RESEARCH PAPER

The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches

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Abstract

Ethylene has long been regarded as the main regulator of ripening in climacteric fruits. The characterization of a few tomato mutants, unable to produce climacteric ethylene and to ripen their fruits even following treatments with exogenous ethylene, has shown that other factors also play an important role in the control of climacteric fruit ripening. In climacteric peach and tomato fruits it has been shown that, concomitant with ethylene production, increases in the amount of auxin can also be measured. In this work a genomic approach has been used in order to understand if such an auxin increase is functional to an independent role played by the hormone during ripening of the climacteric peach fruits. Besides the already known indirect activity on ripening due to its up-regulation of climacteric ethylene synthesis, it has been possible to show that auxin plays a role of its own during ripening of peaches. In fact, the hormone has shown the ability to regulate the expression of a number of different genes. Moreover, many genes involved in biosynthesis and transport and, in particular, the signalling (receptors, Auxin Response Factors and Aux/IAA) of auxin had increased expression in the mesocarp during ripening, thus strengthening the idea that this hormone is actively involved in the ripening of peaches. This study has also 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.

Key words: Auxin–ethylene cross-talk, auxin-related genes, ethylene-related genes, fruit ripening, gene expression, peach, peach microarray, Prunus persica.

Introduction

The series of modifications that transform a mature green fruit into a ripe fruit occur during a limited period of time and involve many different metabolic pathways. This means that they have to be strictly regulated and highly co-ordinated in order to lead to a successful result. Therefore, the factors that control the transition of a fruit from the end of growth to the start of ripening are of primary importance. For such a purpose, many years ago it was discovered that two types of fleshy fruits can be recognized, based on the fact that a peak in the rate of respiration can be measured (climacteric fruits) or not (non-climacteric fruits) before the visible onset of the ripening process. In the climacteric fruits, a peak of ethylene production is also normally observed concomitant with the climacteric respiration. Accordingly, the hormone has been considered as the main signal for the regulation of ripening in these fruits (Abeles et al., 1992).

The easy availability of ethylene, in addition to the fact that tomatoes are climacteric fruits, led to an outburst of research dealing with the ripening of climacteric fruits in general, and tomato in particular. It was thus possible to understand many molecular details of the ripening process. The availability of mutants [either natural or induced by genetic transformation: Nr (Wilkinson et al., 1995) and Gr (Barry et al., 2005; Barry and Giovannoni, 2006) mutants; ACS antisense tomatoes (Oeller et al., 1991); ACO antisense tomatoes (Hamilton et al., 1990), and melons (Ayub et al., 1996)] has also helped researchers to understand the mechanisms underlying the ripening process and to confirm the role of ethylene in the ripening of climacteric fruits.

However, the very great availability of tomato mutants has also yielded some specimens (e.g. mutants rin, nor, cnr) whose fruits are unable to ripen even when treated...
with exogenous ethylene, although they are not impaired in the hormone signal transduction pathway. The nor mutant gene has not yet been made public, but it has been reported to code for a transcription factor (Adams-Phillips et al., 2004). The mutated cnr gene has recently been demonstrated to code for an SBP-box transcription factor where a natural epigenetic mutation has occurred (Manning et al., 2006), while the RIN gene has been shown to encode a MADS-box transcription factor (Vrebalov et al., 2002). The characterization of the rin, nor, and cnr mutants is particularly relevant because it demonstrates that other factors, generally named ‘developmental factors’, act upstream of ethylene and their control of the ripening process is no less important than that played by the hormone (Giovanonni, 2004). In the case of tomato, light has also been shown to play a role in the ripening of fruits (Alba et al., 2000).

To add further complexity, Jones et al. (2002) reported in tomato fruits a differential expression of both ARF (Auxin Response Factors) and Aux/IAA encoding genes in tomato fruits which, as their name suggests, are linked to the auxin signalling pathway. In particular, the latter data suggest that auxin might also be part of the mechanisms that control the ripening of climacteric fruits.

