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Characterization of the Stimulation of Ethylene Production by Galactose in Tomato (*Lycopersicon esculentum* Mill.) Fruit[1]

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ABSTRACT

We have characterized the stimulation of ethylene production by galactose in tomatoes (*Lycopersicon esculentum* Mill.). The effect of concentration was studied by infiltrating 0, 4, 40, 100, 200, 400, or 800 micrograms galactose for each gram of fresh fruit weight into mature green 'Rutgers' fruit. Both 400 and 800 micrograms per gram fresh weight consistently stimulated a transient increase in ethylene approximately 25 hours after infiltration; the lower concentrations did not. Carbon dioxide evolution of fruit infiltrated with 400 to 800 micrograms per gram fresh weight was greater than that of lower concentrations. The ripening mutants, *rin* and *nor*, also showed the transient increase in ethylene and elevated CO_2 evolution by 400 micrograms per gram fresh weight galactose. 1-Aminocyclopropane-1-carboxylic acid (ACC) content and ACC-synthase activity increased concurrently with ethylene production. However, galactose did not stimulate ACC-synthase activity *in vitro*. The infiltrated galactose in pericarp tissue was rapidly metabolized, decreasing to endogenous levels within 50 hours. Infiltrated galacturonic acid, dulcitol, and mannose stimulated transient increases in ethylene production similar to that of galactose. The following sugars produced no response: sucrose, fructose, glucose, rhamnose, arabinose, xylose, raffinose, lactose, and sorbitol.

A substantial loss of cell wall galactosyl residues occurs during ripening of tomato fruit (10) as well as in other ripening fruits (9, 15). The loss of galactosyl residues may involve a reduced synthesis of wall galactan (16) in conjunction with the enzymic hydrolysis of β-1,4-galactan by β-galactosidase II (23), resulting in a 4- to 6-fold increase in free, monomeric galactose during tomato ripening (6, 8). Galactose has been shown to elicit various responses in plant tissues (5) such as: the stimulation of IAA-mediated ethylene production in mung bean hypocotyls (4), inhibition of auxin-induced cell wall elongation in *Avena* coleoptiles (30), promotion of ethylene production in tobacco leaf discs (21), reduction of auxin movement in bean hypocotyl segments (14), and changes in IAA synthesis in *Avena* coleoptiles (2). In addition, certain carbohydrates have been shown to stimulate ethylene production via an auxin-induced increase in ACC[2] synthase activity (22).

It was recently shown that exogenously applied galactose stimulated ethylene production and promoted ripening of mature green tomatoes (7). This suggested the possibility that free galactose, solubilized from tomato cell wall polysaccharides during ripening and which accumulates in the tissue in its free, monomeric form, may potentially be involved in fruit ripening through the stimulation of ethylene biosynthesis. Therefore, we undertook a study to characterize the stimulation of ethylene production in mature green tomato fruit by galactose.

MATERIALS AND METHODS

Plant Material. Tomato (*Lycopersicon esculentum* Mill.) plants were grown in a glasshouse using standard cultural practices. Flowers were pollinated by a mechanical vibrator and tagged at anthesis. Only one or two fruit were permitted to develop on each cluster to allow for uniform fruit development. Normal ripening (cv 'Rutgers'), *nor* (nonripening, isogenic to 'Rutgers'), and *rin* (ripening-inhibitor, isogenic to 'Rutgers') fruits were harvested when mature green at 34 ± 2 d postpollination.

Effect of Galactose Concentration on Ethylene and CO_2 Production. Infiltration of sterile galactose solutions was carried out as previously described (7). Immediately after harvest, 'Rutgers' fruit were rinsed with distilled water and air-dried. Fruit were then vacuum-infiltrated with 0.5 to 1.0 ml of galactose in sterile distilled H_2O, allowing for 0, 4, 40, 100, 200, 400, and 800 μg galactose/gfw of fruit after infiltration. Individual fruit were kept in 470 ml glass jars at 20°C. Ethylene and carbon dioxide production were monitored using an automatic sampling, flow-through system as described previously (29). All handling procedures of the freshly harvested fruit were carefully undertaken to avoid any wounding of the fruit.

