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Involvement of nitric oxide and auxin in signal transduction of copper-induced morphological responses in *Arabidopsis* seedlings

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• *Background and Aims* Plants are able to adapt to the environment dynamically through regulation of their growth and development. Excess copper (Cu^{2+}), a toxic heavy metal, induces morphological alterations in plant organs; however, the underlying mechanisms are still unclear. With this in mind, the multiple signalling functions of nitric oxide (NO) in plant cells and its possible regulatory role and relationship with auxin were examined during Cu^{2+}-induced morphological responses.
• *Methods* Endogenous auxin distribution was determined by microscopic observation of X-Gluc-stained DR5::GUS arabidopsis, and the levels of NO, superoxide and peroxynitrite were detected by fluorescence microscopy. As well as wild-type, NO-overproducer (*nox1*) and -deficient (*nia1nia2* and *nia1nia2noa1-2*) arabidopsis plants were used.
• *Key Results* Cu^{2+} at a concentration of 50 μM resulted in a large reduction in cotyledon area and hypocotyl and primary root lengths, accompanied by an increase in auxin levels. In cotyledons, a low Cu^{2+} concentration promoted NO accumulation, which was arrested by nitric oxide synthase or nitrate reductase inhibitors. The 5-μM Cu^{2+}-induced NO synthesis was not detectable in *nia1nia2* or *nia1nia2noa1-2* plants. In roots, Cu^{2+} caused a decrease of the NO level which was not associated with superoxide and peroxynitrite formation. Inhibition of auxin transport resulted in an increase in NO levels, while exogenous application of an NO donor reduced DR5::GUS expression. The elongation processes of *nox1* were not sensitive to Cu^{2+}, but NO-deficient plants showed diverse growth responses.
• *Conclusions* In plant organs, Cu^{2+} excess results in severe morphological responses during which the endogenous hormonal balance and signal transduction are affected. Auxin and NO negatively regulate each other's level and NO intensifies the metal-induced cotyledon expansion, but mitigates elongation processes under Cu^{2+} exposure.

Key words: *Arabidopsis thaliana*, auxin, copper, morphological responses, nitric oxide.

INTRODUCTION

Copper (Cu^{2+}), a heavy metal, is an essential microelement for plants, but it is toxic at high concentrations. It can accumulate in various plant organs, directly causing a reduction in photosynthetic activity, enhancement of carbohydrate content, damage to lipids, proteins and DNA and cell death (Shao *et al.*, 2010). Furthermore, Cu^{2+} is known to induce morphological changes in plant organs at an early stage in the growth cycle, e.g. a reduction in cotyledon area, inhibition of primary-root elongation via arrest of root apical meristem cell division, induction of new meristem formation or reorganization of root-hair development (Pasternak *et al.*, 2005; Potters *et al.*, 2009). At the whole-plant level, auxin, ethylene and reactive oxygen species have been identified as major components of morphological alterations (Potters *et al.*, 2009).

The plant hormone auxin (indole-3-acetic acid, IAA) has long been known to promote developmental processes in the stem and root system. Root-cell elongation is enhanced by extremely low IAA concentrations, while hypocotyl elongation proved to be less sensitive to auxins. Alterations in auxin homeostasis induced by different stress factors (e.g. salinity, osmolarity, paraquat) can be partly responsible for morphological responses (Wang *et al.*, 2009; Kolbert *et al.*, 2008; Pasternak *et al.*, 2005).

Nitric oxide (NO) is a highly reactive, diffusible, lipophilic gas which acts in many tissues, regulating different physiological and biochemical processes in plants. In plant cells there are two major ways in which enzymatic NO is produced: as mammalian nitric oxide synthase (NOS)-like enzyme and nitrate reductase (NR). The animal NOS enzyme catalyses the oxidation of L-arginine to L-citrulline and NO; this activity has been reported in several plant species. Recently, an active NOS enzyme in the green alga *Ostreococcus tauri* was characterized by Foresi *et al.* (2010). Competitive inhibition of NOS by L-arginine analogues [e.g. L-nitro-arginine methyl ester (L-NAME)] implicated enzyme activity in, for example, arabidopsis or tobacco (references in Hasanuzzaman *et al.*, 2011)]. Earlier it was clearly shown that plant cells can produce NO via nitrite reduction by NR (Desikan *et al.*, 2002) and the

activity of this enzyme was considered to be the major NO source in plants (Xu and Zhao, 2003). The NR double mutant (*nia1nia2*) of arabidopsis possesses only 1 % NR activity of the wild type and has also significantly reduced endogenous NO levels (Wilkinson and Crawford, 1993; Lozano-Juste and León, 2010). The recently generated *nia1nia2noa1-2* triple mutant [impaired in NR- and NO-associated 1 (AtNOA1)-mediated pathways), having reduced levels of NO, confirms the existence of both NO biosynthetic pathways in plant cells (Lozano-Juste and León, 2010). Nitric oxide is considered to be a general plant signal, since it regulates both normal developmental processes and biotic or abiotic stress responses. In tomato, PR elongation was inhibited and lateral root (LR) generation was induced by NO, and the involvement of this molecule in auxin signal transduction has also been published (Pagnussat *et al.*, 2002; Correa-Aragunde *et al.*, 2006). Auxin-regulated LR or root-hair development and gravitropic bending could be inhibited by an NO scavenger, reflecting the relationship between auxin and NO action (Correa-Aragunde *et al.*, 2004; Hu *et al.*, 2005; Lombardo *et al.*, 2006). During abiotic stress such as a Cu^{2+} excess, plant cells respond with alterations to their NO status; however, the background mechanisms are not yet understood (references in Xiong *et al.*, 2010). Nitric oxide may act as an antioxidant by elimination of superoxide radical and by formation of the less-toxic peroxynitrite, or it plays a role in signal transduction leading to gene expression. However, the high amounts of NO generated can induce serious damage to plant cells during abiotic stress (Hasanuzzaman *et al.*, 2011).

