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Suppression of Ripening-Associated Gene Expression in Tomato Fruits Subjected to a High CO_2 Concentration

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High concentrations of CO_2 block or delay the ripening of fruits. In this study we investigated the effects of high CO_2 on ripening and on the expression of stress- and ripening-inducible genes in cherry tomato (*Lycopersicon esculentum* Mill.) fruit. Mature-green tomato fruits were submitted to a high CO_2 concentration (20%) for 3 d and then transferred to air. These conditions effectively inhibited ripening-associated color changes and ethylene production, and reduced the protein content. No clear-cut effect was observed on the expression of two proteolysis-related genes, encoding polyubiquitin and ubiquitin-conjugating enzyme E2, respectively. Exposure of fruit to high CO_2 also resulted in the strong induction of two genes encoding stress-related proteins: a ripening-regulated heat-shock protein and glutamate decarboxylase. Induction of these two genes indicated that high CO_2 had a stress effect, most likely through cytosolic acidification. In addition, high CO_2 blocked the accumulation of mRNAs for genes involved in the main ripening-related changes: ethylene synthesis (1-aminocyclopropane-1-carboxylic acid synthase and 1-aminocyclopropane-1-carboxylic acid oxidase), color (phytoene synthase), firmness (polygalacturonase), and sugar accumulation (acid invertase). The expression of ripening-specific genes was affected by CO_2 regardless of whether their induction was ethylene- or development-dependent. It is proposed that the inhibition of tomato fruit ripening by high CO_2 is due, in part, to the suppression of the expression of ripening-associated genes, which is probably related to the stress effect exerted by high CO_2.

The ripening of climacteric fruits is delayed or inhibited by a variety of compounds or treatments including inhibitors of ethylene action, such as Ag^+ (Davies et al., 1988), high temperature stress (Picton and Grierson, 1988), the end product of fermentation, ethanol (Saltveit, 1989), and cool temperatures. The use of low O_2 and/or high CO_2 atmospheres can also effectively delay ripening (Brecht, 1980; Kader et al., 1989).

Depending on the species, the physiological state, and the conditions of storage, most fruits tolerate continuous exposure to moderately high CO_2 concentrations, ranging from 1 to 5%. At these levels, CO_2 probably retards the ripening of climacteric fruits through its effect on ethylene (Sisler and Wood, 1988), which plays a central role in the control of climacteric fruit ripening. CO_2 reduces ethylene synthesis (Sisler and Wood, 1988; Rothan and Nicolas, 1994) and counteracts its action (Burg and Burg, 1967; Mathooko, 1996). Most fruits develop severe physiological disorders during long-term storage under higher CO_2 concentrations (Kader et al., 1989). However, various fruits can tolerate short exposures or repeated intermittent exposures to CO_2 concentrations higher than 10% (Wang and Mellenthin, 1975; Marcellin and Chaves, 1982; Nicolas et al., 1989). Kiwifruit has a tolerance limit of 5% CO_2 (Kader et al., 1989) and yet may be stored for up to 8 months under intermittent 30% CO_2 treatments without loss of quality (Nicolas et al., 1989). These high CO_2 concentrations (10–30%) also affect fruit metabolism in addition to their well-known effect on ethylene. The CO_2-mediated reduction in the activities of glycolysis and TCA cycle enzymes (Brecht, 1980; Monning, 1983; Kerbel et al., 1988), or the modifications of cytosolic pH (Bown, 1985; Siriphanich and Kader, 1986) might further contribute to the inhibition of ripening.

Tomato (*Lycopersicon esculentum*) fruit is the model of choice for studying the ripening of climacteric fruits. Investigations at both the biochemical and molecular levels have identified ripening-associated mRNAs that encode proteins involved in the ripening process (Gray et al., 1992). These include mRNAs encoding enzymes involved in ethylene biosynthesis such as ACS (Van der Straeten et al., 1990; Rottmann et al., 1991) and ACO (Hamilton et al., 1990), in texture changes such as PG (Grierson et al., 1986), in carotenoid biosynthesis such as PSY (Bird et al., 1991), and in sugar accumulation such as AI (Elliott et al., 1993). In recent years the different genetic strategies used to impair ethylene production have produced slow- or non-ripening transgenic tomatoes (Hamilton et al., 1990; Klee et al., 1991; Oeller et al., 1991) and have ultimately shown that both ethylene-dependent and -independent pathways are involved in the triggering of ripening (Theologis, 1992).

As a step toward understanding the mechanism of action of very high CO_2 concentrations, we have investigated the effect of 20% CO_2 on the expression of ripening-associated genes in ripening MG tomato fruit. We present evidence that in preclimacteric tomato fruit high CO_2 induces the expression of certain stress-related genes and blocks the expression of both ethylene-dependent and -independent ripening-associated genes. These results indicate that CO_2

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Abbreviations: ACO, ACC oxidase; ACS, ACC synthase; AI, acid invertase; GAD, glutamate decarboxylase; HSP, heat-shock protein; MG, mature-green; PG, polygalacturonase; PSY, phytoene synthase; UBC, ubiquitin-conjugating enzyme; UBI, polyubiquitin.

delays fruit ripening at least in part through down-regulation of genes involved in ripening-related changes and that this delaying effect is probably related to the acidification effect exerted by high CO_2.

