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Research article

Early response of wheat seminal roots growing under copper excess

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ABSTRACT

Growth reduction caused by copper excess during plant photoautotrophic metabolism has been widely investigated, but information regarding early responses of root apical meristem (RAM) to toxic concentrations of this metal at the initial heterotrophic stage is certainly scarce. We analysed some determinants of seminal root growth in developing wheat seedlings germinated in the presence of 1, 5 and 10 μM $CuCl_2$, focussing on oxidative damage to cell membrane and to proteins, and investigated the expression patterns of some genes relevant to cell cycle progression and cell expansion. The proliferation zone of the RAM was shorter under 5 and 10 μM $CuCl_2$. Cyclin D and CDKA levels remained unchanged in the root apexes of wheat seedlings grown under these Cu^{2+} concentrations, but more carbonylated levels of both proteins and less ubiquitinated-cyclin D was detected under 10 μM $CuCl_2$. Increased levels of ROS were revealed by fluorescent probes at this Cu^{2+} dose, and severe cell membrane damage took place at 5 and 10 μM $CuCl_2$. Several genes related to retinoblastome phosphorylation and therefore involved in the transition from G1 to S cell cycle stage were found to be downregulated at 10 μM $CuCl_2$, while most expansin genes here analysed were upregulated, even at a non-toxic concentration of 1 μM.

These results together with previous findings suggest that a "common" signal which involves oxidative posttranslational modifications of specific cell cycle proteins may be necessary to induce root growth arrest under Cd^{2+} and Cu^{2+} stress.

1. Introduction

Copper is one of the transition metals that are essential for plant growth and development (Yruela, 2005). This metal can exist in different oxidation states (Cu^{+} and Cu^{2+}) and as a redox-active element, it is capable of directly generate reactive oxygen species (ROS) in the metal catalysed Haber–Weiss reaction (Stohs and Bagchi, 1995).

Copper participates as cofactor in proteins with enzyme activity (i.e: superoxide dismutase, oxidases and oxygenases) and in proteins involved in electron-transfer, like plastocyanin (Solomon et al., 1996). However, the excess of copper has become a serious environmental and economic problem nowadays (Kopittke et al., 2010). The concentration of copper is increasing in the environment, and copper may enter the food chain endangering people's health (Arora et al., 2008). In metal-contaminated environments, the root system is the main site of copper accumulation (Gajewska and Skłodowska, 2010; Lequeux et al., 2010; Martinez-Peñalver et al., 2012).

An interesting point regarding plant mineral acquisition is that even when metal uptake could be performed through the entire root by means of metal transporters; the lack of the Caspary band at the root tip allows direct xilema load. Analysis of Cd flux profile along a wheat root showed that metal influx at root apex was significantly higher than that at more distal positions back from the apex (Piñeros et al., 1998). So, root apex might be particularly prone to suffer toxic effects caused by metals.

Cellular proliferation in all eukaryotes including plants is a coordinated process governed by cell cycle. Cell cycle progression involves a protein complex consisting of cyclin dependent kinases (CDKs) associated with positive regulators called cyclins, which act as multiple regulating proteins (Inzé, 2005). During the G1-S transition stage in the cell cycle, the cyclin-dependent kinase CDKA interacts with D-type cyclin to form a complex which further phosphorylates retinoblastoma protein (Rb). The resulting

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phosphorylated Rb loses its binding affinity for the family of E2F-DP transcription factors, allowing cell cycle progression to S phase (Vandepoele et al., 2005; Hirano et al., 2008). In plants, D-type cyclins (CycD) link cell cycle to extracellular and developmental signals (Dewitte et al., 2007; Riou-Khamlichi et al., 1999, 2000) and, as it happens with many other critical proteins involved in cell proliferation, their half-life is regulated by the ubiquitin-proteasome system (UPS) (Inzé, 2005).

Along with cell proliferation, cell expansion is responsible for organ growth in plants (Beemster et al., 2003; Shishkova et al., 2008). Plant expansins comprise a large multigene family identified as mediators of acid-induced wall extension. Four families of genes encoding expansins have been identified and are known as alpha-expansins (EXPA), beta-expansins (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Choi et al., 2008). These are cell wall loosening proteins that induce stress relaxation and extension of plant cell walls (Cosgrove, 2000).

Growth reduction caused by copper excess during photoautotrophic metabolism has been demonstrated, and was mainly related to damage on photosynthetic system, which in turn alters plant carbon balance (Hattab et al., 2009; Martínez-Peñalver et al., 2012; Galmés et al., 2013). It has also been reported that exposure to a high Cu^{2+} dose lead to root system architecture remodelling (Potters et al., 2007, 2009). Root apex includes the root apical meristem (RAM), where plant primary (axial) growth takes place. At RAM level, high copper concentrations altered structure (Doncheva, 1998; Liu et al., 2009b), damaged cell integrity in the root transition zone (Madejón et al., 2009), caused cell death (Yeh et al., 2003), impacted on hormone distribution and mineral profile (Lequeux et al., 2010) and affected microtubules polymerization (Liu et al., 2009a).

Information regarding the early response of wheat seminal root growth under copper excess is rather limited, and its impact on RAM when seedlings are still under chemoheterotrophic metabolism has not been investigated in depth. In this study we focused our interest in some determinants of wheat root growth and propose a ROS-mediating mechanism involved in early cell cycle arrest through oxidative protein damage. We also investigated the expression profile of some expansin genes.

