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Ethylene and Glycosidase Promotion in GA_3- and IAA-treated Tomato Fruit (*Lycopersicon esculentum* Mill.)

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Abstract

Transient (1 h) treatment of breaker tomatoes (*Lycopersicon esculentum* Mill. cv. Bonanza) with exogenous GA_3 or IAA at a high concentration (20 mM) resulted in a two- to four-fold increase compared with the control in ethylene biosynthesis during a 9-day experiment. This sharp increase in ethylene emission is characteristic of a stress response. Both phytohormones promoted the activity of 1-aminocyclopropane-1-carboxylate synthase that probably accounts for most of the enhanced ethylene synthesis. GA_3 and IAA also stimulated total α- and β-galactosidase and α-L-arabinofuranosidase activity but showed some potential to delay ripening parameters, among them, fruit softening, chlorophyll loss, and total carotenoid synthesis. GA_3- or IAA-treated fruit did not respond to exogenous 100 ppm C_2H_4 with an increase of autocatalytic ethylene production. Moreover, GA_3 or IAA applied alone showed a faster increase in ethylene biosynthesis than that achieved by exogenous C_2H_4. The combination of GA_3 and C_2H_4-supplemented atmosphere did not result in synergistic effects on glycosidase activity except for a few cases. IAA-treated fruit exposed to C_2H_4-supplemented atmosphere did not promote additional glycosidase activity but rather seemed to have antagonistic effects on β-galactosidase during the first few days of the experiment. Glycosidase response to GA_3 and IAA treatments did not correlate with changes in tomato pericarp firmness, thus suggesting that some isoforms may have no role in tomato fruit softening.

Key words: ACC synthase; α-Arabinofuranosidase; Ethylene; α- and β-Galactosidase; Gibberellic acid; Indole-3-acetic acid

Introduction

α- and β-galactosidase (α- and β-Gal; EC 3.2.1.22 and EC 3.2.1.23, respectively) and α-L-arabinofuranosidase (α-Arab; EC 3.2.1.55) are major glycosidases that may remove galactose and arabinose units from different tomato fruit cell wall polysaccharides. There are important reasons to study these glycosidases in relation to fruit growth and ripening: galactose and arabinose are two of the most dynamic cell wall glycosyl residues in fruits (Gross and Sams 1984). The activity of these enzymes may account for cell wall modification and modulation of neutral sugar levels, presumably important to fruit ripening (Priem and Gross 1992; Priem and others 1993).

α- and β-Gal and α-Arab activities are present at high levels in rapidly growing immature tomato fruit (Sozzi and others 1998a; Fraschina and others

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2000). Regulation of these enzymes during climacteric fruit ripening is fairly well known because ethylene plays a key role as a ripening promoter (Sozzi and others 1998a). The cloning of cDNA (pTomβgal 4) that apparently encodes for tomato β-Gal II, has been reported (Smith and others 1998), and its gene expression is up-regulated by ethylene treatment in mature green tomato fruit (K. Gross and D. Smith, personal communication). On the other hand, other endogenous controls on these glycosidases could be operating in tomato fruit during growth or ripening.

The growth regulators gibberellins and auxins are involved in a number of important physiologic processes in fruits, including stimulation of cell division and increase in cell expansion (Gillaspy and others 1993). There is evidence suggesting that these phytohormones could also be involved in fruit ripening (Brady 1987), and this fact could lead to interesting postharvest applications (Ben-Arie and others 1995). It is well established that exogenous gibberellic acid$_3$ (GA_3) inhibits tomato fruit ripening by delaying total chlorophyll (Chl) degradation, lycopene (Lyc) biosynthesis, and the climacteric respiratory rise (Abdel-Kader and others 1966; Babbitt and others 1973); moreover, the action can be reversed when GA_3-pretreated fruit are treated with ethylene (Dostal and Leopold 1967). Since these early reports of the inhibitory effect of exogenous GA_3 on tomato fruit ripening, different responses have been observed in other species (Ben-Arie and others 1996).

Indole-3-acetic acid (IAA) present in tomato fruit during ripening (Buta and Spaulding 1994; Catalá and others 1992; Riov and Bangerth 1992) may also play a role in regulation of ripening metabolism (Cohen 1996). Conflicting results have been obtained upon treatment of tomato fruit with auxins, probably because of the different systems (intact fruit, tissue disks or in vitro cultures) and application levels used. When using intact fruit, IAA was found to delay the ripening process (Abdel-Kader and others 1966; Babbitt and others 1973), but an advance in softening, flavor, and color development was observed when using 2,4-D (Vendrell 1985). A different pattern was shown by studies using tissue disks (Yunovitz and Gross 1994) probably because of IAA oxidation (a high peroxidative activity is known to degrade IAA; Catalá and others 1994) and wound ethylene formation (Campbell and others 1990).

Auxins and gibberellins usually exert physiologic effects within a concentration range of 10^{-5} to 10^{-9} M, but higher concentrations are generally required to elicit a response in intact ripening fruit when exogenously applied (Ben-Arie and Ferguson 1991; Ben-Arie and others 1996; Reddy and others 1990) because of the effects of degradation and limited diffusion into the tissue. These regulators are frequently applied to delay softening and extend shelf life. Nevertheless, alterations on cell wall glycosidases caused by these treatments have not been evaluated. Treatments with high concentrations of these hormones can promote ethylene production (Ben-Arie and others 1995) as a chemical stress response. In this study, we analyze the effect of high concentrations of applied IAA and GA_3 on ethylene production, the activity of 1-aminocyclopropane-1-carboxylate synthase (ACC-S) and three glycosidases (α- and β-Gal and α-Arab). Firmness and pigment changes were measured as ripening indicators.