The aim of this study was to investigate the possible involvement of auxin and NO in the signal transduction of Cu^{2+}-induced morphological changes in *Arabidopsis thaliana* seedlings and, to clarify the relationship between auxin and NO during organ development, NO-overproducer and -deficient arabidopsis plants were used to study the role of this signal molecule during Cu^{2+}-induced growth responses.

MATERIALS AND METHODS

Plant material, in vitro *culture*

The experiments were performed using 7-d-old wild-type (Col-0), DR5::GUS, *nox1*, *nia1nia2* and *nia1nia2noa1-2* mutant *Arabidopsis thaliana* seedlings. To investigate auxin-dependent gene expression, the DR5::GUS-type arabidopsis, in which the highly auxin-sensitive DR5 response element is fused to the β-glucuronidase gene (Ulmasov *et al.*, 1997) is a useful tool. *Nox1* (*cue1*) is an NO-overproducer mutant which has a larger amount of L-arginine, L-citrulline and NO content compared with the wild type. CUE1 is the chlorophyll *a/b* binding protein-underexpressed 1 gene that encodes the phosphoenolpyruvate/phosphate translocator in the plastid inner envelope (Crawford and Guo, 2005). In arabidopsis, the NR enzyme is encoded by the *NIA1* and *NIA2* genes. The *nia1nia2* double mutant has a point mutation in *NIA1* and a deletion in *NIA2* resulting in only 1 % of the enzyme activity of the wild type (Wilkinson and Crawford, 1993). The recently created n*ia1nia2noa1-2* triple mutant possesses a lower NO level in roots than in the wild type, because it is impaired in NOS (AtNOA1)- and NR (NIA/NR)-mediated NO biosynthesis (Losano-Juste and León, 2010). Seeds of all plant lines were surface sterilized with 5 % (v/v) sodium hypochlorite for 20 min and rinsed five times with sterile distilled water before being transferred to half-strength MS (Murashige and Skoog, 1962) medium [1 % (w/v) sucrose and 0·8 % (w/v) agar] supplemented with $CuSO_4$ at 0-, 5-, 25- or 50-μM concentrations. The Petri dishes were stratified at 4 °C for 24 h and then placed vertically in a growth chamber (FITOCLIMA S66PLH; Aralab, Portugal), where plants were grown under controlled conditions at a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1} (16/8 h day/night period), at a relative humidity of 55–60 % and a temperature of 25 ± 2 °C. Seven-day-old plants were treated with 100-μM sodium nitroprusside (SNP), an NO donor, or 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxid potassium salt (cPTIO), an NO scavanger. As a polar auxin transport inhibitor, naphthylphthalamic acid (NPA) was applied at a concentration of 10 μM. All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Morphological measurements

Primary root length (mm) was measured manually using a scale; the hypocotyl length (mm) was determined under a Zeiss Axiowert 200M microscope using at ×5 magnification. Cotyledon diameters (mm) were measured under the microscope and radii were calculated. Cotyledons of 7-d-old arabidopsis can be perceived as round-shaped organs; therefore, their area (mm^2) was estimated by the formula $r^2\pi$.

Histochemical staining

β-Glucuronidase activity in transgenic DR5::GUS plants was visualized by incubating whole seedlings for 15 h in a solution containing 1 mM X-gluc (5-bromo-4chloro-3-indolyl-β-D-glucuronic acid), 0·1 M phosphate buffer (pH 7·0), 10 mM EDTA, 0·1 % (v/v) Triton X-100 and 1 mM $K_3Fe(CN)_6$ according to Jefferson *et al.* (1987). Samples were washed with 70 % (v/v) ethanol and prepared on microscopic slides. For the experiments, a Zeiss Axiowert 200M inverted microscope and a Zeiss Axioskope 2000-C stereomicroscope (Carl Zeiss, Jena, Germany) were used.

Fluorescence microscopy

Nitric oxide levels in cotyledons and roots of arabidopsis were visualized by an NO-specific fluorescent dye, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) according to Corpas *et al.* (2009). Whole seedlings were vacuum infiltrated for 10 min and incubated for 30 min in 10-μM DAF-FM DA (in 10 mM Tris–HCl, pH 7·4) solution in the dark at 25 ± 2 °C and were washed twice within 30 min with Tris–HCl. Dihydroethidium (10 μM in Tris–HCl) was used to detect superoxide radical in arabidopsis plants (Corpas *et al.*, 2009). Whole seedlings were incubated in dye solution at 37 °C for 30 min and washed twice with Tris–HCl buffer. To detect the peroxynitrite ion, plants were dyed with 10-μM aminophenyl fluorescein (Molecular Probes) for 60 min in the dark at room temperature and were washed twice with

Tris–HCl buffer (Corpas *et al.*, 2009). Observations were carried out with a Zeiss Axiowert 200M microscope (Carl Zeiss) equipped with a high resolution digital camera (Axiocam HR, HQ CCD) and filter set 10 (exc., 450–490 nm; em., 515–565 nm) or filter set 9 (exc., 450–490 nm; em., 515–∞ nm). The FLUAR ×5/0·12 NA and FLUAR ×10/0·25 objective lens were employed. Fluorescent intensities were measured on digital images within circular areas, 60 μm or 120 μm in radius, using Axiovision Rel. 4·8 software. The radii of the circles were not modified during the experiments. The selected fluorescent images are representative of similar results from the two repetitions.

