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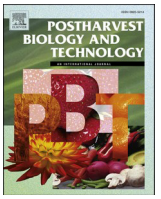

# Postharvest abscisic acid treatment modulates the primary metabolism and the biosynthesis of t-zeatin and riboflavin in zucchini fruit exposed to chilling stress

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ABSTRACT

Abscisic acid (ABA) plays an important role in the regulation of several stress responses such as drought, high salinity and low temperature being also proved as a key phytohormone for the acquisition of postharvest cold tolerance in zucchini fruit. Therefore, it would be of great interest to unravel the mechanisms implicated in the ABA response, using a metabolomic approach. The aim of this work has been to use a combination of metabolomic tools to identify the main metabolic pathways involved in ABA-mediated regulation of chilling tolerance in zucchini fruit. As a result of this study, it was found that ABA modulates the primary metabolism inducing the accumulation of some sugars, organic acids such as succinic acid and amino acids including histidine, serine, phenylalanine, glutamic acid and γ-aminobutyric acid, and that are involved in low-temperature tolerance. ABA treatment also activates the t-zeatin and riboflavin biosynthesis during the first days of cold storage which can be important signals in the ABA-mediated cold response to induce tolerance in zucchini fruit.

## 1. Introduction

An increasing consumption of fresh fruit and vegetables around the world has given rise to a searching for improved storage and transport conditions, which is crucial to maintain the quality of fruit throughout the postharvest period (Brummell and Harpster, 2001). Understanding how the quality maintenance of fruit and vegetables occurs, along postharvest period, is the first step to avoid the deterioration of the commodities. Storing at low temperatures is a widely used technique to maintain fruit quality (Kader, 2003). However, when fruit from tropical or subtropical origin, such as zucchini fruit (*Cucurbita pepo*), are stored at low, but not freezing temperatures, a physiological disorder called chilling injury (CI) appears and lead to changes in the cell wall and epicuticular wax composition (Carvajal et al., 2021; Carvajal et al., 2015a). In zucchini fruit during postharvest cold storage, the antioxidant defense plays a main role in fruit quality maintenance; this defense includes enzymatic and non-enzymatic systems (Carvajal et al., 2015b; Castro-Cegrí et al., 2023b; Palma et al., 2014b, 2016). Different treatments have been carried out to reduce CI in zucchini fruit, including physical treatments such as temperature preconditioning (Carvajal et al., 2015b) or edible coatings (Castro-Cegrí et al., 2023a), and the application of abscisic acid, putrescine, γ-aminobutyric acid, and nitric oxide (Carvajal et al., 2017; Castro-Cegrí et al., 2023b; Jiménez-Muñoz et al., 2021; Palma et al., 2019, 2015).

Abscisic acid (ABA) plays an important role in the regulation of several stress responses such as drought, salinity, and low temperature (Sah et al., 2016). It has been described that ABA alleviates CI in cold-stored fruit, activating the antioxidant enzymatic pathway and inducing the accumulation of osmoprotectants, such as soluble sugars (Tang et al., 2022; Zhao et al., 2022). Specifically in zucchini fruit, ABA has been proved as a key molecule for the acquisition of postharvest cold tolerance (Benítez et al., 2022; Carvajal et al., 2017; Castro-Cegrí et al., 2023b). Findings reported in these papers have shown that during postharvest cold storage in zucchini fruit ABA induces the expression of genes responsible for the biosynthesis of epicuticular waxes, as well as the synthesis of non-enzymatic antioxidant compounds. Having in mind

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that ABA is implicated in the acquisition of chilling resistance in zucchini, it is of utmost interest to unravel the metabolic changes that take place in the fruit during cold postharvest due to the application of this phytohormone.

Comparing tolerant against sensitive varieties of zucchini fruit in previous studies has been described the importance of several pathways of primary metabolism against cold stress (Palma et al., 2014a, 2014b), thus could be of a great interest to study the ABA role in the regulation of primary and secondary metabolism in fruit.

In summary, the aim of this work has been firstly the use of a metabolomic approach to characterize compositional changes of known polar primary metabolites in zucchini fruit during cold postharvest induced after an ABA treatment. Nevertheless, a wide variety of unknown metabolites could be potentially involved in the chilling tolerance induced by ABA in zucchini fruit, thus was also important to identify key secondary metabolic pathways for chilling tolerance mediated by ABA-treatment in zucchini fruit, as are t-zeatin biosynthesis, related with changes in cuticle composition (Gutiérrez et al., 2021), and riboflavin (vitamin B2) metabolism, important in the regulation of abiotic stress (Deng et al., 2014) through activating phenylpropanoid pathway (Taheri and Tarighi, 2011).

## 2. Material and methods

### 2.1. Fruit material, treatments, and storage conditions

Zucchini fruit (*Cucurbita pepo* L. morphotype Zucchini) of the cultivar 'Sinatra' (Clause-Tezier) was provided by the company Fruits & Vegetables La ÑECA S.A.T. Healthy and uniform fruit were separated into three replicates of 6 fruits each one, per treatment and storage period. Freshly-harvested fruit of 'Sinatra' were submerged at 20 ºC for 20 min in 0.5 mM of ABA or distilled water as the control, all fruit were then placed on a desiccant paper in trays and allowed to dry during 2 h, subsequently the replicates were stored in a temperature-controlled chamber and in permanent darkness at 4 ºC and 85–90% relative humidity (RH) for 1 d, 5 d and 14 d. The exocarp of each fruit was removed, mixed per replicate, frozen, powdered in liquid nitrogen and stored at − 80 ºC.

### 2.2. Weight loss, chilling-injury index and electrolyte leakage (%)

The percentage of weight loss of each fruit was calculated using the following formula: % weight loss = (Wi − Wf)/Wi × 100, being Wi the initial fruit weight and Wf the final fruit weight.