Materials and Methods

Plant Material, Treatments, and Reagents

Tomatoes (*L. esculentum* Mill. cv. Bonanza) were grown in Salta, Argentina, under standard cultural practices and hand-harvested at the mature green stage. Fruits were immediately transported to the laboratory by refrigerated truck (14 ± 1°C) and treated 1 day after harvesting. On arrival, defect-free fruits of uniform color (breaker stage), shape, and size (ranging from 150–200 g) were washed in a fungicide solution containing 0.1% (w/v) benomyl and then air-dried. Selected fruits were divided into six batches at random, and submitted to the following treatments: lots T_{0A} and T_{0E} were treated only with a solution containing 0.05% (v/v) Tween 80 (polyoxyethylenesorbitan monooleate) for 1 h; lots T_{1A} and T_{1E} were treated with the preceding wetting agent plus 20 mM GA_3 for 1 h; lots T_{2A} and T_{2E} were dipped in a solution consisting of 0.05% (v/v) Tween 80 and 20 mM IAA for 1 h. Then, fruits were enclosed in 3-L flasks (4 fruits per flask) and exposed to different gaseous mixtures using a constant flow-through gas system (Sozzi and others 1999). T_{0A}, T_{1A}, and T_{2A} lots were ventilated with air, whereas T_{0E}, T_{1E}, and T_{2E} lots received 100 ppm C_2H_4 in air at a flow rate of 50 mL min^{-1}. In addition, to ensure that CO_2 would not exceed 0.5%, flask atmosphere was completely renovated twice a day using a flow rate of 2000 mL min^{-1} for 3 min. The different atmospheres were bubbled through water to provide flows of humidified gas (~90% RH). The continuous ethylene treatment (fruits were treated for up to 9 days) was performed at 24 ± 2°C, and 20 tomatoes per treatment and date were randomly sampled for different analyses. All chemicals were from Sigma Chemical Co. (St. Louis, MO) except for acetone, which was from Merck (Argentina).

Determination of Ethylene Production

To study ethylene production, a static system was used. Ethylene treatment was stopped after various durations of up to 9 days, and C_2H_4-treated fruits were placed in a 60 m^3 dark ripening room ventilated with humidified ethylene-free air at a rate equivalent to one third of the empty room volume per minute to facilitate diffusion of absorbed exogenous C_2H_4 (Sozzi and others 1999). Then, ethylene production was determined by sealing individual fruit in a 1-L glass container for 1 h. One milliliter headspace gas samples were removed through rubber septa inserted in the container lids with a syringe. Ethylene concentration in the gas sample was quantified on a gas chromatograph (Hewlett Packard 5890 Series II) as previously described (Sozzi and others 1999).

ACC-S Extraction and Assay

Pericarp tissue (30 g) was homogenized at 4°C in a Waring blender with 1 mL g^{-1} of extraction media, consisting of 400 mM potassium phosphate, pH 8.5, 1 mM EDTA, 0.5% (v/v) 2-mercaptoethanol, and 0.01 mM pyridoxal 5-phosphate. The homogenate was filtered through three layers of cheesecloth and then centrifuged at 28,000 × *g* for 30 min. The supernatant was discarded, the pellet was resuspended in extraction media and once again centrifuged at 28,000 × *g* for 20 min; and the supernatant was discarded. The resulting pellet was resuspended and ACC-S activity was assayed according to Yip and others (1991).

Firmness Determination and Pigment Content

Fruit firmness was estimated after removal of an epidermal disk, with a hand-held Effegi firmness penetrometer (Model FT 327) fitted with an 11-mm diameter plunger tip as previously described (Sozzi and others 1996). After firmness determination, whole fruits were sliced, seeds and placental tissue were removed, and the unpeeled pericarp was immediately processed for assays except for material intended for pigment measurements, which was frozen at -20°C for later use.

Chl and total carotenoids (Car) were extracted five times from 2-g longitudinal strips of frozen pericarp with 50 mL of acetone in a glass homogenizer while covering the tubes with aluminum foil. The combined mixtures were finally extracted on a reciprocal shaker at 140 rpm for 30 min. The acetone extract was centrifuged at 12,000 × *g* for 15 min. Chl and Car content was calculated as described by Lichtenthaler (1987).

Glycosidase Extraction and Assay

Glycosidases were extracted and assayed as per Sozzi and others (1998b), with minor modifications. Composite samples of 75 g were homogenized in 1 vol of cold 1.4 M NaCl with a Waring blender, and pH was adjusted to 6 with 1 M NaOH. All the subsequent steps were performed at 4°C. The suspension was then shaken for 1 h and filtered through cheesecloth. The filtrate was centrifuged at 12,000 × *g* for 30 min. Extract aliquots were desalted using PD-10 columns (Pharmacia Biotech, Uppsala, Sweden) previously equilibrated in 20 mM sodium acetate/acetic acid buffer, pH 4.75. Columns were eluted with the same buffer.

