Nitric oxide contributes to copper tolerance by influencing ROS metabolism in Arabidopsis

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ORIGINAL PAPER

Nitric oxide contributes to copper tolerance by influencing ROS metabolism in *Arabidopsis*

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Abstract
Key message **Nitric oxide improves copper tolerance via modulation of superoxide and hydrogen peroxide levels. This reflects the necessity of a well-coordinated interplay between NO and ROS during stress tolerance.**
Abstract Copper (Cu) excess causes toxicity and one probable consequence of this is the disturbance of cell redox state maintenance, inter alia, by reactive oxygen- (ROS) and nitrogen species (RNS). The objective of this paper was to examine the role of nitric oxide (NO) in Cu stress tolerance and its relationship with ROS in *Arabidopsis*. In agar-grown seedlings, concentration-dependent Cu accumulation was observed. The 5 μM Cu resulted in reduced cell viability in the NO overproducing *nox1* and *gsnor1-3* root tips compared to the wild-type (WT). In contrast, 25 and 50 μM Cu caused higher viability in these mutants, while in the NO-lacking *nia1nia2* lower viability was detected than in the WT. The exogenous NO donor enhanced cell viability and scavenging endogenous NO decreased it in Cu-exposed WT seedlings. Besides, SNP in *nia1nia2* roots led to the improvement of viability. The ascorbic acid-deficient mutants (*vtc2-1*, *vtc2-3*) possessing slightly elevated ROS levels proved to be Cu sensitive, while *miox4* showing decreased ROS production was more tolerant to Cu than the WT. In *nox1* and *gsnor1-3,* Cu did not induce superoxide formation, and H_2O_2 accumulation occurred only in the case of NO deficiency. Based on these, under mild stress NO intensifies cell injury, while in the case of severe Cu excess it contributes to better viability. ROS were found to be responsible for aggravation of Cu-induced damage. NO alleviates acute Cu stress via modulation of $O_2^{\cdot-}$ and H_2O_2 levels reflecting the necessity of a well-coordinated interplay between NO and ROS during stress tolerance.

Keywords *Arabidopsis thaliana* L. · Copper stress tolerance · Nitric oxide · Reactive oxygen species

Introduction

Copper (Cu) is a vital trace element for normal root and shoot development; however, its excess can be toxic for most plants causing symptoms such as chlorosis and necrosis, stunting and serious growth inhibition (Reichman 2002). The reasons for Cu toxicity can be its binding to sulfhydryl groups in proteins, thus inhibiting protein functions, induction of nutrient deficiencies, impaired cell transport processes and disturbance in cell redox homeostasis (Yruela 2009). The redox status of plant cells is maintained by redox-active compounds (such as reactive oxygen- and nitrogen species and ascorbic acid), their oxidation/reduction states and interactions (Potters et al. 2010). Being a transition metal ion Cu catalyse, the Fenton and Haber–Weiss reactions, thereby directly inducing the production of different reactive oxygen species (ROS). The excessive amount of ROS is responsible for the damage to DNA, lipids and proteins, thus a tightly regulated ROS level is important for normal cell functions (Opdenakker et al. 2012). According to Opdenakker et al. (2012), hydrogen peroxide (H_2O_2) can be produced by the dismutation of superoxide anion ($O_2^{\cdot-}$) formed by NADPH

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oxidases or via the Fenton reactions in roots of Cu-treated *Arabidopsis*. Besides, hydrogen peroxide can act as a signal molecule inducing OXI1-MAPK cascade leading to the regulation of gene expression or it can cause oxidative damage to the membrane lipids through the formation of the highly toxic hydroxyl radical (OH·). Nitric oxide (NO) is a redox-active reactive nitrogen species (RNS) playing a role in normal plant development and also in stress acclimation processes. It has three redox forms (NO radical, nitrosonium cation and nitroxyl anion), which can be rapidly converted to each other in biological systems. Heavy metals, such as Cu, influence NO levels in plant organs. In general, a short-term heavy metal treatment induces a rapid NO burst and a long treatment directly or indirectly decreases NO generation. Although it has to be mentioned that different heavy metal concentrations, duration of treatment and plant age also determine the effect of the metal on NO production (Xiong et al. 2010). Chemical properties of RNS enable them to act as a signal molecule in abiotic stress responses. For example, in favourable conditions, peroxynitrite can regulate cell signalling by the modification of lipids and/or proteins through tyrosine nitration or by interfering with phosphorylation cascades (Vandelle and Delledonne 2011). Reactive nitrogen species also induce the reversible posttranslational modification of thiol-containing proteins by *S*-nitrosylation leading to changes in enzyme activity during signalling processes (Astier et al. 2012). In fact, NO can have pro- or antioxidative effect during heavy metal stress depending on its actual concentration. The antioxidant activities of NO involve its ability to maintain the cellular redox balance and regulate ROS levels, thus controlling their toxicity. Nitric oxide is able to scavenge the superoxide anion in a chemical reaction leading to peroxynitrite ($ONOO^-$) formation as it was observed in the roots of heavy metal-treated *Lupinus luteus* (Kopyra and Gwózdz 2003). This reaction is a key element of ROS and RNS interplay, which determines the different cell signalling pathways through the regulation of the steady-state levels of ROS and RNS (Molassiotis and Fotopoulos 2011). However, peroxynitrite is a strong oxidant in the animal systems, but it seems to be non-toxic for plant cells even at higher concentrations (Delledonne et al. 2001). Furthermore, NO is able to regulate superoxide production by the inhibition of NADPH oxidases via *S*-nitrosylation (Yun et al. 2011). Nitric oxide also induces the activity and expression of antioxidant enzymes such as Cu/zinc superoxide dismutase (Cu/Zn SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and the metallothionein synthesis resulting decreased ROS production and alleviation of Cu toxicity (Hu et al. 2007; Wang et al. 2010).

