



Article

Gene Expression in Zucchini Fruit Development

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Abstract: The study of fruit development in zucchini via gene expression has proven to be applicable in breeding programs. Phenotypic and transcriptomic studies of fruit set and parthenocarpy have been previously developed and some relevant genes have been reported. From these studies, three genotypes (MUCU-16, Whitaker, and Cavili) and six genes (*CpAUX22*, *CpIAA4*, *CpIAMT-1*, *CpPIN5*, *CpCYCD6-1*, and *CpEXPLB1*) were selected. The expression of these genes was analyzed in each genotype under three different treatments (pollination, auxin-treatment and non-treatment) during one week post anthesis. Also, a phenotyping analysis was conducted. The different nature of the samples and the genes selected allowed associations between different fruit traits and fruit development stages. There was a rapid response of *CpAUX22* and *CpIAA4* to the auxin treatment. Also, these genes and the *CpIAMT-1* became more overexpressed in pollinated samples over time. The *CpPIN5* gene increased its expression over time in all genotypes while *CpCYCD6-1* was overexpressed in the early stages of fruit development in all samples. The *CpEXPLB1* was highly up-regulated in non-treated samples, suggesting a relationship with fruit abortion. The overexpression of *CpAUX22* and the non-overexpression of *CpEXPLB1* in early stages may be associated with fruit growth in zucchini.

Keywords: expansin; auxin; fruit set; parthenocarpy; squash; *Cucurbita*



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1. Introduction

One of the most economically valuable morphotypes of the *Cucurbita pepo* specie, Zucchini (*Cucurbita pepo* subsp. *pepo*) is harvested immature, unlike other squashes [1]. If the plant has been pollinated, artificially induced (e.g., by auxins application), or if it is a parthenocarpic genotype, the growth of the zucchini to a marketable size occurs within 3–4 days post anthesis (dpa) [2]. With regard to pollination, the fruit set could be negatively affected by unfavorable environmental conditions, such as low/high temperature or inadequate humidity, which affects pollen viability [3]. Moreover, zucchini pollination depends on insects that during winter conditions are less active, so the application of growth regulators such as hormones are common practices with low temperatures [2]. Nevertheless, there are alternatives that overcome this problem, such as parthenocarpic genotypes, which develop marketable-sized fruits in absence of fertilization. There are fewer examples of vegetative parthenocarpy in zucchini than in other cucurbits such as cucumber [4]. Some commercial hybrid zucchini genotypes that presented high fruit growth without pollination are Argo, Cavili, and Partenon [5]. Furthermore, zucchini accessions CpCAL112, CM-37, E-27, PI261610, and V-185 with high parthenocarpic potential have also been identified [5]. The vegetative parthenocarpy of these accessions differs from the hybrids as it is not associated with the conversion of the females into bisexual flowers or with the attached flowers phenomena [5]. Likewise, the important role of hormones during fruit set of zucchini has been demonstrated, establishing the activating role of auxins and gibberellins against the inhibitory role of ethylene [2,5,6].

A plant that produces fruit of a large caliber and a marketable shape, without pollination, is often referred to as vegetatively parthenocarpic, although this development can also be induced by hormones (induced or artificial parthenocarpy) [7]. In parthenocarpic fruits, there is no seed formation that is obtained through triploid induction, which is also an important trait in other cucurbits such as watermelon [8]. Additionally, it is possible to find fruit development with aborted seeds (stenospermocarpy), as in table grapes [9]. In this sense, different methods that result in the development of fruit according to market standards, without pollination, do not activate the same physiological process and could be controlled by different genes.

Hormone regulation and parthenocarpy are two key factors to consider in the development of a marketable fruit. Functional analysis of RNA-seq data has revealed different candidate genes that are involved in fruit set and could be useful as markers for parthenocarpic selection [6]. From this study, six genes that showed differential expression in zucchini fruit set have been selected. These genes were *CpAUX22* (auxin-induced protein AUX22-like), *CpIAA4* (auxin-responsive protein IAA4-like), *CpIAMT-1* (indole-3-acetate O-methyltransferase 1-like), and *CpPIN5* (auxin efflux carrier PIN-FORMED 5-like), and they were related to auxin pathways, while *CpCYCD6-1* (cyclin D6-1-like) was related to cell division and *CpEXPLB1* (expansin-like B1) was related to cell expansion. Both *CpAUX22* and *CpIAA4* are considered short-lived transcriptional factors that rapidly respond to changes in auxin levels. These genes belong to the AUX/IAA superfamily, which have domains that interact with auxin response factors (ARFs), regulating numerous processes in plant development, such as fruit ripening [10]. The *CpIAMT-1* gene encodes a protein that catalyzes the methylation of plant hormone indole-3-acetic acid (IAA) that controls the IAA activity, which regulates plant development and auxin homeostasis [6]. PIN-FORMED (PIN) proteins are auxin-specific efflux carriers localized in auxin transport-competent cells [11]. Unlike other types of the PIN-FORMED (PIN), PIN5 may not have a direct role in cell-to-cell transport, and it seems to regulate intracellular auxin homeostasis and metabolism [12]. D-type cyclins play an important role in the cell cycle responses of external signals by forming the regulatory subunit of cyclin-dependent kinase complexes [13]. In Arabidopsis, the CYCD6-1 is involved in cell cycle regulation and its expression alteration produces division defects [14]. The expansins are required in many plant physiological development aspects from germination to fruiting [15]. These proteins cause loosening and extension of plant cell walls by disrupting non-covalent bonding between cellulose microfibrils and matrix glucans [16].

The objective of this work is the gene expression study of six selected genes (*CpAUX22*, *CpIAA4*, *CpIAMT-1*, *CpPIN5*, *CpCYCD6-1* and *CpEXPLB1*), in three *Cucurbita pepo* subsp. *pepo* genotypes (MUCU-16, Whitaker, and Cavili) under three treatments (unpollinated, pollinated, and treated with auxins) for one week. Moreover, the expression profiles and the phenotypic analysis could be associated with different phases of zucchini fruit development and fruit growth until a marketable size.

