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RNA-seq based analysis of transcriptomic changes associated with ABA-induced postharvest cold tolerance in zucchini fruit

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ABSTRACT

Given that external treatments with ABA had been proved to alleviate chilling injury in the immature zucchini fruit, an RNAseq analysis was conducted to gain insight into the molecular mechanisms that are involved in ABA-induced postharvest cold tolerance. Fruit from the cold sensitive cultivar Sinatra were treated with ABA and then stored for 14 d at 4 ºC. Exocarp samples from control and ABA-treated fruit were taken at 1, 5 and 14 d of cold storage, assessing the transcriptional changes during postharvest storage relative to freshly harvested fruit. The RNAseq analysis produced 229 million high-quality reads from a total of about 23,000 unigenes. Pairwise comparisons of differentially expressed genes (DEGs) in treated and untreated samples at each cold storage time resulted in 852, 793 and 1120 DEGs that were specifically found in the ABA-treated but not in the control fruit at either 1, 5 or 14 d of cold storage. This ABA-specific DEG list was subjected to a GO and KEGG enrichment analysis, as well as to a clusterization of gene expression profiles. This revealed the significance of certain metabolic and signaling pathways participating in ABA-induced postharvest cold tolerance, highlighting the relevance of the Ca^{2+} signaling pathway, as well as the positive regulation mediated by certain hormones such as ethylene and jasmonate, and the negative regulation mediated by others like auxins and brassinosteroids. A number of DEGs were also found in the ABA-treated fruit that code for transcription factors, as well as for genes involved in oxidative stress response and in membrane and cell wall metabolism. Data indicates that ABA-induced cold tolerance is not mediated by CBF-like genes but involve the up- and down-regulation of several transcription factors in the *BZIP*, *GRAS*, *MYB*, *MYC*, *NAC* and *ZAT* families that are known to participate as positive and negative regulators in the cold defensive response. Moreover, ABA regulates different genes responsible for reducing oxidative stress damage, inducing the biosynthesis of cuticular wax and repressing the biosynthesis of lignin, as well as protecting membrane and cell wall integrity in fruit cells during postharvest cold storage.

1. Introduction

Zucchini is a vegetable fruit that is immaturely consumed worldwide. The poor development of the fruit at harvest, together with its subtropical origin, makes this fruit very sensitive to chilling injury (CI) during postharvest storage (Martínez-Tellez et al., 2002; Balandrán-Quintana et al., 2003). The optimal storage temperature for this immature fruit oscillates between 10 and 13 °C, as CI is induced at temperatures below 8–9 °C (Valenzuela et al., 2017). When stored at 4 °C, the fruit of most current zucchini commercial varieties shows a series of CI symptoms, which include pitting and sunken areas on the fruit surface, as well as an increased loss of weight and firmness, making the fruit to lose its marketable value in 4–5 d (Megías et al., 2014). Zucchini varieties respond differentially to cold storage, showing the fruit of some varieties a delay in the onset of CI symptoms during postharvest cold storage (Carvajal et al., 2011; Megías et al., 2015, 2017).

Zucchini CI symptoms were found to be associated with an

Abbreviations: ABA, Abscisic acid; TF, Transcription factor; FC, Fold-Change; DEG, Differentially Expressed Gene.

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accumulation of reactive oxygen species (ROS) and subsequent chilling oxidative stress, which induces membrane lipid peroxidation and increases in MDA content, as well as with changes in membrane permeability (Carvajal et al., 2011; Megías et al., 2015; Valenzuela et al., 2017). Microscopical analysis of pitting areas and surface surken indicates that they are caused by cell death and cell collapse, associated with solubilisation of pectin and cell wall degradation (Carvajal et al., 2015). Ethylene is highly induced in the cold-damaged fruit upon transferring from the cold storage chambers to room temperature (Megías et al., 2014, 2016, 2017). The production of this cold-induced ethylene is the consequence of cold damage and was higher produced in the most cold-sensitive varieties (Megías et al., 2017). However, the basal ethylene in this non-climacteric fruit must play a regulatory role in the onset of CI symptoms, since postharvest fruit treatments with the ethylene inhibitor 1-MCP and the ethylene insensitive mutation *etr2b* of *C. pepo* were able to reduce fruit CI symptoms (Megías et al., 2016; García et al., 2020).

Different postharvest technologies have been developed to alleviate CI and to reduce zucchini postharvest losses. Individual shrink wrapping (ISW) is a very effective treatment since it reduces weight loss to less than 1%, while pitting symptoms were abolished at 7 d and reduced to less than 25% when compared to control fruit at 14 d of cold storage. ISW is accompanied by a reduction of respiration rate, cold induced ethylene and oxidative stress metabolites production (Megías et al., 2015). Temperature preconditioning, in combination or not with heat shock treatments, is also effective in alleviating CI symptoms by reducing H_2O_2, MDA and ascorbic acid contents, and by inducing the activity of antioxidant enzymes (Wang, 1994, 1995; Carvajal et al., 2015). Different hormonal treatments have also been reported to alleviate CI in zucchini (Valenzuela et al., 2017). 1-MCP, an anti-ethylene agent, avoids fruit weight loss, together with a reduction in the respiration rate, cold-induced ethylene and ethylene pathway gene expression (Megías et al., 2014, 2016). Polyamines are able to induce APX, CAT and GR activities, fatty acid desaturase expression, together with the accumulation of betaine and proline, and changes in the biochemical GABA shunt (Martínez-Tellez et al., 2002; Palma et al., 2015). Finally, cytokinin applications slow the deterioration and dehydration of cell walls by decreased pectin and sugar solubility and increase phenolic compound accumulation (Massolo et al., 2014).

The phytohormone abscisic acid (ABA) plays a crucial role in integrating various environmental stress signals, including drought, salinity and cold, and coordinating the response to stress of the plant (Zhu, 2016). Wang (1991) firstly reported that postharvest temperature conditioning treatments in fruit increased the accumulation of ABA in the zucchini fruit, and that ABA treatments were effective in reducing zucchini postharvest chilling injury. High relative humidity (HRH) storage of zucchini fruit was also found to alleviate chilling injury by promoting the accumulation of proline and ABA (Zuo et al., 2021). Recently, it has been found that ABA content and ABA signaling pathway components are induced upon cold storage in the cold-tolerant zucchini variety Nature (Carvajal et al., 2017). Moreover, ABA treatments can improve the cold tolerance of the CI sensitive variety Sinatra, while treatments with tungstate, an inhibitor of ABA biosynthesis, reduce the cold tolerance in the cold tolerant variety Natura (Carvajal et al., 2017). To gain insight into the molecular mechanisms that regulate cold tolerance in zucchini, in this paper we study the transcriptomic changes underlying the alleviation of CI symptoms in ABA-treated fruit stored for 1, 5 and 14 d at 4 °C.

2. Materials and methods

2.1. Biological samples: treatment and storage

Zucchini fruit of the cold-sensitive variety 'Sinatra' was sampled for the RNA-seq study. Immediately after harvest, 3 independent exocarp samples were obtained, each one taken from 4 freshly harvested fruit. The rest of the fruit was separated into two sets of 36 fruit. The control fruit set was submerged in distilled water at 20 °C for 20 min while the set of ABA-treated fruit was submerged in 0.5 mM of ABA (Merck, CAS 14375–45–2) for the same time. This ABA concentration was known from previous experiments. Fruit was then placed on desiccant paper for 2 h before storage in a temperature-controlled chamber and in permanent darkness at 4 °C and 85–90% RH for 14 d. After 1, 5 and 14 d of cold storage, 3 independent exocarp samples were taken from both ABA-treated and control fruit at each storage time. A total of 18 independent exocarp samples were so obtained, each one taken from 4 fruit.

2.2. Weight loss, chilling injury index and electrical conductivity

Weight loss, chilling injury index and electrical conductivity was measured for ABA-treated and control fruit at either 0, 1, 5 and 14 d of cold storage following the method described by Carvajal et al. (2017). Weight loss was calculated as:

$$Weight\ loss = \frac{Wf - Wi}{Wi} \times 100$$

being Wf and Wi, final weight and initial weight respectively.

CI was determined by a scale designed following the description done in Martínez-Tellez et al. (2002) about CI in *Cucurbita pepo*. That scale was made up of four categories: 0, no pitting; 1, slightly pitted (10% or less); 2, medium pitting (10–20%); 3, severe pitting (> 20%). CI was calculated as the summation of each pitting scale grade (0−3) multiplied by the corresponding number of fruit in the condition, dividing the result by the total number of fruit estimated.

To evaluate cellular integrity, electrolyte leakage was determined in three replicates of fruit exocarp from control and ABA-treated fruit at either 0, 1, 5 and 14 days of cold storage. Each replicate consisted of ten exocarp discs of 11 mm. Discs were rinsed with deionized water three times for 3 min and then incubated in deionized water for 30 min under continuous gentle agitation (100 rpm). The electrical conductivity in the resulting solution was measured at room temperature using a conductimeter. Total conductivity was also determined after boiling the flasks for 10 min and cooling at room temperature. The electrolyte leakage was expressed as a percentage of total conductivity.

2.3. RNA extraction and sequencing

RNA was extracted from each exocarp sample. Frozen tissue at –80 °C was grinded using stainless steel beads, previously cooled with dry ice. For RNA extraction, GeneJET Plant RNA Purification Kit (Thermo Scientific™) was used following its distributor protocol. After finishing all the purification steps, RNA was eluted in nuclease-free water and immediately prepared for sequencing. The sequencing platform used was Illumina NovaSeq 6000 Sequencing System, generating 150 pb pair-end reads. All the generated raw reads were made publicly available in the NCBI database (https://www.ncbi.nlm.nih.gov/) with identification number PRJNA838740.

2.4. Statistical analysis

In order of checking the quality of the sequenced reads, FastQC (Andrews, 2010) tool was used. Therefore, FastQScreen (Wingett and Andrews, 2018) and Trimmomatic (Bolger et al., 2014) were used to remove likely contaminants, delete adapters and trim low quality bases within the data. Mapping of these high-quality reads and transcriptome assembly were carried out by HISAT2 (Pertea et al., 2016) and STRINGTIE (Kovaka et al., 2019; Pertea et al., 2015).

Differential expression analysis was performed using the total count matrix, making use of edgeR ver. 3.28 (Robinson et al., 2010; McCarthy et al., 2012) and limma-voom ver. 3.42.2 (Ritchie et al., 2015; Law et al., 2014) packages in R (R Core Team, 2020). Voom function, available in

the limma package, was applied during data treatment. Adjusted p-value for each gene was calculated using the Benjamini & Hochberg (BH) method (Benjamini and Hochberg, 1995). Only those genes with a value of |log2 fold change| > 1.0 and an associated adjusted P.value < 0.05 were considered as Differentially Expressed Genes (DEGs). Those selected DEGs were then graphically compared using Venn diagrams.

2.5. Functional analysis

For gene annotation, NCBI (https://www.ncbi.nlm.nih.gov/) and Cucurbit Genomics (http://cucurbitgenomics.org/) Databases were used. Gene Ontology enrichment analysis was performed through a homemade R script using topGO package (Alexa and Rahnenfuhrer, 2021). For the *runTest* function, the algorithm used was "weight01″, while the statistics option selected was "fisher". At the same time, pathway analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000). Gene expression profiles were clustered using the Stem software (Ernst and Bar-Joseph, 2006).

