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The use of microarray μPEACH1.0 to investigate transcriptome changes during transition from pre-climacteric to climacteric phase in peach fruit

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Abstract

The transition from pre-climacteric to climacteric phase is a critical step during fruit development. A holistic approach to study this transition has been undertaken using the first available peach microarray (μPEACH1.0) containing about 4800 oligonucleotide probes corresponding to a set of unigenes most of them expressed during the last stages of fruit development. Microarray hybridizations indicated that among the genes present in the microarray slide, 267 and 109 genes are up- and down-regulated, respectively. Genes have been classified according to the TAIR Gene Ontology into three main categories based on cellular localization, molecular function and biological process. Considering the cellular localization, the most significant up- and down-regulated gene products belong to cell wall and chloroplast compartments. Within the molecular function and biological process categories, a dramatic up-regulation has been detected for genes encoding transcription factors and enzymes involved in ethylene biosynthesis and action. A new member of ETR peach family (*Pp-ETR2*) has been characterized: this gene shows high similarity to *Arabidopsis EIN4*, tomato *Le-ETR4*, and strawberry *Fa-ETR2*. Transition from S3 to S4 is paralleled by changes in expression of 19 genes encoding transcription factors (TFs) belonging to several families including MADS-box, AUX/IAA, bZIP, bHLH, HD, and Myb. Differential expression of genes involved in specific quality traits has also been observed: besides confirming previous data on cell wall-related gene expression, a new pectin-methyl esterase and two new expansins have been identified. Several genes encoding enzymes acting in the isoprenoid biosynthetic pathway appeared to be strongly induced at S3/S4 transition. Among those involved in carotenoid biosynthesis we found also a β-carotene hydroxylase, responsible for the formation of β-cryptoxanthin, the most abundant carotenoid of ripe yellow peaches.

Keywords: Ethylene; Gene expression profiling; Isoprenoids; *Prunus persica*; Ripening; Softening; Transcription factors

1. Introduction

Gene expression is one of the key regulatory mechanisms used by living cells to sustain and execute their functions. During ripening, fleshy fruits undergo a number of physico-chemical and physiological changes affecting pigmentation, texture, flavour, and aroma making the fruit more attractive and edible. All these changes are the results of a co-ordinated and programmed modulation of gene expression regulated by complex and inter-related mechanisms affected by internal and external factors. In recent years, studies on the genetic regulation of the ripening process have dramatically increased particularly in tomato, considering the high number of mutants available in this species, the application of the most updated genomic techniques and the in-progress genome sequencing programme [1]. Transcriptome analysis represents an important approach that, in combination with other techniques, allows elucidation and better understanding of complex physiological processes and their genetic regulation [2]. Among the tools developed for large-scale gene expression analysis, microarrays are rapidly and successfully spreading because of their features and advantages mainly (but not exclusively) represented by the possibility of carrying out a massive gene analysis with a single experiment, thus avoiding the limits of the traditional single gene approaches [3]. Following the

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first study carried out in strawberry by Aharoni et al. [4] who identified a gene involved in flavour biogenesis, this technique has been used mainly to monitor changes of gene expression pattern throughout fruit development in different fruit species as tomato [2,5], strawberry [6], pear [7], citrus [8], and grape [9,10]. Taken together, these studies confirmed the potential of this technique for large-scale transcriptome analysis in fleshy fruits and pointed out that, in general, the most dramatic changes in expression profiles occur in correspondence of crucial developmental phases as fruit set, growth cessation, maturation, and the onset of ripening. Thus, the transition from pre-climacteric to climacteric phase in which ethylene acts as enhancer of the ripening syndrome appears of particular interest. Due to peculiar aspects of ripening such as rapid softening, late ethylene climacteric, short post-harvest life, and the increased body of evidences concerning the basic mechanisms operating at this stage regarding ethylene physiology and cell wall metabolism [11], peach is becoming a good model for both ripening and genomic analysis of fruit tree species. In the last years, peach has been a target for genomic studies and EST collections are now available (http://www.genome.clemson.edu/projects/peach/est; http://linuxbox.itb.cnr.it/ESTree). A preliminary analysis carried out on about 1000 ESTs expressed in climacteric peach mesocarp identified some transcription factors (TFs) belonging to MADS, bZIP, and bHLH families that, as observed in other fruit species, might play a regulatory role also in the ripening of peaches [12]. As result of this genomic approach, the first oligo-based peach microarray named μPEACH1.0 has been constructed [13] and used, in the present work, to analyze the transcription profile changes occurring during the transition from pre-climacteric to climacteric stages.

