agriculture

MDPI

Article

Effect of Ethylene-Insensitive Mutation *etr2b* on Postharvest Chilling Injury in Zucchini Fruit

Alicia García [1], Encarnación Aguado [1], Gustavo Cebrián [1], Jessica Iglesias [1], Jonathan Romero [1], Cecilia Martínez [1], Dolores Garrido [2], María del Mar Rebolloso [3], Juan Luis Valenzuela [1,*] and Manuel Jamilena [1,*]

1 Department of Biology and Geology, Higher Engineering School, Research Centres CIAIMBITAL and CeiA3, University of Almería, 04120 Almería, Spain; alicia.garcia@ual.es (A.G.); encarniaguado@ual.es (E.A.); gcebrianc@ual.es (G.C.); yim100@ual.es (J.I.); jrmasegosa@ual.es (J.R.); cmartinez@ual.es (C.M.)
2 Department of Plant Physiology, University of Granada, 18071 Granada, Spain; dgarrido@ugr.es
3 Department of Agronomy, Higher Engineering School, Research Centres CIAIMBITAL and CeiA3, University of Almería, 04120 Almería, Spain; mfuentes@ual.es
* Correspondence: jvalenzu@ual.es (J.L.V.); mjamille@ual.es (M.J.)

Received: 8 October 2020; Accepted: 4 November 2020; Published: 6 November 2020

Abstract: Zucchini is a vegetable fruit that is very susceptible to postharvest chilling injury, and fruit ethylene production is correlated with chilling injury sensitivity, such that the more tolerant the cultivar, the lower is its ethylene production. It is expected that zucchini fruit with reduced sensitivity to ethylene would have a higher chilling injury tolerance. In this study, we compared the postharvest fruit quality of wild type and ethylene-insensitive mutant *etr2b*, in which a mutation was identified in the coding region of the ethylene receptor gene *CpETR2B*. Flowers from homozygous WT (wt/wt), mutant plants in homozygous (*etr2b*/*etr2b*) and heterozygous (wt/*etr2b*) were hand-pollinated, and all fruits were harvested with the same length, at about 8 days after pollination. After harvesting, fruit of each genotype was randomly divided in 3 batches of 12 fruits each (four replications with three fruits each), and then stored at 4 °C and 95% RH. At 0, 7, and 14 days after cold storage, each batch was used to assess ethylene production, respiration rate, weight and firmness loss, chilling injury, and oxidative stress metabolites. The results showed a lower chilling injury associated with lower cold-induced ethylene production in the mutant fruit, in comparison with the WT fruit. These data demonstrated that the ethylene-insensitive *etr2b* mutant fruit was more tolerant to chilling injury, confirming that basal ethylene in the still undamaged fruit could function as a modulator of post-harvest chilling injury. Moreover, the higher chilling tolerance of the *etr2b* mutant fruit was not associated with MDA content, but was concomitant with a reduction in the accumulation of hydrogen peroxide in the refrigerated mutant fruit.

Keywords: *Cucurbita pepo*; ethylene insensitivity; ethylene production; respiration rate; oxidative stress

1. Introduction

Cold storage is the most important postharvest technology for fruit and vegetable conservation. However, below the optimal temperature, perishable plant commodities might shorten their shelf life and reduce their postharvest quality, suffering significant chilling injury (CI) symptoms, such as damage on their surface or pitting, and increased weight loss and dehydration.

Zucchini is a vegetable fruit that is very susceptible to CI, and its postharvest storage at temperatures below 8–10 °C rapidly reduces its quality, as a result of considerable loss of water and fruit surface damage. To improve the zucchini's postharvest life and to reduce economic losses, it is essential to

know the genetic and physiological bases of zucchini CI. Although the network that triggers the onset of CI is very complex, there are some pathways that were discovered. First, there is a net cultivar influence on CI symptoms, indicating that there are some cultivars that are more CI-tolerant than others [1–3]. It was elucidated that CI tolerance is associated with mechanisms that prevent or reduce oxidative stress in the fruit. Thus, the fruit of most tolerant cultivars such as Natura, shows reduced levels of malondialdehyde (MDA) and hydrogen peroxide. In part, this is due to a higher expression and activity of certain antioxidant enzymes such as catalases (CAT), peroxidases, (POX) and superoxide dismutases (SOD) [4].

The production of ethylene after cold storage and rewarming of zucchini fruit is correlated with CI sensitivity, since it was found that the more tolerant the variety, the lower its cold-induced ethylene production [5]. Immediately after harvesting, the zucchini fruit produced little ethylene, but if the fruit was stored at 4 °C or less and then transferred to room temperature, a burst of ethylene was produced on the 7th day of storage [6,7]. The level of cold-induced ethylene depends on the cultivar, the temperature, and the length of the storage period [8]. Since zucchini CI symptoms appear during cold storage and before the fruit is transferred to room temperature (when ethylene is produced), it seems that this cold-induced ethylene is not required to induce CI, but rather resulted from fruit cold damage.

The postharvest technologies reducing CI symptoms in zucchini also reduce cold-induced ethylene. Thus, postharvest preconditioning treatments at 15 °C, for 48 h before cold storage, reduced the CI symptoms and reduced the production of ethylene [9–11]. Likewise, individual shrink wrapping, a postharvest technology that is very useful to reduce zucchini CI, also reduces the production of cold-induced ethylene in the more sensitive cultivars [4,5]. In several fruit species, including persimmon, loquat, and tangerine, the reduction of ethylene production caused by the postharvest application of the ethylene inhibitor 1-MCP, resulted in reduced CI symptoms [12,13]. In the most CI susceptible zucchini cultivars, 1-MCP is also able to alleviate CI symptoms, associated with a reduction in the production of cold-induced ethylene, at 7 days of fruit cold storage [2]. These data indicate that ethylene should be considered a mediator of fruit CI in zucchini.