2.6. Validation of in silico results by quantitative RT-PCR

To validate RNA-seq results, 5 genes involved in ABA biosynthesis and signaling were selected for quantitative reverse transcription (qRT)-PCR on three biological replicates of the samples used for RNA-seq, including samples from fresh harvest fruit, and samples from control and ABA-treated fruit stored for 14 days at 4 ºC. Total RNA from each sample was treated with the RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) following manufacturer's protocol. The RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) with random hexamer primer was used to synthesize cDNA from 2 μg of RNA. Primer pairs for the five selected genes were designed from genomic information available at Cucurbit Genomics Database (Table S1). The qRT-PCR reactions were performed making use of iTaq Universal SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) in Hard-Shell 96 microplates (BIO-RAD, Hercules, CA, USA) and the CFX96 thermal cycler (BIO-RAD, Hercules, CA, USA). Amplification conditions consisted of a first step of 95 ºC for 3 min, followed by 40 cycles of 10 s at 95 °C, 15 s at 55 °C and 15 s at 60 °C. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method using EF1α as the internal reference gene to normalize the data. As in RNA-seq, fresh-harvest fruit (fruit at 0 d of cold storage) were used as calibrator samples to calculate the relative gene expression values, which were reported as $\log_2$ fold change ($\log_2$FC). To assess the consistency between RNA-seq and qRT-PCR analyses, a linear regression was done using Microsoft Excel.

3. Results

3.1. ABA induces cold tolerance in zucchini fruit

Postharvest fruit quality parameters such as weight loss, CI index, and electrolyte leakage were measured at harvest and 1, 5 and 14 d after ABA treatments and storage at 4 ºC (Fig. 1). The postharvest ABA treatment was effective in reducing weight loss, CI symptoms in fruit surface and electrolyte leakage in the fruit of the sensible variety Sinatra. Although at 1 day of cold storage the same weight loss was observed in both treated and non-treated fruit, at 5 and 14 d control fruit showed higher weight loss than ABA-treated fruit (Fig. 1A). Likewise, the effects of cold storage on CI index started to be visible after 5 d of cold storage, and CI index of ABA-treated fruit was reduced at days 5 and 14 of cold storage (Fig. 1B), which indicated a protective effect of ABA in the onset of fruit chilling injury. Electrolyte leakage in fruit peel increased upon fruit cold storage time, but it was lower in the ABA-treated fruit (Fig. 1C). These results support the protective effect of ABA against postharvest cold damage in zucchini fruit.

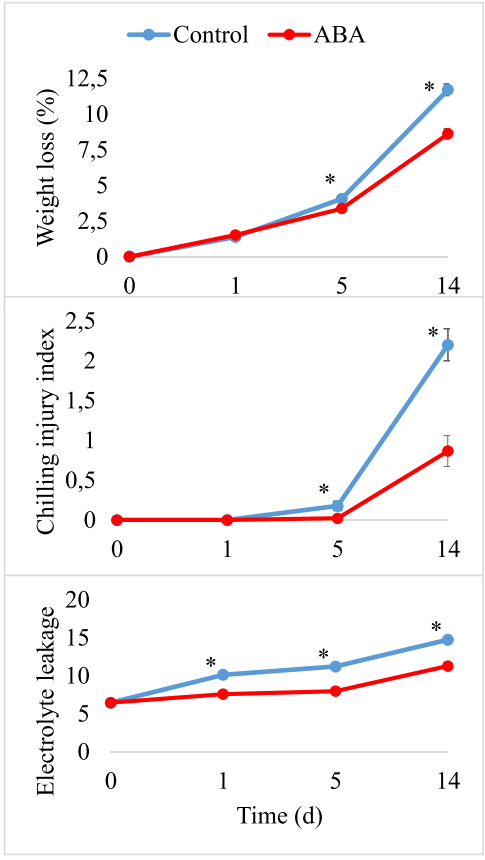


Fig. 1. Effect of ABA postharvest treatments on quality parameters of zucchini fruit under cold storage. a) weight loss, b) chilling-injury index, and c) electrolyte leakage of cv. Sinatra fruit treated with water (control) or ABA and then stored for 14 days at 4 ºC. Data are means of 18 fruit ± SE for each treatment a sampling time. Asterisks indicate significant differences between control and ABA treated fruit at the same storage time according to Fisher's test ($p < 0.05$).

3.2. RNA-seq validation and differential expression analysis of zucchini fruit in response to ABA and cold storage

An RNA-seq analysis was performed to determine the transcriptomic changes that were associated with the higher cold tolerance in the ABA-treated fruit. Sequencing was carried out in 21 fruit samples, accomplishing seven treatments and three repetitions per sample: fresh harvest fruit (FH), and control (C) and ABA-treated fruit stored at 1, 5 and 14 d at 4 ºC. FastqC analysis on raw data showed that all samples added up to 251 million reads. After removing contaminant sequences and adapters, 229 million high quality reads were mapped to the *C. pepo* v4.1 genome (Table 1). The quality of filtered sequencing data fully met the requirements of subsequent analyses.

The generated gene count matrix was used for a Principal Component Analysis (PCA) over the expression data of the 21 fruit samples (Fig. 2). This provided insight into the overall clustering analyses of samples based on the expression patterns of genes that were regulated in the fruit under cold stress and ABA treatment. PC1 and PC2 explained 43.7% and 17.6% of the gene expression variation, respectively. A tight grouping of the three replicates of each sample (Fig. 2) indicated that experimental data is reliable for further analysis. Samples were mainly separated based on cold storage time, but differences between ABA-treated and non-treated samples for the same cold storage time were more reduced (Fig. 2). Differences in gene expression between FH and fruit stored for 5 d and 14 d were explained by both PC1 and PC2, while differences in gene expression between FH and 1 day of cold storage were outlined by PC2.

Table 1
Number of raw reads and percentage of reads after cleaning.

Treatment	Sample	No. of raw reads	No. of HQ reads	Clean reads (%)	Mapped reads (%)
Fresh harvest	1	13907515	13122161	94.35	97.81
	2	10269643	9672604	94.19	97.68
	3	13389047	12630754	94.37	97.70
Control day 1	4	12548469	11766230	93.77	97.29
	5	13401963	12569416	93.79	97.41
	6	10940248	10326561	94.39	97.70
ABA day 1	7	11143192	10411072	93.43	97.34
	8	10271151	9726120	94.69	97.87
	9	10719457	9976035	93.06	97.36
Control day 5	10	9939211	9361832	94.19	97.62
	11	11023478	10374623	94.11	97.51
	12	13201930	12315339	93.28	97.23
ABA day 5	13	14251659	13408034	94.08	97.62
	14	11097042	10414801	93.85	97.45
	15	14179778	13350232	94.15	97.71
Control day 14	16	10677148	10030731	93.95	97.22
	17	11574374	10948112	94.59	97.71
	18	11592818	3661467	88.1	97.22
ABA day 14	19	13845907	12913544	93.27	97.24
	20	11165994	10592991	94.87	97.28
	21	12144574	11336125	93.34	97.22

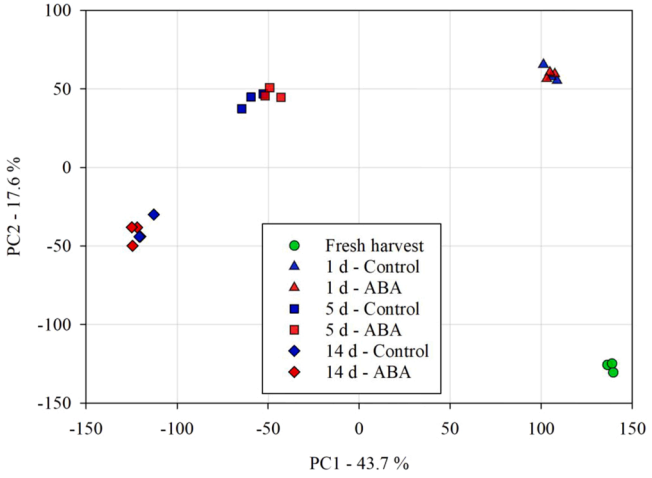


Fig. 2. Principal Component Analysis. Blue icons are used to indicate non-treated samples, while those in red indicate ABA-treated ones. Circles refer to fresh harvested samples and triangles, squares and diamonds represent samples that were stored for 1, 5 and 14 days at 4 ºC, respectively. PC1 and PC2 explained 43.7% and 17.6% of the gene expression variation.

Differential expression analysis was done by pairwise analysis, using FH samples as reference. DEGs were filtered and only genes with a |log-fold change| > 1 and an adjusted P-value < 0.05 were included in the analysis. On this basis, we compared the transcriptomic changes of either control or ABA-treated fruit from FH to 1, 5 or 14 d of storage at 4 ºC. The specific changes in the gene expression profiles due to ABA treatment were then identified by comparing DEGs in both control and ABA fruit at each cold storage time (Fig. 3). Consistently with PCA results, most of the DEGs were common for control and ABA-treated fruit at each storage time: 3069 (64%) genes at day 1, 7982 (81%) genes day 5, and 9444 (84%) at day 14 of cold storage. ABA treatment, however, changed the transcriptomic profiles by specifically modifying the expression of 852 (18%) genes at day 1, 793 (8%) at day 5, and 1120 (10%) genes at day 14 of cold storage (Fig. 3A-C). This set of genes were called ABA-specific DEGs.

To gain insight into ABA inducing cold tolerance in zucchini fruit, we compared ABA-specific DEGs at days 1, 5 and 14 (Fig. 3D). Most of the DEGs that were regulated by the ABA treatment were also highly influenced by time of storage, finding 747, 687 and 1002 DEGs were exclusive of the ABA treatment at 1, 5 and 14 d of cold storage, respectively. Only a reduced number of DEGs (45, 58 and 57) were found to be regulated by the ABA treatment at two storage times, and only three DEGs were regulated by ABA at the three periods of cold storage (Fig. 3D). Those genes were, *Cp4.1LG11g11200*, a Avr9/Cf-9 rapidly elicited protein; *Cp4.1LG01g03670*, UDP-D-glucuronate 4-epimerase 4; and *Cp4.1LG05g02160* a ferredoxin family protein. All of them were down-regulated during the whole period of cold storage.

Table 2 includes ABA-specific DEGs that were up- and down-regulated. Most of the ABA-specific DEGs were down-regulated during early response (73% of DEGs at day 1) whereas only 49% and 45% of DEGs were down-regulated by ABA at days 5 and 14, respectively (Table 2). Moreover, many common ABA-specific DEGs at two or at the three storage times were also mainly down-regulated: 64% ABA-specific DEGs at days 1 and 5 of cold storage, 53% of ABA-specific DEGs at days 5 and 14, and the three ABA-specific DEGs at the three days of cold storage (Table 2).

To validate the results of transcriptomic profiling, the expression of 5 DEGs in the ABA biosynthesis and signaling pathways was analysed by quantitative RT-PCR (Fig. S1). Linear regression analysis of Log_2fold change (log_2FC) obtained by either RNA-seq or qRT-PCR showed a high positive correlation between expression assessed by the two approaches ($R^2 = 0.98$), thus validating the transcriptomic results obtained by RNAseq.

