

Aquaporins involvement in the regulation of melon (*Cucumis melo* L.) fruit cracking under different nutrient (Ca, B and Zn) treatments.

Alvaro Lopez-Zaplana^a, Gloria Bárzana^a, Lei Ding^b, François Chaumont^b and Micaela Carvajal^{a*}

*Corresponding author: mcarvaja@cebas.csic.es

^a Aquaporins Group. Plant Nutrition Department, Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Campus Universitario de Espinardo, Edificio 25, 30100 Murcia, Spain.

^b Louvain Institute of Biomolecular Science and Technology, UC Louvain, 1348 Louvain-la-Neuve, Belgium

ABSTRACT

Melon cracking is a physiopathy that is associated with both internal and external changes by the alteration of the water balance and nutrient homeostasis in the fruit. Aquaporins are channels for water and other small solutes, and they are potentially involved in the regulation of melon cracking. In this work, we studied the mineral concentration and expression of all aquaporins in non-cracked and cracked melon pulp in control and after the application of foliar mineral treatments (Ca+B+Zn and B+Zn) in field conditions. Also, we measured the mineral transport of some aquaporins to connect it to cracking. The results showed that both treatments could ameliorate the incidence of cracking. Mineral elements determination showed increases in B, Ca, Si and Zn content in non-cracked Ca+B+Zn treated melons. In control conditions, only *NIP2;2* and *NIP5;1* had a significant increase in expression in cracked melons compared with non-cracked ones pointing to their involvement in cracking. Furthermore, we were able to verify that the high expression of *PIP1;1*, *PIP1;2* and *TIP1;1*, which are efficient water channels, was involved in the changes observed in cracking incidence in both foliar mineral treatments. Finally, transport assays in oocytes were performed with selected isoforms, highlighting the water channel activity of *NIP2;2* and *NIP5;1*, the B channel activity of *TIP1;3*, *TIP1;2*, *NIP1;1* and *NIP2;2*, and the Si channel activity of *NIP2;2*. In conclusion, both effective foliar treatments for avoiding cracking point to the *PIP1;1*, *PIP1;2*, *TIP1;1* and *NIP5;1* and *NIP2;2* aquaporins as possible molecular markers.

Keywords

Cucumis melo L., cracking, fruit splitting, aquaporin, foliar nutrition, oocyte expression.

1. Introduction

Aquaporins are membrane channels that facilitate the diffusion of water and small molecules across biological membranes (Maurel et al., 2015). Aquaporins occur as multiple isoforms, reflecting a high diversity in cellular localization, transport selectivity, and regulation properties. Currently, five subfamilies have been found in land plants, PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin26-like intrinsic proteins) (Chaumont et al., 2001), SIPs (small basic intrinsic proteins) (Johanson and Gustavsson, 2002) and XIPs (X-intrinsic proteins) (Gupta and Sankararamakrishnan, 2009). Furthermore, some plant aquaporins can facilitate the diffusion different substrates depending their structure and sequence. For example, some PIPs can also transport H₂O₂ (Hooijmaijers et al., 2012) and CO₂ (Uehlein et al., 2003), some TIPs can transport nitrogen (N) compounds such as ammonium (Loqué et al., 2005) and urea (Gerbeau et al., 1999), and some NIPs are usually permeable to some beneficial mineral nutrients such as boron (B), silicon (Si) or selenium (Se) and other toxic elements such as arsenic (As) or antimony (Sb) (Bienert et al., 2008).

Given the importance of aquaporins in regulating essential water and solute transport through cell membranes, it is not surprising that the regulation of these proteins is associated with tolerance to numerous stresses (Barzana et al., 2021; Cabañero and Carvajal, 2007; Yepes-Molina et al., 2020) and physiopathies (Hagassou et al., 2019; Shen et al., 2014). One of the multiple physiopathies related with water is the cracking or splitting of fruits, which consists in the appearance of cracks in fruit due to an imbalance between internal and external pressure (Matas et al., 2004). In this way, some transcriptomic studies have associated cracking with several aquaporins genes such *LcAQP*, *LcPIP*, *LcNIP*, or *LcSIP* in *Litchi chinensis* Sonn (Li et al., 2014). In general, the appearance of fruit cracking has been associated to weather events (intense rainfall, changes in temperature, or high solar radiation), soil conditions (deficit of some nutrient) (Khadivi-Khub, 2015; Li and Chen, 2017; Peet, 1992; Winkler et al., 2016) or other internal causes that can affect the balance of cations, water content or other factors (Capel et al., 2017; Cline and Trought, 2007; Joshi et al., 2018; Qi et al., 2015).

Cracking is a physiopathy with a growing interest as can be seen in the numerous articles that have published in recent years for different fruits including cherries (Fresno and Munné-Bosch, 2021; Gutiérrez et al., 2021; Michailidis et al., 2020) tomato (Capel et al., 2017; Liu et al., 2022; Zhang et al., 2020), jujube (Hou et al., 2022; Li et al., 2020; Ren et al., 2017) or pomegranate (Chater and Garner, 2018; Davarpanah et al., 2018; Draie and Aboras, 2021). But also, one of the plants that can suffer cracking and it has a relevant economic impact is the

melon, *Cucumis melo* L., a member of the Cucurbitaceae family that has a high economic impact in global market, being the fourth most important fruit in the world (De Campos et al., 2017). Among the main causes of melon cracking, differences between abrupt temperatures between day and night, high exposure to the sun during ripening, or high levels of humidity have been described (Fernández-Trujillo et al., 2013), as well as drastic increases in water flow, which alter the standard water flow throughout the plant (Lopez-Zaplana et al., 2020a; Measham et al., 2010). Despite the economic losses that are generated by the splitting of melons (Khadivi-Khub, 2015), not many studies focusing on the expression of genes in this fruit are available, perhaps due to the difficulty in measuring their expression, as the amount of water, secondary metabolites and concentration of sugars in the fruit are very high, making it difficult to work with this genetic material (De Campos et al., 2017).

A typical manner to address this physiopathy has been based on the application of foliar treatments composed of B (Ihsan ul and Abdur, 2012), zinc (Zn) (Chater and Garner, 2018) or some other macronutrient such as calcium (Ca), which have already been shown in numerous studies to reduce the incidence of cracking in different plants (Bakshi, 2018; Breia et al., 2020; Davarpanah et al., 2018; Dinesh et al., 2017). B is a very important nutrient implicated with the formation of cell walls (O'Neill et al., 2004) and, therefore, an increase in B could stimulate the formation of new walls and allow better fruit growth, preventing any mismatch between the internal and external pressure of the fruit. On the other hand, Ca is involved in the stabilization of the membrane systems and the formation of Ca pectate, increasing the rigidity of the middle lamella and cell wall (Mignani et al., 1995). Furthermore, Ca could prevent the incidence of cracking by regulating the expression of some aquaporins (Breia et al., 2020). Finally, Zn could act as an inhibitor of aquaporin activity (Németh-Cahalan et al., 2007; Yukutake et al., 2009), leading to a decrease in water uptake and reduction in the internal pressure.

The present work characterized the cumulated cracking incidence with mineral concentration and the expression of aquaporins, for a period of 5 weeks after the application of two foliar treatments composed of Ca+B+Zn (in the forms of CaCl_2 , H_3BO_3 , and ZnSO_4) and B+Zn (in the forms of H_3BO_3 and ZnSO_4). Our initial hypothesis is that water could be involved in the appearance of cracking, due to an imbalance in the expression of aquaporins, mainly by PIPs. In addition, as some of these aquaporins can also transport different metalloids such as B or Si, we will also verify their transport in vivo, since they may be related to the formation of cell walls and, in some way, with the rigidity and then, to the appearance of cracking. To prove that, we studied the expression (mRNA levels) of all the melon aquaporins identified in cracked

and non-cracked melon fruits. We also tested the channel activity to water, Si and B of selected aquaporins (CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1), to prove their involvement in nutrient and water transport linked to cracking.

2. Material and methods

2.1. Experimental orchard conditions and foliar treatments

The experiments were conducted in the summer growing season in commercial fields in Manzanares (Ciudad Real, Spain), under continental climate conditions of stable temperatures during the summer (July-August). The average temperature in this area during these two months was 27.5 °C, with an average minimum of 17.6 °C at night and an average maximum of 37.3 °C, during the day. The average relative humidity was 34%. The seedlings of *C. melo* cv. Grand Riado (Sakata) were transplanted on May 16th, 2020 on soil, and the fruit harvest was carried out three months later. All plants were drip irrigated as done commercially, with fertirrigation containing KNO₃ (5.2 g m²).

Two months after plant transplant, three foliar treatments were applied once a week for three weeks. The first foliar mineral treatment, Ca+B+Zn, consisted of 10 mM CaCl₂, 7 mM H₃BO₃ and 2 mM ZnSO₄. The second foliar mineral treatment called B+Zn, consisted of 7 mM H₃BO₃ and 2 mM ZnSO₄. As a control foliar treatment, deionized water was used. Each plant received 200 mL of these solutions in each of the three applications.

The experimental design was a completely randomized design. The three foliar treatments were distributed randomly in four different lines within the cultivation field with 6 plots per line (24 total plots). The spatial arrangement of the treatments was random so that it did not affect the results and included one line of plants as border between treatments. The experiment was carried out in 58-65 plants per treatment, distributed in 8 plots per treatment, with 7-9 plants per plot, for a total of 230-250 melons per treatment.

2.2. Incidence of cracked melons

After the third application of the treatments, the total number of cracked and non-cracked melons was counted for the first time and, from that moment on, they continued to be counted, once a week, for 5 weeks. To take a melon into consideration, it had to have a commercial size, greater than 2.5 kg, leaving out those of smaller size. Once a cracked melon appeared, it was

separated from the plant and discarded so that it would not be counted again in the following weeks. In this way, we reduced the appearance of new cracking caused by non-genetic factors, such as insects or animals that are attracted to these first cracks and can affect the other fruits. To consider that a melon was cracked for our harvesting and study, the cracking should be greater than 1 cm but smaller than 3 cm in width, through which the pulp could be seen.

2.3. B, Ca, Si and Zn concentration

Five weeks after application of foliar treatments, in order to know the amounts of the elements applied in the foliar treatments per melon, the pulps of three independent melons with the same characteristics (cracked or not cracked and type of foliar treatment applied) were analyzed. To separate the pulp from the rind, the first 3 cm were considered as rind, with the rest being the pulp. The pulp area near the crack was collected for cracked melon samples, but not the exposed area. For non-cracked melon samples, the same part of the pulp was collected. All the samples were dried and then finely ground in a mill grinder (model A10, IKA, Staufen, Germany) and digested with HNO₃–HClO₄ (2:1) in a microwave oven (CEM Mars Xpress, Mattheus, NC, USA). In addition, Si concentration was determined. All the samples were digested using 20% NaOH. The analysis of the elements was carried out using a Perkin–Elmer (Waltham, MA, USA) 5500 model ICP emission spectrophotometer (Iris Intrepid II, Thermo Electron Corporation, Franklin, TN, USA).

2.4. RNA extraction and cDNA synthesis

Five weeks after the application of foliar treatments, total RNA was extracted from the internal part of the pulp using the NZY Total RNA Isolation kit (Nzytech, Lisbon, Portugal). For cracked melons, the tissue close to the cracked area was sampled, avoiding the exposed part of the crack. Once the sample was crushed in liquid nitrogen, a total of 50 mg of pulp was processed, by means of a vortex (20 s) and a centrifugation (13000 gs, 2 min), to separate nucleic acids from cellular material, passing the resulting sample through the first kit column. The rest of the procedure was similar to the manufacturer's protocol. The quantity and purity of RNA were measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and the integrity of the RNA was verified by agarose gel electrophoresis. Genomic DNA was removed using DNase from the NZY Total RNA Isolation kit (Nzytech, Lisbon, Portugal) according to the manufacturer's instructions. The RNA was stored at -80 °C until use. The High-

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesise cDNA from 2 µg of total RNA, according to the manufacturer's protocol.

2.5. Quantitative real-time PCR (RT-qPCR) analyses

The expression of all the melon aquaporin genes were checked by RT-qPCR with the gene-specific primers already published by our group (Lopez-Zaplana et al., 2020b). It was performed using 2 µL of 1:2 or 1:5 diluted cDNA samples, increasing the concentration for lower expression genes, 500 nM of primers and 5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in 10 µL of total volume reaction. The equipment used was a QuantStudio™ 5 Flex Real-Time qPCR system (Applied Biosystems, Foster City, CA) and the qPCR program consisted of 10 min initial denaturation at 95 °C and then amplification in a two-step procedure: 15 s of denaturation at 95 °C and 60 s of annealing and extension at a primer-specific temperature for 40 cycles, followed by a dissociation stage. Data collection was carried out at the end of each round in step two. These conditions were used for both target and reference genes and the absence of primer-dimers was checked in controls lacking templates. Five housekeeping genes were tested as reference genes (RPL, RAN, ACT, ADP and GAPC2) (Kong et al., 2014) selecting the most stable gene in our conditions, in this case RPL (Ribosomal protein L). Whole specific primers used for RT-qPCR are listed in **Supplementary table 1**. RT-qPCR analysis was performed on 3 independent samples for each treatment (biological replicates) and each sample reaction was carried out in triplicate (technical replicates) in 96-well plates. The transcript levels were calculated using the relative expression method comparing with a housekeeping gen. Negative controls without cDNA were used in all PCR reactions.

2.6. Heterologous expression in *Xenopus laevis* oocytes and transport assays.

The coding region of *CmTIP1;3*, *CmTIP2;2*, *CmNIP1;1*, *CmNIP2;2* and *CmNIP5;1* were PCR amplified and cloned into the USER vector pNB1u and pNB1-YFPu using the USER system (uracil-specific excision reagent cloning technique) compatible with *X. laevis* oocytes expression system (Bienert et al., 2011; Fetter et al., 2004; Nour-Eldin et al., 2006). The pNB1-YFPu vector contains the cDNA encoding the Yellow Fluorescent Protein (YFP) that will be fused to the N-terminus the protein of interest. These genes were selected according to the qPCR results, showing significant differences between treatments or non-cracked/cracked melons or

their increase of expression in the fruit respect other tissues. The gene-specific primers used are listed in **Supplementary table 2**. The generated constructs were verified by Sanger sequencing (Microsynths AG, Switzerland). *In vitro* transcription was performed using T7 RNA polymerase (Promega, USA), treated with Rq1 RNase-Free DNase (Promega, USA) according to the manufacturer's protocol and purified with phenol/chloroform method (Bienert et al., 2011).

50 nL of complementary RNA (cRNA) encoding the aquaporins of interest were injected into each *X. laevis* oocyte (Fetter et al., 2004), at increasing concentrations of 0.2, 0.4 and 0.8 ng nL⁻¹. The selected injected oocytes were kept at 20°C for 2-3 days in Barth's buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM Hepes-NaOH, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, pH 7.4, and ~ 210 mOsm kg⁻¹) to allow protein expression. The YFP signal was detected by confocal microscopy (LSM 710, Carl Zeiss, Oberkochen, Germany) in oocytes microinjected with 40 ng of cRNA (*YFP-CmTIP1;3*, *YFP-CmTIP2;2*, *YFP-CmNIP1;1*, *YFP-CmNIP2;2* and *YFP-CmNIP5;1*). The oocytes were fixed with 4% (p/v) paraformaldehyde and kept at 4 °C until YFP detection (Fetter et al., 2004). The YFP was excited with the 514 nm wavelength of an argon multilaser and the emitted YFP fluorescence detected between 530 and 570 nm.

For the water transport assay, an individual oocyte was transferred from full to 1/2 Barth's buffer to induce swelling. Three independent experiments were performed with three different amounts of injected cRNA (10 ng, 20 ng and 40 ng). In total, 30 to 40 oocytes were tested for each gene and each concentration of cRNA injected. As a positive control, 5 ng of *ZmPIP2;5* cRNA were injected for the swelling assay. The oocyte swelling change was recorded and the water permeability coefficient (P_f) was calculated using the equation

$$P_f = V_0[d(V/V_0)/dt]/[S \cdot V_w(Osm_{in} - Osm_{out})],$$

with an initial volume (V₀) of 9 × 10⁻⁴ cm³, an initial oocyte surface area (S) of 0.045 cm², and a molar volume of water (V_w) of 18 cm³/mol (Fetter et al., 2004; Zhang and Verkman, 1991).

For the boric acid transport assay, oocytes were microinjected with 40 ng cRNA of each gene. As a positive control, *NtXIP1.1* cRNA (12.5 ng) was microinjected per oocyte. After 2-3 days, they were transferred from Barth's buffer to modified iso-osmotic Barth's solutions in which NaCl was replaced with 178 mM H₃BO₃. The oocyte swelling was monitored according to the above-mentioned water swelling assay. The relative volume change (V_t/V₀) was

calculated (Gerbeau et al., 1999; Tanaka et al., 2008). Two independent experiments were performed with a total of 30 to 40 oocytes.

For the Si transport assay, 40 ng cRNA were injected into an oocyte for each gene. The oocytes were exposed to 1 mM GeO₂ in Barth buffer (pH 7.6) to generate Ge(OH)₄, which was used as a chemical analogue of Si(OH)₄ (Jian et al., 2006). At time 0 and 30 min, the oocytes were collected and washed five times with Barth solution. For each gene, 8-14 oocytes were mixed as one group and five groups were digested with 10% HNO₃, heated to 150 °C, and analyzed by ICP-MS (Thermo model ICAP-QC), with a total of 50-60 oocytes for each gene and each time of incubation.

2.7. Statistical analysis

The statistical analysis was performed by using the SPSS 25.0.0.1 software package and R software (R Core Team, 2018). The incidence of cracking, element analysis, RT-qPCR analyses and Si transport assay were analyzed with a two-way ANOVA, followed by the *post hoc* Tukey's multiple comparison test. The P_f was analyzed by one-way ANOVA, followed by the *post hoc* Tukey's multiple comparison test. Boric acid transport assay in oocytes was analyzed with Student's *t* test. The values presented are the means ± SE. Significant differences between the values of all the parameters were determined at $p \leq 0.05$ (significance level of 5%).

3. Results

3.1. Incidence of cracking

After one week, the cumulated incidence of cracking (**Fig. 1**) was 1.92% for the control, 0.82% for the Ca+B+Zn treatment and 0.76% for the B+Zn treatment and no significant differences were observed between the different treatments. In the second week, the incidence of cracking was 3.86% for the control, 2.36% for the Ca+B+Zn treatment and 1.59% for the B+Zn treatment and they were not significantly different. In the third week, an increase of the incidence of cracking can be observed, with a 9.60% for the control, 5.48% for the Ca+B+Zn treatment and 4.11% for the B+Zn treatment and they were not significantly different. The fourth week showed the first significant difference between the control, 12.91%, and the B+Zn treatment, 4.45%. Ca+B+Zn treatment had a cracking incidence of 7.43% with no significant changes. Finally, the fifth week we had a significant decrease of incidence of cracking respect

the control fruits, with incidences of 17.61% for control, 8.17% for Ca+B+Zn and 5.71% for B+Zn. Two-way ANOVA results showed that the differences obtained could be explained by the time factor ($2e^{-16}$), the treatment factor ($8.56e^{-12}$), and the interaction between them (0.000514). If we consider only the time factor, we can see differences from the third week onwards. If we only consider the treatment factor, significant differences were observed between the control and the two treatments, but not between the treatments.

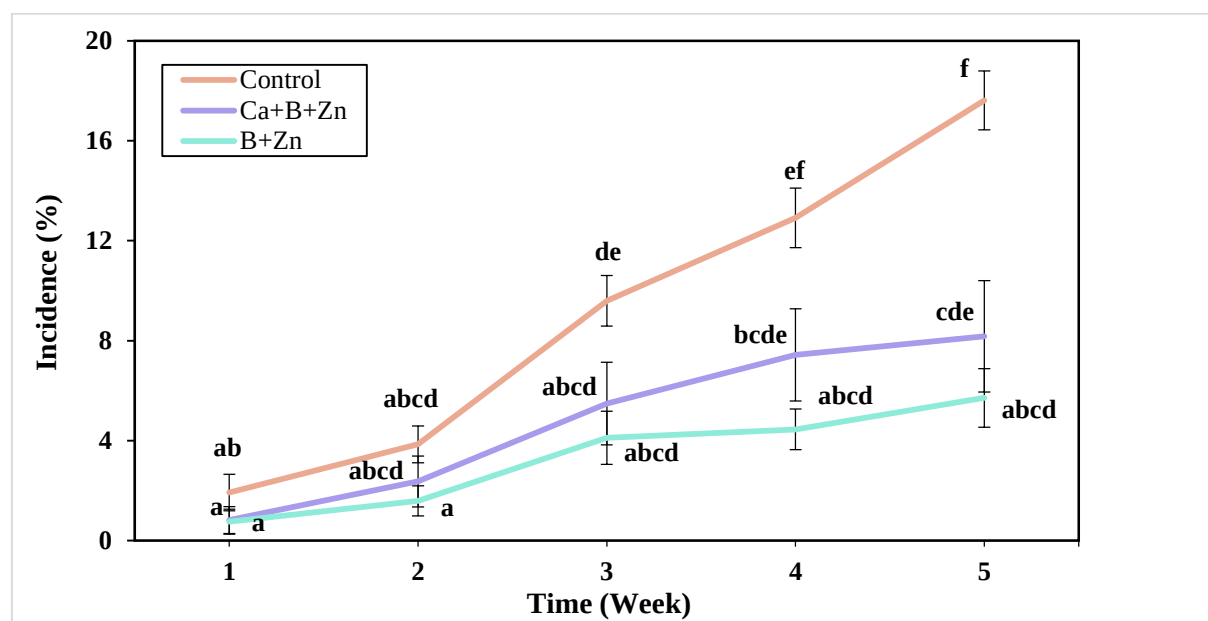


Fig. 1. Evolution of the cumulated incidence of cracking per week. Each value represents the percent of cracked melons from the total of melons in each treatment. Each value represents 8 different plots. Different letters mean significant differences ($p < 0.05$) between control and treatments, (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.2. B, Ca, Si and Zn concentrations in pulp under different foliar mineral treatments

B content in the pulp (**Fig. 2a**) was stable in all the fruits and treatments, except a significantly higher level in the non-cracked melons from the Ca+B+Zn foliar treatment with respect to other melons under different treatments. Calcium content (**Fig. 2b**) increased significantly in both non-cracked and cracked melons from the Ca+B+Zn treatment, with respect to the non-cracked melons from control treatment. Further, a significant increase of Ca concentration was also observed in the cracked melons from B+Zn treatment when compared

to the non-cracked melons from control and B+Zn treatments. Content of Si (**Fig. 2c**) and Zn (**Fig. 2d**) showed a similar response than B. Also, a significant increase of in non-cracked melons from Ca+B+Zn treatment were observed when compared with the melons from control treatment.