2. Materials and methods

2.1. Plant material

Trees of *Prunus persica* (L.) Batsch cv. Fantasia were grown at the experimental farm 'Francesco Dotti' of the University of Milano, Italy. This nectarine is a freestone, mid-season variety taking about 115 days to complete the fruit developmental cycle. The growth curve of peach fruit displays a double sigmoid pattern that can be divided into four stages (S1–S4). These phases are determined on the basis of the first derivate of the growth curve, expressed as the transversal diameter, according to Tonutti et al. [14]. Peach is a climacteric fruit in which ethylene evolution peaks at late S4 (climacteric phase). Samples of mesocarp at the four stages were collected and stored at −80 °C until use.

2.2. RNA extraction and Northern analyses

Total RNA was extracted using the protocol described by Ruperti et al. [15]. Ten micrograms of total RNA was fractionated on a 1.2% agarose denaturing gel for Northern blot and further hybridized with ^{32}P DNA labeled probe as described by Tonutti et al. [14]. To evaluate equal loading, blots were probed with a PCR fragment encoding peach ribosomal RNA (18S rRNA).

2.3. Preparation of the μPEACH1.0 microarray

In the framework of an Italian consortium for the development of peach genomics (ESTtree, see the web site http://linuxbox.itb.cnr.it/ESTree), several thousands of ESTs have been produced. These sequences, together with others available in public databases (mainly at GDR, Genome Database for Rosaceae at Clemson University Genomics Institute, http://www.genome.clemson.edu/gdr/projects/prunus/unigene/), for a total of 11,201 independent entries were pre-processed and clustered using Seqman II software (Lasergene DNASTAR). After clustering, the 4818 unigenes have been matched against *Arabidopsis* proteome using the BLASTX algorithm [16] and manually annotated.

From the unigene set, 4806 specific 70-mer oligos have been designed based on the cross-hybridization identity (maximum 70%), GC content (maximum 60%), and T_m (minimum 70 °C). The probes have been synthesized by Operon (from which the Peach Array-Ready Oligo Set is commercially available at www.operon.com). Each of the 4806 oligos, harbouring a 5′ amino linker, was deposited onto glass slides (Perkin-Elmer, USA) at CRIBI (University of Padova) using GenpakARRAY 21 spotter (Genetix Inc., Massachusetts, USA) in 32 subgrids (4 columns × 8 rows) with a replicate in the same subgrid. To facilitate image analysis reference spots have been deposited in each first line/column of each subgrid. Distance between spots was 135 μm on either axes and spot average diameter was ranging from 70 to 80 μm (for further details and general features about μPEACH1.0 preparation see Ref. [13]).

2.4. cDNA synthesis and labeling

Total RNA (20 μg) from S3 (pre-climacteric) and S4 (climacteric) stages was converted into target cDNA by reverse transcription using the SuperScript™ Indirect cDNA Labeling System (Invitrogen, USA) following manufacturer instruction. To label the cDNA, this indirect system incorporates aminoallyl- and aminohexyl-modified nucleotides instead of fluorescent nucleotides in the first stranded cDNA synthesis reaction, to avoid the low incorporation that may result from direct labeling systems [2]. The amino-modified cDNA was coupled to a monoreactive *N*-hydroxysuccinimide (NHS)-ester fluorescent dye: the green-fluorescent cyanine3 (Cy3) and the red-fluorescent cyanine5 (Cy5) (Amersham Biosciences, UK). A final purification step removed any unincorporated dye. The purity and yield of the labeled cDNA was calculated from the OD values obtained by means of a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences) using the formulas reported in the SuperScript™ Indirect cDNA Labeling System (Invitrogen) instruction manual.

2.5. Microarray hybridization

The pre-hybridization and hybridization steps were carried out in Corning® hybridization chambers with some drops of 0.3× SSC to maintain internal humidity and salts concentration, immersed in a water bath at 48 °C. The μPEACH1.0 was

pre-hybridized for 2 h with a solution containing 5× SSC, 0.1% SDS, 1× Denhardt's, and 100 ng/μl DNA carrier. Then the slides were washed once with 0.2× SSC solution and isopropanol, and dried by centrifuging for 2 min at 2000 rpm.

