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ORIGINAL PAPER

Long-term copper (Cu^{2+}) exposure impacts on auxin, nitric oxide (NO) metabolism and morphology of *Arabidopsis thaliana* L.

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Abstract Plants are able to dynamically adapt to their environment by reprogramming of their growth and development. Copper (Cu^{2+}) excess modifies shoot and root architecture of plants by a lesser known mechanism, therefore the involvement of a major hormone component (auxin) and a signal molecule (nitric oxide) in Cu^{2+}-induced morphological responses were studied in *Arabidopsis* using microscopic methods. Auxin-inducible gene expression was visualized in *DR5::GUS Arabidopsis* and nitric oxide (NO) levels were detected by DAF-FM fluorophore in the stem and root system. Copper excess caused the inhibition of stem and root growth of *Arabidopsis*, during which cell elongation, division and expansion were also affected. The symptoms of stress-induced morphogenic response were found in the root system of 25 µM Cu^{2+}-treated plants. In both organs, the decrease of auxin-dependent gene expression was found, which can partly explain the growth inhibitions. Besides hormonal system, nitric oxide metabolism was also affected by Cu^{2+}. In root tips, copper excess induced NO generation, while NO content in lateral roots was not affected by the treatments. Using *nia1nia2* mutants, nitrate reductase enzyme as a putative source of Cu^{2+}-induced NO was identified in *Arabidopsis* primary roots.

Keywords Auxin · Copper · Nitric oxide · Plant morphology

Introduction

Copper (Cu^{2+}) is able to accumulate in soils as a result of both natural and anthropogenic mechanisms such as application of fertilizers or industrial activities (Pilon-Smits and Pilon 2002). Although, copper is an essential microelement for plants, its excess leads to irreversible damages such as inhibition of photosynthesis and respiration. In the presence of toxic Cu^{2+} concentrations, the reduction of root and shoot biomass, chlorosis and necrosis can be seen (Yruela 2009). Similar to other transition metals, copper catalyzes the formation of reactive oxygen species (ROS) causing oxidative stress (Halliwell and Gutteridge 1984). Plants possess relevant physiological plasticity; hence they can respond to suboptimal environmental conditions by growth redistribution of their organs (stress-induced morphogenic response, SIMR; Potters et al. 2009).

The development of the SIMR phenotype is controlled by growth substance gradients (e.g. auxin distribution) and signal molecules at the organ and organismal levels (Potters et al. 2009). The best known morphogens are auxins, which are responsible for controlling embryogenesis, phyllotaxis, leaf and root development (Zhao 2010). Alterations in auxin metabolism and/or sensitivity induced by various stress factors (e.g. aluminium, salinity) can be partly responsible for morphological responses (Kollmeier et al. 2000; Wang et al. 2009).

Nitric oxide (NO) is a diffusible gas signal in plants having a wide range of physiological roles. Besides regulating normal development it plays a role also in different biotic and abiotic stress responses; hence NO is considered to be a general signal molecule in plant cells. Nitric oxide positively regulates auxin signalling during adventitious or lateral root (LR) and root hair development (Pagnussat

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et al. 2002; Correa-Aragunde et al. 2004; Lombardo et al. 2006), but it is a negative regulator of primary root (PR) elongation in tomato and of the growth of *Arabidopsis* seedlings treated with copper (Pagnussat et al. 2002; Pető et al. 2011). There are two major enzymatic sources of this signal molecule in plant cells: mammalian nitric oxide synthase (NOS)-like enzyme and nitrate reductase (NR). The activity of NOS-like enzyme has been reported in some plant species and an active NOS enzyme was purified and characterized in *Ostreococcus tauri* (Foresi et al. 2010). Recently, Shi et al. (2012) reported that *Arabidopsis* expressing rat neuronal NOS showed increased endogenous NO content. Nitric oxide can generate through the reduction of nitrite by NR and this enzyme was considered to be the major NO source in plants (Xu and Zhao 2003). The NR-deficient *Arabidopsis* mutant (*nia1nia2*) shows only 1 % NR activity of the wild type and has significantly reduced NO levels (Wilkinson and Crawford 1993) which reflects the involvement of NR in endogenous NO synthesis.

The aim of this work was to examine the morphological changes induced by long-term copper exposure in *Arabidopsis* plants and to study the putative involvement of auxin and NO in the signal transduction of developmental alterations. With the help of *nia1nia2 Arabidopsis* mutants, the enzymatic source of copper-induced NO was also investigated.

