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Differential Aquaporin Response to Distinct Effects of Two Zn Concentrations after Foliar Application in Pak Choi (*Brassica rapa* L.) Plants

Hamideh Fatemi [1], Chokri Zaghdoud [2], Pedro A. Nortes [3], Micaela Carvajal [4] and Maria del Carmen Martínez-Ballesta [4,5,*]

1 Department of Horticulture, University of Mohaghegh Ardabili, Ardabil 56199-13131, Iran; ha.fatemi@yahoo.com
2 Dry Land Farming and Oasis Cropping Laboratory, Institute of Arid Regions of Medenine, University of Gabes, Medenine 4119, Tunisia; chokri_zaghdoud@yahoo.fr
3 Departament of Irrigation, Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), P.O. Box 164, 30100 Espinardo (Murcia), Spain; panortes@cebas.csic.es
4 Department of Plant Nutrition, Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), P.O. Box 164, 30100 Espinardo (Murcia), Spain; mcarvaja@cebas.csic.es
5 Department of Agronomy Engineering, Universidad Politécnica de Cartagena, Paseo Alfonso XIII, 48, 30203 Cartagena (Murcia), Spain
* Correspondence: mcarmen.ballesta@upct.es; Tel.: +34-968-325457

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Abstract: Zinc (Zn) is considered an essential element with beneficial effects on plant cells; however, as a heavy metal, it may induce adverse effects on plants if its concentration exceeds a threshold. In this work, the effects of short-term and prolonged application of low (25 μM) and high (500 μM) Zn concentrations on pak choi (*Brassica rapa* L.) plants were evaluated. For this, two experiments were conducted. In the first, the effects of short-term (15 h) and partial foliar application were evaluated, and in the second a long-term (15 day) foliar application was applied. The results indicate that at short-term, Zn may induce a rapid hydraulic signal from the sprayed leaves to the roots, leading to changes in root hydraulic conductance but without effects on the whole-leaf gas exchange parameters. Root accumulation of Zn may prevent leaf damage. The role of different root and leaf aquaporin isoforms in the mediation of this signal is discussed, since significant variations in *PIP1* and *PIP2* gene expression were observed. In the second experiment, low Zn concentration had a beneficial effect on plant growth and specific aquaporin isoforms were differentially regulated at the transcriptional level in the roots. By contrast, the high Zn concentration had a detrimental effect on growth, with reductions in the root hydraulic conductance, leaf photosynthesis rate and Ca^{2+} uptake in the roots. The abundance of the PIP1 isoforms was significantly increased during this response. Therefore, a 25 μM Zn dose resulted in a positive effect in pak choi growth through an increased root hydraulic conductance.

Keywords: aquaporin; *Brassica rapa*; gas exchange parameters; growth; root hydraulic conductance; zinc

1. Introduction

Zinc (Zn) is an essential element for plants and animals [1]. In plants, as a co-factor of at least 300 metalloenzymes [2], Zn plays an important role in protein structure [3]. Another important role of Zn is the scavenging, for oxidation, of reactive oxygen species (ROS) in cells under normal and stress conditions [4]. However, when the Zn concentration exceeds a threshold in the cells, it can induce stress

in the plants. Thus, at high concentrations, an important decrease in some physiological functions in plants—such as photosynthesis, respiration and reproductive performance—has been observed [5–7].

Zinc fertilization has beneficial effects for crops, since this microelement is involved in the synthesis of tryptophan, a precursor of indole acetic acid (IAA), responsible for growth stimulation [8]. However, shoot growth was significantly reduced at Zn concentrations above 25 mg L^{-1} in nutrient solution or above 170 mg kg^{-1} in the soil. The sensitivity to Zn toxicity differed among other crops, being sensitivity higher in celery > Chinese cabbage > pak choi. But pak choi can accumulate high levels of Zn in their edible parts with negative impact for human health [9]. The threshold between optimal Zn dose for plant development and the amount in which Zn leads to plant toxicity symptoms or human damage needs to be study in this new crop. However, the application of Zn to the soil results less efficient than foliar supply, due to soil and roots limitations and the poor Zn mobility in the phloem [10]. For this reason, repeated foliar sprays of Zn are frequent during vegetables cultivation.

The aquaporins (AQPs) are a family of small (24–30 kDa), pore-forming, integral membrane proteins, and are involved in the transport of water and small solutes such as urea, CO_2, ammonia, silicic acid and boric acid [11–13]. Plant AQPs are subdivided into plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and uncharacterised intrinsic proteins (XIPs) [14–16]. AQPs are responsible for 75%–95% of water passage in the plant [17–19] and are the target of many heavy metals and ions within the cells, including Zn. It has been shown that exposure to heavy metals (Zn, Cd or Cu) decreases root and leaf AQPs expression, in order to avoid water loss and maintain the plant water status [20,21]. Furthermore, in *Mesembryanthemum crystallinum*, exposure to 500 µM $ZnSO_4$ caused differential changes in water status, depending on the duration of the treatment [22]. Thus, reductions in the root to stem water flow, leaf water content, relative water content in leaf tissues, transpiration rate, leaf osmotic potential and expression of AQP genes in the root and leaf tissues were observed after 24 and 72 h of Zn treatment. However, the expression of the *PIP1;4* isoform was unaffected, being continuous and stable, while the expression of other AQP genes—such as those of the plasma membrane (*McPIP1;1; McPIP2;1; McPIP2;3*) or tonoplast AQPs (*McTIP1;2 and McTIP2;2*)—was decreased, showing the roles of different AQPs isoforms in the heavy metal response as key elements with specific involvement. Additionally, Gitto and Fricke [21] reported the effects of 0.1 and 1 mM Zn on water relations in barley (*Hordeum vulgare* L.) plants in relation to AQPs expression. They found that the decline in expression of three AQPs (*HvPIP1;2*, *HvPIP2;4*, *HvPIP2;5*) was stronger (46%–77%) with the application of 0.1 mM Zn than with 1 mM Zn (20%–50%); the simultaneous reductions in plant transpiration rate and root hydraulic conductivity (Lpr), of 24% (0.1 mM Zn) and 58% (1 mM Zn), respectively, would have limited the transport of Zn to the shoot to avoid major toxicity.

Zinc affects not only AQPs expression, but also their functionality, through the binding of the metal to the thiol groups of the protein, inducing a conformational change in the structure and the closure of the pore [23]. Additionally, heavy metals have been found to diminish water transport in *Actinidia deliciosa* protoplasts [24]. Yukutake et al. [25] showed that the water permeability mediated by AQPs was rapidly and reversibly regulated by dynamic changes in the intracellular Zn^{2+} concentration linked to a disturbed cellular redox state. In addition, there is evidence that Zn may be an integral part of biomembranes, and thus required for the stability and control of the lateral mobility of membrane molecules [26].