Before hybridization, 100–150 pmol of S3-Cy3 and S4-Cy5 (or S4-Cy3 and S3-Cy5 for the swap procedure) probes were mixed to have an equal amount of each fluorescent dye, ethanol precipitated with ammonium acetate (final 2 M) and resuspended in 37 μl of hybridization solution (5× SSC, 0.1% SDS, 25% formamide, and 40 ng/μl DNA carrier). The probe was denaturated for 30 s, distributed on the spotted area of the slide, and covered with a glass cover slip. After 48 h of hybridization the slides were washed once in 1× SSC 0.2% SDS and in 0.1× SSC 0.2% SDS for 5 min and twice in 0.2× SSC for 5 min to eliminate completely SDS residues that may cause shadows on the spotted area. The probe design and these hybridization/washing conditions allow the detection of specific genes even within gene families.

The two cDNA were labeled with inverted fluorophores in order to increase accuracy and to correct for dye-related bias [4]. Biological replicates were also created, repeating three times the same combination of targets.

2.6. Data analysis

The microarrays were scanned with a two-channel confocal microarray scanner (ScanArray® Lite, Perkin-Elmer) using its dedicated software (ScanArray Express 3.0.0., Perkin-Elmer). The laser power was set between 67 and 79% of maximum and the photomultiplier tube (PMT) was set between 69 and 79% of maximum. The excitation/emission settings were 543/570 nm for Cy3 and 633/670 nm for Cy5. After laser focusing and balancing of the two channels, scans were conducted at a resolution of 5 μm. For any scan, two separate 16-bit TIFF images were produced.

The images were then processed using the software TIGR Spotfinder 2.2.3. (www.tigr.org) using the Otsu algorithm. The spots were also visually examined to delete the non-uniform ones. The mean intensity of the randomly negative controls present on the array for each dye was calculated to estimate the background intensity caused by non-specific binding of the labeled cDNA. The 60% of this value was arbitrarily attributed to those spots having in a single channel a fluorescence value less than the mean of the negative control. This procedure has been applied to avoid over-evaluation of the ratio between the two channels for those genes showing a sudden transcriptional switch between the two development stages.

Raw data were imported to Genespring Software demo (Agilent Tech., USA) and Locally Weighted Regression Scatter Plot Smoothing (LOWESS) approach has been used to normalize and transform the expression ratios. After normalization, the ratios were transformed to their $\log_2$ values. A $\log_2$ transformation converts the expression values to an intuitive linear scale that represents two-fold differences [2].

The final step was to identify the genes that are differentially expressed during the transition between S3 and S4. A Student's *t*-test ($p < 0.05$) was used to select the genes with an expression ratio different from 1. Genes showing $\log_2$ ratio >1 (up-regulated) and <-1 (down-regulated) were annotated following the Gene Ontology categories (GO) developed by TAIR [17].

3. Results

3.1. Ontology of genes differentially expressed during S3/S4 transition

Hybridization of μPEACH1.0, carried out with cDNA probes obtained from mesocarp at S3 (pre-climacteric phase, ethylene evolution 0.5 nl gfw^{-1} h^{-1}) and S4 (climacteric phase, ethylene evolution 8 nl gfw^{-1} h^{-1}) stages, allowed the identification, on the basis of ratio intensity, of 267 up-regulated, 1775 unaffected, and 109 down-regulated genes at S3/S4 transition (data supplement). Considering up- and down-regulated genes, 56 do not show any significant matches with the *Arabidopsis* proteome. Interestingly, to this group belongs Ctg_1 that is the most abundantly represented sequence with 552 copies corresponding to about 5% of total EST number in the EST peach repertoire [13]. The product of this gene shows the highest similarity with plant proteins belonging to Rosids as a putative allergen in apricot and a ripening-related polypeptide in grape. All other genes have been annotated according to the three standard ontologies strictly linked in order to establish a relation among the cellular localization, the gene product and the biological process [17]. Each category is represented by the number of up- and down-regulated genes during the transition from S3 to S4 (Fig. 1). All the major cellular compartments show a significant higher amount of up-regulated genes in comparison with the down-regulated ones (Fig. 1A) but for plasma membrane, ribosome, and Golgi apparatus. The few genes (10) targeted to these compartments are mainly down-regulated. Considering the molecular functions (Fig. 1B), with the exception of genes encoding proteins with either structural or nucleic acid binding activity, a general up-regulation is present at S3/S4 transition. The up-regulation of genes encoding proteins with hydrolase, kinase, transferase, and other enzyme activity appears of particular relevance. Also considering the GO biological processes (Fig. 1C), a general up-regulation is observed with a marked increase of genes belonging to three generic categories (development, other cellular processes, and other metabolic processes).

GO grouping has allowed the selection of specific genes strictly related to the regulation of the ripening syndrome and to the development of some fruit quality traits as firmness, colour, and nutraceutical properties. Some of these genes have been chosen to perform northern experiments to validate the microarray data. Expression profiling throughout peach fruit development (stages S1–S4) confirms the pattern observed in the array experiment, validating the microarray analysis (Fig. 2).