Chilling injury index (CI) was evaluated using a subjective scale of visual symptoms described by Carvajal et al. (2011). Each fruit was rated according to the following scale: 0, no pitting; 1, slight (10% or less of pitting in fruit surface); 2, medium (10–20% of pitting in fruit surface); and 3, severe pitting (>20% of pitting in fruit surface).

Electrolyte leakage was determined in the exocarp of fruit, 10 discs were taken from each replicate with an 11 mm diameter stainless-steel cork borer and 1–1.5 mm height. Five replicates from each treatment were measured. Each replicate was rinsed with 50 mL of deionized water three times for 3 min. After being incubated for 30 min and shaken at 100 rpm in 50 mL of deionized water, this solution was measured for conductivity at room temperature using a conductimeter. Total conductivity was determined after boiling the flasks for 10 min and cooling at room temperature. The electrolyte leakage was expressed as percentage of total conductivity.

### 2.3. Profiling primary metabolites by GC-TOF-MS

Polar primary metabolites were extracted and derivatized following the protocol of Lisec et al. (2006). These metabolites were measured by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) as described in Osorio et al. (2012).

Quantities of metabolites are expressed as relative intensity, based on peak integration, and relative to the internal standard. Data were normalized and scaled for principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) using MetaboAnalyst 5.0.

### 2.4. Untargeted metabolic profiling by UPLC-QTOF/ESI-MS

For each sample 500 mg of powdered exocarp were extracted with 3 mL MeOH/$H_2O$ (80/20, v/v). The mixture was shaken for 1 h at 4 ºC, centrifuged at 10000 xg for 15 min at 4 ºC. The supernatant was diluted with $H_2O$ (1/3, v/v), filtered with 0.22 μm nylon filter and transferred into vials. The untargeted metabolic profiling was performed using Ultra High-Performance Liquid Chromatography coupled with Mass Spectrometer-Ion Mobility-Time of Flight UPLC-IM-QToF-MS (Acquity H-Class quaternary flow solvent manager with Synapt G2, Waters Co.). The mass spectrometer was operated in both negative and positive ionization modes, the acquisition parameters for both modes were: capillary voltage 3 kV; cone gas flow 30 L $h^{-1}$, desolvation gas flow 800 L $h^{-1}$; source temperature 100 ºC, using an Acquity UPLC class system for solvent delivery and sample introduction. Each sample was injected into a column (Acquity UPLC BEH C18 1.7 μm, 2.1 mm × 50 mm). The injection volume of each sample was 10 μl with autosampler, the solvent flow was 0.3 mL $min^{-1}$ with a run time of 22 min, and the elution gradient was prepared with eluent A (water) and eluent B (acetonitrile). The gradient profile was applied as follow (t (min); % A): (0; 90%), (1.5; 90%), (16; 10%), (16.1; 0%), (19; 0%), (19.1; 10%).

The mass chromatographic raw data were visualised and processed using MarkerLynxXS™software (Waters, Manchester, UK). Parameters set to analyse ESI (+) mode data were: retention time (Rt) range of 1–20 min of the MS chromatogram, mass range of 50–1200 Da, Rt window of 0.01 min and mass tolerance of 0.008 Da. Peak detection and alignment were achieved using the MarkerLynx patented ApexPeakTrack algorithm. Data normalisation was performed using total ion intensities of each defined peak, if a peak was not detected in a sample the matrix was filled with a zero, and a supervised orthogonal partial least squared discriminant anaylsis (OPLS-DA) was done using the MarkerLynx software. S-plot from PLS-DA dataset was done to visualize the correlation matrix, pairwise metabolite correlations were calculated by Pearson's correlation coefficient ($r_{ij}$), and the level of significance was set as $r \geq 0.85$. The accurate mass, fragmentation patterns, elemental composition, Rt, and online database searches were used to facilitate the allocation of the correct chemical structure to each selected feature. Online databases used for metabolite identification were KEGG (e), ChemSpider (e), PlantCyc(e) and MetaCyc (e). To realise a pathway mapping, the Metabolomic Pathway Analysis (MetPA) tool, a component of MetaboAnalyst 5.0, was used.

### 2.5. Riboflavin, FMN and FAD determination

Riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were extracted using 0.3 g of exocarp grounded in liquid nitrogen and 1.2 mL of cold extraction medium (ethanol/chloroform/water) (12/5/1; v/v/v). The homogenate was centrifuged at 12000g and 4 ºC during 10 min, then 1 mL of chloroform and 0.5 mL of water were added to separate into aqueous and chloroform phases. Riboflavin, FMN and FAD were determined from aqueous phase, which was dried under nitrogen flow, resuspended in 0.6 mL of ammonium acetate 2.5 mM pH 6 and filtered with nylon filter (0.22 μm).

The chromatographic analysis was based on the method proposed by (Sunarić et al., 2020), but with important modifications for adapting to the tissue used. Riboflavin, FMN and FAD were analysed by HPLC using an Agilent 1260 Infinity system equipped with a Zorbax Eclipse Plus C18 (4.6 mm × 150 mm, 5 μm). Solvent flow was 1 mL $min^{-1}$, and the elution gradient was prepared with eluent A (ammonium acetate 2.5 mM pH 6) and eluent B (acetonitrile). 0.05 mL of each sample were

injected and the gradient profile was applied as follow (t (min); % A): (0; 90%), (1.5; 70%), (6.5; 70%), (7.5; 0%), (13; 0%) and (14; 90%), having FAD, FMN and riboflavin a retention time of 2.32, 3.24 and 3.65 min respectively. Detection was done with a fluorometer using excitation and emission wavelengths of 440 nm and 520 nm, respectively.

