**Title Page**

**Title:** Possible role of HMA4a TILLING mutants of *Brassica rapa* in cadmium phytoremediation programs

**Author names and affiliations:** Eloy Navarro-León\*†, Jhonnatan Oviedo-Silva†, Juan Manuel Ruiz and Begoña Blasco

†E. Navarro-León and J. Oviedo-Silva have contributed equally, so should be considered as joint first authors

Department of Plant Physiology, Faculty of Sciences, University of Granada, 18071 Granada, Spain

**E-mail address of the corresponding author (\*):** enleon@ugr.es

**Telephone number:** + 00 34 958 243255

**E-mail addresses:**

**E. Navarro-León:** enleon@ugr.es

**J. Oviedo-Silva:** jhoviedo@correo.ugr.es

**J.M. Ruiz:** jmrs@ugr.es

**B. Blasco:** bblasco@ugr.es

**Abstract**

Cadmium (Cd) is a dangerous transition element that causes environmental and health problems due to its high mobility in the soil-plant system. In plants, Cd causes serious alterations in physiological processes, affecting different vital functions such as photosynthesis. Species such as *Brassica juncea* and *Brassica rapa* have been selected as suitable plants for phytoremediation purposes due to their ability to tolerate the toxic effect of heavy metals. In order to improve this strategy, techniques of plant mutagenesis such as TILLING (Targeting Induced Local Lessions in Genomes) have been employed. In the present work we studied the role of the *HMA4* gene in the tolerance to Cd toxicity (100 μM CdCl2) using a TILLING mutant of *B. rapa* (*BraA.hma4a-3*). These mutant plants presented a lower biomass reduction and a higher Cd concentration in leaves. An increase in the GSH / GSSG ratio, in the content of photosynthetic pigments and a reduction of oxidative stress was observed, as well as a better photosynthetic index, confirming that *BraA.hma4a-3* plants showed a higher tolerance to Cd. In conclusion, according to the results obtained in this work, *BraA.hma4a-3* plants could be used for phytoremediation purposes of Cd contaminated soils.

*Keywords: Brassica rapa*;cadmium; nutrients; photosynthesis; phytoremediation; TILLING

*Abbreviations:* Chl, chlorophyll; DC, distribution coefficient; HMA, heavy metal associated; LOX, lipoxygenase; MDA, malondialdehyde; NPSHs,non-protein thiols; PCs, phytochelatins; ROS, reactive oxygen species; TILLING, targeting induced local lesions in genomes.

**1. Introduction**

Nowadays*,* several toxic heavy metals are present at low concentrations in the environment mainly derived from anthropogenic activities. Among these heavy metals, Cd is a potentially dangerous metal that causes environmental and health problems due to its high mobility in the soil-plant system. Indeed, Cd is considered as one of the major heavy metals that reach the food chain through several activities. Contamination by this heavy metal in soils is a major threat to plant growth and productivity, especially in urbanized areas (Clemens and Ma, 2016).

Despite of Cd is not an essential element it can enter into the plant through LCT1 and IRT1 transporters (Clemens et al., 1998; Guerinot, 2000). Once inside the plant cell Cd causes severe alterations in physiological plant processes affecting different vital functions, water relations and mineral uptake (Gallego et al., 2012). Some symptoms of Cd toxicity are growth delay, leaf chlorosis, and inhibition of photosynthesis and respiration. In addition, Cd promotes premature chloroplast senescence, which is accompanied by stomatal closure, inhibition of carbon assimilation and photosynthetic electron transport (Gallego et al., 2012). At the cellular level, one of the major consequences of Cd toxicity is increased production of reactive oxygen species (ROS) and the inhibition of ROS detoxifying enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) (Sandalio et al., 2009; Gratão et al., 2012; Ali et al., 2014). Because of this ROS overproduction, the most detrimental oxidative effect is membrane lipid peroxidation by inducing lipoxygenase (LOX) activity, which results in the concurrent malondialdehyde (MDA) production (Karuppanapandian et al., 2011).

Only few plant species are able to tolerate Cd given its extreme phytotoxicity. There are two strategies in plants to overcome toxicity by heavy metals: the exclusion strategy, in which plants try to avoid the entry of heavy metals into roots and the tolerance strategy. In the latter, potentially toxic metals accumulate mainly in the aerial part, which is a fundamental requirement for phytoextractive plants used in soil phytoremediation. These hyperaccumulator plants grow in soils contaminated by heavy metals and tolerate from 100 to 1000-fold higher concentrations than other species. Plant species such as *Brassica juncea* and *Brassica rapa* (2n) show this metal-hyperaccumulator behaviour, so they are employed as model plants in experimental studies (Ali-Zade et al., 2010).

One of the main purposes of phytoremediation research is the selection of suitable plants for pollutant accumulation. In recent years researchers are paying increasing attention to the search for new genotypes with higher storage capacity of heavy metals (Ali-Zade et al., 2010). In order to improve this strategy, mutagenesis induction techniques have recently been developed, generating thousands of new crop varieties in hundreds of species. In this sense, TILLING (Targeting Induced Local Lesions in Genomes) technique has recently been used, which is a high-performance agronomic tool that generates and identifies unique nucleotide mutations in a specific gene region. This technique allows the identification of new variations in target genes that may be useful for the development of germplasm with improved characteristics (Barkley and Wang, 2008).