It has been postulated that a hydraulic signal sent from the root to the shoot could be responsible for the reduction in leaf turgor after root abiotic stress perception [27]. Similarly, the disruption of the water status during heavy metal stress could be a consequence of a decreased number of stomata and/or their closure for water preservation. Plants must adjust their whole water status to the constant demand of the aerial parts and AQPs may play an important role in the hydraulic balance [21]. The number of stomata was reduced by heavy metals such as copper, cadmium and Zn in *Phaseolus vulgaris* L. [28] and *Beta vulgaris* L. [29]. In Zn-treated plants, the stomata were round in shape and smaller than in control plants [29]. Generally, heavy metals disrupt water flow not only by reductions in stomatal conductance

(Gs), but also by reducing the flow of solutes; this can be a major cause of heavy metals toxicity and the decline in plant biomass at higher concentrations. Interestingly, decreased Gs values have also been reported under Zn deficiency [30], in chickpea plants; their Gs increased when treated with Zn (2.5 μg g^{-1} soil) and they were able to maintain membrane integrity [30]. The values of gas exchange parameters were also increased after Zn application in cotton (*Gossypium hirsutum* L.), but intercellular CO_2 decreased in Zn-treated plants, compared to controls [31].

Pak choi (*Brassica rapa* L. ssp. *chinensis*), also known as Chinese cabbage, is a popular leafy vegetable, grown and consumed worldwide. It is an annual crop that has optimal growth at temperatures ranging from 15 to 20 °C [32]. Although this vegetable is cultivated mainly in Asia, it could be grown in areas of Central Europe from spring to winter, due to its short vegetative growth period and lack of thermal requirements. Pak choi is not frequently found on supermarket shelfs, opening new perspectives for small producers [33].

Pak choi is well known to be tolerant of heavy metals such as Cd [34] and Zn [35], and it accumulates metal ions mainly in the leaves. In addition, the involvement of calcium (Ca) ions in its Zn tolerance has been reported [36]. Related to this, coordinated increases in Zn and Ca accumulation have been described in *Silene maritima* L., the tolerance of the Zn-tolerant population being associated with the tissue Ca levels and its presence in the leaf tissues [37].

Although the short- and long-term effects of heavy metals on plant water transport have been reported in different works [38,39], as well as the effects of low and high Zn concentrations [21], there are no reports describing the short- and long-term, concentration-dependent effects of Zn application in pak choi plants. In spite that pak choi plants may accumulate higher amounts of metals in their leaves, the adverse effects of these elements at molecular level are not well documented and often poorly understood, in particular those related to the changes in root water transport properties.

Thus, because of the importance of the Zn concentration and the treatment duration regarding the effects on plant water relations and their regulation by distinct AQPs isoforms, the specific aim of this work was to study: (1) The effect of the long-term application of Zn at two concentrations on the water status of pak choi plants, in order to identify the target AQPs isoforms and evaluate their differential responses in the beneficial or toxic effects that were induced in an ion-concentration-dependent way; and (2) the effect of the short-term application of Zn at two concentrations, in order to compare the water balance response with that under long-term Zn application and discern the hydraulic signals moving from shoot to root, with the involvement of AQP isoforms in the signalling process.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seeds of pak choi (*Brassica rapa* L. ssp. *chinensis*) were pre-hydrated with de-ionised water and aerated continuously for 24 h. After this, the seeds were germinated in vermiculite, in the dark at 28 °C, for 2 days. They were then transferred to a controlled-environment chamber with a 16 h light and 8 h dark cycle, and temperatures of 20 and 15 °C and relative humidity of 60% and 80%, respectively. Photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1} was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Jena, Germany and Sylvania F36 W/GRO, Manchester, NH, USA) and metal halide lamps (OsramHQI, T 400 W, Berlin, Germany). After 5 days, the seedlings were placed in 15 L containers with continuously-aerated Hoagland nutrient solution [40].

2.2. Experimental Design

Two types of experiment were conducted. In the first, after one week of growth, a fixed amount of solution (18 mL per 6 plants container) of low (25 μM) and high (500 μM) Zn concentration (as $ZnSO_4$) was applied by foliar spraying during 15 days (long-term application), three times in total (1, 7 and 14 days before harvesting). Zn was applied using a 1 mL L^{-1} Tween-20 spraying solution allowing leaf spray retention and an alumni paper section under each spray leaf was used in order to avoid the

sprayed Zn to reach the root nutrient solution. Plants were 21 days-old when they were harvested. Distilled water spraying was used as the control.

In the second experiment, after 20 days of plant growth, low (25 μM) and high (500 μM) Zn concentrations (as $ZnSO_4$) were applied by foliar spraying to half of the leaves (+F), leaving the other half without Zn spraying (−F). After 15 h (short-term application) the 21-day-old plants were harvested for measurements. Leaves and roots were separated for determinations in both experimental procedures. The measurements were made in the middle of the photoperiod in order to obtain the highest values for gas exchanges parameters and all samples were collected at this time for the rest of determinations.

2.3. Root Hydraulic Conductance

The root hydraulic conductance (L_0) of the plants was measured by pressurising the roots in a Scholander pressure chamber (UGT: Umwelt-Geräte-Technik GmbH, Freising-Weihenstepha, Germany), as described in Javot et al. [41]. The aerial parts of the plant were removed and the freshly-excised roots were inserted into the pressure chamber, in a plastic tube with the same nutrient solution used for their growth. A gradual increase in pressure (from 0.1 to 0.4 MPa) was applied to the detached roots. The sap that accumulated in this pressure range during a certain time, according to the treatment, was collected in a graduated glass micropipette. The sap flow (Jv) was expressed in mg g (root fresh weight)$^{-1}$ h^{-1} and plotted against pressure (MPa), the slope being the L_0 value in mg g (root fresh weight)$^{-1}$ h^{-1} MPa^{-1}.