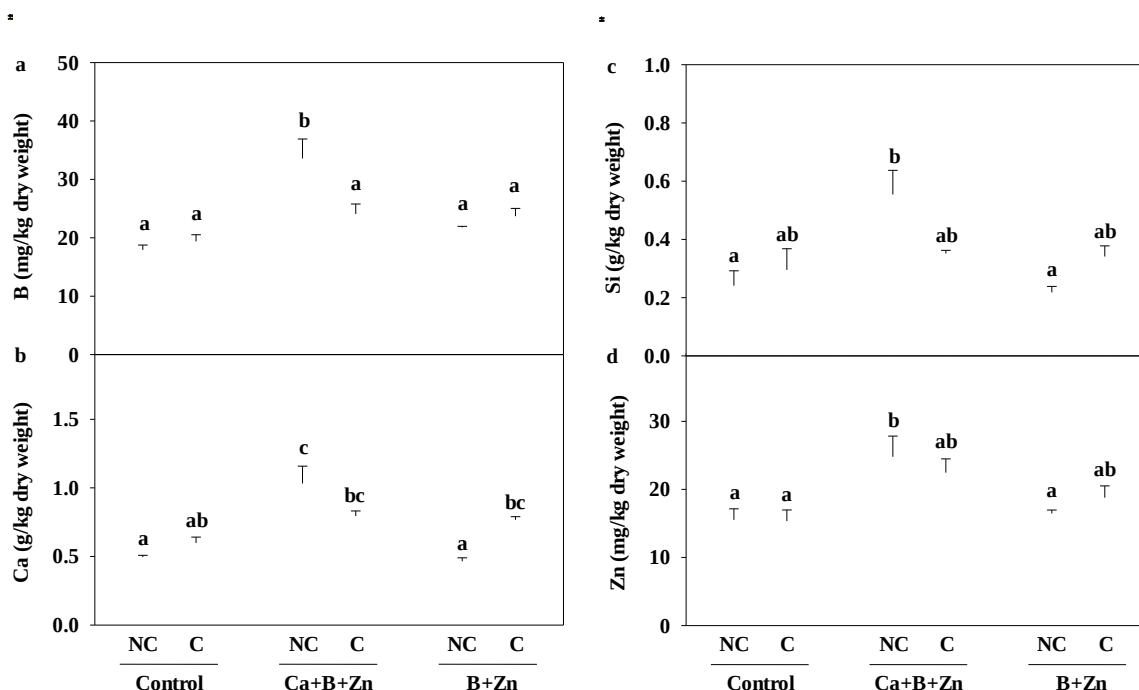
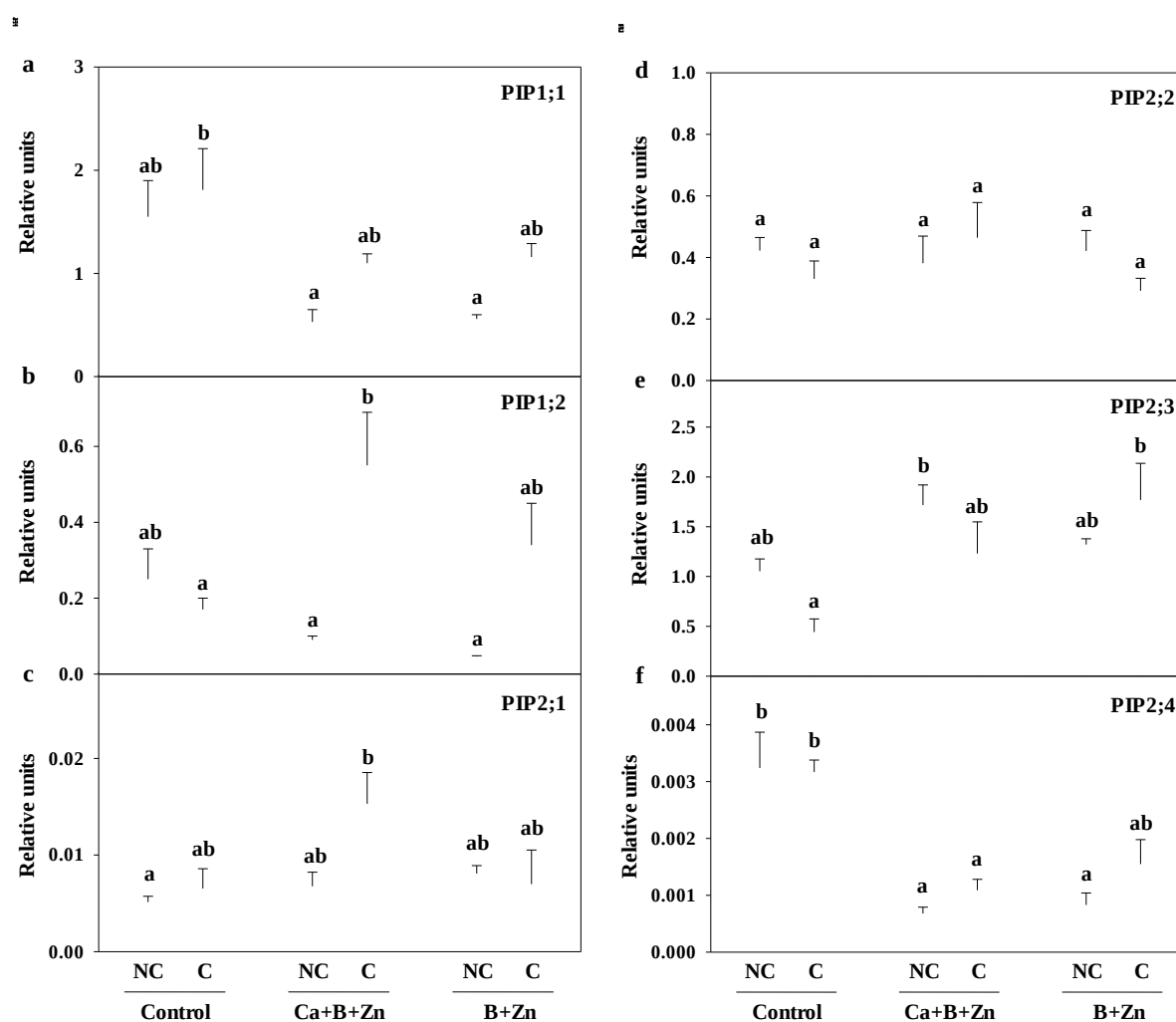


Fig. 2. B (mg) (**a**), Ca (g) (**b**), Si (g) (**c**) and Zn (mg) (**d**) per kg dry weight of pulp in NC (non-cracked melons) and C (cracked melons). Each value represents 3 biological replicates. Different letters mean significant differences ($p < 0.05$) between control and treatments, (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.3. Analysis of PIPs

The expression of the twelve identified *PIP* genes in melon was detected and quantified in fruits (**Fig. 3**). *PIP1;1* (**Fig. 3a**) showed significant decrease in expression in both foliar mineral treatments (Ca+B+Zn and B+Zn) non-cracked melons with respect to control cracked melons. Expression of *PIP1;2* (**Fig. 3b**) showed a significant increase of Ca+B+Zn cracked melons compared with cracked control melons and non-cracked Ca+B+Zn and B+Zn treatments melons. Expression of *PIP2;1* (**Fig. 3c**) showed a significant increase in cracked melons from the Ca+B+Zn treatment with respect to non-cracked control melons. Other aquaporin genes had stable expression levels without significant differences regardless of whether it was a non-cracked/cracked melon or if the foliar treatments were considered, as it

was the case for *PIP2*;2 (**Fig. 3d**), *PIP2*;5 (**Fig. 3g**), *PIP2*;7 (**Fig. 3i**) and *PIP2*;10 (**Fig. 3l**). *PIP2*;3 (**Fig. 3e**) did not show any significant difference in expression between cracked and non-cracked melons within the same foliar treatment, but a significant difference between the control cracked melons, non-cracked Ca+B+Zn treated melons and cracked B+Zn foliar treated melons were found, observing a significant increase in the expression of the last two. Expression of *PIP2*;4 (**Fig. 3f**) showed lower levels of mRNA in both foliar mineral treatments non-cracked melons and cracked Ca+B+Zn treatment respect to the control foliar treatment. However, no differences were observed between cracked and non-cracked melons in any foliar treatment. Expression of *PIP2*;6 (**Fig. 3h**) and *PIP2*;8 (**Fig. 3j**) showed similar response, a significant increase of expression in cracked Ca+B+Zn treatment melons with respect all other treatments and cracked or non-cracked melons. Expression of *PIP2*;9 (**Fig. 3k**) did not show significant differences within the same foliar treatment, but significant increase was found in cracked Ca+B+Zn treatment melons with respect to both cracked and non-cracked in control and cracked B+Zn treated melons.



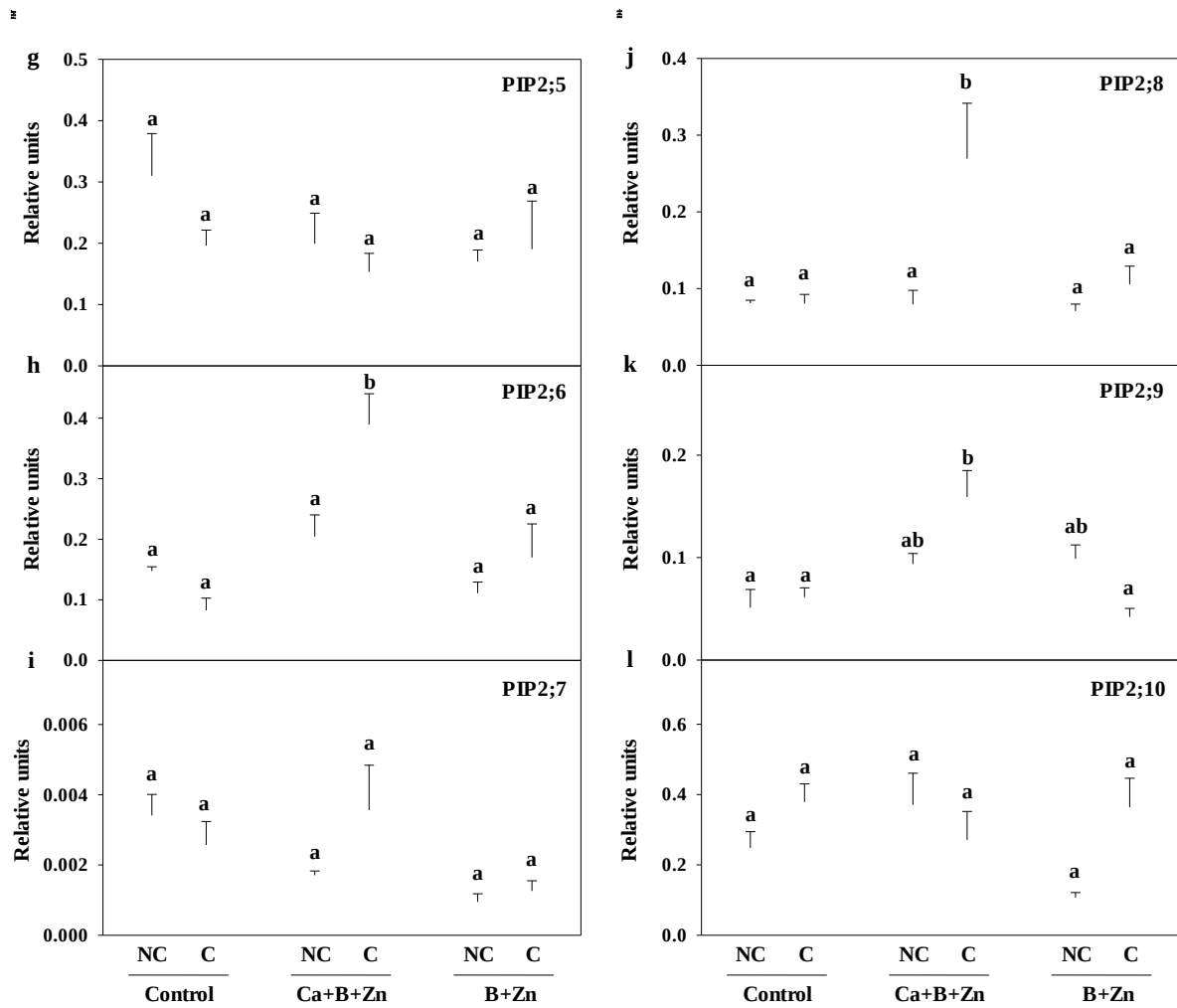


Fig. 3. Plasma membrane Intrinsic Proteins (PIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates of each measurement. Different letters mean significant differences ($p < 0.05$) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.4. Analysis of TIPs

The expression of the eight identified melon *TIP* genes were also measured in fruits (**Fig. 4**). *TIP1;1* (**Fig. 4a**) showed an increase in expression in cracked melons from the Ca+B+Zn treatment as compared to non-cracked melons. *TIP1;2* (**Fig. 4b**) showed a significant decrease in expression in non-cracked melons from the B+Zn treatment with respect to the non-cracked melons of the control treatment. *TIP1;3* (**Fig. 4c**) showed the lowest expression in cracked control melons and the highest in non-cracked B+Zn fruits with significant differences between them. Expression of *TIP2;1* (**Fig. 4d**) and *TIP2;2* (**Fig. 4e**) showed a significant increase in

cracked and non-cracked melons with Ca+B+Zn treatment with respect to cracked control melons. *TIP3;1* (**Fig. 4f**) did not show any significant differences in expression in cracked or non-cracked melons between treatments. *TIP4;1* (**Fig. 4g**) showed significant decrease in expression in the non-cracked melons in both Ca+B+Zn and B+Zn treatments respect to the cracked control melons. *TIP5;1* (**Fig. 4h**) showed a significant increase in expression in cracked melons in the Ca+B+Zn treatment with respect to non-cracked melons of the same treatment.

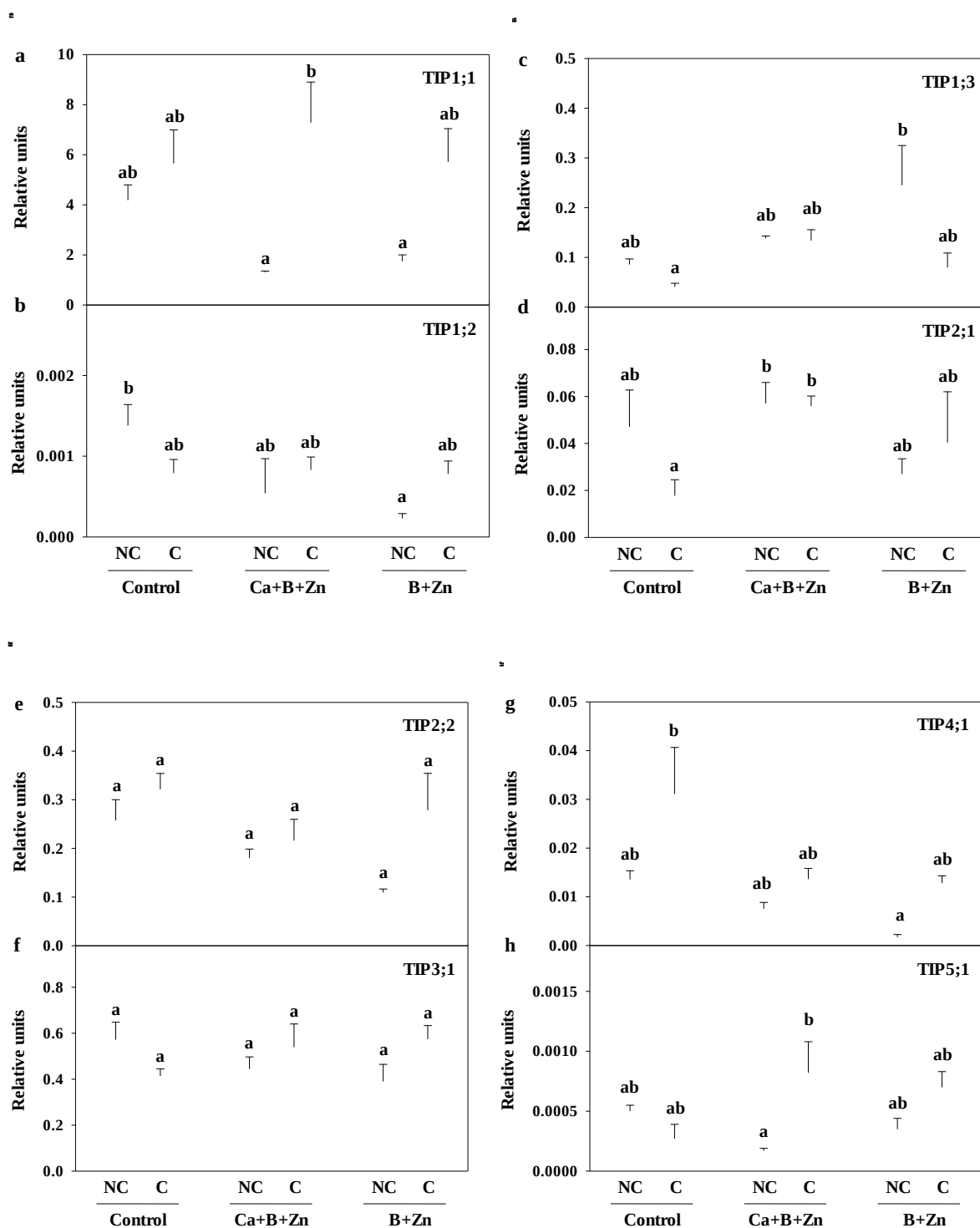


Fig. 4. Tonoplast Intrinsic Proteins (TIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates of each measurement. Different letters mean significant differences ($p < 0.05$) between control and treatments (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.5. Analysis of NIPs

The expression of the eight identified melon NIPs was detected and quantified in fruit (**Fig. 5**). *NIP1;1* (**Fig. 5a**) showed the highest expression in the Ca+B+Zn treatment, being significant only in non-cracked melons as compared with the control treatment. *NIP2;1* (**Fig. 5b**) showed a significant increase in expression in cracked melons from the Ca+B+Zn treatment with respect to the non-cracked melons of control treatment and the cracked melons with B+Zn treatment. *NIP2;2* (**Fig. 5c**) showed a significant increase in expression in cracked melons from the control treatment with respect to non-cracked ones and a decrease was observed in cracked melons of the B+Zn treatment with respect to cracked control melons. *NIP4;1* (**Fig. 5d**) showed the highest values of expression in cracked melons from the Ca+B+Zn treated, being significantly higher than non-cracked control and cracked B+Zn treated. *NIP5;1* (**Fig. 5e**) showed a significant increase in expression in cracked melons in the control and B+Zn treatments with respect to their non-cracked controls, while no changes were observed in the cracked or non-cracked melons from the Ca+B+Zn treatment. Inversely, *NIP5;2* (**Fig. 5f**) showed a significant increase in expression in cracked melons from the Ca+B+Zn treatment with respect to the non-cracked melons, while no differences were found between cracked and non-cracked melons in the control or B+Zn treatments. *NIP6;1* (**Fig. 5g**) did not show any significant differences in expression. *NIP7;1* (**Fig. 5h**) showed the highest values in both cracked and non-cracked melons from the Ca+B+Zn treated plants, being significant respect cracked control and cracked and non-cracked B+Zn treated plants.

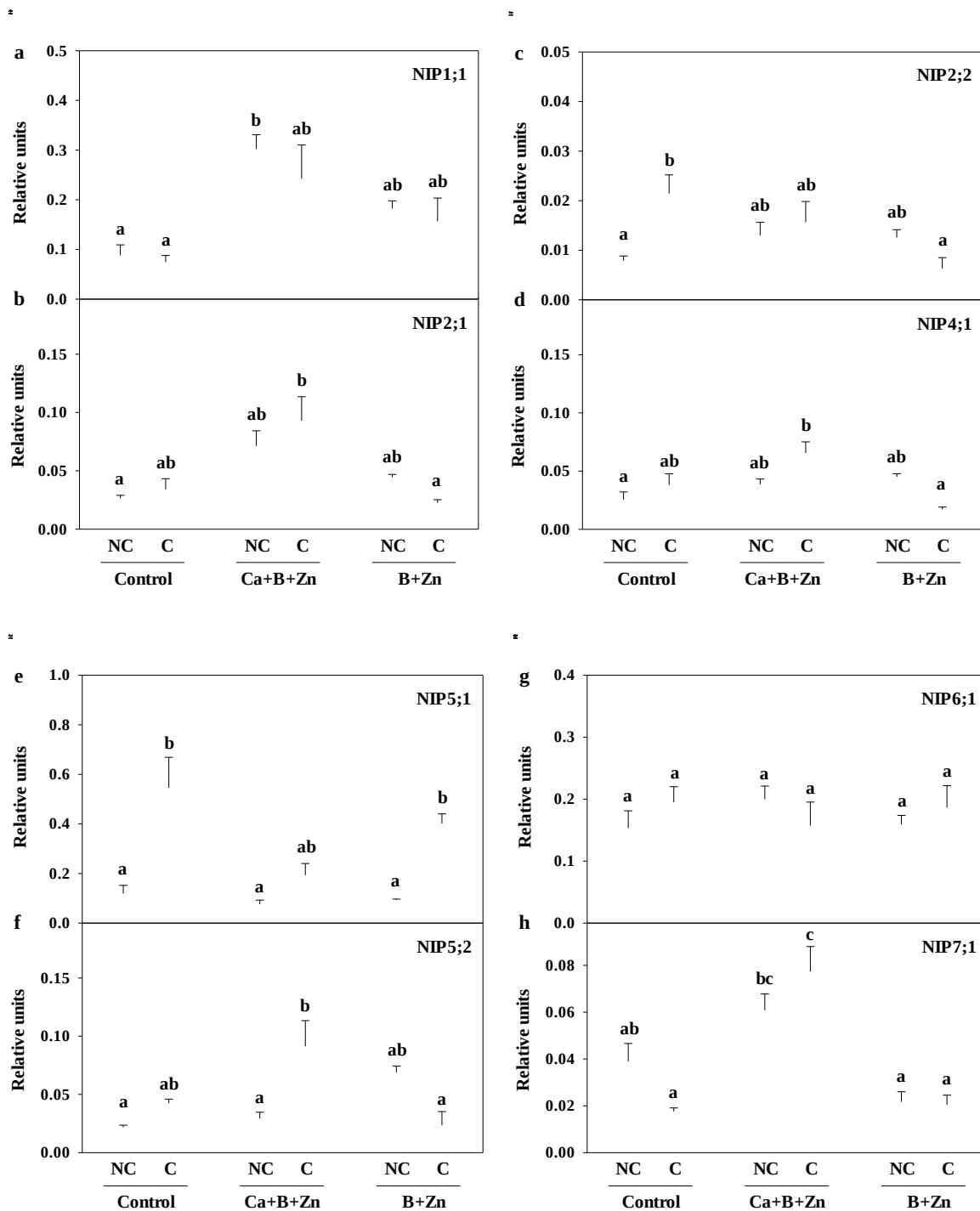


Fig. 5. Nodulin 26-like Intrinsic Proteins (NIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates for each measurement. Different letters mean significant differences ($p < 0.05$) between control and treatments (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.6. Analysis of SIPs and XIP

The expression of the two SIPs and one XIP known in melon were also detected and quantified in fruit (**Fig. 6**). *SIP1;1* (**Fig. 6a**) and *XIP1;1* (**Fig. 6c**) did not show any significant differences between any treatment or cracked/non-cracked melons. *SIP2;1* (**Fig. 6b**) showed a significant increase in expression in cracked melons from the Ca+B+Zn treatment with respect to the non-cracked melons from control treatment.

