Transcriptome analysis and postharvest behavior of the kiwifruit '*Actinidia deliciosa*' reveal the role of ethylene-related phytohormones during fruit ripening

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Abstract

Kiwifruit are climacteric fruit, so they must be harvested before they are fully ripe, allowing for the extension of their shelf-life via cold storage. Therefore, an adequate knowledge about how ethylene-induced fruit senescence is required to avoid significant economic losses. The main goal of the present study was to investigate the kiwifruit ripening process at the physiological and molecular levels by RNA-seq after 1-MCP (ethylene inhibitor) and Ethrel® (ethylene stimulator) treatments. The results showed that Ethrel® (ethephon) treatment induced more accelerated fruit ripening, leading to rapid fruit senescence, meanwhile 1-MCP (1-Methylcyclopropene) caused a slowing flesh softening, and thus a longer shelf-life period. The RNA-seq was carried out on the fruit after 4 and 13 days, considering day 4 as the most determinant in terms of differentially

expressed genes (DEGs). The sequencing achieved 70.7 % alignment with the 'Hongyang' genome, obtaining 18,036 DEGs. The protein-protein interaction (PPI) network shows the interaction between different pathways in two main clusters: (1) pentose and glucoronate interconversions, citrate cycle, glycolysis and gluconeogenesis or starch and sucrose metabolism and (2) porphyrin and chlorophyll metabolism. The first cluster is mainly interconnected by G6PD1 (pentose pathway); E1 ALPHA and ACLB-2 (citrate cycle); Achn209711 (pentose and glucoronate); LOS2 (glycolysis); HKL1 and HXK1 (glycolysis – starch and sucrose); and PHS2 (starch and sucrose). In the second cluster GUN5 through PORA is interacting with CRD1 and NYC1 which were overexpressed by 1-MCP in the porphyrin and chlorophyll metabolism. In addition, genes linked to PSBY and PSBP photosynthesis-linked proteins in photosystem 2 were overexpressed by 1-MCP which is undoubtedly related to chlorophyll degradation and fruit senescence. These results suggest that in kiwifruit the main pathways that are regulated by ethylene-induced senescence comprise sugar catabolism and chlorophyll degradation.

Keywords

Actinidia deliciosa, Kiwifruit, Postharvest, Ethylene, 1-MCP, RNA-seq

Introduction

Kiwifruit belong to the *Actinidia* genus, and due to its sensory and health attributes is highly appreciated by consumers. Among kiwifruit, *Actinidia chinensis* Planchon, *A. deliciosa* (*A. chinensis* var. deliciosa A. Chevalier), *A. arguta* (Siebold and Zuccarini) Planchon ex Miquel, and *A. eriantha* Bentham are the most widely cultivated species around the world (Datson & Ferguson, 2011; Huang et al. 2013). However, the hexaploid 'Hayward' variety (2n = 6x) is by far the most commercialized green fleshy kiwifruit. *Actinidia* genera holds different polyploid species with a haploid number of chromosomes (x = 29), among them *A. deliciosa*, a hexaploid (6n = 174), and *A. chinensis* (2n = 58) (Atkinson et al. 1997). It has recently been reported that the draft genome sequence of heterozygous kiwifruit, known as 'Hong Yang' (*A. chinensis*), and the assembled genome have a total length of 616.1 Mbp and contain 39,040 genes (Huang et al. 2013). Therefore, this first genome is considered an essential reference for researchers, allowing the scientific community an useful molecular tool for analyzing important postharvest and fruit quality traits.

Kiwifruit is climacteric, meaning that the fruit will continue to ripen after harvest. Therefore, kiwifruit is harvested when still unripe and is then kept in cold storage for long periods. Rhodes et al. (1980) defined the climacteric phase as the moment in which fruit experience biochemical changes, starting with autocatalytic ethylene production. Thus, the beginning stage of ethylene emission is involved in the changes that initiate the fruit senescence, which promotes an increase in respiration and flesh softening. Seymour et al. (1993) and Wills et al. (1998) noted that non-climacteric fruit show a gradual decrease in respiration, while climacteric fruit exhibit a peak in their respiratory rates. In some cases, the climacteric phase involves an increase in respiration, thereby increasing ethylene production. This is the case with both pepper (Tadesse et al. 2002) and kiwifruit (Viera

et al. 2010; Park et al. 2015). Other authors have shown that the major ethylene rise may take place before, just after, or close to the respiratory peak (Lim et al. 2016).

Therefore, the use of ethylene biosynthesis inhibitors, such as 1-Methylcyclopropene (1-MCP), would delay ripening. This approach is mainly applied to climacteric fruit in order to block the ethylene receptors. 1-MCP have been used in peach for alleviating chilling injury symptoms (e.g., internal browning and flesh mealiness), as well as for maintaining a high level of quality in different fruit species (Jin et al. 2011). Following 1-MCP treatment, reduced ethylene production, reduced respiration rate, and delayed fruit softening have been commonly observed effects in kiwifruit (*Actinidia deliciosa*), apricot (*Prunus armeniaca* L.), "aprium" fruit (*Prunus armeniaca* × *salicina* L. cv. Xingmei), and 'Bartlett' pears (*Pyrus communis* L) (Ilina et al. 2010; Muñoz et al. 2012; Ma et al. 2014; Wang and Sugar et al. 2015).

In other cases, however, it may be desirable to accelerate the ripening of immature fruit, depending on market requirements. Ethephon, known as acid-2-chloroethyl-phosphonic (Ethrel®), is known for accelerating ripening in climacteric fruit, thus promoting the development of fruit color and flesh softening. However, as Zhang et al. (2012) have discussed, despite the usefulness of ethephon, its effects on the physicochemical properties and on kiwifruit quality have not been fully unveiled.

Ethylene-coding genes have been intensely investigated in different fruit species, including kiwifruit, *Actinidia deliciosa* (Ikoma et al. 1999, 1998; Sun et al. 2010; Xu et al. 1998, 2000). In recent years, researchers have made some progress regarding the possible genes related to the production of ethylene in kiwifruit using different 1-MCP treatments. It has also been reported that the inhibition of the expression of KWACO1 and KWACS1 genes by 1-MCP is related to the inhibition of ethylene production (Ilina et al. 2010). In addition, an increase of AC-ACO1, AC-ACO2, AC-SAM1, and AC-

SAM2 transcripts before the induction of AC-ACS1, as well as an increase in ethylene production after propylene treatments, have also been reported (Mworia et al. 2010).

Even though today there are many available sequencing technologies, there are few studies that have focused on the genes related to kiwifruit ripening based on RNA-seq. RNA-seq technology utilizes pyro-sequencing, and it has been shown to sequence cDNA efficiently (Shendure and Ji, 2008; Wang et al. 2009). Therefore, transcriptomic analysis is a widely used tool in studies of differential gene expression, using contrasting conditions or treatments, as well as different plant tissues, in order to discern genes related to the fruit ripening process or genes involved in the metabolism of ethylene. Minas et al. (2018) confirmed that 1-MCP and ozone acted as repressive modulators of kiwifruit ripening, and Gunaseelan et al. (2019) reported new copy variants in ETHYLENE RESPONSE FACTOR/APETALA2 (ERF/AP2) related to kiwifruit ripening associated to cold and ethylene response.

In peach (*Prunus persica* L.), Wang et al. (2017a) recently used basic local alignment search tool (BLAST) analysis of sequences between tomato proteins (*Solanum lycopersicum* L.) and the peach genome database, allowing the identification of 15 genes involved in ethylene signal transduction, including an ethylene ETR receptor (Prupe.1G034300) and five AP2/ERF genes (Prupe.1G037700, Prupe.2G289500, Prupe.3G240000, Prupe.5G061800, and Prupe.7G194400). Furthermore, previous analyses of the fruit transcriptome of peach treated with 1-MCP and using microarrays (μPEACH1.0) confirmed an ethylene receptor (ETR2) and three ethylene-sensitive factors (ERF), together with other transcription factors (TFs) and dependent genes of ethylene, involved in changes in ripening parameters (Ziliotto et al. 2008). Additionally, studies on differential gene expression related on fruit treated with 1-MCP and Ethrel® have been reported in papaya (*Carica papaya* L.) (Shen et al. 2017). Ultimately, 20 genes

related to the cell wall and 18 related genes with chlorophyll and carotenoids were identified—related to chlorophyll degradation and carotenoid metabolism inhibition—were shown to play an important role in papaya coloration. In the case of persimmon, fruit treated with ethylene showed a faster expression of the DKEIL, DKERF2, DKERF5, and DKERF8 genes at 25 °C than at 15 °C (Park et al. 2017). In cherry tomato (*Solanum lycopersicum* L.), 1-MCP significantly affected the biosynthesis of aromatic compounds, as well as postharvest storage at low temperatures (Zou et al. 2018). In the specific case of *Actinidia*, few studies have been focused on RNA-seq analysis as the work of Li et al. (2015), where the gene expression profile of anthocyanin was thoroughly investigated in 'Hong Yang' kiwifruit. Moreover, using 'Hong Yang' kiwifruit, Tang et al. (2016) identified thousands of differentially expressed genes (DEGs) that were associated with sugar metabolism, organic acid, and main amino acids.

The aim of the present study was to evaluate the physiological response of kiwifruit, as well as the molecular mechanisms involved, under diverse conditions in ripening and ethylene biosynthesis. To achieve this goal, we generated a transcriptomic reference framework in the response of *Actinidia deliciosa* kiwifruit vis-à-vis harvest time and the presence of 1-MCP ethylene antagonist and Ethrel®.

Materials and methods

Plant material and assays

In this work, 'Hayward' kiwifruits (*Actinidia deliciosa*) were collected from six kiwifruit vines previously marked in an orchard nurseries Biotecnia© located in Teno, Chile.

Two ripeness stages (5.2 % and 8.0 % soluble sugar content) were defined, and 850 fruit were used for each ripeness stage (Tables S1 and S2). In the first treatment (1-MCP), fruits were placed into two 70 L watertight polypropylene

chambers, and 1-Methylcyclopropene (1-MCP) was gasified as a Smart Fresh™ product 0.14 % (625 ppb = 0.625 µl L^{-1}) during 16 h, which was homogenized by constant airflow. In the second treatment (Ethrel®), fruits were treated by dipping them into a water solution with ethephon (acid-2-chloroethyl-phosphonic), applied as Ethrel® 48 SL (300 µl L^{-1}). A total of twenty fruits per treatment (1-MCP and Ethrel®) and day including a control (not treated fruit) were stored at 20 °C for 29 d. For each treatment and control, 12 kiwifruits were characterized at the beginning of the trial period (at harvest= day 0; 48 hours after harvest= day 1) in the middle of the study period (after 15 d), and at the end of the trial period (after 29 d or at eating ripeness, when flesh reached 13 N). Fruit weight (g), diameter (three axes), the skin and flesh chlorophyll index (I_{AD}), the skin and flesh color (L*a*b*), dry matter content (%) and soluble solids (%) were measured. Additionally, the firmness evolution of kiwifruits was monitored every 2 days during this period, including fruit firmness by penetration (7.9 mm diameter plunger) of the whole fruit after removing the skin from both 'cheeks'; flesh firmness by punction (2 mm diameter plunger) of the half fruit; and core firmness by punction (2 mm diameter plunger) which corresponds to the placental tissue. Respiration rate (ml CO_2 kg^{-1} h^{-1}) as well as kiwifruit firmness evolution was monitored every two days. Finally, levels of vitamin C—as ascorbic acid (mg mL^{-1})—and organic sugar content, including glucose, fructose, and sucrose (mg mL^{-1}), were also quantified.

I_{AD} was measured by a DA-meter (Gottardi et al. 2009); the skin and flesh colors were measured with a Minolta colorimeter (CIE L*a*b*); the firmness was quantified in Newton (N) by a texture analyzer (TA.XT plus); and soluble solid content (SSC) was determined by a handheld ATAGO® refractometer, calibrated as the percentage of sucrose at 20 °C. The respiration rate was determined by using HP 5890 SERIES II Gas

Chromatography System (Hewlett-Packard, 1989) using five biological replicates (five fruits). As for levels of vitamin C—as ascorbic acid (mg mL^{-1})—and organic sugar content, including glucose, fructose, and sucrose (mg mL^{-1}), samples were prepared from three fruit halves per day, treatment, and ripeness stage, and then conserved at -80 °C. Juice samples were mechanically extracted with sterile gauze and stored in 2 mL tubes. Subsequently, the samples were filtered using a physical filter (SLGVX13NL Millex-GV, Durapore PVDF hidrof. of 0.22 µm) and a chemical filter (OASIS HLB of 6 cm^3 and 30 µm), and stored again at -80 °C for the ascorbic acid and sugars measurements. A Hitachi HPLC using Kromasil® C18 was used for the sugar determinations, and NH_2 columns were used for the ascorbic acid determinations. For the sugar measurements, 5 mL of the sample was prepared with 1 mL of juice, 0.5 mL of glycerol standard, and 3.5 mL of doubly distilled and deionized water using 80 % acetonitrile as the mobile phase under isocratic conditions according to Vicente et al. (1991).

These samples were transferred to amber vials to inject a volume of 20 uL using an injection time of 15 min and a mobile phase flow of 1 mL min^{-1}. For the ascorbic acid measurements, pure juice samples were directly transferred into amber vials for injection into the HPLC. The mobile phase was used in the phosphate buffer and acetonitrile 60:40 (v/v) according to El Gindy et al. (2006), and the volume injected per sample was 10 uL using an injection time of 6 min and a mobile phase flow of 0.8 mL min^{-1}. For the organic sugar and ascorbic acid determination three samples were injected per treatment and day.

Finally, five fruit samples for each treatment and evaluation day were frozen for RNA-seq analysis.

Phenotypic data analysis

Analysis of variance (ANOVA) per treatment and per day for each ripeness stage was performed by comparing the mean difference by Tukey test ($\alpha < 0.05$). Pearson

correlation coefficients were also analyzed, and the effect of the treatments and time over the evaluated traits was calculated by principal component analysis (PCA). In addition, a general linear mixed model (GLMM) analysis was performed using the restricted maximum likelihood method (REML), taking the time as the fixed variable (evaluation day) and the treatments (not treated, 1-MCP and Ethrel®) as the random variables. This procedure allowed us to gauge the effects of the treatments in an independent way over time using best linear unbiased predictors (BLUPs). All statistical analyses were calculated and edited using INFOSTAT v16 software, with the exception of the GLMM analysis, which was calculated by an interface between INFOSTAT and R.

Library construction and sequencing

Total RNA was extracted from the fruits for each treatment (not treated, 1-MCP and Ethrel®) and day (D4 and D13) using a Sigma Spectrum ™ Plant Total RNA Kit. Tissues for RNA extraction included flesh, core, and seeds, and three independent biological replicates were used for each treatment and day, which supposed 18 RNA samples for the libraries' construction. In order to quantify the RNA, a Qubit ® RNA BR Assay Kit was used, and to verify the RNA quality, a Fragment Analyzer Automated CE System (AATI) was used. A TruSeq RNA HT Sample Prep Kit was used for library construction using one microgram of the RNA sample. The libraries were sequenced using a HiSeq 2500 platform according to HiSeq 2500 System User 'Guide Part 15011190 Rev. V HCS 2.2.70'. Raw images were generated by an Illumina HiSeq using HiSeq Control Software v2.2 for system control and base calling through an integrated primary analysis software called RTA (Real Time Analysis. v1.18). The base binary calls were converted into FASTQ by utilizing Illumina package bcl2fastq (v1.8.4). The quality reads sequencing obtained by the Illumina HiSeq 2500 were analyzed (pre-trimming) with the software FastQC1 for determining the quality of sequencing. FLEXBAR2 software was

used for trimming, as well as to filter the low-quality reads (i.e., a Phred value of less than 25), removing the adapters, sequences with many Ns, and reads smaller than 100 bp from the sequencing libraries.