### 2.6. ABA, t-Zeatin and t-zeatin riboside determination

Abscisic acid (ABA), t-Zeatin (t-Z) and t-Zeatin Riboside (t-ZR) were quantified utilizing a selective hormone extraction method, which consisted of 0.5 g of liquid nitrogen grounded exocarp and 1 mL of cold extraction method (Isopropanol/HCl 0.1 N) (10/5; v/v), and then it was shaken during 1 h at 4 ºC. The extract was centrifuged at 12000g and 4 ºC for 10 min, then 0.5 mL of dichloromethane was added to each sample and strongly shaken, and the organic phase was dried under nitrogen flow and stored at − 20 ºC under an inert atmosphere.

An Acquity UPLC class system was used for solvent delivery and sample introduction. Each sample was injected into a column (Acquity UPLC BEH C18 1.7 μm, 2.1 mm × 50 mm). Solvent flow was 0.5 mL $min^{-1}$ and the elution gradient was prepared with eluent A (0.1% formic acid) and eluent B (acetonitrile plus 0.1% formic acid). 0.001 mL of each sample were injected and the gradient profile was applied as follow (t (min); % A): (0; 90%), (6; 5%), (8; 5%), (8.1; 90%), having t-ZR, t-Z and ABA a retention time of 0.72, 1.45 and 3.30 min respectively.

Eluates were detected using a Xevo TQ-S triple quadrupole mass spectrometer (Waters) in the positive electrospray ionization (ESI) mode. The ion spray voltage was set at 2500 V, the source temperature at 100 ºC and desolvation temperature 500 ºC.

Abscisic acid, t-Zeatin and t-Zeatin Riboside were detected in the multiple reaction monitoring mode of the tandem mass spectrometer with the following transitions: *abscisic acid, m/z* 263.10 → 153.00; *t-zeatin, m/z* 220.08 → 135.73; *t-zeatin riboside m/z* 352,31 → 135,69.

### 2.7. Statistical analysis of quantified compounds

The experiments were totally randomized. The statistical analysis was performed by ANOVA using the SPSS 26.0 program (SPSS Inc.). Means were compared by Duncan's least significant differences test ($p < 0.05$).

## 3. Results

ABA treatment maintains zucchini fruit quality during cold storage, diminishing the percentage of weight loss and the CI after 14 d of cold storage, and also reducing the percentage of electrolyte leakage by around 30–40% as it was observed in Table 1.

### 3.1. Profiling primary metabolites

A total of 57 polar primary metabolites were detected, including 25 amino acids, 16 sugars and sugar derivates, and 10 organic acids. Principal component analysis (PCA) was assessed to explore the changes in primary metabolites induced by the application of ABA before the cold storage. PCA scores revealed a total described variation of 47% considering overall PC 1 and PC 2 (Fig. 1A). ABA-treated fruit appeared clearly separated from control fruit after 1 d and 5 d of cold storage, whereas after 14 d both treated and non-treated fruit grouped together. Except for the 14 d samples, ABA-treated fruit were located in the left half part of the graph, pointing the importance of the PC1 in their separation. The PCA loadings plot allowed discriminating which metabolites contributed to the clustering (Fig. 1B). In the upper left quadrant we found the metabolites related with the separation of the ABA-treated fruit after 1 d at 4 ºC, among them homoserine, glycine, citric acid and glycerol 3-phosphate. The metabolites displayed in the lower left quadrant were more relevant in the fruit treated with ABA after 5 d of cold storage, such as ornithine, glutamic, pyruvic, and galacturonic acid, whereas putrescine, 4-hydroxyproline, tryptophane, γ-aminobutyric acid (GABA), and phosphate were situated in the lower right quadrant corresponding to zucchini metabolism after long-term cold storage.

To analyse the importance of the different group of polar primary metabolites in the metabolic profiling, independent PCA were conducted using the data corresponding to amino acids, sugars and their derivates as well as organic acids. Results showed that the amino acids group was the most important for the clustering of ABA-treated from non-treated fruit (Fig. S1A).

Partial least discriminant analysis (PLS-DA) was carried out using the measured metabolites as predictor variables, and the development of CI or the application of ABA as response variables. Using CI as response, the scores plot of the PLS-DA model clustered separated the injured from the non-injured samples and the percentage of variance explained using 2 components was around 45% (Fig. 2 A). Variable importance in projection (VIP) score shows the contribution of the predictor variables, the measured metabolites in this case, to the model. VIP score plot for the most important metabolites identified by PLS-DA is shown in Fig. 2B. Valine, phosphate, putrescine, 4-hydroxyproline, and GABA showed a positive correlation with the development of CI. On the contrary, glyceric acid, β-alanine, serine, aspartic acid, glycine, asparagine, homoserine, succinic acid, glycerol 3-phosphate, 3,4-dihydroxy-trans-cinnamate, citric acid, glucaric acid, o-methyl-α-mannopyranoside, citramalic acid and glycerol showed a higher content in non-injured fruit (Fig. 2B). Changes in these metabolites along the storage in control and ABA-treated fruit are summarized in Fig. S2.

The scores of the PLS-DA model using the ABA treatment as response variable separated most of the treated from the non-treated samples, except for those corresponding to 14 d of storage at 4 ºC (Fig. 2 C). This behaviour is due to the similarities found in the primary metabolite profile presented by these samples, as showed in the PCA scores plot (Fig. 1A). The percentage of explained variance using two components was in this case around 43%. Some compounds important for the PLS-DA model positively related with the application of ABA were histidine, o-acetylserine, phenylalanine, glutamic acid, tyramine, ornithine, maltotriose, fucose, and urea. Other metabolites that also present a positive correlation with ABA treatment and a higher content in non-damaged fruit in the the PLS-DA model built using CI as response were o-methyl-α-mannopyranoside, glycerol, succinic, glucaric and glyeric acids, and the amino acids serine, asparagine and β-alanine (Fig. 2D). On the contrary, the amino acids glutamine and valine presented negative correlation with the ABA treatment. Valine also presented a high VIP score in the PLS-DA model positively correlated with the appearance of CI. The changes in the levels of these metabolites

**Table 1**
Changes in weight loss (%), chilling injury index and electrolyte leakage (%) due to ABA treatment throughout cold storage period.