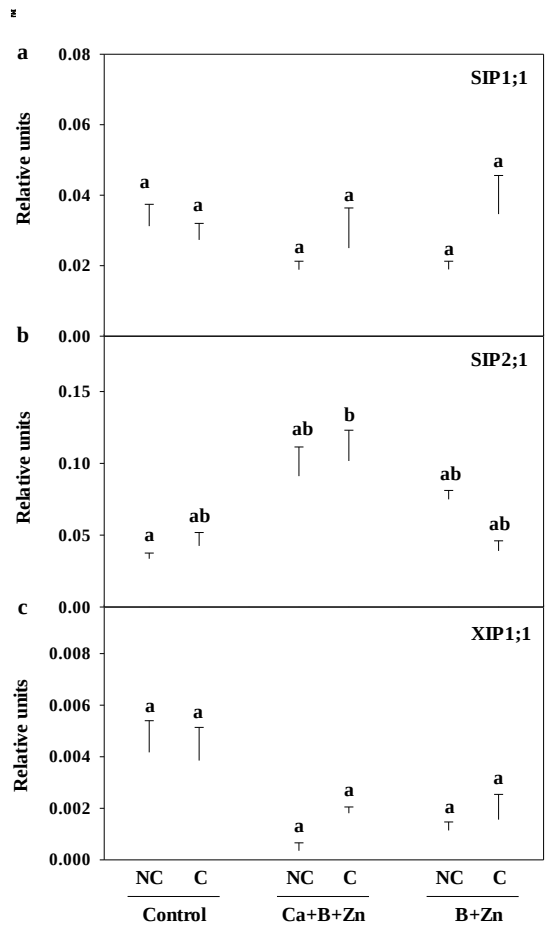


Fig. 6. Small and basic Intrinsic Proteins (SIPs) and X-intrinsic proteins (XIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates for each measurement. Different letters mean significant differences ($p > 0.05$) between control and treatments (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

To find out which variable is responsible for the changes in expression that were found, a two-way ANOVA was performed, checking the p values of each variable, treatment and cracked/uncracked, separately and combined (**Table 1**). In some genes, we observed that the

expression changed due to the effect of cracking, as is the case of PIP1;1, PIP1;2, PIP2;8, PIP2;10, TIP1;1, TIP1;3, TIP2;2, TIP4;1, TIP5; 1 and NIP5;1. Other genes had changes due to treatments, such as PIP1;1, PIP2;3, PIP2;4, PIP2;6, PIP2;7, PIP2;8, PIP2;9, TIP1;3, TIP4;1, NIP1;1, NIP2;1, NIP4;1, NIP5;1, NIP7;1, SIP2;1 and XIP1;1. Finally, some genes showed significant changes that can be explained by the cracking-treatment interaction, PIP1;2, PIP2;6, PIP2;8, PIP2;9, TIP5;1, NIP2;2, NIP4;1 and NIP7;1.

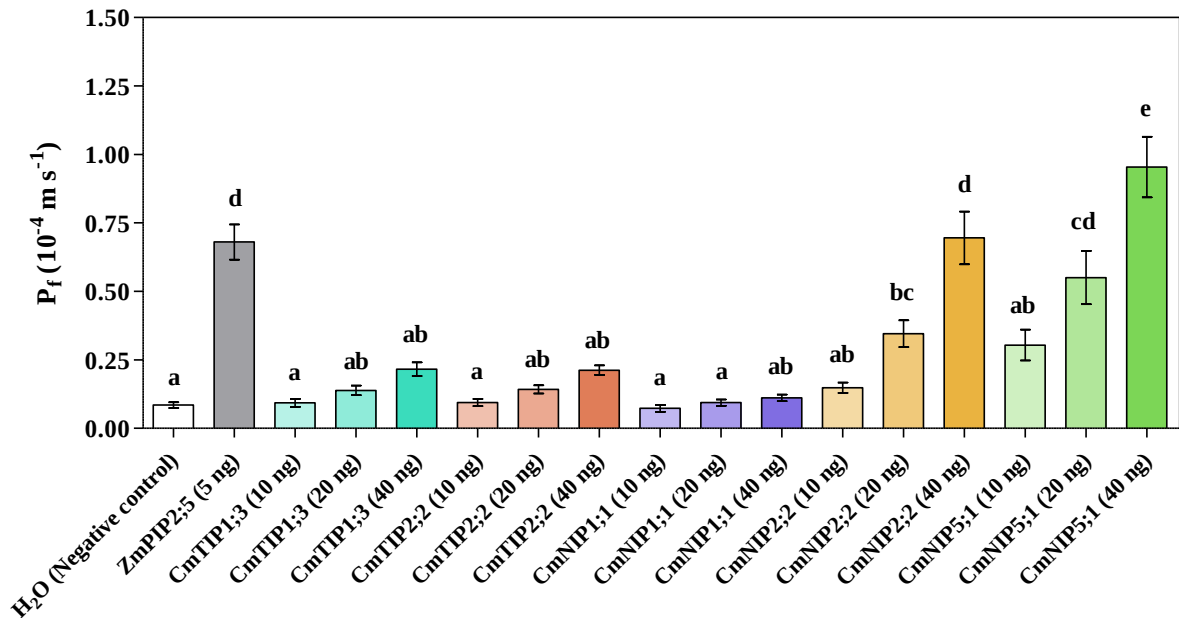
Table 1. P values obtained from aquaporin expression after two-way ANOVA analysis and *post hoc* Tukey's multiple comparison test. Significance codes: *** if p value < 0.001, ** if p value < 0.01, * if p value < 0.05, ns (non-significant) if p value > 0.05.

Gen	P value cracked	P value treatments	P value cracked*treatments
PIP1;1	0.02869 *	0.00468 **	0.71597 ns
PIP1;2	0.00464 **	0.26716 ns	0.01534 *
PIP2;1	0.0563 ns	0.1463 ns	0.1152 ns
PIP2;2	0.464 ns	0.660 ns	0.337 ns
PIP2;3	0.2715 ns	0.00782 **	0.07613 ns
PIP2;4	0.196762 ns	0.000134 ***	0.754925 ns
PIP2;5	0.286 ns	0.272 ns	0.428 ns
PIP2;6	0.063938 ns	0.000539 ***	0.015160 *
PIP2;7	0.4259 ns	0.0316 *	0.1636 ns
PIP2;8	0.0156 *	0.0241 *	0.0281 *
PIP2;9	0.62595 ns	0.00161 **	0.00671 **
PIP2;10	0.0239 *	0.4167 ns	0.0908 ns
TIP1;1	0.0008 ***	0.5428 ns	0.1402 ns
TIP1;2	0.6568 ns	0.0783 ns	0.0771 ns
TIP1;3	0.02082 *	0.00464 **	0.25585 ns
TIP2;1	0.578 ns	0.127 ns	0.250 ns
TIP2;2	0.0341 *	0.1059 ns	0.3446 ns
TIP3;1	0.4847 ns	0.9870 ns	0.0721 ns
TIP4;1	0.00514 **	0.00856 **	0.41849 ns
TIP5;1	0.0346 *	0.5825 ns	0.0198 *
NIP1;1	0.301609 ns	0.000993 ***	0.818327 ns
NIP2;1	0.782027 ns	0.000593 ***	0.161971 ns
NIP2;2	0.1735 ns	0.1557 ns	0.0137 *
NIP4;1	0.47955 ns	0.01207 *	0.00354 **
NIP5;1	0.000194 ***	0.017777 *	0.062764 ns
NIP5;2	0.18759 ns	0.06593 ns	0.00112 ns
NIP6;1	0.710 ns	0.978 ns	0.316 ns
NIP7;1	0.7150 ns	0.000181 ***	0.0405 *
SIP1;1	0.332 ns	0.601 ns	0.426 ns
SIP2;1	0.62143 ns	0.00278 **	0.18297 ns
XIP1;1	0.48950 ns	0.00919 **	0.59333 ns

3.7. Transport activity in *Xenopus Oocytes*

Due to their significant increase in expression in fruit with respect to leaves or roots (Lopez-Zaplana et al., 2020b) and the significance observed in cracked/non cracked fruits TIP1;3 and TIP2;2 were selected. On the other hand, due to the possible importance in fruit cracking, treatments applied and the possible implication in the transport of metalloids, NIP1;1, NIP2;2 and NIP5;1 were selected. The channel activity of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 were tested after their expression in *X. laevis* oocytes. First, the osmotic water permeability coefficient (P_f) (**Fig. 7a**) was determined, and we observed that the expression of CmNIP2;2 and CmNIP5;1 increased P_f significantly as compared with the water-injected oocytes, similarly to oocytes expressing the ZmPIP2;5 cRNA, indicating that CmNIP2;2 and CmNIP5;1 are active water channels. On the other hand, the expression of CmTIP1;3, CmTIP2;2, CmNIP1;1 did not result in any P_f increase. We also tested the expression and localization of the proteins fused to YFP. A fluorescent signal was detected mostly in the oocyte plasma membrane (**Fig. 7b-g**), indicating that all the proteins were well expressed and targeted to the membrane, and that the absence of swelling probably reflected their inability to facilitate water diffusion.

a



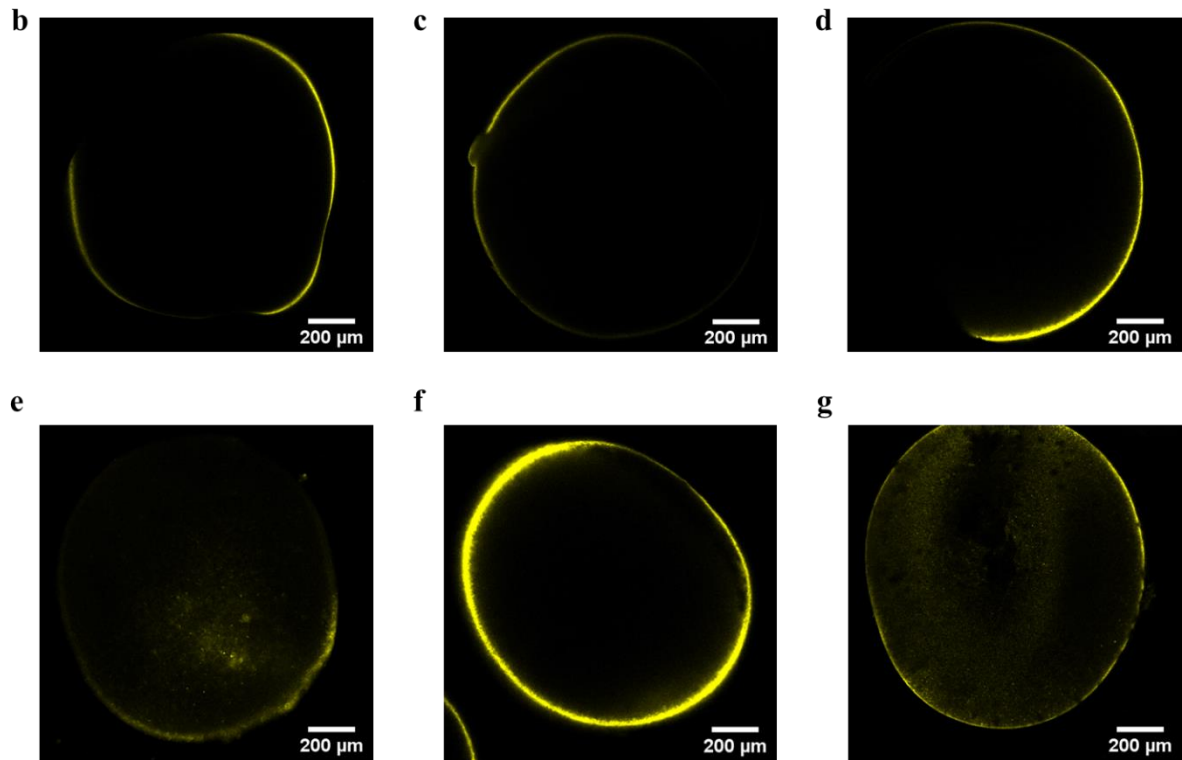


Fig. 7. Water permeability coefficient (P_f) measurements **(a)** of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1. Water-microinjected oocytes were used as negative control and ZmPIP2;5 as a positive control. Each value represents the means $\pm$ SE of 10-26 biological replicates, in two to three times with independent frogs and consistent results. Different letters mean significant differences ($p < 0.05$) according to the *post hoc* Tukey's multiple comparison test. **(b-g)** Confocal pictures of oocytes microinjected with 5 ng ZmPIP2;5 **(b)** and 40 ng CmTIP1;3 **(c)**, CmTIP2;2, **(d)**, CmNIP1;1 **(e)**, CmNIP2;2 **(f)** and CmNIP5;1 **(g)**.

The B transport activity was determined using an iso-osmotic swelling assay in presence of boric acid (**Fig. 8**). A significant increase in V/V_0 was observed for oocytes expressing the positive control NtXIP1;1, CmTIP1;3, CmTIP2;2, CmNIP1;1 and CmNIP2;2, but not for oocytes expressing CmNIP5;1 or water injected oocytes, suggesting that the former isoforms are able to facilitate the diffusion of B.

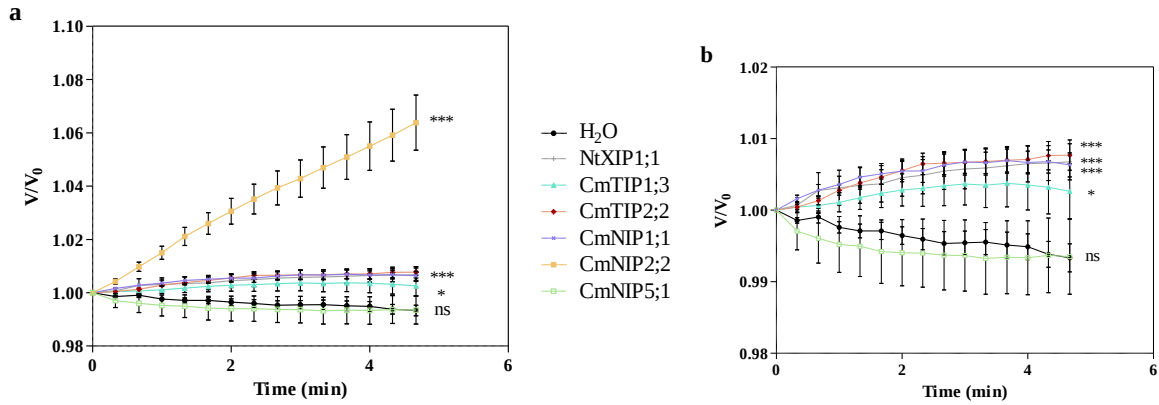


Fig. 8. (a) Osmotic boric acid swelling assay with CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 (40 ng), NtXIP1;1 (12.5 ng) as positive control and water microinjection as negative control. **(b)** Detail of the same experiment with CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP5;1 and NtXIP1;1. Data shown are means $\pm$ SE of 17-24 oocytes measured every 20 seconds. * means significant differences ($p < 0.05$), *** ($p < 0.001$) and ns, non-significant differences between the gene and the negative control in the last point according to Student's t-test.

The Si channel activity of the aquaporins was deduced from the uptake of germanium (Ge) for 30 min and its quantification by ICP-MS (**Fig. 9**). Interestingly, only the NIP2;2 expression induced a significant accumulation of Ge in the oocytes as compared to the water-injected oocytes, suggesting that this NIP2;2 isoform can act as a Si channel.

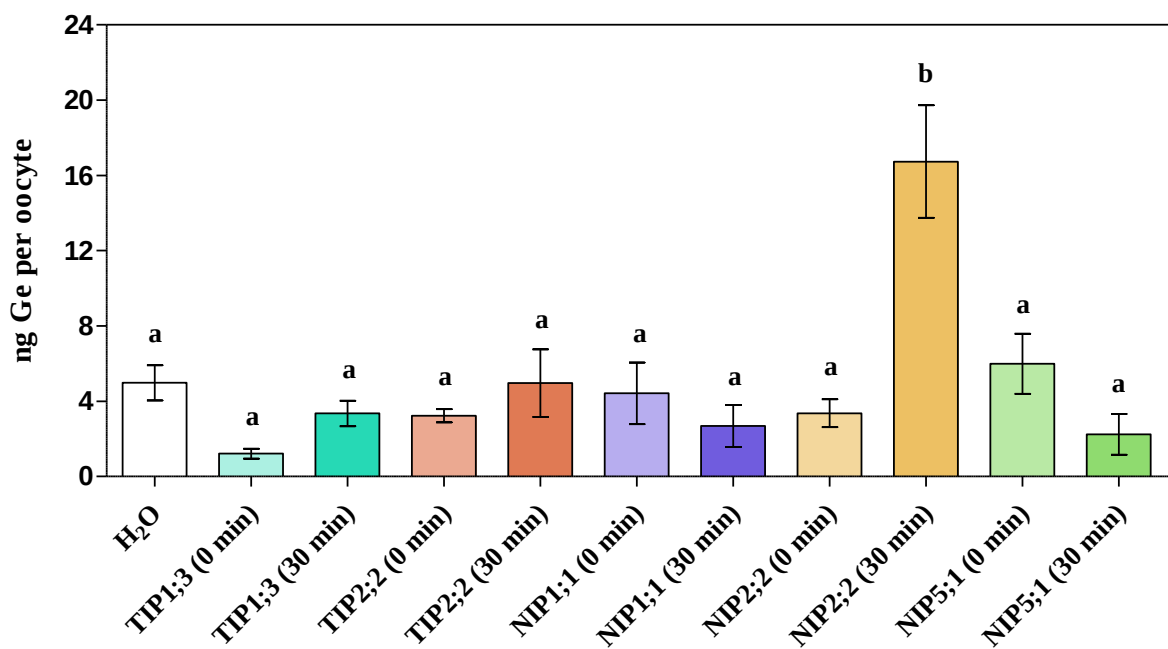


Fig. 9. Permeability of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 (40 ng) to Germanic acid (a chemical analogue of silicic acid) in *Xenopus* oocytes direct uptake assays. Oocytes microinjected with 40 ng of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 were exposed to 1mM GeO₂ (which forms Ge(OH)₄ in solution, as the analogue of Si(OH)₄), for 30 min in a modified Barth's buffer. Bar charts represent means $\pm$ SE of 5 pools of oocytes (n=7-12 oocytes per pool). Different letters mean significant differences (p>0.05) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

4. Discussion

4.1. Element composition and relation in cracked melons

In this study, we focused on the nutrients most utilized to reduce melon cracking that are Ca, B and Zn. Ca is the most common treatment used to prevent the appearance of cracking in fruits, with positive incidence in some species such as blueberry (Marshall et al., 2019), lemon (Bakshi, 2018), litchi (Dinesh et al., 2017), pomegranate (Davarpanah et al., 2018) or sweet cherry (Breia et al., 2020). On the other hand, Ca has been proven to improve the growth of melon plants, increasing the number of leaves, the relative water content, the number of melons per plant, and the properties of the fruit such as increasing its diameter, thinness and firmness (Khoravi mashizi and Sarcheshmehpour, 2015).

Furthermore, the microelements applied in our treatments, B and Zn, are typically used in the prevention of cracking (Bakshi, 2018; Chater and Garner, 2018; Dinesh et al., 2017; Draie and Aboras, 2021) and both of them are associated with cell wall and membrane integrity, and nutrient transport (Barzana et al., 2021). B is an important structural microelement, as it is associated with the apiosyl residue of rhamnogalacturonan-II (RGII) and the cross-linking of two monomers (Ishii and Matsunaga, 1996). It is therefore a fundamental element needed for the correct biosynthesis of cell walls and the development of new tissues (Lewis, 2019). In previous studies, it has been described that B deficiency may increase fruit cracking incidence (Khadiji-Khub, 2015), and resistant cultivars have higher levels of B and Ca (Yang et al., 2016). This makes sense, given that B is required, next to Ca, for maintaining the pectin network and the "egg-box" structure (Shi et al., 2017). Zn is necessary for maintaining the integrity of membranes, their phospholipid levels and the correct ion transport system, which affects the capacity of water uptake (Dang et al., 2010; Kasim, 2007). Altogether, these elements could have an influence on cracking reduction.

In our study, we observed that in the first two weeks after application of the Ca+B+Zn and B+Zn treatments, there were no significant changes in terms of the accumulate incidence of cracking. These results are in agreement with previous results (Lopez-Zaplana et al., 2020a), in which similar treatments, composed of Ca and micronutrients or only micronutrients, did not lead to any significant differences in cracking after one week of treatment. However, in the present work, significant differences were found from the third week of treatment, with a decrease in the incidence of cracking in both foliar mineral treatments with respect to the control, highlighting the relationship between greater fruit ripening process and increased incidence of cracking already described by other authors (Domínguez et al., 2012; Jiang et al., 2019).

In this study, we also wanted to analyze Si, although it was not applied in the treatments, as its involvement as a modulator of the response to abiotic stress is known (Ma and Yamaji, 2006; Seal et al., 2018). In melon, it has been described as an important element associated with the resistance to high temperature stress (Lopez-Zaplana et al., 2022), which could be related with cracking. In addition, Si is a substrate of some aquaporins (Lopez-Zaplana et al., 2020b; Pommerrenig et al., 2015).

In this study, a significant increase in B, Si and Zn content was only observed in the non-cracked melons from the Ca+B+Zn treatment, pointing to the fact that Ca could be involved in the regulation of their movement. In this sense, some studies have shown that the activity of aquaporins is positively regulated by Ca through phosphorylation (Alleva et al., 2006; Johansson et al., 1996), by increasing passive diffusion of water and other solutes, or by directly increasing the activity of specific aquaporins such as NIPs, which are able to transport B or Si (Bienert et al., 2008; Lopez-Zaplana et al., 2020b).

