Euphytica (2017) 213:9
DOI 10.1007/s10681-016-1805-0

Postharvest cold tolerance in summer squash and its association with reduced cold-induced ethylene production

Zoraida Megías · Susana Manzano · Cecilia Martínez · Alicia García ·
Encarnación Aguado · Dolores Garrido · María del Mar Rebolloso ·
Juan Luis Valenzuela · Manuel Jamilena

Received: 6 July 2016 / Accepted: 15 November 2016 / Published online: 9 December 2016

Abstract Tolerance to postharvest chilling injury (PCI) is becoming an ever more essential trait for export-oriented vegetables requiring refrigerated transport and/or storage. Summer squash, *Cucurbita pepo*, is highly subject to PCI. We screened a collection consisting of 80 long-fruited accessions of morphotypes zucchini, cocozelle, and vegetable marrow for cold tolerance. Of these, we selected the most cold tolerant and some of the least cold tolerant for further scrutiny. Fruits from each accession were stored for 7 and 14 days at 4 °C before evaluating PCI, weight loss (WL) and ethylene production. Several accessions, including CpCAL003, CpCAL053 and CpCAL051, showed tolerance to PCI, with a lower percentage of the fruit surface suffering injury and WL at 4 °C. The mode of inheritance of tolerance to PCI index and WL was investigated by crossing the tolerant accession designated CpCAL003 with the susceptible CpCAL112, and observing their filial- and backcross-generation progenies. The results indicate that cold tolerance is not conferred by a single gene, but rather is quantitatively inherited trait. Cold-induced ethylene production co-segregated with PCI susceptibility. Thus, ethylene production under cold storage can be used as physiological indicator in selecting for PCI tolerance in summer squash.

Keywords *Cucurbita pepo* · Postharvest chilling injury · Fruit quality · Ethylene

Introduction

The *Cucurbitaceae* include a number of major cultivated species that are produced and distributed worldwide, such as cucumber, melon, watermelon, pumpkins and squash. *Cucurbita pepo* L., which includes zucchini and other summer squash, is one of the most important cucurbit crop species due to its wide distribution and high economic value (Nee 1990). Summer squash fruits are non-climacteric, but are harvested very young, only 2–5 days past anthesis, and therefore are highly perishable. Consequently, extending their shelf life for as long as possible is critical for exporting to distant markets, such as southeastern Spain to markets in western and central

---

Z. Megías · S. Manzano · C. Martínez ·
A. García · E. Aguado · M. del Mar Rebolloso ·
J. L. Valenzuela · M. Jamilena (&)
Departamento de Biologia y Geología, Agrifood Campus
of International Excellence (CeiA3), CIAIMBITAL,
Universidad de Almería, La Cañada de San Urbano s/n,
04120 Almería, Spain
e-mail: mjamille@ual.es

D. Garrido
Departamento de Fisiología Vegetal, Facultad de
Ciencias, Universidad de Granada, Fuentenueva s/n,
18071 Granada, Spain

Europe. Cold storage has been used to reduce decay development as in many other commodities, but summer squash are very sensitive to postharvest chilling injury (PCI), and cold storage generates considerable postharvest losses.

When summer squash are stored under suboptimal temperatures, they develop small surface depressions called ‘‘pitting’’, and they dehydrate and soften (Martínez-Tellez et al. 2002; Balandran-Quintana et al. 2003; Carvajal et al. 2011; Megías et al. 2014). These appreciable symptoms are associated with alterations in cell membrane permeability, favoring ion leakage and lipid peroxidation (Gualanduzzi et al. 2009; Carvajal et al. 2011), with an increase in the production of reactive oxygen species (ROS), and an inhibition of the activity of antioxidant enzymes (Gualanduzzi et al. 2009; Carvajal et al. 2011).

Various postharvest technology approaches have been used to induce chilling tolerance during storage of summer squash at low temperatures. Temperature preconditioning treatments (2 days at 15 or 12 °C before cold storage at 4 °C) are effective in reducing the incidence of PCI in this fruit (Wang 1995; Megías et al. 2014; Carvajal et al. 2015). Individual shrink packaging (ISW) was found to be an excellent technology to delay fruit senescence and induce cold tolerance in zucchini, concomitantly with a reduction in ethylene production, respiration rate and ROS accumulation (Megías et al. 2015). Treatment with superatmospheric oxygen has also given good results to enhance zucchini cold tolerance by increasing the oxygen radical absorbance capacity (ORAC) values and the total phenolic content (Zheng et al. 2008).

Many plant growth regulators have been considered to have possible protective effects against PCI. For example, the application of methyl jasmonate (MJ) in zucchini increased abscisic acid (ABA) and polyamines (PA) levels, stabilizing the cellular membrane and avoiding the development of PCI symptoms (Wang 1994). The role of PA in the development of PCI has been studied due to their capacity to bind phospholipids. Their antioxidant properties could protect cell membranes from lipid peroxidation. This physiological response depends on the dose and the type of PA concerned (Martínez-Tellez et al. 2002). Thus, in zucchini fruits, treatments with PA, or with low oxygen atmospheres and high CO_2, which induce an increase in PA, have been correlated with a decrease in PCI symptoms (Wang and Ji 1989; Wang 1995 Serrano et al. 1998; Martínez-Tellez et al. 2002; Palma et al. 2014, 2015). As PA levels affect ethylene production, as they share the same precursor pathway *S*-adenosylmethionine (SAM), the role of ethylene in PCI has been widely discussed. In zucchini fruit, cold storage induces a high peak of ethylene production after rewarming. We have observed that PCI appears in cold chambers, with ethylene not affecting the development of PCI, but rather being a consequence of the stress generated (Megías et al. 2014). However, our latest data concerning the inhibition of ethylene perception by the use of 1-MCP suggested that ethylene is involved in the onset of PCI symptoms in zucchini fruit, and that 1-MCP can retard and reduce PCI in the more sensitive varieties (Massolo et al. 2013; Megías et al. 2016).

Cold tolerance is controlled by different molecular and environmental factors (Sanghera et al. 2011). The molecular factors includes the induction of genes of the COR family, transcription factors such as CBFs (CRT/DRE-binding factors) and DREB (DRE binding factors) (Thomashow 2001; Riechmann and Meyerowitz 1998), or heat shock proteins (HSP) (Sevillano et al. 2009). Up to now, however, the number of genes or molecular factors regulating PCI tolerance is not well understood (Cushman and Bohnert 2000). Genomics studies of fruit species have become important potential tools to understand the complexity of PCI symptoms in fruit stored under low temperature conditions (Yun et al. 2012), opening new opportunities for reducing postharvest losses of fruits and vegetables (Pech and Latché 2013).