| Days of storing at 4 °C | 0 | 1 | | 5 | | 14 | |
|---|---|---|---|---|---|---|---|
| | At harvest | Control | ABA | Control | ABA | Control | ABA |
| Weight loss (%) | $0 \pm 0^{f}$ | $1.38 \pm 0.1^{e}$ | $1.48 \pm 0.06^{e}$ | $4.06 \pm 0.1^{c}$ | $3.37 \pm 0.08^{d}$ | $11.99 \pm 0.4^{a}$ | $8.39 \pm 0.7^{b}$ |
| Chilling Injury | $0 \pm 0^{c}$ | $0 \pm 0^{c}$ | $0 \pm 0^{c}$ | $0.18 \pm 0.06^{c}$ | $0.02 \pm 0.02^{c}$ | $2.23 \pm 0.2^{a}$ | $0.69 \pm 0.16^{b}$ |
| Electrolyte leakage (%) | $6.45 \pm 0.2^{e}$ | $10.13 \pm 0.4^{c}$ | $7.57 \pm 0.15^{d}$ | $11.22 \pm 0.25^{b}$ | $7.98 \pm 0.06^{d}$ | $14.74 \pm 0.3^{a}$ | $11.28 \pm 0.5^{b}$ |

Data presented are means ± SD of triplicate samples of six fruit each. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

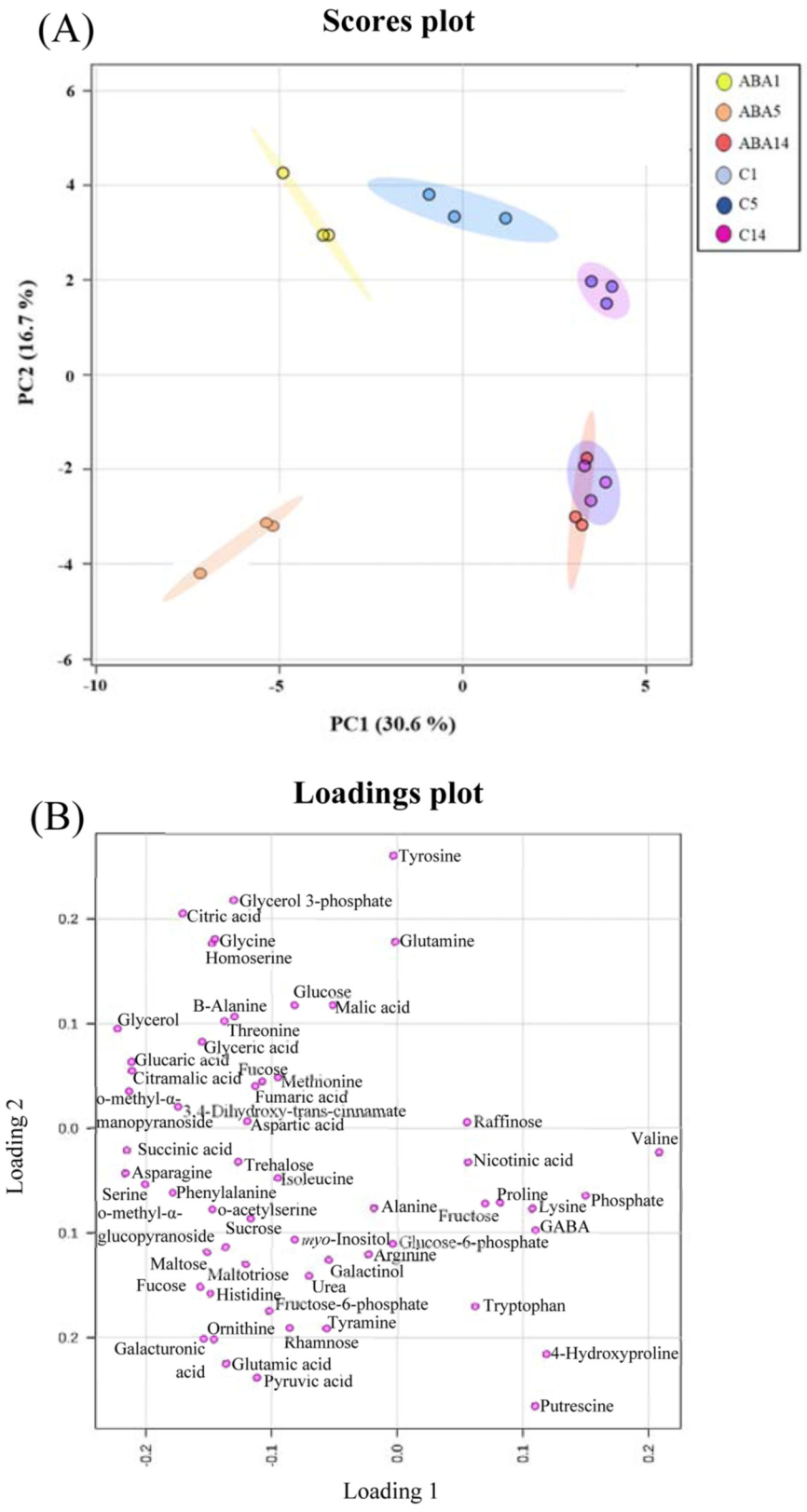


**Fig. 1. :** Principal component analysis (PCA) of the effect of ABA treatment on primary metabolites in the exocarp of zucchini fruit during postharvest cold storage. (A) PCA scores plot. Values in parentheses are the percentage of variance explained by each principal component (PC). (B) PCA loadings plot. Three biological replicates (6 fruit each) were used (n = 3).

