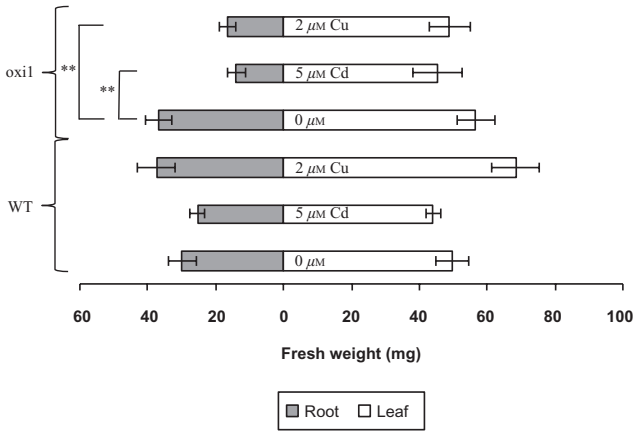

Figure 1. Leaf and root weights (mg plant^{-1}) of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ over a 24 h period or grown under control conditions. Values are mean ± SE of ± 20 biologically independent replicates (significance levels: $^{**}P < 0.05$). WT, wild type.

overall effects (effect of the treatment, effect of the genotype and an interaction effect of treatment and genotype) as well as differences between groups are discussed. The statistical analysis was performed using the MIXED procedure in SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Growth parameters and elemental profile

In wild-type plants, no significant effects on root weight were detected between the conditions tested. However, the roots of *oxi1* mutants exposed to 5 μM Cd and 2 μM Cu showed a significant weight reduction as compared with the unexposed plants. In the leaves of both wild type and *oxi1* mutants, no significant effects on weight were noticed (Fig. 1).

Elemental profiles were determined, but no significant differences in Cd accumulation were observed when comparing wild-type plants and *oxi1* mutants after metal exposure (results not shown). The Cd content increased significantly in both roots and leaves of all Cd-exposed plants (Cd contents of about 3500 mg kg^{-1} and 600 mg kg^{-1} in roots and leaves, respectively). On the other hand, when comparing Cu contents, genotype differences were observed. In roots, Cu exposure led to increased Cu levels in both genotypes, with a higher increase in wild types as compared with the *oxi1* mutants (475 mg kg^{-1} and 303 mg kg^{-1}, respectively, Fig. 2a). The Cu concentration in the leaves of Cu-exposed *oxi1* mutants did not change, whereas an increase was observed in wild-type plants (Fig. 2b).

Level of pro-oxidants

In the roots of wild-type plants, the H_2O_2 content increased significantly after Cd exposure, whereas Cu exposure did not affect the H_2O_2 level (Fig. 3a). Cd exposure had no effect on the H_2O_2 content in the roots of *oxi1* mutants, whereas an exposure to 2 μM Cu resulted in a significant H_2O_2 decrease as compared with the unexposed *oxi1* mutants (Fig. 3a).

Increased H_2O_2 levels were observed in leaves of Cd-exposed wild types and *oxi1* mutants (Fig. 3b), whereas no significant differences were observed in the leaf H_2O_2 content of Cu-exposed plants (Fig. 3b). Apparently, the effect of Cu exposure depended on the genotype, and a significant interaction effect (treatment–genotype: $P = 0.02$) was detected in the leaves.

Lipid peroxidation was investigated by determining tissue levels of TBA-reactive metabolites (TBArm) (Fig. 4). In contrast to what we found in other ecotypes (Cuypers *et al.* 2011), no significant differences were observed between untreated and metal-exposed plants neither in roots nor in leaves.

Gene expression levels of ROS-producing enzymes

In the roots of Cd-exposed plants, significant increases in *LOX1* (lipoxygenase) and *RBOHD* (respiratory burst

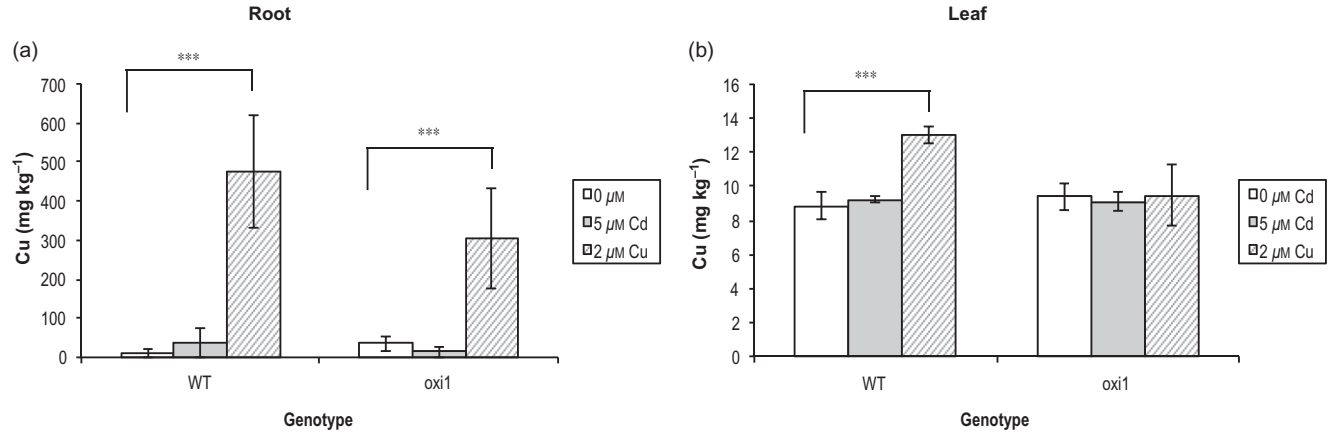

Figure 2. Cu content (mg kg^{-1} DW^{-1}) in roots (a) and leaves (b) of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ over a 24 h period or grown under control conditions. Values are mean ± SE of six biologically independent replicates (significance levels: $^{***}P < 0.01$). DW, dry weight; WT, wild type.