3.3. Functional analysis of ABA-specific DEGs: GO and KEGG enrichment analyses

To identify the biological processes (BP), molecular functions (MF) and cellular components (CC) contributing to ABA-induced cold tolerance in zucchini, Gene Ontology (GO) enrichment analysis was performed with up- and down-regulated ABA-specific DEGs at 1, 5 and 14 d of cold storage (Fig. 4 and Table S2). After 1 day of cold storage, the top most abundant up-regulated BP and MF terms were “regulation of gene expression” and “heterocyclic compound binding” while the most abundant down-regulated term were “response to stimulus” and “acetylglucosaminyltransferase activity” (Fig. 4). Interestingly, terms like “auxin influx” and “cellular response to acid chemical” were up-regulated, confirming the role of auxin and ABA in fruit cold response (Table S2).

BP and MF terms were also found to be enriched in the set of ABA-specific DEGs at 5 d of cold storage. The terms “nucleotide phosphorylation” and “regulation of transcription and protein kinase activity” were up-regulated, while the terms “protein phosphorylation”, and “protein ubiquitination” were down-regulated (Fig. 4). Remarkable is the up-regulation of “photosynthetic acclimation”, “response to photo-oxidative stress” and “ethylene receptor activity” terms, as well as the down-regulation of “abscisic acid binding” and “calcium-dependent protein kinase activity” categories (Table S2). Finally, at 14 d of cold-storage ABA-treated fruit was specifically induced in DEGs included in BP and MF terms like “response to stimulus”, while repressed in terms like “starch metabolic process” and “response to abscisic acid” (Fig. 4).

ABA-regulated DEGs were also mapped on the KEGG pathway database. Plant hormone signal transduction, MAPK signaling, and carotenoid biosynthesis were the most significant pathways associated with cold tolerance in the fruit exposed to ABA (Fig. S2 and 5). The ABA treatment changed the expression of many genes in hormone and MAPK signaling pathways, indicating a crosstalk between ABA and other hormones involved in abiotic stress responses, Ca^{2+}, H_2O_2 and other ROS MAPKs signaling pathways (Fig. 5). The transcriptomic changes were mainly found in fruit that were stored at 4 ºC for 5 and 14 d after the ABA

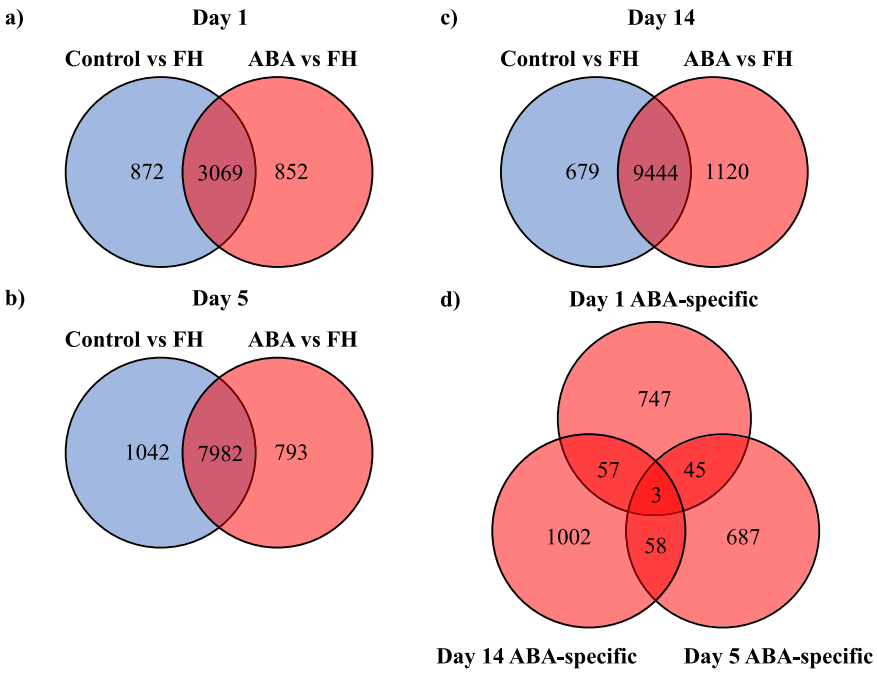


Fig. 3. Venn Diagrams depicting differentially expressed genes between fresh harvest (FH) and control and ABA-treated fruit after 1 (a), 5 (b) and 14 (c) days of cold storage. In (d) ABA-specific DEGs for each cold storage day were compared.

Table 2
Up and down-regulated ABA-specific DEG list at different days of cold storage.

Cold storage	UP	DOWN	TOTAL
Day 1	228	624	852
Day 5	407	386	793
Day 14	617	503	1120
Early regulated DEGs (days 1 and 5)	16	29	45
Late regulated DEGs (days 5 and 14)	27	31	58
Regulated at three storage days (1, 5 and 14)		3	3

treatment, but few changes were also found in fruit one day after the treatment (Fig. 5). Carotenoids can provide precursors for the biosynthesis of ABA and strigolactones. Genes involved in the biosynthesis of carotenes (1.3.5.5; *Cp4.1LG08g06310*), lutein (1.14.141.58; *Cp4.1LG19g03680*) and ABA (1.2.3.14; *Cp4.1LG05g05600*) were up-regulated 5 d after the treatment in cold stored fruit (Fig. S2), indicating that the postharvest application of ABA may increase endogenous ABA biosynthesis in the fruit (Fig. S2). DEGs were also found in the ABA signaling pathway in response to ABA treatment. At 1 and 5 d after the ABA treatment and cold storage, genes *SnRK2* (*Cp4.1LG05g11310*) and *ABF* (*Cp4.1LG01g21530*) were down-regulated, but 14 d after the

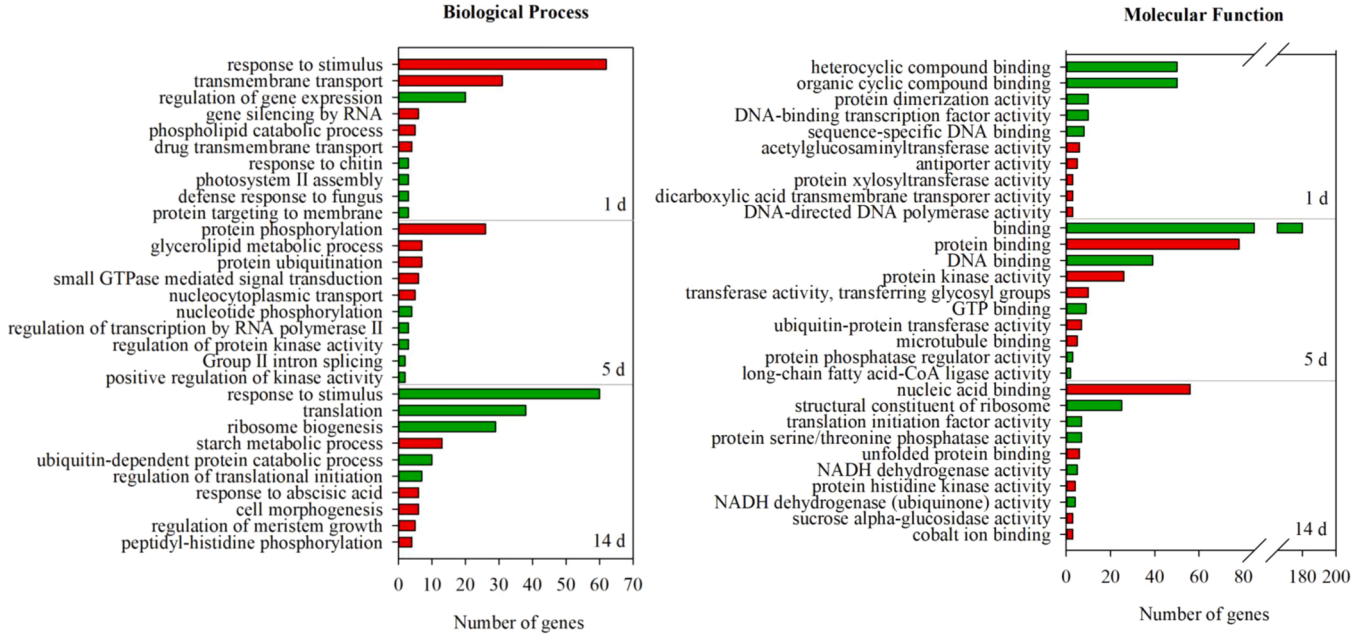


Fig. 4. Gene Ontology (GO) terms of categories Biological Process and Molecular Function enriched in the ABA-specific DEGs. Significantly enriched GO terms were selected based on a FDR < 0.05. Down- and up-regulated GO terms are depicted in red and green, respectively.

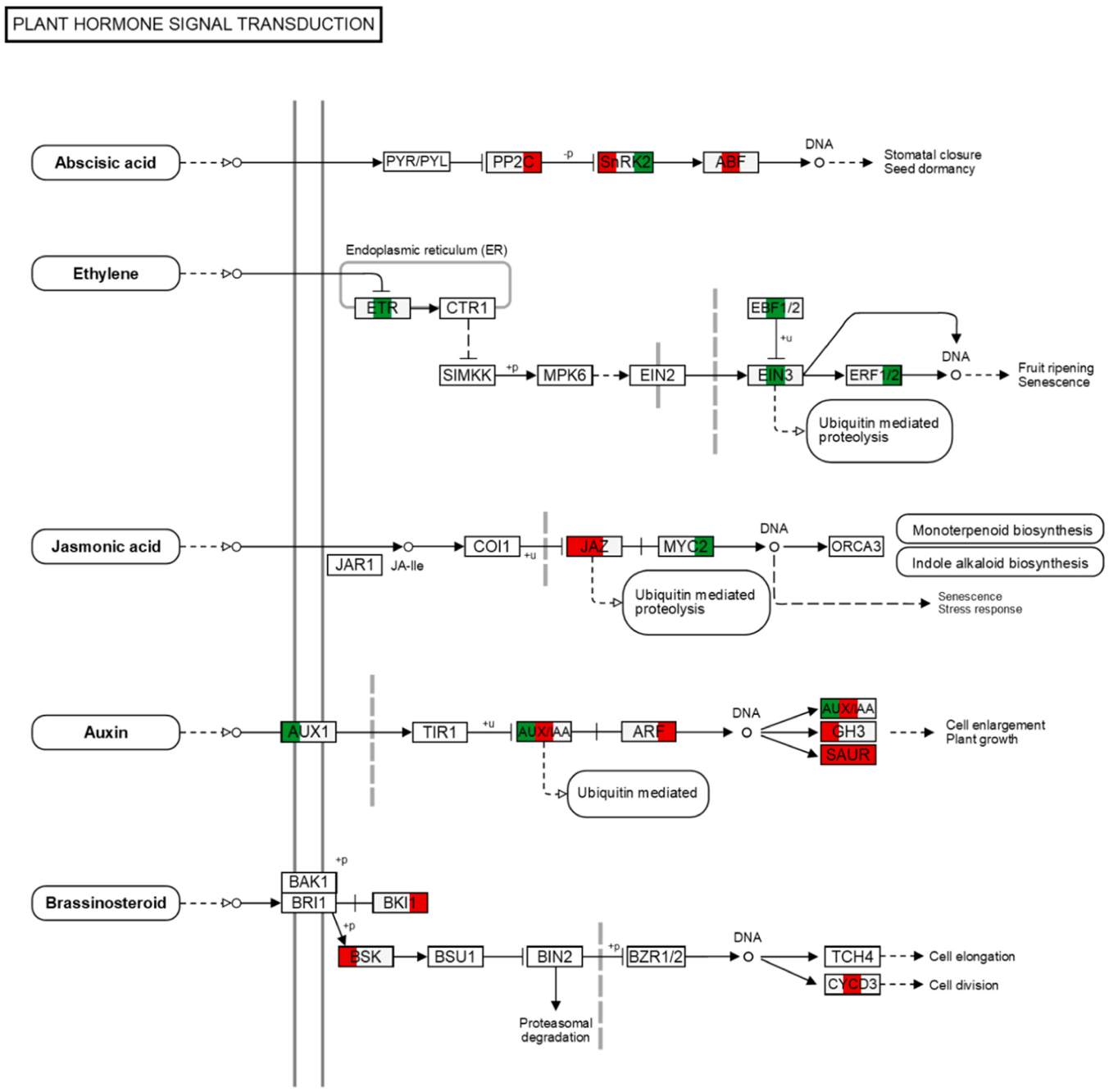


Fig. 5. Plant hormone signal transduction KEGG map of ABA-specific DEGs during the postharvest storage of zucchini fruit at 4 °C. Each box is divided in 3 segments, each one representing the fruit at 1, 5 and 14 days of cold storage. Red and green highlighted boxes indicate that the gene is respectively down-regulated or up-regulated in ABA-treated fruit compared with fresh harvest fruit. Box and segments lacking color indicate no gene expression change at that specific storage time in comparison with fresh harvest fruit.