A sustainable solution to reduce zucchini PCI would be the development of cold-tolerant varieties. Therefore the objectives of our work were to identify cold tolerant squash germplasm, to investigate the mode of inheritance of cold tolerance, and to determinate whether or not PCI and ethylene production are co-inherited. The ultimate goal is the introgression of PCI tolerance in commercial varieties, as has been done in the commercial breeding programs of other horticultural crops such as peach (Cantín et al. 2010) and cucumber (Gordon and Staub 2014).

Materials and methods

Plant material and culture conditions

In 2012, we screened 80 accessions, mostly old open-pollinated Spanish cultivars having elongated fruits

(Table 1), from the morphotypes zucchini, cocozelle, and vegetable marrow (Paris 1986). The fruit of each accession was stored for 14 days at 4 °C and then evaluated for PCI. After this screening, the sensitivity to PCI of the accessions was classified as low, medium or high. Of these 80 accessions, we selected 20, from those showing the highest PCI tolerance as well as some having low PCI tolerance, for further analysis. In spring 2013, the fruit of the selected accessions were evaluated for PCI, weight loss (WL) and ethylene production, as were fruit of five zucchini commercial hybrids, including ‘Alexander’, ‘Victoria’, ‘Cavili’ and ‘Natura’ and ‘Sinatra’, which were found to be more tolerant and sensitive, respectively, to PCI in previous trials (Carvajal et al. 2011; Meg´ıas et al. 2014). Seeds of each accession were germinated on nursery trays under high humidity conditions for 2 days, and then grown under standard conditions for 15 days before transplanting to soil in a greenhouse. The fruit to be used in each evaluation was harvested from plants growing under the same typical greenhouse conditions in the south-eastern Spain regarding fertilization and disease and pest control.

Genetic analysis of cold tolerance in summer squash

After the first screening of the 20 summer squash accessions, two that were recorded as PCI tolerant (CpCAL003 and CpCAL053) and three that were sensitive to PCI (CpCAL044, CpCAL112 and CpCAL097), were re-evaluated for postharvest cold tolerance in the winter campaign of 2013–2014. After this evaluation, accessions of contrasting PCI tolerance were crossed (CpCAL003 9 CpCAL112, CpCAL 003 9 CpCAL0097, and CpCAL003 9 Cp005).

In spring 2014, the F_1 generation of the cross CpCAL003 9 CpCAL112 was evaluated and the F_2 offspring of each cross was obtained by selfing the F_1. The backcrossing generations BC1(P1) and BC1(P2) were also obtained by crossing the F_1 with each of the parental lines CpCAL003 [BC1(P1)] and CpCAL112 [BC1(P2)].

In the winter campaign of 2014–2015, the parental lines CpCAL003 and CpCAL112 (20 plants each) and their offspring F_1 (40 plants), F_2 (61 plants), BC1(P1) (60 plants) and BC1(P2) (73 plants), were grown together with the hybrids ‘Natura’ and ‘Sinatra’ (20 plants each), the latter as controls of PCI tolerance and sensitivity, respectively. The fruit of each plant, especially in the segregating populations, was harvested individually and then stored for 14 days at 4 °C to evaluate PCI tolerance in individual plants. Only plants producing more than five fruits were recorded. After scoring individual plants for PCI index and WL in F_2 and BC generations, those having the lowest (PCI \ 2) and the highest (PCI C 4) scores for PCI, representing the most and least PCI tolerant plants in the respective segregating populations, were selected for ethylene production measurements and cosegregation analysis between PCI and fruit ethylene production.

Measurements of PCI, WL and ethylene production

To evaluate PCI tolerance, fruits with uniform width (4–5 cm maximum diameter) of each accession were harvested with about 1.5 cm of peduncle and randomly divided in different lots before storage at 4 °C and 60% RH in controlled chambers for a total of 14 days. At days 7 and 14, 12 fruits per accession and storage period were transferred to 20 °C for 6 h before evaluating postharvest parameters and processing of the samples. For each genotype, four replications of three fruits each were used for WL, PCI and ethylene production measurements.

The percentage of WL during storage was assessed by weighing 12 individual fruits from each genotype at 0, 7 and 14 days of cold storage. The percentage of WL of each fruit was calculated according to the following equation:

$$\%\ \text{Weight loss}\ ¼\ \frac{Wi - Wf}{Wi} \times 100$$

where wi and wf are the initial and final fruit weights, respectively.

To assess PCI we evaluated the surface of the fruit affected by pitting and the severity of pitting symptoms. The fruit surface affected by pitting was used to classify each fruit according to the following scale: 0 = no damage, 1 = C5% damage, 2 = 6–15% damage, 3 = 16–25% damage, 4 = 26–50% damage, and 5 = C50% damage (Meg´ıas et al. 2014). To assess the severity of pitting symptoms, the scale was 0 = no damage, 1 = very superficial damage, 2 = superficial damage, 3 = moderate damage, 4 = severe damage, 5 = very severe damage. These two

Table 1 Evaluation of PCI tolerance in 25 accessions of *Cucurbita pepo* subsp. *pepo* having elongated fruit