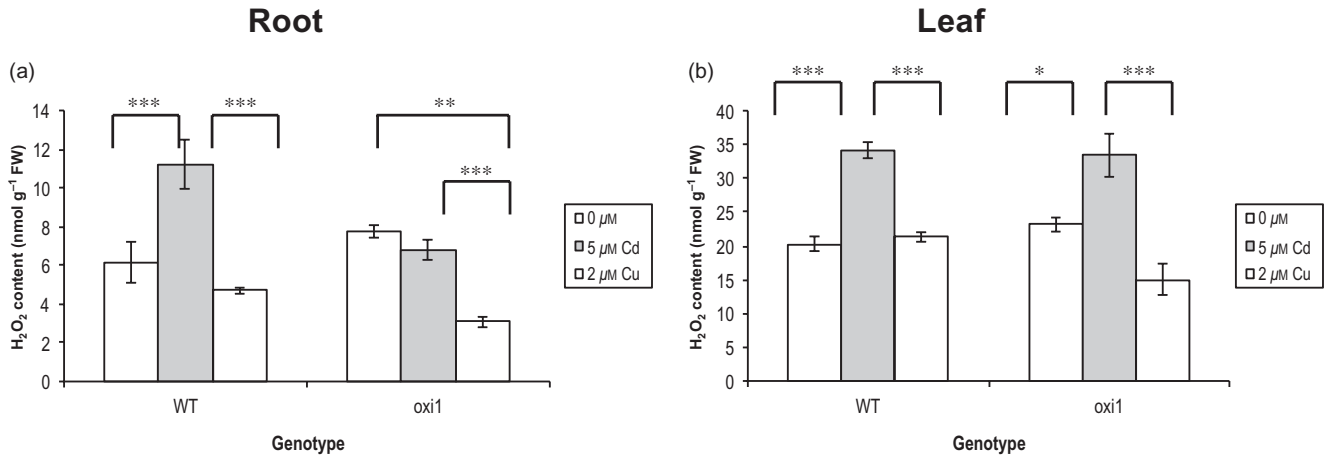

Figure 3. H_2O_2 content (nmol g^{-1} FW) in roots (a) and leaves (b) of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ over a 24 h period or grown under control conditions. Values are mean ± SE of three biologically independent replicates (significance levels:*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$). FW, fresh weight; WT, wild type.

oxidase homolog) expression were observed for both genotypes, as compared with the unexposed plants (Table 2). Transcript levels of both genes were also enhanced under Cu stress. A genotypic difference in *LOX2* expression was observed, as it was significantly higher in the unexposed *oxi1* mutants. Whether OXI1 is involved in the regulation of specific components during metal stress can be detected via genotype–treatment interaction effects. This was observed in the roots of *oxi1* mutants where *LOX1* gene transcripts were less induced after exposure to Cd and Cu as compared with the wild type (Table 2) and where an obvious genotype–treatment interaction effect ($P < 0.05$) was noticed in both conditions.

Cd exposure significantly induced *LOX2* and *RBOHC* expression in the leaves of wild-type plants, whereas this induction was less pronounced or even absent in leaves of *oxi1* mutants (Table 3). In addition, *RBOHE* transcript levels were up-regulated in leaves of both genotypes when exposed to Cd (Table 3). No significant changes were observed in the expression level of the ROS-producing genes in the leaves of both genotypes exposed to Cu. A genotype–treatment interaction effect, however, was observed for *RBOHD* expression under Cu stress with opposing trends in wild-type plants and *oxi1* mutants (Table 3).

Gene expression levels of ROS-scavenging enzymes

Transcript levels of several antioxidative genes were significantly altered in roots of metal-exposed plants (Table 2). *CAT1* (catalase), *APX2* (ascorbate peroxidase) and *GST2* (glutathione-S-transferase) expressions were significantly increased for both exposures. Metal-specific effects were observed in superoxide dismutase (SOD) expression.

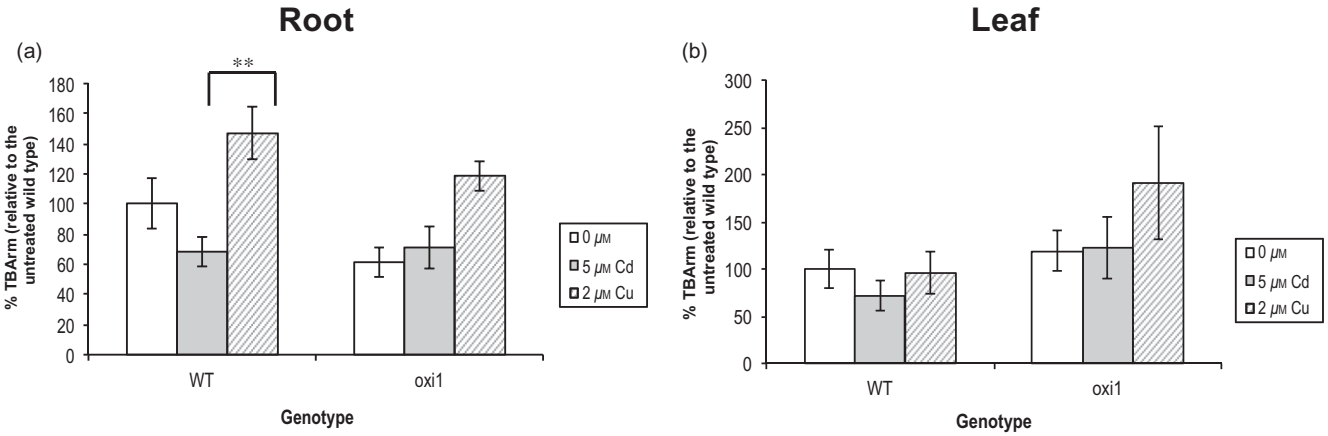

Figure 4. Lipid peroxidation measurement was based on the amount of thiobarbituric acid-reactive metabolites (TBArm) and was analysed in roots (a) and leaves (b) of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ over a 24 h period or grown under control conditions (= 100%). Values are mean ± SE of three (roots) or four (leaves) biologically independent replicates (significance levels:*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$). WT, wild type.

