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**Title:** Study of Zn accumulation and tolerance of HMA4 TILLING mutants of *Brassica rapa* grown under Zn deficiency and Zn toxicity

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**ABSTRACT**

Nowadays, Zinc (Zn) deficiency is the most widespread micronutrient deficiencybut simultaneously Zn toxicity is produced due to environmental pollution. A potential method to alleviate Zn deficiency and to reduce Zn concentration in soils is through the generation of plants with enhanced capacity for Zn accumulation and higher tolerance. This could be achieved through the modification of HMA4 transporter. *BraA.hma4a-3* is a TILLING mutant plant that presents one modification in HMA4 transporter. Thus, in this study we analyzed the potential of *BraA.hma4a-3* for Zn accumulation and Zn deficiency and toxicity tolerance. *BraA.hma4a-3* and parental R-o-18 plants were grown with different Zn doses: 1 µM ZnSO4 (Control), 0.01 µM ZnSO4 (Zn deficiency) and 100 µM ZnSO4 (Zn toxicity). Parameters of biomass, Zn concentration, photosynthesis, oxidative stress, N metabolism and amino acids (AAs) were measured. *BraA.hma4a-3* did not affect plant biomass but did increase Zn accumulation in leaves under an adequate Zn supply and Fe under control and Zn deficiency doses. Regarding stress tolerance parameters and N metabolism, *BraA.hma4a* did not produce alterations under control conditions. In addition, under Zn toxicity, parameters suggest a greater tolerance. Briefly, the obtained results point to *BraA.hma4a-3* as a useful mutant to increase Zn accumulation.

*Keywords*: Amino acids, Ionome, N metabolism, Oxidative stress, TILLING, Zinc

*Abbreviations*: AAs, amino acids; AAT, aspartate aminotransferase; Chl, chlorophyll; DC, distribution coefficient; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; HMA, Heavy Metal-ATPase; LOX, lipoxygenase; MDA, malondialdehyde; NR, nitrate reductase; TILLING, targeting induced local lesions in genomes; ZnUpE, Zn uptake efficiency; ZnUtE, Zn utilisation efficiency

**1. Introduction**

Nowadays, problems with the availability of zinc (Zn) are present in crops. There are cases of both deficiency and toxicity all over the world. Zn deficiency is the most widespread micronutrient deficiency in crops animals and humans,being the 25 to 30% of the world’s population afflicted by Zn deficiency.The main cause is the low Zn bioavailability for crops, so there are a poor consumption of this essential micronutrient in human diets [1, 2]. However, at the same time, Zn toxicity is produced in some areas mainly due to environmental pollution originated by industrial and agricultural activities.Zn is a cofactor and structural element necessary for the activity of hundreds of proteins including ROS detoxifying enzymes.Crop yield is negatively affected by Zn deficiency and toxicity due to Zn is an essential micronutrient within plant physiology. An inadequate Zn supply affects negatively to photosynthesis performance, reduce carbonic anhydrase activity, the chlorophylls (Chls) biosynthesis and alter the cell membrane integrity and the protein synthesis [1, 3]. In addition, this element is necessary for a correct N metabolism performance due to Zn disorders are often correlated with an altered N uptake and assimilation and the synthesis of N protective compounds such as proline [4]. On the other hand, Zn imbalance produce ROS accumulation and displaces other elements from their active sites in proteins[5]. Likewise, Zn interacts with many other elements in plants so Zn deficiency or toxicity usually lead to the alteration of the homeostasis of the other elements such as Fe, Ca and P [6].

One potential method to alleviate Zn deficiency and increase nutrient concentration is through biofortification. This is an strategy to increase the content of nutrients in edible parts of crops [2]. Molecular biology and crop breeding allow the generation of new genotypes with enhanced Zn accumulation. In the case of Zn biofortification the increase in xylem loading could increase Zn transport to the shoot [7].On the other hand, a higher capacity for Zn transport to the shoot could be very useful for phytoremediation when Zn concentration in the medium is found at toxic levels. Phytoremediation is an environmentally friendly strategy to reduce the presence of toxic heavy metals in soils [8]. This technique uses hyperaccumulator plants that are capable of grow on soils with high concentration of metal and besides, they accumulate this metals in its tissues. Therefore, for the success in phytoremediation we must selects species with a high biomass production, a high tolerance and a good transport capacity to the shoot.Among these plants Brassicacea species appear to be good candidates [9].