In addition, Ca also plays an important role at the cell structure level. A recent theory argues that Ca may reduce fruit cracking through the inhibition of cell wall swelling by its accumulation in the pectin network (Yu et al., 2020). Indeed, Ca crosslinks the carboxyl groups of the homogalacturonan domains and forms an “egg-box” structure, accounting for 60-75% of the total cell wall (Aghdam et al., 2012). Furthermore, in numerous studies, it has been observed that the fruits with cracking presented lower levels of Ca (Shi et al., 2017; Yang et al., 2016). But nevertheless, we could find significant increases in cracked melons in both foliar treatments respect non-cracked control melons. Bearing in mind that Ca movement depends on the flow of water from transpiration in the apoplastic pathway (Gilliam et al., 2011), we could think both apoplastic and symplastic pathway, mediated by aquaporins (Yaaran and Moshelion,

2016), both routes are altered and there is an increase in generalized water transport. Finally, abiotic stresses trigger Ca signals, that convergent with abscisic acid (ABA) modulating the common targets (Edel and Kudla, 2016), being the ABA involved in different process like fruit cuticle integrity (Gutiérrez et al., 2021) or root hydraulic conductance via aquaporin expression modulation (Mahdieh and Mostajeran, 2009).

4.2. Aquaporin expression patterns in cracked melons

Aquaporins are an interesting object of study regarding the cracking physiopathy, which is related to water (Fernández-Trujillo et al., 2013; Lopez-Zaplana et al., 2020a). Studying the proteins responsible for its membrane diffusion makes sense, although this study could be complex due to the wide variety of aquaporin subfamilies and members (Maurel et al., 2015).

When comparing non-cracked and cracked melons, we highlighted that in the control treatment, which showed the highest incidence of cracking, no differences in the expression of most aquaporin genes were found. The only two exceptions were *NIP2;2* and *NIP5;1* (both exhibiting water channel activity), with both increasing their expression in the cracked control melons as compared with non-cracked ones.

Upon both foliar treatments, several aquaporins showed very specific patterns. This suggests that the ability to reduce melon cracking by adding Ca, B or Zn is directly associated to the control of aquaporins. In general, we could find an increase in expression in cracked melons as compared with non-cracked ones, therefore demonstrating their involvement in the cracking process, either as a cause or an effect. This was the case for *PIP1;1*, *PIP1;2* and *TIP1;1*, which showed the highest mRNA levels in cracked melons in all treatments, being this relation significant if we study the cracked non-cracked variable in the two-way ANOVA.

PIP1s have been mainly associated to water transport functions and modulation of PIP2s activity, principally increasing the cell P_f when PIP1s are co-expressed with PIP2s (Berny et al., 2016; Bienert et al., 2018; Fetter et al., 2004; Postaire et al., 2010; Zelazny et al., 2007). In our experiment, we observed a significant decrease in *PIP1;1* and *PIP1;2* expressions in non-cracked melons in both treatments, but not in the control treatment. This points to a possible decrease in the water membrane permeability in this treatment possibly leading to the decreased incidence of cracking we observed. Recently, the aquaporin with the highest expression in sweet cherry, *PIP1;4*, was shown to be overexpressed when the plant was treated with Ca, a

treatment decreasing cracking (Breia et al., 2020). In our study, the highest expressed PIP aquaporin was the *PIP1;1*, which showed an increased expression in cracked fruits with respect non-cracked fruits (p value of 0.02869), and the same response was observed for *PIP1;2* (p value of 0.00464). Therefore, the relationship between this two PIP1s and cracking incidence in melon could be established.

In the same manner, *TIP1;1* showed a similar expression pattern as PIP1s. *TIP1;1* has been characterized as the main aquaporin responsible for water transport in the tonoplast of maize (Barrieu et al., 1998) and melon (Lopez-Zaplana et al., 2020b), being the most expressed aquaporin in melon fruit. Furthermore, it could be able to transport urea, ammonia or hydrogen peroxide (Schüssler et al., 2008), with the latter being an important molecule for stress signaling (Bienert and Chaumont, 2014). The significant decrease in *TIP1;1* expression in non-cracked melons as compared with cracked melons in Ca+B+Zn treatments suggests that a decrease in water accumulation in the tonoplast is a possible effect of our treatments. Furthermore, the two-way ANOVA point the importance of the increase of expression in cracked melons with a p value of 0.0008 respect cracked/non-cracked variable.

Apart this 3 water related aquaporins (*PIP1;1*, *PIP1;2* and *TIP1;1*), the increase in oocyte P_f after expression of *NIP2;2* and *NIP5;1* demonstrated their ability to facilitate water diffusion, and they could be associated with the incidence of cracking respect water transport imbalance. In this sense, the increased expression of some aquaporin genes in cracked fruits was previously described in a transcriptomic study where they identified different transcripts involved in water transport in cracked fruits in *Litchi chinensis* Sonn, such as *LcAQP*, *LcPIP*, *LcNIPs* and *LcSIPs* (Li et al., 2014), and other studies on melon cracking indicated that its cause was due to a very rapid and abundant uptake of water into the fruit (Lopez-Zaplana et al., 2020a). Although it seems obvious that there was an imbalance in water transport in plants that suffer cracking and that this process is mediated by some aquaporins, some studies pointed out a correlation between the increased expression of some aquaporins and a reduction in the number of cracked fruits (Breia et al., 2020; Joshi et al., 2018). Joshi et al. (2018) described how an increase in aquaporin expression reduced the incidence of cracking in apples, as was the case of *PIP2A*.

If we compared the treatments, it is noteworthy that there were a large number of aquaporins had their expression modified by the Ca+B+Zn treatment, with the highest values observed in cracked melons. This was the case for *PIP1;2*, *PIP2;1*, *PIP2;6*, *PIP2;8*, *PIP2;9*, *TIP1;1*, *TIP5;1*, *NIP2;1*, *NIP4;1*, *NIP5;2* and *NIP7;1*. Among them, *PIP1;2*, *PIP2;6*, *PIP2;8*, *TIP1;1*, *TIP5;1* and *NIP5;2* showed a significant increase in expression in cracked melons in

the Ca+B+Zn treatment as compared with non-cracked melons for the same foliar treatment. Here, we found many aquaporins associated with transport of water, but also other compounds such as B or Si (Lopez-Zaplana et al., 2020b). Therefore, it seems that Ca played a role in altering the expression patterns, not only affecting the transport of water, but also other important nutrients necessary for the proper intracellular balance and structural development of the melon fruits.

The expression of most aquaporins was unaffected by the B+Zn treatment in non-cracked melons, although it did affect aquaporins in relation to cracking occurrence. Indeed, only *PIP2;4* and *TIP1;2* expressions were reduced in non-cracked melons in this condition, but *TIP1;2* were unaffected by Ca+B+Zn treatment. Therefore, the presence of Zn or B seems to influence *TIP1;2* expression depending on the presence or absence of Ca in the treatment solution. It was suggested that the foliar application of Zn decreased aquaporin activity, mainly through post-translational modifications, while its effect on the transcriptional level was variable (Barzana et al., 2021), but this hypothesis needs to be proven with activity analysis. For example, *PIP2;4* expression decreased in all melons treated with Ca+B+Zn and with B+Zn compared with the control foliar treatment, suggesting that B+Zn reduced its expression. Other studies reported a decrease in *HvPIP1;2*, *HvPIP2;4*, *HvPIP2;5* expression (Gitto and Fricke, 2018) and *AQUA1* (Ariani et al., 2019), or the non-modification in the transcription of aquaporins (Devi et al., 2016), depending on Zn concentrations. Furthermore, Zn was also associated with an increase in the water channel activity and the expression of several aquaporins in pak choi roots and leaves (Fatemi et al., 2020). Since Ca enhanced Zn accumulation in non-cracked melons, it could be argued that *TIP1;2* expression could be affected by Zn content in fruit, but its possible involvement in the aquaporin regulation needs further investigation.

Notwithstanding the fact that all the known aquaporins were detected in melon fruit, some of them (*PIP2;1*, *PIP2;4*, *PIP2;7*, *TIP1;2*, *TIP4;1*, *TIP5;1*, *NIP2;2* and *XIP1;1*) had a very low expression level, suggesting that their expression levels may not be related to the significant changes in water or mineral transport (**Supplementary figure 1**). On the other hand, *PIP1;1*, *PIP1;2*, *PIP2;2*, *PIP2;3*, *PIP2;5*, *PIP2;6*, *PIP2;10*, *TIP1;1*, *TIP2;2*, *TIP3;1*, *NIP5;1* and *NIP6;1* had the highest expression level (**Supplementary figure 1**). When compared with their expression in roots and leaves (Lopez-Zaplana et al., 2020b), we observed for most of the aquaporin genes a similar trend, with some interesting differences. For instance, *TIP1;3* and *TIP2;2* were mostly expressed in melon fruit, while they were almost undetectable in other

parts of the plant, highlighting their relevance in fruit development. As for the cracking incidence, *TIP1;3* had the lowest expression level in control cracked plants, while it increased in the non-cracked B+Zn melons. Its possible involvement in the transport of B was previously predicted (Lopez-Zaplana et al., 2020b) and based our B swelling results it could be one of its main functions in melon fruits.

Finally, the expression of *PIP2;2*, *PIP2;5*, *PIP2;7*, *PIP2;10*, *TIP2;2*, *TIP3;1*, *NIP6;1*, *SIP1.1* and *XIP1.1* was not affected by either of the foliar treatments in cracked or non-cracked melon.

4.3. Transport activity in *X. laevis*

The only two aquaporins that showed a significant increase in expression in cracked melon as compared with non-cracked melons in control conditions were *NIP2;2* and *NIP5;1*, suggesting that their regulation is related with cracking. When expressed in *Xenopus* oocytes, *NIP2;2* showed the highest B channel activity amongst all the studied aquaporins, but it also acted as a water channel, and was the only isoform transporting Si, in accordance with previous predictions (Lopez-Zaplana et al., 2020b). Si has a mechanical barrier function, defending tissues against abiotic stress, pathogens, and colonization by fungi (Ma and Yamaji, 2006; Rémus-Borel et al., 2005; Rodrigues et al., 2004). In this sense, its increase in expression in cracked control melons could be a response for protecting the fruit from the outside (Rémus-Borel et al., 2005; Rodrigues et al., 2004). *NIP5;1* showed the highest expression amongst *NIP* isoforms and facilitated membrane diffusion of water but not B or Si. Both *NIP2;2* and *NIP5;1* increased the P_f when we increased the amount of RNA microinjected according with other studies (Bienert et al., 2018; Fetter et al., 2004). These results strongly support the importance of water transport in cracking incidence as proposed in numerous articles (Breia et al., 2020; Fernández-Trujillo et al., 2013; Joshi et al., 2018; Li et al., 2014; Lopez-Zaplana et al., 2020a). *NIP1;1* did not show any transport activity for water nor Si, but we could detect some activity in facilitating B diffusion. Interestingly, *NIP1;1* showed an expression increase in both mineral treatments, reaching maximum values in the presence of Ca+B+Zn, and was the only aquaporin with a significant increase in expression in non-cracked melons with Ca+B+Zn as compared with non-cracked control melons, supporting the idea that the B transport activity of *NIP1;1* was favored by Ca in melon.

In contrast, *TIP1;3* and *TIP2;2*, which showed a high expression levels in melon fruit as compared to the expression in leaf or root (Lopez-Zaplana et al., 2020b), did not exhibit very clear results regarding their water channel activity, even if a slight increase in P_f was observed when injecting more cRNA. They did not facilitate Si diffusion, in accordance with our previous predictions (Lopez-Zaplana et al., 2020b), although *TIP1;3* showed a slight transport of B. The case of *TIP2;2* was especially striking, as it had a high specificity in the transport of NH_3 (Lopez-Zaplana et al., 2020b) and, as we showed here, it also facilitated B diffusion. The decrease in its expression in non-cracked melons from the B+Zn treatment compared with the control treatment, and the large increase in expression in the cracked melons from the same B+Zn treatment again suggest a regulation of this isoform by Zn. In any case, it seems quite evident that the regulation of B content is especially important in fruit development.

5. Conclusions

From this work, we can conclude that both foliar mineral treatments were able to reduce the cumulative incidence of cracked melons from the third week. Moreover, we demonstrated a relation between aquaporin expression and cracked fruit, mainly for water active channels, which can facilitate the entry of water (probably as a response to changes in ambient conditions), producing cracked fruits. In the cracked melons without foliar mineral treatments, *NIP2;2* and *NIP5;1* were overexpressed in the cracked part, indicating a basal regulation of these aquaporins. Furthermore, *NIP2;2* is a channel for B and Si, indicating the possible need to increase the concentration of these nutrients in the cracked fruits. Similar observation can be done for *PIP1;1* and *PIP1;2* water channels, with high expression levels in the fruit and with significantly higher levels in cracked melons in both foliar mineral treatments, suggesting a regulation of the water uptake, which could lead to an increase in the internal pressure of the fruit. The melon could be increasing the expression of *TIP1;1*, the most expressed TIP in melon fruit, to decrease the amount of water in the cytosol. Regarding the role of the elements, Ca could be involved in the regulation of the increased transport of other nutrients and water flow. With the changes in the expression levels of some aquaporins such as *TIP1;3*, *TIP2;2*, *NIP1;1* and *NIP2;2*, it seems that both mineral treatments induced the entry and storage of B. Finally, the possible role of Zn in *TIP2;2* regulation and Ca in *NIP1;1* function was strongly suggested.

Until now, not many studies have been found which deeply analyzed the expression patterns of all aquaporins in a fruit and their involvement in cracking. Thus, the present work

is an important starting point for the promotion and development of aquaporins as molecular markers, as they seem to have a considerable influence on the appearance of this physiopathy. In the future, it would be interesting to simplify the foliar treatments to unique elements, incorporating different cultivars, or species resistant or susceptible to cracking, and to analyze how the expression patterns of aquaporin are affected.

Funding

This work was supported by the Spanish Ministry of Science, Innovation and Universities (RTC-2017-6119-2) and developed in the Spanish Higher Council for Scientific Research (CSIC). The stay of A.L.-Z. in F.C. laboratory was possible thanks to the scholarship EIDUM-CMN of Murcia University. L.D. was a postdoctoral researcher supported by the Belgian National Fund for Scientific Research (FNRS).

Author contributions

Conceptualisation: G.B., F.C. and M.C. Methodology and Investigation: A.L.-Z., L.D. and G.B. Formal analysis, Data Curation and Visualization: A.L.-Z., L.D. and G.B. Writing—Original draft: A.L.-Z. and L.D. Writing-Review & editing: L.D., G.B., F.C. and M.C. Supervision: F.C. and M.C. Validation, Project administration and Funding acquisition: F.C. and M.C. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors thank Sakata Seeds Ibérica S.L.U. for providing the melon seeds and fields, Mr. Mario G. Fon the language correction, Jahed Ahmed for collecting the oocytes from *Xenopus* and Lucia Yepes for the statistical analysis correction.

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Aquaporins involvement in the regulation of melon (*Cucumis melo* L.) fruit cracking under different nutrient (Ca, B and Zn) treatments.

Alvaro Lopez-Zaplana^a, Gloria Bárzana^a, Lei Ding^b, François Chaumont^b and Micaela Carvajal^{a*}

*Corresponding author: mcarvaja@cebas.csic.es

^a Aquaporins Group. Plant Nutrition Department, Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Campus Universitario de Espinardo, Edificio 25, 30100 Murcia, Spain.

^b Louvain Institute of Biomolecular Science and Technology, UC Louvain, 1348 Louvain-la-Neuve, Belgium

ABSTRACT

Melon cracking is a physiopathy that is associated with both internal and external changes by the alteration of the water balance and nutrient homeostasis in the fruit. Aquaporins are channels for water and other small solutes, and they are potentially involved in the regulation of melon cracking. In this work, we studied the mineral concentration and expression of all aquaporins in non-cracked and cracked melon pulp in control and after the application of foliar mineral treatments (Ca+B+Zn and B+Zn) in field conditions. Also, we measured the mineral transport of some aquaporins to connect it to cracking. The results showed that both treatments could ameliorate the incidence of cracking. Mineral elements determination showed increases in B, Ca, Si and Zn content in non-cracked Ca+B+Zn treated melons. In control conditions, only *NIP2;2* and *NIP5;1* had a significant increase in expression in cracked melons compared with non-cracked ones pointing to their involvement in cracking. Furthermore, we were able to verify that the high expression of *PIP1;1*, *PIP1;2* and *TIP1;1*, which are efficient water channels, was involved in the changes observed in cracking incidence in both foliar mineral treatments. Finally, transport assays in oocytes were performed with selected isoforms, highlighting the water channel activity of *NIP2;2* and *NIP5;1*, the B channel activity of *TIP1;3*, *TIP1;2*, *NIP1;1* and *NIP2;2*, and the Si channel activity of *NIP2;2*. In conclusion, both effective foliar treatments for avoiding cracking point to the *PIP1;1*, *PIP1;2*, *TIP1;1* and *NIP5;1* and *NIP2;2* aquaporins as possible molecular markers.

Keywords

Cucumis melo L., cracking, fruit splitting, aquaporin, foliar nutrition, oocyte expression.

1. Introduction

Aquaporins are membrane channels that facilitate the diffusion of water and small molecules across biological membranes (Maurel et al., 2015). Aquaporins occur as multiple isoforms, reflecting a high diversity in cellular localization, transport selectivity, and regulation properties. Currently, five subfamilies have been found in land plants, PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin26-like intrinsic proteins) (Chaumont et al., 2001), SIPs (small basic intrinsic proteins) (Johanson and Gustavsson, 2002) and XIPs (X-intrinsic proteins) (Gupta and Sankararamakrishnan, 2009). Furthermore, some plant aquaporins can facilitate the diffusion different substrates depending their structure and sequence. For example, some PIPs can also transport H₂O₂ (Hooijmaijers et al., 2012) and CO₂ (Uehlein et al., 2003), some TIPs can transport nitrogen (N) compounds such as ammonium (Loqué et al., 2005) and urea (Gerbeau et al., 1999), and some NIPs are usually permeable to some beneficial mineral nutrients such as boron (B), silicon (Si) or selenium (Se) and other toxic elements such as arsenic (As) or antimony (Sb) (Bienert et al., 2008).

Given the importance of aquaporins in regulating essential water and solute transport through cell membranes, it is not surprising that the regulation of these proteins is associated with tolerance to numerous stresses (Barzana et al., 2021; Cabañero and Carvajal, 2007; Yepes-Molina et al., 2020) and physiopathies (Hagassou et al., 2019; Shen et al., 2014). One of the multiple physiopathies related with water is the cracking or splitting of fruits, which consists in the appearance of cracks in fruit due to an imbalance between internal and external pressure (Matas et al., 2004). In this way, some transcriptomic studies have associated cracking with several aquaporins genes such *LcAQP*, *LcPIP*, *LcNIP*, or *LcSIP* in *Litchi chinensis* Sonn (Li et al., 2014). In general, the appearance of fruit cracking has been associated to weather events (intense rainfall, changes in temperature, or high solar radiation), soil conditions (deficit of some nutrient) (Khadivi-Khub, 2015; Li and Chen, 2017; Peet, 1992; Winkler et al., 2016) or other internal causes that can affect the balance of cations, water content or other factors (Capel et al., 2017; Cline and Trought, 2007; Joshi et al., 2018; Qi et al., 2015).

Cracking is a physiopathy with a growing interest as can be seen in the numerous articles that have published in recent years for different fruits including cherries (Fresno and Munné-Bosch, 2021; Gutiérrez et al., 2021; Michailidis et al., 2020) tomato (Capel et al., 2017; Liu et al., 2022; Zhang et al., 2020), jujube (Hou et al., 2022; Li et al., 2020; Ren et al., 2017) or pomegranate (Chater and Garner, 2018; Davarpanah et al., 2018; Draie and Aboras, 2021). But also, one of the plants that can suffer cracking and it has a relevant economic impact is the

melon, *Cucumis melo* L., a member of the Cucurbitaceae family that has a high economic impact in global market, being the fourth most important fruit in the world (De Campos et al., 2017). Among the main causes of melon cracking, differences between abrupt temperatures between day and night, high exposure to the sun during ripening, or high levels of humidity have been described (Fernández-Trujillo et al., 2013), as well as drastic increases in water flow, which alter the standard water flow throughout the plant (Lopez-Zaplana et al., 2020a; Measham et al., 2010). Despite the economic losses that are generated by the splitting of melons (Khadivi-Khub, 2015), not many studies focusing on the expression of genes in this fruit are available, perhaps due to the difficulty in measuring their expression, as the amount of water, secondary metabolites and concentration of sugars in the fruit are very high, making it difficult to work with this genetic material (De Campos et al., 2017).

A typical manner to address this physiopathy has been based on the application of foliar treatments composed of B (Ihsan ul and Abdur, 2012), zinc (Zn) (Chater and Garner, 2018) or some other macronutrient such as calcium (Ca), which have already been shown in numerous studies to reduce the incidence of cracking in different plants (Bakshi, 2018; Breia et al., 2020; Davarpanah et al., 2018; Dinesh et al., 2017). B is a very important nutrient implicated with the formation of cell walls (O'Neill et al., 2004) and, therefore, an increase in B could stimulate the formation of new walls and allow better fruit growth, preventing any mismatch between the internal and external pressure of the fruit. On the other hand, Ca is involved in the stabilization of the membrane systems and the formation of Ca pectate, increasing the rigidity of the middle lamella and cell wall (Mignani et al., 1995). Furthermore, Ca could prevent the incidence of cracking by regulating the expression of some aquaporins (Breia et al., 2020). Finally, Zn could act as an inhibitor of aquaporin activity (Németh-Cahalan et al., 2007; Yukutake et al., 2009), leading to a decrease in water uptake and reduction in the internal pressure.

The present work characterized the cumulated cracking incidence with mineral concentration and the expression of aquaporins, for a period of 5 weeks after the application of two foliar treatments composed of Ca+B+Zn (in the forms of CaCl₂, H₃BO₃, and ZnSO₄) and B+Zn (in the forms of H₃BO₃ and ZnSO₄). Our initial hypothesis is that water could be involved in the appearance of cracking, due to an imbalance in the expression of aquaporins, mainly by PIPs. In addition, as some of these aquaporins can also transport different metalloids such as B or Si, we will also verify their transport in vivo, since they may be related to the formation of cell walls and, in some way, with the rigidity and then, to the appearance of cracking. To prove that, we studied the expression (mRNA levels) of all the melon aquaporins identified in cracked

and non-cracked melon fruits. We also tested the channel activity to water, Si and B of selected aquaporins (CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1), to prove their involvement in nutrient and water transport linked to cracking.