MATERIALS AND METHODS

Plant Material and Treatments

Cherry tomato (*Lycopersicon esculentum* Mill., cv Evita) plants were grown in a greenhouse under standard conditions. Advanced MG tomato fruit about 22 mm in diameter and weighing 6.3 g were harvested before the onset of ethylene synthesis and of ripening, when the distal end of the fruit lightened due to chlorophyll loss. Randomly constituted samples of at least five tomato fruit were enclosed at 20°C within 2 h after harvest in 1-L sealed glass jars. A flow of either humidified air or a mixture of 20% CO_2/20% O_2/60% N_2 was maintained at 3 L h^{-1}. Fruits kept in air were used as control. After 3 d CO_2-treated fruits were transferred to air for 4 additional d. All experiments were repeated. Results presented are from a typical experiment.

Color and Ethylene Measurements

Color of fruits was measured with a chromameter (MicroColor, Lange, Düsseldorf, Germany). Color was expressed in the L*a*b color system where "a" chromaticity coordinate expresses color changes from green to red. Values represent the average of six measurements around the equatorial diameter of the fruit. Ethylene production rates were determined immediately after harvest on fruits individually enclosed for 30 min in 30-mL glass tubes sealed with a rubber septum. A 0.5-mL gas sample was withdrawn with a syringe and analyzed on a gas chromatograph equipped with an activated alumina column and a flame ionization detector.

DNA Probes

The ACS, ACO, and GAD cDNA clones were obtained by reverse transcription-PCR using 5 μg of DNase-treated total RNA extracted from turning (ACS and ACO) or immature-green (GAD) tomato fruits. For ACS, the following amplimers based on the published sequence of tomato *ACS2* (Rottmann et al., 1991) were used: ACS2–3′, 5′-CTCAACACCTACGAACCTCCG-3′, was used for the first-strand cDNA synthesis by reverse transcription and ACS2–5′, 5′-AAAATGGGATTTGAGATTGC-3′, was used in conjunction with the former primer for subsequent PCR amplification. For ACO, the following amplimers based on the published sequence of tomato *ACO1* (Köck et al., 1991) were similarly used: ACO-3′, 5′-CTGCATCACTTCCTGGATTG-3′, and ACO-5′, 5′-TGGAGATGAGAGAGCCAACAC-3′. For GAD, the following amplimers based on the published sequence of tomato ERT D1 (Gallego et al., 1995) were used: GAD-3′, 5′-CCGCTGGTCTTCTTCCCTGC-3′, and GAD-5′, ATGGTGTTAACAACGACGTC-3′. Following amplification, the PCR fragments were purified, blunt-ended, and subcloned into the *Sma*I site of pUC18; a partial sequencing of the PCR fragments was performed to confirm their identity. The ripening-related tomato cDNA clones of the pTOM series (pTOM 6, pTOM 36, and pTOM 66 encoding, respectively, PG, a protein of unknown function, and a small HSP; Slater et al., 1985) were a gift from Don Grierson (Nottingham University, Loughborough, UK). The pepper cDNA clone for PSY (Römer et al., 1993) was a gift from Bilal Camara (IBMP, Strasbourg, France). The tobacco cDNA clone 6pU11–2 (UBI) (Genschik et al., 1992) for UBI was a gift from Jacqueline Fleck (IBMP, Strasbourg, France). The carrot cDNA clone for soluble invertase isoenzyme II (AI) (Unger et al., 1994) was a gift from Arnd Sturm (Friedrich Miescher-Institut, Basel, Switzerland). The Arabidopsis cDNA clone (YAP 161) for UBC E2 was a gift from Michel Delseny (unpublished results). A rice ribosomal rDNA clone (pRR217) was used as a control for equal loading of RNA sample in northern-blot analysis.

Nucleic Acid Isolation

Following ethylene and color measurements, the pericarp of the tomato fruits was immediately cut with a sterile razor blade, frozen, and ground to a fine powder in liquid nitrogen. The extraction of total RNA was performed according to the method of Verwoerd et al. (1989) with modifications. Hot phenol (80°C) extraction buffer (0.1 M LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS [1:1]) was added to the powdered tissue at a ratio of 4 mL buffer g^{-1} tissue. The mixture was homogenized during 45 s with a Polytron (Kinematica, Luzern, Switzerland) at a medium setting and centrifuged at 30,000*g* for 10 min. The aqueous phase was then removed and the polysaccharides were precipitated during 30 min on ice with the addition of 1/20 volume of ethanol and 1/10 volume of 5.0 M potassium acetate (pH 5.2). The mixture was further extracted with 5 mL of chloroform:isoamylic alcohol (24:1, v/v) and centrifuged at 30,000*g* for 10 min. RNA was precipitated overnight at 4°C with 2.5 M LiCl. The RNA pellet was recovered by centrifugation at 10,000*g* for 20 min, dissolved in 300 μL of diethyl pyrocarbonate-treated water, and precipitated again by the addition of 1/10 volume of 3.0 M sodium acetate (pH 5.2) and 3.0 volumes of ethanol for 1 h at −80°C. The resulting RNA pellet was washed with ice-cold 70% ethanol and finally dissolved in 50 μL of diethyl pyrocarbonate-treated water.

