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RESEARCH PAPER

Inhibition of the ethylene response by 1-MCP in tomato suggests that polyamines are not involved in delaying ripening, but may moderate the rate of ripening or over-ripening

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Abstract

Ethylene initiates the ripening and senescence of climacteric fruit, whereas polyamines have been considered as senescence inhibitors. Ethylene and polyamine biosynthetic pathways share *S*-adenosylmethionine as a common intermediate. The effects of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, on ethylene and polyamine metabolism and associated gene expression was investigated during ripening of the model climacteric fruit, tomato (*Solanum lycopersicum* L.), to determine whether its effect could be via polyamines as well as through a direct effect on ethylene. 1-MCP delayed ripening for 8 d compared with control fruit, similarly delaying ethylene production and the expression of 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase and some ethylene receptor genes, but not that of ACC oxidase. The expression of ethylene receptor genes returned as ripening was reinitiated. Free putrescine contents remained low while ripening was inhibited by 1-MCP, but increased when the fruit started to ripen; bound putrescine contents were lower. The activity of the putrescine biosynthetic enzyme, arginine decarboxylase, was higher in 1-MCP-treated fruit. Activity of *S*-adenosylmethionine-decarboxylase peaked at the same time as putrescine levels in control and treated fruit. Gene expression for arginine decarboxylase peaked early in non-treated fruit and coincident with the delayed peak in putrescine in treated fruit. A coincident peak in the gene expression for arginase, *S*-adenosylmethionine-decarboxylase, and spermidine and spermine synthases was also seen in treated fruit. No effect of treatment on ornithine decarboxylase activity was detected. Polyamines are thus not directly associated with a delay in tomato fruit ripening, but may prolong the fully-ripe stage before the fruit tissues undergo senescence.

Key words: Ethylene, fruit ripening, 1-MCP, polyamines, tomato.

Introduction

Tomato is the primary model for climacteric fruit ripening for a combination of scientific and agricultural reasons. Ripening of climacteric fruit is accompanied by a peak in respiration and a concomitant burst of ethylene that is needed to trigger several processes associated with ripening, such as the dramatic changes in colour, texture, flavour, and aroma of the fruit flesh. A large number of studies have been published on the biochemical, molecular, and genetic regulation of tomato ripening, especially in relation to ethylene (Giovannoni, 2004). Ethylene production in plant tissues results from the metabolism of methionine (Yang, 1985). The rate-limiting steps in fruit ethylene biosynthesis include the conversion of *S*-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) via ACC synthase (ACS), and the subsequent metabolism of ACC to ethylene by ACC oxidase (ACO) (Fig. 1). In tomato, both steps are highly transcriptionally regulated and encoded by

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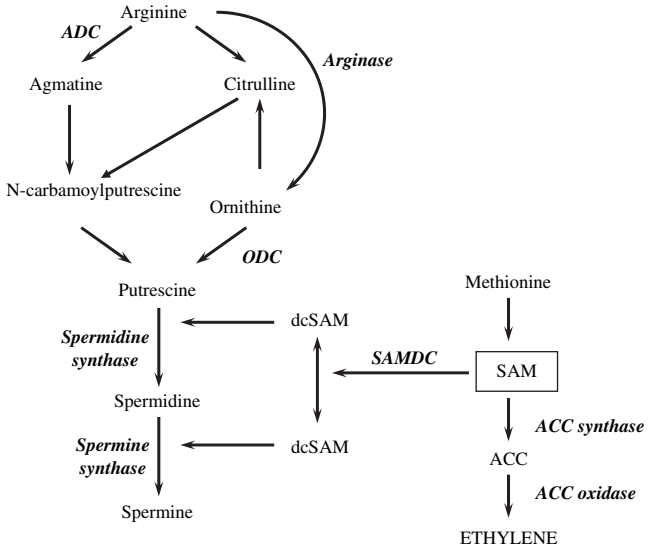

Fig. 1. Pathways and enzymes of ethylene and polyamine biosynthesis.

multigene families. At least four *ACS* genes are expressed in tomato fruit (Barry *et al*., 2000; Pech *et al*., 2004), while two different *ACO* genes (*LeACO1* and *LeACO3*) are expressed and accumulate to various degrees at distinct fruit developmental stages (Barry *et al*., 1996; Pech *et al*., 2004). Ethylene is perceived by ethylene receptors (ETRs) and six different genes have been identified in tomato (Klee, 2002; Klee and Tieman, 2002; Klee and Clark, 2004). The protein structures of ETRs seem to be quite divergent, exhibiting less than 50% identity in the primary sequence at the extremes. Tomato *LeETR1* and *LeETR2* genes are expressed at constant levels in all tissues, including the fruit throughout development, with *LeETR1* expressed at about 5-fold higher than *LeETR2* (Tieman and Klee, 1999). *NR* (*Never Ripe*, also referred to as *LeETR3*) was the first identified ethylene receptor in tomato; because in the mutant form it lacks the receiver domain of the protein it confers dominant insensitivity to ethylene. *NR* expression was shown to increase significantly during fruit ripening (Wilkinson *et al*., 1995). The *LeETR4*, *LeETR5*, and *LeETR6* genes are expressed abundantly in reproductive tissues (flowers and fruit) (Klee, 2002) and, in particular, the levels of *LeETR4* and *LeETR5* increase as fruit mature and ripen.

Aliphatic polyamines (PAs) [putrescine (Ptc), spermidine (Spd), and spermine (Spm)] are ubiquitously distributed compounds implicated in a large range of growth and developmental processes (Bagni, 1989). In plants, Ptc can be synthesized from arginine or ornithine by arginine and ornithine decarboxylase (ADC and ODC), respectively (Fig. 1) and changes in both enzyme activities generally seem to regulate Ptc synthesis (Bagni and Tassoni, 2001). Spd and Spm derive by the subsequent addition of an aminopropyl moiety, derived from decarboxylated *S*-adenosylmethionine (dcSAM), to Ptc or Spd, respectively. These reactions are catalysed by Spd synthase or Spm synthase, respectively, and the dcSAM is derived from SAM by *S*-adenosylmethionine decarboxylase (SAMDC) (Bagni and Tassoni, 2001). Polyamines share with ethylene the common intermediate SAM, which is both the substrate for ACS in ethylene biosynthesis and for SAMDC in PA biosynthesis. While ethylene action is associated with senescence and ripening, PAs, on the other hand have been considered senescence inhibitors. They can in fact delay senescence by inhibiting the rise in RNase, protease and peroxidase and by inducing DNA synthesis and mitotic activity (Dumbroff, 1990).

The levels of PAs and biosynthetic enzyme activities have been determined in tomato fruit of several ripening stages and in ovaries (Dibble *et al*., 1988; Rastogi and Davies, 1991; Belles *et al*., 1993; Egea-Cortines *et al*., 1993; Alabadí *et al*., 1996; Antognoni *et al*., 2002; Fos *et al*., 2003). In general, Ptc is the predominant PA during tomato ripening, its levels being high at the immature-green stage and then declining throughout the ripening process. Spd is present at a lower amount than Ptc and its levels do not seem to change during ripening, while Spm content is always very low. High levels of free PAs at anthesis and during the initial stages of fruit development have been reported (Cohen *et al*., 1982; Teitel *et al*., 1985; Egea-Cortines *et al*., 1993). A rapid and transient increase in the amount of the free PAs and a decrease in the conjugated PAs after pollination and during early parthenocarpic fruit development of the *pat* mutant (Antognoni *et al*., 2002) or induced by auxin and gibberellin treatment (Alabadí *et al*., 1996) have also been observed. Cultivars possessing slow-ripening and longer-keeping fruit qualities (Alcobaca and Daniela) have a greater amount of Ptc than normal-ripening cultivars (Rutgers and Breton), leading to the hypothesis that PAs play a role with regard to their ripening and storage characteristics (Dibble *et al*., 1988; Saftner and Baldi, 1990; Martinez-Madrid *et al*., 1996). Following the decline in Ptc during fruit maturation, the level of Ptc in Alcobaca, a landrace with slow ripening and a very delayed over-ripening process, rises from the mature green stage through ripe, whereas the level remains low in the normal ripening fruit (Dibble *et al*., 1988). The activity of the Ptc biosynthetic enzyme ADC is also elevated during the early stages of fruit development in Alcobaca, as compared with normal tomato (Rastogi and Davies, 1991). In addition, the exogenous application of polyamines to mature-green tomato fruit leads to a delay in ripening (Law *et al*., 1991). The expression of ODC and Spd synthase genes is up-regulated during early fruit development after pollination, 2,4-D, and gibberellin application (Alabadí and Carbonell, 1998). In addition, a correlation between maximal ODC transcript levels and maximal ODC activity has been observed (Alabadí *et al*., 1996), supporting the idea that ODC is the primary enzyme involved in Ptc biosynthesis during early fruit development in tomato (10 d

