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ORIGINAL ARTICLE

Glutathione and transpiration as key factors conditioning oxidative stress in *Arabidopsis thaliana* exposed to uranium

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Abstract Although oxidative stress has been previously described in plants exposed to uranium (U), some uncertainty remains about the role of glutathione and tocopherol availability in the different responsiveness of plants to photo-oxidative damage. Moreover, in most cases, little consideration is given to the role of water transport in shoot heavy metal accumulation. Here, we investigated the effect of uranyl nitrate exposure (50 μM) on PSII and parameters involved in water transport (leaf transpiration and aquaporin gene expression) of *Arabidopsis* wild type (WT) and mutant plants that are deficient in tocopherol (*vte1*: null α/γ-tocopherol and *vte4*: null α-tocopherol) and glutathione biosynthesis (high content: *cad1.3* and low content: *cad2.1*). We show how U exposure induced photosynthetic inhibition that entailed an electron sink/source imbalance that caused PSII photoinhibition in the mutants. The WT was the only line where U did not damage PSII. The increase in energy thermal dissipation observed in all the plants exposed to U did not avoid photo-oxidative damage of mutants. The maintenance of control of glutathione and malondialdehyde contents probed to be target points for the overcoming of photoinhibition in the WT. The relationship between leaf U content and leaf transpiration confirmed the relevance of water transport in heavy metals partitioning and accumulation in leaves, with the consequent implication of susceptibility to oxidative stress.

Keywords Arabidopsis · Chlorophyll fluorescence · Photosynthesis · Plant hormones · Transpiration · Tocopherol · Uranium

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Introduction

Heavy metals (HM) is a collective term that applies to the group of metals and metalloids with a density over 4 g cm^{-3}. This group is made up of compounds such as uranium (U), cadmium (Cd), lead (Pb) and others. Unlike most organic pollutants, heavy metals are elements found naturally in the earth's crust. In Europe, the amount of waste generated (excluding agricultural waste) reaches 1,300 million tonnes (of which 36 million tonnes are

hazardous waste) (European Environment Agency 1999). As described by the European Environment Agency (EEA), in 1999 in Western Europe 1,500,000 contaminated areas were estimated and 300,000 were identified (European Environment Agency 1999). In the case of France, the European agency indicates that there are 700,000 potential cases and 895 confirmed contamination sites. However, the report also notes that most European countries are in an early stage of identification and registration of contaminated sites.

The origins of the pollutants are the combustion of fossil fuels (cars, etc.), industry, slurry and waste, and raw materials (applied to agriculture) and phosphates (Battarbee et al. 1988). Uranium is a naturally occurring radionuclide and heavy metal with an average concentration in the continental crust of 1.7 ppm (Wedepohl 1995). In the soluble form, U may be taken up by plants and can therefore contaminate the food chain (Neves et al. 2012). U is a chemotoxic and a radiotoxic element: chemical toxicity is particularly significant in compounds containing natural uranium, whereas its radiological toxicity is directly related to ionizing radiation effects of enriched U (Ribera et al. 1996 and references therein).

Although the effect of U exposure has been previously tested in human and animal species (Ribera et al. 1996), information on the effect of U exposure in plants is scarce (Vanhoudt et al. 2008, 2011a, b, c; Viehweger et al. 2011). Similar to descriptions for other heavy metals such as Cd (López-Millán et al. 2009) and Cr (Rodriguez et al. 2012), U has been characterised as an inducing oxidative stress agent in plants (Vanhoudt et al. 2008, 2011a, b, c; Viehweger et al. 2011). Most of these studies have been focused on the expression of genes involved in the removal of reactive oxygen species (ROS). The steady-state level of ROS in cells needs to be tightly regulated to avoid their accumulation in excess that might induce oxidative damage. Under stress conditions it has been described (Schützendübel and Polle 2002; Villiers et al. 2011) to be an increase/decrease in the capacity of the antioxidative defence system. Plants have developed three main mechanisms to diminish photooxidation. First, although under high-energy donation conditions might cause photo-oxidative damage, in some cases, the plants might prevent the production of ROS by diminishing the electron transport chain. Second, the plants might diminish photooxidation of the photosystem II light-harvesting antenna through the xanthophyll cycle-dependent thermal dissipation and changes in photosynthetic light-harvesting complex II antennae (Verhoeven et al. 1999; Janik et al. 2013). The third mechanism consists in the capacity to scavenge ROS formed by an integrated system of enzymatic (e.g. superoxide dismutase, catalase, ascorbate peroxidase; Mittler et al. 2004) and non-enzymatic antioxidants [e.g. ascorbate (AsA), glutathione (GSH) and tocopherols Asada (1999)].

Glutathione (GSH) is the major non-protein thiol source in plants and among its many functions it serves as one of the major scavengers of peroxides (May et al. 1998). It can act as an antioxidant by scavenging radicals, resulting in the oxidation of GSH to glutathione disulphide (GSSG). GSH is related to ascorbate (AsA) through the ascorbate–glutathione cycle (AsA–GSH cycle). The ascorbate–glutathione cycle is the most important antioxidant cycle in plants (Foyer and Noctor 2011). Higher ascorbate levels in uranium-exposed plants have been suggested to increase antioxidant defences via the ascorbate–glutathione pathway (Vanhoudt et al. 2008, 2011a, b, c). Tocopherols also have been described as important antioxidants (Cela et al. 2011). Tocopherols are lipophilic antioxidants that are part of the vitamin E group and are only synthesized in plants (Munné-Bosch 2005). Stress-tolerant plants show increased tocopherol levels, whereas in stress-sensitive plants tocopherol content decreases with consequent oxidative damage (Munné-Bosch and Alegre 2002). In cooperation with other antioxidants, tocopherol plays a part in reducing ROS levels (Munné-Bosch 2005). α-Tocopherol can physically quench, and therefore deactivate $^1O_2$ in chloroplasts. It has been estimated that before being degraded, one molecule of α-tocopherol can deactivate up to 120 $^1O_2$ molecules by resonance energy transfer (Fahrenholzt et al. 1974). In addition, α-tocopherol can chemically scavenge $^1O_2$ and lipid peroxyl radicals. Although previous studies (Munné-Bosch and Alegre 2002; Ricciarelli et al. 2001) reflect the relevance of tocopherols in oxidative stress regulation, up to the present time its role in plants exposed to HM has only been investigated under challenge with cadmium and copper (Collin et al. 2008).

The implications of oxidative stress in light capture and gas exchange parameters have received little attention in studies analysing the U effect on plant performance. However, this is a matter of major concern because photosynthesis represents a major energy sink, and because keeping control of photosynthetic rates is essential for plant development. Changes in C assimilation require modifications in the partitioning of the absorbed energy between heat dissipation and photochemistry. When photosynthesis decreases and the light excitation energy is in excess, over-excitation of the photosynthetic pigments in the antenna can occur. Impairment of photosynthetic function will lead to excessive excitation energy in photosystem II (PSII), leading to the accumulation of ROS, and resulting in oxidative stress (Chaves et al. 2009; Lawlor and Tezara 2009). Research has indicated that stomatal closure is involved in the inhibition of photosynthesis in plants exposed to different HM, such as Cr (Subrahmanyam 2008; Rodriguez et al. 2012) and Cd (López-Millán et al. 2009). However, even if those studies describe a reduction in stomatal conductance of between 25 and 60 %, the mechanisms leading to

regulation of water transport in plants exposed to HM have been poorly questioned.