Materials and methods

Plant material

The experiments were carried out using 17-day-old wild type (*Col-0*), *DR5::GUS* and *nia1nia2* mutant *Arabidopsis thaliana* L. plants. Seeds were surface sterilized with 5 % (v/v) sodium hypochlorite for 20 min and rinsed with sterile distilled water before transferring them to half-strength MS (Murashige and Skoog 1962) medium (1 % (w/v) sucrose, and 0.8 % (w/v) agar) which was supplemented with 0, 5, 25 or 50 µM $CuSO_4$. The Petri dishes were placed in a greenhouse at photo flux density of 150 µmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55–60 % with 25 ± 2 °C.

Morphological measurements

Primary root length (mm) was measured manually using a scale; the length of the hypocotyls (mm) and the petioles (mm) were determined under Zeiss Axiowert 200 M (Carl Zeiss, Jena, Germany) microscope using 5× magnification. The area of the oldest real leaf (mm^2) was measured using software (AnalyzingDigitalImages) and the developmental stages of the lateral roots were determined in X-Gluc-stained *DR5::GUS*-type *Arabidopsis* according to Malamy and Benfey (1997).

Histochemical staining

The β-glucuronidase activity was visualized in *DR5::GUS* plants by incubating whole plants for 15 h in a staining solution (containing 1 mM 5-bromo-4chloro-3-indolyl-β-D-glucuronic acid) according to Jefferson et al. (1987). After washing with 70 % (v/v) ethanol samples were examined under Zeiss Axiowert 200 M inverted microscope and Zeiss Axioscope 2000-C (Carl Zeiss, Jena, Germany) stereomicroscope.

Fluorescent microscopy and image analysis

Seventeen-day-old *Arabidopsis* plants were dyed with a NO-specific fluorophore, 4-amino-5-methylamino-2′,7′-difluoroflorescein (10 µM DAF-FM DA in Tris–HCl buffer, pH 7.4) for 30 min at room temperature in darkness at 25 ± 2 °C. After 5 min vacuum infiltration, samples were washed with Tris-buffer solution twice within 30 min. Intensities of nitric oxide-dependent fluorescence were detected with Zeiss Axiowert 200 M microscope equipped with a high resolution digital camera (Axiocam HR, HQ CCD) and filter set 10 (exc.: 450–490 nm, em.: 515–565 nm). FLUAR 5x/0.12 NA and FLUAR 10x/0.25 objective lenses were applied for the investigations. Fluorescent intensities were measured on digital images within area of circles (45 µm radii for the root and 600 µm radii for the leaf) using Axiovision Rel. 4.8 software. The selected microscopic images are representatives of similar results from the two repetitions. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Statistical analysis

All experiments were performed at least twice and 10 samples were measured in each experiment. Statistical analysis was performed using SigmaStat 11. software (ANOVA, $P < 0.05$, Duncan-test).

Results

Low concentration of copper exposure (5 µM) did not affect the leaf number and petiole length of *Arabidopsis*