Accesion name	Morphotype[a]	Source[b]	Country of origin[c]	PCI index[d]		% Weight loss[d]		PCI tolerance[e]
				7 days	14 days	7 days	14 days	
CpCAL044	ZU	BSUAL	Spain, Valencia	4.92 ± 0.5	5 ± 0	10.76 ± 2.5	20.32 ± 3.2	Low
Sinatra	ZU	Clause-Tezier		4.25 ± 0.4	5 ± 0	8.2 ± 1.3	14.9 ± 2.3	Low
CpCAL112	ZU	BSUAL	Spain, Valencia	4 ± 0.5	4.8 ± 0.4	8.67 ± 3.1	17.61 ± 3.4	Low
CpCAL005	VM	BSUAL	Spain, Andalusia	4 ± 0.27	4.5 ± 0.32	7.1 ± 2.75	23 ± 5.3	Low
CpCAL059	VM	BSUAL	Spain, Cantabria	4 ± 0.35	4.5 ± 0.55	11.53 ± 3.8	21.73 ± 9.1	Low
C CpCAL052	VM	BSUAL	Spain, Catalonia	4 ± 0.4	4.5 ± 0.3	8 ± 3.27	15.98 ± 2.7	Low
CpCAL072	CO	USDA	Spain, C. León	3.9 ± 0.5	4.5 ± 0.5	11.14 ± 3.1	20.19 ± 4.9	Low
CpCAL022	CO	BSUAL	Spain, C. León	3.85 ± 0.5	4.5 ± 0.3	8.25 ± 1.25	16.8 ± 3.7	Low
CpCAL097	CO	BSUAL	Spain, Valencia	3.85 ± 0.8	4.85 ± 0.5	9.05 ± 1.6	18.61 ± 3.4	Low
CpCAL048	ZU-VM	BSUAL	Spain, Valencia	3.83 ± 1	4.16 ± 0.8	7.16 ± 3.3	14.65 ± 5.5	Low
CpCAL046	CO	BSUAL	Spain, Valencia	3.14 ± 0.9	4.83 ± 0.4	7.51 ± 3.2	19.16 ± 3.6	Low
Cavili	ZU	NUNHEM BV		2.93 ± 0.8	4.55 ± 0.5	6.95 ± 3.0	14.83 ± 0.1	Low
CA-84	VM	COMAV	Spain, Canary Island	2.7 ± 0.3	5 ± 0	7 ± 1.9	15.1 ± 3.9	Low
Victoria	ZU	Clause-Tezier		2.55 ± 0.8	3.8 ± 0.5	9.48 ± 1.8	16.02 ± 3.3	Low
CpCAL070	VM	USDA	Turkey	2.5 ± 0.3	4.3 ± 0.4	9.69 ± 3.33	20.3 ± 5.18	Low
CpCAL094	ZU	BSUAL	Spain, Murcia	2.5 ± 0.5	5 ± 0	7.46 ± 1.3	15.69 ± 2.1	Low
Natura	ZU	Enza Zaden		2.5 ± 0.4	4 ± 0.3	5.8 ± 1.6	9.7 ± 3.3	Intermediate
CpCAL058	ZU-CO	BSUAL	Spain, Valencia	2.38 ± 0.3	3.57 ± 0.6	5.96 ± 1.2	13.98 ± 4.2	Intermediate
AN-CU-92	VM	COMAV	Spain, Andalusia	2.3 ± 0.3	3.5 ± 0.7	4.3 ± 1.9	8.1 ± 5.1	Intermediate
Alexander	ZU	Diamond Seeds		2.28 ± 0.4	4.27 ± 0.6	8.05 ± 1.7	14.24 ± 2.1	Intermediate
CpCAL056	ZU-CO	BSUAL	Spain, Valencia	1.77 ± 0.6	4.55 ± 0.7	6.12 ± 3.4	11.32 ± 5.6	Intermediate
CpCAL064	ZU	BSUAL	Greek A. Paraskeis	1.3 ± 0.5	4.33 ± 0.4	4.43 ± 2.6	12.67 ± 3.5	Intermediate
CpCAL053	ZU-VM	BSUAL	Morocco, Khmelat	1.2 ± 0.4	3.25 ± 0.5	6.71 ± 2.9	12.08 ± 5.5	High
CpCAL051	ZU-VM	BSUAL	Spain, Catalonia	0.8 ± 0.4	3.2 ± 0.6	4.15 ± 2.3	8.55 ± 4.8	High
CpCAL003	VM	BSUAL	Spain, Andalusia	0.58 ± 0.5	1.71 ± 0.5	2.63 ± 0.8	7.17 ± 2.6	High

[a] Fruit shape morphotypes: *ZU* Zucchini, *VM* Vegetable Marrow, *CO* Coccozelle (Paris 1986)

[b] COMAV Germoplam Bank at the Polytechnic University of Valencia; USDA United States Department of Agriculture; BSUAL Seed Bank at the University of Almería

[c] Origin data obtained from COMAV (http://www.comav.upv.es/BancogermoplasmaUPV/consulta2sesion.php), USDA/GRIN (http://www.ars-grin.gov/cgi-bin/npgs/html/tax_acc.pl) and BSUAL germoplasm bank at UAL

[d] PCI index and weight loss scores represent the mean ± standard deviation of at least three biological repetition of three-four fruits each. PCI values are the mean score ranged from 0 to 5, and weight loss is expressed in (%)

[e] PCI tolerance classification in Spring 2012. High tolerance, PCI = 0–2; intermediate tolerance, PCI = 2.1–2.4; low tolerance PCI = 2.5–5 at 7 days of cold storage

parameters were evaluated in 12 fruits per genotype and storage time at 0, 7 and 14 days of cold storage. The final PCI index was the average of both parameters.

Ethylene production was determined at 0, 7 and 14 days of cold storage. Twelve fruits were analyzed for each time and storage temperature, i.e. four replicates of three fruits each. Once removed from storage chambers, fruit was enclosed in sealed 10-l containers for 6 h at 20 °C. After this incubation period, gas samples were taken and ethylene content was determined three times by gas chromatography in a Varian 3900 GC fitted with a flame ionization detector (FID). Ethylene production was expressed as $\mathrm{nl\ g^{-1}\ FW\ 6\ h^{-1}}$.

Statistical analysis

In order to evaluate the differences in postharvest fruit quality parameters and ethylene production among accessions and hybrids, and between plants in the segregating populations, the means and standard deviation of each parameter were calculated. Dependence between variables was studied by regression. A formal genetic analysis followed by a v^2 test was used to study the inheritance of PCI tolerance in CpCAL003. Differences between genotypes and between groups in the segregating populations were determined by analysis of variance (ANOVA), followed by least significant difference test (LSD) with significance level at P B 0.05. ANOVA was performed using the statistical software Statgraphic Centurion XVI (STATGRAPHICS. Statpoint Technologies, Inc., Warrenton, VA). Normality of distribution was verified by the Kolmogorov–Smirnov test.

Results

Screening for postharvest cold tolerance in *C. pepo*

A collection of 80 accessions and five commercial hybrids of *C. pepo* were grown under the same conditions and screened for PCI tolerance. From this first screening, each accession's tolerance to PCI was scored as low, intermediate or high. The most promising accessions were selected for a second screening, evaluating two of the most important postharvest parameters: WL and PCI. Table 1 shows PCI and WL in the fruit of the 20 selected accessions and five commercial hybrids after 7 and 14 days of storage at 4 °C. Accessions whose fruit showed an average PCI of below 2 after 7 days of cold storage and below 3.5 at 14 days of storage were considered to have high tolerance (Table 1).

After 7 days of cold storage, the commercial hybrid 'Sinatra', which was used as a control of PCI sensitivity, showed one of the highest PCI indexes, while the hybrid 'Natura', used as a control of PCI tolerance, showed an intermediate PCI index. Some of the accessions in our trial (CpCAL056, CpCAL064, CpCAL051, CpCAL053 and CpCAL003) had a higher tolerance to PCI than 'Natura', demonstrating the existence of a source of PCI tolerance for breeding the current commercial varieties. At 14 days of cold storage it was difficult to establish differences among the different accessions, except for CpCAL003, which was the most PCI tolerant accession of those assayed (Table 1). After 14 days of cold storage at 4 °C, the fruit of most of the accessions showed a PCI index of over 3, indicating that more than 30% of the fruit's surface was damaged and that the lesions were moderate-to-severe; therefore the fruit had lost its market value. Only the accession CpCAL003 showed a PCI score of below 2 at 14 days of cold storage (Table 1). This was considered the most tolerant accession in our collection.

