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Arabidopsis thaliana thymidine kinase 1a is ubiquitously expressed during development and contributes to confer tolerance to genotoxic stress

José Antonio Pedroza-García · Manuela Nájera-Martínez · María de la Paz Sanchez · Javier Plasencia

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Abstract Thymidine kinase catalyzes the first step in the nucleotide salvage pathway by transferring a phosphate group to a thymidine molecule. In mammals thymidine kinase supplies deoxyribonucleotides for DNA replication and DNA repair, and the expression of the gene is tightly regulated during the cell cycle. Although this gene is phylogenetically conserved in many taxa, its physiological function in plants remains unknown. The genome of the model plant *Arabidopsis thaliana* has two thymidine kinase genes (*AtTK1a* and *AtTK1b*) and microarray data suggest they might have redundant roles. In this study we analyzed the *TK1a* function by evaluating its expression pattern during development and in response to genotoxic stress. We also studied its role in DNA repair by the characterization of a mutant that contained the T-DNA insertion in the promoter region of the *TK1a* gene. We found that *TK1a* is expressed in most tissues during plant development and it was differentially induced by ultraviolet-C radiation because *TK1b* expression was unaffected. In the mutant, the T-DNA insertion caused a 40 % rise in transcript levels and enzyme activity in *Arabidopsis* seedlings compared to wild-type plants. This elevation was enough to confer tolerance to ultraviolet-C irradiation in dark conditions, as determined by root growth, and meristem length and structure. *TK1a* overexpression also provided tolerance to genotoxins that induce double-strand break. Our results suggest that thymidine kinase contributes to several DNA repair pathways by providing deoxythymidine triphosphate that serve as precursors for DNA repair and to balance deoxyribonucleotides pools.

Keywords Thymidine kinase · Nucleotide metabolism · UVC tolerance · DNA damage · DNA repair

Introduction

The plant genome is constantly subjected to endogenous and environmental genotoxic stress. Reactive oxygen species (ROS) damage DNA through several mechanisms and are generated by physiological events such as respiration and photosynthesis. Besides ozone, low-level ionizing radiation and ultraviolet (UV) light provide additional ROS (Roldán-Arjona and Ariza 2009). Because of their sessile lifestyle plants are continually exposed to the UV-B radiation (290–320 nm) from sunlight that causes the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidinone dimers (6-4PPs) in the DNA double strand. Such modification inhibits DNA and RNA synthesis and, if they go unrepaired, mutations occur (Tuteja et al. 2009). Therefore, several DNA repair mechanisms operate to contend with this and other types of genotoxic stress (Britt 2002; Hays 2002).

Most DNA repair mechanisms include an excision step in which the damaged DNA strand is removed and new DNA fills the gap. The efficiency of these mechanisms depends on DNA synthesis, thus the levels of its monomeric units (deoxyribonucleotides; dNTPs) are crucial for adequate repair and genome stability. When DNA is damaged, an

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J. A. Pedroza-García · M. Nájera-Martínez · J. Plasencia (✉)
Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, 04510 México, D.F., México
e-mail: javierp@unam.mx

M. de la Paz Sanchez
Departamento de Ecología Funcional, Instituto de Ecología, Universidad Nacional Autónoma de México, 04510 México, D.F., México

increase in the levels of the four dNTPs occurs, indicating a close relationship between the regulation of dNTP synthesis and DNA repair response (Tuteja et al. 2009). Imbalances in dNTPs pools facilitate nucleotide misincorporation during the excision repair synthesis thus facilitating mutations (Kunz et al. 1994). The dNTPs might derive from simple molecules—via de novo pathway—or from preformed precursors through the salvage pathway (Kafer et al. 2004). The synthesis of a pyrimidine deoxiribonucleotide requires nine enzymatic steps, from glutamine and HCO_3^- to yield cytidine triphosphate (CTP). While the de novo pathway involves multiple enzymatic steps to generate a dNTP, the salvage pathway only needs three reactions to produce a new dNTP; this pathway recycles the nucleosides and free bases derived from the intracellular degradation of the DNA and RNA nucleotides. For example, pyrimidine nucleosides—uridine/cytidine and thymidine—are phosphorylated by specific nucleoside kinases (Moffatt and Ashihara 2002; Stasolla et al. 2003; Zrenner et al. 2006).

In the salvage pathway, thymidine kinase (TK) catalyzes the first step by transferring a phosphate group to a thymidine molecule. In mammals, TK contributes with the dNTPs pools for DNA replication and DNA repair, and the expression of the gene is tightly regulated during the cell cycle (Gasparri et al. 2009). Although plant cells also contain the gene for TK, its physiological function is unknown. In *Oryza sativa*, *TK1* gene expression is independent of cell-cycle regulation as the transcript is present in all developmental stages, and it is even more abundant in non-proliferating tissues (Ullah et al. 1999). In *Hevea brasiliensis*, the rubber tree, up-regulation of the *TK1* gene is closely associated with resistance to mechanical wounding (Venkatachalam et al. 2010). *Arabidopsis thaliana* genome contains two genes—*AtTK1a* and *AtTK1b*—that encode for enzymes with TK activity and share a 63 % similarity. While mutants for each *TK1* gene show normal growth, the double mutant develops poorly and plantlets die at an early stage, indicating that the function of TK1 is essential for plant development (Clausen et al. 2012).

Here, we aimed to study the function of *AtTK1a* by evaluating its expression pattern during development and in response to UV radiation and other genotoxic agents. We also characterized a T-DNA insertion mutant that overexpresses the *TK1a* gene and compare its response to genotoxic stress with wild-type plants.

Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type plant throughout this study. The SALK_037113 mutant was provided by the *Arabidopsis* Biological Resource Center. Seeds were surface disinfected with a solution containing 5 % sodium hypochlorite and 0.1 % Tween-20, for 15 min and rinsed four times with sterile water and stratified at 4 °C for 2 days. Seeds were sown on Petri plates containing GB-5 agar media (Gamborg's B-5 Basal Medium Sigma-Aldrich Co.) supplemented with 2 % sucrose and transferred to a growth chamber at 22 ± 0.5 °C. Light was supplied by cool white fluorescent bulbs in a photoperiod of 8 h light (125 μmol/m^2/s), and 16 h dark. Plantlets were grown in a 3:1:1 mixture of Sunshine mix #3 (SunGro), agrolite and vermiculite.

Genotoxic treatments

Seeds were grown in vertically-oriented plates containing GB-5 agar media. The position of the root tips of 4-day-old seedlings were marked before the treatment with UV-C irradiation. The seedlings were irradiated for 6 min with 10 J/m^2/s—determined with a IL1700 Research Radiometer—(International Light Technologies) of UV-C light (254 nm) using a UV lamp (Model UVGL-25, UVP), and then the plates were incubated under dark conditions for 3 days to prevent photoreactivation. New primary root growth was measured. Furthermore, to measure the length of root meristem, seedling roots were stained with a lugol solution for 2 min, and then clarified with a chloral hydrate solution (80 % chloral hydrate and 10 % glycerol). Roots were observed under Olympus CH30 optic microscope in the 10× objective equipped with a Moticam 1000. Images were analyzed using the Moticam Images Plus 2.0 software. Roots of untreated controls and UVC-irradiated seedlings were also stained with a propidium iodide (PI) solution (5 μg/mL) for 1 min before imaging under an Olympus FV1000 confocal microscope. PI was excited using a 543 nm laser and emission light was collected through a long-pass 560 nm filter. Green fluorescent protein (GFP) fluorescence was analyzed using 488 nm and 505–545 nm as excitation and emission wavelengths respectively. Images were captured with the 40× objective and GFP/propidium iodide images were overlapped.