Alignment and gene expression analysis

The sequence alignment was performed with Bowtie v2 software (Langmead et al. 2012), using 'Hong Yang' (*Actinidia chinensis*) as the reference genome (National Center for Biotechnology Information, NCBI). Samtools4 v0.1.19 was used to convert the files in sequence alignment/map (SAM) format into binary alignment map (BAM) format. SAM format is a generic format used to store alignments of nucleotide sequences, while BAM is the compressed version of the SAM format. Transcript abundance and the transcriptome assembly of each sample were created using Cufflinks5 v.2.1.13, using the BAM files resulting from the alignment. The transcriptomes assembled, the annotated genome, and the genome sequence of *Actinidia chinensis* (NCBI) were merged into a single gtf format using the Cuffmerge tool. The Cuffdiff by differential gene expression analysis and the results obtained were loaded with the CummeRbund6 visualization package in order to manage, visualize, and integrate all the data produced by the Cuffdiff analysis, using the statistical information environment R7 (version 3.3.3). The CummeRbund6 visualization package shows the quantity versus the dispersion of all genes for the different conditions (Fig. S3), the density of genes for each condition (Fig. S4), the dispersion diagrams according to $\log_{10}$ of fragments per kilobase million (FPKM) (Fig. S5), and boxplots for FPKM distributions, including three biological replicates (Fig. S6). Finally, we defined the cutoff point between $\log_{10}$(p-value) and $\log_2$(fold change) for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13) at p-value < 0.005, obtaining 18,036 DEGs from a total of 79,614 genes (Fig. S7). FPKM

were used to measure the number of transcripts of each gene, and only doubly expressed genes were considered as DEGs for each treatment comparison.

Gene Ontology and network analysis

Gene-act-network analysis was performed to identify the interactive network among the Gene Ontology (GO) terms enriched in DEGs based on the GO database using the Biological Networks Gene Ontology (BiNGO) tool in Cytoscape version 3.7.2 (Maere et al. 2005; Shannon et al. 2003), which calculates overrepresented GO terms in the network and displays them as a network of significant GO terms. For analysis of the enrichment of the metabolic pathways, the public Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Bindea et al. 2009, 2013) and the pathways were analyzed in Cytoscape (Shannon et al. 2003) using the ClueGO (Kanehisa et al. 2016, 2017) plug-in. Besides, PPI of the DEGs—associated with cellular and metabolic processes, and the translation of hormonal signals,— was analyzed using the search tool for the retrieval of interacting genes (STRING) (Szklarczyk et al. 2017), where a score > 0.400 was considered for the DEGs. Cytoscape was used to visualize the PPI for the DEGs and to identify the key genes (Shannon et al. 2003).

Verification of qRT-PCR

The oligonucleotide primers were designed from sequences of genes previously aligned to the *Actinidia* reference genome (Huang et al. 2013) using the software Primer3 version 0.4.0 (Untergasser et al. 2007) (Table S12). The AGB1 (Achn005971) and ACTIN 7 (Achn107351) genes were used as references. Internal data from the RNA-seq was used for reference gene selection following the criteria established by Zhou et al. (2017). RT-qPCR was carried out using a KAPA SYBR® FAST Universal qPCR Kit (Kapa Biosystems) on an Eco Real-Time PCR System (Illumina). Each reaction mixture contained 1.0 μL of cDNA, KAPA SYBR® FAST Universal qPCR Kit 5 μL, KAPA

SYBR® ROX Low (50X) 0.2 µL, PCR direct primer (10 µmol L^{-1}) 0.2 µL PCR, reverse primer (10 µmol L^{-1}) 0.2 µL, and 3.4 µL ddH_2O, for a final volume of 10 µL. The PCR conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s, and 72 °C for 35 s. The quantification of the expression level of the genes was carried out using a relative quantification method, Pfaffl (Satyanarayana et al. 1984), and each sample was amplified in duplicate.

Results

Fruit phenotyping

'Hayward' kiwifruit were harvested in two ripeness stages (i.e., E1, ≈ 5 % of SSC; and E2, ≈ 8 % of SSC) after four postharvest periods— at harvest (day 0), 48 h after harvest (day 1), 15 d after harvest (day 15), and 29 d after harvest (day 29) (Fig. 1 and Tables S1-S2)—and treated with 1-MCP and Ethrel® in order to test the effect of different ethylene biosynthesis conditions in terms of fruit physiology. In general, the fruit of the first harvest (E1) were more responsive to the treatments in most of the evaluated traits (i.e., the skin I_{AD}, flesh I_{AD}, SSC, and flesh color) than the E2 fruit, showing greater differences over time among the treatments (Fig. 1). Thus, the skin and flesh I_{AD} of 1-MCP treatment showed a lower decrease due to the 1-MCP effect, indicating lower chlorophyll degradation, in contrast to Ethrel®, which produced greater chlorophyll degradation, and therefore, faster fruit senescence. Similar results were observed for flesh color, particularly in terms of luminosity (L*; CIE L*a*b*), demonstrating that the fruit from 1-MCP treatment had a lower luminosity decrease for both ripeness stages with respect to not treated and Ethrel® treated fruit due to the 1-MCP effect (Fig. 1). Nonetheless, it is noteworthy that in all cases, not treated and Ethrel® treated fruit exceeded 1-MCP treatment in terms of SSC, which could suggest that the 1-MCP effect might decrease or delay fruit sugar accumulation.

Regarding respiration rate, not treated and Ethrel® fruit produced a respiratory increase in CO_2 production, unlike the 1-MCP, especially in E1 (Fig. 2). However, E2 showed no differences among the treatments in terms of respiratory rate, which was probably due to the advanced ripeness stage. According to maximum treatment differences, we highlighted Ethrel® and not treated fruit, which reached their highest respiratory rate at day 4 and day 6, respectively, with values over 30 ml CO_2 kg^{-1} h^{-1}; meanwhile, 1-MCP as expected for an ethylene inhibitor remained at a low and constant respiratory rate for both ripeness stages (Fig. 2). As for firmness evolution, fruit, flesh, and core firmness showed significant differences between 1-MCP and Ethrel® treatments, especially in E1 (Fig. 3). Regarding fruit firmness in E1, all treatments started with similar values of around 80 N. It is interesting to note that 1-MCP maintained a constant firmness level for several days, ranging between 70 N and 80 N, but then a change in this trend starting on day 15 was noted, and ultimately reaching a firmness of 50 N on day 29. On the other hand, the firmness in not treated and Ethrel® fruit decreased considerably throughout the whole period. The greatest loss of firmness was experienced by the Ethrel® treated fruit, which reached their consumption maturity (10-15 N) on around day 25, while the not treated fruit reached their consumption maturity on day 29. It should be noted that the postharvest life of the fruit was much higher for the 1-MCP treatment, which did not reach consumption maturity after 29 d. In addition, the greatest differences in firmness between the treatments were observed on day 13, when there was a difference of up to 50 N between 1-MCP and Ethrel® treatments (Fig. 3). Regarding E2, the differences between the treatments were lower than E1, with almost no significant differences except for day 20, where Ethrel® showed a difference of around 20 N compared to not treated and 1-MCP fruit (Fig. 3).

In terms of for sugar content, we observed that 1-MCP produced a lower increase in contrast to the not treated or Ethrel® fruit, as these differences were lower in the E2 ripeness stage (Fig. S1). In both cases, the highest concentrations ranged from 7 to 8 mg mL^{-1} of glucose and fructose. Additionally, we observed no differences between the fructose and glucose levels, which showed similar concentrations (Fig. S1). As for vitamin C, determined as ascorbic acid (mg mL^{-1}), there was neither a clear trend among the treatments nor over time, always ranging from 0.5 to 0.7 mg mL^{-1} (Fig. S1).

According to Pearson correlations, the highest value was obtained between fruit firmness and SSC for both ripeness stages (-0.93 and -0.84) as well as there was another important correlation in the E1 between flesh color (L*) and fruit firmness (0.89) (Tables S3 and S4). Principal component analysis (PCA) of the fruit trait data showed that most of the variability was explained by the first component (PC1) in terms of both treatments and time, reaching 74.6 % and 92 %, respectively (Fig. 4); a significant effect of the treatment over traits was noted, especially in E1, where Ethrel® treatment was related to a major SSC increase, including in fructose and glucose levels, while 1-MCP had a greater effect on fruit firmness, implying a lesser degree of softening and chlorophyll degradation (Fig. 4).

In summary, after analyzing the data for all evaluated traits and ripeness stages, treatments showed a greater effect in the E1 stage. This observation was supported by GLMM analysis, since obtained BLUPs' coefficients indicated an inverse effect of 1-MCP compared to not treated and Ethrel® fruit (Table S5). This situation resulted in a less fruit softening for 1-MCP treatment and a major SSC and sugars content for not treated and Ethrel® fruit. Thus, we found that the most suitable ripeness stage to evaluate the differential gene expression due to treatments was E1, because of the critical differences observed. Therefore, for the RNA-seq analysis, we decided to choose fruit

from E1 because there were greater differences between treatments and days compared to E2. Taking into account that fruit firmness and respiration rate were the traits most significantly affected by the treatments in E1 (Fig. 4 and Tables S3-S4), we selected day 4, according to the maximum respiration rate (Fig. 2), as well as day 13, for its maximum firmness difference between the treatments (Fig. 3), since we considered.

Sequencing and alignment results

Supposing an average value of 37,233,093 reads per sample, we sequenced a total of 670,195,682 raw reads. We learned that, on average, more than 95 % of the reads showed a Phred quality score over 20 (Q20 %), and more than 90 % of the reads had a quality score of over Q30 (Table S6). Thus, a total of 315,537,899 filtered reads were aligned to the *Actinidia chinensis* genome (Huang et al. 2013), obtaining a 70.7 % alignment, while 29.3 % were not aligned (Tables 1 and S7). In addition, 50.7 % of the aligned sequences (35.87 %) were located in a unique position, while the remaining sequences (34.84 %) were aligned to multiple positions. All raw reads in FASTQ format, including paired-end and replicates, are available from the NCBI Short Read Archive (SRA) database under BioProject number PRJNA638129.

Differentially expressed genes (DEGs) and functional annotation

As we previously observed, the 1-MCP and Ethrel® treatments produced an expected effect that, respectively, extended and reduced the fruit's shelf-life potential. A total of 18,036 genes (Fig. 5a and Table S8) were differentially expressed at p-value < 0.005. Moreover, in the PCA (Fig. S2), we noted more differences between the treatments on day 4. In order to obtain the most significant genes, we only considered as DEGs those doubly expressed in any treatment comparisons, obtaining 8,626 upregulated genes of 1-MCP treatment (T1) over Ethrel® treatment (T2) in day 4, while in day 13, the number of up- or downregulated genes was similar for both treatment comparisons (Fig. 5b). In

addition, we found that the number of genes per range expression was reduced considerably on day 13 (Fig. S8).

The functional annotation of the DEGs was performed using the Gene Ontology resource, whereby the genes were classified according to three criteria: biological process, cellular component, and molecular function. The number of DEGs on day 4 was quantitatively higher than those on day 13; however, they showed a proportional similarity (Fig. 6 and Table S9). The difference was marked by treatment comparisons, generating a 1-MCP (T1) versus Ethrel® (T2) enrichment on day 4, and a not treated fruit (T0) versus 1-MCP (T1) on day 13. In addition, the graphic absence of a 1-MCP (T1) versus Ethrel® (T2) on day 13 was due to the low number of DEGs. The terms of cellular and metabolic processes were observed more frequently in the ontology of biological processes; meanwhile, the terms' cell, intracellular, cytoplasm, and membrane were most frequently observed in the ontology of the cellular components and in the molecular functions, catalytic activity and binding activity (Fig. 6).

Enrichment analysis of pathways

Additionally, KEGG assignments were used to classify the functional annotations for a better understanding of the biological functions and to enrich the biological path of each DEG-derived transcript. At least 473 genes (Table S10) were related to different metabolic pathways, *e.g*, photosynthesis, porphyrin, and chlorophyll metabolism; plant hormone signal transduction; starch and sucrose metabolism; amino sugar nucleotide sugar metabolism; RNA transport; and spliceosome (Figs. 7 and S9-S16). The results showed that the DEGs that participated in the route of photosynthesis and starch and sucrose metabolism decreased in day 13, probably because the fruit experienced a transitional period of starch accumulation characterized by the activity of key enzymes in the metabolic route of sucrose, which decreased in parallel to the decrease in starch levels.

Plant hormone signal transduction, amino sugar nucleotide sugar metabolism, RNA transport, and spliceosome were significantly enriched on day 4 after treatment (Fig. 7 and Table S10).

Protein-protein interaction network of DEGs and related pathways

The putative function and DEGS levels were integrated into a network of PPIs using the STRING biological database, with an interaction score of > 0.7. G6PD1, PGL1 and PRSI from pentose phosphate pathway were identified (Fig. 8) being PGL1 (K01057) and PRSI (K00948) overexpressed in Ethrel® and 1-MCP respectively and interacting with the key genes of tricarboxylic acid cycle [TCA] cycle such as E1 ALPHA, mtLPD1, ACO3 and ACLB-2 being ACO3 (K01681) especially overexpressed in 1-MCP (Fig. 9 and Fig. S10). In turn, TCA related genes interact with GAPC2, GAPC1, and LOS2 (Fig. 8), which participate in glycolysis being LOS2 (K01689) overexpressed by 1-MCP at the previous synthesis stage of 2-phosphoenolpyruvate (PEP) (Fig. S11). In addition, LOS2 interacts with genes belonging to starch and sucrose metabolism such as HKL1 and APL3, and within this group, PHS2, TRE, and HXK1 were also identified (Fig. 8). In this instance, APL3 (K00975) and PHS2 (K00688) were especially overexpressed in 1-MCP (Fig. S12). As for pentose and glucuronate interconversions ATPME3, PMR6, UGD2 and Achn209711 were identified (Fig. 8). In addition, Achn209711 (K01805) which is overexpressed in 1-MCP, is in a key position interacting with glycolysis and starch and sucrose metabolism (HXK1 and HKL1) while the provider of nucleotide sugars for the cell wall UGD2 (K00012) is downregulated in 1-MCP and upregulated in Ethrel® (Fig. S13). Moreover, PMR6 (K01728, K22539) and Achn330311 (K01051) were overexpressed in Ethrel® in the pentose and glucuronate interconversions, which in turn are related to the disassemble of the fruit cell wall mediated by polygalacturonase (Fig.

S13). Therefore, if we consider the most differentially expressed genes by metabolic pathway, the polygalacturonase enzyme is overexpressed in Ethrel® (Fig. 9).