after anthesis). PAs induce a partial parthenocarpy when applied to wild-type unpollinated tomato ovaries, whereas the parthenocarpic ability of *pat-2* mutants depends on elevated PA levels in unpollinated tomato ovaries, and these high PA levels seem to correlate with the activation of ODC and Spd synthase genes (Fos *et al*., 2003). However, it was determined that in the later stages of fruit development ADC activity was higher than ODC (Teitel *et al*., 1985), while in the slow-ripening Alcobaca cultivar, it was found that even though ODC activity was higher than ADC during the later stages of fruit development, ADC activity correlated with the high Ptc levels detected during the later stages of tomato ripening indicating that the increase of Ptc is probably due to ADC activity (Rastogi and Davies, 1991). This hypothesis was confirmed by Alabadí *et al*. (1996) who noted a drastic decrease in arginase activity associated with the initiation of cell division and cell expansion in fruit growth, indicating a redirection of nitrogen metabolism to the synthesis of arginine. Expression analysis of the ADC transcript during later tomato fruit maturation demonstrated a higher level of expression at the breaker stage (Rastogi *et al*., 1993).

The synthetic gaseous compound 1-methylcyclopropene (1-MCP) has proved commercially valuable to extend storability of several plant products (Watkins, 2002; Blankenship and Dole, 2003; Watkins and Miller, 2005), as well as providing a powerful tool in research programmes which attempt to understand and provide new insight into ethylene action and responses (Sisler and Serek, 1997). 1-MCP is thought to occupy ethylene receptors, preventing ethylene binding and thus its action (Sisler and Serek, 1997). The affinity of 1-MCP for the receptors is approximately ten times greater than that of ethylene and, therefore, compared with ethylene, 1-MCP is active at much lower concentrations. 1-MCP also influences ethylene biosynthesis by exerting a feedback inhibition on ACS and ACO enzyme expression (Blankenship and Dole, 2003).

Tomato fruit ripening is inhibited by 1-MCP, although effective concentrations vary among different cultivars, exposure periods, and fruit eventually may recover from ripening inhibition (Sisler *et al*., 1996; Wills and Ku, 2002; Hoeberichts *et al*., 2002; Mostolfi *et al*., 2003). Most of these studies have focused on the potential for commercial application, but have included analyses of colour development (chlorophyll and lycopene), softening (polygalacturonase activity), and ethylene production in tomato fruit harvested at mature-green, breaker, and orange stages (Hoeberichts *et al*., 2002; Mostolfi *et al*., 2003). It is generally assumed that 1-MCP binds permanently to ethylene receptors present at the time of treatment and the recovery of ethylene sensitivity seems to be due to the appearance of new binding sites (Feng *et al*., 2004), such that a continued effect can only be achieved by repeated treatments.

1-MCP treatment of tomato fruit at different ripening stages decreases the mRNA levels of some ripening-related enzymes such as phytoene synthase and expansin 1 (Hoeberichts *et al*., 2002), and most importantly inhibited the expression of some of the ACO and ACS isoforms (Nakatsuka *et al*., 1997, 1998; Hoeberichts *et al*., 2002). In particular, the transcript accumulation of *LeACS2*, *LeACS4*, *LeACO1*, and *LeACO4*, which normally increase during tomato ripening, was greatly inhibited by exposure of the mature-green to pink stage fruit to 1-MCP (Nakatsuka *et al*., 1998).

The relationships between ethylene and PAs are not well understood, despite the fact that their biosynthetic pathways share a common intermediate, *S*-adenosylmethionine. The objective of this study was to analyse the role of PAs in tomato fruit ripening further and to determine whether the effect of 1-MCP in temporarily stopping tomato fruit ripening could be ascribed to the anti-senescence properties of PAs. The effect of 1-MCP treatment on ripening tomato fruit has been examined for both ethylene production and PA content, as well as the activity of several enzymes of the PA biosynthesis pathway, and the transcription of genes encoding these enzymes, the enzymes of ethylene biosynthesis, and the ethylene receptor proteins.

Materials and methods

Plant material

Tomato fruit [*Solanum lycopersicum* L. (formerly *Lycopersicon esculentum* Miller) cv. Trust], grown in a commercial greenhouse (21–29 °C day, >13 °C night) in a peat/perlite mix (Perlmix, Premier Horticulture, Quakertown, PA, USA) with constantly supplied liquid nutrients (Peters, 15/11/29 (Scotts Horticulture, Marysville, OH, USA) with double Ca^{2+}) in solar-radiation-regulated automatic watering, were collected at the breaker stage in June 2004 from plants about 5 months from sowing.

1-MCP treatment

1-MCP was applied to tomato fruit on the day of harvest. Three grams of a 1-MCP-releasing powder (SmartFresh™, 0.14% of active ingredient by weight; Rohm and Haas, Springhouse, PA, USA) was dissolved in 50 ml of water to provide a final gas concentration of 20 µl l^{-1} of 1-MCP and used to treat 40 tomatoes, of approximately 150 g each, in a 75 l container, which was rapidly closed and the airtight lid taped to ensure a tight seal. The treatment was performed for 4 h at 20–25 °C in the dark and control tomatoes were kept under identical conditions in air. After 1-MCP treatment the containers were vented and thereafter the tomatoes were kept in darkness at 20 °C and 70% relative humidity. Three different fruit both for control and 1-MCP-treated tomatoes were collected at alternate days up to 16 d after harvest and used to assess firmness, colour change (six fruits), and ethylene production before samples of the pericarp tissue were frozen with liquid N_2 and stored at −80 °C for subsequent analyses.

Determination of firmness, colour change, and ethylene production

A modified Precision Scientific penetrometer (Fisher Scientific Inc., NH, USA) was used for non-destructive firmness measurements. The probe was replaced with a 5 cm diameter Plexiglas disc. The fruit was held in a hollow, inverted triangular pyramid, with a 90° basal angle, such that the fruit was held in position with three contact points.

A 500 g force was applied to the fruit for 5 s and the compression measured in mm. The change in fruit colour was determined both at the equator level and the blossom end by using a Minolta Chroma Meter, Model CR-300 (Minolta, Mahwah, NJ, USA) in terms of lightness (*L*), a green (negative values) to red (positive values) scale (*a*), and saturation, a blue (negative values) to yellow (positive values) scale (*b*). Three different readings were collected from both equator region and blossom end of each fruit. The changes in colour were expressed by the tomato colour index (TCI) according to the formula:

$$2000a/L\ (a^2+b^2)^{1/2}$$

(Richardson and Hobson, 1987).

For ethylene measurement fruit were sealed daily in 1.0 l containers for 1 h each day and headspace ethylene concentrations were determined in duplicate using a Hewlett Packard 5890 series II gas chromatograph (Hewlett Packard Co., Wilmington, Delaware, USA) equipped with a stainless steel column packed with 60/80 mesh alumina F-1 (2 m×4 mm, i.d.) and a flame ionization detector.

Data are from three replicates from each of three different fruits, except the colour measurement was from six fruits. Experiments were performed twice with similar results.

Polyamine analysis by HPLC

Polyamine analyses were performed according to Tassoni *et al*. (2000). Tomato samples (about 0.2 g FW) were homogenized in 10 vols of 4% (v/v) cold perchloric acid (PCA) and centrifuged at 20 000 *g* for 30 min at 4 °C. The pellet was washed three times and resuspended to the original volume with PCA 4%. Triplicates of this suspension and of the supernatant were hydrolysed with 6 N HCl in flame-sealed glass ampoules at 110 °C for 20 h in order to release PAs from their conjugates. Aliquots (0.2 ml) of supernatant, hydrolysed supernatant, and hydrolysed pellet were derivatized with dansyl-chloride (3 mg ml^{-1} of acetone), extracted with toluene and analysed by HPLC (Beckman 110B (Fullerton, CA, USA) pumps, Perkin Elmer (Norwalk, CT, USA) 650–10LC flow-through fluorescence spectrophotometer and EZChrom (San Ramon, CA, USA) pump control and data analysis software) with a reverse phase C_{18} column (Spherisorb ODS2, 5 μM particle diameter, 4.6 mm×250 mm, Phenomenex, Torrance, CA, USA) as described by Dibble *et al*. (1988) with a 60–95% aqueous methanol gradient over 23 min recording fluorescence at 365 nm activation and 510 nm emission.