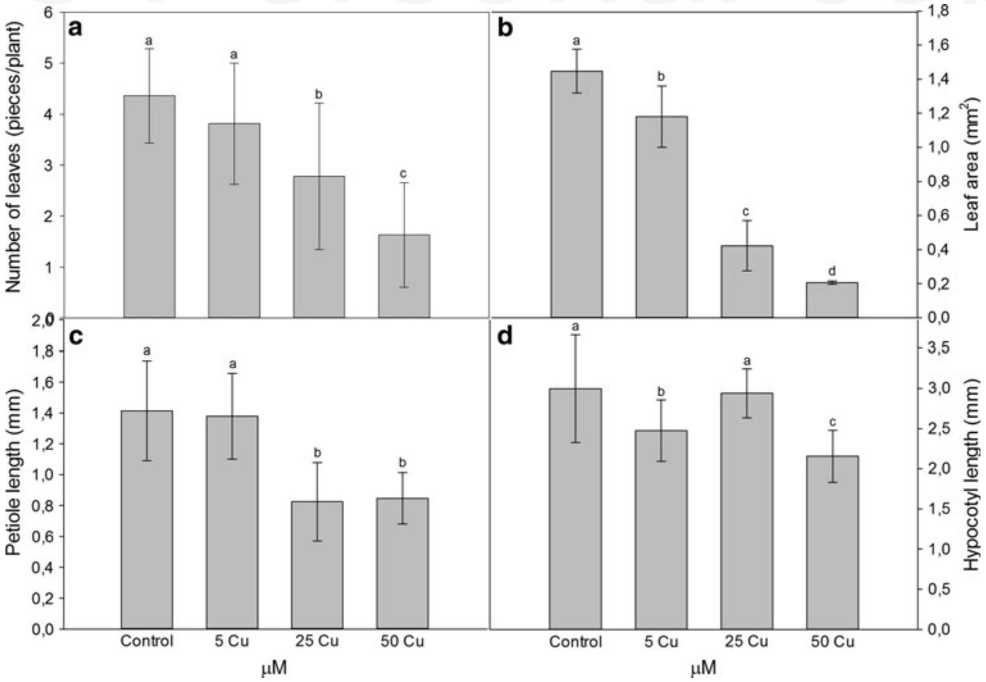


Fig. 1 Leaf number (pieces/plant, **a**), leaf area (mm^2, **b**), petiole (**c**) and hypocotyl length (mm, **d**) of 0, 5, 25 and 50 µM copper-treated *Arabidopsis*. Values are means of 10 plants ±SE. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's test

plants; however it caused significant inhibition of leaf expansion and hypocotyl elongation (Fig. 1a–d). The *Arabidopsis* plants treated with 25 or 50 µM $CuSO_4$ in agar medium showed serious alterations in their stem morphology. Besides the decrease in leaf number (Fig. 1a), copper also resulted in a significant reduction of leaf area (Fig. 1b). In the stem system, cell elongation processes were affected by higher copper concentrations, since petiole and hypocotyl elongation showed a notable inhibition (Fig. 1c, d). Copper excess also affected root morphology; length of the primary root slightly decreased in 5 and 50 µM Cu^{2+}-treated plants; however 25 µM Cu^{2+} had the most serious effect on root elongation (Fig. 2a). Initiation process of lateral roots was significantly inhibited by the treatments (Fig. 2b), although the later phase of LR development was induced by 25 µM but it was inhibited by 50 µM copper concentration (Fig. 2c). Figure 3 shows the Cu^{2+}-induced reduction of stem growth, root hair length and number. Moreover, as a consequence of PR reduction the root hairs were visible closer to the tip.

The auxin-inducible *DR5::GUS* gene expression was also studied in the stem and root system of *Arabidopsis* plants. Under control conditions the expression of the reporter gene fusion was found in the shoot apical meristem, in leaf primordia and at the top of the older leaves (Fig. 4a). Lower copper concentrations had a slight effect on auxin-induced gene expression (Fig. 4b, c), while with regard to the effect of 50 µM Cu^{2+}, the expression level of *DR5::GUS* decreased or almost disappeared from the stem (Fig. 4d). Within control root tips, the auxin responsive *DR5* gene expressed in the root cap and the meristem. This phenomenon was not affected by 5 µM Cu^{2+} (Fig. 4e, f). In contrast, the more serious copper excess lead to the notable decrease of auxin-dependent gene expression in the PR tip (Fig. 4g, h). The *DR5::GUS* expression was also examined in LRs of different developmental stages (smaller than stage VII, stage VII and larger than stage VII). The auxin responsive gene expression proved to be intense in LR primordia at all degrees of development in the control plants and showed a heavy decrease in case of 50 µM Cu^{2+} exposure. However, in the tip of older laterals (larger than stage VII) the *DR5* expression remained, which makes further growth possible (Fig. 5).

The level of nitric oxide as a component of auxin signal transduction was studied in leaves, primary and lateral roots by fluorescent microscopic methods. In the leaves of copper-treated plants, no significant changes were detected compared to control (data not shown) and nitric oxide levels in differentially developed laterals of copper-treated plants proved to be similar to that of wild type (Fig. 6a). However, in the root meristem (MZ) 50 µM copper resulted in a significant enhancement of NO content (Figs. 6b, 7). Under controlled conditions, the elongation zone of the primary root showed higher NO fluorescence than the MZ and 50 µM Cu caused a notable elevation of it (Figs. 6b, 7). In both zones of *nia1nia2* primary roots,