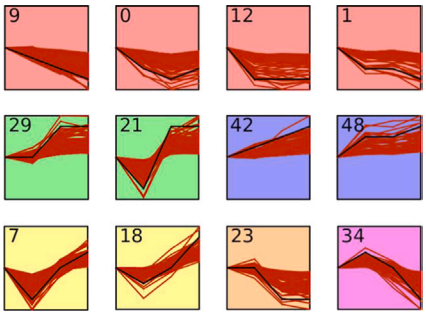
treatment ABA signaling was activated by a down-regulation of the inhibitor *PP2C* (*Cp4.1LG13g11070*) and the up-regulation of the activator *SnRK2* (*Cp4.1LG05g12260*) (Fig. 5). Ethylene and jasmonic acid signaling pathways were also found to be induced in the ABA-treated fruit, especially through ethylene mediated transcription factors such as *EIN3* (*Cp4.1LG18g01370*), *ERF1/2* (*Cp4.1LG08g03650*; *Cp4.1LG12g01490*) and *EBF1/2* (*Cp4.1LG11g10740*) and the JA-mediated MYC2 (*Cp4.1LG08g08520*) (Fig. 5). By contrast, the signaling genes in the auxin and the brassinosteroid pathways were mainly repressed, since the ABA treatment down-regulated genes *AUX/IAA* (*Cp4.1LG03g14120* at day 1; *Cp4.1LG08g03670* and *Cp4.1LG12g08260* at day 5), *ARF* (*Cp4.1LG13g00970*), and *SAUR* (*Cp4.1LG16g02890* and *Cp4.1LG20g07120* at day 1; *Cp4.1LG06g00730* at day 5; and *Cp4.1LG03g15990* at 14 d) in the auxin signaling pathway, and genes *BKI1* (*Cp4.1LG11g01690*), *BSK* (*Cp4.1LG05g03490* and *Cp4.1LG08g02230*) and *CYCD3* (*Cp4.1LG19g11010*) in the brassinosteroid signaling pathway (Fig. 5).

A number of genes in the MAPK signaling pathway, which are induced in response to biotic and abiotic stresses, were also induced in response to ABA postharvest treatment and cold storage (Fig. S2). The *MAKKK ANP1* (*Cp4.1_scaffold000727g00010*), *MAPK3/6* (*Cp4.1_scaffold000480g00030* and *Cp4.1LG02g12910*) and MAPK substrate *MKS1* (*Cp4.1LG05g10550*) were up-regulated in response to ABA and cold storage at 5 and 14 d of cold storage, while *RbohD* genes

(*Cp4.1_scaffold000170g00090*, *Cp4.1LG01g00110* and *Cp4.1LG08g06540*), which act at the end of the MAPK cascade to maintain ROS homeostasis, were also induced in the fruit at 14 d of cold storage (Fig. S2).

3.4. Gene expression profiles

Since it is likely that genes sharing the same expression profile can also share functionality, the annotated matrix with ABA-specific DEGs was used as input for clustering genes with similar gene expression patterns. Genes were so clustered in 50 different groups, but 12 clusters had a statistically significant number of genes assigned respect to the number expected (Fig. 6). Searching for ABA signaling genes in the more significant clustered profiles, a number of genes were found in the profile 29 that could be involved in ABA-induced postharvest cold tolerance. Profile 29 comprises 233 genes with a progressive up-regulation from harvest to 14 d of cold storage (Fig. 6). It includes the *GEM*-like genes *Cp4.1LG09g10910* and *Cp4.1LG03g13070*, which are part of the ABA signaling pathway, and *Cp4.1LG08g03650*, an ethylene-responsive factor 1B (*ERF1B*) in the plant hormone and MAPK signaling pathways. Other genes in ABA and cold stress response in profile 29 include calcium-related genes like CDPK *Cp4.1LG09g03630* and the calmodulins *Cp4.1LG01g13290* and *Cp4.1_scaffold019707g00010*, a few glutaredoxin (*Cp4.1LG01g26090*, *Cp4.1LG11g06470*, *Cp4.1LG16g06630*) and reductase (*Cp4.1LG15g03980*) genes, which are part of oxidative stress response (Table 3), and different transcription factors of the MYB (*Cp4.1LG12g0846*, *Cp4.1LG02g17290*), NAC (*Cp4.1LG05g15540*) and ZAT (*Cp4.1LG10g08860*) families. Profile 21, which is in the same cluster than profile 29, revealed more calmodulin, reductase, NAC and ZAT genes, but also the transcription factor ICE1 *Cp4.1LG03g06610* and the phospholipase *Cp4.1LG03g03580* (Table 3). All these genes, as well as others involved in the cold stress response pathways, including Ca^{2+} signaling, cold induced transcription factors, as well as oxidative stress and membrane and cell wall degradation responses, are considered in more depth below. Moreover, a comprehensive list comprising all the ABA-specific DEGs is supplied as the Table S3.



Profile ID	Functional Group	CuGenDB ID
Profile 29	GRAM	Cp4.1LG09g10910
		Cp4.1LG03g13070
	Kinase	Cp4.1LG09g03630
	Calcium binding	Cp4.1LG01g13290
		Cp4.1_scaffold019707g00010
	Glutamate	Cp4.1_scaffold000895g00020
	Glutaredoxin	Cp4.1LG01g26090
		Cp4.1LG11g06470
		Cp4.1LG16g06630
	Reductase	Cp4.1LG15g03980
	MYB	Cp4.1LG12g08460
		Cp4.1LG02g17290
	NAC	Cp4.1LG05g15540
	ZAT	Cp4.1LG10g08860
Profile 21	Reductase	Cp4.1LG05g12240
		Cp4.1LG04g06090
	NAC	Cp4.1LG18g09070
	ICE1	Cp4.1LG03g06610
	Phospholipase	Cp4.1LG03g03580
	Calcium binding	Cp4.1LG14g00130
	ZAT	Cp4.1LG19g04400

Fig. 6. Clustering of gene expression profiles of ABA-regulated genes in zucchini fruit under cold storage. Each cluster comprises the ABA-specific DEGs with similar gene expression patterns in zucchini fruit from harvest to 14 d of cold storage. Clusters sharing background-color shade are clustered together. Table shows DEGs from profiles 29 and 21 that are known to be regulated by ABA in other systems.

3.5. Calcium pathway in ABA-mediated cold response

A search was conducted to identify genes that are known to participate in the ABA signaling pathway and that can be responsible for the ABA-mediated fruit cold tolerance. 42 genes in the calcium-mediated pathway were found in the set of ABA-specific DEGs from cold stored fruit (Table 3). A high number of calmodulin (CaM) genes, as well as genes including calmodulin's characteristic EF-hand motif sequences and numerous calmodulin-binding proteins (CaMBP) were found to be regulated by ABA in the refrigerated fruit (Table 3). Although the expression of some of these calcium-related genes also changed in response to cold storage in control fruit, they were specifically or more down- or up-regulated in the ABA-treated and cold stored fruit (Table 3). For example, the Ca2 + binding proteins *Cp4.1LG11g01520*, *Cp4.1LG04g16040*, *Cp4.1_scaffold000209g00020* and *Cp4.1LG20g00700* were specifically induced between 4 and 8 times more in the ABA-treated fruit at 14 d of cold storage (Table 3). Other CaM, CaMBP and calcineurins, including *Cp4.1LG18g07230* and *Cp4.1LG03g16210* were down-regulated by cold in both control and ABA-treated fruit, but changes in gene repression were higher in response to ABA (Table 3). A number of genes coding for Ca^{2+}-dependent lipid-binding (CaLB) and C2 calcium-dependent membrane targeting proteins, including *Cp4.1LG03g16730*, *Cp4.1LG01g14580* and *Cp4.1LG17g00890*, which are involved in plant stress signaling cascades, showed a specific activation in response to ABA-treatment in the fruit (Table 3).

The same was true for calcium/calmodulin receptors like kinases (CRLK) and calcium-dependent protein kinases (CDPK). Genes *Cp4.1LG13g08000* and *Cp4.1LG14g00060*, sharing a high homology with Arabidopsis *AT5G15730* (CRLK2) and *AT5G54590* (CRLK1) respectively and taking part in the molecular response to cold, were up- or down-regulated, respectively, in response to ABA and cold storage of the zucchini fruit. Four CDPK genes were also specifically found in ABA-treated and refrigerated fruit. *Cp4.1LG09g03630* and *Cp4.1LG19g11410* were up-regulated, showing the former a logFC around 8 in the fruit after 5 and 14 d of cold storage. The other two CDPK-like genes, *Cp4.1LG16g08740* and *Cp4.1LG10g07900*, were more down-regulated in the fruit after ABA treatment and cold storage (Table 3).

3.6. Transcription factors in ABA-mediated cold response

Annotated transcription factors (TF) for C. pepo were retrieved from PlantTFDB, and 209 TFs identified in the ABA-specific list of DEGs were further annotated. They represent multiple TF families, including AP2, bZIP, bHLH, C2H2, NAC and MYB genes. Many of the identified genes were down-regulated, but several others were up-regulated. From the AP2 family we revealed two ethylene responsive transcription factor genes, *Cp4.1LG04g13010* and *Cp4.1LG01g14110*, that were earlier and higher induced in the ABA-treated fruit at the 1, 5 and 14 d of cold storage (Table 3). *Cp4.1LG01g14110* is known to be a positive regulator of ABA response in Arabidopsis. Nine transcription factors were recognised as bZIP (Table 3). *Cp4.1LG01g21530*, which was down-regulated in the ABA-treated fruit after 5 d of cold storage, encoded for an Abscisic acid-insensitive 5-like protein, the bZIP factors

Table 3
List of selected genes that may be responsible for the ABA-mediated fruit cold tolerance. Red and green shaded cells highlight those values which are under and over 1 logFC respectively.