ACC Determination and ACC Synthase Assay. Ethylene production was monitored from fruit treated with 400 μg galactose/gfw, and three fruit were taken at selected times based on the course of C_2H_4 production as described in Figure 1. Fruit were cut in half equatorially and the pericarp tissue from the top half was frozen and lyophilized for subsequent ACC determination. Dry-powdered tissue, corresponding to 5 g of fresh tissue, was extracted with 10 ml of 80% ethanol and centrifuged at 27,000g for 15 min. The supernatant was made up to 20 ml with 80% ethanol and 4 ml of the extract was kept for free galactose determination. The remaining 16 ml of the ethanolic extract was taken to dryness under N_2 at 50°C and the residue dissolved in 1 ml of distilled H_2O. ACC analysis was performed as described

[1] Use of a company or product name by the United States Department of Agriculture does not imply approval or recommendation of the product to the exclusion of others which may also be suitable. From a dissertation to be submitted to the graduate school, University of Maryland, by Mr. Jongkee Kim in partial fulfillment of the requirements for the Ph.D. degree in Horticulture.

[2] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, S-adenosyl-methionine; gfw, gram fresh weight.

by Lizada and Yang (18).

Measurement of ACC synthase activity was carried out according to the method described previously (3, 32). Fresh pericarp tissue, including columella tissue (20 gfw), was homogenized with a mortar and pestle with 20 ml of 100 mM HEPES-KOH buffer (pH 8.2), containing 5 mM DTT, 0.5 μM pyridoxal phosphate, and 0.1% PVP. After centrifugation at 27,000*g* for 15 min, the supernatant was dialyzed overnight against 10 mM HEPES buffer (pH 8.2), containing 0.5 mM DTT and 0.5 μM pyridoxal-P. The buffer solution was changed twice during dialysis. The procedure was carried out at 0 to 4°C. The reaction mixture (0.6 ml) of the enzyme assay consisted of 0.4 ml of desalted enzyme extract, 50 μM SAM, 1.0 μM pyridoxal-P, and 0.2 ml of 50 mM HEPES-KOH buffer (pH 8.2). Samples were incubated at 28°C for 3 h and the amount of ACC formed was determined by the method of Lizada and Yang (18). Controls without SAM were run for each enzyme assay.

***In Vitro* Assay of ACC Synthase with Various Sugar Solutions.** Pericarp tissue (20 gfw) from light red 'Rutgers' fruit was sliced into 2 mm sections and kept at 23°C under aseptic conditions to allow for wounding-induced ACC synthase formation (13). After 4 h incubation, the sliced tissue was extracted, dialyzed, and assayed for ACC synthase activity as described above. The desalted enzyme extract was assayed in the presence of the following sugar solutions; 0.1, 1.0, and 10.0 mM galactose and 1.0 mM sucrose, mannose, glucose, galacturonic acid, or dulcitol.

Soluble Galactose Determination. Free, monomeric galactose from the pericarp tissue which was infiltrated with 400 μg/gfw was determined as previously described with a few modifications (6). Ethanolic extracts, saved from ACC analysis, were evaporated to dryness at 50°C under a stream of N_2. Soluble monosaccharides were made into their aldononitrile acetate derivatives (17) and quantified using a GC-MS equipped with a 25 m WCOT fused silica 5% phenylmethylsilicone capillary column (0.2 mm i.d.). Selected ion monitoring (*m/e* 145) was used for quantification; allose was used as an internal standard.

