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ORIGINAL PAPER

Enhanced osmotic stress tolerance in *Medicago truncatula* plants overexpressing the DNA repair gene *MtTdp2α* (tyrosyl-DNA phosphodiesterase 2)

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Abstract No information is currently available in plants concerning the tyrosyl-DNA phosphodiesterase 2 (Tdp2) enzyme which in animals is involved in the removal of DNA topoisomerase II-mediated DNA damage and cell proliferation/differentiation signaling. Bioinformatic investigation revealed the occurrence in the plant kingdom of three distinct Tdp2 isoforms, named α, β and γ. The *MtTdp2α* gene from *Medicago truncatula* Gaertn., encoding a protein with putative nuclear localization signal and chloroplast transit peptide, was significantly up-regulated in response to osmotic stress induced by polyethylene glycol. The transgenic *M. truncatula* lines Tdp2α-13C and Tdp2α-28 overexpressing the *MtTdp2α* gene were characterised by enhanced tolerance to both osmotic and photo-oxidative stress. According to single cell gel electrophoresis, *MtTdp2α* gene overexpression prevented accumulation of double strand breaks in absence and presence of osmotic stress. Interestingly, the *MtMRE11*, *MtRAD50* and *MtNBS1* genes involved in double strand break sensing/repair were significantly up-regulated in the *MtTdp2α*-overexpressing plants grown under physiological conditions and no further up-regulation occurred in response the osmotic agent. The Tdp2α-13C and Tdp2α-28 lines also showed significant up-regulation of several genes essential for the control of DNA topology and genome maintenance, such as *MtTdp1α*, *MtTop2* (DNA topoisomerase II) and *MtGYR* (DNA gyrase). The role of *MtTdp2α* gene in enhancing the plant response to genotoxic injury under osmotic stress is discussed.

Keywords DNA repair · Double strand breaks · *Medicago truncatula* · Osmotic stress · Transgenic plant · Tyrosyl-DNA phosphodiesterase 2

Abbreviations

APX	Ascorbate peroxidase
CFX	Ciprofloxacin
SOD	Superoxide dismutase
DSB	Double strand break
Gyr	Gyrase
MRE	Meiotic recombination
MT	Metallothionein
NBS	Nijmegen breakage syndrome
NLS	Nuclear localisation signal
PCD	Programmed cell death
PEG	Polyethylene glycol
PQ	Paraquat
QRT-PCR	Quantitative realtime polymerase chain reaction
RAD	Radiation-sensitive
SCGE	Single cell gel electrophoresis

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Tdp	Tyrosyl-DNA phosphodiesterase
hTdp	Human tyrosyl-DNA phosphodiesterase
Topo	DNA topoisomerase
TTRAP	TRAF and TNF receptor-associated protein

Introduction

Topological problems associated with DNA replication, transcription, recombination and accumulated during chromatin remodeling in the DNA molecules are solved by DNA topoisomerases I (topo I) and DNA topoisomerases II (topo II) through the controlled and transient breakage of single or double DNA strands (SSBs, DSBs), respectively (Vos et al. 2011). Specifically, during its catalytic cycle, topo II becomes covalently linked to the 5′-terminus of DNA by a phosphotyrosyl bond. Covalent complexes are normally resolved, unless they are formed in close proximity of damaged DNA sites or in the presence of topo II inhibitors. In human cells, the TTRAP (TRAF and TNF receptor-associated protein) enzyme is able to break the 5′-phosphotyrosyl bond, restoring the intact 5′-phosphate DNA termini (Cortes Ledesma et al. 2009). TTRAP is a member of the Mg^{2+}/Mn^{2+}-dependent family of phosphodiesterases and its depletion is associated with increased levels of topo II-mediated DNA damage. The enzyme, recently named tyrosyl-DNA phosphodiesterase 2 (Tdp2) (EC number 3.1.4.-), is an essential protein for cell survival in response to the accumulation of DSBs induced by topo II (Zeng et al. 2011). In a recent review, Li et al. (2011a) have provided an overall description of the pleiotropic functions played by the human Tdp2 enzyme which is involved in DNA repair, in transcriptional regulation and in signaling processes. Several transcription factors playing a role in the regulation of cell proliferation/differentiation but also in apoptosis, associate with Tdp2 and there is an increasing body of literature highlighting the complex network of Tdp2-mediated signaling pathways (Li et al. 2011a). In mammalian cells, the Tdp2 protein acts as an adaptor molecule within a non-canonical signaling pathway which triggers the Transforming Growth Factor-β (TGF-β)-mediated apoptosis (Varady et al. 2011). The Tdp2 protein, which has been localized also in the nucleolar cavities, associated with rRNA biogenesis, might be involved in the cross-talk between cytoplasm and nucleolus under stress conditions (Vilotti et al. 2012).

The Tyrosyl-DNA phosphodiesterase family includes also the Tdp1 enzyme able to break the covalent linkage (3′-phosphotyrosyl bond) between the DNA termini and the catalytic tyrosine residue of topo I, thus removing the stabilized topo I/DNA covalent complexes (Interthal et al. 2001). In animal cells, Tdp1 contributes to enhance the fidelity of non homologous end joining (NHEJ) mechanism (Bahmed et al. 2010) and participates in base excision repair (BER) (Lebedeva et al. 2011). Furthermore, Das et al. (2010) reported that the human Tdp1 protein is required for the efficient repair of DNA oxidative damage in mitochondria.

By contrast, information on plant tyrosyl-DNA phosphodiesterases is still limited. Macovei et al. (2010) reported for the first time in plants on the *Tdp1* gene family from *Medicago truncatula* Gaertn. composed of *MtTdp1α* and *MtTdp1β* genes that were up-regulated in response to heavy metal and osmotic stresses (Macovei et al. 2010). The *MtTdp1* genes were also significantly up-regulated during seed imbibition when DNA repair is required to preserve genome integrity and improve seed vigour (Balestrazzi et al. 2011a, b; Ventura et al. 2012). An *Arabidopsis thaliana Tdp1* mutant was described and the catalytic function of the protein demonstrated by Kim et al. (2012). More recently, an intron-spliced hairpin RNA approach was used for the targeted silencing of the *MtTdp1α* gene encoding the MtTdp1α isoform (Donà et al. 2013a). RNA-Seq analysis, carried out in the *MtTdp1α*-depleted *M. truncatula* plants revealed different levels of transcriptional modulation, e.g. differential expression and alternative splicing in genes involved in DNA damage sensing, DNA repair and chromatin remodeling (Donà et al. 2013a).

The plant response to environmental stresses involves metabolic networks that are activated following damage perception and provide appropriate defences, among which DNA repair represents a unique route to preserve genetic information (Balestrazzi et al. 2012). Despite the literature available on the the plant response to genotoxic stress, several open questions still remain, such as those concerning the use of DNA repair genes as tools to improve the field response in crops (Tuteja et al. 2009; Ahmad et al. 2010). Genes involved in DNA and RNA metabolism are regulated in response to environmental stresses, as reported in the case of the *Pisum sativum* DNA helicase which, expressed in tobacco, provided high salinity tolerance. Excess salt induces osmotic stress accompanied by ROS accumulation and most researchers have focused their interest on the antioxidant genes activated under salt stress (Mhadhbi et al. 2011). However, DNA repair genes that are significantly up-regulated under salt stress might represent interesting tools to improve plant tolerance as exemplified by the work carried out on plant DEAD-box helicases (Vashisht and Tuteja 2006; Macovei et al. 2012).

In this paper, the *MtTdp2α* gene from *M. truncatula* Gaertn. has been isolated and characterised, for the first time in plants. The potential involvement of the *MtTdp2α* gene in the plant response to genotoxic injury caused by

exposure to osmotic stress was investigated using transgenic *M. truncatula* plants overexpressing the *MtTdp2α* gene. The reported data open new perspectives for improving crop tolerance to adverse environmental conditions.

Materials and methods

Plant materials and treatments

Plantlets of *Medicago truncatula* Gaertn. (genotype R108-1) (Trinh et al. 1998) were grown in vitro in sterile vessels (Micropoli, Cesano Boscone, Italy) on a medium containing macrosalts and microsalts 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). *M. truncatula* Gaertn. cv. Jemalong (M9-10a genotype) plants were micropropagated in vitro as described by Confalonieri et al. (2009). The *M. truncatula* plants were 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 μMol/m^2 s^{-1} under a cool white fluorescence lamp. For osmotic stress treatments, polyethylene glycol (PEG6000, Duchefa Biochemie, The Netherlands) was added at the indicated concentrations. Paraquat (PQ) treatments were carried out as described by Balestrazzi et al. (2009) with the following modifications. Leaflets were excised from 20-days-old barrel medic plants grown in pots and transferred to Petri dishes containing 10 ml of a solution with 0.5 μM PQ and 0.1 % Tween 20 as a surfactant. Incubation was carried out under continuous light (27–33 μMol/m^2/s) at 25 °C for 24 h. Ciprofloxacin (CFX; Sigma-Aldrich, Milan, Italy) treatments were carried out as described by Wall et al. (2004). Leaflets were excised from 20-day-old barrel medic plants grown in pots and transferred to Petri dishes containing 10 mL of a solution with 25 μM CFX. Incubation was carried out as previously described. For expression profile analysis, plants were grown in greenhouse, in pots containing a mixture of peat and soil (1:2). Cultivation was performed following standard procedures. Plants were not supplied with any additional nutrients and watered when needed. Tissues were collected from plants and stored in liquid N_2.

Chlorophyll content

In order to determine the chlorophyll content, three leaf discs per treatments were analysed as follows. Extraction with 1 mL of 80 % (v/v) acetone was carried out and the total chlorophyll content was determined by measuring the absorbance of the supernatant at 664 and 647 nm (Wellburn 1994).