Table 2. Transcript levels of reactive oxygen species (ROS)-producing enzymes, antioxidative enzymes and regulatory proteins, expressed in the roots of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ during 24 h

	UNTREATED		CADMIUM			COPPER		
ROOT	WT	*oxi1*	WT	*oxi1*	Interaction	WT	*oxi1*	Interaction
LOX1	100 ± 11	110 ± 21	**1741 ± 563**	**757 ± 238**	***P* < 0.05**	**3489 ± 898**	**1568 ± 553**	***P* < 0.05**
LOX2	100 ± 37	224 ± 51	170 ± 75	87 ± 49	NO	277 ± 55	291 ± 53	NO
RBOHC	100 ± 08	79 ± 15	88 ± 36	108 ± 59	NO	27 ± 08	107 ± 79	NO
RBOHD	100 ± 20	137 ± 20	402 ± 181	378 ± 165	NO	326 ± 52	213 ± 50	NO
RBOHE	100 ± 17	98 ± 19	187 ± 43	250 ± 69	NO	149 ± 07	124 ± 09	NO
RBOHF	100 ± 15	87 ± 16	154 ± 49	215 ± 77	NO	100 ± 17	109 ± 30	NO
CSD1	100 ± 20	93 ± 10	103 ± 15	100 ± 20	NO	269 ± 48	199 ± 26	NO
CSD2	100 ± 13	102 ± 35	85 ± 13	116 ± 22	NO	156 ± 19	97 ± 09	NO
FSD1	100 ± 32	48 ± 19	2775 ± 667	4476 ± 976	NO	**7 ± 03**	**30 ± 07**	***P* < 0.05**
CAT1	100 ± 10	159 ± 43	1105 ± 199	854 ± 210	NO	**3230 ± 40**	**915 ± 360**	***P* < 0.05**
APX1	100 ± 8	89 ± 6	101 ± 36	93 ± 25	NO	100 ± 16	85 ± 10	NO
APX2	100 ± 11	66 ± 9	1035 ± 308	934 ± 186	NO	254 ± 72	261 ± 59	NO
GST2	100 ± 20	197 ± 80	3957 ± 1260	1100 ± 230	NO	2811 ± 166	2589 ± 567	NO
OXI1	100 ± 20	–	280 ± 80	–	NO	453 ± 37	–	NO
ANP1	100 ± 09	75 ± 09	157 ± 31	119 ± 18	NO	**121 ± 14**	**91 ± 08**	***P* < 0.05**
ANP2	100 ± 07	84 ± 08	153 ± 64	341 ± 188	NO	198 ± 21	191 ± 21	NO
MEKK1	100 ± 13	112 ± 05	268 ± 136	221 ± 85	NO	**182 ± 03**	**107 ± 09**	***P* < 0.05**
MKK2	100 ± 12	104 ± 13	295 ± 53	399 ± 122	NO	265 ± 28	181 ± 17	NO
MPK3	100 ± 25	129 ± 31	152 ± 12	183 ± 38	NO	**163 ± 21**	79 ± 08	***P* < 0.1**
MPK4	100 ± 08	99 ± 19	224 ± 36	170 ± 26	NO	113 ± 02	96 ± 10	NO
MPK6	100 ± 07	98 ± 10	139 ± 43	89 ± 18	NO	144 ± 38	79 ± 04	NO
CDPK1	100 ± 11	106 ± 21	249 ± 48	274 ± 84	NO	131 ± 21	122 ± 23	NO
SOS3	100 ± 17	87 ± 18	375 ± 81	559 ± 130	NO	312 ± 44	230 ± 41	NO
SOS2	100 ± 18	135 ± 08	175 ± 22	109 ± 26	NO	**173 ± 26**	**79 ± 26**	***P* < 0.05**
SOS1	100 ± 20	102 ± 08	2 ± 1	5 ± 4	NO	230 ± 182	75 ± 10	NO
WRKY22	100 ± 16	120 ± 16	50 ± 18	40 ± 6	NO	347 ± 23	167 ± 17	NO
WRKY25	100 ± 12	129 ± 10	289 ± 28	1937 ± 156	NO	**156 ± 18**	**61 ± 14**	***P* < 0.01**
WRKY29	100 ± 05	91 ± 09	308 ± 104	214 ± 102	NO	208 ± 21	182 ± 60	NO

Significant up- or downregulation: $P < 0.1$; $P < 0.05$; $P < 0.01$.

Genotype effects in unexposed conditions are represented in the first and second column (significant differences between wild type and *oxi1* mutant = coloured). Treatment effects (between unexposed and exposed plants within one genotype) are represented in the first columns of each treatment, values are relative to the unexposed genotype (=100%). Each point represents the mean of 4 or 5 biological replicates ± SE (significance levels: indicated below table). Genotype*treatment interaction effects are indicated in the last column of each treatment, and genes with specific interaction effects are highlighted (**bold**). Significant interaction effects are treatments effects that differ between the different genotypes.

ROS-producing enzymes: LOX, lipoxygenase; RBOH, respiratory burst oxidase homolog.

Antioxidative enzymes: APX, ascorbate peroxidise; CAT, catalase; CSD, copper zinc superoxide dismutase, FSD, iron superoxide dismutase; GST, glutathione-S-transferase.

Regulatory proteins: CDPK, calcium-dependent protein kinase; MAPK, mitogen-activated protein kinase; MEKK, MAPkinase kinase kinase; MKK, MAPkinase kinase; NIG, NaCl-inducible gene; RBOH, respiratory burst oxidase homolog; SOS, salt overly sensitive protein (CBL-interacting protein kinase); WRKY, DNA-binding protein (transcription factor).

Whereas *FSD1* (Fe SOD) was up-regulated in case of Cd stress, Cu exposure significantly decreased *FSD1* transcription. *CSD1* (Cu/Zn SOD) expression was significantly elevated during Cu stress, albeit only in the wild-type plants. The expression of some of these genes was also genotype-dependent under control conditions. In *oxi1* plants, *FSD1* was significantly down-regulated, whereas *CAT1* was up-regulated as compared with the wild type. For these genes, genotype–treatment interaction effects were also observed during Cu stress.

In leaves, only *GST2* was significantly up-regulated upon both metal exposures (Table 3). A down-regulation of *CSD2* was observed in leaves of Cd-exposed plants, whereas in case of Cu-exposed *oxi1* plants, *CSD1* gene transcripts were significantly increased. On the other hand, *FSD1* expression was significantly down-regulated in the leaves of Cu-exposed plants. No genotype–treatment interaction effects were observed.