Statistical analysis

Results are expressed as mean ± s.e. Statistical analysis was performed with SigmaStat 11. software using analysis of variance (ANOVA, $P < 0{\cdot}05$) and Duncan's test for multiple comparison analyses. All experiments were carried out at least twice. In each treatment at least ten samples were measured.

RESULTS

Cu^{2+} impacts on stem and primary root growth in arabidopsis seedlings

Treatment of 1-week-old arabidopsis seedlings with Cu^{2+} resulted in altered stem and root growth. Compared with the control situation, a low Cu^{2+} concentration (5 μM) did not significantly increase the cotyledon area, while 50-μM Cu^{2+} resulted in a reduction in this parameter. In the case of 25-μM Cu^{2+}, enhancement of cotyledon size was observed (Fig. 1A). Elongation of the hypocotyl significantly increased as a result of low Cu^{2+} concentration and it was not affected by 25-μM Cu^{2+}, while the higher metal concentration resulted in reduced hypocotyl length (Fig. 1B). The length of the primary root significantly decreased after treatment with 25- or 50-μM Cu^{2+} (Fig. 1C).

Cu^{2+} modifies auxin and NO homeostasis

Changes in auxin homeostasis were investigated using the DR5::GUS arabidopsis reporter line. In cotyledons of control plants, DR5 expression was restricted to the tips, while in Cu^{2+}-treated plants it extended to the whole cotyledon blade (Fig. 2A–H). A Cu^{2+} excess resulted in an enhancement of DR5::GUS expression suggesting increased auxin levels in primary root apices (Fig. 2I–L). In both organs, the effect of Cu^{2+} proved to be concentration dependent. The levels of the general signal molecule, NO, were also influenced by Cu^{2+} treatments. Interestingly, a low Cu^{2+} concentration (5 μM) caused a significant increase in NO-specific fluorescence in cotyledons, although 25- and 50-μM Cu^{2+} did not significantly reduce NO levels compared with the control (Fig. 3A, B). However, in the primary root meristem the level of NO was not influenced by 25- and 50-μM Cu^{2+} and the NO content in the elongation zone significantly decreased (Fig. 3C, D). To examine the possible enzymatic source of Cu^{2+}-induced NO in cotyledons, biochemical (treatments of wild-type plants with L-NAME or tungstate) and genetic (*nia1nia2* and *nia1nia2noa1-2* mutants) experiments were carried out. Application of the L-arginine analogue L-NAME or the NR inhibitor tungstate significantly reduced the NO levels in Cu^{2+}-treated plants compared with plants which were treated with Cu^{2+} alone (Fig. 4A). In cotyledons of untreated *nia1nia2* and *nia1nia2noa1-2* arabidopsis, lower NO levels were found compared with wild type, and Cu^{2+}-induced NO accumulation could not be detected in cotyledons of mutant lines (Fig. 4B). To test the possibility, that under Cu^{2+} excess, superoxide formation and its reaction with NO yielding peroxynitrite may contribute to a reduction in NO levels in root tips, O_2^- and $ONOO^-$ were detected within the primary root of arabidopsis using fluorescent staining methods. Superoxide-dependent fluorescence showed maximal intensities in primary root meristem and no O_2^- accumulation was observed in the elongation zone as an effect of Cu^{2+} (Fig. 5A). The presence of peroxynitrite was detected in the differentiation zone at the sites of root hairs and Cu^{2+} did not induce the accumulation of this radical in the elongation zone of the primary root (Fig. 5B). The tissue distribution of the different molecules examined is presented in Fig. 5C.

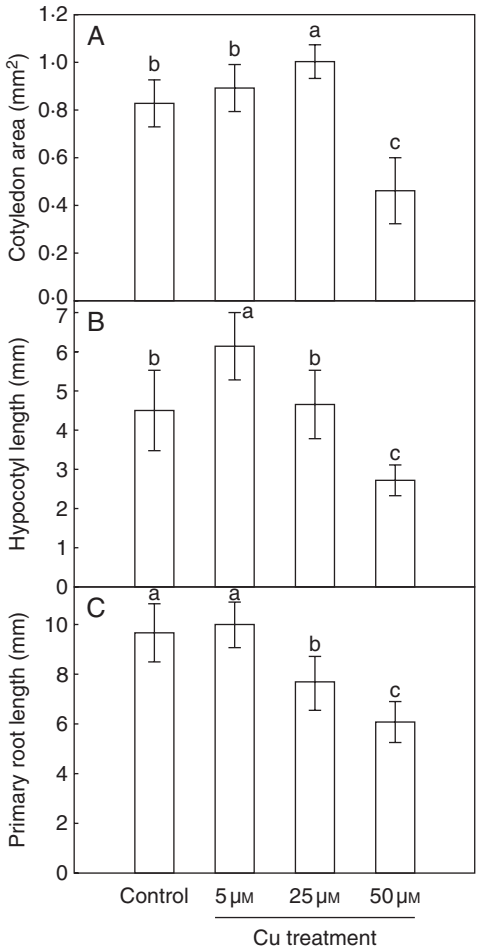

FIG. 1. (A) Cotyledon area, (B) hypocotyl length and (C) primary root length of wild-type arabidopsis seedlings grown in the presence of 0, 5, 25 or 50 μM copper for 7 d. Values are means of ten plants ± s.e. Different letters indicate significant differences according to Duncan's test ($P < 0{\cdot}05$).