The role of glutathione and tocopherol in the responsiveness of plants to U exposure has not been explored. Furthermore, the consequences of oxidative stress in photosynthetic performance remain to be elucidated in U-treated plants. The aim of this study was to characterize the role of these two major antioxidants (glutathione and tocopherol) in the response of *Arabidopsis thaliana* plants to U exposure. Furthermore, we also investigated the mechanisms leading to the limitations in water transport described in other HM studies by following hormone and antioxidant contents together with analyses of the expression of genes encoding aquaporins. To clarify these aspects, we examined the effect of U on Arabidopsis mutants with altered abilities in accumulating glutathione (*cad1.3, cad2.1*) (Howden et al. 1995) and tocopherol biosynthesis (*vte1*) (Porfirova et al. 2002) by completing a physiological and photosystem II chlorophyll fluorescence characterization.

Materials and methods

Experimental design

In this study, we worked with Arabidopsis wild type (Col-0, WT), together with tocopherol (*vte1*: null α/γ-tocopherol content and *vte4*: null α-tocopherol) (Porfirova et al. 2002) and glutathione (*cad1.3*: high content and *cad2.1* low content) mutants (Howden et al. 1995). The seeds were germinated by placing one seed per seed holder (Araponics S.A, Liège, Belgium) filled with agar solution (0.65 %). Plants were grown in hydroponically controlled pots of 350 ml. For each treatment combination, four pots containing our four plants per pot were grown. Nutrient solution was replaced every 2 days. The photoperiod was 10 h, with a photosynthetic photon flux density (PPFD) of ≈110 µmol photons m^{-2} s^{-1}. The day/night temperature was 22/20 °C and the relative humidity was maintained close to 65 %. The mineral solution was Hoagland's. The pH of the medium was checked and adjusted daily to 5.3–5.6. When plants were 50 days old, half of them were selected randomly and were placed into a new solution containing uranyl nitrate (50 µM) dissolved in milliQ water solution. The plants were grown in this media over 48 h. After U exposure, roots were first rinsed with 10 mM Na_2CO_3, then with water, and finally samples were stored at −80 °C. The experiment was repeated three times to improve the repeatability of obtained results.

Uranium quantification

U was determined in shoot (about 100 mg of fresh material) samples dried for one night at 80 °C and mineralized in 9 ml of 65 % (v/v) HNO_3 (Suprapur; Merck) and 3 ml of 30 % (v/v) HCl (Suprapur, Merck) at 125 °C. After complete evaporation of the mixture, residual material was dissolved in 1 % (v/v) HNO_3. The U concentration in the extract was then determined using ICP-MS (HP4500 ChemStation ICP-MS device; Yokogawa Analytical Systems) following ^{238} U.

Glutathione and ascorbate content and determination of oxidative damage to lipids

For determination of glutathione content and oxidative damage to lipids, aliquots of leaves were homogenized in sulfosalicilic acid 5 % (w/v) in an ice-cold Potter–Elvehjem homogenizer and centrifuged at 20,000*g*, 10 min, 0–4 °C. The supernatants were kept at −70 °C for subsequent determinations. Ascorbic acid (AsA) and dehydroascorbic acid (DHA), which were extracted with *m*-phosphoric acid (6 %) and 0.2 mM diethylenetriaminepentaacetic acid (DTPA), were determined using the ascorbate oxidase assay as described (Turcsányi et al. 2000). The oxidized state was calculated as AsA/(AsA + DHA) × 100.

One millilitre of supernatant was neutralized by 1.5 ml 0.5 M K-phosphate buffer (pH 7.5). The standard incubation medium was a mixture of: 100 µl 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 50 µl 6 mM 5,5-dithiobis-(-2-nitrobenzoic acid), 25 µl 2 mM NADPH, and 25 units of glutathione reductase (GR). The reaction was initiated by the addition of 25 µl glutathione standard or extract. The change in absorbance at 412 nm was recorded for 4 min. The calibration curve was made using oxidized glutathione (GSSG) in the range of 0–100 µmol.

Lipid peroxidation was estimated as the content of 2-thiobarbituric acid reactive substances (TBARS) and expressed as equivalents of malondialdehyde (MDA). The chromogen was formed by mixing 200 µl of supernatants with 1 ml of a reaction mixture containing 15 % (w/v) trichloroacetic acid (TCA), 0.375 % (w/v) 2-thiobarbituric acid (TBA), 0.1 % (w/v) butyl hydroxytoluene, and 0.25 N HCl, and by incubating the mixture at 100 °C for 30 min (Minotti and Aust 1987). After cooling to room temperature, tubes were centrifuged at 800*g* for 5 min and the supernatant was used for spectrophotometric reading at 532 and 600 nm. The calibration curve was made using MDA in the range of 0.1–10 µmol. A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 0.25 N HCl. In all cases, 0.1 % (w/v) butyl hydroxytoluene was included in the reaction mixtures to prevent artifactual formation of TBARS during the acid-heating step of the assay.

Gas exchange and chlorophyll fluorescence determinations

Fully expanded apical leaves were enclosed in a LI-COR 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). The gas exchange determinations were conducted at 25 °C with a photosynthetic photon flux density of 90 μmol m^{-2} s^{-1}. Photosynthetic activity was calculated using equations developed by von Caemmerer and Farquhar (1981). Stomatal conductance (g_s) was determined as described by (Harley et al. 1992). The electron transport rate (ETR) was measured as described by Harley et al. (1992).

Steady-state modulated chlorophyll fluorescence was determined with a portable fluorimeter (Mini-PAM; Walz, Effeltrich, Germany). Light-adapted components of chlorophyll fluorescence were measured: Steady-state fluorescence (F), maximal fluorescence (F'_m), variable fluorescence F'_v equivalent to ($F'_m - F$) and quantum yield of PS II photochemistry (ΦPSII) equivalent to ($F'_m - F$)/F'_m. Leaves were then dark adapted to obtain F_o (minimum fluorescence), F_m (maximum fluorescence), F_v variable fluorescence (equivalent to $F_m - F_o$) and F_v/F_m (maximum quantum yield of PSII photochemistry, equivalent to ($F_m - F_o$)/F_m). Leaves were dark adapted for at least 20 min, after which F_v/F_m values reach about 95 % of the pre-dawn values. F'_o was estimated as F_o/((F_{vc}/F_m) + (F_o/F'_m)). F'_o was used to calculate photochemical quenching (qP), photochemical quenching of fluorescence (equivalent to $F'_m - F$)/($F'_m - F'_o$)), and F'_v/F'_m, the intrinsic efficiency of open PSII centres during illumination (equivalent to ($F'_m - F'_o$)/F'_m). Three different leaves of each individual were measured. All data were corrected for changes in the fluorescence detector sensitivity induced by temperature variation of the Mini-Pam. Photochemical quenching was estimated as ($F'_m - F_s$)/F'_v. Non-photochemical quenching (NPQ) was calculated as ($F_m - F'_m$) − 1. The variable F'_v/F'_m was calculated and was an indicator of absorbed light dissipated thermally.