To evaluate the tolerance to genotoxic agents, *Arabidposis* seeds were sown in GB-5 agar media supplemented with 10 μM zeocin (ZEO) or 15–25 μM mitomycin C(MMC) and the plates were incubated for 5 days at 4 °C in dark. To evaluate the effects of these genotoxins, we recorded the fresh weight of 17-day-old plantlets exposed to ZEO and the root length of 3-day-old seedlings growing in presence of MMC.

DNA damage analysis

The levels of pyrimidine dimers generated by UVC radiation and the degree of repaired DNA was quantified by

ELISA using the TDM-2 and 64M-2 monoclonal antibodies (Cosmo Bio Co. LTD Japan), which recognize CPDs and 6-4PPs, respectively. We followed the protocol recommended by the manufacturer. Rosette leaves were immersed in water through the peciole in 24-well plates and irradiated with UV-C, and then incubated under dark conditions. Samples were harvested at different time intervals after irradiation. Genomic DNA was extracted using the DNeasy plant mini kit (Qiagen). DNA concentration was determined by measuring the absorbance at 260 nm in a Nanodrop spectrophotometer. A 50 µL aliquot of the extracted DNA sample (at a final concentration of 0.2 ng/µL for CPDs and 0.5 ng/µL for 6,4-PPs) was used in each well. CPDs and 6-4PPs were detected at 492 nm in an Epoch, Bio Tek ELISA plate reader. For each sample, the mean value of four replica wells was calculated and the background was subtracted.

Promoter activity

A fragment of 1,080 bp from *AtTK1a* gene, comprising nucleotides −1,077 to +3 with respect to the ATG initiation codon, was amplified by PCR from genomic DNA using specific primers (AtTK1aF/R, Supplementary Table 1), and cloned into the pGEM-T Easy (Promega Corp. WI). This plasmid was used as template for amplification using a primer set with *att*B extensions (TK1a/attB1 and TK1a/attB2, Suppl Table 1). Afterward the *att*B sequences were completed with a second PCR with primers attB1 and attB2. The DNA fragment flanked by the *att*B sequences was cloned in the pDONR221 vector by BP-reaction according to manufacturer's instructions to generate the entry clone (Invitrogen). The promoter fragment from *AtTK1a* was subsequently subcloned into the Gateway destination vector pMDC163 using a conventional LR clonase reaction to yield the pMDC163/*promTK1a::GUS* construct. This vector was introduced into *Agrobacterium tumefaciens* strain PGV2260/C58 and transformed bacteria were used to obtain transgenic *Arabidopsis* plants by the floral-dip method (Clough and Bent 1998). The same strategy was employed to isolate and clone a 1,086-bp fragment of the *AtTK1b* gene promoter using the AtTK1bF/R primers (Suppl. Table 1).

To select *promTK1a/b::GUS* transgenic plants, seeds were grown on plates containing GB-5 agar media supplemented with hygromycin (15 µg/mL). T2 seeds from transgenic lines exhibiting 3:1 segregation for hygromycin resistance were selected and T3-independent homozygous lines exhibiting 100 % hygromycin resistance were recovered. T3 and T4 plants were used to analyze β-glucuronidase (GUS) activity in three independent lines. A similar strategy was employed to obtain *promTK1a::GFP* transgenic plants but the pGWB4 plasmid was employed.

GUS activity was detected by histochemical staining following the procedure of Jefferson et al. (1987). Tissues were immersed in GUS staining buffer (100 mM sodium phosphate, pH 7.0, Tween 20 0.1 %, and 0.5 mg/mL of 5-*bromo*-4-*chloro*-3-indolyl-β-D-glucuronide substrate predissolved in 0.5 % *N*-dimethylformamide), and incubated for approximately 16 h at 37 °C. The staining solution was removed and the samples were clarified in absolute ethanol to remove chlorophyll.

TK1 activity measurement

Soluble proteins were extracted from 7-day-old seedlings. Following grinding in liquid nitrogen, plant tissue samples were homogenized in protein extraction buffer (70 mM Tris pH 7.5, 1 mM $MgCl_2$, 25 mM KCl, 5 mM $Na_2EDTA\ 2H_2O$ pH 8.0, 0.25 mM sucrose, 15 mM β-mercaptoethanol, 0.1 % Triton ×-100, protease inhibitor cocktail, Complete Mini, Roche). The homogenized tissue was kept on ice all the time and centrifuged at 16,200×*g* for 30 min to obtain the cytosolic soluble fraction. Total protein was quantified according to Bradford (1976) using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc.).

TK enzymatic activity was determined following the protocol reported by Wolcott and Colacino (1989). The reaction mixture for this assay was prepared with 500 µL of reaction buffer (100 mM Tris, 10 mM $MgCl_2$, 10 mM NaF, 10 mM ATP, 2 mM β-mercaptoethanol), 2 µM thymidine (0.75 µM of [methyl-^{3}H]-thymidine and 1.25 µM of thymidine), 150 µg of soluble protein extract, and deionized water to complete 1,000 µL. The reaction mixture was incubated at 37 °C for 90 min. At the end of the incubation time, 200 µL of the reaction mixture was mixed with 1 mL of precipitation solution (100 mM $LaCl_3$; 5 mM triethanolamine), and centrifuged for 10 min at 2,000×*g*; this step was repeated twice. The precipitate was dissolved in 300 µL of 0.05 N HCl and 3 mL of Bray scintillation liquid. Total cpm were recorded in a Beckman LS 6000IC scintillation counter and specific activity was calculated.

Semi-quantitative RT-PCR and RT-qPCR

Total RNA was isolated from approximately 200 mg of 7-day-old seedlings or adult rosette leaves using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quality and quantity of isolated RNA was assessed by denaturing gel electrophoresis and spectrophotometric analysis respectively. Reverse transcription was performed with 1 µg of RNA in 25 µL using ImProm-II reverse transcriptase (Promega) and 2.5 µM oligo-dT. For each PCR 1 µL cDNA was used as template

for the reaction with gene-specific primers of *AtTK1a* (At3TK1F/R) and *AtTK1b* (At5TK1F/R). The *AtAPT1* gene was used as control (primers AtAPT1F/R, Suppl. table 1). To detect relative differences in transcript levels, the number of cycles needed to reach an exponential accumulation of the amplicon was determined for each gene. PCR products were separated on 2 % (w/v) agarose gels and the intensities of ethidium bromide-stained bands were determined using Carestream 5.0 software. Band intensities of *TK1a* and *TK1b* were corrected according to the relative quantity of *AtAPT1* product.

Real time RT-PCR was carried out using the 7500 DNA analyzer (Applied Biosystems, Foster, CA). A specific primer set were designed to amplify a cDNA fragment of *AtTK1a* and *AtTK1b* gene respectively (At3TK1F/At3TK1R and At5TK1Fq/At5TK1Rq primers, Suppl. table 1). Standard curves were generated from duplicated series of five DNA template dilutions to test PCR efficiencies. To verify amplification specificity, melting curves were determined and end-point PCR carried out. The PCR was performed with 2 μL of cDNA, 0.15 μM of each primer, 10 μL SYBR® Green PCR master mix (Applied Biosystems) in 20 μL. The cycling parameters were as follows: one cycle of 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, The *AtAPT1* gene transcript was used as internal control (Santiago et al. 2008), amplified with the primers AtAPT1Fq and AtAPT1Rq. Transcript levels were quantified using the comparative method known as $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001). The software used for quantitative RT-PCR analysis was ABI PRISM 7000SDS.