*HMA4* gene is a potential target for TILLING because HMA4 transporters are able to transport Cd besides Zn to the shoot. There are experiments in which *HMA4* expression was modifiedand as a result, Zn levels increased in leaves (Mills et al., 2010). HMA4 belongs to the group of P1B ATPases, also called heavy metal ATPases or HMA (Heavy Metal Associated). Indeed, the role of AtHMA2 and AtHMA4 in Zn and Cd transport from the root to the shoot was demonstrated in *Arabidopsis thaliana* plants (Wong and Cobbett, 2008). Therefore, the main objective of the present work was to study the tolerance and Cd accumulation of *B. rapa* (*BraA.hma4a-3*) mutant plants generated by TILLING technique.

**2. Materials and methods**

*2.1. Mutant obtention* and *growth conditions*

TILLING technique (Till et al., 2003) was employed to obtain the seeds employed in the experiment. For the present work were used plants of the parental line of *B. rapa* ssp. Trilocularis ‘R-o-18’ and M3 generation mutant plants obtained from the R-o-18 TILLING population (*BraA.hma4a-3*). Mutant plants were obtained and identified as described by Lochlainn et al. (2011) and Graham et al. (2014). *B. rapa* ssp. Trilocularis ‘R-o-18’ seeds were treated with 0.4% EMS to produce mutations (M0 generation). M0 was self-crossed threefold to obtain M1, M2, and M3 generations. M3 generation was homozygous for the *BraA.HMA4a-3* mutation. The mutation caused a cysteine to tyrosine change at amino acid 31. Mutations in the *BraA.HMA4a* gene were identified as described by Lochlainn et al. (2011) using the RevGenUK service (http://revgenuk.jic.ac.uk). A 1 kb fragment, including the transcriptional start site and the first exon, was used as the target for TILLING. M3 line with mutation were back-crossed to the R-o-18 parent line of the TILLING population. The *BraA.BC1HMA4a* plants were grown and individual plants genotyped using high-resolution melt analysis (Lochlainn et al., 2011), modified to include MeltDoctor HRM Master Mix (Applied Biosystems) according to the manufacturer’s instructions. Plants heterozygous for their mutation were self-crossed, and 20 individual *BraA.S1BC1HMA4a* plants were grown and genotyped. Homozygous mutant lines were identified, selfed to *BraA.S2BC1HMA4a* and used in the experiment.

Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9 cm Petri dishes. The dishes were incubated in the dark for 1 d at 4ºC before transferring to pots filled with vermiculite. These pots where placed in a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 14/10-h photoperiod at a photosynthetic photon flux density of 350 µmol m-2s-1 (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Throughout the experiment the plants received a growth solution composed of 4 mM KNO3, 3 mM Ca(NO3)2 • 4H2O, 2 mM MgSO4 • 7 H2O, 6 mM KH2PO4, 1 mM NaH2PO4 • 2 H2O, 2 μM MnCl2 • 4 H2O, 0.25 μM CuSO4 • 5 H2O, 0.1 μM Na2MoO4 • 2 H2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H3BO3. This solution, with a pH of 5.5–6.0, was renewed every three days.

*2.2. Experimental design and treatments*

The experimental design consisted of randomized complete block with four treatments, arranged in individual benches with eight plants per treatment and three replications each. Treatments were applied 30 days after germination and were maintained for 21 days. The treatments were: (1) R-o-18 Control (nutrient solution without CdCl2); (2) R-o-18 CdCl2 (Nutrient solution + 100 µM CdCl2); (3) *BraA.hma4a-3* Control (nutrient solution without CdCl2) and (4) *BraA.hma4a-3* CdCl2 (Nutrient solution + 100 µM CdCl2).

*2.3. Plant sampling*

After 51 days after germination, the plant material was sampled. The leaves and roots were washed with distilled water, dried on filter paper and weighed to determine fresh weight (FW). Half of the plant material from each treatment was frozen at -30 °C for subsequent biochemical tests and the other half of the plant material was lyophilized to measure dry weight (DW) and nutrient concentrations.

*2.4. Analysis of Cd concentration*

Samples were mineralized by wet digestion according to Wolf (1982). For that, 0.1 g of lyophilized leaves were mineralized with HNO3 and H2O2 30% at a temperature of 90-100 °C. From the resulting mineralization, 20 mL of deionized water were added and finally the concentration of Cd was quantified by ICP-MS.

*2.5. Distribution coefficient of Cd*

Distribution coefficient (DC) was calculated as the ratio of leaf Cd concentration and root Cd concentration (Zhu et al., 2003).

*2.6. MDA and H2O2 concentrations and LOX activity*

H2O2 concentration was measured colorimetrically according Mukherjee and Choudhuri (1983). 0.1 g of leaves was homogenized in 1 ml of cold acetone. The yellow color intensity of the supernatant was measured at 415 nm. The result of H2O2 concentration was expressed as μg g−1 DW.

For the MDA assay, 0.1 g of leaves was homogenized in 1 ml of a 0.25% thiobarbituric acid solution in 10% trichloroacetic acid. The mixture was heated at 95 °C for 30 min and then cooled in an ice bath. Subsequently the samples were centrifuged at 8300 x *g* rpm for 10 min. The MDA concentration in the supernatant was quantified by measuring absorbance at 532 nm. The non-specific absorbance value at 600 nm was obtained to correct the turbidity. MDA concentration was calculated using 155 mM-1 cm-1 as extinction coefficient (Fu and Huang, 2001).

LOX activity was measured according to Minguez-Mosquera et al. (1993) using 50 mM potassium phosphate buffer (pH 6.0) for extraction. The reaction mixture consisted of 25 μL of enzymatic extract and 200 μL of 0.5 mM linoleic acid in potassium phosphate buffer (pH 6.0). Absorbance changes were recorded at a wavelength of 234 nm.

