RESEARCH PAPER

Transcriptome profiling of ripening nectarine (*Prunus persica* L. Batsch) fruit treated with 1-MCP

Fiorenza Ziliotto[1], Maura Begheldo[1], Angela Rasori[1], Claudio Bonghi[1,*] and Pietro Tonutti[2,*]

[1] *Department of Environmental Agronomy and Crop Science, University of Padova, Viale dell' Università 16, I-35025 Legnaro (Padova), Italy*

[2] *Sant'Anna School of Advanced Studies, Piazza Martiri della Libertà 33, I-56127 Pisa, Italy*

Abstract

A large-scale transcriptome analysis has been conducted using μPEACH1.0 microarray on nectarine (*Prunus persica* L. Batsch) fruit treated with 1-methyl-cyclopropene (1-MCP). 1-MCP maintained flesh firmness but did not block ethylene biosynthesis. Compared with samples at harvest, only nine genes appeared to be differentially expressed when fruit were sampled immediately after treatment, while a total of 90 targets were up- or down-regulated in untreated fruit. The effect of 1-MCP was confirmed by a direct comparison of transcript profiles in treated and untreated fruit after 24 h of incubation with 106 targets differentially expressed. About 30% of these targets correspond to genes involved in primary metabolism and response processes related to ethylene, auxin, and other hormones. In treated fruit, altered transcript accumulation was detected for some genes with a role in ripening-related events such as softening, colour development, and sugar metabolism. A rapid decrease in flesh firmness and an increase in ethylene production were observed in treated fruit maintained for 48 h in air at 20 °C after the end of the incubation period. Microarray comparison of this sample with untreated fruit 24 h after harvest revealed that about 45% of the genes affected by 1-MCP at the end of the incubation period changed their expression during the following 48 h in air. Among these genes, an ethylene receptor (ETR2) and three ethylene-responsive factors (ERF) were present, together with other transcription factors and ethylene-dependent genes involved in quality parameter changes.

Introduction

In climacteric fruit, ethylene is required for ripening and the control of this developmental process mainly relies on the possibility of affecting ethylene production and/or action. This can be achieved through genetic manipulation, by modulating environmental parameters (temperature and atmosphere composition) during storage, or using specific ethylene inhibitors. In general, the result of these approaches is an altered gene transcription pattern and, as a consequence, modified ripening behaviour. 1-methylcyclopropene (1-MCP) is thought to interact with ethylene receptors thus preventing ethylene-dependent processes (Sisler and Serek, 1997). Research carried out using 1-MCP report that the chemical, by affecting ethylene production, respiration, softening, colour change, and aroma production, delays ripening and increases the post-harvest life of numerous climacteric fruits. However, treatment efficacy is variable and depends on several factors including the concentration of 1-MCP used, storage condition and duration, and maturity of the fruit before application (for a review on 1-MCP effects see Watkins, 2006). In addition, 1-MCP induces variable responses in different climacteric fruit species and even in varieties of the same species (Blankenship and Dole, 2003), thus suggesting the presence of different mechanisms controlling ripening in climacteric fruit. This is particularly evident when comparing two important fruit species

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* To whom correspondence should be addressed. E-mail: claudio.bonghi@unipd.it, pietro.tonutti@sssup.it

such as apple and peach. Following 1-MCP treatment, ethylene production is strongly reduced, ripening is inhibited or delayed for many days, and storage prolonged in apples (Watkins *et al.*, 2000; Fan and Mattheis, 2002; Jiang and Joyce, 2002; Bai *et al.*, 2005). On the other hand, the effects of 1-MCP treatments on peaches and nectarines are limited to the incubation period and a few hours afterwards, when the maintenance of flesh firmness is associated with an altered gene expression pattern as observed for endo-polygalacturonase (*endo-PG*) (Dong *et al.*, 2001; Dal Cin *et al.*, 2005). Recovery of ripening occurs within a few days after the end of treatment in both peaches and nectarines which soften rapidly, especially at room temperature (Mathooko *et al.*, 2001; Fan *et al.*, 2002; Ziliotto *et al.*, 2003). These features make apple and peach (both belonging to the Rosaceae family) interesting models for elucidating the molecular mechanisms responsible for the different ripening patterns and responses to ethylene and its inhibitors. In a comparative work, Dal Cin *et al.* (2006) pointed out that, whereas ethylene biosynthesis is markedly inhibited by 1-MCP in apples, its production in peaches was not reduced by the chemical. This suggests that the variable responses to the inhibitor of ethylene perception might be due to differences in terms of ratio, expression pattern, turnover of the ethylene receptors, and/or mechanisms leading to altered chemical binding of 1-MCP.

The genomics approach and the development of high throughput technologies for large-scale analyses of transcriptomes represent powerful tools for unravelling the molecular mechanisms of complex processes, such as fruit ripening, and elucidating, on a large-scale basis, the role and effects of endogenous and/or exogenous factors. In particular, microarray analyses have been performed for transcriptome profiling during the transition from the pre-climacteric to the climacteric stage and the role of ethylene in ripening pear (Fonseca *et al.*, 2004), tomato (Alba *et al.*, 2005), and peach (Trainotti *et al.*, 2006) fruit. In this last species, a dramatic up-regulation has been detected for genes encoding transcription factors and others involved in ethylene biosynthesis, perception, and signal transduction. Using the same microarray, Trainotti *et al.* (2007) identified genes involved in biosynthesis, transport, and signalling of auxin that show, in peach mesocarp, an increased expression at ripening, and demonstrated the existence of an important cross-talk between auxin and ethylene, with genes in the auxin domain regulated by ethylene, and genes in the ethylene domain regulated by auxin.

The results of a transcriptomic approach, undertaken with the aim of elucidating molecular mechanisms and identifying candidate genes involved in the responses of nectarine fruit to the inhibitor of ethylene action 1-MCP are reported here.

Materials and methods

Plant material and experimental design

Nectarine (*Prunus persica* L. Batsch, cv. Super Crimson Gold) fruit were harvested at commercial ripening stage (about 60 N flesh firmness) and immediately transferred to the Post-harvest Laboratory of the Faculty of Agriculture, University of Padova, Italy, where they were maintained at room temperature (20 °C) throughout the experiments. They were then divided in two groups of 40 fruit each: the first group was enclosed in gas-tight glass jars and treated with $1 \, \mu l \, l^{-1}$ of 1-MCP for 24 h. The second group (control) was enclosed for 24 h in sealed jars of the same volume without 1-MCP. To avoid excessive CO_2 accumulation, KOH was added to the jars. At the end of the 24 h incubation period fruit were removed from the jars and transferred to air at 20 °C for a further 48 h (72 h from harvest, 72MCP and 72AIR). Samplings were performed at the beginning of the experiment (T0), at 24 (24MCP and 24AIR, end of the incubation period), 48 and 72 (72MCP and 72AIR, 48 h from the end of the incubation period) hours after harvest.