It is known that plants can produce ethylene by either a System-1 or a System-2 biosynthesis, and that the latter is active when climacteric ethylene has to be produced (McMurchie et al., 1972; Oetiker and Yang, 1995). Interestingly, auxin can stimulate the synthesis of more climacteric ethylene (Bleecker and Kende, 2000) through its inductive action on the expression of the key enzyme ACS (Abel and Theologis, 1996). Therefore, in such conditions, any effect of auxin on the ripening process would be indirect and mediated by ethylene.

Evaluations of the auxin contents in Redhaven peach fruits (i.e. the same cultivar studied in the present work) were made some years ago in another laboratory (Miller et al., 1987). Those analyses demonstrated that concomitant with the climacteric ethylene production a significant increment of the IAA content could be measured in the mesocarp tissues. Could it be that such an increase in auxin content would only be needed to stimulate the synthesis of ethylene further? Unpublished data obtained in our laboratory suggested that such a ripening-specific auxin increase might be correlated to an autonomous role played by the hormone in the regulation of the climacteric fruit ripening. In this work, a genomic approach has been used to study the possibility that, in the climacteric peach fruits, the hormone auxin plays a role of its own in the regulation of ripening.

Materials and methods

Plant material and hormone treatments

Plants of Prunus persica (L.) Batsch cv. Redhaven were grown in a field near Padua. Fruits at various stages of development [S1, S2, S3L, S3H, S4I, and S4II; see Zanchin et al. (1994), corresponding to 40, 65, 85, 95, 115, 120–125 d after full bloom, respectively] were collected and used either without or with a hormone treatment. The ethylene treatment was provided by placing whole fruits (attached to a branch for all stages but for the S4s) in a sealed chamber and flushing them with ethylene (10 μl l⁻¹) in air at a flow rate of approximately 6 l h⁻¹. The auxin treatment was performed by dipping whole fruits in 1-naphthalene acetic acid [NAA, 2 mM added with Silwet L-77 (200 μl l⁻¹) as surfactant] for 15 min; thereafter, fruits were sprayed with the NAA solution every 12 h over a period of 48 h. Flowers were collected at full bloom, most likely after pollination. Leaves were collected in mid-summer, fully expanded and without any evident signs of senescence. Both treated and untreated samples were frozen in liquid nitrogen and stored at −80 °C for subsequent use.

RNA extraction

Total RNA was extracted from leaves, flowers, and fruits as described in Chang et al. (1993). RNA yield and purity was checked by means of UV absorption spectra, whereas RNA integrity was ascertained by means of electrophoresis in agarose gels followed by ethidium bromide staining on the ribonucleic acid.

Microarray experiments

cDNA synthesis and labelling: Total RNA (15 μg) from peach fruits was converted into target cDNA by reverse transcription using the SuperScript™ Indirect cDNA Labelling System (Invitrogen, USA) following the manufacturer’s instructions, as already described in Trainotti et al. (2006).

Microarray hybridization: Microarray experiments were carried out using the μPEACH 1.0 platform, developed within the ESTTree consortium (ESTree, see the web site http://www.itb.cnr.it/estree/). μPEACH 1.0 is an oligonucleotide microarray carrying 4806 gene-specific probes (70 bases long), selected on cDNA sequences mostly obtained from fruit libraries (ESTree Consortium, 2005; Trainotti et al., 2006). Prehybridizations were carried out by soaking whole glass slides in a solution containing 5× SSC, 0.1% SDS, 5× Denhardt’s solution, and 100 ng μl⁻¹ DNA carrier at 48 °C for at least 2 h. Then the slides were washed once with a 0.2× SSC solution, rinsed with isopropanol, and dried by centrifuging for 2 min at 2000 rev. min⁻¹.