Additionally, in a separate cluster, genes involved in porphyrin and chlorophyll metabolism were identified (Fig. 8), such as GUN5, ALB1, NYC1, CLH2, PORA, and PORB. Genes related to multifunctional protein GUN5 (K03403), PORA (K00218), and PORB (K00218) were overexpressed in 1-MCP as well as CLH2 (K08099) and NYC1 (K13606) which were involved in the previous steps of chlorophyll a and b synthesis (Fig. S14). Therefore, we can hypothesize that 1-MCP slowed the chlorophylls degradation. Moreover, GUN5 is interacting with OST1 (K14498) which links porphyrin and chlorophyll metabolism with plant hormone signal transduction. EIN2 and ETR2 participate in plant hormonal signal transduction (Fig. 8) specifically in the cysteine and methionine metabolism. In this case, ETR2 (K14509), as expected were downregulated by 1-MCP and upregulated by Ethrel® in day 4 which confirms the 1-MCP and Ethrel® effect over ethylene emission through both proteins (Fig. 9 and Fig. S15). As for carotenoid biosynthesis, CHY2 and B2 (K15746) were interconnected in a separate cluster being B2 downregulated by 1-MCP for the day 4 as it´s shown in Fig. 9 and Fig. S16.

Finally, if we analyze the genes that showed higher expression per metabolic pathway in the 1-MCP vs Ethrel® comparison (Fig. 9 and Table S11), we could appreciate genes encoding proteins linked to photosynthesis in photosystems a and b, resulting in an overexpression by 1-MCP for PSBY (K02723) and PSBP (K02717), which could show evidences of the chlorophyll–photosynthesis interrelation in fruit senescence.

Confirming genes' expression using RT-qPCR

In addition to the physical and chemical changes described above, a set of genes involved in ripening was investigated. Fruit ripening implies physiological changes, such

as flesh softening, color change, and taste development, among other process. In particular, fruits treated with 1-MCP showed differences in the expression of several genes related to ripening (Fig. 10), suggesting that this endogenous ethylene inhibitor acts largely independently in kiwifruit. After the application of 1-MCP, the expression of certain negative ethylene regulators genes involved in the starch and sucrose metabolism (ATSPS4F, Achn218701), porphyrin and chlorophyll (CHLM, Achn271251) and citrate cycle (ACO3, Achn152281) increased.

In addition, as we have shown the application of 1-MCP can delay the degradation of chlorophyll being fruit color largely determined by the presence of chlorophyll and carotenoids (McGhie et al. 2002). This delay in chlorophyll degradation is associated with inhibition of ethylene production by suppressing the gene expression of PAO, NYC (Achn069361), NOL, and SGR1, which are closely associated with the chlorophyll catabolic pathway (Cheng et al. 2012), thus increasing the expression of CHLM (Achn271251) and CRD1 (Achn328501).

Ethrel® treatment was found to stimulate polygalacturonase (Achn051381) synthesis, while 1-MCP treatment delayed fruit maturation and polygalacturonase synthesis. Besides, PRF3 (Achn163471) linked to regulation of actin cytoskeleton was overexpressed in day 4 of 1-MCP treatment since it would be participating in the polymerization of actin filaments.

Discussion

Fruit phenotyping

According to the phenotyping results discussed above, most of the evaluated traits, including skin I_{AD}, flesh I_{AD}, SSC, and flesh color, showed higher differences. This was particularly the case for E1, the first ripeness stage, probably due to the more advanced

physiological ripeness stage in E2. Moreover, not treated and Ethrel® treated fruit favored a major SSC, including the main sugars in *Actinidia deliciosa*, fructose and glucose (Nishiyama et al. 2008), as well, since greater fruit softening and chlorophyll degradation is contrary to 1-MCP treatment. A previous study in 'Qinmei' kiwifruit, suggested that applications of high concentrations of 1-MCP affected consumer acceptance due to excessive sourness, but not because of fruit firmness or SSC (Deng et al. 2015). As for ascorbic acid content, it did not show a clear trend either between treatments or between days. Lim et al. (2016) claimed that the decrease in ascorbic acid and phenolic compounds in kiwifruit is associated with longer cold storage times.

In addition, in the present study, a significant respiratory increase was shown for not treated and Ethrel® treated fruit in days 4 and 6 compared to 1-MCP treatment, which remained at a low and constant respiratory rate. This respiratory increase may be related to the beginning of ethylene emission, which can occur either before or after it (Lim et al. 2016). An increase of ethylene approximately one week after harvest was reported by Ilina et al. (2010) in 'Hayward' kiwifruit, which coincides with the respiratory increase obtained for the not treated fruit observed in this trial. Thus, the results we obtained allowed us by BLUP coefficients to select fruit firmness and respiratory rate as the most determining traits in the fruit maturation process. For sequencing analysis, we selected fruit from day 4 in E1, which coincided with the maximum respiratory rate, and day 13, when the maximum fruit firmness difference was shown. As for E2, no fruit were selected due to the small differences between the treatments.

Sequencing and alignment results

In terms of sequencing and alignment results, we based our bioinformatics work on one of the few currently available molecular tools, i.e., the diploid kiwifruit 'Hong Yang' (*A. chinensis*), used as a reference genome (Huang et al. 2013; Wu et al. 2019).

Although there is a ploidy difference between this cultivar and commercially important varieties, there are many transcriptomic studies that have attempted to bring a greater fundamental understanding with respect to disease resistance, most especially *Pseudomonas syringae* (Wang et al. 2017b, 2018; Michelotti et al. 2018; Song et al. 2019). However, from the fruit quality or postharvest points of view, there are few studies available (Tang et al. 2016; Zhang et al. 2018; Gan et al. 2020). The sequencing results of our work have shown to be of high quality according to the Phred values, even obtaining a high alignment result (≈ 70 %) with respect to the diploid reference genome, which indicates a high synteny between *Actinidia chinensis*, even between different species, such as *A. eriantha* (Tang et al. 2019), despite the hexaploid character of the 'Hayward' variety.

Differentially expressed genes (DEGs) and functional annotation

Gene expression analysis provided a total of 18,036 DEGs, most of which were expressed by day 4. This suggests that the most important changes in the fruit maturation process at the physiological and biochemical levels are taking place in the first few days after harvest. In contrast, the differences between 1-MCP and Ethrel® treatments were minimal for day 13, indicating a reduced effect of 1-MCP during the postharvest period. In order to gain a better understanding the biological functions related to the most important genes, KEGG pathways were used to relate each DEG-derived transcript being several genes linked to starch and sucrose metabolism; amino sugar nucleotide sugar metabolism; photosynthesis, porphyrin, and chlorophyll metabolism; plant hormone signal transduction; RNA transport; or spliceosome. The most significant difference between treatments we found was in the porphyrin and chlorophyll metabolism activity for day 4, indicating a major fruit softening rate due to the chlorophyll breakdown in not treated and Ethrel® treated fruit, which also occur in the ripening process of many fruit,

including banana (*Musa acuminata*) and tomato (*Solanum lycopersicum*) (Guyer et al. 2014). Contrary to other fruit crops, green-fleshed varieties of kiwifruit only lose some of their chlorophyll during prolonged storage (Burdon and Lallu, 2011) or when applying accelerating treatments of ethylene biosynthesis by using Ethrel®, as we did in this study.

Protein-protein interaction network of DEGs and related pathways

PPI network revealed significative interconnections differentiated into two main clusters: (1) pentose phosphate pathway, citrate cycle, glycolysis and gluconeogenesis, starch and sucrose metabolism and pentose glucoronate interconversions and (2) porphyrin and chlorophyll metabolism. E1 ALPHA, mtLPD1, ACO3 and ACLB-2 from citrate cycle interact with GAPC2, GAPC1, and LOS2 from glycolysis and gluconeogenesis. LOS2 (K01689) was overexpressed by 1-MCP at the previous synthesis stage of 2-phosphoenolpyruvate (PEP) while the pyruvate decarboxylase enzyme PDC2 (K01568) was found to be overexpressed for the Ethrel® treatment. Therefore, both related genes are linked to the oxidative decarboxylation process of pyruvic acid to acetaldehyde (pyruvate decarboxylase) and pyruvate to acetate (pyruvate dehydrogenase) is catalyzed by both enzymes releasing CO_2. For this reason, we found differences in the kiwifruit's CO_2 emissions between the 1-MCP and Ethrel® treatments, indicating that the 1-MCP treatment can interfere in the transcriptional control of respiration by maintaining lower rates in kiwifruit.

As for starch and sucrose metabolism APL3 (K00975) and PHS2 (K00688) were overexpressed in 1-MCP, even ATSPS4F (K00696) which could be correlated with sucrose synthesis during ripening; this is possibly due to the accumulation of starch in the flesh, which, through a disassembly mechanism, can provide a carbon source for the synthesis of sugars during ripening, including sucrose (Nogueira et al. 2012).

According to pentose and glucuronate interconversions, in the PPI network Achn209711 (K01805) was overexpressed by 1-MCP treatment and this gene interacts with glycolysis and starch and sucrose metabolism (HXK1 and HKL1) while UGD2 (K00012) is overexpressed by Ethrel® treatment favoring the supply of nucleotide sugars for the cell wall. The pectinesterase enzyme Achn330311 (K01051) is the first enzyme in the process by which Poly (1,4-α-galacturonide) is converted into Poly (1,4-α-galacturonide) (n) as a prior step to its conversion to digalacturonate through the polygalacturonase (K01184 [3.2.1.15]). In our trial, the enzyme pectinesterase was overexpressed by the Ethrel® treatment. This enzyme belongs to the pectinases group, which, as reported by Paniagua et al. (2014), is usually involved in fruit softening, whereby pectins play an important role in the cell wall disassembly. In addition, lower cell wall degradation has been described in plums after 1-MCP treatment especially for pectinesterase, polygalacturonase, cellulase, and β-galactosidase enzymes and a higher content of cell wall polysaccharide was detected (Lin et al. 2018). In addition, polygalacturonase enzymes that degrade the cell wall (EC 3.2.1.15) are involved in flesh softening. A kinetics study related to ethylene evolution and polygalacturonase synthesis demonstrated that the evolution of ethylene preceded the synthesis of polygalacturonase at 20 h (Grierson and Tucker, 1983).

The green color of the kiwifruit pericarp is mainly determined by the different concentrations and proportions of carotenoids and chlorophyll (McGhie and Ainge, 2002). The applications of ethylene affected the color of the fruit, showing changes in the color of the flesh and a loss of luminosity, through the inhibition of chlorophyll metabolism. Chlorophyll biosynthesis enzymes are part of this process, such as protochlorophyllide reductase A (PORA), protoporphyrinogen oxidase (HEMG2), magnesium-chelatase subunit ChlH (CHLH), and cardiolipin synthase (CRD1), as well

as chlorophyll degradation enzymes, such as chlorophyll degradation (CLH2) and robbable chlorophyll (ide) b reductase NYC1 (NYC1). In contrast, we found that the application of 1-MCP increased the transcription of these enzymes. However, in the route of carotenoid biosynthesis, the gene related to BETA-HIDROXILASE 1 protein (B2) was downregulated after treatment with 1-MCP, decreasing its expression in 1-MCP, and increasing its expression via ethylene in Ethrel® treatment.

Plant hormone signal transduction and the MAPK signaling pathway are both other metabolic pathways affected by treatments. In our study, the kiwifruit gene Achn067861 (K14509) was overexpressed after the Ethrel® treatment, contrary to 1-MCP. This gene encodes the ethylene receptor [ETR2; EC2.7.13], which is involved in the metabolism of cysteine and methionine, catalyzing the onset of serine/threonine-protein kinase (CTR1; [EC: 2.7.11.1]), and it is responsible for fruit senescence. Other studies have reported several TFs that are also involved in fruit ripening and ethylene biosynthesis, some of which include MADS-box, AP2 / ERF, NAC domain, and homeobox HD-Zip family proteins (Liu et al. 2015; Ma et al. 2014; Wang et al. 2017a).

Finally, we could assert that fruit ripeness is a very complex process which implies many pathways such as pentose and glucoronate interconversions, glycolysis or starch and sucrose metabolism on the one side and porphyrin and chlorophyll metabolism in the other hand. Moreover, the interrelations within them through plant hormonal signal transduction are critical especially for cysteine and methionine metabolism where ethylene is finally released triggering a series of biological processes that favor fruit senescence. In addition, the evidence of genes that encode proteins linked to photosynthesis in the present study as PSBY, PSBA or PSBP are indicating that the slowdown in the degradation of chlorophylls by 1-MCP would also affect other photosystem II-related proteins even though the fruit is not in a photosynthetic stage. In

previous studies, the photosystems' involvement in the fruit maturation process has not yet been clarified at pre-harvest level. However, at post-harvest level a possible implication of photosystems would not make sense so far. For example, in tomato, Carrara et al. (2001) found that the fruit showed no CO_2 assimilation in the pre-harvest period; however, these authors found that the fruit tissues exhibited consistent photochemical activity. In a more recent study, Lytovchenko et al. (2011) asserted that tomato fruit photosynthesis is apparently not important in the ripeness process, but that it does play a significant role in seed development. Even more recently, Ceusters et al. (2018) summarized evidence showing that ethylene acts as a regulator of photosynthesis and its associated components, such as stomatal conductance, chlorophyll, light reactions, carboxylation, carbohydrate partitioning, and age-related leaves senescence of *Arabidopsis*, which could explain the chlorophyll degradation and the fruit senescence from the Ethrel® treatment, as well as the opposite effect from the 1-MCP treatment. Therefore, the degree of involvement of these phytohormones on photosynthesis and their interrelations with fruit ripening are still unknown. Thus, the study of non-coding RNA's like miRNA could be an interesting approach because in climacteric fruit as tomato some miRNA targets play an important role on fruit development and ripening (Karlova et al., 2013) as well as in non-climacteric fruit such as strawberry (Xu et al., 2013) which identified miRNA targets associated to fruit senescence through NAC transcription factors or Auxin response factors (ARF) and Myb transcription factors.

Conclusions

This study allowed us to identify genes related to the fruit ripening of 'Hayward' kiwifruit. This work is useful for creating a gene reference framework to focus on new trials of other *Actinidia* species. Fruit ripening is a complex process that requires the participation of biochemical, genetic, and hormonal signals. Application of exogenous

ethylene accelerates ripening, as it triggers the metabolic processes responsible for the fruit's growth and maturation. This step also involves the disassembly of the cell wall, which is a result of the breaking up of the sugar chains, the pigment changes, and the release of aromatic compounds. In the present study, 1-MCP proved to be effective in delaying fruit ripening by inhibiting the ethylene receptors, which prevents the coupling of ethylene. These changes are also expressed at the molecular level, involving many related pathways which are grouped in two main clusters: (1) pentose and glucoronate, citrate cycle, glycolysis and gluconeogenesis or starch and sucrose metabolism and (2) porphyrin and chlorophyll metabolism. Thus, increased knowledge of enzymes or proteins variations related to chlorophyll content caused by ethephon, 1-MCP or even over time during kiwifruit shelf-life period, could allow for a more complete understanding of fruit ripening. Therefore, it would be an interesting approach to consider studies on the chlorophyll evolution and even photosynthetic activity on fruit species throughout the development of the fruit in the pre-harvest period, which could be related to a longer or shorter fruit shelf-life in the post-harvest period. This could be an interesting line of investigation for more concretely discerning the physiological mechanisms involved in the fruit maturation process.

Ultimately, our results allow us to visualize the key genes involved in this process. However, since fruit ripening is a complex process and involves more than one metabolic pathway, the miRNA analysis, and even an epigenetic approach, such as methylation, could complement our findings here, creating a much more complete picture of the entire fruit ripening process.