2. Material and methods

2.1. Plant material and growing conditions

Fifteen seeds of wheat (*Triticum aestivum* L. cv 75 Aniversario supplied by Buck, Argentina) were placed in flasks containing 30 ml of demineralised water (control) or different amounts of $CuCl_2$ salt to give final concentrations of 1, 5, 10 and 100 μM. Flasks were placed in a rotary shaker at 24 ± 2 °C in darkness at 100 rpm. After 48 h, root length was assessed by measuring the radicle of each seedling. Dry weight (DW) was determined after drying the roots at 80 °C for 7 days or up to constant weight. Seedlings were gently washed with distilled water and determinations were performed on 5 mm root segments obtained from the root apex. Experiments were repeated three times with five replicates per treatment.

2.2. Root cell length measurements

Roots were fixed overnight in FAA (paraformaldehyde:ethanol:glacial acetic acid; (1:4:1) and processed by the inclusion of paraffin technique. Root sections were stained with safranin-fast green and mounted with DPX. Cell lengths and numbers were measured using the software analysis package, ImageJ.

2.3. Proline

Proline content was determined in root extracts prepared in 3% (w/v) 5-sulfosalicylic acid, according to the method described by Bates et al. (1973). After centrifuging the homogenates at 3000 × g for 15 min, the supernatant fractions were used for the assay.

2.4. Imaging of ROS

The presence of ROS was determined in wheat root apexes using 25 μM of 2′,7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and 10 μM of dihydroethidium (DHE) prepared in 10 mM Tris–HCl (pH 7.4) as probes for H_2O_2/peroxides and $O_2^{\bullet-}$, respectively (Rodríguez-Serrano et al., 2009). After 48 h of Cu^{2+} treatment, the root apex was incubated for 30 min at 37 °C in darkness with the corresponding probe. Where indicated, roots were incubated with 1 mM ascorbic acid (ASC) (a H_2O_2 scavenger) or 4 mM $MnCl_2$ (a $O_2^{\bullet-}$ dismutating agent) prior to the H_2DCFDA and DHE treatments, respectively, in order to verify that the fluorescent signal was actually due to H_2O_2/peroxides and $O_2^{\bullet-}$, respectively. A microscope Olympus *BX50* (Olympus Optical Co., Tokyo, Japan) with an excitation filter of 490 nm was used to detect green fluorescence of DCF-DA (485 nm excitation, 530 nm emission) and red fluorescence of DHE (490 nm excitation, 520 nm emission). Exposure times were equal for all samples. Autofluorescence was not observed in unstained controls.

2.5. Evans blue root assay

Cell membrane integrity was determined by Evans blue staining method (Baker and Mock, 1994). This staining is considered an indicator of cell death, as the dye may only accumulate inside cells if membranes are damaged.

Seedlings were submerged in 0.25% (w/v) aqueous Evans blue solution for 20 min at room temperature and then washed with distilled water to remove the excess of stain. Then seedlings were incubated in the distilled water overnight, after which 5 mm-segments of the root tips (apical region) were excised from 10 randomly selected seedlings. To release trapped Evans blue from cells, root were submerged in 50% (v/v) methanol–1% (w/v) SDS for 1 h at 50 °C and then centrifuged at 3000 g for 3 min. Optical density of the supernatant was determined spectrophotometrically at 595 nm.

2.6. Oxidative damage to lipids

The amount of thiobarbituric acid reactive substances (TBARS) was determined as an index of lipid peroxidation according to Heath and Packer (1968). Root apexes (0–5 mm) samples of 0.1 g were homogenised in 1 mL of 20% (w/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 3000 × g for 20 min. To 1 mL aliquots of the supernatant, 1 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA and 100 μL 4% (w/v) butylhydroxytoluene (BHT) in ethanol were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The homogenates were centrifuged at 10,000 × g for 15 min and the absorbance was measured at 532 nm. The concentration of TBARS was calculated using an ε of 155 mM^{-1} cm^{-1}.

2.7. Western blot determinations

Protein extracts were prepared by homogenizing root apexes (0–5 mm) samples of 0.1 g in 1 mL of 100 mM phosphate buffer (pH7.4) containing 120 mM KCl and 1 mM EDTA. The homogenates were centrifuged at 10,000 × g for 20 min and the supernatants

Table 1
Primers designed for *Triticum aestivum* L. to amplify several cDNA fragments.

Gene	Sense and antisense primers	PCR conditions	Amplicon size (pb)	Accession number (GenBank ID)
pcna	5′-CACCAAGGAGGGTGTCAAGT 5′-GATCTTGGGGTGCCAGATAA	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 58 °C, 30 s 72 °C; 10 min 72 °C	367	AK335595
rdr	5′-TTCCCCATCCGGTTCCCGCA 5′-TGAGCCCGCGCTTCTTGAGC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 45 s 72 °C; 10 min 72 °C	508	CK204951
mcm2	5′-ACGACGGCGCCACCGTTATC 5′-TTGCGATGAAGCGCCGGACT	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 58 °C, 45 s 72 °C; 10 min 72 °C	552	AY532594
EXPB8	5′-GGTTGTTCATAGTCGACCAATATGG 5′-AGAGCAGGAAGGGTTGTTGG	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 50 °C, 30 s 72 °C; 10 min 72 °C	351	AY543542
EXPB10	5′-GAAGACCTGTAGTGCCAATATGGCTGG 5′-GTCGGTGATGACGATCCTCC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 50 °C, 30 s 72 °C; 10 min 72 °C	373	AY543544
EXPA5	5′-CGACGACATGGCGGTCATCAAGATG 5′-CCCGTTGATGGTGAACCTGA	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	499	AY543531
EXPA6	5′-ATGGCAGCTGGGATGCGCTTCCT 5′-CGGTAGATGGCGAGGTTCTC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	422	AY543532
EXPA8	5′-CGAGCATCTCCGCACTTGCAAATTCAAG 5′-GCCTGGGCGATCTTAGTGAA	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	482	AY543534
Actin	5′-GGATCGGTGGCTCTATTTTG 5′-TGTACCCCTTATTCCTCTGAGG	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	224	AB181991

pcna: proliferating cell nuclear antigen; *rdr*: ribonucleotide reductase small subunit; *mcm2*: minichromosomal maintenance factor; *EXPB*: beta-expansins; *EXPA*: alpha-expansins.

were used for further analysis.