Gene expression levels of signal transduction components

In the roots of Cd-exposed plants, expression of genes related to the MAPK pathway showed limited changes (Table 2). Only the *MPK4* transcript level was significantly increased in the roots of Cd-exposed wild-type plants. Cu, on the other hand, significantly induced the transcription level of several MAPK genes, although this induction was

Table 3. Transcript levels of reactive oxygen species (ROS)-producing enzymes, antioxidative enzymes and regulatory proteins, expressed in the leaves of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ or 2 μM $CuSO_4$ during 24 h

	UNTREATED		CADMIUM			COPPER		
LEAF	Ws	*oxi1*	Ws	*oxi1*	Interaction	Ws	*oxi1*	Interaction
LOX1	100 ± 14	144 ± 25	133 ± 35	84 ± 06	NO	99 ± 18	106 ± 18	NO
LOX2	100 ± 13	184 ± 34	214 ± 31	122 ± 19	NO	205 ± 62	152 ± 37	NO
RBOHC	100 ± 27	185 ± 91	3266 ± 1618	1815 ± 1437	NO	344 ± 231	82 ± 08	NO
RBOHD	100 ± 12	208 ± 43	86 ± 14	89 ± 12	NO	**196 ± 29**	**78 ± 04**	***P* < 0.05**
RBOHE	100 ± 22	184 ± 67	527 ± 103	1110 ± 349	NO	126 ± 23	89 ± 14	NO
RBOHF	100 ± 19	153 ± 50	185 ± 56	92 ± 30	NO	71 ± 07	146 ± 28	NO
CSD1	100 ± 18	109 ± 18	44 ± 11	94 ± 36	NO	329 ± 26	265 ± 64	NO
CSD2	100 ± 23	85 ± 21	35 ± 06	46 ± 17	NO	140 ± 06	173 ± 49	NO
FSD1	100 ± 13	87 ± 17	254 ± 45	108 ± 40	NO	4 ± 02	39 ± 15	NO
CAT1	100 ± 09	111 ± 14	155 ± 37	132 ± 22	NO	117 ± 12	120 ± 03	NO
APX1	100 ± 13	100 ± 24	115 ± 38	274 ± 108	NO	105 ± 08	150 ± 10	NO
APX2	100 ± 20	112 ± 21	157 ± 30	124 ± 30	NO	202 ± 45	184 ± 44	NO
GST2	100 ± 06	1152 ± 56	3730 ± 1273	2032 ± 1141	NO	373 ± 69	294 ± 20	NO
OXI1	100 ± 24	–	3035 ± 1841	–	NO	164 ± 33	–	NO
ANP1	100 ± 14	73 ± 14	93 ± 08	101 ± 9	NO	**102 ± 11**	**160 ± 23**	***P* < 0.05**
ANP2	100 ± 11	95 ± 06	241 ± 89	121 ± 43	NO	75 ± 10	164 ± 24	NO
MEKK1	100 ± 13	123 ± 12	152 ± 29	154 ± 22	NO	113 ± 20	95 ± 06	NO
MKK2	100 ± 06	114 ± 14	301 ± 61	483 ± 1841	NO	149 ± 19	127 ± 09	NO
MPK3	100 ± 13	155 ± 19	162 ± 27	288 ± 53	NO	213 ± 25	119 ± 18	NO
MPK4	100 ± 08	98 ± 10	227 ± 29	175 ± 32	NO	130 ± 13	115 ± 07	NO
MPK6	100 ± 07	100 ± 06	104 ± 16	110 ± 21	NO	113 ± 17	135 ± 08	NO
CDPK1	100 ± 18	169 ± 29	223 ± 75	242 ± 60	NO	374 ± 244	71 ± 05	NO
SOS3	100 ± 14	117 ± 21	166 ± 24	197 ± 91	NO	114 ± 12	113 ± 08	NO
SOS2	100 ± 11	117 ± 25	**109 ± 26**	**49 ± 11**	***P* < 0.05**	113 ± 11	113 ± 07	NO
SOS1	100 ± 19	158 ± 24	**262 ± 46**	**95 ± 13**	***P* < 0.05**	135 ± 19	179 ± 28	NO
WRKY22	100 ± 25	103 ± 12	55 ± 11	131 ± 37	NO	246 ± 37	185 ± 39	NO
WRKY25	100 ± 13	161 ± 26	1119 ± 281	680 ± 267	NO	168 ± 37	267 ± 52	NO
WRKY29	100 ± 25	71 ± 16	84 ± 22	91 ± 25	NO	80 ± 09	96 ± 18	NO

Significant up- or downregulation: $P < 0.1$; $P < 0.05$; $P < 0.01$.

Genotype effects in unexposed conditions are represented in the first and second column (significant differences between wild type and *oxi1* mutant = coloured). Treatment effects (between unexposed and exposed plants within one genotype) are represented in the first columns of each treatment, values are relative to the unexposed genotype (=100%). Each point represents the mean of 4 or 5 biological replicates ± SE (significance levels: indicated below table). Genotype*treatment interaction effects are indicated in the last column of each treatment, and genes with specific interaction effects are highlighted (**bold**). Significant interaction effects are treatments effects that differ between the different genotypes.

ROS-producing enzymes: LOX, lipoxygenase; RBOH, respiratory burst oxidase homolog.

Antioxidative enzymes: APX, ascorbate peroxidise; CAT, catalase; CSD, copper zinc superoxide dismutase; FSD, iron superoxide dismutase; GST, glutathione-S-transferase.

Regulatory proteins: ARG, argonaute; CDPK, calcium-dependent protein kinase; MAPK, mitogen-activated protein kinase; MEKK, MAPkinase kinase kinase; MKK, MAPkinase kinase; NIG, NaCl-inducible gene; RBOH, respiratory burst oxidase homolog; SOS, salt overly sensitive protein (CBL-interacting protein kinase); WRKY, DNA-binding protein (transcription factor).

less significant in the *oxi1* mutants. Moreover, an opposite trend was observed for *MPK3* and *MPK6* gene transcripts in the *oxi1* mutants when compared with the Cu-exposed wild-type plants.

The Ca^{2+}-dependent signalling components CDPK1 and SOS3 were transcriptionally up-regulated in roots of both Cd-exposed genotypes as compared with the unexposed plants (Table 2). *SOS1*, however, was strongly reduced during Cd stress. Cu significantly induced *SOS3* expression in both genotypes.

Transcript levels of the WRKY transcription factors were affected by both metal exposures. On one hand, the WRKY25 transcript level was up-regulated in roots of both metal-exposed genotypes. The transcript level of *WRKY22*, on the other hand, was reduced in Cd-exposed plants and up-regulated in Cu-exposed plants. These effects were only significant in the *oxi1* mutants. Only in the wild-type plants, WRKY29 expression was significantly up-regulated during Cu stress. Whereas no genotype–treatment interaction was detected in roots of Cd-exposed plants, this was the case for *ANP1, MPK3, SOS2* and *WRKY25* in roots of Cu-exposed plants.