According to previous research, it is expected that zucchini fruit with a reduced ethylene production or sensitivity could have a better postharvest performance, because of a higher CI tolerance. To test this hypothesis, in this study, we compared the postharvest fruit quality of WT and ethylene-insensitive mutant *etr2b*. This and other ethylene-insensitive mutants were previously identified from a *C. pepo* mutant collection by the ethylene triple response assay [14]. The mutations also affect sex determination and female fertility, which precludes self-fertilization and the maintenance of the mutation in homozygous conditions.

In this study, we selected the ethylene-insensitive mutant *etr2b* because it was possible to multiply and obtain seeds of the mutant line without obtaining mutant plants from a segregated population. In addition, we had already identified the *etr2b* mutation in the coding region of the ethylene receptor gene *CpETR2B* [15], allowing WT, heterozygous, and homozygous *etr2b* plants to be separated unambiguously. Results confirmed the role of ethylene in the onset of CI of zucchini fruit, establishing that mutants that produce less ethylene or are more insensitive to ethylene could be a source of tolerance to CI, in zucchini breeding programs.

2. Materials and Methods

2.1. Plants and Growth Conditions

All fruits used in this study were harvested from WT and *etr2b* mutant plants derived from a BC_2S_1 populations. These populations were obtained by backcrossing each *etr* mutant with the background genotype MUC16 twice, therefore eliminating mutations, except for *etr2b* in each line, and by selfing the heterozygous BC_2 plants. The homozygous WT (wt/wt), as well as mutant plants in homozygous (*etr2b*/*etr2b*), or heterozygous (wt/*etr2b*) conditions were selected by genotyping with TaqMan probes,

and then selfed for their maintenance. All plants were grown in greenhouses under the typical cultural practices for zucchini crops in the experimental field of the University of Almería, Almería, Spain.

Flowers were hand-pollinated, and the fruit harvested with the same length for about 8 days after pollination. After harvesting, fruit of each genotype was randomly divided in four replications of three fruits each, and then stored at 4 °C and 95% RH for 14 days. Four replications with three fruits each were used to assess ethylene production, respiration rate, weight and firmness loss, CI and oxidative stress metabolites in WT, heterozygous and homozygous fruit on days 0, 7, and 14, after cold storage.

2.2. Genotyping etr2b Mutation Using TaqMan Probes

Genotyping analysis was performed to identify the homozygous WT (wt/wt), heterozygous (wt/*etr2b*) and homozygous mutant (*etr2b*/*etr2b*) plants from BC2S1 populations. DNA from young leaves of each individual plant was isolated and used for genotyping, by using real-time PCR with TaqMan probes. The multiplex PCRs were done using the SensiFAST™ Probe No-ROX Kit (Meridian Bioscience, Inc. Menphis, TN, USA) a set of forward and reverse primers, amplifying the polymorphic sequence, and two allele-specific probes descriptive of the SNP of interest (C–T). The WT probe was labelled with FAM dye, while the mutant probe was labelled with HEX reporter dye. The BHQ1 quencher molecule was used in both probes. Primers and probes sequences were previously described [15].

2.3. Ethylene Production and Respiration Rate

For each replications, 3 fruits were enclosed in 10 L air-tight container for 6 h at 20 °C, then the ethylene content was determined three times, by means a gas chromatograph model Varian 3900GC (Varian analytical Instruments, Walnut Creek, CA, USA), using an FID detector, and the ethylene content was expressed as nL ethylene g^{-1}FW 6 h^{-1}.

Respiration rate was assessed by analyzing the CO_2 three time, by means of a Check Mate II Headspace Analysers (Dansensor), and the respiration rate was expressed as ml of CO_2 kg^{-1} FW 6 h^{-1}.

2.4. Weight Loss, Firmness, and Chilling Injury Index

Twelve individual fruits of each genotype (WT and heterozygous and homozygous) were weighed on days 0, 7, and 14 of cold storage, assessing the percentage of weight loss in each fruit. Fruit firmness was determined by a penetration test in the distal region of the fruits, employing a TA.TX Plus Texture analyzer (Stable Micro-System, Godalming, Surrey, UK). The penetration test was performed by using a 4 mm-diameter probes, with a speed of 2 mm·s^{-1} and a depth of penetration of 10 mm. Each fruit was punctured three times. The firmness was expressed as maximum force reached (N).

To calculate the Chilling Injury Index (CI), the surface and severity of pitting of 12 fruits per genotype were evaluated. The surface affected by pitting was estimated according to the following scale: 0 = no damage, 1 = 5% damage, 2 = 6–15% damage, 3 = 16–25% damage, 4 = 26–50% damage, and 5 = 50% or more damage. The severity of pitting was estimated using the following scale: 0 = no damage, 1 = very superficial damage, 2 = superficial, 3 = moderate damage, 4 = severe damage, and 5 = very severe damage. Then, the averages of both parameters were pondered at 50% each for assessing the CI Index [16].

2.5. MDA and H_2O_2 Contents

Malonyldialdheyde (MDA) was determined, following the methodology proposed by Carvajal et al. [1], with some modifications [4]. Exocarp was homogenized in 1:10 (*w*/*v*) trichloroacetic acid (TCA) and 0.25% (*w*/*v*) of a solution of 2-thiobarbituric acid (TBA) 1:10 (*w*/*v*), and then heated at 95 °C in a water bath for 30 min, followed by immediate cooling in ice and centrifugation at 4000× *g*, for 20 min at 4 °C. The absorbance of the supernatant was measured at 532 and 600 nm. MDA content was expressed as nmol MDA g^{-1} FW.