2.4. Gas Exchange Measurements

A LI-6400XT photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA), equipped with a LI-6400-40 Leaf Chamber Fluorometer (Li-Cor, Inc., Lincoln, NE, USA) and a LICOR 6400-01 CO_2 injector (Licor Bioscience, Lincoln, NE, U.S.A.), was used to measure the net photosynthetic rate (A), stomatal conductance (Gs) and leaf transpiration (T). Leaf gas exchange was measured in a 2 cm^2 leaf cuvette. During these measurements, the air CO_2 concentration was controlled using the injection system and compressed CO_2-cylinders with a CO_2 concentration of 400 μmol mol^{-1} CO_2. Measurements were made at a PAR of 500 μmol m^{-2} s^{-1}, and at ambient air temperature and relative humidity. The air flow was set to 400 μmol s^{-1}. The third fully-expanded leaf was chosen for the analysis, after 15 h or 15 d of treatment. The measurements were made in the middle of the photoperiod in order to obtain the highest values.

2.5. RNA Extraction and Reverse Transcription

Total RNA was extracted from fully-expanded leaves of both, short and long-term treated plants and they were frozen and ground to a fine powder in liquid nitrogen using a prechilled mortar and pestle. The ground tissues were stored at −80 °C until use. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Contaminated DNA from samples was removed with DNase I, using the DNA-free Kit (Ambion, Applied Biosystems, Austin, TX, USA), and the RNA concentration was quantified with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA USA). The extracted RNA was stored at −80 °C until use.

cDNA was synthesised from 2 mg of total RNA, using M-MLV reverse transcriptase from the RETROscript Kit (Ambion, Applied Biosystems, Austin, TX, USA). Reverse transcription was carried out with heat denaturation of the RNA, according to the manufacturer's instructions.

2.6. Quantitative Real-Time PCR (QRT-PCR) Analyses

To compare the expression of *PIP1* and *PIP*2 under different treatments, QRT-PCR analyses were performed as described previously [42], in an Applied Biosystems 7500 Real-Time PCR system (ThermoFisher Scientific S.L, Madrid, Spain).

Gene-specific primers of *Brassica rapa* L. ssp. *pekinensis* genes [43] were used for different PIP isoforms. The primers and the lengths of the amplicons are described in Supplementary Table S1. *B. rapa* var. *italica*, 18s ribosomal RNA [43] was used as the reference gene for standardisation of each sample. After denaturation at 95 °C for 10 min, amplification occurred in a two-step procedure: 15 s of denaturation at 95 °C and 1 min of annealing and extension at 60 °C for 40 cycles, followed by a dissociation stage. Data collection was carried out at the end of each round in step 2. These conditions were used for both target and reference genes and the absence of primer–dimers was checked in controls lacking templates. The amplifications were performed on three independent samples for each treatment (biological replicates) and triplicate reactions were carried out for each sample (technical replicates) in 96-well plates. The transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method [44]. Standard curves (log of the cDNA dilution vs. Ct) using serial 10-fold dilutions of cDNA were built for each pair of selected primers, obtaining 95% PCR efficiency, corresponding to a slope of −3.44.

2.7. Zinc and Calcium Tissue Analyses

Zinc and Ca were analysed in the oven-dried root and leaf tissues (ca. 100 mg DW). The samples were digested, after HNO_3-H_2O_2 (2:1) addition [45], in a microwave oven (CEM Mars Xpress, North Carolina, USA) and analysed by ICP spectrometry (Iris Intrepid II, Thermo Electron Corporation, Franklin, TN, USA).

2.8. Statistical Analysis

The data were analysed statistically, using the SPSS 13.0 software package (IBM, Armonk, NY, USA), by analysis of variance (ANOVA) and by Tukey's test. Significant differences were determined at $p < 0.05$.

3. Results

3.1. Plant Biomass

A preliminary experiment was carried out in order to determine the effect of different foliar-supplied Zn concentrations on biomass, which allowed the selection of the appropriate Zn concentrations for the subsequent experiments. Plant growth was significantly influenced by long-term (15 days) foliar Zn fertilisation, depending on the concentration (Table 1). Thus, at 25 µM Zn, a marked increase in the total plant fresh (FW) and dry (DW) weights (28.48% and 11.85%, respectively) was observed, compared to the controls. By contrast, significant reductions in these parameters were found at 500 µM Zn (30.62% and 25.69% in plant FW and DW, respectively). At 50 and 100 µM Zn there were no significant changes in the fresh and dry weights with regard to the control and at 1 mM Zn an extreme toxic effect in the plants was observed. Therefore, 25 and 500 µM Zn were chosen as low and high concentrations, respectively, for foliar applications in the short and long term (Figure 1).

Table 1. Effect of long-term exposure to different zinc concentrations on the biomass of pak choi (*Brassica compestris*). Values are means ± SE ($n = 5$).

Zinc Treatments	Shoot Fresh Weight	Root Fresh Weight	Shoot Dry Weight	Root Dry Weight
Control	77.87 ± 5.23b	14.71 ± 1.25b	5.51 ± 0.51b	0.99 ± 0.15a
25 µM	98.83 ± 4.74a	20.12 ± 1.46a	6.02 ± 0.57a	1.25 ± 0.10a
50 µM	66.06 ± 5.09cb	11.08 ± 0.77cb	5.10 ± 0.54cb	0.72 ± 0.08b
100 µM	69.00 ± 5.70cb	11.21 ± 1.38cb	5.28 ± 0.26b	0.72 ± 0.11b
500 µM	54.32 ± 3.08c	9.91 ± 0.47c	4.19 ± 0.20c	0.64 ± 0.08b
1 mM	41.89 ± 4.82c	8.83 ± 1.13c	3.74 ± 0.44d	0.54 ± 0.10c

The different letters indicate significant differences among treatments according to Duncan's test ($p < 0.05$).

Figure 1. Pak choi plants treated with Zn (0, 25 and 500 µM).

3.2. *Effect of Zn on Root Hydraulic Conductance (L_0)*

After short-term (15 h) partial foliar application of Zn, the behaviour of L_0 at the low (25 µM) concentration was the opposite of that at the high (500 µM) concentration (Figure 2A), the values being significantly increased (35.16%) and decreased (25.13%), respectively, compared to control plants. In the long-term foliar Zn application, a significant effect on L_0 was only recorded at the high concentration, with the values being lower than those of the short-term application (the reduction reached 57.51%, relative to the controls) (Figure 2B).