The relationship between the reactive oxygen and nitrogen species in heavy metal stress tolerance has been investigated mainly using biochemical methods; therefore, in this study beyond the biochemical level the metabolism of reactive molecules was examined also genetically in the root system of Cu-treated WT and mutant *Arabidopsis thaliana* seedlings.

Materials and methods

Plant material and growth conditions

During the experiments, seven-day-old wild-type (WT) and mutant *Arabidopsis thaliana L.* seedlings were used. *Nox1 (cue1)* is an NO overproducing mutant having higher L-arginine, L-citrulline and NO contents as compared to the WT. *CUE1* is the chlorophyll a/b binding protein under expressed 1 gene that encodes the phosphoenolpyruvate/phosphate translocator in the plastid inner envelope (He et al. 2004). The *S*-nitrosoglutathione reductase (GSNOR) activity of *gsnor1-3 Arabidopsis* plants is reduced by 80 % compared to WT and this mutant has higher total *S*-nitrosothiol, nitrate and NO levels (Feechan et al. 2005; Rustérucci et al. 2007; Lee et al. 2008). In *Arabidopsis*, nitrate reductase enzyme is encoded by the NIA1 and NIA2 genes. The *nia1nia2* is a nitrate reductase-deficient mutant, with a point mutation in NIA1 and a deletion in NIA2 gene, having only 0.5 % of the NR enzyme activity of the WT (Wilkinson and Crawford 1993). The mutant also possesses lower arginine and nitrite levels (Modolo et al. 2006). *Arabidopsis* plants with low (*vtc2-1* and *vtc 2-3*) or high (*miox4*) ascorbic acid contents were also used. The *vtc2-1* plants contain 25–30 % of WT ascorbic acid (Conklin et al. 2000) and *vtc2-3* shows 40–50 % of the WT AsA level (Conklin 2001). The *myo*-inositol oxygenase (*MIOX4)* gene is overexpressed in *miox4* transgenic *Arabidopsis* resulting 2–3-fold ascorbic acid accumulation in the leaves (Lorence et al. 2004). All *Arabidopsis* lines were of the ecotype Columbia (Col) background. The seeds of all plant lines were surface sterilised with 5 % (v/v) sodium hypochlorite and transferred to half-strength Murashige and Skoog medium (1 % (w/v) sucrose and 0.8 % (w/v) agar) supplemented with 0, 5, 25 or 50 μM $CuSO_4$. The Petri dishes were kept in a greenhouse at a photo flux density of 150 μmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55–60 % and 25 ± 2 °C. As an NO scavenger, 50 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid potassium salt (cPTIO) was used. Also, sodium nitroprusside (SNP) as an NO donor was applied at a concentration of 10 μM. These chemicals were added to the nutrient media before the seeds were planted.

Element analysis by inductively coupled plasma mass spectrometry (ICP-MS)

Fourteen-day-old WT *Arabidopsis* plants were exposed to different Cu concentrations (0, 5, 25 and 50 μM $CuSO_4$), then roots and shoots were separated and washed with distilled water. Approximately 1,000 seedlings were used for a treatment; each measurement was repeated twice. After drying (70 °C, 72 h), nitric acid [HNO_3, 65 % (w/v)] and hydrogen peroxide [H_2O_2, 30 % (w/v)] were added to the dry material and the samples were destructed by microwave-assisted digestion (MarsXpress CEM, Matthews, USA) at 200 °C on 1,600 W for 15 min. The samples were cooled and diluted with distilled water and the element analysis was carried out by ICP-MS (Thermo Scientific XSeries II, Asheville, USA). Values of Cu and other microelement (Fe, Zn, Mn, Mo, B) concentrations are given in μg g^{-1} dry weight (DW) and from the data shoot to root ratio was calculated.

Morphological observations

Seedling morphology of WT and mutant *Arabidopsis* was observed under Zeiss Axioskope 200-C stereomicroscope (Carl Zeiss, Jena, Germany). Fresh weights (FW) of the whole plants were measured using a balance and were expressed as average weight of ten seedlings.

Fluorescence microscopy

Nitric oxide levels in *Arabidopsis* roots were analysed by 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) according to Pető et al. (2011). Whole seedlings were incubated for 30 min in 10 μM dye solution (prepared in 10 mM Tris–HCl, pH 7.4) and were washed twice within 30 min with Tris–HCl. Dihydroethidium (DHE, 10 μM) in Tris–HCl buffer was used to visualise superoxide radical in *Arabidopsis* plants (Lehotai et al. 2011). To detect total intracellular ROS, 10 μM 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used at 37 °C for 15 min, then the samples were washed four times in 20 min with 2-N-morpholine-ethansulphonic acid/potassium chloride MES/KCl (10^{-3} M, pH 6.15) buffer. For in situ H_2O_2 detection, 10-acetyl-3,7-dihydroxyphenoxazine (ADHP or Ampiflu^{TM}) fluorescent dye was used. Seedlings were incubated in small Petri dishes with 2 ml of 50 μM ADHP dye solution (prepared in 50 mM sodium phosphate buffer, pH 7.5) for 30 min and washed once with the buffer (Lehotai et al. 2012). Fluorescein diacetate (FDA) was used for the determination of cell viability according to Lehotai et al. (2011). Investigations were carried out using a Zeiss Axiowert 200M-type inverted-fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR, HQ CCD) with filter set 10 (excitation 450–490 nm, emission: 515–565 nm), filter set 20HE (excitation: 535–585 nm, emission: 600–655 nm) and filter set 9 (excitation: 450–490 nm; emission: 515–∞ nm). Fluorescence intensities (pixel intensity) were measured on digital images within circular areas of 45 μm radii using Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

Statistical analysis

Results are expressed as mean ± SE. Multiple comparison analyses were performed with SigmaStat 11 software using analysis of variance (ANOVA, $P < 0.05$) and Duncan's test. In some cases, Microsoft Excel 2010 and Student's *t* test were used ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). All experiments were carried out at least two times. In each treatment, at least 10 samples were measured.