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Data Archiving Statement

Raw reads of the present RNAseq study has been uploaded in the National Center for Biotechnology (NCBI), including a total of eighteen libraries in FASTQ format. These libraries include paired-end reads and three biological replicates for treatments (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and days (D4 and D13). The database is available in the NCBI Short Read Archive (SRA) under BioProject number PRJNA638129.

References

Atkinson RG, Cipriani G, Whittaker DJ, Gardner RC (1997) The allopolyploid origin of kiwifruit, *Actinidia deliciosa* (Actinidiaceae). Pl Syst Evol 205, 111–124. https://doi.org/10.1007/BF00982801

Bindea G, Galon J, Mlecnik B (2013) CluePedia Cytoscape plugin: pathway insights using integrated experimental and in silico data. Bioinformatics 29, 661–3. https://doi.org/10.1093/bioinformatics/btt019

Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pagès F, Trajanoski Z, Galon J (2009) ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091–3. https://doi.org/10.1093/bioinformatics/btp101

Burdon J, Lallu N (2011) Kiwifruit (*Actinidia spp*.). In: Yahia EM (ed) Postharvest biology and technology of tropical and subtropical fruit, vol 3. Cocona to mango. Woodhead Publishing Limited, Cambridge, England, pp 326–360

Carrara S, Pardossi A, Soldatini GF, Tognoni F, Guidi L (2001) Photosynthetic Activity of Ripening Tomato Fruit. Photosynthetica 39, 75–78. https://doi.org/10.1023/A:1012495903093

Ceusters J, Van de Poel B (2018) Ethylene Exerts Species-Specific and Age-Dependent Control of Photosynthesis. Plant Physiology 176, 2601 LP – 2612. https://doi.org/10.1104/pp.17.01706

Cheng Y, Dong Y, Yan H, Ge W, Shen C, Guan J, Liu L, Zhang Y (2012) Effects of 1-MCP on chlorophyll degradation pathway-associated genes expression and chloroplast ultrastructure during the peel yellowing of Chinese pear fruits in storage. Food Chem. 135, 415–422. https://doi.org/10.1016/j.foodchem.2012.05.017

Datson PM, Ferguson AR (2011) *Actinidia*, in: Wild Crop Relatives: Genomic and Breeding Resources. Springer Berlin Heidelberg, pp. 1–20. https://doi.org/10.1007/978-3-642-20447-0_1

Deng L, Jiang CZ, Mu W, Wang Q (2015) Influence of 1-MCP treatments on eating quality and consumer preferences of 'Qinmei' kiwifruit during shelf life. Journal of Food Science and Technology 52, 335–342. https://doi.org/10.1007/s13197-013-0986-y

El Gindy A, Emara S, Mostafa A (2006) Application and validation of chemometrics-assisted spectrophotometry and liquid chromatography for the simultaneous determination of sixcomponent pharmaceuticals. J Pharm Biomed Anal. 41(2): 421–30

Gan Z, Shan N, Fei L, Wan C, Chen J (2020) Isolation of the 9-cis-epoxycarotenoid dioxygenase (NCED) gene from kiwifruit and its effects on postharvest softening and ripening. Scientia Horticulturae 261, 109020. https://doi.org/https://doi.org/10.1016/j.scienta.2019.109020

Gottardi F, Noferini M, Fiori G, Barbanera M, Mazzini C, Costa G (2009) "The index of absorbance difference (IAD) as a tool for segregating peaches and nectarines into homogeneous classes with different shelf-life and consumer acceptance," in Proceedings of the 8th Pangborn Sensory Science Symposium. Firenze

Grierson D, Tucker GA (1983) Timing of ethylene and polygalacturonase synthesis in relation to the control of tomato fruit ripening. Planta 157, 174–179. https://doi.org/10.1007/BF00393652

Gunaseelan K, McAtee PA, Nardozza S, Pidakala P, Wang R, David K, Burdon J, Schaffer RJ (2019) Copy number variants in kiwifruit ETHYLENE RESPONSE FACTOR/APETALA2 (ERF/AP2)-like genes show divergence in fruit ripening associated cold and ethylene responses in C-REPEAT/DRE BINDING FACTOR-like genes. PLoS One. 14(5):e0216120. doi: 10.1371/journal.pone.0216120. PMID: 31083658; PMCID: PMC6513069.

Guyer L, Schelbert S, Christ B, Lira B, Rossi M, Hortensteiner S (2014) Different Mechanisms Are Responsible for Chlorophyll Dephytylation during Fruit Ripening and Leaf Senescence in Tomato. Plant physiology 166. https://doi.org/10.1104/pp.114.239541

Hewlett-Packard (1989) Floating measurements and guarding. Application note number 123, 2nd edn, Hewlett-Packard, Palo alto, CA.

Huang S, Ding J, Deng D, Tang W, Sun H, et al. (2013) Draft genome of the kiwifruit *Actinidia chinensis*. Nat. Commun. 4, 1–9. https://doi.org/10.1038/ncomms3640

Ikoma Y, Yano M, Chuan Xu Z, Ogawa K (1998) Reduction in ethylene synthesis in parthenocarpic *Actinidia deliciosa* fruit induced by N-(2-chloro-4-pyridyl)-N'-phenylurea. Postharvest Biol. Technol. 13, 121–129. https://doi.org/10.1016/S0925-5214(98)00002-7

Ikoma Y, Yano M, Xu ZC, Ogawa K (1999) Isolation of a cDNA encoding active protein for kiwifruit ACC synthase and its specific expression in the outer pericarp. J. Japanese Soc. Hortic. Sci. 68, 286–288. https://doi.org/10.2503/jjshs.68.286

Ilina N, Alem HJ, Pagano EA, Sozzi GO (2010) Postharvest Biology and Technology Suppression of ethylene perception after exposure to cooling conditions delays the progress of softening in 'Hayward' kiwifruit. Postharvest Biology and Technology, 55, 160–168. https://doi.org/10.1016/j.postharvbio.2009.11.005

Jin P, Shang H, Chen J, Zhu H, Zhao Y, Zheng Y (2011) Effect of 1-Methylcyclopropene on Chilling Injury and Quality of Peach Fruit during Cold Storage. Journal of Food Science, 76, 0–6. https://doi.org/10.1111/j.1750-3841.2011.02349.x

Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M (2016) KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44, D457–D462. https://doi.org/10.1093/nar/gkv1070

Kanehisa, M, Furumichi M, Tanabe M, Sato Y, Morishima K (2017) KEGG: New perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45, D353–D361. https://doi.org/10.1093/nar/gkw1092

Karlova, R., van Haarst, J.C., Maliepaard, C., van de Geest, H., Bovy, A.G., Lammers, M., Angenent, G.C., de Maagd, R.A., 2013. Identification of microRNA targets in tomato fruit development using high-throughput sequencing and degradome analysis. Journal of Experimental Botany 64, 1863–1878. https://doi.org/10.1093/jxb/ert049

Langmead B, Salzberg S (2012) Fast gapped-read alignment with Bowtie 2. Nature Methods. 2012, 9:357-359. https://doi.org/10.1038/nmeth.1923

Li W, Liu Y, Zeng S, Xiao G, Wang G, Wang Y, Peng M, Huang H (2015) Correction: Gene Expression Profiling of Development and Anthocyanin Accumulation in Kiwifruit (*Actinidia chinensis*) Based on Transcriptome Sequencing. PLoS One 10, e0138743. https://doi.org/10.1371/journal.pone.0138743

Lim S, Hyun S, Kim J, Jun H, Gu J, Jin E (2016) Inhibition of hardy kiwifruit (*Actinidia aruguta*) ripening by 1-methylcyclopropene during cold storage and anticancer properties of the fruit extract. Food Chem. 190, 150–157. https://doi.org/10.1016/j.foodchem.2015.05.085

Lin Yifen, Lin Yixiong, Lin H, Lin M, Li H, Yuan F, Chen Y (2018) Effects of paper containing 1-MCP postharvest treatment on the disassembly of cell wall polysaccharides and softening in Younai plum fruit during storage. Food Chem. 264, 1–8. https://doi.org/10.1016/j.foodchem.2018.05.031

Liu Y, Li D, Yan L, Huang H (2015) The Microgeographical Patterns of Morphological and Molecular Variation of a Mixed Ploidy Population in the Species Complex *Actinidia chinensis* 1–15. PLoS ONE 10(2): e0117596. https://doi.org/10.1371/journal.pone.0117596

Lytovchenko A, Eickmeier I, Pons C, Osorio S, Szecowka M, Lehmberg K, Arrivault S, Tohge T, Pineda B, Anton M, Hedtke B, Lu Y, Fisahn J, Bock R, Stitt M, Grimm B, Granell A, Fernie A (2011) Tomato Fruit Photosynthesis Is Seemingly Unimportant in Primary Metabolism and

Ripening but Plays a Considerable Role in Seed Development. Plant physiology 157, 1650–1663. https://doi.org/10.1104/pp.111.186874

Ma L, Cao J, Xu L, Zhang X, Wang Z, Jiang W (2014) Scientia Horticulturae Effects of 1-methylcyclopropene in combination with chitosan oligosaccharides on post-harvest quality of aprium fruits. Sci. Hortic. (Amsterdam). 179, 301–305. https://doi.org/10.1016/j.scienta.2014.09.052

Maere S, Heymans K, Kuiper M (2005) BiNGO: a Cytoscape plugin to assess overrepresentation of Gene Ontology categories in Biological Networks. Bioinformatics 21, 3448–3449. https://doi.org/10.1093/bioinformatics/bti551

McGhie TK, Ainge GD (2002) Color in fruit of the genus *Actinidia*: Carotenoid and chlorophyll compositions. J. Agric. Food Chem. 50, 117–121. https://doi.org/10.1021/jf010677l

Michelotti V, Lamontanara A, Buriani G, Orrù L, Cellini A, Donati I, Vanneste JL, Cattivelli L, Tacconi G, Spinelli F (2018) Comparative transcriptome analysis of the interaction between *Actinidia chinensis var. chinensis* and *Pseudomonas syringae pv. actinidiae* in absence and presence of acibenzolar-S-methyl. BMC Genomics 19, 585. https://doi.org/10.1186/s12864-018-4967-4

Minas IS, Tanou G, Krokida A, Karagiannis E, Belghazi M, Vasilakakis M, Papadopoulou KK, Molassiotis A (2018) Ozone-induced inhibition of kiwifruit ripening is amplified by 1-methylcyclopropene and reversed by exogenous ethylene. BMC Plant Biol. 18(1):358. doi: 10.1186/s12870-018-1584-y. PMID: 30558543; PMCID: PMC6296049.

Muñoz-Robredo P, Rubio P, Infante R, Campos-Vargas R, Manríquez D, González-Agüero M, & Defilippi BG (2012) Ethylene biosynthesis in apricot: Identification of a ripening-related 1-aminocyclopropane-1-carboxylic acid synthase (ACS) gene. Postharvest Biology and Technology, 63(1), 85–90. http://doi.org/10.1016/j.postharvbio.2011.09.001

Mworia EG, Yoshikawa T, Yokotani N, Fukuda T, Suezawa K, Ushijima K, Nakano R, Kubo Y (2010) Characterization of ethylene biosynthesis and its regulation during fruit ripening in kiwifruit, *Actinidia chinensis* 'Sanuki Gold'. Postharvest Biology and Technology, 55(2), 108–113. https://doi.org/10.1016/j.postharvbio.2009.08.007

Nishiyama I, Fukuda T, Shimohashi A, Oota T (2008) Sugar and Organic Acid Composition in the Fruit Juice of Different *Actinidia* Varieties. Food Science and Technology Research, 14(1), 67–73. https://doi.org/10.3136/fstr.14.67

Nogueira SB, Labate CA, Gozzo FC, Pilau EJ, Lajolo FM, Oliveira do Nascimento JR (2012) Proteomic analysis of papaya fruit ripening using 2DE-DIGE. J. Proteomics 75, 1428–1439. https://doi.org/10.1016/j.jprot.2011.11.015

Paniagua C, Posé S, Morris VJ, Kirby AR, Quesada MA, Mercado JA (2014) Fruit softening and pectin disassembly: an overview of nanostructural pectin modifications assessed by atomic force microscopy. Annals of botany 114, 1375–1383. https://doi.org/10.1093/aob/mcu149

Park D, Tilahun S, Heo JY, Jeong C (2017) Quality and expression of ethylene response genes of 'Daebong' persimmon fruit during ripening at different temperatures. Postharvest Biology and Technology 133, 57–63. https://doi.org/10.1016/j.postharvbio.2017.06.011

Park YS, Im MH, Gorinstein S (2015) Shelf life extension and antioxidant activity of 'Hayward' kiwi fruit as a result of prestorage conditioning and 1-methylcyclopropene treatment. J Food Sci Technol, 52(5):2711-20. https://doi.org/10.1007/s13197-014-1300-3

Rhodes MJC (1980) Respiration and Senescence of Plant Organs, in: Metabolism and Respiration. Elsevier, pp. 419–462. https://doi.org/10.1016/b978-0-12-675402-5.50016-7

Satyanarayana P, Ossakow SL, Huba JD, Guzdar PN (1984) Rayleigh-Taylor Instability in the Presence of a Stratified Shear Layer. J. Geophys. Res. 89, 2945–2954. https://doi.org/10.1029/JA089iA05p02945

Seymour GB, Taylor JE, Tucker GA (1993) Biochemistry of Fruit Ripening | G.B. Seymour Springer. https://doi.org/10.1007/978-94-011-1584-1

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–504. https://doi.org/10.1101/gr.1239303

Shen YH, Lu BG, Feng L, Yang FY, Geng JJ, Ming R, Chen XJ (2017) Isolation of ripening-related genes from ethylene/1-MCP treated papaya through RNA-seq. BMC Genomics 18, 1–13. https://doi.org/10.1186/s12864-017-4072-0

Shendure J, Ji H (2008) Next-generation DNA sequencing. Nat. Biotechnol. https://doi.org/10.1038/nbt1486

Song Y, Sun L, Lin M, Chen J, Qi X, Hu C, Fang J (2019) Comparative transcriptome analysis of resistant and susceptible kiwifruits in response to *Pseudomonas syringae pv. Actinidiae* during early infection. PLOS ONE 14, e0211913

Sun JJ, Li F, Li X, Liu XC, Rao GY, Luo JC, Wang DH, Xu ZH, Bai SN (2010) Why is ethylene involved in selective promotion of female flower development in *cucumber*? Plant Signal. Behav. 5, 1052–1056. https://doi.org/10.4161/psb.5.8.12411

Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, Santos A, Doncheva NT, Roth A, Bork P, Jensen LJ, Von Mering C (2017) The STRING database in 2017: Quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res. 45, D362–D368. https://doi.org/10.1093/nar/gkw937

Tadesse T, Hewett EW, Nichols MA, Fisher KJ (2002) Changes in physicochemical attributes of sweet pepper cv. Domino during fruit growth and development. Scientia Horticulturae 93(2):91-103. doi: 10.1016/S0304-4238(01)00317-X

Tang W, Sun X, Yue J, Tang X, Jiao C, Yang Y, Niu X, Miao M, Zhang D, Huang S, Shi W, Li M, Fang C, Fei Z, Liu Y (2019) Chromosome-scale genome assembly of kiwifruit *Actinidia eriantha* with single-molecule sequencing and chromatin interaction mapping. Giga Science 8. https://doi.org/10.1093/gigascience/giz027

Tang W, Zheng Y, Dong J, Yu J, Yue J, Liu F, Guo X, Huang S, Wisniewski M, Sun J, Niu X, Ding J, Liu J, Fei Z, Liu Y (2016) Comprehensive transcriptome profiling reveals long noncoding RNA expression and alternative splicing regulation during fruit development and ripening in kiwifruit (*Actinidia chinensis*). Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.00335

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35. doi: 10.1093/nar/gkm306.

