

# Copper-mediated genotoxic stress is attenuated by the overexpression of the DNA repair gene *MtTdp2 $\alpha$* (tyrosyl-DNA phosphodiesterase 2) in *Medicago truncatula* plants

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## Abstract

**Key message** Our study highlights the use of the DNA repair gene *MtTdp2 $\alpha$*  as a tool for improving the plant response to heavy metal stress.

**Abstract** Tyrosyl-DNA phosphodiesterase 2 (Tdp2), involved in the removal of DNA topoisomerase II-mediated DNA damage and cell proliferation/differentiation signalling in animal cells, is still poorly characterised in plants. The *Medicago truncatula* lines Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 overexpressing the *MtTdp2 $\alpha$*  gene and control (CTRL) line were exposed to 0.2 mM CuCl<sub>2</sub>. The DNA diffusion assay revealed a significant reduction in the

percentage of necrosis caused by copper in the aerial parts of the Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 plants while neutral single cell gel electrophoresis highlighted a significant decrease in double strand breaks (DSBs), compared to CTRL. In the copper-treated Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines there was up-regulation (up to 4.0-fold) of genes encoding the  $\alpha$  and  $\beta$  isoforms of Tyrosyl-DNA phosphodiesterase 1, indicating the requirement for Tdp1 function in the response to heavy metals. As for DSB sensing, the *MtMRE11*, *MtRAD50* and *MtNBS1* genes were also significantly up-regulated (up to 2.3-fold) in the *MtTdp2 $\alpha$* -overexpressing plants grown under physiological conditions, compared to CTRL line, and then further stimulated in response to copper. The basal antioxidant machinery was always activated in all the tested lines, as indicated by the concomitant up-regulation of *MtCytSOD* and *MtCpSOD* genes (cytosolic and chloroplastic Superoxide Dismutase), and *MtMT2* (type 2 metallothionein) gene. The role of *MtTdp2 $\alpha$*  gene in enhancing the plant response to genotoxic injury under heavy metal stress is discussed.

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## Introduction

Heavy metals are naturally present in traces in the soil but modern agricultural and industrial practices have so far contributed to release huge amounts of these elements as environmental pollutants. Heavy metal accumulation in plant tissues leads to genotoxic stress and genome instability, with the occurrence of chromosomal aberrations and micronuclei (Rodriguez et al. 2011). DNA repair genes

have been reported to be up-regulated in response to heavy metal stress, such as the *MSH3* and *MLH1* (*MutS* and *MutL* Homologue, respectively) encoding enzymes involved in Mismatch Repair (MR) (Liu et al. 2008), *OGG1* (8-oxoguanine DNA glycosylase/lyase) and *FPG* (Formamido-pyrimidine-DNA glycosylase) (Macovei et al. 2011a), *TFIIIS* involved in Transcription-Coupled-Nucleotide Excision Repair (TC-NER) (Macovei et al. 2010) and Tdp1 encoding tyrosyl-DNA phosphodiesterase 1 (Macovei et al. 2011b).

Tyrosyl-DNA phosphodiesterases (TDPs) are involved in the repair of DNA damage induced by DNA topoisomerases, key enzymes that regulate DNA topology (Pommier 2013). These enzymes release the covalent bond originating between the DNA molecule and stalled DNA topoisomerases in the presence of oxidised nucleotides or specific inhibitors. Tdp1 cleaves the 3'-phosphotyrosyl bond between DNA topoisomerase I (topo I) and DNA (Interthal et al. 2001), while Tdp2 is able to resolve the 5'-phosphotyrosyl bonds between stalled DNA topoisomerase II (topo II) and DNA (Cortes Ledesma et al. 2009). In animal models, Tdp2 is also known as a factor interacting with components of different signalling pathways (Varady et al. 2011). In plants, information concerning TDPs is still limited, although it is rapidly expanding. The small *Tdp1* gene family was first identified *in planta* in the model legume *M. truncatula* by Macovei et al. (2010). Both the *MtTdp1 $\alpha$*  and *MtTdp1 $\beta$*  genes were up-regulated in response to heavy metal and osmotic stress and during seed imbibition, when Reactive Oxygen Species (ROS) are accumulated and DNA repair genes are induced to maintain genome stability (Balestrazzi et al. 2011a, b; Ventura et al. 2012). The involvement of plant TDPs in the complex response of *M. truncatula* cell suspension cultures to ionising radiation, as a function of dose rate, has been recently demonstrated by Donà et al. (2013a). In *M. truncatula*, depletion of *MtTdp1 $\alpha$*  gene impairs ribosome biogenesis, thus highlighting a role of *MtTdp1 $\alpha$*  enzyme in nucleolar metabolism (Donà et al. 2013b). As regards the Tdp2 function in plants, *M. truncatula* lines overexpressing the *MtTdp2 $\alpha$*  gene were characterised by increased biomass and chlorophyll content under physiological and osmotic stress conditions. Moreover, these lines showed reduced DNA damage accumulation in response to osmotic stress and up-regulation of both antioxidant and topology-related genes (Confalonieri et al. 2013).

The present work focuses on the molecular characterisation of *MtTdp2 $\alpha$* -overexpressing *M. truncatula* lines challenged with toxic copper concentrations. According to the reported data, *MtTdp2 $\alpha$*  gene plays a relevant role in the DNA damage response against heavy metal stress and, thus, it could represent a promising tool for biotechnological applications meant to improve crop productivity.