Proteins were separated by 10% (w/v) SDS–PAGE (Laemmli, 1970). To assess oxidative damage to proteins, samples were first derivatised with 2,4-dinitrophenylhydrazine (2,4-DNPH) (Levine et al., 1990). One gel was used for protein staining with Coomassie Brilliant Blue R-250. Two others (with non derivatised proteins and derivatised with 2,4-DNPH, respectively) were transferred onto polyvinylidene difluoride (PVDF) membranes. The first one was detected with rabbit anti-ubiquitin-protein conjugates (Sigma–Aldrich, St Luis, USA); the second one with rabbit anti-DNP primary antibodies (Sigma–Aldrich, St Louis, USA). Bands corresponding to ubiquitinated and oxidised proteins were visualised by secondary goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase 1:2000 (DakoCytomation), using 3,3'-diaminobenzidine (DAB) as substrate (information available on http://www.encorbio.com/protocols/blotting.htm). Membranes were photographed with Fotodyn (Fotodyn Inc.) and analysed with GelPro Analyzer 4.0 software, based on absolute integrated optical density of each line.

For specific cyclin D and CDKA proteins, samples (50 μg proteins) were subjected to electrophoretic analysis using 15% (w/v) SDS-PAGE (Laemmli, 1970). For determination of oxidised proteins, extracts were previously derivatised with 2,4-dinitrophenylhydrazine (2,4-DNPH) as described above. Oxidised and polyubiquitinated proteins were separated by affinity chromatography. Antibodies anti-DNP or anti-ubiquitin-protein conjugates were linked to cyanogen bromide-activated sepharose 4% agarose matrix (100 mg) from Sigma–Aldrich (St Luis, USA). Samples were incubated overnight at 4 °C with an excess of resin and then centrifuged for 5 min at 10,000 × g. Resin beads were washed 3 times with Tris-buffered saline (TBS), pellets were resuspended in 100 mM glycine-HCl (50 μL, pH 2.5). After centrifugation, pellets were discarded and supernatants pH adjusted to 6.8 with 0.5 M Tris–HCl buffer (5 μL, pH 8.8), prior to immunodetection.

Gels were transferred onto PVDF membranes. Polyclonal antibodies rose against cyclin D1 (Santa Cruz Biotechnology, Inc.), CDK2 (Santa Cruz Biotechnology, Inc.) –proteins closely related to cyclin D and A-type CDK from plants, respectively–were employed. Bands were subsequently visualised using a secondary goat antibody conjugated with horseradish peroxidase (HRP) and revealed by using 3,3′-diaminobenzidine (DAB) as substrate. Membranes were photographed with a Fotodyn, analysed with GelPro software and expressed as arbitrary units (assuming control value equal to 100 units), based on absolute integrated optical density of each band.

2.8. RT-PCR assays

In order to analyse the effect of copper on the expression pattern of certain genes relevant to cell cycle regulation, the accumulation of transcripts related to the Rb/E2f pathway was investigated. Some expansin genes were also determined. Total RNA was extracted from the root apical and subapical region (0–10 mm) using a modified TRIzol (Invitrogen; Carlsbad, CA, USA) procedure, treated with DNase I (Promega) and then converted to cDNAs with oligo (dT)15 using the RevertAid™ MMuLV Reverse Transcriptase (Fermentas). PCR primers and conditions for amplifications are described in Table 1. PCR reactions were performed using a programmable Termocycler T 18 (Ivema). The PCR products were electrophoresed through 1.5% (w/v) agarose and visualised with ethidium bromide. Fragments of wheat actin gene were amplified as internal standards to normalise for differences of total RNA amounts. Gels were photographed with Fotodyn and analysed with GelPro software; data were expressed as arbitrary units (assuming control value equal to 100), based on absolute integrated optical density of each band. Each expression profile presented was representative of at least three experiments.

2.9. Total protein determinations

Total protein concentrations were determined according to Bradford (1976) using bovine serum albumin as standard.

2.10. Statistics

Values are expressed as mean ± SEM. Differences among treatments were analysed by 1-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

3. Results

3.1. Copper decreased root growth and altered RAM structure

Copper was toxic for plant growth and biomass accumulation at a concentration of 5 μM or higher. As shown on Fig. 1, root length

was significantly reduced at 48 h post-imbibition, with length decreases respect to control values of 36% and 56% under 5 or 10 μM Cu^{2+}. Despite 100 μM Cu^{2+} allowed seed germination (defined as radicle protrusion from the seed coat), 48 h of metal exposure resulted in seedlings growth arrest (Fig. 1). Decreases in root length were associated to reductions of root biomass of 28% and 52% for 5 and 10 μM Cu^{2+}, respectively, remaining similar to the control their relative dry mass (RDM), calculated as 1-[(FW-DW)/FW] × 100 (Table 2). Viability of seeds in control medium was always above 90%.

To determine the effects of copper on RAM structure, we counted the number of cortex cells in a file extending from the quiescent center (QC) to the first elongated cell, as a measurement of meristem size (Dello Ioio et al., 2007). Seeds germinated in the presence of a copper toxic concentration (10 μM) showed a shorter RAM, as can be observed on Fig. 2, where the number of cells in the proliferation/transition zone was lower than in the control. Equal number of cells in the apex meristem was counted in control and 1 μM Cu^{2+}-treated seedlings (Fig. 2). Additionally, partial detachment of outer cortex and rhizodermis cells was observed at 10 μM.