Results

Copper uptake, translocation and microelement homeostasis in WT *Arabidopsis*

Microelement concentrations of control, 5, 25 and 50 μM Cu-treated WT *Arabidopsis* plants were measured by ICP-MS and shoot to root ratio was calculated to draw conclusions about element distribution. In control plants, the root showed higher Cu concentrations than the shoot, however; as the effect of the metal treatment the Cu accumulation was more significant within the shoot system. Decreased iron content was observed in the shoots of the treated plants, since the concentration of this microelement in the root was significantly enhanced, thus the shoot to root ratio was modified by Cu. Zinc homeostasis of the roots was also affected by Cu excess, because reduced concentrations were measured compared to the control leading to significant increase of shoot to root ratio. Mild Cu stress (5 μM) resulted in an increase of manganese and molybdenum contents within the root system. The concentration of the latter element in the shoot was decreased, thus the distribution of it altered within the Cu-exposed seedlings compared to non-treated plants. Moreover, Cu treatment led to the reduction of boron content in both organs (Table 1).

Different growth and copper sensitivity of nitric oxide- (*nox1*, *gsnor1-3*, *nia1nia2*) and ascorbic acid (*vtc2-1*, *vtc2-3*, *miox4*) mutant *Arabidopsis*

First, the NO, H_2O_2 and total, intracellular ROS levels were detected in the primary root tips of the mutant

Table 1 Microelement concentrations (μg g^{-1} DW) and shoot: root ratios of 0, 5, 25 and 50 μM copper-treated *Arabidopsis*

$CuSO_4$	Shoot						
	0 μM	5 μM		25 μM		50 μM	
Cu	17.39 ± 0.43	82.23 ± 0.70	***	234.50 ± 1.96	***	514.20 ± 1.27	***
Fe	628.70 ± 3.31	438.60 ± 1.43	***	494.60 ± 3.20	***	431.20 ± 2.35	***
Zn	204.00 ± 5.05	169.80 ± 0.95	**	168.50 ± 1.36	**	196.60 ± 0.45	n.s.
Mn	129.20 ± 0.26	136.90 ± 0.35	***	113.90 ± 0.60	***	120.20 ± 0.11	***
Mo	12.91 ± 0.02	9.68 ± 0.03	***	9.60 ± 0.01	***	7.56 ± 0.04	***
B	80.31 ± 0.15	66.20 ± 0.58	***	40.19 ± 0.32	***	55.87 ± 0.36	***
$CuSO_4$	Root						
	0 μM	5 μM		25 μM		50 μM	
Cu	26.14 ± 0.54	78.93 ± 0.91	***	194.10 ± 16.51	***	280.81 ± 5.99	***
Fe	1860.00 ± 6.52	2151.00 ± 18.99	***	3409.00 ± 10.55	***	2692.02 ± 9.41	***
Zn	269.30 ± 1.31	144.00 ± 0.88	***	121.50 ± 0.07	***	160.10 ± 0.89	***
Mn	146.40 ± 0.94	173.10 ± 0.66	***	124.53 ± 0.94	***	129.50 ± 0.24	***
Mo	13.71 ± 0.04	16.92 ± 0.04	***	14.78 ± 0.10	***	13.86 ± 0.09	n.s.
B	123.92 ± 0.89	79.95 ± 0.69	***	25.82 ± 0.39	***	71.87 ± 1.18	***
$CuSO_4$	Shoot:Root						
	0 μM	5 μM		25 μM		50 μM	
Cu	0.665	1.041	***	1.208	**	1.831	***
Fe	0.338	0.203	***	0.145	***	0.160	***
Zn	0.757	1.179	***	1.386	***	1.227	***
Mn	0.882	0.790	***	0.914	***	0.928	**
Mo	0.942	0.572	***	0.649	***	0.545	***
B	0.648	0.828	***	1.557	***	0.777	**

The lack of significancy (*n.s.*) or significant differences according to Student's *t*-test ($n = 10$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) are indicated

Microelement concentration (μg g^{-1} ± SE)

Arabidopsis seedlings. In *nox1* and *gsnor1-3* plants, approximately twofold NO accumulation was found compared to the WT and the nitrate reductase-deficient *nia1-nia2* roots showed only ~40 % of the NO level of the WT (Table 2a). In the primary root tips of the ascorbic acid-deficient *vtc2-1* mutant, slightly but not significantly elevated H_2O_2 and total ROS levels were observed, while *vtc2-3* roots showed more pronounced ROS accumulation. In contrast, slightly reduced H_2O_2 and total ROS-dependent fluorescence were detected in *miox4* mutants (Table 2b).