3.2. Ethylene biosynthesis and perception

Considering genes responsible for the ethylene biosynthesis (Fig. 3), microarray data confirm, as previously reported

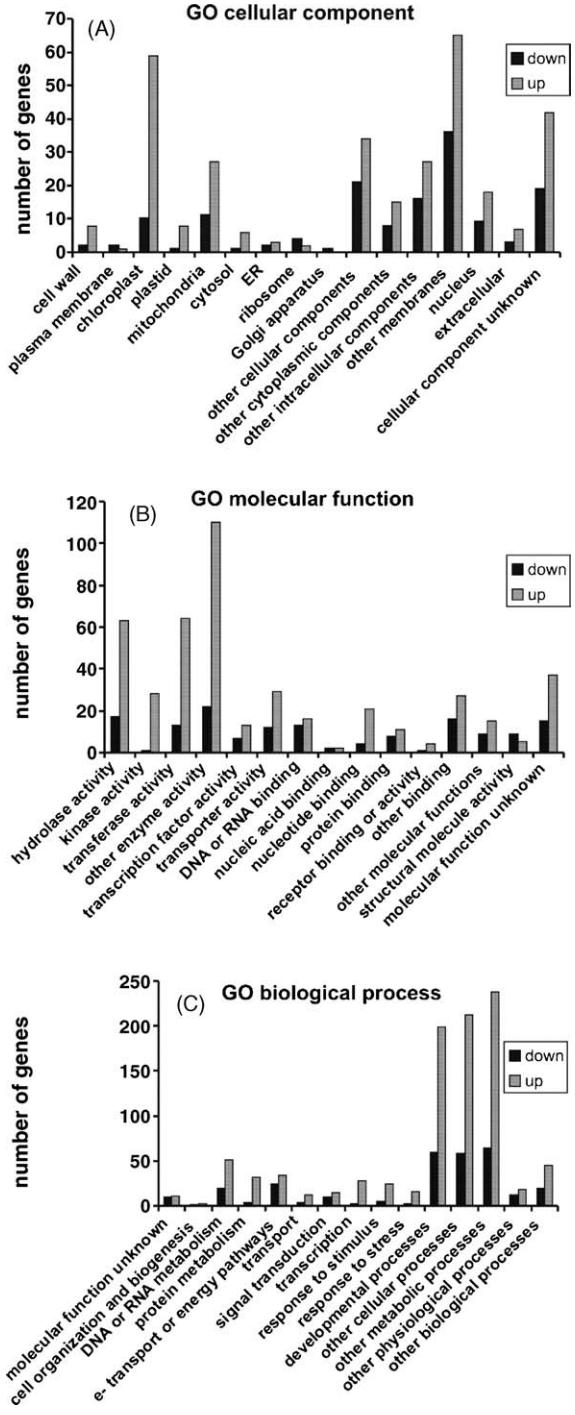

Fig. 1. Distribution of up- and down-regulated genes, based on their homology to the *Arabidopsis* proteome, and grouped according to the terms used in the GO cellular component (A), molecular function (B) and biological process (C) vocabularies.

[15,18], that *Pp-ACS1* (Ctg_489) and *Pp-ACO1* (Ctg_64) are largely up-regulated in S4 fruit. These two genes are involved in system2 of ethylene biosynthesis typical of climacteric fruits [19] in which ACS and ACO gene transcription is positively regulated by the hormone. Among the genes involved in ethylene perception, the microarray approach indicates that, besides the up-regulation of *Pp-ERS1* (Ctg_356) and the constitutive expression of *Pp-ETR1* [20], the transition from S3

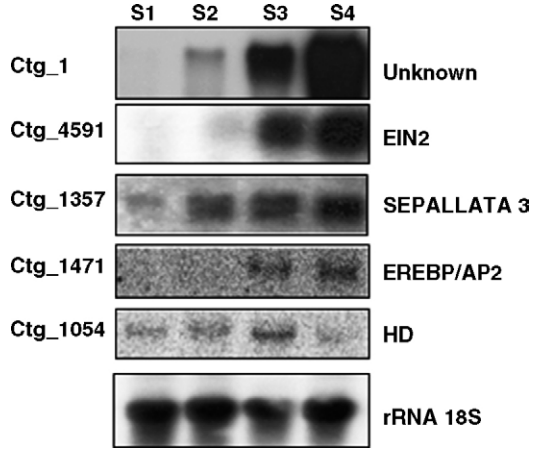

Fig. 2. Northern analysis of selected Contigs throughout peach mesocarp development (S1–S4). The amount of RNAs was checked using 18S rRNA as internal standard.

to S4 is accompanied by an increase of a new member of the ETR peach family, named *Pp-ETR2* (Ctg_4109) (Fig. 3). This gene shows homology to *Arabidopsis EIN4* [21], tomato *Le-ETR4* [22], and strawberry *Fa-ETR2* [23]. Microarray analysis highlights also an up-regulation of a cDNA homologous to *EIN2* (Ctg_4591), the first positive regulator in the ethylene signalling cascade acting downstream of CTR1 [24].