Cloning procedure and sequence analysis

RNA isolation was carried out using the Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Milan, Italy), according to manufacturer's instructions. cDNAs were obtained with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Monza, Italy) according to manufacturer's suggestions. The oligonucleotide primers MtTDP2-F1 (5′-CGGGGTACCCATGTCTTGGTCATGC AAAAAATGC-3′) and MtTDP2-R1 (5′-CGCGGATCCCC TTAGACCCAACAATAGCACAGT-3′) were used to amplify the full length *MtTdp2* cDNA. Amplification was obtained using the following PCR conditions: 94 °C 50 s, 59 °C 50 s, 72 °C 2 min (35 cycles) in a T-Gradient PCR apparatus (Biometra GmBH, Goettingen, Germany), using the TaKaRa Ex Taq™ DNA Polymerase (Takara Bio Inc., Otsu-Shiga, Japan). PCR products were purified from agarose gel (Duchefa Biochemie) using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Milan, Italy) and subsequently cloned into the TA cloning vector pTZ57R/T with the InsTA Clone™ PCR Cloning Kit (Fermentas, Cornaredo, Italy). The recombinant plasmid pTZ57R-Tdp2 was then purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Milan, Italy). Sequence analysis was performed by BMR Genomics (Padua, Italy).

Construction of pTdp2α plasmid, plant genetic transformation and in vitro regeneration

The full length *MtTdp2α* cDNA (Phytozome Database Accession N° Medtr8g146980) was amplified from the pTZ57R-Tdp2α plasmid by PCR carried out with the oligonucleotide primers MtTDP2-F1 and MtTDP2-R1 as described in the text. The PCR product was purified using the Wizard™ SV Gel and PCR Clean UP Kit (Promega) and subsequently digested with the restriction enzymes *Kpn*I and *Bam*HI (Fermentas), according to manufacturer's instructions, in order to allow unidirectional ligation into the multiple cloning site of the pHannibal vector (CSIRO Plant Industry, Camberra, Australia). The resulting 35SCaMV-*Tdp2α*-OCS cassette was excised from the pHannibal vector by restriction with *Not*I (Fermentas) and then inserted into the binary vector pART27 (CSIRO). Sequencing of the pART-Tdp2α construct was performed by BMR Genomics. The binary vector pART-Tdp2α was subsequently transferred by electroporation into EHA105 *Agrobacterium tumefaciens* strain containing the hypervirulent disarmed plasmid pTIBo542 (Hood et al. 1993) and the resulting engineered strain EHA105-pTdp2α was used to transform the *M. truncatula* leaf explants. *Agrobacterium tumefaciens*-mediated transformation of M9-10a leaf explants and in vitro regeneration were performed as

described by Confalonieri et al. (2009). All the regenerated pTdp2α and control transgenic lines were maintained in vitro on a modified (10 g/L sucrose) MS030A medium and propagated using stem nodal explants with one or two axillary buds to obtain replicated plantlets for molecular analyses. Plantlets were maintained in a climate chamber with a 16 h-photoperiod (photon flux density 65–75 μMol/m^2/s, 24 °C; dark, 22 °C).

Molecular analyses of transgenic Tdp2α *M. truncatula* lines

Genomic DNA extraction from *M. truncatula* leaves, standard PCR analysis carried out to assess the presence of the construct sequences and QRT-PCR analyses performed to measure the transgene copy number are described in detail in Supplemental Data. For gene expression analysis, QRT-PCR was carried out with the SsoFast™ EvaGreen® Supermix (Bio-Rad) in a final volume of 20 μl 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 ELF1α* (GenBank Accession N° EST317575) as reference gene. Gene-specific oligonucleotide primers for the *M. truncatula MtTdp2α* (Phytozome Database Accession N° Medtr8g146980), *MtTdp1α* (XM_003622639), *MtTop2* (Phytozome Database Accession N° Medtr3g103270), *MtMRE11* (Phytozome Database Accession N° Medtr2g081100), *MtRAD50* (Phytozome Database Accession N° Medtr3g084300), *MtNBS1* (Phytozome Database Accession N° Medtr5g076180), *MtAPX* (DFCI ID TC115331), *MtSOD* (BQ255311), *MtMT2* (AC147202.14), *MtGyrA* (Phytozome Database Accession N° Medtr1g034980), and *MtGyrB* (Phytozome Database Accession N° Medtr7g106810) 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).

Single cell gel electrophoresis (SCGE)

Nuclei were extracted from *M. truncatula* cell suspension cultures as described by Donà et al. (2013b). The suspension containing purified nuclei and a solution of 1 % low melting point agarose in phosphate-buffered saline (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 μL DAPI (1 μgMmL, 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).

Bioinformatic analysis

The *M. truncatula* sequences were obtained from the Phytozome Database (http://www.phytozome.net). Comparison of amino acid sequences was performed using the Expasy SIB BLAST Network Service (http://www.expasy.ch/tools/blast//) and the EMBL-EBI Clustal W2 Multiple Sequence Alignement tool (http://www.ebi.ac.uk/tools/msa/clustalw2). The search for choroplast transit peptide was carried out using the ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/). The search for the nuclear localization signal was carried out using the NucPred program (http://www.sbc.su.se/~maccallr/nucpred/cgi-bin/single.cgi). The Motif Scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and Prosite (http://expasy.org/cgi-bin/nicedoc.pl?PS01358) were used to identify the other reported motifs within the Tdp2α protein sequence. The phylogenetic investigation was carried out using the Plaza 2.5 tool (bioinformatics.psb.ugent.be/plaza/) (Van Bel et al. 2012).

Telomere length analysis

Telomere length was evaluated with a QRT-PCR assay as described by Cawthon (2002) with the following modifications. Two distinct QRT-PCR reactions were performed in order to amplify the telomere (T) and single copy gene *MtIRE* (S), respectively. The T reaction was carried out in triplicate in a total volume of 30 mL containing *M. truncatula* genomic DNA (20 ng), 1 × BioEasy SYBR Green I Mix (Bioer Technology, Hangzhou, China), Tel-FW (5′-CGGTTTGTTGTGGGTTGTGGGTTGTGGGTTGTGGGTTGTGGGTT-3′) and Tel-RV (5′-GGCTTGTCCTGACCCTTGACCCTTGACCCTTGACCCTTGACCCT-3′) primers (100 nM each). The S reaction was carried out as previously described, using the MtIRE-FW (5′-TCCTCAAGCAAGCACATACG-3′) and MtIRE-RV (5′-CAAGATCGCTGAGTTGGTGA-3′) primers (300 nM each). Cycle

conditions were: denaturation at 95 °C for 10 min; 40 s at 95 °C, 60 s at 63 °C with fluorescence acquisition (40 cycles). Reactions were carried out in a Rotor-Gene 6000 PCR apparatus (Corbett Robotics). Standard curves for both telomere and *MtIRE* were set up using 5-fold serial dilutions of DNA from the CTRL line, ranging from 125 to 1 ng. Relative concentrations, expressed in ng per reaction, were calculated with the Rotor-Gene proprietary software. Telomere length was then expressed as the adimensional number T/S ratio, which represents the relative abundance of telomeric sequence per genome.

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

Plants own three putative Tdp2 isoforms

A detailed search in plant databases highlighted the presence of three distinct isoforms of the Tdp2 protein (hereby named Tdp2α, Tdp2β and Tdp2γ), all of them characterised by a putative endonucleases/exonuclease/phosphodiesterase family profile spanning the central and C-terminal regions. Plant Tdp2 isoforms differ in their N-terminal portion since the α isoform owns two Zinc finger RanBP2-type domains, the β isoform has only a single Zinc finger RanBP2-type domain while the γ isoform lacks the motif (Fig. 1). It is worth noting that the human Tdp2 protein lacks the Zinc finger RanBP2-type domain.

The three Tdp2 isoforms are also differently distributed among plant species, as listed in Fig. 1. Several Dicotyledons, such as the model plant *A. thaliana* and the closely related species *Arabidopsis lyrata*, and the legumes *M. truncatula*, *Glycine max* and *Lotus japonicus* possess only the Tdp2α isoform. In all of them *Tdp1α* is a single-copy gene, except for *G. max* that contains two gene copies. The Tdp2α isoform is found also in tropical species, e.g. *Theobroma cacao* and *Carica papaya*, as well as in the Euphorbiaceae *Manihot esculenta* (cassava) and *Ricinus communis* (castor bean). The Tdp2α isoform is present in *Populus trichocarpa* and *Vitis vinifera* (Fig. 1). The Tdp2β isoform is present in *Phaseolus vulgaris*. The Monocot *Oryza sativa* spp. japonica owns two genes encoding the α and β isoforms while *Oryza sativa* spp. indica is characterised by three genes, encoding the α, β and γ isoforms. The *Poaceae* family includes also *Zea mays* and *Sorghum bicolor*, both characterised by the presence of the β and γ Tdp2 isoforms, and the model grass *Brachipodium distachyon* with five *Tdp2* genes all encoding the γ isoform. The latter is also the unique Tdp2 protein found in some green algae such as *Chlamydomonas reinhardtii*, *Volvox carteri*, *Ostreococcus* and *Micromonas* (Fig. 1). Finally, the moss *Physcomitrella patens* (Briophytae) owns a single copy Tdp2 gene, coding for the β isoform.