3.2. Copper increased proline root content and modified cell membrane integrity

Metal treatments increased proline accumulation in root of wheat seedlings even at a non-toxic concentration of 1 μM (Fig. 3). However, at this Cu level cell membrane integrity was preserved, while higher Cu levels resulted also in disturbance of plasma membrane integrity, as determined by the income of Evans blue dye (Fig. 3).

3.3. Copper induced ROS production and oxidation of total and specific proteins in root apexes, but caused no lipid peroxidation

High levels of green fluorescence originated by ROS-induced oxidation of H_2DCFDA were detected when seeds were germinated in the presence of 10 μM Cu^{2+}. Similar results were obtained when 5 μM were added to the incubation medium (not shown). This fluorescence level almost disappeared when roots were previously treated with ascorbic acid (Fig. 4, upper panel). In the same

Table 2
Effect of copper on seminal root biomass. Wheat seedlings were germinated and grown for 48 h in flasks with 30 ml of demineralised water alone (control) or containing 1, 5 and 10 μM $CuCl_2$. Fresh and dry weights (FW and DW, respectively) are expressed in mg by seedling. Relative dry weight (RDM) was calculated as 1-[(FW-DW)/FW] × 100.

	C	1	5	10
		μM $CuCl_2$		
Fresh weight	12.10 ± 0.69a	12.40 ± 1.12a	8.65 ± 0.95b	5.82 ± 0.52c
Dry weight	0.79 ± 0.20a	0.91 ± 0.14a	0.60 ± 0.10b	0.38 ± 0.09c
RDW%	6.51a	7.30a	6.91a	6.53a

Each value represents the means ± SEM of three different experiments (n = 150). Means with the same letters between columns are not significantly different at P < 0.05, according to Tukey's Multiple Range.

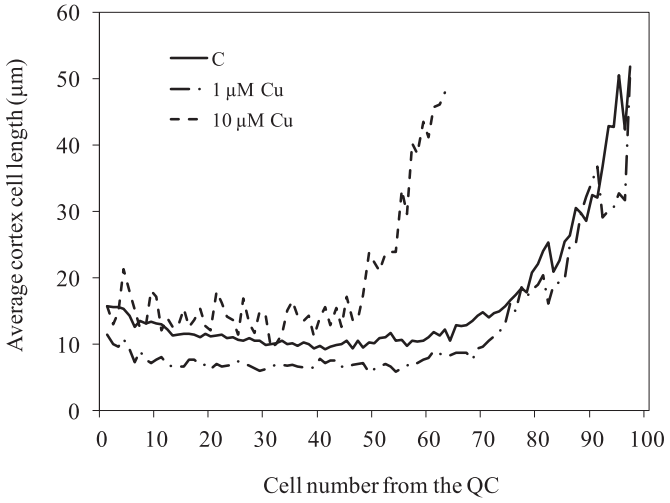

Fig. 2. Cortex cell lengths and numbers in different zones of the root apical meristem. Wheat seeds (15 per flask) were germinated in 30 mL of demineralised water (control) or water containing 1 or 10 μM $CuCl_2$ for 48 h.

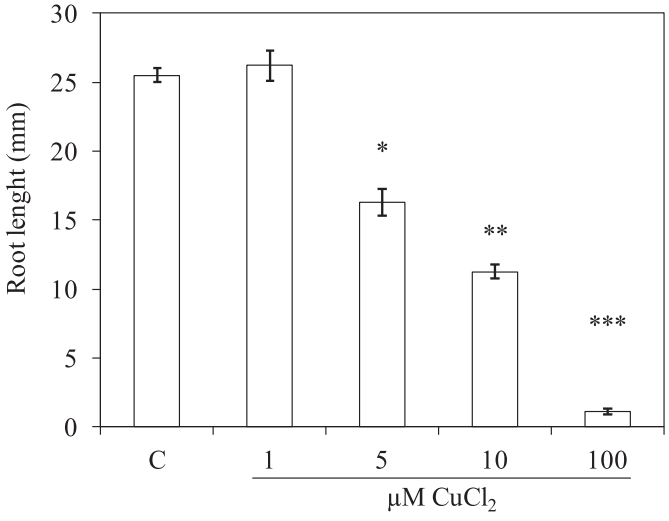

Fig. 1. Effect of copper on wheat seminal root growth. Wheat seeds (15 per flask) were germinated in 30 ml of demineralised water (control) or water containing different concentrations of $CuCl_2$ for 48 h. Significant differences with respect to control at *P < 0.05, **P < 0.01 and ***P < 0.001 according to Tukey's multiple range test.