H_2O_2 content was determined by using the procedure in Alexieva et al. [17], with minor modifications. The exocarp was homogenized in TCA and cold centrifugated, then 0.5 mL supernatant was added with a potassium buffer (pH 7) and potassium iodide. The mixture was kept in the dark for 60 min, after which, the absorbance at 390 nm was measured. H_2O_2 content was expressed as μmol H_2O_2 g^{-1} FW. All reagents and chemicals were obtained from Sigma-Aldrich, Madrid, Spain.

2.6. Statistical Analysis

For statistical analysis, STATGRAPHICS® Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA) was used. The data were treated by ANOVA, followed by a least significant difference test with a significance level of 95%. A Kolmogorov-Smirnov test was used to check the normality of data distribution. When normality failed, the variables were transformed and a non- parametric Kruskal-Wallis test at <0.05 significance level was used.

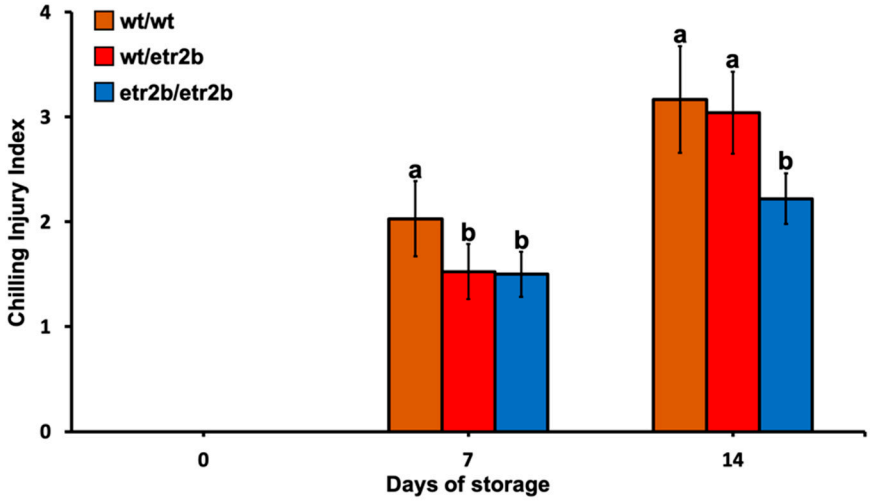
3. Results

3.1. Response of Zucchini Ethylene-Insensitive Mutant Fruit to Cold Storage

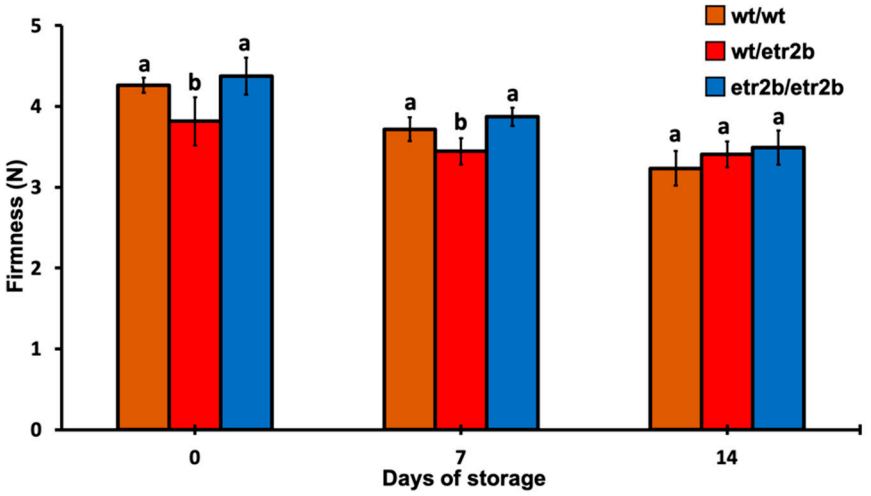
Figure 1 shows the effect of the ethylene-insensitive mutation *etr2b* on fruit phenotype, at harvest and after 14 days of cold storage. The mutation enables the flower to retain its green color and to be attached to the fruit at harvest, indicating that the reduced ethylene sensitivity of the *etr2/etr2* mutant delays floral organ abscission. After cold storage, the *etr2/etr2b* flowers gradually dries up and senesces, but the fruits remain fresher and show much less CI than the WT fruit.

Figure 1. General appearance of WT and the *etr2b/etr2b* fruit at harvest and after 14 days of storage, at 4 °C. Note that the mutant fruit maintain the flower attached at harvest. After cold storage, the flower is senescent but the fruit have a reduced percentage CI.

The effects of the ethylene-insensitive mutation on different postharvest fruit-quality parameters are shown in Figure 2. Cold storage at 4 °C induced pitting on the surface of zucchini fruits, with a noticeable increase in the CI index, after 14 days of storage (Figure 1). However, cold storage affected the WT and mutant fruits differentially. On the 7th day of storage, the WT fruit was more sensitive to cold than the heterozygous and homozygous *etr2b* fruits. On day 14 of storage, however, only the homozygous mutant fruits *etr2b/etr2b* reduced the CI index, compared to the WT and heterozygous fruits (Figure 1). This alleviation of CI was not accompanied by a reduced weight loss or firmness, showing that the WT and *etr2b* fruits had statistically similar values for these two parameters (Figure 1). At the time of harvest, the heterozygous fruits presented a slightly lower value of firmness, but as the storage time went by, the differences in firmness between the fruits of the different genotypes were reduced, until no differences were found after 14 days of cold storage (Figure 1).