2. Materials and Methods

2.1. Plant Materials, Growing Conditions, and Treatments

Cucurbita pepo subsp. *pepo* MUCU-16 (COMAV-UPV) and Whitaker (New York State Agriculture Experiment Station) and the hybrid Cavili (Numhens BV) were used for both phenotypical and expression analysis. The plants were grown in 25 L pots filled with perlite in a greenhouse at the IFAPA research center in Almeria (Spain). The temperature range was 12–30 °C and the relative humidity was 60–80%, controlled by an automatic cooling system. The temperature was under 30 °C to avoid the attached flower phenomenon in Cavili. This trait occurs when the fruit is large enough to be harvested but it still retains the flower, and it is usually favored by high temperatures.

Plants were randomly distributed in lines of 30 plants in the greenhouse, 3 lines per genotype, avoiding the environmental effect of the border with plants that were not included in the analysis. This system was carried out in duplicate. Among both trials,

a total of 180 plants of the Cavali, Whitaker, and MUCU-16 genotypes were planted and developed for 1 month following standard local practices for plant nutrition and disease control. This number of plants was necessary to ensure the synchrony between the female and the male flowering at the same day for each genotype and treatment. The pollination, synthetic auxins (0.5 mL of 0.8% Fruitone SL, Bayer CropSciences) and control (0.5 mL distilled water) treatments were applied similar to Pomares-Viciano et al. 2017 [2]. Only one fruit was obtained per plant, the rest of the plant fruits were removed before and after the treatment.

Market standards for length and weight categorized as S (small: 7–14 cm; 50–100 g), M (medium: 14–21 cm; 100–225 g), and L (large: 21–30 cm; 225–450 g) were considered according to the zucchini exportation standards of Spain. Fruit weight, length, and its central and apical diameter were measured on at least 8 fruits collected on 1 day pre-anthesis and from 0 to 5 dpa for the three treatments (pollination, auxin-treated, and control). The relation of the apical versus central diameter ($\text{cm}_{\text{apical}}/\text{cm}_{\text{central}}$ or %Apical) showed the rounded shape of the fruits. Also, the mesocarp width ($\text{cm}_{\text{mesocarp}}/\text{cm}_{\text{central}}$ or %Mesocarp) was measured because it was the tissue used for RNA extraction. All the fruits used in the phenotypical characterization and in the RNA extraction were treated and harvested in a time span of two weeks in order to increase the correlation between both analyses. The selected fruits for the fruit phenotype comparison were the most representative for each genotype and treatment. The data of the pollinated and auxin-treated fruits were relativized with the mean of the untreated control to allow the comparison of the treatment effect between genotypes.

2.2. Gene Selection, Sample Collection, and RNA Expression Analysis

Previous results from a RNA-Seq analysis in zucchini [6] pointed out some genes that could be involved in the fruit set in zucchini, and six of them were selected according to their relationship in different fruit development stages and their responses to the treatments. The *CpAUX22*, *CpIAA4*, *CpIAMT-1*, *CpPIN5*, *CpCYCD6-1*, and *CpEXPLB1* transcripts (Table 1) were targeted in the NCBI GenBank database. The design of the primers was obtained with Primer3 software and the expected size of each amplicon was checked with agarose gel (1.5%).

Table 1. Primers used for the analysis.

Gene Name ¹	Target mRNA	Primer Sequence (5'-3')		Size (pb)	Tm ² (°C)
<i>CpAUX22</i>	XM_023693420.1	GATCTTGCTGTGCTCTTGAGAAG	CTTTGTCCTCGTAAATGGGAACG	103	80.1
<i>CpCYCD6-1</i>	XM_023657092.1	CAAACAGAGCACAATCTCTTCG	TCCCAAGATGAGAGATTCCATTC	93	78.3
<i>CpEXPLB1</i>	XM_023698474.1	GCGACTTTATAATGACCCGAAG	AATAACACCGAGGGCTAACAAA	89	83.5
<i>CpIAA4</i>	XM_023671421.1	AGGAACACCCCTCAATCAAAGA	GGAGAAGCTCAGGGTAACCTTTG	140	82.4
<i>CpIAMT-1</i>	XM_023680319.1	TTCCCGTGTATGCTCCTAGTTTG	TCGTCAGGCTGGTTCACTACTA	124	80.5
<i>CpPIN5</i>	XM_023683116.1	GGGAATCCCAACAATCTGTG	GGAGGGCCATTAACCTTTC	103	79.1

¹ *CpAUX22* (auxin-induced protein AUX22-like), *CpIAA4* (auxin-responsive protein IAA4-like), *CpIAMT-1* (indole-3-acetate O-methyltransferase 1-like), *CpPIN5* (auxin efflux carrier PIN-FORMED 5-like), *CpCYCD6-1* (cyclin D6-1-like), and *CpEXPLB1* (expansin-like B1). ² Melting temperature. UFP (ubiquitin fusion protein) and EF-1 α (elongation factor- α) were used as reference genes [17].

Samples from one day pre-anthesis, 0 dpa (6 h after treatments), 1 dpa, 3 dpa, 5 dpa, and 7 dpa were collected in the same day. The mesocarp samples were obtained from three parts of each fruit (apical, central, and basal) due to the polarization and the gradient expression of some genes involved in fruit development [14]. Three out of five of the most representative fruits for each treatment and genotype were visually pre-selected prior the tissue extraction. The mesocarp samples extracted from recently plant-removed fruits were instantly frozen in liquid nitrogen. A total of nine samples (the three mesocarp areas from the three most representative fruits) were mixed in three biological replicates before RNA extraction. Samples were homogenized with a mortar with liquid nitrogen and the RNA was extracted using TRIzol reagent (Ambion, Thermo Fisher Scientific, Waltham,

MA, USA). The RNA quality and the concentration were checked by electrophoretic gel and NanoDrop2000c (Thermo Scientific, Waltham, MA, USA). To remove the remaining genomic material, DNase I (Invitrogen, Waltham, MA, USA) was applied and transformed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). The obtained cDNA was quantified in a LightCycler 96 (Roche Diagnostic, Basel, Switzerland) using the PowerUP SYBR green master mix kit (Applied Biosystems, Waltham, MA, USA) with three technical replicates. All the reagents were used following manufacturer's instructions. The qPCR conditions were: 50 °C 2'; 95 °C 10'; 40 × (95 °C 15'', 60 °C 1''); and melting 95 °C 15'', 60 °C 1', 95 °C 1''. Similar melting peaks curves for the same gene for the different samples were checked with Tm calling analysis (LightCycler 96 Software, Basel, Switzerland).