Pigment content

Extracts for pigment analysis were prepared by grinding 100 mg fresh weight in a cold mortar with 10 ml of ethanol (95 %, v/v). The homogenate was centrifuged at 3,165*g* for 10 min at 4 °C. An aliquot of 1 ml from the supernatant was taken, 4 ml of 95 % ethanol were added, and the absorbance measured at 750, 665, 649 and 470 nm. Absorbance determinations were carried out with a Spectronic 2000 (Bausch and Lomb, Rochester, USA) spectrophotometer. Chlorophyll *a*, *b*, total and carotenoid (Chl_a, Chl_b, Chl_{a+b} and C_{x+c}, respectively) contents were determined according to Liechenthaler (Lichtenthaler 1987). In brief, $Chl_a = 13.36 \times A_{665} - 5.19 \times A_{649}$; $Chl_b = 27.43 \times A_{649} - 8.12 \times A_{665}$, $Chl_{a+b} = 5.24 \times A_{664} + 22.24 \times A_{649}$, $C_{x+c} = (1{,}000 \times A_{470} - 2.13\ Chl_a - 97.46\ Chl_b)/198$.

Hormones and tocopherols

Phytohormones, including ABA, JA and SA were measured by UPLC-MS/MS as described (Müller and Munné-Bosch 2011). Deuterium-labelled hormones were used to estimate recovery rates for each sample. Tocopherols were measured by HPLC as described by Cela et al. (2011).

Expression of plasma membrane intrinsic proteins isoforms (*PIP1.1; PIP1.2; PIP2.1; PIP2.5* and *PIP2.6*)

RNA isolation

Total RNA was isolated from *Arabidopsis* leaves by phenol/chloroform extraction (Kay et al. 1987). DNase treatment of total RNA was performed according to Qiagen's protocol (Quantitect Reverse Transcription KIT Cat#205311, Qiagen, CA).

Quantitative real-time RT-PCR

The expression of each *plasma membrane intrinsic protein* (*PIP*) gene was studied by real-time PCR using an iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 2.5 μg of total DNase-treated RNA in a 20 μl reaction containing oligo$(dT)_{15}$ primer (Promega, Madison, WI), 10 mM dNTP (Invitrogen, Carlsbad, California, USA), 0.1 M DTT (Invitrogen, Carlsbad, CA, USA), 40 U of RNase inhibitor (Promega, Madison, WI), 5X first strand buffer (Invitrogen) and 200 U of Superscript II Reverse Transcriptase (Invitrogen) with the temperature recommended by the enzyme supplier. The primer sets used, including that of reference gene *actin*, to amplify each studied gene in the synthesized cDNAs are shown in supplemental Table 1.

Each 23 μl reaction contained 3 μl of a 1:10 dilution of the cDNA, 10.5 μl of Master Mix (Bio-Rad Laboratories S.A, Madrid), 8.6 μl of deionised water and 0.45 μl of each primer pair. The PCR programme consisted of a 3-min incubation at 95 °C to activate the hot-start recombinant Taq DNA polymerase, followed by 31 cycles of 30 s at 94 °C, 30 s at the established annealing temperature and 30 s at 72 °C, where the fluorescence signal was measured, followed by 1 cycle of 1 min at 95 °C, 1 min at 70 °C and 60 cycles of 10 s at 70 °C. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70 to 100 °C) after the final cycle of the PCR.

Real-time PCR experiments were carried out four times with biological independent samples, with the threshold cycle (C_T) determined in triplicate. The relative levels

Table 1 Uranium (U) exposure (50 μM) effect on leaf net photosynthesis (A_n), electron transport rate (ETR) and the ETR/A_n ratio of *Arabidopsis thaliana* wild type (WT, ecotype Columbia), tocopherol (null α/γ-tocopherol: *vte1* and *vte4*: null in α-tocopherol) and glutathione (high content: *cad1.3* and low content: *cad2.1*) mutants

Line	Treatment	A_n (μmol CO_2 $m^{-2}s^{-1}$)	ETR (μmol e^{-2} s^{-1})	ETR/A_n (μmol CO_2 μmol e^{-2})
WT	Control	9.63 ± 0.77 a	176.61 ± 8.21 a	18.63 ± 2.01 f
	U	0.08 ± 0.24 e	191.33 ± 12.59 a	700.48 ± 166.54 a
Vte 1	Control	8.28 ± 0.46 ab	175.89 ± 5.11 a	20.80 ± 1.74 f
	U	0.95 ± 0.14 e	155.55 ± 4.87 ab	122.69 ± 35.76 d
Vte 4	Control	6.71 ± 0.07 bc	175.42 ± 9.27 a	26.77 ± 1.50 f
	U	0.46 ± 0.06 e	148.24 ± 5.28 b	334.61 ± 68.74 bc
Cad 1.3	Control	5.65 ± 0.69 c	181.31 ± 4.69 a	32.2 ± 6.25 f
	U	1.32 ± 0.78 e	157.75 ± 10.77 ab	251.18 ± 95.03 c
Cad 2.1	Control	3.5 ± 0.25 d	188,03 ± 3.10 a	58.57 ± 3.18 e
	U	0.43 ± 0.08 e	169.06 ± 5.12 ab	393.37 ± 4.45 b

Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

of transcription were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), using actin gene as reference with CT variation among treatments <1. All the primers used presented efficiencies close to 1.9 calculated as described Pfaffl 2001). Negative controls without cDNA were used in all PCR reactions.

Statistic analyses

Data were processed by two-way analysis of variance (ANOVA) to test the effects of U and mutation using the statistical software package, SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The results were accepted as significant at $P < 0.05$. When differences between treatments were significant according to the ANOVA analysis, least significant differences (LSD) were evaluated using Fisher's LSD test ($P < 0.05$).

Results

Shoot uranium content

The amounts of U present in the shoots showed that the U content was quite low. Interestingly, the lowest U values were observed in the WT and the *cad2.1* mutant (7.7 and 4.4 μg g^{-1} of dry matter, respectively).

Antioxidant contents in Arabidopsis mutants affected in glutathione and α-tocopherol biosynthesis

Antioxidant molecule quantifications including α- and γ-tocopherol, glutathione, ascorbate and dehydroascorbate were performed in leaves of the different Arabidopsis mutants and in the WT used in this study (Fig. 1). In accordance with the literature, our analyses confirmed that: (1) no α-tocopherol was detected in *vte1* and *vte4* plants and no γ-tocopherol was detected in *vte1* plants (Porfirova et al. 2002); and (2) the *cad2.1* mutant deficient in glutathione synthetase contained less glutathione than the wild type (Howden et al. 1995), whereas an increase in glutathione content was observed in the mutant affected in phytochelatin synthase (*cad1.3*) (Howden et al. 1995). Under non-stressed conditions, the *cad1.3* mutant had the largest α-tocopherol content, indicating that an increase in glutathione content is accompanied by an increase in α-tocopherol. Interestingly, the *vte4* mutant that contained no α-tocopherol is the mutant that contains less glutathione, suggesting a link between the biosynthesis of these two molecules. U affected α-tocopherol content negatively in the WT and the *cad* mutants. Concerning γ-tocopherol characterization, the largest values were observed in the *vte4* mutant followed by the WT. The lowest γ-tocopherol levels were observed in the *cad1.3* and *cad2.1* mutants (Fig. 1). U diminished the γ-tocopherol content in the WT. On the other hand, *vte1* mutants had the lowest ascorbate content in control conditions. The treatment with uranyl nitrate provoked a strong decrease in ascorbate content, regardless of the plant analysed (Fig. 1), whereas no significant differences were detected in dehydroascorbate (DHA) contents between the different lines and treatments. The ascorbate oxidized state was dramatically low in the U-treated plants (Fig. 1). *vte1* and *cad1.3* (as mentioned before) were the lines with the largest glutathione content in control conditions, followed by the WT and *cad2.1*. Glutathione availability was not significantly affected by U in the WT, whereas its content decreased in the *vte1*, *cad1.3* and *cad2.1* mutants, and surprisingly increased in the *vte4* mutant.