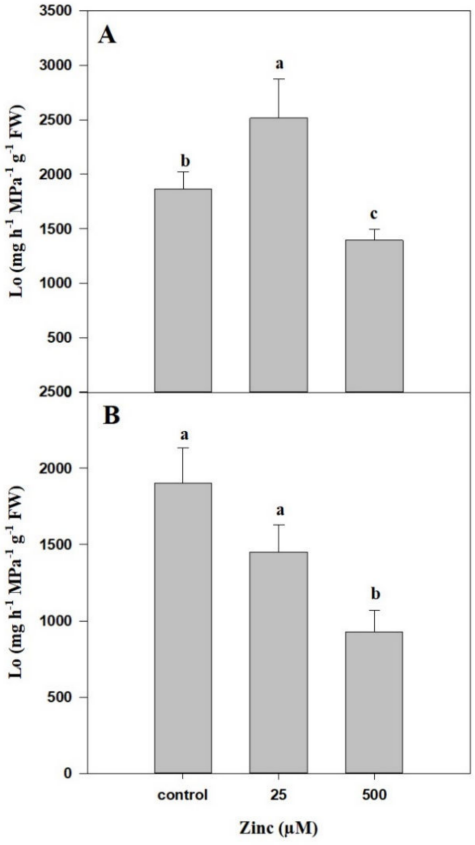

Figure 2. Effect of zinc concentration (0, 25 and 500 µM) on root hydraulic conductance (L_0) of pak choi plants, after foliar application. (**A**) Short-term experiment (15 h) in which half of the leaves were sprayed. (**B**) Long-term experiment (15 days). Values are means ± SE (n = 5). Bars with different letters represent significant ($p < 0.05$) differences after ANOVA and an LSD (Least significant difference) test.

3.3. Effect of Zn on Gas Exchange Parameters

At both Zn concentrations, the stomatal conductance (Gs), transpiration rate (E) and net photosynthetic rate per unit leaf area (A) remained without significant changes after the short-term treatment, in both types of leaf (with and without foliar Zn spraying) (Figure 3A–C). After the long-term treatment, Gs was decreased (42.46%) at 500 µM $ZnSO_4$, whereas A was increased and decreased at 25 and 500 µM, respectively, but not E, relative to untreated plants (Figure 3D–F).

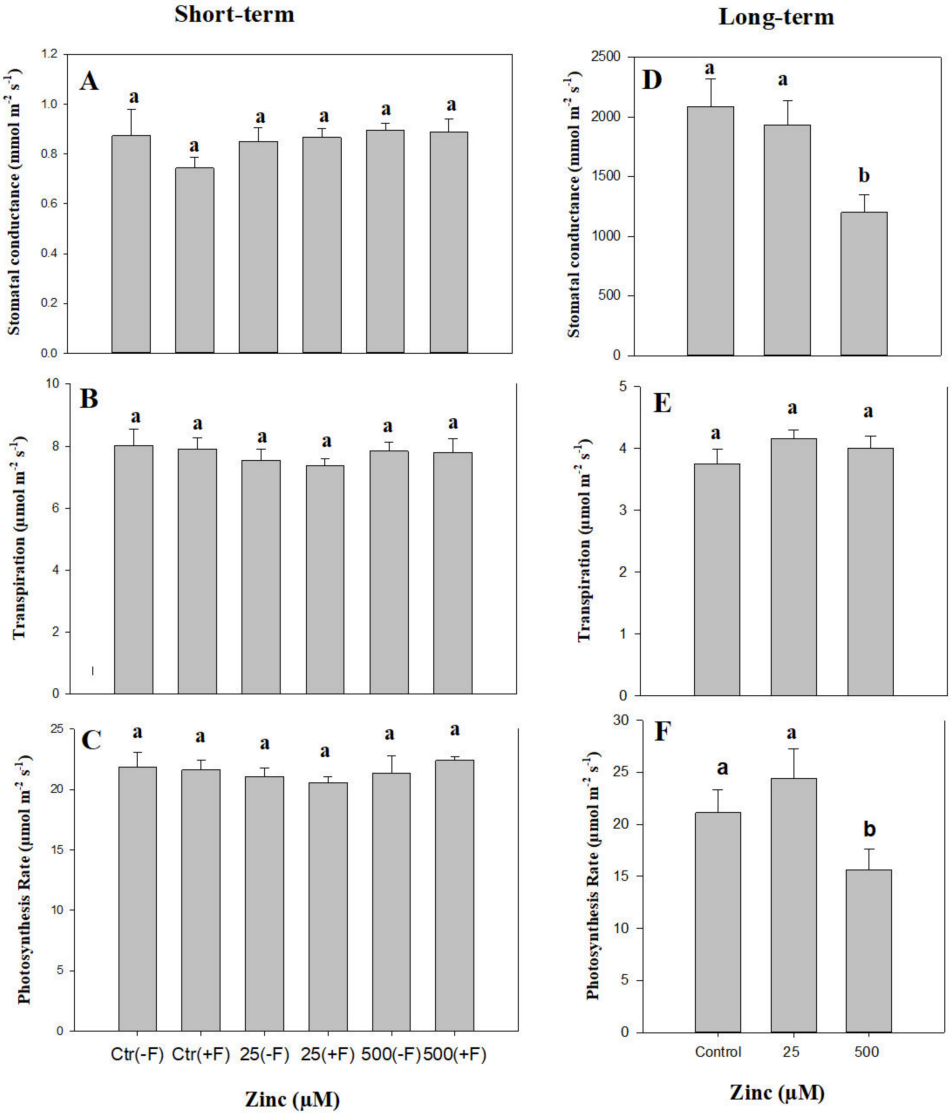

Figure 3. Effect of zinc concentration (0, 25 and 500 µM) on gas exchange parameters of pak choi plants. After short-term application: (**A**) Stomatal conductance, (**B**) transpiration, (**C**) photosynthesis rate, where –F represents the measurements in the leaves that were not sprayed and +F represents the measurements in the leaves that were sprayed. After long-term application: (**D**) Stomatal conductance, (**E**) transpiration, (**F**) carbon assimilation. Values are means ± SE (n = 5). Bars with different letters represent significant ($p < 0.05$) differences after ANOVA and an LSD (Least significant difference) test.

3.4. Tissue Contents of Zn and Ca

Macro and micro nutrients were determined in both experiments. However, in addition to Zn, only Ca showed significant differences between treatments.

After 15 h of the partial foliar Zn treatment, the Zn^{2+} content in leaves showed a similar, significant increase at both Zn concentrations, compared to control plants, the values being significantly lower in

non-sprayed leaves than in sprayed ones (about 2.2- and 2.90-fold at 25 and 500 µM Zn, respectively) (Figure 4A). However, in roots the Zn^{2+} content significantly increased with the spray concentration of Zn; thus, at 500 µM $ZnSO_4$ this increase reached 2.25-fold relative to the control value (Figure 4B). A pattern of variation similar to that of Zn was observed for the leaf Ca content following the short-term fertilisation (Figure 4C), without differences between the control plant leaves and the non-sprayed leaves of the Zn-treated plants. In roots, the Ca content was significantly increased (by 15.88%) and decreased (by 20.97%) at 25 and 500 µM Zn, respectively, after 15 h of treatment (Figure 4D).