A



B

Figure 2. *Cont.*

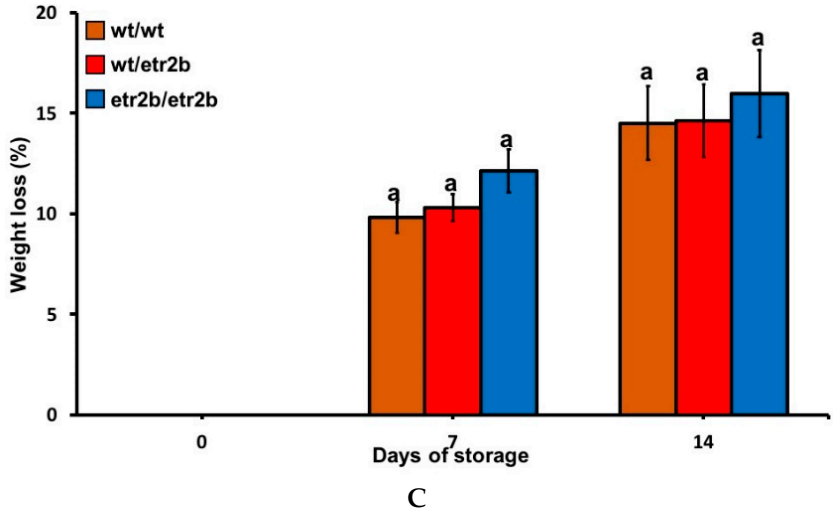


Figure 2. Comparing postharvest quality in WT and *etr2b* mutant zucchini fruits during cold storage for 14 days. (**A**) Chilling injury index, (**B**) firmness, and (**C**) weight loss. Heterozygous and homozygous *etr2b* were harvested at a similar stage of development, when stored for 0, 7, and 14 days at 4 °C, and then rewarmed at 20 °C for 6 h performing measurements. The results represent the mean and standard error of four independent replicates for each sample. Different letters indicate significant differences between genotypes, for each storage time (p-value < 0.05).

3.2. *Effect of Ethylene-Insensitive Mutations on Ethylene and CO_2 Production in Cold-Stored Zucchini Fruit*

Figure 3 compares the pattern of ethylene production and respiration rate of the WT and *etr2b* fruits, throughout cold storage. The ethylene production profile was in agreement with what was previously observed in the zucchini fruit. At harvest time, ethylene production was very low and did not show statistical differences between WT and the heterozygous and homozygous *etr2b* plants. After 7 days of cold storage and a rewarming period of 6 h, a burst of ethylene was found to be more significant in the WT than in the mutant fruit. After 2 weeks of cold storage, ethylene production dropped sharply to levels almost as low as those achieved at harvest time (Figure 3). The ethylene-insensitive mutation significantly reduced cold-induced ethylene on day 7 of storage, both in the heterozygous and homozygous conditions (Figure 3).

No statistical differences were found between the respiration rate of WT and *etr* mutant fruit on day 0, 7, and 14 of cold storage, indicating that a reduction in ethylene production and sensitivity under cold storage was not able to alter the fruit's respiration pattern in terms of the respiration rate.

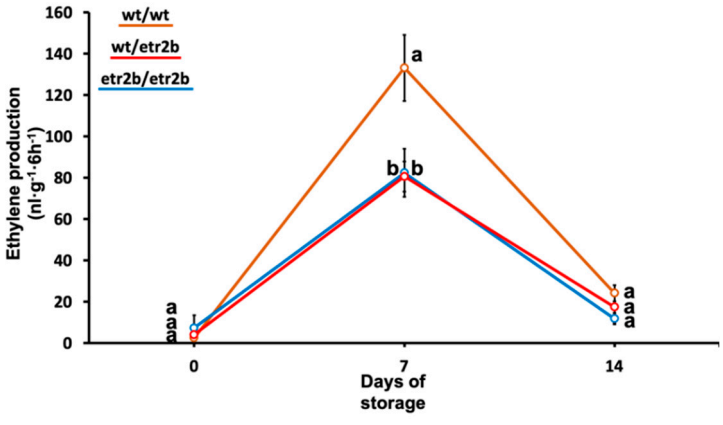
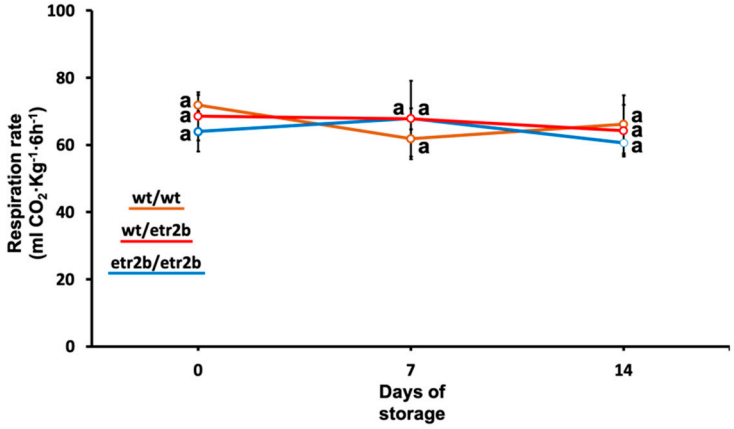


Figure 3. *Cont.*



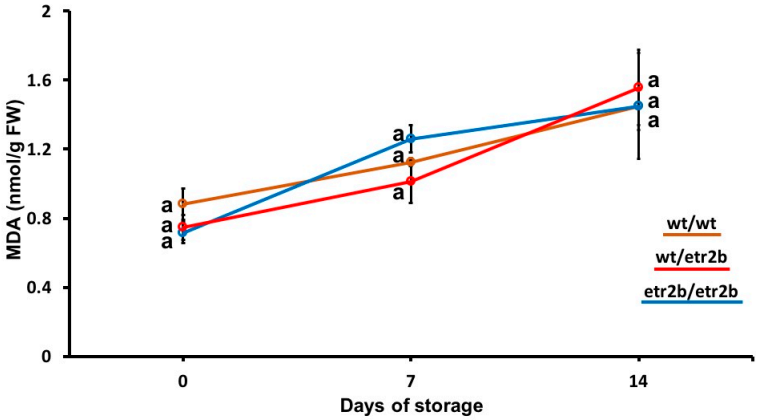
B

Figure 3. Evolution of ethylene (**A**) and CO_2 (**B**) in WT (wt/wt) and heterozygous (wt/*etr2b*) and homozygous (*etr2b*/*etr2b*) mutant fruits. Fruit was stored for 0, 7, and 14 days at 4 °C and then rewarmed at 20 °C for 6 h before measurements. The results represent the mean of four independent replicates for each sample. Different letters indicate significant differences between genotypes for each storage time (p-value < 0.05).