*2.7. Concentration of photosynthetic pigments*

The concentration of photosynthetic pigments was analysed following the method of Wellburn (Wellburn, 1994). 0.1 g of frozen vegetable material was macerated in 1 mL of methanol. It was then centrifuged for 5 minutes at 2200 x *g*. Absorbance was measured at 3 different wavelengths: 653 nm, 666 nm and 470 nm. Pigments concentration was calculated as follows:

Chlorophyll *a* = 15.65 X A666 nm - 7.34 X A653 nm

Chlorophyll *b* = 27.05 X A653 nm - 11.21 X A666 nm

Carotenoids = (1000 X A470 nm - 2.86 X Chl *a* - 129.2 X Chl *b*) / 221

*2.8. Chl a fluorescence and analysis of the fluorescence transients*

Plants were adapted to dark for 30 min before measurements using a special leaf clip holder that was allocated in each leaf. Chl *a* fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King’s Lynn, Norfolk, UK); the OJIP transients were induced by red light (650 nm) with 3000 µmol photons m-2s-1 light intensity and recorded by the instrument. OJIP transients data were analysed using the JIP-test (Strasser et al., 2000). Measurements were conducted with six plants of fully expanded leaves at midstem position. Parameters employed in this research to study the energy flow and photosynthetic activities by JIP-test were: initial fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv = Fm – Fo), maximum quantum yield for primary photochemistry (ΦPo = Fv/Fm), the area over the fluorescence curve between Fo and Fm, the number of times that QA is reduced from time 0 to the time that the maximum fluorescence (N), performance index for energy conservation from photons absorbed by PSII antenna to the reduction of QB (PIABS), proportion of active reaction centres (RCs) (RC/ABS), the efficiency/probability with which a PSII trapped electron is transferred from QA to QB (ΨEo), and maximum quantum yield of electron transport (ΦEo = ETo/ABS) (Strasser et al., 2000).

*2.9. Determination of the different GSH forms*

Reduced GSH, GSSG and total GSH (reduced GSH + GSSG) were assayed according to the GSSG-recycling method (Griffith, 1980). Leaves material were homogenized in liquid N2 with metaphosphoric acid at 5% (w/v) and centrifuged at 13,500 × *g* and 4ºC for 15 min. GSSG and total GSH were assayed in the same extract. A standard curve was analysing in the same manner as for the extracts. Reduced GSH levels were estimated as the difference between total GSH and GSSG.

*2.10. Non-protein thiols and phytochelatins (PCs) determination*

Non-protein thiols (NPSHs) were determined as described by Garg and Kaur (2013). 100 µl of the supernatant was taken in a microfuge tube, to which 0.5 ml reaction buffer (100 mM K-phosphate buffer (pH 7.0), 0.5 mM EDTA and 1 mM DTNB were added. The reaction mixture was incubated for 10 min, and absorbance was read at A412 nm. Values were corrected for the absorbance by preparing a blank without extract. A standard curve was prepared from varying concentrations of cysteine to calculate NPSHs content in samples. PCs levels were estimated from the difference between NPSHs and reduced GSH concentrations (PCs = NPSHs – GSH) (Hartley-Whitaker et al., 2001).

*2.11. Statistical analysis*

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI program. A two-tailed ANOVA was applied to ascertain whether the mutation (M), the Cd application (C), or the interaction of the two factors (M \* C) significantly affected the results. Means were compared by Fisher’s least significant differences (LSD). The significance levels for both analyses were expressed as \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, or NS (not significant).

**3. Results and discussion**

*3.1. Biomass and Cd* *concentration*

The presence of Cd in plants interfere in different metabolic processes such as photosynthesis, respiration, transpiration and nutritional content (Gallego et al., 2012). D’Alessandro et al. (2013) observed phytotoxicity symptoms in *B. juncea* plants when Cd was applied at a dose higher than 25 µM CdCl2 and they observed the inhibition of different metabolic processes and the reduction in plant growth was proportional to CdCl2 concentration. In the present study,biomass decreased significantly in both the shoot and root parts in genotype R-o-18 and *BraA.hma4a-3* plants when Cd was applied. However, *BraA.hma4a-3* presented a lower biomass reduction (56% lower) compared with its control than the parental R-o-18 (62% lower) (Fig. 1). Despite this, mutant plants reached a lower DW until sampling time (7 weeks after germination) (Fig.1). Previous experiments observed different effects of *HMA* mutations on plants. Mills et al. (2010), in their studies in *A. thaliana* plants, observed that *hma2-4* and *hma4-2* single mutations presented no alteration in growth in comparison to the WT genotype. In contrast, in *hma2-4 hma4-2* double mutant’s growth was significantly lower compared to WT plants. In addition, Liedschulte et al. (2017) observed a biomass reduction by 50% in tobacco mutant plants (HMA4.1 Q293\*/HMA4.2 Q561\*) after 5 weeks of growth; however, after 11 weeks mutant plants reached a similar biomass than WT plants. Therefore, it is possible that the biomass of *BraA.hma4a-3* mutants reaches similar values to R-o-18 plants after a large culture period.