2. Material and methods

2.1. Experimental orchard conditions and foliar treatments

The experiments were conducted in the summer growing season in commercial fields in Manzanares (Ciudad Real, Spain), under continental climate conditions of stable temperatures during the summer (July-August). The average temperature in this area during these two months was 27.5 °C, with an average minimum of 17.6 °C at night and an average maximum of 37.3 °C, during the day. The average relative humidity was 34%. The seedlings of *C. melo* cv. Grand Riado (Sakata) were transplanted on May 16th, 2020 on soil, and the fruit harvest was carried out three months later. All plants were drip irrigated as done commercially, with fertirrigation containing KNO₃ (5.2 g m²).

Two months after plant transplant, three foliar treatments were applied once a week for three weeks. The first foliar mineral treatment, Ca+B+Zn, consisted of 10 mM CaCl₂, 7 mM H₃BO₃ and 2 mM ZnSO₄. The second foliar mineral treatment called B+Zn, consisted of 7 mM H₃BO₃ and 2 mM ZnSO₄. As a control foliar treatment, deionized water was used. Each plant received 200 mL of these solutions in each of the three applications.

The experimental design was a completely randomized design. The three foliar treatments were distributed randomly in four different lines within the cultivation field with 6 plots per line (24 total plots). The spatial arrangement of the treatments was random so that it did not affect the results and included one line of plants as border between treatments. The experiment was carried out in 58-65 plants per treatment, distributed in 8 plots per treatment, with 7-9 plants per plot, for a total of 230-250 melons per treatment.

2.2. Incidence of cracked melons

After the third application of the treatments, the total number of cracked and non-cracked melons was counted for the first time and, from that moment on, they continued to be counted, once a week, for 5 weeks. To take a melon into consideration, it had to have a commercial size, greater than 2.5 kg, leaving out those of smaller size. Once a cracked melon appeared, it was

separated from the plant and discarded so that it would not be counted again in the following weeks. In this way, we reduced the appearance of new cracking caused by non-genetic factors, such as insects or animals that are attracted to these first cracks and can affect the other fruits. To consider that a melon was cracked for our harvesting and study, the cracking should be greater than 1 cm but smaller than 3 cm in width, through which the pulp could be seen.

2.3. B, Ca, Si and Zn concentration

Five weeks after application of foliar treatments, in order to know the amounts of the elements applied in the foliar treatments per melon, the pulps of three independent melons with the same characteristics (cracked or not cracked and type of foliar treatment applied) were analyzed. To separate the pulp from the rind, the first 3 cm were considered as rind, with the rest being the pulp. The pulp area near the crack was collected for cracked melon samples, but not the exposed area. For non-cracked melon samples, the same part of the pulp was collected. All the samples were dried and then finely ground in a mill grinder (model A10, IKA, Staufen, Germany) and digested with HNO₃–HClO₄ (2:1) in a microwave oven (CEM Mars Xpress, Mattheus, NC, USA). In addition, Si concentration was determined. All the samples were digested using 20% NaOH. The analysis of the elements was carried out using a Perkin–Elmer (Waltham, MA, USA) 5500 model ICP emission spectrophotometer (Iris Intrepid II, Thermo Electron Corporation, Franklin, TN, USA).

2.4. RNA extraction and cDNA synthesis

Five weeks after the application of foliar treatments, total RNA was extracted from the internal part of the pulp using the NZY Total RNA Isolation kit (Nzytech, Lisbon, Portugal). For cracked melons, the tissue close to the cracked area was sampled, avoiding the exposed part of the crack. Once the sample was crushed in liquid nitrogen, a total of 50 mg of pulp was processed, by means of a vortex (20 s) and a centrifugation (13000 gs, 2 min), to separate nucleic acids from cellular material, passing the resulting sample through the first kit column. The rest of the procedure was similar to the manufacturer's protocol. The quantity and purity of RNA were measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and the integrity of the RNA was verified by agarose gel electrophoresis. Genomic DNA was removed using DNase from the NZY Total RNA Isolation kit (Nzytech, Lisbon, Portugal) according to the manufacturer's instructions. The RNA was stored at -80 °C until use. The High-

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to synthesise cDNA from 2 µg of total RNA, according to the manufacturer's protocol.

2.5. Quantitative real-time PCR (RT-qPCR) analyses

The expression of all the melon aquaporin genes were checked by RT-qPCR with the gene-specific primers already published by our group (Lopez-Zaplana et al., 2020b). It was performed using 2 µL of 1:2 or 1:5 diluted cDNA samples, increasing the concentration for lower expression genes, 500 nM of primers and 5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in 10 µL of total volume reaction. The equipment used was a QuantStudio™ 5 Flex Real-Time qPCR system (Applied Biosystems, Foster City, CA) and the qPCR program consisted of 10 min initial denaturation at 95 °C and then amplification in a two-step procedure: 15 s of denaturation at 95 °C and 60 s of annealing and extension at a primer-specific temperature for 40 cycles, followed by a dissociation stage. Data collection was carried out at the end of each round in step two. These conditions were used for both target and reference genes and the absence of primer-dimers was checked in controls lacking templates. Five housekeeping genes were tested as reference genes (RPL, RAN, ACT, ADP and GAPC2) (Kong et al., 2014) selecting the most stable gene in our conditions, in this case RPL (Ribosomal protein L). Whole specific primers used for RT-qPCR are listed in **Supplementary table 1**. RT-qPCR analysis was performed on 3 independent samples for each treatment (biological replicates) and each sample reaction was carried out in triplicate (technical replicates) in 96-well plates. The transcript levels were calculated using the relative expression method comparing with a housekeeping gen. Negative controls without cDNA were used in all PCR reactions.

2.6. Heterologous expression in *Xenopus laevis* oocytes and transport assays.

The coding region of *CmTIP1;3*, *CmTIP2;2*, *CmNIP1;1*, *CmNIP2;2* and *CmNIP5;1* were PCR amplified and cloned into the USER vector pNB1u and pNB1-YFPu using the USER system (uracil-specific excision reagent cloning technique) compatible with *X. laevis* oocytes expression system (Bienert et al., 2011; Fetter et al., 2004; Nour-Eldin et al., 2006). The pNB1-YFPu vector contains the cDNA encoding the Yellow Fluorescent Protein (YFP) that will be fused to the N-terminus the protein of interest. These genes were selected according to the qPCR results, showing significant differences between treatments or non-cracked/cracked melons or

their increase of expression in the fruit respect other tissues. The gene-specific primers used are listed in **Supplementary table 2**. The generated constructs were verified by Sanger sequencing (Microsynths AG, Switzerland). *In vitro* transcription was performed using T7 RNA polymerase (Promega, USA), treated with Rq1 RNase-Free DNase (Promega, USA) according to the manufacturer's protocol and purified with phenol/chloroform method (Bienert et al., 2011).

50 nL of complementary RNA (cRNA) encoding the aquaporins of interest were injected into each *X. laevis* oocyte (Fetter et al., 2004), at increasing concentrations of 0.2, 0.4 and 0.8 ng nL⁻¹. The selected injected oocytes were kept at 20°C for 2-3 days in Barth's buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM Hepes-NaOH, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, pH 7.4, and ~ 210 mOsm kg⁻¹) to allow protein expression. The YFP signal was detected by confocal microscopy (LSM 710, Carl Zeiss, Oberkochen, Germany) in oocytes microinjected with 40 ng of cRNA (*YFP-CmTIP1;3*, *YFP-CmTIP2;2*, *YFP-CmNIP1;1*, *YFP-CmNIP2;2* and *YFP-CmNIP5;1*). The oocytes were fixed with 4% (p/v) paraformaldehyde and kept at 4 °C until YFP detection (Fetter et al., 2004). The YFP was excited with the 514 nm wavelength of an argon multilaser and the emitted YFP fluorescence detected between 530 and 570 nm.

For the water transport assay, an individual oocyte was transferred from full to 1/2 Barth's buffer to induce swelling. Three independent experiments were performed with three different amounts of injected cRNA (10 ng, 20 ng and 40 ng). In total, 30 to 40 oocytes were tested for each gene and each concentration of cRNA injected. As a positive control, 5 ng of *ZmPIP2;5* cRNA were injected for the swelling assay. The oocyte swelling change was recorded and the water permeability coefficient (P_f) was calculated using the equation

$$P_f = V_0[d(V/V_0)/dt]/[S \cdot V_w(Osm_{in} - Osm_{out})],$$

with an initial volume (V₀) of 9 × 10⁻⁴ cm³, an initial oocyte surface area (S) of 0.045 cm², and a molar volume of water (V_w) of 18 cm³/mol (Fetter et al., 2004; Zhang and Verkman, 1991).

For the boric acid transport assay, oocytes were microinjected with 40 ng cRNA of each gene. As a positive control, *NtXIP1.1* cRNA (12.5 ng) was microinjected per oocyte. After 2-3 days, they were transferred from Barth's buffer to modified iso-osmotic Barth's solutions in which NaCl was replaced with 178 mM H₃BO₃. The oocyte swelling was monitored according to the above-mentioned water swelling assay. The relative volume change (V_t/V₀) was

calculated (Gerbeau et al., 1999; Tanaka et al., 2008). Two independent experiments were performed with a total of 30 to 40 oocytes.

For the Si transport assay, 40 ng cRNA were injected into an oocyte for each gene. The oocytes were exposed to 1 mM GeO₂ in Barth buffer (pH 7.6) to generate Ge(OH)₄, which was used as a chemical analogue of Si(OH)₄ (Jian et al., 2006). At time 0 and 30 min, the oocytes were collected and washed five times with Barth solution. For each gene, 8-14 oocytes were mixed as one group and five groups were digested with 10% HNO₃, heated to 150 °C, and analyzed by ICP-MS (Thermo model ICAP-QC), with a total of 50-60 oocytes for each gene and each time of incubation.

2.7. Statistical analysis

The statistical analysis was performed by using the SPSS 25.0.0.1 software package and R software (R Core Team, 2018). The incidence of cracking, element analysis, RT-qPCR analyses and Si transport assay were analyzed with a two-way ANOVA, followed by the *post hoc* Tukey's multiple comparison test. The P_f was analyzed by one-way ANOVA, followed by the *post hoc* Tukey's multiple comparison test. Boric acid transport assay in oocytes was analyzed with Student's *t* test. The values presented are the means ± SE. Significant differences between the values of all the parameters were determined at $p \leq 0.05$ (significance level of 5%).

3. Results

3.1. Incidence of cracking

After one week, the cumulated incidence of cracking (**Fig. 1**) was 1.92% for the control, 0.82% for the Ca+B+Zn treatment and 0.76% for the B+Zn treatment and no significant differences were observed between the different treatments. In the second week, the incidence of cracking was 3.86% for the control, 2.36% for the Ca+B+Zn treatment and 1.59% for the B+Zn treatment and they were not significantly different. In the third week, an increase of the incidence of cracking can be observed, with a 9.60% for the control, 5.48% for the Ca+B+Zn treatment and 4.11% for the B+Zn treatment and they were not significantly different. The fourth week showed the first significant difference between the control, 12.91%, and the B+Zn treatment, 4.45%. Ca+B+Zn treatment had a cracking incidence of 7.43% with no significant changes. Finally, the fifth week we had a significant decrease of incidence of cracking respect

the control fruits, with incidences of 17.61% for control, 8.17% for Ca+B+Zn and 5.71% for B+Zn. Two-way ANOVA results showed that the differences obtained could be explained by the time factor ($2e^{-16}$), the treatment factor ($8.56e^{-12}$), and the interaction between them (0.000514). If we consider only the time factor, we can see differences from the third week onwards. If we only consider the treatment factor, significant differences were observed between the control and the two treatments, but not between the treatments.

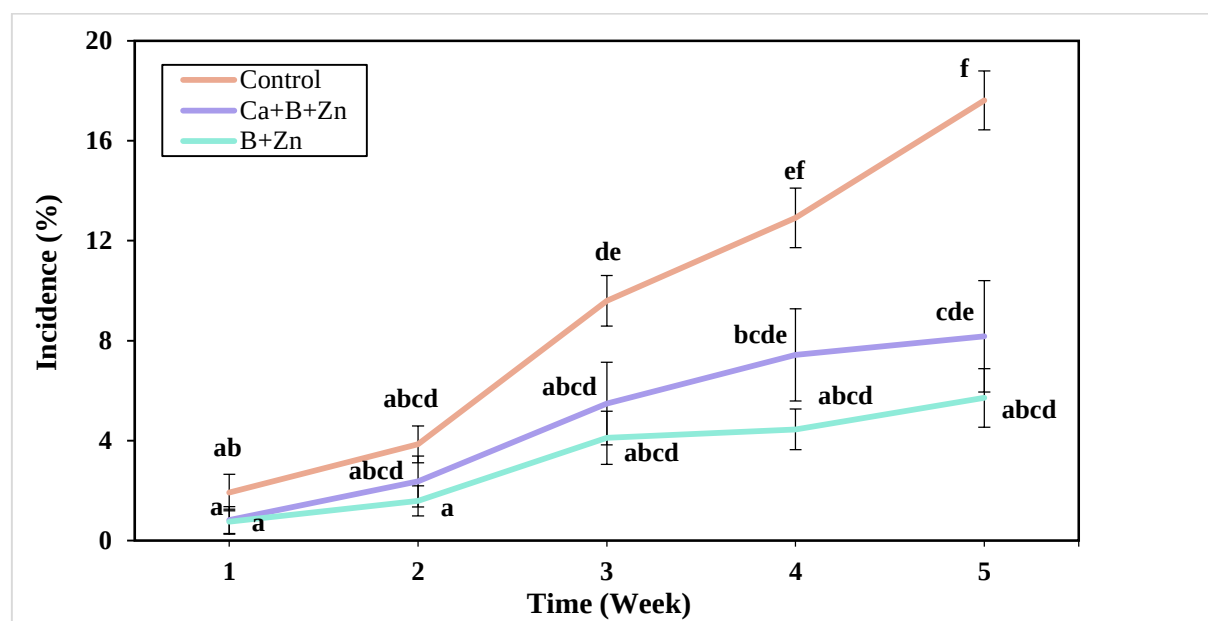


Fig. 1. Evolution of the cumulated incidence of cracking per week. Each value represents the percent of cracked melons from the total of melons in each treatment. Each value represents 8 different plots. Different letters mean significant differences ($p < 0.05$) between control and treatments, (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.2. B, Ca, Si and Zn concentrations in pulp under different foliar mineral treatments

B content in the pulp (**Fig. 2a**) was stable in all the fruits and treatments, except a significantly higher level in the non-cracked melons from the Ca+B+Zn foliar treatment with respect to other melons under different treatments. Calcium content (**Fig. 2b**) increased significantly in both non-cracked and cracked melons from the Ca+B+Zn treatment, with respect to the non-cracked melons from control treatment. Further, a significant increase of Ca concentration was also observed in the cracked melons from B+Zn treatment when compared

to the non-cracked melons from control and B+Zn treatments. Content of Si (**Fig. 2c**) and Zn (**Fig. 2d**) showed a similar response than B. Also, a significant increase of in non-cracked melons from Ca+B+Zn treatment were observed when compared with the melons from control treatment.

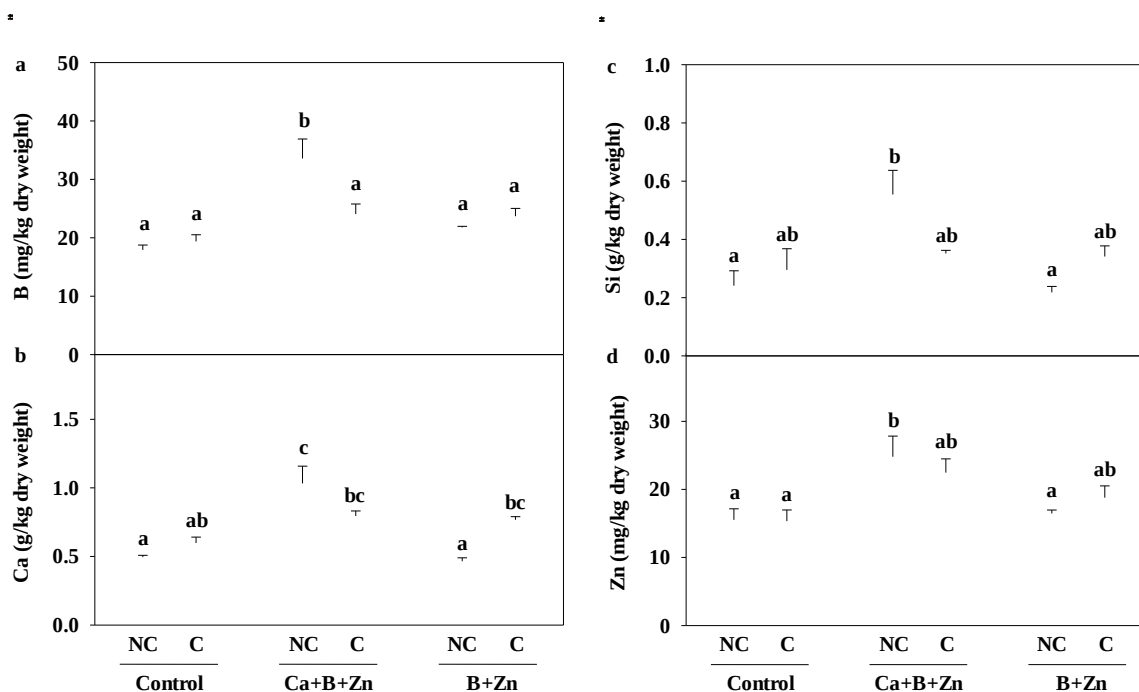
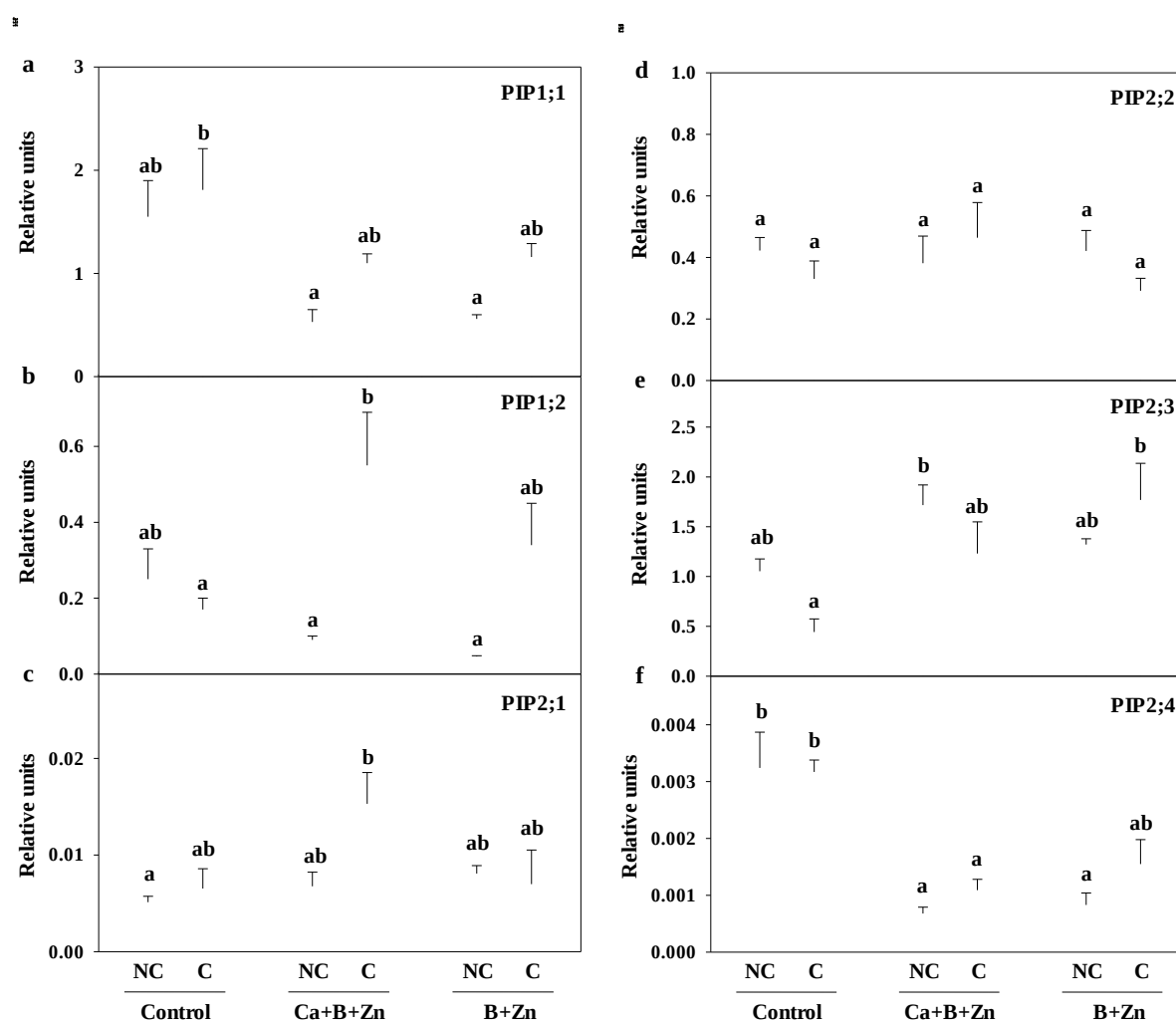


Fig. 2. B (mg) (**a**), Ca (g) (**b**), Si (g) (**c**) and Zn (mg) (**d**) per kg dry weight of pulp in NC (non-cracked melons) and C (cracked melons). Each value represents 3 biological replicates. Different letters mean significant differences ($p < 0.05$) between control and treatments, (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.3. Analysis of PIPs

The expression of the twelve identified *PIP* genes in melon was detected and quantified in fruits (**Fig. 3**). *PIP1;1* (**Fig. 3a**) showed significant decrease in expression in both foliar mineral treatments (Ca+B+Zn and B+Zn) non-cracked melons with respect to control cracked melons. Expression of *PIP1;2* (**Fig. 3b**) showed a significant increase of Ca+B+Zn cracked melons compared with cracked control melons and non-cracked Ca+B+Zn and B+Zn treatments melons. Expression of *PIP2;1* (**Fig. 3c**) showed a significant increase in cracked melons from the Ca+B+Zn treatment with respect to non-cracked control melons. Other aquaporin genes had stable expression levels without significant differences regardless of whether it was a non-cracked/cracked melon or if the foliar treatments were considered, as it

was the case for *PIP2*;2 (**Fig. 3d**), *PIP2*;5 (**Fig. 3g**), *PIP2*;7 (**Fig. 3i**) and *PIP2*;10 (**Fig. 3l**). *PIP2*;3 (**Fig. 3e**) did not show any significant difference in expression between cracked and non-cracked melons within the same foliar treatment, but a significant difference between the control cracked melons, non-cracked Ca+B+Zn treated melons and cracked B+Zn foliar treated melons were found, observing a significant increase in the expression of the last two. Expression of *PIP2*;4 (**Fig. 3f**) showed lower levels of mRNA in both foliar mineral treatments non-cracked melons and cracked Ca+B+Zn treatment respect to the control foliar treatment. However, no differences were observed between cracked and non-cracked melons in any foliar treatment. Expression of *PIP2*;6 (**Fig. 3h**) and *PIP2*;8 (**Fig. 3j**) showed similar response, a significant increase of expression in cracked Ca+B+Zn treatment melons with respect all other treatments and cracked or non-cracked melons. Expression of *PIP2*;9 (**Fig. 3k**) did not show significant differences within the same foliar treatment, but significant increase was found in cracked Ca+B+Zn treatment melons with respect to both cracked and non-cracked in control and cracked B+Zn treated melons.