2.3. Statistical Analysis

Significant differences were obtained by analysis of variance (ANOVA, $p < 0.05$), and they were indicated with letters by Tukey HSD all-pairwise comparison test, using software Statistix v.9 (Tallahassee, FL, USA). Data normal distribution and homoscedasticity were checked by Shapiro–Wilk and Levene tests. The fruits obtained from the non-treated Whitaker that reached large caliber (16%) were not incorporated into the phenotype analysis in order to increase the correlation with the gene expression analysis, as none of them appeared in this test. For the RNA quantification, the data was processed with efficiency correction, reference gene normalization, and the use of error propagation rules [2,18] using UFP (ubiquitin fusion protein) and EFP-1a α (elongation factor- α) as reference genes [17]. The data obtained was expressed as normalized relative quantities (NRQ) and, prior to the analysis between treatments, the data were transformed by $\text{Log}_{10}(\text{NRQ}_{\text{treatment}}/\text{NRQ}_{\text{control}})$.

3. Results

3.1. Phenotyping of Genotypes and Treated-Fruits

The Cavili genotype and the pollination and auxin treatments produced fruits with higher length and weight compared to the Whitaker and the MUCU-16 genotypes and the untreated fruits, respectively (Figure 1). The non-treated fruits of MUCU-16 and Whitaker genotypes were of small caliber standards of length and weight after 5 dpa, while the non-treated Cavili fruits were of large caliber by 4 dpa (Figure 1). The fruit growth until large caliber of weight and length was observed in 95% of the non-treated Cavili fruits and in 16% of the non-treated Whitaker fruits. No untreated MUCU-16 fruit reached the large standards.

On the other hand, the auxin-treated and pollinated Cavili fruits experienced such rapid exponential growth that it exceeded market standards of weight from 4 dpa (Figure 1). This confirmed that the auxin-treated and pollinated fruits of Cavili had only a single day of ideal marketable harvest at 3 dpa (Figure 1). Fruit growth differences among samples started at 3 dpa ($p = 0.002$). Moreover, the fruit weight and the fruit length increase had a higher value between 2 and 3 dpa ($p < 0.001$). These results suggested that fruit abortion could be detectable at 3dpa due to fruit weight and length.

To analyze the influence of genotype and treatment factors, the data were normalized with respect to the non-treated control of each genotype (Figure 2). Significant differences ($p < 0.001$) were observed among genotypes in weight and length after 5 dpa (Figure 2). The auxin-treated and pollinated fruits of Whitaker and MUCU-16 increased their differences in weight and length with respect to the control over time (Figure 2). In Cavili, these were minor variances with respect to the non-treated fruits (Figure 2). The difference in length and in weight compared to the untreated control of pollinated and auxin-treated samples was significantly higher in Whitaker from 3 dpa (Figure 2). However, no significant differences between pollination and auxin treatments were observed in fruit length and weight.