Cd exposure significantly enhanced *MKK2* and *WRKY25* expression in the leaves of both genotypes (Table 3). *MPK4* and *SOS1* expressions were only induced in the leaves of the Cd-exposed wild-type plants, whereas *MPK3* transcript levels were significantly induced in the leaves of Cd-exposed *oxi1* mutants. Genotype–treatment interactions were noticed

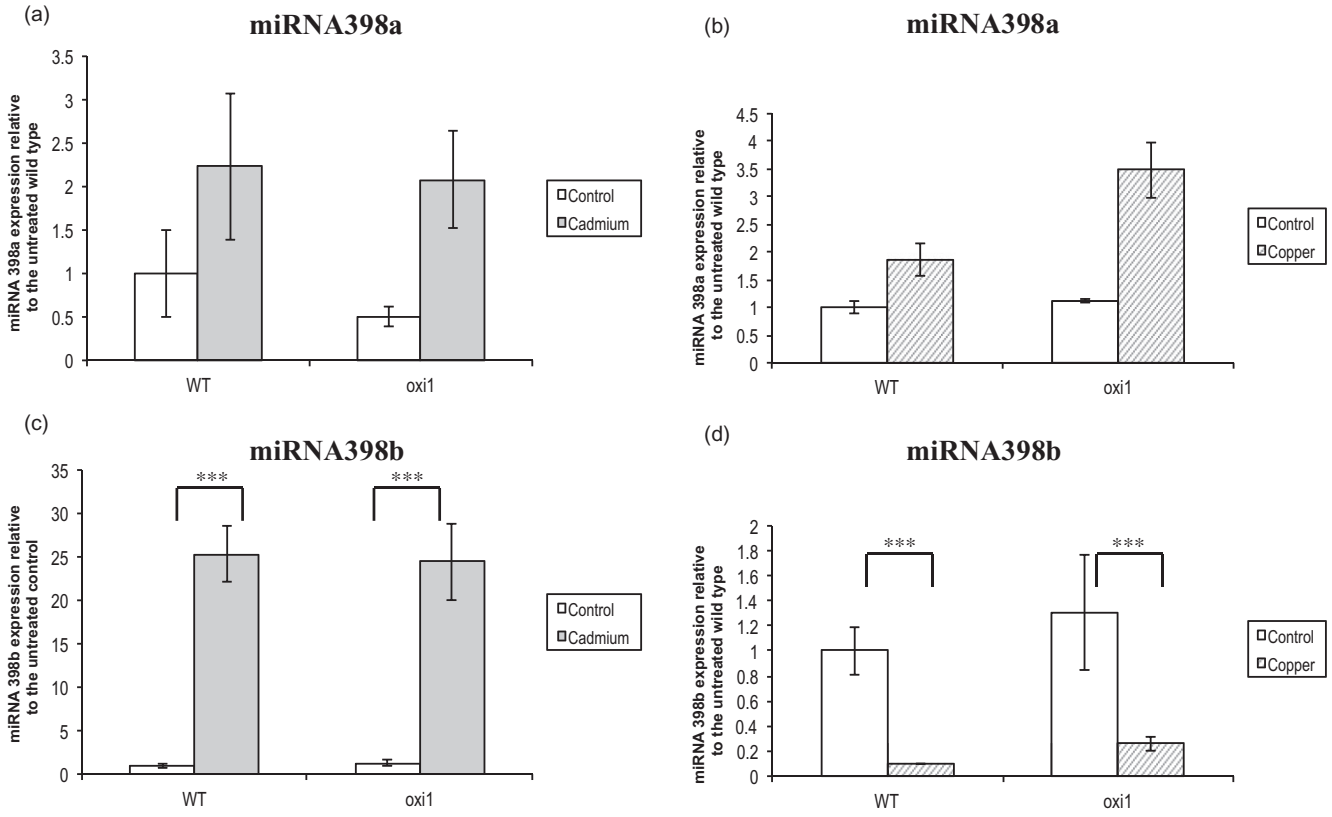

Figure 5. The expression level of miRNA398a (a + b) and miRNA398b (c + d) in the roots of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ (a + c) or 2 μM $CuSO_4$ (b + d) over a 24 h period or grown under control conditions (= 1). Values are mean ± SE of four biologically independent replicates (significance levels:*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$). WT, wild type.

for *SOS1/2* under Cd exposure. In the leaves of Cu-exposed plants, only *WRKY22* expression was significantly induced in both genotypes. In the leaves of Cu-exposed *oxi1* mutants, a genotype–treatment interaction effect was detected for the kinase ANP1 (Table 3).

miRNA expression profiles

In the roots of Cd-exposed plants, miRNA398b expression was significantly higher for both genotypes (Fig. 5c). In the roots of both genotypes exposed to Cu, the expression of this miRNA398b was significantly down-regulated (Fig. 5d). No statistically significant effects were observed on the expression of miRNA398a (Fig. 5a,b).

In the leaves of both genotypes, Cd significantly up-regulated miRNA398a expression (Fig. 6a). miRNA398b level, on the other hand, was only significantly induced in the leaves of Cd-exposed *oxi1* mutants (genotype–treatment interaction: $P = 0.0094$) (Fig. 6c). In leaves of Cu-exposed wild-type plants, miRNA398b levels were significantly reduced (Fig. 6d), whereas in leaves of Cu-exposed *oxi1* mutants, no significant changes in the expression levels of these miRNAs were detected. A genotype–treatment interaction, however, was noticed for miRNA398a ($P = 0.08$) (Fig. 6b,d).

DISCUSSION

Comparison between Cd- and Cu-induced redox and signal transduction signatures

Both Cd and Cu affect the cellular redox homeostasis in roots and leaves of *A. thaliana* (Fig. 7) (Cuypers *et al.* 2000; Smeets *et al.* 2008a, 2009; Cuypers, Smeets & Vangronsveld 2009). The levels of pro- and antioxidants and their subcellular localization characterize a redox signature. Our results show a significant increase of H_2O_2 amounts during Cd exposure in both roots and leaves, possibly via NADPH oxidases localized in the plasma membrane (originating from the conversion of superoxide) (Fig. 3, Tables 2 & 3). Cu exposure also induces NADPH oxidase expression (Table 2) (Remans *et al.* 2010; Cuypers *et al.* 2011), but can reduce H_2O_2 directly via the Fenton reaction. This explains the relatively stable H_2O_2 production during Cu exposure as compared with control plants (Fig. 3). In addition, the increased amount of TBArm in Cu-exposed roots, indicating lipid peroxidation, can be due to Cu-induced production of hydroxyl radicals (°OH). Alternatively, this can be a result of the oxygenation of fatty acids via lipoxygenases (LOXes).