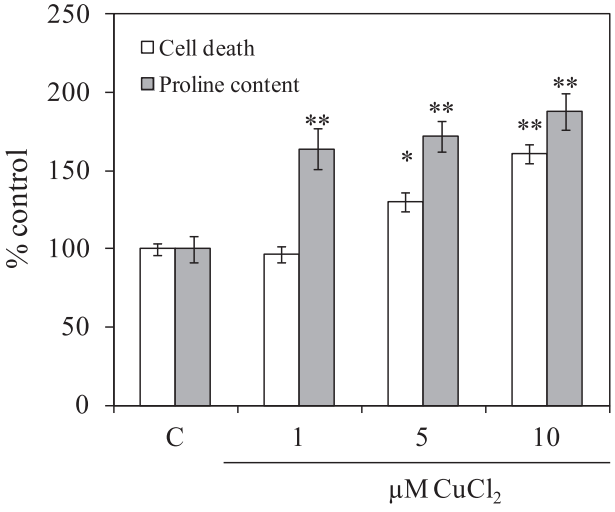

Fig. 3. Proline content and cell membrane integrity (estimated by Evans Blue staining method) in wheat root apexes. Wheat seeds (15 per flask) were germinated in 30 ml of demineralised water (control) or water containing $CuCl_2$ for 48 h. Data are expressed as percentage of control (assuming a control value equal to 100%). Mean proline content of control: 69 μg g^{-1} FW. Significant differences with respect to control at **P < 0.01 according to Tukey's multiple range test.

manner, a red fluorescence related to DHE oxidation was detected in the root apexes of 5 (not shown) and 10 μM Cu^{2+}-treated seedlings (Fig. 4, lower panel). Preincubation of root apexes with $MnCl_2$ prevented the increment of the red fluorescence.

Total soluble proteins (Fig. 5A) and ubiquitin-conjugated proteins (Fig. 5B) obtained from the root apex of Cu-treated seedlings showed similar qualitative patterns as compared to control seedlings. Accumulation of soluble carbonylated proteins, however, took place in this tissue at 5 μM and 10 μM Cu^{2+} (Fig. 5C and D).

Cyclin D and CDKA abundance did not change upon copper treatment (Fig. 6A and B, first rows); however, 10 μM Cu^{2+} treatment resulted in increased levels of carbonylation of both proteins (Fig. 6A and B, second rows, and Fig. 6C and D, white bars). Although no modification in total Ub-conjugated proteins was detected (Fig. 5B), 10 μM Cu^{2+} decreased ubiquitination of cyclin D, but not that of CDKA (Figs. 6A and 7B, third rows, and Fig. 6C and D, black bars).

TBARS content in the root apex of Cu-treated seedlings showed no significant changes (data no shown).

3.4. Copper affected cell cycle and expansin transcripts accumulation

E2f transcription factor biosynthetic rate in the root meristem, based on the abundance of *pcna*, *rdr* and *mcm2* transcripts, remained similar to controls in wheat seeds germinated under 1 μM Cu^{2+}, but was found to be significantly downregulated in those germinated under 10 μM of this metal (Fig. 7A). Additionally, copper increased transcript accumulation of all expansin analysed except for EXPB8 at 1 μM (Fig. 7B). Increases were less pronounced but still significant at 10 μM for most expansin genes, except again for EXPB8, which was significantly downregulated at this copper concentration (Fig. 7B).

4. Discussion

Plant roots show high susceptibility to metal ions toxicity. In this sense, the ability to maintain a relatively high rate of root elongation has been regarded as an indicator of metal tolerance in plants (Ernst et al., 1992; Barcelo and Poschenrieder, 1996). However, the mechanisms involved in the root growth inhibition frequently observed under metal stress have not been yet clearly established.

In our experimental conditions, copper levels of 5 μM Cu^{2+} or greater had negative effects on seminal root growth in wheat. Thus, wheat plants showed similar basal tolerance to copper ions than other plant species such as rice, maize and *Arabidopsis thaliana* (Doncheva, 1998; Jiang et al., 2001; Madejón et al., 2009; Lequeux et al., 2010; Pető et al., 2011). Toxic copper concentrations resulting in root growth reduction also lead to ROS accumulation in the root tip, as demonstrated by *in vivo* detection using fluorescent dyes. Copper, as a redox active metal, may directly catalyse ROS production (Aust et al., 1985). In addition, Sgherri et al. (2007) proposed that early ROS production (particularly $O_2^{\cdot-}$) induced by Cu^{2+} in the roots of *Triticum durum* could have an apoplastic origin through NADPH oxidase. The induction of the antioxidant system has been described as an adaptative response of plants to oxidative stress caused by copper (Thounaojam et al., 2012), where proline accumulation in Cu-stressed seedlings would provide an additional defense against oxidative stress.

Current information is indicating that plant development plasticity in response to environmental challenges is linked to ROS. Redox/hormone balance form part of a complex signalling hub that regulates normal RAM physiology (Pasternak et al., 2005; De Tullio et al., 2010). However, a detailed analysis of RAM response regarding metal toxicity is still lacking. Wheat seedlings as well as *Arabidopsis* plants treated with either paraquat or H_2O_2 had short primary roots (Pasternak et al., 2005; Pena et al., 2012b).

Proteins are one of the main targets of ROS deleterious action, which results in the covalent modification of polypeptides, and in