Aliquots of crude extract were assayed for total α- and β-Gal and α-Arab activity using *p*-nitrophenyl-glycosides as substrates as previously described (Sozzi and others 1998b). An incubation period of 15 min was selected for α- and β-Gal and of 60 min for α-Arab. After incubation at 37°C, the reaction was stopped by adding 2 mL of 0.2 M (for α- and β-Gal) or 0.13 M (for α-Arab) sodium carbonate. One unit of each glycosidase was defined as the amount that hydrolyzed 1 nmol min^{-1} of *p*-nitrophenyl glycoside. Activity was expressed on a tissue fresh mass basis. Free *p*-nitrophenol was used as standard.

Statistical Analysis

Statistical significance was determined by analysis of variance. In the case of a significant F-value, data were subjected to Tukey's test for comparison of treatments within each date. Significance was determined at $p < 0.05$. Details for replication of the experiments are given in figure legends.

Results

Ethylene Biosynthesis Rate and ACC-S Enzyme Activity

In control fruit, the ethylene climacteric peak occurred at day 6 with an ensuing decline, but measurable quantities of ethylene were detected from the start of the experiment (Figure 1a). Treatment with Tween 80 did not affect ethylene production (data not presented). Dissimilar effects involving ethylene production on treatment with surfactants (Tween, Triton) have been observed in other plant tissues, probably because of the application levels used (Ciaccio and Hodges 1987; Lownds and Buko-

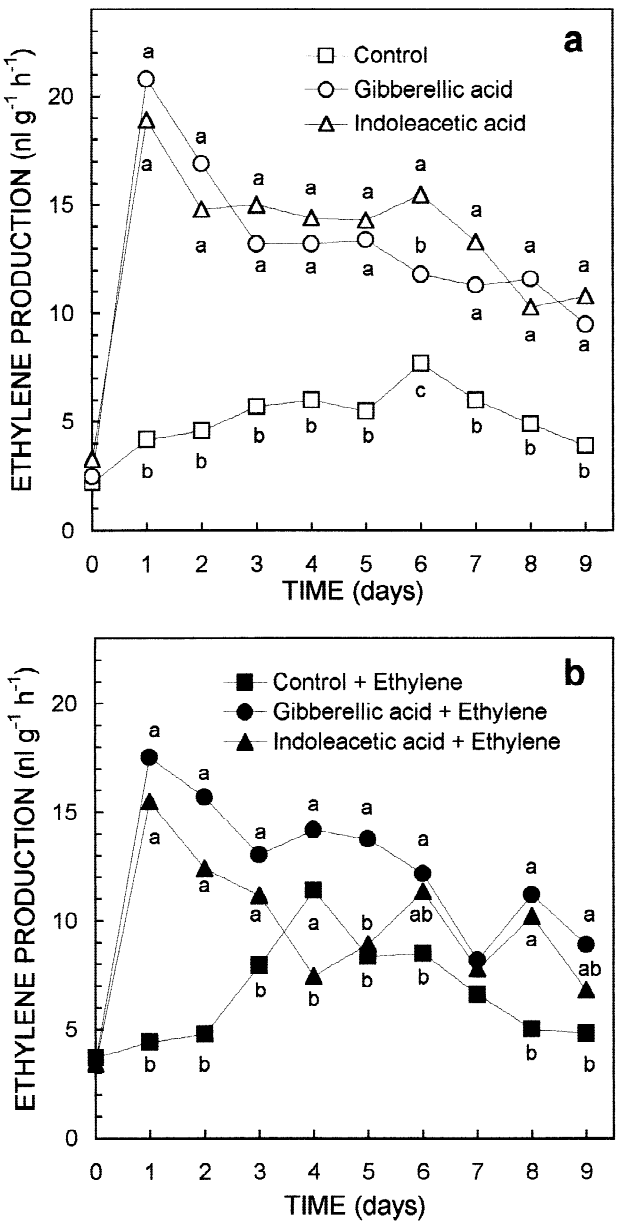

Figure 1. Effects of (a) GA_3 and IAA and (b) C_2H_4, GA_3+C_2H_4 and IAA+C_2H_4 on the rate of ethylene production. Each point represents the average of eight replications. Means with different letters at each time interval are significantly different according to Tukey's test ($p < 0.05$). When points are not accompanied by letters, there are no significant differences between treatments at the corresponding storage period.

vac 1989). A 1-h dip treatment of breaker fruit in 20 mM solution of GA_3 or IAA stimulated ethylene production six- to sevenfold after 1 day (Figure 1a) and remained higher than control fruit over the 9-day period. A similar trend was observed when fruits were treated with GA_3 or IAA followed by C_2H_4 treatment (Figure 1b). Moreover, ethylene production by control fruit subsequently treated with 100 ppm of ethylene was similar to that of IAA+C_2H_4-treated fruit after day 4 (Figure 1b). GA_3- and IAA-treated fruit did not respond to exogenous C_2H_4 with an increase of autocatalytic ethylene production. GA_3 or IAA individually acted more quickly than C_2H_4 in increasing ethylene biosynthesis (Figure 1). Treatment with GA_3 or IAA significantly increased in vitro ACC-S activity compared with the control after 24 h (Table 1).