Organisms respond to a disturbed redox status by altering their antioxidative defence system in order to reach a new cellular equilibrium. An antioxidative reaction is provoked at

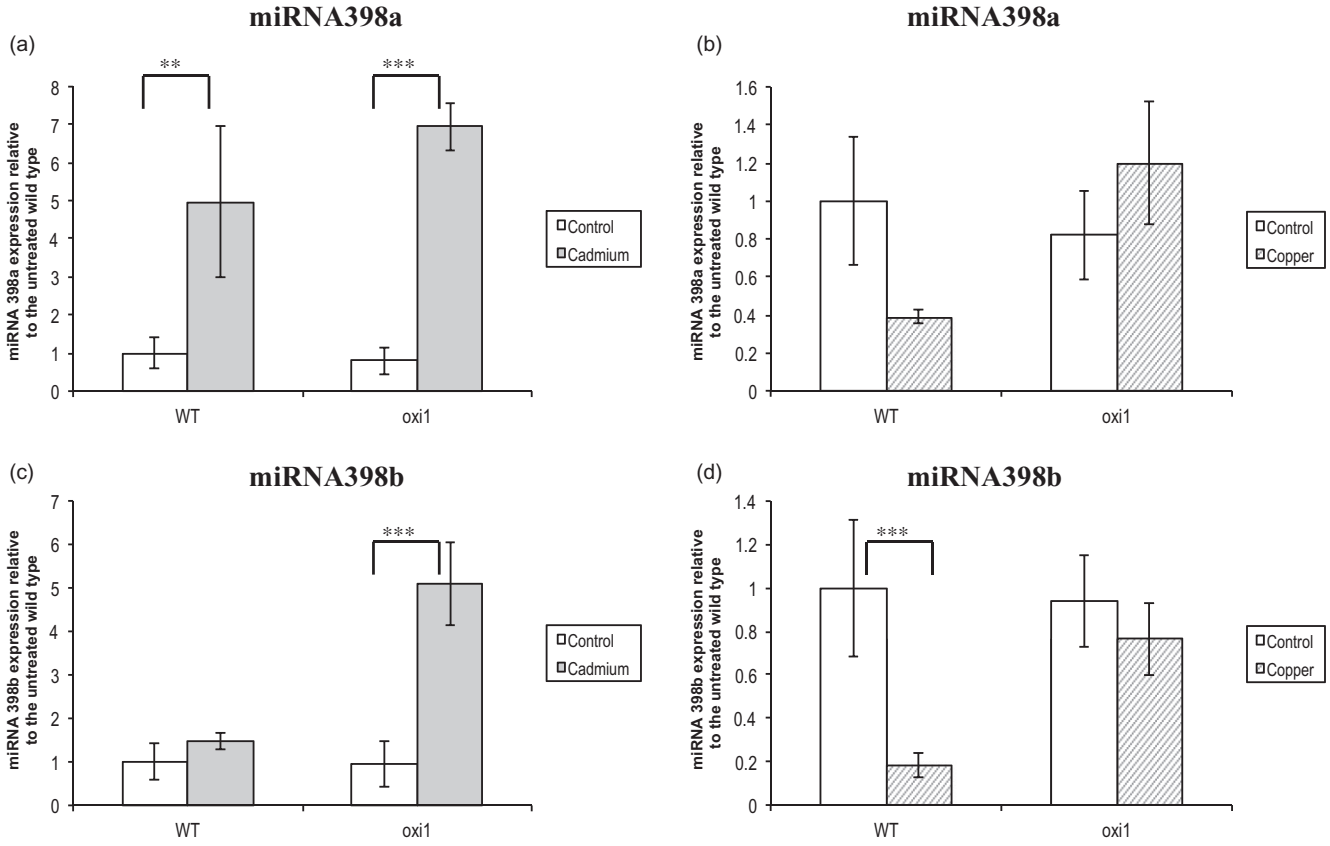

Figure 6. The expression level of miRNA398a (a + b) and miRNA398b (c + d) in the leaves of 3-week-old *Arabidopsis thaliana* genotypes exposed to 5 μM $CdSO_4$ (a + c) or 2 μM $CuSO_4$ (b + d) over a 24 h period or grown under control conditions (= 1). Values are mean ± SE of four biologically independent replicates (significance levels:*$P < 0.1$; **$P < 0.05$; ***$P < 0.01$). WT, wild type.

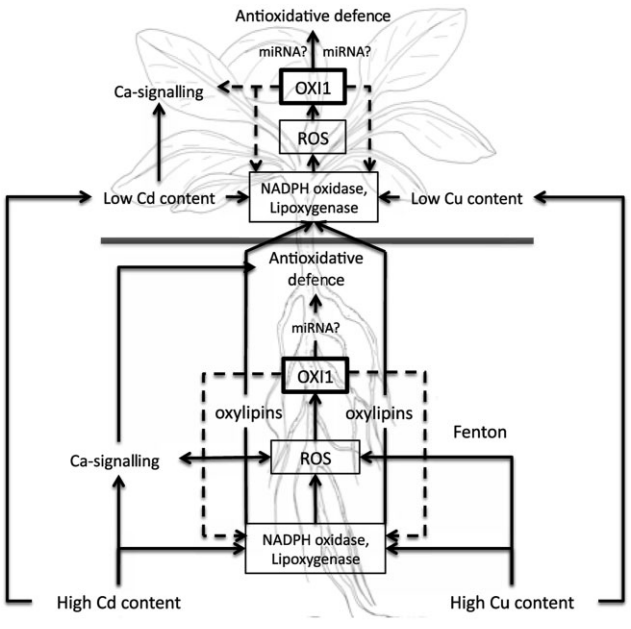

Figure 7. The role of OXI1 in the dynamic process of cellular interactions during metal stress.

multiple biological levels, as shown for Cd and Cu stress in previous studies (Smeets *et al*. 2008a, 2009; Cuypers *et al*. 2011). Corresponding to this work and that of others (Ding & Zhu 2009), the most important metal-specific differences in the regulation of antioxidative defence were related to the SOD. The responses were opposite for the gene transcripts of *FSD1* versus *CSD2* and its corresponding miRNAs (Table 2, Fig. 5).