Isolation and sequence analysis of the *M. truncatula* *MtTdp2α* cDNA

A bioinformatic investigation carried out in the *M. truncatula* database revealed the presence of the *Tdp2* sequence. The full-length *MtTdp2α* cDNA (1,593 bp) isolated by RT-PCR, contains a 5′-untranslated region (156 bp), followed by an open reading frame (ORF, 1,314 bp) and a 3′-untranslated end (117 bp) (data not shown). The ORF encodes a protein of 437 amino acids (aa) with a predicted molecular mass of 49.5 kDa (Fig. 2). A putative chloroplast transit peptide (MSWSCKKCTFVNPPSQISECEICFSSP PHPSSSSATSSSSSSPKWSCKS, aa 1–49) has been identified at the N-terminal region (Fig. 2). The protein contains also a putative nuclear localization signal (NLS) characterised by a short stretch of basic amino acid residues (KRKA, aa 98–102) and two Zinc finger RanBP2-type domains (SWSCKKCTFVNPPSQISECEICFSSPPH, aa 2–29; WSCKSCTLFNSYKNPICHLC, aa 45–64) frequently found in proteins involved in nuclear transport or localized to the nuclear envelope (Mattaj and Englmeier 1998) (Fig. 2).

Bioinformatic investigations revealed the presence of a putative endonucleases/exonuclease/phosphodiesterase family profile spanning the central and C-terminal regions (aa 169–436) of the *Mt*Tdp2α protein (Supplemental Fig. S1). The domain is found in a large number of proteins, including the Mg^{2+}-dependent endonucleases and phosphatases involved in intracellular signaling (Dlakic 2000) and DNA repair (Mol et al. 1995). The same domain is also known as the AP endonucleases family 1 profile which includes Exonuclease III and AP endonuclease 1 related enzymes, both with a role in the DNA repair by cleaving phosphodiester bonds at apurinic or apyrimidinic (AP) sites and producing 5′-ends that are base-free deoxyribose 5-phosphate residues. A typical feature of these proteins is the presence of metal binding sites (MBS). As shown in Supplemental Fig. S1, six highly conserved putative metal binding sites are present within the *Mt*Tdp2α and *At*Tdp2α sequences as well as within the human protein. The metal binding sites correspond to the following amino acid residues: N175, E207, D326, N328, D426 and H427

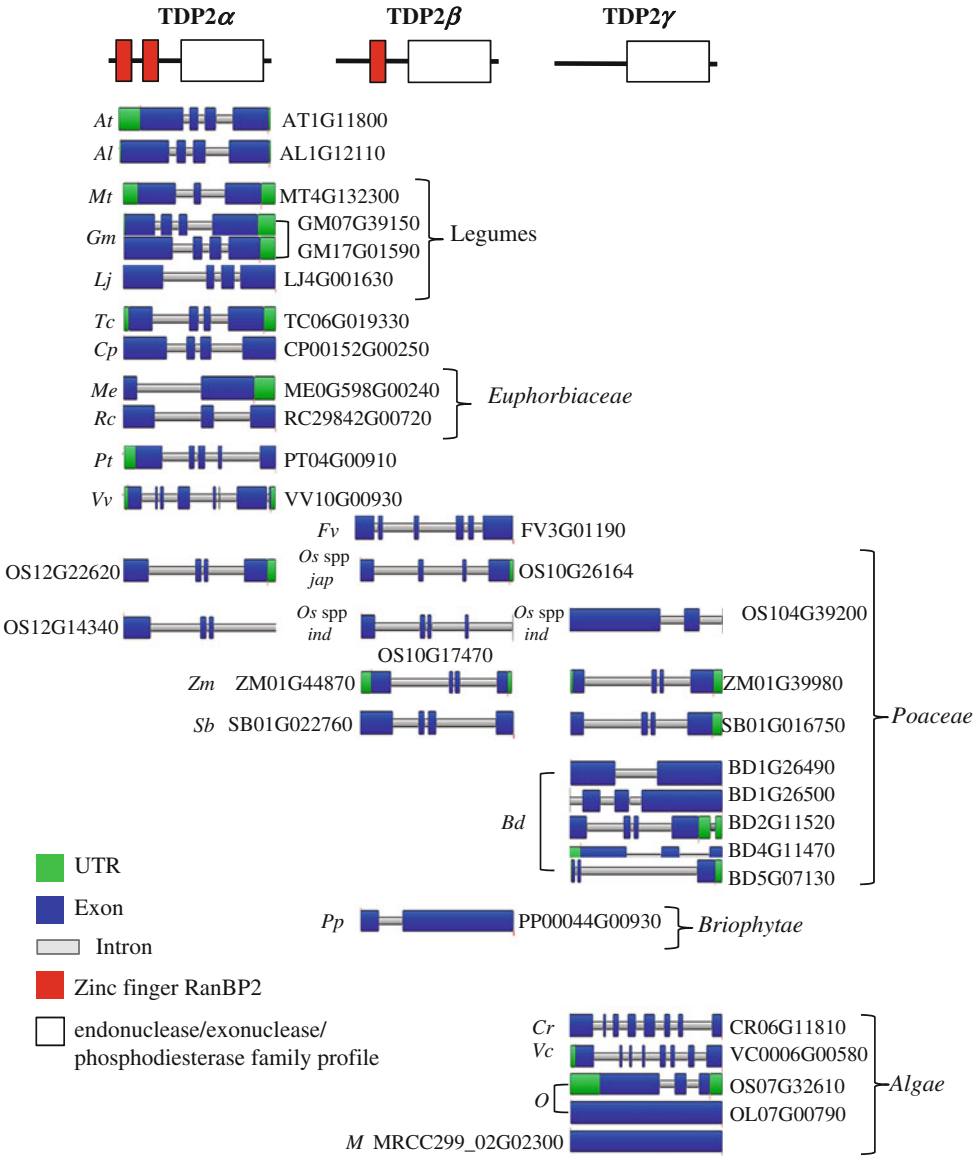

Fig. 1 Occurrence of the three different Tdp2 isoforms (α, β and γ) within the plant kingdom. For each species, the *Tdp2* gene structure (intron, exon, untranslated region) and the corresponding Genbank Accession N° are shown. At, *Arabidopsis thaliana*; Al, *Arabidopsis lyrata*; Mt, *Medicago truncatula*; Gm, *Glycine max*; Lj, *Lotus japonicus*; Tc, *Theobroma cacao*; Cp, *Carica papaya*; Me, *Manihot esculenta*; Rc, *Ricinus communis*; Pt, *Populus trichocarpa*; Vv, *Vitis vinifera*; Fv, *Fragaria visca*; Os, *Oryza sativa* (spp. Japonica; spp. Indica); Zm, *Zea mays*; Sb, *Sorghum bicolor*; Bd, *Brachypodium distachyon*; Pp, *Physcomitrella patens*; Cr, *Chlamydomonas reinhardtii*; Vc, *Volvox carteri*; O, *Ostreococcus*; M, *Micromonas*

(*M. truncatula*); N179, E211, D330, N332, D430 and H431 (*A. thaliana*); N120, E152, D262, N264, D350 and H351 (*H. sapiens*). The TWN motif (T/S W/F/Y N) corresponding to aa 174–176 (SYN) of the *Mt*Tdp2α protein is required for the proper orientation of the catalytic residues while the LQE motif (FQE, aa 205–207), particularly the Glu residue, is involved in the coordination of the Mg^{2+} or Mn^{2+} cation (Cortes Ledesma et al. 2009). The GDXN motif (where X is in most cases an hydrophobic residue), located at aa 325–328 (GDMN) of the *Mt*Tdp2α protein (Supplemental Fig. S1), is required for the interaction of the enzyme with the phosphate group of the substrate (Cortes Ledesma et al. 2009). The SDH motif (aa 425–427) is involved in the acid–base catalysis and stabilization of the transition state (Whisstock et al. 2000).

The comparison between the Tdp2α amino acid sequences from *M. truncatula* (Phytozome Database Accession N° Medtr8g146980), *A. thaliana* (Phytozome Database Accession N° AT1G11800) and *Homo sapiens* (GenBank Accession N° NP_057698) is shown in Supplemental Fig. S1. The overall similarity of the *Mt*Tdp2α, *At*Tdp2α and *Hs*Tdp2 amino acid sequences is extremely low (13.9 %) while the two plant Tdp2α amino acid sequences share a higher similarity (57.8 %). The putative chloroplast transit peptide and the NLS signal are evidenced in both the *M. truncatula* and *A. thaliana* sequences.