WL also differed between accessions (Table 1). After 7 days of cold storage at 4 °C, the percentage of WL ranged from 2.63% in CpCAL003 to 11.14% in 'CpCAL0072', and after 14 days at 4 °C the percentage of WL increased to 23% in the accession CpCAL005; once again the accession CpCAL003 showed the lowest value for the percentage of WL (7.17%). The fruit of 'Sinatra' and 'Natura' used as control, showed intermediates values for WL (8.2 and 5.8% at 7 days, and 14.9 and 9.7% at 14 days, respectively).

By using PCI and WL we have, therefore, identified different sources of cold tolerance in *C. pepo*. Three accessions, CpCAL003, CpCAL0051, CpCAL0053, could be used as a source of PCI tolerance in commercial breeding programs of summer squash.

Inheritance of the cold tolerance trait of CpCAL003

A formal genetic analysis was performed to determine the inheritance pattern of PCI tolerance in CpCAL003. The tolerant accession CpCAL003 was crossed with the PCI sensitive accessions CpCAL005, CpCAL97 and CpCAL112, and the F_1 offsprings evaluated for both PCI and WL (Table S1). The hybrids 'Sinatra' and 'Natura' were also used in the essay as commercial controls for PCI sensitivity and tolerance, respectively. After evaluating the three F_1 generations (Table S1) we selected the cross CpCAL003 9 CpCAL112 for producing the F_2, and BC1(P1) and BC1(P2), generations. To perform plant crossings female flowers were covered with paper bags the day before anthesis. The next day, the stigma of a female flower was lightly rubbed with stamens of a male flower in anthesis, and covered again with the paper bag for 2–3 days to avoid insect pollination.

Figures 1 and 2 show PCI and WL at 7 days of cold storage of F_1, F_2 and BC1(P1) and BC1(P2),progenies of the cross CpCAL003 9 CpCAL112. In the segregating populations [F_2, BC1(P1) and BC1(P2)] plants were evaluated individually, and then separated in different phenotypic classes to analyze the segregating distributions. Regarding PCI, the F_1 showed an intermediate average value between the two parental lines (Fig. 1), and in the continuous variation of the F_2 and BC1(P1), plants were found to havea lower PCI than not only the tolerant hybrid 'Natura', but also the tolerant parental line CpCAL003 (Fig. 1). These data suggest that PCI tolerance is a quantitative polygenic trait, but that it is likely to select plants having a higher PCI tolerance derived from the accession CpCAL003 (Fig. 1).

The inheritance of WL was very similar to that of PCI (Fig. 2). The F_1 had an intermediate average value between the two parental lines CpCAL003and CpCAL112, and in the segregating populations F_2, BC1(P1), and BC1(P2),plants showed a continuous segregation pattern as a quantitative polygenic trait (Fig. 2). Plants in the F_2 and BC1(P1) generations could be selected that have a lower WL than the commercial hybrid 'Natura' and the PCI tolerant accession CpCAL003 (Fig. 2).

Association between ethylene production and PCI

Summer squash is a non-climacteric fruit that hardly produces ethylene at harvest and at non-chilling storage temperature. Nevertheless, when stored under chilling and rewarmed at room temperature (RT), they produce a burst of ethylene that peaks at 7 days of cold storage and decreases to its original value after 14 days of storage (Fig. 3; Meg´ıas et al. 2014). Nineteen accessions studied from the first screening were used to establish the possible correlation between WL, PCI and ethylene production during the postharvest of summer squash stored at 4 °C (Fig. 3). Cold storage induced the production of ethylene in all of the accessions, although the level of ethylene production was markedly different among accessions (Fig. 3). After 7 days of cold storage at 4 °C, the fruit of those accessions that were more tolerant to PCI produced less ethylene than fruit of those accessions that were more sensitive to PCI (Fig. 3).

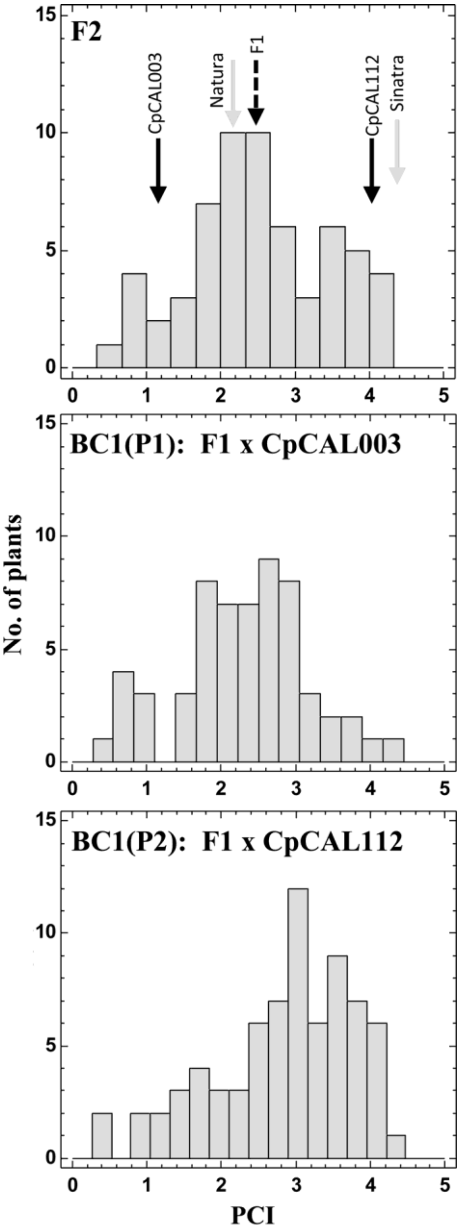

Fig. 1 Distribution of PCI in different segregating populations of the cross CpCAL003 9 CpCAL112. Evaluation was carried out on fruit stored 7 days at 4 °C from individual plants of the F_2, BC1(P1) and BC1(P2). Only those plants with at least five scored fruits were considered in the analysis. *Black arrows* indicate the means of the two parental lines, while the *dashed line arrow* indicates the mean of the F_1. The *grey arrows* represent the mean of the control hybrids 'Natura' and 'Sinatra', which were used as positive and negative control of PCI tolerance in zucchini