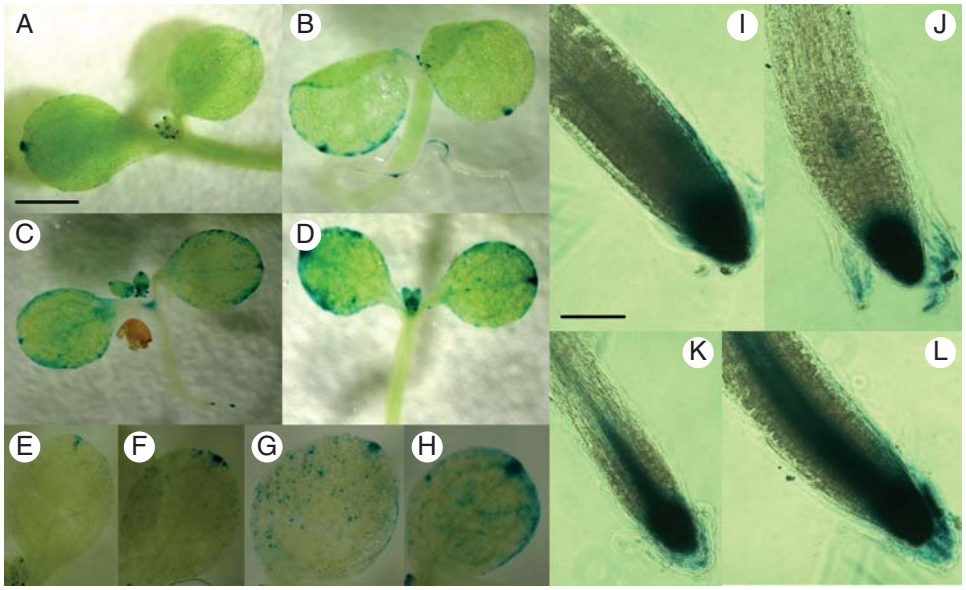

FIG. 2. (A–H) Cotyledons and (I–L) primary root tips of GUS-stained DR5::GUS arabidopsis treated with 0-, 5-, 25- or 50-μM $CuSO_4$ for 7 d: (A, E and I) control; (B, F and J) 5-μM Cu; (C, G and K) 25-μM Cu; (D, H and L) 50-μM Cu. Scale bars: (A–H) 1 mm; (I–L) = 0·5 mm.

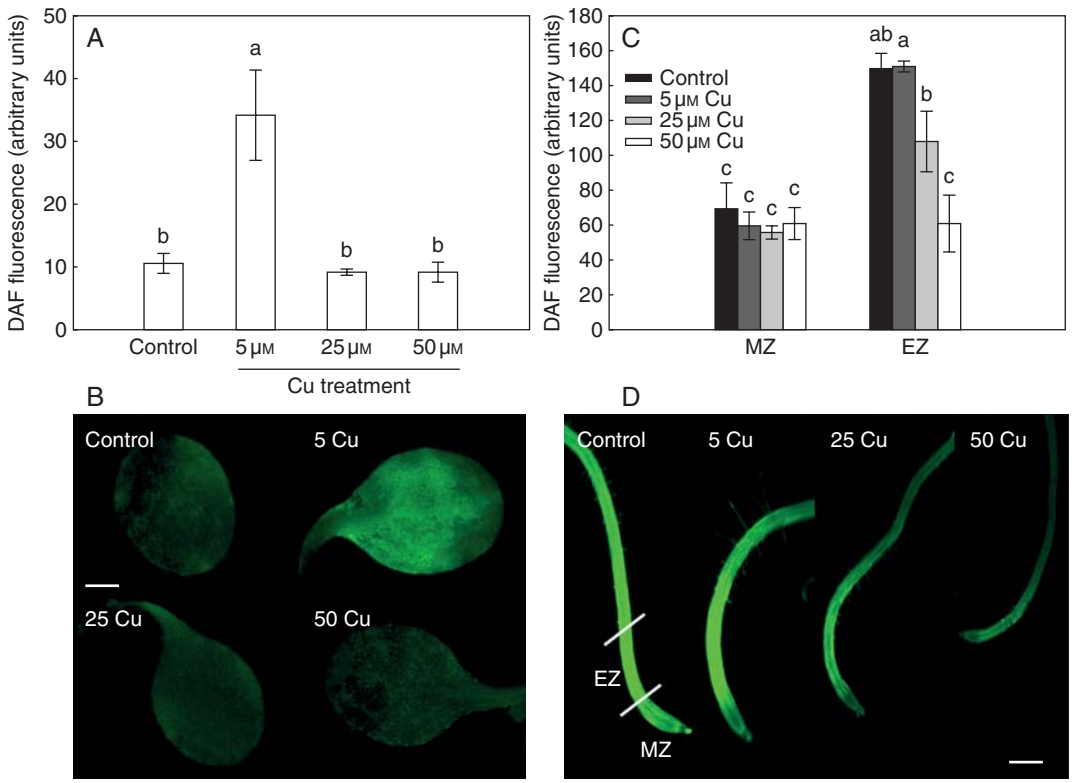

FIG. 3. NO production indicated by DAF fluorescence in (A) cotyledons and (C) meristematic zone (MZ) and elongation zone (EZ) of the primary root of wild-type arabidopsis. Values are means of ten plants ± s.e. Different letters indicate significant differences ($P < 0·05$) according to Duncan's test. (B, D) Representative fluorescent microscopic images of cotyledon and primary root, respectively. Scale bars = 1 mm.