3.3. *Effect of Ethylene-Insensitive Mutations on Oxidative Stress*

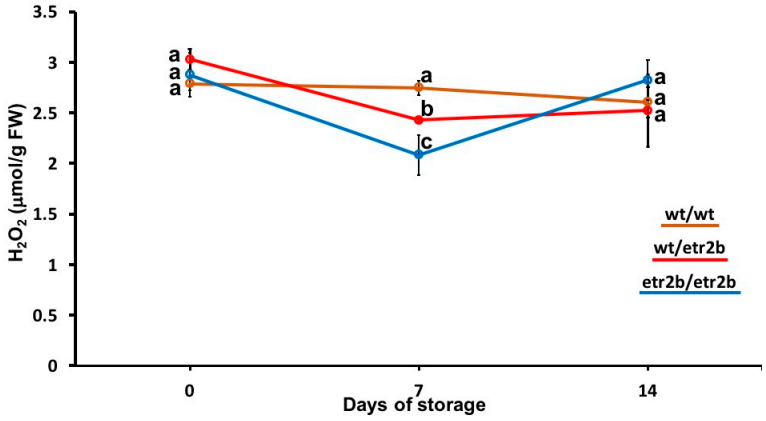
We previously observed that CI tolerance in Zucchini fruit was associated with a reduction in oxidative stress. Given that MDA is caused by lipid peroxidation, and is therefore an indicator of cell membrane integrity, we compared the MDA content in the exocarp of WT and *etr2b* fruits (Figure 4). During cold storage, MDA increased in the WT and heterozygous and homozygous mutant fruits, but the content of MDA in the mutant and WT fruits was similar.

The content of H_2O_2 in the fruit exocarp of the different genotypes was also very similar throughout the storage period, but after 7 days of cold storage, statistical differences were found between the H_2O_2 content of the WT and *etr2b* mutant fruit. The reduction in ethylene production after 7 days of cold storage in the homozygous *etr2b*/*etr2b* fruit, was therefore associated with a diminution of the of H_2O_2 content (Figure 4). As expected, the heterozygous wt/*etr2b* fruit, whose plants have an intermediate sensitivity to ethylene [14,15], showed an intermediate content of H_2O_2 at 7 day of cold storage, with respect to the WT and the homozygous mutant fruits (Figure 4).



A

Figure 4. *Cont.*



B

Figure 4. Contents of MDA (**A**) and H_2O_2 (**B**) in WT, and the heterozygous and homozygous *etr2b* mutant fruits. Samples were collected after rewarming the fruit at 20 °C for 6 h. The results represent the mean of four independent replicate samples for each genotype and storage time. Different letters indicate significant differences between genotype means at each specific storage time (p-value < 0.05).

4. Discussion

During cold storage, zucchini showed significant CI symptoms, the most important of which are pitting and weight loss [4,16]. The relationship between the incidence of pitting and increased weight loss found in this and other works might indicate that surface damage and evapotranspiration are both dependent on cold storage time [18]. Moreover, pitting damage caused by cold in zucchini surface can affect membrane integrity, favoring greater weight loss [8,19]. Similar water loss in WT and ethylene-insensitive *etr2b* fruits could be due to the presence of floral organs in the *etr2b* mutant fruit (Figure 1). The loss of water associated with the senescence of the petals in the mutant fruit with the attached flower, could cause this to lose the same water as the WT fruit, despite the former being more cold-tolerant. On the other hand, zucchini is very sensitive to cold storage, but fruit postharvest firmness was hardly affected in the WT and the *etr2b* fruits. It was reported that zucchini firmness was more dependent on the cultivar than on other factors, such as cold damage or weight loss [1,2]. The reduced metabolism and the lower activity of cell wall degrading enzymes, such as polygalacturonase (PG) and pectin methylesterase (PME) under cold storage [20–22], might account for the maintenance of zucchini fruit firmness in our experiments.

Zucchini is a non-climacteric fruit with a very low ethylene production at harvest and during its storage period at 20 °C [16]. When the fruit is stored cold for 7 days, and then transferred to room temperature for a few hours, it produces a burst of ethylene. This cold-induced ethylene disappears when cold storage continues up to 14 days, before rewarming [2,16]. A clear association was found between this cold-induced ethylene and CI susceptibility in zucchini fruit, since the fruits of cultivars and plants from segregating populations that are CI-tolerant, always produce less ethylene than those that are more susceptible to CI [5] and the treatments that lessen CI in zucchini, including individual shrink wrapping and 1-MCP, concomitantly reducing the production of cold-induced ethylene on day 7 of cold storage [2,4]. In this study, we also found that reduced CI in the ethylene-insensitive *etr2b* fruit compared to the WT fruit on days 7 and 14 of cold storage, was also correlated with a reduced production of cold-induced ethylene. The production of ethylene is also induced after chilling storage and rewarming in a number of non-climacteric fruits, such as cucumber, citrus, pear, and kiwi [23–26]. However, this cold-induced ethylene is not required in zucchini for the onset of CI in zucchini squash, since it was demonstrated that CI appears in the refrigerated chamber before rewarming of the fruit at room temperature, and therefore, before the burst of ethylene production [2,16]. Accordingly, the cold-induced ethylene is actually the fruit's response to cold damage, and the lower production of

this cold-induced ethylene in *etr2b* mutants might indicate that their fruit suffers less cold damage than that of WT, and is therefore, more tolerant to CI.