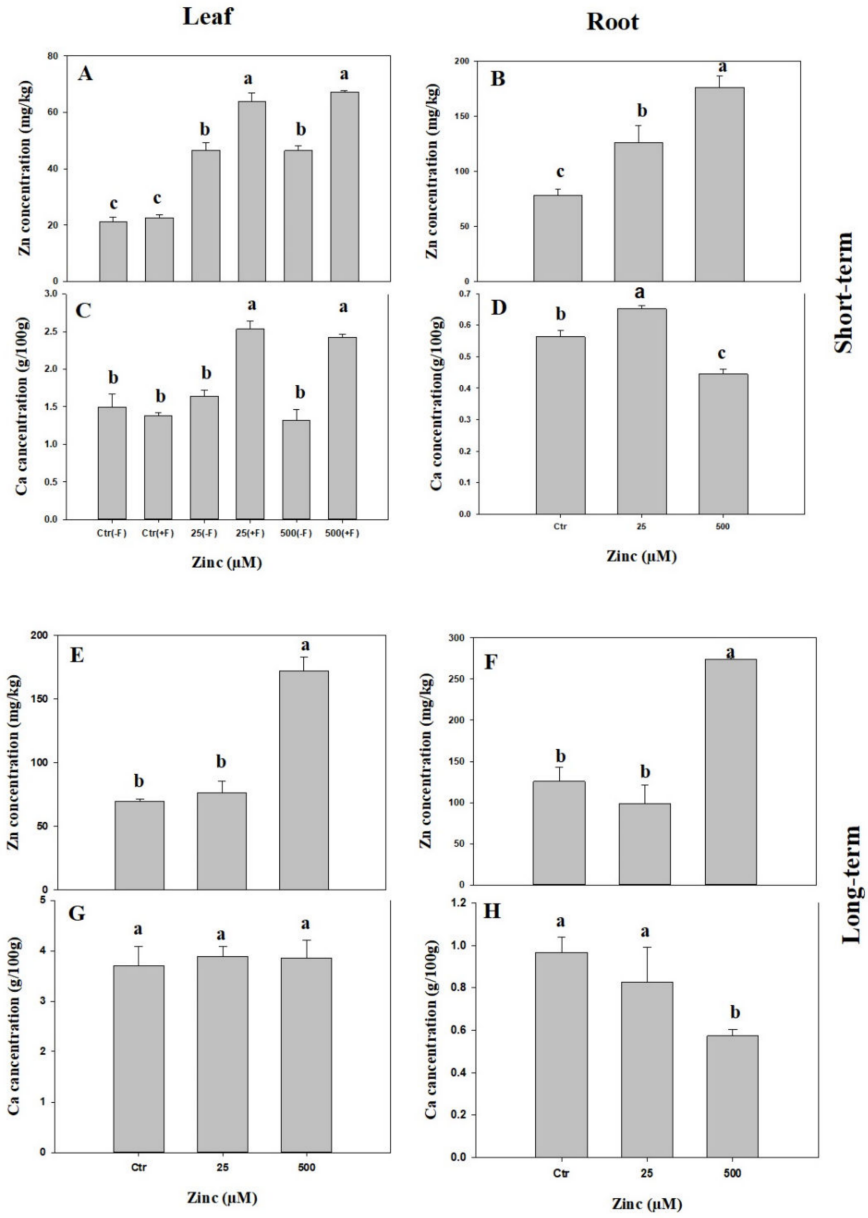

Figure 4. Effect of zinc concentration (0, 25 and 500 µM) on Zn and Ca concentrations of pak choi plants. After short-term application: (**A**) Zn concentration in leaf, (**B**) Zn concentration in root, (**C**) Ca concentration in leaf, (**D**) Ca concentration in root, where –F represents the measurements in the leaves that were not sprayed and +F represents the measurements in the leaves that were sprayed. After long-term application: (**E**) Zn concentration in leaf, (**F**) Zn concentration in root, (**G**) Ca concentration in leaf, (**H**) Ca concentration in root. Values are means ± SE (n = 5). Bars with different letters represent significant ($p < 0.05$) differences after ANOVA and an LSD (Least significant difference) test.

In the long-term study the Zn content in the leaves and roots was significantly increased only by the highest Zn concentration, increasing 2.47- and 2.18-fold in leaves and roots, respectively, relative to the controls (Figure 4E,F). Under these conditions, the Ca content was maintained in the leaves, but decreased in the roots (by 40.77%), at 500 µM Zn (Figure 4G,H).

3.5. PIP1 and PIP2 Isoforms Expression in Root and Leaf Tissues

Differences between plant organs in the PIP1 and PIP2 gene expression patterns due to Zn exposure were observed, depending on the experiment duration.

After short-term partial foliar Zn spraying only the highest Zn concentration (500 µM) significantly increased the transcript levels of the aquaporins PIP1;1 and PIP1;2 in leaves (3.71- and 2.42-fold, respectively) (Figure 5A), and of PIP1;4 in roots (3.45-fold), above the control levels (Figure 5B). The changes in these isoforms were specific to the toxic Zn concentration. In addition, while at both Zn concentrations, 25 and 500 µM, the expression levels of PIP2;1 and PIP2;5 in leaves were increased and that of PIP2;2 in roots was decreased, different behaviour of the expression pattern regarding the low and high Zn concentrations was observed for PIP2;2 in leaves and PIP2;1, PIP2;3 and PIP2;7 in roots (Figure 5C,D).