The NO–auxin relationship during organ development under Cu^{2+} excess

The polar auxin-transport inhibitor NPA was applied to reduce the auxin content in tissues. In the cotyledons NPA reduced Cu^{2+} (5 μM)-induced NO accumulation, but in the case of higher metal concentrations NPA treatment caused a significant elevation in NO levels (Fig. 6A). Treatment of seedlings with Cu^{2+}, together with NPA,

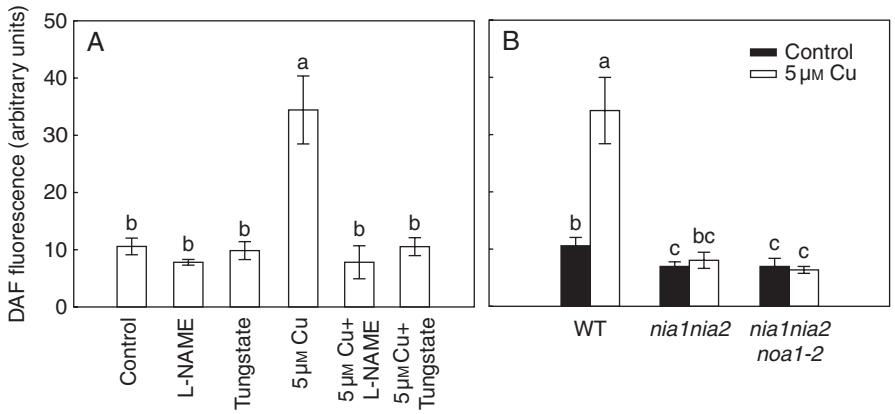

FIG. 4. (A) NO production indicated by DAF fluorescence in cotyledons of control and treated (7 d) wild-type arabidopsis. (B) DAF fluorescence in cotyledons of control and 5 μM Cu-treated wild-type (WT), *nianial2* and *nia1nia2noa1-2* mutant arabidopsis. Values are means of ten plants ± standard error. Different letters indicate significant differences according to Duncan's test ($P < 0.05$).

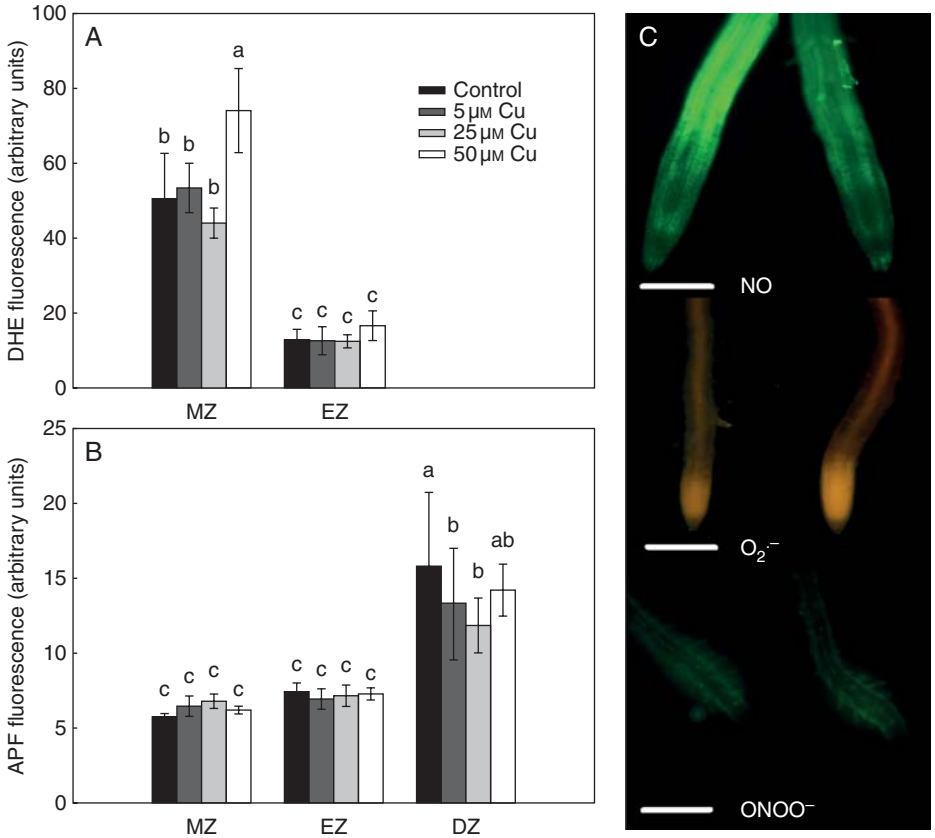

FIG. 5. (A) Superoxide [dihydroethidium, DHE) fluorescence, and (B) peroxynitrite (aminophenyl fluorescein, APF) fluorescence levels in meristematic (MZ), elongation (EZ) and differentiation (DZ) primary root zones of control and copper-treated arabidopsis. Values are means of ten plants ± s.e. Different letters indicate significant differences according to Duncan's test ($P < 0.05$). (C) Different tissue localization of NO, O_2^- and $ONOO^-$ within the primary root: left, control; right, 50-μM Cu-treated. Scale bars: ($ONOO^-$ and $O_2^{\cdot-}$) = 1 mm; (NO) = 0·5 mm.

resulted in significantly higher NO levels in the elongation zone of primary roots (Fig. 6B). Exogenously applied NO (in the form of SNP) considerably reduced the Cu^{2+}-induced *DR5* gene expression in cotyledons and primary roots, while in the case of + cPTIO the X-gluc staining pattern was more extended in the primary root tips or was similar to the control in the cotyledons (Fig. 7A–L).