At each sampling time, ethylene production of 10 individual fruit was determined using a gas chromatograph (DANI 3200) as described by Tonutti *et al.* (1997) and flesh firmness measured with a penetrometer (TR, Forlì, Italy) equipped with a 8 mm probe. For each sampling time (T0, 24AIR, 24MCP, 72AIR and 72MCP) two representative fruit (biological replicates) have been used for transcript analyses.

RNA extraction, microarray preparation, and hybridization

Total RNA was extracted using the protocol described by Ruperti *et al.* (2001). RNA yield and purity was checked by means of UV absorption spectra, whereas RNA integrity was ascertained by means of electrophoresis in agarose gels.

Total RNA (20 μg) from T0, air-, and 1-MCP-treated fruits was converted into target cDNA by reverse transcription using the SuperScript™ Indirect cDNA Labeling System (Invitrogen, USA) following the manufacturer's instructions.

The features, preparation, and hybridization protocols of the peach microarray μPEACH 1.0 are described in Trainotti *et al.* (2006).

Data analysis

The microarrays were scanned as described by Trainotti *et al.* (2006). The TM4 (www.tm4.org) package developed at TIGR (www.tigr.org; Saeed *et al.*, 2003) was used to analyse microarray data. The images were processed using the Spotfinder 2.2.3. software by means of the Otsu algorithm. The expression data extracted by Spotfinder were normalized by MIDAS 2.18 using the LOWESS (Locally Weighted Regression Scatter Plot Smoothing; Cleveland, 1979) algorithm with the 'block mode', keeping the Cy3 channel as reference. After normalization, data from each slide were split in two as each probe is spotted twice on μPEACH1.0. Thereafter, each spot value was considered to be independent. Normalized split data were loaded in MeV 3.1 and for each comparison (24MCPvsT0, 24AIRvsT0, 24MCPvsAIR; 72MCPvs24AIR, at least three experiments) a 66% cut-off was imposed to select genes differentially expressed by one-class unpaired SAM (Significance Analysis of Microarrays; Tusher *et al.*, 2001) analysis. Clones with significant changes in expression (threshold ratios, expressed as $\log_2$, higher than 1 and lower than –1 for up- and down-regulation, respectively) were identified at delta values giving a 0% of false discovery rate (FDR).

Quantification of mRNA via qRT-PCR

To validate microarray data, transcript accumulation of *AUX/IAA*, β-carotene hydroxylase, catalase, ethylene receptor *ETR2*, *ERF*-like,

and trehalose-6-phosphatase genes was evaluated via qRT-PCR. The cDNA single strand used for RT-PCR was obtained as follows: 50 µg of total RNA were treated with 10 units of RQ1 RNase-Free DNase (Promega) and 1 unit of RNAguard (RNase INHIBITOR, GE Healthcare) for 30 min, then purified by phenol–chloroform. One microgram of total DNA-free RNA was reverse-transcribed with 200 U of M-MLV Reverse Transcriptase (Promega), 1 unit of RNAguard (RNase INHIBITOR) and 2.5 µM oligo-dT$_{12–18}$ as primer at 37 °C for 90 min in a final volume of 20 µl, as described in Sambrook *et al.* (1989). The single strand cDNA obtained (1 µl) was subjected to real-time PCR in a final volume of 10 µl containing 2× Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA) and specific primers (3 pmol) (see Supplementary Table S1 at *JXB* online). Three technical replicates for each sample were run on an ABI 7500 Real Time PCR System Sequence Detection machine (PE Applied Biosystems) programmed to heat for 10 min at 95 °C, followed by cycling conditions (melting step for 30 s at 95 °C, annealing for 30 s at 64 °C, and extension for 35 s at 72 °C) repeated for 40 cycles. An additional dissociation cycle (15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C) was carried out. The amplified cDNA fragments were cloned into pBluescript II KS+ vector (Fermentas International, Burlington, Canada) and sequenced. The expression values were calculated following the mathematical model proposed by Livak and Schmittgen (2001) using 18S as housekeeping.

Results

Compared with the control, which softened rapidly, 1-MCP-treated fruit maintained higher flesh firmness at the end of the 24 h incubation period (Fig. 1). A difference between samples was still present after transferring treated fruit to air for 24 h and 48 h (48 h and 72 h from harvest, respectively) when treated fruit softened. A similar increasing pattern of ethylene biosynthesis was observed in both samples throughout the experiment. At the end of the incubation period and 24 h afterwards, treated fruit synthesized ethylene at the same level as the control, while at 72 h a higher production was detected in treated fruit (Fig. 1).
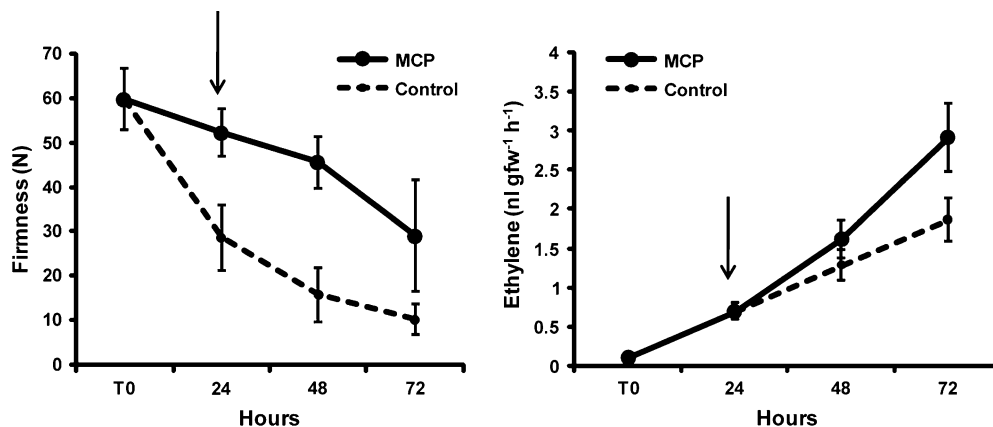
Transcript profiling at the end of 1-MCP treatment