Statistical analysis

All experiments were repeated at least three times. Results are shown as mean ± standard deviation. Data were compared by Student's *t* test. *P* values of 0.05 or less were considered to be statistically significant.

Results

AtTK1 is ubiquitously expressed during plant development

Arabidopsis thaliana contains two potential genes—At3g07800 and At5g23070—coding TK, designated *AtTK1a* and *AtTK1b*, respectively (Clausen et al. 2012). According to Genevestigator (www.genevestigator.com/gv/plant.jsp; Hruz et al. 2008) database, both genes have a ubiquitous expression during plant development, from seed germination to flower maturity (Suppl. Table 2). However, in all tissues transcript levels of *AtTK1a* are higher than those of *AtTK1b*. To corroborate this expression pattern of *TK1a*, we detected GUS activity in transgenic plants containing the *AtTK1a* promoter–GUS gene fusion. During normal conditions, we observed a strong staining at the early stages of germination upon testa rupture (Fig. 1a) and throughout seedling development; high GUS activity was detected in the cotyledons and foliar primordium (Fig. 1b). In young leaves, foci of GUS activity were noted in the trichomes (Fig. 1c, e). In 2-day old seedlings GUS activity was high in almost every organ except the root meristem (Fig. 1f, h); this was also the case in the secondary root primordium (Fig. 1d, g). Stems showed low GUS staining (Fig. 1i). In reproductive organs, intense GUS was revealed in stigma, petals (Fig. 1l) anthers and pollen (Fig. 1m). In developing siliques, GUS activity was found only in the pedicel (Fig. 1j) and the septum (Fig. 1n). The *AtTK1a* promoter also drove GUS expression in the vasculature of the rosette leaves (Fig. 1p) as well as in the pedicel and base (Fig. 1o). All together, these results point out that *AtTK1a* is expressed ubiquitously in *Arabidopsis* and are consistent with Genevestigator database (Suppl. Table 2).

AtTK1a and *AtTK1b* are differentially expressed upon UV-C radiation treatment

Since UV radiation is the main genotoxin at which plants are constantly exposed, we tested whether *AtTK1a* and *AtTK1b* were induced by such insult and found that in 7-day-old seedlings irradiated with UV-C light, only *AtTK1a* expression was induced (Fig. 2a). Quantitative RT-PCR analysis showed a three-fold induction of *AtTK1a* at 3 h post-irradiation (hpi) and a 5.5-fold increase at 12 hpi. In contrast, transcript levels of *AtTK1b* remained unchanged during this time course (Fig. 2b). Transgenic plants expressing promoter–GUS fusions for both genes confirmed these results; seedlings that contained the *AtTK1a* promoter displayed a strong induction of GUS activity in the roots, also present in the root apical meristem (Fig. 2c), whereas in *AtTK1b* remained unchanged (Supplementary Figure 1).

T-DNA insertion in SALK_037113 line caused overexpression of *AtTK1a* gene

To study the role of *AtTK1a* in the plant response to UV radiation we searched the ABRC database and at the time this study was performed only a few mutants containing a T-DNA within an intron, were identified. A distinct type of mutant, SALK_037113 line (Fig. 3a), that contains an insertion in the 5′- region, 312-bp upstream of the start codon (Fig. 3b), was available as well, so we decided to further characterize it. The promoter region contains various potential regulatory elements, among them a putative

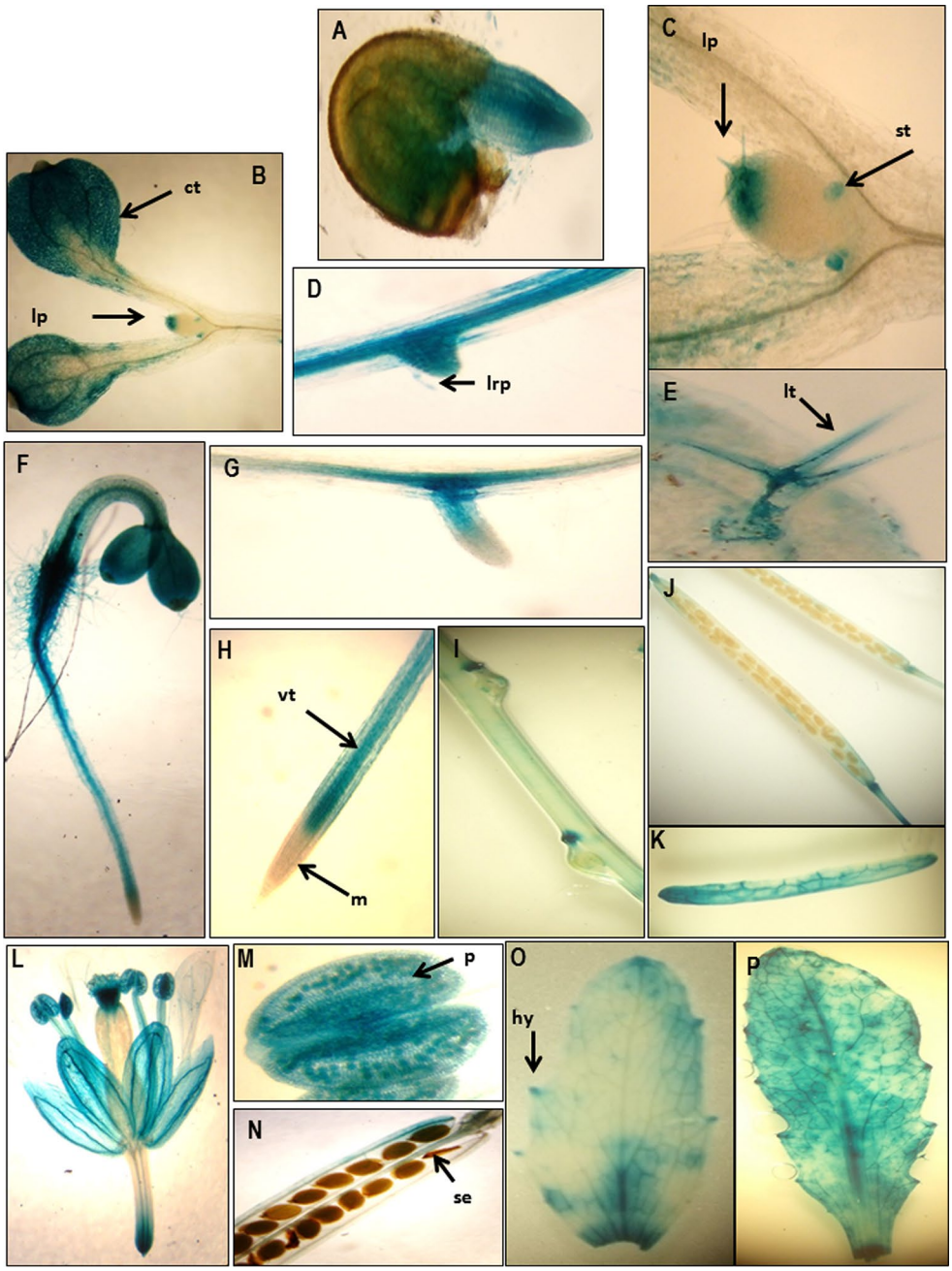

Fig. 1 Histochemical localization of GUS activity directed by the *AtTK1a* promoter in transgenic *A. thaliana*. **a** Radicle protrusion at 48 h post-imbibition. **b** Cotyledons (ct) and leaf primordium (lp) in 7-day-old seedlings. **c** Stipule (st) in leaf primordium. **d** Lateral root primordium (lrp). **e** Leaf trichome (lt). **f** Two-day old seedling. **g** Lateral root. **h** Root vascular tissue (vt), and meristem (m) depicted by *arrows*. **i** Stem. **j** Developing siliques. **k** Dry, empty siliques. **l** Anthers and gynoecium in mature flowers. **m** Anther full of pollen (p). **n** Siliques depicting the septum (se). **o** Caulinar leaf depicting the hydathode (hy). **p** Rosette leaf