*NO mutants (*nox1*,* nia1nia2*,* nia1nia2noa1-2*) show altered morphology under Cu^{2+} excess*

To explore the possible involvement of NO in the signal transduction of a Cu^{2+}-induced morphological response, observations on the mutant lines were carried out. Nitric oxide-overproducing (*nox1*) and -deficient (*nia1nia2* and *nia1nia2noa1-2*) arabidopsis lines were treated with Cu^{2+} and the resulting morphological parameters were determined. Interestingly, under control conditions, a significant difference was established in cotyledon areas of the NO-overproducer (*nox1*) and NO-deficient (*nia1nia2noa1-2*) mutants, although their differences were not significant compared with the wild type. In *nox1* plants 50-μM Cu^{2+} caused a serious decrease in cotyledon size, whereas in NO-deficient seedlings the Cu^{2+}-induced decline seemed to be moderate (Fig. 8A). Hypocotyl lengths of control *nox1* and triple mutant seedlings were significantly shorter than those of wild type, while, in the case of *nia1nia2* hypocotyls, no significant difference compared with wild type was found. In NO-deficient mutants, all the applied metal concentrations resulted in decreased hypocotyl length, although in *nox1* Cu^{2+} had no effect on hypocotyl elongation (Fig. 8B). Under control conditions, significantly shorter primary roots were observed in all the mutant lines examined than in the wild type; however, no significant difference was established among them. In the case of wild-type and NO-deficient *nia1nia2* plants a notable decrease of primary root length was noticed, but in *nox1* and *nia1nia2noa1-2* plants this phenomenon was not prominent (Fig. 8C).

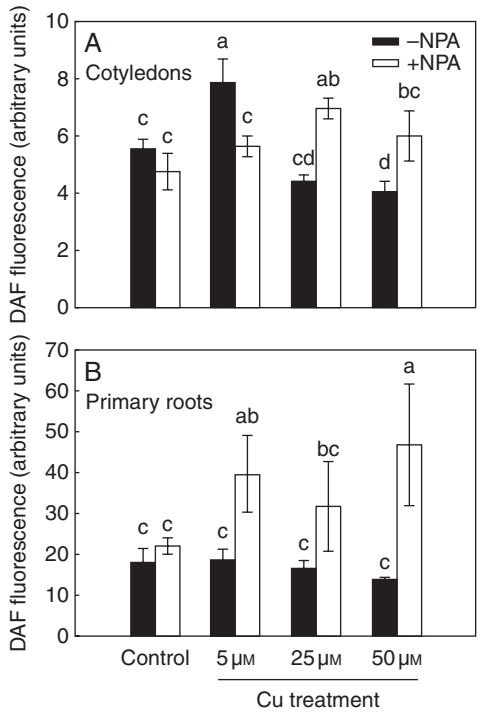

FIG. 6. NO production indicated by DAF fluorescence in (A) cotyledons and (B) primary roots of arabidopsis treated with 0-, 5-, 25- or 50-μM copper with or without 10-μM NPA for 7 d. Values are means of ten plants ± s.e. Different letters indicate significant differences according to Duncan's test ($P < 0{\cdot}05$).

DISCUSSION

Exposure to copper induces morphological alterations in the stem system of the plant, as was shown in Cu^{2+}-treated arabidopsis, in which the number and size of leaves and the rosette diameter were reduced (Pasternak *et al.*, 2005). Copper also blocks the division of root apical meristem cells, hence elongation of the primary root is inhibited. The size of the elongation zone and the root hair density are also affected by Cu^{2+} (Pasternak *et al.*, 2005). Similar morphological changes were found under mild osmotic or salt stress and beta-amino-butyric acid treatment in pea and arabidopsis (Kolbert *et al.*, 2008, 2010; Wu *et al.*, 2009; Zolla *et al.*, 2010). During the present study, Cu^{2+}-induced alterations in the stem and root system were determined in 7-d-old arabidopsis seedlings: the lowest Cu^{2+} concentration (5 μM) resulted in a slightly increased cotyledon area and hypocotyl and primary root length, while a more serious Cu^{2+} excess (50 μM) caused a significant inhibition in stem and root development (Fig. 1). The root system proved to be more sensitive to Cu^{2+} exposure, since its growth was affected by 25-μM Cu^{2+}, while in the case of stem parameters this Cu^{2+} concentration had no effect. Similar results were obtained by Pasternak *et al.* (2005),

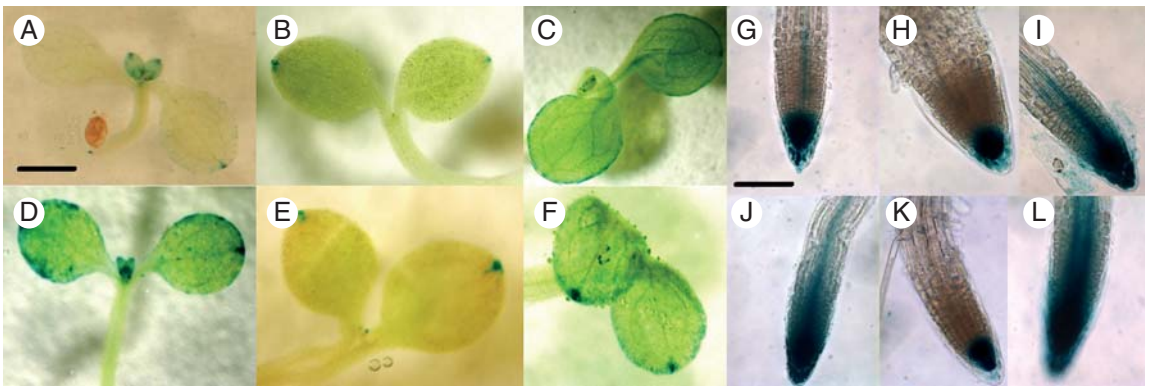

FIG. 7. Expression of the auxin-responsive reporter gene DR5::GUS in cotyledons (A–F) and primary root tips (G–L) of 7-d-old DR5::GUS arabidopsis reporter line grown in the presence of Cu: (A, G) control; (B, H) 100-μM SNP; (C, I) 100-μM cPTIO; (D, J) 50-μM Cu; (E, K) 50-μM Cu + SNP; (F, L) 50-μM Cu + cPTIO. Scale bars = 1 mm.