Vicente C, Mateos JL, Pedrosa MM, Legaz ME (1991) High-performance liquid chromatographic determination of sugars and polyols in extracts of lichens and sugarcane juice. Journal of Chromatography. 553, 271–283. https://doi.org/10.1016/S0021-9673(01)88498-3

Vieira M, Argenta L, Steffens C, Vieira A (2010) Postharvest quality preservation of "Bruno" kiwifruit by ethylene control. Revista Brasileira de Fruticultura 32, 309–406. https://doi.org/10.1590/S0100-29452010000200008

Wang T, Wang G, Jia ZH, Pan DL, Zhang JY, Guo ZR (2018) Transcriptome Analysis of Kiwifruit in Response to *Pseudomonas syringae pv. actinidiae* Infection. International journal of molecular sciences 19, 373. https://doi.org/10.3390/ijms19020373

Wang X, Ding Y, Wang Y, Pan L, Niu L, Lu Z, et al. (2017a) Genes involved in ethylene signal transduction in peach (*Prunus persica*) and their expression profiles during fruit maturation. Scientia Horticulturae. 224:306–16. https://doi.org/10.1016/j.scienta.2017.06.035.

Wang Y, Sugar D (2015) Postharvest Biology and Technology 1-MCP efficacy in extending storage life of 'Bartlett' pears is affected by harvest maturity, production elevation, and holding temperature during treatment delay. Postharvest Biol. Technol. 103, 1–8. https://doi.org/10.1016/j.postharvbio.2015.02.013

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: A revolutionary tool for transcriptomics. Nat. Rev. Genet. https://doi.org/10.1038/nrg2484

Wang Z, Liu Y, Li L, Li D, Zhang Q, Guo Y, Wang S, Zhong C, Huang H (2017b) Whole transcriptome sequencing of *Pseudomonas syringae pv. actinidiae*-infected kiwifruit plants reveals species-specific interaction between long non-coding RNA and coding genes. Scientific reports 7, 4910. https://doi.org/10.1038/s41598-017-05377-y

Wills RBH (1998) Enhancement of senescence in non climacteric fruit and vegetables by low ethylene levels. Acta Hort. 464, 159_/162.

Wu H, Ma T, Kang M, Ai F, Zhang J, Dong G, Liu J (2019) A high-quality *Actinidia chinensis* (kiwifruit) genome. Horticulture Research 6, 117. https://doi.org/10.1038/s41438-019-0202-y

Xu X, Yin L, Ying Q, Song H, Xue D, et al. (2013) High-Throughput Sequencing and Degradome Analysis Identify miRNAs and Their Targets Involved in Fruit Senescence of *Fragaria ananassa*. PLOS ONE 8(8): e70959. https://doi.org/10.1371/journal.pone.0070959

Xu ZC, Hyodo H, Ikoma Y, Yano M, Ogawa K (2000) Relation between ethylene-producing potential and gene expression of 1-aminocyclopropane-1-carboxylic acid synthase in *Actinidia chinensis* and *A. deliciosa* fruits. J. Japanese Soc. Hortic. Sci. 69, 192–194. https://doi.org/10.2503/jjshs.69.192

Xu ZC, Ikoma Y, Yano M, Ogawa K, Hyodo H (1998) Varietal differences in the potential to produce ethylene and gene expression of ACC synthase and ACC oxidase between "Kui mi" and "Hong xin" of Chinese kiwifruit. J. Japanese Soc. Hortic. Sci. 67, 204–209. https://doi.org/10.2503/jjshs.67.204

Zhang A, Wang W, Tong Y, Li M, Grierson D, Ferguson I, Chen K, Yin X (2018) Transcriptome Analysis Identifies a Zinc Finger Protein Regulating Starch Degradation in Kiwifruit. Plant Physiology 178, 850 LP – 863. https://doi.org/10.1104/pp.18.00427

Zhang L, Li S, Liu X, Song C, Liu X (2012) Postharvest Biology and Technology Effects of ethephon on physicochemical and quality properties of kiwifruit during ripening. Postharvest Biol. Technol. 65, 69–75. https://doi.org/10.1016/j.postharvbio.2011.11.004

Zhou Z, Cong P, Tian Y, Zhu Y (2017) Using RNA-seq data to select reference genes for normalizing gene expression in apple roots. PLoS One 12, 1–17. https://doi.org/10.1371/journal.pone.0185288

Ziliotto F, Begheldo M, Rasori A, Bonghi C, Tonutti P (2008) Transcriptome profiling of ripening nectarine (*Prunus persica* L. Batsch) fruit treated with 1-MCP. J. Exp. Bot. 59, 2781–2791. https://doi.org/10.1093/jxb/ern136

Zou J, Chen J, Tang N, Gao Y, Hong M, Wei W, Cao H, Jian W, Li N, Deng W, Li Z (2018) Transcriptome analysis of aroma volatile metabolism change in tomato (*Solanum lycopersicum*) fruit under different storage temperatures and 1-MCP treatment. Postharvest Biol. Technol. 135, 57–67. https://doi.org/10.1016/j.postharvbio.2017.08.017

TABLE AND FIGURE LEGENDS

Table 1. Summary of alignment results of filtered reads per treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13).

Fig. 1. Fruit chlorophyll (Skin I_{AD} and Flesh I_{AD}), flesh color (L*), and soluble solids content (%) changes in 'Hayward' kiwifruit during 29 d of storage at 20 °C at two ripeness stages (E1 and E2) and four postharvest periods: day 0 (at harvest), day 1 (48 h after harvest), day 15 (after 15 days) and day 29 (after 29 days). In red (not treated fruit), blue (1-MCP), and purple (Ethrel®) are shown. Standard error of each treatment and day is shown and significative differences between treatments per day are shown by Tukey test at p-value < 0.05.

Fig. 2. CO_2 emissions (ml kg^{-1} h^{-1}) of 'Hayward' kiwifruit during 29 d of storage at 20 °C at two ripeness stages (E1 and E2). In red (not treated fruit), blue (1-MCP), and purple

(Ethrel®). Standard error of each treatment and day is shown and significative differences between treatments per day are shown by Tukey test at p-value < 0.05.

Fig. 3. Changes in fruit firmness (P7.9), flesh firmness (P2), and core firmness (P2) in 'Hayward' kiwifruit during 29 d of storage at 20 °C at two ripeness stages (E1 and E2). In red (not treated fruit), blue (1-MCP), and purple (Ethrel®). Standard error of each treatment and day is shown and significative differences between treatments per day are shown by Tukey test at p-value < 0.05.

Fig. 4. Principal component analysis (PCA), including all evaluated traits per treatment (above: T0, not treated fruit; T1, 1-MCP; and T2, Ethrel®) and time (below: A, day 1; B, day 15; and C, day 29).

Fig. 5. a) Number of DEGs by FPKM range in 'Hayward' kiwifruit between treatments (T0, not treated fruit; T1, 1-MCP; and T2, Ethrel®) and days (D4 and D13) including 18,036 DEGs at p-value < 0.005. b) Number of up- and downregulation genes with a differential expression of at least double in day 4 and day 13 for each treatment comparison (T0vsT1, T0vsT2, and T1vsT2).

Fig. 6. Functional enrichment analysis performed for all DEGs in kiwifruit with the BiNGO plug-in in Cytoscape. Functional enrichment analysis performed for all DEGs in kiwifruit under the different treatments (T0, not treated fruit; T1, 1-MCP; and T2, Ethrel®), for day 4 (D4) and day 13 (D13), with the BiNGO complement in Cytoscape. Venn diagrams (A and E) are showing the number of DEGs while the assigned GO terms were used to classify the functions of the DEGs according to biological processes (B and F), cellular components (C and G) and molecular functions (D and H). The colors on the edges of the nodes represents the p-value, as shown by the legend in the graph.

Fig. 7. KEGG pathway analysis of DEGs. Bubble chart shows the enrichment of DEGs in the signaling pathways. The y-axis label represents the pathways, and the x-axis label represents the rich factor. The size of the bubble represents the amount of DEGs enriched, and the color shows the Q value to each pathway.

Fig. 8. DEGs' IPP network. The network is showing the protein-protein interaction nodes. The size of the node is proportional to the number of genes that interact with proteins, and the color of the node represents each pathway. The colors on the edges of the nodes represents the p-value, as shown by the legend in the graph.

Fig. 9. Summary of the top hit genes up and down-regulated considering not treated fruit (T0) and contrasting treatments (T1, 1-MCP vs T2, Ethrel®) in the main pathways involved. Red color indicates a minimum value of FPKM while light yellow indicates a maximum FPKM.

Fig. 10. The expression levels of the genes revealed by RT-qPCR and RNA-seq. In each panel, the post treatment days (day 4 and day 13) are indicated, where the bars arranged from left to right represent the treatments (T0, not treated fruit; T1, 1-MCP; and T2, Ethrel®) to which the kiwifruit were subjected. The annotation of the selected genes are as follows: Achn069361: NYC (porphyrin and chlorophyll); Achn218701: ATSPS4F (starch and sucrose); Achn271251: CHLM (porphyrin and chlorophyll); Achn152281: ACO3 (citrate and glycosylate); Achn219321: PDC2 (glycolysis and gluconeogenesis); Achn281901: PPa1 (oxidative phosphorylation); Achn051381: EC: 3.2.1.15 (pentose and glucuronate); Achn253411: EC: 2.7.1.159 2.7.1.134 (inositol phosphate); Achn358091: PRF3 (actin cytoskeleton); Achn163471: POR (porphyrin and chlorophyll); Achn383491: EC 6.1.1.17 (porphyrin and chlorophyll); Achn330311: ATPME3 (pentose and glucuronate); Achn328501: CRD1 (porphyrin and chlorophyll); Achn372361: APL3 (starch and sucrose); and Achn282431: EC: 1.5.3.1 1.5.3.7 (lysine degradation).

Achn005971 (AGB1) and Achn107351 (ACTIN 7) were used as the reference genes. The error bars in each column indicate the SD of two replicates. The different asterisks in the bars indicate statistically significant differences with a $P < 0.05$ (unidirectional ANOVA, Tukey tests).

SUPPLEMENTARY MATERIAL

Fig. S1. Fructose, glucose, and ascorbic acid content by treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and days (D1, D15 and D29). Standard error of each treatment and day is shown. Significative differences between fructose and glucose (above) per day and treatment and significative differences for ascorbic acid content (below) between treatments for each day are shown by Tukey test at p-value < 0.05.

Fig. S2. Principal components analysis (PCA) of the RNA-seq for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13).

Fig. S3. Quantity versus dispersion of the genes for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13).

Fig. S4. Gene density for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13). Average values (left), and including the three biological replicates (right), per each condition.

Fig. S5. Dispersion diagrams for identifying global changes and trends in gene expression among treatments (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and days (D4 and D13).

Fig. S6. Boxplots of fragments per kilobase million (FPKM) for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13). Average values (left), and including the three biological replicates (right), per each condition.

Fig. S7. Volcano plots of the significant relationship between log10(p-value) and log2(fold change) for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2)

and day (D4 and D13). This graph defines the genes' significant cutoff points (alpha value or p-value). In this case, the alpha is fixed at 0.005, obtaining 18,036 DEGs from a total of 79,614 genes. The non-significant genes are shown in black, and the significant genes are shown in red.

Fig. S8. Number of genes by count range for each treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 on the left, and D13 on the right).

Fig. S9. Pentose phosphate KEGG pathway.

Fig. S10. Citrate cycle (TCA cycle) KEGG pathway.

Fig. S11. Glycolysis-Gluconeogenesis KEGG pathway.

Fig. S12. Starch and sucrose metabolism KEGG pathway.

Fig. S13. Pentose phosphate KEGG pathway.

Fig. S14. Porphyrin and chlorophyll metabolism KEGG pathway.

Fig. S15. Plant hormone signal transduction KEGG pathway.

Fig. S16. Carotenoid biosynthesis KEGG pathway.

Table. S1. Summary of evaluated traits for ripeness stage 1 at harvest.

Table. S2. Summary of evaluated traits for ripeness stage 2 at harvest.

Table. S3. Pearson correlations of ripeness stage 1.

Table. S4. Pearson correlations of ripeness stage 2.

Table. S5. BLUP coefficients for both ripeness stages.

Table. S6. Raw data RNA-seq statistics.

Table. S7. Total filtered reads and alignment results by replicate.

Table. S8. Test genes in different treatments and days.xlsx

Table. S9. Gene Ontology_Bingo.xlsx

Table. S10. Analysis of enrichment of pathways_Kegg.xls

Table. S11. PPI string_1mcp.xlsx

Table. S12. RT-qPCR.xlsx

Table 1. Summary of alignment results of filtered reads per treatment (not treated fruit, T0; 1-MCP, T1; and Ethrel®, T2) and day (D4 and D13).