Data are from three replicates from each of three different fruits. Experiments were performed twice with similar results.

ADC, ODC, SAMDC, and Spd synthase enzyme activities

Arginine decarboxylase (ADC; EC 4.1.1.19), ornithine decarboxylase (ODC; EC 4.1.1.17), and *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50) activities were determined by a radiochemical method as described by Tassoni *et al*. (2000), but using 0.5 ml of 1 M KOH as the $^{14}CO_2$ trapping compound. To determine ADC and ODC activities, tomato tissues (1 g FW) were homogenized in an ice-cold mortar with 3 ml of the assay buffer (100 mM TRIS–HCl (pH 8.5), 50 μM pyridoxal phosphate) and centrifuged at 20 000 *g* for 15 min at 4 °C. Aliquots of 0.3 ml of both supernatant and resuspended pellet (containing cell wall, nuclei, plastids, and mitochondria) were used (0.5 ml final assay volume). The ADC assay was performed by measuring the $^{14}CO_2$ evolution from 0.2 μCi of L-[U-^{14}C]arginine (specific activity 318 mCi $mmol^{-1}$, MP Biochemicals Inc., Irvine, CA, USA) over 2 h, while the ODC assay was performed by measuring the $^{14}CO_2$ evolution from 0.2 μCi of D,L-[1-^{14}C]ornithine (specific activity 56 mCi $mmol^{-1}$, Amersham Biosciences, UK) over 2 h. The percentage of arginase (EC 3.5.3.1) activity was calculated from the difference between the ADC activities performed in the presence of 0.2 μCi of [^{14}C]arginine in the absence and in the presence of 10 mM unlabelled ornithine.

To determine SAMDC activity, tomato samples (1 g FW) were homogenized in 5 vols of 100 mM TRIS–HCl (pH 7.6), 50 μM EDTA, and 25 μM pyridoxal phosphate and centrifuged at 20 000 *g* for 15 min at 4 °C. The supernatant and the resuspended pellet (0.3 ml aliquots) were incubated separately with 0.1 μCi [1-^{14}C]*S*-adenosylmethionine (specific activity 61 mCi $mmol^{-1}$, Amersham Biosciences, UK) and $^{14}CO_2$ evolution was measured over 2 h.

Spd synthase activity (Spd synth, EC 2.5.1.16) was determined according to Franceschetti *et al*. (2004) with minor modifications. Tomato tissue (1 g FW) was homogenized in 3 ml of 100 mM potassium phosphate (pH 7.5), 10 mM 2-mercaptoethanol buffer, in an ice-cold mortar. The homogenate was centrifuged at 20 000 *g* for 15 min at 4 °C, the supernatant was collected, and the pellet resuspended with the same buffer to the original volume. Both supernatant and pellet samples were incubated with 100 μM SAM and 0.2 μCi [1-4–^{14}C]Ptc (specific activity 110 mCi $mmol^{-1}$, American Radiolabeled Chemical Inc., St Louis, MO, USA). The reaction (350 μl total volume) was carried out at 37 °C for 2 h and then stopped with 150 μl of cold 5% (v/v) PCA. The denatured proteins were removed by centrifugation at 9 000 *g* for 20 min and the supernatants were used for quantifying the reaction products. Labelled Spd was separated by HPLC according to Rastogi and Davies (1990), and the quantification of the radioactivity of the corresponding peak was performed in a scintillation counter (Beckman LS1801, Fullerton, CA, USA) after the collection of 1 ml fractions.

The protein content of supernatant and pellet fractions was determined according to Bradford, (1976) with bovine serum albumin as standard.

All experiments were performed twice with three replicate samples each.

RT-PCR expression analysis

Tomato pericarp was frozen in liquid N_2 and powdered using a mortar and pestle. About 100 mg of tissue powder was used for total RNA extraction with the TRIAZOL Reagent system according to the manufacturer's instructions (Gibco BRL, Invitrogen, Carlsbad, CA, USA). Concentration of total RNA samples was routinely determined from absorption at 260 nm and 280 nm and the integrity checked by 1% (w/v) agarose gel (Sambrook *et al*., 1989).

First strand cDNAs were synthesized from 50 ng of total RNA with 120 ng of oligo(dT) primer (18 mers) and 20 U of StrataScript reverse transcriptase (ProSTAR™ Ultra HF RT-PCR System, Stratagene, La Jolla, CA, USA) in a final volume of 20 μl. The reverse transcription was carried out in an Amplitron II PCR (Thermolyne, Dubuque, IA, USA) with the following temperature parameters: 5 min at 95 °C followed by 30 min at 42 °C. The amount of each cDNA sample used in the following amplification reactions was calibrated by PCR using degenerate primers amplifying members of the actin gene family, as described by Testa *et al*. (2002) (Table 1).

Based on database published sequences, a pair of specific primers was designed for each tomato ethylene biosynthesis and receptor gene and PA biosynthesis gene of interest (Table 1). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The sequence alignments were performed with the program ClustalW (EMBL-EBI, Heidelberg, Germany). Actin, ADC, and ODC were as used in Testa *et al*. (2002) and Antognoni *et al*. (2002). ACC synthase degenerate primers were designed according to the conserved regions of the aligned database tomato *ACS* gene isoforms *LeACS2*, *LeACS4*, and *LeACS6*. The other known *ACS* isoforms seem to be expressed only in the very early stages, are not expressed during fruit development, or are not affected by 1-MCP treatment (Nakatsuka *et al*., 1998). ACC oxidase degenerated primers were designed according to the conserved aligned regions of the gene isoforms *LeACO1*, *LeACO3*, and *LeACO4*; *LeACO2* seems not to be

Table 1. *Primers for RT-PCR amplification of cDNA fragments*

The annealing temperature (Ta) and number of cycles are indicated. The degenerated primers were designed on conserved regions of aligned database sequences (see text for details). The nucleotide mixtures are indicated by the following one letter code: R: a, g; Y: c, t; M: a, c; K: g, t; S: g, c; W: a, t; D: g, a, t.

Amplified cDNA	Primers	Ta; cycles
Actin[a]	**ACT1:** 5′-GAY TCT GGD GAT GGK STS AS-3′	55 °C
(500 bp)	**ACT2:** 5′-ATY TTC ATG CTR CTD GGA GC-3′	25 or 30 cycles
ACC synthase (ACS)	**AT200:** 5′-GCT GAT CCT GGY GAT GCW TT-3′	58 °C
(440 bp)	**AT201:** 5′-ACY CKA AAT CCT GGW AAM CCT-3′	25 cycles
ACC oxidase	**AT202:** 5′-AAR AAG TGC ATG GAA CAR AGG-3′	57 °C
(ACO) 507 bp	**AT203:** 5′-GRC CAC TSA CTT TGT CAT CTT-3′	25 cycles
ETR 1 and 2	**AT204:** 5′-ATT CTG CTG GAC CTG GRC TT-3″	55 °C
(517bp)	**AT205:** 5′-AGA RTA ACT CCA TCC ATW CCA A-3	30 cycles
Never ripe (NR)	**AT206:** 5′-GTC TAG GGC TTG CCA TTT G-3′	55 °C
(365 bp)	**AT207:** 5′-GAC AAA CTG TGC CAT TTG GA-3′	30 cycles
ETR 4, 5 and 6	**AT208:** 5′-CTG TKG CTG CWA TTM GGA TG-3′	58 °C
(375 bp)	**AT209:** 5′-CWT CYT GMA AYA WGG AGA GCA-3′	30 cycles
ODC	**M104:** 5′-CCC AAT GGG TCC AAA ATA C-3′	52 °C
(700 bp)[b]	**M105:** 5′-AAG GTG AGT AAC AAT GGC G-3′	30 cycles
ADC	**M102:** 5′-TCC TGT TGG CGA TGA ACT G-3′	55 °C
(430 bp)[b]	**M103:** 5′-CAG AGT AAA TCT GAG TGG CCT CAC-3′	25 cycles
Arginase	**AT214:** 5′-GCC TCA ACG TCT CTT CTT GG-3′	57.5 °C
(546 bp)	**AT215:** 5′-TCG CAT TTC ATA TTG CTC CA-3′	25 cycles
SAMDC	**M108bis:** 5′-TCT GCC ATT GGT TTT GAA G-3′	54 °C
(310 bp)	**M109bis:** 5′-CGG GTA TAC CTC ACG TCT TG-3′	25 cycles
Spd synthase	**AT210:** 5′-TGC TTG TTT TGG ATG GTG TG-3′	57.5 °C
(592 bp)	**AT211:** 5′-GCA GGT CCC TCA GTA GAG CA-3′	25 cycles
Spm synthase	**AT212:** 5′-AGG AGA AGC ACA TTC CCT GA-3′	57.5 °C
(547 bp)	**AT213:** 5′-ACA TGC TCT CTG CCA TGT TG-3′	25 cycles