RESULTS AND DISCUSSION

Effect of Galactose Concentration on Ethylene and CO_2 Production. Infiltration of various concentrations of galactose into mature green 'Rutgers' tomato resulted in different responses in ethylene and CO_2 production (Table I). No consistent response was observed in fruit infiltrated with less than 400 μg galactose/gfw. Although the time course and the rate of ethylene and CO_2 evolution varied somewhat between fruit, presumably due to slight differences in maturity and/or galactose penetration, most fruit showed a wounding-induced ethylene peak due to vacuum-infiltration after 6 ± 2 h (Fig. 1). Similar to previous results on promotion of ripening by galactose (7), fruit applied with 400 or 800 μg/gfw of galactose showed a second, transient peak 25 ± 5 h after infiltration, ranging from 4.4 to 10.2 μl C_2H_4/kg·h, whereas fruit treated with 0, 4, or 40 μg/gfw of galactose did not. In addition to the stimulation of ethylene production, CO_2 production was also stimulated by the higher amounts of galactose concurrently with the increase in ethylene production, ranging from 48.2 to 65.7 mg CO_2/kg·h. The amount of free, monomeric galactose in mature green and red ripe 'Rutgers' fruit is approximately 30 and 220 μg/gfw, respectively (6). Therefore, it is noteworthy that only 400 μg/gfw of galactose was able to stimulate both ethylene and CO_2 production. Furthermore, we have observed that in some fruit as little as 100 or 200 μg/gfw of galactose is able to induce the second, transient ethylene production peak (Table I).

Table I. *Effect of Galactose Concentration on Ethylene Production by Infiltrated Mature Green 'Rutgers' Fruit*

Ethylene production during the second, galactose-induced, transient peak ranged from 4.4 to 10.2 μl/kg·h. Carbon dioxide production was stimulated concurrently with ethylene, ranging from 48.2 to 65.7 mg/kg·h.

Galactose Amount	No. of Fruit Tested	No. of Fruit Showing Ethylene Stimulation	Percent Showing Response
(μg/gfw)			
0	16	0	0
4	8	0	0
40	12	0	0
100	8	1	13
200	8	2	25
400	16	14	88
800	8	7	88

Changes in ACC Content and ACC Synthase Activity after Galactose Infiltration. Galactose infiltrated into mature green 'Rutgers' tomatoes stimulated ACC synthase activity and consequently enhanced ethylene production (Fig. 1) whereas H_2O-infiltration did not. When intact fruit were infiltrated with 400 μg/gfw of galactose, the amount of ACC increased 2.5-fold during the second ethylene peak. The ACC content in mature green tomato has been shown to be relatively low, ranging from 0.1 to 1.0 nmol/gfw (12, 26) and to increase proportionally to the climacteric rise in ethylene production (12, 26). The same trend has been reported for ACC synthase activity (13, 26). In the present study, the increase in ACC synthase activity by galactose-infiltration closely paralleled the pattern of ethylene production, while ACC content increased less. This is probably due to the fact that mature green tomato fruit, which were sampled at 5 ± 2 d before the onset of the climacteric rise in ethylene production, have a low level of ACC (12, 26) and the conversion of SAM to ACC is known to be a rate-limiting step in ethylene biosynthesis (3, 31). Thus, the increased ACC due to enhanced ACC synthase

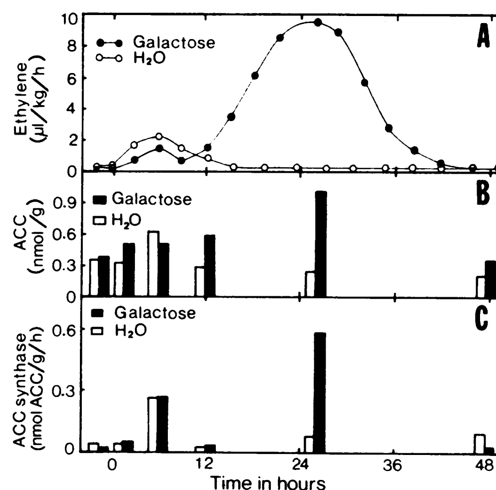

FIG. 1. Effect of exogenous galactose on ACC content (B) and ACC synthase activity (C) during the stimulation of ethylene production (A). Whole intact fruit (mature green) were infiltrated with 400 μg/gfw of galactose or sterile distilled H_2O alone. A typical pattern of ethylene production from individual fruit after galactose or H_2O infiltration is shown. ACC content and ACC synthase activity were measured in fruit sampled immediately before infiltration, immediately after infiltration, during the first ethylene peak, before onset of the second ethylene peak, during the second ethylene peak, and after ethylene production had returned to baseline levels after the second ethylene peak. Time 0 refers to time of infiltration. Data shown are the average from three individual fruit.

activity would be readily converted to ethylene by EFE (ethylene forming enzyme) (25, 31).