ID*	Filtered reads	Reads not aligned	Reads (=1)	Reads >1	Reads aligned
T0_D4	17,428,301	5,077,644	6,295,065	6,055,592	12,350,657
T1_D4	17,807,074	5,085,540	6,546,095	6,175,439	12,721,534
T2_D4	16,956,703	4,742,136	6,119,174	6,095,393	12,214,567
T0_D13	16,972,564	5,101,475	6,007,613	5,863,476	11,871,089
T1_D13	17,073,064	5,058,979	6,095,167	5,918,918	12,014,085
T2_D13	18,941,593	5,729,116	6,685,738	6,526,738	13,212,476
Average	17,529,883 (100%)	5,132,482 (29.3%)	6,291,475 (35.9%)	6,105,926 (34.8%)	12,397,401 (70.7%)

Data for each treatment and day are from three biological replicates (*). Reads aligned to unique position (=1) and reads aligned to multiple positions (<1)

Fig. 1

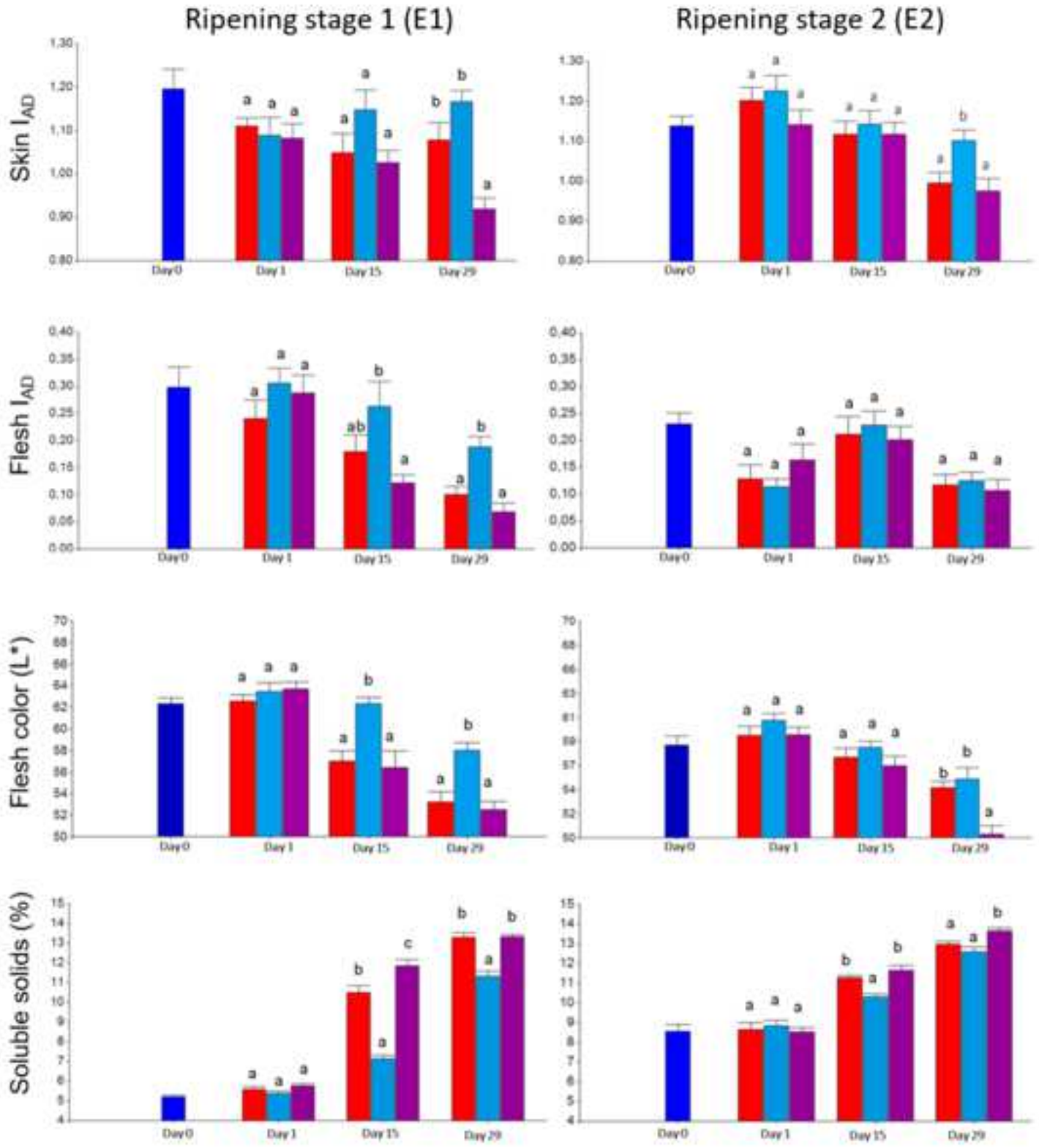

Fig. 2

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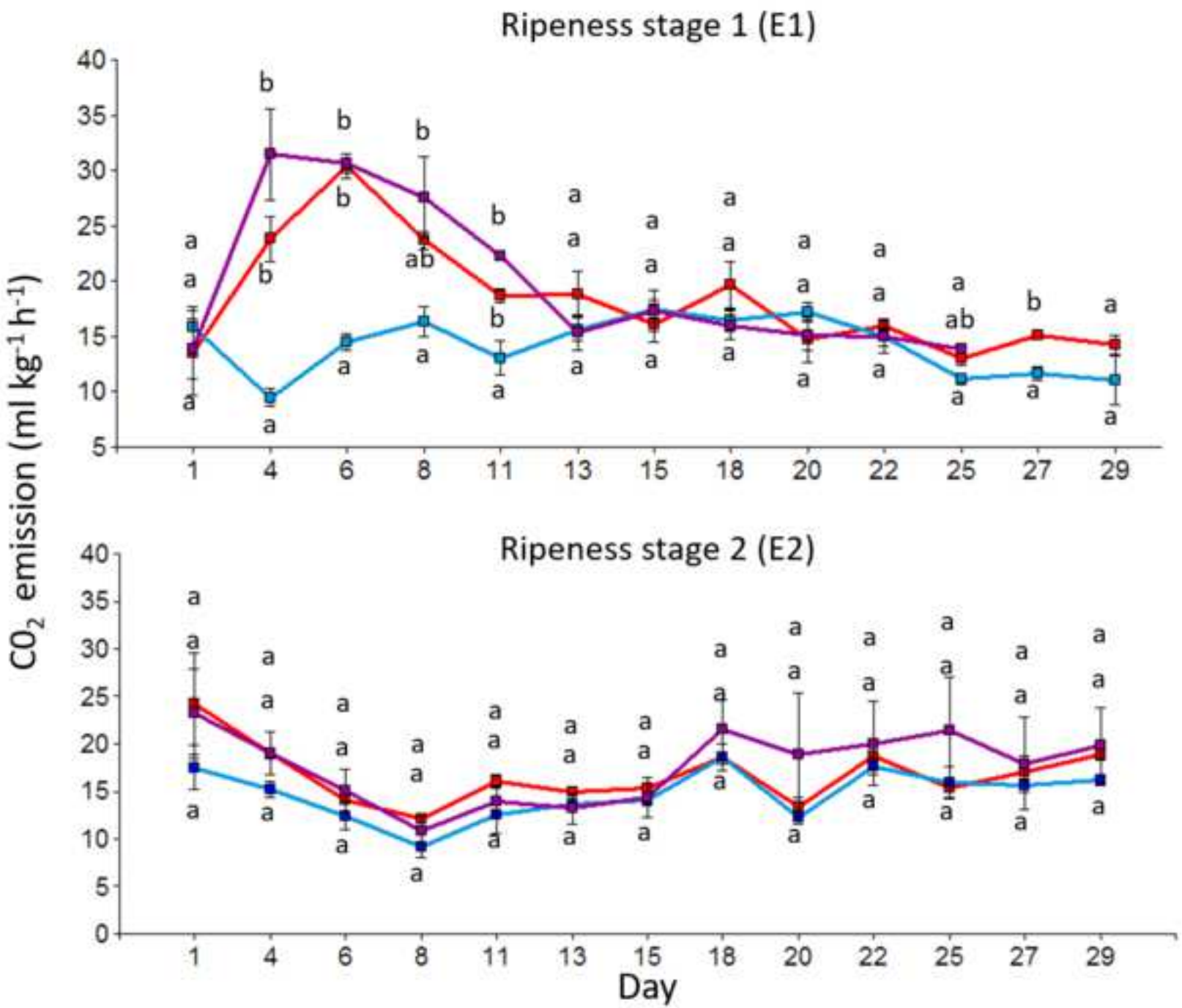

Fig. 3

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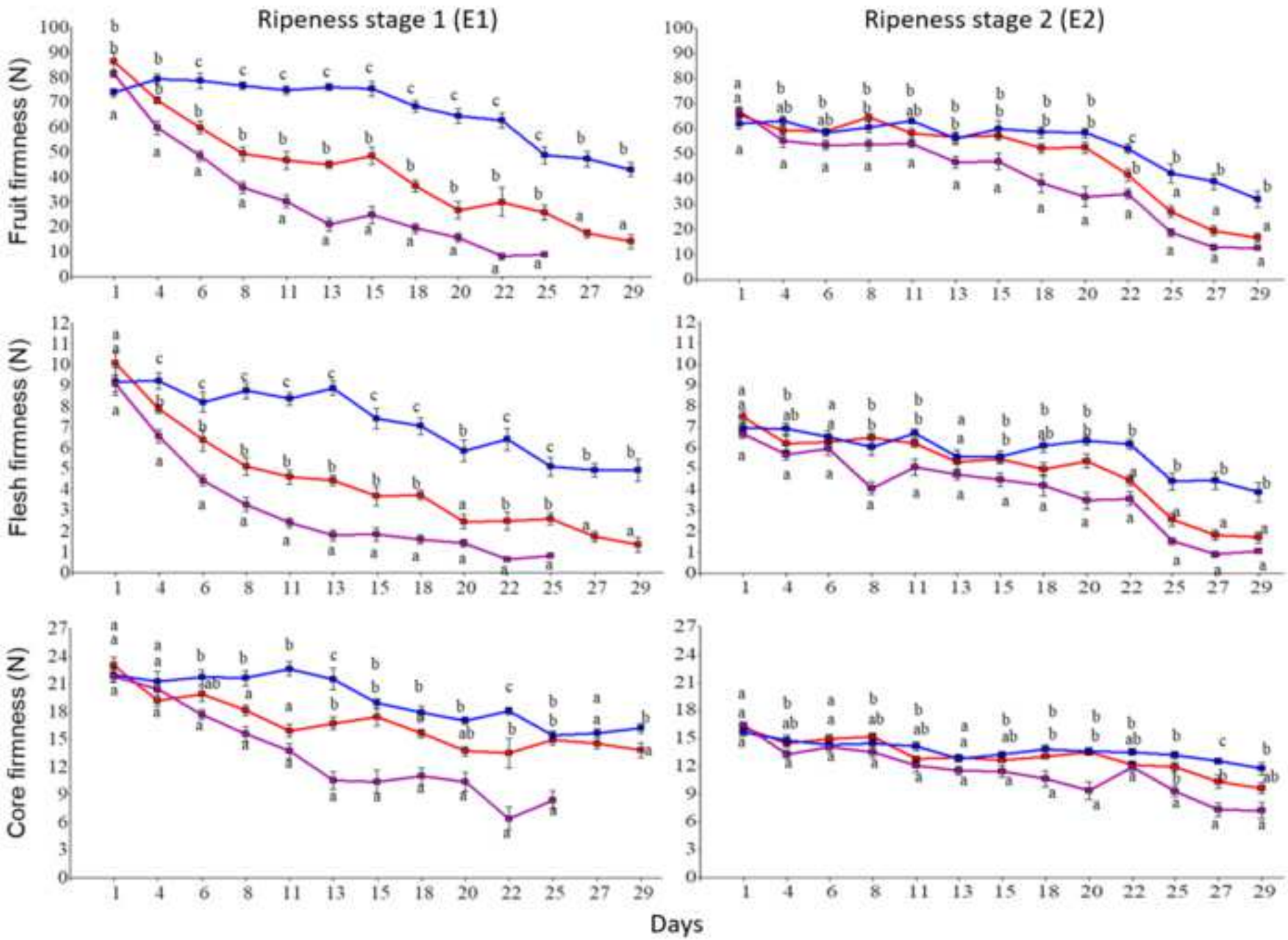

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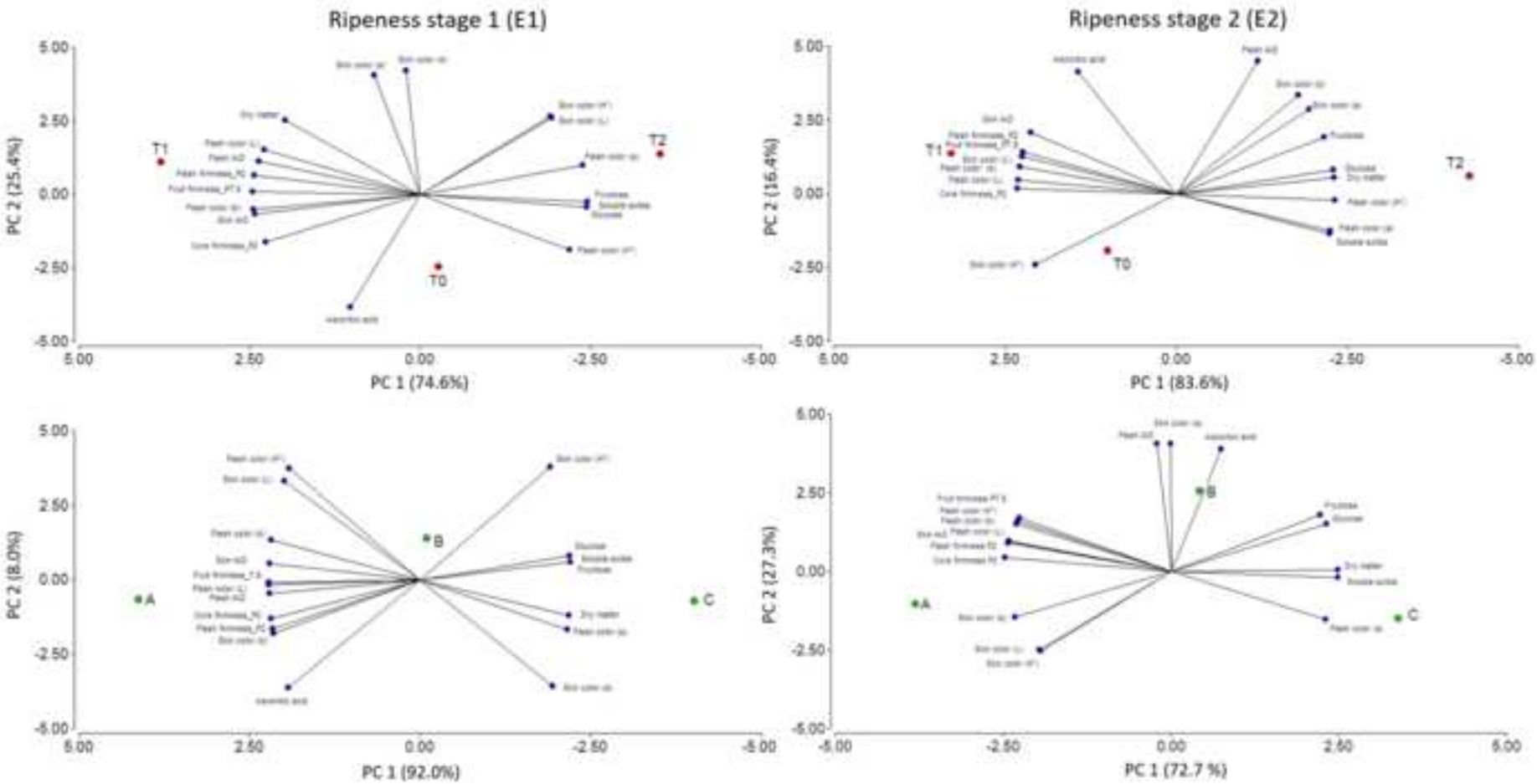