Table 1. GA_3- and IAA-Induced ACC Synthase Activity Changes During the First 24 h of the Experiment

	ACC Synthase Activity (nmol ACC g^{-1} FW h^{-1})	
Treatment	1 h After Immersion	24 h After Immersion
Control	1.08 ± 0.45^a	1.15 ± 0.31^a
GA_3	0.98 ± 0.41^a	3.32 ± 0.05^b
IAA	0.92 ± 0.30^a	3.20 ± 0.61^b

The values are means ± SD of three replications. Values in the same column with different letters differ significantly according to Tukey's test ($p < 0.05$).

Firmness and Pigment Content

GA_3 and IAA treatments delayed loss in flesh firmness (Figure 2a). Only fruit treated with GA_3+C_2H_4 continued softening after day 4 at a rate significantly higher than that for fruit treated with GA_3 alone (Figure 2). No significant differences were detected between fruit submitted to IAA+C_2H_4 and those treated only with IAA.

A similar trend was observed for Chl loss (Figure 3): ethylene treatment only marginally increased Chl loss. GA_3 treatment slightly delayed Car accumulation and ethylene treatment did not markedly affect this (Figure 4), whereas control and IAA-treated fruit accumulated Car in a similar fashion with or without supplemental ethylene treatment.

Glycosidase Activity

In GA_3-treated fruit, α-Gal activity showed an increasing pattern toward the end of the experimental period: by the eighth day, α-Gal was 26% higher in tomatoes treated with GA_3 than in control fruit (Figure 5a). When fruits with and without GA_3 were exposed to C_2H_4, a significant difference (19%) was detected after 48 h (Figure 5b). When compared

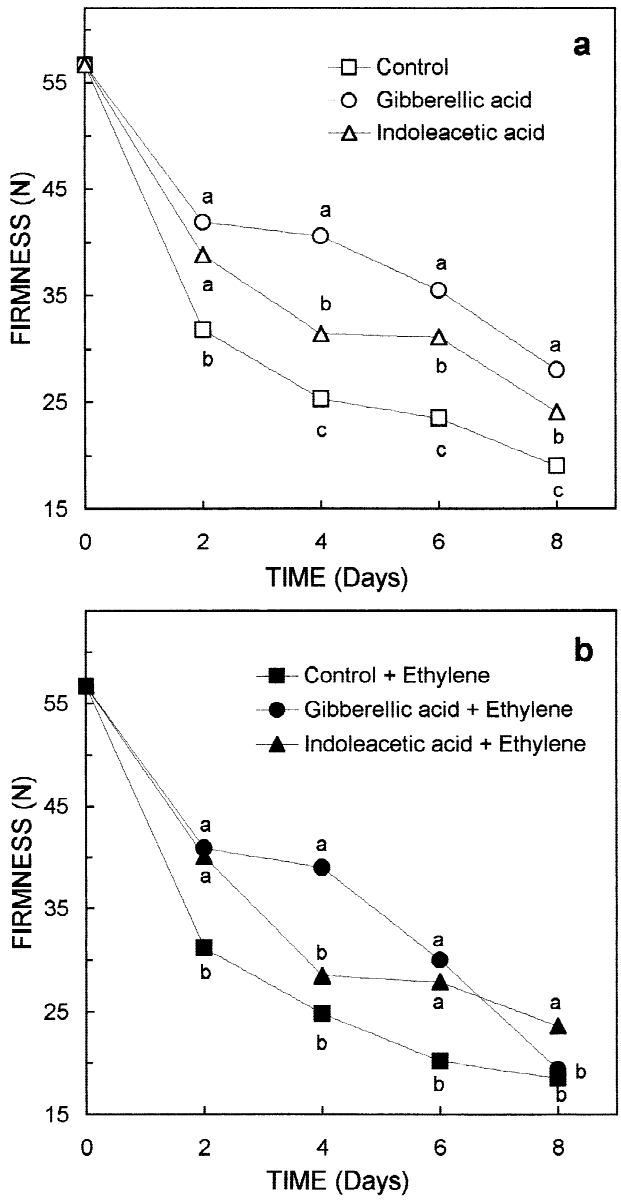

Figure 2. Changes in tomato fruit firmness after different hormonal treatments. Each point represents the average of eight replications. Letters are as in Figure 1.