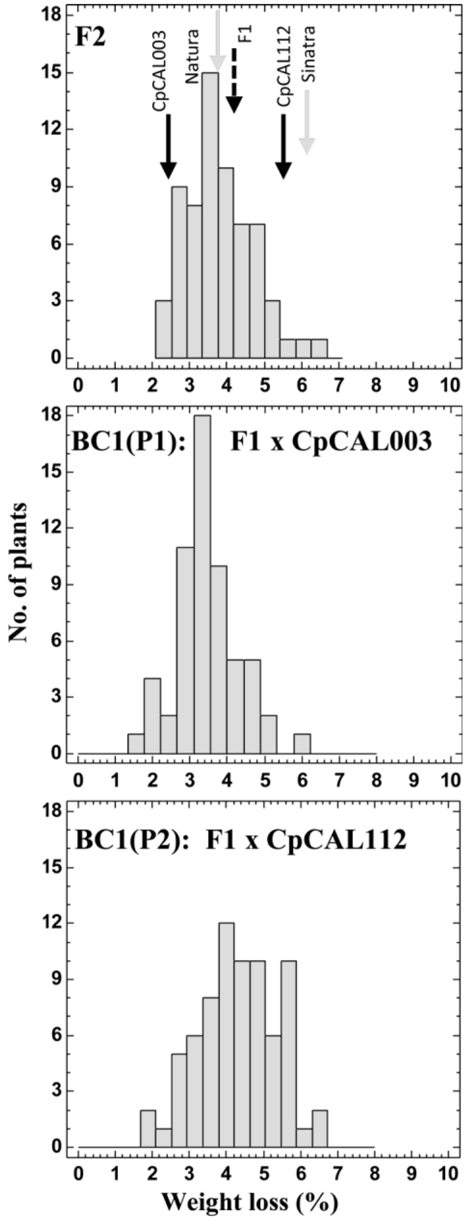

Fig. 2 Distribution of WL (%) in different segregating populations of the cross CpCAL003 9 CpCAL112. Evaluation was carried out on fruit stored 7 days at 4 °C from individual plants of the F_2, BC1(P1) and BC1(P2). Only those plants with at least five scored fruits were considered in the analysis. *Black arrows* represent the means of the two parental lines, while the *dashed arrow* represents the mean of the F_1. The *grey arrows* indicate the mean % WL of control hybrids ‘Natura’ and ‘Sinatra’ fruit

Regression analyses were performed between PCI, WL and cold-induced ethylene production (Fig. 4). As expected, WL was found to be correlated with PCI (r = 0.8016; P = 0.0000) in the different accessions analyzed (Fig. 4a). Moreover, a significant positive correlation was detected between the incidence of PCI and the production of ethylene after 7 days of cold storage at 4 °C (r = 0.8218; P = 0.0000) (Fig. 4c), as well as a positive correlation between ethylene production and WL (r = 0.7039; P = 0.0008) (Fig. 4b). Thus there is a highly significant correlation between the production of ethylene and occurrence of PCI during the postharvest storage of summer squash fruit.

To further determine whether low cold-induced ethylene production could serve as a reliable indicator for selection of PCI-tolerant summer squash, we have analyzed whether ethylene production in the refrigerated fruit was actually linked to PCI, we have analyzed the production of ethylene in the fruit from the more PCI sensitive and tolerant plants of the segregating populations F_2, BC1(P1) and BC1(P2),of the cross CpCAL003 9 CpCAL112 (Fig. 5). The segregating plants having the lowest and the highest scores for PCI were selected for ethylene production analyses. CpCAL003, the most tolerant accession to PCI showed the lowest ethylene production (11.63 nl g^{-1} 6 h^{-1}), even less than ‘Natura’, which was the most PCI tolerant control hybrid (Fig. 5). CpCAL112, on the other hand, produced much more ethylene (92.71 nl g^{-1} 6 h^{-1}), although less than the PCI sensitive control hybrid ‘Sinatra’ (Fig. 5). In the more PCI tolerant plants of the segregating populations [F_2, BC1(P1) and BC1(P2)], the fruit produced much less ethylene than in the more sensitive plants, indicating that the level of ethylene production cosegregates with PCI tolerance in the three analyzed populations (Fig. 5).

Discussion

Plant sensitivity to chilling temperatures produces important economic losses worldwide. Much research has focus on understanding the downstream plant response mechanisms to cold stress, as well as to establish the basis of cold tolerance in seedlings, crops and fruits (dos Reis et al. 2012; Miura and Furumoto 2013; Klay et al. 2014; Kosova´ et al. 2015). Past and current breeding programs pay little attention to postharvest cold tolerance, a trait of major importance for export-oriented fruits and vegetables requiring

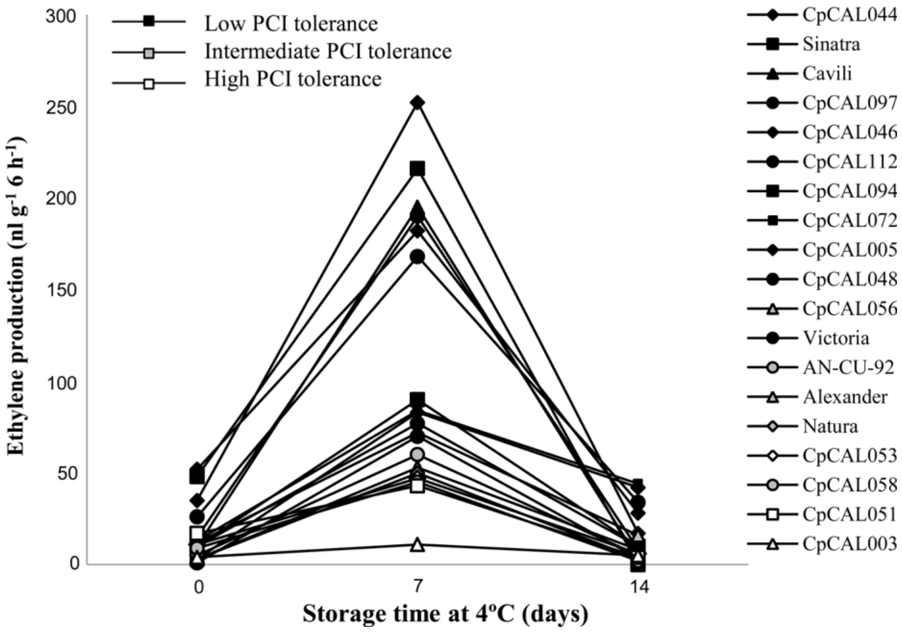

Fig. 3 Ethylene production in 19 accessions of *C. pepo* during 14 days of storage at 4 °C. Fruit was stored for 0, 7 and 14 days at 4 °C and then rewarmed at 20 °C for 6 h before ethylene production measurements. The results represent the mean of four independent replicates for each sample. Accessions were classified as having high, intermediate and low tolerance to PCI. Note that the accessions producing the least cold-induced ethylene are those that showed the highest PCI tolerance

refrigeration during transport and/or storage. Although cold storage preserves commodities during large storage periods, summer squash are very sensitive to PCI, which is expressed as pitting, water loss, and loss of turgor.