Several experiments observed that variations in Heavy Metal-ATPase 4 (HMA4) are responsible for different Zn accumulation capacity in the shoot [10]. Therefore, the enhancement in the accumulation of Zn in aerial tissue can be achieved through the modification of HMA4 transporter [7]. HMA4 are P1B ATPases, also known as heavy metal ATPases or HMA (Heavy Metal Associated) and its main function is the transport of Zn to the xylem vessels from root to shoot [11]. HMA4 drives the efflux of Zn out of the cell to the cell wall. In experiments in which HMA4is enhanced, Zn concentration increased in the shoot [12]. Experiments in tobacco, tomato and *Arabidopsis thaliana* with higher HMA4 activity confirmed this enhanced Zn transport capacity regardless the Zn dose applied [13, 14, 15]. A higher activity of HMA4 transporter also is able to affect to Fe accumulation and root to shoot distribution in tomato [16]. Employing TILLING Targeting Induced Local Lessons in Genomes) technique, researchers generated a *Brassica rapa* ssp. trilocularis ’R-o-18’ mutant for HMA4 transporter (*BraA.hma4a-3*). TILLING (allows the screening of new variations in target genes that may be useful to obtain plants with improved characteristics [17]. Thus, the aim of this study is to evaluate the accumulation of Zn in *BraA.hma4a-3* mutant to evaluate its potential for biofortification and phytoremediation programs and Zn deficiency and toxicity tolerance.

**2. Material and methods**

*2.1. Mutant obtention and growth conditions*

TILLING technique [17] was employed to obtain the seeds used 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. [18] and Graham et al. [19]. *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. [18] 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 [18], 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 started 30 days after germination and were kept for 21 days. Plants were grown with different Zn doses: 1 µM ZnSO4 (Control), 0.01 µM ZnSO4 (Zn deficiency), 100 µM ZnSO4 (Zn toxicity). The two factors involved in the experiment were the Zn dose applied (Z) and the genotype employed (G).

*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 mineral nutrients and N forms*

Sulphur (S), phosphorus (P), potassium (K), Ca, Mg, Fe, copper (Cu), manganese (Mn), zinc (Zn) and boron (B) were determined after a sample of 150 mg dry material was subjected to a process of mineralization by wet digestion [20]. To carry out this assay, dry material was ground and mineralized with a mixture of nitric acid (HNO3)/perchloric acid (HClO4) (v/v) and H2O2 at 30%. From the resulting mineralization, and after the addition of 20 ml of deionized H2O, elements concentrations were determined by ICP-MS.

NO3- was analyzed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100-µl aliquot was taken for NO3- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO3- concentration by spectrophotometry [21]. NH4+ was analyzed from the aqueous extraction and total reduced N were obtained from digested samples and both were determined by the method described by Krom [22]. Total N was obtained as the sum of NO3- plus total reduced N.

*2.5. Zn efficiency parameters (ZnUE) and distribution coefficient (DC)*

ZnUE parameters were calculated as follow:

Zn uptake efficiency (ZnUpE) was calculated as total Zn accumulation divided by root DW (mg Zn g−1 RDW) [23].

Zn utilization efficiency (ZnUtE) was calculated as leaf tissue DW divided by Zn concentration (g2 LDW mg−1 Zn) [24].

DC was calculated as the quotient between Zn concentration in leaves and Zn concentration in roots [25].

*2.6. Pigment concentrations, SPAD and performance index (PIABS)*

Total chlorophyll (Chl) and carotenoid were extracted in methanol and centrifuged at 5000 × g for 5 min. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm. The Chl a, Chl b, and carotenoids were estimated by using the equation of Lichtenthaler [26]. Total Chl was calculated as the sum of Chl a and Chl b.

SPAD value was measured using meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Three measurements were made in each leaf and average was calculated.

PIABS was obtained through the Chl a fluorescence analysis. For this, plants were adapted to dark for 30 min before measurements using a leaf clip holder that was allocated in each fully expanded leaf. Chl a fluorescence was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King’s Lynn, Norfolk, UK) [27]. Measurements were conducted with six plants of fully expanded leaves at midstem position.