## Materials and methods

### Plant material and treatments

*Medicago truncatula* Gaertn. cv. Jemalong (M9-10a genotype) plants were grown *in vitro* in sterile vessels (Micropoli, Cesano Boscone, Italy) on a medium containing macrosalts and microsals MS (Murashige and Skoog 1962), vitamin SH (Schenk and Hildebrandt 1972), 20 g/L sucrose and 4 g/L Gelrite™ (Duchefa Biochemie, Haarlem, The Netherlands) and maintained in a climate chamber at 22–24 °C with a 16-h light/8-h dark cycle photoperiod and a photosynthetic photon flux of 65–70  $\mu\text{Mol/m}^2/\text{s}$  under a cool white fluorescent lamp (Confalonieri et al. 2013). For heavy metal stress treatments, 0.2 mM  $\text{CuCl}_2$  (Sigma-Aldrich, Milan, Italy) was added to the above reported growth substrate as described by Macovei et al. (2010). For molecular analyses, tissues were collected from plants and stored in liquid  $\text{N}_2$ .

### Chlorophyll content

To determine the chlorophyll content, three leaflets per treatment were excised from 10-day-old *M. truncatula* plants grown *in vitro* in presence/absence of 0.2 mM  $\text{CuCl}_2$  and analysed as follows. Extraction with 1 ml of 80 % (v/v) acetone was performed and the total chlorophyll content was determined by measuring the absorbance of the supernatant at 663 and 646 nm (Wellburn 1994). Spectrum profiles of leaf pigments were determined performing a spectrophotometric assay on ethanol extracts of leaf tissues of untreated and treated (0.2 mM  $\text{CuCl}_2$ ) plantlets. Spectrum measurements were carried out between 400 and 550 nm using a V-530 spectrophotometer (Jasco Europe S.r.l., Cremella, Italy).

### Quantitative real-time polymerase chain reaction

QRT-PCR was carried out with the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories S.r.l., Segrate, Italy) in a final volume of 20  $\mu\text{l}$  according to supplier's indications, and using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia). Amplification conditions were as follows: initial denaturation step at 95 °C for 30 s, and subsequently 95 °C for 5 s, 59 °C for 30 s, 72 °C for 30 s (40 cycles). Quantification was carried out using the *M. truncatula* *ELF1 $\alpha$*  (GenBank Accession N° EST317575) as reference gene. Gene-specific oligonucleotide primers for the *M. truncatula* *MtTdp2 $\alpha$*  (Phytozome Database Accession N° Medtr8g146980), *MtTdp1 $\alpha$*  (XM\_003622639), *MtTdp1 $\beta$*  (GenBank Accession N° AC141864.7), *MtMRE11* (Phytozome Database Accession N° Medtr2g081100), *MtRAD50* (Phytozome Database

Accession N° Medtr3g084300), *MtNBS1* (Phytozome Database Accession N° Medtr5g076180), *MtctySOD* (Cu/Zn-SOD isoform) (Phytozome Database Accession N° Medtr7g114240), *MtcpSOD* (Cu/Zn-SOD isoform) (Phytozome Database Accession N° Medtr4g076170), *MtMT2* (AC147202.14) coding sequences were designed using the Real-Time PCR Primer Design program from GenScript (<https://www.genscript.com/ssl-bin/app/primer>) (Supplemental Table S1). For each oligonucleotide set, a no-template water control was used.  $C_t$  values and QRT-PCR efficiency values, obtained by the Rotor-Gene 6000 Series Software 1.7 (Corbett Robotics, Brisbane, Australia), were analysed and statistically validated using the REST2009 Software V2.0.13 (Qiagen GmbH, Hilden, Germany) (Pfaffl et al. 2002).

#### DNA diffusion assay

The DNA diffusion assay (Singh 2003) was performed to evaluate cell death (PCD versus necrosis). Three leaflets per treatment were excised from 10-day-old *M. truncatula* plants grown in vitro in presence/absence of 0.2 mM  $\text{CuCl}_2$  and analysed. Nuclei were extracted as described by Donà et al. (2013b). Agarose-precoated slides were prepared by spreading 1 ml of 1 % agarose on each slide and drying them at room temperature. Aliquots (300  $\mu\text{l}$ ) of nuclei suspension were mixed with 200  $\mu\text{l}$  of 1 % low melting point agarose (Sigma-Aldrich, Milan, Italy) in phosphate-buffered saline (PBS) at 37 °C and gently transferred onto slides. The gel was covered with a cover glass, slides were cooled on ice for 1 min. Cover glasses were removed and slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl pH 7.5) for 20 min at room temperature. After lysis, slides were washed twice in neutral solution TBE (89 mM Tris Base, 89 mM Boric Acid, 2 mM EDTA, pH 8.3) for 5 min, rinsed in 70 % ethanol (v/v) for 10 min at room temperature. Slides were air dried and stored at room temperature, stained with 20  $\mu\text{l}$  DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich, 1  $\mu\text{g ml}^{-1}$ ) and covered with a cover slip for 10 min. One hundred nuclei were analysed per slide. Cells undergoing PCD or necrosis were distinguished from viable cells as indicated by Singh (2003). In case of PCD, cell nuclei own a homogeneous outline without any clear boundary due to nucleosomal-sized DNA diffusing into the agarose. Necrotic cell nuclei are bigger and poorly defined with a non-homogeneous halo appearance. For each treatment, three replicated samples were analysed in two independent experiments.