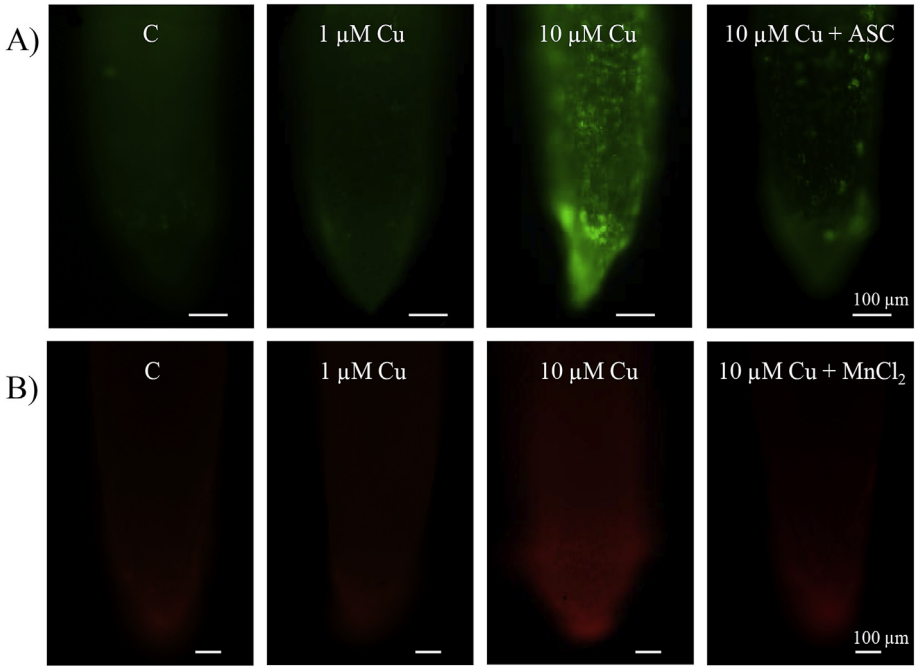

Fig. 4. ROS accumulation in wheat root apexes. Wheat seeds (15 per flask) were germinated in 30 mL of demineralised water (control) or water containing 1 or 10 μM $CuCl_2$ for 48 h. After treatments, wheat roots were incubated with (A) 25 μM H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) or (B) 10 μM DHE (dihydroethidium) as probes for H_2O_2/peroxides and $O_2^{\cdot-}$, respectively. Where indicated, roots were incubated with 1 mM ascorbic acid (ASC) or 4 mM $MnCl_2$ prior to the H_2DCFDA and DHE treatments, respectively. Images are representative of several similar experiments.

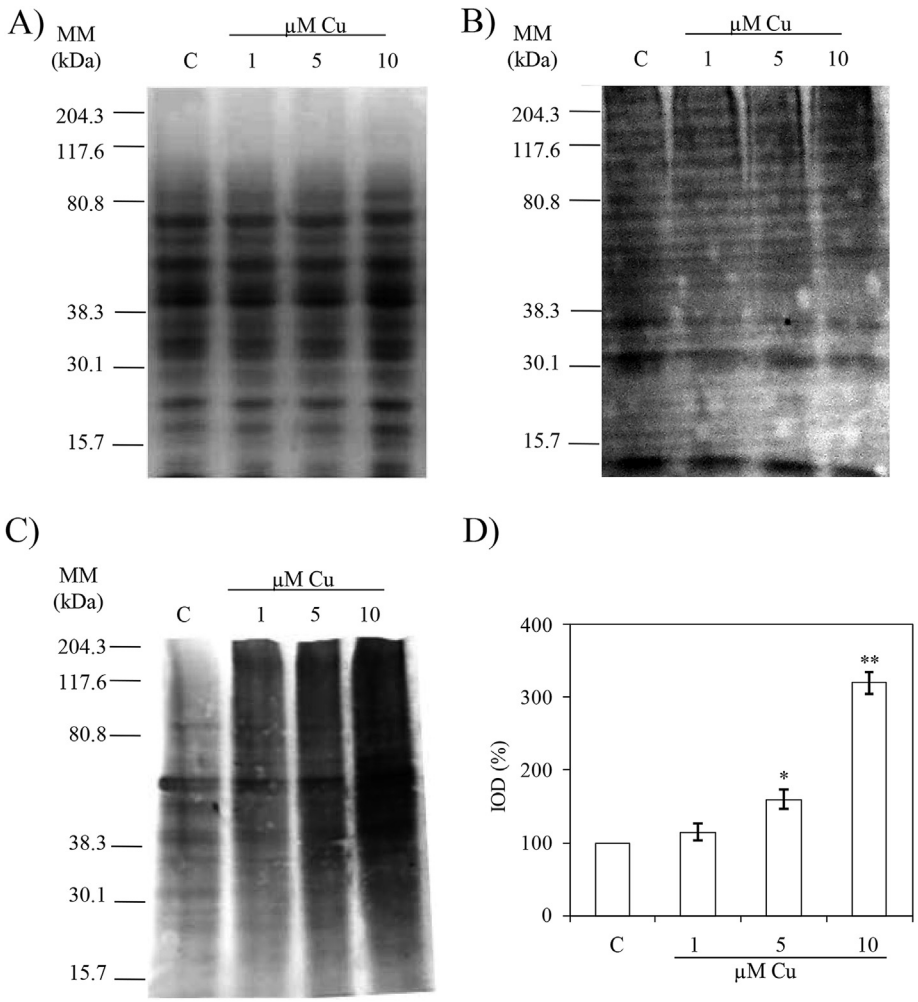

Fig. 5. Western blot of oxidative and ubiquitinated modified proteins from wheat root apexes. Wheat seeds (15 per flask) were germinated in 30 ml of demineralised water (control) or water containing 1, 5 or 10 μM $CuCl_2$ for 48 h. (A) Proteins (50 μg) were subjected to SDS–PAGE (10% w/v) and were stained with Coomassie Brilliant Blue R-250. (B) Ubiquitinated proteins, performed as was described in Material and Methods. (C) The DNPH-derivatised proteins (50 μg of total protein) were subjected to an SDS–PAGE (10% w/v). Western blotting was performed using anti-DNP antibody, bands were visualised as described in Material and Methods. (D) Quantification of oxidised proteins expressed in arbitrary units (assuming control value equal to 100%), based on absolute integrated optical density (IOD) of each lane. The position of molecular mass markers (in kDa) is shown on the left. Photographs of membrane and gel shown are representative of four Western blots and electrophoreses performed.

this regard, the oxidative damage to proteins is generally considered an indicator of oxidative stress (Pena et al., 2012a). In this investigation, copper concentrations that caused impaired root growth and ROS accumulation also increased protein oxidation in the root apex. We hypothesised that oxidation of specific proteins may be relevant to the regulation of the cell cycle by affecting the expression of cell cycle genes. Therefore, we evaluated carbonylation of specific proteins: cyclin D and CDKA. Immunoblot analysis showed that cyclin D and CDKA levels remained unchanged in the root apexes of Cu-treated seedlings; however, these proteins underwent oxidative posttranslational modifications. The oxidation of proteins has been associated with loss of functionality (Nyström, 2005; Møller et al., 2011). Cyclin D is a rate-limiting component for activation of the Rb-E2F pathway, in turn responsible for the G1/S cell cycle transition (Dewitte et al., 2007).