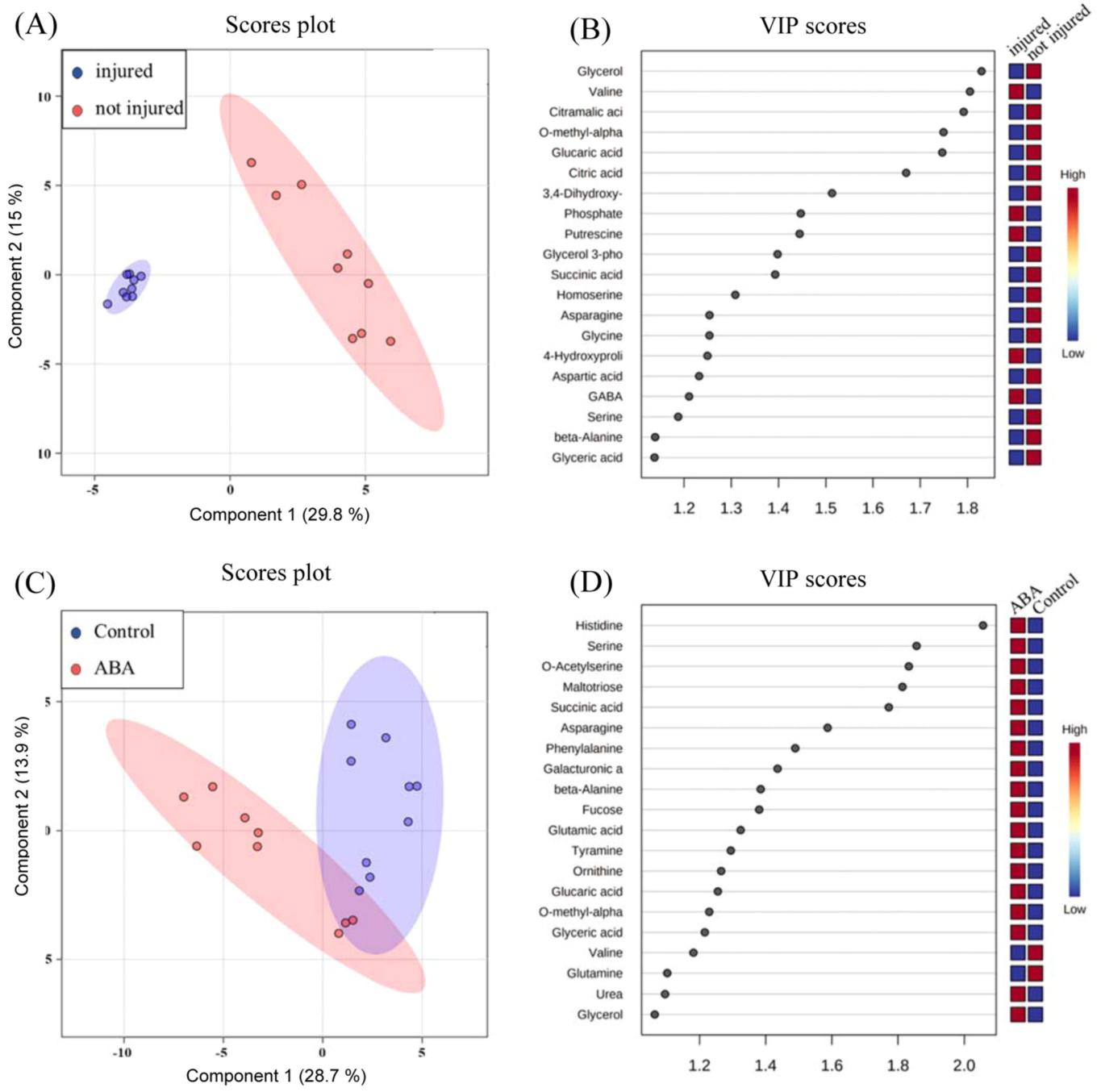


**Fig. 2. :** Partial least square discriminant analysis (PLS-DA) of development of CI or the application of exogenous ABA in the exocarp of zucchini fruit during postharvest cold storage. (A) PLSDA scores plot of the model built using CI as variable response. (B) Variable importance in projections (VIP) scores of top metabolites identified by PLS-DA model for CI. (C) PLS-DA scores plot of the model built using ABA treatment as variable response. (D) VIP scores of top metabolites identified by PLS-DA model for ABA treatment. Values in parentheses are the percentage of variance explained by each component. Three biological replicates (6 fruit each) were used (n = 3), for Control and ABA-treatment at 1, 5 and 14 days of cold storing.

during the cold storage are represented in Fig. S3.

### 3.2. Untargeted metabolic profiling

Initial UPLC-IM-QToF-MS analysis from all the samples detected a total of 3990 features. However, 186 discriminant ions were only selected by Pearson's correlation coefficient ($r \geq 0.85$) for metabolite identification. Data processing allowed us to putatively identify 78 metabolites that were modified by ABA treatment. An accurate description of these metabolites was listed in table S2, including *m/z*, fragmentation patterns information and KEGG IDs. In order to clarify the connection between these compounds a pathway mapping was performed.

### 3.3. Pathway mapping

A Metabolomic Pathway Analysis (MetPA) was used to unravel the implication of ABA in the modification of several pathways associated with the putatively identified metabolites along cold storage. The 10 pathways that were more altered by ABA treatment were listed in Table 2, being zeatin biosynthesis, riboflavin metabolism and pyrimidine metabolism the most important pathways with $p < 0.05$ and impact

**Table 2**
List of the 10 metabolic pathways most altered by the application of exogenous ABA to zucchini fruit along cold storage as generated by Metabolic Pathway Analysis (MetPA). 'Match status' indicates the number of metabolites found in each particular pathway. The '*p*' represents *p*-value calculated from the enrichment analysis, '-log(*p*)' shows the negative logarithm of *p*-value and finally the 'Impact' represent the importance of each pathway calculated from the pathway topology analysis.