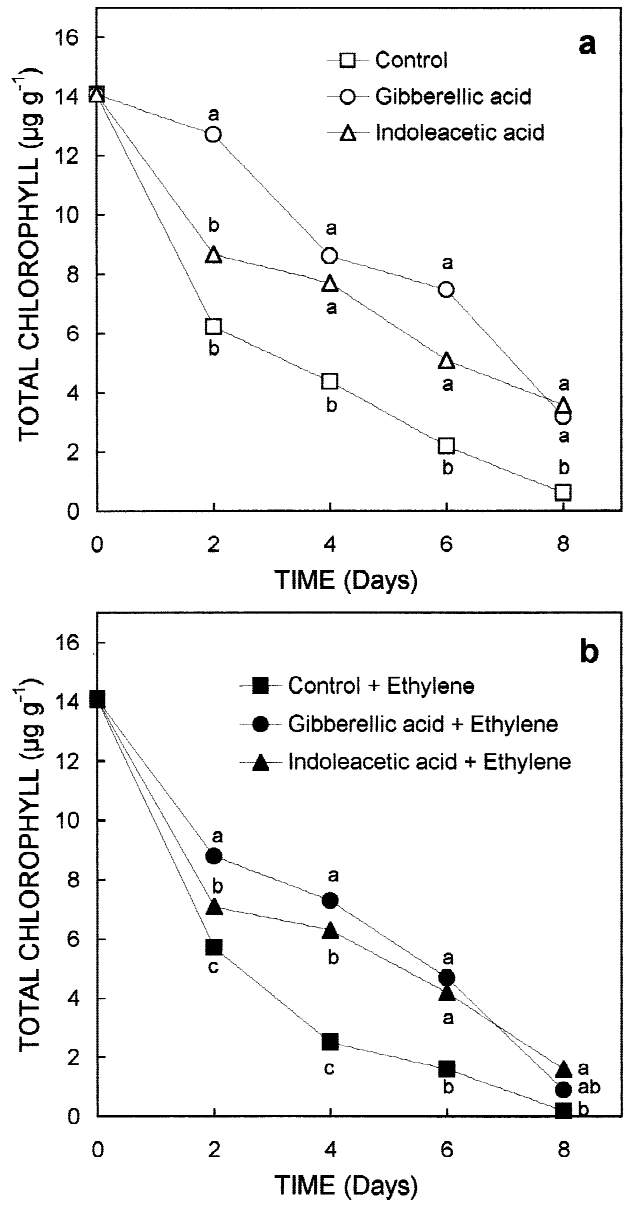

Figure 3. Chlorophyll degradation after different hormonal treatments. Each point represents the average of five replications. Letters are as in Figure 1.

with that of control fruit, α-Gal activity of IAA- and IAA+C_2H_4-treated fruit did not significantly change until 4 days after treatment but was higher during the rest of the experimental period, with a pronounced peak on day 6 (Figure 5).

β-Gal activity was significantly enhanced in GA_3-treated fruit during the first days of the experiment, both in the presence (37%) or absence (21%) of C_2H_4, but dropped to the initial value by day 8 (Figure 6). During the first 2 days, β-Gal activity in IAA-treated fruit reached a maximum 42% higher than the corresponding level in control fruit; after a slight decrease during the following 2 days, activity remained constant (Figure 6a). In IAA-treated tomatoes submitted to an C_2H_4-supplemented atmosphere, β-Gal activity gradually increased to reach a maximum at the end of the experiment (Figure 6b).

Levels of α-Arab activity in GA_3-treated fruit, both with and without C_2H_4, peaked at 48 h after treatment (activity in GA_3-treated fruit was 33% higher than that in control fruit) but fell dramatically toward the end of the experiment, showing no significant differences with the control in the later stages (Figure 7a). In IAA-treated fruit, α-Arab ac-

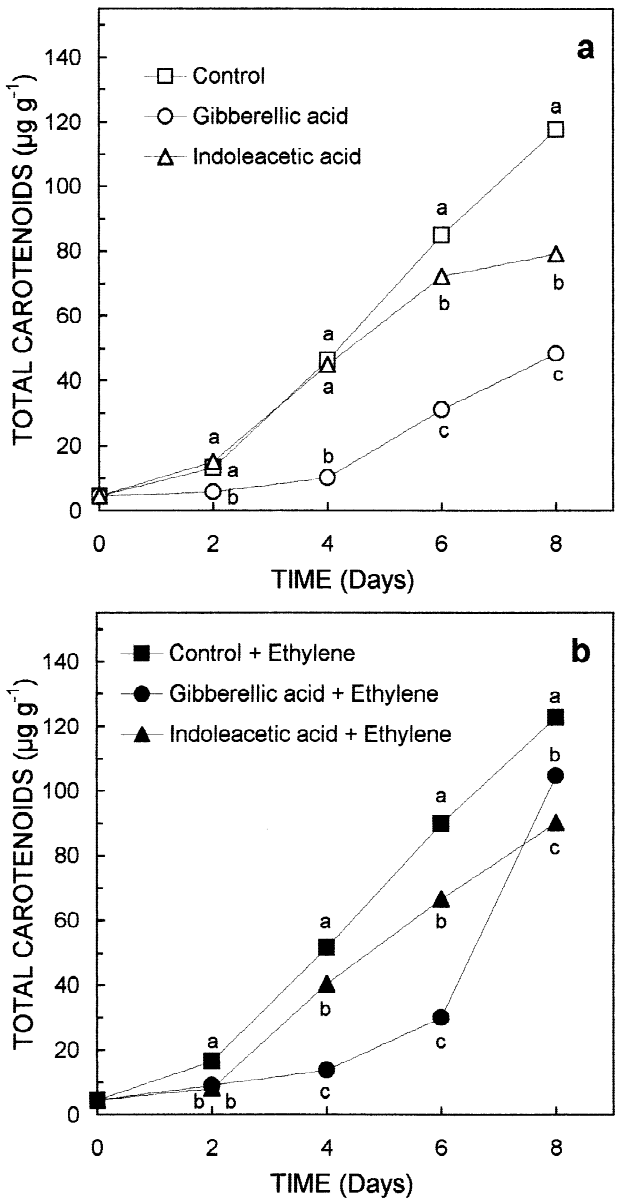

Figure 4. Carotenoid synthesis after different hormonal treatments. Each point represents the average of five replications. Letters are as in Figure 1.