3.3. Transcription factors

Transition from S3 to S4 is paralleled by changes in expression of 19 genes encoding transcription factors (TFs) belonging to several families (Fig. 4). Some members of the AP2 family appear to be up-regulated (Ctg_1868 and

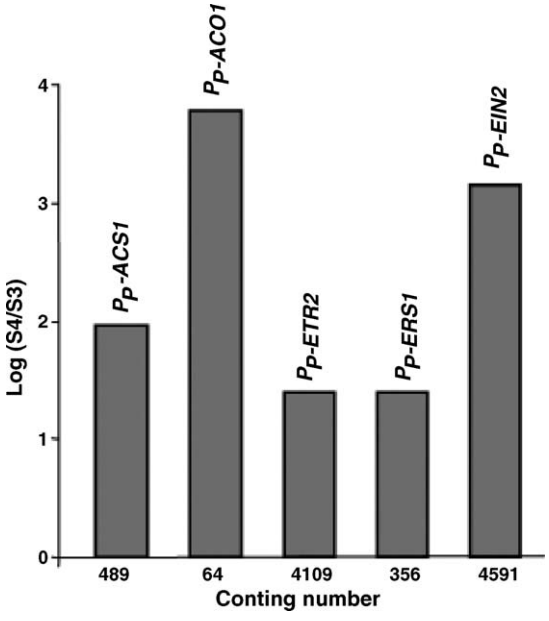

Fig. 3. Up-regulation of genes involved in ethylene biosynthesis (*Pp-ACS1* and *Pp-ACO1*), perception (*Pp-ETR2* and *Pp-ERS1*), and action (*Pp-EIN2*) assessed by microarray analysis occurring at S3/S4 transition.

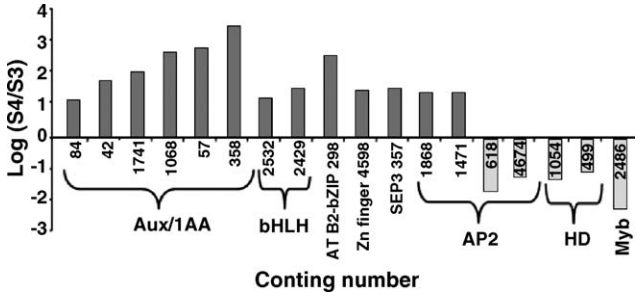

Fig. 4. Up- and down-regulation of transcription factors belonging to AUX/IAA, bHLH, b-ZIP, Zn finger proteins, MADS-box (*SEP3*), *APETALA2*, homeo domain (HD), and MYB families assessed by microarray analysis occurring at S3/S4 transition.

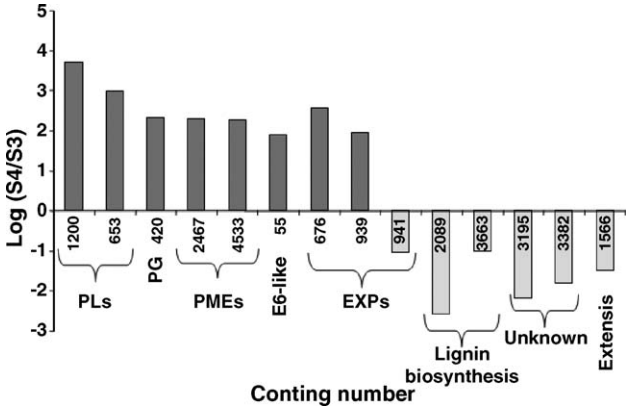

Fig. 5. Up- and down-regulation of genes involved in cell wall metabolism assessed by microarray analysis occurring at S3/S4 transition. PL, pectate lyase; PG, polygalacturonase; PME, pectinmethylesterase; EXP, expansin.

Ctg_1471), whereas some others are down-regulated (Ctg_618 and Ctg_4674), despite the fact that genes of both groups encode Ethylene Responsive Elements Binding Proteins (EREBPs) [25]. Ctg_1357, up-regulated in S4, shows homology to Arabidopsis *Sepallata3* (*SEP3*), a TF of MADS-box type belonging to group E, involved in the carpel development [26].