E2F binding site (TTTGGCCC) in the reverse strand at 635-bp upstream of the ATG codon. We found that the insertion affected gene expression, as *AtTK1a* transcript levels in 7-day old seedlings were 40 % higher than in wild-type plantlets (Fig. 3c). Raise in gene expression was also reflected in enzyme activity; the seedlings showed 65 % higher TK specific activity (Fig. 3d). In adult plants the difference in transcript level between this insertion mutant and wild-type plants was exacerbated as the former contained up to 2.5-fold higher levels than Col (Supplementary Fig. 2). Because the T-DNA insertion in the promoter region of the *TK1a* gene lead to higher transcript levels, we named this line *over-expression tk1a* (*oetk1a*) mutant.

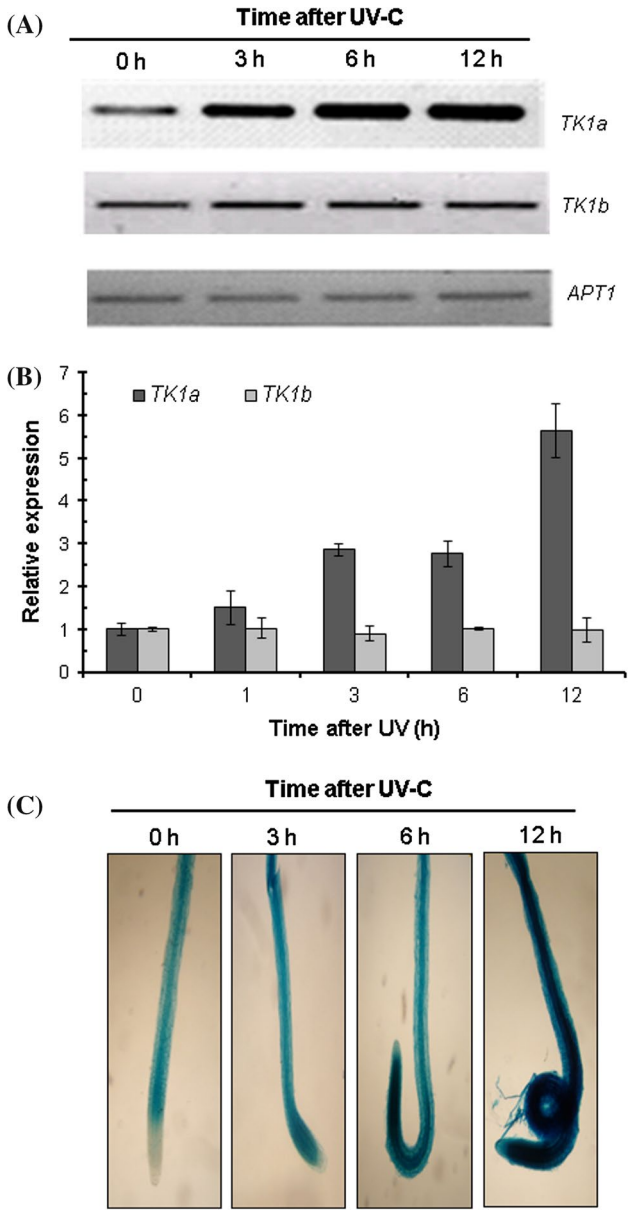

Fig. 2 Differential response of *AtTK1a* and *AtTK1b* genes to UV-C radiation. **a** *AtTK1a* and *AtTK1b* transcript levels evaluated by end-point RT-PCR in *Arabidopsis* seedlings before and 3, 6 and 12 after UV-C radiation. **b** *AtTK1a* and *AtTK1b* transcript levels evaluated by real-time RT-PCR in *Arabidopsis* seedlings before and after UV-C radiation. **c** GUS activity in the root of prom*TK1a*::GUS seedlings before and after UV-C radiation. In all cases, 7-day-old seedlings were irradiated for 6 min with UV-C light (10 J/m^2/s) and sampled at several time points post-irradiation

AtTK1a overexpression confers tolerance to genotoxic stress in *Arabidopsis* seedlings

In order to study the role of TK during DNA repair events, we submitted wild-type and *oetk1a* plants to various types of genotoxic stress. Four-days-old seedlings were irradiated with UV-C light and incubated under dark conditions to prevent photoreactivation for 3 days. A 70 % inhibition in root length was recorded in the wild-type genotype but only a 40 % reduction was observed in the oe*tk1a* mutant plants (Fig. 4a, b). When plantlets were grown under a photoperiod regime after UV treatment, improved recovery was observed due to photolyase activity in both genotypes but still the oe*tk1a* mutant showed increased tolerance as only 20 % inhibition occurred in contrast to 50 % reduction in wild-type seedlings (Supplementary Fig. 3).

Inhibition of root growth by genotoxic stress is associated with reduction of meristem length (Adachi et al. 2011). When we analyzed the meristematic zone of the primary root, we observed a reduction in the length of root meristem only in wild-type plants irradiated with UV-C light (Fig. 4c). In fact, wild-type plants showed a 40 % drop in the meristem length whereas in *oetk1a* mutants a minor reduction was recorded (Fig. 4d).

Furthermore, seedling roots were stained with propidium iodide and observed with confocal microscopy. Figure 4e shows representative images of the roots of irradiated and non-irradiated wild-type and *oetk1a* seedlings. We observed that the number of cells in the cortex (16 ± 2.5 in Col vs. 19 ± 4 in *oetk1a*) and the length (Fig. 4d) of the area of the meristem of *oetk1a* did not differ in comparison to its non-irradiated control. In contrast, in the wild-type roots a reduction of the meristem was observed in irradiated seedlings (Fig. 4d) that correlated with a decrease in the number of cells in the cortex (9.5 ± 2.3 in Col vs. 14 ± 5.6 in *oetk1a*; $P < 0.01$). Although the T-DNA insertion in the promoter region caused a slight increase in transcript level and enzyme activity, it was sufficient to provide tolerance to UV-C radiation.

Thymidine kinase is readily induced in *oetk1a* mutant plants by UV-C

We evaluated whether *TK1a* gene was induced by UV-C in the *oetk1a* mutant in a similar manner as in wild-type plants. UV-C-irradiated seedlings were kept in the dark and sampled for RNA extraction. In intact *oetk1a* seedlings, *TK1a* transcript levels were 35 % higher than in wild-type seedlings. At 1 hpi, a 100 % elevation of transcript levels occurred in *oetk1a* seedlings while a 40 % induction was registered in the wild-type. However, by 3, 6 and 12 hpi both genotypes showed similar *TK1a* transcript augmentation pattern (Fig. 5a). In mutant plants enzymatic activity was 40 % higher than in wild-type seedlings and upon UV treatment, a small but significant induction was observed in both genotypes at 3 hpi. The *oetk1a* seedlings showed higher enzymatic activity at all times measured (Fig. 5b).