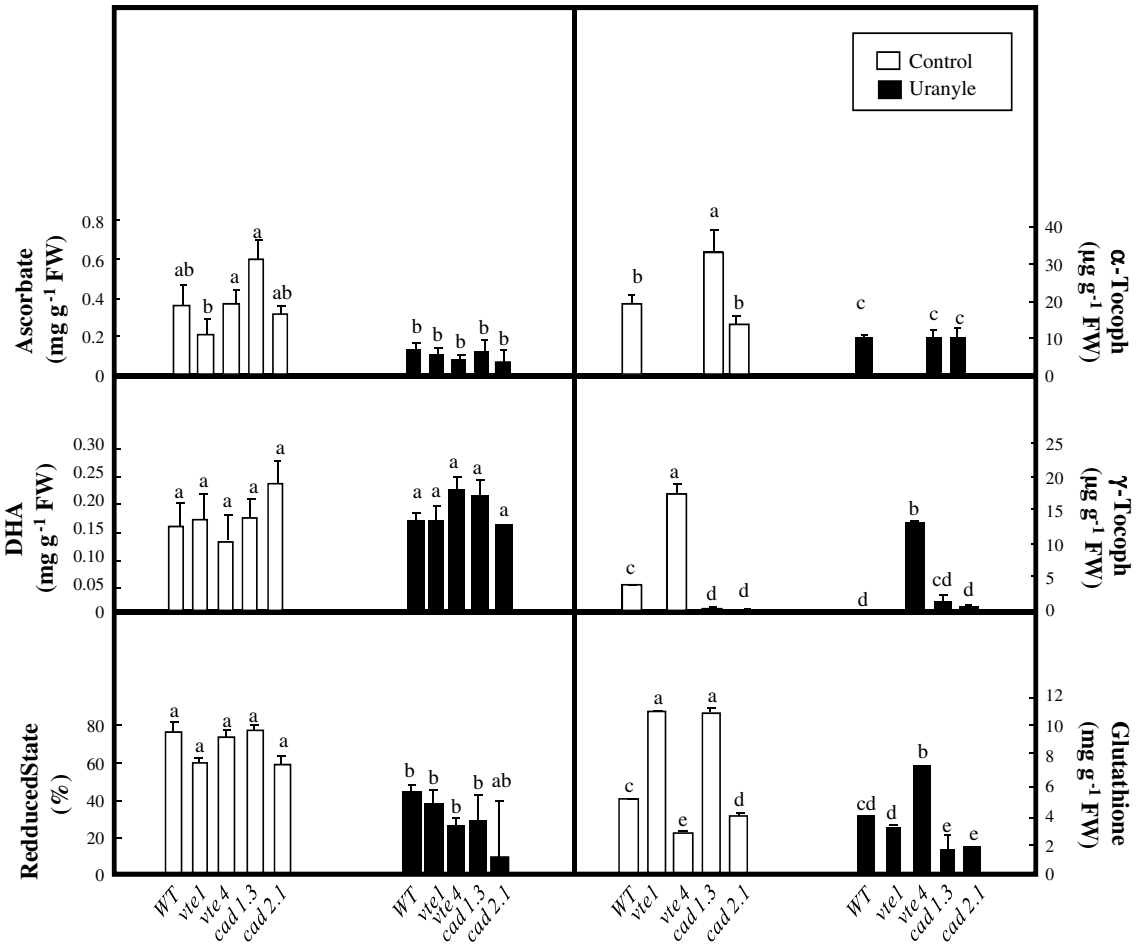

Fig. 1 Uranium exposure (48 h to 50 µM uranyl nitrate) effect on ascorbate, dehydroascorbate (DHA), ascorbate reduced state, α-tocopherol, γ-tocopherol and glutathione of *Arabidopsis thaliana* wild type (WT, ecotype Columbia) and Arabidopsis mutants affected in tocopherol biosynthesis (no α/γ-tocopherol: *vte1* and *vte4*: no α-tocopherol) and in glutathione (high content: *cad1.3* and low content: *cad2.1*). Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

Malondialdehyde (MDA), a marker of lipid peroxidation, was also quantified in the different lines stressed or not stressed with U. Figure 2 shows that no significant differences were detected in plant grown under control conditions, whereas an increase in lipid peroxidation was observed in U-treated plants, especially in *cad2.*1. In contrast, in *cad1.3* the phenomenon was less pronounced.

Gas exchange, chlorophyll fluorescence and pigment content parameters

Leaf photosynthesis was followed during the experiment so as to check whether the exposure to applied U did cause a physiological response. The main parameters are shown in Table 1. Regardless of the lines analysed, after 48 h of exposure to 50 µM uranyl nitrate, U strongly inhibited the photosynthetic rates (A_n) of plants. With the exception of *vte4* (where its values decreased), ETR was not affected by U (Table 1). ETR/A_n ratio increased in plants exposed to U, being the WT (followed by the *cad2.1*) the plants were the largest values were observed. Although the lower stomatal conductance (g_s) measured in the presence of U indicates stomatal closure and suggests that a lower CO_2 concentration could be the cause of photosynthetic inhibition in U-treated plants, the measured accumulation of intercellular CO_2 concentration (C_i) (Table 2) refuted such a hypothesis. Nevertheless, stomatal closure was confirmed by the diminished ^{13}C discrimination ($\Delta^{13}C$) observed in plants exposed to U and by the decrease in leaf transpiration (T_r) measured in stressed plants (Table 2).

As shown in Fig. 3, even in the case of control conditions, chlorophyll fluorescence data did not reveal significant differences among Arabidopsis lines. Exposure to U affected the light capture and processing machinery in a

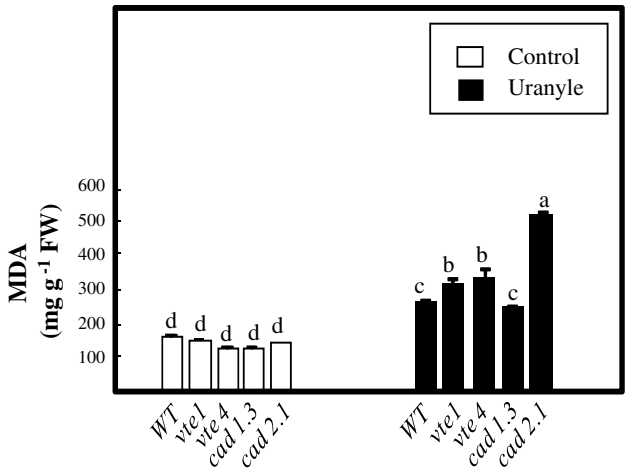

Fig. 2 Uranium exposure (48 h to 50 μM uranyl nitrate) effect on malondialdehyde (MDA), of *Arabidopsis thaliana* wild type (WT, ecotype Columbia) and Arabidopsis mutants affected in tocopherol biosynthesis (no α/γ-tocopherol: *vte1* and *vte4*: no α-tocopherol) and in glutathione (high content: *cad1.3* and low content: *cad2.1*). Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

different manner. Although the maximum quantum yield of the PSII photochemistry (F_v/F_m) and photochemical PSII efficiency (Φ_{PSII}) were not affected in the WT, exposure to U affected this parameter negatively in *vte1*, *vte4* and *cad2.1* (Fig. 3). In *cad1.3* while Φ_{PSII} was not altered by U, F_v/F_m was negatively affected by the exposure to the heavy metal. Even though no significant differences were observed in the WT, in all the Arabidopsis mutants, Fv'/Fm' decreased in stressed plants. No significant differences were also observed for photochemical quenching (qP) (Fig. 3). No significant differences were detected in the non-photochemical quenching (NPQ) between plants and treatments. Exposure to U had no significant effect on chlorophyll content (Chl_{a+b}), carotenoids (Car) and Car/Chl (Fig. 4). Chl_a/Chl_b diminished in *vte1*, *vte4* and *cad2.1*, whereas in the WT and *cad1.3* no significant differences were detected.