During control conditions, NO overproducer and deficient mutants showed reduced cotyledon and root sizes as compared to WT. In contrast, the shoot and root sizes of the non-stressed *vtc2-1*, *vtc2-3* and *miox4* were similar to Col-0 (Fig. 1a). Unlike the WT, fresh weight of the NO overproducer plants slightly increased as an effect of mild Cu stress (5 μM $CuSO_4$). More serious metal excess caused growth inhibition and decrease of fresh weight in *nox1* and *gsnor1-3* mutants to a similar extent as in the WT. In 50 μM Cu-treated *nia1nia2* plants, the loss of fresh weight was more pronounced than in Col-0. In the case of the two *vtc* mutants, low Cu concentrations led to a slight increase in FW, while more serious metal excess caused fresh weight loss similar to that in the WT. The fresh weight diminution of the *myo*-inositol oxygenase overexpressing plants (*miox4*) was similar to that of Col-0, but proved to be milder than in the NO-deficient *nia1nia2* (Fig. 1b). In root meristem cells of WT plants, only 50 μM Cu caused significant loss of viability, while in the NO overproducing *nox1* mutant, the lowest concentration reduced and the more serious Cu excess only slightly decreased cell viability. In *gsnor1-3* roots, similar tendency was observed, since higher Cu concentrations did not affect cell viability. More pronounced Cu sensitivity was observed in *nia1nia2* plants, because all the applied concentrations triggered loss of viability in the root apex (Fig. 1c). In primary root tips of the AsA-deficient *vtc2-1* and *vtc2-3*, 25 and 50 μM Cu

Table 2 (a) Nitric oxide levels (pixel intensities of DAF-FM fluorescence) in the primary root tips of untreated WT, *nox1*, *gsnor1-3* and *nia1nia2 Arabidopsis* seedlings. (b) H_2O_2 and total ROS levels (pixel intensities of resorufin and DCF fluorescence) in the primary root tips of untreated WT, *vtc2-1*, *vtc2-3* and *miox4* seedlings

(a)	NO (WT %)	(b)	H_2O_2 (WT %)	Total ROS (WT %)
WT	100 ± 3.04	WT	100 ± 6.97	100 ± 6.17
nox1	215.70 ± 2.15***	*vtc2-1*	111.64 ± 8.09 n.s.	119.07 ± 12.49 n.s.
gsnor1-3	170.20 ± 2.32***	*vtc2-3*	133.87 ± 6.73*	138.20 ± 9.03 n.s.
nia1nia2	39.45 ± 0.99***	*miox4*	90.17 ± 3.83 n.s.	90.69 ± 13.64 n.s.

The data are expressed as percentage of the wild-type. The lack of significancy (*n.s.*) or significant differences according to Student's *t*-test ($n = 10$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) are indicated

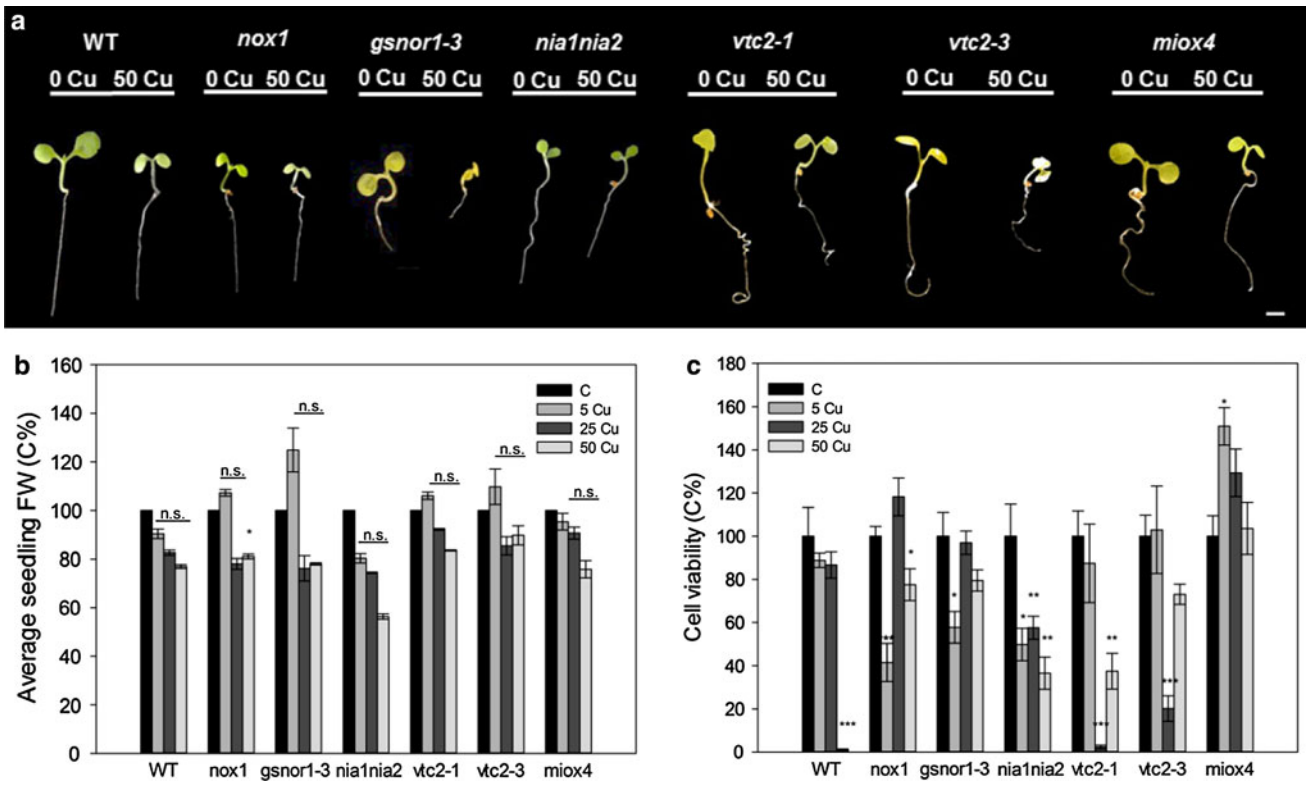

Fig. 1 **a** Representative stereomicroscopic images of seven-day-old WT and mutant *Arabidopsis* seedlings treated with 0 or 50. *Bar* 1 mm **b** Average fresh weight (control %) of WT, *nox1*, *gsnor1-3*, *nia1nia2*, *vtc2-1*, *vtc2-3* and *miox4* seedlings treated with 0, 5, 25 and 50 μM Cu. **c** Cell viability in root meristem of control and Cu-treated WT, NO and ascorbic acid mutant *Arabidopsis.* The lack of significancy (*n.s.*) or significant differences according to Student's *t*-test ($n = 10$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) are indicated

significantly decreased cell viability. Cu excess did not reduce the viability of *miox4* plants having lower ROS levels; moreover, low metal excess resulted in enhanced viability (Fig. 1c).