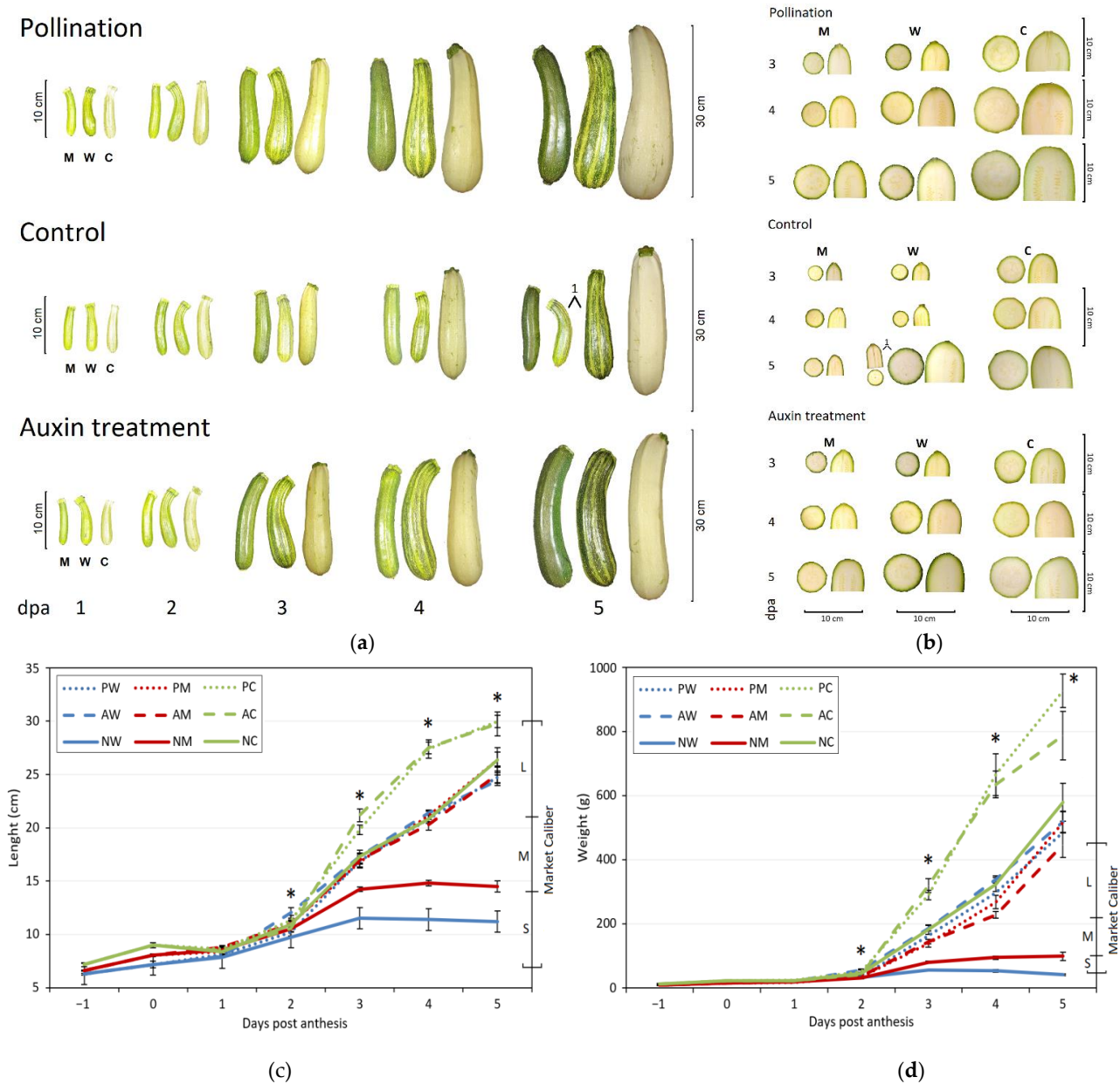


Figure 1. Morphological changes in Cavili, MUCU-16, and Whitaker genotypes after auxin, pollination, and control treatments for 5 days post anthesis. Whole fruit (a) and central (transversal cut) and stylar (longitudinal cut) sections (b). ¹ In Whitaker, both phenotypes were found, mostly the smallest phenotype (84%). Mean length (c) and weight (d) of each sample for 5 dpa with the market standards categorized in S (small: 7–14 cm; 50–100g), M (medium: 14–21 cm; 100–225 g) and L (large: 21–30 cm; 225–450 g). Pollinated (P), Auxin-treated (A), Non-treated (N) Whitaker (W), MUCU-16 (M) and Cavili (C) genotypes. * Significant differences between samples ($p < 0.005$). Error bars are standard errors ($n \geq 8$).

The auxin-treated and pollinated fruits of the three genotypes had a more rounded apical shape compared with the non-treated fruits (Figures 1 and 2). This difference increased over time (Figure 2) but without significant differences among genotypes at 5 dpa (Figure 2). This indicated that despite the non-treated Cavili fruits reaching a large market-caliber length and weight, its fruit shape was still less rounded compared to the pollinated and auxin-treated fruits. Additionally, minor differences were observed between pollination and auxin treatments 5 dpa; only the Whitaker genotype showed a significant rounder shape in pollinated fruits (Figure 2). The auxin-treated samples presented wider

mesocarp compared with the pollinated samples, with significant differences (Figure 2) in the MUCU-16 and Cavili genotypes by 5 dpa. Furthermore, the auxin- and pollination-treatment produced a higher increase in the mesocarp width in MUCU-16 than in the other genotypes (Figure 2).

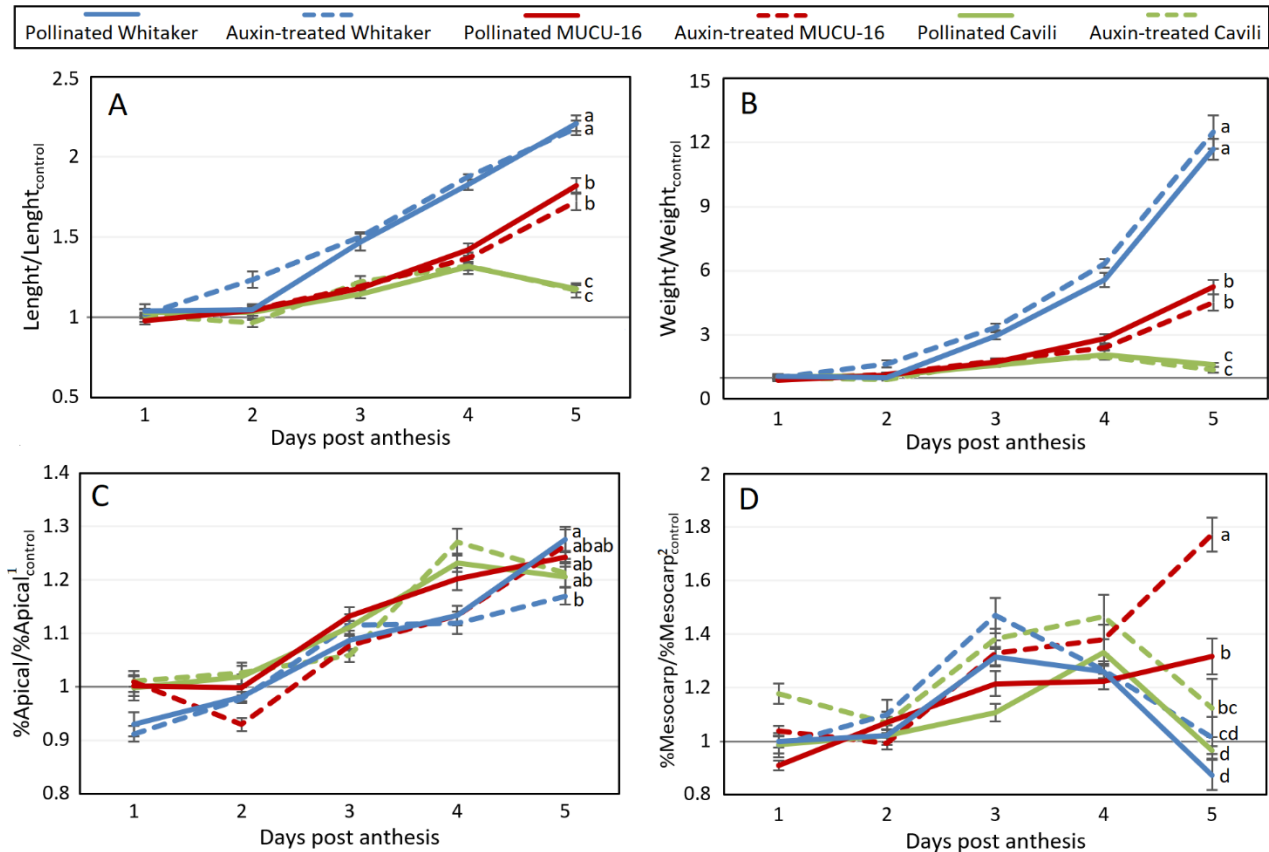


Figure 2. Fruit morphological changes versus non-treated fruits. Length (A), Weight (B), Apical shape (C) and Mesocarp width (D) of the auxin-treated (A) and pollinated (P) fruits compared with the non-treated fruits, in the Whitaker (W), MUCU-16 (M) and Cavili (C) genotypes for 5 dpa. ¹ %Apical ($cm_{apical}/cm_{central}$). ² %Mesocarp ($cm_{mesocarp}/cm_{central}$). The line corresponded with the non-treated control value of each sample. Significant differences 5 dpa (ANOVA $p < 0.05$) are indicated with letters (Tukey HSD). Error bars are standard errors ($n \geq 8$).