Plasma membrane intrinsic proteins (PIP) gene expression

The expression of several PIP member genes analysed by quantitative real-time RT-PCR (Fig. 5) revealed that in all cases the *cad1.3* line had the largest *PIP* expression levels, whereas the *vte1* line and especially the WT had the lowest levels under control conditions. When plants were exposed to U the gene expression of *PIP1.1*, was not affected in the WT and *vte1* mutants, while it increased in the *vte4* and *cad1.3* lines. In the case of *cad2.1*, *PIP1.1* was up-regulated (Fig. 5). With respect to *PIP1.2*, its expression increased in the WT exposed to U and no significant effect was detected in the *vte1* and *cad2.1* lines. In the other mutants such as the *vte4* and *cad1.3*, *PIP1.2* was down-regulated. *PIP2.1* expression was also down-regulated in *vte4* and *cad1.3* stressed plants, but no significant effect was detected in WT, *vte1* and *cad2.1*. Figure 5 also shows that while the expression of gene *PIP2.5* was not significantly affected by U in WT and *cad2.1* (where no differences were observed), the expression of this gene was down-regulated in the other lines. Finally, regarding the expression of *PIP2.6*, two tendencies were detected. In *cad2.1* the expression of this gene was up-regulated by U exposure. In *vte4* and *cad1.3* U decreased the expression of this plasma membrane protein.

Table 2 Uranium (U) exposure (50 μM) effect on leaf transpiration (T_r), stomatal conductance (g_s, mol CO_2 $m^{-2}s^{-1}$), intercellular [CO_2] concentration (C_i, μmol CO_2 mol^{-1}air) and ^{13}C isotopic discrimination ($\Delta^{13}C$, ‰) of *Arabidopsis thaliana* wild type (WT, ecotype Columbia), tocopherol (null α/γ-tocopherol: *vte1* and *vte4*: null in α-tocopherol) and glutathione (high content: *cad1.3* and low content: *cad2.1*) mutants

Line	Treatment	T_r	g_s	C_i	$\Delta^{13}C$
WT	Control	1.48 ± 0.55 a	666.50 ± 114.91 a	246.00 ± 48.06 c	14.88 ± 0.10 a
	U	0.40 ± 0.14 bc	187.25 ± 33.18 d	427.75 ± 70.03 b	12.62 ± 0.33 b
vte1	Control	1.98 ± 0.58 a	681.66 ± 50.71 a	244.00 ± 62.96 c	14.89 ± 0.20 a
	U	0.76 ± 0.09 bc	333.01 ± 21.86 bc	340.75 ± 8.46 bc	12.51 ± 0.44 b
vte4	Control	1.35 ± 0.61 a	559.50 ± 31.21 abc	266.51 ± 23.44 c	17.09 ± 1.50 a
	U	0.69 ± 0.20 ab	296.75 ± 43.51 cd	353.75 ± 32.29 bc	13.76 ± 0.13 b
cad1.3	Control	1.07 ± 0.39 a	608.25 ± 26.38 abc	230.71 ± 47.76 c	14.54 ± 0.14 ab
	U	0.69 ± 0.28 bc	354.80 ± 83.81 cd	376.55 ± 36.84 bc	12.53 ± 0.70 b
cad 2.1	Control	1.13 ± 0.43 a	587.53 ± 69.5 abc	262.75 ± 10.40 c	14.45 ± 0.03 ab
	U	0.22 ± 0.09 c	92.57 ± 17.82 d	667.75 ± 153.77 a	12.81 ± 0.32 b

Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

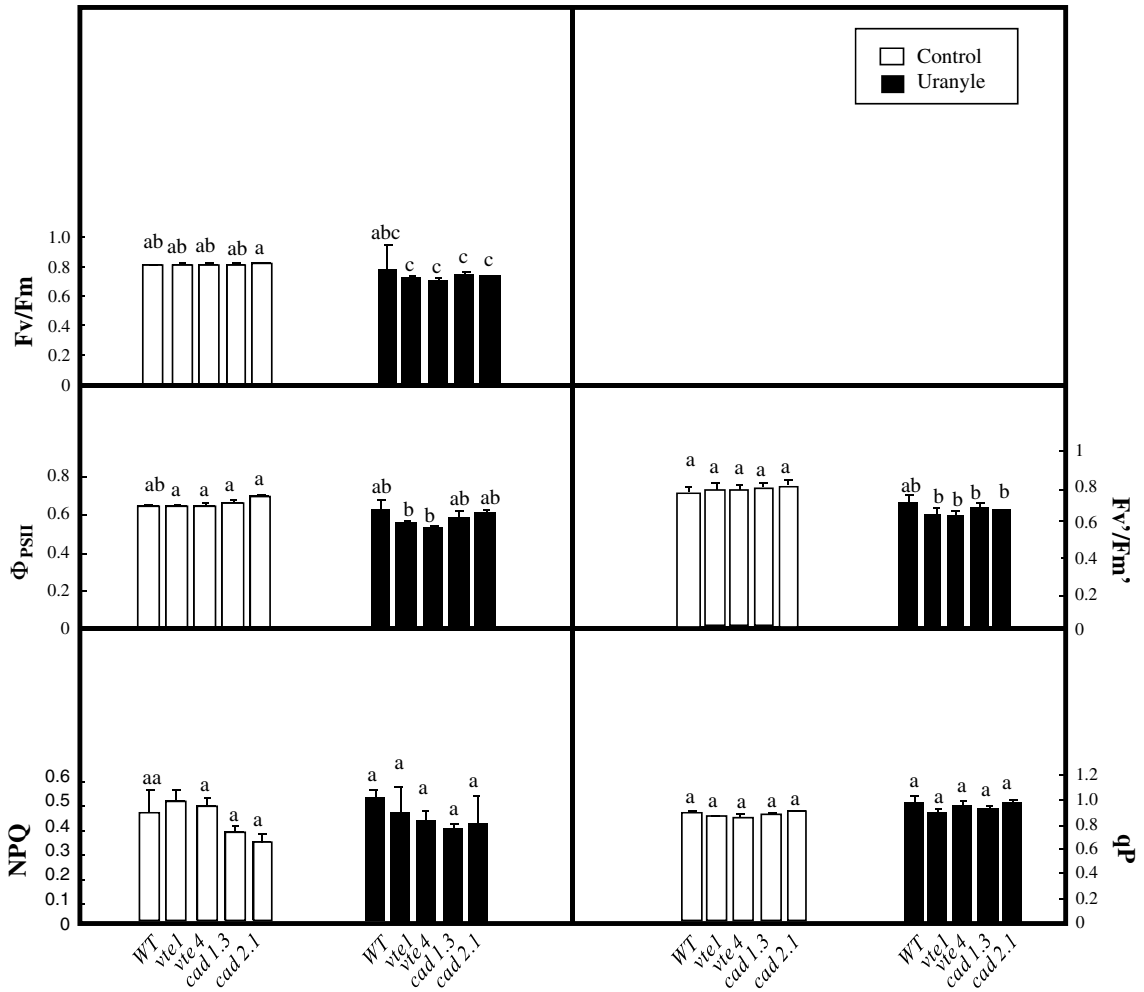

Fig. 3 Uranium exposure (48 h to 50 μM uranyl nitrate) effect on maximum quantum yield of PSII photochemistry (F_v/F_m), quantum yield of PS II photochemistry (ΦPSII), non-photochemical quenching (NPQ), the intrinsic efficiency of open PSII centres during illumination (F'_v/F'_m) and photochemical quenching (qP) of *Arabidopsis thaliana* wild type (WT, ecotype Columbia), tocopherol (*vte1* and *vte4*) and glutathione (high content: cad1.3 and low content: cad2.1) mutants. Each value represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