To associate the increase in gene expression and enzyme activity with DNA repair capacity we irradiated wild-type

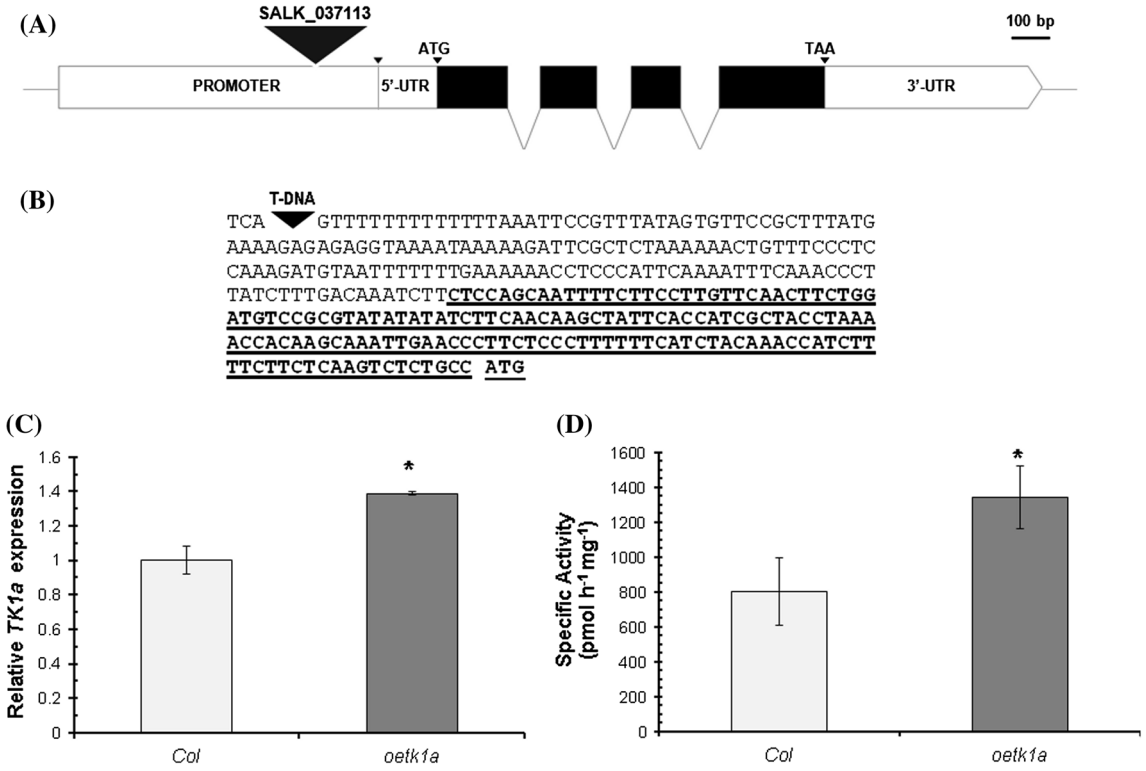

Fig. 3 T-DNA insertion in the promoter region of the *AtTK1a* gene causes overexpression. **a** Scheme of *AtTK1a* gene. The predicted *AtTK1a* open reading frame contains four exons (*black bar*) and three introns (*zig-zag lines*). The T-DNA insert in SALK_037113 (*black triangle*) is located in the promoter region of the gene. **b** T-DNA insert is positioned 312-bp upstream of the start codon ATG in the *AtTK1a* gene. The 5′-UTR is depicted in *bold letters*. **c** *TK1a* transcript levels, quantified by real-time RT-PCR, in wild-type and *oetk1a* mutant plants. **d** Thymidine kinase total activity in wild-type and *oetk1a* mutant plants. For transcript and activity analyses, 7-day-old seedlings were used. Three independent pools of seedlings for each genotype were used and the *asterisk* denotes significant difference (Student *t* test; $P < 0.05$)

and *oetk1a* plants with UV-C light and measured the repair rates of two photoproducts (CPDs and 6-4PPs). Plant tissue was incubated under dark conditions post-irradiation to prevent photorepair and evaluate exclusively repair mechanisms that require DNA synthesis. Repair of both photoproducts showed a similar pattern, and the *oetk1a* mutants exhibited higher levels of repair than wild-type at each time point for CPDs reaching up to a 60 % reduction (Fig. 5c). Dark repair of 6-4 PPs was more efficient in wild-type plants at 30 min post-irradiation (52 vs. 65 % remaining) but in *oetk1a* plants a meaningful decline of photoproducts was recorded at later time-points (Fig. 5d).

AtTK1a is also induced in response to various types of genotoxic stress

To study the expression and role of *AtTK1a* in response to other type of genotoxic stress that also require DNA synthesis, we treated the *Arabidopsis* genotypes with MMC, a DNA crosslinker, and ZEO a DNA intercalator. As it occurred with UV-C radiation, *oetk1a* mutants were more tolerant to these agents. Figure 6a shows *Arabidopsis* seedlings growing in presence of 10 μM ZEO that caused an important reduction in root growth of the wild-type plants, whereas growth of the *oetk1a* plants was barely affected. The genotoxic agent caused a 40 % reduction in plant biomass, determined by fresh weight, in the wild-type genotype while only a 14 % decrease occurred in *oetk1a* mutants (Fig. 6b). Similar to what we observed with UV-C treatments, transgenic plants expressing the *AtTK1a* promoter–GUS fusion revealed that the *AtTK1a* gene was strongly induced upon ZEO treatment (Fig. 6c). Likewise, increased tolerance to MMC was observed by the root length assay; while wild-type seedlings treated 15 μM MMC showed a 50 % inhibition in root elongation, *oetk1a* seedlings displayed a 36 % inhibition ($P < 0.05$). The *oetk1a* plants grow more vigorously than wild type plants and the prom*TK1a*::GUS seedlings showed that a strong gene induction occurred upon MMC treatment (Supplementary Fig. 4). To study *TK1a* expression at cell level we generated transgenic plants expressing the GFP under *TK1a* promoter control (*promTK1a::GFP*) and examine the