3.2. Gene Expression in Non-Treated Samples

Significant differences among genotypes were found in the untreated samples in most of the genes tested (Figure 3). The *CpAUX22* gene was significantly more expressed in Cavili than in Whitaker and in MUCU-16 (Figure 3). Likewise, Cavili expressed significantly more *CpIAA4* than Whitaker (0, 3, 5, 7 dpa) and MUCU-16 (0–1 dpa) (Figure 3). Moreover, Whitaker genotype expressed significantly less *CpIAA4* from 3 to 7 dpa than MUCU-16 and Cavili (Figure 3). The *CpIAMT-1* was more expressed in MUCU-16 on days 1 pre-anthesis and 0 post anthesis, and less expressed in Whitaker on days 3 and 7 post anthesis (Figure 3).

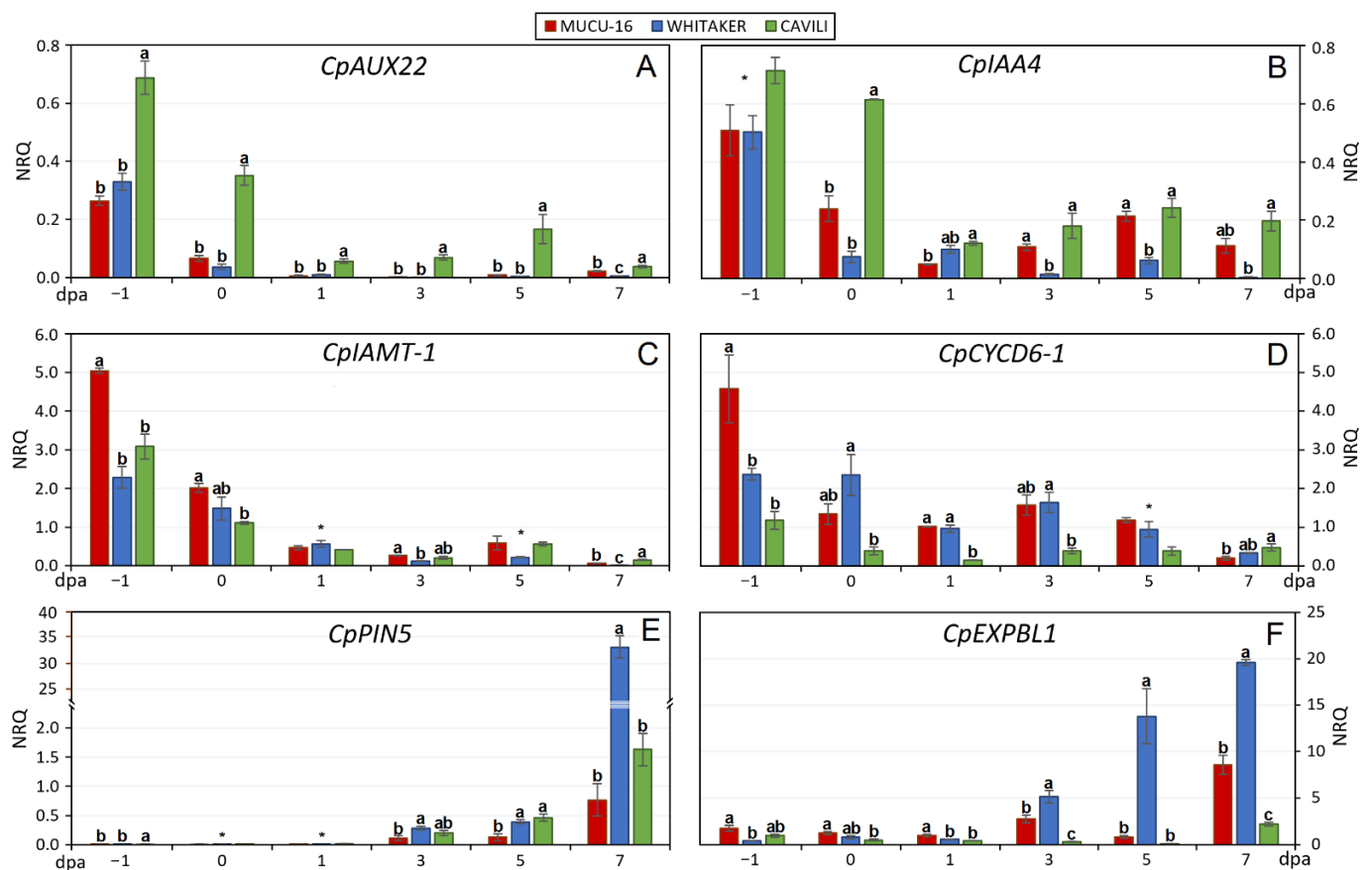


Figure 3. Normalized relative quantities (NRQ) in non-treated samples. Data represents the NRQ for genotypes MUCU-16 (red), Whitaker (blue) and Cavili (green) and days post anthesis -1 , 0 , 1 , 3 , 5 and 7 in each gen: (A), *CpAUX22* (auxin-induced protein AUX22-like); (B), *CpIAA4* (auxin-responsive protein IAA4-like); (C), *CpIAMT-1* (indole-3-acetate O-methyltransferase 1-like); (D), *CpPIN5* (auxin efflux carrier PIN-FORMED 5-like); (E), *CpCYCD6-1* (cyclin D6-1-like); (F), *CpEXPBL1* (expansin-like B1). Significant differences (ANOVA, $p < 0.05$) are indicated with letters (Tukey HSD). * No significant differences. Error bars are standard errors (n = 3).

The *CpCYCD1-6* expression in Cavili was significantly lower than in Whitaker and in MUCU-16 on 0 , 1 , and 3 dpa (Figure 3). The differences in the *CpPIN5* expression among genotypes appeared from 3 dpa (Figure 3). Besides, the *CpPIN5* expression was lower in MUCU-16 and similar between Whitaker and Cavili until 7 dpa, when *CpPIN5* became significantly more expressed in Whitaker (Figure 3). Similar to *CpPIN5*, the differences in the *CpEXPBL1* expression among genotypes were higher from 3 dpa until 7 dpa (Figure 3). The *CpEXPBL1* was significantly more expressed in Whitaker than in MUCU-16 and in Cavili, which presented the lowest *CpEXPBL1* expression from 3 dpa (Figure 3). The expression of *CpAUX22*, *CpIAA4*, *CpIAMT-1*, and *CpCYCD6-1* was higher between day 1 pre-anthesis and 0 dpa in all genotypes, while the *CpPIN5* and *CpEXPBL1* expression increased over time in all genotypes (Figure 3).