It was suggested that ethylene plays an essential role in CI development [27,28]. In fact, it is long known that methods that induce a reduction in ethylene synthesis leads to an improvement in cold damage. Thus, ACO-anti-sense melons showed a higher tolerance to cold damage than WT, when kept at 2 °C [29]. In the opposite direction and even more interestingly, Obando et al. [30] observed that crossing two non-climacteric lines of melons obtained a line of climacteric melons, and that this character was associated with a higher sensitivity to chilling damage. In zucchini, the burst of cold-induced ethylene is not required for the onset of CI, as mentioned above [2,5,16], but ethylene must be involved in a positive regulation of CI. Thus, we previously found that ethylene is slightly induced in refrigerated fruit before rewarming [2]. Moreover, external treatments with the ethylene inhibitor 1-MCP reduced CI in the fruit of cold-susceptible zucchini cultivars [11]. Here, we demonstrated that the mutation of ethylene receptor CpETR2B, leading to ethylene insensitivity [14,15], are also able to reduce postharvest CI, concomitantly with a reduction of ethylene (Figure 3). In cold-sensitive grapefruit, an induction of certain ethylene receptor isoforms (ETRs) was observed, as well as ethylene response factors (ERFs), suggesting a close relationship between ethylene perception and signaling with cold damage [28,31]. Huang et al. [32] found that the levels of expression of ethylene signal transduction genes *PgERF1* and *PgERF2* in pomegranate, correlated with increased cold damage. However, no clear linear relationship was found between *PgETR* expression and chilling injury. The fact that the inhibition of ethylene response in the *etr2b* mutants was able to reduce CI in zucchini also indicates an active role of ethylene in the control of cold damage. Given that 1-MCP only alleviate CI in the most cold-susceptible cultivars, and that the reduction of CI in the *etr2b* mutant is not very pronounced, it is likely that other cooperating factors participate together with ethylene, in the regulation of fruit chilling response in zucchini squash. The effect of the individual mutation *etr2b* on CI is even smaller than that obtained by 1-MCP treatments [2], because 1-MCP would surely affect all ethylene receptors in the fruit, while the individual ethylene insensitivity *etr2b* mutation would only affect just one specific ethylene receptor, resulting in 43.5% and 63.7% of ethylene insensitivity in wt/*etr2b* and *etr2b*/*etr2b*, respectively [15].

CI alleviation in the *etr2b* mutant fruit was not associated with a reduction in the respiration rate, but significantly reduced the accumulation of oxidative stress metabolites such as hydrogen peroxide. Several postharvest treatments that alleviate CI symptoms in zucchini reduce the fruit respiration rate and oxidative stress [33,34]. The reported effect of 1-MCP on the respiration rate of refrigerated fruit was variable, and only the fruit of more susceptible zucchini cultivars was able to reduce its respiration [2]. Moreover, the onset of CI in refrigerated non-climacteric fruit is concomitant with an increase in the production of Reactive Oxygen Species (ROS) and oxidative damage of cell membranes and MDA accumulation [4]. It was demonstrated that cold tolerance of the zucchini fruit is associated with a reduction in MDA content [4,8]. The fact that the *etr2b* mutant fruit showed reduced CI symptoms and H_2O_2 content, but accumulated a similar level of MDA than WT fruit (Figure 4) could indicate that the role of ethylene signaling in lipid peroxidation is limited. The role of ethylene receptors and signaling components in oxidative stress is not clear. In Arabidopsis, it was demonstrated that the ethylene-insensitive *ein2-1* mutation was able to alleviate plant oxidative stress [35]. In four ethylene-insensitive mutants of tobacco plants, however, an increased MDA content was found when the plants were undergoing oxidative stress, in response to drought [36], and in the ethylene-insensitive mutant *Never ripe* of tomato, plants showed an enhanced MDA content in roots and leaves, in response to NaCl and $CdCl_2$ stress [37].

5. Conclusions

Comparison of postharvest quality of WT and *etr2b* mutant fruit during cold storage showed that ethylene could modulate cold damage in zucchini vegetable fruit. The mutation was able to reduce plant ethylene sensitivity, concomitantly, with a reduced production of cold-induced ethylene, and an

enhanced chilling tolerance of refrigerated fruit. The ethylene-insensitive mutation also reduced the accumulation of hydrogen peroxide, but had no effect on the MDA content.

Author Contributions: Conceptualized, conceived and designed the experiments: A.G. and M.J.; Managed crop and greenhouse evaluations: A.G., E.A., J.I., J.R., and G.C.; Performed the experiments and formal analysis: A.G., C.M., E.A., J.L.V., and M.d.M.R.; Writing original draft preparation: A.G., J.L.V., and M.J.; Writing, review and editing: D.G.; J.L.V., and M.J.; Supervision, grant management, and funding acquisition, M.J. All authors read and agreed to the published version of the manuscript.