| Pathway Name | Match status | *p* | -log (*p*) | Impact |
|---|---|---|---|---|
| Zeatin biosynthesis | 6/21 | 5.78E-4 | 3.238 | 0.336 |
| Riboflavin metabolism | 3/11 | 0.018 | 1.740 | 0.252 |
| Other antibiotics | 2/6 | 0.037 | 1.423 | 0.5 |
| Pyrimidine metabolism | 5/38 | 0.050 | 1.303 | 0.225 |
| Pentose phosphate pathway | 3/19 | 0.079 | 1.105 | 0.002 |
| Betalain biosynthesis | 1/3 | 0.153 | 0.814 | 1 |
| Tryptophan metabolism | 3/28 | 0.189 | 0.723 | 0.148 |
| Isoquinoline alkaloid biosynthesis | 1/6 | 0.283 | 0.547 | 0 |
| Fructose and mannose metabolism | 2/20 | 0.294 | 0.532 | 0.202 |
| Carbon fixation in photosynthetic organisms | 2/21 | 0.314 | 0.502 | 0.031 |

> 0.2. This tool mapped the metabolites and presented graphically the data in Fig. 3, observing the metabolic pathways associated to our untargeted analysis, on the y-axis is represented -log10 (*p*) associated to p-value calculated from the enrichment analysis, and the impact values on the x-axis (Fig. 3A). Zeatin biosynthesis resulted as the most significantly altered pathway, also presenting a high pathway impact due to the finding of six compounds differently accumulated (Fig. 3B), riboflavin metabolism was also important, because three compounds of this pathway were found (Fig. 3C). Subsequently, to corroborate the results obtained in the pathway mapping, endogenous ABA and the main metabolites of both pathways were quantified.

### 3.4. Compound quantification from untargeted profiling metabolomics

Abscisic acid (ABA), t-zeatin, t-zeatin riboside, riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were quantified at all times of experimentation. As expected, the addition of ABA increased the endogenous ABA content in the exocarp of treated fruit, observing the largest differences at 1 d of cold storage. At the same time, t-zeatin and riboflavin content also increased with ABA application, both compounds almost doubled their content respect to untreated fruit (Figs. 4B and 5A). ABA-treated fruit also showed more content in riboflavin than control fruit at 5 d of cold storage (Fig. 5A). ABA treatment diminished the t-zeatin riboside content at 1 d and 5 d of cold stress compared to freshly harvested fruit (Fig. 4C). However, there were not significant differences after 1 d of low-temperature storage between control fruit and freshly harvested fruit. Results obtained showed a rise in FMN amount in untreated fruit respect to freshly harvested fruit at 1 d of cold storage, whereas ABA-treated fruit maintained FMN levels as at harvest (Fig. 5B). In the case of FAD, no significant changes were detected throughout the experimentation time (Fig. 5C).

## 4. Discussion

Untargeted and targeted metabolomic approaches have been carried out throughout postharvest period in several crops such as blackberries (Kim et al., 2019), bananas (Yuan et al., 2017), and some Cucurbitaceae's like pumpkin (Huang et al., 2019) or zucchini (Abreu et al., 2018). ABA has been proved as a key molecule in cold tolerance of zucchini fruit (Carvajal et al., 2017), producing important transcriptomic changes that involve regulation by signalling components, specific transcription factors or phytohormones (Benítez et al., 2022), possibly implicated in the production of bioactive compounds with antioxidant capacity such as carotenoids, phenolic compounds or ascorbate, increasing the nutritional value and the postharvest cold tolerance of zucchini fruit (Castro-Cegrí et al., 2023b). Unravelling the changes in the metabolome of zucchini fruit along cold storage due to ABA would contribute to select markers of tolerance to low temperature. Thus, in this work, metabolomic approaches were carried out for elucidating the role of ABA in the regulation of metabolic pathways involved in chilling tolerance of zucchini fruit.

Primary metabolism analysis revealed that application of ABA induced important metabolic changes in the zucchini fruit during the first days of cold storage, as pointed out by PCA analysis (Fig. 1). However, no differences were found in primary metabolites after 14 d at low temperature. These results indicate that the defense mechanisms triggered by ABA take place at short-term storage and, subsequently, primary metabolism in treated fruit changes, resembling that of the non-treated ones.

Accumulation of amino acids has been described as a defense mechanism under several stress conditions and specifically during postharvest management of fruit (Bang et al., 2019; Brizzolara et al., 2018). In our study, we found through PLS-DA analysis an important number of amino acids related with lower incidence of CI and the application of ABA (Fig. 2). Histidine accumulation has been reported in