We corroborated that oxidation of certain proteins implicated in the cell cycle occurred concomitantly with a downregulation of some transcriptional targets of E2F, such as *mcm2*, *rdr* and *PCNA*. Thus, the loss of functionality of the cyclin D-CDKA complex could have been, at least in part, responsible for the shortening of the cell proliferation zone of the RAM when 5 or 10 μM Cu^{2+} was included in the incubation medium. This is consistent with previous reports which indicate that Cu^{2+} decreased the nucleus in S stage in RAM of maize (Doncheva, 1998).

RAM phenotype observed in Cu-treated wheat seedlings agree with the model proposed by Potters et al. (2007, 2009), in which sublethal stress conditions lead to a morphogenic response designated as stress-induced morphogenic response (SIMR). According to this model, plant growth is redirected to diminish exposure to the stress factor as part of an overall strategy of acclimation.

Oxidative modification of specific cell cycle proteins by ROS could be part of a general mechanism through which, under stress conditions, the cells rapidly block cell cycle progression (West et al., 2004). Cells presumably prevent their entry to S-state, when cell components are particularly vulnerable to damage, and allow the cellular defence system to be activated. The results presented in this work add information to those suggested by Rinalducci et al. (2008), who proposed that oxidative posttranslational modifications triggered during cellular stress could be of significance in the

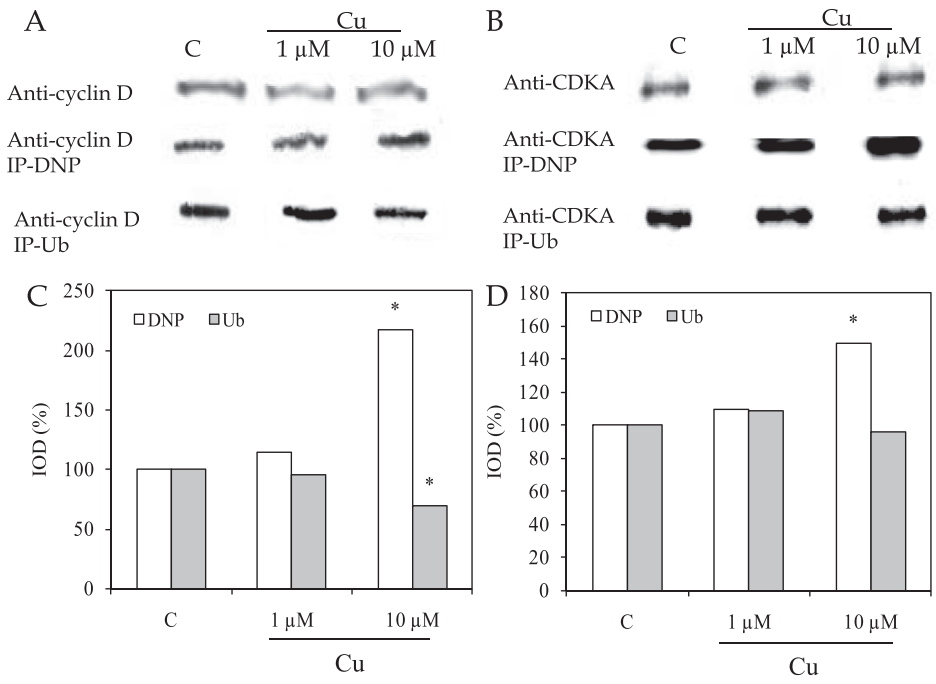

Fig. 6. Effect of copper on cyclin and CDK and identification of oxidised and ubiquitinated forms. Wheat seeds (15 per flask) were germinated in 30 ml of demineralised water (control) or water containing 1 or 10 μM $CuCl_2$ for 48 h. Extracts for determinations were prepared from root apexes. (A) Western blotting with anti-cyclin D antibody (Anti-cyclin D), after derivatization with 2,4-DNPH followed by immunoseparation with anti-DNP (Anti-cyclin D IP-DNP), and after immunoseparation with anti-Ub (Anti-cyclin D IP-Ub). (B) Western blotting using anti-CDKA antibody (Anti-CDKA), after derivatization with 2,4-DNPH followed by immunoseparation with anti-DNP (Anti-CDKA IP-DNP), and after immunoseparation with anti-Ub (Anti-CDKA IP-Ub). Bands were photographed with a Fotodyn, analysed with GelPro software. Quantification of the bands is expressed in arbitrary units (assuming control value equal to 100%), based on absolute integrated optical density (IOD) of each band. As loading controls of western blotting with anti-cyclin D and anti-CDKA antibodies, Ponceau S-stainings were used. The western blots shown are representative of several replicates. Significant differences with respect to control at $^*P < 0.05$ according to Tukey's multiple range test.

physiology of the stress response, and not merely a secondary consequence of it.