A first set of microarray hybridizations was carried out using a simple loop/flipped dye design (Fig. 2), to compare T0, 24MCP, and 24AIR samples. In the 24AIRvsT0 comparison, a total of 215 targets were significant at SAM analysis, whereas 212 targets were identified in both 24MCPvsT0 and 24MCPvs24AIR comparisons. According to the threshold log ratio (>1 for induced; <–1 for repressed), 90 (43 induced and 47 repressed) and nine unigenes (three induced and six repressed) were expressed differentially in 24AIRvsT0 and 24MCPvsT0, respectively. Compared with the 24AIR sample, a total of 106 targets showed different (54 greater and 52 lower) transcript accumulation in treated fruit (24MCP) (Fig. 2). This clearly indicates that the presence of 1-MCP in the atmosphere surrounding nectarine fruit is effective in altering the expression of specific genes, resulting in a block of the ripening process as demonstrated by the maintenance of high flesh firmness values (Fig. 1).
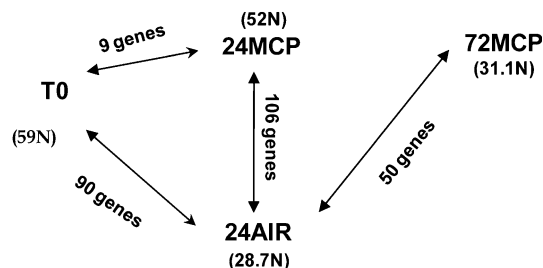
Fig. 2. Experimental design of microarray analyses. In the simple loop, six slides (12 technical replicates) were used for the comparison 24MCPvsT0 and three (six technical replicates) for 24AIRvsT0, 24MCPvs24AIR, and 72MCPvs24AIR. Dye flip was performed for each comparison (three for 24MCPvsT0 and one for 24AIRvsT0, 24MCPvs24AIR, and 72MCPvs24AIR). For each comparison the number of genes showing differential expression, identified by SAM analysis, is reported. Flesh firmness value (N) is also indicated.

Fig. 1. Flesh firmness (left panel) and ethylene biosynthesis (right panel) in 1-MCP-treated (solid line) and control (dotted line) nectarine fruit. Arrows indicate the end of the incubation period. Data are means of 10 fruit. Vertical bars represent ±SD.

A list of targets that were significant by SAM analysis and those showing differential expression and/or transcript accumulation is reported in Table S2 at *JXB* online. Among targets that were more abundant in 24MCP than in 24AIR, two are involved in auxin metabolism (ctg_57 and ctg_1741), and two correspond to genes related to cell wall metabolism (expansin *PpEXP2*, ctg_941; and mannan endo-1,4 β-mannosidase, ctg_1954). Higher transcript levels of ctg_1024 and ctg_5424, corresponding to a catalase and a lipoxygenase, respectively, and ctg_1751, representing a glucose acyltransferase were also observed at the end of 1-MCP treatment. Of the 54 genes that more were expressed in 1-MCP-treated fruit, 36 (66.6%) appeared down-regulated in the 24AIRvsT0 comparison microarray (see Supplementary Table S2 at *JXB* online), suggesting that these genes are involved in the ripening process of nectarines.

Among the 52 targets showing significantly lower (log ratio <–1) transcript accumulation in the 24MCPvs24AIR comparison (see Supplementary Table S2 at *JXB* online), specific cell wall-related genes are present as *endo-PG* (ctg_420), pectin acetylesterase (*PAE)* precursor (ctg_1816), one putative pectin methylesterases (*PME*) (ctg_4533), expansin *PpEXP3* (ctg_676), and *EGase* (*PpEG1*, ctg_ 2197). Genes related to quality parameters such as colour, flavour development, and sugar metabolism have also been identified: β-carotene hydroxylase (ctg_711), omega-6 fatty acid desaturase (ctg_835), putative invertase inhibitor (ctg_4499), pyruvate decarboxylase (ctg_112), sucrose synthase (ctg_61), glucose acyltransferase (ctg_1752), and trehalose-6-phosphate phosphatase, *TPPA* (ctg_4621). With respect to hormone metabolism, not only two auxin-related genes (*AUX/IAA* protein, ctg_768, auxin response factor, *ARF*, ctg_1991) and a 9-*cis*-epoxycarotenoid dioxygenase2 (ctg_2980) involved in ABA biosynthesis, but a number of genes with a role in ethylene production, perception, and signal transduction accumulated lower transcripts in 1-MCP-treated fruit: 1-aminocyclopropane-1-carboxylate (ACC) oxidase (*ACO1*, ctg_64), ethylene receptor *ETR2* (ctg_4109), ethylene-response factors (*ERF1*, ctg_3350; *ERF2*, ctg_2116; *ERF*-like, ctg_2757). Two other transcription factors, a MADS-box homologue (ctg_1511), and HD-ZIP protein (ctg_2443) were identified, as well as three targets corresponding to pathogenesis-related proteins (ctg_1026, ctg_1069, and ctg_4524) and one ripening-related protein (ctg_938). Of the 52 genes showing lower transcript accumulation at the end of the 1-MCP incubation period, 27 (51.9%) appeared up-regulated in the 24AIRvsT0 comparison (see Supplementary Tables S2 at *JXB* online). This implies a role for these genes in nectarine ripening.

As reported above, only nine targets (three induced and six repressed) resulted as being differentially expressed in 24MCP compared with the T0 sample. Of the three targets up-regulated, one putatively corresponds to a sucrose synthase gene, and one of the six repressed genes refers to an auxin-induced protein (data not shown).

Transcript profiling in the post-treatment phase

After transferring fruit to air at the end of the 24 h incubation period, rapid softening occurred at 72 h (Fig. 1). This was accompanied by a marked increase in ethylene biosynthesis. To elucidate this behaviour at a molecular level, the microarray hybridization design was implemented to perform an additional comparison: 72MCP (31.1 N flesh firmness) versus 24AIR (28.7 N flesh firmness). Of the 54 targets showing greater transcript accumulation in the 24MCPvs24AIR comparison, 12 appeared to be expressed at a similar level in 72MCP and 24AIR samples, indicating a decreasing transcription activity during the post-treatment phase, whereas 36 targets (66.6%) still displayed a higher transcript accumulation 48 h after the end of 1-MCP treatment (see Supplementary Table S2 at *JXB* online). Among these targets, catalase (ctg_1024) was selected to validate, via qRT-PCR, the microarray data: as reported in Fig. 3A, expression analysis confirmed that 1-MCP induced an increased expression of this gene at the end of the incubation period and this behaviour was also observed 48 h later. Considering the 52 targets showing a reduced transcript accumulation at the end of the 24 h treatment period (24MCPvs24AIR), only 13 still maintained a reduced transcript level in the 72MCPvs24AIR comparison, and for 35 (67.3%) log ratio values higher than <–1 were detected (see Supplementary Table S2 at *JXB* online). This would indicate that a number of targets negatively affected by 1-MCP at the end of the 24 h incubation period recovered their expression 48 h later. The lower amount of transcript accumulation in 1-MCP-treated samples at the end of the incubation period and the recovery pattern at 72 h was confirmed by qRT-PCR analysis of ctg_711 (β-carotene hydroxylase), ctg_768 (AUX/IAA protein), ctg_4109 (*ETR2*), and ctg_4621 (*TPPA*) (Fig. 3B, C, D, E). A marked reduced transcript level at 24 h and slight recovery at 72 h was also observed via qRT-PCR in treated fruit for ctg_2757 (*ERF*-like) (Fig. 3F), one of the 13 targets still maintaining log ratio value <–1 in the 72MCPvs24AIR comparison.