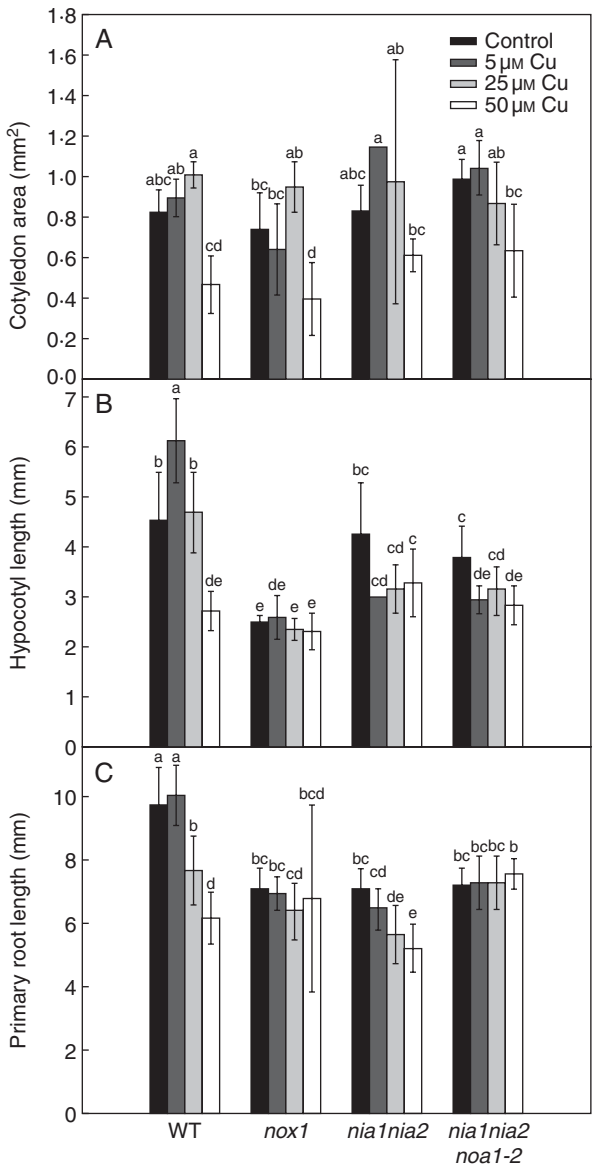

FIG. 8. (A) Cotyledon area, (B) hypocotyl length and (C) primary root length of 7-d-old wild-type, *nox1*, *nia1nia2* and *nia1nia2noa1-2* mutant arabidopsis grown in the presence of 0-, 5-, 25- or 50-μM copper. Values are means of ten plants ± s.e. Different letters indicate significant differences according to Duncan's test ($P < 0{\cdot}05$).

where Cu^{2+} had a relatively small inhibitory effect on cotyledon expansion. The greater sensitivity of the root can be explained by the larger proportion of the Cu^{2+} taken up and accumulating in the root system (Lequeux *et al.*, 2010).

Alteration of auxin metabolism and transport plays an important role in developmental changes induced by heavy metals (Potters *et al.*, 2009). In cotyledons of Cu^{2+}-treated seedlings, increased DR5 expression was found compared with control. Similar to results of Lequeux *et al.* (2010), the activity of the DR5 promoter in Cu^{2+}-treated seedlings was more evident in root regions above the meristem. In microscopic images with higher magnification, X-Gluc staining was also seen to be pronounced in meristematic root zones of plants treated with 25- or 50-μM Cu^{2+} (Fig. 2), similar to root tips during 150-mM NaCl treatment (Wang *et al.*, 2009). Transcriptome analysis of Cu^{2+}-regulated genes revealed that the expression of auxin biosynthetic genes (e.g. IAA amide synthase, tryptophan synthase) is induced in response to Cu^{2+} treatment (Zhao *et al.*, 2009), which might be responsible for an increase in auxin levels under Cu^{2+} excess.

As the effect of Cu^{2+}, changes in NO levels were evoked in both organs. In cotyledons, 5-μM Cu^{2+} caused a significant NO accumulation, while more serious excess reduced the NO content (Fig. 3). As a result of Cu^{2+} treatment, NO generation was detected also in *Chlamydomonas reinhardtii* and *Brassica juncea*, *Pisum sativum* and *Panax ginseng* roots (Bartha *et al.*, 2005; Tewari *et al.*, 2008; Zhang *et al.*, 2008). Under control conditions, a notable tissue specificity of NO was found within the primary root, because much higher NO levels were detected in the elongation zone compared with in the meristem. Similar results to this elevated NO-dependent fluorescence in the distal part of the transition zone compared with meristem were found by Illéš *et al.* (2006). In the elongation zone of the primary root, the Cu^{2+} treatment caused a significant NO decrease, while the NO content of meristematic zone was not affected (Fig. 3). A heavy metal-induced decrease of NO levels was observed, *inter alia*, in pea leaves and roots (Rodriguez-Serrano *et al.*, 2009); however, it should be noted that the concentration of the heavy metal applied, the treatment conditions, the age of the plant and the variety of tissues examined all affect NO production (Xiong *et al.*, 2010). The possible mechanisms leading to the NO level changes in both organs were biochemically and genetically examined, and the results showed that in cotyledons both L-arginine- and NR-dependent biosynthetic pathways can be responsible for Cu^{2+}-induced NO accumulation. Involvement of both NO synthetic pathways in cotyledons was also confirmed by detecting a reduced NO level in *nia1nia2noa1-2* plants compared with wild type (Fig. 4). Although NR mainly functions in the roots, there is evidence for NR-dependent NO synthesis in the aerial parts of the plant as well (Bright *et al.*, 2006; Sang *et al.*, 2008; Xu *et al.*, 2010). Related to the decrease in the NO content in the root elongation zone, it is tempting to hypothesize that Cu^{2+}-induced superoxide radicals eliminate NO by the reaction yielding peroxynitrite. The rate constant for the reaction between NO and O_2^- is controlled at near diffusion values (Yamasaki *et al.*, 2011), therefore NO and superoxide will most likely react if they have similar tissue localization. The present results do not support the hypothesis, because neither an elevation of the superoxide level in elongation zone, nor any co-localization of NO, O_2^- or $ONOO^-$ was observed within the primary root under Cu^{2+} excess (Fig. 5). The background mechanism of Cu^{2+}-induced NO content decrease may be the down-regulation of either or both NO biosynthetic pathways (L-arginine- and/or nitrate-dependent), as has been found under aluminium exposure (Tian *et al.*, 2007; Wang *et al.*, 2010).