Functional group		CuGenDB ID	log2 Fold Change					
			1 d		5 d		14 d	
			Control	ABA	Control	ABA	Control	ABA
Calcium	Calcium binding	Cp4.1LG18g07230						-3.08
		Cp4.1LG07g02180	-1.38	-1.33	-1.70	-1.45	-0.99	-1.39
		Cp4.1LG03g10260	-2.79	-4.50		-1.28	-1.05	
		Cp4.1_scaffold000102g00210	-1.42					-1.01
		Cp4.1LG02g07030	1.00	1.12	0.84	1.05		
		Cp4.1LG01g13290			1.33	1.01	0.98	1.03
		Cp4.1LG14g00130			1.22	1.05		1.03
		Cp4.1LG09g05540	0.56	0.77	0.59	0.40	0.80	1.23
		Cp4.1LG18g08810	-3.83					1.44
		Cp4.1LG03g05830	-1.51	-1.32	1.07			2.01
		Cp4.1LG03g11850	0.90	1.02	1.00	0.98	1.55	2.04
		Cp4.1LG11g01520						2.16
		Cp4.1LG04g16040						2.69
		Cp4.1_scaffold000686g00030			0.91	1.61		
		Cp4.1LG18g09300	0.56	0.46	0.85	1.01	0.97	1.07
		Cp4.1_scaffold019707g00010			2.07	1.47		1.42
		Cp4.1LG04g16160			1.82			2.36
		Cp4.1_scaffold000209g00020						3.06
	Calmodulin binding	Cp4.1LG20g00700						3.14
		Cp4.1LG10g04260			-4.99	-2.83		-4.55
		Cp4.1LG16g02030				-2.68	-4.37	
		Cp4.1LG03g00960	-0.71	-1.15	-1.27	-1.17	-0.52	-0.63
		Cp4.1LG12g03040		-0.21	-0.82	-1.03	-0.66	-0.62

(*continued on next page*)

Table 3 (*continued*)

		Cp4.1LG08g10260			-0.79	-1.24	-0.50	-0.60
		Cp4.1LG04g10770	-3.91	-4.53		-1.90		
		Cp4.1LG08g13490			-0.96	-1.38		
	Calcineurin	Cp4.1LG15g02970	-0.80	-1.21	-1.39	-1.06	-1.71	-1.10
		Cp4.1LG03g16210	-1.26	-1.80		-1.76	-1.42	-2.22
	CaLB	Cp4.1LG03g16730						1.48
		Cp4.1LG01g14580						2.28
	Calcium membrane targeting	Cp4.1LG17g00890			4.74			3.99
	Kinase	Cp4.1LG10g09580	-1.44	-1.25	-0.93	-1.15	-0.76	-0.60
		Cp4.1LG19g11410	1.44	1.39	1.39	1.24	0.77	1.00
		Cp4.1LG09g07900	-6.04	-2.70		-1.55	-3.41	-5.30
		Cp4.1LG16g08740			-0.98	-1.21	-4.28	-4.29
		Cp4.1LG09g03630				8.28		8.19
		Cp4.1LG14g00060			-0.72	-1.31	-1.55	-1.38
		Cp4.1LG13g08000				1.46	2.44	2.55
	Transporter	Cp4.1_scaffold000829g00020		-6.63	-1.88	-7.26	-2.16	-1.97
		Cp4.1LG03g03930	-0.64	-1.55			0.55	
		Cp4.1LG20g08690	0.22		0.56	0.50	0.98	1.07
		Cp4.1LG10g02960	2.34	1.96	2.62	2.11		2.39
Glutamate	**Glutamate**	Cp4.1LG07g06070	-0.78			-1.16	-0.71	-0.67
		Cp4.1LG19g08570	-0.82	-1.04	-2.73	-2.80	-2.68	-2.91
		Cp4.1LG06g06620	-0.98	-1.11	-2.93	-3.07	-2.79	-2.75
		Cp4.1LG08g13340	0.73	0.65	0.87	1.01	0.54	0.47
		Cp4.1_scaffold000895g00020				3.50	4.34	3.89
		Cp4.1LG11g11650	0.93	1.23	2.81	2.73	2.95	3.02
Oxidative stress	**Catalase**	Cp4.1LG07g05850	-1.15	-1.11	-1.15	-0.80	-0.89	-1.34
	Peroxidase	Cp4.1LG07g03070	-0.97	-1.01		-0.38		-0.41
		Cp4.1LG09g08150		-4.45				
		Cp4.1LG03g01810	-5.82	-4.26	-5.91	-3.06		-2.87

(*continued on next page*)

Table 3 (*continued*)

		Cp4.1LG01g13160	0.92	1.11	1.53	1.61	2.36	2.15
		Cp4.1LG15g08990				2.35	4.18	4.53
		Cp4.1LG16g04260		-3.17	-2.35			
		Cp4.1LG12g11780					0.95	1.02
	Glutathione S-transferase	Cp4.1LG03g18240						2.38
		Cp4.1LG08g02950	-0.79	-1.63				
		Cp4.1LG14g03330	-2.49			-1.76	-2.79	-5.07
		Cp4.1LG03g18220	-4.12			-5.85	-3.77	-2.73
		Cp4.1LG20g00370						-2.02
		Cp4.1LG17g07080						-1.32
		Cp4.1LG13g11730	-0.86	-1.01	-0.77	-0.89		
		Cp4.1LG01g10570	-1.06			-1.07	-1.74	-1.61
	Glutaredoxin	Cp4.1LG01g26090	-0.70		0.74	1.02	1.75	1.66
		Cp4.1LG02g05990				1.20		
		Cp4.1LG16g06630			1.73	1.28	0.66	1.02
		Cp4.1LG11g06470			0.99	1.34	1.17	1.19
		Cp4.1LG04g01400		3.53	3.35	2.98		
	Reductase	Cp4.1LG06g00040					0.99	1.16
		Cp4.1LG04g16350	-0.79	-1.17	-0.83	-0.86	-0.76	-0.71
		Cp4.1LG11g03790	-1.13	-1.22	-1.22	-0.96	-1.00	-1.16
		Cp4.1LG05g09710	-3.63	-1.83	-2.46	-2.76		-2.06
		Cp4.1LG02g16280	-0.42		0.52		0.65	1.03
		Cp4.1LG05g12240	-0.48	-0.61	0.61	0.57	0.97	1.15
		Cp4.1LG15g03980			1.34	1.00	0.54	1.33
		Cp4.1LG01g16850	1.19	1.35		1.03		
		Cp4.1LG04g06090	-0.55	-0.51	1.40	1.17	0.97	1.13
	RBOH	Cp4.1LG08g06540	0.78	0.89	2.39	1.91	0.72	1.35
		Cp4.1LG01g00110					0.88	1.08
		Cp4.1_scaffold000170g00090			2.95			3.53
Membrane and cell	**Fatty acid**	Cp4.1_scaffold002	0.67	0.60	0.93	0.89	-0.78	-1.31

(*continued on next page*)

Table 3 (*continued*)

wall degradation	**desaturases**	129g00010						
		Cp4.1LG08g13790	0.60	0.54	0.82	0.81	-0.87	-1.19
	Phospholipases	Cp4.1LG15g03050				-3.27		
		Cp4.1LG03g03580			0.78	1.12	1.24	1.60
		Cp4.1LG17g06140	-1.36	-1.74		-1.45	-2.48	
		Cp4.1LG11g03250	-0.90	-1.23	-2.23	-2.60	-2.24	-1.59
		Cp4.1LG05g13190	-0.86	-1.08	-2.57	-2.22	-4.44	-4.70
		Cp4.1LG05g07640	1.28	1.14				-2.08
		Cp4.1LG01g05330	0.91	0.97	0.89	1.02	0.43	0.33
		Cp4.1LG06g08410	0.91	1.04	1.32	1.63	2.82	2.69
	Pectato lyase	Cp4.1LG15g01230			-2.30	-3.32		-1.95
		Cp4.1LG01g23700				-4.86		
	β-glucosidase	Cp4.1LG15g07190		-1.10	-2.19	-2.16	-3.20	-2.54
		Cp4.1LG06g07180	-0.98	-1.06	-1.93	-2.30	-3.02	-3.37
	Glucan endo-1.3-β-glucosidase	Cp4.1LG07g01580						1.47
		Cp4.1LG11g10550				-1.34		
		Cp4.1LG09g07400		-3.32			-3.58	-2.80
		Cp4.1LG02g02390						-3.30
		Cp4.1LG05g00510		-2.63	-2.87	-2.03	-5.37	-2.28
		Cp4.1LG03g12250						-4.34
		Cp4.1LG04g10430	-0.83	-0.79	-1.55	-1.90	-0.86	-1.20
	Expansin B3	Cp4.1LG05g15010				-5.78	-4.07	-4.74
		Cp4.1LG19g03470	1.90	1.94				1.30
	Xyloglucan	Cp4.1LG03g01540		-2.98				
		Cp4.1LG05g00910	-0.82	-1.24	-0.76	-0.84	-1.76	-1.93
		Cp4.1LG13g06580	1.46	1.27	1.35	1.32	0.97	1.03
	LACS	Cp4.1LG13g01110		-3.15			2.08	2.04
		Cp4.1LG08g04330	0.88	0.85	0.93	1.19	1.24	1.15
		Cp4.1LG02g03430	0.34	0.34	0.96	1.01	1.46	1.37
		Cp4.1LG04g04220						1.09
	Lignin	Cp4.1LG16g06940	-0.92	-1.22	1.03	0.47		

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Table 3 (*continued*)

	biosinthesis	Cp4.1LG04g06600	-0.94	-1.15	-4.21	-4.43	-4.78	-6.29
		Cp4.1LG16g01990						-1.20
	AP2	Cp4.1LG04g13010	1.68	1.75	0.78	1.13		-0.72
		Cp4.1LG01g14110						2.92
	bZIP	Cp4.1_scaffold000271g00070	-2.83	-3.13	-2.15	-2.52	-0.74	-1.08
		Cp4.1LG03g05090	0.61	0.61	0.98	0.80	0.95	1.25
		Cp4.1LG15g05880	0.60	0.49	0.36		-0.53	-1.04
		Cp4.1LG20g07970		-3.10	-3.88	-6.47	-1.76	-1.63
		Cp4.1LG05g09970	-0.98	-1.08			-1.48	-1.21
		Cp4.1LG06g00920		1.07	2.37	2.18		
		Cp4.1LG07g09470		-4.61		-3.34		
		Cp4.1LG01g22220	1.68					-3.37
		Cp4.1LG01g21530			-0.92	-1.57		-0.80
Transcription Factors	**MYB**	Cp4.1LG03g06350	-0.99	-1.16	-1.20	-1.42	-3.85	-4.17
		Cp4.1LG08g04800	0.76	0.89	0.89	1.23	1.56	1.33
		Cp4.1LG04g15360	0.63	0.69	1.26	1.11	0.65	1.22
		Cp4.1LG03g11950	0.92	1.29	-1.45			
		Cp4.1LG20g04160	0.96	0.75	-1.00	-1.03	-5.88	-4.11
		Cp4.1LG10g02110		-0.43	-0.71	-0.80	-0.77	-1.01
		Cp4.1LG07g02300		1.87				1.86
		Cp4.1LG10g01790		3.04	4.55	4.27	3.51	4.33
		Cp4.1LG02g17050		1.90	-3.37	-4.29	-3.70	-4.28
		Cp4.1LG19g10410		2.37				
		Cp4.1LG16g01100						-4.44
		Cp4.1LG04g10590			-0.60	-1.06		
		Cp4.1LG01g23480				-1.35		
		Cp4.1LG12g08460			0.64	0.69	0.96	1.20
		Cp4.1LG20g08440					-0.92	-1.17
		Cp4.1LG11g05310				-2.46	-3.55	-5.13
		Cp4.1LG10g09350						1.79

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Table 3 (*continued*)