*2.7. Malondialdehyde (MDA), O2.−, and H2O2 concentrations and LOX activity*

Determination of O2.− in leaf extracts was based on the ability to reduce nitroblue tetrazolium (NBT) [28]. Absorbance was measured at 580 nm and the O2.− concentration was expressed as μg g−1 DW.

H2O2 concentration in leaf extracts was colorimetrically measured as described by Mukherjee and Choudhuri [29]. Leaf samples were extracted with cold acetone to determine H2O2 levels. The intensity of yellow colour of the supernatant was measured at 415 nm. The result of H2O2 concentration was expressed as μg g−1 DW.

For 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 samples were centrifuged at 9500 rpm for 10 min. 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 [30].

LOX activity in leaf extracts was measured according to Minguez-Mosquera et al. [31] using 50 mM K–phosphate buffer (pH 6.0) containing 5 mM EDTA and 1% PVP for extraction. LOX activity was calculated following the rise in the extinction at *A*234 using an extinction coefficient of 25,000 M−1cm−1.

*2.8. N metabolism enzyme extractions and assays*

Leaves were ground at 0ºC in 50 mM KH2PO4 buffer (pH 7.5) containing 2 mM EDTA, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 30,000 *× g* for 20 min. The resulting extract was used to measure enzyme activity of nitrate reductase (NR), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH). NR assay followed the methodology of [32] Kaiser and Lewis (1984). GDH and GOGAT activities were assayed spectrophotometrically by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance [33] and Singh and Srivastava [34].

Glutamine synthetase (GS) was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis [32]. Leaves were ground at 0ºC in 50 ml maleic acid-KOH buffer (pH 6.8) containing 100 mM sucrose, 2% (v/v) β-mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000 *× g* for 20 min. The resulting extract was used to measure enzyme activity of GS. After incubation at 28ºC for 30 min, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride.

Aspartate aminotransferase (AAT) activity was assayed spectrophotometrically at 340 nm using the method published by Gonzalez et al. [35]. AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 4 mM MgCl2, 10 mM aspartic acid, and enzyme extract.

The protein concentration of the extracts was determined according to the method of Bradford [36] using bovine-serum albumin as the standard.

*2.9. Soluble AAs analysis*

Soluble AAs were extracted following the method of Bieleski and Turner [37] with some modifications. 0.1 g of fresh leaves were homogenised in 1 ml of MCW (methanol: chloroform: water, 12:5:1). 50 μl of L-2 aminobutyric acid was added as an internal standard. The mixture was centrifuged at 2,300 *× g* for 10 min. To the resulting supernatant were added 700 μl of Milli-Q water and 1.2 ml of chloroform and incubated 24 h at 4 °C. Then, the aqueous phase was obtained, which was lyophilized and the resulting extract was diluted with 0.1 M HCl. Instrumental analysis of soluble AAs was carried out using the precolumn AccQ Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). LC fluorescence analysis was performed on the Waters Acquity® UPLC System equipped with the Acquity fluorescence detector. UPLC separation was performed on the AccQ Tag Ultra column (2.1 x 100 mm, 1.7 μm) from Waters. The flow rate was 0.7 mL min-1, and the column temperature was kept at 55ºC. The injection volume was 1 µL, and the detection was set at a 266-nm excitation wavelength and a 473-nm emission wavelength. The solvent system consisted of two eluents: 1:20 Dilution of AccQ Tag Ultra eluent A concentrate and AccQ Tag Ultra eluent B.