Copper tolerance is associated with altered nitric oxide levels in the roots

The basal NO content of the WT and mutant roots was biochemically modified by NO donor (SNP) and/or scavenger (cPTIO) treatment to determine whether, Cu tolerance or sensitivity is associated with NO levels. Exogenous NO prevented WT root tips from Cu-induced viability loss, while treatment with the NO scavenger resulted in decreased viability (Fig. 2a). Quenching of NO in *nox1* mutant resulted in higher cell viability, especially in the 5 and 50 μM Cu-treated roots (Fig. 2b). In the *gsnor1-3* mutants treated with 50 μM Cu plus cPTIO showed elevated viability compared to those treated with Cu alone. However, the notable viability loss in 5 μM Cu-treated roots was not ameliorated by reducing NO levels of *gsnor1-3* (Fig. 2c). When the low NO content of the Cu-treated *nia1nia2* mutants was enhanced by SNP addition, cell viability of root meristem increased (Fig. 2d).

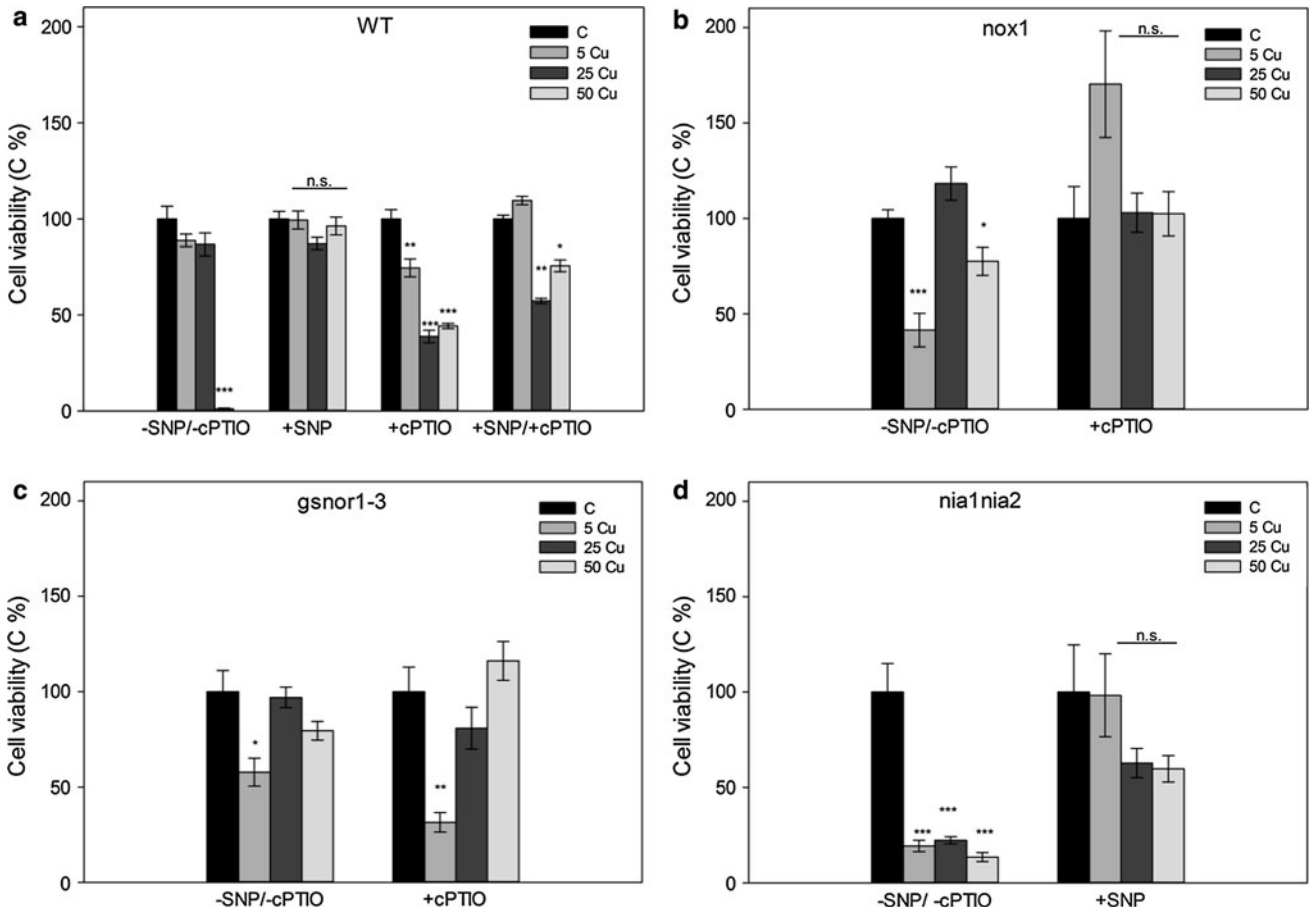

Fig. 2 **a–d** Cell viability in root tips of WT, *nox1*, *gsnor1-3* and *nia1nia2 Arabidopsis* treated with different copper concentrations without (−SNP/−cPTIO) or with 10 μM SNP (+SNP) or 50 μM cPTIO (+cPTIO) or with SNP and cPTIO together (+SNP/+cPTIO). The lack of significancy (*n.s.*) or significant differences according to Student's *t*-test ($n = 10$, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$) are indicated