When desalted ACC synthase extracts were assayed in the presence of galactose, no increase in activity was observed at any of the concentrations tested (Table II). Enzyme activity in the absence of any carbohydrate was 1.8 nmol ACC/gfw·h, somewhat lower than a previous report (13). None of the carbohydrates tested stimulated the activity of the enzyme *in vitro*. Although exogenously applied galactose stimulates ACC synthase activity and, subsequently, ethylene production in intact fruit (Table I; Fig. 1), it does not seem to be acting as an allosteric effector of ACC synthase. Thus, the results in Figure 1 and Table II suggest that exogenous galactose may possibly be involved in the induction of *de novo* synthesis of ACC synthase. Further studies using ACC synthase protein determination are necessary to clarify this possibility.

Galactose also stimulated ethylene production in *rin* (Fig. 2) and *nor* fruit (data not shown). When mature green *rin* fruit (34 ± 2 d postpollination) were infiltrated with 400 μg/gfw of galactose, glucose, or H_2O alone, only fruit treated with galactose showed a second, transient ethylene peak. However, the extent of the stimulation of ethylene production in mutant fruit was lower compared to that of 'Rutgers' fruit (Table I; Fig. 1). The

Table II. *Effect of Various Monosaccharides on the Activity of ACC Synthase in Vitro*

Pericarp tissue from light red tomato fruit ('Rutgers') was sliced and kept 4 h under aseptic conditions. ACC synthase activity was determined in preparations of desalted enzyme extracts. Data presented were obtained from three replications of each sugar solution. See "Materials and Methods" for details about assay conditions.

Carbohydrate	Sugar Concentration	Activity[a]
	mM	*% of control*
Galactose	0.1	100.0 ± 8.11
	1.0	98.6 ± 3.88
	10.0	92.0 ± 4.19
Mannose	1.0	110.8 ± 2.70
Dulcitol	1.0	104.7 ± 2.70
Glucose	1.0	98.6 ± 4.86
Sucrose	1.0	94.9 ± 3.38
Fructose	1.0	94.9 ± 3.38
Galacturonic acid	1.0	79.1 ± 11.76

[a] Control (without any sugar) activity of the enzyme extract was 1.8 nmol ACC/gfw·h.

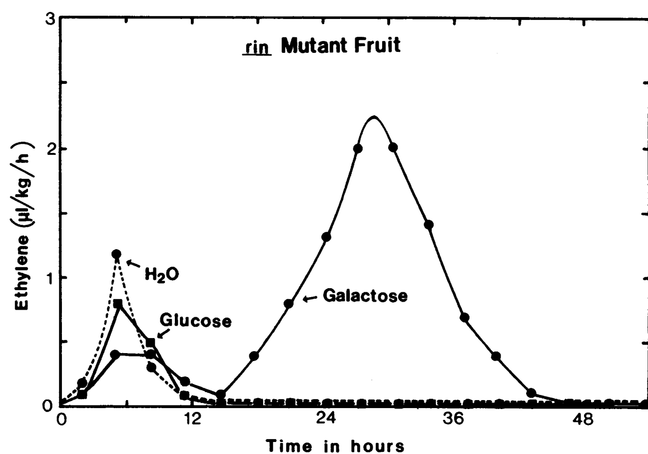

FIG. 2. Ethylene evolution from mature green *rin* mutant fruit. Fruit, 34 ± 2 d postpollination, were infiltrated with 400 μg/gfw of galactose, glucose, or H_2O alone. The ethylene production profile shown is representative of four individual fruit per treatment.