#### Single cell gel electrophoresis (SCGE)

Three leaflets per treatment were excised from 10-day-old *M. truncatula* plants grown in vitro in presence/absence of

0.2 mM  $\text{CuCl}_2$  and analysed. Nuclei were extracted from *M. truncatula* cells as described by Donà et al. (2013b). The suspension containing purified nuclei and a solution of 1 % low melting point agarose in PBS at 37 °C were mixed in equal volume. Two drops of the resulting suspension were then pipetted onto agarose-precoated slides and solidified on ice. For neutral SCGE, slides were then incubated 20 min at room temperature in high salt lysis buffer (2.5 M NaCl, 100 mM Tris-HCl pH 7.5, 100 mM EDTA) to disrupt the nuclear membrane and subsequently electrophoresed 8 min at 1 V/cm in TBE. After electrophoresis, slides were washed in 0.4 M Tris-HCl pH 7.5 three times for 5 min, rinsed in 70 % ethanol (v/v) three times for 5 min at 4 °C and dried overnight at room temperature. Subsequently, slides were stained with 20  $\mu\text{l}$  DAPI (1  $\mu\text{gMmL}$ , Sigma-Aldrich). For each slide, one hundred nucleoids were scored, using a fluorescence microscope with an excitation filter of 340–380 nm and a barrier filter of 400 nm. Nucleoids were classified and results were expressed in arbitrary units according to Collins (2004).

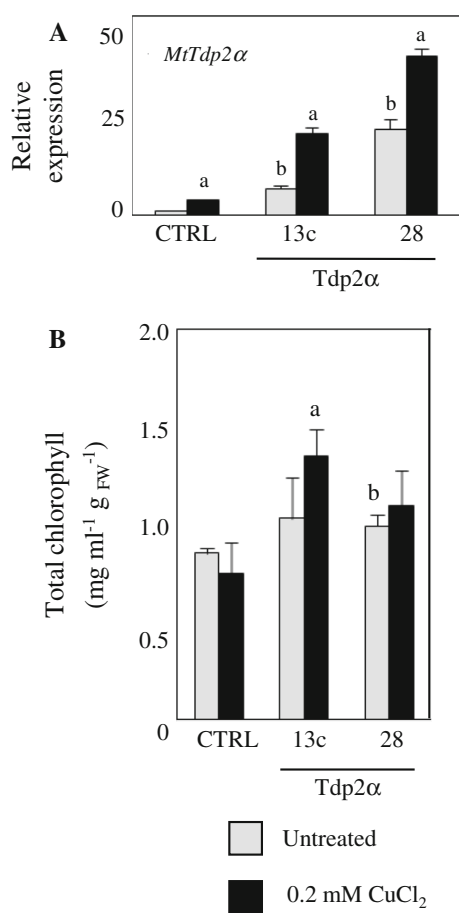
#### Statistical analysis

Three replicated plantlets from each treatment combination were randomly selected for tissue analysis. Results were subjected to Analysis of Variance (ANOVA) and the means were compared by Holm-Sidak test. Percentage data were transformed to  $\arcsin\sqrt{x}$  before statistical analysis. Statistical significance of differences was determined using Student's *t* test ( $P < 0.05$ ).

## Results

#### Chlorophyll content in *MtTdp2 $\alpha$* -overexpressing *M. truncatula* plants exposed to copper

To assess the response to heavy metal toxicity, the Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines and the control (CTRL) line carrying the empty vector were grown in vitro for 10 days in presence/absence of 0.2 mM  $\text{CuCl}_2$ . The latter was able to induce visible toxic effects on growth rates in *M. truncatula* plants (Macovei et al. 2010). The amount of *MtTdp2 $\alpha$*  was measured by QRT-PCR in the CTRL, Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines grown in presence/absence of copper. Under physiological conditions, the overexpression of *MtTdp2 $\alpha$*  gene was 6.8-fold (Tdp2 $\alpha$ -13c) and 21.7-fold (Tdp2 $\alpha$ -28), compared to CTRL line. Exposure to copper caused a further enhancement in the amount of *MtTdp2 $\alpha$*  transcript not only in the CTRL (3.9-fold) but also in the *MtTdp2 $\alpha$* -overexpressing lines (3.1-fold, Tdp2 $\alpha$ -13c; 1.8-fold, Tdp2 $\alpha$ -28) (Fig. 1a). The estimated increase in



**Fig. 1** Expression profiles of *MtTdp2α* gene assessed by QRT-PCR (a) and total chlorophyll content (b) in leaves of 10-day-old plants (CTRL and transgenic lines Tdp2α-13c and Tdp2α-28) grown in vitro in presence/absence of 0.2 mM CuCl<sub>2</sub> for 10 days. For each treatment combination, data represent the mean values ± SD of at least two independent experiments each of them carried out using three technical replications. *a* Significant difference between copper treatment and physiological conditions. *b* Significant difference between Tdp2α-overexpressing line and CTRL line

*MtTdp2α* mRNA was lower in the Tdp2α-28 line characterised by higher overexpression of the gene of interest.

The chlorophyll content was analysed in leaflets of the CTRL, Tdp2α-13c and Tdp2α-28 lines exposed to 0.2 mM CuCl<sub>2</sub> (Fig. 1b). In the untreated CTRL line, the amount of total chlorophyll was  $0.86 \pm 0.03 \text{ mg ml}^{-1} \text{ gFW}^{-1}$  while a non significant ( $P = 0.108$ ) increase occurred in the Tdp2α-13c line ( $1.03 \pm 0.21 \text{ mg ml}^{-1} \text{ gFW}^{-1}$ ) and contrarily a significant ( $P < 0.0001$ ) increase ( $0.99 \pm 0.06 \text{ mg ml}^{-1} \text{ gFW}^{-1}$ ) was detected in the Tdp2α-28 line. Under heavy metal stress, no significant changes ( $P = 0.0453$ ) in chlorophyll content were observed in the CTRL line ( $0.75 \pm 0.16 \text{ mg ml}^{-1} \text{ gFW}^{-1}$ ) while a significant increase was present in the Tdp2α-13c line ( $1.35 \pm 0.13 \text{ mg ml}^{-1} \text{ gFW}^{-1}$ ) (Fig. 1b). The observed response might be due to compensatory mechanisms for copper-mediated stress