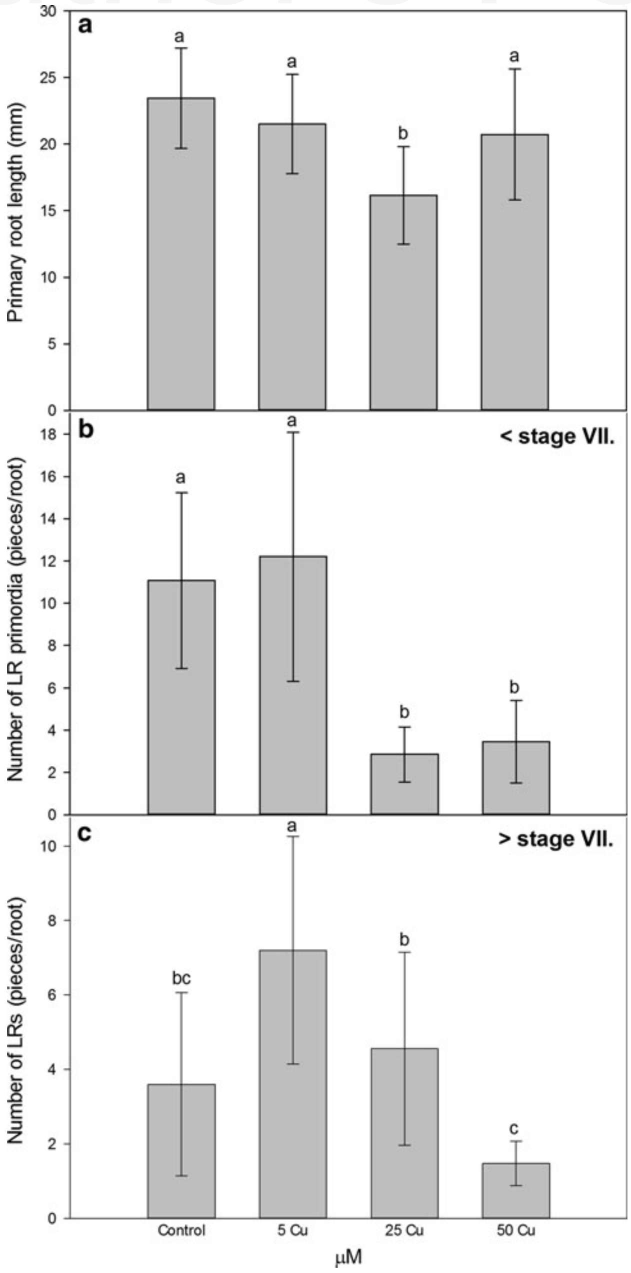


Fig. 2 Primary root length (RL, mm, **a**) and lateral root number (**b**, **c**) of *Arabidopsis* treated with 0, 5, 25 and 50 µM copper for 17 days. Developmental stages of laterals were determined according to Malamy and Benfey (1997). Values are means of 10 plants ±SE. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's test

NO content was lower compared to wild type and these roots showed no increase in their NO content due to the effect of copper (Fig. 7).

Discussion

During environmental stress conditions such as copper excess the basic term of plant survival is the rearrangement of means from growth and development to defence mechanisms. Therefore, one of the most characteristic symptoms of heavy metal exposure is the growth inhibition of most plant organs. Within the stem system, leaf number, leaf size, petiole length and hypocotyl length were decreased by 25 and 50 µM copper (Fig. 1), which suggests serious Cu^{2+}-induced inhibition of cell division, cell expansion and cell elongation processes (Jiang et al. 2000; Alaoui-Sossé et al. 2004). The heavy decrease of stem growth may be the result of the carbohydrate content increase and the simultaneous reduction of photosynthesis induced by copper (Maksymiec and Baszynski 1998). However, Cu^{2+} is taken up and accumulated by the roots; PR was only slightly affected by it. In contrast to elongation, cell division allowing LR initiation proved to be sensitive to Cu^{2+} excess (Fig. 2). In the root system of 25 µM Cu^{2+}-treated plants, the symptoms of the stress-induced morphological response (Pasternak et al. 2005; Potters et al. 2009) were visible: inhibition of elongation, consequent formation of root hairs near apex and elevated number of lateral roots (>stage VII) (Fig. 3). Similar results were published by Lequeux et al. (2010) where the total lateral root density of *Arabidopsis* enhanced upon copper treatment; however the different LR developmental stages were not examined separately. In our study, it was found that the early steps of LR development (driven by cell divisions) are more and the later events (mainly cell elongation) are less sensitive to copper in *Arabidopsis* roots. The SIMR phenotype also appeared under mild osmotic or salt stress in pea and *Arabidopsis* (Kolbert et al. 2008; Zolla et al. 2010); however it has to be noted that the development of SIMR depends on the concentration and duration of copper treatment. Presumably, the appearance of 25 µM copper-induced SIMR may be a basic element of the acclimation process, since the reduced leaf size and the enhanced number of lateral roots can contribute to the better water supply and the survival of the plant.