	Cp4.1LG01g22460						-4.01
	Cp4.1LG02g04920						3.17
	Cp4.1LG02g17290			0.95	1.09	0.94	1.02
bHLH	Cp4.1LG08g08520						1.07
ICE1	Cp4.1LG03g06610			1.92	2.15		1.56
GRAS	Cp4.1LG04g15890	4.10		3.70	2.91		2.81
	Cp4.1LG20g08470	-1.34	-1.77			0.78	1.28
	Cp4.1LG12g09770	2.55	2.21		-2.02	-5.25	-5.84
	Cp4.1LG19g09550				-1.41		
NAC	Cp4.1LG17g09240	1.24	1.35	1.36	1.47	0.89	1.10
	Cp4.1LG04g06830		1.39			-2.61	-6.66
	Cp4.1LG12g04350		-2.74			-5.31	-5.90
	Cp4.1LG16g07040						-4.31
	Cp4.1LG02g01620		-2.31	-4.06	-5.14	-4.25	-3.99
	Cp4.1LG20g00280						-3.10
	Cp4.1LG13g05510				-1.74	-2.73	-2.38
	Cp4.1LG18g03850	0.99	1.04		0.88	-2.85	-1.39
	Cp4.1LG09g02760	-0.98	-1.33	-1.65	-1.66	-1.32	-1.19
	Cp4.1LG03g01200			-0.96	-1.04	-0.81	-1.09
	Cp4.1_scaffold000968g00010	-1.80	-2.24	-1.41	-2.02	-0.76	-1.08
	Cp4.1LG08g00180	-0.39	-0.51	-0.57	-1.07	-0.69	-0.90
	Cp4.1LG03g12850	0.65	0.77	0.66	1.20		-0.40
	Cp4.1LG07g10520	0.62	0.97	0.70	1.15		
	Cp4.1LG02g10300	1.47	1.40	0.91	1.29	1.54	1.14
	Cp4.1LG05g15540			1.50	1.35	0.77	1.17
	Cp4.1LG18g09070	-1.62	-1.73	2.41	2.09	0.97	1.59
	Cp4.1LG04g06190			0.91	1.19	1.91	1.97
	Cp4.1LG19g05060		2.48		1.99	2.49	2.08
ZAT	Cp4.1LG03g15550		-3.42		-4.29	-4.73	-3.78
	Cp4.1LG16g05900	-2.73					2.35

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Table 3 (*continued*)

		Cp4.1LG05g08890						3.54
		Cp4.1LG02g06710		1.52				
		Cp4.1LG02g06720	1.00	1.21	0.90	1.02		
		Cp4.1LG02g06530	1.26	1.30	0.83	1.17	0.54	0.83
		Cp4.1LG10g08860			2.85	1.89		1.59
		Cp4.1LG19g04400			2.93			3.76
Auxin	**Auxin**	Cp4.1LG10g08010		1.99			-4.12	-4.71
Profile 29	**GRAM**	Cp4.1LG09g10910	-0.35	-0.38	1.11	0.74	0.97	1.33
		Cp4.1LG03g13070			0.57	0.64	0.95	1.04
	Kinase	Cp4.1LG09g03630				8.28		8.19
	Calcium binding	Cp4.1LG01g13290			1.33	1.01	0.98	1.03
		Cp4.1_scaffold019707g00010			2.07	1.47		1.42
	Glutamate	Cp4.1_scaffold000895g00020				3.50	4.34	3.89
	Glutaredoxin	Cp4.1LG01g26090	-0.70		0.74	1.02	1.75	1.66
		Cp4.1LG11g06470			0.99	1.34	1.17	1.19
		Cp4.1LG16g06630			1.73	1.28	0.66	1.02
	Reductase	Cp4.1LG15g03980			1.34	1.00	0.54	1.33
	MYB	Cp4.1LG12g08460			0.64	0.69	0.96	1.20
		Cp4.1LG02g17290			0.95	1.09	0.94	1.02
	NAC	Cp4.1LG05g15540			1.50	1.35	0.77	1.17
	ZAT	Cp4.1LG10g08860			2.85	1.89		1.59
Profile 21	**Reductase**	Cp4.1LG05g12240	-0.48	-0.61	0.61	0.57	0.97	1.15
		Cp4.1LG04g06090	-0.55	-0.51	1.40	1.17	0.97	1.13
	NAC	Cp4.1LG18g09070	-1.62	-1.73	2.41	2.09	0.97	1.59
	ICE1	Cp4.1LG03g06610			1.92	2.15		1.56
	Phospholipase	Cp4.1LG03g03580			0.78	1.12	1.24	1.60
	Calcium binding	Cp4.1LG14g00130			1.22	1.05		1.03
	ZAT	Cp4.1LG19g04400			2.93			3.76

Cp4.1LG20g0797, *Cp4.1LG07g09470* and *Cp4.1LG01g22220* were highly repressed in the ABA-treated fruit at either 1, 5 and 14 d of cold storage. The MYB family was largely represented, with 20 TFs in the dataset of genes that responded to ABA in cold stored fruit. Special mention deserves the MYB genes *Cp4.1LG19g10410* and *Cp4.1LG07g02300*, both specifically induced in ABA-treated fruit at 1 day of cold storage (Table 3). The MYB-like *Cp4.1LG16g01100* was also found to be highly regulated by ABA, since it was more than 16 times down-regulated in the ABA-treated fruit at 14 d of cold storage (Table 3).

The c-repeat/DRE binding factor (CBF) family and the so-called cold-responsive (COR) genes are involved in plant cold acclimation. We found that *Cp4.1LG03g06610*, homologous to the TF Inducer of CBF Expression 1 (ICE1) was up-regulated in response to ABA after 5 and 14 d of cold storage, although it was also induced in the control fruit at 5 d (Table 4). There are other two ICE1-like genes in the *C. pepo* genome, *Cp4.1LG04g06330* and *Cp4.1LG09g09410*, although they did not specifically respond to the ABA treatment, they were respectively induced and repressed by cold in both ABA-treated and untreated fruit (Table 4). Therefore, it seems that cold induction of some of the ICE1-like genes in the zucchini fruit is independent of ABA. Moreover, none of the CBF-like genes was found in the ABA-specific DEG list, although 13 CBF-like genes were detected in the common DEGs of ABA-treated and untreated fruit (Table 4), indicating that their expression was induced in response to cold storage independently of ABA. Only four of those CBF-like genes, *Cp4.1LG10g07720*, *Cp4.1LG02g03600*, *Cp4.1LG12g00930* and *Cp4.1LG05g14940*, were up-regulated in the fruit, but the remaining 9 CBFs were down-regulated (Table 4).

Some other transcription factors that are known to participate in the cold response in other systems were also found to be differentially expressed in the zucchini fruit in response to ABA and cold storage. *Cp4.1LG04g15890*, a DELLA-like transcription factor of the *GRAS* family, which is a negative regulator of the ICE1/CBF pathway, was up-regulated in both ABA-treated and control cold stored fruit at 5 d, although its induction was maintained in ABA-treated fruit at 14 d. The GRAS-like genes *Cp4.1LG19g09550* and *Cp4.1LG12g09770* were, however, more down-regulated in the ABA-treated fruit at 5 d of cold storage (Table 3). The ZAT gene family, some of whose members participate as positive or negative regulators of the CBFs in other species, comprised 8 genes within the ABA-specific DEG list. The ZAT-like *Cp4.1LG03g15550* was found to be strongly down-regulated at 1, 5 and 14 d of cold storage in the ABA-treated fruit (Table 3). Other ZAT genes like *Cp4.1LG02g06710*, *Cp4.1LG10g08860* *Cp4.1LG19g04400*, *Cp4.1LG16g05900*, *Cp4.1LG05g08890* were specifically up-regulated at either 1, 5 or 14 d of cold stress in response to ABA and cold storage (Table 3). Similar expression profiles were found in the 19 NAC transcription factors. The NAC-like gene *Cp4.1LG04g06830* was specifically induced in the ABA fruit after 1 day of cold storage but was later repressed at 14 d of cold storage in both control and ABA-treated fruit (Table 3). The NAC-like *Cp4.1LG19g05060* was also earlier induced by ABNA, but later it was up-regulated in both control and ABA fruit (Table 3). Taken together, the differential expression of all these transcription factor genes in the ABA-treated fruit indicate that they can function as positive or negative regulators of the ABA-mediated postharvest cold tolerance in zucchini, but that regulation seems to occur in a CBF-independent manner.

3.7. Glutamate and oxidative stress regulation in ABA-mediated cold tolerance

Glutamate has a key role in cold tolerance as was proved for the glutamate receptors *AtGLR1.2* and *AtGLR1.3* in *Arabidopsis.* In the present study, six glutamate genes were found within the ABA-specific DEGs in cold stored zucchini fruit. Three GLR genes, *Cp4.1LG06g06620*, *Cp4.1LG19g08570* and *Cp4.1LG07g06070*, were down-regulated in control and ABA-treated fruit at 1, 5 and 14 d of cold storage, although their repression seems to be higher in the ABA-treated fruit at some storage times. Moreover, fruit induced the production of glutamate in response to ABA, since *Cp4.1LG08g13340* and *Cp4.1_scaffold000895g00020*, which encode for a glutamate synthase and a glutamate-glyoxylate aminotransferase 2-like, respectively, were found to be induced upon fruit cold storage, but were earlier and more highly induced in ABA-treated fruit (Table 3).

A total of 30 genes that were related with oxidative stress were identified in the ABA-specific gene list from cold stored fruit (Table 3). The catalase gene *Cp4.1LG07g05850* was down-regulated in response to cold storage in both ABA and control fruit, although it was less repressed in the ABA-treated fruit at day 5 of cold storage. Several peroxidase genes were downregulated in both control and ABA-treated fruit upon cold storage. However, the peroxidases *Cp4.1LG01g13160* and *Cp4.1LG15g08990* were found to be earlier and highly induced in the ABA-treated fruit at either 1, 5 or 14 d of cold storage. Furthermore, from 8 glutathione S-transferase genes found in the ABA-specific DEG set, only the gene *Cp4.1LG03g18240* was specifically induced by the ABA treatment at 14 d of cold storage. The rest of the glutathione S-

Table 4
CBF-related DEGs in control and ABA-treated fruit after cold storage at either 1, 5 or 14 d of cold storage.