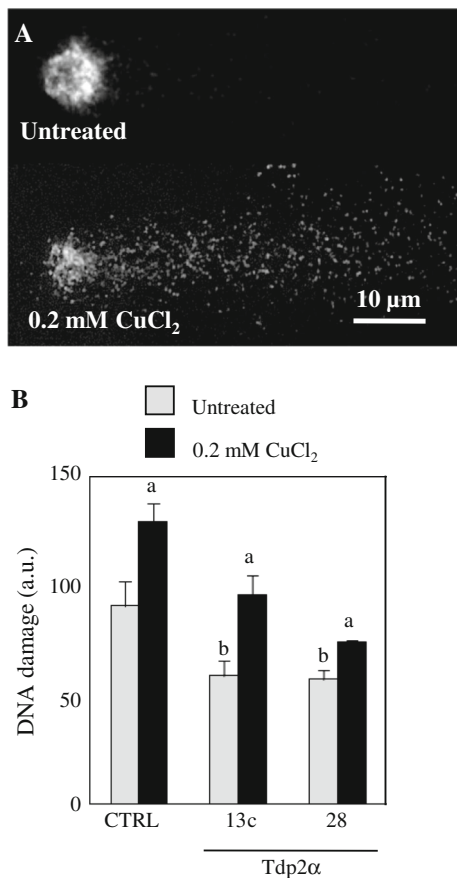
occurring at the photosynthetic apparatus, already described in *M. truncatula* (Macovei et al. 2010). The absorbance spectra of leaf carotenoids were also determined and, as shown in Supplemental Fig. S1, the Tdp2α-13C and Tdp2α-28 lines exposed to heavy metal stress were able to maintain significantly ( $P < 0.05$ ) higher levels of these antioxidant compounds, compared to CTRL. As for the average plant biomass, exposure to copper caused an estimated decrease of 60 % in the CTRL line. The recorded average biomass of the Tdp2α-13c and Tdp2α-28 plants was reduced under heavy metal stress up to 28 % (data not shown).

#### Evaluation of cell viability, programmed cell death and necrosis in response to heavy metal stress

Occurrence of cell death was monitored in leaves of *M. truncatula* plants grown in vitro in presence/absence of 0.2 mM CuCl<sub>2</sub>, using the DNA diffusion assay. Three distinct nuclear morphologies were evidenced, corresponding to viable cells, PCD and necrosis events (Supplemental Fig. S2). Statistical significance of differences was determined using Student's *t* test ( $P < 0.05$ ). As shown in Supplemental Table S2, under physiological conditions, the percentage of viable cells in the CTRL leaves was 75.0 % while slight different values were recorded for Tdp2α-13c (72.5 %) and Tdp2α-28 (81.0 %). PCD was predominant in CTRL (15.0 %), Tdp2α-13c (19.5 %) and Tdp2α-28 (15.0 %) while the necrosis events, estimated 10.0 % in the CTRL line, were only slightly reduced in Tdp2α-13c (8.0 %) and significantly decreased in Tdp2α-28 (4.0 %) lines. This feature became more evident under heavy metal stress (Supplemental Table S2). In the CTRL leaves exposed to copper there was an overall reduction in cell viability (57.5 %) (Supplemental Table S2). However, the PCD events only slightly changed (17.5 %), compared to the untreated sample while necrosis accounted for 26.5 %. By contrast, both Tdp2α-13c and Tdp2α-28 lines treated with copper maintained almost unaltered viability values (75.0 %, Tdp2α-13c; 79.0 %, Tdp2α-28) when compared to the untreated sample. Under oxidative stress, the estimated percentage of PCD events was 17.0 % (Tdp2α-13c) and 15.5 % (Tdp2α-28) while necrosis accounted for 8.0 % (Tdp2α-13c) and 5.5 % (Tdp2α-28). The observed decrease in cell death events well correlated with the level of *MtTdp2α* gene overexpression. When comparing CTRL and the *MtTdp2α*-overexpressing lines, the calculate decrease in necrosis frequency was 25 % (Tdp2α-13c) and 20 % (Tdp2α-28).

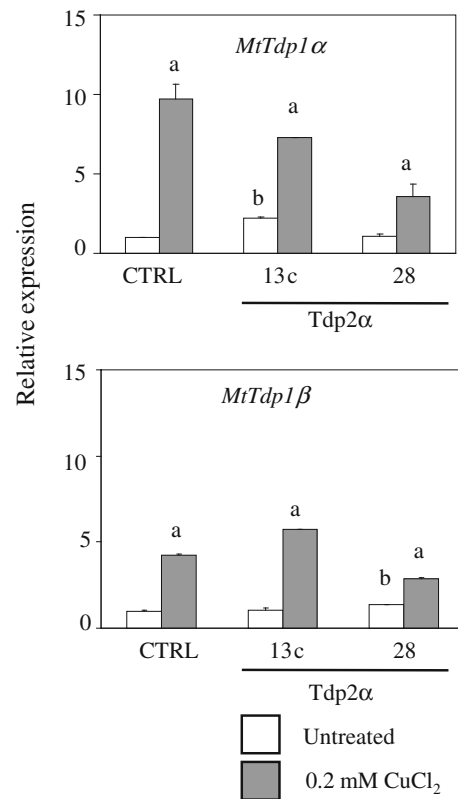
#### Double strand break accumulation in *MtTdp2α*-overexpressing and CTRL lines