Considering the AUX/IAA family, six genes numbered as Ctg_42, Ctg_57, Ctg_84, Ctg_1741, Ctg_358, and Ctg_1068, and showing the homology to *At-IAA17*, *16*, *4*, *13*, *9*, and *19*, respectively, are up-regulated being Ctg_358 and Ctg_57 those expressed at the highest level. Ctg_298, similar to the soybean b-ZIP ATB2, Ctg_2532 and Ctg_2429, homologous to members of *Arabidopsis* basic helix-loop-helix (bHLH) family, and Ctg_4598, similar to a Zinc-finger protein, are also present among the group of up-regulated TFs (Fig. 4). Ctg_499 and Ctg_1054, containing a homeobox-leucine zipper domain (HD), and Ctg_2486, with a Myb domain, are identified within the down-regulated TFs.

3.4. Quality

Firmness is one of the most important quality traits defining ripening. It has been shown that several cell wall hydrolases are expressed during peach fruit softening and that this complex process implies the sequential action of different enzymes: for instance, endo-β-1,4-glucanase, pectate lyases (PL), and expansins (EXP) appear to be involved in the early softening, while endopolygalacturonase (endo-PG) and pectin-methyl esterase (PME) are responsible for the later melting process [27].

The microarray analysis confirms the results previously obtained on cv. Redhaven and using a more limited EST collection [27]. Furthermore, it is here reported for the first time the up-regulation of one new PME (Ctg_2467) and the differential expression of two new expansins, the first one being up-regulated (Ctg_676), while the second one appears to be down-regulated (Ctg_941) (Fig. 5). The latter is homologous to *Arabidopsis* EXP1 (At1g69530), whose transcription is induced by jasmonate (http://arabidopsis.org/tools/bulk/microarray/index.jsp), a growth regulator effective in delaying fruit ripening [28]. In addition to Ctg_3195 and Ctg_3382 encoding proteins with unknown function, genes involved in phenolic biosynthesis (Ctg_2089 and Ctg_3663) and one extensin (Ctg_1566) appear to be down-regulated during ripening.

Several genes (Ctg_1672, Ctg_3687, Ctg_407, Ctg_3668, Ctg_2420, Ctg_4052, and Ctg_711) encoding enzymes involved in isoprenoid biosynthesis were strongly induced at S3/S4 transition (Fig. 6). Ctg_711 is homologous to a β-carotene hydroxylase (β-O4), an enzyme involved in the oxidative metabolism leading to the formation of β-cryptoxanthin [29]. Considering the isoprenoid pathway in more detail, the stimulation of 1-deoxy-D-xilulose 5-phosphate synthase (DOXP, Ctg_1672), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECS, Ctg_3687), and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase (HDS, Ctg_407) may drive carbon groups to the biosynthesis of isopentenyl-diphosphate (IPP). The enhanced IPP availability and the increment of phytoene synthase (PSY, 3668), ζ-carotene desaturase (ZDS, Ctg_2420), lycopene β cyclase (LCYB, Ctg_4052), and β-carotene hydroxylase (β-O4, Ctg_711) may result in a stimulation of β-carotene and β-cryptoxanthin biosynthesis, pigments responsible for the yellow flesh colour.

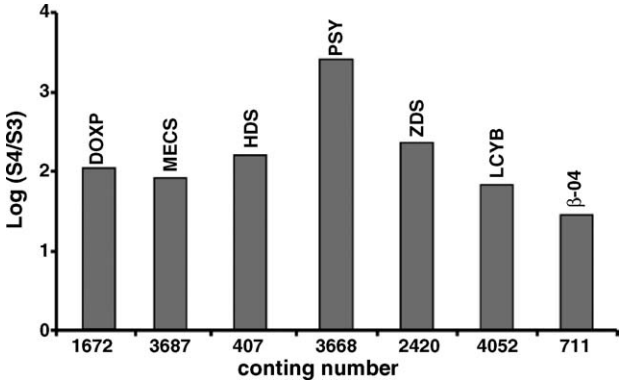

Fig. 6. Up-regulation of genes involved in isoprenoids biosynthesis and metabolism assessed by microarray analysis occurring at S3/S4 transition. DOXP, 1-deoxy-D-xilulose 5-phosphate synthase; MECS, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; PSY, phytoene synthase, ZDS, ζ-carotene desaturase; LCYB, lycopene β cyclase; β-O4, β-carotene hydroxylase.

