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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