With the aim of better describing molecular aspects that characterize the response of nectarine fruit to 1-MCP, targets present in the μPEACH1.0 array (and being shown as significant with SAM analysis) were grouped according to their putative function and microarray data resulting from different comparisons (24MCPvs24AIR, 24AIRvsT0, and 72MCPvs24AIR) were plotted together. Considering genes involved in ethylene physiology (Fig. 4), as stated above, lower (<–1) transcript accumulation was detected for *ACO1* (ctg_64), *ETR2* (ctg_4109), *ERF1* (ctg_3350), *ERF2* (ctg_2116), and *ERF*-like (ctg_2757) genes in
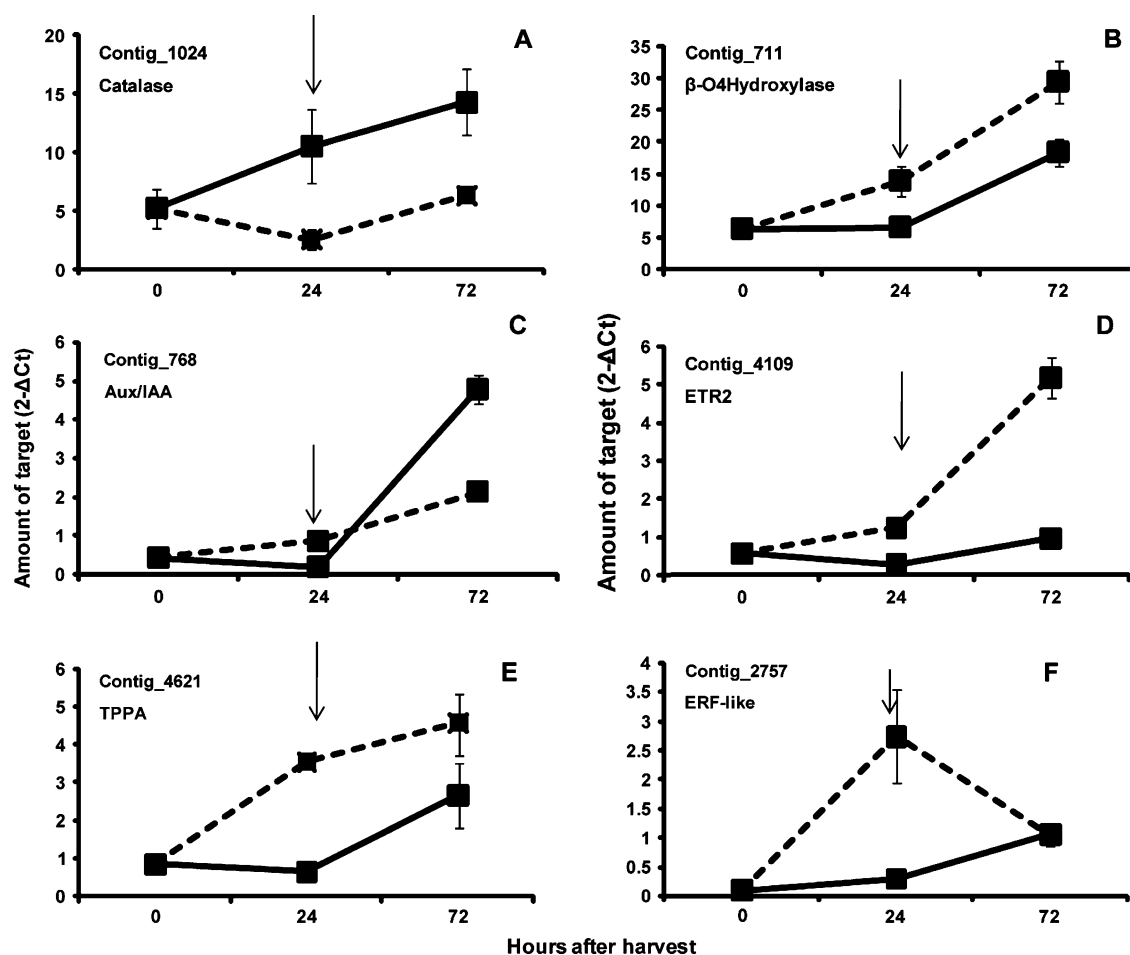
Fig. 3. Expression profiles throughout the experiment of selected genes showing higher (A, catalase) or lower (B, β-carotene hydroxylase; C, *AUX/IAA*; D, *ETR2*; E, *TPPA*; F, *ERF*-like) transcript accumulation in the 24MCPvs24AIR comparison microarray. Solid lines indicate 1-MCP-treated fruit and dotted lines control fruit. Bars represent ±SE of three independent replicates. Arrows indicate the end of 1-MCP treatment.

24MCP samples compared with 24AIR, whereas no significant difference was observed for ACC synthase (*ACS1*, ctg_489), *ETR1* (ctg_1436), *ERS* (ctg_356), *EIN2* (ctg_4591), and *EIL1* (ctg_4073). All the targets showing lower transcript accumulation at 24 h in treated fruit displayed a recovering expression at 72 h (higher log ratio value in 72MCPvs24AIR than in 24MCPvs24AIR). This behaviour was demonstrated by qRT-PCR analyses for ctg_4109 (*ETR2*) and ctg_2757 (*ERF*-like) (Fig. 3D, F). Interestingly, an increase in transcript accumulation at 72 h was also detected for *ACS1* and, although not statistically significant, for *EIN2* (Fig. 4). Worthy of note is the fact that at the early ripening in air (24AIRvsT0) a significant increase of transcript was only observed for *ACO1* and *ETR2*.