[a] From Testa *et al*. (2002).
[b] From Antognoni *et al*. (2002)

expressed in any of the fruit development stages (Barry *et al*., 1996). Three different pairs of degenerate primers were designed for ethylene receptor (ETR) genes according to their sequence similarity (respectively for *LeETR1* and *LeETR2*; *NR; LeETR4*, *LeETR5*, and *LeETR6*) (Tieman and Klee, 1999) (Table 1). ADC, ODC SAMDC, and Spd synthase amplification primers were designed on the basis of the following GenBank database sequence accession numbers: L16582 (ADC), AF029349 (ODC), AW154887 (EST for SAMDC), AJ006414 (Spd synth). The Spm synthase amplification primers were designed according to a putative tomato Spm synthase complete mRNA sequence (accession number AY335900), and the arginase primers were designed on the basis of the alignment between an EST sequence (putative arginase, accession number CD003536) and a database tomato mRNA complete sequence with unknown function (accession number BT013286) that showed 100% sequence identity. ACO, ACS, arginase, Spd synthase, and Spm synthase were prechecked with the BLAST programme for the specificity of both primers.

Depending on the sample, 0.3–1 μl of the first-strand reaction were used as a template for PCR amplification together with 30 pmol of each primer, 1.5 mM dNTPs, 1 U of PfuTurbo® DNA polymerase (ProSTAR™ Ultra HF RT-PCR System, Stratagene, La Jolla, CA, USA). PCR amplifications were performed with the following temperature parameters: 5 min at 95 °C; followed by 25 or 30 cycles of 45 s at 95 °C, 1 min at the annealing temperature (Table 1), 1 min at 68 °C; final extension 10 min at 68 °C. The PCR products were then separated on 1% (w/v) agarose gel in TRIS-acetate/EDTA electrophoresis buffer and the band were stained with GelStar® Nucleic acid Gel Stain (Cambrex Bio Science Rockland Inc., Rockland, Maine, USA). A 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as a molecular weight standard in each gel. Densitometry of the gels was performed by the image analysis software Gel Pro Analyser 3.1 (Media Cybernetics, Silver Spring, MD, USA). The relative amount of the band was reported as the absolute integrated absorbancy normalized to the relative actin band obtained after the same number of amplification cycles. All the PCR reaction produced a clear single band.

All PCR analyses were repeated twice with similar results.

Results

Firmness and colour change

Tomato fruit at the breaker stage (the first sign of colouration at the distal end) were treated with 20 μl l^{-1} 1-MCP for 4 h about 2 h after removal from the plant and stored at 20 °C and 70% relative humidity. Treatment of nominally mature-green fruit resulted in excessive variability because of an inability to determine the exact stage, so slightly older breaker-stage (first sign of orange colouration at the distal end) fruit were used. Five μl l^{-1} was found to produce too small an effect for definitive analyses so 20 μl l^{-1} was chosen. The firmness of three different fruit for both control (air only) and 1-MCP-treated tomatoes was assessed on alternate days for 16 d after harvest. The compression of untreated control fruit increased slowly for the first 4 d after harvest and then more rapidly to reach 1.5 mm of compression at day 16 (Fig. 2A). 1-MCP-treated fruit did not soften appreciably until day 12.

Colour change as indicated by the tomato colour index (TCI) was determined both at the equator level (Fig. 2B)

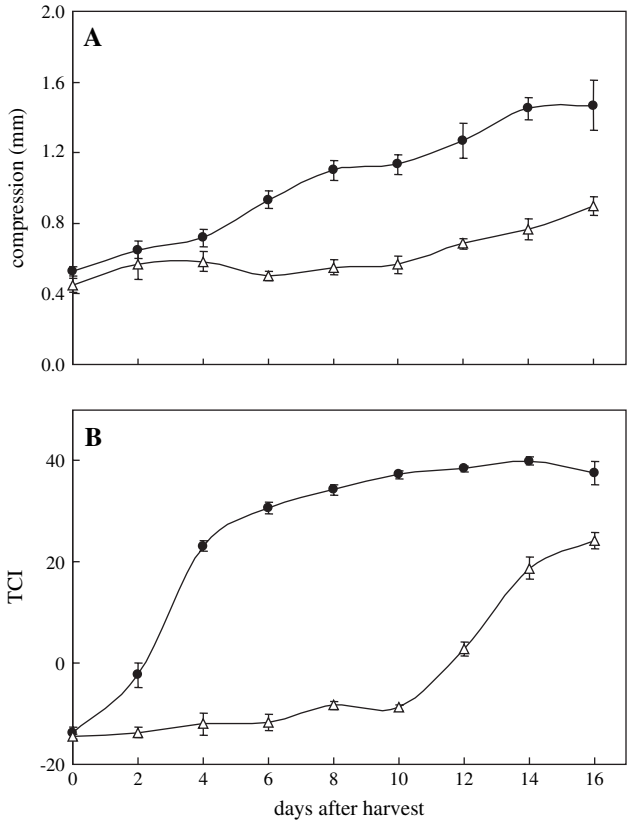

Fig. 2. Firmness (compression in mm) and colour (tomato colour index, TCI) of tomato fruit harvested at breaker stage and then untreated (filled circles) or treated with 20 µl l^{-1} 1-MCP (open triangles). Fruit were treated with 1-MCP the same day of harvesting and then kept at 20 °C and 70% relative humidity for 16 d after harvest. (A) Firmness. Increasing values indicate increased softening of the fruit; (B) colour change at the fruit equator. For firmness, each data point represents the mean ±SE of three replicates from each of three different fruits, while for colour changes each data represents the mean ±SE of three replicates from each of six different fruits.

and the blossom end (data not shown). Colour changes associated with ripening take place first at the blossom end of tomato and then spread to the whole fruit, but similar trends for colour change were determined for equator and blossom end measurements. Colour change of the control fruit was rapid over the first 4 d as they ripened. 1-MCP-treated fruit did not show any colour variation until 10–12 d after harvest and then the TCI increased at a slower rate than had occurred in the control. The colour level of 1-MCP-exposed tomatoes at day 16 was comparable with that of the control fruit at day 4.

Total protein content remained approximately constant over time in both control and 1-MCP-treated fruit (ranging around 1.1 mg protein g^{-1} FW) (data not shown).

Ethylene metabolism

Maximum ethylene production occurred in control fruit on day 4 after harvest (Fig. 3), gradually decreasing from day 6 to day 16. By contrast 1-MCP-treated fruit showed a small

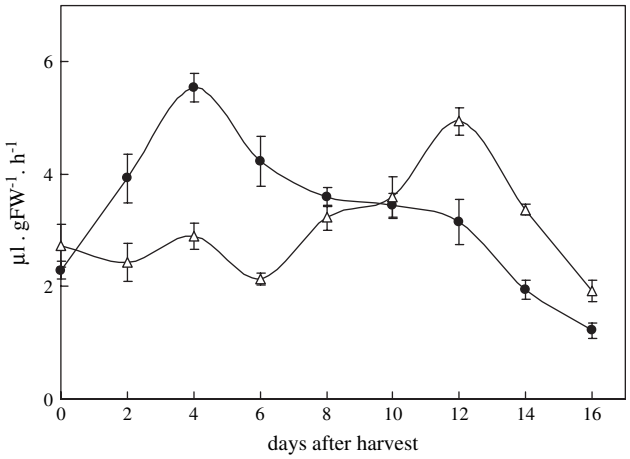

Fig. 3. Ethylene production (µl g^{-1} FW h^{-1}) during ripening of tomato fruit harvested at breaker stage and then untreated (filled circles) or treated with 20 µl l^{-1} 1-MCP (open triangles). Data represent the mean ±SE of three replicates from each of three different fruits.

peak in ethylene production at the same time as the control on day 4, followed by a decline to the original rate before increasing rapidly from about day 8 to reach a maximum on day 12, similar in magnitude to the ethylene peak in the control.