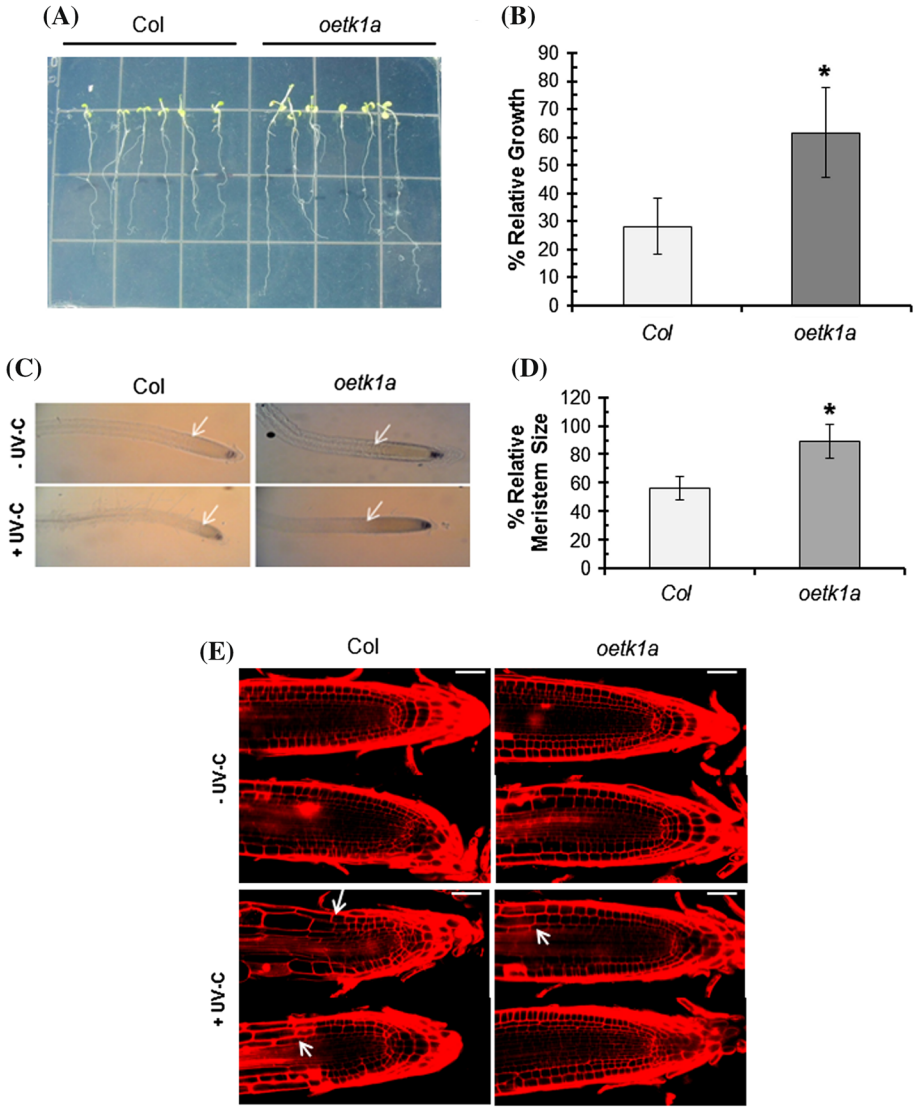

Fig. 4 Overexpression of *TK1a* enhances tolerance to UV-C radiation. **a** UV-C sensitivity of wild-type and *oetk1a* mutant plants. Four-day-old seedlings were irradiated with 10 J/m^2/s and incubated under dark conditions. **b** Root length of wild-type (Col) and *oetk1a* mutants was measured and results are expressed as the percentage with respect of the corresponding non-irradiated genotypes. **c** Effect of UV-C radiation on the root meristem. Irradiated and non-irradiated seedlings were stained with lugol solution and the roots examined under an optical microscope. The *white arrow* indicates the edge of the root differentiation zone. **d** Meristem length of wild-type (Col) and *oetk1a* mutants was measured and results are expressed as the percentage with respect of the corresponding non-irradiated genotypes. **e** Effect of UV-C radiation on the root apex structure. Irradiated and non-irradiated seedlings were stained with propidium iodide solution and the roots examined under confocal microscope. The *white arrow* indicates the edge of the root differentiation zone (*scale bars* 30 μm). For **b**, **c**, the *asterisk* denotes significant difference (Student *t* test; $P < 0.05$; n = 12)

roots by confocal microscopy after exposure to genotoxic stress. Supplementary Figure 5 shows representative confocal images and the propidium iodide overlap of the same tissue. In the root apex of unexposed control plants, GFP levels are very low and a slight induction is observed 6 hpi, but only in the endodermis cell layer. In contrast, treatment with MMC and ZEO strongly induced TK1a gene expression as GFP was observed in different cell layers, mainly in the epidermis layer and the root vascular zone.

Discussion

We found that the *TK1a* gene is constitutively expressed during *A. thaliana* development, and it is induced upon UV radiation and contributes to DNA repair of pyrimidine dimers. The T-DNA insertion in the promoter of *TK1a* gene in SALK_037113 caused increased transcription, probably by interrupting one or a string of negative regulatory elements. Elevation in TK activity was associated to increased tolerance to genotoxic stress, compared to wild-type plants. *TK1a* gene was strongly induced by ZEO—a DNA intercalating agent that induces double-strand breaks—, by MMC—a DNA crosslinker—and by UV-C radiation—a thymine dimer inducer. Although the lesions caused by the three agents are repaired by distinct mechanisms, all require DNA polymerization thus adequate nucleotide levels are needed for accurate DNA synthesis.

Most studies aimed to determine the function of eukaryotic TK have been carried out in cultured cells and cell lines (McKenna and Hickey 1981; Chen et al. 2010) so it is relevant to explore it in a full organism. In mammals, the only study with such characteristics was performed with homozygous knockout (TK1$^{-/-}$) mice that showed the enzyme is required for development, as KO animals display sclerosis of kidney glomeruli and spleen abnormalities.

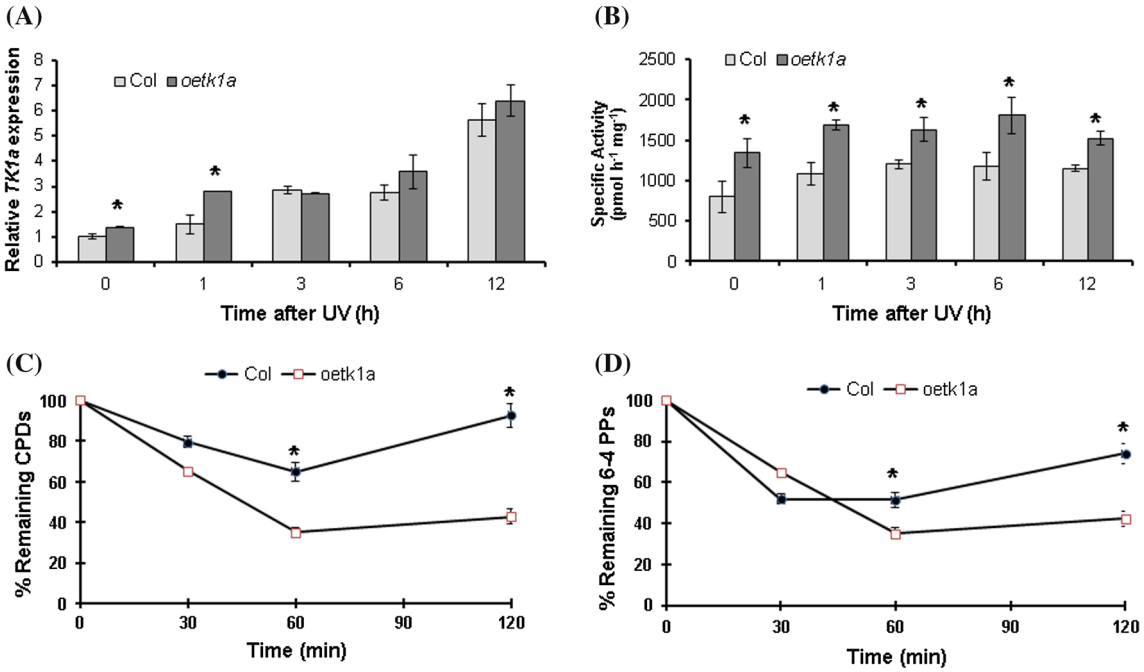

Fig. 5 *oetk1a* mutants have a heightened and early response to UV-C light that contributes to DNA photoproduct repair. **a** *TK1a* transcript levels evaluated by quantitative real-time RT-PCR in wild-type and *oetk1a* mutant *Arabidopsis* seedlings before and after UV-C exposure. **b** Total TK enzymatic activity determined in wild-type and *oetk1a* mutant *Arabidopsis* seedlings before and after UV-C exposure. **c** CPD photoproduct repair in wild-type and *oetk1a* mutant plants after UV-C irradiation. **d** Pyrimidine (6-4) dimer photoproduct repair in wild-type and *oetk1a* mutant plants after UV-C irradiation. For C and D, data are shown as mean % photoproduct remaining ± SD (n = 4). For each time point the *asterisk* denotes significant difference (Student *t* test; $P < 0.05$)

Mice die before 1 year of age (Dobrovolsky et al. 2003). *Arabidopsis* insertion mutants for each *TK1* gene are unaffected during development. However, the double mutant seeds germinate and elongate but die at the seedling stage (Clausen et al. 2012). These results suggest that both proteins catalyze the same reaction and that *TK1* is essential for development.