****

**Fig. 1.** Leaf biomass (A), and root biomass (B) in R-o-18 and *BraA.hma4a-3* genotypes subjected to control and Cd toxicity treatments. Columns represent mean values ± standard error (n = 6), and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

HMA transporters, in particular P1B-2 type, are known to transport Zn to the shoot. However, researchers demonstrated the role of *AtHMA2* and *AtHMA4* in *A. thaliana* plants in the discharge of Cd from the root to the stem. Thus, the silencing of tobacco *HMA4*, directly reduced the Cd content in the shoot proving the role of HMA4 in Cd transport to the shoot (Wong and Cobbett, 2008; Liedschulte et al., 2017). Our results indicated that Cd concentration in leaves significantly increased in mutant plants (46%) compared with genotype R-o-18 (Table 1). In roots, Cd concentration was not affected by the mutation in comparison to R-o-18 when Cd was applied (Table 1). These results show that the distribution coefficient towards the shoot was higher in mutant plants (Table 1). In addition, Zn concentration in leaves greatly increased in mutant plants subjected to both treatments (data not shown). Therefore, the Cd and Zn accumulation in leaves could be attributed to a greater HMA4 transporter activity in *BraA.hma4a-3* plants. Another possibility is that the mutation of an allele is compensated by the expression of another allele or other Cd transporters such as *HMA*2 and *HMA3* (Wong and Cobbett, 2008; Liedschulte et al., 2017).

**Table 1**. Cd concentration in leaves, roots and its distribution coefficient (DC) in R-o-18 and *BraA.hma4a-3* genotypes subjected to Cd toxicity

|  |  |  |  |
| --- | --- | --- | --- |
|  | Leaf Cd concentration(μg g-1 DW) | Root Cd concentration(μg g-1 DW) | DC of Cd |
| R-o-18 | 72.35±4.23 | 683.45±89.25 | 0.10±0.01 |
| *BraA.hma4a-3* | 105.87±6.01 | 667.01±102.35 | 0.16±0.12 |
| *p*-value | \*\*\* | NS | \*\*\* |
| LSD0.05 | 21.67 | 87.40 | 0.02 |

Values are means (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.2. Oxidative stress indicators (LOX, H2O2* *and MDA)*

The presence of Cd in plants triggers ROS generation that causes oxidative stress. An excessive ROS production is very harmful since it causes the degradation of photosynthetic structures and, in turn, photosynthesis inhibition. Likewise, one of the most important effects of oxidative stress is lipid peroxidation in membranes, which impairs its permeability due to the induction of LOX activity and the consequent MDA production (Karuppanapandian et al., 2011). Furthermore, Noriega et al. (2012) proved that Cd inhibits ROS-detoxifying enzymes such as SOD and APX*.* Surprisingly, in the present experiment, no significant differences were observed in the H2O2 production between treatments, genotypes or the interaction of both. It was observed a reduction of LOX activity in mutant plants compared to R-o-18 (Table 2). According to Zong et al. (2017), *B. rapa* plants grown with a 50 µM Cd treatment showed an increase of more than 50% in MDA content compared to control plants. Our results indicated a decrease in MDA concentration in the R-o-18 genotype, while in mutant plants MDA increased as a result of Cd treatment. However, MDA concentration in *BraA.hma4a-3* plants was lower compared to R-o-18 plants. This might be related to the decreased LOX activity in mutant plants, which can result in lower membrane lipid peroxidation (Table 2).

**Table 2**. Oxidative stress indicatorsin R-o-18 and *BraA.hma4a-3* genotypes subjected to CdCl2 treatment

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | H2O2(μg g-1 FW) | LOX(ΔAbs mg prot-1min-1) | MDA(μM g-1 FW) |
| R-o-18 | Control | 0.54±0.05 | 0.17±0.01 | 11.20±0.39 |
|  | 100 μM Cd | 0.49±0.05 | 0.19±0.03 | 9.17±0.30 |
|  | *p*-value | NS | NS | \*\*\* |
|  | LSD0.05 | 0.10 | 0.07 | 1.05 |
|  |  |  |  |  |
| *BraA.hma4a-3* | Control | 0.54±0.02 | 0.11±0.01 | 6.60±0.11 |
|  | 100 μM Cd | 0.54±0.01 | 0.10±0.01 | 7.68±0.11 |
|  | *p*-value | NS | NS | \*\*\* |
|  | LSD0.05 | 0.04 | 0.48 | 0.34 |
| Analysis of variance |
| Mutation (M) |  | NS | \*\*\* | \*\*\* |
| Cd (C) |  | NS | NS | NS |
| M x C |  | NS | NS | \*\*\* |
| LSD0.05 |  | 0.05 | 0.04 | 0.50 |

Values are means (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.3. Pigments concentration and photosynthetic performance*

Vital processes such as photosynthesis and respiration are strongly affected in plants growing in medium containing large heavy-metals concentrations (Ali-Zade et al., 2010). Experiments in *Zea mays* plants showed a net photosynthesis inhibition, while transpiration, stomatal conductance and CO2 content increased due to Cd application (Zhang et al., 2011). Indeed, one of the most affected compounds by Cd are photosynthetic pigments (Gallego et al., 2012). In contrast, our results showed no significant differences of chlorophyll *a*, *b* and carotenoids concentration in R-o-18 plants subjected to Cd toxicity in comparison to control plants. Even *BraA.hma4a-3* plants presented higher concentration of these pigments when Cd was applied (Table 3). These results indicate that mutant plants might be able to preserve photosynthetic pigments against toxicity by this metal. Regarding Chl *a*/*b* ratio, it was not affected by Cd treatment in both lines (Table 3). Chl *a* mostly is associated with core antenna proteins of PSII and RCs while Chl *b* is mainly present in light-harvesting complexes (LHCII). Thus, Chl *a*/*b* ratio is an indicator of RC/LHCII proportion. When Chl *a*/*b* increases, this usually indicates a conversion of Chl *b* to Chl *a* to maintain Chl *a* levels and thereby active RCs (Nyongesah et al., 2015). However, our results did not show any difference regarding Chl *a*/*b* ratio, so neither Cd nor *HMA* mutation affected to the Chls proportion in RC and LHCII (Table 3).