The level of Double strand breaks (DSBs) was evaluated in leaf tissues of CTRL, Tdp2α-13c and Tdp2α-28 lines



**Fig. 2** Comet images of intact (untreated control) and damaged (0.2 mM CuCl<sub>2</sub>) DNA from *M. truncatula* leaf tissues (a) and level of DSBs measured by neutral SCGE in leaves excised from 10-day-old Tdp2 $\alpha$ -13c, Tdp2 $\alpha$ -28 and control (CTRL) plantlets grown in vitro in presence/absence of 0.2 mM CuCl<sub>2</sub> (b). For each treatment combination, data represent the mean values  $\pm$  SD of at least two independent experiments each of them carried out using three technical replications. a.u. arbitrary units. a Significant difference between copper treatment and physiological conditions. b Significant difference between Tdp2 $\alpha$ -overexpressing line and CTRL line

challenged with 0.2 mM CuCl<sub>2</sub> using neutral SCGE (Fig. 2). Morphologies of nuclei extracted from viable, PCD and necrotic cells are shown in Suppl. Fig. S2. In the absence of copper, the amount of DSBs in leaf tissues was  $91.0 \pm 11.0$  a.u. while a significantly ( $P < 0.0001$ ) lower level of DNA damage was recorded in the Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines ( $59.0 \pm 7.0$  and  $57.3 \pm 4.1$  a.u., respectively). In response to copper treatment, the amount of DSBs was significantly ( $P < 0.0001$ ) enhanced ( $129.3 \pm 8.5$  a.u.) in CTRL leaves in comparison with the untreated sample and a similar response was observed in the Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines, which showed a DSB level of  $96.0 \pm 9.0$  a.u. and  $74.5 \pm 0.6$  a.u., respectively (Fig. 2). Heavy metal-induced DNA damage increased up to 1.0-fold in the CTRL line while there was a limited increase (0.7- and 0.4-fold, respectively) in the Tdp2 $\alpha$ -13c



**Fig. 3** Expression profiles of the *MtTdp1 $\alpha$*  and *MtTdp1 $\beta$*  genes in the *MtTdp2 $\alpha$* -overexpressing lines Tdp2 $\alpha$ -13c, Tdp2 $\alpha$ -28 and control (CTRL) line assessed by QRT-PCR. Leaves were excised from 10-day-old plants grown in vitro in presence/absence of 0.2 mM CuCl<sub>2</sub>. For each treatment combination, data represent the mean values  $\pm$  SD of at least two independent experiments each of them carried out using three technical replications. a Significant difference between copper treatment and physiological conditions. b Significant difference between Tdp2 $\alpha$ -overexpressing line and CTRL line

and Tdp2 $\alpha$ -28 lines. The Tdp2 $\alpha$ -13c line with lower levels of *MtTdp2 $\alpha$*  gene overexpression, accumulated significantly higher DSB amounts, compared to the Tdp2 $\alpha$ -28 line.

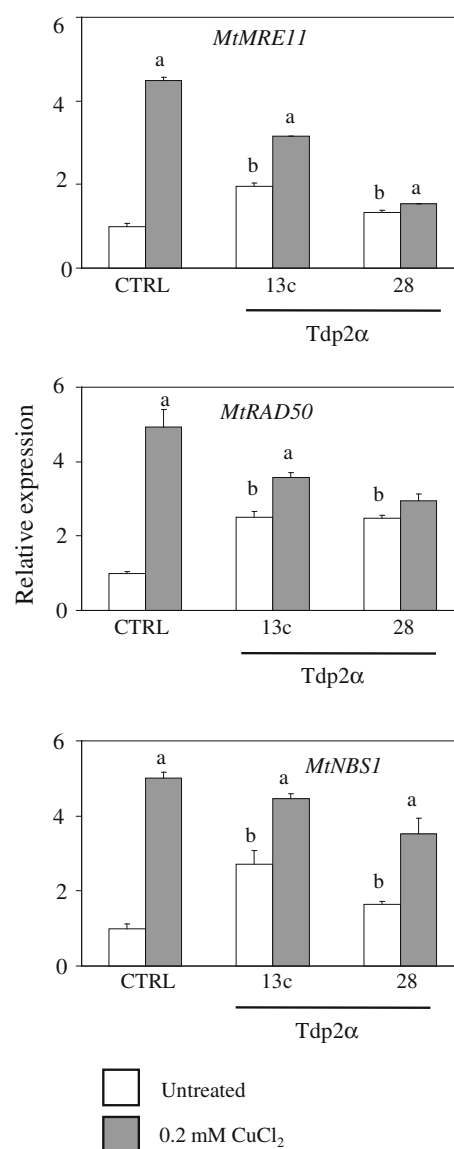
#### Expression profiles of *Tdp1* genes