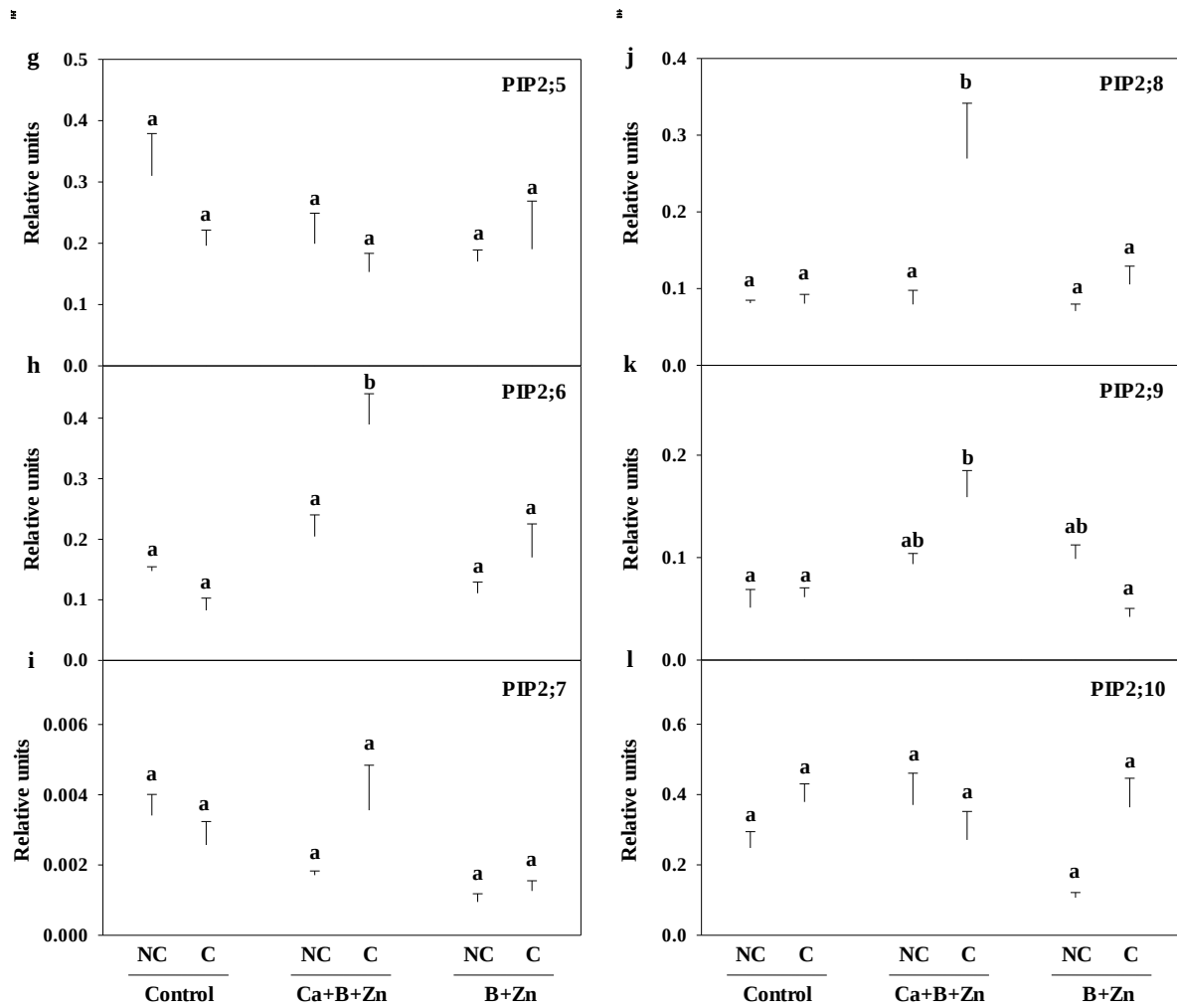


Fig. 3. Plasma membrane Intrinsic Proteins (PIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates of each measurement. Different letters mean significant differences ($p < 0.05$) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.4. Analysis of TIPs

The expression of the eight identified melon *TIP* genes were also measured in fruits (Fig. 4). *TIP1;1* (Fig. 4a) showed an increase in expression in cracked melons from the Ca+B+Zn treatment as compared to non-cracked melons. *TIP1;2* (Fig. 4b) showed a significant decrease in expression in non-cracked melons from the B+Zn treatment with respect to the non-cracked melons of the control treatment. *TIP1;3* (Fig. 4c) showed the lowest expression in cracked control melons and the highest in non-cracked B+Zn fruits with significant differences between them. Expression of *TIP2;1* (Fig. 4d) and *TIP2;2* (Fig. 4e) showed a significant increase in

cracked and non-cracked melons with Ca+B+Zn treatment with respect to cracked control melons. *TIP3;1* (Fig. 4f) did not show any significant differences in expression in cracked or non-cracked melons between treatments. *TIP4;1* (Fig. 4g) showed significant decrease in expression in the non-cracked melons in both Ca+B+Zn and B+Zn treatments respect to the cracked control melons. *TIP5;1* (Fig. 4h) showed a significant increase in expression in cracked melons in the Ca+B+Zn treatment with respect to non-cracked melons of the same treatment.

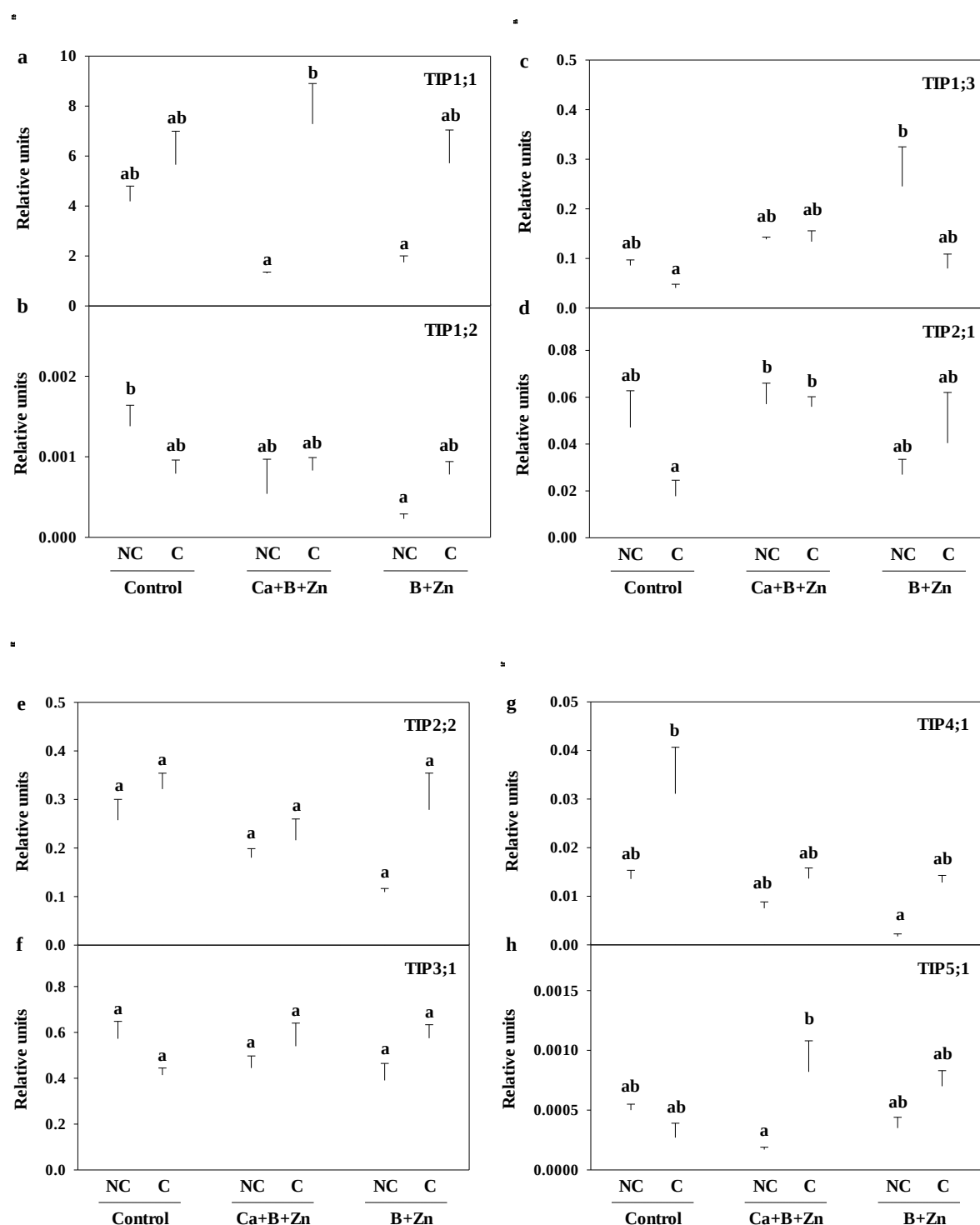


Fig. 4. Tonoplast Intrinsic Proteins (TIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates of each measurement. Different letters mean significant differences ($p < 0.05$) between control and treatments (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.5. Analysis of NIPs

The expression of the eight identified melon NIPs was detected and quantified in fruit (**Fig. 5**). *NIP1;1* (**Fig. 5a**) showed the highest expression in the Ca+B+Zn treatment, being significant only in non-cracked melons as compared with the control treatment. *NIP2;1* (**Fig. 5b**) showed a significant increase in expression in cracked melons from the Ca+B+Zn treatment with respect to the non-cracked melons of control treatment and the cracked melons with B+Zn treatment. *NIP2;2* (**Fig. 5c**) showed a significant increase in expression in cracked melons from the control treatment with respect to non-cracked ones and a decrease was observed in cracked melons of the B+Zn treatment with respect to cracked control melons. *NIP4;1* (**Fig. 5d**) showed the highest values of expression in cracked melons from the Ca+B+Zn treated, being significantly higher than non-cracked control and cracked B+Zn treated. *NIP5;1* (**Fig. 5e**) showed a significant increase in expression in cracked melons in the control and B+Zn treatments with respect to their non-cracked controls, while no changes were observed in the cracked or non-cracked melons from the Ca+B+Zn treatment. Inversely, *NIP5;2* (**Fig. 5f**) showed a significant increase in expression in cracked melons from the Ca+B+Zn treatment with respect to the non-cracked melons, while no differences were found between cracked and non-cracked melons in the control or B+Zn treatments. *NIP6;1* (**Fig. 5g**) did not show any significant differences in expression. *NIP7;1* (**Fig. 5h**) showed the highest values in both cracked and non-cracked melons from the Ca+B+Zn treated plants, being significant respect cracked control and cracked and non-cracked B+Zn treated plants.

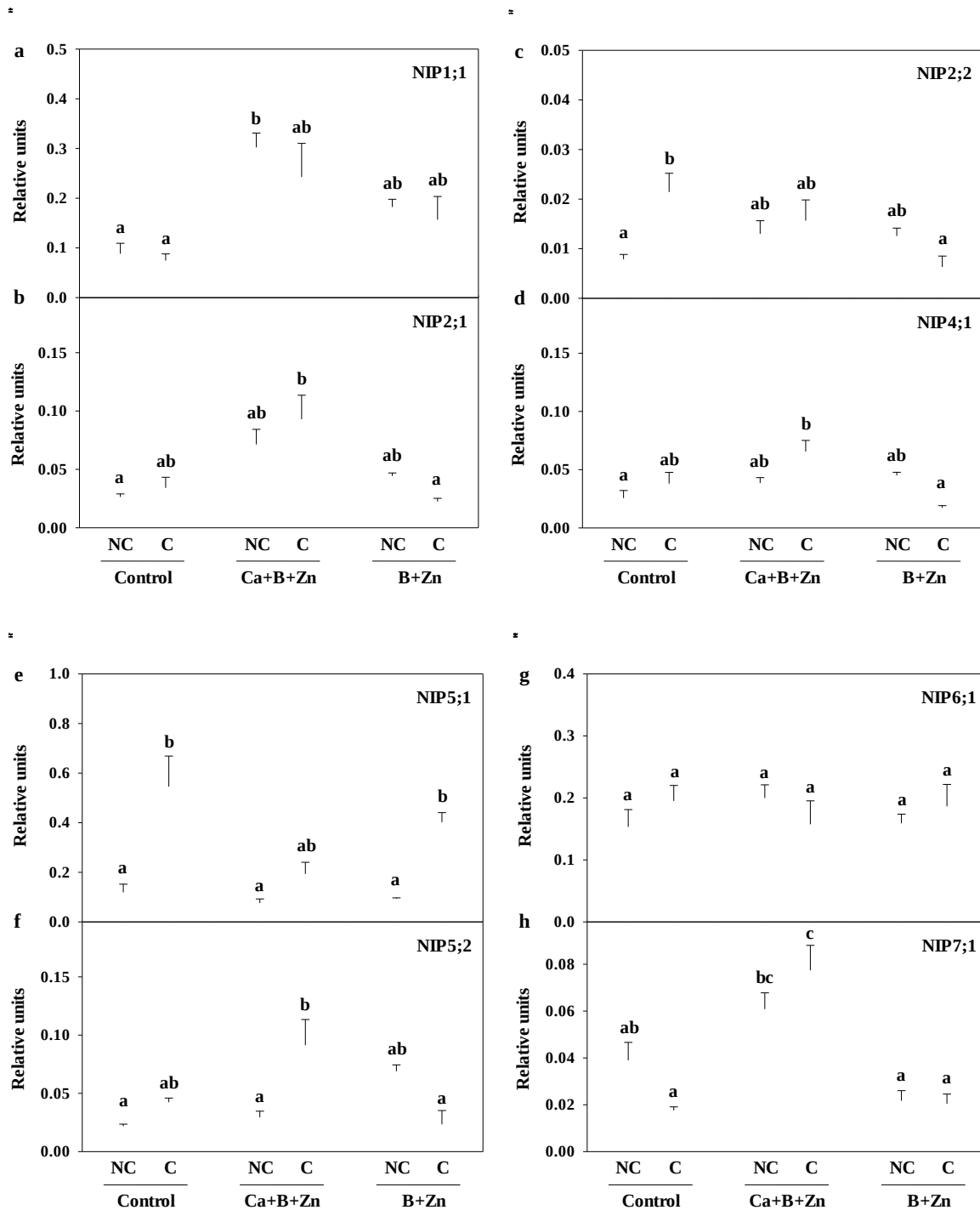


Fig. 5. Nodulin 26-like Intrinsic Proteins (NIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates for each measurement. Different letters mean significant differences ($p < 0.05$) between control and treatments (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

3.6. Analysis of SIPs and XIP

The expression of the two SIPs and one XIP known in melon were also detected and quantified in fruit (**Fig. 6**). *SIP1;1* (**Fig. 6a**) and *XIP1;1* (**Fig. 6c**) did not show any significant differences between any treatment or cracked/non-cracked melons. *SIP2;1* (**Fig. 6b**) showed a significant increase in expression in cracked melons from the Ca+B+Zn treatment with respect to the non-cracked melons from control treatment.

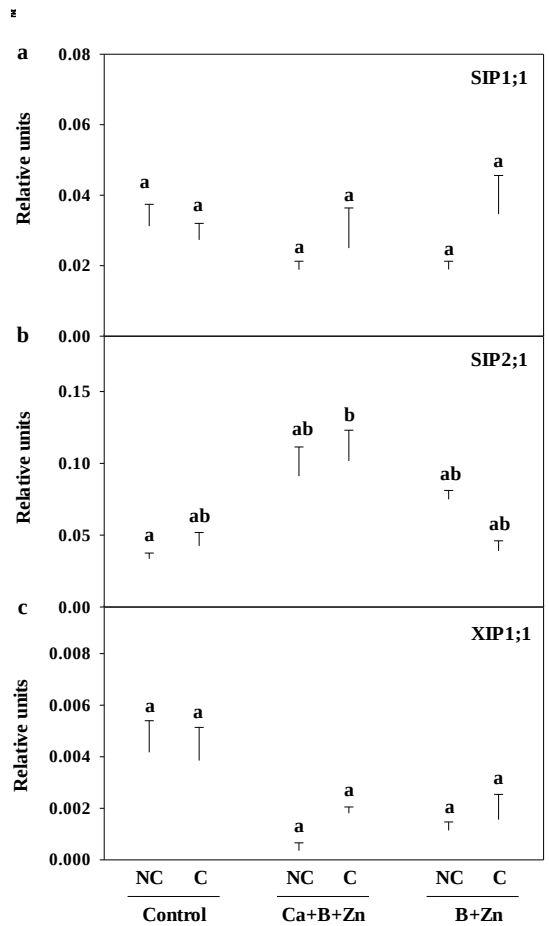


Fig. 6. Small and basic Intrinsic Proteins (SIPs) and X-intrinsic proteins (XIPs) relative expression in non-cracked melons (NC) and cracked melons (C) for control, Ca+B+Zn, and B+Zn treatments. Each value represents the means $\pm$ SE of 3 biological replicates, with 3 technical replicates for each measurement. Different letters mean significant differences ($p > 0.05$) between control and treatments (Ca+B+Zn and B+Zn) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

To find out which variable is responsible for the changes in expression that were found, a two-way ANOVA was performed, checking the p values of each variable, treatment and cracked/uncracked, separately and combined (**Table 1**). In some genes, we observed that the

expression changed due to the effect of cracking, as is the case of PIP1;1, PIP1;2, PIP2;8, PIP2;10, TIP1;1, TIP1;3, TIP2;2, TIP4;1, TIP5; 1 and NIP5;1. Other genes had changes due to treatments, such as PIP1;1, PIP2;3, PIP2;4, PIP2;6, PIP2;7, PIP2;8, PIP2;9, TIP1;3, TIP4;1, NIP1;1, NIP2;1, NIP4;1, NIP5;1, NIP7;1, SIP2;1 and XIP1;1. Finally, some genes showed significant changes that can be explained by the cracking-treatment interaction, PIP1;2, PIP2;6, PIP2;8, PIP2;9, TIP5;1, NIP2;2, NIP4;1 and NIP7;1.

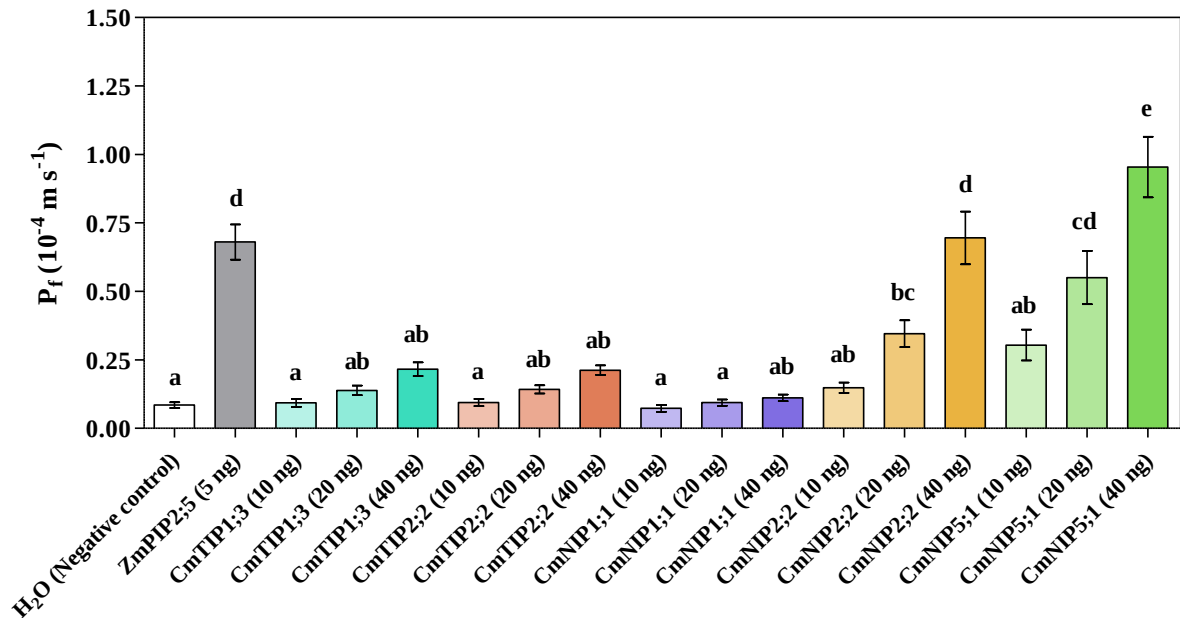
Table 1. P values obtained from aquaporin expression after two-way ANOVA analysis and *post hoc* Tukey's multiple comparison test. Significance codes: *** if p value < 0.001, ** if p value < 0.01, * if p value < 0.05, ns (non-significant) if p value > 0.05.