Screening for postharvest cold tolerance in *C. pepo*

Our data confirmed the high sensitivity of the immature summer squash fruit to low storage temperatures, which clearly reduced their commercial value in almost all analyzed accessions of *C. pepo* after few days of storage at 4 °C. WL and the surface PCI symptoms substantially decrease the fruit's marketability (Table 1). To search for new useful sources of PCI tolerance in squash, we have analyzed over than 80 accessions of *C. pepo*, mainly derived from the core collection of the COMAV germplasms bank at the Polytechnic University of Valencia, and the BSUAL germplasm bank at the University of Almería, along with previously analyzed commercial hybrids (Carvajal et al. 2011; Megías et al. 2014, 2015). The selection criterion for summer squash PCI tolerance was the ability to maintain postharvest quality for a longer time, without major impact by chilling injury, i.e. pitting and WL associated with fruit dehydration. Several accessions, including CpCAL0051, CpCAL0053 and CpCAL003, showed a higher PCI tolerance than previously detected in commercial hybrids such as 'Natura', which is very promising for breeding this trait in the current breeding programs of squash. As previously detected, the variability found in Spanish accessions was higher than in commercial varieties (Formisano et al. 2012; Martínez et al. 2014). The different origin of the identified PCI tolerant accessions (Andalusia, Catalonia and Morocco) suggests that they represent different sources of PCI tolerance in *C. pepo*. The introgression of the trait in the zucchini commercial hybrids will provide a long term solution for PCI.

Inheritance of the cold tolerance trait of CpCAL003

The genetic basis of summer squash PCI tolerance has been studied by analyzing major cold tolerance symptoms (pitting and WL) in the contrasting lines CpCAL003 and CpCAL112, as well as in their F_1, F_2 and BC progenies. The normal distributions of both PCI index and WL not only in the F_1, but also in the

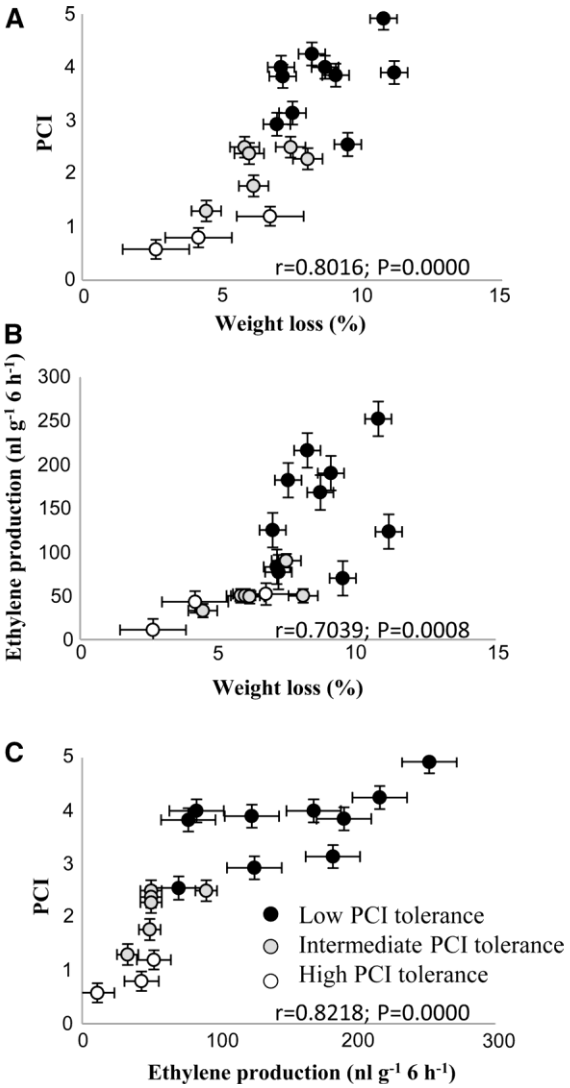

Fig. 4 Regression analyses for postharvest parameters in 19 accessions of *C. pepo*: PCI, weight loss (%) and cold-induced ethylene production (nl g^{-1} 6 h^{-1}) after 7 days of cold storage at 4 °C. a PCI versus weight loss. b Ethylene production versus weight loss. c PCI versus ethylene production

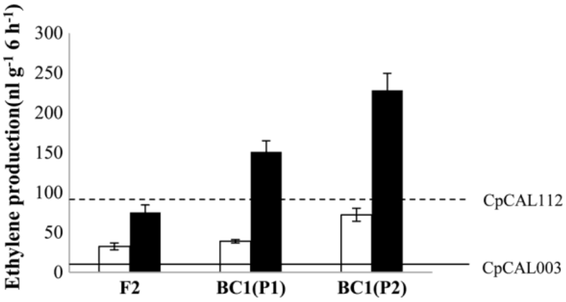

Fig. 5 Ethylene production in the F_2, and BC1(P1) and BC1(P2) from the cross CpCAL003 9 CpCAL112. Fruit was stored for 7 days at 4 °C and then rewarmed for 6 h at 20 °C before ethylene production measurements. Results represent the mean and standard deviation (*error bars*) of four independent replicates for each sample. In each segregating population, *black bars* represent ethylene production in PCI sensitive plants from the segregating populations (average PCI C 4 in at least five fruits/plant), and *white bars* represent ethylene production in PCI tolerant plants (average PCI B 2 in at least five fruits/plant). *Lines* indicate the average ethylene production of parental lines

segregating populations F_2 and BC1(P1) and BC1(P2), suggest that the PCI tolerance of CpCAL003 is a polygenic quantitative trait. Tolerance to other abiotic stresses, including tolerance to salt, drought, and cold was also found to have a polygenic control in other plant species (Vij and Tyagi 2007; Pérez-Clemente et al. 2013). In cucumber, another cucurbit fruit that is harvested at an immature stage of development, it has been reported that the response to PCI is controlled by cytoplasmic and nuclear factors (Chung et al. 2003; Gordon and Staub 2011), in such a way that PCI tolerance is dominant and regulated by paternal genetic factors (Ali et al. 2014). Similar results were reported for fruit quality and chilling tolerance in peach (Cant´ın et al. 2010).

Cold tolerance is already known to be a very complex trait controlled by different genetic and environmental factors (Sanghera et al. 2011). Although the number and the nature of the genes involved is not well known, the network includes transcription factors and regulatory genes that control multiple defense enzymes and proteins pathways (Cushman and Bohnert 2000). A recent study has revealed that the response to PCI in peach is regulated by ABA, auxins, and ethylene (Pons et al. 2014). Different activation patterns of these hormones could be key factors involved in cold tolerance (Pons et al. 2014). In zucchini squash it has been reported that cold tolerance, i.e. a reduction of visible PCI symptoms on the fruit surface and the loss of weight and firmness during the storage period at 4 °C, was accompanied by a reduction in fruit respiration rate, ethylene production and ethylene biosynthesis genes *CpACS1* and *CpACO1* (Meg´ıas et al. 2014, 2015, 2016), an inhibition of oxidative stress and oxidative damage processes (Carvajal et al. 2011; Meg´ıas et al. 2015), and a decrease in the cell wall degradation process (Carvajal et al. 2015). The chilling tolerance induced by PA in zucchini fruit was also associated with a reduction in the respiration rate and ethylene production in the fruit (Martínez-Tellez et al. 2002; Palma et al. 2015). Taken together all these data suggest the existence of multiple molecular mechanisms in the regulation of chilling tolerance in the immature fruit of squash.