**Table 3**. Photosynthetic pigments in R-o-18 and *BraA.hma4a-3* genotypes subjected to CdCl2 treatment

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | Chl *a*(mg g-1 FW) | Chl *b*(mg g-1 FW) | Carotenoids(μg g-1 FW) | Chl *a*/*b* Ratio |
| R-o-18 | Control | 0.117±0.002 | 0.055±0.001 | 14.99±0.30 | 2.129±0.023 |
|  | 100 μM Cd | 0.118±0.001 | 0.054±0.001 | 15.36±0.40 | 2.184±0.022 |
|  | *p*-value | NS | NS | NS | NS |
|  | LSD0.05 | 0.006 | 0.003 | 1.56 | 0.066 |
|  |  |  |  |  |  |
| *BraA.hma4a-3* | Control | 0.099±0.001 | 0.045±0.001 | 13.50±0.12 | 2.178±0.012 |
|  | 100 μM Cd | 0.107±0.002 | 0.050±0.001 | 14.21±0.25 | 2.148±0.007 |
|  | *p*-value | \*\*\* | \*\*\* | \* | NS |
|  | LSD0.05 | 0.004 | 0.002 | 0.09 | 0.030 |
| Analysis of variance |  |
| Mutation (M) |  | \*\*\* | \*\*\* | \*\*\* | NS |
| Cd (C) |  | \* | NS | NS | NS |
| M x C |  | \* | \*\* | NS | \* |
| LSD0.05 |  | 0.003 | 0.002 | 0.1 | 0.035 |

Values are means (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

The study of the chlorophyll fluorescence provides useful information about the functioning of the photosynthetic apparatus. Thus, stress conditions can disturb or block the photosynthetic electron transport, which reflects in a fluorescence increase. JIP test is used for the quantification of Chl fluorescence, providing parameters indicating *in vivo* PSII functioning (Strasser et al., 2000). Taking into account all fluorescence parameters, it was determined that Cd negatively affects photosynthetic activity mainly in R-o-18 plants; this is shown by reductions in Fv, Fv/Fm, Area, PIABS, Ψo and ΦEO in comparison to control plants (Table 4). In contrast, only RC/ABS decreased in *BraA.hma4a-3* plants and the rest of parameters presented similar values than control plants. The parameters that showed a higher decrease in R-o-18 plants were PIABS and Area (Table 4). PIABS indicates the vitality of the plant and the ability to withstand external pressures and it depends on 3 factors: the ratio of active PSII RCs, Fv/Fm, and the efficiency of electron transport beyond QA (Ψo). On the other hand, the Area is defined as the area over the fluorescence curve between Fo and Fm, which represents the size of electron acceptors (QA) pool. Thus, during stress, electron transference from RCs is blocked and area is reduced (Strasser et al., 2000). The results for Fv and Fv/Fm suggest that Cd is reducing the energy that reaches to RCs, whereas the results for Area, Ψo and ΦEO showed that Cd blocked the electron transfer through PSII. Conversely, *BraA.hma4a-3* photosynthetic performance parameters indicated a greater efficiency and resilience to stress caused by Cd. This is also supported by the higher N value in *BraA.hma4a-3* (Table 4). N value represents the number of times QA is reduced from time 0 to the time that the maximum fluorescence is reached.

**Table 4.** Values of Chl *a* fluorescence parameters derived from the JIP test in R-o-18 and *BraA.hma4a-3* genotypes subjected to CdCl2 treatment

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Fo | Fm | Fv | Fv/Fm | Área | N | PIABS | RC/ABS | Ψo | **Φ**EO |
| R-o-18 | Control | 265±4.26 | 1779±19.62 | 1436±25.14 | 0.84±0.00 | 58556±1949 | 39.95±0.99 | 7.87±0.52 | 0.76±0.02 | 0.66±0.00 | 0.55±0.00 |
|  | 100 μM Cd | 264±2.50 | 1743±13.25 | 1248±61.73 | 0.83±0.00 | 49577±1864 | 37.85±1.19 | 5.81±0.53 | 0.71±0.02 | 0.61±0.01 | 0.51±0.01 |
|  | *p*-value | NS | NS | \*\* | \*\*\* | \*\* | NS | \* | NS | \*\* | \*\* |
|  | LSD0.05 | 12.10 | 46.87 | 119.74 | 0.00 | 5762 | 3.88 | 1.57 | 0.08 | 0.02 | 0.02 |
|  |  |  |  |  |  |  |  |  |  |  |  |
| *BraA.hma4a-3* | Control | 261±6.66 | 1706±18.82 | 1449±15.18 | 0.847±0.00 | 66706±1058 | 52.94±1.10 | 8.31±0.41 | 0.78±0.01 | 0.65±0.00 | 0.55±0.00 |
|  | 100 μM Cd | 267±0.84 | 1683±15.44 | 1416±15.12 | 0.844±0.00 | 64588±1855 | 50.53±0.93 | 7.40±0.32 | 0.72±0.01 | 0.64±0.00 | 0.54±0.00 |
|  | *p*-value | NS | NS | NS | NS | NS | NS | NS | \*\* | NS | NS |
|  | LSD0.05 | 14.96 | 54.25 | 47.74 | 0.00 | 4760 | 3.19 | 1.16 | 0.04 | 0.01 | 0.01 |
| Analysis of variance |  |  |  |  |  |  |  |  |  |  |
| Mutation (M) |  | NS | \*\* | \* | \*\*\* | \*\*\* | \*\*\* | NS | NS | NS | NS |
| Cd (C) |  | NS | NS | \*\* | \*\*\* | \*\* | NS | \*\* | \* | \*\*\* | \*\* |
| M x C |  | NS | NS | \* | \* | \* | NS | NS | NS | \* | \* |
| LSD0.05 |  | 9.13 | 34.58 | 74.13 | 0.00 | 3885 | 2.61 | 1.03 | 0.04 | 0.01 | 0.01 |