Hybridizations were carried out in 200 μl of hybridization solution (5× SSC, 0.1% SDS, 25% formamide) containing 90–100 pml of Cy3- and Cy5-labelled target cDNAs. The hybridization solution was kept in place by means of a microarray gene frame (ABgene, UK), while the glass slides were placed in a hybridization chamber (HybChamber, by Genomic Solutions, USA) kept on a rotary oven for at least 36 h. Then the slides were briefly rinsed with 1× SSC 0.1% SDS and washed once with the same solution for 5 min. Three additional washes (one with 0.2× SSC solution, rinsed with isopropanol, and dried by centrifuging for 2 min at 2000 rev. min⁻¹) were performed before drying the glass slides with a brief centrifugation.

The probe design and these hybridization/washing conditions allow the detection of specific genes even within gene families.

Data analysis: The microarrays were scanned with a two-channel confocal microarray scanner (ScanArray® Lite, Perkin Elmer, USA) using its dedicated software (ScanArray Express 3.0.0., Perkin Elmer). The laser power and the photomultiplier tube (PMT) were set between 75% and 85% 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.

Software from 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. Spots were also visually examined to delete the non-uniform ones.

The expression data extracted by Spotfinder were normalized by MICAAS 2.18 using the LOWESS (Locally Weighted Regression Scatter Plot Smoothing; Cleveland, 1979) algorithm with the ‘block mode’, keeping as reference the C3 channel.

After normalization, data from each slide were split in two, by using Microsoft Excel, since each probe is spotted twice on mPEACH1.0. Thereafter, each spot value was considered to be independent.

Normal split data were loaded in MeV 3.1 and subjected to SAM (Significance Analysis of Microarrays; Tusher et al., 2001) analyses. Since each comparison (S3II→S4I, S3II0→S3IIet, S3IIair→S3IIet, S3II0→S3IIAA, S3IIair→S3IIAA) was repeated at least twice, there were at least four values for each gene to be used in the SAM analyses. Lists of clones with significant changes in expression among at least two experimental samples were identified at delta values that gave a false discovery rate (FDR) of 0%.

Expression analyses by real-time PCR

Two different protocols were used to prepare the cDNAs to be used as templates for real-time PCR. The RNA samples obtained from leaves, flowers, and fruits at different developmental stages were converted to cDNAs by means of the SuperScript II Reverse Transcriptase (Invitrogen) polymerase chain reaction (PCR) using dT18V as primer, while cDNAs from the hormone-treated fruits were synthesized by means of the SuperScript (Invitrogen) polymerase chain reaction (PCR) using the ‘block mode’, keeping as reference the C3 channel.

After normalization, data from each slide were split in two, by using Microsoft Excel, since each probe is spotted twice on mPEACH1.0. Thereafter, each spot value was considered to be independent.

Normal split data were loaded in MeV 3.1 and subjected to SAM (Significance Analysis of Microarrays; Tusher et al., 2001) analyses. Since each comparison (S3II→S4I, S3II0→S3IIet, S3IIair→S3IIet, S3II0→S3IIAA, S3IIair→S3IIAA) was repeated at least twice, there were at least four values for each gene to be used in the SAM analyses. Lists of clones with significant changes in expression among at least two experimental samples were identified at delta values that gave a false discovery rate (FDR) of 0%.

Results

Possible independent roles played by the hormones ethylene and auxin during the transition to ripening [i.e. from the preclimacteric (S3II) to the climacteric (S4I) stage] were studied in peach fruits. To accomplish this, transcriptome changes were first monitored in untreated fruits during the above transition. Then, hormone untreated and treated fruits were compared. The stage S3II of development had previously been identified as the most convenient stage to investigate the hormonal effect on gene expression during the transition from preclimacteric (S3II) to climacteric (S4I) phases (Trainotti et al., 2003, 2006). Thus, fruits harvested at the S3II stage (in this case indicated as S3II0) and either kept in air (S3IIair) or treated with either ethylene (S3IIet) or the auxin analogue NAA (1-naphthalene acetic acid, S3IIAA) were used to prepare the RNA for the microarray study.