Since developmental processes are mainly triggered by morphogens such as auxin, we examined the in vivo expression of the auxin-inducible *DR5* promoter using *DR5::GUS* transgenic *Arabidopsis*. The expression pattern in control stem system proved to be similar to the results of Aloni et al. (2002). Like copper, other environmental stress factors (e.g. salt, cadmium) induce the decrease of *DR5* expression in *Arabidopsis* leaves (Xu et al. 2009). Similar to results of Lequeux et al. (2010), reduction of the auxin-dependent gene expression was found in PR root meristems of copper-treated plants, which suggests the copper-induced decrease of auxin levels (Fig. 4). As well as in PR tips, copper treatments resulted in a serious decrease of *DR5* expression in differentially developed LRs (Fig. 5). The connection of Cu^{2+} stress with auxin signal

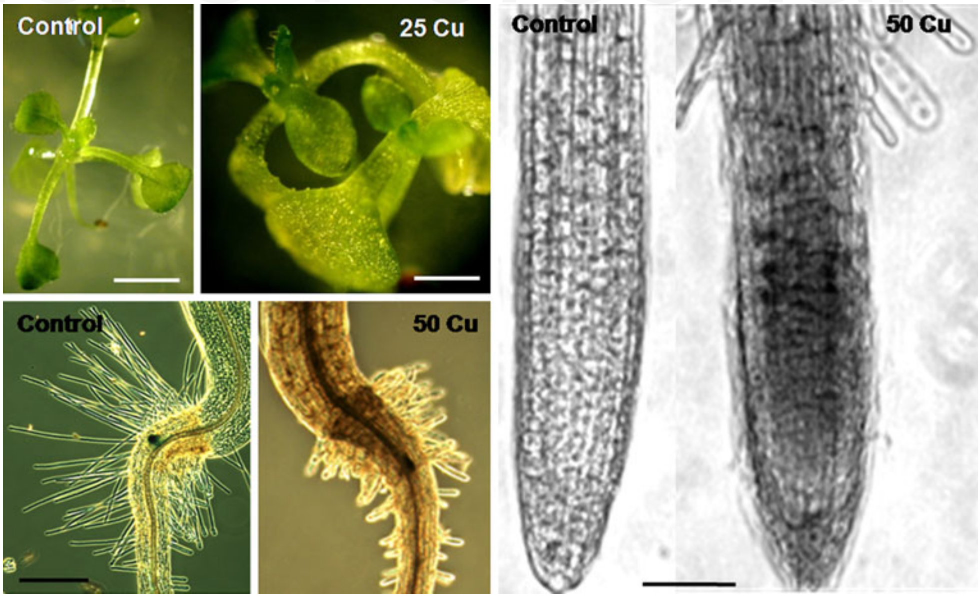


Fig. 3 Representative images of stem system, root hairs and PR tips of control and 25 or 50 μM Cu^{2+}-treated *Arabidopsis*. *Bars* = 5, 1 or 0.5 mm