Figure 4A shows the combined level of expression of ACS isoforms *LeACS2*, *LeACS4*, and *LeACS6*. The expression levels of *ACSs* in the control and 1-MCP fruit mimic the levels of ethylene production; control fruit had the *ACS* highest expression level from day 2 to day 6, subsequently decreasing, while in treated samples *ACSs* showed a small expression peak at day 4 and a major amount of transcript at day 12 after harvest. The combined expression levels of *ACO* genes (*LeACO1*, *LeACO3*, and *LeACO4*) showed two maxima, at day 2 and day 12, in the control fruit, whereas in 1-MCP fruit a steady increase in *ACO* mRNA levels took place from day 2 to day 16 (Fig. 4B).

The mRNA levels for the ETRs were determined by using three different primer duplexes designed according to their nucleotide sequence similarities (ClustalW program, EMBL-EBI, Heidelberg, Germany). Pronounced changes (about 10-fold) in the expression of the ethylene receptors *LeETR4*, *LeETR5*, and *LeETR6* occurred following the initiation of ripening (Fig. 5B), with peaks at day 2 in control fruit and at day 12 in 1-MCP fruit. In particular, control fruit had a rapid increase of *LeETR4/5/6* expression at day 2 which remained almost constant until day 10, while in the same period 1-MCP fruit expressed these receptors only at a basal level, with an increase in their production at day 8 to a peak at day 12 (the same day in which ethylene production was maximal and *ACSs* genes were expressed at the highest level). Changes in the expression of receptor genes *LeETR1* and *LeETR2* were minor (Fig. 5A) with slight peaks of expression at a similar time to *LeETR4/5/6*.

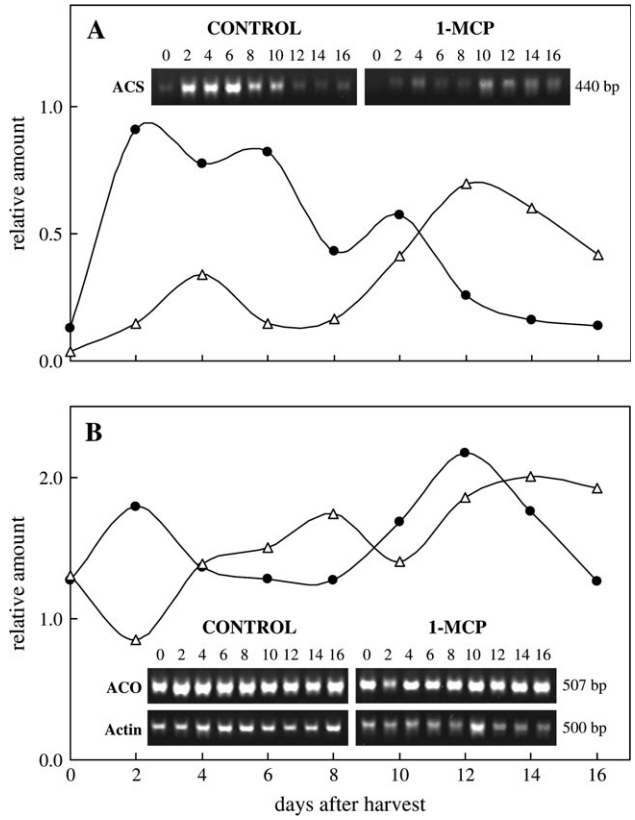

Fig. 4. RT-PCR analysis of expression levels of ACC synthases and ACC oxidases. The degenerated primers were designed in order to amplify the combined level of expression of *ACS* isoforms *LeACS2*, *LeACS4*, and *LeACS6* (A) and the combined expression level of *ACO* isoforms *LeACO1*, *LeACO3*, and *LeACO4* (B). The reported relative signal amount was normalized to the actin signal (inset) both obtained after 25 cycles of RT-PCR amplification. (filled circles) Control; (open triangles) 1-MCP-treated fruit.

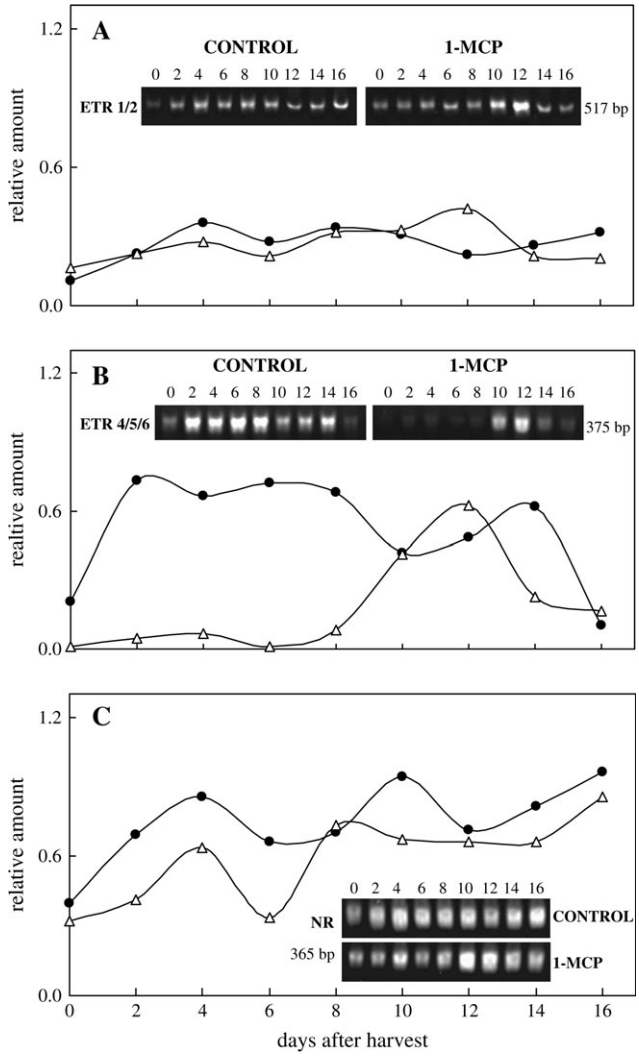

Fig. 5. RT-PCR analysis of ethylene receptor (ETR) expression levels. Three different sets of primers were designed in order to amplify separately the expression level of *LeETR1* and *LeETR2* (A), *LeETR4*, *LeETR5*, and *LeETR6* (B), and *NR* (C) isoforms of the receptor genes. The reported relative signal amount was normalized to the actin signal (data not shown) obtained after 30 cycles of RT-PCR amplification. (filled circles) Control; (open triangles) 1-MCP-treated fruit.

NR receptor expression patterns were similar in control and 1-MCP-treated fruit and did not follow those for ethylene production (Fig. 5C).

Polyamine metabolism

The content of Ptc, Spd, and Spm was determined for the free, PCA-soluble bound and PCA-insoluble bound fractions separately from three control and treated fruit. In the control fruit, the free Ptc content varied little throughout the post-harvest period (Fig. 6A), while in the 1-MCP-treated fruit a 50% increase in the free Ptc occurred from day 8 to day 14, decreasing to the same level as the control at day 16. The PCA-soluble bound Ptc showed an opposite trend with respect to the free Ptc, with an increase in control fruit from day 6 to day 12 and a decrease in 1-MCP samples during the same period. The levels of soluble-bound Ptc were equal in control and treated fruit starting from day 14 (Fig. 6B).

Expression levels of Ptc-forming enzymes were analysed by RT-PCR (Fig. 7). The ODC mRNA amount (Fig. 7A) was relatively low (analysis performed after 30 amplification cycles) and did not show any different pattern between control and 1-MCP-treated fruit with the exception of a small peak at day 4 in 1-MCP fruit not present in control samples. ADC (Fig. 7B) had a higher level of expression than ODC (analysis performed after 25 amplification cycles), with a distinct difference in timing between control and 1-MCP-treated fruit. Control fruit showed a maximum of expression at day 6 whereas 1-MCP-exposed fruit had a peak of ADC expression at day 12. The arginase mRNA (Fig. 7C) amount in control fruit did not change throughout the post-harvest period, while 1-MCP-treated fruit showed a peak of arginase expression at day 12 coincident with the expression peak for ADC.