The transcriptome variations were analysed by means of the mPEACH1.0 oligonucleotide microarray (Trainotti et al., 2006). The S3II–S4I transition was investigated using a direct comparison experimental design, with four repetitions of which one was a dye swap. As each probe was spotted twice on the mPEACH1.0 array, the following SAM (Significance Analysis of Microarrays; Tusher et al., 2001) analysis was performed on a dataset of eight values for each gene. The hormonal effect on gene transcription was monitored with a double loop experimental design so that the S3II0 and S3IIair samples were the common references. Each comparison (S3II0–S3IIet, S3IIair–S3IIet, S3II0–S3IIAA, S3IIair–S3IIAA) was repeated at least twice, so that at least four values for each gene were used in the subsequent SAM analyses. Since the S3II sample used for the comparison with S4I corresponded to the S3II0 used in the hormone experiment, the whole dataset could be subjected to a ‘multi class’ study design with five classes (S3II0/S4I; S3II/S3IIet; S3IIair/S3IIet; S3II0/S3IIAA; S3IIair/S3IIAA). Using a delta of 0.2 and a median false discovery rate (FDR) of 0.00%, the analysis yielded 703 genes as differentially expressed (see Supplementary Table 2 at JXB online). The same dataset was also tested with SAM using a ‘one class’ study design. This analysis was carried out on the following three subgroups of data: S3II/S4I, S3II/S3IIet, and S3II/S3IIAA because the experiment with the S3II0 used in the hormone experiment, the whole dataset could be subjected to a ‘multi class’ study design with five classes (S3II0/S4I; S3II/S3IIet; S3IIair/S3IIet; S3II0/S3IIAA; S3IIair/S3IIAA). Using a delta of 0.2 and a median false discovery rate (FDR) of 0.00%, the analysis yielded 703 genes as differentially expressed (see Supplementary Table 2 at JXB online). The same dataset was also tested with SAM using a ‘one class’ study design. This analysis was carried out on the following three subgroups of data: S3II/S4I, S3II/S3IIet, and S3II/S3IIAA because the experiment with the S3II0 and S3IIair reference clustered together. Keeping the median FDR to 0.00%, six groups of genes were selected (the up- and down-regulated for each of the three subgroups of data) and crossed with the 703 genes coming from the multi-class analysis. These conservative parameters allowed us to identify: (i) 219 genes up-regulated and 188 down-regulated during the transition to ripening (i.e. the passage from stage S3II to stage S4I); (ii) 196 genes induced and 169 repressed following treatment with exogenous ethylene; (iii) 126 genes induced and 120 repressed by the NAA treatment. By crossing these data, it was possible to show the different up- and down-regulating effects (represented by means of the Venn diagrams shown in panels A and B, respectively, of Fig. 1; a list of genes
with expression values can be found in Supplementary Table 2 at JXB online):

(i) a subset (71) of these gene are either up- (35) or down-regulated (36) in all the experimental situations;
(ii) the S3II–S4I transition showed a specific regulatory effect (i.e. apparently not dependent on either ethylene or auxin) on 205 genes: 109 induced and 96 repressed.
(iii) treatments with ethylene associated with the S3II–S4I transition showed an up-regulating effect on 102 genes and a repressive effect on 80 genes;
(iv) treatments with NAA associated with the S3II–S4I transition induced the expression of 43 and the repression of 48 genes, respectively;
(v) by excluding the genes that are also responsive to ethylene, the auxin treatment and the transition to ripening specifically induced eight genes and repressed 12 genes.

Some of the genes evidenced by the S3II–S4I transition to ripening (e.g. those coding for endopolygalacturonase, expansin, and ACO1; Trainotti et al., 2003) were already known to be ripening-associated and ethylene-regulated. Interestingly, other ripening-associated genes appeared related to the auxin ‘domain’. Among them, a gene (ctg3721, Fig. 6A), encoding a PIN auxin efflux facilitator, was a new finding. The fact that a few genes related to auxin appeared to be up-regulated during the transition to ripening induced a further exploration of the microarray data. Such a study was aimed at obtaining better information about the expression of other auxin-related genes, including those that previously had not been considered to be differentially expressed due to the stringency of the SAM parameters. This analysis showed that a number of genes encoding either ARF (Auxin Response Factors) or Aux/IAA proteins (i.e transcriptional modulators of the hormone response), but also a hormone receptor encoding gene (i.e. TIR1) and a couple of other genes encoding proteins possibly involved in the early steps of auxin biosynthesis (IGPS: indole-3-glycerol phosphate synthase, and W synt: tryptophan synthase beta subunit), exhibited increased expression during the transition to ripening, besides the PIN gene mentioned previously (Fig. 2).