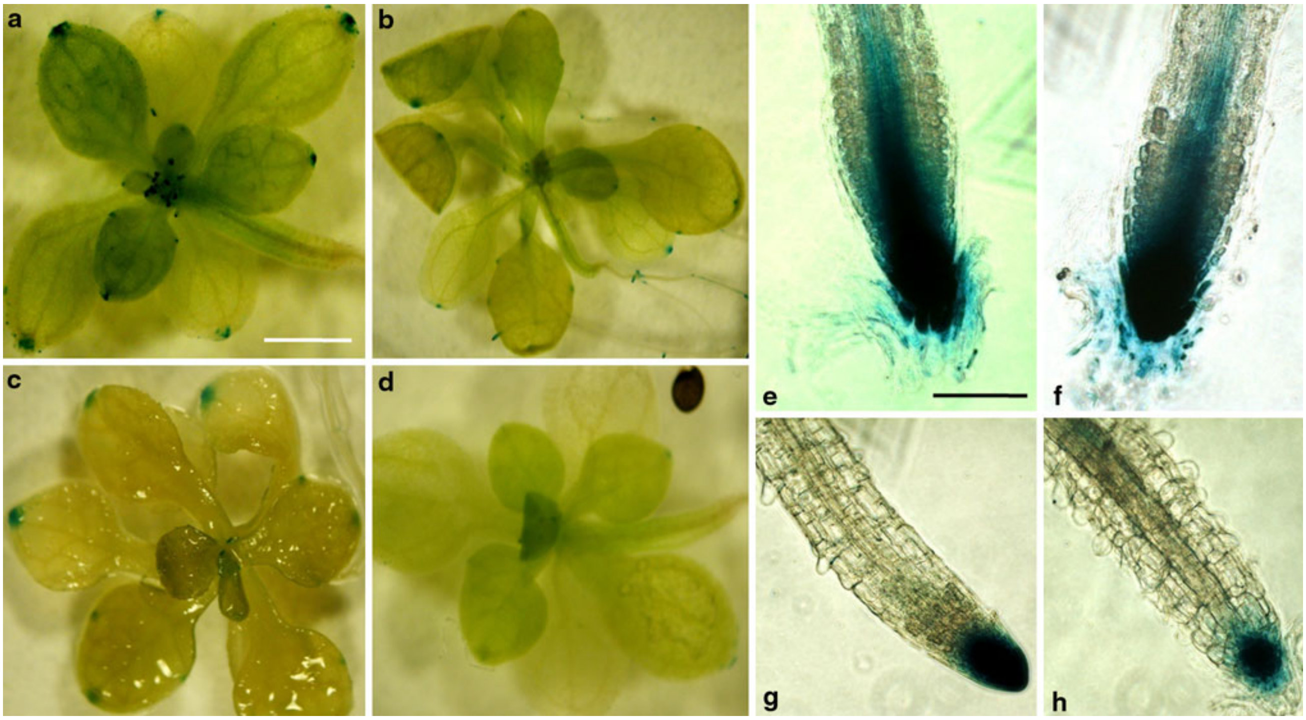
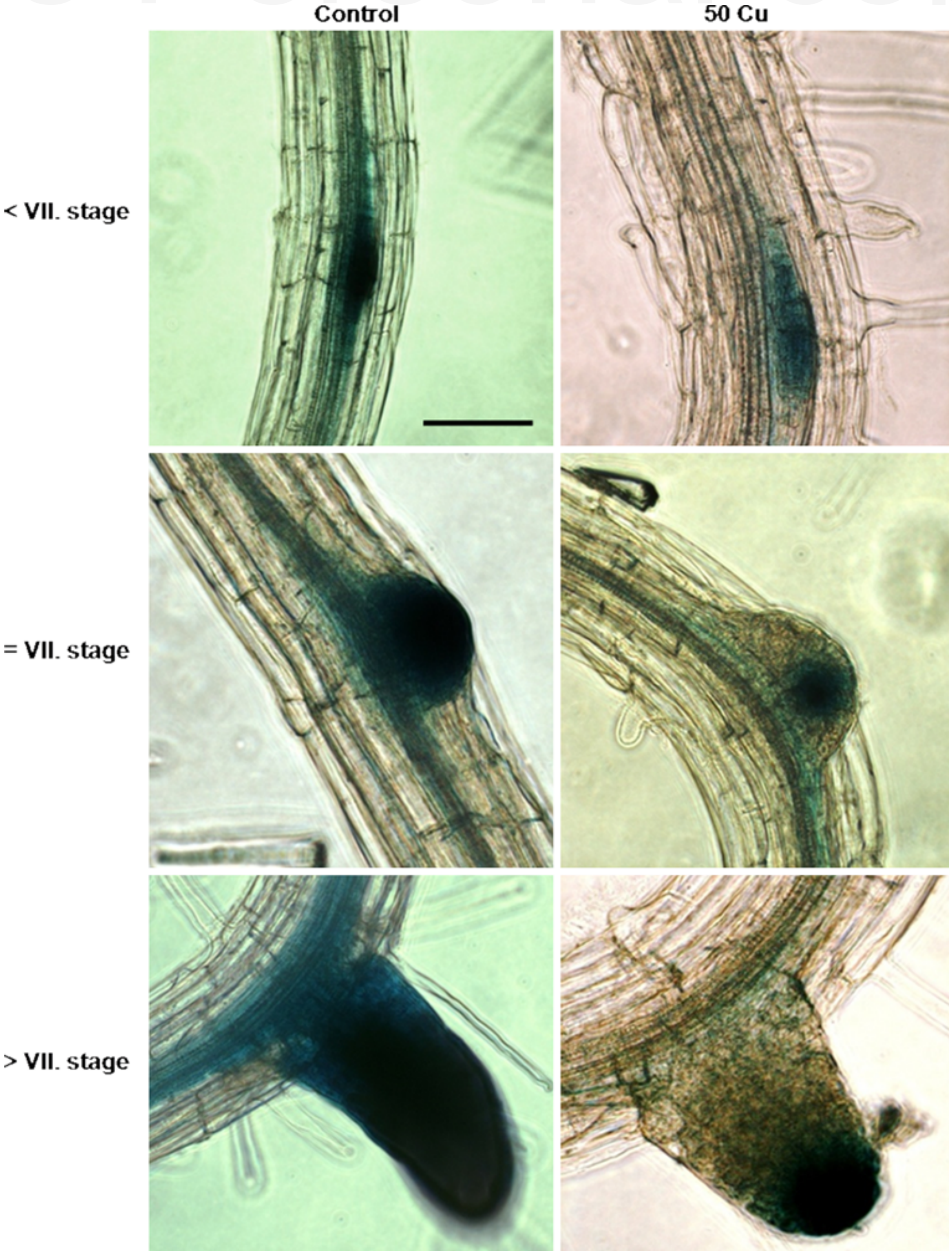