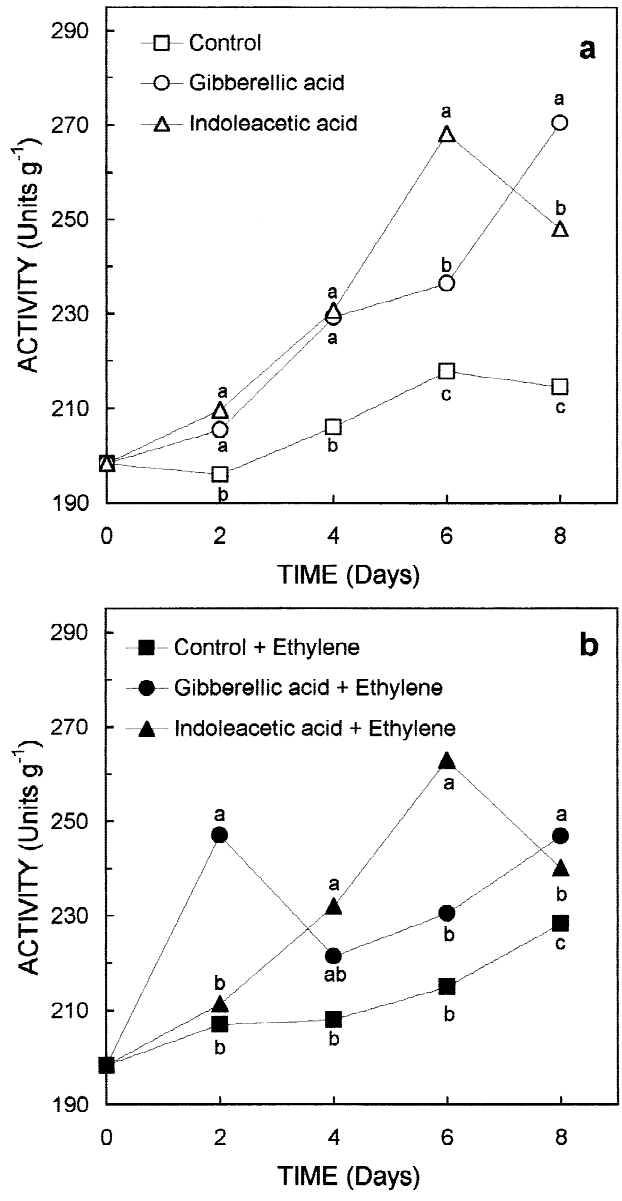

Figure 5. α-Gal activity after different hormonal treatments. Each point represents the average of four replications. Letters are as in Figure 1.

tivity remained higher than in control fruit during all the experimental period and reached a level 57% above that of control fruit by day 8. In fruit treated with the combination of IAA and C_2H_4, α-Arab activity showed a sharp rise in the following 48 h and reached a level 40% higher than in fruit exposed only to C_2H_4 (Figure 7b).

In general, GA_3, IAA, and C_2H_4 promoted glycosidase activity. The combination of GA_3 and C_2H_4 displayed synergistic effects only in a few cases (α-Gal, day 2; β-Gal, days 2–4). IAA-treated fruit treated with C_2H_4-supplemented atmosphere showed no additional promotion of enzyme activity but seemed to have antagonistic effects on β-Gal activity during the first days of the experiment.

Discussion

The sharp increase in ethylene emission in GA_3- and IAA-treated tomatoes compared with control fruit (Figure 1) is typical of a stress response (Abeles and others 1992). Ethylene biosynthesis is enhanced in tomato fruit or plants submitted to different stress conditions: after exposure to extreme temperatures