Fig. 5

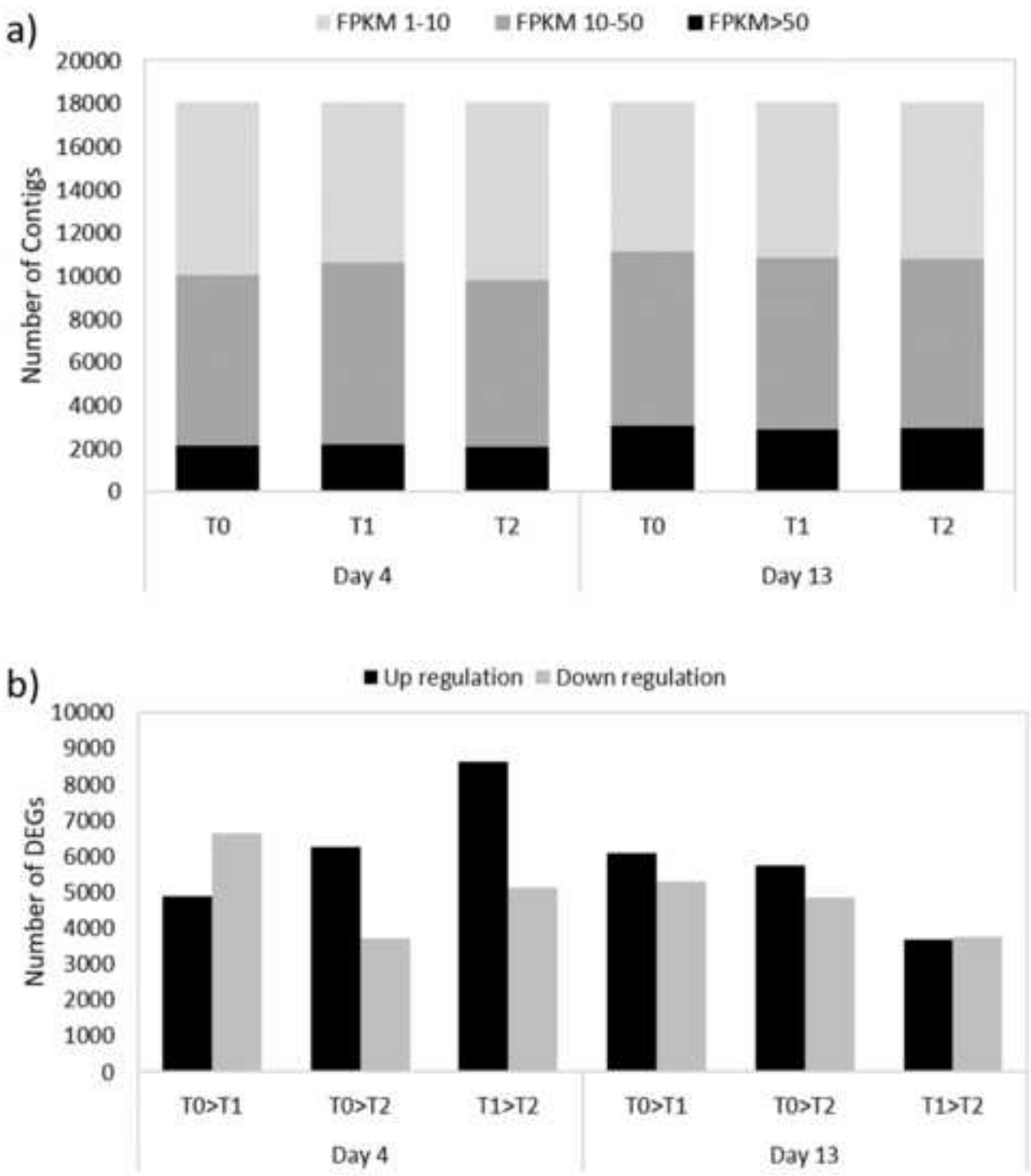

Fig. 6

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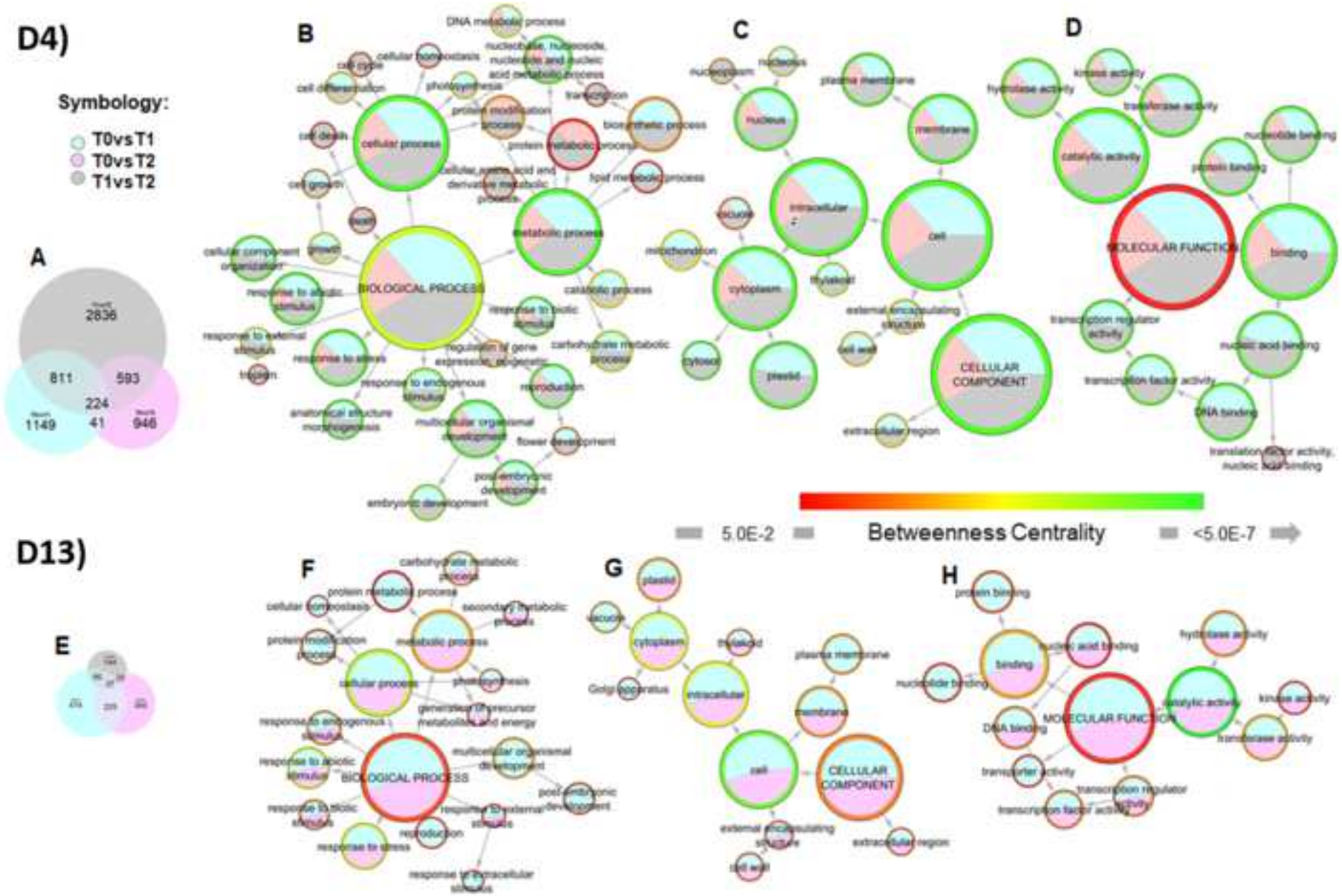

Fig. 7

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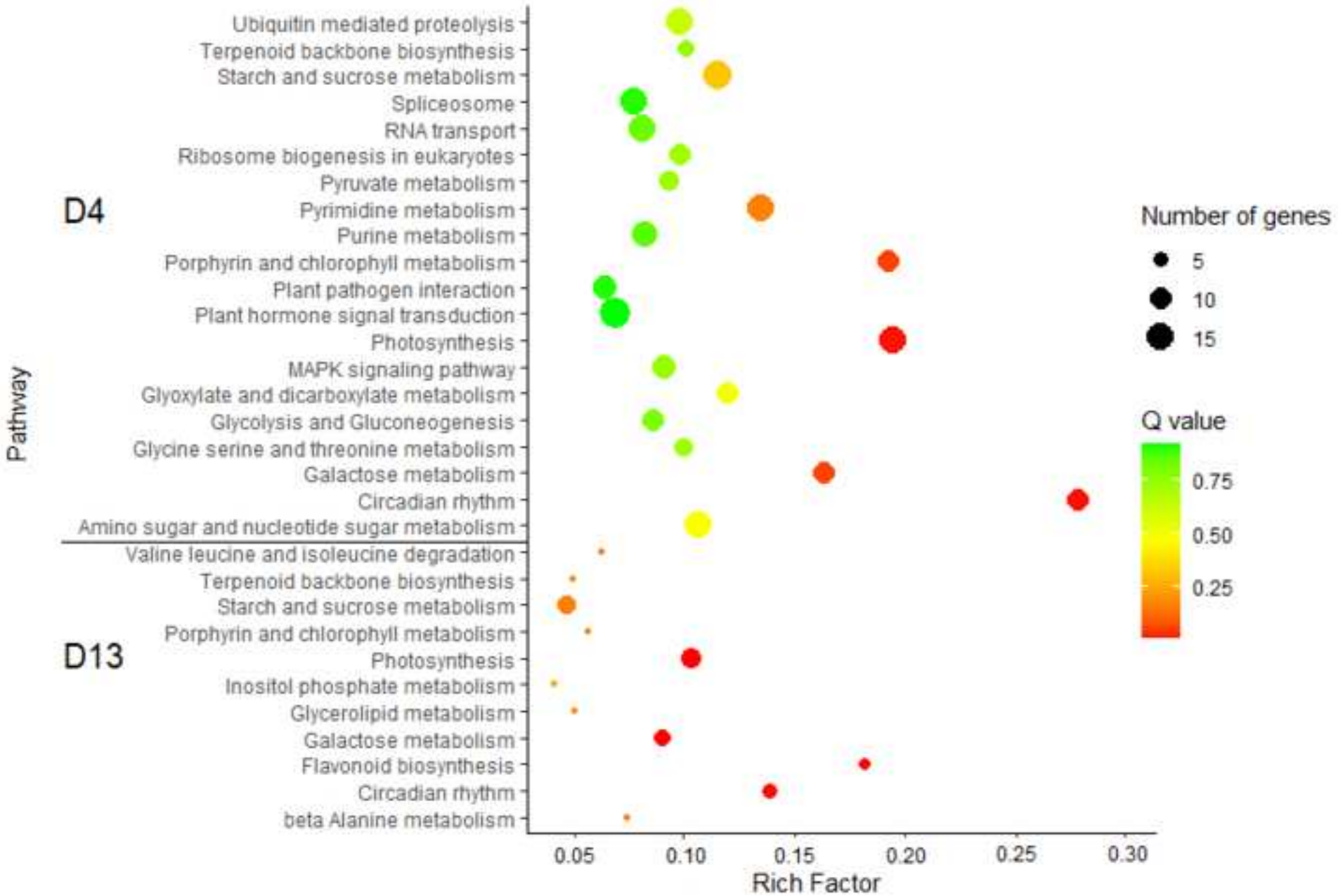

Fig. 8

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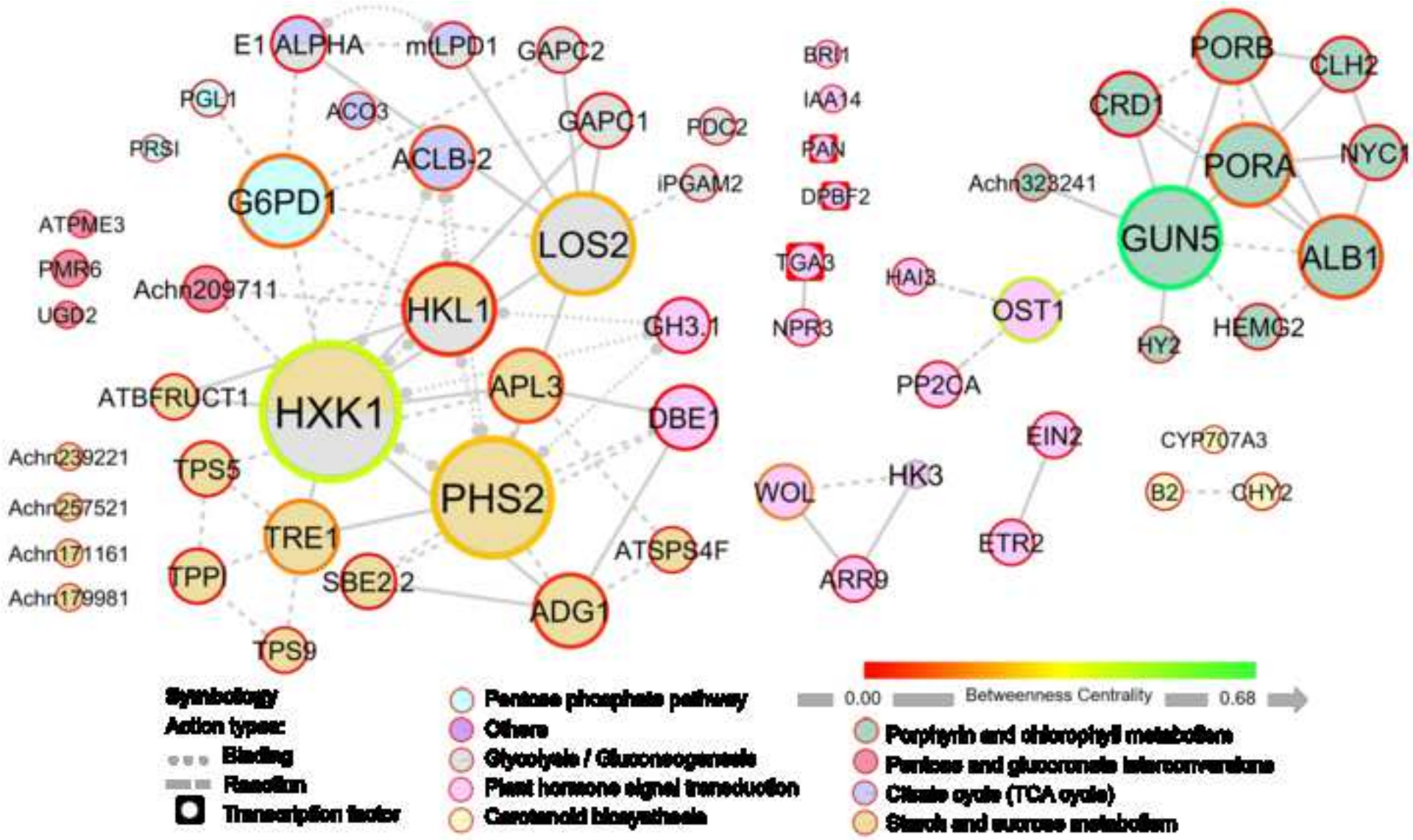