Fig. 4 X-Gluc staining in stem system (**a**–**d**) or root tips (**e**–**h**) of *DR5::GUS Arabidopsis* treated with 0 (**a**, **e**), 5 (**b**, **f**), 25 (**c**, **g**) or 50 μM (**d**, **h**) copper. *Bars* = 1 or 0.5 mm

transduction pathways can be evidenced by the down-regulation of the auxin-controlled *SHY2/IAA3* genes by copper (Zhao et al. 2009).

Nitric oxide being a relevant component of auxin signal transduction pathways regulates diverse developmental processes in plants such as stem growth, PR elongation,

Fig. 5 X-Gluc staining in lateral roots (<stage VII; =stage VII; >stage VII) of *DR5::GUS Arabidopsis* treated with 0 or 50 µM copper. Developmental stages of laterals were determined according to Malamy and Benfey (1997). *Bars* = 0.5 mm



LR or adventitious root formation, gravitropic bending (He et al. 2004; Pagnussat et al. 2002; Correa-Aragunde et al. 2004; Lombardo et al. 2006). Plant cells respond to heavy metal stress with alterations in their NO metabolism; however the background mechanisms are poorly understood (Xiong et al. 2010). Copper-induced NO generation was found in *Chlamydomonas reinhardtii* cell suspension, *Panax ginseng* and *Pisum sativum* roots (refs. in Xiong et al. 2010). In the elongation zone of *Arabidopsis* roots, one-week Cu^{2+} treatment significantly decreased NO levels (Pető et al. 2011), but longer Cu^{2+} exposure (17 days) resulted in a notable increase (Fig. 6b). This phenomenon can be explained by the fact that the concentration and duration of heavy metal treatment determine the effect on NO generation (Xiong et al. 2010). Nitric oxide negatively regulates PR elongation and elevated levels of this signal molecule may contribute to the inhibition of root growth by reducing PIN FORMED 1 (PIN1)-mediated auxin transport and *DR5* expression (Fernández-Marcos et al. 2011). In roots, the major enzymatic source of NO is nitrate reductase and this enzyme produces NO under stress conditions such as aluminium exposure,