*2.10. 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 Zn treatment (Z), the mutation (M), or the interaction of the two factors (Z \* M) 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. Plant Biomass and Zn accumulation*

In previous experiments a reduction in biomass is observed when plants are subjected to both Zn deficiency and toxicity. This reduction is due to Zn imbalances that affect crucial processes in plant physiology [1, 3]. We observed a biomass reduction around 40% in Zn deficient plants and around 50% in Zn toxicity plants in comparison to control conditions. However, these reductions were similar in both genotypes and no significant differences were observed between R-o-18 and *BraA.hma4a-3* plants regardless the Zn dose applied (Fig. 1). In other studies, when HMA4 activity is modified, diverse effects on biomass are produced depending on the transgenic mutation and the species. Thus, tomato plants expressing *AtHMA4* [12, 16] and *AhHMA4* [14] and *AtHMA4* in tobacco [38] showed lower biomass under Zn toxicity than Wt plants. However, *hma4-2* mutation in *A. thaliana* and tobacco TILLING mutations did not cause effects on growth [39]. In Verret et al. [40] study, tomato plants presented higher tolerance and the expression ofAhHMA4p1::AhHMA4 increased productivity [41].Therefore, the effect of HMA4 mutation on plant biomass depend on the specific mutation and our TILLING approach has the advantage of no negative effects on growth.

Previous experiments proved that a reduction in HMA4 activity clearly produce a decrease in Zn concentration in the shoot [11]. In addition, the effects of HMA4 activity modification depends on the Zn dose applied. Thus, in experiments with plants with higher HMA4 activity, it was observed that Zn accumulation increase in the shoot only under a moderate Zn supply [13, 14, 41]. In the present experiment, under control and Zn toxicity conditions, *BraA.hma4a-3* increased Zn accumulation in leaves presenting higher Zn concentration and total Zn accumulation (Fig. 2A-B). The higher Zn accumulation was supported by a great uptake capacity (Fig. 2C) and by a higher translocation capacity to the shoot (Fig. 2D). In contrast, under Zn deficiency conditions, Zn accumulation was similar in both genotypes (Fig. 2A-B) which means that the higher Zn accumulation capacity requires an adequate Zn supply. The optimum dose for biofortification programs would be 1 µM ZnSO4 (control conditions) because it increased the TZnA by 41% respect R-o-18 whereas Zn toxicity only increased by 11% (Fig. 2B). This is especially due to a lower ZnUtE efficiency in mutant plants under this Zn dose (Fig. 2E). *BraA.hma4a-3* presented higher translocation capacity regardless the Zn dose applied (Fig. 2D) which is reflected in lower root Zn concentration in the mutants (Fig. 2F). These results suggest that *BraA.hma4a-3* mutation provide a higher HMA4 activity and thereby, higher translocation activity.

*3.2. Mineral nutrients concentration*

Being that Zn interacts with many other elements in plants, Zn deficiency or toxicity usually alter the homeostasis of other elements such as Fe, Ca and P [6]. Indeed, Shetty et al. [42] suggested that the lower growth caused by Zn disbalances is due to lower P concentration. In contrast, in the present study we did not observe any clear alteration in mineral nutrients concentration except for Zn, under Zn deficiency or toxicity in comparison to control conditions (Table S1). Comparing between genotypes and regarding leaf macronutrients, under control conditions we did not observe significant differences between R-o-18 and mutant plants, except for an increase in Mg and N elements in mutants. Under Zn deficiency, *BraA.hma4a-3* plants registered lower N, P, S and Ca but higher K levels and Mg did not show differences. Likewise, under Zn toxicity all macronutrients were lower in mutant plants except N that was higher in mutants and K that did not show significant differences (Fig. 3A; Table S1). In spite of the reduction of some *BraA.hma4a-3* macronutrients concentration, this is not of enough magnitude to affect growth since their levels were similar to R-o-18 plants (Fig. 1). The reduction could be caused by mineral shoot transport competition. Thus, several studies proved that Zn toxicity decrease the translocation and reduce the concentrations in the shoot of N, Mg, K, P and Ca [5, 43].

Micronutrients play a key role in plant metabolism and their availability directly affects their physiological functioning and also the crop quality [44]*.* The most known effect of Zn imbalances on micronutrients concentration is its negative effect on Fe accumulation. Thus, Zn compete with Fe in the chelation during Fe uptake [45]and it reduce its accumulation in the shoot [5]. In the present experiment, *BraA.hma4a-3* showed lower Mn under all Zn doses. This is probably due to the antagonistic relationship between Zn and Mn [5]. The higher Zn transport rate to the shoot appear to reduce Mn transport to the shoot in *BraA.hma4a-3* plants although this does not appear to affect to plant growth. On the other hand, under control conditions mutant plants showed higher Fe and Cu levels. Under Zn deficiency, *BraA.hma4a-3* also presented higher Fe concentration whereas when Zn toxicity was applied, mutants presented lower Cu concentrations (Fig. 3A; Table S1). In other experiments the increment in HMA4 activity by ectopic expression of HMA4 orthologs caused the reduction in Fe accumulation [14, 16]. In contrast, *BraA.hma4a-3* plants accumulated more Fe, so they appear to overcome the antagonistic relationship between Zn and Fe and this is a positive result especially for biofortification programs.