Besides the ethylene-related Transcription Factors (TFs), several other targets corresponding to TFs are present in the μPEACH1.0 array and some of these appeared to be affected by 1-MCP. The effect of the ethylene inhibitor appeared to be variable: at the end of

the incubation period, it significantly reduced the accumulation of transcripts of a MADS box homolog (ctg_1511), *ARF* (ctg_1991), *AUX/IAA* (ctg_768), and an *HD-ZIP* (ctg_2443), and increased that of another *HD-ZIP* (ctg_3293) and two *AUX/IAA* TFs (ctg_57 and ctg_1741) (Fig. 5). The effect of 1-MCP was maintained at 72 h for ctg_57, ctg_1741, and ctg_3293, whereas a recovery of gene expression was detected for ctg_1511, ctg_2443, and ctg_768, which displayed a dramatic increase in transcript accumulation (Fig. 5), as demonstrated for ctg_768 by qRT-PCR analysis (Fig. 3C). In addition, higher transcript levels in treated fruit at 72 h were detected for ctg_84 (*AUX/IAA*) (Fig. 5).

Three other groups of targets involved in quality-related metabolic processes were identified. The first deals with sugar metabolism (Fig. 6): four targets (ctg_112, pyruvate decarboxylase; ctg_1752, glucose acyltransferase; ctg_4499, putative invertase inhibitor; ctg_4621, TTPA) were up-regulated during early ripening and significantly less represented in treated fruit at the end of the 1-MCP
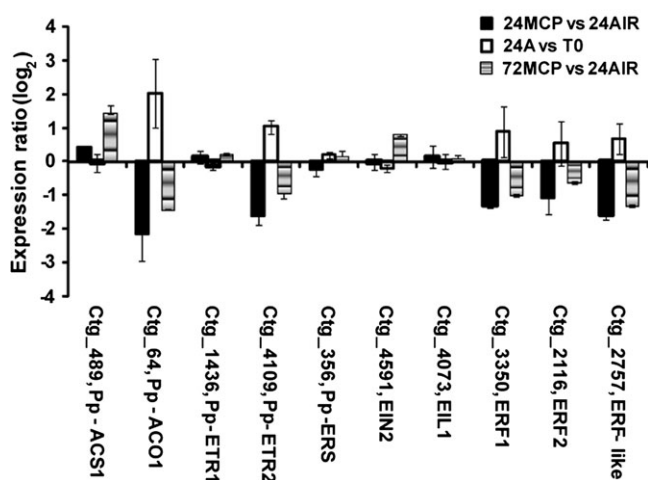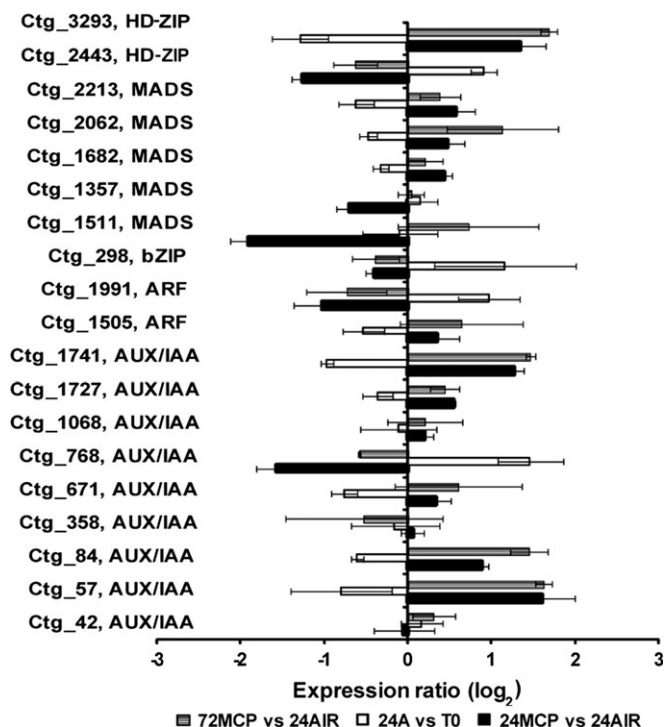
Fig. 4. Hybridization intensity ratio, reported as $\log_2$, of probes spotted on μPEACH1.0 and significant at SAM analysis corresponding to genes involved in ethylene biosynthesis, perception, and signal transduction. The ratio has been calculated by comparing, in three microarray experiments, the hybridization signal of cDNAs corresponding to transcripts of fruit sampled at harvest (T0), after 24 h in air (24A) or in 1-MCP (24MCP), and 48 h in air from the end of 1-MCP treatment (72MCP). Log value is the average of a minimum of four replicates. Bars indicate standard deviation. Abbreviations: ACO1, 1-aminocyclo-propane-1-carboxylate oxidase; ACS1, 1-aminocyclopropane-1-carboxylate synthase; EIL, EIN3-like protein; ERF, ethylene response factors; ERS, ethylene receptor ERS-like; ETR, ethylene receptor ETR type.

incubation period, showed a recovery of their expression at 72 h (as demonstrated for ctg_4621 by qRT-PCR analysis, Fig. 3E). Another putative glucose acyltransfer-ase gene (ctg_1751) appeared to be down-regulated during the early ripening stage and showed a higher transcript level in treated fruit at both 24 h and 72 h.

The second group comprises targets involved in cell-wall metabolism (Fig. 7): with the exception of ctg_676 (*PpEXP3*), all the targets displaying lower transcript accumulation at 24 h in treated fruit and an up-regulation during the early stages of ripening (ctg_420, *endo-PG*; ctg_2197, *PpEG1*; ctg_4533, *PME*; ctg_1816, *PAE*) were characterized by a significant recovery of expression at 72 h. Interestingly, *PpEXP2* (ctg_941), down-regulated at early ripening stage and showing higher transcript levels in treated fruit at 24 h, did not undergo any expression change at 72 h. A similar behaviour has been observed for ctg_1954 representing a mannan endo-1,4-β-mannosidase (Fig. 7).