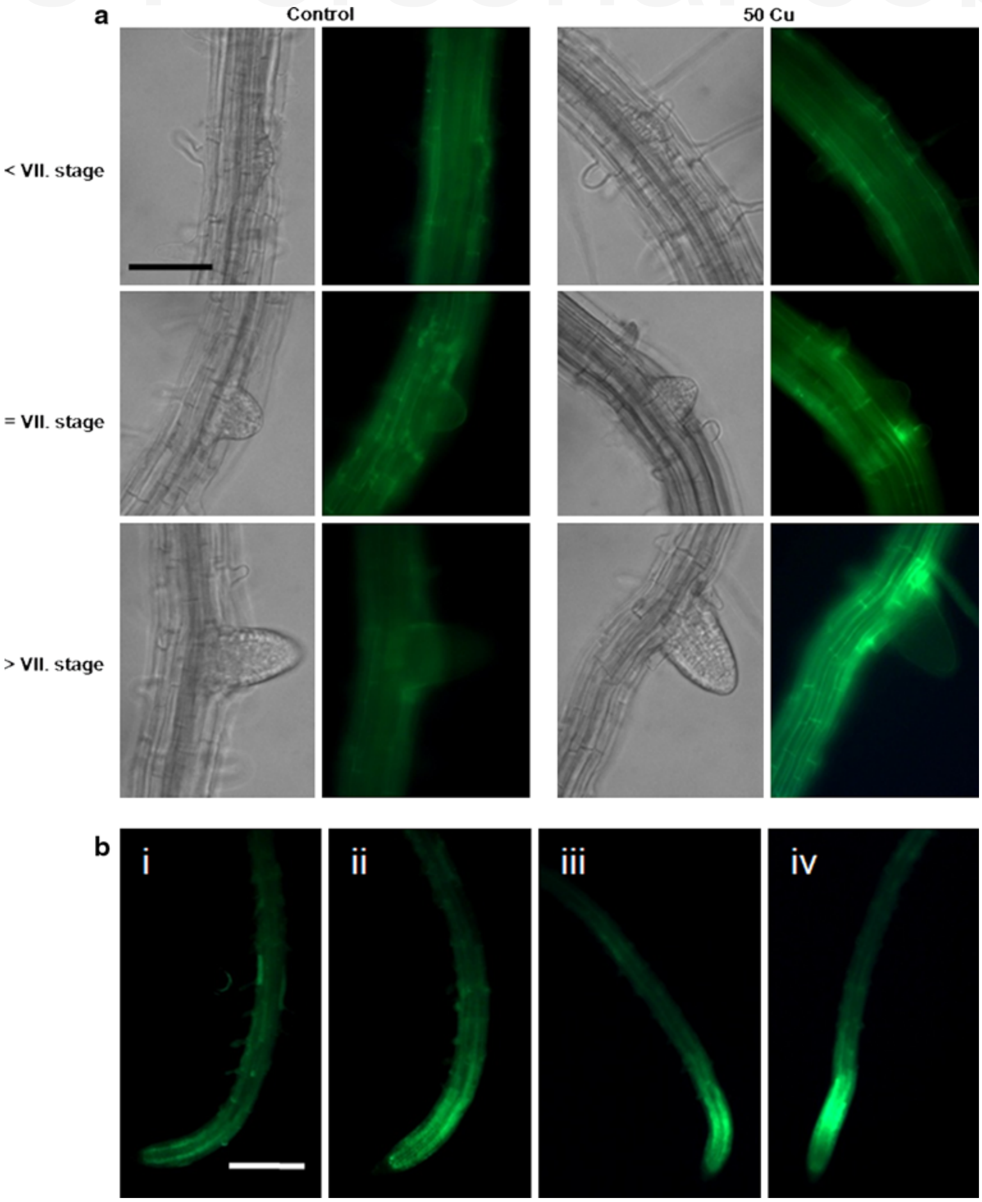


Fig. 6 **a** Nitric oxide levels (DAF-FM fluorescence) in differentially developed lateral roots (<stage VII; =stage VII; >stage VII) of control and 50 µM copper-treated *Arabidopsis*. Developmental stages of laterals were determined according to Malamy and Benfey (1997). *Bars* = 0.5 mm. **b** Nitric oxide levels in PRs of *Arabidopsis* treated with 0 (*i*), 5 (*ii*), 25 (*iii*) or 50 µM (*iv*) copper. *Bars* = 1 mm

osmotic stress or pathogen attack (Wang et al. 2010; Kolbert et al. 2010; Oliveira et al. 2010). Therefore, we investigated the possible involvement of this enzyme in Cu^{2+}-induced NO production using genetic method. In contrast to wild type, copper-induced NO production was not detected in NR-deficient *nia1nia2* primary roots, which suggests the role of NR in the synthesis of copper-induced NO (Fig. 7).

Based on these, long-term (17 d) copper excess lead to a serious inhibition of stem and root development of *A. thaliana*, during which cell elongation, division and expansion were also affected. In case of 25 µM Cu^{2+} treatment the symptoms of stress-induced morphogenic response were discovered in the root system, which may contribute to stress acclimation. The growth alterations can be partly explained by the altered auxin homeostasis in both organs. Besides hormonal system nitric oxide metabolism was also affected and the high levels of this signal molecule can contribute to the copper-induced PR shortening by inhibiting PIN1-mediated auxin transport. In roots of *Arabidopsis*, nitrate reductase enzyme may be responsible for Cu^{2+}-induced NO production.

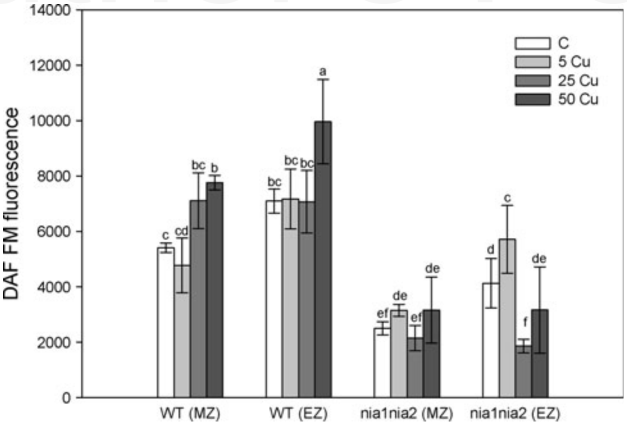


Fig. 7 Nitric oxide levels (DAF-FM fluorescence) in PR meristem (MZ) and elongation zone (EZ) of wild type (WT, *Col-0*) and *nia1nia2* roots. Values are means of 10 plants ±SE. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's test

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