Hormone content

ABA content was not significantly affected by U, with the exception of the *cad2.1* line which presented the highest ABA content under control conditions. As shown in Fig. 6, jasmonate (JA) and salicylic acid (SA) contents were not the same, but were comparable among the plant lines and were not significantly affected by U.

Discussion

In the current study, we characterised the effect of U exposure on the oxidative state of *A. thaliana* plants and the role of two major antioxidants (glutathione and tocopherol) in maintaining oxidative equilibrium. U can be found in immobile and mobile forms, so to ensure that uranyl ions are more available for the plants, we decided to apply U in a water solution (Doustaly et al. unpublished data). To characterize the role of glutathione and tocopherol in the oxidative state of Arabidopsis leaves, in addition to the determination of the main enzymatic and non-enzymatic antioxidants, we worked with mutant Arabidopsis plants with altered tocopherol (*vte1*: null α/γ-tocopherol content and *vte4*: null in α-tocopherol) (Porfirova et al. 2002) and glutathione (*cad1.3*: high content and *cad2.1* low content) (Howden et al. 1995) contents.

Imbalanced light absorption and photochemistry requirements induce oxidative stress

As mentioned previously, the maintenance of equilibrium between light capture and photochemistry requirements is

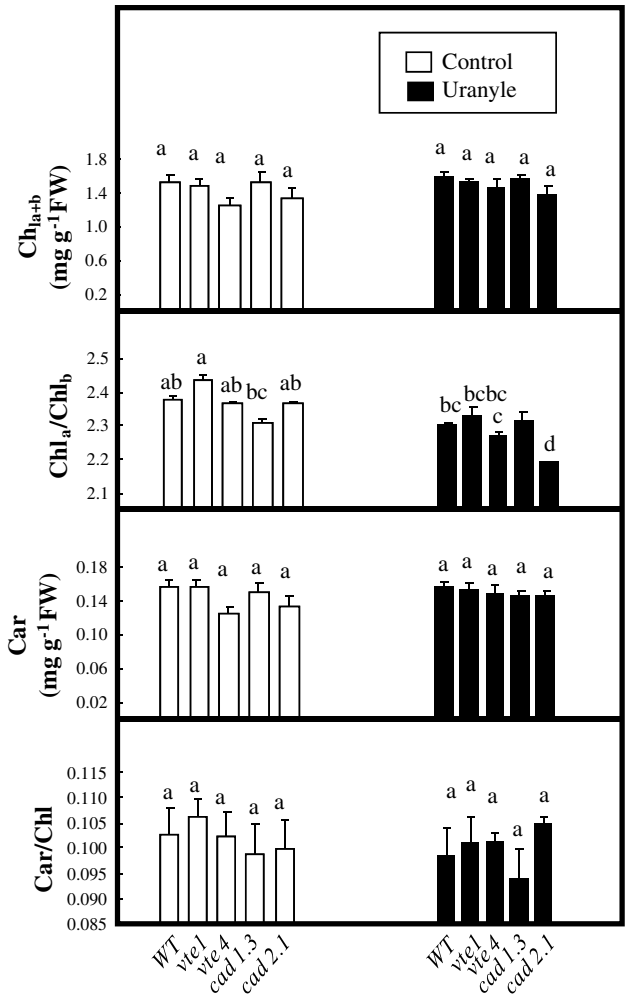

Fig. 4 Uranium exposure (48 h to 50 μM uranyl nitrate) effect on chlorophyll *a* and *b* (Chl_{a+b}), chlorophyll *a* and *b* ratio (Chl_a/Chl_b), carotenoid (Car) and carotenoid chlorophyll ratio (Car/Chl) of *Arabidopsis thaliana* wild type (WT, ecotype Columbia), tocopherol (*vte1* and *vte4*) and glutathione (high content: *cad1.3* and low content: *cad2.1*) mutants. Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

a key point for the avoidance of ROS (Niinemets and Kull 2001). The dramatic decrease in photosynthetic activity (A_n, a major electron sink) in U-treated plants suggests that, as observed in the case of exposure to other heavy metals (López-Millán et al. 2009), photosynthetic machinery was a primary target of high U. Although photosynthetic activity has been previously described in plants exposed to Cd (López-Millán et al. 2009) and Cr (Subrahmanyam 2008), to our knowledge this is the first time that gas exchange characterization (together with the above mentioned chlorophyll fluorescence determinations) has been conducted in plants exposed to U. Our data showed that the strong inhibition of photosynthetic activity in U-treated plants implied the inhibition of a major electron sink. The large ETR/A_n ratio detected in plants exposed to U suggests that those plants were subjected to an increase in the proton gradient, with the consequent susceptibility to generate ROS. Depleted F_v/F_m and Φ_{PSII} confirmed that U induces an oxidative stress in Arabidopsis leaves like other HM such as Cd (Clemens 2006; López-Millán et al. 2009; Villiers et al. 2012; Vanhoudt et al. 2008, 2011a, b, c). However, our study revealed that U exposure-derived PSII damage was focused in the tocopherol (*vte1* and *vte4*) and glutathione (*cad1.3* and *cad2.1*) mutants, with no significant effect in the WT. Our finding is supported by previous studies (Vanhoudt et al. 2008, 2011a, b, c) conducted with *Arabidopsis* where the cellular redox balance was strongly disrupted during U stress, but at relatively high concentration (100 μM). The present study supports the fact that the inhibition of a major electron sink (as ETR/A_n suggest) would have been involved in the PSII damage. The presence of the same chlorophyll content in all the plants meant that the concept of reductions in light absorbance as a protection mechanism against photoinhibition could be discarded (Galmés et al. 2007). On the other hand, the absence of significant differences in qP and NPQ highlighted the fact that Φ_{PSII} diminished as a consequence of the diminished F'_v/F'_m.

Leaf stomatal conductance (g_s) and transpiration (T_r) in U-treated plants indicated the stomatal closure induced by U. Carbon isotope discrimination ($\Delta^{13}C$) has been widely described as integrating stomatal opening, transpiration efficiency and the ratio of net photosynthesis to water transpired (Araus et al. 2003). The lower $\Delta^{13}C$ detected in U-treated plants confirmed the stomatal closure of those plants. Although stomatal conductance (g_s) decreased in U plants, the larger intercellular CO_2 content (C_i) discarded CO_2 limitations as being the cause of photosynthetic inhibition. Our data suggest that the photosynthetic inhibition of U-treated plants was related to the inhibition of the Bassham, Benson and Calvin (BBC) cycle, maybe at the level of Rubisco activity. Experiments need to be performed to confirm that Rusbisco is a primary enzyme target of the BBC cycle.