Depending on the specificities of the stress situation, a different redox signature leads to different cellular and physiological responses (Smeets *et al*. 2009). This can be caused by the activation and interplay of signalling networks (Jonak *et al*. 2004; Yeh *et al*. 2007; Thevenod 2009; Cuypers *et al*. 2011). As such, Ca-responding cascades were most strongly induced in roots of Cd-exposed plants (Tables 2 & 3), an effect that can be explained by the chemical resemblance between Cd and Ca (Garnier *et al*. 2006; Rodriguez-Serrano *et al*. 2009). In addition, concerning the MAPK signalling pathway, a clear differentiation is obvious between Cd- and Cu-exposed seedlings (Tables 2 & 3). At the protein level, Jonak *et al*. (2004) already reported an induction of distinct MAPK pathways because of excess amounts of Cd and Cu. At the transcript level, *MPK4* and its corresponding transcription factor *WRKY25* were strongly up-regulated in both

roots and leaves during Cd exposure (Tables 2 & 3). An alternative MAPK pathway is stimulated in Cu-exposed plants and was mainly restricted to the roots (Tables 2 & 3). A similar response to both metals, however, was observed for OXI1 expression (Tables 2 & 3), which was highly induced. OXI1 is known as a key link between oxidative burst signals (such as H_2O_2) and downstream responses (Rentel *et al*. 2004). Therefore, its specific role in metal stress was further elucidated via the use of *oxi1* null mutants. An overview of these responses is presented in Fig. 7, showing a working model for Cd/Cu phytotoxicity in which the redox signature is interconnected to signalling pathways with special attention for OXI1 herein.

OXI1 as a principal component during Cu stress

OXI1 signalling seems most important at the place of metal exposure, that is, the roots. Previous studies have shown that *oxi1* mutants are impaired in the activation of the MAPKs MPK3 and MPK6 upon oxidative stress (Rentel *et al*. 2004). Complementary to these findings, our data show decreased transcript levels of these and other MAPKs in the roots of Cu-exposed mutants, suggesting a substantial contribution of OXI1 in Cu-induced responses (Fig. 7, Table 2). We hypothesize that in roots OXI1 regulates downstream transcription factors such as WRKY25 via the MEKK1-MKK2 cascade during Cu stress; these interactions were also described in other stress situations (Pitzschke *et al*. 2009). In addition, OXI1-independent pathways are regulating downstream responses in Cd-exposed plants. Ca^{2+}-signalling pathways remain activated during Cd stress in the roots of *oxi1* mutants, or other MAPK cascades are able to bypass OXI1 signalling functions during Cd stress (Table 2). As a result, *WRKY25* expression remains strongly up-regulated, even though *MPK4* expression was lower in the Cd-exposed *oxi1* mutants as compared with wild-type plants (Table 2).

OXI1 is not only activated upon redox changes (Anthony *et al*. 2006), but also has a strong influence on redox characteristics themselves (Fig. 7) (Mittler *et al*. 2004; Rentel *et al*. 2004). As such, H_2O_2 concentrations were lower in the roots of metal-exposed *oxi1* mutants as compared with exposed wild-type plants (Fig. 3). Whether NADPH oxidases play a role in the H_2O_2 production downstream of OXI1 cannot be concluded from our results (Table 2). However, based on significant genotype effects and genotype–treatment interactions (Fig. 7, Table 2), lipoxygenases (LOXes) were identified as possible downstream targets of OXI1. No genotype differences were observed in lipid peroxidation amounts, and we presume that metal-induced LOXes function in inter- and intracellular signalling via the production of oxylipins (Fig. 7).

Concerning enzymes involved in antioxidative defence, transcript levels of *FSD1* and *CAT1* were significantly altered after OXI1 knockout in roots of unexposed plants (Table 2). Genotype–treatment interactions, that were only significant for Cu, suggest that both *FSD1* and *CAT1* are regulated via the OXI1 pathway in normal circumstances as well as during metal stress.

In leaves, OXI1 dependence was less obvious, although the redox signature was influenced via a few specific pro-oxidative and antioxidative responses. Like in roots, LOX transcription seems regulated by OXI1, since both *LOX1* and *LOX2* are up-regulated after *OXI1* knockout in the untreated mutants. *LOX2* expression was influenced by the genotype in the Cd-exposed plants as well (Table 3). These data confirm our earlier hypothesis of LOXes as a potential downstream target of OXI1, which is supported by Anthony *et al*. (2006), since they already demonstrated a key role for OXI1 in the complex network of lipid signalling.

No direct link was observed between OXI1 and downstream antioxidative defence in the leaves (Table 3), but miRNA398 expression was strongly altered in metal-exposed *oxi-1* mutants as compared with wild-type plants (Fig. 6). miRNA398 expression is already known to be redox-sensitive, but our data show an involvement of OXI1 in the miRNA-related fine-tuning of the redox signature during metal stress (Fig. 7). As a result, metal-specific effects are obvious on both cellular as well as physiological (Figs 1 & 2) level.

In conclusion, our results indicate a highly important role for OXI1 in the cellular signalling during Cu-induced toxicity in the roots of *A. thaliana*. As a result, Cu-exposed *oxi* mutants respond differently to redox-induced changes. Cd-exposed plants rely on other signalling pathways, among which Ca-based cascades. Overall, H_2O_2 and LOXes, but especially miRNA398, were revealed as potential downstream targets of OXI1 under both stress conditions. As limited differences in the composition of a redox signature are often the underlying cause of significant stress-specific responses, it is of great importance to further elucidate these strong fluctuations in miRNA expression as well as their upstream and downstream targets.

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REFERENCES

Anthony R.G., Henriques R., Helfer A., Meszaros T., Rios G., Testerink C., Munnik T., Deak M., Koncz C. & Bogre L. (2004) A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in Arabidopsis. *EMBO Journal* **23,** 572–581.

Anthony R.G., Khan S., Costa J., Pais M.S. & Bogre L. (2006) The Arabidopsis protein kinase PTI1-2 is activated by convergent phosphatidic acid and oxidative stress signaling pathways downstream of PDK1 and OXI1. *Journal of Biological Chemistry* **281,** 37536–37546.

Cuypers A., Vangronsveld J. & Clijsters H. (2000) Biphasic effect of copper on the ascorbate-glutathione pathway in primary leaves of Phaseolus vulgaris seedlings during the early stages of metal assimilation. *Physiologia Plantarum* **110,** 512–517.

Cuypers A., Smeets K. & Vangronsveld J. (2009) Heavy metal stress in plants. In *Plant Stress Biology* (ed. H. Hirt), pp. 161–178. WILEY-VCH Verlag GmbH & Co, Weinheim, Germany.