All PA biosynthetic enzyme activities were assayed both in supernatant and pellet fractions every 4 d after harvest

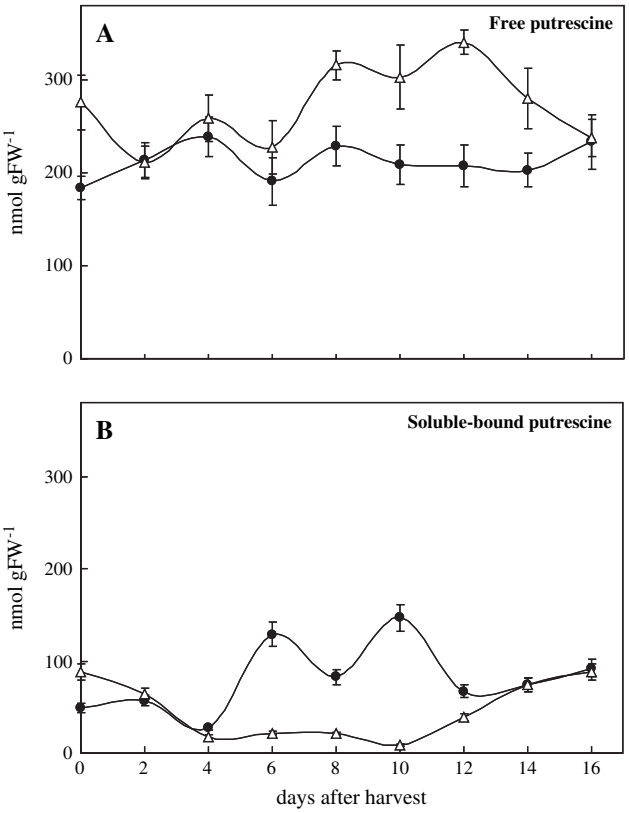

Fig. 6. Free (A) and PCA-soluble bound (B) Ptc content (nmol g^{-1} FW) from control and 1-MCP (20 µl l^{-1}) treated tomato fruit. The fruit were treated with 1-MCP the same day of harvesting and then stored at 20 °C, 70% relative humidity. (filled circles) Control; (open triangles) 1-MCP-treated fruit. Data are the mean ±SE of three replicates from each of three different fruits.

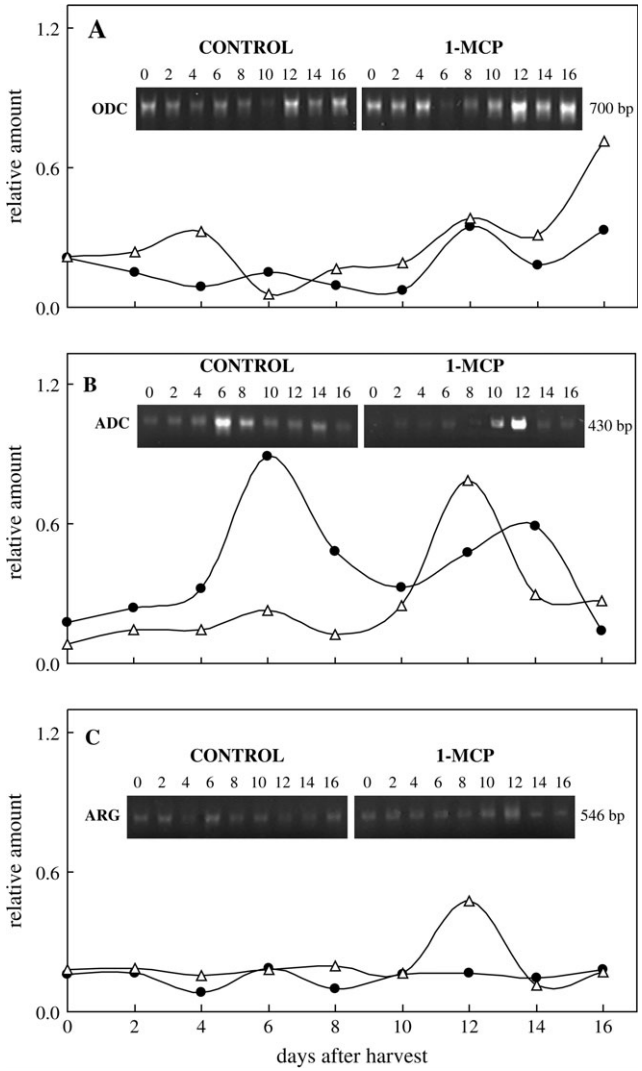

Fig. 7. RT-PCR analysis of ornithine decarboxylase (A) (ODC), arginine decarboxylase (B) (ADC), and arginase (C) (ARG) expression levels. The relative signal amount was obtained after 25 amplification cycles for ADC and ARG and after 30 amplification cycles for ODC and normalized to the actin signal obtained after the same amplification cycles. (filled circles) Control; (open triangles) 1-MCP-treated fruit.

with the addition of the samples at day 6 for control and at day 14 for 1-MCP-treated fruit. In contrast to expression level data (Fig. 7), ODC enzyme activity (Fig. 8A) was higher than that of ADC (Fig. 8B) in all analysed samples, with a higher activity amount in the pellet fraction (Fig. 8). ODC activity in the control increased during the post-harvest period, while 1-MCP samples showed a small peak of activity at day 4 and a larger one at day 12 (mainly localized in the pellet fraction). Both peaks of ODC activity in 1-MCP-treated fruit seemed to be in relation to an increase of ODC gene expression (Fig. 7A). ADC activity was low in control fruit with a small increase around day 6 to 8, while in 1-MCP fruit a generally higher ADC activity was found with peaks at day 4 and day 12 (Fig. 8B), the latter one in accordance with the ADC mRNA expression levels (Fig. 7B). Figure 9 shows the percentage of arginase activity calculated as the difference between ADC activities measured in the absence and in the presence of 10 mM unlabelled ornithine. The most relevant result seemed to be the higher percentage of arginase activity in the control samples as compared with the 1-MCP-treated samples from day 8 to day 16. An attempt to evaluate the percentage of ornithine to arginine conversion was also performed by adding 10 mM unlabelled arginine to the ODC assay, but no clear results were obtained probably due to the involvement in this process of several enzymes, among which ornithine transcarbamoylase (data not shown).

In general free Spd (Fig. 10A) and free Spm (Fig. 10C) levels were, respectively, 10-fold and 100-fold lower than free Ptc (Fig. 6A) and, similar to free Ptc, a higher amount of free Spd and free Spm was measured from day 6 to day 12 in 1-MCP-treated samples with respect to the control ones (Fig. 10A, C). PCA-soluble Spd (Fig. 10B) and Spm (Fig. 10D) were generally higher in the control than in the 1-MCP samples.

The mean insoluble-bound Ptc and Spd concentration from days 0–16 was lower in the MCP-treated fruits

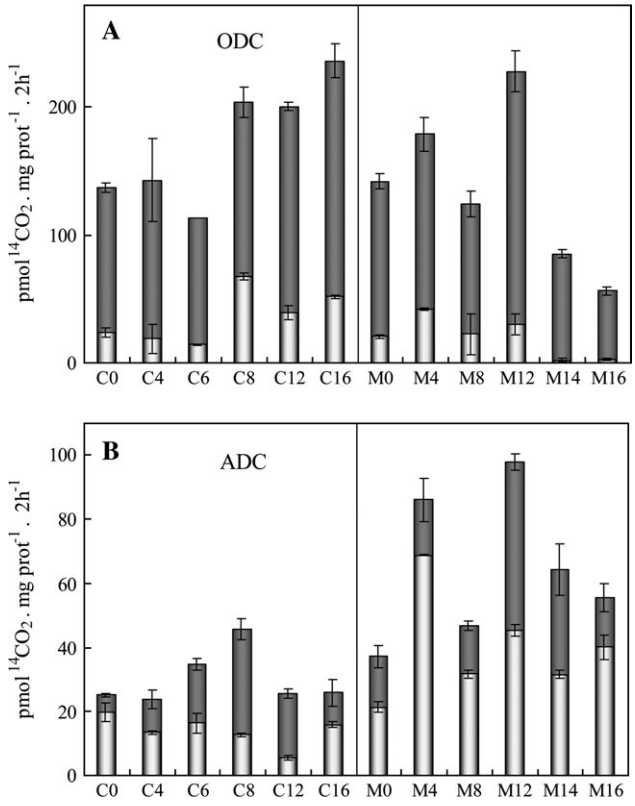

Fig. 8. ODC (A) and ADC (B) activities (pmol $^{14}CO_2$ mg^{-1} protein 2 h^{-1}) of tomato fruit treated or not treated with 20 µl l^{-1} 1-MCP. C, control fruit; M, 1-MCP exposed fruit. The numbers indicate the day after harvest. (filled squares) Supernatant fraction; (open squares) pellet fraction. Data are the mean ±SE of three replicates from each of three different fruits.