3.3. Gene Expression and Treatments

The *CpAUX22* and the *CpIAA4* genes had an analogous expression pattern in the three genotypes when pollination and auxin treatment data were normalized with the non-treatment (Figure 4). Initially, on day 0 (6 h after the treatment), both genes were significantly more expressed in the presence of auxin treatment in Whitaker and MUCU-16 (Figure 4). However, in Cavili, the response was similar between pollinated and auxin treated fruits 6 h after the treatment application (Figure 4). After 24 h, the overexpression

of *CpAUX22* and *CpIAA4* was high in both pollinated and auxin-treated samples (Figure 4). However, this initial overexpression in auxin-treated samples decreased over time and, from 5 (*CpAUX22*) and 7 (*CpIAA4*) dpa, they were significantly more expressed in pollinated samples (Figure 4). The pollinated samples maintained the overexpression of *CpAUX22* and *CpIAA4* over time, while the auxin-treated fruits decreased their expression until they reached the control levels (Figure 4). The *CpIAMT-1* gene was more expressed in pollinated and auxin-treated samples over time with respect to the non-treated. However, similar to *CpAUX22* and *CpIAA4* on 7 dpa, the expression in the pollinated samples was significantly higher than in the auxin-treated samples (Figure 4).

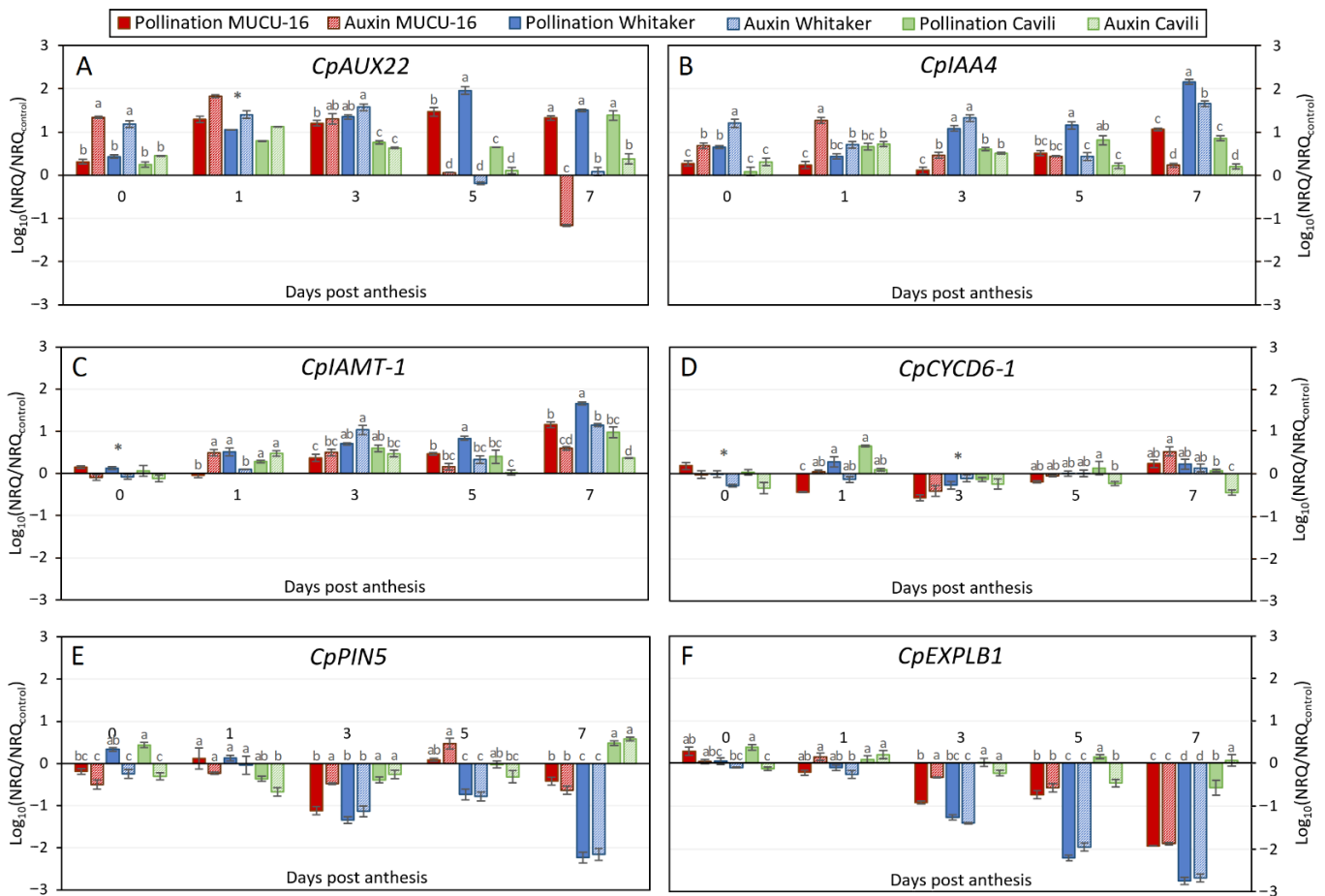


Figure 4. Differential gene expression after treatments in each genotype by day post anthesis. Data is represented by the Normalized relative quantities (NRQ) relativization of pollination and auxin-treatment versus the non-treated (NRQ control) with a logarithmic transformation: $\text{Log}_{10}(\text{NRQ}_{\text{treatment}}/\text{NRQ}_{\text{control}})$. Positive values represent more expression in each sample than in the non-treated control, while negative values represent more expression in the non-treated control. Each unit is a 10-fold expression differential. (A) (*CpAUX22*, auxin-induced protein AUX22-like), (B) (*CpIAA4*, auxin-responsive protein IAA4-like), (C) (*CpIAMT-1*, indole-3-acetate O-methyltransferase 1-like), (D) (*CpPIN5*, auxin efflux carrier PIN-FORMED 5-like), (E) (*CpCYCD6-1*, cyclin D6-1-like) and (F) (*CpEXPLB1*, expansin-like B1). Significant differences (ANOVA, $p < 0.05$) are indicated with letters (Tukey HSD). * No significant differences. Error bars are standard errors (n = 3).