TK1a is ubiquitously expressed during plant development

TK1a promoter–GUS fusions showed that the gene is ubiquitously expressed during plant development and our data are consistent with the microarray database. We observed intense GUS staining during the first 24 h post-imbibition after dormancy break-up, just before radicle protrusion. This implies high *TK1a* promoter activity and suggests a relevant role of the salvage pathway during germination. During seed germination, nucleotides are required as carriers of chemical energy, as components of some enzyme cofactors and as precursors of nucleic acids synthesis. This fact implies a strict coordination between the de novo and salvage pathways to establish the ribonucleotide and deoxynucleotides pools during seed imbibiton (Stasolla et al. 2003). During germination, the free bases and nucleosides, released from storage tissues, are transformed into nucleotides through the salvage pathway (Moffatt and Ashihara 2002). For pyrimidine nucleotides, the salvage pathway contributes during early development before the de novo pathway takes over (Stasolla et al. 2003).

In dry wheat embryos dNTPs are undetected and dCTP and TTP levels reach a plateau after 16–20 h of germination. Likewise, TK activity shows a ten-fold increase at 20 h after water imbibition from the dry embryos (Castroviejo et al. 1979). Moreover, in *Arabidopsis*, the uridine salvage pathway is required during early plant development as uracil phosphoribosyltransferases mutants exhibit growth retardation, a pale-green to albino phenotype and flimsy roots with poor branching (Mainguet et al. 2009). *TK1a* was also expressed in the root primordium that leads to lateral root formation. Pyrimidine salvage is required for primordium formation and in general for organogenesis (Stasolla et al. 2003).

TK1a promoter was also active in reproductive tissues such as anthers and pollen; we found several *cis* elements localized in the promoter regions that specifically regulate gene expression in these tissues. For example the CArG (AP1) box that is involved in *APETALA3* gene expression in *Arabidopsis* flowers (Hill et al. 1998), and it also correlates with an active nucleotide synthesis (Kafer et al. 2004). *TK1a* promoter activity was also high in

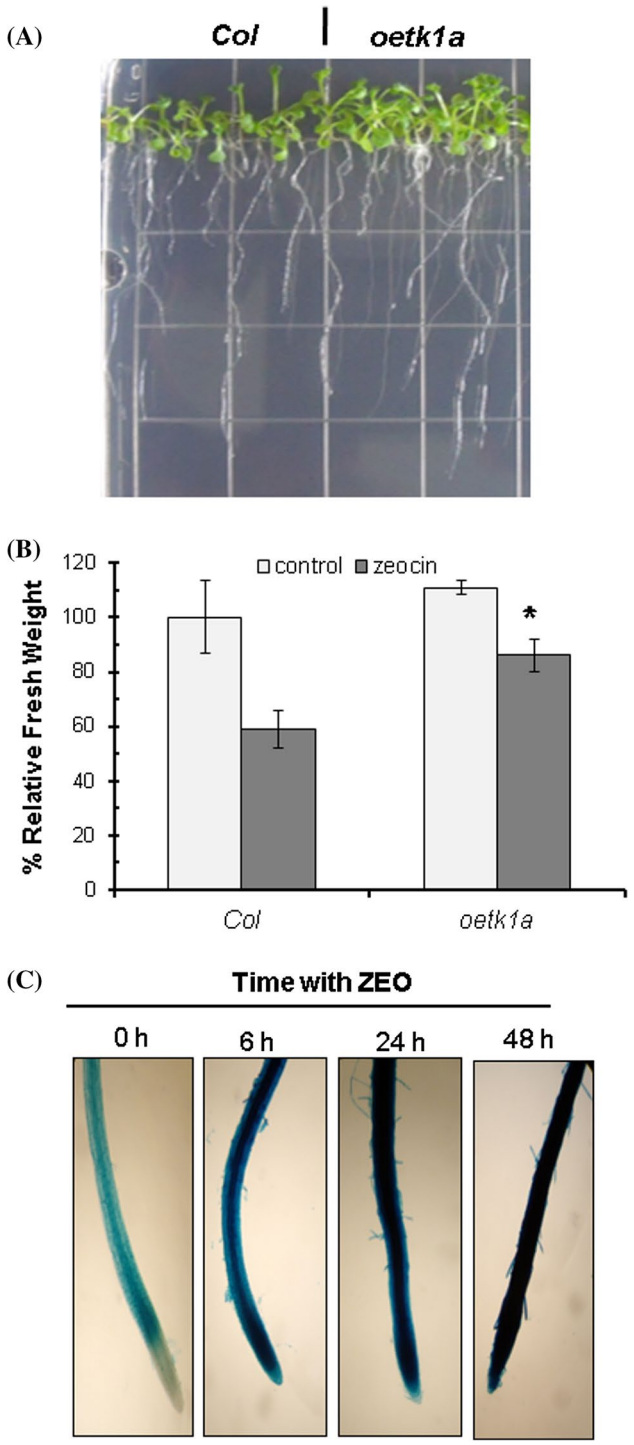

Fig. 6 Overexpression of *TK1a* also improves tolerance to DNA intercalating agents. **a** Zeocin sensitivity of wild-type (Col) and *oetk1a* mutants. Seeds were sown on GB-5 agar media supplemented with 10 μM zeocin. **b** Fresh weight of seedling was determined after 17 days. Results are expressed as the ratio (%) of the fresh weight to the non-treated wild-type plants. The *asterisk* denotes significant difference between the wild-type and the *oetk1a* mutant (Student *t* test; $P < 0.05$; $n = 16$). **c** GUS activity in the root of prom*TK1a*::GUS seedlings before and after zeocin exposure. Four-day-old seedlings were transferred to media containing the genotoxin and sampled for GUS staining

trichomes-specialized cells that go through several rounds of endoreduplication—that have a high nucleotide demand (Bramsiepe et al. 2010). *TK1a* promoter activity was absent during embryogenesis and data are lacking about the role of the salvage pathway at this stage in other species. During somatic embryogenesis of *Picea glauca*, about 80 % of the nucleotides are synthesized through the de novo pathway (Stasolla et al. 2003).