4. Discussion

Goal of this study was to draw a preliminary and global overview of the transcriptome changes occurring during the transition from the pre-climacteric to the climacteric phase of peach fruit using the peach microarray (μPEACH1.0) containing 4806 oligos corresponding to genes expressed in peach fruit throughout development [13].

Data of microarray analysis, validated through the expression patterns of few but representative genes, pointed out that, as observed in other species using the same technique [2,6–10] major changes in expression profiles characterize the transition of peach fruit from immature to ripe stages. Ripening is a complex syndrome and this complexity is well represented by the three ontology categories showing high frequencies of genes differentially expressed at S3/S4 transition. The higher number of up-regulated genes versus down-regulated ones might be due to a high frequency of oligos spotted on the chip corresponding to EST S4 repertoire. Taking into account GO molecular function, the marked increase of up-regulated genes encoding proteins with enzymatic activity clearly demonstrates that changes in metabolic pathways represent an important feature of the ripening syndrome. It must be stressed that among the differentially expressed genes, 12% were classified as unknown according to BLASTX score against the *Arabidopsis* proteome: the elucidation of their function relays on further investigations based also on comparative genomic approaches with other fruit species.

Microarray data confirmed the involvement of *Pp-ACO1* and *Pp-ACS1* in system2 of ethylene biosynthesis as previously reported by Ruperti et al. [15] and Mathooko et al. [18]. The autocatalytic role of ethylene, accompanied by an up-regulation of *Pp-ACO1* is in agreement with the organization of the *Pp-ACO1* promoter [30] in which three EREs have been identified. Considering ethylene perception and action, microarray analyses highlighted the involvement of a new receptor, named *Pp-ETR2*, beside those (*Pp-ERS1* and *Pp-ETR1*) already characterized [20]. The deduced protein belongs to the subfamily2 of ethylene receptors [31] as tomato *Le-ETR4* [22], pear *Pc-ETR5* [32], and strawberry *Fa-ETR2* [23]. The expression of these genes increases with the progression of ripening and upon the ethylene treatment. In *Arabidopsis*, all the receptors are channelled through *EIN2* [33] and the up-regulation of a peach *At-EIN2* homologue (Ctg_4591) has been detected during the transition from pre-climacteric to climacteric stage. EIN2 is thought to play a central role in ethylene responses, since in the loss-of-function mutant of *Arabidopsis* (*At-ein2*) ethylene responses are completely blocked [34]. *EIN2* is involved in petunia, as well as in *Arabidopsis*, in the regulation of ethylene-mediated processes such as flower senescence, fruit ripening, and adventitious root and root hair formation [35]. However, differently from *Arabidopsis* [24] and tomato [36], *EIN2* expression is up-regulated by ethylene in petunia [35] and peach (Ziliotto et al., unpublished).

In peach, as in other climacteric fruits, the transition from pre-climacteric to climacteric phase is paralleled by significant changes in the transcript profile of genes that are differentially regulated by ethylene. Microarray analyses indicated that TFs related to ethylene action (as EREBPs) can be either induced (Ctg_1868 and Ctg_1471) or repressed (Ctg_618 and Ctg_4674) at S3/S4 transition. This is in agreement with the analysis carried out by means of a digital expression approach on ripening tomato fruit in which three and one EREBPs are induced and repressed, respectively [37]. In addition, the product of Ctg_1471 shows high similarity to the *Arabidopsis* gene *AtEBP/RAP2.3* [38]. This gene is induced by ethylene suggesting that it may play an important role in the response of *Arabidopsis* to the hormone [39]. It has been reported that other EREBPs proteins, named Ethylene Responsive Factors (ERFs) act either as transcriptional activators or repressors in plant [40]. Data presented in this paper and those obtained by Fei et al. [37] suggest that EREBPs may participate in the dynamic regulation of fruit ripening through antagonizing mechanisms.

During peach ripening many members of the Aux/IAA family are up-regulated. These genes encode short-lived nuclear proteins that may function as repressors of IAA-responsive genes in the presence of low hormone level [41]. An increase of transcription of an AUX/IAA gene, named DR3, has been reported in ripening tomatoes [42]. This tomato gene is induced by ethylene as well as its peach orthologue (Ctg_358, Zanin and Ziliotto, unpublished). These data are interesting because they strengthen a possible role of some of the AUX/IAA genes in the regulation of fruit ripening, probably by cross-talking with the ethylene regulatory pathway, as demonstrated by the presence of auxin-responsive elements within the *Pp-ACO1* promoter [30].