The *CpCYCD6-1* gene expression was similar between treatments and their control, without an expression pattern clearly associated with any treatment or day post anthesis (Figure 4). On the other hand, *CpPIN5* and *CpEXPLB1* were down-regulated in MUCU-16 and Whitaker for both treatments, while minor differences with respect to the non-treated control were found in Cavili (Figure 4). These down-regulations were significantly higher

in Whitaker from 5 dpa in both pollinated and auxin-treated samples (Figure 4) due to the large expression in the non-treated samples (Figure 3). No significant differences were found between pollination and auxin treatment in *CpPIN5* and in *CpEXPLB1*.

4. Discussion

4.1. Fruit Development Stages

In most plants, after pollination, early fruit development can be divided into three phases: fertilization, cell division, and cell expansion [19] (Figure A1). The first phase occurs as the pollen tube reaches and fertilizes the ovule [20]. In this phase, fruit growth is slow, which is consistent with the 0–1 dpa data that did not show differences between treatments (Figure 1) [2]. Also, the fruit set is established, and the ovary could start developing with further fruit cell division or be aborted [2]. This indicates that by 3 dpa it could be deduced whether a fruit will abort, as it was easily observable due to the size differences obtained (Figure 1).

In the second phase, fruit development is primarily due to cell division, and the fruit experiences exponential growth [19]. This exponential growth can be achieved by pollination or auxin treatment, and it is applicable in other horticultural crops [21]. In this study, the exponential growth in zucchini started at approximately 2–3 dpa, and it occurred until at least day 5 in all treated fruits (Figure 1). This agreed with the observation of Pomares-Viciano et al. [2]. Also, genes such as *CpIAMT-1*, *CpPIN5*, *CpEXPBL1* started to be differentially expressed from 3 dpa (Figures 3 and 4), corresponding to the dpa when phenotypic differences among treatments were observable (Figure 1). In addition, the high initial expression of *CpCYCD6-1* (Figure 3) that decreases over time could indicate a relationship between this gene and the proliferation stage, since it has been described as a cell cycle regulator gene [14]. In cucumber, it has been observed that the cell division and the pre-exponential growth occurs between 0 and 4 days post anthesis after the pollination [22].

During the third phase, fruit growth continues, but mostly by cell expansion (Figure A1), until the fruit reaches its final development [19]. The Cavili auxin-treated and the pollinated fruits presented the fastest growth, but their exponential growth decreased at 5 dpa (Figure 2), which may be related to a phase change. Taking this into account, it was possible that auxin-treated and pollinated Cavili fruits shorten their proliferation stage in favor of the expansion stage, since they were already more developed than the Whitaker and the MUCU-16 genotypes from day 5 (Figure 1). This would explain why only at 7 dpa these fruits expressed more expansins than Whitaker and MUCU-16 only in pollinated and auxin-treated samples (Figure A2). In cucumber, there is a peak in the cell exponential expansion 8 days after the pollination [22], which may be related to these observations. In addition, results also suggested that the fruits that are going to abort do not follow the fruit development model proposed (Figure A1). The aborted fruit could be in an early expansion phase given the higher *CpEXPLB1* expression in untreated samples of both MUCU-16 and Whitaker, observable from day 3 (Figure 3).

It should be noted that in our study only one fruit was left on the plant, which may favor fruit development. Also, commercial genotypes use to have higher fruit growth than non-commercial lines, so length and weight market parameters could be reached easier without treatment, as observed with the Cavili (Figure 1). However, no significant differences among genotypes in non-treated fruits were found in the rounded shape (Figure 2), also a market desirable trait. Among the three genotypes tested here, Cavili presented the largest fruits, in agreement with Martinez et al. 2014, who suggested that Cavili presented a high parthenocarpy potential [5]. It has been reported that the pollination treatment produces more cylindrical and rounded fruits than auxin-treated and non-treated fruits in *Cucurbita pepo* [2]. Here, we have also observed the less round shape of non-treated fruits, but we have found only significant differences between pollinated and auxin treated samples in Whitaker (Figure 2).

4.2. Gene Expression Responses

No phenotypic differences among samples were found until 2 dpa (Figure 1). However, molecular differences appeared by 0 dpa, 6 h after the treatment, as occurred with the overexpression of *CpAUX22* and *CpIAA4* in auxin-treated samples (Figure 4). The differential gene expression of the targeted genes corresponded with the RNAseq observations [6]. In that study [6], after 2 dpa in zucchini, *AUX22*, *IAA4*, *IAA16*, and *IAA14* genes were more expressed in auxin-treated and in the pollinated than in the controls. Here, the *CpAUX22* expression seemed to be strongly linked to an initial auxin response and the pollination treatment, since they were highly overexpressed (more than 10-fold) with respect to the non-treated samples (Figure 4). Both *IAA4* and *AUX22* genes, as members of the AUX/IAA superfamily, are described as repressors of early auxin response, by forming heterodimers with the ARFs [23], so their quick expression could be explained in this context. The pattern under auxin treatment of rapid response of *CpAUX22* and *CpIAA4* within 6 h that decreased over time (Figure 4) has also been described in *Arabidopsis thaliana* [24]. The response to pollination could appear in 24 h, as in the case of *CpAUX22*, but this overexpression was constant until day 7 (Figure 4). Although visually both pollination and auxin treatments led to similar fruit development, the addition of hormones does not initiate the same molecular processes as pollination [25,26], as in the case of *CpAUX22*, *CpIAA4*, and *CpIAMT-1* genes (Figure 4). The results also suggested that *CpAUX22*, *CpIAA4*, and *CpIAMT-1* may be related to mature fruit development due to their higher expression in the pollinated fruits 7 dpa. Also, the *CpIAMT-1* gene could be involved in the fruit's apical round shape formation because it was more expressed in both auxin and pollination treatments than in the control for the three genotypes but without significant differences between non-treated fruits. The *CpIAA4* and the *CpIAMT-1* genes did not seem to be as closely associated to fruit growth as *CpAUX22* as this gene was also more expressed in Cavili than in the other genotypes (Figure 3). This could explain the Cavili lower peak response to the auxin treatment 0 dpa (Figure 4). In blueberry, the *AUX22* was up-regulated after the auxin treatment, and it has been associated with root growth [27], so it is likely involved in the development of other plant organs.

The *CpPIN5* expression was especially high in untreated Whitaker samples (Figure 3). However, it was more expressed in non-treated samples in MUCU-16 (3 and 7 dpa) and in Cavili (1 and 3 dpa) than in pollinated and auxin-treated samples (Figure 4). These results were also obtained previously, where both MUCU-16 and Whitaker samples also expressed more *PIN5* in untreated fruits [6]. The *CpPIN5*, despite its low initial expression, increased its expression over time from 3 dpa in all genotypes (Figure 3). This was also observed in *Solanum lycopersicum*, where the *PIN5* expression level increased gradually after fertilization [14].