Funding: This research was funded by grants AGL2014-54598-C2-1-R, AGL2017-82885-C2-1-R, and UAL18-BIO-B017B, which were partly funded by the European Regional Development Fund and partly by the Spanish Ministry of Science and the University of Almería. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgments: A.G., J.I., and G.C. acknowledge the FPI and FPU scholarship programs from MEC, Spain.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Carvajal, F.; Martinez, C.; Jamilena, M.; Garrido, D. Differential response of zucchini varieties to low storage temperature. *Sci. Hortic.* **2011**, *130*, 90–96. [CrossRef]
2. Megías, Z.; Martínez, C.; Manzano, S.; García, A.; Rebolloso-Fuentes, M.; Valenzuela, J.L.; Garrido DJamilena, M. Ethylene biosynthesis and signaling elements involved in chilling injury and other postharvest quality traits in the non-climacteric fruit of zucchini (*Cucurbita pepo*). *Postharvest Biol. Technol.* **2016**, *113*, 48–57. [CrossRef]
3. Megías, Z.; González-Rodríguez, L.J.; Aguado, E.; Garcia, A.; Manzano, S.; Rebolloso, M.M.; Valenzuela, J.L.; Jamilena, M. Effect of cold storage time on chilling injury in two zucchini cultivars. *Acta. Hortic.* **2018**, *1194*, 479–486. [CrossRef]
4. Megías, Z.; Martínez, C.; Manzano, S.; García, A.; Rebolloso-Fuentes, M.M.; Garrido, D.; Valenzuela, J.L.; Jamilena, M. Individual Shrink Wrapping of Zucchini Fruit Improves Postharvest Chilling Tolerance Associated with a Reduction in Ethylene Production and Oxidative Stress Metabolites. *PLoS ONE* **2015**, *10*, e0133058. [CrossRef] [PubMed]
5. Megías, Z.; Manzano, S.; Martínez, C.; García, A.; Aguado, E.; Garrido, D.; Valenzuela, J.L.; Jamilena, M. Postharvest cold tolerance in summer squash and its association with reduced cold-induced ethylene production. *Euphytica* **2017**, *213*, 9. [CrossRef]
6. Balandrán-Quintana, R.R.; Mendoza-Wilson, A.M.; Gardea-Béjar, A.A.; Vargas-Arispuro, I.; Martínez-Téllez, M.A. Irreversibility of chilling injury in zucchini squash (*Cucurbita pepo* L.) could be a programmed event long before the visible symptoms are evident. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 553–557. [CrossRef]
7. Megías, Z.; Manzano, S.; Martínez, C.; Valenzuela, J.L.; Garrido, D.; Jamilena, M. Ethylene production by fruits of zucchini cultivars differing in postharvest fruit quality and tolerance to chilling injury. In Proceedings of the 10th EUCARPIA Meet Genet Breed Cucurbitaceae, Antalya, Turkey, 15–18 October 2012; pp. 638–642.
8. Valenzuela, J.; Manzano, S.; Palma, F.; Carvajal, F.; Garrido, D.; Jamilena, M. Oxidative Stress Associated with Chilling Injury in Immature Fruit: Postharvest Technological and Biotechnological Solutions. *Int. J. Mol. Sci.* **2017**, *18*, 1467. [CrossRef] [PubMed]
9. Wang, C.Y. Combined treatment of heat shock and low temperature conditioning reduces chilling injury in zucchini squash. *Postharvest Biol. Technol.* **1994**, *4*, 65–73. [CrossRef]
10. Carvajal, F.; Palma, F.; Jamilena, M.; Garrido, D. Cell wall metabolism and chilling injury during postharvest cold storage in zucchini fruit. *Postharvest Biol. Technol.* **2015**, *108*, 68–77. [CrossRef]
11. Megías, Z.; Barrera, A.; Manzano, S.; Martínez, C.; Garrido, D.; Valenzuela, J.L.; Jamilena, M. Physical and chemical treatments enhancing postharvest fruit quality in zucchini. *Acta. Hortic.* **2015**, *1091*, 141–146. [CrossRef]
12. Salvador, A.; Arnal, L.; Monterde, A.; Cuquerella, J. Reduction of chilling injury symptoms in persimmon fruit cv. 'Rojo Brillante' by 1-MCP. *Postharvest Biol. Technol.* **2004**, *33*, 285–291. [CrossRef]