RNA-Blot Analysis

Total RNA (5–10 μg per sample according to the probe used) was separated by electrophoresis on a 1.2% agarose gel containing 6.6% formaldehyde, blotted onto a nylon membrane (Hybond-N; Amersham), and fixed by UV irradiation. Hybridization was carried out at 65°C for 16 h as described by Sambrook et al. (1989), with probes labeled by random priming (Gibco BRL) using α-[^{32}P]dCTP. Following hybridization, the membrane was washed twice for 20 min in 2 × SSC, 0.1% SDS at 65°C and then once for 20 min in 0.1 × SSC, 0.1% SDS at 65°C. Following exposure of the membrane (Sambrook et al., 1989), spots on the membrane

corresponding to positive signals on the autoradiogram were excised and quantified by liquid-scintillation analysis.

Protein Extraction

Frozen powdered pericarp tissue (0.2 g) was thawed in 1 mL of extraction buffer containing 100 mM Tris-HCl buffer (pH 8.0), 0.2 M NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 10% (v/v) glycerol, and 0.5% (v/v) Triton X-100. After centrifugation (10,000*g*, 15 min), the total amount of proteins in the supernatant was determined by the method of Bradford (1976) using BSA as a standard (Bradford microassay; Bio-Rad).

RESULTS

Delayed Ripening and Ethylene Production of CO_2-Treated Fruit

No color development occurred while fruits were kept under 20% CO_2, indicating an inhibition of ripening (Fig. 1). Ripening of the CO_2-treated fruit eventually resumed 2 d after transfer to air. However, when compared with the air-ripened fruit (control), which showed a typical pattern of ripening, the CO_2-treated fruit remained less colored after 4 additional d in air.

Within 1 h after picking, the fruit exhibited a high ethylene production rate (Fig. 2), which may be attributed to the stress of harvest. Both control and CO_2-treated MG fruit reached a basal preclimacteric ethylene level within 1 d. Control fruit demonstrated a typical ethylene climacteric rise during ripening. In contrast, the synthesis of ethylene in CO_2-treated fruit was completely prevented during the 3 d of storage under CO_2 as previously shown (Marcellin and Chaves, 1982). When CO_2-treated fruits were transferred to air, ethylene synthesis recovered after a lag period

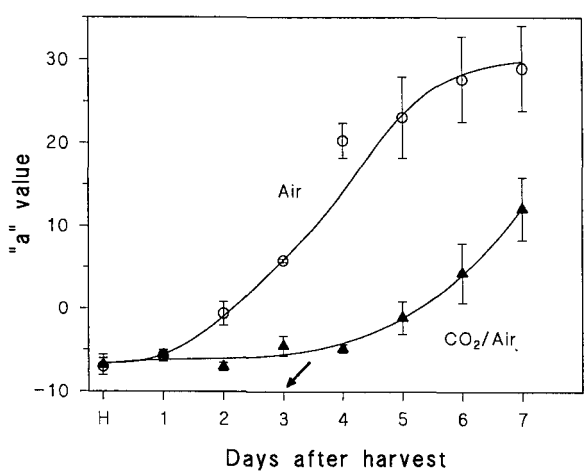

Figure 1. Color changes in the pericarp from air-ripened and CO_2-treated tomato fruit. Fruits harvested at the MG stage were ripened at 20°C in air during 7 d (O) or submitted to 20% CO_2 during 3 d and then transferred to air for 4 additional d (▲) as indicated in "Materials and Methods." H, Harvest. Arrow indicates the time of transfer of the fruit from CO_2 to air. Data are the mean (± SD) of at least five fruits.

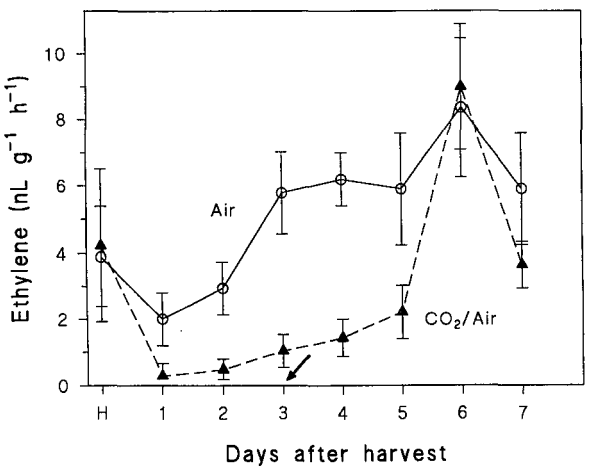

Figure 2. Ethylene evolution from air-ripened and CO_2-treated tomato fruit. Fruits harvested at the MG stage were ripened at 20°C in air during 7 d (O) or submitted to 20% CO_2 during 3 d and then transferred to air for 4 additional d (▲) as indicated in "Materials and Methods." H, Harvest. Arrow indicates the time of transfer of the fruit from CO_2 to air. Data are the mean (± SD) of at least five fruits.

of 2 d concomitant with the onset of ripening (Figs. 1 and 2). Thus, high CO_2 had a profound effect on the ripening of MG fruit because the CO_2 treatment significantly delayed both the changes in color and ethylene production that normally occur during tomato fruit ripening.