Real-time qRT-PCR experiments were carried out to investigate further the interplay between auxin and ethylene in ripening peaches. It is known that auxin may stimulate ethylene biosynthesis by increasing the transcription of ACS genes. This also happened in our system, where an ACS gene (Mathooko et al., 2001) was induced more by NAA than by exogenous ethylene treatments (Fig. 3A). By contrast, the steady-state level of an ACO gene (ACO1; Ruperti et al., 2001) appeared almost insensitive to NAA, and was greatly induced by ethylene (Fig. 3B). Ethylene receptor encoding genes were either insensitive to both hormones (ETR1 ctg1436, Fig. 3C and ERS ctg356, Fig. 3D) or showed a fruit-specific, ripening-induced, and ethylene-dependent expression (ETR2 ctg2025, Fig. 3E). Among the genes involved in the ethylene ‘domain’, most interesting was the expression of an ethylene-response-factor2 (ctg2116), which was found to be almost fruit specific. In particular, it showed a dramatic increase during the melting phase (i.e. S4II, see Fig. 3F), and because of such a late expression, no variations in gene expression was detected by microarray analysis. Interestingly, its expression could be induced much more by NAA than by ethylene, thus suggesting
that auxin might also be important during late ripening of peaches. On the other hand, ethylene could stimulate the expression of auxin-related genes. All the Aux/IAA considered in this work (Fig. 4) were fruit-specific and ripening-induced, while the hormonal regulation was different. Two of them (ctg1741 and ctg1727; Fig. 4A, B) were clearly up-regulated only by NAA. Two others (ctg768 and ctg1068; Fig. 4C, D) were up-regulated by NAA and, to a lesser extent, by ethylene. In other cases, Aux/IAA genes were either up- (as ctg671 and ctg42; Fig. 4E, F) or down-regulated (as ctg57 and ctg84; Fig. 4H, I) by both hormones. Finally, ctg358 (Fig. 4G) appeared more or less insensitive to the hormone treatments.

In the case of the ARF encoding genes, all with increased expression from stage S3II onwards (Fig. 5), three of them (ctg1505, ctg3678, and ctg1991; Fig. 5A, B, C) were ripening-induced while ctg4040 (Fig. 5D) was almost constitutive. Only ctg1991 expression, which peaked at the melting phase (i.e. S4II), was slightly induced by ethylene, while the expression of the other ARFs was reduced by the exogenous hormones. The ethylene effect was particularly strong on ctg3721, a PIN-like gene whose expression was fruit specific and ripening-induced (Fig. 6A). Two auxin receptors (ctg2713 and ctg1541) have been found to be ripening-related, while the hormone treatments slightly decreased their expression (Fig. 6B, C, respectively). Genes belonging to both the tryptophan dependent (W synthase, ctg3371, Fig. 6E) and independent (IGPS, indole-3-glycerol phosphate
Discussion

The genomic approach used in this work has confirmed that ethylene can affect the expression of many genes, as expected since peach is a climacteric fruit. These experiments have also shown that a great number of genes appear to be specifically regulated by the S3II–S4I phase transition. This effect is apparently independent from either ethylene or NAA, therefore the expression of such genes might be regulated by other, as yet unknown, factors.