Association between ethylene production and PCI

In this paper we have confirmed that the deterioration of the refrigerated summer squash fruit is associated with a peak of ethylene production at 7 days of cold storage (Megías et al. 2014, 2016). This cold-induced ethylene is only produced after the fruit is stored for 7 days at 4 °C and then rewarmed for several hours at RT (Megías et al. 2014). To determine whether this cold-induced ethylene is associated with PCI symptoms we have studied the correlation between ethylene production in fruit stored for 7 days at 4 °C and fruit WL and pitting in 19 accessions of *C. pepo* with different levels of PCI tolerance. The results clearly show that there is a significant positive correlation between ethylene production and the development of PCI. The varieties that show more PCI in the fruit are those that produce more ethylene while those that are more tolerant produce much less ethylene. Moreover, the correlation between PCI and cold-induced ethylene is also maintained in plants of the segregating populations (BCs and F_2), which demonstrates a high degree of co-inheritance between PCI symptoms and ethylene production in summer squash. Since ethylene explosion is produced after rewarming, and therefore after the onset of visible pitting symptoms, we have previously reported that ethylene is produced in the fruit as a consequence of chilling damage (Megías et al. 2014). However, we have also reported that the inhibition of ethylene response by 1-MCP is able to reduce PCI symptoms in several zucchini cultivars, demonstrating that ethylene may also play an active role in the onset of visible pitting symptoms as well as in cold-induced fruit dehydration (Megías et al. 2016). The association between cold-induced ethylene and the occurrence of PCI can be used as a criterion to select cold-tolerant genotypes and individual segregating plants in squash breeding programs. The fruits producing the lowest level of ethylene during storage at 4 °C for 7 days are those derived from the most tolerant plants and genotypes.

Our data are consistent with previous reports indicating that ethylene production is negatively correlated with cold tolerance in the model species *Arabidopsis thaliana* (Shi et al. 2012) and *Medicago truncatula* (Zhao et al. 2014), and also in other fruits and vegetables such as peach (Pons et al. 2014), grapefruit (Lado et al. 2015), loquat (Pareek et al. 2014), different genotypes of maize (Janowiak and Dorffling 1995), cucumber (Cabrera et al. 1992) and some *solanaceous* crops such as tomato and tobacco (Zhang and Huang 2010), among others. The relationship between ethylene production and the occurrence of PCI has been widely discussed in the literature, as has the issue of whether ethylene plays a role as an inducer of PCI or is the consequence of the stress generated by cold storage. Zou et al. (2014) has studied the relationship between ethylene related genes and the disorders caused by PCI in papaya, concluding that ethylene plays a fundamental role in PCI. The reduction of ethylene and PCI symptoms by using 1-MCP has confirmed the active role of this hormone for the development of PCI in other cucurbit crops such as melon (Alves et al. 2005) and cucumber (Chen et al. 2014). In zucchini squash the effect of 1-MCP is genotype-dependent for both the reduction of ethylene production in the fruit, associated with a down regulation of ethylene biosynthesis genes, and for the alleviation of PCI symptoms (Megías et al. 2016). Here we found that the cold-induced ethylene production was correlated with the two most important postharvest parameters, visible PCI symptoms and WL, indicating that ethylene is involved in the control of both pitting and fruit dehydration.

Acknowledgements This work was supported by Grants AGL2011-30568-C02/ALI and AGL2014-54598-C2-1-R from the Spanish Ministry of Science and Innovation, and P12-AGR-1423 from Junta de Andalucía, Spain. Z.M. acknowledges FPU program scholarships from MEC, Spain. S.M. is funded by Grant PTA2011-479-I from the Spanish Ministry of Science and Innovation.

References

Ali A, Bang SW, Yang EM, Chung Staub JE (2014) Putative paternal factors controlling chilling tolerance in Korean market-type cucumber (*Cucumis sativus* L.). Sci Hortic 167:145–148

Alves RE, Filgueiras HAC, Almeida AS, Machado FLC, Bastos MSR, Lima MAC, Terao D, Silva EO, Santos EC, Pereira

MEC, Miranda MRA (2005) Postharvest use of 1-MCP to extend storage life of melon in Brazil—current research status. Acta Hortic 682:2233–2238

Balandran-Quintana RR, Mendoza-Wilson AM, Gardea-Bejar AA, Vargas-Arispuro I, Mart´ınez-Tellez MA (2003) 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 307:553–557

Cabrera RM, Saltveit ME, Owens K (1992) Cucumber cultivars differ in their response to chilling temperatures. J Am Soc Hortic Sci 117:802–807

Cant´ın CM, Crisosto CH, Ogundiwin EA, Gradziel T, Torrents J, Moreno MA, Gogorcena Y (2010) Chilling injury susceptibility in an intra-specific peach [*Prunus persica* (L.) Batsch] progeny. Postharvest Biol Technol 58:79–87

Carvajal F, Martinez C, Jamilena M, Garrido D (2011) Differential response of zucchini varieties to low storage temperature. Sci Hortic 130:90–96

Carvajal F, Palma F, Jamilena M, Garrido D (2015) Preconditioning treatment induces chilling tolerance in zucchini fruit improving different physiological mechanisms against cold injury. Ann Appl Biol 166:340–354

Chen J, Zhao Y, Chen X, Peng Y, Hurr BM, Mao L (2014) The role of ethylene and calcium in programmed cell death of cold-stored cucumber fruit. J Food Biochem 38:337–344

Chung S-, Staub JE, Fazio G (2003) Inheritance of chilling injury: a maternally inherited trait in cucumber. J Am Soc Hortic Sci 128:526–530

Cushman JC, Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Curr Opin Plant Biol 3:117–124

dos Reis SP, Lima AM, de Souza CRB (2012) Recent molecular advances on downstream plant responses to abiotic stress. Int J Mol Sci 13:8628–8647

Formisano G, Roig C, Esteras C, Ercolano MR, Nuez F, Monforte AJ, Picó MB (2012) Genetic diversity of Spanish *Cucurbita pepo* landraces: an unexploited resource for summer squash breeding. Genet Resour Crop Evol 59:1169–1184

Gordon VS, Staub JE (2011) Comparative analysis of chilling response in cucumber through plastidic and nuclear genetic effects component analysis. J Am Soc Hortic Sci 136:256–264

Gordon VS, Staub JE (2014) Backcross introgression of plastomic factors controlling chilling tolerance into elite cucumber (*Cucumis sativus* L.) germplasm: early generation recovery of recurrent parent phenotype. Euphytica 195:217–234