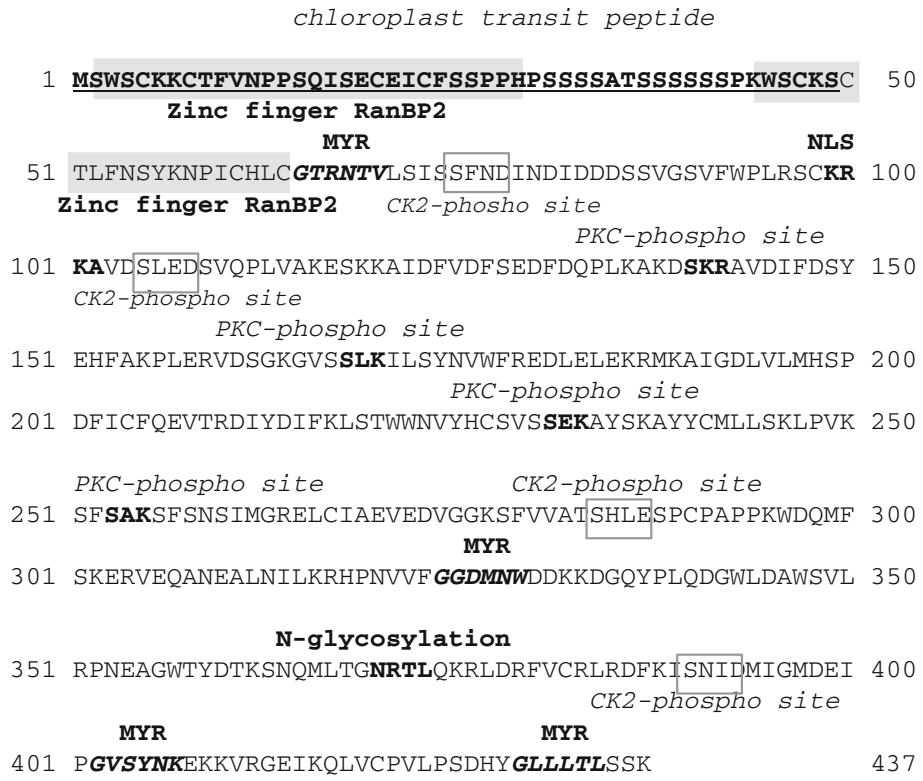

Fig. 2 *Mt*Tdp2α amino acid sequence (Phytozome Database Accession N° Medtr8g146980). The putative chloroplast targeting peptide (MSWSCKKCTFVNPPSQISECEICFSSPPHPSSSSATSSSSSSPKW SCKS) is represented in *bold* and *underlined*. The Ran-BP2 zinc finger domains (SWSCKKCTFVNPPSQISECEICFSSPPH; WSCKS CTLFNSYKNPICHLC) are shown within *gray boxes*. Putative casein kinase 2 (CK2) phosphorylation sites (SFND, SLED, SHLE, SNID) are highlighted by a *grey* frame while putative protein kinase C (PKC) phosphorylation sites (SKR, SLK, SEK, SAK) are shown in *bold*. The putative N-myristoylation sites (MYR) (GTRNTV, GGDMNW, GVSYNK, GLLLTL) and the N-glycosylation site (NRTL) are in *bold*. *NLS* nuclear localisation signal

The *MtTdp2α* gene is up-regulated in response to oxidative stress

The *MtTdp2α* gene was constitutively expressed in *M. truncatula* plants grown in greenhouse at the reproductive (two-months old) growth stage as evidenced by QRT-PCR analysis (Fig. 3a). The highest expression level was detected in leaves while significantly ($P < 0.0001$) lower amounts of *MtTdp2α* transcript were measured in roots and nodules, compared to leaves. The *MtTdp2α* mRNA was barely detected in flower and pods.

Since DNA repair genes are involved in plant responses to genotoxic stress, an understanding of the *Mt*Tdp2α function *in planta* may be relevant to a deeper knowledge of the DNA repair activation as a consequence of oxidative stress conditions. Consistent with this idea, two different treatments inducing oxidative stress in plants were applied to *M. truncatula* and the expression analysis of the *MtTdp2α* gene was carried out by QRT-PCR. The expression profiles were evaluated in both aerial parts and roots of *M. truncatula* plants grown in vitro in the presence of increasing concentrations (0, 50, 100 and 150 g/L) of the osmotic agent PEG6000 (Fig. 3b). The water potential of the culture medium was estimated −0.30 MPa (0 g/L PEG 6000), −0.60 MPa (50 g/L PEG 6000), −0.66 MPa (100 g/L PEG 6000) and −1.0 MPa (150 g/L PEG 6000). As for the expression in aerial parts, a significant ($P = 0.00461$) up-regulation (approximately 1.7-fold) of *MtTdp2α* was observed in response to 50 g/L PEG6000, compared to the untreated control, while treatment with 100 g/L PEG 6000 resulted in a non significant ($P = 0.0156$) up-regulation. At the highest concentration of osmotic agent (150 g/L), there was a significant ($P < 0.0001$) up-regulation of the *MtTdp2α* gene (3.6-fold), compared to the untreated control (Fig. 3b). Similarly, the level of *MtTdp2α* mRNA was enhanced in roots of *M. truncatula* plants challenged with PEG6000. Significant up-regulation (6.7- and 9.0-fold, $P = 0.0006$ and $P < 0.0001$, respectively) was evidenced following exposure to 50 and 100 g/L PEG6000, compared to the untreated control. With the highest concentration of osmotic agent (150 g/L), the up-regulation of the *MtTdp2α*

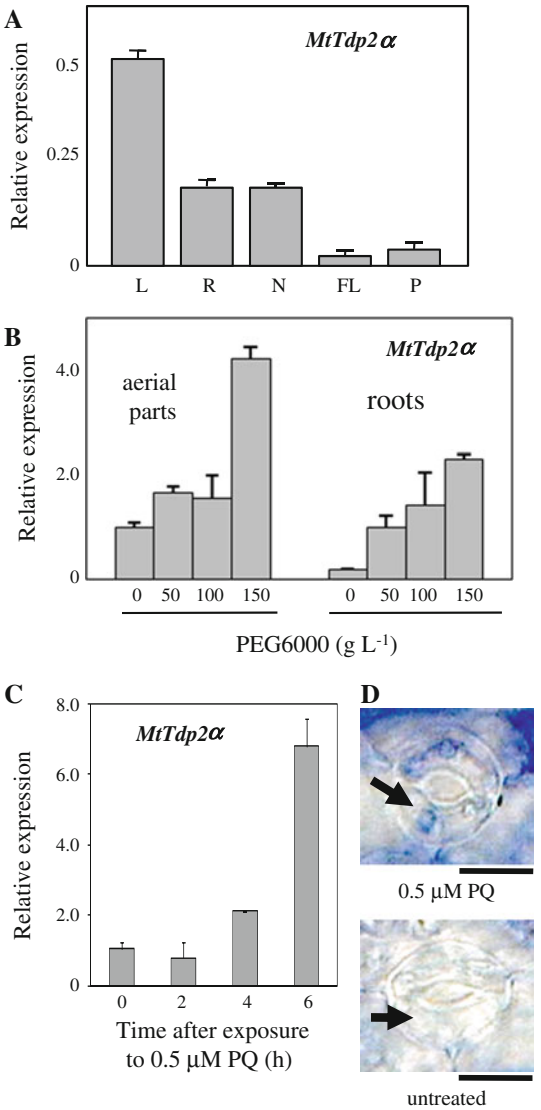

Fig. 3 **a** Expression profiles of *MtTdp2α* gene evaluated by QRT-PCR analysis in different tissues of two-months old *M. truncatula* plants. Values are expressed as mean ± SD of three independent replicated plants. L, leaf. R, roots. N, nodule. FL, flower. P, pods. **b** Expression profiles of *MtTdp2α* gene in response to osmotic stress. Results from QRT-PCR analyses carried out on aerial parts and roots of *M. truncatula* plants grown in vitro for 30 days in presence/absence of increasing PEG6000 concentrations are shown. **c** Expression profiles of *MtTdp2α* gene in response to photo-oxidative stress. Results from QRT-PCR analyses carried out on *M. truncatula* leaf discs exposed to 0.5 µM PQ and collected at 0, 2, 4 and 6 h following treatment are shown. *Asterisks* indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$). **d** Nitroblue tetrazolium (NBT) staining was used to evidence superoxide radical accumulation in chloroplasts of *M. truncatula* leaves exposed to PQ. The untreated control is also shown. For each treatment combination, data represent the mean values ± SD of three independent replications

gene was significantly ($P < 0.0001$) increased up to 13-fold compared to the untreated control (Fig. 3b). The reported data highlight the involvement of the *MtTdp2α* function during the *M. truncatula* response to osmotic stress conditions, both at the level of aerial parts and root apparatus.

QRT-PCR analyses were carried out on leaf tissues incubated with 0.5 µM PQ under continuous light in order to assess the response of *MtTdp2α* gene to photo-oxidative stress. Results from these experiments are shown in Fig. 3c. The expression profile of the *MtTdp2α* gene was evaluated at 0, 2, 4 and 6 h following incubation with PQ. Significant ($P < 0.0001$) up-regulation of *MtTdp2α* occurred at 4 h (2.1-fold) and 6 h (7.0-fold). ROS accumulation in chloroplasts of PQ-treated leaf discs was evidenced by nitroblue tetrazolium (NBT)-staining. Reduction of NBT to the unsoluble diformazan salt resulted in a change in colour of chloroplasts (Fig. 3d), indicating that, at the concentration utilised, PQ induces accumulation of superoxide radical (O_2^-), as a consequence of the block of photosystem II (PSII) electron transport.

Molecular characterisation of transgenic *M. truncatula* lines overexpressing the *MtTdp2α* gene

Overexpression is a powerful tool to investigate candidate genes for biotechnological application aimed at improving crop ability to withstand environmental stresses. In order to analyse the role played by the *MtTdp2α* function *in planta* and reveal the contribution of this specific gene in the response to genotoxic stress, transgenic *M. truncatula* lines overexpressing the *MtTdp2α* gene were obtained following *Agrobacterium tumefaciens*-mediated genetic transformation with the pTdp2α construct carrying the 35SCaMV-*MtTdp2α*-*Ocs* cassette (Fig. 4a). Nine independent pTdp2α kanamycin resistant lines (1, 2, 2d, 7b, 9b, 13c, 14b, 20 and 28) were regenerated. A control *A. tumefaciens* strain, carrying the empty vector, was used in a parallel co-cultivation experiment to obtain the control line (CTRL). PCR analysis confirmed the presence of the construct (Supplemental Fig. S2) and the *nptII* copy number was measured by QRT-PCR (Supplemental Table S2). For each line, the amount of *MtTdp2α* mRNA was evaluated by QRT-PCR in leaves excised from 10-days-old plantlets grown in vitro (Fig. 4b). The Tdp2α-13c line showed a significant ($P < 0.0001$) enhancement (2.2-fold) in the *MtTdp2α* mRNA. Similarly a significant ($P < 0.0001$) increase occurred in the Tdp2α-9b and Tdp2α-28 lines (8.0- and 7.0-fold, respectively). The Tdp2α-13c and Tdp2α-28 lines showing a 2.2- and 7.0-fold increase in the level of *MtTdp2α* transcript were selected for further studies. The Tdp2α-9b line, having the highest transgene expression level, showed an abnormal phenotype with reduced growth rates and for this reason it was not considered for investigation (data not shown).