A considerable body of evidences is available concerning the involvement of MADS-box members in fruit development and ripening [1]. Microarray data highlighted an up-regulation of Ctg_1357 that encodes a protein homologous to *SEP3* of *Arabidopsis*. Recently, an expression profiling of all MADS-box genes during siliqua development in *Arabidopsis* revealed an accumulation of *SEP3* transcripts 16 days after pollination and a higher level in the *empty siliques* partenocarpic mutant [43]. An up-regulation of the *SEP3*-orthologue during the transition to the veraison stage has been observed also in grape berries [44]. Thus, *SEP3* could be a putative regulatory element shared by different fruit typologies (dry and fleshy, climacteric and non-climacteric).

Another TF that appears to be involved in regulatory mechanism common to many fruit types is the one encoding a homologue to the soybean *ATB2* (Ctg_298). Transcripts of orthologues of this gene increase during ripening also in tomato and grape [37].

TFs repressed at S3/S4 transition include Ctg_1054 and Ctg_499 homologous to *Arabidopsis HAT5* and *AtHB6* homeobox-leucine zipper (HD), respectively. The *AtHB6* promoter region drives the expression of high level of *GusA* reporter gene in dividing cells of developing cotyledons, leaves, roots, and carpels, but not in those undergoing differentiation. These expression data suggest that *AtHB6* might have a function related to cell division of developing organs [45]. As far as fleshy fruits are concerned, a decrease in mRNA accumulation was observed for *MDH1* during apple fruit

development [46]. In addition, the same authors demonstrated that in *Arabidopsis*, the over-expression of *MDH1* induced plant dwarfing, reduced fertility and irregular development of the siliqua, thus confirming the negative role of the gene in the last phases of fruit development. Nevertheless, digital expression carried out on ripe tomato pointed out that two genes encoding homebox domain proteins were up-regulated [47], while other four genes showed a steady-state expression. Thus, these data indicate that also the HD TFs may affect fruit development and ripening possibly through a complex of overlapping/antagonistic actions.

Peach firmness deeply affects fruit quality. Firmer flesh helps in harvesting and handling of fruits prior their consumption although consumers prefer soft and juicy peaches. This state is reached when a complex battery of cell wall modifying enzymes has accomplished its task. The physiological on-tree softening of the fruits of the cv. Fantasia is very similar that previously described for cv. Redhaven [27]. The pectolytic enzymes are predominant and the most abundant proteins probably acting on xyloglucans are expansins. It has been demonstrated that, in peach, the loss of firmness starts before the climacteric rise [14] when the early expressed genes controlling softening are down-regulated [27]. Moreover, some cell wall modifying enzymes expressed in S3 could be involved in cell enlargement associated with the vigorous fruit growth, as it has been described for the expansin *PpExp2* [48,49]. Several attempts to relate the expression of cell wall modifying enzymes to the development of mealiness have been done in the past [50]. It was not the goal of this research to investigate mealiness at the molecular level, but the availability of this microarray could help in the systematic search of molecular markers associated with this and other disorders.

Fruit carotenogenesis has been extensively studied in tomato in which the concentrations of these isoprenoids increase from 10- up to 14-fold during ripening. This increase is due mainly to the accumulation of lycopene [51], but very few fruit, besides tomato, accumulate this molecule. In fact, in peach, nectarine, orange, and persimmon the major carotenoid is β-cryptoxanthin [52]. This difference is confirmed by peach microarray analysis that showed an increase of the Ctg_711, homologous to β-carotene hydroxylase responsible for hydroxylation of β-carotene to β-cryptoxanthin [53]. Carotenoid biosynthesis is regulated by a complex mechanism poorly understood [54]. Our data, showing an accumulation of PSY, ZDS, LCYB, and β-04 transcripts during peach mesocarp ripening, suggest that, besides flower [55] and plastid development [56], a co-ordinated induction and regulation of gene expression appears to be an important feature of carotenoid accumulation also during fruit ripening. In apricot, a peach closely related species, expression of upstream (*PSY* and phytoene desaturase) and downstream (*ZDS*) genes of carotenoid biosynthetic pathway appears to be differently regulated by ethylene [57]. Carotenoids are just one class of isoprenoids, thus the study of their biosynthetic regulation should take into account all branches of the isoprenoids pathway. In fact, as already observed in tomato ripening [58], in ripe peach a higher accumulation of DOXP synthase (Ctg_1672), a key enzyme of the early steps of isoprenoids biosynthesis, occurred. Products of this enzymatic reaction may enter into the biosynthesis of Vitamins B1 and B6 indicating a crucial role of DOXP synthase in the accumulation of nutraceutical compounds.

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