Interplay between NO and ROS during copper stress in *Arabidopsis* roots

To explore a possible relationship between ROS and NO metabolism during Cu stress, the levels of different ROS forms (superoxide radical and hydrogen peroxide) were studied in NO overproducing and deficient mutants and vice versa. Superoxide levels in control root tips were lower in mutants having elevated NO contents (*nox1, gsnor1-3*) and were higher in the NO-deficient *nia1nia2*. As an effect of Cu exposure, superoxide levels increased in WT *Arabidopsis* roots; however, they decreased in the NO overproducing *nox1* and the deficient *nia1nia2* mutants. Besides, in *gsnor1-3* roots, a non-significant $O_2^{\cdot-}$ depletion was detected (Fig. 3a, c). A consequent Cu-induced H_2O_2 accumulation was observed only in the case of the NO deficient *nia1nia2 Arabidopsis*. In the WT and NO overproducing plants, H_2O_2 decreased as an effect of Cu treatment (Fig. 3b, c). In non-treated AsA mutants, significantly reduced NO levels were measured compared to the WT; however, the mutants possessing reduced or elevated ROS levels, did not significantly differ from each other. The effect of Cu on NO metabolism did not prove to be significant in the mutants showing altered ascorbate levels (Fig. 3d).

Discussion

Copper uptake, translocation and microelement homeostasis in wild-type *Arabidopsis*

Concentrations of microelements (Cu, Fe, Zn, Mn, Mo, B) in the shoot and root system and their organismal-level distributions were determined in *Arabidopsis* grown on nutrient agar medium (Table 1). Based on the results, *Arabidopsis* seedlings are able to uptake and accumulate a portion of the Cu and translocate it into the shoot system. Presumably, Cu makes complexes with nicotianamine and is transported via xylem vessels into the shoot system