Values are means (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.4. Glutathione forms, PCs and thiols*

Plants possess various antioxidant systems to eliminate ROS and therefore increase stress tolerance (Pitzschke et al., 2006). Thus, GSH contributes to H2O2 elimination, prevents lipid peroxidation caused by heavy metals and acts as a PC biosynthesis precursor (Szalai et al., 2009). Furthermore, GSH is considered a biochemical indicator of heavy-metal toxicity in plants (Tausz et al., 2004). Garg and Kaur (2013) observed an increase in GSH synthesis in an experiment applying Cd to *Cajanus cajan* plants. In contrast, in the present experiment, both the R-o-18 plants and *BraA.hma4a-3* mutants showed a similar tendency in terms of their GSH concentrations and the proportion of their forms when Cd was applied to the medium. Cd treatment reduced the total GSH concentration mainly due to the decrease in its oxidized form because the reduced form did not change with respect to control. The decrease in GSSG form increased the GSH/GSSG ratio and in the GSH redox state (Table 5). These results disagree with what Hendry et al. (1992) observed because during stress ascorbate and glutathione are mostly in their oxidized forms and their redox states decrease. In our plants, the higher GSH/GSSG ratio might contribute to a higher Cd stress tolerance.

**Table 5.** GSH forms (μg g-1 FW), ratio GSH/GSH, and GSH redox state in R-o-18 and *BraA.hma4a-3* genotypes subjected to CdCl2 treatment

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | Total GSH | GSH | GSSG | Ratio GSH/GSSG | GSH Redox State |
| R-o-18 | Control | 33.02±1.04 | 27.76±1.17 | 5.25±0.42 | 5.65±0.62 | 83.96±1.37 |
|  | 100μM Cd | 26.63±1.33 | 28.02±2.84 | 1.66±0.28 | 15.79±3.33 | 93.87±1.20 |
|  | *p*-value | \* | NS | \*\*\* | \*\* | \*\*\* |
|  | LSD0.05 | 3.59 | 6.51 | 1.15 | 7.18 | 3.86 |
|  |  |  |  |  |  |  |
| *BraA.hma4a-3* | Control | 35.37±1.84 | 30.66±1.82 | 4.71±0.36 | 6.91±0.82 | 86.45±1.11 |
|  | 100 μM Cd | 29.82±1.47 | 27.81±1.52 | 2.00±0.22 | 15.64±2.22 | 93.12±0.85 |
|  | *p*-value | \* | NS | \*\*\* | \*\* | \*\*\* |
|  | LSD0.05 | 5.00 | 5.03 | 0.91 | 5.02 | 2.97 |
| Analysis of variance |  |  |
| Mutation (M) |  | NS | NS | NS | NS | NS |
| Cd (C) |  | \*\*\* | NS | \*\*\* | \*\*\* | \*\*\* |
| M x C |  | NS | NS | NS | NS | NS |
| LSD0.05 |  | 2.95 | 3.95 | 0.74 | 4.20 | 2.34 |

Values are means (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

In addition to the role of GSH in oxidative stress, GSH has the ability to join heavy metals such as Cd through its thiol group which is considered as a specific defence strategy against heavy-metal toxicity (Szalai et al., 2009). These types of compounds that possess a thiol group are called NPSHs and include mainly GSH and PCs. PCs are synthesized by transpeptidation of Glu-Cys residues in GSH on another GSH molecule or by phytochelatin synthase enzyme which is post-translationally activated by heavy metals (Grill et al., 2007). Therefore, due to PCs synthesis is related to heavy metals stress in plants, its analysis is used to evaluate the toxicity as a metal biomarker (Vázquez et al., 2006). The results showed a reduction in PCs concentration when Cd was applied to R-o-18 plants. Conversely, this concentration increased in mutant plants, which can chelate Cd and block its solubility in the plant to prevent toxic effects (Fig. 2A). In this sense, Roxas et al. (2000) showed that overexpression of enzymes that promote PCs synthesis improved growth in transgenic tomato under abiotic stress conditions. The lower NPSHs concentration observed in R-o-18 plants could be due to the decrease in PCs and GSH levels under Cd toxicity. In contrast, this did not occur in mutant plants that maintained NPSHs levels (Fig. 2B), which might be useful to counteract Cd toxicity.