*3.3. Chl concentration and photosynthesis*

Photosynthesis is a basic metabolic process that directly determines the biomass production and is highly sensitive to stress such as nutrients imbalances [46]. Thus, Zn accumulation interfere with Fe and Mg ions, reduce Chl concentration and alter the composition of chloroplast membranes [47]. However, Zn is necessary because the repair processes of PSII and several reactions in photosynthetic metabolism are Zn-dependent [5]. In our study, *BraA.hma4a-3* plant showed lower SPAD Chl values when were grown under optimum Zn supply, while under Zn deficiency and toxicity no differences were found between the two genotypes. Regarding total Chls we only found differences in plants grown under low Zn supply presenting the *BraA.hma4a-3* plants higher concentrations (Table 1). Chl a/b ratio is an indicator of RC/ LHCII proportion as Chl a is principally in RC and core antenna proteins whereas Chl b is in light-harvesting complexes. Thus, a higher Chl a/b usually indicates a conversion of Chl b to Chl a to maintain active RCs under stress conditions [48]. Under Zn deficiency, R-o-18 presented higher Chl a/b ratio whereas under Zn toxicity this parameter was greater in *BraA.hma4a-3* plants (Table 1). On the other hand, PIABS is a parameter derived from the Chl a fluorescence measure and indicate the in vivo PS II performance. Higher PIABS indicate higher capacity to withstand a stress [27]. PIABS values were not affected neither by *HMA4* mutation nor the Zn doses. In general, we did not observe important effects of the different Zn supply on photosynthetic pigments and performance. Likewise, *HMA4* mutation did not affect neither SPAD values nor PIABS. However, total Chls concentration increased under Zn-deficiency conditions. This increment was especially due to an increase of Chl b concentration. Conversely, under Zn toxicity conditions this ratio was higher in *BraA.hma4a-3* (Table 1) suggesting a better use of the energy and lower stress.

*3.4. Oxidative stress indicators*

Oxidative stress parameters are good indicators of plant tolerance against a stress. In the present study we assessed MDA, O2-, H2O2 concentrations and LOX activity because they are key elements in oxidative stress. An excessive ROS production causes cell damage, affects DNA, and produce lipid peroxidation [49].Zn toxicity produce oxidative stress accumulating ROS and leading to lipid peroxidation which increase membrane permeability [1, 2]. In the present experiment, both Zn deficiency and toxicity increased MDA and ROS concentrations in both genotypes in comparison to control conditions. However, in *BraA.hma4a-3* plants the values were lower than in R-o-18 plants. LOX activity correlated well with MDA concentration being also lower in *BraA.hma4a-3* grown under Zn deficiency and toxicity. Likewise, under Zn deficiency, *BraA.hma4a-3* plant presented higher O2- and H2O2 concentrations than R-o-18 plants. In contrast, under Zn toxicity H2O2 were similar in both genotypes and O2- levels were lower in *BraA.hma4a-3* plants (Table 2). In spite of the higher Zn accumulation in *BraA.hma4a-3*, these plants appear to have enhanced defenses against oxidative stress due to the lower O2- and peroxidation levels. Zn induce the expression of ROS scavenging enzymes and compounds. A higher Cu/Zn SOD activity appear to be key in a higher Zn efficiency under Zn deficiency conditions in wheat [50]. However, these defenses are ineffective under Zn deficiency since mutant plants presented higher ROS levels (Table 2).