Uranium exposure effect in water and U transport

Although HM-induced stomatal closure has been previously described (López-Millán et al. 2009; Subrahmanyam 2008) information related to the involvement of HM in water transport of plants is scarce. HM are taken up by root cells and transported through xylem towards the stem and leaves. Transpiration (T_r), which is the dominant process that controls water transport in plants, has been

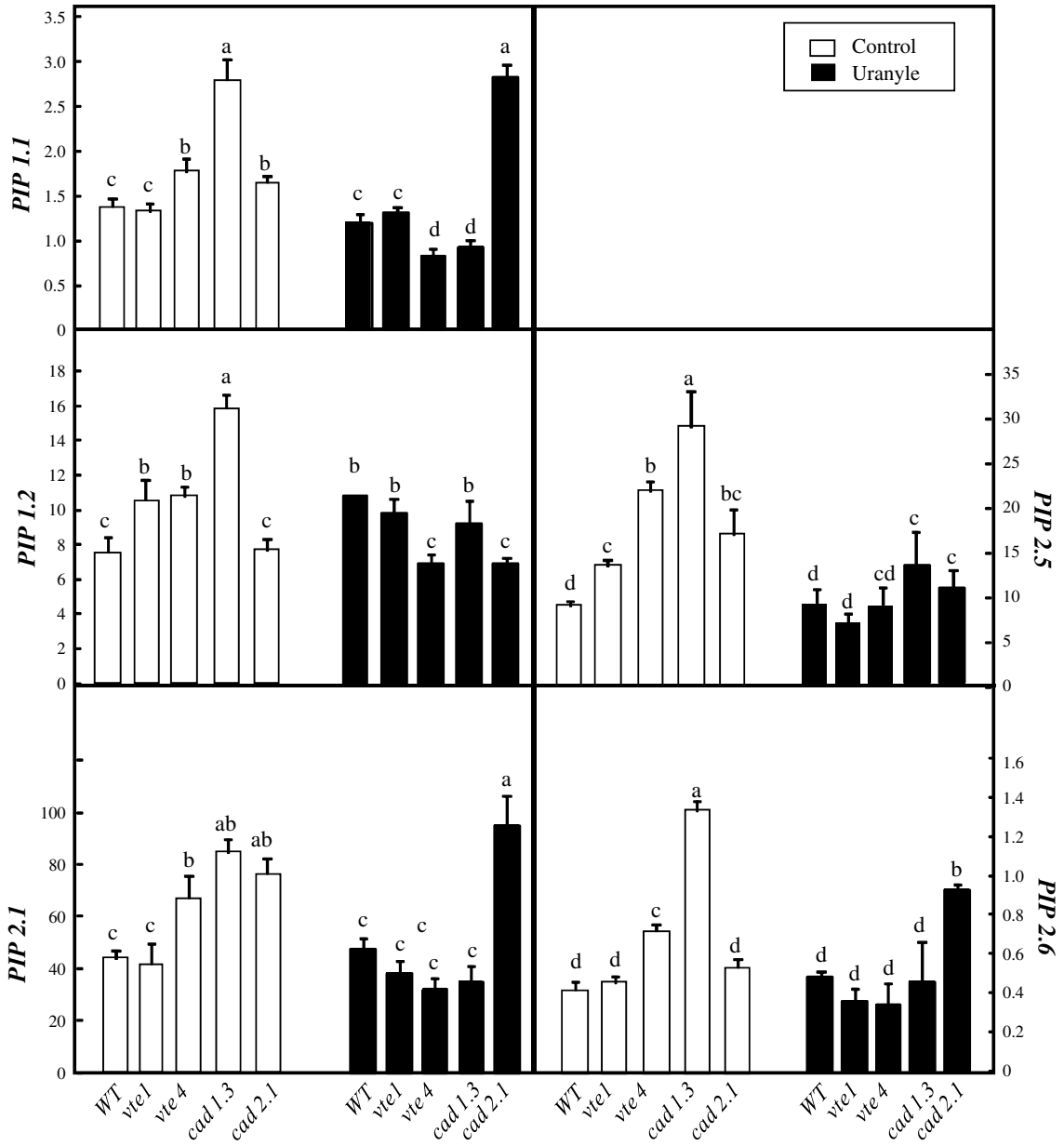

Fig. 5 Uranium exposure (48 h to 50 μM) effect on the expression of plasma membrane intrinsic proteins isoforms (PIP1.1; PIP1.2; PIP2.1; PIP2.5 and PIP2.6) of *Arabidopsis thaliana* wild type (WT, ecotype Columbia), tocopherol (null α/γ-tocopherol: *vte1* and *vte4*: null in α-tocopherol) and glutathione (high content: *cad1.3* and low content: *cad2.1*) mutants. Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P < 0.05$) were followed by a different letter according to the LSD test parameters

described as a key parameter conditioning HM transport in plants (Bartoli et al. 2012; Claus et al. 2013). The fact that in plants where T_r was lower (*cad2.1* and WT), the U content was also lower, might be explained by the fact that lower transpiration rates were linked to lower absorption and translocation of HM towards shoots (Renkema et al. 2012). This hypothesis was also supported by the observation that in *vte1* (that showed the highest transpiration rates) the highest leaf U levels were detected. Even when photosynthesis is diminished by stomatal closure, water needs to circulate between the phloem and xylem (Morillon and Chrispeels 2001). Furthermore, when T_r decreases, cell-to-cell water movement has been described as being stimulated (Aroca et al. 2012; Morillon and Chrispeels 2001). Aquaporins (AQP) are central components in plant–water relations at all levels of organization (cell, tissue,

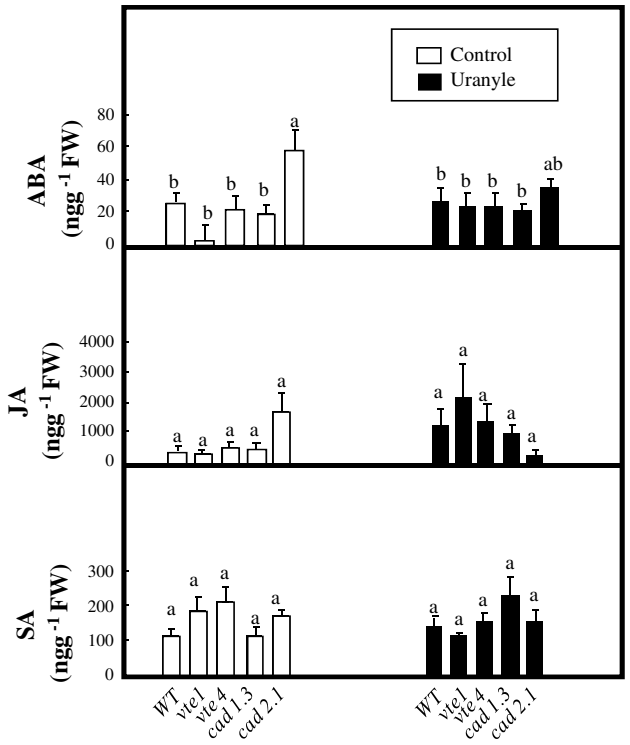

Fig. 6 Uranium exposure (48 h to 50 μM) effect on abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) of *Arabidopsis thaliana* wild type (WT, ecotype Columbia), tocopherol (null α/γ-tocopherol: *vte1* and *vte4*: null in α-tocopherol) and glutathione (high content: *cad1.3* and low content: *cad2.1*) mutants. Each *value* represents the mean of 4 replicates ± SE. Statistical analysis was made by a two-factor analysis of the variance (ANOVA). When significant differences were detected in ANOVA, LSD analysis was applied. Means that differed significantly ($P > 0.05$) were followed by a different letter according to the LSD test parameters