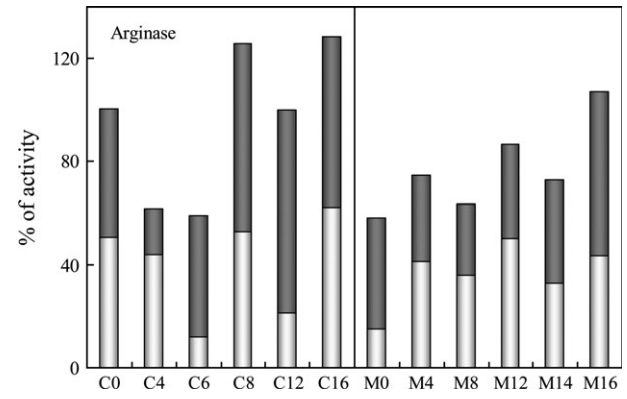

Fig. 9. Percentage of arginase activity calculated as the difference between the ADC activities measured with 0.2 µCi [^{14}C]arginine in the absence or in the presence of 10 mM unlabelled ornithine. C, control fruit; M, 1-MCP exposed fruit. The numbers indicate the day after harvest. (open squares) Supernatant fraction; (filled squares) pellet fraction. Data are the mean ±SE of three replicates from each of three different fruits.

(34.9±2.9 and 2.7±0.5 nmol g^{-1} FW, respectively) than in the control fruit (50.4±4.1 and 4.0±0.6 nmol g^{-1} FW, respectively). Insoluble-bound Spm was present only in trace amounts.

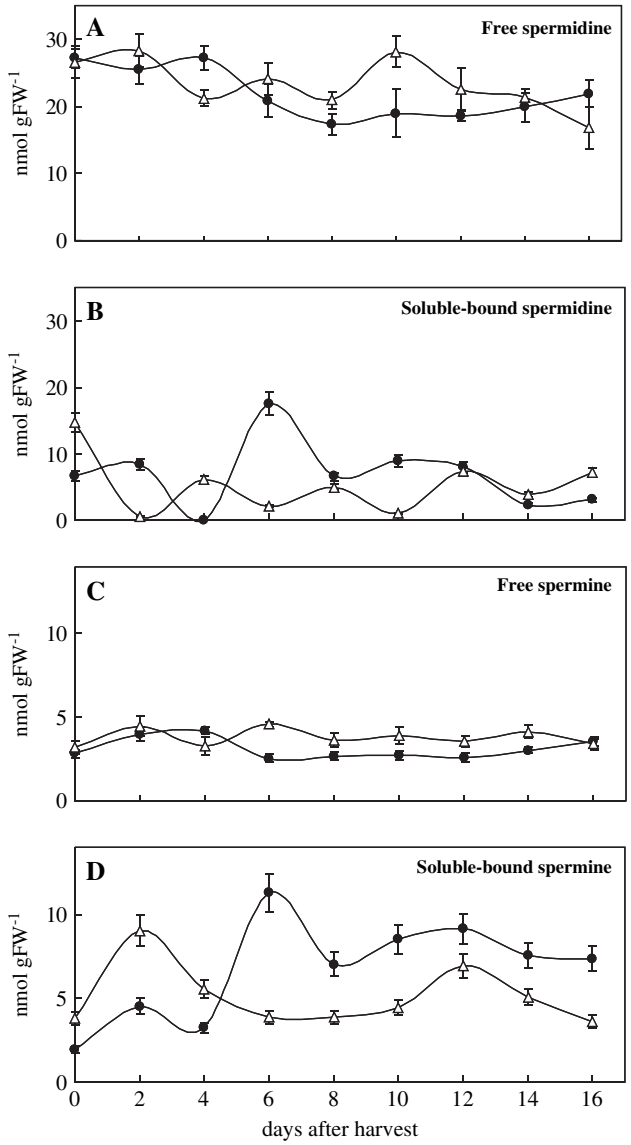

Fig. 10. Free (A), PCA-soluble bound (B) Spd and free (C) and PCA-soluble bound Spm (D) content (nmol g^{-1} FW) from tomato control and 1-MCP (20 µl l^{-1}) treated fruit. The fruit were exposed to 1-MCP on the same day as harvesting and then stored at 20 °C, 70% relative humidity. (filled circles) Control; (open triangles) 1-MCP-treated fruit. Data are the mean ±SE of three replicates from each of three different fruits.

SAMDC, Spd synthase, and Spm synthase gene expression levels (Fig. 11A, B, C, respectively) were all affected by 1-MCP treatment, showing a maximum peak of expression at day 12 in 1-MCP fruit. A lower level of maximal expression of SAMDC was, by contrast, detected at day 6 in control fruit, whereas Spd and Spm synthase enzymes showed almost no variation in the control samples. The overall amount of Spm synthase transcript was lower than that of Spd synthase (Fig. 11B, C).

SAMDC activity was measured as $^{14}CO_2$ evolution in the supernatant and pellet fraction of both control and 1-MCP treated fruit (Fig. 12A). Control fruit showed a maximum at day 4, when SAMDC activity was mainly

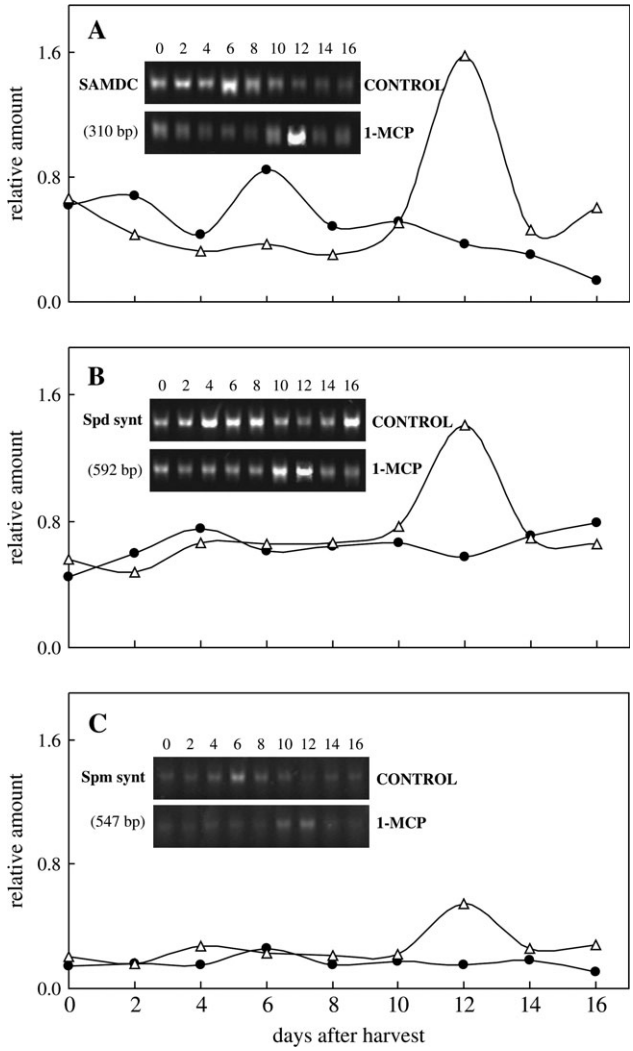

Fig. 11. RT-PCR analysis of *S*-adenosylmethionine decarboxylase (A) (SAMDC), Spd synthase (B), and Spm synthase (**C**) expression levels. The relative signal amount was obtained after 25 amplification cycles and normalized to the actin signal obtained after the same amplification cycles. (filled circles) Control; (open triangles) 1-MCP-treated fruit.