*3.5. N Metabolism*

A direct relationship between NO3- and foliar N concentrations and plant growth has been proved [51]. On the other hand, Zn is crucial for N uptake and assimilation and hence the degree of sensitivity to Zn deficiency can be correlated with alterations in N reduction. Thus, Navarro-León et al. [4] observed that Zn deficiency reduced NO3- concentration and NR activity. However, in other experiments Zn deficiency did not produce changes in NR activity [50].In the present experiment, under Zn deficiency, we observed lower NH4+concentration (Table 3) and lower GDH activity in comparison to control conditions (Fig. 4A). Vallee and Auld [52] proved that Zn is necessary for GDH activity. On the other hand, Zn toxicity also have a detrimental effect on N metabolism. For instance, Zn toxicity also reduced NR in radish [47]. We observed a decrease in NR, and AAT activity and in NH4+ concentration when toxic Zn concentrations were applied (Table 3; Fig 4D). These results indicate a negative effect of Zn on N metabolism which could contribute to the lower biomass in comparison to control conditions.

Comparing between genotypes, *BraA.hma4a-3* presented lower NO3- concentration under Zn deficiency and higher NO3- concentration in comparison to R-o-18 when plants were grown under control and toxic Zn supplies. On the other hand, NR activity was higher under Zn deficiency in *BraA.hma4a-3* plants while it was lower under Zn toxicity and no differences were found respect NH4+ concentration. Nevertheless, total reduced N did not show differences between genotypes under Zn deficiency or toxicity (Table 3). Regarding GDH activity, it only showed differences between genotypes under Zn deficiency being lower in *BraA.hma4a-3* plants (Fig. 4A). Likewise, *BraA.hma4a-3* showed lower GS values under control and deficiency conditions but it was higher under Zn toxicity (Fig. 4C). In addition, AAT activity was higher in *BraA.hma4a-3* under both Zn deficiency and toxicity (Fig. 4D). Overall, there is no great difference between the two genotypes regarding N metabolism, except for an improved N assimilatory enzyme activities in *BraA.hma4a-3* which could contribute to a higher Zn tolerance. Navarro-León et al. [4] also observed higher N assimilatory enzyme activities in *B. oleracea* that presented higher Zn efficiency in comparison to *L. sativa* that was more Zn inefficient.

*3.5. Amino acid profile*

Several studies proved that AAs profile is affected by Zn imbalances in plants [47, 54, 55]. In the present experiment, it was observed an increase in total soluble AAs when plants were subjected to Zn deficiency in comparison to control conditions (Fig. 3B; Table S2). An stress that reduce biomass usually is accompanied by an increase in free AAs because of a decrease in the protein synthesis/degradation ratio [55]. Comparing between genotypes, *BraA.hma4a-3* registered lower concentrations of most of the AAs which reflects in a lower total AAs concentration when plants were grown under optimal Zn supply. However, we observed the opposite under Zn deficiency conditions with increments in all AAs except for Pro, Gly and Glu that did not present significant differences between genotypes and Asp that showed lower concentration. Indeed, *BraA.hma4a-3* presented a great increment in Thr and Arg concentrations (Fig. 3B; Table S2). Other studies pointed out that Zn efficient rice genotypes presented lower Asp and higher Arg levels [3, 57]. However, *BraA.hma4a-3* did not presented a higher Zn efficiency parameter (Fig. 2). On other hand, under Zn toxicity conditions, in general AAs did not differ between the two genotypes although, *BraA.hma4a-3* presented lower Pro, Thr, and Asp concentrations (Fig. 3B; Table S2).

In plants Zn is transported in the xylem join to different compounds and one of them is His[57]. His can also chelate the excess of Zn in cells under Zn toxicity [5]. This prevent Fe deficiency and ROS production [1].In *BraA.hma4a-3* the increase in His could contribute to increase Zn transport to the shoot. However, under Zn toxicity did not fulfil an important role. Indeed, any of the AAs analyzed could be responsible for a higher Zn accumulation in *BraA.hma4a-3* mutants since any of them increased its concentration at the same time under control and Zn toxicity conditions (Fig. 3B; Table S2). On the other hand, Navarro-León et al. [4] suggested that Gly accumulation could be responsible for the higher Zn uptake capacity in lettuce plants. Zn-Gly complexes increase Zn uptake and transport [58]. In the present study the increment in Gly is also observed as a result of Zn deficiency although it was similar in mutant and R-o-18 plants (Table S2).