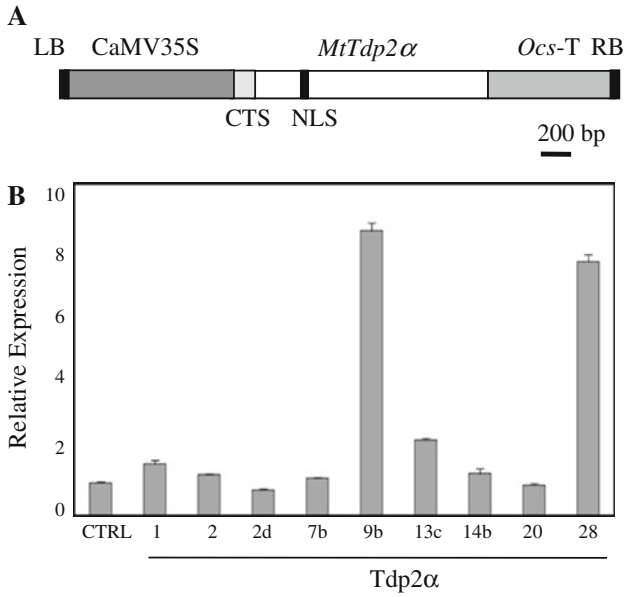

Fig. 4 **a** Schematic representation of pTdp2α construct used for the overexpression of *MtTdp2α* gene in *M. truncatula* Gaertn. 35SCaMV, cauliflower mosaic virus 35S promoter. Tdp2, tyrosyl-DNA phosphodiesterase 2. nptII, neomycin phosphotransferase II gene. Ocs-T, octopine synthase terminator LB, Left Border. RB, Right Border. **b** QRT-PCR analysis was carried out on leaves excised from 20-days-old plantlets grown in vitro of the transgenic Tdp2α lines and control (CTRL) line carrying the empty vector. Data represent the mean values ± SD of three replications from two independent experiments. *Asterisks* indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$)

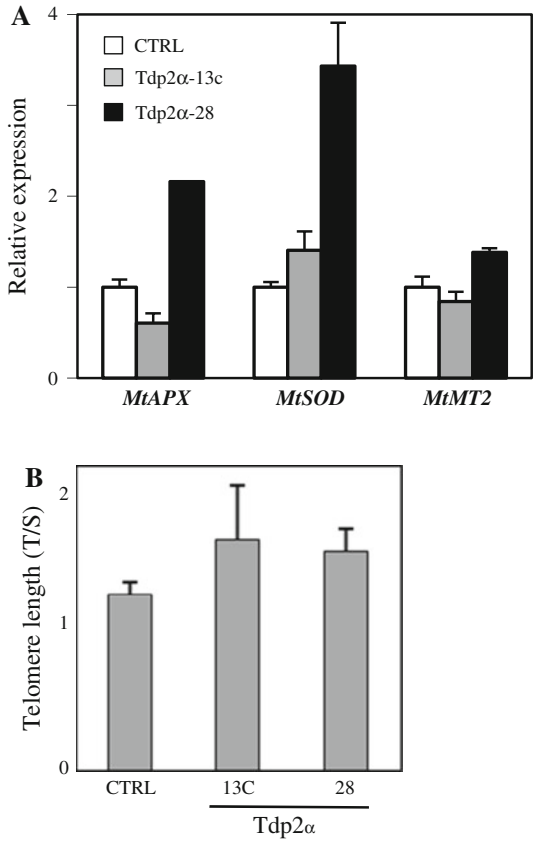

Fig. 5 **a** Expression profiles of the *MtAPX, MtSOD* and *MtMT2* genes in the *MtTdp2α*-overexpressing lines Tdp2α-13c, Tdp2α-28 and control (CTRL) line were assessed by QRT-PCR. Leaves were excised from 20-day old plants grown in vitro under physiological conditions. Data represent the mean values ± SD of three independent replications. *Asterisks* indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$). **b** Effects of *MtTdp2α* gene overexpression on telomere homeostasis. Telomere length measurement was carried out by QRT-PCR in leaves of 20-day old plants grown in vitro. T/S represents the ratio between the copy number of the target telomeric sequence and the copy number of the single copy gene used as reference. Values are expressed as means ± SD of three independent experiments. *Asterisk* indicates significant difference ($P < 0.05$, Student's *t*-test)

Expression profiles of antioxidant genes and telomere length in *MtTdp2α*-overexpressing lines

The plant antioxidant system include several enzymes that respond in a coordinated manner when ROS are generated under stress conditions. SOD catalyses the dismutation of O_2^- to O_2 and H_2O_2 while APX is an efficient scavenger of H_2O_2 under stress conditions (Mhadhbi et al. 2011). In addition, metallothioneins have been recognised as efficient ROS scavengers able to prevent DNA damage (Coyle et al. 2002). A preliminary characterisation of the Tdp2α-13c and Tdp2α-28 lines was carried out by investigating the expression profiles of the *MtAPX* and *MtSOD* genes, encoding the cytosolic isoforms of Ascorbate Peroxidase and Superoxide Dismutase, and the *MtMT2* gene encoding a type 2 metallothionein. As shown in Fig. 5a, significant ($P < 0.0001$) up-regulation of the *MtAPX* gene was observed in the leaf tissues of Tdp2α-28 line (2.2-fold) compared to CTRL. Similarly, the *MtSOD* gene turned out to be significantly ($P < 0.0001$) up-regulated in both Tdp2α-13c (1.4-fold) and Tdp2α-28 (3.4-fold) lines, compared to CTRL. As for the *MtMT2* gene expression profiles, a significant ($P < 0.0001$) increase (1.4-fold) in the amount of *MtMT2* mRNA was evident only in the Tdp2α-28 line (Fig. 5a). The observed up-regulation of genes encoding ROS scavengers in *MtTdp2α*-overexpressing lines grown under physiological conditions might help reducing the ROS-mediated genotoxic injury.

The recent work by Donà et al. (2013a) has underlined for the first time the link between the Tdp1α function and telomere homeostasis in plants. In the present study, telomere length was measured in leaves of *MtTdp2α*-overexpressing and CTRL lines grown under physiological conditions. Results are shown in Fig. 5b. The estimated telomere length in leaf cells from the CTRL line was 1.04 ± 0.07 T/S ratio. As for Tdp2α-28, telomere length increased (up to 1.30 ± 0.21 T/S ratio) under physiological

Table 1 Average fresh weights of *M. truncatula* plantlets of the control (CTRL) and transgenic lines Tdp2α-13C and Tdp2α-28 incubated in presence/absence of PEG6000 (50 g L^{-1}) for 10 days

	Untreated (g_{FW})	PEG6000 (g_{FW})	Estimated change caused by PEG6000 (%)
CTRL	93.62 ± 1.9	55.89 ± 1.4	−40.3
Tdp2α-13C	102.86 ± 1.7	63.02 ± 1.3	−38.7
Tdp2α-28	100.08 ± 1.5	72.96 ± 1.1	−27.1

The reduction in fresh weight observed as a consequence of stress treatments, expressed as percentage of the value (100 %) measured in the untreated sample, is also shown

Table 2 Total chlorophyll content measured in leaflets excised from *M. truncatula* plantlets of the control (CTRL) and transgenic lines Tdp2α-13C and Tdp2α-28 grown in presence/absence of PEG6000 (50 g L^{-1}) for 10 days

	Untreated (mg g^{-1} $_{FW}$)	PEG6000 (50 g L^{-1}) (mg g^{-1} $_{FW}$)	Estimated change caused by PEG6000 (%)
CTRL	0.85 ± 0.03	0.55 ± 0.01	−35.8
Tdp2α-13C	1.03 ± 0.2	0.74 ± 0.01	−28.7
Tdp2α-28	0.99 ± 0.05	0.78 ± 0.03	−20.9

The change in total chlorophyll content observed as a consequence of stress treatments, expressed as percentage of the value (100 %) measured in the untreated sample, is also shown

conditions, although this value was not significantly different ($P = 0.0856$) from that reported for CTRL. A significant ($P = 0.004$) increase in telomere length (1.54 ± 0.37 T/S ratio) occurred in leaves of the Tdp2α-13c line.

Overexpression of *MtTdp2α* gene in *M. truncatula* confers tolerance to osmotic stress

The CTRL, Tdp2α-13C and Tdp2α-28 lines were grown in vitro for 10 days in presence/absence of PEG6000 (50 g/L) and subsequently analysed (Table 1). Under physiological conditions, the average biomass in the CTRL was 93.62 ± 1.9 g_{FW} while there was a significant ($P < 0.05$) increase in both Tdp2α-13C and Tdp2α-28 lines (102.86 ± 1.7 and 100.08 ± 1.5 g_{FW}, respectively), compared to CTRL. The average biomass of the CTRL line was significantly ($P < 0.05$) lowered (55.89 ± 1.4 g_{FW}; −40.3 %) following exposure to PEG6000. Osmotic stress also caused a significant ($P < 0.05$) decrease in the average biomass of Tdp2α-13C and Tdp2α-28 lines (63.02 ± 1.3 and 72.96 ± 1.3 g_{FW}, respectively, with an estimated reduction of −38.7 and −27.1 %) (Table 1).