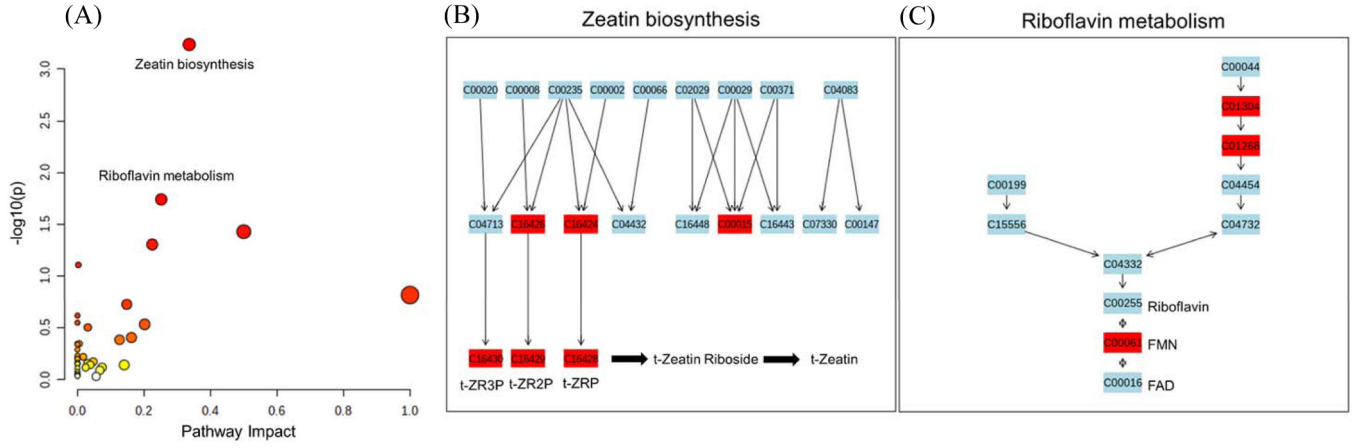


**Fig. 3. :** The pathway analysis gave as result this overview of the several metabolic mathways (MetPA) that were mapped from identified metabolites and showed a significant accumulation. Graph A represents the metabolome view represented with the -log10(*p*) on the y-axis and the impact values from the pathway topology analysis on the x-axis. The colour gradient that fill up each circle (pathways) from white until dark red indicates from the lowest to the highest intensity respectively. Two of the most altered pathways were zeatin biosynthesis (B) and riboflavin metabolism (C), both of them showing in red some of the compounds putatively identified in this untargeted analysis.

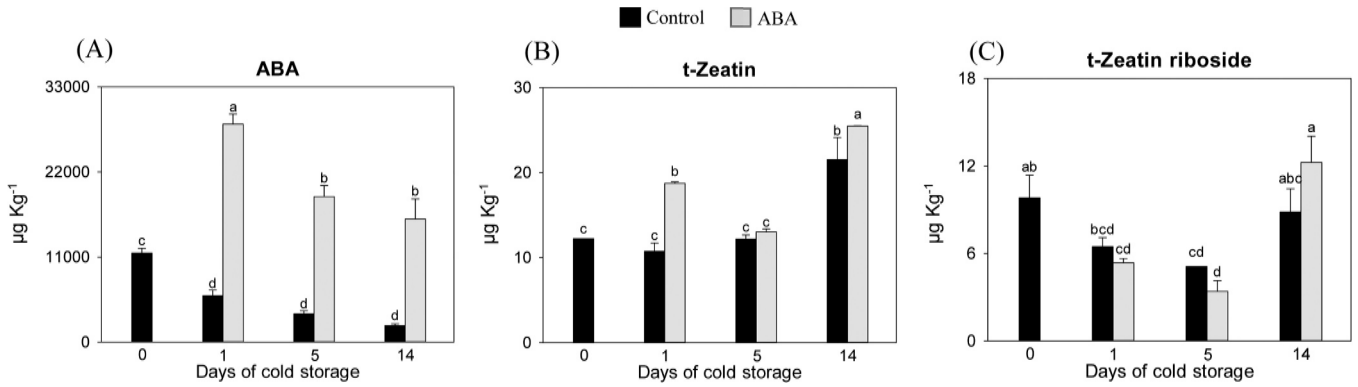


**Fig. 4. :** Determination of content of ABA (A), t-Zeatin (B) and t-Zeatin riboside (C) in the exocarp of control and ABA-treated fruit, at 0, 1, 5 and 14 d of cold storage. Data presented are means ± SE of triplicate samples of six fruit each, expressed as µg per Kg of fresh weight. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

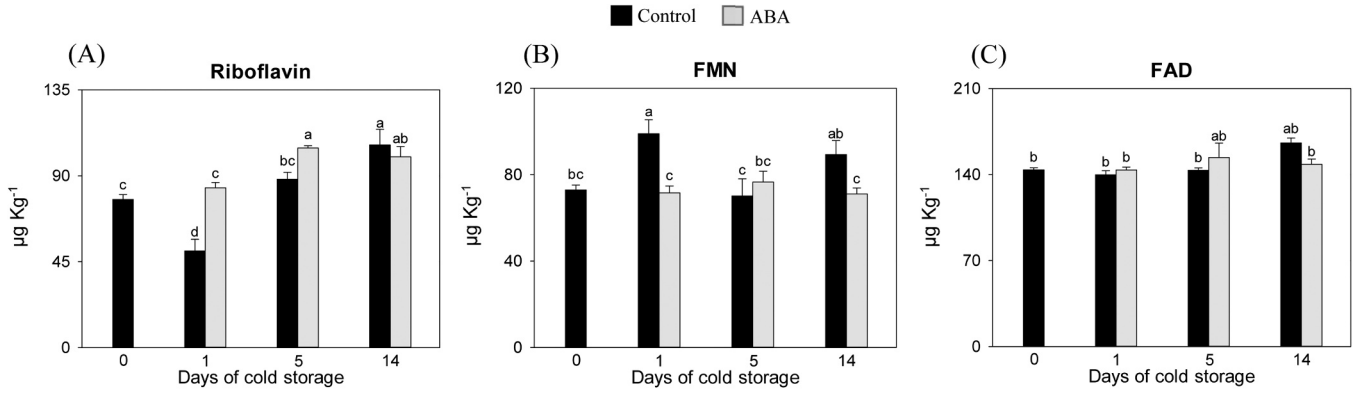


**Fig. 5. :** Determination of content of riboflavin (A), flavin mononucleotide (FMN) (B) and flavin adenin dinucleotide (FAD) (C), in the exocarp of control and ABA-treated fruit, at 0, 1, 5 and 14 d of cold storage. Data presented are means ± SE of triplicate samples of six fruit each, expressed as µg per Kg of fresh weight. Different letters indicate significant differences according to Duncan's test ($p < 0.05$).

plants exposed to cadmium and long-term drought stress, playing a role reducing oxidative damage, maintaining membrane integrity, and water status (Khan et al., 2019; Zemanová et al., 2014). Zucchini fruit treated with ABA presented significantly higher levels of this amino acid along the storage, being able to have a similar role in the adaptation of the fruit to low temperatures. Another relevant amino acid in our study is serine, whose content in ABA-treated fruit increased sharply after the exposition to low temperature. Serine is involved in the adaptation to different stress conditions including low temperature (Watanabe et al., 2021). The accumulation of phenylalanine, meanwhile, is important due to its role as precursor of phenylpropanoid pathway (Barros et al., 2016; Kumar Patel et al., 2023), increasing the synthesis and accumulation of phenolic compounds, helping to the defense against CI in zucchini fruit (Castro-Cegrí et al., 2023b).