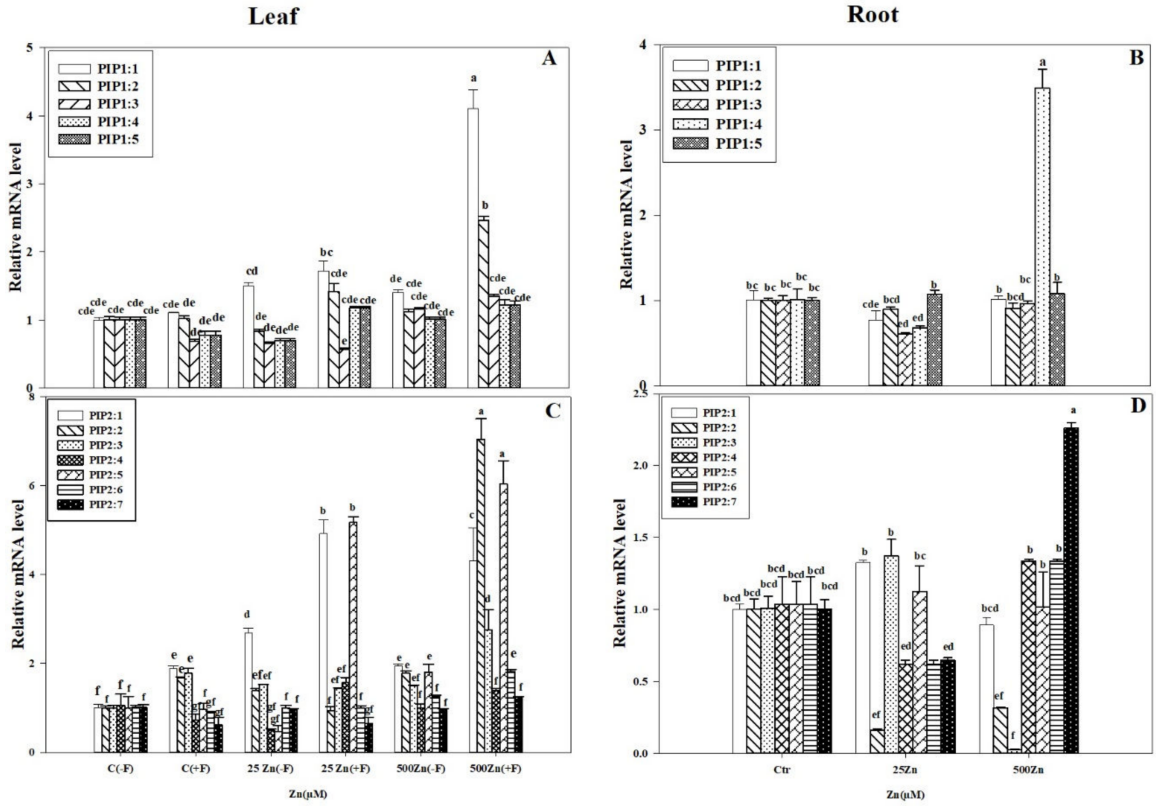

Figure 5. Relative expression level of aquaporin isoforms after short-term application of Zn: (**A**) PIP1 in root, (**B**) PIP1 in leaf, (**C**) PIP2 in root, (**D**) PIP2 in leaf, determined by Q-RT-PCR (quantitative real time-polymerase chain reaction) in control plants and plants treated with 25 or 500 µM Zn. In leaf tissues, gene expression was determined in both non-sprayed (−F) and sprayed (+F) leaves. Mean values and standard errors are shown (n = 3). Bars with different letters represent significant ($p < 0.05$) differences after ANOVA and an LSD test.

The plant hydraulic regulation after long-term foliar Zn spraying showed the implication of other aquaporins isoforms. Thus, in roots, while the PIP1;1 isoform remained up-regulated only at 500 µM Zn (1.84-fold), down-regulation of PIP1;3 and PIP2;4 (1.64-fold) was observed only at 25 µM Zn, relative to the levels of control plants (Figure 6A,B). Interestingly, the expression levels of the

PIP2;6 and PIP2;7 isoforms were the ones most significantly affected by long-term Zn exposure, in both plant organs, without differences between the low and high Zn doses (Figure 6C,D). At 500 µM Zn, PIP2;6 and PIP2;7 were up-regulated, by 4.31- and 2.57-fold, respectively, in leaves; whereas, in roots, they were decreased and increased, respectively, 50- and 5.60-fold, compared to the controls.

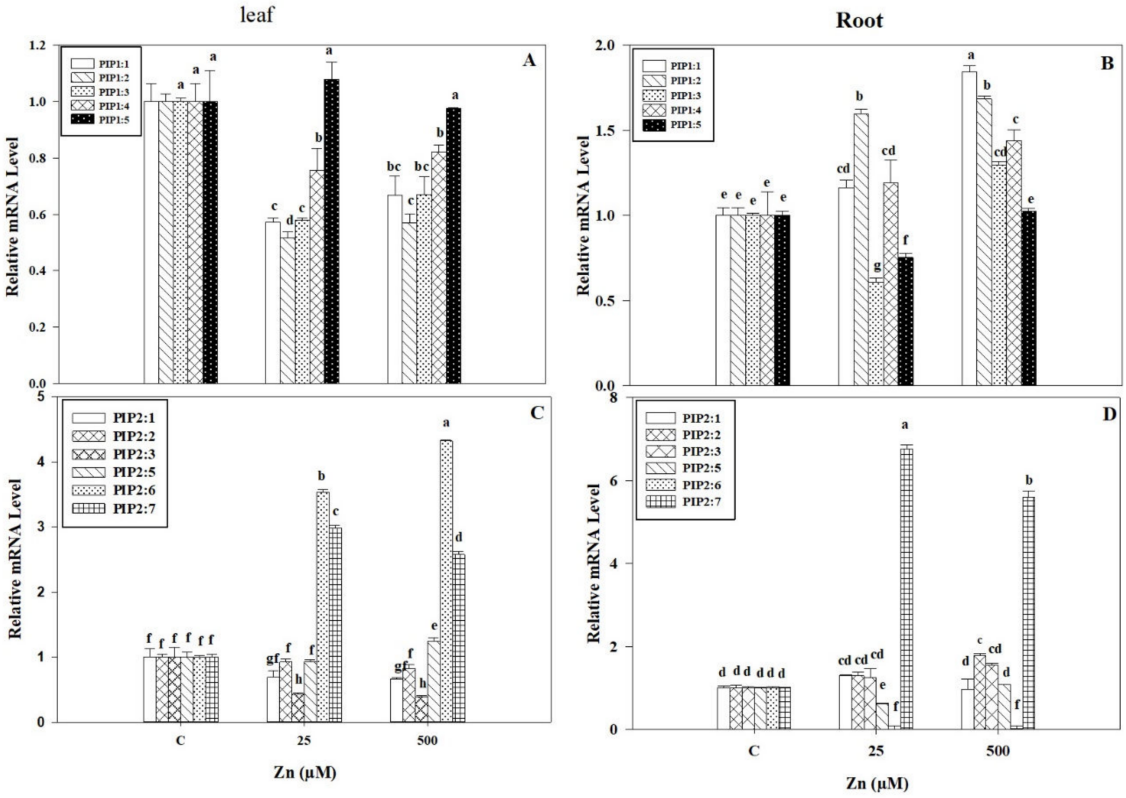

Figure 6. Relative expression level of aquaporin isoforms after long-term application of Zn: (**A**) leaf PIP1, (**B**) leaf PIP2 (**C**)root PIP1 (**D**)root PIP2 genes, determined by Q-RT-PCR in control plants and plants treated with 25 or 500 µM Zn. Mean values and standard errors are shown (n = 3). Bars with different letters represent significant ($p < 0.05$) differences after ANOVA and an LSD (Least significant difference) test.