Expression of Stress-Related Genes during and following CO_2 Treatment

Under the conditions used in this experiment (20% CO_2 at 20°C for 3 d), high CO_2 may stress tomato fruit (Kader et al., 1989), thus slowing all aspects of tomato fruit ripening. Therefore, we investigated the potential stress effect of high CO_2 on tomato fruit by examining the changes in protein content and the accumulation of mRNAs in which the expression is related to stress. The amount of extractable proteins in the pericarp from MG tomato dropped by one-third while fruits were kept 2 d under 20% CO_2 (Fig. 3). These results are in agreement with the previous obser-

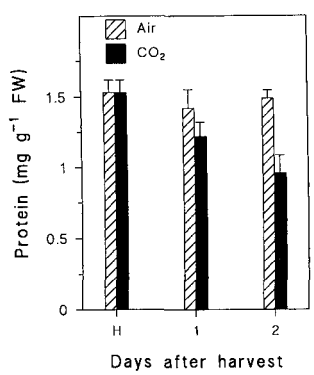

Figure 3. Changes in the amount of extractable proteins in MG tomato fruit ripened during 2 d at 20°C, in air or under 20% CO_2. Proteins were extracted from pooled pericarp tissue obtained from five fruits. H, Harvest. Data are the mean (± SD) of three replicates.

vations of Kerbel et al. (1988) using pear fruit. Transcripts for UBI and the UBC E2, involved in the selective proteolysis of stress-damaged proteins (Vierstra, 1993), were taken as putative stress indicators. We also followed the expression of mRNAs encoding the low-molecular-weight HSP, encoded by pTOM 66, which is also developmentally regulated in tomato fruit (Picton and Grierson, 1988).

The major UBI transcript of tomato, which increased during ripening in control fruit, was slightly delayed in CO_2-treated fruit (Fig. 4). The levels of UBI transcript in CO_2-treated fruit did not differ from the levels of UBI transcript in control fruit. UBC mRNAs seemed to be constitutively expressed in control fruit. The level of UBC transcript was slightly reduced while fruits were kept under 20% CO_2 but remained otherwise unaffected (Fig. 4). In contrast, transcript encoding the low-molecular-weight HSP were induced in CO_2-treated tomato fruit within 1 d. HSP transcript reached a high level at the end of CO_2 treatment and remained at a level comparable to the one reached in control fruit after subsequent transfer to air.

The stress resulting from the 20% CO_2 treatment, suggested by the strong accumulation of HSP transcript (Fig. 4), may possibly reflect the acidification of the cytosol (Bown, 1985; Kurkdjian and Guern, 1989). CO_2-treated fruit show some indirect indications of acid stress, such as the accumulation of γ-aminobutyrate, in which the formation is catalyzed by GAD (Caroll et al., 1994; Kader et al., 1989). To determine whether the CO_2 treatment would affect the synthesis of this enzyme, we examined the accumulation of GAD transcript in air- and CO_2-treated tomato fruit. A putative GAD, highly expressed during ripening of the slow-ripening *rin* tomato mutant but mostly expressed at early breaker stage in wild-type fruit, has recently been characterized (Gallego et al., 1995). We similarly found that the level of mRNA homologous to the tomato GAD ERT D1 (Gallego et al., 1995) peaked after the MG stage in control fruit (Fig. 5A). In CO_2-treated fruit the GAD transcript accumulated during the 3 d of exposure to CO_2. Quantification of transcript accumulation indicated that the level of GAD mRNA was twice as high in CO_2-treated fruit, as compared with control fruit (Fig. 5B). After transfer of CO_2-treated fruit to air, the level of GAD mRNA declined and eventually reached the normal air level at d 5 after harvest (Fig. 5B). Thus, the CO_2 treatment apparently exerts a stress effect on tomato fruit, as indicated by the strong expression of the two stress-related genes.