CuGenDB ID	Functional Annotation	log2 Fold Change					
		1 d		5 d		14 d	
		Control	ABA	Control	ABA	Control	ABA
Cp4.1LG03g06610[a]	ICE1			1.92	2.15		1.56
Cp4.1LG04g06330	ICE1	0.88	0.82	1.19	1.22	0.76	0.92
Cp4.1LG09g09410	ICE1			-1.37	-0.95	-2.74	-2.86
Cp4.1LG14g05940	CBF	-6.82	-5.79				
Cp4.1LG05g06700	DREB	-8.33	-8.14	-4.67	-6.93	-6.62	-3.60
Cp4.1LG13g01840	DREB	-6.28	-6.13	-3.19	-6.99	-7.36	-3.36
Cp4.1LG05g03760	DREB	-7.12	-3.59	-1.71	-4.85	-5.96	-1.74
Cp4.1LG11g02220	DREB					-4.92	-4.50
Cp4.1LG10g07720	DREB			0.92	0.85	1.63	1.61
Cp4.1LG18g04100	DREB	-2.00	-2.26	-1.77	-1.65	-7.53	-6.95
Cp4.1LG04g10090	DREB					-5.81	-3.25
Cp4.1LG02g03600	DREB	2.60	2.87	3.88	3.89	4.30	3.63
Cp4.1LG01g00420	DREB			-2.26	-2.68	-4.38	-4.60
Cp4.1LG12g00930	DREB	4.69	5.12	5.48	5.54	4.56	4.11
Cp4.1LG05g14940	DREB					3.55	3.37
Cp4.1LG13g07480	DREB	-1.06	-1.07	-1.41	-1.53	-1.42	-1.66

[a] The gene *Cp4.1LG03g06610* is the only one that is specifically induced in the ABA-treated fruit at 14 d of cold storage. The rest of the genes are similarly induced or repressed in control and ABA-treated fruit. ICE1, Transcription factor Inducer of CBF expression 1; CBF, Cold Binding Factor DREB, Dehydration Responsive Element Binding Transcription Factor. Only statically significant gene expression changes in relation to fresh harvest fruit are indicated.

transferase genes were down-regulated in both control and ABA-treated fruit, although *Cp4.1LG14g03330* and *Cp4.1LG03g18220* were not repressed in the ABA fruit 1 day after the treatment (Table 3). Interestingly, the five glutaredoxin genes found in the ABA-specific DEG list, known to be regulators of oxidative stress response and abiotic stress tolerance in other systems, were up-regulated in both control and ABA-treated fruit, but the induction was higher in response to the ABA treatment (Table 3). On the other hand, three respiratory burst oxidase homologues (RBOHs) genes, which integrate Ca^{2+} and ROS signaling pathways, were highly induced in the ABA-treated fruit immediately after the cold storage, and the *Cp4.1_scaffold000170g00090* was only induced in ABA-treated fruit at 1, 5 and 14 d of cold storage (Table 3).

3.8. Regulation of membrane and cell wall degradation in response to ABA and cold

Major adverse effects of cold stress are associated with membrane damage, in such a way that the mechanisms that maintain the fluidity and integrity of membranes can be responsible for cold tolerance. The fluidity is better maintained in membranes enriched in unsaturated fatty acids. In this sense, we detected two genes for fatty acid desaturases, *Cp4.1_scaffold002129g00010* and *Cp4.1LG08g13790*, that were induced at 1 and 5 d of cold storage, although they were later repressed at 14 d of cold storage. Moreover, eight phospholipases (PL), responsible for degradation of membrane phospholipids and to produce phosphatidic acid (PA) as a secondary messenger molecule in cold stress, were identified in the ABA-specific DEG list. The phospholipase A1 *Cp4.1LG15g03050* was only repressed by ABA at 5 d of cold storage, and the expression of the other PLs also changed in response to cold storage (Table 3). No lipoxygenase (LOX), which catalyses the generation of hydroperoxy derivatives from polyunsaturated fatty acids, was identified in the ABA-specific DEG list.

Genes coding for cell wall-degrading enzymes were also differentially regulated by ABA during the postharvest cold storage of zucchini fruit. The down-regulation of the pectate lyases *Cp4.1LG15g01230* and *Cp4.1LG01g23700*, glucan endo-1,3-beta-glucosidases *Cp4.1LG11g10550*, *Cp4.1LG09g07400*, *Cp4.1LG05g00510* and *Cp4.1LG03g12250*, the xyloglucan endotransglucosylase/hydrolase *Cp4.1LG03g01540* and the expansin B3-like *Cp4.1LG05g15010* was higher in the ABA-treated fruit upon cold storage (Table 3), suggesting that the repression of these enzymes may reduce cell wall degradation and fruit softening during postharvest storage.

Moreover, an induction of Long-Chain Acyl-CoA Synthetase (LACS) genes (*Cp4.1LG13g01110, Cp4.1LG08g04330*, *Cp4.1LG02g03430* and *Cp4.1LG04g04220*), that are involved in the biosynthesis of wax cuticle, as well as a repression of lignin biosynthesis enzymes, as well as the cinnamate-4-hydroxylase gene *Cp4.1LG16g06940* and Cinnamoyl-CoA reductase genes *Cp4.1LG04g06600* and *Cp4.1LG16g01990,* were identified in the zucchini fruit in response to ABA (Table 3).

4. Discussion

Zucchini fruit is very sensitive to chilling injury, displaying postharvest deterioration and reduced market value after storage at temperatures below 10 °C (Valenzuela et al., 2017). In this paper we have confirmed previous reports indicating that ABA was able to induce a cold tolerant response in the zucchini fruit, resulting in a reduction in surface pitting, fruit weight loss and electrolyte leakage (Carvajal et al., 2017). Cold storage induces numerous transcriptomic changes in zucchini fruit, but the molecular mechanisms involved in ABA-induced cold tolerance still need to be explored. In this paper we found that ABA-mediated chilling tolerance was related to more specific and narrowed transcriptomics changes than those produced by the postharvest cold storage itself. However, several pathways and genes have been discovered that were exclusively or more down- or up-regulated in response to the ABA treatment; these can be responsible for the ABA-induced postharvest cold tolerance.

4.1. Hormone and Ca^{2+} signalling pathways in ABA-mediated postharvest cold tolerance

GO and KEGG enrichment analyses have indicated that the postharvest ABA treatment on zucchini impaired hormone and Ca^{2+} signaling pathways. ABA biosynthesis and signaling genes were up-regulated in response to the postharvest ABA treatment in cold stored fruit. Moreover, genes in the ethylene and jasmonate (JA) signaling pathways were induced, while those in the auxin and brassinosteroid pathways were repressed in response to ABA-treatment and cold storage, suggesting that JA and ethylene are positive regulators while auxin and brassinosteroids are negative regulators of ABA-mediated cold tolerance in zucchini fruit. External treatments with JA and ABA were known to reduce postharvest CI symptoms in zucchini (Wang and Buta, 1994; Carvajal et al., 2017) and other fruit (Ding et al., 2002; Zhang et al., 2019). Both ABA and JA can induce the cold defensive response through both CBF-dependent and CBF-independent pathways (Hu et al., 2013; Sharma and Laxmi, 2016; Eremina et al., 2016). However, ethylene was reported to be a positive regulator of CI in this species, since 1-MCP treatments and ethylene insensitive mutations are able to alleviate postharvest CI (Megías et al., 2016; García et al., 2020). Although it is not excluded a possible positive role of ethylene in ABA-induced cold tolerance of zucchini during the first d of cold storage, the observed induced ethylene response was observed at 5 and 14 d of cold storage and could be likely associated with the cold-induced ethylene production occurring in the zucchini fruit as a symptom of CI (Megías et al., 2014, 2017). In other plants, ethylene also mediates a general mechanism by which plants respond to cold stress, including fruit during cold storage (Pons et al., 2014; Lado et al., 2015; Pareek et al., 2014), but the question whether it has a positive or a negative regulatory role in cold tolerance is not yet clear (Eremina et al., 2016). The role of auxins and brassinosteroids (BR) as negative regulators of postharvest cold tolerance was not documented in zucchini. In other species, however, pre- and post-harvest BR treatments increase cold tolerance in either seedlings or fruit (Ali et al., 2019; Jiang et al., 2013; Li et al., 2018; Singh et al., 2012), but its positive control of cold stress response is not well-defined (Kim et al., 2010). Less is known on the role of auxin in postharvest cold stress response, but auxin concentration and distribution are altered by inhibiting auxin transporters such as PIN3 (Shibasaki et al., 2009). In zucchini, we have also found that ABA and cold hardly repress the expression of different PIN-like auxin efflux carriers like *Cp4.1LG09g03360* and *Cp4.1LG14g10690* (Table S1) which may alter the distribution of auxin in the fruit. This crosstalk between ABA and auxin in cold response was also reported in rice, where ABA deficient mutants had reduced IAA content and exhibited increased cold tolerance (Du et al., 2012, 2013).

Ca^{2+} has been recognized to play a key role as a second messenger coupling various environmental and hormonal stimuli to plant adaptive response, including cold (Hepler, 2005). The ABA postharvest treatment of the zucchini fruit before cold storage clearly mispaired Ca^{2+} signaling, suggesting that ABA-induced cold tolerance is mediated by Ca^{2+}. A high number of Calmodulins (CaMs), Calmodulin binding proteins (CaMBPs) and calcium dependent protein kinases (CDPKs), which are major Ca^{2+} sensors, were specifically up- or down-regulated in response to ABA treatment in the refrigerated fruit. CaM and CaMBP are regulated by ABA in response to different environmental stimuli (Delk et al., 2005; Xu et al., 2011; Zeng et al., 2015), and the ABA pathway is interconnected with Ca^{2+} on different levels of signal processing (Edel and Kudla, 2016). Therefore, the CaM and CaMBPs found in the ABA-specific set of DEGS are likely involved in transducing Ca^{2+} signals in response to ABA and cold storage of zucchini fruit. The down- and up-regulation of CaM genes in the ABA-treated fruit also indicates that they can act as negative or positive regulators of ABA signaling. Many Arabidopsis CaM and CaM-like participate as positive regulators of cold

response (Zeng et al., 2015), but others like CaM3 function as negative regulators of cold responsive genes (Townley and Knight, 2002). The specific up-regulation of the CDPK *Cp4.1LG09g03630* and the down-regulation of *Cp4.1LG09g07900* in ABA-treated fruit also indicate that these Ca^{2+} sensors are positive and negative regulators of ABA-mediated cold tolerance in zucchini fruit. CDPKs participate in many phytohormone signaling pathways, especially in Ca^{2+} -mediated ABA signaling pathway (Shi et al., 2018a, 2018b), where they can act as positive or negative effectors of Ca^{2+} signaling (Choi et al., 2005; Mori et al., 2006; Geiger et al., 2010; Franz et al., 2011;) and have distinctive functions in different abiotic stresses (Boudsocq and Sheen, 2013; Shi et al., 2018a, 2018b).

Regarding downstream effectors of Ca^{2+} signaling, two Calmodulin Receptor Like Kinase (CRLK) genes were found to be up- and down-regulated in the ABA-treated and cold stored zucchini fruit. These two genes share a high homology with Arabidopsis Ca^{2+}/CaM-regulated *CRLK1* and *CRLK2*, two kinases that specifically activate a MAP kinase cascade, including MPK6 and MPK4, that elicits the cold response (Yang et al., 2010; Zhao et al., 2017). Given that we also found that several MAPK genes were up-regulated in the fruit in response to ABA and cold treatment, it is likely that zucchini CRLKs also modulate the ABA-induced cold response in the fruit by means of the MAP kinase signaling pathway. One of the three genes that were specifically down-regulated by ABA at either 1,5 and 14 days of cold storage (*Cp4.1LG11g11200*), and that encoded for an Avr9/Cf-9 rapidly elicited protein, could also be related to multiple MAP kinase cascades that can interlink not only biotic but also abiotic stresses (Romeis et al., 1999).