As pointed out in the Introduction, a significant increase in IAA content had been measured by Miller et al. (1987) in the mesocarp of Redhaven peaches concomitant with the production of climacteric ethylene. In connection with a possible autonomous role played by auxin, at least nine different Aux/IAA genes have been found to have increased expression at the onset of ripening (i.e. S3II–S4I transition), and could therefore be regarded as involved in this process, although a few of them showed a decrease in expression during late ripening (i.e. S4II; Figs 2, 4). In particular, two genes (ctg1741 and ctg1727; Fig. 4A, B) were significantly up-regulated by NAA, six other genes (ctg768, ctg1068, ctg671, ctg42, ctg57, and ctg84; Fig. 4C, D, E, F, H, I, respectively) could be regulated (either up or down) by both hormones, while the remaining gene ctg358 (Fig. 4G) appeared to be up-regulated mostly by the S3II–S4I transition.

The Aux/IAA proteins share a four domain structure, and domains III and IV allow them to interact either with each other or with similar domains of Auxin Response Factor (ARF) proteins. While ARFs have an N-terminal DNA-binding domain, and can thus regulate the expression of auxin-responsive genes, Aux/IAA proteins have no such domain and can therefore act as transcriptional regulators through their interaction with ARF proteins (Berleth et al., 2004; Tiwari et al., 2004).

Interestingly, besides the aforementioned Aux/IAA genes, four genes encoding different Auxin Response Factors (ARFs) have also been found to be expressed in the fruit mesocarp (Fig. 5). One of them (ctg4040; Fig. 5D)

Fig. 4. Relative expression profiles of some peach Aux/IAA genes in different plant tissues and during fruit development and ripening (left) and after hormone treatment of S3II fruits (right). Values (means of the normalized expression) have been obtained by means of real-time qRT-PCR. Numbers in the top left of each panel indicate the peach contig name. Stages S1–S4II encompass the development and ripening of peach fruits. Hormone treatments (et, ethylene; NAA, 1-naphthalene acetic acid) lasted for 48 h. Bars are the standard deviations from the means.
did not show any significant variation in expression during ripening, by contrast with the other three genes whose transcripts appeared significantly increased during ripening proper (Fig. 5A, B, C). But for ctg1991 whose expression seemed to be slightly increased by both hormones (Fig. 5C), it was the S3II–S4I transition to ripening that apparently increased the transcription levels of the remaining three ARFs.

The embryo that is developing inside the peach stone might be a source of the IAA that is present in the mesocarp. However, the IAA increment measured by Miller et al. (1987) in the mesocarp tissues from 67 d to 88 d after anthesis coincided with a net decrease in the embryo IAA content measured in the same time interval, thus suggesting that the mesocarp might synthesize its own IAA.

In agreement with such an idea, a gene coding for a tryptophan synthase β subunit was found to be expressed in the mesocarp in increasing amounts from stage S3I until late ripening (ctg 3371; Fig. 6E). Tryptophan is known to be the most common IAA precursor, and it is interesting to note that the expression of the above gene appears to be up-regulated by hormone treatments, especially by NAA.

Another gene, encoding a putative indole-3-glycerol phosphate synthase (IGPS), showed increased expression from the end of growth until late ripening. By contrast with the tryptophan synthase β subunit gene, the
expression of the IGP synthase-encoding gene seems to be especially dependent from the S3II–S4I phase transition (Fig. 6D). Besides being a precursor for indole biosynthesis, IGPS can also represent a branch point for IAA synthesis through the tryptophan-independent pathway (Taiz and Zeiger, 2006). Therefore, the above expression data support the hypothesis that the observed increment in mesocarp IAA content is likely to have been produced by an endogenous biosynthesis of the hormone.

The idea that an active IAA-related metabolism might occur in the fruit mesocarp is further strengthened by the expression of two putative TIR1 genes [i.e. genes coding for auxin receptors (Dharmasiri et al., 2005; Kepinski and Leyser, 2005)]. One of them (ctg1541; Fig. 6C) showed a maximum at the S3II stage and slowly decreased thereafter while the other gene (ctg2713; Fig. 6B) exhibited a marked increase during the transition to ripening and slowly decreased during late ripening. For both genes the expression seems to be particularly up-regulated by the transition to ripening while the effect of the two hormones does not appear particularly relevant.