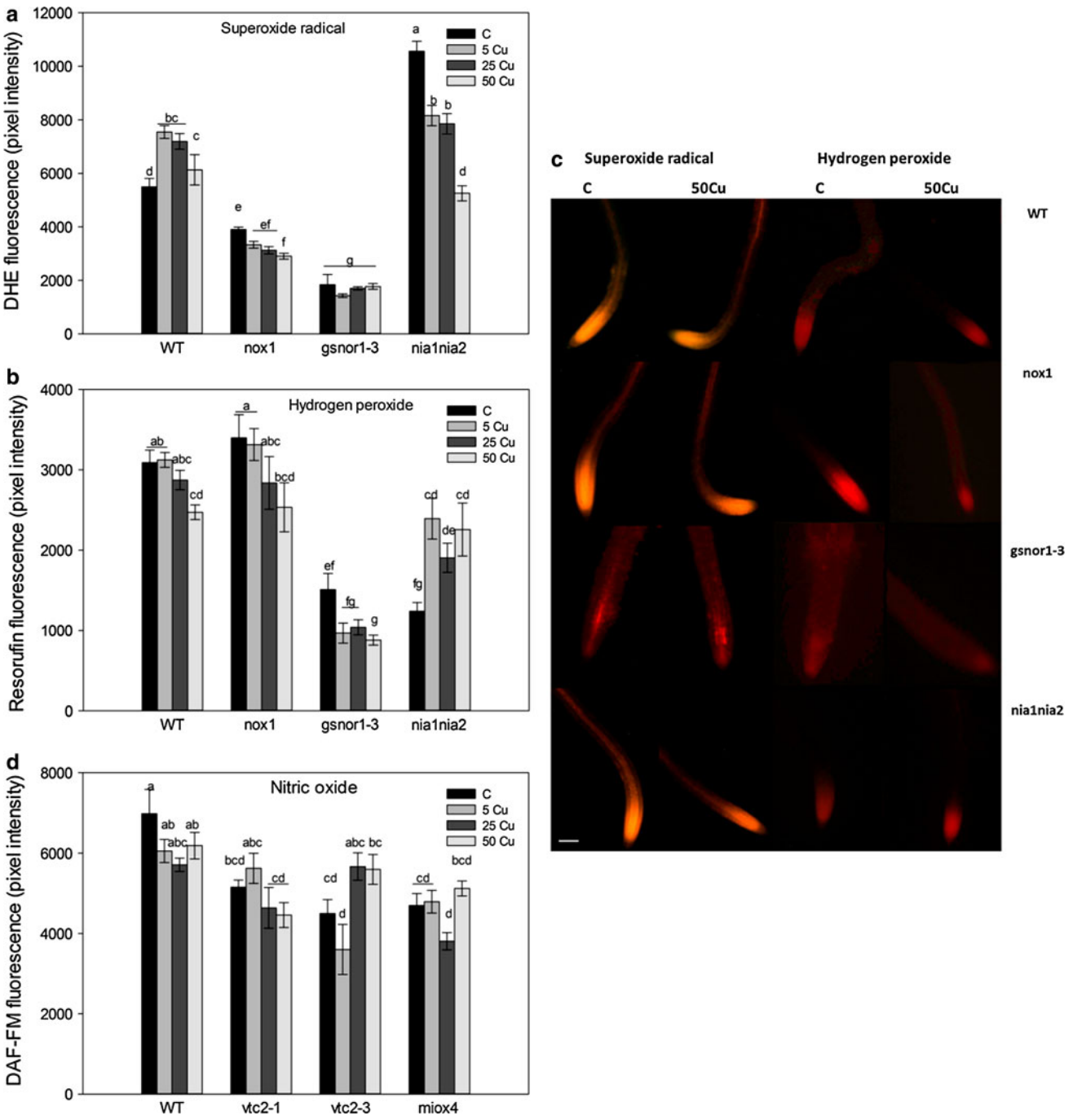

Fig. 3 Superoxide (pixel intensities of DHE fluorescence, **a** and H_2O_2 (pixel intensities of resorufin fluorescence, **b** levels in WT and NO mutant *Arabidopsis*. **c** Representative images of control and 50 μM copper-treated WT and mutant *Arabidopsis* root tips stained by DHE or Ampiflu™, respectively. *Bar* 100 μm. Nitric oxide levels (pixel intensities of DAF-FM fluorescence, **d** in root meristems of WT and ascorbic acid mutant *Arabidopsis* treated with 0, 5, 25 and 50 μM copper. *Different letters* indicate significant differences according to Duncan-test ($n = 10$, $P \leq 0.05$)

(Burkhead et al. 2009). Cu excess notably modifies microelement homeostasis of the seedlings. The reduced shoot iron content reflects the antagonism between Cu and Fe due to their competition during the uptake (Lequeux et al. 2010). The main symptom of iron deficiency is intervenial chlorosis (Taylor and Foy 1985), which was clearly visible in the cotyledons of *Arabidopsis* seedlings (see Fig. 2a). Elevated iron concentrations in the roots of Cu-treated plants could be explained by the fact that Cu is able to intensify iron availability by displacing it from Fe-EDTA complexes in the nutrient medium (Lequeux et al. 2010). Root zinc status was negatively affected by Cu

excess, since both ions use the same transporter molecules, ZINC-REGULATED TRANSPORTERS, IRON-REGULATED TRANSPORTER-LIKE PROTEINS (ZIP, Wintz et al. 2003). The reducing effect of Cu on manganese, molybdenum and boron contents can be explained by the fact that Cu may change their uptake rates (Lidon and Henriques 1993). In general, Cu exposure significantly modified the shoot–root distribution of the examined microelements within the seedlings. The serious disturbances of microelement homeostasis can result in growth inhibition of Cu-exposed *Arabidopsis* seedlings.

Different growth and copper sensitivity of nitric oxide- (*nox1*, *gsnor1-3*, *nia1nia2*) and ascorbic acid (*vtc2-1*, *vtc2-3*, *miox4*) mutant *Arabidopsis*

First and foremost, the NO and ROS levels were verified in the root tips of the mutants to justify the effect of the mutations on the production of these molecules (Table 2). In the root tip of *nox1,* enhanced NO levels were detected due to the probable oxidative NO synthesis following from elevated L-arginine concentration in this mutant (He et al. 2004). The *gsnor1-3* plants are defective in NO removal mediated by GSNO reductase (Feechan et al. 2005), which results in highly elevated (170 % of the WT) NO levels. The nitrate reductase-deficient *nia1nia2* double mutant showed significantly reduced NO levels compared to the WT, which suggests the involvement of this enzyme in NO synthesis of non-stressed *Arabidopsis* roots. Similarly, decreased NO level was published in *nia1nia2* roots by Lozano-Juste and Léon (2010). Interestingly, the *vtc2-3* mutant containing higher (40–50 % of the WT) AsA concentration than *vtc2-1* (Conklin 2001) showed more pronounced enhancement of H_2O_2 (and total ROS), although the difference showed only a low level of significance. Similarly, slight differences in hydrogen peroxide concentrations were measured in whole seedlings of *vtc* mutants (Barth et al. 2010). In contrast, the *miox4* roots possessing elevated ascorbic acid contents showed reduced levels of H_2O_2 and total intracellular ROS. Considering the statistically non-significant differences, it can be concluded that ascorbic acid participates in the regulation of ROS levels in non-stressed *Arabidopsis* roots; however, other elements of the antioxidant system (e.g. glutathione and enzymes) can also be involved in this process. The mutant seedlings possessing altered NO metabolism (*nox1*, *gsnor1-3* and *nia1nia2*) showed reduced shoot and root sizes and fresh weights as compared to the WT, suggesting the necessity of an optimal NO level for the appropriate growth (Fig. 1). Moreover, the semidwarf phenotype of *gsnor1-3* reflects that the GSNOR-dependent NO removal is necessary for the optimal development (Lee et al. 2008). The results are supported by the work of He et al. (2004), where high NO level (exogenous sodium nitroprusside treatment) resulted in growth inhibition of *Arabidopsis* and an optimal SNP concentration for promoting shoot growth was determined. Furthermore, contrasting effects were observed also in pea leaves depending on the actual tissue concentration of NO (Leshem and Haramaty 1996). Also in the root system, the growth inducing effect of NO is dose-dependent, since higher levels of exogenously applied NO (as chemical NO donor) caused growth inhibition (Gouvêa et al. 1997; Pagnussat et al. 2002). In accordance with the results of Dowdle et al. (2007), *vtc2-3* showed WT-like shoot and root size under control conditions. Also, non-stressed *miox4* plants had a size similar to WT. These results are similar to those of Alford (2009), where WT-like hypocotyl and root length of *miox4* was measured during control circumstances. These suggest that modified ascorbate contents and consequently slightly altered ROS levels do not significantly influence the early development of *Arabidopsis* seedlings. According to Epstein and Bloom (2005) basal Cu levels in plants ranged from 2 to 50 μg g^{-1} DW, and in agar medium Cu concentrations above 20 μM (1.27 ppm) is considered to be toxic (Murphy and Taiz 1995). Based on these, the lowest $CuSO_4$ concentration (5 μM) applied in this study resulted in slightly elevated Cu level (~80 μg g^{-1} DW, see Table 1) and it proved to be not toxic for Col-0 seedlings. What is more, 5 μM Cu treatment had a slight growth promoting effect (non-significant elevation of FW), though elevated NO and ROS levels were needed for this. In the case of NO deficiency, Cu had a more pronounced growth-reducing effect than in the WT, reflecting the role of this molecule in the maintenance of growth under abiotic stress conditions. In the same experimental setup, the *nia1nia2* seedlings showed serious Cu-induced reduction in cell elongation during hypocotyl and primary root growth (Pető et al. 2011).