4. Discussion

4.1. Growth, Root Hydraulic Conductance and Gas Exchange Parameters

It has been reported that Zn may promote growth at optimal concentration but at higher or low levels induced a growth reduction by interfering with plant metabolic activities [46]. Similarly, in pak choi plants 25 µM Zn had a beneficial effect on plant growth whereas a 500 µM Zn concentration decreased plant biomass at both, shoot and root level. In addition, water relations and photosynthetic rate may result affected by Zn concentration and duration of Zn exposition.

After short-term Zn spraying, no consistent relationship between L_0 and Gs was observed and whereas L_0 was increased and decreased, respectively, at the low and high Zn concentrations, Gs was maintained under all treatments. Thus, in our plants, the Zn applied by foliar spraying was rapidly (after 15 h) and substantially transported to the roots. This supports previous results obtained in wheat, for which foliar-applied Zn was translocated to leaves, both above and below the treated leaf, as well as to the root tips via the phloem [47,48]. Moreover, the higher content of Zn in root tissues relative to leaves was a mechanism to protect photosynthetic tissues, as no changes in the gas exchange parameters were observed in plants after the short-term foliar treatment, independently of the Zn concentration and zone of spraying.

It has been proposed that the movement of water from root to shoot through the xylem was decreased after metal treatment as consequence of structural responses in the cells [49]. However, in the leaves of *A. rubrum,* the relative water content (RWC) was maintained after metal exposition. The authors indicated that plants were able to compensate the effect of the stress and maintain leaf water status [49]. Similar results were found in pak choi leaf tissues after short-term Zn exposition and root hydraulic conductance did not modulate transpiration. This fact indicated that L_0 and Gs were uncoupled processes under these experimental conditions. In *Beta vulgaris*, an ability to rapidly lose (4 h) and gain (24 h) turgor in plants under abiotic stress (200 mM NaCl) was observed, with reductions in Lp_r consistent with Gs decreases [50], but different stresses and genotypes were described. In any case, it is plausible that L_0 in pak choi plants responded to a rapid shoot to root sensing action of Zn that in addition triggers differential regulation of aquaporin gene expression.

In the long-term Zn experiment, no changes were observed in L_0, Gs or E in plants treated with the low Zn concentration, but growth was correlated to an enhanced photosynthetic rate (A) at the optimal Zn (25 μM) dose. It has been previously reported that photosynthesis, carbonic anhydrase activity and chlorophyll concentrations were correlated with Zn nutrition at low levels [51,52]. However, at high Zn concentration a reduced stomatal conductance was in consonance with L_0 reductions and a photosynthetic rate decrease in pak choi. Similarly, it has been reported that high levels of Zn (200–500 μM) inhibited CO_2 assimilation due to structural and functional disturbances in the photosynthetic process [6,53,54]. Thus, photosynthetic response in our plants to long-term Zn could be attributed to an alteration of the photosystem and pigments rather than to the transpiration change. In pea plants, 1 mM cadmium (Cd) had no statistically significant effect on the transpiration rate but it decreased this parameter a 23.1% regarding control in barley plants [55], pointing out the genotype-dependence of the effect of metals on transpiration.

4.2. Calcium and Zinc Concentrations in Pak Choi Plants after Treatments

The short-term Zn accumulation in our plants was accompanied by significant increases in the Ca^{2+} contents only in sprayed leaves—to a similar extent at both Zn concentrations—and in roots at the low dose. Calcium is a crucial intracellular messenger and its homeostasis can be modified rapidly by hormonal and environmental stimuli, including metal [56]. The correlation of Zn and Ca in Zn-tolerant and non-tolerant populations of *Silene maritima* was demonstrated by Baker [37]. The important role of Ca in the alleviation of heavy metals toxicity in plants occurs through the prevention of a decrease in the negative charge on the plasma membrane, decreasing the activity of heavy metal ions on the plasma membrane surface, and the maintenance of Ca-related signalling pathways in the presence of the toxicant(s) [57–59]. Davis and Parker [36] also reported that Zn toxicity was highly correlated with the Ca:Zn ratio and reduced stem biomass. According to this, in our pak choi plants the root Ca increment at the low Zn concentration reflects the lack of toxicity of this heavy metal at low doses after a short-term foliar application. By contrast, the decrease in the root Ca content in response to the short-term application of a high Zn concentration is indicative of Zn toxicity and is related to the antagonistic relationship between Zn and Ca in plants [60].

In the long-term experiment the Zn and Ca contents in both organs of plants sprayed with low Zn returned towards control values, compared to the short-term treatment. These results suggest the involvement of an efficient Zn-detoxification mechanism, maintained over time after the low Zn-dose application. Other brassicas, such as *Brassica campestris* L. and *Raphanus sativus* L., have been found to act as hyper-accumulator plants, with higher Zn concentrations in their leaves—relative to the levels of other heavy metals—after irrigation with sewage water [61].

4.3. PIP Aquaporin Expression Remove

With the short-term Zn application, no aquaporin expression changes were found in non-spraying leaves and only in the sprayed ones there were significant increases in *PIP1* and *PIP2* expression after 15 h and depending on the Zn dose. This was concomitant with a similar Ca accumulation at both Zn

doses. The maintenance of critical Ca levels in the apoplast may be required to keep membrane stability and the water channels open through phosphorylation [62–64], reinforcing previous view that AQP phosphorylation may be among the initial targets of Zn toxicity [65]. In fact, Przedpelska-Wasowicz and Wierzbicka [66] showed that, in *Allium cepa* L., the fast (10 min) toxic effects of heavy metals at the cellular level involved an AQP gating mechanism and this response cannot be ruled out in pak choi plants, being gene expression regulation a compensatory mechanism of the water channel gating.