Stressed plants accumulate certain AAs that protect against stress such as Pro. This AA contribute to a higher stress tolerance because it is involved in the adjusting of the osmotic potential, ROS detoxification, the protection of membranes and the stabilization of enzymes and proteins [59]. The relationship between Pro and Zn depend on the species assessed. For instance, under Zn deficiency Pro decreased in *Phaseolus vulgaris* [54] and increased in cabbage plants (*B. oleracea*) [53]. Under Zn toxicity, Pro decrease in *B. olearacea* and increased in lettuce, being *B. oleracea* a more tolerant species [60]. Therefore, the lower Pro in *BraA.hma4a-3* mutants (Fig. 3B; Table S2) could indicate lower stress in spite of the higher Zn concentration which also is reflected in lower oxidative stress indicators (Table 2).

**4. Conclusions**

According to the results, *BraA.hma4a-3* did not cause any negative effect on plant biomass as *BraA.hma4a-3* presented similar biomass reduction than R-o-18 under Zn deficiency and toxicity. However, *BraA.hma4a-3* did increase Zn accumulation in leaves but only under an adequate Zn supply. Results suggest a possible increase HMA4 activity that could provide higher Zn translocation capacity. With respect other nutrients *BraA.hma4a* appear to accumulate lower macronutrients and Mn under Zn toxicity and deficiency although it accumulated more Fe. Regarding stress tolerance parameters and N metabolism, *BraA.hma4a* did not produce relevant alterations when control Zn dose was applied. In addition, under Zn toxicity conditions parameters suggest a better Zn stress tolerance as presented a higher Chl a/b ratio, lower oxidative stress indicators, higher N assimilation activities and lower Pro concentration. Briefly, the obtained results point to *BraA.hma4a-3* as a useful mutant to increase Zn accumulation that could be used in biofortification and phytoremediation programs.

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**Table 1** Photosynthetic indicators in R-o-18 and *BraA.hma4a-3* genotypes subjected to three different Zn doses

Values are means (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 (\*\*\*).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | SPAD chlorophyll | Total Chls mg g-1 FW | Chl a/b | PIABS |
| Control | R-o-18 | 42.05 | 32.34 | 2.32 | 10.68 |
|  | *BraA.hma4a-3* | 36.42 | 32.53 | 2.29 | 9.23 |
|  | *p*-value | \*\* | NS | NS | NS |
|  | LSD0.05 | 3.04 | 1.80 | 0.03 | 1.47 |
| Zn deficiency | R-o-18 | 35.86 | 32.53 | 2.34 | 9.40 |
| *BraA.hma4a-3* | 35.05 | 37.02 | 2.20 | 9.27 |
|  | *p*-value | NS | \*\*\* | \*\*\* | NS |
|  | LSD0.05 | 2.71 | 1.75 | 0.06 | 2.40 |
| Zn toxicity | R-o-18 | 40.39 | 33.60 | 2.29 | 9.63 |
|  | *BraA.hma4a-3* | 41.20 | 32.61 | 2.42 | 8.67 |
|  | *p*-value | NS | NS | \*\*\* | NS |
|  | LSD0.05 | 4.74 | 1.02 | 0.03 | 1.07 |
| Analysis of variance | | | | | |
| Zn treatment (Z) |  | \*\*\* | \*\*\* | \*\*\* | NS |
| Mutation (M) |  | NS | \*\* | \* | NS |
| Z x M |  | \* | \*\*\* | \*\*\* | NS |
| LSD0.05 |  | 1.99 | 0.86 | 0.02 | 0.90 |