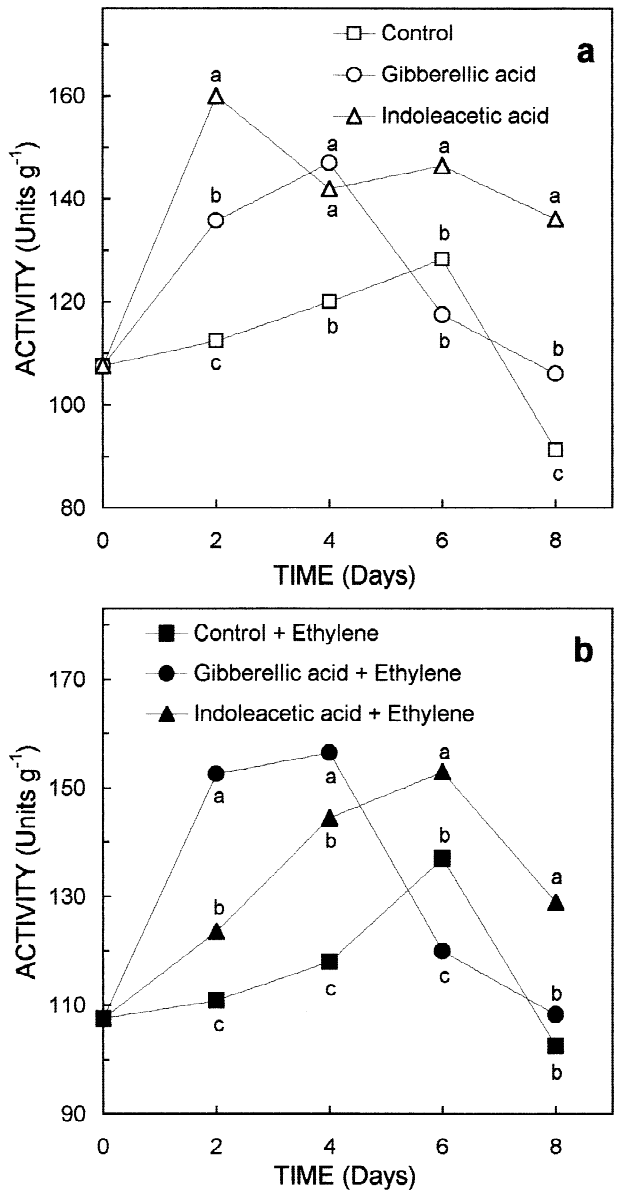

Figure 6. β-Gal activity after different hormonal treatments. Each point represents the average of four replications. Letters are as in Figure 1.

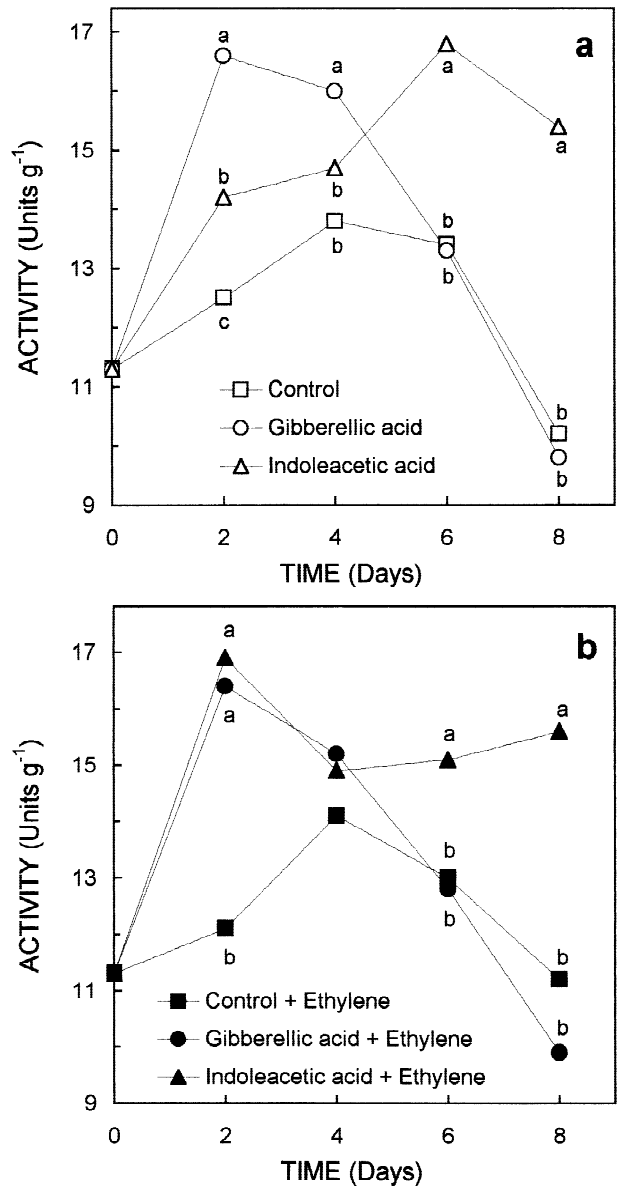

Figure 7. α-Arab activity after different hormonal treatments. Each point represents the average of four replications. Letters are as in Figure 1.

(Biggs and others 1988; Lurie and Klein 1991) or excessive water (Jackson and Campbell 1975), wounding (MacLeod and others 1976; Moretti and others 1998), irradiation (Larrigaudière and others 1990), fungi attack (Pegg and Cronshaw 1976), or injection of fungal pectinases (Baldwin and Pressey 1989), modification of atmospheric gas composition (Klieber and others 1996; Kubo and others 1990) or ozone exposure (Tuomainen and others 1997). Moreover, a similar stimulus in ethylene production has been detected in response to other mild toxicants (Abeles and others 1992).

We observed considerable differences in the relative increase of ethylene production produced by dipping in GA_3 or IAA when using different commercial cultivars. However, in all cases these hormones caused a significant false climacteric (data not shown). The cultivar effect may be due to the relative presence or absence of various surface diffusion barriers. Although the agricultural surfactant Tween 80 improves penetration, this enhancement appears to depend on fruit anatomy and morphology (pedicel type, abscission zone quality, cutin covering the epidermal surface, epicarp thickness).

ACC-S is known to mediate the transduction of stress stimuli, leading to greater ethylene formation. Our results show that high concentrations of GA_3 or IAA applied to breaker tomatoes increase ACC-S activity, and this presumably enhances the ability of fruit tissue to turn *S*-adenosylmethionine into ACC. This in turn is the most probable basis for the faster rates of ethylene production by the hormone-treated fruit. Increased activity of ACC-S is often observed in plants subjected to different environmental stressors and seems to be the key regulatory step in stress ethylene production (Abeles and others 1992). It is well known that several ACC-S genes are present in plant tissues and that the ones activated by stress are different from those activated by auxin-induced and normal basal metabolism (Nakajima and others 1990; Van der Straeten and others 1990). Therefore, it is debatable whether IAA at high concentrations acts as a stressor.