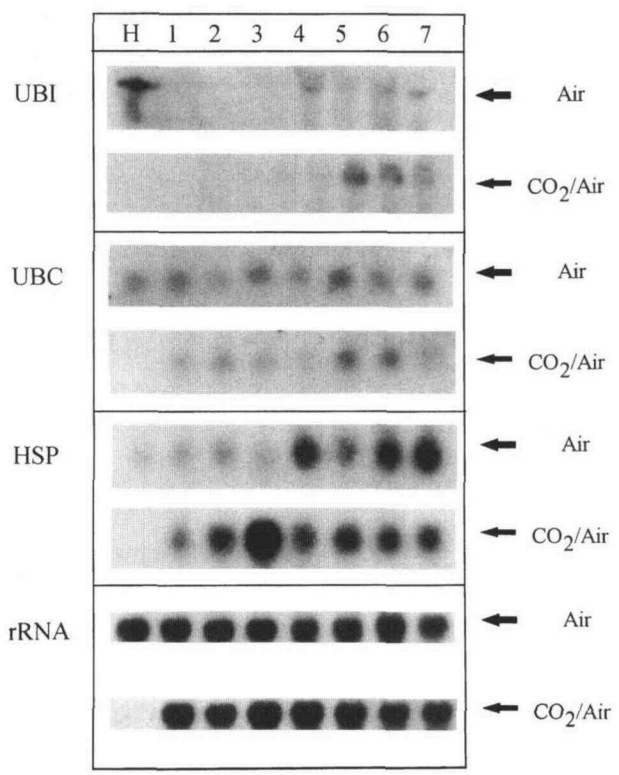

Figure 4. Expression of stress-related genes in air-ripened and in CO_2-treated MG tomato fruit. Fruits harvested at the MG stage were ripened at 20°C in air during 7 d (Air) or submitted to 20% CO_2 during 3 d and then transferred to air for 4 additional d (CO_2/Air) as indicated in "Materials and Methods." Lane H, Harvest; lanes 1 through 7 indicate the number of days after harvest. Total RNA was isolated from pooled pericarp tissue obtained from five fruits. A northern blot (8 μg for each sample) was probed for UBI, UBC E2, and TOM 66 (HSP) mRNAs. A ribosomal probe was used as a control of relative loadings of RNA in each lane (25S rRNA is shown).

Expression of Genes Involved in Ethylene Biosynthesis during and following High CO_2 Treatment

We have shown that high CO_2 markedly down-regulated ethylene production in MG tomato fruit. Thus, the steady-state accumulation of transcripts encoding ACS and ACO, the two key enzymes involved in ethylene synthesis (Yang and Hoffman, 1984), was examined in MG tomato fruit exposed to 20% CO_2. The levels of mRNAs homologous to *ACS2* (Rottmann et al., 1991) and *ACO1* (Köck et al., 1991) were strongly increased during ripening of MG tomato fruits in air at 20°C (Fig. 6A), as previously reported (Slater et al., 1985; Rottmann et al., 1991). By contrast, under high CO_2 the induction of both ACS and ACO mRNAs was notably delayed until 5 d after harvest, although ACO mRNA was present at a low constitutive level. Quantification of transcript accumulation indicated that at d 5 after harvest ACS and ACO mRNA levels in the CO_2-treated MG fruit were reduced to approximately 15 and 25%, respectively, of mRNA levels in control fruit (Fig. 6, B and C). Furthermore, ACS and ACO mRNAs accumulated from d 5 at a much lower rate in CO_2-treated fruit than in control fruit (Fig. 6, A–C). Thus, the time course for the accumulation of mRNAs encoding enzymes for ethylene biosynthesis was similar to the evolution of ethylene (Fig. 2). These results indicate that high CO_2 delayed, at least in part, the climacteric rise in ethylene production by affecting the transcriptional activation of ACS and ACO genes.

Prevention by High CO_2 of the Expression of Ripening-Related Genes

In addition to its effect on ethylene formation, CO_2 is known to prevent or to reduce the extent of changes in firmness, color, and flavor that are associated with tomato

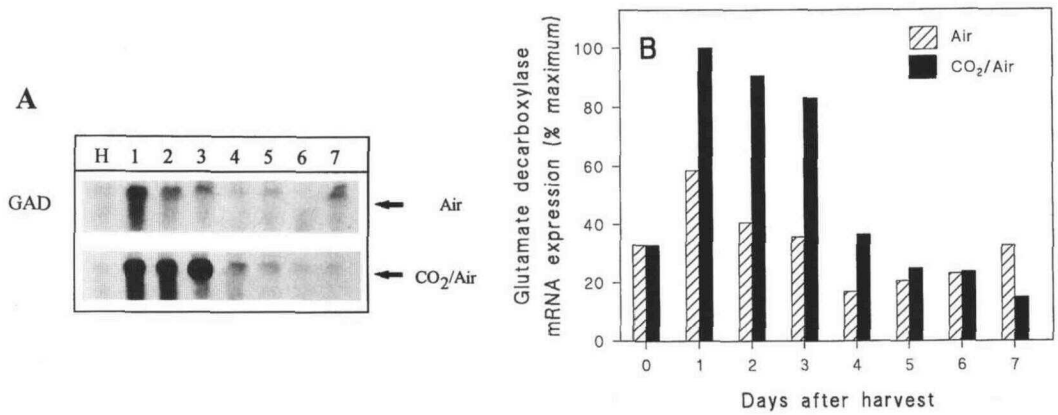

Figure 5. GAD mRNA expression in air-ripened and in CO_2-treated MG tomato fruit. Conditions are as for Figure 4. A, Northern-blot analysis of RNA samples (8 μg) probed for GAD mRNA. Lane H, Harvest; lanes 1 through 7 indicate the number of days after harvest. B, Quantification of GAD transcript level.