Cuypers A., Smeets K., Ruytinx J., *et al*. (2011) Oxidative stress as a modulator in Cd and Cu toxicity in *Arabidopsis thaliana* seedlings. *Journal of Plant Physiology* **168,** 309–316.

Cuypers A., Keunen E., Bohler S., *et al*. (2012) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling. In *Metal Toxicity in Plants: Perception, Signalling and Remediation* (eds D.K.G. Gupta & L.M. Sandalio), pp. 65–90. Springer-Verlag GmbH, Berlin, Heidelberg, Germany.

Czechowski T., Stitt M., Altmann T., Udvardi M.K. & Scheible W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* **139,** 5–17.

Desikan R., Mackerness S.A.H., Hancock J.T. & Neill S.J. (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. *Plant Physiology* **127,** 159–172.

Ding Y.F. & Zhu C. (2009) The role of microRNAs in copper and cadmium homeostasis. *Biochemical and Biophysical Research Communications* **386,** 6–10.

Dugas D.V. & Bartel B. (2008) Sucrose induction of Arabidopsis miR398 represses two Cu/Zn superoxide dismutases. *Plant Molecular Biology* **67,** 403–417.

Garnier L., Simon-Plas F., Thuleau P., Agnel J.P., Blein J.P., Ranjeva R. & Montillet J.L. (2006) Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant, Cell & Environment* **29,** 1956–1969.

Jonak C., Nakagami H. & Hirt H. (2004) Heavy metal stress: activation of distinct mitogen-activated protein kinase pathways by copper and cadmium. *Plant Physiology* **136,** 3276–3283.

Keunen E., Remans T., Bohler S., Vangronsveld J. & Cuypers A. (2011) Metal-induced oxidative stress and plant mitochondria. *International Journal of Molecular Sciences* **12,** 6894–6914.

Koga M., Zwaal R., Guan K.L., Avery L. & Ohshima Y. (2000) A Caenorhabditis elegans MAP kinase kinase, MEK-1, is involved in stress responses. *EMBO Journal* **19,** 5148–5156.

Krznaric E., Verbruggen N., Wevers J.H.L., Carleer R., Vangronsveld J. & Colpaert J.V. (2009) Cd-tolerant Suillus luteus: a fungal insurance for pines exposed to Cd. *Environmental Pollution* **157,** 1581–1588.

Liu X.M., Kim K.E., Kim K.C., *et al*. (2010) Cadmium activates *Arabidopsis* MPK3 and MPK6 via accumulation of reactive oxygen species. *Phytochemistry* **71,** 614–618.

Mishra N.S., Tuteja R. & Tuteja N. (2006) Signaling through MAPK pathways in plants. *Archives of Biochemistry and Biophysics* **452,** 55–68.

Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7,** 405–410.

Mittler R., Vanderauwera S., Gollery M. & Van Breusegem F. (2004) Reactive oxygen gene network of plants. *Trends in Plant Science* **9,** 490–498.

Nakagami H., Pitzschke A. & Hirt H. (2005) Emerging MAP kinase pathways in plant stress signalling. *Trends in Plant Science* **10,** 339–346.

Opdenakker K., Remans T., Keunen E., Vangronsveld J. & Cuypers A. (2012) Exposure of Arabidopsis thaliana to Cd or Cu excess leads to oxidative stress mediated alterations in MAPKinase transcript levels. *Environmental and Experimental Botany* **83,** 53–61.

Pitzschke A., Djamei A., Bitton F. & Hirt H. (2009) A major role of the MEKK1-MKK1/2-MPK4 pathway in ROS signalling. *Molecular Plant* **2,** 120–137.

Remans T., Smeets K., Opdenakker K., Mathijsen D., Vangronsveld J. & Cuypers A. (2008) Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. *Planta* **227,** 1343–1349.

Remans T., Opdenakker K., Smeets K., Mathijsen D., Vangronsveld J. & Cuypers A. (2010) Metal-specific and NADPH oxidase dependent changes in lipoxygenase and NADPH oxidase gene expression in *Arabidopsis thaliana* to cadmium or excess copper. *Functional Plant Biology* **6,** 532–544.

Rentel M.C., Lecourieux D., Ouaked F., *et al*. (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature* **427,** 858–861.

Rodriguez-Serrano M., Romero-Puertas M.C., Zabalza A., Corpas F.J., Gomez M., Del Rio L.A. & Sandalio L.M. (2006) Cadmium effect on oxidative metabolism of pea (Pisum sativum L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. *Plant, Cell & Environment* **29,** 1532–1544.

Rodriguez-Serrano M., Romero-Puertas M.C., Pazmino D.M., Testillano P.S., Risueno M.C., del Rio L.A. & Sandalio L.M. (2009) Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. *Plant Physiology* **150,** 229–243.

Smeets K., Ruytinx J., Semane B., Van Belleghem F., Remans T., Van Sanden S., Vangronsveld J. & Cuypers A. (2008a) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environmental and Experimental Botany* **63,** 1–8.

Smeets K., Ruytinx J., Van Belleghem F., Semane B., Lin D., Vangronsveld J. & Cuypers A. (2008b) Critical evaluation and statistical validation of a hydroponic culture system for *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* **46,** 212–218.

Smeets K., Opdenakker K., Remans T., Van Sanden S., Van Belleghem F., Semane B., Horemans N., Guisez Y., Vangronsveld J. & Cuypers A. (2009) Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. *Journal of Plant Physiology* **166,** 1982–1992.

Sunkar R., Kapoor A. & Zhu J.K. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. *The Plant Cell* **18,** 2051–2065.

Thevenod F. (2009) Cadmium and cellular signaling cascades: to be or not to be? *Toxicology and Applied Pharmacology* **238,** 221–239.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. & Speleman F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3,** Research0034.

Verbeke G. & Molenberghs G. (2000) *Linear Mixed Models for Longitudinal Data*. Springer Verlag, New York, NY, USA.

Yang T.W., Xue L.G. & An L.Z. (2007) Functional diversity of miRNA in plants. *Plant Science* **172,** 423–432.

Yeh C.M., Hung W.C. & Huang H.J. (2003) Copper treatment activates mitogen-activated protein kinase signalling in rice. *Physiologia Plantarum* **119,** 392–399.

Yeh C.M., Chien P.S. & Huang H.J. (2007) Distinct signalling pathways for induction of MAP kinase activities by cadmium and copper in rice roots. *Journal of Experimental Botany* **58,** 659–671.

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