organ and whole plant). It is now widely known that most (75–95 %) water transport is mediated by aquaporins (Maurel 1997). Therefore, the open/closed state of AQP and their regulation is essential in maintaining cell water balance and the adjustment of plant–water relations. In our case, the responsiveness of several PIP aquaporins varied depending on the lines analysed. In v*te1*, *vte4* and *cad1.3*, PIP transcript content decreased, whereas in the WT no significant effect was observed and in the *cad2.1* line its expression increased. HM-induced inhibition of aquaporin content has been previously described in plants exposed to Cd (Yamaguchi et al. 2010), Cu, Hg, Pb, Zn (Przedpelska-Wasowicz and Wierzbicka 2011). The inhibition in transcription of PIP aquaporin observed in U-treated plants would contribute to the above-mentioned restriction of U assimilation and transport in *vte1*, *vte4* and *cad1.3*. In this sense, it is remarkable that *PIP* transcription in the WT was least affected by U exposure. As observed by (Sakurai-Ishikawa et al. 2011), the sensitivity of *PIP* expression to T_r rates differs depending on the AQP analysed. In agreement with Sakurai-Ishikawa et al. (2011), our study showed that *PIP2.5* was the most sensitive to changes in T_r. Finally, we would also like to highlight that the increase in *PIP1.1* and *PIP2.6* observed in the plants (*cad2.1*) where the T_r in U-treated conditions was the lowest and the intercellular CO_2 concentration the highest. Such data suggest the idea that that as described for NtAQP1 aquaporin (Flexas et al. 2006), PIP1.1 and PIP2.6 improved CO_2 transport instead of H_2O transport in plants exposed to U.

Previous studies conducted with Cd showed that HM affect ABA content and root permeability to water (Przedpelska-Wasowicz and Wierzbicka 2011). However, in our case, ABA content was only decreased in *cad2.1*, which was the line with the greatest reduction in stomatal conductance. Regulation of stomatal opening through ABA has been linked to the role of this hormone in H_2O_2 production (Zhang et al. 2001). Although not significant, such tendency was only observed in the *cad2.1* mutants. It is also worth noting that H_2O_2 is also conditioned by ascorbate content. Indeed, our data match other studies (Chen and Gallie 2004) in suggesting that the lower leaf ascorbate content of stressed plants could reflect a higher H_2O_2 content that leads to stomatal closure and transpiration decrease.

Glutathione and tocopherol in oxidative state maintenance

The fact that the WT was the only *Arabidopsis* line that kept control levels of glutathione and ascorbate under U-exposed conditions, underscores the relevance of these antioxidants as target points in maintaining oxidative status. Concerning the glutathione mutants, although *cad1.3* showed higher glutathione content in control conditions, the fact that it was not capable of maintaining these values under U exposure explained the oxidative stress of these plants. Moreover, the fact that the glutathione deficient mutant (*cad2.1*) had the highest MDA and least reduced (although no statistically significant) levels of ascorbate, highlighted the role of this antioxidant in oxidative stress avoidance. As observed by Viehweger et al. (2011), keeping high levels of reduced glutathione (GSH) levels is very important to avoid oxidative stress. The authors showed that, whereas exposure to 10 μM of U did not affect GSH content (no being oxidative stress), when the plants were exposed to 50 μM of U, high GSH levels were detected to probably support the leaf ROS detoxification processes (Viehweger et al. (2011).

Even though tocopherols have been described as playing a role as ROS scavengers and quenchers (Munné-Bosch 2005), to our knowledge the relevance of these antioxidants has not been tested in U-stressed plants. It is remarkable that the lack of tocopherols in the *vte1* line was not reflected in a worse PSII status (compared to *cad1.3*

and *cad2.1*). *vte1* mutants were capable of maintaining high glutathione content, but similar to the observations in *cad1.3*, U exposure strongly decreased glutathione levels. Although *vte1* mutants lack both α/γ-tocopherol and α-tocopherol, these mutants accumulate plastoquinol, an important lipid-soluble antioxidant, which in addition to tocopherols protects chloroplasts from oxidative stress (Szymanska and Kruk 2010). It is therefore likely that plastoquinol accumulation substituted the lack of tocochromanol in *vte1*. Responsiveness to U was similar in γ-tocopherol mutants (*vte4*). Furthermore, although these mutants lack α-tocopherol, they have been described as accumulating γ-tocopherol in leaves (Mène-Saffrané et al. 2010). The fact that γ-tocopherol might be as efficient as α-tocopherol (Cela et al. 2011) suggests that a large amount of γ-tocopherol could have helped overcome α-tocopherol deficiency (Falk and Munné-Bosch 2010) in U-treated plants. Stress-related phytohormones such as abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) have been implicated in oxidative stress avoidance (Larkindale and Knight 2002). Moreover, tocopherol content has been described as being conditioned by these hormones. In rice, several genes related to tocopherol biosynthesis contain ABRE elements (related with ABA response) in their promoter regions (Chaudhary and Khurana 2009) and in *Cistus creticus,* both ABA and α-tocopherol biosynthetic pathways have a similar response to water stress (Munné-Bosch et al. 2009). It was suggested that JA levels can be regulated by α-tocopherol levels (Munné-Bosch 2007; Munné-Bosch et al. 2009), and SA treatments during drought periods improve the levels of α-tocopherol, ascorbic acid and glutathione in *Ctenanthe setosa* (Kadioglu et al. 2011). The absence of a U effect on ABA, JA and SA reveals that the general depletion of α-tocopherol could have been linked to the lower ascorbate content of U-treated plants. It must be mentioned that the quantification of the signalling hormones was performed after a 48 h exposure. Although the current experimental design cannot confirm it, it could be possible that hormone responsiveness to U, after 48 h, was different from that during the initial hours of exposure (Maksymiec et al. 2007).

However, other mechanisms that may operate in these mutants cannot be excluded. A recent study conducted by Vanhoudt et al. (2011a) in *Arabidopsis* plants exposed to U revealed that H_2O_2 detoxification was tightly linked to ascorbate content. Although ascorbate content was maintained under control levels in U-treated WT, *vte1* and *cad2.1* plants, the fact that photoinhibition was only avoided in the WT revealed that the availability of this antioxidant, together with the ascorbate/dehydroascorbate redox balance, was not by itself enough to avoid photo-oxidative damage.

Conclusion

This study revealed the relevance of maintaining a regulated electron sink/source balance in *Arabidopsis* for the correct functioning of PSII in plants exposed to U. The WT plants were the only ones capable of overcoming photo-oxidative stress. The fact that the WT was the only line where PSII was not damaged shows the importance of keeping in control glutathione and malondialdehyde contents for the overcoming of photoinhibition in the WT. Interestingly, our data also suggest that lower transpiration rates, together with the down-regulation of aquaporin expression, were linked to U absorption and translocation. Stomatal closure decreased leaf transpiration and consequently reduced U accumulation in leaves with the consequent effect in shoot oxidative stress.

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