Gen	P value cracked	P value treatments	P value cracked*treatments
PIP1;1	0.02869 *	0.00468 **	0.71597 ns
PIP1;2	0.00464 **	0.26716 ns	0.01534 *
PIP2;1	0.0563 ns	0.1463 ns	0.1152 ns
PIP2;2	0.464 ns	0.660 ns	0.337 ns
PIP2;3	0.2715 ns	0.00782 **	0.07613 ns
PIP2;4	0.196762 ns	0.000134 ***	0.754925 ns
PIP2;5	0.286 ns	0.272 ns	0.428 ns
PIP2;6	0.063938 ns	0.000539 ***	0.015160 *
PIP2;7	0.4259 ns	0.0316 *	0.1636 ns
PIP2;8	0.0156 *	0.0241 *	0.0281 *
PIP2;9	0.62595 ns	0.00161 **	0.00671 **
PIP2;10	0.0239 *	0.4167 ns	0.0908 ns
TIP1;1	0.0008 ***	0.5428 ns	0.1402 ns
TIP1;2	0.6568 ns	0.0783 ns	0.0771 ns
TIP1;3	0.02082 *	0.00464 **	0.25585 ns
TIP2;1	0.578 ns	0.127 ns	0.250 ns
TIP2;2	0.0341 *	0.1059 ns	0.3446 ns
TIP3;1	0.4847 ns	0.9870 ns	0.0721 ns
TIP4;1	0.00514 **	0.00856 **	0.41849 ns
TIP5;1	0.0346 *	0.5825 ns	0.0198 *
NIP1;1	0.301609 ns	0.000993 ***	0.818327 ns
NIP2;1	0.782027 ns	0.000593 ***	0.161971 ns
NIP2;2	0.1735 ns	0.1557 ns	0.0137 *
NIP4;1	0.47955 ns	0.01207 *	0.00354 **
NIP5;1	0.000194 ***	0.017777 *	0.062764 ns
NIP5;2	0.18759 ns	0.06593 ns	0.00112 ns
NIP6;1	0.710 ns	0.978 ns	0.316 ns
NIP7;1	0.7150 ns	0.000181 ***	0.0405 *
SIP1;1	0.332 ns	0.601 ns	0.426 ns
SIP2;1	0.62143 ns	0.00278 **	0.18297 ns
XIP1;1	0.48950 ns	0.00919 **	0.59333 ns

3.7. Transport activity in *Xenopus Oocytes*

Due to their significant increase in expression in fruit with respect to leaves or roots (Lopez-Zaplana et al., 2020b) and the significance observed in cracked/non cracked fruits TIP1;3 and TIP2;2 were selected. On the other hand, due to the possible importance in fruit cracking, treatments applied and the possible implication in the transport of metalloids, NIP1;1, NIP2;2 and NIP5;1 were selected. The channel activity of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 were tested after their expression in *X. laevis* oocytes. First, the osmotic water permeability coefficient (P_f) (Fig. 7a) was determined, and we observed that the expression of CmNIP2;2 and CmNIP5;1 increased P_f significantly as compared with the water-injected oocytes, similarly to oocytes expressing the ZmPIP2;5 cRNA, indicating that CmNIP2;2 and CmNIP5;1 are active water channels. On the other hand, the expression of CmTIP1;3, CmTIP2;2, CmNIP1;1 did not result in any P_f increase. We also tested the expression and localization of the proteins fused to YFP. A fluorescent signal was detected mostly in the oocyte plasma membrane (Fig. 7b-g), indicating that all the proteins were well expressed and targeted to the membrane, and that the absence of swelling probably reflected their inability to facilitate water diffusion.

a



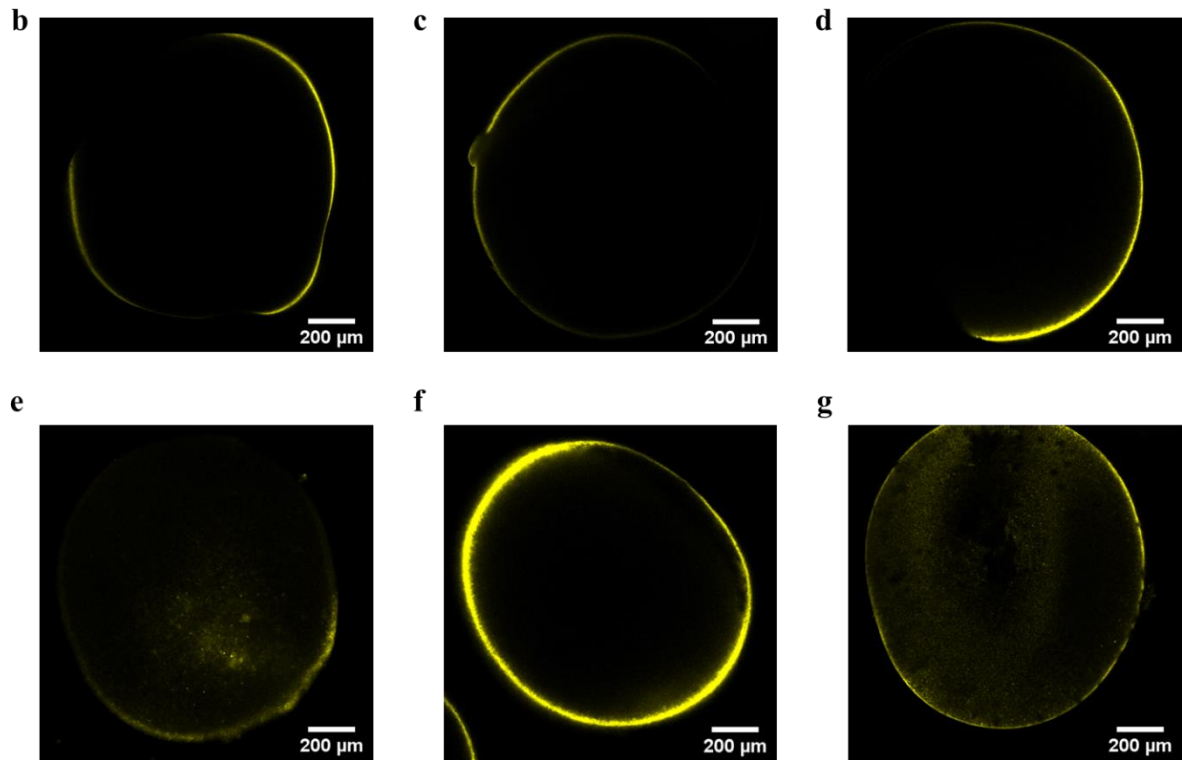


Fig. 7. Water permeability coefficient (P_f) measurements **(a)** of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1. Water-microinjected oocytes were used as negative control and ZmPIP2;5 as a positive control. Each value represents the means $\pm$ SE of 10-26 biological replicates, in two to three times with independent frogs and consistent results. Different letters mean significant differences ($p < 0.05$) according to the *post hoc* Tukey's multiple comparison test. **(b-g)** Confocal pictures of oocytes microinjected with 5 ng ZmPIP2;5 **(b)** and 40 ng CmTIP1;3 **(c)**, CmTIP2;2, **(d)**, CmNIP1;1 **(e)**, CmNIP2;2 **(f)** and CmNIP5;1 **(g)**.

The B transport activity was determined using an iso-osmotic swelling assay in presence of boric acid (**Fig. 8**). A significant increase in V/V_0 was observed for oocytes expressing the positive control NtXIP1;1, CmTIP1;3, CmTIP2;2, CmNIP1;1 and CmNIP2;2, but not for oocytes expressing CmNIP5;1 or water injected oocytes, suggesting that the former isoforms are able to facilitate the diffusion of B.

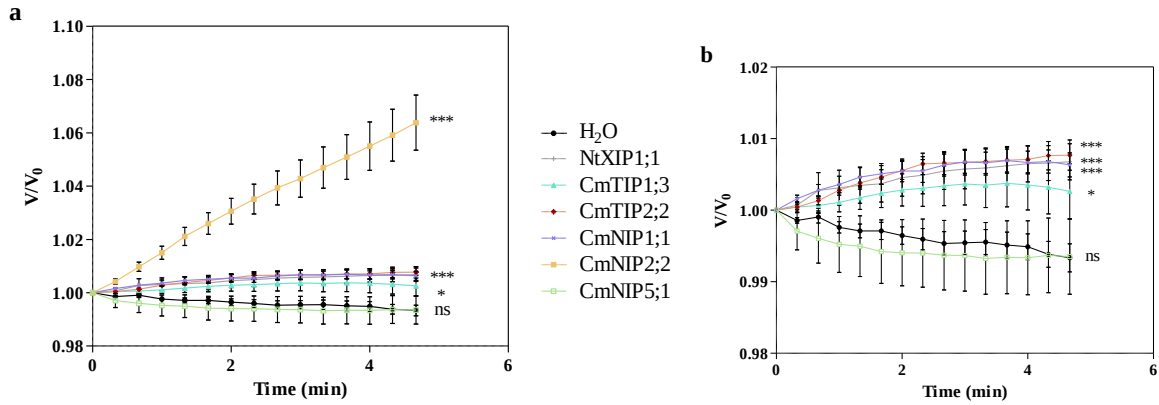


Fig. 8. (a) Osmotic boric acid swelling assay with CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 (40 ng), NtXIP1;1 (12.5 ng) as positive control and water microinjection as negative control. **(b)** Detail of the same experiment with CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP5;1 and NtXIP1;1. Data shown are means $\pm$ SE of 17-24 oocytes measured every 20 seconds. * means significant differences ($p < 0.05$), *** ($p < 0.001$) and ns, non-significant differences between the gene and the negative control in the last point according to Student's t-test.

The Si channel activity of the aquaporins was deduced from the uptake of germanium (Ge) for 30 min and its quantification by ICP-MS (**Fig. 9**). Interestingly, only the NIP2;2 expression induced a significant accumulation of Ge in the oocytes as compared to the water-injected oocytes, suggesting that this NIP2;2 isoform can act as a Si channel.

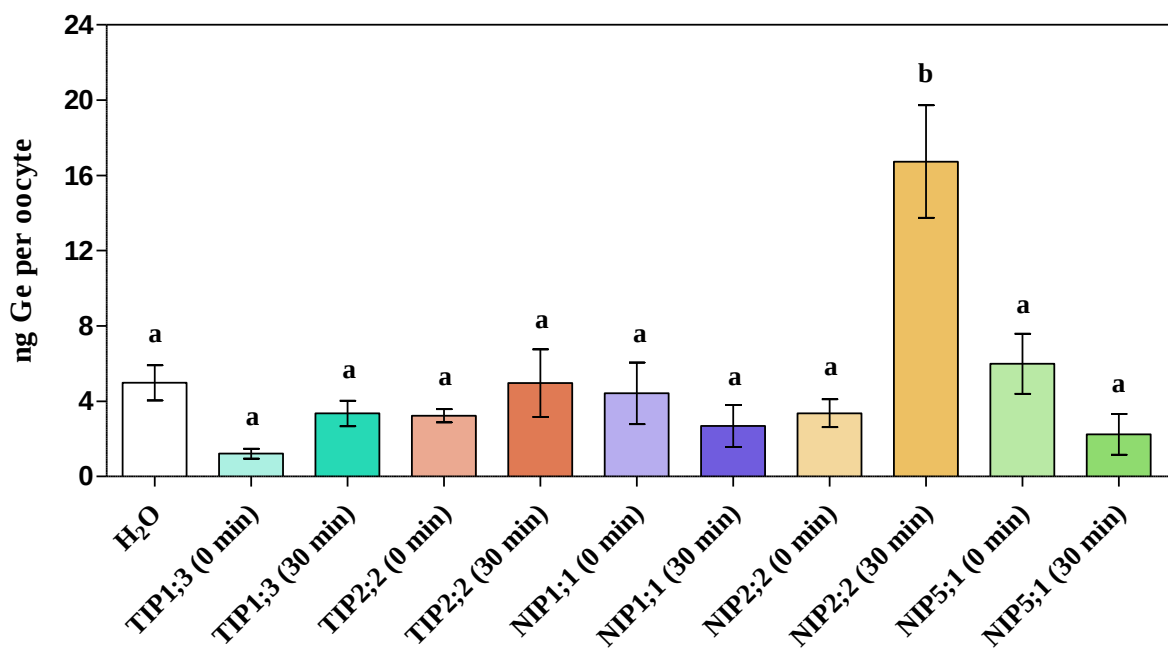


Fig. 9. Permeability of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 (40 ng) to Germanic acid (a chemical analogue of silicic acid) in *Xenopus* oocytes direct uptake assays. Oocytes microinjected with 40 ng of CmTIP1;3, CmTIP2;2, CmNIP1;1, CmNIP2;2 and CmNIP5;1 were exposed to 1mM GeO₂ (which forms Ge(OH)₄ in solution, as the analogue of Si(OH)₄), for 30 min in a modified Barth's buffer. Bar charts represent means ± SE of 5 pools of oocytes (n=7-12 oocytes per pool). Different letters mean significant differences (p>0.05) according to the two-way ANOVA test followed by the *post hoc* Tukey's multiple comparison test.

4. Discussion

4.1. Element composition and relation in cracked melons

In this study, we focused on the nutrients most utilized to reduce melon cracking that are Ca, B and Zn. Ca is the most common treatment used to prevent the appearance of cracking in fruits, with positive incidence in some species such as blueberry (Marshall et al., 2019), lemon (Bakshi, 2018), litchi (Dinesh et al., 2017), pomegranate (Davarpanah et al., 2018) or sweet cherry (Breia et al., 2020). On the other hand, Ca has been proven to improve the growth of melon plants, increasing the number of leaves, the relative water content, the number of melons per plant, and the properties of the fruit such as increasing its diameter, thinness and firmness (Khoravi mashizi and Sarcheshmehpour, 2015).

Furthermore, the microelements applied in our treatments, B and Zn, are typically used in the prevention of cracking (Bakshi, 2018; Chater and Garner, 2018; Dinesh et al., 2017; Draie and Aboras, 2021) and both of them are associated with cell wall and membrane integrity, and nutrient transport (Barzana et al., 2021). B is an important structural microelement, as it is associated with the apiosyl residue of rhamnogalacturonan-II (RGII) and the cross-linking of two monomers (Ishii and Matsunaga, 1996). It is therefore a fundamental element needed for the correct biosynthesis of cell walls and the development of new tissues (Lewis, 2019). In previous studies, it has been described that B deficiency may increase fruit cracking incidence (Khadiji-Khub, 2015), and resistant cultivars have higher levels of B and Ca (Yang et al., 2016). This makes sense, given that B is required, next to Ca, for maintaining the pectin network and the "egg-box" structure (Shi et al., 2017). Zn is necessary for maintaining the integrity of membranes, their phospholipid levels and the correct ion transport system, which affects the capacity of water uptake (Dang et al., 2010; Kasim, 2007). Altogether, these elements could have an influence on cracking reduction.

In our study, we observed that in the first two weeks after application of the Ca+B+Zn and B+Zn treatments, there were no significant changes in terms of the accumulate incidence of cracking. These results are in agreement with previous results (Lopez-Zaplana et al., 2020a), in which similar treatments, composed of Ca and micronutrients or only micronutrients, did not lead to any significant differences in cracking after one week of treatment. However, in the present work, significant differences were found from the third week of treatment, with a decrease in the incidence of cracking in both foliar mineral treatments with respect to the control, highlighting the relationship between greater fruit ripening process and increased incidence of cracking already described by other authors (Domínguez et al., 2012; Jiang et al., 2019).

In this study, we also wanted to analyze Si, although it was not applied in the treatments, as its involvement as a modulator of the response to abiotic stress is known (Ma and Yamaji, 2006; Seal et al., 2018). In melon, it has been described as an important element associated with the resistance to high temperature stress (Lopez-Zaplana et al., 2022), which could be related with cracking. In addition, Si is a substrate of some aquaporins (Lopez-Zaplana et al., 2020b; Pommerrenig et al., 2015).

In this study, a significant increase in B, Si and Zn content was only observed in the non-cracked melons from the Ca+B+Zn treatment, pointing to the fact that Ca could be involved in the regulation of their movement. In this sense, some studies have shown that the activity of aquaporins is positively regulated by Ca through phosphorylation (Alleva et al., 2006; Johansson et al., 1996), by increasing passive diffusion of water and other solutes, or by directly increasing the activity of specific aquaporins such as NIPs, which are able to transport B or Si (Bienert et al., 2008; Lopez-Zaplana et al., 2020b).

In addition, Ca also plays an important role at the cell structure level. A recent theory argues that Ca may reduce fruit cracking through the inhibition of cell wall swelling by its accumulation in the pectin network (Yu et al., 2020). Indeed, Ca crosslinks the carboxyl groups of the homogalacturonan domains and forms an “egg-box” structure, accounting for 60-75% of the total cell wall (Aghdam et al., 2012). Furthermore, in numerous studies, it has been observed that the fruits with cracking presented lower levels of Ca (Shi et al., 2017; Yang et al., 2016). But nevertheless, we could find significant increases in cracked melons in both foliar treatments respect non-cracked control melons. Bearing in mind that Ca movement depends on the flow of water from transpiration in the apoplastic pathway (Gilliam et al., 2011), we could think both apoplastic and symplastic pathway, mediated by aquaporins (Yaaran and Moshelion,

2016), both routes are altered and there is an increase in generalized water transport. Finally, abiotic stresses trigger Ca signals, that convergent with abscisic acid (ABA) modulating the common targets (Edel and Kudla, 2016), being the ABA involved in different process like fruit cuticle integrity (Gutiérrez et al., 2021) or root hydraulic conductance via aquaporin expression modulation (Mahdieh and Mostajeran, 2009).

4.2. Aquaporin expression patterns in cracked melons

Aquaporins are an interesting object of study regarding the cracking physiopathy, which is related to water (Fernández-Trujillo et al., 2013; Lopez-Zaplana et al., 2020a). Studying the proteins responsible for its membrane diffusion makes sense, although this study could be complex due to the wide variety of aquaporin subfamilies and members (Maurel et al., 2015).

When comparing non-cracked and cracked melons, we highlighted that in the control treatment, which showed the highest incidence of cracking, no differences in the expression of most aquaporin genes were found. The only two exceptions were *NIP2;2* and *NIP5;1* (both exhibiting water channel activity), with both increasing their expression in the cracked control melons as compared with non-cracked ones.

Upon both foliar treatments, several aquaporins showed very specific patterns. This suggests that the ability to reduce melon cracking by adding Ca, B or Zn is directly associated to the control of aquaporins. In general, we could find an increase in expression in cracked melons as compared with non-cracked ones, therefore demonstrating their involvement in the cracking process, either as a cause or an effect. This was the case for *PIP1;1*, *PIP1;2* and *TIP1;1*, which showed the highest mRNA levels in cracked melons in all treatments, being this relation significant if we study the cracked non-cracked variable in the two-way ANOVA.

PIP1s have been mainly associated to water transport functions and modulation of PIP2s activity, principally increasing the cell P_f when PIP1s are co-expressed with PIP2s (Berny et al., 2016; Bienert et al., 2018; Fetter et al., 2004; Postaire et al., 2010; Zelazny et al., 2007). In our experiment, we observed a significant decrease in *PIP1;1* and *PIP1;2* expressions in non-cracked melons in both treatments, but not in the control treatment. This points to a possible decrease in the water membrane permeability in this treatment possibly leading to the decreased incidence of cracking we observed. Recently, the aquaporin with the highest expression in sweet cherry, *PIP1;4*, was shown to be overexpressed when the plant was treated with Ca, a

treatment decreasing cracking (Breia et al., 2020). In our study, the highest expressed PIP aquaporin was the *PIP1;1*, which showed an increased expression in cracked fruits with respect non-cracked fruits (p value of 0.02869), and the same response was observed for *PIP1;2* (p value of 0.00464). Therefore, the relationship between this two PIP1s and cracking incidence in melon could be established.

In the same manner, *TIP1;1* showed a similar expression pattern as PIP1s. *TIP1;1* has been characterized as the main aquaporin responsible for water transport in the tonoplast of maize (Barrieu et al., 1998) and melon (Lopez-Zaplana et al., 2020b), being the most expressed aquaporin in melon fruit. Furthermore, it could be able to transport urea, ammonia or hydrogen peroxide (Schüssler et al., 2008), with the latter being an important molecule for stress signaling (Bienert and Chaumont, 2014). The significant decrease in *TIP1;1* expression in non-cracked melons as compared with cracked melons in Ca+B+Zn treatments suggests that a decrease in water accumulation in the tonoplast is a possible effect of our treatments. Furthermore, the two-way ANOVA point the importance of the increase of expression in cracked melons with a p value of 0.0008 respect cracked/non-cracked variable.

Apart this 3 water related aquaporins (*PIP1;1*, *PIP1;2* and *TIP1;1*), the increase in oocyte P_f after expression of *NIP2;2* and *NIP5;1* demonstrated their ability to facilitate water diffusion, and they could be associated with the incidence of cracking respect water transport imbalance. In this sense, the increased expression of some aquaporin genes in cracked fruits was previously described in a transcriptomic study where they identified different transcripts involved in water transport in cracked fruits in *Litchi chinensis* Sonn, such as *LcAQP*, *LcPIP*, *LcNIPs* and *LcSIPs* (Li et al., 2014), and other studies on melon cracking indicated that its cause was due to a very rapid and abundant uptake of water into the fruit (Lopez-Zaplana et al., 2020a). Although it seems obvious that there was an imbalance in water transport in plants that suffer cracking and that this process is mediated by some aquaporins, some studies pointed out a correlation between the increased expression of some aquaporins and a reduction in the number of cracked fruits (Breia et al., 2020; Joshi et al., 2018). Joshi et al. (2018) described how an increase in aquaporin expression reduced the incidence of cracking in apples, as was the case of *PIP2A*.

If we compared the treatments, it is noteworthy that there were a large number of aquaporins had their expression modified by the Ca+B+Zn treatment, with the highest values observed in cracked melons. This was the case for *PIP1;2*, *PIP2;1*, *PIP2;6*, *PIP2;8*, *PIP2;9*, *TIP1;1*, *TIP5;1*, *NIP2;1*, *NIP4;1*, *NIP5;2* and *NIP7;1*. Among them, *PIP1;2*, *PIP2;6*, *PIP2;8*, *TIP1;1*, *TIP5;1* and *NIP5;2* showed a significant increase in expression in cracked melons in

the Ca+B+Zn treatment as compared with non-cracked melons for the same foliar treatment. Here, we found many aquaporins associated with transport of water, but also other compounds such as B or Si (Lopez-Zaplana et al., 2020b). Therefore, it seems that Ca played a role in altering the expression patterns, not only affecting the transport of water, but also other important nutrients necessary for the proper intracellular balance and structural development of the melon fruits.

The expression of most aquaporins was unaffected by the B+Zn treatment in non-cracked melons, although it did affect aquaporins in relation to cracking occurrence. Indeed, only *PIP2;4* and *TIP1;2* expressions were reduced in non-cracked melons in this condition, but *TIP1;2* were unaffected by Ca+B+Zn treatment. Therefore, the presence of Zn or B seems to influence *TIP1;2* expression depending on the presence or absence of Ca in the treatment solution. It was suggested that the foliar application of Zn decreased aquaporin activity, mainly through post-translational modifications, while its effect on the transcriptional level was variable (Barzana et al., 2021), but this hypothesis needs to be proven with activity analysis. For example, *PIP2;4* expression decreased in all melons treated with Ca+B+Zn and with B+Zn compared with the control foliar treatment, suggesting that B+Zn reduced its expression. Other studies reported a decrease in *HvPIP1;2*, *HvPIP2;4*, *HvPIP2;5* expression (Gitto and Fricke, 2018) and *AQUA1* (Ariani et al., 2019), or the non-modification in the transcription of aquaporins (Devi et al., 2016), depending on Zn concentrations. Furthermore, Zn was also associated with an increase in the water channel activity and the expression of several aquaporins in pak choi roots and leaves (Fatemi et al., 2020). Since Ca enhanced Zn accumulation in non-cracked melons, it could be argued that *TIP1;2* expression could be affected by Zn content in fruit, but its possible involvement in the aquaporin regulation needs further investigation.