The last selected group includes transcripts correspond-ing to genes involved in the isoprenoid pathway and the development of typical yellow pigmentation in this nectarine variety (Fig. 8). The only gene appearing signifi-cantly affected by 1-MCP at the end of the 24 h incubation period was β-carotene hydroxylase (ctg_711), up-regulated during the early ripening stages. The effect of the ethylene inhibitor on this gene was confirmed by qRT-PCR (Fig. 3B): this analysis pointed out, as also
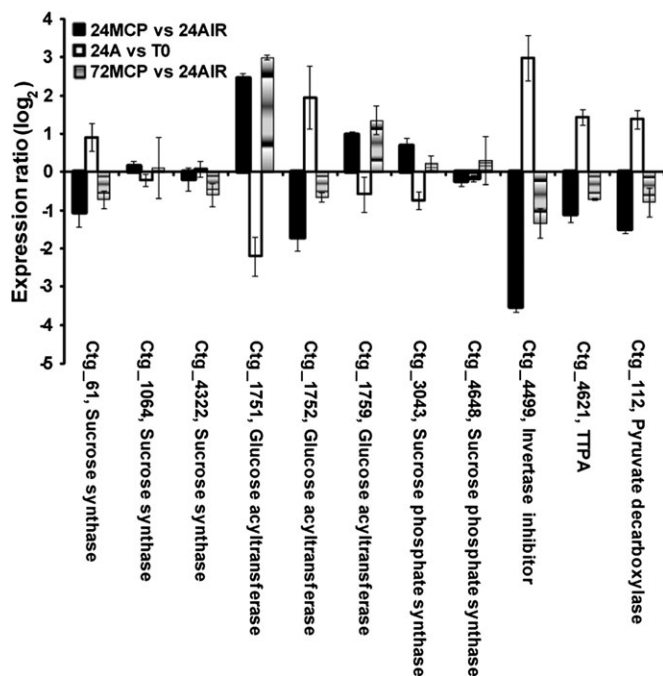

Fig. 5. Hybridization intensity ratio, reported as $\log_2$, of probes spotted on μPEACH1.0 and significant at SAM analysis corresponding to members belonging to transcriptional regulator gene families. The ratio has been calculated by comparing, in three microarray experiments, the hybridization signal of cDNAs corresponding to transcripts of fruit sampled at harvest (T0), after 24 h in air (24A) or in 1-MCP (24MCP), and 48 h in air from the end of 1-MCP treatment (72MCP). Log value is the average of a minimum of four replicates. Bars indicate standard deviation.

indicated by microarray data, a recovery of the expression of this gene at 72 h.

Discussion and conclusions

The discovery of 1-MCP as a powerful antagonist of ethylene action is providing great opportunities for scientists to gain insight into the fundamental mechanisms involved in many plant processes including fruit ripening. It is confirmed here that, differently from other climacteric species, the responses of peaches and nectarines to 1-MCP treatment are confined to the incubation period and a few hours thereafter. The large-scale transcriptome analysis performed using μPEACH1.0 indicates that the quick ripening observed after transferring treated peaches to air is not due to a limited physiological effect of 1-MCP during incubation. In fact, at the end of the 24 h treatment period, the maintenance of high firmness (52.0 N) is accompanied by marked changes in transcript profiling if compared to control fruit (24AIR). Only nine genes showed significant changes in 24MCPvsT0 microarray

Fig. 6. Hybridization intensity ratio, reported as $\log_2$, of probes spotted on μPEACH1.0 and significant at SAM analysis corresponding to genes involved in sugar metabolism. The ratio has been calculated by comparing, in three microarray experiments, the hybridization signal of cDNAs corresponding to transcripts of fruit sampled at harvest (T0), after 24 h in air (24A) or in 1-MCP (24MCP), and 48 h in air from the end of 1-MCP treatment (72MCP). Log value is the average of a minimum of four replicates. Bars indicate standard deviation. Abbreviations: TPPA, trehalose-6-phosphate phosphatase.
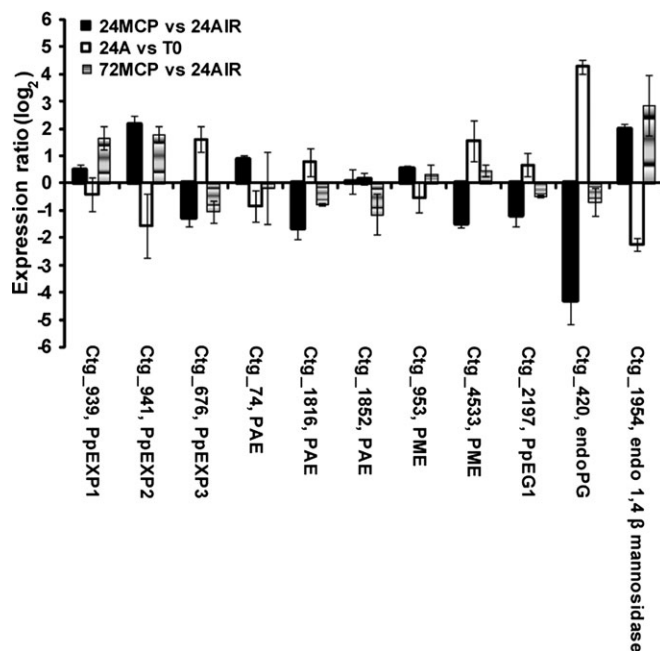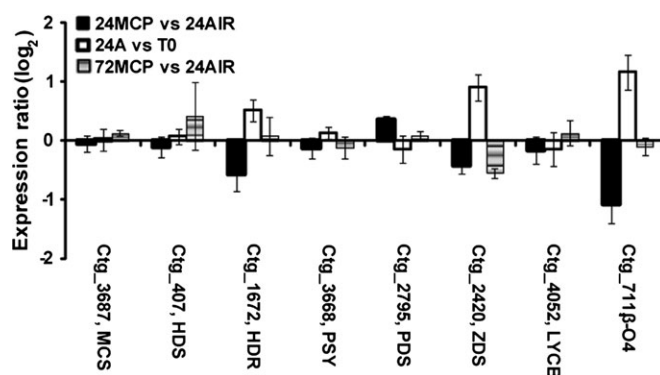

Fig. 7. Hybridization intensity ratio, reported as $\log_2$, of probes spotted on μPEACH1.0 and significant at SAM analysis corresponding to genes involved in cell wall metabolism. The ratio has been calculated by comparing, in three microarray experiments, the hybridization signal of cDNAs corresponding to transcripts of fruit sampled at harvest (T0), after 24 h in air (24A) or in 1-MCP (24MCP), and 48 h in air from the end of 1-MCP treatment (72MCP). Log value is the average of a minimum of four replicates. Bars indicate standard deviation. Abbreviations: EG, endo-β-1,4-glucanase; endoPG, endopolygalacturonase; EXP, expansin; PAE, pectin acetylesterase; PME, pectin methylesterase.

analysis compared with 90 targets displaying differential expression during 24 h ripening in air (24AIRvsT0). Such results indicate that the presence of 1-MCP, by altering ethylene perception, induces a block of ripening as demonstrated by the maintenance of flesh firmness value. This physiological effect is confirmed by the 106 genes that were differentially expressed when comparing 24MCP and 24AIR samples, and the fact that a number of affected targets correspond to genes with a role in hormone (ethylene, but also auxin and ABA) metabolism and in regulating transcription. Considering that 66.6% and 51.9% of transcripts displaying greater and lower transcript accumulation, respectively, at the end of the 1-MCP incubation period show an opposite trend in ripening fruit kept in air (24AIRvsT0 comparison), it might be hypothesized that these targets correspond to ripening-related and ethylene-dependent genes.