****

**Fig. 2.** Phytochelatins (A), and NPSHs (B) in R-o-18 *and BraA.hma4a-3* genotypes subjected to control and Cd toxicity treatments. Columns represent mean values ± standard error (n = 9), and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

**4. Conclusions**

In conclusion, *BraA.hma4a-­‐3* plants showed lower biomass compared to R-­‐o-­‐18 plants, but the mutagenized plants presented a lower loss of biomass under Cd-­‐toxicity conditions, therefore suggesting a better tolerance to Cd. In addition, *HMA4* mutation allowed a higher Cd accumulation in leaves, probably due to an enhanced transport from the root probably caused by an increased HMA4 activity in mutants plants. The increase in photosynthetic pigments concentration, the lower oxidative stress, and the better photosynthetic performance, suggest that *BraA.hma4a-3* plants exhibit increased Cd tolerance. This could be explained by an increase in PCs synthesis that could block Cd toxic effects. Therefore, *BraA.hma4a-3* plants could be a potential resource for phytoremediation purposes of Cd-contaminated soils.

**Acknowledgements**

We would like to thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) for the scholarship of J.O.S for the PhD Training Stay at University of Granada.

**Funding sources**

This work was supported by the PAI programme (Plan Andaluz de Investigación, Grupo de Investigación AGR282), by a grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL (FPU14/01858) and by a grant from CONACYT awarded to J.O.S. (CVU 710705/420648).

**References**

Ali-Zade, V., Alirzayeva, E., Shirvani, T., 2010. Plant Resistance to Anthropogenic Toxicants: Approaches to Phytoremediation, in: Plant Adaptation and Phytoremediation. Springer Netherlands, Dordrecht, pp. 173–192. https://doi.org/10.1007/978-90-481-9370-7\_9

Ali, B., Gill, R.A., Yang, S., Gill, M.B., Ali, S., Rafiq, M.T., Zhou, W., 2014. Hydrogen sulfide alleviates cadmium-induced morpho-physiological and ultrastructural changes in *Brassica napus*. Ecotoxicol. Environ. Saf. 110, 197–207. https://doi.org/10.1016/j.ecoenv.2014.08.027

Barkley, N., Wang, M., 2008. Application of TILLING and EcoTILLING as reverse genetic approaches to elucidate the function of genes in plants and animals. Curr. Genomics 9, 212–226. https://doi.org/10.2174/138920208784533656

Clemens, S., Antosiewicz, D.M., Ward, J.M., Schachtman, D.P., Schroeder, J.I., 1998. The plant cDNA LCT1 mediates the uptake of calcium and cadmium in yeast. Proc. Natl. Acad. Sci. U. S. A. 95, 12043–8. https://doi.org/10.1073/PNAS.95.20.12043

Clemens, S., Ma, J.F., 2016. Toxic heavy metal and metalloid accumulation in crop plants and foods. Annu. Rev. Plant Biol. 67, 489–512. https://doi.org/10.1146/annurev-arplant-043015-112301

D’Alessandro, A., Taamalli, M., Gevi, F., Timperio, A.M., Zolla, L., Ghnaya, T., 2013. Cadmium stress responses in *Brassica juncea* : Hints from proteomics and metabolomics. J. Proteome Res. 12, 4979–4997. https://doi.org/10.1021/pr400793e

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45, 105–114. https://doi.org/10.1016/S0098-8472(00)00084-8

Gallego, S.M., Pena, L.B., Barcia, R.A., Azpilicueta, C.E., Iannone, M.F., Rosales, E.P., Zawoznik, M.S., Groppa, M.D., Benavides, M.P., 2012. Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. Environ. Exp. Bot. 83, 33–46. https://doi.org/10.1016/j.envexpbot.2012.04.006

Garg, N., Kaur, H., 2013. Response of antioxidant enzymes, phytochelatins and glutathione production towards Cd and Zn stresses in *Cajanus cajan* (L.) Millsp. genotypes colonized by arbuscular mycorrhizal fungi. J. Agron. Crop Sci. 199, 118–133. https://doi.org/10.1111/j.1439-037X.2012.00533.x

Graham, N.S., Hammond, J.P., Lysenko, A., Mayes, S., O Lochlainn, S., Blasco, B., Bowen, H.C., Rawlings, C.J., Rios, J.J., Welham, S., Carion, P.W.C., Dupuy, L.X., King, G.J., White, P.J., Broadley, M.R., 2014. Genetical and comparative genomics of Brassica under altered Ca supply identifies Arabidopsis Ca-transporter orthologs. Plant Cell 26, 1–14. https://doi.org/10.1105/tpc.114.128603

Gratão, P.L., Monteiro, C.C., Carvalho, R.F., Tezotto, T., Piotto, F.A., Peres, L.E.P., Azevedo, R.A., 2012. Biochemical dissection of diageotropica and never ripe tomato mutants to Cd-stressful conditions. Plant Physiol. Biochem. 56, 79–96. https://doi.org/10.1016/j.plaphy.2012.04.009

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212. https://doi.org/10.1016/0003-2697(80)90139-6

Grill, E., Mishra, S., Srivastava, S., Tripathi, R.D., 2007. Role of Phytochelatins in Phytoremediation of Heavy Metals, in: Environmental Bioremediation Technologies. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 101–146. https://doi.org/10.1007/978-3-540-34793-4\_5

Guerinot, M.L., 2000. The ZIP family of metal transporters. Biochim. Biophys. Acta - Biomembr. 1465, 190–198. https://doi.org/10.1016/S0005-2736(00)00138-3

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W. Ten, Schat, H., Meharg, A.A., 2001. Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. Plant Physiol. 126, 299–306

Hendry, G.A.F., Finch-Savage, W.E., Thorpe, P.C., Atherton, N.M., Buckland, S.M., Nilsson, K.A., Seel, W.E., 1992. Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. New Phytol. 122, 273–279. https://doi.org/10.1111/j.1469-8137.1992.tb04231.x

Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K., Kim, W., 2011. Reactive oxygen species in plants: Their generation, signal transduction, and scavenging mechanisms. Aust. J. Crop Sci. 5, 709–725.