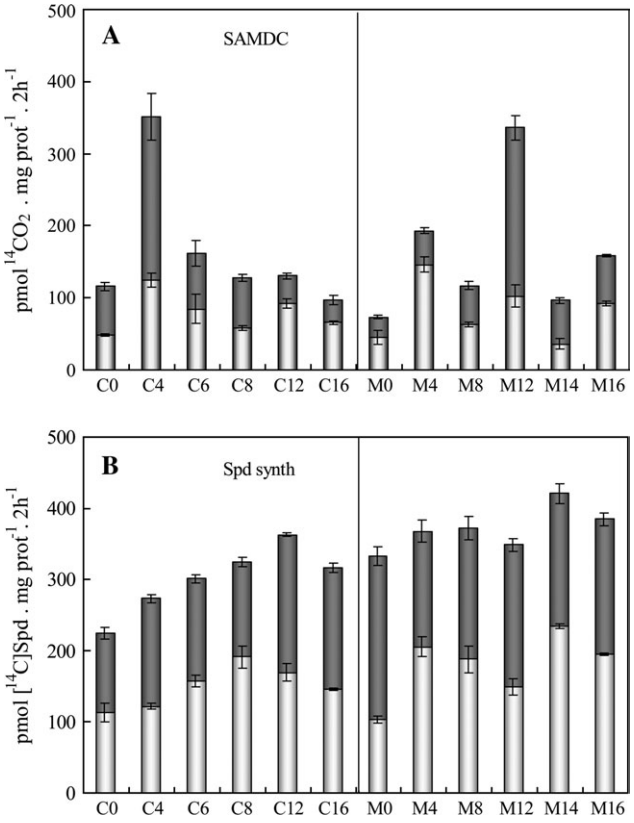

Fig. 12. SAMDC (pmol $^{14}CO_2$ mg protein^{-1} 2 h^{-1}) (**A**) and Spd synthase (pmol [^{14}C]Spd mg protein^{-1} 2 h^{-1}) (B) activities on tomato fruit treated or not treated with 20 µl l^{-1} 1–MCP. C, control fruit; M, 1-MCP exposed fruit. The numbers indicate the day after harvest. (open squares) Supernatant fraction; (filled squares) pellet fraction. Data are the mean ±SE of three replicates from each of three different fruits.

localized in the pellet fraction, while 1-MCP fruit had a small peak of activity at day 4, mainly localized in the supernatant fraction of the cell, and a maximum of activity at day 12, principally due to the pellet fraction. Spd synthase activity was measured as the amount of [^{14}C]Spd produced in 2 h after having supplied labelled Ptc to both the supernatant and the pellet fraction of both control and 1-MCP-treated tissues (Fig. 12B). The amount of [^{14}C]Spd produced seemed to increase slightly until day 12–14 of both control and 1-MCP samples and was almost equally distributed between the supernatant and pellet fractions.

Discussion

As the content of polyamines does not rise with the prevention of ripening by 1-MCP it is clear that polyamines do not play an effective role in the delay of the ripening process. However, the polyamine level did rise as ripening started. Together with the previous findings that applied polyamines can delay ripening (Law *et al*., 1991) and that a tomato type with delayed ripening has a higher polyamine content (Dibble *et al*., 1988) it is suggested that polyamines, rather than regulating ripening *per se*, may modulate the rate of ripening and over-ripening by counteracting the ripening promotion by ethylene.

In addition, it is demonstrated that the resumption of ripening, following the delay of tomato fruit ripening induced by 1-MCP, is associated with a renewed expression of the genes encoding the ethylene receptors.

Changes in ethylene production, biosynthesis, and receptor gene transcription following 1-MCP treatment

The short-term application of 1-MCP to breaker-stage tomato fruit delayed autocatalytic ethylene production and reddening by about 8 d. The peak of ethylene production in 1-MCP-treated fruit occurred at day 12, at a time when the ethylene production in untreated fruit is on its 8th day of decline. This effect is consistent with other reports for

tomato that show that 1-MCP causes a temporary delay in ripening and not a complete inhibition (Hoeberichts *et al*., 2002; Wills and Ku, 2002; Mostolfi *et al*., 2003). A delayed increase in ethylene production is associated with delays in the rise of the transcription of the genes for the enzymes of ethylene biosynthesis, especially *LeACS*, and to a lesser extent *LeACO*, as previously noted by Hoeberichts *et al*. (2002), and Nakatsuka *et al*. (1997, 1998).

These results demonstrate that the reacquisition of ripening competence coincides with the recovery of ethylene receptor gene transcription. 1-MCP is assumed to act by binding to the ethylene receptor and thereby blocking the ethylene binding sites. It is therefore interesting that this also suppresses the immediate transcription of ethylene receptor genes rather than enhancing this transcription to compensate for the lack of functional receptors. The renewed production of new ethylene receptors, to which 1-MCP would not be bound, is thus the cause of the renewed sensitivity to the freshly produced ethylene leading to the progression of ripening. While this has previously been assumed to be the reason that 1-MCP produces only a temporary delay in ripening (Feng *et al*., 2004), it has not previously been so demonstrated. The delayed rise in ethylene receptor gene transcription, however, does not consist of a rise in the transcription of all ethylene receptor genes, but only of those particularly associated with ripening, namely *LeETR4*, *5*, and *6* (Klee and Tieman, 2002), whereas no relationship was found in *LeETR1* and *2*, which are expressed in all tissues during development (Tieman and Klee, 1999). Although *NR* (*LeETR3*) (Wilkinson *et al*., 1995) expression peaked during ripening it failed to show any strong relationship with the delay produced by 1-MCP. The reason for the re-start in ethylene gene transcription cannot be determined from current data. Once LeETR4, 5, and 6 receptors are in place they probably induce the transcription of *LeACS*. *LeACS* transcription peaks at day 12 in 1-MCP-exposed fruit, compared with days 2–6 in the untreated fruit, in which *ACS* transcription is declining by day 12. A slight rise in *LeACO* transcription also occurs a little later.

Polyamine content and the transcription of polyamine biosynthetic genes following 1-MCP treatment

Polyamine level or biosynthesis did not increase during the period of delayed fruit ripening as might be expected if PAs were ripening inhibitors. On the contrary, there is a delay in the rise in the level of Ptc and Ptc biosynthesis; the level of Ptc rises during the later period of ripening whose delay is induced by the 1-MCP. Therefore, the delay in ripening is entirely ethylene associated and no involvement of PAs is suggested by these data.

The rise in Ptc in 1-MCP-treated fruit is associated with a rise in *ADC* transcription and ADC activity, but has less relationship to *ODC* transcription and ODC activity. Rastogi and Davies (1991) have previously shown that the rise in Ptc in long-keeping Alcobaca tomato is caused by a rise in the activity of ADC and not ODC. Arginase activity and the transcription of arginase also appear coincident with the peak in Ptc content, possible indications of some increased biosynthesis of Ptc via ODC if ODC is not limiting. The rise in free Ptc in 1-MCP-treated fruit is associated with a lower level of soluble bound Ptc than in the control tissues from 6–10 d after treatment. Only the free polyamines are considered to be active whereas conjugated PAs are generally thought to be inactive, but interconversions could be a homeostatic mechanism for regulating active PA levels. In contrast to the treated fruit, the clear peak in ADC transcription at days 6–8 in control tissue is coincident with the elevated level of bound rather than free Ptc, although this rise is less obvious in ADC activity at that time. It is therefore possible that more of the newly synthesized Ptc remains in the free form in the treated fruit instead of being conjugated, as appears to occur in the control tissues.

Following 1-MCP treatment, a rise in SAMDC activity and gene transcription, and Spd synthase and Spm synthase gene transcription but not Spd synthase activity, all occurred coincident with the peak in ethylene production on day 12; no such rise was seen in control fruit either coincident with the earlier rise in ethylene in these fruit or when the rise occurred in 1-MCP fruit. However, no clearly related rise took place in Spd (which is present at one-tenth the level of Ptc) or Spm (which is present at about one-hundredth the level of Ptc). The peak in these enzymes therefore appears to be associated in the delay in ripening by 1-MCP, but shows no relation to the level of the content of their products so any significance remains obscure.

Proposed role of polyamines in fruit ripening

It is clear that PAs are not directly associated with a delay in tomato fruit ripening, but rather with a prolonging of the fully-ripe stage before the fruit tissues undergo senescence. For example, in the long-keeping land-race cv. Alcobaca there is no elevation of the PA content at the start of ripening, whereas the level of Ptc starts to rise as ripening progresses, to show a small but significant increase in Alcobaca by the mature-green stage and a doubling by the ripe stage (Dibble *et al*., 1988). Perhaps PAs, specifically Ptc, can be seen as moderators of the ripening and over-ripening processes. In so doing they would prevent a 'runaway progress' of overall senescence, confining the ethylene effects to the biosynthesis of colour, aroma, and flavour compounds, and to some specific moderation of the cell walls, rather than an immediate overall induction of senescence processes that start to occur during the over-ripening phase.

Ripening and over-ripening are induced by the biosynthesis of ethylene with the ethylene detected by the ethylene receptors. Following the initiation of ripening by

ethylene the level of Ptc rises to dampen the processes of tissue senescence. However, this rise in Ptc occurs only in long keeping (e.g. Alcobaca) and 1-MCP-treated fruit, but not following the start of ripening in normal untreated fruit. This could be associated with the slower ripening and delay of over-ripening in Alcobaca and 1-MCP-treated fruit. Alternatively, the rise in Ptc could be a delayed reaction to the inhibition of ripening, here blocked by 1-MCP. However, if this is the case, there could be a delay of the effect of about 10 d, which would be substantial.

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