GA_3 and IAA have some potential for delaying major ripening processes such as Chl degradation, Car synthesis, and fruit softening (Figures 2–4). Our results confirm and demonstrate that those climacteric ripening processes are not exclusively linked to the ethylene burst because GA_3 and IAA-treated fruit not only increased ethylene production but also delayed ripening. GA_3 treatment also appears to disrupt the close relationship between ethylene biosynthesis and different ripening-related events when an open pericarp disk system is used (Ben-Arie and others 1995). It appears that 20 mM GA_3 or IAA may render cells insensitive to ethylene, including the extra ethylene that these hormones inevitably bring about. This fact could generate some kind of protection from the senescence-promoting influence of ethylene. The standard explanation of the well-known ability of IAA to inhibit different phenomena (e.g., abscission) despite a vigorous stimulation of ethylene formation (Abeles and others 1992) may readily be transferred to fruit ripening.

The increase in α- and β-Gal and α-Arab in response to hormonal treatments (Figures 5–7) is particularly notable. Glycosidases are not commonly associated with stress responses in fruits although an increase in total β-Gal activity was reported for irradiated tomatoes (El Assi and others 1997). β-Gal exists in at least three forms in tomato fruit (Pressey 1983) but our study addresses total activities regardless of specific isoenzymes. All these glycosidases are known to increase their activity in response to ethylene (Sozzi and others 1998a; Fraschina and others 2000). The response of total α- and β-Gal and α-Arab to GA_3 and IAA treatments does not parallel changes in tomato pericarp firmness, and their involvement in the softening process does not seem to be essential; glycosidase activity shows transient or permanent increases, whereas normal softening is impaired. Perhaps a decrease in the activity of a ripening-related isoenzyme (such as β-Gal II) may be masked in the crude extract. Two- to 3-week-old fruit display high total α- and β-Gal activity levels, but this activity involves β-Gal isoenzymes different from β-Gal II (Sozzi and others 1998a). These peaks in α- and β-Gal activity appear after the first surge in the auxin level and coincide with the second peak of gibberellin accumulation (Gillaspy and others 1993); this is consistent with a model in which the action of these enzymes could be required for subsequent expansion or reorganization of the cell wall but not necessarily with fruit softening (Sozzi and others 1998a). Moreover, studies in ripening kiwifruit disks suggest no alignment between softening and cell wall galactose decline (Redgwell and Harker 1995). The loss of neutral sugars from the cell wall, primary galactose and arabinose, are a characteristic feature of ripening fruit (Gross and Sams 1984) and have been associated with loss of wall galactans and disassembly of the pectin macromolecular matrix in which β-Gal II may be involved (Carey and others 1995; Pressey 1983). Ben-Arie and others (1996) found that the decrease in arabinose and galactose was less pronounced in GA_3-treated persimmons than in untreated fruit. Thus, it should be interesting to determine whether β-Gal II activity and different cell wall fractions are also affected in GA_3- and IAA-treated tomato fruit, too.

The use of GA_3 and IAA may be a useful framework of analysis to check the supposed ripening-related role of certain wall-bound enzymes and their hormonal regulation. The action of these phytohormones in fruit tissue causes a dual effect, namely an increase in ethylene production (which may enhance certain enzyme presence) and a prevailing simultaneous delay in fruit ripening (incompatible with an increase in softening-responsible enzymes). For example, GA_3 decreases the rate of softening by 40% in comparison with control fruit and causes a similar reduction in cellulase activity (Babbitt and others 1973). Moreover, it almost totally blocks the rise of polygalacturonase in intact fruit (Babbitt and others 1973) and in pericarp discs (Mignani and others 1995) despite polygalacturonase gene expression being ethylene dependent (Sitrit and Bennett 1998) and ethylene production increasing in GA_3-treated intact fruit (Figure 1) and disks (Ben-Arie and others 1995).

Endogenous GA and IAA levels drop throughout tomato fruit expansion to very low levels (Buta and Spaulding 1994; Gillaspy and others 1993); thus, IAA- and GA-deficient tissues carry out the tomato

ripening program (Cohen 1996). This fact raises the question of the physiologic relevance of endogenous GA and IAA during tomato ripening. Only artificially elevated levels of said phytohormones appear to alter tomato ripening. However, assays using exogenous IAA and GA_3 may be a useful contribution to postharvest physiology and technology because IAA and GA_3 can delay whole tomato ripening and softening (Abdel-Kader 1966; Babbitt and others 1973) and become the biologically active factors partially displacing ethylene in the ripening program control. High levels of GA_3 and IAA make tomato fruit tissues relatively insensitive to ethylene as a promoter of the normal ripening process.

In this study, it is not clear whether exogenous GA_3 and IAA are directly responsible for the increase in ripening-related glycosidase activities or indirectly responsible for it because of the induced rise in ethylene. Nevertheless, our results suggest that activities of some glycosidase isoforms may have little to do with fruit softening though soluble neutral sugars do increase during tomato ripening according to previous works (Gross and Sams 1984; Kim and others 1991).

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