Gualanduzzi S, Baraldi E, Braschi I, Carnevali F, Gessa CE, De Santis A (2009) Respiration, hydrogen peroxide levels and antioxidant enzyme activities during cold storage of zucchini squash fruit. Postharvest Biol Technol 52:16–23

Janowiak F, Dorffling K (1995) Chilling-induced changes in the contents of 1-aminocyclopropane-1-carboxylic acid (ACC) and its *N*-malonyl conjugate (MACC) in seedlings of two maize inbreds differing in chilling tolerance. J Plant Physiol 147:257–262

Klay I, Pirrello J, Riahi L, Bernadac A, Cherif A, Bouzayen M, Bouzid S (2014) Ethylene response factor Sl-ERF.B.3 is responsive to abiotic stresses and mediates salt and cold stress response regulation in tomato. Sci J 2014:167681

Kosová K, Vítámvás P, Urban MO, Klíma M, Roy A, Tom Prášil I (2015) Biological networks underlying abiotic stress tolerance in temperate crops-a proteomic perspective. Int J Mol Sci 16:20913–20942

Lado J, Rodrigo MJ, Cronje P, Zacar´ıas L (2015) Involvement of lycopene in the induction of tolerance to chilling injury in grapefruit. Postharvest Biol Technol 100:176–186

Mart´ınez C, Manzano S, Meg´ıas Z, Garrido D, Pico B, Jamilena M (2014) Sources of parthenocarpy for Zucchini breeding: relationship with ethylene production and sensitivity. Euphytica 200:349–362

Martínez-Téllez MA, Ramos-Clamont MG, Gardea AA, Vargas-Arispuro I (2002) Effect of infiltrated polyamines on polygalacturonase activity and chilling injury responses in zucchini squash (*Cucurbita pepo* L.). Biochem Biophys Res Commun 295:98–101

Massolo JF, Concellón A, Chaves AR, Vicente AR (2013) Use of 1-methylcyclopropene to complement refrigeration and ameliorate chilling injury symptoms in summer squash. CYTA J Food 11:19–26

Meg´ıas Z, Martinez C, Manzano S, Barrera A, Rosales R, Luis Valenzuela J, Garrido D, Jamilena M (2014) 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 57:194–199

Meg´ıas Z, Mart´ınez C, Manzano S, Garc´ıa A, Del Mar Rebolloso-Fuentes M, Garrido D, Valenzuela JL, Jamilena M (2015) Individual shrink wrapping of zucchini fruit improves postharvest chilling tolerance associated with a reduction in ethylene production and oxidative stress metabolites. PLoS ONE 10(7):e0133058

Meg´ıas Z, Mart´ınez C, Manzano S, Garc´ıa A, del Mar Rebolloso-Fuentes M, Valenzuela JL, Garrido D, Jamilena M (2016) 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 113:48–57

Miura K, Furumoto T (2013) Cold signaling and cold response in plants. Int J Mol Sci 14:5312–5337

Nee M (1990) The domestication of *Cucurbita*. Econ Bot 44(3 suppl):56–68

Palma F, Carvajal F, Jamilena M, Garrido D (2014) Contribution of polyamines and other related metabolites to the maintenance of zucchini fruit quality during cold storage. Plant Physiol Biochem 82:161–171

Palma F, Carvajal F, Ramos JM, Jamilena M, Garrido D (2015) 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 99:131–140

Pareek S, Benkeblia N, Janick J, Cao S, Yahia EM (2014) Postharvest physiology and technology of loquat (*Eriobotrya japonica* Lindl.) fruit. J Sci Food Agric 94:1495–1504

Paris H (1986) A proposed subspecific classification for *Cucurbita pepo*. Phytologia 61:133–138

Pech JC, Latche´ A (2013) Contribution of genomics to postharvest biology. Stewart Postharvest Rev 9(4):1–6

Pérez-Clemente RM, Vives V, Zandalinas SI, López-Climent MF, Muñoz V, Gómez-Cadenas A (2013) Biotechnological approaches to study plant responses to stress. BioMed Res Int 2013:654120

Pons C, Mart´ı C, Forment J, Crisosto CH, Dandekar AM, Granell A (2014) A bulk segregant gene expression analysis of a peach population reveals components of the underlying mechanism of the fruit cold response. PLoS ONE 9(3):e90706

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem 379:633–646

Sanghera GS, Wani SH, Hussain W, Singh NB (2011) Engineering cold stress tolerance in crop plants. Curr Genomics 12:30–43

Serrano M, Pretel MT, Martinez-Madrid MC, Romojaro F, Riquelme F (1998) CO_2 treatment of zucchini squash reduces chilling-induced physiological changes. J Agric Food Chem 46:2465–2468

Sevillano L, Sanchez-Ballesta MT, Romojaro F, Flores FB (2009) Physiological, hormonal and molecular mechanisms regulating chilling injury in horticultural species. Postharvest technologies applied to reduce its impact. J Sci Food Agric 89(4):555–573

Shi Y, Tian S, Hou L, Huang X, Zhang X, Guo H, Yang S (2012) Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-A ARR genes in *Arabidopsis*. Plant Cell 24:2578–2595

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiol 125:89–93

Vij S, Tyagi AK (2007) Emerging trends in the functional genomics of the abiotic stress response in crop plants. Plant Biotechnol J 5:361–380

Wang CY (1994) Combined treatment of heat shock and low temperature conditioning reduces chilling injury in zucchini squash. Postharvest Biol Technol 4:65–73

Wang CY (1995) Effect of temperature preconditioning on catalase, peroxidase, and superoxide-dismutase in chilled zucchini squash. Postharvest Biol Technol 5:67–76

Wang CY, Ji ZL (1989) Effect of low-oxygen storage on chilling injury and polyamines in zucchini squash. Sci Hortic 39:1–7

Yun Z, Jin S, Ding Y, Wang Z, Gao H, Pan Z, Xu J, Cheng Y, Deng X (2012) Comparative transcriptomic and proteomics analysis of citrus fruit, to improve understanding of the effect of low temperature on maintaining fruit quality during length post-harvest storage. J Exp Bot 63(8):2873–2893

Zhang Z, Huang R (2010) Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor TERF2/LeERF2 is modulated by ethylene biosynthesis. Plant Mol Biol 73:241–249

Zhao M, Liu W, Xia X, Wang T, Zhang W- (2014) Cold acclimation-induced freezing tolerance of *Medicago truncatula* seedlings is negatively regulated by ethylene. Physiol Plant 152:115–129

Zheng Y, Fung RWM, Wang SY, Wang CY (2008) Transcript levels of antioxidative genes and oxygen radical scavenging enzyme activities in chilled zucchini squash in response to superatmospheric oxygen. Postharvest Biol Technol 47:151–158

Zou Y, Zhang L, Rao S, Zhu X, Ye L, Chen W, Li X (2014) The relationship between the expression of ethylene-related genes and papaya fruit ripening disorder caused by chilling injury. PLoS ONE 9:e116002