4.2. CBF and other transcription factors in ABA-mediated postharvest cold tolerance

Expression profiles of ICE1- and CBF-like genes under cold stress have indicated that the ICE1/CBF pathway is not essential for ABA-induced cold tolerance in zucchini fruit. In Arabidopsis, ICE1 is one of the transcription factors that induce CBF genes (Chinnusamy et al., 2003; Ding et al., 2015). CBFs are then able to bind to the promoter of the COR genes, so activating plant response to cold stress (Shi et al., 2018a, 2018b; Ding et al., 2019). Only one ICE1 was induced in response to ABA, although a number of ICE1- and CBF-like genes responded to cold in both ABA-treated and untreated fruit. These data indicate that although ICE1/CBF pathway can play an active role in conferring postharvest cold tolerance in Zucchini fruit, the response is not dependent on ABA but is directly induced by cold stress. Some other CBF-like genes in the *C. pepo* genome did not change their expression patterns in response to ABA and cold storage, or were even down-regulated, suggesting that not all CBF genes were involved in the cold response, at least in the fruit. This differential regulation of CBF-like genes was also found in other fruit like grape and peach, where some of the CBF-like genes respond to cold stress and others not (Liang et al., 2013; Vazquez-Hernandez et al., 2017). In citrus, the expression of CBF1-like gene, but not that of CBF2- and CBF3-like, was correlated with a reduction in fruit chilling injury (Salvo et al., 2021).

The different transcription factors that were found to be regulated by ABA in the zucchini fruit do not seems to connect ABA and Ca2 + signaling with CBF pathway as described in other systems (Eremina et al., 2016). Among the transcription factors that were specifically induced by the postharvest ABA treatment it is worth mentioning the Ethylene-responsive transcription factor *Cp4.1LG01g14110*, the MYB-like factors *Cp4.1LG07g02300*, *Cp4.1LG19g10410*, *Cp4.1LG10g09350* and *Cp4.1LG02g04920*, the MYC2-like factor *Cp4.1LG08g08520* and the ZAT factors *Cp4.1LG05g08890* and *Cp4.1LG02g06710*, while among those that are only repressed in ABA-treated fruit include the BZIP *Cp4.1LG07g09470*, MYB-like *Cp4.1LG16g01100*, *Cp4.1LG01g23480* and *Cp4.1LG01g22460* factors, the GRAS-like factor *Cp4.1LG19g09550*, NAC like factors *Cp4.1LG16g07040* and *Cp4.1LG20g00280* and the ZAT-like *Cp4.1LG16g05900*. All these transcription factors respond specifically to ABA and should act as positive or negative mediators of ABA-induced postharvest cold tolerance. *Cp4.1LG01g14110* is highly homologous to *WRINKLED1* (*WRI1*), a AP2 transcriptional activator involved in the biosynthesis of fatty acids, triacylglycerol and cuticular waxes in seeds and plant organ surface, including fruit (Cernac and Benning, 2004; Hao et al., 2017). This *C. pepo WRI1* gene could be responsible of activating the Long-Chain Acyl-CoA Synthetase (LACS) genes observed in the ABA-treated zucchini fruit (Table 3), so taking part in biosynthesis of fruit wax cuticle and alleviating the water loss associated with postharvest cold storage of zucchini fruit. On the other hand, the specific activation of MYC2, a central factor in jasmonate signal transduction pathway, but also in the crosstalk with other hormones, including ABA (Kazan and Manners, 2013), could be likely involved in postharvest cold tolerance in zucchini as observed in other systems like apple (Wang et al., 2019) and tomato (Ding et al., 2021). ZAT genes are known to be positive and negative regulators of cold tolerance by CBF-dependent and CBF-independent pathways (Liu et al., 2019). In the zucchini fruit, however, the ABA-induced and repressed ZATs are likely to participate in cold tolerance in a CBF independent manner. Among the transcriptional repressors of fruit cold tolerance, *Cp4.1LG16g01100* and *Cp4.1LG01g23480* are MYB330- and MYB308-like transcriptions factors of Arabidopsis that repress phenolic acid metabolism and lignin biosynthesis (Tamagnone et al., 1998). We have also observed a repression of lignin biosynthesis enzymes cinnamate-4-hydroxylase and Cinnamoyl-CoA reductase in the Zucchini fruit in response to ABA (Table 3). On the other hand, the transcriptional repressor *Cp4.1LG01g22460* is homologous to MYB59, which has been reported to be involved in plant growth and stress responses by acting as negative regulator of Ca^{2+} homeostasis and signaling in Arabidopsis (Fasani et al., 2019).

4.3. Oxidative stress avoidance and reduced membrane and cell wall damage in response to ABA and cold storage

At high concentration ROS can induce oxidative stress and damage of different type of biomolecules, but at moderate and low concentrations, ROS can serve as signal transduction molecules to control a number of biological processes, including biotic and abiotic tolerance responses (Valenzuela et al., 2017; Waszczak et al., 2018). The induction of three respiratory burst oxidases (RBOH) indicates that the ROS produced by these enzymes can act as a second messenger in ABA and Ca^{2+} signaling pathways during postharvest cold storage of the zucchini fruit. RBOHs are key signaling nodes which integrate different signal transduction pathways with ROS signaling in response to biotic and abiotic stimuli, and in the control of different developmental processes (Suzuki et al., 2011; Mhamdi and Van Breusegem, 2018).

On the other hand, ABA and cold storage induced the plant defense system against oxidative stress. DEGs for antioxidant enzymes in the ABA-treated fruit, including peroxidases, glutathione S-transferases and glutaredoxins, can counteract the increase in ROS after a long cold storage time and protect cells from oxidative damage. Glutathione S-transferases (GSTs) inactivate reactive molecules by the conjugation of glutathione (GSH) and their overexpression enhances cold and other abiotic stress tolerance (Kumar and Trivedi, 2018). However, it has been reported that certain GSTs have a negative role in abiotic stress tolerance, as found for the Arabidopsis GSTU17 (Chen et al., 2012), as may occur for some of the GSTs that were found to be negatively regulated by ABA and cold during zucchini postharvest storage. Some peroxidases were also down-regulated in response to ABA and cold, but those that were induced in response to ABA-treated and cold stored fruit may also neutralise the harmful effect of ROS on cell damage. However, all the glutaredoxins found in the ABA-specific list were induced upon ABA treatment and cold storage. Glutaredoxins comprise a family of oxido-reductases which use glutathione to reduce disulfide bonds of target proteins and to maintain cellular redox homeostasis. They participate in

biotic and abiotic stress tolerance responses, including chilling stress, and in different developmental processes (Rouhier et al., 2008; Hu et al., 2015). The earlier or specific induction of glutaredoxins Cp4.1LG02g05990 and Cp4.1LG04g01400 in zucchini fruit in response to ABA suggests that these enzymes can mediate the ABA-induced cold tolerance response in the fruit by reducing oxidative damage.

Cp4.1LG05g02160, one of the three ABA-specific genes that were down-regulated in the fruit during the whole cold storage period, is a ferredoxin (FD), a important protein for electron transfer in a wide range of metabolic reactions, but also for redox signaling and maintenance of redox balance. The function of FD in plant abiotic stresses response is still unclear. The overexpression of Fd in plants confers tolerance to biotic and abiotic stresses, probably by inducing ROS signaling (Huang et al., 2020), but the level of Fd transcripts and protein is repressed under environmental stresses in some plants (Zhang et al., 2015), as occurs for Cp4.1LG05g02160 in the zucchini fruit under cold storage. The future silencing of *Cp4.1LG05g02160* gene will give us a more precise view of its role on postharvest cold tolerance.

The enhanced cold tolerance of ABA-treated zucchini fruit, including reduced ion leakage, was also found to be associated with membrane and cell wall protection. The increase in fatty acid unsaturation levels, mediated by the activation of fatty acid desaturases (FADs), can likely adjust membrane fluidity in response to low temperature (Uemura et al., 1995). In fact, the overexpression of FADs induces cold tolerance in different plant species (Nishida and Murata, 1996; Orlova et al., 2003). Moreover, as occurs in zucchini, several FAD genes are transitorily induced in cucumber as a short-term response to cold, although their expressions are reduced in the long-term (Dong et al., 2016). On the other hand, the remodeling of membrane lipids by phospholipases (PLs) also plays a critical role in cold tolerance. PLs catalyse the hydrolysis of membrane phospholipids to phosphatidic acid (PA) and free head groups, serving PA and derived products as second messengers in biotic and abiotic stress defensive responses (Hou et al., 2016). Nevertheless, the hydrolysis of membrane phospholipids can also compromise membrane stability and integrity, triggering chilling injury (Liu et al., 2020). Therefore, the up- and down-regulation of different phospholipases in the ABA-treated and cold stored fruit suggests that they can hydrolyse different lipid species and have different functions in ABA-mediated cold response. So, the specific down-regulation of *PLA1 Cp4.1LG15g03050* in the ABA-treated fruit may avoid membrane damage and subsequent chilling injury occurrence, as occurs in cucumber (Mao et al., 2007) or banana (Liu et al., 2020). The suppression of *PLDα1* induces freezing tolerance in Arabidopsis by modulating the cold-responsive genes and accumulation of osmolytes (Rajashekar et al., 2006). Similarly, cell wall hydrolases, including six from a total of seven Glucan endo-1, 3-β-glucosidase DEGs, one expansin and two pectate lyases, were down-regulated in response to ABA treatment and cold storage, suggesting that ABA-induced cold tolerance is mediated by cell wall protection (Carvajal et al., 2015). The down regulation of pectin biosynthesis key enzyme UDP-D-glucuronate 4-epimerase 4 (Cp4.1LG01g03670) during the whole storage period also suggested a reduced pectin synthesis or cell wall regeneration during postharvest cold storage of zucchini fruit (Zhang et al., 2006).

4.4. Glutamate's response to cold storage

Glutamate is a protein amino acid that has an impactful role in plant growth and stimuli response (Qiu et al., 2020; Li et al., 2019). Different biotic and abiotic response processes are mediated by glutamate, including cold stress (Qiu et al., 2020). Moreover, glutamate achieves its regulatory functionality through GLRs, which are the glutamate receptors present in plants similar to those ionotropic glutamate receptors in animals (Lam et al., 1998; López-Bucio et al., 2018; Qiu et al., 2020). As previously reported, *Arabidopsis thaliana* seedlings mutants *glr1.2* and *glr1.3* resulted to be sensitive to cold stress, while the overexpression of *AtGLR1.2* and *AtGLR1.3* improved the chilling tolerance through higher levels of endogenous jasmonate and downstream enhancement of the CBF cold response pathway (Zheng et al., 2018). The same occurs for *GLR3.3* and *GLR3.5* in *Solanum lycopersicum* (Li et al., 2019). In ABA-treated and cold stored zucchini fruit we found three GLR coding genes, but all were down-regulated in response to ABA and cold, suggesting that these three genes are regulated by ABA, but they seem to function as negative regulators of ABA-induced cold tolerance. Further research would be necessary to assess the mechanism of action of these glutamate receptor genes in zucchini cold tolerance.

5. Conclusion

The transcriptomic changes of *C. pepo* fruit in response to ABA application and cold storage have demonstrated that the ABA-induced postharvest cold tolerance is mediated by several specific pathways, including Ca^{2+} and MAPK signaling components, specific transcription factors excluding CBFs, and membrane and cell wall degrading enzymes. The regulation is also enhanced by jasmonate and ethylene and inhibited by auxin and brassinosteroids. All these identified genes are susceptible to be used by biotech approaches for improving the postharvest cold tolerance in zucchini.

CRediT authorship contribution statement

Álvaro Benítez: Investigation, Software, Formal analysis, Visualization, Writing – original draft. **Jessica Iglesias:** Investigation. **María Segura:** Investigation, Visualization. **Fátima Carvajal:** Investigation. **Francisco Palma:** Investigation. **Dolores Garrido:** Conceptualization, Funding acquisition. **Cecilia Martínez:** Investigation. **Manuel Jamilena:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.112023.

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