The chlorophyll content was analysed in leaflets of the CTRL, Tdp2α-13C and Tdp2α-28 lines exposed to osmotic stress (Table 2). In the untreated CTRL line, the estimated amount of total chlorophyll was 0.85 ± 0.03 mg g^{-1} $_{FW}$ while the level of total chlorophyll was slightly but significantly ($P = 0.041$) enhanced in the Tdp2α-13C (1.03 ± 0.2 mg g^{-1} $_{FW}$) while there was no significant ($P = 0.018$) change in Tdp2α-28 (0.99 ± 0.05 mg g^{-1} $_{FW}$). The CTRL line treated with PEG6000 underwent a significant ($P < 0.001$) reduction in total chlorophyll content (0.55 ± 0.01 mg g^{-1} $_{FW}$, −35.8 %), compared to the untreated sample. In the Tdp2α-13C line overexpressing the *MtTdp2α* gene, the total chlorophyll amount was significantly decreased ($P < 0.001$) 0.74 ± 0.01 mg g^{-1} $_{FW}$ (−28.7 %) as well as in the Tdp2α-28 line 0.78 ± 0.03 mg g^{-1} $_{FW}$ ($P = 0.0013$) (−20.9 %) (Table 2). Carotenoids are required for effective photoprotection and indeed the Tdp2α-13C and Tdp2α-28 lines exposed to osmotic stress were able to maintain significantly ($P < 0.05$) higher levels of these antioxidant compounds compared to CTRL line (Supplemental Fig. S3).

According to the reported data, overexpression of the *MtTdp2α* gene correlated with resistance to osmotic stress, resulting in limited biomass reduction and limited decrease in chlorophyll and carotenoids content compared to control line.

Overexpression of *MtTdp2α* gene prevents DSBs accumulation

The effects of *MtTdp2α* gene overexpression on genome integrity were assessed by measuring the level of DSBs in leaf tissues of CTRL, Tdp2α-13C and Tdp2α-28 lines challenged with the osmotic agent PEG6000 (50 g/L). Results from neutral SCGE are shown in Fig. 6a. When CTRL plantlets were grown for 10 days in the absence of osmotic agent, the estimated amount of DSBs in leaf tissues was 90.5 ± 0.9 a.u. while a significantly ($P < 0.0001$) lower level of DNA damage was recorded in the Tdp2α-13C and Tdp2α-28 lines (57.3 ± 4.1 and 59.0 ± 7.0 a.u., respectively). In response to PEG6000 treatment, the amount of DSBs was significantly ($P < 0.0001$) enhanced (163.0 ± 8.0 a.u.) in the CTRL leaf cells in comparison with the untreated sample. A similar response was observed in the Tdp2α-13C and Tdp2α-28 lines which showed a significant ($P < 0.0001$ and $P = 0.0004$, respectively) increase in DSBs level (81.0 ± 1.4 and 100.0 ± 2.0 a.u., respectively) (Fig. 6a). The estimated DNA damage resulting from osmotic stress increased up to 1.8-fold in the CTRL line while there was a limited increase (1.4- and 1.7-fold, respectively) in the Tdp2α-13C and Tdp2α-28 lines.

The reported data highlight that the overexpression of *MtTdp2α* gene provides increased ability to withstand

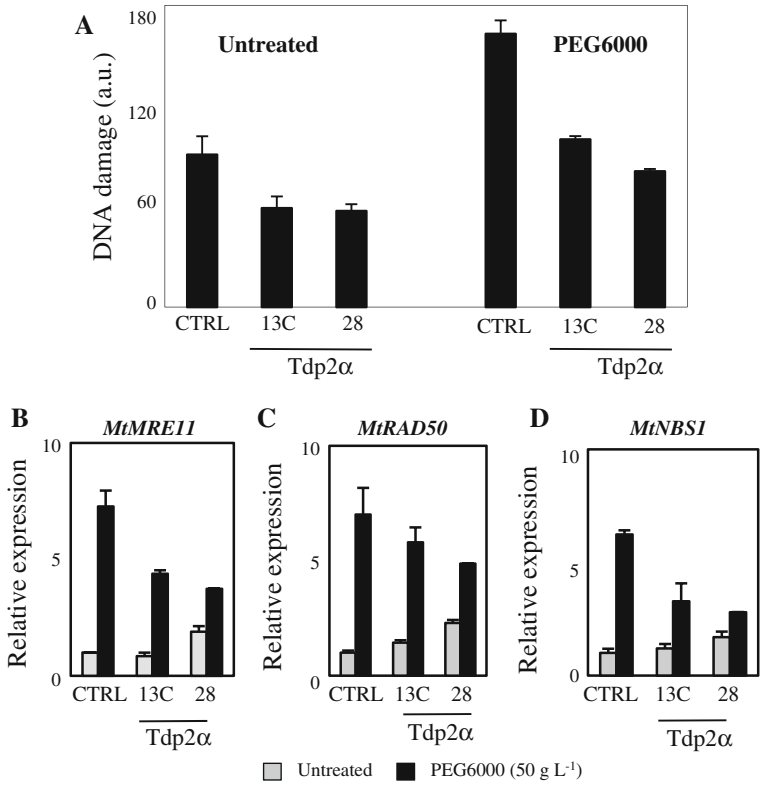

Fig. 6 **a** Neutral SCGE was used to evaluate the level of DSBs in leaves excised from 10-day old Tdp2α-13c, Tdp2α-28 and control (CTRL) plantlets grown in vitro in presence/absence of the osmotic agent PEG6000 (50 g L^{-1}). Data represent the mean values ± SD of three replications from two independent experiments. a. u., arbitrary units. *Asterisks* indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$). Expression profiles of the *MtMRE11* (**b**), *MtRAD50* (**c**) and *MtNBS* (**d**) genes in *M. truncatula* leaves of 10-day old plants grown in vitro in presence/absence of the osmotic agent PEG6000 (50 g L^{-1}) were assessed by QRT-PCR analysis. For each treatment combination, data represent the mean values ± SD of three independent replications. *Asterisks* indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$)

genotoxic stress in *M. truncatula* plants grown under physiological conditions since a 40 % decrease in DSBs was observed, compared to CTRL. Genome stability is preserved also under osmotic stress conditions, considering the limited enhancement in DSBs evidenced in the *MtTdp2α*-overexpressing plants.

Genes involved in DSB sensing/repair are up-regulated in *MtTdp2α*-overexpressing *M. truncatula* lines

In plants, as in animals, the MRN (MRE11-RAD50-NBSI) 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, the RAD50 protein characterised by ATPase and DNA binding activity interacts with MRE11 while NBS1 directs the nuclear localization of the complex (Daoudal-Cotterell et al. 2002). The expression profiles of *MtMRE11*, *MtRAD50* and *MtNBS1* genes were evaluated by QRT-PCR in leaf tissues of CTRL and *MtTdp2α*-overexpressing lines. In the Tdp2α-28 line, the amount of *MtMRE11* mRNA was significantly higher (1.9-fold, $P = 0.0006$) under physiological conditions compared to CTRL. Exposure to PEG6000 resulted in a significant increase in the level of *MtMRE11* transcript in the CTRL (7.3-fold, $P < 0.0001$), Tdp2α-13c (5.2-fold, $P < 0.0001$) and Tdp2α-28 (2.0-fold, $P < 0.0001$) lines (Fig. 6b). The *MtRAD50* gene was significantly up-regulated under physiological conditions in the Tdp2α-28 (2.3-fold, $P = 0.00379$) line, compared to CTRL. Exposure to osmotic stress always caused the significant ($P < 0.0001$) enhancement of *MtRAD50* gene expression with an estimated accumulation of *MtRAD50* transcript of 7.0-fold (CTRL), 4.0-fold (Tdp2α-13c) and 2.1-fold (Tdp2α-28) (Fig. 6c). The *MtNBS1* gene was significantly up-regulated (1.7-fold, $P = 0.0154$) under physiological conditions in the Tdp2α-28 line, compared to CTRL. In plants exposed to osmotic stress, the estimated up-

Table 3 Total chlorophyll content measured in leaflets excised from *M. truncatula* plantlets of the control (CTRL) and transgenic lines Tdp2α-13C and Tdp2α-28 incubated in presence/absence of 0.5 μM Paraquat (PQ) and 50 μM Ciprofloxacin (CFX), respectively

	Untreated (mg g^{-1} $_{FW}$)	0.5 μM PQ (mg g^{-1} $_{FW}$)	Estimated change caused by PQ (%)	50 μM CFX (mg g^{-1} $_{FW}$)	Estimated change caused by CFX (%)
CTRL	0.91 ± 0.08	0.48 ± 0.07	−47.5	0.13 ± 0.01	−85.1
Tdp2α-13C	0.99 ± 0.08	0.71 ± 0.06	−28.2	0.37 ± 0.02	−62.7
Tdp2α-28	0.98 ± 0.04	1.16 ± 0.11	+ 18.3	0.61 ± 0.02	−37.6

The change in total chlorophyll content observed as a consequence of stress treatments, expressed as percentage of the value (100 %) measured in the untreated sample, is also shown

regulation of *MtNBS1* gene was 6.2-fold (CTRL), 2.7-fold (Tdp2α-13c) and 1.7-fold (Tdp2α-28) (Fig. 6d).

The enhanced expression of the DSB sensing/repair genes observed in *MtTdp2α*-overexpressing *M. truncatula* lines under physiological conditions well correlates with the reduced level of DSBs revealed by SCGE analysis. On the other hand, it should be hypothesized that osmotic stress did dot result in *MtMRE11*, *MtRAD50* and *MtNBS1* mRNA accumulation as high as that found in CTRL due to the low DSB levels occurring in the *MtTdp2α*-over-expressing *M. truncatula* lines.