To assess the overall contribution in the plant response to heavy metal stress resulting from Tyrosyl-DNA phosphodiesterases, the expression profiles of *MtTdp1 $\alpha$*  and *MtTdp1 $\beta$*  genes were also investigated in leaves of CTRL and *MtTdp2 $\alpha$* -overexpressing plants. As shown in Fig. 3, the *MtTdp1 $\alpha$*  gene was significantly ( $P < 0.0001$ ) up-regulated (9.7-fold) in the CTRL leaves exposed to 0.2 mM CuCl<sub>2</sub>. The Tdp2 $\alpha$ -13c line, showing a significant ( $P = 0.0116$ ) increase in *MtTdp1 $\alpha$*  transcript levels (2.3-fold compared to CTRL) under physiological conditions, was responsive to copper since there was a further significant ( $P < 0.0001$ ) enhancement (3.1-fold compared to the

untreated sample). The Tdp2 $\alpha$ -28 line resulted into a significant ( $P < 0.0001$ ) accumulation of the *MtTdp1 $\alpha$*  transcript (3.5-fold compared to the untreated sample) only under heavy metal stress (Fig. 3). The *MtTdp1 $\beta$*  gene was significantly ( $P < 0.0001$ ) up-regulated (4.2-fold) in the CTRL leaves exposed to 0.2 mM CuCl<sub>2</sub>. The Tdp2 $\alpha$ -13c line, showed a significant ( $P < 0.0001$ ) increase in *MtTdp1 $\beta$*  transcript levels (5.7-fold compared to CTRL) in response to copper while the Tdp2 $\alpha$ -28 line resulted into a significant ( $P < 0.0001$ ) accumulation of the *MtTdp1 $\beta$*  transcript (2.1-fold compared to the untreated sample) under heavy metal stress (Fig. 3). The reported data indicate that the Tdp1 function plays a key role in preserving genome stability in response to copper. The Tdp2 $\alpha$ -28 line, showing the highest levels of *MtTdp2 $\alpha$*  transcript, revealed lower accumulation of *MtTdp1 $\alpha$*  and *MtTdp1 $\beta$*  transcripts compared to Tdp2 $\alpha$ -13c line.

#### Expression profiles of genes involved in DSB sensing in *MtTdp2 $\alpha$* -overexpressing lines

The expression profiles of *MtMRE11*, *MtRAD50* and *MtNBS1* genes were evaluated by QRT-PCR in leaf tissues of CTRL and *MtTdp2 $\alpha$* -overexpressing lines exposed to copper. As shown in Fig. 4, the *MtMRE11* gene was significantly ( $P < 0.0001$ ) up-regulated (4.5-fold) in the CTRL leaves exposed to 0.2 mM CuCl<sub>2</sub>. The Tdp2 $\alpha$ -13c line, showing a significant ( $P < 0.0001$ ) increase in *MtMRE11* transcript levels (2.0-fold compared to CTRL) under physiological conditions, was responsive to copper since there was a further significant ( $P < 0.0001$ ) enhancement (1.6-fold compared to the untreated sample). The Tdp2 $\alpha$ -28 line resulted into a significant ( $P < 0.0001$ ) up-regulation of *MtMRE11* gene, both under physiological conditions (1.3-fold) and copper stress (1.5-fold). The *MtRAD50* gene was significantly ( $P < 0.0001$ ) up-regulated (4.9-fold) in the CTRL leaves exposed to 0.2 mM CuCl<sub>2</sub>. Both the Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines showed a significant ( $P < 0.0001$ ) increase in *MtRAD50* transcript levels (2.5-fold in both lines) under physiological conditions. Exposure to copper triggered a further transcript accumulation (1.4- and 1.2-fold compared to the untreated sample). The *MtNBS1* gene was significantly ( $P < 0.0001$ ) up-regulated (5.0-fold) in the CTRL leaves exposed to 0.2 mM CuCl<sub>2</sub>. The Tdp2 $\alpha$ -13c line, showing a significant ( $P < 0.0001$ ) increase in *MtNBS1* transcript levels (2.7-fold compared to CTRL) under physiological conditions, was responsive to copper since there was a further significant ( $P < 0.0001$ ) enhancement (1.6-fold compared to the untreated sample). A similar response was observed for the Tdp2 $\alpha$ -28 line which showed a significant ( $P = 0.0082$ ) increase in *MtNBS1* transcript levels (1.6-fold compared to CTRL) under physiological conditions, and a further

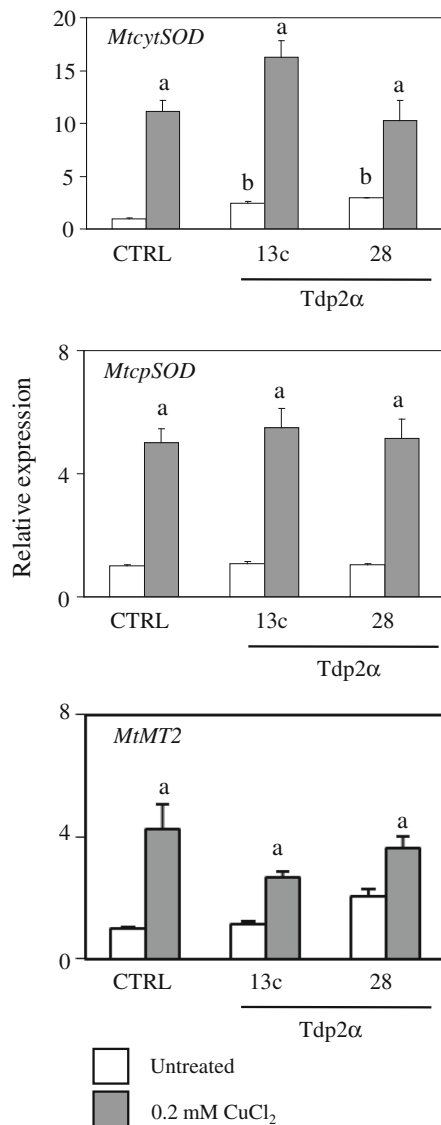


**Fig. 4** Expression profiles of the *MtMRE11*, *MtRAD50* and *MtNBS1* genes in *MtTdp2 $\alpha$* -overexpressing lines Tdp2 $\alpha$ -13c, Tdp2 $\alpha$ -28 and control (CTRL) line assessed by QRT-PCR. Leaves were excised from 10-day-old plants grown in vitro in presence/absence of 0.2 mM CuCl<sub>2</sub>. For each treatment combination, data represent the mean values  $\pm$  SD of at least two independent experiments each of them carried out using three technical replications. *a* Significant difference between copper treatment and physiological conditions. *b* Significant difference between Tdp2 $\alpha$ -overexpressing line and CTRL line

significant ( $P < 0.0001$ ) enhancement (2.2-fold compared to the untreated sample) (Fig. 4).