**Table 2** Oxidative stress indicatorsin R-o-18 and *BraA.hma4a-3* genotypes subjected to three different Zn doses

Values are means (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 (\*\*\*).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | MDA  (μM g-1 FW) | LOX  (ΔAbs mg prot-1min-1) | O2-  (μg g-1 FW) | H2O2  (μg g-1 FW) |
| Control | R-o-18 | 4.34 | 0.08 | 5.11 | 23.96 |
|  | *BraA.hma4a-3* | 4.38 | 0.08 | 4.51 | 15.95 |
|  | *p*-value | NS | NS | NS | \*\* |
|  | LSD0.05 | 0.57 | 0.02 | 1.03 | 4.82 |
| Zn deficiency | R-o-18 | 6.25 | 0.08 | 6.96 | 27.14 |
| *BraA.hma4a-3* | 5.59 | 0.06 | 10.36 | 42.08 |
|  | *p*-value | \* | \* | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.50 | 0.02 | 0.91 | 5.12 |
| Zn toxicity | R-o-18 | 6.75 | 0.09 | 6.41 | 30.52 |
|  | *BraA.hma4a-3* | 4.86 | 0.06 | 4.40 | 35.05 |
|  | *p*-value | \*\*\* | \* | \*\*\* | NS |
|  | LSD0.05 | 0.98 | 0.02 | 0.58 | 4.83 |
| Analysis of variance | | | | | |
| Zn treatment (Z) |  | \*\*\* | NS | \*\*\* | \*\*\* |
| Mutation (M) |  | \*\*\* | \*\* | \*\*\* | \*\*\* |
| Z x M |  | \*\*\* | NS | \*\*\* | \*\*\* |
| LSD0.05 |  | 0.39 | 0.01 | 0.39 | 2.70 |

**Table 3** N forms and NR activityin *BraA.hma4a-3* and R-o-18 supplied with three different Zn doses

Values are means (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 (\*\*\*).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | NO3-  (mg g-1 DW) | NR  (µM NO2 mg prot-1 min-1) | NH4+  (mg g-1 DW) | Total Reduced N (mg g-1 DW) |
| Control | R-o-18 | 61.99 | 2.96 | 2.83 | 44.89 |
|  | *BraA.hma4a-3* | 84.69 | 2.70 | 2.93 | 54.31 |
|  | *p*-value | \* | NS | NS | \* |
|  | LSD0.05 | 18.67 | 0.45 | 0.14 | 6.94 |
| Zn deficiency | R-o-18 | 107.12 | 4.04 | 2.51 | 56.77 |
| *BraA.hma4a-3* | 89.44 | 6.96 | 2.44 | 52.62 |
|  | *p*-value | \* | \*\*\* | NS | NS |
|  | LSD0.05 | 14.15 | 0.82 | 0.12 | 7.90 |
| Zn toxicity | R-o-18 | 69.81 | 1.13 | 2.58 | 51.80 |
|  | *BraA.hma4a-3* | 88.53 | 0.40 | 2.52 | 53.47 |
|  | *p*-value | \* | \*\*\* | NS | NS |
|  | LSD0.05 | 17.42 | 0.15 | 0.20 | 4.83 |
| Analysis of variance | | | | | |
| Zn treatment (Z) |  | \*\*\* | \*\*\* | \*\*\* | NS |
| Mutation (M) |  | NS | \*\*\* | NS | NS |
| Z x M |  | \*\* | \*\*\* | NS | \* |
| LSD0.05 |  | 8.92 | 0.30 | 0.08 | 3.53 |

**Fig. 1** Leaf biomass in *BraA.hma4a-3* mutants and R-o-18 plants grown under three Zn2+ doses. Columns are mean ± SE (n = 9) and differences between means were compared by Fisher´s least-significance test (LSD; P=0.05).

**Fig. 2** Effect of Zn dose and *BraA.hma4a-3* mutation on foliar (A) and root (B) Zn concentrations, DC (C), TZnA (D), ZnUtE (E) and ZnUpE (F). Columns are mean ± SE (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 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

m

**Fig. 3** Heat map showing the effect of Zn dose and *BraA.hma4a-3* mutation on leaf nutrients (A) and leaf free amino acids concentrations (B). Color scale corresponds to the logarithmic transformation (log10) of measured values (higher levels are shown in red, lower levels in blue and intermediate levels in white colors). The names of parameters are indicated on the top, genotype and Zn doses on the right side of clusters. For interpretation of the references to color in this figure legend, the reader is referred to Supplementary tables.

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**Fig. 4** Effect of Zn dose and *BraA.hma4a-3* mutation on N assimilation enzyme activities: GDH (A), GS (B), GOGAT (C) and AAT (D). Values are expressed as means ± standard error (n=9). Columns are mean ± SE (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 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

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