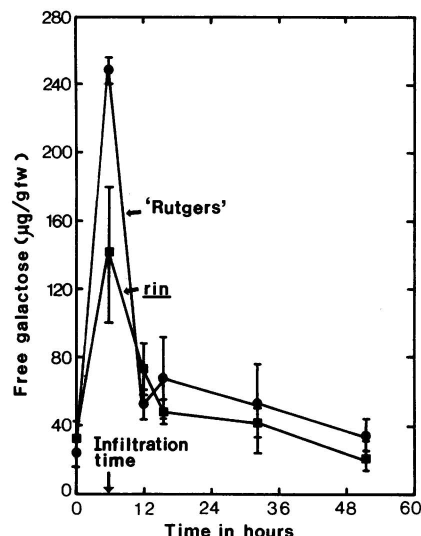

FIG. 3. Changes in soluble galactose content from galactose-infiltrated normal and *rin* tomato fruit. Monomeric galactose was quantified in the pericarp tissue from three fruit sampled at the times after infiltration as described in Figure 1. Bars at each data point represent the mean ± SD of three individual fruit.

galactose stimulation of ethylene production in *rin* was accompanied by an increase in ACC synthase activity during the second ethylene peak after infiltration (data not shown). It has been reported that wounding pericarp tissue from *rin* tomato stimulated ethylene production (11) which was due to an increase in ACC synthase activity (13). The mutant fruit in the present study did not initiate ripening after infiltration; they were monitored for 2 weeks, which is sufficient time for 'Rutgers' fruit to reach the fully red-ripe stage. As discussed previously (20, 24), *nor* and *rin* fruit lack autocatalytic ethylene production and do not have the ability to ripen. Thus, these mutant fruit apparently do not have the ability to respond to ethylene, *i.e.* lack tissue sensitivity to this gaseous hormone (27).

Changes in Free Galactose after Infiltration. Infiltrated galactose was rapidly metabolized in mature green tomatoes (Fig. 3). The amount of free, monomeric galactose in the pericarp tissues from both 'Rutgers' and *rin* fruit was approximately 30 μg/gfw prior to infiltration. The content of this monosaccharide increased to 250 μg/gfw in 'Rutgers' fruit immediately after infiltration and decreased rapidly within 6 h, subsequently returning to its endogenous level in 50 h. Mutant fruit (*rin*) showed a pattern similar to that of 'Rutgers' fruit. Exogenously applied galactose is thought to be converted to galactose-1-P which has been suggested to competitively inhibit the conversion of glucose-1-P to UDP-glucose by hexose 1-P uridyltransferase (19), resulting in the inhibition of cell wall polymer synthesis in *Avena* coleoptiles (30).

In vegetative tissues, evidence has been reported that various carbohydrates including galactose, sucrose, and glucose can stimulate ethylene production (4, 21). We have also observed that carbohydrates other than galactose stimulate ethylene production in mature green 'Rutgers' tomato (data not shown). Among the sugars tested, dulcitol, galacturonic acid, and mannose, at 400 μg/gfw, stimulated ethylene production, whereas arabinose, fructose, glucose, lactose, raffinose, rhamnose, sorbitol, sucrose, and xylose had no effect.

The mechanism by which carbohydrates stimulate ethylene production in tobacco leaf discs (21) may involve an increase in ACC synthase activity via the enhancement of the hydrolysis of IAA conjugates to yield free IAA (1). Further study is needed to determine if the galactose stimulation of ethylene production in

tomatoes may possibly be associated with an IAA-induced enhancement of ethylene production via ACC synthase (28).

The ability of exogenous galactose to stimulate ACC synthase activity in mature green tomatoes (Fig. 1) in concert with the observations that a substantial loss of galactosyl residues from cell wall polysaccharides (10, 16) and a 4- to 6-fold increase in free, monomeric galactose occurs during tomato fruit ripening (6, 8), suggest that it is of importance to study galactose metabolism and cell wall turnover and their temporal relationship to ethylene biosynthesis during tomato fruit ripening.

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