Recent investigations suggest that inhibition of root elongation induced by copper is not a direct result of loss of membrane integrity induced by oxidative conditions and subsequent cell death. Madejón et al. (2009) observed that root elongation decreases and the loss of membrane integrity in the root apical meristem did not always match with the plant sensitivity to Cu in different maize cultivars. On another hand, Kulikova et al. (2011) found reduction of root growth of soybean plants exposed to Cu and lipid peroxidation, not always in correlation with impairment of membrane integrity and cell death.

Although the overall ubiquitination process was not altered in copper treatments, conjugation of cyclin D to Ub decreased upon copper exposure. A possible mechanism underlying this result involves oxidation of lysine residues. Ubiquitin binds to the ε-amino groups of lysine in the target protein. Lysine is one of the most sensitive aminoacids to be carbonylated (Møller et al., 2011). Thus, the oxidative modification of lysine residues could directly alter protein conjugation to Ub.

In addition to cell division, cell expansion is also involved in the growth of a plant organ (Woolhouse, 1983). Expansins are proteins located in the cell wall that are implicated in cell wall plasticity. Expression levels of expansins increased in different plant species in response to environmental stresses such as heat, shading, anaerobic conditions and infections of pathogens (Lü et al., 2013). Five genes showing high levels of expression in roots but low levels in leaves (TaEXPA5, TaEXPA6, TaEXPA8, TaEXPB8 and TaEXPB10) were analysed by Lin et al. (2005). We observed a complex expression pattern of expansins when copper was included in the incubation medium, even at low concentrations. Overexpression of TaEXP23 in tobacco resulted in accelerated growth of leaves and internodes in early stages of development (Xing et al., 2009), and also increased stress tolerance (Li et al., 2011; Han et al., 2012). Interestingly, the same expansin expressed in *A. thaliana* under a stress-inducible promoter had no effect on growth, but conferred stress tolerance (Li et al., 2013).

Tobacco plants overexpressing EXPA1 of *Brassica juncea* (BjEXPA1) were susceptible to stress generated by cadmium. The BjEXPA1 promoter was induced by gibberellin and exogenous auxins, but inhibited by abscisic acid, cadmium and polyethylene glycol 6000 (Sun et al., 2011). Thus, despite all expansins are involved in cell wall plasticity modifications, they seem not to have redundant functions, and some expansins would be related to the normal physiology while others may be more linked to stress response. Lizana et al. (2010) suggested that the increased expression of a particular expansin gene may reflect specific interactions between the protein and the cell wall composition, which may vary on considering different tissues, organs and developmental stages. For example, the amount and type of phenolic acids present (Sasayama et al., 2011), or the relative abundance of polysaccharides (Lizana et al., 2010), could modify the susceptibility of the cell wall to expansins.

The results obtained in this research, where copper (a redox active metal) effects on wheat germinating plants were investigated, together with those obtained in a previous report in which cadmium (a non-redox active element) effects on developing wheat seedlings were assessed (Pena et al., 2012b), allow us to propose that a "common" signal which involves oxidative post-translational modification of specific cell cycle proteins may be necessary to induce root growth arrest under Cd and Cu stress. Thus, oxidation of specific proteins by ROS could be part of a large

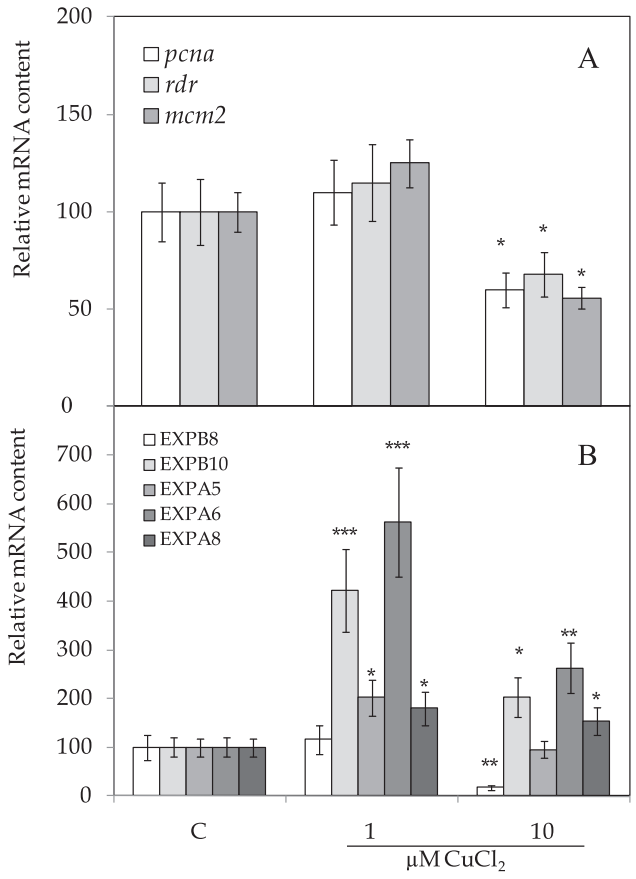

Fig. 7. Semiquantitative RT PCR analysis in RNA samples obtained from root apexes using specific primers for A) *pcna*, *mcm2* and *rdr*, B) *TaEXPA5*, *TaEXPA6*, *TaEXPA8*, *TaEXPB8* and *TaEXPB10*. *Actin* transcript was used as housekeeping gene and to calculate the mRNA values. Data are expressed as percentage of control (assuming a control value equal to 100%). Significant differences with respect to control at *P < 0.05 according to Tukey's multiple range test.

assortment of mechanisms that allow plants to perceive and transduce external signals, in order to halt proliferation processes and to trigger adaptative responses.

Contributions

Conceived and designed the experiments: LP, SG.
Performed the experiments: LP, AM, CM.
Analyzed the data: LP, MZ, SG.
Supervised the experimental procedure and wrote the paper: MZ, SG.

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