This gene expression up-regulation over time also occurred in *CpEXPLB1* but mainly in the untreated samples (Figures 3 and A2), which resulted in a down-regulation over time in both treatments with respect to the untreated fruits (Figure 4). These differences were lower in Cavili (Figure 4). The expression profile of *CpEXPLB1* suggested that its expression was inversely related to early fruit growth (3–5 dpa). During 3–7 dpa, non-treated fruits of Whitaker and MUCU-16 fruits expressed more *CpEXPLB1* than Cavili (Figures 3 and A2). Additionally, when the exponential growth of pollinated and auxin-treated fruits of Cavili decreased (Figure 2), this gene was more expressed in these samples (Figure A2). This also suggested that expansins could be associated with the fruit development cell expansion phase, as occurs in tomato [28], but further analyses are required. It is clear that the incorporation of expansins in crop breeding programs presents a potential tool but there are contradictions about their role [15,29,30]. Some of the type-B expansins influenced root hair formation [31] and its overexpression produced longer petioles [32], while other results pointed out that its overexpression could reduce plant development [30]. In *Arabidopsis*, *RhEXPA4* has been reported to negatively affect plant development when expressed at very high levels [30], similar to our results (Figures 3 and 4). In any case, it should be noted that both cell division and cell expansion could produce growth. However, in some

plant organs such as the fruit, the moment when these expansins are expressed could determine the fruit set [19]. This means that expansin overexpression could be associated with early fruit abortion (during cell division phase) or with late fruit development (during cell expansion phase).

In *Arabidopsis thaliana*, CYCD6-1 gene expression alteration produces cell division defects [14]. Also, in transgenic tomato plants, the cyclin AtCycD2 elevated overall growth rates because it reduced the length of the cell-cycle G1 phase, which leads to a faster cell division rate [33]. In the *Cucumis sativus* fruit, a cell number increase was observed in the days before anthesis, while the cellular size was the same [22]. This could explain why we found the highest basal expression of *CpCYCD6-1* in early stages (Figure 3), at the time of proliferation phase (Figure A1). Furthermore, in *Cucumis sativus*, the CsCYCD6-1 expression decreases over time [34], and we have also observed this situation in *Cucurbita pepo* (Figure 3). The Cavili *CpCYCD6-1* was downregulated with respect to MUCU-16 and Whitaker until 5 dpa for non-treated samples (Figure 3). However, unlike for *CpEXPBL1*, the *CpCYCD6-1* gene down-regulation in Cavili (Figure 3) was not exclusively associated with non-treated samples (Figure 4), so its lower expression was not as related to fruit growth as in the *CpEXPBL1* case.

The overexpression of *CpAUX22* and the non-overexpression of *CpEXPLB1* in early stages (day 3 post anthesis) could be related to fruit growth. Gene silencing or mutation studies are necessary to explore their possible relationship with this trait. In addition, it is probable that these results could be useful in other crops with similar fruit development stages.

5. Conclusions

Further information about the key genes involved in fruit development has been obtained. In addition, the association between gene expression profiles at different stages of fruit development has been discussed. Differences with respect to the untreated control can be observed visually between days 2–3, while differences in genes such as *CpAUX22* and *CpIAA4* appeared within a few hours at a molecular level. Since one of the major objectives of zucchini breeding programs is fruit growth, it is important to note that the overexpression of genes such as *CpAUX22* and the non-overexpression of *CpEXPBL1* in the early stages of fruit development may be related to this trait.

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Appendix A

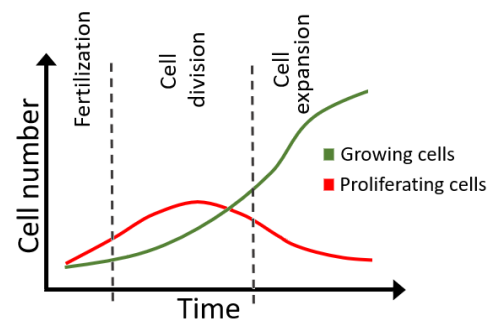


Figure A1. Early fruit development phases. Initially, the number of cells that are proliferating is greater and, with the passage of time, the majority of cells are expanding. Adaptation from tomato fruit development studies [19,35]. Tomato and zucchini could share early fruit development stages since both fruits are anatomically berries. In cucumber, the cell division phase is described until day 4 post pollination, and the cell expansion peak is 8 days post pollination [22].

Appendix B

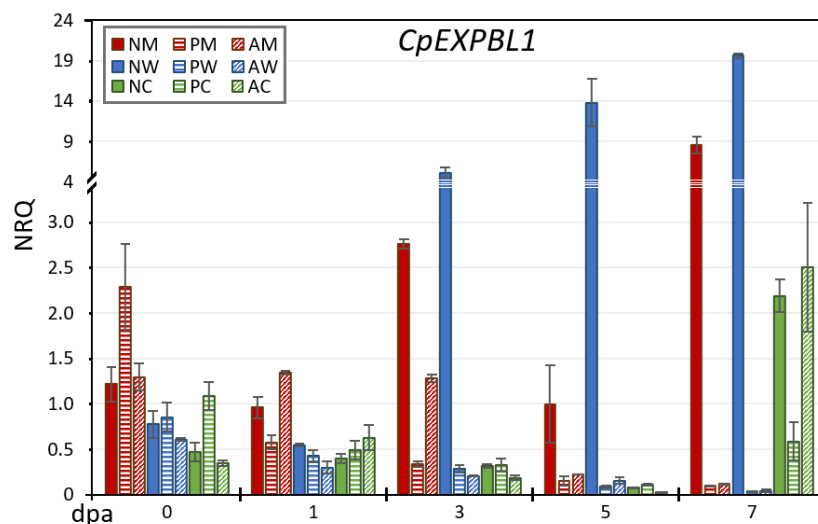


Figure A2. *CpEXPBL1* normalized relative quantities for all treatments and genotypes. Auxin-treated (A), Pollinated (P), Non-treated (N); Genotype MUCU-16 (M), Whitaker (W) and Cavili (C). *CpEXPBL1* (expansin-like B1). Error bars are standard error ($n = 3$).

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