13. Salvador, A.; Carvalho, C.P.; Monterde, A.; Martínez-Jávega, J.M. 1-MCP effect on chilling injury development in 'Nova' and 'Ortanique' mandarins. *Food Sci. Technol. Int.* **2006**, *12*, 165–170. [CrossRef]
14. García, A.; Aguado, E.; Parra, G.; Manzano, S.; Martínez, C.; Megías, Z.; Cebrián, G.; Romero, J.; Beltrán, S.; Garrido, D. Jamilena. Phenomic and Genomic Characterization of a Mutant Platform in Cucurbita pepo. *Front Plant Sci.* **2018**, *9*, 1049. [CrossRef]
15. García, A.; Aguado, E.; Martínez, C.; Loska, D.; Beltrán, S.; Valenzuela, J.L.; Garrido, D.; Jamilena, M. The ethylene receptors CpETR1A and CpETR2B cooperate in the control of sex determination in *Cucurbita pepo*. *J. Exp. Bot.* **2020**, *71*, 154–167. [CrossRef]
16. Megías, Z.; Martínez, C.; Manzano, S.; Barrera, A.; Rosales, R.; Valenzuela, J.L.; Garrido DJamilena, M. Cold-induced ethylene in relation to chilling injury and chilling sensitivity in the non-climacteric fruit of zucchini (*Cucurbita pepo* L.). *LWT-Food. Sci. Technol.* **2014**, *57*, 194–199. [CrossRef]
17. Alexieva, V.; Sergiev, I.; Mapelli, S.; Karanov, E. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant. Cell. Environ.* **2001**, *24*, 1337–1344. [CrossRef]
18. Lara, I.; Belge, B.; Goulao, L.F. The fruit cuticle as a modulator of postharvest quality. *Postharvest Biol. Technol.* **2014**, *87*, 103–112. [CrossRef]
19. Fernández-Trujillo, J.P.; Martínez, J.A. Ultrastructure of the onset of chilling injury in cucumber fruit. *J. Appl. Bot. Food Qual.* **2006**, *80*, 100–110.
20. Brummell, D.A.; Harpster, M.H. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. In *Plant Cell Wall*; Carpita, N.C., Campbell, M., Tierney, M., Eds.; Springer: Dordrecht, The Netherlands, 2001; pp. 311–340. [CrossRef]
21. Guzmán, M.; Sánchez, A.; Salas, M.C.; Del Moral, F.; Valenzuela, J.L. Relationship between pectin-methyl esterase activity and softening in "Raf" tomato fruit. *Acta. Hortic.* **2012**, *934*, 1291–1296. [CrossRef]
22. Rugkong, A.; Rose, J.K.; Lee, S.J.; Giovannoni, J.J.; O'Neill, M.A.; Watkins, C.B. Cell wall metabolism in cold-stored tomato fruit. *Postharvest Biol. Technol.* **2010**, *57*, 106–1013. [CrossRef]
23. Wang, C.Y.; Adams, D.O. Chilling-Induced Ethylene Production in Cucumbers (*Cucumis sativus* L.). *Plant Physiol.* **1982**, *69*, 424–427. [CrossRef]
24. Hyodo, H.; Fukasawa, R. Ethylene Production in Kiwifruit. *J. Jpn. Soc. Hortic. Sci.* **1985**, *54*, 209–215. [CrossRef]
25. McCollum, T.G.; McDonald, R.E. Electrolyte leakage, respiration, and ethylene production as indices of chilling injury in grapefruit. *HortScience* **1991**, *26*, 1191–1192. [CrossRef]
26. Lafuente, M.T.; Zacarias, L.; Martínez-Téllez, M.A.; Sánchez-Ballesta, M.T.; Dupille, E. Phenylalanine Ammonia-lyase As Related to Ethylene in the Development of Chilling Symptoms during Cold Storage of Citrus Fruits. *J. Agric. Food Chem.* **2001**, *49*, 6020–6025. [CrossRef]
27. Pons, C.; Martí, C.; Forment, J.; Crisosto, C.H.; Dandekar, A.M.; Granell, A. A bulk segregant gene expression analysis of a peach population reveals components of the underlying mechanism of the fruit cold response. *PLoS ONE* **2014**, *9*, e90706. [CrossRef]
28. Lado, J.; Rodrigo, M.J.; Zacarías, L. Analysis of ethylene biosynthesis and perception during postharvest cold storage of Marsh and Star Ruby grapefruits. *Food Sci. Technol. Int.* **2015**, *21*, 537–546. [CrossRef]
29. Ben-Amor, M.; Flores, B.; Latché, A.; Bouzayen, M.; Pech, J.C.; Romojaro, F. Inhibition of ethylene biosynthesis by antisense ACC oxidase RNA prevents chilling injury in Charentais cantaloupe melons. *Plant Cell Environ.* **1999**, *22*, 1579–1586. [CrossRef]
30. Obando, J.; Miranda, C.; Jowkar, M.M.; Moreno, E.; Sour, M.K.; Martínez, J.A.; Arús, P.; García-Mas, J.; Monforte, A.J.; Fernández-Trujillo, J.P. Creating climacteric melon fruit from nonclimacteric parentals: Postharvest quality implications. In *Advances in Plant Ethylene Research*; Ramina, A., Chang, C., Giovannoni, J., Klee, H., Perata, P., Woltering, E., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 197–205. [CrossRef]
31. Lado, J.; Manzi, M.; Sainz, M.M.; Sotelo, M.; Zacarías, L. Involvement of Plant Hormones in Cold Stress Tolerance. In *Plant Hormones under Challenging Environmental Factors*; Ahammed, G., Yu, J.Q., Eds.; Springer: Dordrecht, The Netherlands, 2016; pp. 23–49. [CrossRef]
32. Huang, R.; Li, W.; Guan, X.-W.; Xie, B.; Zhang, S.-M. Molecular cloning and characterization of genes related to the ethylene signal transduction pathway in pomegranate (*Punica granatum* L.) under different temperature treatments. *J. Biosci.* **2019**, *44*, 137. [CrossRef]

33. Martínez-Téllez, M.; Ramos-Clamont, M.; Gardea, A.; Vargas-Arispuro, I. Effect of infiltrated polyamines on polygalacturonase activity and chilling injury responses in zucchini squash (*Cucurbita pepo* L.). *Biochem. Biophys. Res. Commun.* **2002**, *295*, 98–101. [CrossRef]
34. Palma, F.; Carvajal, F.; Ramos, J.M.; Jamilena, M.; Garrido, D. Effect of putrescine application on maintenance of zucchini fruit quality during cold storage: Contribution of GABA shunt and other related nitrogen metabolites. *Postharvest Biol. Technol.* **2015**, *99*, 131–140. [CrossRef]
35. Cao, S.; Jiang, S.; Zhang, R. Evidence for a role of Ethylene-Insensitive 2 gene in the regulation of the oxidative stress response in Arabidopsis. *Acta Physiol. Plant* **2006**, *28*, 417–425. [CrossRef]
36. Wang, H.; Wang, F.; Zheng, F.; Wang, L.; Pei, H.; Dong, C.-H. Ethylene-insensitive mutants of Nicotiana tabacum exhibit drought stress resistance. *Plant Growth Regul.* **2016**, *79*, 107–117. [CrossRef]
37. Monteiro, C.C.; Carvalho, R.F.; Gratão, P.L.; Carvalho, G.; Tezotto, T.; Medici, L.O.; Peres, L.E.P.; Azevedo, R.A. Biochemical responses of the ethylene-insensitive Never ripe tomato mutant subjected to cadmium and sodium stresses. *Environ. Exp. Bot.* **2011**, *71*, 306–320. [CrossRef]