Our results showed a short-term modulation of leaf *PIP2;1* and *PIP2;5*, which were up-regulated at both Zn concentrations. Exposure of plants to heavy metals (Cd, Ni and Zn) is known to induce water deficit in plant organs [67]. Thus, the leaf *PIP2;1* and *PIP2;5* isoforms may play a key role in water dynamics as part of a rapid Zn-induced sensing response and osmotic stress prevention. The over-expression of these two AQPs isoforms was previously observed to favour water transport into the inner leaf tissues, preventing a fall in leaf water potentials and reducing xylem tensions [68,69]. By contrast, in this study, an up-regulation of *PIP1;1*, *PIP1;2* and *PIP2;2* in pak choi leaves was only observed after short-term application of 500 µM Zn. This fact suggests the implication of these isoforms in the regulation of water homeostasis under a rapid and toxic Zn effect, as reported in several studies for different abiotic stresses [70–73]. Thus, co-expression of *PIP1;2* and *PIP2;5* resulted in increased water-channel activity in *Zea mays* plants [74]. In addition, transgenic rice plants over-expressing *PIP1;1* or *PIP2;2* developed enhanced tolerance to 200 mM mannitol and mild salt stress [70]. In our plants, upregulation of a few individual PIPs in the leaves may redirect water flow into specific cells—which is crucial for plant survival, maintaining gas exchange mechanisms, as it was observed from our data and according to those observed by Alexandersson et al. [75] under water deficit.

Regarding root AQPs expression, in our short-term experiment, among the root *PIP1* genes, only *PIP1;4* showed overexpression at the highest Zn dose. In *Arabidopsis thaliana*, the transcription level of *PIP1;4* isoforms increased more than five-fold over the first 48 h of drought stress (250 mM mannitol), in leaves and roots, as well as in response to salt (150 mM NaCl) and cold stresses [14]. However, expression of this gene was unaffected during *M. crystallinum* adaptation to 1 d of Cu, Zn [22] or salinity [76] stress. After rapid application of a high Zn dose to pak choi plants, *PIP1;4* may function as promotor of water transport from roots to the aerial parts, to maintain water relations and gas exchange in the plants, even at low L_0. The fact that only AQPs located in the plasma membrane of sprayed leaves and roots had modified gene expression suggests the involvement of these proteins in the rapid shoot-to-root hydraulic communication, previous to the Zn effect on leaf transpiration [77].

Additionally, in short-term experiment, root *PIP2;2* and *PIP2;3* showed significant sensitivity to Zn application, especially at high Zn concentration. However, the functional redundancy within AQPs [78] represents a challenge to the determination of the overall role of any given type of AQP in the response to Zn stress. In fact, the decline of both *PIP2;2* and *PIP2;3*—which had high homology, showing 96.8% amino acid identity—could have been compensated by the enhancement of *PIP2;7*. In other reports, a transient up-regulation of *PIP2;3* gene expression upon short-term (2 to 96 h) salt stress has been also reported, implying a possible influence of *PIP2;3* on the short-term response to ionic or purely-osmotic stress [14,79,80]. In addition, rapid (1 to 24 h) up-regulation of *PIP2;7* was observed to be involved in the osmoregulation in plants only under high-toxicity stress [81,82], and the efflux of metalloids from roots to avoid toxicity under excessive levels [83,84], which could explain its overexpression in our pak choi roots only at 500 µM Zn, when root accumulation of Zn^{2+} ions was high enough. In any case, similar to that which occurs with other stresses and genotypes, *PIP2;2, PIP2;3* and *PIP2;7* showed a coordinated expression in pak choi plants exposed to Zn.

In the long-term application, by contrast to the short-term experiment, *PIP2;7* was up-regulated in both plant organs, independently of the Zn dose. Apart from its role in plant growth, facilitating water transport into the rapidly-elongating root cells [85], *PIP2;7* facilitates H_2O_2 diffusion across the plasma membrane [86,87], which is an important signal in the regulation of multiple genes associated with abiotic stress tolerance [88,89]. Thus, in pak choi plants *PIP2;7* acts as a sensitive target element

for Zn in prolonged heavy metal exposure and its role as part of this signalling response to Zn must be elucidated.

In addition, reductions in root *PIP1;3*, *PIP1;5*, *PIP2;5* and *PIP2;6* expression in plants treated with 25 µM Zn were not correlated with the maintenance of L_0, but other significant increments in the AQP mRNA levels were observed such as for *PIP1;1; PIP1;2, PIP1;4* and *PIP2;7* which may contribute to L_0. After the long-term 500 µM -Zn treatment, when Zn^{2+} ions were largely accumulated in leaves and to a greater extent in roots, the majority of *PIP1* isoforms were upregulated in roots. However, L_0 was significantly decreased in these conditions, concomitant with a reduction in the root Ca^{2+} content. This fact pointing out the role of PIP2 subfamily to water transport contribution and the aquaporin regulation through other mechanisms alternative to a transcriptional control as Ca^{2+} dependent-protein phosphorylation. There were also concomitant reductions in A and Gs after long-term exposure to high Zn, indicating severe injury to the photosynthetic apparatus, which finally induced a reduction in plant FW and DW. These results were in consonance with the similar effects of excess of Zn in *Jatropha* plants [90].

5. Conclusions

Pak choi plants have a differential response to Zn depending on the treatment intensity and duration. A rapid shoot-to-root hydraulic signal was involved in the response to short-term partial foliar Zn application, affecting root hydraulic conductance, that it was increased or decreased depending on the Zn dose applied. However, in these plants, L_0 was a no-coordinated process with leaf transpiration and the closure of the stoma under these conditions. Similar root patterns of variation for L_0 and Ca ions with distinct Zn concentrations pointed out the effect of Zn on Ca availability and the importance that this element could have in processes such as aquaporin gating. Leaf *PIP2;1* and *PIP2;5* aquaporin isoforms play a key role sensing the rapid Zn-induced response at leaf level and may act in controlling water dynamics as part of this rapid Zn-induced sensing response preventing osmotic stress. By contrast, *PIP1;1* and *PIP2;2* could be involved in the regulation of leaf water homeostasis, specifically under a toxic Zn effect.

Long-term exposition to low-Zn dose had a beneficial effect on plant growth through an increased leaf photosynthesis rate and maintaining plant water balance. However, high Zn concentrations induced a stomatal closure that together with a decrease in the photosynthesis rate and water transport uptake led to a reduced plant biomass. Differential pattern of aquaporin isoforms reveals that almost all *PIP1* isoforms and *PIP2;6* and *PIP2;7* were involved in sensing the long-term response to Zn treatments reflecting a major Zn dose dependence the PIP1 subfamily. Based on the transcriptional response, other mechanisms of aquaporin regulation at protein level need to be elucidated for the isoforms involved in the response to low and high Zn concentrations and particular function of each isoform in the Zn response have to be deeper studied.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/3/450/s1, Table S1: Primer sequence used for real time and RT-PCR amplification of Aquaporin genes of *B. rapa*.

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