Tolerance to paraquat and ciprofloxacin in *MtTdp2α*-overexpressing *M. truncatula* lines

The ability of *MtTdp2α*-overexpressing lines to face stress conditions was further investigated using paraquat (PQ) and ciprofloxacin (CFX). Tolerance to photo-oxidative stress was assessed in the transgenic Tdp2α-13C and Tdp2α-28 lines by measuring the total chlorophyll content in leaf discs incubated for 24 h under continuous light in presence of 0.5 μM PQ (Table 3). The leaf discs from the CTRL line were visibly damaged after exposure 0.5 μM PQ, while the Tdp2α-13C and Tdp2α-28 leaf tissues remained green, showing a marked ability to withstand the treatment (data not shown). Incubation under light for 24 h in distilled water did not significantly affect the total chlorophyll content in both CTRL and transgenic lines (data not shown). In the untreated CTRL line, the estimated amount of total chlorophyll was 0.91 ± 0.08 mg g^{-1} $_{FW}$ while the level of total chlorophyll was slightly enhanced in the Tdp2α-13C (0.99 ± 0.08 mg g^{-1} $_{FW}$) and Tdp2α-28 (0.98 ± 0.04 mg g^{-1} $_{FW}$) lines. The CTRL leaflets exposed to PQ underwent a significant ($P < 0.0001$) reduction in total chlorophyll content corresponding to 0.48 ± 0.07 mg g^{-1} $_{FW}$ (−48 %, compared to the untreated sample) while in the Tdp2α-13C line the total chlorophyll amount was 0.71 ± 0.06 mg g^{-1} $_{FW}$ (−28.2 %, compared to the untreated sample). By contrast, in the Tdp2α-28 line there was significantly ($P < 0.0001$) enhanced accumulation (1.16 ± 0.11 mg g^{-1} $_{FW}$) with an estimated increase of 15.6 % (Table 3). It is worth noting that the increase in chlorophyll accumulation in aerial parts of *M. truncatula* plants has been proposed as a compensatory mechanism in response to damage occurring at the photosyntesis machinery (Nunes et al. 2008). This provides evidence that the transgenic lines Tdp2α-13C and Tdp2α-28 were more resistant to the photo-oxidative stress induced by PQ compared to the control line.

DNA gyrase is a type II topoisomerase found in plant organelles where it plays a key role in DNA replication and transcription (Wall et al. 2004). *M. truncatula* leaves were exposed to ciprofloxacin (CFX), a second generation fluoroquinolone that specifically inhibits the organellar enzyme without affecting the nuclear enzyme topo II. The fluoroquinolones form a complex with the enzyme and DNA in which both strands of the DNA backbone are cleaved and covalently linked to the protein. Two molecules of fluoroquinolone are found in each complex and the resulting stabilised DNA/enzyme complex has cytotoxic effects. The CTRL line and the Tdp2α-13C and Tdp2α-28 lines overexpressing the *MtTdp2α* gene were also tested for their ability to withstand the cytotoxic effects of CFX. As shown in Table 3, a significant ($P < 0.0001$) reduction in the amount of total chlorophyll was observed in all the lines, following exposure to 50 μM CFX, compared to the untreated control. The estimated reduction in total chlorophyll content was 85.5 % for the CTRL line. Both the Tdp2α-13C and Tdp2α-28 lines showed a depletion in total chlorophyll corresponding to 62.8 and 37.62 %, respectively.

Overexpression of *MtTdp2α* gene positively affects the expression of genes involved in the control of DNA topology

It should be hypothesized that the protective effects resulting from *MtTdp2α* gene overexpression *in planta* might involve other genes playing key roles in genome stability. For this reason, the expression profiles of genes involved in the regulation of DNA topology, *MtTdp1α*, *MtTop2*, *MtGYRA* and *MtGYRB*, were analysed. It is worth

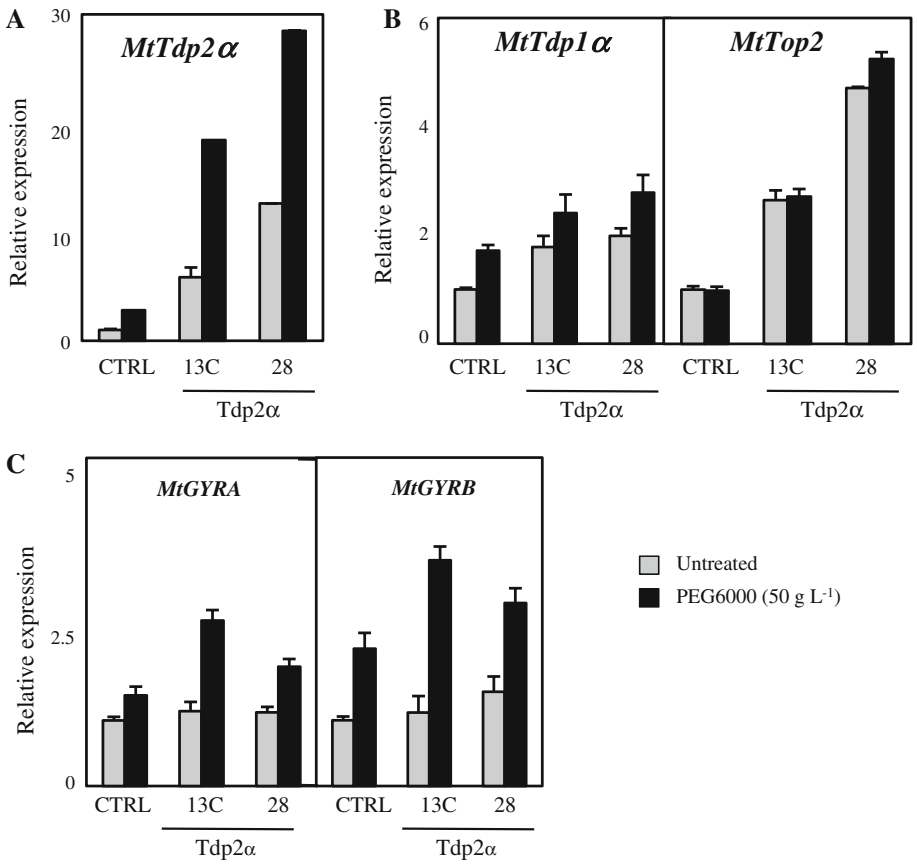

Fig. 7 Expression profiles of *MtTdp2α* and *MtTdp1α* genes (**a**), *MtTop1β* and *MtTop2* genes (**b**), *MtGyrA* and *MtGyrB* genes (**c**) in leaves of 10-day old plants grown in vitro in presence/absence of the osmotic agent PEG6000 (50 g L^{-1}) were assessed by QRT-PCR analysis. For each treatment combination, data represent the mean values ± SD of three independent replications. *Asterisks* indicate statistical significance of differences determined using Student's *t*-test ($P < 0.05$)

noting that in the Tdp2α-13c and Tdp2α-28 lines exposed to PEG6000 there was a further significant ($P < 0.0001$) enhancement in the amount of *MtTdp2α* transcript (3.2-fold, Tdp2α-13c and 2.3-fold, Tdp2α-28), due to the up-regulation of the endogenous gene. As expected, the CTRL line also showed a significant ($P < 0.0001$) up-regulation (2.8-fold) of the *MtTdp2α* gene in response to osmotic stress (Fig. 7a).

In animal cells, tyrosyl-DNA phosphodiesterase 1 is responsible for the removal of DNA damage induced by both topo I and topo II enzymes (Murai et al. 2012). Under physiological conditions, the overexpression of *MtTdp2α* gene was associated with enhanced accumulation of *MtTdp1α* mRNA in both Tdp2α lines. As shown in Fig. 7b, the estimated up-regulation of *MtTdp1α* gene was 1.8- and 2.0-fold ($P = 0.0002$ and $P < 0.0001$) in the Tdp2α-13c and Tdp2α-28 lines, compared to CTRL. A further increase in the amount of *MtTdp1α* transcript occurred in response to osmotic stress. However, in the CTRL line the exposure to PEG6000 resulted in a 1.7-fold increase ($P = 0.0002$) in *MtTdp1α* mRNA while in both the *MtTdp2α* overexpressing lines the estimated enhancement was only 1.4-fold ($P = 0.0002$ and $P < 0.0001$) (Fig. 7b). Similarly, both Tdp2α-13c and Tdp2α-28 lines showed significant ($P < 0.0001$) up-regulation (2.6-fold and 4.7-fold, respectively) of the *MtTop2* gene encoding topo II under physiological conditions, compared to CTRL. Following exposure to PEG6000, no further up-regulation of *MtTop2* gene was observed in the CTRL and Tdp2α-13c lines while a slight increase occurred in Tdp2α-28 (Fig. 7b).

As for *MtGyrA* and *MtGyrB* genes encoding the subunits A and B of DNA gyrase, no significant up-regulation was observed in the *MtTdp2α* overexpressing lines, under physiological conditions, compared to CTRL. In response to PEG6000, the estimated increase of *MtGyrA* mRNA was 1.4-fold (CTRL, $P = 0.0002$), 2.2-fold (Tdp2α-13c, $P < 0.0001$) and 1.6-fold (Tdp2α-28, $P < 0.0001$) (Fig. 7c). Exposure to osmotic stress resulted in accumulation of *MtGyrB* mRNA in all the tested lines, with a significant ($P < 0.0001$) increase of 2.1-fold (CTRL), 3.1-fold (Tdp2α-13c) and 1.9-fold (Tdp2α-28). Overall, the amount of *MtGyrA* and *MtGyrB* transcripts was always significantly higher in the *MtTdp2α* overexpressing lines challenged with stress, compared to CTRL (Fig. 7c).