Still consistent with an active IAA-related metabolism, a gene encoding a putative auxin efflux facilitator protein PIN1 (Paponov et al., 2005) was also found to be expressed in the mesocarp particularly during ripening proper (i.e. stages S4I and S4II, see Fig. 6A). However, the expression of this gene appears to be significantly up-regulated by ethylene rather than by auxin. This finding is particularly interesting because it demonstrates that in peaches as well an active cross-talk between auxin and ethylene is also important for the regulation of ripening, besides the independent roles played by each hormone. Until now, cross-talk between auxin and ethylene had mostly been observed in vegetative tissues (Chae et al., 2000; Rahman et al., 2002; Swarup et al., 2002; McDonald and Visser, 2003; Stepanova et al., 2005; Muday et al., 2006), while less was known about its existence in fruits (Jones et al., 2002).

An interplay between the two hormones was also found when the expression of genes was analysed on the ethylene side. As expected, biosynthesis genes like ACS and ACO1 showed a ripening-specific expression with transcript amounts that increased significantly during stages S4I and S4II. However, while the expression of ACO1 (Rupert et al., 2001) seems to be up-regulated mostly by ethylene, in the case of the ACS gene (Mathooko et al., 2001) the opposite occurs, so that the highest up-regulating effect is caused by auxin (Fig. 3B, A, respectively), the latter finding being in agreement with previous data from other plants (Abel and Theologis, 1996; Beckman et al., 2000; Yamagami et al., 2003; Tsuchisaka and Theologis, 2004).

The expression of a gene encoding a putative Ethylene Response Factor 2 (ctg2116) was observed to be late-ripening specific and highly up-regulated by auxin although, to a lower extent, ethylene also had an inductive effect on its expression (Fig. 3F). As regards ethylene receptors, the two already described by Rasori et al. (2002) appeared to be expressed either during growth and ripening without significant variations (ETRI) or at increasing amounts during ripening (ERS1). In both cases treatments with either one or the other of the two hormones did not significantly affect their expression compared with what had been observed in samples kept in air for the same period of time (Fig. 3A, B). Interestingly, a third receptor gene (i.e. ETR2; Trainotti et al., 2006), whose expression had not yet been described, showed a clear ripening-specific expression pattern (Fig. 3E). In this case the greatest inductive effect on its expression was yielded by ethylene, while the more limited increase in transcript amount observed following a treatment with auxin was probably indirect and mediated by ethylene.

Ethylene has long been known to be of paramount importance for the ripening of climacteric fruits, and it continues to be used worldwide to control their ripening. Besides confirming this common knowledge, the approach used in this work has shown a novel aspect of the regulatory networks that operate during the ripening of climacteric peach fruits. In particular, it has been found that auxin, whose role was considered relevant mostly for fruit set (Spena and Rotino, 2001) and the early stages of fruit growth (Nitsch, 1950), may also play a relevant and independent role during the ripening of climacteric fruits.

For the latter purpose, the authors are aware that the conclusions that can be drawn from the present findings in peach fruits cannot be extended to other climacteric fruits without supporting experimental data. However, a possible similar role for auxin in the ripening of tomato fruits does not appear so unlikely. In fact, Jones et al. (2002) have shown that both Auxin Response Factors (ARF) and Aux/IAA genes were differentially expressed in tomato fruits. Moreover, an Aux/IAA gene (DR3) showed maximum accumulation in early red fruits and had ethylene-inducible expression, the latter finding demonstrating that, analogous to peach fruits, an auxin–ethylene cross-talk is also present in tomato fruits. Finally, similarly to what was found in peaches by Miller et al. (1987), in ripening tomato fruits increases in auxin content have also been reported to parallel those in climacteric ethylene production (Gillaspy et al., 1993).

**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 about the data of the microarray experiments for all the 4806 probes present on μPEACH1.0. Genes positive to SAM analysis and belonging to ‘groups i-vi’ are labelled.
Moreover, gene annotations and probe unique identifiers are provided.

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