Fig. 9

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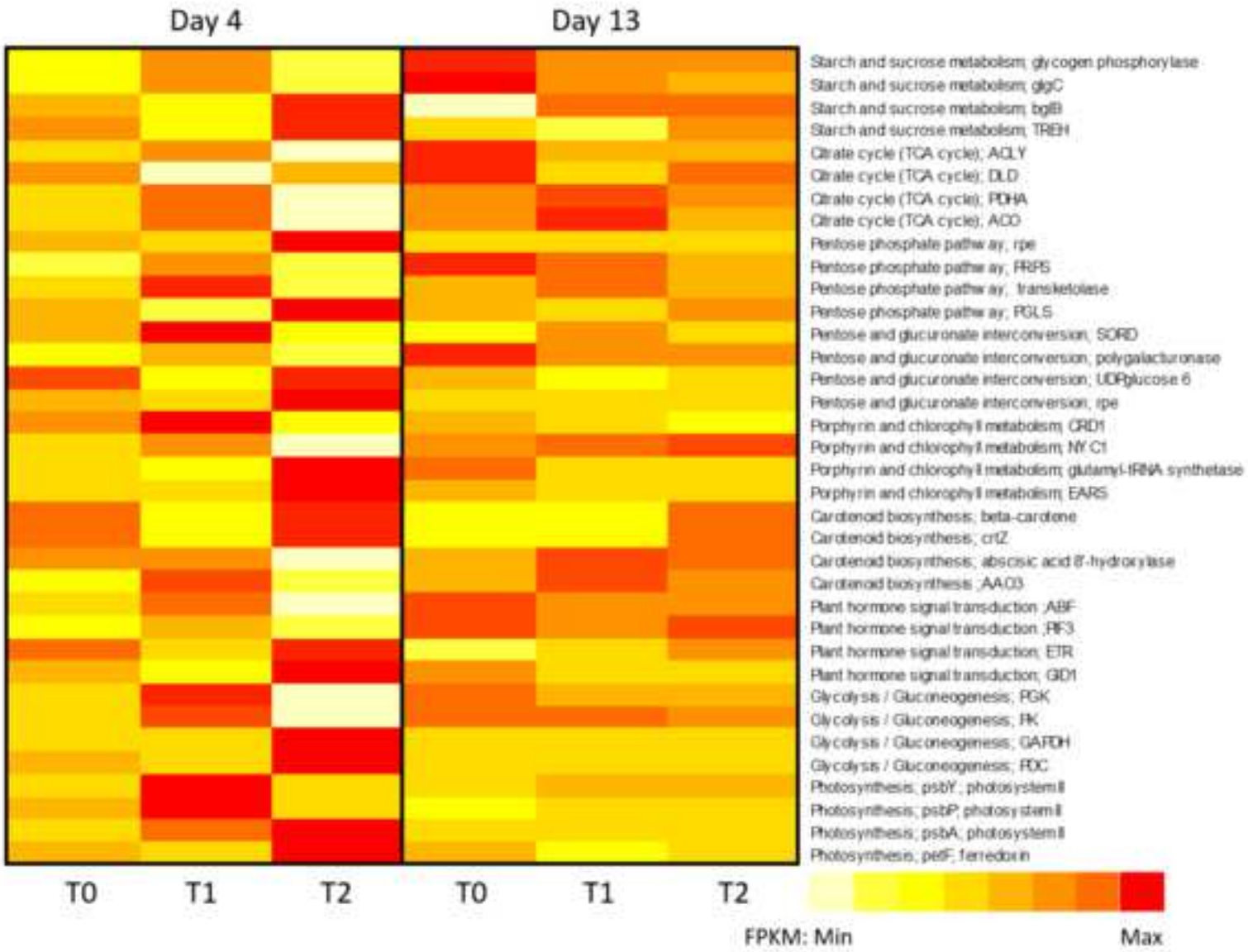

Fig. 10

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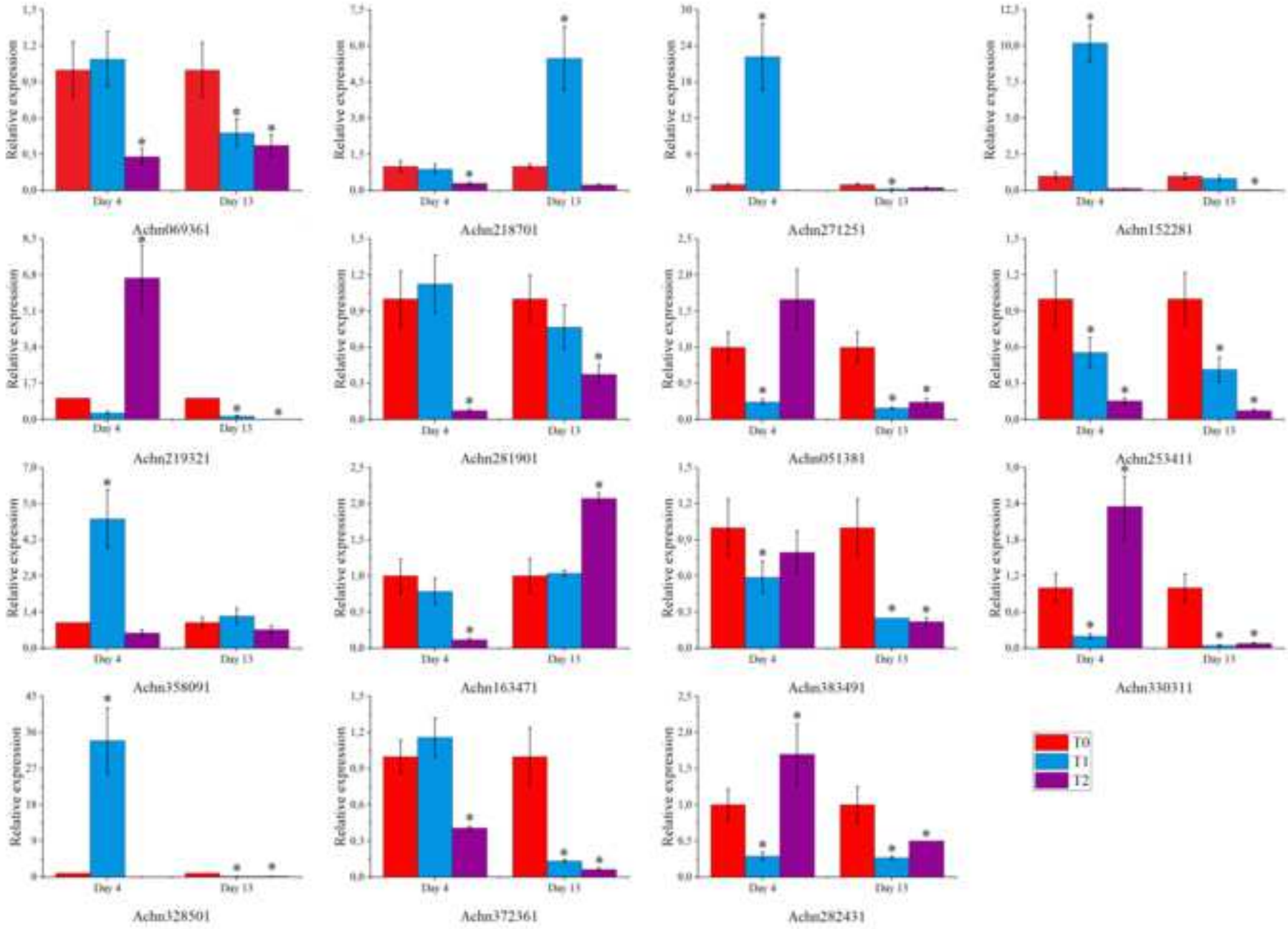

Fig. S1

Fig. S2

Fig. S3

Fig. S4

Fig. S5

Fig. S6

Fig. S7

Fig. S8

Fig. S9

Fig. S10

Fig. S11

Fig. S12

Fig. S13

Fig. S14

Fig. S15

Fig. S16

Table S9

Table S10

Click here to access/download

Supplementary Material

Table S10. Analysis of enrichment of pathways_Kegg.xls

Table S11

Table S12

Comments for the Author:

AE: The manuscript requires minor revisions but is overall a good quality manuscript fitting the scope of TGGE, it could be published after corrections following the reviewers' recommendations, below.

Reviewer #1: The present work is interesting as it investigates the main protagonists of the ripening of kiwifruits in the post-harvesting steps. It is a very big and complex work that I really appreciated. Thus, I have only some minor suggestions for the authors to improve their paper:

a) the title should be revised because unclear.

The title was modified for: "Transcriptome analysis and postharvest behavior of the kiwifruit 'Actinidia deliciosa' reveal the role of ethylene-related phytohormones during fruit ripening"

b) in the abstract what are 1-MCP and Ethrel should be reported.

In the Abstract section, 1-MCP and Ethrel® treatments have been specified as ethylene inhibitor and ethylene stimulator respectively (lines: 32-33)

c) how was identified the plant genetic identify at variety level?

The 'Hayward' variety identity was corroborated through "Viveros Biotecnia" in an orchard in Teno (line 154)

d) No citation is reported for the methods applied to measure vitamin C content, sugar content, etc. Please, add them.

Methodology references for sugar measurements (Vicente et al., 1991) and for vitamin C content (El Gindy et al., 2006) were cited (lines 196 and 201).

e) Why did not the authors consider RNA smaller than 100 bp? It could be interesting to also investigate presence and variation of microRNA that recently have been documented to possess a key role in the determination of plant development. I strongly suggest the authors to make a further study, in future, and recover the data of the small RNAs from their libraries and check putative differences and microRNAs with potential function in kiwifruit ripening. In the present paper, thus, they should state that also microRNA could have an important activity in regulating kiwifruit maturation and that it could be investigated. In particular, they should also underline the role that plant microRNA could also have on the consumers of these fruits. I suggest to read and cite some papers to support this evidence: Journal of experimental botany, 2013, 64.7: 1863-1878;. Scientific reports, 2019, 9.1: 1-14; PLoS One, 2013, 8.8: e70959.

I agree, in the following studies we will consider this fact and we will recovery small RNAs in order to find evidence about how this small RNA is affecting ripening in kiwifruit. In addition, as you have suggested we have cited the papers above (Karlova et al., 2013 in Journal of Experimental Botany and Xu et al., 2013 in PLOS ONE) in the discussion section, considering further analysis using small RNA (lines: 620-621).

f) line 281 (and others) I do not understand the meaning of the symbol reported as a full stop in "10 µmol * L-1". Please, remove it.

This symbol was removed (new lines 283 and 284). Therefore, "µmol * L^{-1}" was replaced by "µmol L^{-1}"

g) The patronymic of all cited plant species should be reported. Morevoer, the scientific names of plants should be reported in italics (even in reference list).

The patronymic of all plant species was cited (lines: 92-95) and plant scientific names have been reported in italics even in the reference list

Reviewer #2: The several gene networks related to kiwifruit ripening were found after treatments by 1-mcp and Ethrel, and the author also verified the gene expression by qRT-PCR analysis. These results provide some important reference information for extending the shelf-life of kiwifruit using 1-MCP and Ethrel. The grammar is also good.

(1) In the section of plant materials, genus and species names of kiwifruit are required.

In this section and in all manuscript genus and species names of kiwifruit and other species were added.

(2) In the section of discussion, the things discussed are not very clear. It is better divided into several subsections.

This section was divided in several subsections as follows: fruit phenotyping (line 476), sequencing and alignment results (line 501), differentially expressed genes (DEGs) and functional annotation (line 516), Protein-protein interaction network of DEGs and related pathways (line 534)

Reviewer #3: In their manuscript "Postharvest behavior and the expression of 1-MCP and ethylene responses in genes of the kiwifruit 'Actinidia deliciosa' during ripening", Juan Salazar and colleagues analyze the effect of 1-MCP and Ethrel® treatment on the kiwifruit at the physiological and molecular level. First the authors analyzed different parameters (chlorophyll and sugar content, CO2 emission, firmness…) at different time points (0, 1, 15 and 29 days after harvesting) after mock, 1-MCP or Ethrel® treatment made at two different ripeness stages (5.2% (E1) and 8.0% (E2) soluble sugar content) to evaluate the effect of the treatment on fruit ripening and post-harvest behavior. As expected, 1-MCP (ethylene biosynthesis inhibitor) and Ethrel® (climacteric fruit ripening accelerator) treatments led to

opposite effects, with delay and accelerated ripening respectively (reported by changes of chlorophyll content, fruit flesh color and firmness, and increase of soluble sugar concentration), compared to mock treatment. Their analyses revealing a bigger effect in the E1 condition and the biggest differences being at 4 days (burst of respiration rate) and 13 days (highest fruit firmess difference) they carried out a RNAseq analysis on fruits in the different conditions at these two stages. RNAseq analyses revealed as expected a high number of differentially expressed genes with many genes involved in the central carbon and chlorophyll metabolism in relation with the timing difference in the cholorophyll degradation and fruit ripening between the treatments. Taken together, these data provides a very complete description of the role of ethylene in kiwifruit ripening at a physiological and gene expression level and will be of great interest to the scientific community. However I think that the manuscript could be improved and I address my concerns in several comments below. I hope that my comments are constructively contributing to improve the quality of the manuscript. In my opinion, the manuscript is suitable for publication in TGGE, after the authors have addressed the following comments and questions:

Scientific concerns:

- The effect of the treatments on fruit ethylene production has not been demonstrated in this study, ethylene production measurements should have been done to prove that the respiratory burst is linked to an increased ethylene production at day 4 and to demonstrate that 1-MCP treatment is blocking this ethylene burst.

I agree with the comment, but although we did not measure ethylene emission, in the 'Hayward' case, in previous studies such as Ilina et al. (2010), the respiratory burst is linked to an ethylene increase. However, despite we should have taken into account the ethylene rate measurement, the results show gene expression differences on ripening or ethylene-related genes. In further analysis of kiwifruit or other climactic fruits the ethylene emission will be considered.

- Some enzymes activity (involved in the glycolysis for example) could have been measured to confirm that the transcriptional regulation is effectively affecting the metabolism.

In the gene validation by RT-qPCR (Fig.10) is included the gene Achn219321 linked to PDC2 protein being this protein involved in the glycolysis. Anyway, in order to clarify the metabolic pathway of each gene, I've added the linked pathway between parenthesis.

- The last reference of the result part is wrong, the publication referenced is about MCP-1 for Monocyte chemoattractant protein-1 (Kim et al., 2014), the authors should be more careful.

Ok, this reference was eliminated because it was generating some confusion

The writing and organization of the manuscript could be improved:

- I suggest to call the three different treatments differently, calling them T0, T1 and T2 is confusing. I would suggest to call them not treated, 1-MCP and Ethrel® treatment instead.

As you have suggested T0, T1 and T2 were replaced by not treated, 1-MCP and Ethrel® treatments respectively throughout the manuscript.

- Why 48h after harvest is called day 1 and not day 2?

We consider to evaluate the fruit after 48 hours to facilitate the healing of the peduncular zone of the fruit, considering day 1 after 48h

- The last paragraph of the results is a discussion, it should be removed from the result part.

The last paragraph refered to gene validation (section: Confirming genes' expression using RT-qPCR) was modified, and some information in the discussion section was included (lines: 567-571).

- The title of the manuscript is unclear, I suggest "Transcriptome analysis and postharvest behavior of the kiwifruit 'Actinidia deliciosa' reveal the role of ethylene during fruit ripening" or similar.

The title was replaced by: "Transcriptome analysis and postharvest behavior of the kiwifruit 'Actinidia deliciosa' reveal the role of ethylene-related phytohormones during fruit ripening"

Several modifications of the formatting of the figures could greatly improved the reading of the manuscript:

- The color code should be mentioned on each figure and be more consistent between figures.

The color code or the description of each treatment and day is described in the legends

- I suggest to keep the same formatting in main and supplemental figure, for example keeping the color code red, blue purple in Supp. Fig1.

The formatting of Supp. Fig 1. has been remained because a color change in the color code does not favor the aesthetics of the figure. The rest of the figures were modified as suggested by the reviewer.

- Figure 3.
  - Top right panel: the blue color is not the same
  - Bottom panel: y-axis should start at 0

Blue color and y-axis have been modified in Fig. 3

- Figure 5 and 6 could be combined

Figures 5 and 6 were combined as Fig. 5.

- Figure 10. I suggest to go from White (min) to Red (max).

FPKM range was changed from White (min) to Red (max) and figures has been renamed to Figure 9

- Figure 11.

o I suggest to use the same color code as in figure 1 (blue, red and purple)
o The correspondence f the green to red colors is not clearly explained in the figure legend.

Figure 11 has been renamed to Figure 10 and I have replaced color code as in figure 1 (blue, red and purple) and raw Z-score were not considered in this figure.

Several mistakes and misformulations should be corrected, I suggest to the author to do a careful proofreading of the main text.

All of mistakes and misformulations were corrected after a detailed reading