Cell wall-related genes

The maintenance of high firmness values at the end of the incubation period appears to be the result of a down-regulation of cell wall-related genes, involved, in particular, in pectin metabolism, such as *endo-PG*. Microarray data on *endo-PG* confirm specific expression analysis

Fig. 8. Hybridization intensity ratio, reported as $\log_2$, of probes spotted on μPEACH1.0 and significant at SAM analysis corresponding to genes involved in isoprenoid pathway. The ratio has been calculated by comparing, in three microarray experiments, the hybridization signal of cDNAs corresponding to transcripts of fruit sampled at harvest (T0), after 24 h in air (24A) or in 1-MCP (24MCP), and 48 h in air from the end of 1-MCP treatment (72MCP). Log value is the average of a minimum of four replicates. Bars indicate standard deviation. Abbreviations: β-O4: β-carotene hydroxylase; HDR, 14-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase; HDS, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; LCYB, lycopene β cyclase; MCS, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; PDS, phytoene dehydrogenase; PSY, phytoene synthase; ZDS, ζ-carotene desaturase.

(Ziliotto *et al*., 2003; Dal Cin *et al*., 2005), showing a reduction of *endo-PG* transcript accumulation at the end of 1-MCP treatment and further emphasize the crucial role played by *endo-PG* in peach fruit softening, as clearly demonstrated in non-melting (Callahan *et al*., 2004; Morgutti *et al*., 2006) and stony hard (Hayama *et al*., 2006*a*, *b*; Begheldo *et al*., 2008) genotypes.

Considering the strict relationship existing between *endo-PG* and ethylene (Sitrit and Bennett, 1998), the marked reduction of *endo-PG* transcript accumulation in treated fruit (log ratio value –4.29, the second lowest value among affected targets) indicates that ethylene action is markedly inhibited by 1-MCP in ripening nectarines. One of the targets showing higher transcript level in 1-MCP-treated fruit is *PpEXP2* and this would confirm that this gene, down-regulated by ethylene, is related to peach fruit growth and, differently from *PpEXP3* (ctg_676), which displayed a reduced level of transcript accumulation in 1-MCP treated fruit, is not responsible for softening as reported by Hayama *et al*. (2001, 2003).

Regulation of ethylene biosynthesis

When evaluating the list of targets showing lower transcript accumulation at the end of the 1-MCP incubation period, it is interesting to notice that, of the two elements involved in the last ethylene biosynthetic steps, only *ACO1* is present, whereas *ACS1* does not appear to be negatively affected by 1-MCP. In addition, microarray data of the 24AIRvsT0 comparison confirm that, in *P. persica* fruit, *ACO1* is induced earlier than *ACS1* at the onset of ripening as previously reported (Tonutti *et al*., 1997; Begheldo *et al*., 2008). Considering ethylene receptors, *ETR2* but not *ETR1* and *ERS1* show lower transcript levels after 24 h treatment with 1-MCP. These data are in agreement with results published by Dal Cin *et al*. (2006) indicating that *ACS1*, *ETR1*, and *ERS1* expressions are not or only slightly affected by 1-MCP in peaches, and suggest that *ETR2*, induced at the very early steps of climacteric (Fig. 4) and displaying a ripening-related expression pattern (Trainotti *et al*., 2006), plays a crucial role in the modulation of ethylene responses in peach fruit. This hypothesis is reinforced by the fact that, in ripening peaches, a great inductive effect on *ETR2* is yielded by exogenous ethylene treatment (Trainotti *et al*., 2007).

As specifically pointed out by Mathooko *et al*. (2001) and Dal Cin *et al*. (2006), *ACS1* expression and activity are not affected by 1-MCP in ripening peaches. Considering that *ACO1* gene expression and activity appear reduced but not blocked, as reported by our microarray data and by Mathooko *et al*. (2001), the result is that ethylene production is not inhibited by 1-MCP in *P. persica* fruit at the end of 1-MCP treatment. The higher level of ethylene production observed in the post-treatment phase (72 h), paralleled by a significant increase in *ACS1* transcript accumulation, confirms previously published data (Dong *et al*., 2001; Rasori *et al*., 2002; Ziliotto *et al*., 2003; Dal Cin *et al*., 2006). *ACO1* gene expression is also only slightly affected in apples but, unlike peaches, *ACS1* transcript accumulation is suppressed by 1-MCP (Dal Cin *et al*., 2006; Tatsuki *et al*., 2007). Taken together, these data would indicate that *ACS1* might represent one crucial factor in the modulation of responses to 1-MCP application. Trainotti *et al*. (2007) report that many genes involved in biosynthesis, transport, and signalling of auxin have an increased expression in peach mesocarp during ripening and some ripening-related genes, including *ACS1*, are more strongly influenced by exogenous auxin (NAA) than by ethylene. The same authors suggest that, in addition to the independent role played by auxins, an active cross-talk between auxin and ethylene modulates peach ripening. Besides the well-known inductive effects of auxin on *ACS1* and the ethylene climacteric (Abel and Theologis, 1996; Bleecker and Kende, 2000), the hypothesis of a crucial interplay between auxin and ethylene action is supported by our microarray experiments showing significant transcription changes at early ripening and an effect of 1-MCP on the expression of some auxin metabolism-related genes (ctg_57, ctg_768, ctg_1741, and ctg_1991). Whether the limited effect of 1-MCP in delaying peach fruit ripening is the result of this interplay between auxin and ethylene remains to be elucidated.