#### Expression profiles of antioxidant genes

A preliminary search in Phytozome and NCBI databases revealed the presence of different superoxide dismutase (SOD) isoforms in *M. truncatula*. Results from this analysis are summarised in see Supplemental Table S3 where



**Fig. 5** Expression profiles of the *MtcytSOD*, *MtcpSOD* and *MtMT2* genes in the *MtTdp2α*-overexpressing lines Tdp2α-13c, Tdp2α-28 and control (CTRL) line assessed by QRT-PCR. Leaves were excised from 20-day-old plants grown in vitro in presence/absence of 0.2 mM CuCl<sub>2</sub>. For each treatment combination, data represent the mean values ± SD of at least two independent experiments each of them carried out using three technical replications. *a* Significant difference between copper treatment and physiological conditions. *b* Significant difference between Tdp2α-overexpressing line and CTRL line

all isoforms (Cu/Zn SOD, Fe SOD and Mn SOD) and relative subcellular localization are indicated. In the present work, the expression profiles of the *MtcytSOD* (Medtr7g114240) and *MtcpSOD* (Medtr4g057240) genes, encoding a cytosolic and a chloroplastic isoform, respectively, were evaluated. Moreover, the *MtMT2* gene encoding a type 2 metallothionein was investigated. As shown in Fig. 5, the *MtcytSOD* gene was significantly ( $P < 0.0001$ ) up-regulated (11.1-fold) in the CTRL leaves exposed to 0.2 mM CuCl<sub>2</sub>. The Tdp2α-13c line, showing a

significant ( $P = 0.0051$ ) increase in *MtcytSOD* transcript levels (2.5-fold compared to CTRL) under physiological conditions, was highly responsive to copper since there was a further significant ( $P < 0.0001$ ) enhancement (6.5-fold compared to the untreated sample). Similarly, the Tdp2α-28 line resulted into a significant ( $P = 0.0002$ ) accumulation of the *MtcytSOD* transcript (3.0-fold compared to the untreated sample) under physiological conditions and a further up-regulation (3.4-fold compared to the untreated sample) under heavy metal stress (Fig. 5). As for the *MtcpSOD* gene, there was a significant ( $P < 0.0001$ ) up-regulation only under heavy metal stress in all the tested lines, with an estimated increase of 5.0-fold (CTRL), 5.5-fold (Tdp2α-13c) and 5.2-fold (Tdp2α-28). Similarly, the *MtMT2* gene showed a significant ( $P < 0.0001$ ) up-regulation only under heavy metal stress in all the tested lines, with an increase of 4.2-fold (CTRL), 2.4-fold (Tdp2α-13c) and 1.8-fold (Tdp2α-28).

## Discussion

Heavy metal pollution originating from anthropogenic sources represents a concern for the environment and the nutritional quality of plant-derived foods. Due to the complexity of DNA repair pathways and antioxidant processes activated *in planta*, detailed investigations are required to sort out genes involved in the removal of heavy metal-mediated DNA damage. The role played by tyrosyl-DNA phosphodiesterases in protecting plants against the genotoxic damage caused by copper has been demonstrated in the present report, using transgenic *M. truncatula* plants overexpressing the *MtTdp2α* gene. To our knowledge, this is the first case in which the overexpression *in planta* of a specific DNA repair gene reinforces the genotoxic stress response.

The protective effects against copper toxicity resulting from the overexpression of *MtTdp2α* gene were clearly evidenced by scoring the frequency of cell death events. In both Tdp2α-13c and Tdp2α-28 lines, cell viability remained significantly higher than in CTRL line, concomitant with the reduction in the percentage of necrotic cells. Interestingly, a lower frequency of necrosis was also recorded in the same *MtTdp2α*-overexpressing lines exposed to osmotic stress (Confalonieri et al. 2013). Since necrosis arises when high stress levels are provided (Reape and McCabe 2008), the reduced amount of necrotic cells found in the *MtTdp2α*-overexpressing lines clearly suggests that the tested copper concentration was perceived as a mild stress treatment in these tissues, contrarily to what was observed in the CTRL plants. It is worth noting that the Tdp2α-28 line, characterised by the highest levels of overexpression, also showed the highest percentage of

viable cells together with the most consistent reduction in frequency of necrosis.

SCGE confirmed that both Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines accumulated significantly lower amounts of DSBs compared to CTRL, even in the absence of heavy metal. The same DSB amount was detected in both the *MtTdp2 $\alpha$* -overexpressing lines, despite the different levels of transgene overexpression, in agreement with Confalonieri et al. (2013). Under physiological conditions, the *M. truncatula* cells may own a damage threshold under which no further DNA repair takes place, an aspect which deserves a deeper investigation. As for DNA damage levels in relation to *MtTdp2 $\alpha$*  overexpression, the Tdp2 $\alpha$ -28 line stands as the best performing. The ability of *MtTdp2 $\alpha$* -overexpressing lines to buffer DSB accumulation under osmotic stress was demonstrated by Confalonieri et al. (2013).