Copper sensitivity was evaluated by studying the viability of root meristem cells using fluorescent microscopy. During mild stress, high NO levels intensified sensitivity, while under severe stress conditions they facilitated tolerance. This means that the role of NO signalling depends on the strength of the stress. Under mild Cu stress, the negative effect on viability can be reversible by scavenging the high NO level in *nox1* (see Fig. 2b). The assent of NO to abiotic or biotic stress tolerance was evidenced by several authors. For instance, the high *S*-nitrosothiol level of *gsnor1-3* was shown to be an important contributor to thermo- or selenite tolerance (Lee et al. 2008; Lehotai et al. 2012) and the NO-deficient *nia1nia2* mutant proved to be less tolerant to other stressors such as water deficit, freezing or selenite excess (Zhao et al. 2009; Lozano-Juste and Léon 2010; Lehotai et al. 2012). Recently, an NO-overproducing tomato mutant (*shr*) was isolated, in which the

disease resistance was associated with the enhanced NO production (Negi et al. 2010). Based on the reduced viability in Cu-exposed *vtc2-1*, *vtc2-3* and the more viable cells in *miox4* roots, enhanced ROS content due to reduced AsA level triggers Cu sensitivity, while decreased ROS concentration as a result of AsA overproduction favours Cu tolerance. Elevated ROS levels induce disturbances in redox state leading to oxidative damage to the cells (Potters et al. 2010), which is likely to intensify Cu sensitivity. The *vtc2* mutants proved to be more susceptible to other environmental stresses such as heat, salt, photooxidative stress and selenite excess (Smirnoff 2000; Müller-Moulé et al. 2004; Larkindale et al. 2005; Lehotai et al. 2012).

Copper tolerance is associated with altered nitric oxide levels in the roots

During biochemical studies, exogenous NO donor (SNP) and scavenger (cPTIO) treatments were applied to verify the involvement of NO in the development of Cu tolerance. Based on the higher viability of NO-treated WT plants, NO ameliorated cell injury induced by severe Cu excess (Fig. 2). The increased Cu tolerance of the NO overproducing mutants and the more pronounced sensitivity of *nia1nia2 Arabidopsis* also confirm the stress mitigating effect of NO (see Fig. 1). Similar results were obtained by Cui et al. (2010), where NO treatment reduced lipid peroxidation and increased the root fresh weight in Cu-treated tomato plants. In another study, *Panax ginseng* plants treated with SNP and subsequently exposed to Cu exhibited a significantly reduced cell death and oxidative damage to the membranes (Tewari et al. 2008). However, the results also show that Cu resistance determined by root cell viability is associated with a strictly regulated NO balance, since higher or lower than the optimal NO level may result in cell damage (Fig. 2b, c).

Interplay between NO and ROS during copper stress in *Arabidopsis* roots

The possible connection between NO and ROS during Cu stress was also examined using microscopic methods. Elevated NO level resulted in reduced superoxide contents of the control *Arabidopsis* root tips and vice versa. It follows that under stress-free conditions, NO is able to negatively regulate superoxide levels possibly by the chemical reaction yielding peroxynitrite formation (Koppenol et al. 1992) or by modifying the activities of antioxidants such as superoxide dismutase (Wang et al. 2010). The NADPH oxidases can be responsible for the Cu-induced superoxide accumulation in WT *Arabidopsis* roots (Opdenakker et al. 2012). In the case of NO excess *(nox1*, *gsnor1-3)*, Cu was not able to enhance superoxide level, probably because of the inactivation of NADPH oxidases by *S*-nitrosylation (Yun et al. 2011). As an effect of Cu, H_2O_2 levels decreased as long as NO was present in excess, therefore, ROS detoxification could happen through the NO-dependent induction of expression of antioxidant genes such as glutathione peroxidase or glutathione transferase (Polverari et al. 2003). These enzymes were shown to be regulated also by NO-dependent *S*-nitrosylation (Lindermayr et al. 2005). Furthermore, in the case of NO deficiency, superoxide reduction was associated with H_2O_2 generation reflecting the possible SOD-dependent detoxification. The altered AsA metabolism and the consequently changed ROS content in *vtc2-1*, *vtc2-3* and *miox4* mutants resulted in reduced NO levels, which suggests the impact of a strictly regulated ROS level on NO metabolism under stress-free conditions. On the basis of Cu–induced non-significant alterations, it can be assumed that under Cu stress the impact of ROS on NO metabolism is negligible (Fig. 3d).

The results of the present study clearly show that *Arabidopsis* seedlings are able to uptake and accumulate Cu from the agar medium and translocate it into the shoot system. Its accumulation within the tissues fundamentally modifies microelement homeostasis of the seedlings, which can partly be responsible for the growth inhibition. Nitric oxide can intensify Cu sensitivity or can facilitate tolerance depending on the strength of the stress. The excess of ROS derived from ascorbic acid deficiency is associated with Cu sensitivity and vice versa. Moreover, NO mitigates Cu stress by regulating ROS ($O_2^{\cdot-}$ and H_2O_2) metabolism. Based on these findings, Cu stress tolerance is regulated by a fine-tuned NO/ROS balance in *Arabidopsis*.

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