Liedschulte, V., Laparra, H., Battey, J.N.D., Schwaar, J.D., Broye, H., Mark, R., Klein, M., Goepfert, S., Bovet, L., 2017. Impairing both *HMA4* homeologs is required for cadmium reduction in tobacco. Plant. Cell Environ. 40, 364–377. https://doi.org/10.1111/pce.12870

Lochlainn, S.Ó., Amoah, S., Graham, N.S., Alamer, K., Rios, J.J., Kurup, S., Stoute, A., Hammond, J.P., Østergaard, L., King, G.J., White, P.J., Broadley, M.R., 2011. High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7, 43. https://doi.org/10.1186/1746-4811-7-43

Mills, R.F., Valdes, B., Duke, M., Peaston, K.A., Lahner, B., Salt, D.E., Williams, L.E., 2010. Functional Significance of AtHMA4 C-Terminal Domain In Planta. PLoS One 5, e13388. https://doi.org/10.1371/journal.pone.0013388

Minguez-Mosquera, M.I., Jaren-Galan, M., Garrido-Fernandez, J., 1993. Lipoxygenase activity during pepper ripening and processing of paprika. Phytochemistry 32, 1103–1108. https://doi.org/10.1016/S0031-9422(00)95073-8

Mukherjee, S.P., Choudhuri, M.A., 1983. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. Physiol. Plant. 58, 166–170. https://doi.org/10.1111/j.1399-3054.1983.tb04162.x

Noriega, G., Cruz, D.S., Batlle, A., Tomaro, M., Balestrasse, K., 2012. Heme oxygenase is involved in the protection exerted by jasmonic acid against cadmium stress in soybean roots. J. Plant Growth Regul. 31, 79–89. https://doi.org/10.1007/s00344-011-9221-0

Nyongesah, M.J., Wang, Q., Li, P., 2015. Effectiveness of photochemical reflectance index to trace vertical and seasonal chlorophyll a/b ratio in *Haloxylon ammodendron*. Acta Physiol. Plant. 37, 2. https://doi.org/10.1007/s11738-014-1747-x

Pitzschke, A., Forzani, C., Hirt, H., 2006. Reactive oxygen species signaling in plants. Antioxid. Redox Signal. 8, 1757–1764. https://doi.org/10.1089/ars.2006.8.1757

Roxas, V.P., Lodhi, S.A., Garrett, D.K., Mahan, J.R., Allen, R.D., 2000. Stress tolerance in transgenic tobacco seedlings that overexpress Glutathione s-transferase/Glutathione peroxidase. Plant Cell Physiol. 41, 1229–1234. https://doi.org/10.1093/pcp/pcd051

Sandalio, L.M., Rodríguez-Serrano, M., del Río, L.A., Romero-Puertas, M.C., 2009. Reactive Oxygen Species and Signaling in Cadmium Toxicity. Springer, Berlin, Heidelberg, pp. 175–189. https://doi.org/10.1007/978-3-642-00390-5\_11

Strasser, R., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples, in: Yunus, M., Pathre, U., Mohanty, P. (Eds.), Probing Photosynthesis: Mechanism, Regulation and Adaptation. Taylor & Francis, London, pp. 443–480.

Szalai, G., Kellős, T., Galiba, G., Kocsy, G., 2009. Glutathione as an antioxidant and regulatory molecule in plants under abiotic stress conditions. J. Plant Growth Regul. 28, 66–80. https://doi.org/10.1007/s00344-008-9075-2

Tausz, M., Šircelj, H., Grill, D., 2004. The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J. Exp. Bot. 55, 1955–1962. https://doi.org/10.1093/jxb/erh194

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E., Henikoff, J.G., Comai, L., Henikoff, S., 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. 13, 524–530. https://doi.org/10.1101/gr.977903

Vázquez, S., Goldsbrough, P., Carpena, R.O., 2006. Assessing the relative contributions of phytochelatins and the cell wall to cadmium resistance in white lupin. Physiol. Plant. 128, 487–495. https://doi.org/10.1111/j.1399-3054.2006.00764.x

Wellburn, A.R., 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. 144, 307–313. https://doi.org/10.1016/S0176-1617(11)81192-2

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Wong, C.K.E., Cobbett, C.S., 2008. HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*. New Phytol. 181, 71–78. https://doi.org/10.1111/j.1469-8137.2008.02638.x

Zhang, L.Y., Zhang, H.Y., Guo, W., Tian, Y.L., Chen, Z.S., Wei, X.F., 2011. Photosynthetic responses of energy plant maize under cadmium contamination stress. Adv. Mater. Res. 356, 283–286. https://doi.org/10.4028/www.scientific.net/AMR.356-360.283

Zhu, Y.-G., Huang, Y.-Z., Hu, Y., Liu, Y.-X., 2003. Iodine uptake by spinach (*Spinacia oleracea* L.) plants grown in solution culture: effects of iodine species and solution concentrations. Environ. Int. 29, 33–37. https://doi.org/10.1016/S0160-4120(02)00129-0

Zong, H., Liu, S., Xing, R., Chen, X., Li, P., 2017. Protective effect of chitosan on photosynthesis and antioxidative defense system in edible rape (*Brassica rapa* L.) in the presence of cadmium. Ecotoxicol. Environ. Saf. 138, 271–278. https://doi.org/10.1016/j.ecoenv.2017.01.009