Notwithstanding the fact that all the known aquaporins were detected in melon fruit, some of them (*PIP2;1*, *PIP2;4*, *PIP2;7*, *TIP1;2*, *TIP4;1*, *TIP5;1*, *NIP2;2* and *XIP1;1*) had a very low expression level, suggesting that their expression levels may not be related to the significant changes in water or mineral transport (**Supplementary figure 1**). On the other hand, *PIP1;1*, *PIP1;2*, *PIP2;2*, *PIP2;3*, *PIP2;5*, *PIP2;6*, *PIP2;10*, *TIP1;1*, *TIP2;2*, *TIP3;1*, *NIP5;1* and *NIP6;1* had the highest expression level (**Supplementary figure 1**). When compared with their expression in roots and leaves (Lopez-Zaplana et al., 2020b), we observed for most of the aquaporin genes a similar trend, with some interesting differences. For instance, *TIP1;3* and *TIP2;2* were mostly expressed in melon fruit, while they were almost undetectable in other

parts of the plant, highlighting their relevance in fruit development. As for the cracking incidence, *TIP1;3* had the lowest expression level in control cracked plants, while it increased in the non-cracked B+Zn melons. Its possible involvement in the transport of B was previously predicted (Lopez-Zaplana et al., 2020b) and based on our B swelling results it could be one of its main functions in melon fruits.

Finally, the expression of *PIP2;2*, *PIP2;5*, *PIP2;7*, *PIP2;10*, *TIP2;2*, *TIP3;1*, *NIP6;1*, *SIP1.1* and *XIP1.1* was not affected by either of the foliar treatments in cracked or non-cracked melon.

4.3. Transport activity in *X. laevis*

The only two aquaporins that showed a significant increase in expression in cracked melon as compared with non-cracked melons in control conditions were *NIP2;2* and *NIP5;1*, suggesting that their regulation is related with cracking. When expressed in *Xenopus* oocytes, *NIP2;2* showed the highest B channel activity amongst all the studied aquaporins, but it also acted as a water channel, and was the only isoform transporting Si, in accordance with previous predictions (Lopez-Zaplana et al., 2020b). Si has a mechanical barrier function, defending tissues against abiotic stress, pathogens, and colonization by fungi (Ma and Yamaji, 2006; Rémus-Borel et al., 2005; Rodrigues et al., 2004). In this sense, its increase in expression in cracked control melons could be a response for protecting the fruit from the outside (Rémus-Borel et al., 2005; Rodrigues et al., 2004). *NIP5;1* showed the highest expression amongst *NIP* isoforms and facilitated membrane diffusion of water but not B or Si. Both *NIP2;2* and *NIP5;1* increased the P_f when we increased the amount of RNA microinjected according with other studies (Bienert et al., 2018; Fetter et al., 2004). These results strongly support the importance of water transport in cracking incidence as proposed in numerous articles (Breia et al., 2020; Fernández-Trujillo et al., 2013; Joshi et al., 2018; Li et al., 2014; Lopez-Zaplana et al., 2020a). *NIP1;1* did not show any transport activity for water nor Si, but we could detect some activity in facilitating B diffusion. Interestingly, *NIP1;1* showed an expression increase in both mineral treatments, reaching maximum values in the presence of Ca+B+Zn, and was the only aquaporin with a significant increase in expression in non-cracked melons with Ca+B+Zn as compared with non-cracked control melons, supporting the idea that the B transport activity of *NIP1;1* was favored by Ca in melon.

In contrast, *TIP1;3* and *TIP2;2*, which showed a high expression levels in melon fruit as compared to the expression in leaf or root (Lopez-Zaplana et al., 2020b), did not exhibit very clear results regarding their water channel activity, even if a slight increase in P_f was observed when injecting more cRNA. They did not facilitate Si diffusion, in accordance with our previous predictions (Lopez-Zaplana et al., 2020b), although *TIP1;3* showed a slight transport of B. The case of *TIP2;2* was especially striking, as it had a high specificity in the transport of NH_3 (Lopez-Zaplana et al., 2020b) and, as we showed here, it also facilitated B diffusion. The decrease in its expression in non-cracked melons from the B+Zn treatment compared with the control treatment, and the large increase in expression in the cracked melons from the same B+Zn treatment again suggest a regulation of this isoform by Zn. In any case, it seems quite evident that the regulation of B content is especially important in fruit development.

5. Conclusions

From this work, we can conclude that both foliar mineral treatments were able to reduce the cumulative incidence of cracked melons from the third week. Moreover, we demonstrated a relation between aquaporin expression and cracked fruit, mainly for water active channels, which can facilitate the entry of water (probably as a response to changes in ambient conditions), producing cracked fruits. In the cracked melons without foliar mineral treatments, *NIP2;2* and *NIP5;1* were overexpressed in the cracked part, indicating a basal regulation of these aquaporins. Furthermore, *NIP2;2* is a channel for B and Si, indicating the possible need to increase the concentration of these nutrients in the cracked fruits. Similar observation can be done for *PIP1;1* and *PIP1;2* water channels, with high expression levels in the fruit and with significantly higher levels in cracked melons in both foliar mineral treatments, suggesting a regulation of the water uptake, which could lead to an increase in the internal pressure of the fruit. The melon could be increasing the expression of *TIP1;1*, the most expressed TIP in melon fruit, to decrease the amount of water in the cytosol. Regarding the role of the elements, Ca could be involved in the regulation of the increased transport of other nutrients and water flow. With the changes in the expression levels of some aquaporins such as *TIP1;3*, *TIP2;2*, *NIP1;1* and *NIP2;2*, it seems that both mineral treatments induced the entry and storage of B. Finally, the possible role of Zn in *TIP2;2* regulation and Ca in *NIP1;1* function was strongly suggested.

Until now, not many studies have been found which deeply analyzed the expression patterns of all aquaporins in a fruit and their involvement in cracking. Thus, the present work

is an important starting point for the promotion and development of aquaporins as molecular markers, as they seem to have a considerable influence on the appearance of this physiopathy. In the future, it would be interesting to simplify the foliar treatments to unique elements, incorporating different cultivars, or species resistant or susceptible to cracking, and to analyze how the expression patterns of aquaporin are affected.

Funding

This work was supported by the Spanish Ministry of Science, Innovation and Universities (RTC-2017-6119-2) and developed in the Spanish Higher Council for Scientific Research (CSIC). The stay of A.L.-Z. in F.C. laboratory was possible thanks to the scholarship EIDUM-CMN of Murcia University. L.D. was a postdoctoral researcher supported by the Belgian National Fund for Scientific Research (FNRS).

Author contributions

Conceptualisation: G.B., F.C. and M.C. Methodology and Investigation: A.L.-Z., L.D. and G.B. Formal analysis, Data Curation and Visualization: A.L.-Z., L.D. and G.B. Writing—Original draft: A.L.-Z. and L.D. Writing-Review & editing: L.D., G.B., F.C. and M.C. Supervision: F.C. and M.C. Validation, Project administration and Funding acquisition: F.C. and M.C. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors thank Sakata Seeds Ibérica S.L.U. for providing the melon seeds and fields, Mr. Mario G. Fon the language correction, Jahed Ahmed for collecting the oocytes from *Xenopus* and **Lucia Yepes for the statistical analysis correction.**

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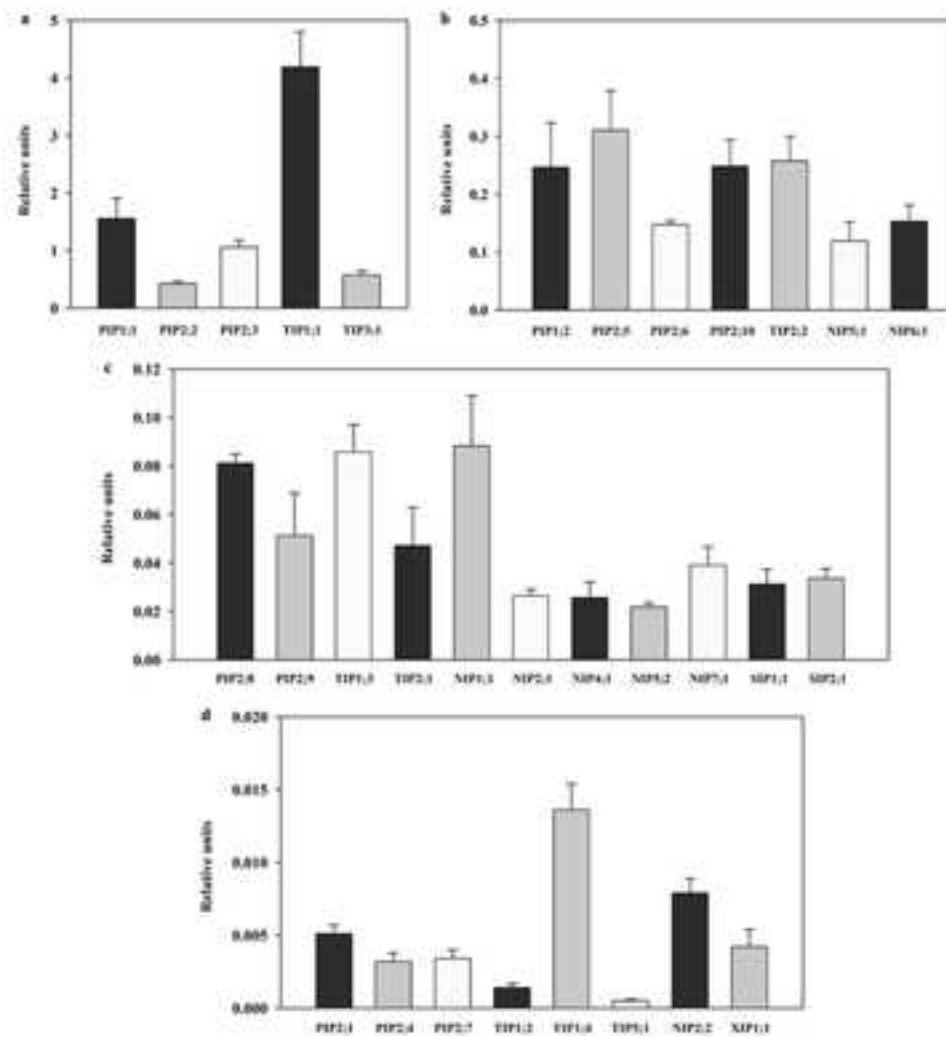
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Conflict of Interest

The authors declare no conflict of interest:

Author statement

Conceptualisation: G.B., F.C. and M.C. Methodology and Investigation: A.L.-Z., L.D. and G.B. Formal analysis, Data Curation and Visualization: A.L.-Z., L.D. and G.B. Writing—Original draft: A.L.-Z and L.D. Writing-Review & editing: L.D., G.B., F.C. and M.C. Supervision: F.C. and M.C. Validation, Project administration and Funding acquisition: F.C. and M.C. All authors have read and agreed to the published version of the manuscript.

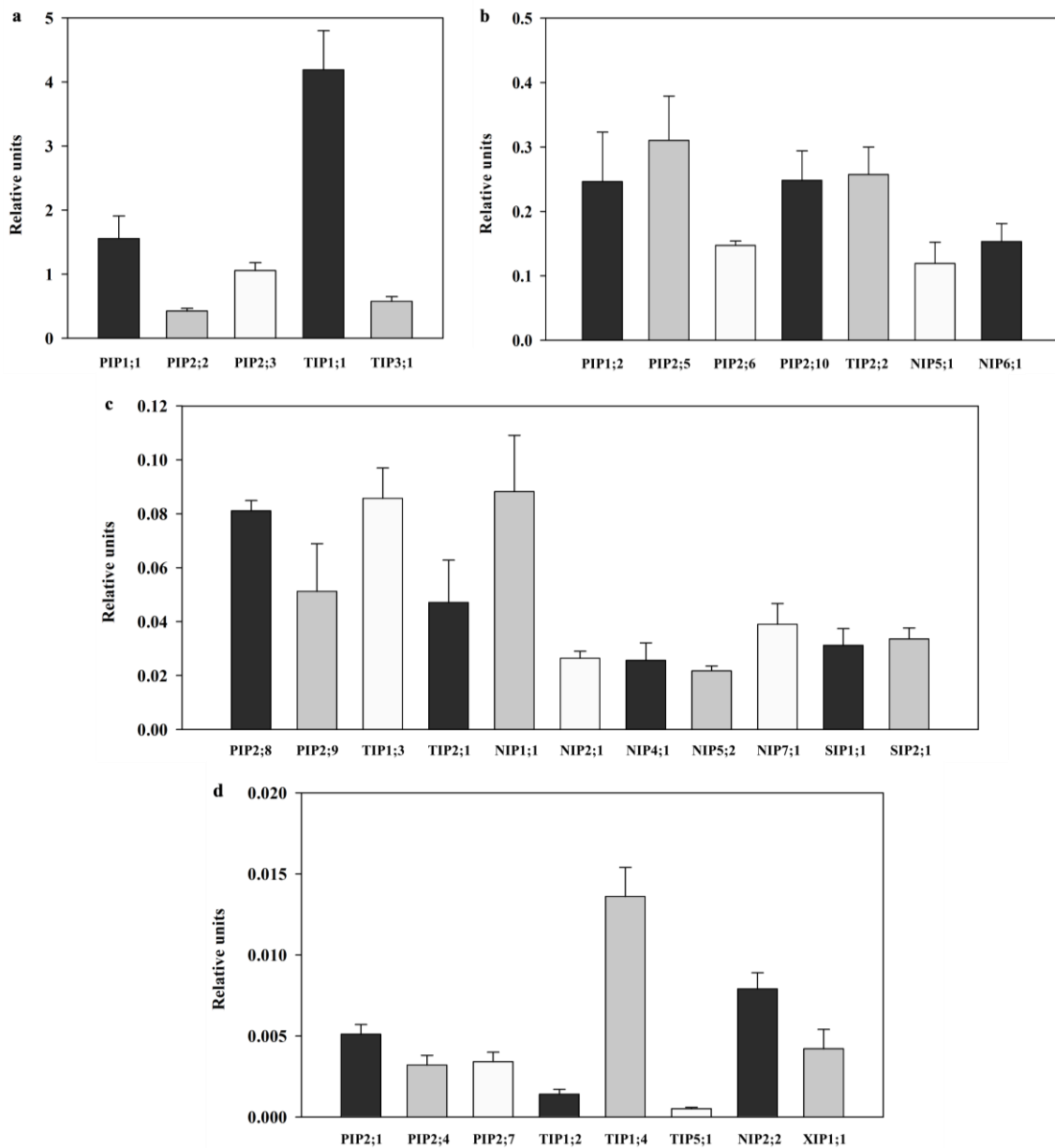
Gene name	Primer sequence 5'→3' (forward/reverse primer)	Amplicon size (pb)
<i>CmRPL</i> #	F: CGACAATACTGGAGCCAAGAA R: CATCACCATATCTCCACACAA	100
<i>CmPIP1;1</i> *	F: GCTTCTTCAATCAATCACAGC R: ACCATTACATAGCTTCATAGCC	181
<i>CmPIP1;2</i> *	F: CATTCTTCCCCAAAAGCAAA R: AGTTTTTCAGAAAGGCAGCCA	179
<i>CmPIP2;1</i> *	F: TTAAGCCTAATAGTTGTGTGC R: AAAAGAGAGCAAAAACCACG	147
<i>CmPIP2;2</i> *	F: GGATCTAAAATGTGTGATTAGG R: ATAAAACCTTCAAAATATGTGC	158
<i>CmPIP2;3</i> *	F: ATCTCTAACATCCATCACTCC R: TAGTTAGCTTGTGGTGGTCCG	94
<i>CmPIP2;4</i> *	F: CTCTCGTCTGCTTTGGTCC R: ATACATAGGATGAGAATGAGC	158
<i>CmPIP2;5</i> *	F: AAGAAGATGATGGTAGAAGTGG R: CATTCAAAGACAATCCCTTCC	120
<i>CmPIP2;6</i> *	F: TCTGCTCTATAAATCTTATCCC R: TAACACACTTCATTAGTTCAGC	144
<i>CmPIP2;7</i> *	F: CCATTCCATAAGCAAAAGACT R: GAACATTAGTAAGCCAAGTGG	177
<i>CmPIP2;8</i> *	F: ACAAACCAAAGAAGTGTTTCG R: GCAGGATCTCAGTGAATGTG	158
<i>CmPIP2;9</i> *	F: CTTCTTCCTTACACTTCATGC R: TTACCCAATTACAAAAGATTGC	119
<i>CmPIP2;10</i> *	F: TTCCTTTTGCTATTGGAATGG R: TTGCTGTACATTACTAGG	89
<i>CmTIP1;1</i> *	F: TCAACCACCACCACCACC R: GACACGACCAAACCCATCC	129
<i>CmTIP1;2</i> *	F: CGTCAACTTCTTTGTTCTACGT R: CAATTTAATACGACATCAAAATGG	90
<i>CmTIP1;3</i> *	F: TCTTGACTTTATTTCAGAGACC R: ATTCTCTTCCTGATTCTTAGC	105
<i>CmTIP2;1</i> *	F: TCCCTTTGTAATAAGAGGAGG R: AAGAAGAGAATCCAATGAACC	133
<i>CmTIP2;2</i> *	F: GTGTAAAAAATGAAACCAAAACG R: TTGAGGGAAAACCGAAGAAGG	150
<i>CmTIP3;2</i> *	F: TTTCTGCTCTATATGTTGTAGG R: CTGTATGACATTTATTACCTTC	144
<i>CmTIP4;1</i> *	F: GTCATCATACTTACCATTTGC R: ACTACAAGAACTGGAAAGG	104
<i>CmTIP5;1</i> *	F: TTTAAGCGTTGGTTTTGTGC R: GATAAAAATTCATGTTAGATACAC	97
<i>CmNIP1;1</i> *	F: CCTTACTTCACATGAACTAGG R: CAGCCATCAAGAAGTTTGG	99
<i>CmNIP2;1</i> *	F: ATAGTTTGAGTGTTTTAATGAGC R: GGCTACTTCTGATACATTGC	124
<i>CmNIP2;2</i> *	F: GAGAAGAATGAATCTGAAATAGG R: GAAAAGAAGAACCAATTTTATGG	115
<i>CmNIP4;1</i> *	F: AAAGGAAGAACATAAACGATAAC R: ATTGAGTCTCAGAAAGAAAGG	86
<i>CmNIP5;1</i> *	F: AGAATAAAGTTGAGAAGAAAAGG R: GGCAAGTAGAAACAATATAGCA	95

<i>CmNIP5;2</i> *	F: TGATAATGATAGTGGTCGTTG R: TTGACATGAAAGTAAAAGGTCG	75
<i>CmNIP6;1</i> *	F: CCCAGAGAACAACCTTTGAACC R: ATACACAATGACCAATACTTGC	137
<i>CmNIP7;1</i> *	F: CCCTCTATATTTCCAGTTGC R: AAGTAAGGTTTAATTTGATTACCG	132
<i>CmSIP1;1</i> *	F: CTCTTAATGATTCCAATGTAGTG R: TGACGATGTTGTCGGATTCC	101
<i>CmSIP1;2</i> *	F: GCAGTTATGTTTAGTTTGATTTC R: CTAATGTCCAAAGTCTATAAGC	92
<i>CmXIP1;1</i> *	F: TCTTCCTCTTTTCTCTCAAGG R: GCAGTCGCTACTAATTCTGTC	101

Supplementary table 1. Primers used to measure *C. melo* fruit aquaporins expression by RT-qPCR. # RPL primers were obtained from Kong et al. (2014). * Aquaporins primer were obtained from Lopez-Zaplana et al. (2020b).

Gen	Sequence 5'-3'	Amplicon size (pb)	T _m (°C)
TIP1;3	F: AAAGGAAGAAAAATGCCAATCCA R: TGAGTCCAAAAATTGCAGCAGA	838	57.43 59.04
TIP2;2	F: GCTGCTCTCAGACCATCCC R: CACACAAACAAAAACAAGCACATTC	852	59.85 59.21
NIP1;1	F: GTCCTTGCTCAACTCTGTTTGCT R: TAAGGACGTTGTTCTGTGTCCA	1061	57.85 58.87
NIP2;2	F: AAGAAATGGCGAGACGCAAC R: GAAGTTCCAGCACGATCCGA	823	59.48 60.11
NIP5;1	F: TCCATCATTCCAACTAAAAACCC R: GCATAGCCAAATTATTGCGACC	998	57.65 58.69
TIP1;3	F: GGCTTAAUATGCCAATCCATAAGTTT R: GGTTTAAUCTAGAAATCATTATACTGAGA	778	64.6 59.1
TIP2;2	F: GGCTTAAUATGGTGAAGTTGGCTATA R: GGTTTAAUTTAAGCATAATCACCGGA	762	63.8 65.3
NIP1;1	F: GGCTTAAUATGGCTGAGATTTTCAGGA R: GGTTTAAUTCAAGATGAACCTCTGCG	840	67.7 68.1
NIP2;2	F: GGCTTAAUATGGCGAGACGCAACGACAA R: GGTTTAAUTCAATGTGACGAGAGTGAATGG	795	76 70.9
NIP5;1	F: GGCTTAAUATGCCGGAATCGGAAAGT R: GGTTTAAUTCAACGCCGGAAGCTCCT	906	71.3 73.3

Supplementary table 2. Primers used to amplify the genes and add the necessary tags for the user system. The first five pairs of primers were used to amplify longer fragments and they were designed using the non-coding regions due to the high similarity between the aquaporins in their coding regions. Then, the ORF (open reading frame) with user cassettes were amplified with the last 5 primers.



Supplementary figure 1. Relative expression levels of all the aquaporins genes in melon fruit in control conditions. All the analyses were performed using qPCR. The values are the means $\pm$ SE of 3 biological replicates. **(a)** Group 1 (0.5–5 relative units): PIP1;1, PIP2;2, PIP2;3, TIP1;1 and TIP3;1. **(b)** Group 2 (0.1–0.5 relative units): PIP1;2, PIP2;5, PIP2;6, PIP2;10, TIP2;2, NIP5;1 and NIP6;1. **(c)** Group 3 (0.02–0.1 relative units): PIP2;8, PIP2;9, TIP1;3, TIP2;1, NIP1;1, NIP2;1, NIP4;1, NIP5;2, NIP7;1, SIP1;1 and SIP2;1. **(d)** Group 4 (0–0.02 relative units): PIP2;1, PIP2;4, PIP2;7, TIP1;2, TIP4;1, TIP5;1, NIP2;2 and XIP1;1.