Previous studies have pointed the importance of the GABA shunt in the acquisition of cold tolerance in zucchini fruit. More resistant zucchini fruit varieties, as well as fruit with induced chilling tolerance due to preconditioning treatment, or by application of putrescine or GABA, presented an induction of this pathway (Carvajal et al., 2015b; Palma et al., 2019, 2015, 2014a). At short-term storage, ABA-treated fruit had higher GABA levels than non-treated fruit, dropping that content after 14 d. We also found an increase in glutamic acid significantly higher in treated fruit. An induction of the biosynthesis of these amino acids could suppose a greater availability to be metabolized in GABA-shunt pathway to generate succinic acid that can enter in the TCA cycle. Succinic acid showed an augment in ABA-treated fruit exposed to cold whereas in control fruit less succinic acid was measured. In zucchini fruit, it has been described that induction of the GABA-shunt pathway provides intermediates of the TCA cycle and, accordingly, higher ATP and NADH contents (Palma et al., 2019). Different treatments that alleviate CI in fruit increase the ATP content (Hu et al., 2023; Wang et al., 2023).

Soluble sugars play a crucial role in stress responses, acting as compatible solutes (Rathinasabapathi, 2000). In a cold-tolerant variety of zucchini, an increase in sugar content has been also observed under cold stress (Palma et al., 2014b). The main sugar detected in ABA treated fruit is maltotriose, a sugar that participates in the adaptation to cold stress in Arabidopsis (Kaplan and Guy, 2005).

In resume, ABA is able to modify the primary metabolism, what could enhance defence pathways against cold stress along postharvest period in zucchini fruit. Therefore, to gain deeper insight into this response, an untargeted metabolomic approach was carried out to search for secondary metabolites involved in ABA-induced cold tolerance. The Metabolomic Pathway Analysis (MetPA) was used to unravel the ABA role over several pathways associated with the putatively identified metabolites. This analysis pointed to zeatin biosynthesis and riboflavin metabolism as the main ABA induced pathways (Fig. 3), therefore some metabolites of both pathways were quantified. After ABA treatment, fruit kept at 4 ºC for 1 d increased their endogenous ABA content in exocarp respect to control fruit, which indicates that the exogenous ABA could effectively remain in this area, and at that day also the content in t-zeatin and riboflavin increased (Fig. 4). It has already been described that the changes in zucchini fruit during the first days of storage at low temperature are key to the induction of chilling tolerance (Carvajal et al., 2017; Palma et al., 2019, 2015). In fact, Carvajal et al. (2021) described an induction in genes responsible for the synthesis of

cuticular waxes also at 1 d of cold storage in a cold-tolerant variety of zucchini, showing this variety a high amount of ABA during the first days of cold storage (Carvajal et al., 2017). Recently, it has been also proved the role of ABA in cuticle integrity by accumulation of cytokinins in sweet cherries (Gutiérrez et al., 2021). These results are similar to the found in our experiment, where the application of ABA induces the accumulation of t-zeatin after 1 d of cold stress, a natural cytokinin. Thus, this response of ABA to low temperature could be mediated by an increase of the zeatin biosynthesis pathway, responsible for changes in the cuticle composition of zucchini fruit that would maintain fruit quality along cold storage.

In the same way, a higher accumulation of riboflavin was also observed in ABA-treated fruit respect to control fruit after 1 d at low temperature. On the contrary, FMN showed in control fruit a higher amount than in ABA treatment at that same time of storage. Riboflavin (Vitamin B2) is the main product of riboflavin biosynthesis pathway and acts as a natural antioxidant compound in vegetal tissues (Fischer and Bacher, 2006). This vitamin is the precursor of FAD and FMN, essential cofactors in many metabolic processes in plants (Sandoval et al., 2008). Riboflavin has been proved as an important regulator of several physiological processes such as drought tolerance (Deng et al., 2014), quality improvement (Pérez-Álvarez et al., 2019) or avoidance of browning (Zha et al., 2022). The mechanisms responsible of these processes involve the riboflavin-mediated regulation of ROS and endogenous antioxidant system (Li et al., 2012). It has been described that riboflavin treatment strengthens the antioxidant system to avoid membrane peroxidation of fresh-cut apples during 8 d of storage at 4 °C (Zha et al., 2022). The application of riboflavin also showed a tendency to increase the amino acid concentration (Pérez-Álvarez et al., 2019) and an upregulation of phenylalanine ammonia-lyase (*PAL*) gene, activating the phenylpropanoid pathway (Taheri and Tarighi, 2011). Castro-Cegrí et al., 2023b have proposed an induction of the phenylpropanoid pathway by ABA treatment in zucchini fruit, so this response could be regulated by the accumulation of riboflavin. The phenolic accumulation is known to cause cell wall fortification (Taheri and Tarighi, 2010), that would be also important for cold tolerance in zucchini fruit. In maize, a mutation in a pyrimidine reductase in riboflavin synthesis produces plants with lower riboflavin and also lower amount of ABA (Dai et al., 2019). By all these mechanisms, the CI could be alleviated by ABA in zucchini fruit.

## 5. Conclusions

A metabolomic approach is highly useful for understanding how the abscisic acid generates cold tolerance in zucchini fruit. This phytohormone modulates the primary metabolism inducing the accumulation of key amino acids in chilling tolerance, and of t-zeatin and riboflavin in first days of low-temperature storage, which indicates that the protective effect of abscisic acid for cold stress could be regulated by riboflavin and t-zeatin in zucchini fruit.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dolores Garrido Garrido reports financial support was provided by Spain Ministry of Science and Innovation.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2023.112457.

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