In addition, we wanted to explore the relationship between hormonal (auxin) and signal (NO) components in the signal transduction of Cu^{2+}-induced morphological responses. Based on the results it can be concluded that auxin transport

is needed for 5-μM Cu^{2+}-induced NO accumulation in cotyledons, i.e. auxin positively regulates NO synthesis under mild Cu^{2+} exposure. However, in the case of higher Cu^{2+} concentrations the lack of auxin resulted in an increase in NO levels. Plants in which the auxin level was reducd by NPA showed significantly higher NO fluorescence in the roots compared with plants treated with Cu^{2+} alone, which suggests a negative regulation of the NO level by auxin in the primary root as well (Fig. 6). These findings seem to conflict with most of the published results, where NO is described as a positive regulator component of auxin signal transduction (see references in Correa-Aragunde *et al.*, 2007). Although, those findings refer to other physiological processes such as adventitious or lateral root development, in the present experimental system exogenous indole-3-acetic acid (10^{-6} M) did not induce NO generation either in cotyledons or in primary roots (data not shown). When endogenous NO levels were enhanced by donor application the auxin-sensitive gene expression notably decreased in cotyledons and primary root tips, which implies an inhibitory link between the hormonal (auxin) and signal (NO) components of Cu^{2+}-induced morphological changes (Fig. 7). These results were also confirmed by genetic studies during which Cu^{2+}-induced growth response was compared in wild-type, NO over-producer (*nox1*) and NO-deficient (*nia1nia2* and *nia1nia2noa1-2*) arabidopsis seedlings. The cytological background mechanisms of the developmental events examined are different: cell division, which is mainly responsible for cotyledon expansion/growth and cell elongation, occurs during hypocotyl and primary root growth. In the case of a NO excess, smaller cotyledon areas were observed, whereas NO-deficient mutants possess slightly larger cotyledons compared with wild-type plants; moreover, Cu^{2+}-induced reduction in cotyledon size was pronounced under NO excess. In contrast, with regards to hypocotyl cell elongation, NO-deficient mutants showed an enhanced sensitivity compared with the wild type, while in *nox1* no morphological response to Cu^{2+} was found. However, *nox1* produces shorter hypocotyl lengths than wild-type plants (Fig. 8), similar to the results of Lee *et al.* (2008). Regarding primary-root elongation, the behaviour of mutants was not obvious. Under control conditions, the primary root length of NO-over-producer and -deficient mutants was smaller than that of the wild type, which can also be supported by data in the literature (He *et al.*, 2004; Lozano-Juste and León, 2010). According to Lozano-Juste and León (2010) the NO level in the primary root tip of the triple mutant was much lower than that of *nia1nia2*; moreover, an enhanced NO content in primary roots of *nox1* was detected during the present experiments (data not shown). Copper exposure did not cause primary root shortening of *nox1* and *nia1nia2noa1-2* mutants; however, it resulted in a heavy reduction in the length of the *nia1nia2* primary root. The different root growth responses of NO-deficient mutants to Cu^{2+} can be explained by the hypothesis that close control of NO status is needed to regulate root architecture. Nitric oxide content being over or under the optimal level results in the inhibition of the Cu^{2+}-triggered root morphological response.

Taken together, these results clearly show that Cu^{2+} excess leads to notable morphological responses in plant organs. During these developmental alterations both the endogenous hormonal balance and signal transduction are affected. It was shown that Cu^{2+}-induced NO accumulation in cotyledons is associated with both putative enzymatic pathways (L-arginine- and NR-dependent), while the NO decrease in primary roots occurs independently from superoxide and peroxynitrite generation. Under mild Cu^{2+} exposure in cotyledons, auxin positively regulates NO synthesis, while NO inhibits auxin-dependent gene expression, which refers to a negative feedback regulation. Under serious Cu^{2+} excess (25 and 50 μM) the hormonal (auxin) and signal (NO) components in signal transduction of morphological changes proved to be negative regulators of each other in both organs. With the help of mutant plants possessing altered NO levels, the possible involvement of this signal molecule in Cu^{2+}-induced morphological responses was demonstrated. In the case of cotyledon growth (cell division), NO excess intensifies the metal-induced growth alterations; but contrary to this, during cell elongation (hypocotyl and primary root growth) enhanced NO levels mitigate growth responses. Moreover, primary-root elongation proved to be strictly regulated by the endogenous NO status under Cu^{2+} exposure.

Since most of the developmental processes are determined by hormonal interactions, additional hormonal actions (e.g. ethylene, cytokinin) must be considered during Cu^{2+}-induced growth responses. For example, cytokinins are considered to be important regulators of cotyledon expansion and are also able to induce rapid NO generation in arabidopsis, parsley and tobacco (Tun *et al.*, 2001); therefore in the future it is crucial to examine hormonal interactions in order to explore the complex signal transduction network of Cu^{2+}-induced morphological responses.

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