Genes involved in ethylene perception and signal transduction

The assumption that 1-MCP irreversibly binds to the ethylene receptors explains the effects of the chemical during the incubation period and this occurs either in systems where ethylene production is blocked (e.g. apple) or not, as in nectarines, where, at the end of the treatment, the delay in ripening is accompanied by altered transcript profiles. It has been postulated that, after 1-MCP treatment, the return of ethylene sensitivity is due to the appearance of new receptors (Sisler and Serek, 1997; Blankenship and Dole, 2003). This appears to be the case for nectarines where the transcription of *ETR1* and *ERS1* appears to be unaffected, in our experimental conditions, by 1-MCP (Fig. 4) and *ETR2* expression recovers when treated fruit are transferred to air (Fig. 3D). In fact, the rapid ripening observed at 72 h (48 h from the end of 1-MCP treatment) is associated with the increased transcript accumulation of well-known ethylene-dependent genes, such as *endo-PG*, and others (ctg_2197, *PpEG1*; and ctg_4533, *PME*) showing an up-regulation during peach fruit ripening and/or following ethylene treatment (Trainotti *et al*., 1997, 2003). This occurs in parallel with the

accumulation of *EIN2* transcripts (ctg_4591) and recovering expression of specific *ERF*s at 72 h (ctg_2116, ctg_2757, and ctg_3350; Figs 3F, 4). Transcript accumulation of *EIN2*, absolutely required for ethylene signalling (Chen and Bleeker, 1995; Alonso *et al.*, 1999), has been associated with ripening peaches (Trainotti *et al.*, 2006; Begheldo *et al.*, 2008). Considering *ERF*s, proteins that specifically bind to the so-called GCC box in promoters of ethylene-responsive genes, the up-regulation observed in ripening fruit (24AIRvsT0), and the reduced amount of transcripts detected at 24 h in treated fruit confirm results obtained in apple and plum, where *ERF1* expression sharply increases with the climacteric peak and 1-MCP treatment inhibits transcript accumulation (El-Sharkawy *et al.*, 2007; Wang *et al.*, 2007). An up-regulation of *ERF2* has been associated with peach fruit ripening and to the presence of ethylene (Trainotti *et al.*, 2007). The relationship between *ERF2* and ethylene is confirmed by our data showing the presence of reduced amounts of specific transcripts in 1-MCP treated fruit. Even though expression of *ERF*s appears to be regulated not exclusively by ethylene in ripening fruit (Wang *et al.*, 2007), the increasing expression trend of the three selected *ERF*s observed at 72 h (Figs 3F, 4) might be interpreted as the result of the recovered perception of ethylene and the reactivation of the signal transduction mechanisms leading to the induction of ethylene-dependent processes.

Colour development and sugar metabolism

Considering the biosynthetic pathway of xanthophylls, pigments responsible for the yellow colour in peaches and nectarines, microarray data indicate that β-carotene hydroxylase is the earliest gene induced at the onset of ripening and appears to be, among the selected targets, the only one significantly affected by 1-MCP (Fig. 8). Ethylene-dependent expression of β-carotene hydroxylase has been observed in orange fruit, another species accumulating xanthophylls at ripening (Rodrigo and Zacarias, 2007), but not in tomato, where lycopene is the main pigment and this gene, together with phytoene synthase (PSY), is expressed in an ethylene-independent manner (Alba *et al.*, 2005). Considering these aspects, it might be hypothesized that different ethylene-related regulatory mechanisms of carotenoid biosynthesis operate in ripening fruit, depending on the genetic background, determining the accumulation pattern and abundance of specific compounds (lycopene, β-carotene, xanthophylls), rather than the presence or not of the ethylene climacteric at ripening. The increasing trend of transcript accumulation detected at 72 h in treated fruit confirms that carotenoid genes are expressed in a ripening-related manner in *P. persica* fruit (Trainotti *et al.*, 2006) and suggests the presence of different factors, including development and ethylene, modulating their transcription.

The complex regulation of carotenoid biosynthesis in *Prunus* spp. species is supported by evidence on *P. mume*, where PSY is constitutively expressed, but ethylene accelerates its induction (Kita *et al.*, 2007), and on *P. armeniaca*, where different effects of 1-MCP have been detected on PSY, phytoene desaturase, and ζ-carotene desaturase (Marty *et al.*, 2005). In addition, stony hard peaches, characterized by a lack of ethylene production at ripening, undergo colour changes during the last developmental stages (Haji *et al.*, 2003) and, if treated with exogenous ethylene, develop a more pronounced yellow colour (P. Tonutti *et al.*, unpublished data), reinforcing the hypothesis that both development and ethylene synergistically contribute to the changes in ground colour of ripening peaches. Considering sugar metabolism, our data indicate that ethylene may induce variable effects even on genes belonging to the same family, as in the case of glucose acyltransferases. Five targets appear to be significantly influenced by 1-MCP: in particular, the expression of a putative invertase inhibitor (ctg_4499), markedly induced at ripening, is strongly affected by the ethylene antagonist. Inhibitor proteins often regulate the activity of enzymes involved in carbohydrate metabolism that are secreted out of the cytoplasmic compartment into the apoplast or to the vacuole (Juge *et al.*, 2004). Examples are represented by invertase and PME inhibitors that are members of a large family of proteins named PMEI-related proteins (Bellincampi *et al.*, 2004; Hothorn *et al.*, 2004). Neutral invertase activity and gene expression (*PpNI1*) decline during peach fruit ripening in parallel with an increase of sucrose, a decrease of glucose+fructose content (Vizzotto *et al.*, 1996; Nonis *et al.*, 2007) and a dramatic up-regulation of a putative invertase inhibitor (Fig. 6). In grape berries, a putative invertase/PME inhibitor protein is highly represented at the prevéraison stage but not during the later stages of fruit development when, differently from peaches, glucose+fructose but not sucrose accumulate (da Silva *et al.*, 2005). Whether the putative invertase inhibitor and the ethylene-dependent modulation of its expression play a major role in sugar metabolism of ripening peaches and nectarines remains to be elucidated.

In conclusion, the genomics approach described in this paper has shed light on mechanisms involved in the limited responses to 1-MCP of nectarine fruit, characterized by a recovery of ripening after the end of the incubation period, and to identify new genes potentially implicated in the ripening process and modulated by ethylene. The quick recovery of nectarine ripening following 1-MCP treatment seems to be related to the limited inhibition of ethylene synthesis by 1-MCP: in this context, *ACS1* and its regulation appear to play a crucial role. However, other mechanisms appear to modulate the expression of ethylene-dependent genes involved in ripening of nectarines. If for some genes (e.g. *endo-PG*,

ETR2) a marked expression recovery has been observed in the post-treatment phase, and this could be imputed to the presence of ethylene, for others (e.g. *PpEXP2*, *PpEXP3*, *HD-ZIP* ctg_3293) no expression changes have been detected after transferring fruit to air for 48 h indicating that the ethylene inhibitor is still effective. Targeted studies and comparative genomics approaches in *Prunus* species characterized by a wide range of ripening phenotypes with different responses to ethylene and its inhibitors will be of great help to elucidate these complex physiological mechanisms.

Supplementary data

Supplementary data are available at *JXB* online. Supplementary Table 1 lists the oligonucleotides used in the qRT-PCR experiments. Supplementary Table 2 reports data of microarray experiments for all the 4805 probes present on μPEACH1.0.

Acknowledgements

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