The reported data highlight a relevant feature associated with the overexpression *in planta* of *MtTdp2α* gene, that is an increased expression of the *MtTdp1α* and *MtTop2* genes both required to overcome topoisomerase-mediated DNA damage, observed even under physiological conditions.

Discussion

Among the wide range of environmental stresses, salinity is a major component affecting approximately 7 % of the world's total land area and one of the negative consequences of the global climate change (Munns and Tester 2008; Ashraf and Akram 2009). Salt stress impairs key functions for plant survival and productivity, among which is photosynthesis, and causes osmotic stress with the consequent oxidative injury.

Within this context, DNA repair genes are gaining in relevance as potential tools for innovative biotechnological applications in which both genetic engineering and traditional breeding are combined. Previous work performed with the model legume *M. truncatula* (Macovei et al. 2010; Balestrazzi et al. 2011a) has allowed gaining insight into the role of the *MtTdp1α* and *MtTdp1β* genes in the plant response to osmotic stress induced by PEG. The relevance of plant Tyrosyl-DNA phosphodiesterases as components of the osmotic stress response is strengthened by the results provided in the present work which reports for the first time the *in planta* characterisation of the *MtTdp2α* gene, encoding tyrosyl-DNA phosphodiesterase 2. Tdp2 is still an uncovered topic in plants and the present work provides for the first time information about the *in planta* functions of this gene.

Differently from animals where a single-copy *Tdp2* gene is present, in the plant kingdom there are three different Tdp2 isoforms. The phylogenetic distribution of the three different isoforms of the plant Tdp2 enzyme suggests that the γ isoform might be the most ancient one, since it is typically associated with several eukaryotic unicellular organisms and multicellular green algae. The γ isoform is present in the *Poaceae* family which also contain the β isoform. The latter is found as well in the moss *Physcomitrella patens* which occupies a relevant phylogenetic position and then important for investigating the evolutionary development in higher plants (Yue et al. 2012). The α isoform has been detected in Legumes, in some tropical tree crops and in members of the *Euphorbiaceae* family.

The *Mt*Tdp2α protein contains a zinc finger RanBP2 domain, which is part of a novel class of RNA binding domain able to bind single strand RNA (Nguyen et al. 2011). This finding suggests for a possible role of *Mt*Tdp2α in the regulation of mRNA processing, since it has been demonstrated that lack of *h*Tdp2 alters rRNA biogenesis, affecting the level of precursor ribosomal RNA (Vilotti et al. 2011). Participation of *h*Tdp2 in rRNA processing requires the SIM (SUMO-Interacting) motif while the 5′-tyrosyl DNA phosphodiesterase activity is dispensable (Vilotti et al. 2011). It could be hypothesized that the function played by the SIM motif, absent from the plant Tdp2α protein, might be replaced by the zinc finger RanBP2 domain. It is worth noting that the link between Tdp1α and ribosome biogenesis has been recently demonstrated in plants by Donà et al. (2013a).

In animal cells, the effects of *hTdp2* gene overexpression are puzzling. It has been reported that *hTdp2* gene overexpression in NSCLC (Non-Small-Cell Lung Carcinoma) enhances cell proliferation by promoting the G_1/S transition (Li et al. 2011b). The same authors evidenced that the MAPK-ERK (Mitogen-Activated Protein Kinase-Extracellular Signal-Regulated Kinase) pathway is one of the downstream targets of the Tdp2-mediated signaling route leading to activation of *myc-c* gene encoding the helix loop helic/leucine zipper transcription factor MYC and other cell-cycle related genes. Interestingly, Zoppoli et al. (2011) demonstrated that the expression of *myc-c* gene directly correlated with that of the *mtTop1* gene encoding the human mitochondrial DNA topoisomerase I. Opposite results have been recently provided by Zhou et al. (2013) who showed that *hTdp2* gene overexpression in osteosarcoma cells induces cell cycle arrest at G_2/M, leading to apoptosis.

As for plants, this is not only the first report dealing with the Tdp2 function but also the first case in which the effects deriving from *in planta* overexpression of a *Tdp* gene are investigated. Indeed, at the moment only Tdp1-depleted plants have been characterised (Kim et al. 2012; Donà et al. 2013a).

In our opinion, it is quite relevant the finding that the *MtTdp2α* gene plays a protective role *in planta* against genotoxic stress induced by different agents but it is as well important the fact that reduced DSB accumulation and significant up-regulation of key genes involved in the maintenance of genome stability and ROS scavenging are observed under physiological conditions. How the overexpression of *MtTdp2α* gene might enhance all these functions essential for cell survival is unclear.

The overexpression of *MtTdp2α* gene enhances the transcript level of the *MRE11*, *RAD50* and *NBS1* genes encoding components of the MRN complex, a key sensor of DSBs, possibly favouring a more effective DNA damage sensing/repair. As for the increased expression of antioxidant genes detected in the *MtTdp2α*-overexpressing lines grown under physiological conditions, information concerning the link between ROS scavenging genes and DNA repair pathways is scanty, even in animal cells. However, Nagano et al. (2002) demonstrated that mice cells defective for the cytoplasmic SOD isoform were impaired in the BER pathway. A system biology study carried out by Bonatto (2007) in yeast suggests that the SOD enzyme may act as a sensor of O_2^- in response to stress, leading to the activation of specific DNA repair mechanisms.

The down-regulation of the MRN components, observed in the *MtTdp2α* overexpressing lines under osmotic stress

conditions, might represent an indirect effect resulting from a negative feedback caused by the decrease in DSBs levels. On the other hand, when considering the expression profiles of genes involved in the control of DNA topology under osmotic stress condition, different patterns are reported. Both the *MtTdp1α* and *MtTop2* genes were investigated, due to the close relationship between these specific functions and the role played by Tdp2 evidenced in animal cells. Indeed, Murai et al. (2012) demonstrated that the Tdp1 enzyme is also implicated in the repair of topo II-mediated DNA damage while no information is currently available concerning the possible perturbation of *Top2* gene expression profiles in animal cells with enhanced Tdp2 activity. It is possible that the *MtTdp1α* and *MtTop2* genes share a common step, regulated in some way by the Tdp2 function, in the related signaling pathways. It is reasonable to hypothesize that the *MtTdp2α* overexpression might in some way affect these genes in a process mediated by specific transcription factors, as described in animal cells (Zoppoli et al. 2011; Zhou et al. 2013). The interest for the *MtGYRA* and *MtGYRB* genes encoding the components of organellar topo II, namely DNA gyrase, is due to the recent findings which describe the involvement of the human Tdp1 enzyme in the repair of mitochondrial DNA damage (Das et al. 2010). Apparently, no changes were observed in the expression profiles of the *MtGYRA* and *MtGYRB* genes in the *MtTdp2α*-overexpressing lines grown under physiological conditions and osmotic stress conditions resulted into *MtGYRA* and *MtGYRB* transcript accumulation in both *MtTdp2α*-overexpressing and CTRL lines. This might suggest the lack of some possible correlation between the *MtTdp2α* function and the DNA repair response occurring within plant chloroplasts. Based on the reported data and considering the role played by the *hTdp2* gene in animal signal transduction pathways, the possible involvement of the *MtTdp2α* gene in plant signaling networks represents a reasonable starting point for future investigations.

In animal cells, no information is currently available concerning the possible involvement of tyrosyl-DNA phosphodiesterases in telomere homeostasis while in plants a direct relationship has been recently demonstrated by Donà et al. (2013a). The usefulness of telomere length as a parameter for assessing the plant response to environmental stress is still debated while the current knowledge needs to be expanded. The dynamics of telomere shortening in relation to seed aging has been recently investigated in *Silene* spp. by Donà et al. (2013c). Increased telomere length was evident in *S. acaulis* seeds characterised by lower antioxidant ability during the early phase of seed imbibition, possibly due to repair mechanisms activated during the early phase of seed imbibition (Balestrazzi et al. 2011a) but there are other reports suggesting the association between increased telomere length and genotoxic stress (Hong et al. 2007). An hypothesis proposed in animal cells states that oxidative damage at telomeres caused by endogenous or exogenous factors can be differently recognised, leading to different responses (Wang et al. 2010). According to these authors, moderate injuries in telomeric sequences caused by cellular metabolism activate telomere lenghtening while extensive damage caused by external factors result in telomere degradation. The response observed in the *MtTdp2α*-overexpressing lines in terms of telomere length evidences only a slight effect of *MtTdp2α* on telomere homeostasis and thus further investigation will be necessary to better assess this aspect.

In our opinion, the present investigation provides a significant advance in the knowledge of the complex molecular bases underlying the genotoxic stress response. Only few reports are available describing transgenic plants with enhanced DNA repair functions and their performance under physiological and stress conditions. Kaiser et al. (2009) demonstrated that *Arabidopsis* lines overexpressing the CPD photolyase gene showed a significant increase in growth rates under physiological conditions. However, reduced growth rates were observed in plants exposed to UV-B light, notwithstanding a significant reduction in the level of CPD lesions. This finding was possibly due to the fact that DNA lesions other than CPDs were accumulated in plant tissues under UV-B light (Kaiser et al. 2009). On the other hand, the overexpression in *Arabidopsis* of *AtOGG1* gene, encoding a DNA glycosylase/AP lyase, resulted in enhanced seed vigor with reduced accumulation of oxidative DNA lesions and the concomitant increase in the ability to withstand oxidative stress caused by different agents, e.g. paraquat, NaCl, mannitol and high temperature (Chen et al. 2012).

Based on the reported data, the possible roles played by the Tdp2α function in plants can be argued both within the DNA damage-induced signaling mechanisms and the DNA repair response.

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