ripening. Recently, it has been shown that the expression of genes involved in ripening-associated changes are differentially regulated in response to ethylene and development (Davies et al., 1988; Oeller et al., 1991; Picton et al., 1993; Yen et al., 1995). Through its stress effect on tomato fruit, the high CO_2 concentration used in our experiment may also have blocked the expression of most ripening-related genes, as already observed for heat shock (this report; Picton and Grierson, 1988). Through the inhibition of ethylene synthesis or action, high CO_2 would only suppress the expression of ethylene-inducible genes (Oeller et al., 1991; Picton et al., 1993; Yen et al., 1995). Therefore, we further investigated the effect of high CO_2 on the expression of four ethylene-dependent or -independent ripening-related genes, encoding PG, PSY, AI, and a protein of unknown function (encoded by pTOM 36; Slater et al., 1985), respectively. In control fruit the accumulation of these transcripts during fruit ripening (Fig. 7) typically occurred after the onset of ethylene production (Fig. 2). In contrast, in CO_2-treated fruit the accumulation of all of the

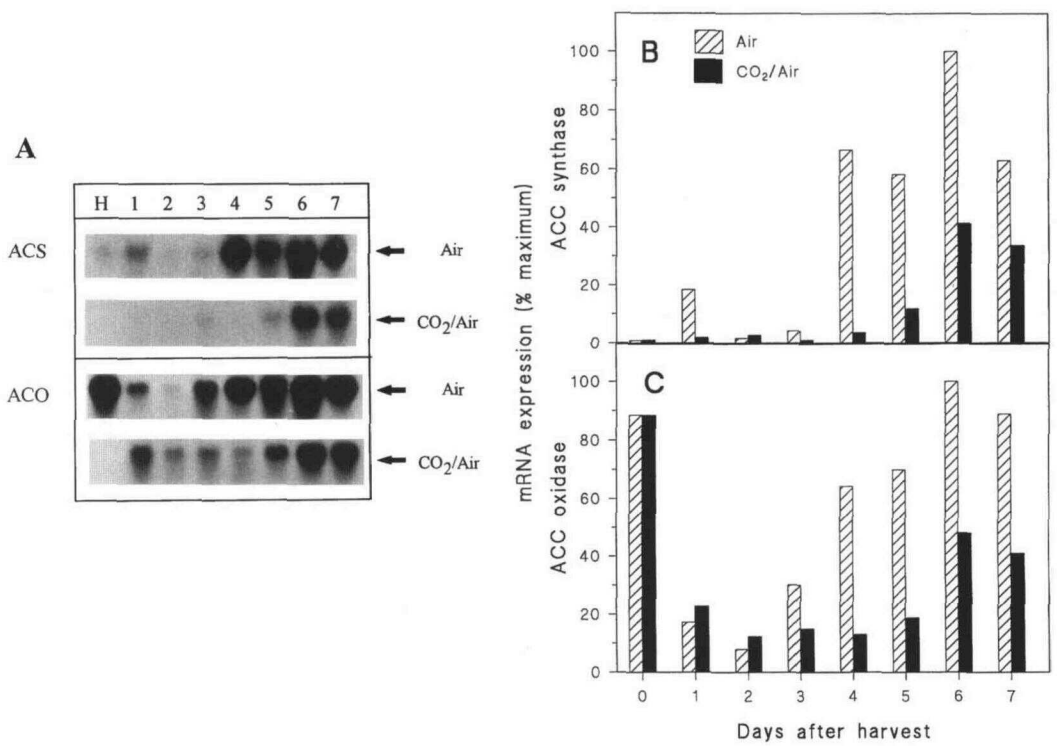

Figure 6. ACS and ACO mRNA expression in air-ripened and in CO_2-treated MG tomato fruit. Conditions are as for Figure 4. A, Northern-blot analysis of RNA samples (5 μg) probed for ACS and ACO mRNAs. Lane H, Harvest; lanes 1 through 7 indicate the number of days after harvest. B, Quantification of ACS transcript level. C, Quantification of ACO transcript level.

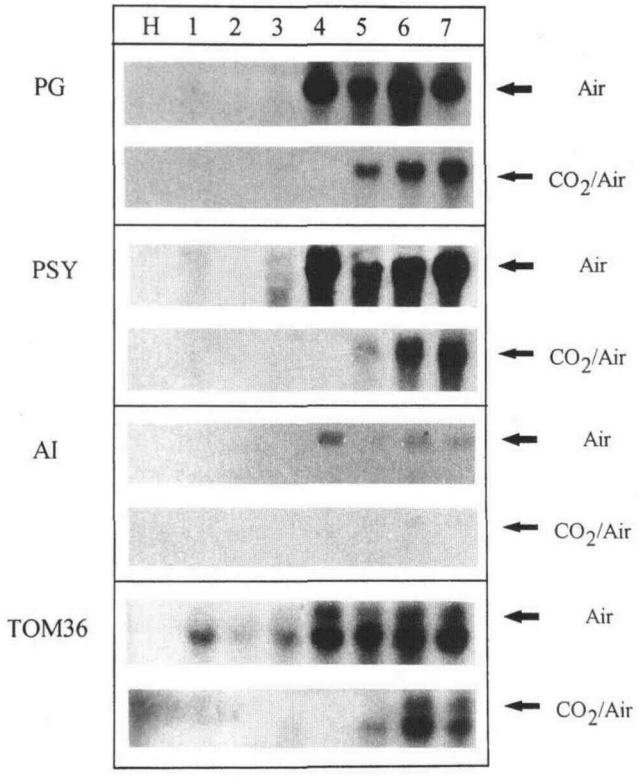

Figure 7. Expression of ripening-related genes in air-ripened and in CO_2-treated MG tomato fruit. Conditions are as for Figure 4. A northern blot (8 μg for each sample) was probed for PG, PSY, AI, and TOM 36 mRNAs. Lane H, Harvest; lanes 1 through 7 indicate the number of days after harvest.