TK1a overexpression confers tolerance to genotoxic stress

Since TK1a was induced by UV-C light radiation and by genotoxins, we examined the phenotype of *oetk1a* seedlings subjected to genotoxic stress. We found that the mutant plants containing the T-DNA insertion in the promoter region were more tolerant to UV-C light under dark conditions, assessed by root growth, meristem length and meristem integrity, than the wild-type genotype. By allowing photoreactivation, both genotypes coped better with stress but still the *oetk1a* line was superior. Likewise, growth in presence of genotoxins that induce double-strand breaks was barely affected in the mutant plants. Although *TK1a* transcripts increased and TK total activity was elevated, these parameters were unequal in their magnitude. Such differences between RNA and activity levels might be due to post-translation regulatory mechanism. The homodimer of human TK1 has low activity while the tetramer has high activity. The conversion between these two forms is reversible and depends on ATP concentration (Munch-Petersen 2009). Such transition does not occur in the tomato TK1, (Mutahir et al. 2011), so far the only plant enzyme studied in this respect. However, other unknown mechanisms might operate in the regulation of plant TK1 activity.

A rapid increase in dTTP levels in response to DNA damage must contribute to several DNA repair pathways that require DNA polymerization and to correct imbalances in dNTP pools. Mammalian cells lines lacking this enzyme are hypersensitive to genotoxic stress. Mouse erythroleukaemia cells—TK minus—show heightened sensitivity to UV radiation (McKenna and Hickey 1981), to genotoxins (ethyl methane sulfonate, *N*-methyl-*N′*-nitrosoguanidine, MMC, and to gamma irradiation; McKenna et al. 1988) than wild-type cells. In general, the mutant cells showed less survival and higher cytogenetic aberrations. Likewise, rat glioma cells that lack *TK* are more sensitive to ionizing radiation than the wild-type cells. The ability to repair sublethal DNA damage was associated with TK expression as introduction of the herpes virus *TK* partially restored tolerance in the mutant cells (Al-Nabulsi et al. 1994). Because TK1 in humans was characterized as an enzyme of the G1 and S phases of the cell cycle i.e. proliferative cells it has been proposed as a biomarker for cancer cells

that proliferate very rapidly (O'Neill et al. 2001). Indeed, increased TK1 activity has been found in the serum of patients with lung cancer (Holdenrieder et al. 2010), and breast cancer (Nisman et al. 2010). The levels of TK1 activity in serum has been proposed as a diagnosis and prognosis marker, and the recent discovery of its role in DNA repair (Chen et al. 2010) suggests that it might be worth exploring its usefulness as a marker for resistance to radio- and chemo-therapy. Since TK activity is tightly regulated it is unlikely that *TK1a* overexpression could skew dNTPs ratios. In fact, the experimental strategies to skew distort pool ratios by excess of dTTP consist in the exogenous application of thymidine (10 mM) at high concentrations (Goncalves et al. 1984).

While photoreactivation contributes in non-proliferative tissues, NER is the main DNA repair mechanism in meristems (Kimura et al. 2004). Although *TK1a* showed low levels in the meristem, it was strongly induced when exposed to genotoxic agents, suggesting a role in DNA repair in these tissues. In situ expression of *TK1a* was analyzed in *promTK1a::GFP* transformed plants. A weak GFP signal was recorded in intact plants, but the genotoxins ZEO and MMC induced a raise of GFP in all cell layers. When the plants were treated with UV-C, GFP was detected only in the endodermis. Such differences in expression site and levels might be due to the levels of damaged DNA caused by each agent. Transcription profiles show that root meristem and bud are enriched in transcripts from genes of DNA repair proteins, thus might be specialized organs for these events (Yadav et al. 2009). *Arabidopsis* lines with mutations in the translesion DNA polymerase genes show highly disorganized cell layers below the quiescent center (QC) when exposed to UV-B radiation, suggesting the relevance of NER on keeping the integrity of the cell layers adjacent to the QC (Curtis and Hays 2007). Response to the genotoxic stress in QC includes a higher rate of cell division to replace the damaged and dead cells (Jiang and Feldman 2005).

ATTK1 genes are differentially expressed in response to UV-C

We showed a differential transcriptional response of the two *AtTK1* genes when *Arabidopsis* seedlings were irradiated with UV-C as only *TK1a* was induced by this replicative stress that causes thymine dimer formation. Because *TK1a* gene was induced under other types of stress we hypothesized that it would play a role in providing dTTPs for DNA excision repair.

In our search for T-DNA insertion mutants to study TK1 function we came across with SALK_037113 line, and found that the insertion within the first 300 bp upstream of the start codon lead to the overexpression of the *TK1a* gene. This mechanism could be explained either by disruption of a negatively regulated element or by the introduction of an element present in the T-DNA sequence that responds positively (Halliday et al. 1999). In any case, the promoter kept the functional elements as mutant *oetk1a* seedlings presented a faster response to UV-C than wild-type plants; at 1 hpr, a three-fold increase was observed and it took 3 hpr for the wild-type genotype to reach comparable mRNA levels.

The *TK1a* promoter contains a canonical E2F-conserved element that is also present in several other genes involved in DNA replication, DNA repair, cell cycle regulation, transcription and stress response (Ramirez-Parra et al. 2003). This appears to be a functional element as *TK1a* is induced 15-fold in transgenic *Arabidopsis* overexpressing the E2Fa and DPa transcription factors (Vandepoele et al. 2005). The E2F element in the *HsTK1* gene regulates transcription during the G1-S transition (Rotheneder et al. 1999), but in plant cells its function might be wider. In *Nicotiana tabacum* E2F participates in the control of ribonucleotide reductase subunit 1b (RNR1b), a key enzyme in the de novo synthesis of nucleotides. Through this control, *RNR1b* is specifically expressed during the S phase of the cell cycle in meristematic tissue (Chabouté et al. 2002). Other DNA repair genes such as uracil DNA glycosylase, DNA mismatch repair protein (MSH6-1) and RAD5 contain conserved E2F motifs in their promoters (Vandepoele et al. 2005).

AtTK1a is also strongly induced (ten-fold) by treatment with bleomycin plus MMC, two agents that also induce double strand breaks (Chen et al. 2003). Mechanisms to perceive damaged DNA are highly conserved in eukaryotic cells through the ATM and ATR proteins. Both are phosphatidylinositol kinase-related proteins that regulate cell response to a variety of DNA-damaging agents. ATM is activated by double-strand breaks, while the ATR pathway is triggered by the presence of single-stranded DNA. Upon γ-irradiation, hundreds of genes are induced and most are dependent on ATM. *AtTK1a* belongs to this group as it is strongly induced (46-fold increase) 1.5 h post-irraditaion in wild-type *Arabidopsis* plants irradiated with 100 Gy, and the induction drops to a 2.3-fold in *atm* mutants. In contrast, the gene is still induced at similar levels in *atr* mutant plants (Culligan et al. 2006). Similar results were obtained by Ricaud et al. (2007) at 5 h post-irradiation. One of the downstream targets of ATM is SOG1, a plant-specific transcription factor, whose pathway and regulation is analogous to the human p53 (Yoshiyama et al. 2014). ATM but not ATR mediates hyperphosporylation of SOG1 during the DNA damage response to γ-irradiation (Yoshiyama et al. 2013). Most (88 %) of the genes induced by γ-irradiation depend on SOG1 as in *sog1-1* mutant plants are not induced. In these experiments, *AtTK1a* gene expression is induced 20-fold in the wild-type plants while its expression is curbed in the *sog1-1* mutant (Yoshiyama et al. 2009). In none of the conditions described in these

microarray experiments *AtTK1b* is induced significantly, thus supporting a selective role of *AtTK1a* in the DNA damage response. Finally, our results are in agreement, and provide functional evidence, with the recent inclusion of *AtTK1a* as one of the core of genes induced by multiple DNA-damaging agents (Yi et al. 2014).

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