Up-regulation of the endogenous *MtTdp2 $\alpha$*  gene was observed in response to copper in all the tested lines, however, the extent of up-regulation was limited in the Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines, compared to the CTRL line. It is possible that the enhanced amounts of *MtTdp2 $\alpha$*  mRNA contribute to improve the plant response to copper-induced genotoxic injury. Although information concerning the contribution of the *Tdp1* family members in heavy metal stress response in plants is scanty, these genes were demonstrated to be copper responsive (Macovei et al. 2010; Balestrazzi et al. 2011a).

Another interesting point is related to the ability of the *MtTdp2 $\alpha$* -overexpressing lines to sense DNA damage accumulation. This capacity should be possibly enhanced since all the three components of the MRN complex, a crucial sensor of DSBs in both animal and plant cells, were up-regulated under physiological conditions and then further stimulated in presence of copper. The MRN (MRE11-RAD50-NBS1) complex plays a key role as a sensor of DSBs, able to activate the DSB-induced cell cycle checkpoints and recognise DSB-repair effectors (Lamarche et al. 2010). MRE11 is a multifunctional nuclease active as 3'-5' exonuclease in DSB repair, while the RAD50 protein characterised by ATPase and DNA binding activity interacts with MRE11. NBS1 directs the nuclear localization of the complex (Daoudal-Cotterell et al. 2002). The expression profiles of MNR components hereby reported are in agreement with those presented by Confalonieri et al. (2013) in the CTRL, Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 lines exposed to osmotic stress. Copper-induced ROS accumulation, particularly the production of hydroxyl radical (OH $\cdot$ ) results in enhanced levels of single strand breaks (SSBs) and DSBs (Chiu et al. 1995). When copper binds directly to DNA, the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) seems to be required for the genotoxic effect to occur (Moriwaki et al. 2008). The enhanced expression of the DSB sensing/repair genes observed in *MtTdp2 $\alpha$* -

overexpressing *M. truncatula* lines under physiological conditions well correlates with the reduced level of DSBs revealed by SCGE. On the other hand, it should be hypothesised that heavy metal stress did not result in *MtMRE11*, *MtRAD50* and *MtNBS1* mRNA accumulation as high as that found in CTRL due to the low DSB levels occurring in the *MtTdp2 $\alpha$* -overexpressing *M. truncatula* lines.

The plant antioxidant system includes several enzymes that respond in a coordinated manner when ROS are generated under stress conditions. SOD catalyses the dismutation of O<sub>2</sub><sup>-</sup> to O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> while metallothioneins have been recognised as efficient ROS scavengers able to prevent DNA damage (Balestrazzi et al. 2009). The plant tolerance to heavy metals is mediated by the antioxidant network and among the enzymatic components SOD plays a key role. Copper toxicity targets chloroplasts by affecting the photosynthetic electron transport and altering the thylakoid membrane structure (Vinit-Dunand et al. 2002). The Cu/Zn-SOD isoforms are found in cytosol and chloroplast, the cytSOD being responsive to 0.2 mM CuCl<sub>2</sub> in aerial parts of *M. truncatula* plants (Macovei et al. 2011). Confalonieri et al. (2013) reported the up-regulation of *MtCytSOD* gene in the aerial parts of Tdp2 $\alpha$ -13c and Tdp2 $\alpha$ -28 plants grown in vitro under physiological conditions and this finding was then confirmed in the present work. However, a significant and consistent up-regulation of *MtCytSOD* gene was observed in all the tested lines exposed to copper, suggesting that the heavy metal treatment caused the induction of the basal antioxidant defence, independent on *MtTdp2 $\alpha$*  gene overexpression. The enhancement of this specific DNA repair function may provide cells with the ability to face genotoxic injury inside the nuclear compartment. On the contrary, outside the nucleus in the surrounding cytosol the incoming copper-mediated oxidative damage triggers the antioxidant machinery. This hypothesis is also strengthened by the response observed with the *MtCytSOD* gene which was as well up-regulated in all the tested lines, independent on *MtTdp2 $\alpha$*  gene overexpression. Thus, the reported data underline the occurrence of compartment-specific antioxidant defences and repair mechanisms in the *MtTdp2 $\alpha$* -overexpressing cells, in agreement with the current literature (Kodicha and Stochaj 2012).

The *MtMT2* gene was up-regulated only in the Tdp2 $\alpha$ -28 line under physiological conditions, in agreement with Confalonieri et al. (2013) while there was significant up-regulation in all the tested lines when copper was provided. The role of metallothionein in preventing the genotoxic injury caused by copper has been demonstrated by Balestrazzi et al. (2009) using transgenic white poplar plants overexpressing the *PsMTa2* gene. However, differently from the transgenic white poplar plants overexpressing the



*PsMTa2* gene, the *M. truncatula* lines hereby investigated possess enhanced DNA repair gene expression which ensures a proper protection within the nuclear compartment. Based on the reported data, it seems that the basal antioxidant defence activated by *M. truncatula* cells, with and without *MtTdp2α* gene overexpression, includes metallothionein.

The overall picture emerging from the present work strongly support for the use of *MtTdp2α* gene as a tool for improving the plant response to heavy metal stress and provides further evidence that the overexpression of this DNA repair gene enhances the plant response to genotoxic injury resulting from different stress agents. Thus, biotechnological approaches involving the overexpression of *MtTdp2α* gene are expected to significantly reinforce the agronomical performance of elite crop varieties under adverse field conditions. However, the reported data also highlight the compartment-specific organisation of the cell response to oxidative injury, a complex as well as flexible way to control the molecular events inside and outside the nucleus. This point needs to be carefully evaluated when designing novel strategies to improve the plant tolerance to environmental stresses, which might possibly require the coordinated interplay between DNA repair functions and ROS scavengers within and outside the nuclear compartment.

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