transcripts studied was severely delayed by at least 1 or 2 d. Furthermore, as already observed for genes involved in ethylene biosynthesis, the normal expression level of the ripening-specific genes was not fully restored upon transfer of fruit from CO_2 to air, at least during the time course of the experiment.

DISCUSSION

Prolonged exposure to a high CO_2 concentration has a striking effect on fruit development, whereby the main ripening-associated changes, color, firmness, and flavor, are delayed (Fig. 1; Kader et al., 1989). The inhibition of ripening is correlated with a delay or reduction in the formation of ethylene (Fig. 2), a hormone crucial in coordinating the ripening process in tomato (Klee et al., 1991; Oeller et al., 1991; Picton et al., 1993). We also showed that a 20% CO_2 treatment may concomittantly prevent the increase during ripening of ACS and ACO mRNA (Fig. 6), which are responsible for ethylene synthesis. CO_2 is also known to competitively inhibit the binding of ethylene to its receptor(s) (Burg and Burg, 1967). Thus, in our experiment, high CO_2 may have blocked tomato fruit ripening through its effect on ethylene synthesis and/or perception. To gain further insight into the mode of action of CO_2, we attempted to determine whether high CO_2 may additionally stress the fruit through a decrease in cytosolic pH, and hence contribute to the inhibition of ripening.

Stress Effect of High CO_2 on Tomato Fruit

High CO_2 has a noticeable effect on the level of extractable proteins (Fig. 3), a finding previously noted for several fruits subjected to high CO_2 treatment (Wang and Mellenthin, 1975; Kerbel et al., 1988). By contrast, high CO_2 does not affect the protein profile of in vitro-translated mRNAs (data not shown), unlike heat shock (Picton and Grierson, 1988). The reduction in extractable proteins may result from the degradation of stress-damaged proteins. Selective proteolysis of damaged proteins is mainly controlled through the ubiquitin-mediated process which involves ubiquitin and several UBCs (Vierstra, 1993). Expression of UBI is strongly increased in heat-shocked tomato fruit (Schaffer and Fischer, 1990). However, high CO_2 apparently only slightly affects the accumulation of tomato fruit UBI mRNA and of tomato E2 mRNA hybridizing with the Arabidopsis UBC E2 studied (Fig. 4). Other ubiquitin-independent proteolytic processes involving specific proteases may degrade the putative stress-damaged proteins, as shown in low temperature-stressed tomato fruit (Schaffer and Fischer, 1990). Furthermore, the strong accumulation of a mRNA (TOM66) encoding a low-molecular-weight HSP by high CO_2 (Fig. 4) suggests that CO_2 exerts a specific stress effect on proteins. In soybean seedlings the class I low-molecular-weight HSPs, which include TOM66 protein (Gray et al., 1992), are involved in the protection of soluble proteins from stress damage during heat shock (Jinn et al., 1995). In the present case, expression of TOM66 may be required to protect proteins against cellular acidosis.

The solubilization of CO_2 in the tissues induces cellular acidosis (Bown, 1985; Kurkdjian and Guern, 1989). A 20% CO_2 concentration may theoretically decrease the cytosolic pH from 7.5 to as low as 6.4 due to the formation of H^+ and HCO_3^- (Heber et al., 1994). However, evidence indicates that various mechanisms involved in the maintenance of pH homeostasis of the cell (Kurkdjian and Guern, 1989; Caroll et al., 1994; Heber et al., 1994) are operating in leaves submitted to 15 to 20% CO_2 (Siriphanich and Kader, 1986; Heber et al., 1994). Whether these mechanisms are able to compensate for CO_2-triggered acid loading in fruit is not known because the techniques currently available to determine cytosolic pH, such as ^{31}P NMR, are difficult to use with the highly vacuolized fruit cells. Nevertheless, indirect evidence, such as accumulation of γ-aminobutyrate, indicates that acid stress may occur in fruits under high CO_2 (Kader et al., 1989). Decarboxylation of glutamate to yield γ-aminobutyrate is a proton-consuming reaction stimulated by the decrease in cytosolic pH (Carroll et al., 1994; Crawford et al., 1994). Response of carrot cells to acidosis involves increased activity of GAD by both activation and increased synthesis of the enzyme (Carroll et al., 1994). We observed a strong accumulation of GAD mRNA in tomato fruit subjected to high CO_2, suggesting acidification of the cytosol (Fig. 5). Tomato fruit appeared to recover from such stress

soon after removal from high CO_2, because GAD mRNA (Fig. 5) as well as HSP mRNA (Fig. 4) strongly decreased the day following transfer to air and ultimately reached a level near that of control fruit (Figs. 4 and 6). The end of this period correlates with the recovery of normal ripening (Fig. 1) and ethylene formation (Fig. 2). Thus, the cytosolic acidification induced by high CO_2 may affect ripening. In particular, the modulation of protein synthesis by cytosolic pH may be analogous to that observed in O_2-deprived maize roots, where translation of normal cellular messages is repressed by the low O_2-induced cytoplasmic acidification (for review, see Gallie, 1993). Levels of proteins and enzymes required for the ripening process may be thereby regulated by the decrease in cytosolic pH.

High CO_2 Affects Both Ethylene- and Development-Dependent Gene Expression

In an attempt to determine whether high CO_2 may have blocked ripening through its stress effect rather than its effect on ethylene synthesis and action, we investigated the effect of high CO_2 on the time-course accumulation of mRNAs that are involved in the main ripening-related changes, and are differentially regulated in response to ethylene and to developmental processes (Oeller et al., 1991; Picton et al., 1993). The regulation of the expression of these genes appears to be complex. By analyzing transgenic and natural tomato mutants with deficient ethylene production or perception, several groups have recently shown that the induction of the ripening process is regulated by at least two different pathways in tomato fruit (Oeller et al., 1991; Gray et al., 1994; Yen et al., 1995).

One pathway is ethylene-dependent and is presently well characterized (Zarembinski and Theologis, 1994). Among the genes in which transcription is regulated by ethylene are ACS and PSY; the latter catalyzing the first step in carotenoid biosynthesis (Oeller et al., 1991; Klee, 1993; Picton et al., 1993). As was expected from the delay and reduction in color development in CO_2-treated fruit (Fig. 1), the accumulation of mRNAs for PSY is blocked by the CO_2 treatment in MG fruit producing low amounts of ethylene (Fig. 7). Similarly, the expression of ACS is affected by the CO_2 treatment (Fig. 6). These observations are thus consistent with the hypothesis of an interference of CO_2 with ethylene signaling.

The other pathway that modulates transcription of ripening-related genes does not require ethylene to regulate gene expression at the transcriptional level, but is development-dependent. How this pathway regulates the expression of ripening-related genes is still poorly understood (Gray et al., 1994; Zarembinski and Theologis, 1994). The expression of PG, which is involved in the degradation of cell wall polyuronides, and ACO is probably mediated via this pathway, although the posttranscriptional processing of the former is ethylene-dependent (Theologis, 1992). The expression of AI and another ripening-specific pTOM gene of an unknown function, pTOM 36 (Slater et al., 1985), is probably also ethylene-independent. They are only slightly affected by the reduction in ethylene formation in ACO antisense fruits (Picton et al., 1993) or by silver ions (Davies et al., 1988), known inhibitors of ethylene action. If we assume that the action of CO_2 is limited to the blocking of ethylene signaling, the CO_2 treatment should only affect the induction of ethylene-dependent genes. Furthermore, the effect of CO_2 on the accumulation of ripening-related mRNAs should be relieved upon transfer to air of the fruits previously submitted to CO_2. On the contrary, the induction of both the ethylene-dependent (ACS and PSY) and -independent (ACO, PG, AI, and TOM36) ripening-related genes is strongly suppressed in MG fruits held under 20% CO_2, whatever their regulation may be (Figs. 6 and 7). Furthermore, the normal accumulation pattern of ripening-related mRNAs is not immediately restored upon transfer of CO_2-treated fruit to air (Figs. 6 and 7). The severity of the stress imposed to the fruit may possibly explain why mRNA levels of CO_2-treated fruit transferred to air remain lower than those of control fruit. On the contrary, tomato mutants with reduced ethylene production (Hamilton et al., 1990; Klee et al., 1991; Oeller et al., 1991) or altered ethylene perception (Yen et al., 1995), which display slow- or nonripening phenotypes, continue to accumulate mRNAs or proteins encoded by ethylene-independent genes (Oeller et al., 1991; Klee, 1993; Picton et al., 1993; Yen et al., 1995). In this way, fruit detached from ACS antisense plants remains firm and green (Oeller et al., 1991), like CO_2-treated fruit, and yet they express PG and ACO mRNA unlike CO_2-treated fruit.

In view of these observations, it appears that high CO_2 affects the induction of both ethylene- and development-dependent genes involved in the control of ripening-associated changes. Delay in the recovery of normal gene expression following transfer to air of fruits previously submitted to CO_2 may explain the residual effect of the 20% CO_2 treatment on ripening. One possibility is that at the concentrations used, CO_2 blocks the expression of ethylene-inducible genes by reducing the synthesis or counteracting the action of ethylene. Since the expressions of early ethylene-inducible genes (ACS) and of ethylene-independent genes are similarly delayed, another possibility is that CO_2 alters the competence of the fruit to respond to yet-undefined developmental signals before the onset of ripening. These results further point out that a stress effect exerted by CO_2 may account for the overall inhibition of fruit ripening. It remains to be seen if CO_2 exerts the same physiological and biochemical effects as those demonstrated in this study, when present at the 1 to 5% concentrations suitable for controlled atmosphere storage of fresh produce.

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