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J. Amer. Soc. Hort. Sci. 109(5):712–717. 1984.

Ripening Behavior of the *Green Ripe* Tomato Mutant

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Additional index words. *Lycopersicon esculentum*, ripening

Abstract. **The fruit ripening behavior of the green ripe mutant (*Gr*) of tomato (*Lycopersicon esculentum* Mill.) was examined. Green ripe fruit are climacteric and evolve increasing amounts of ethylene after harvest; however, the time course for these events is dramatically altered in comparison with 'Rutgers'. Maximal rates of C_2H_4 evolution from *Gr* fruit were achieved 20 days after the initial increase, and 7 to 10 days prior to maximal respiratory rates. Fruit age at harvest did not affect either the rate or the magnitude of these processes. Wavelength scans of pigment extracts from 60 day postharvest *Gr* fruit indicated low levels of carotenoids. Mutant fruit also remain firm a long time after harvest. Polygalacturonase activity in *Gr* fruit increases with fruit age, but reaches only 3% to 5% of the total activity in 'Rutgers'. PG activity was only slightly reduced when extracts were heated to 65°C for 5 min, suggesting that a heat stable isoenzyme of PG is predominant in mature mutant fruit in contrast to 'Rutgers' in which 90% to 95% of PG activity in ripe fruit is heat labile under these conditions. When heterozygous, the *Gr* mutation is dominant in its effects on total PG activity and on the time course from the initiation of C_2H_4 and respiratory increases to their maximal rates of evolution. The magnitude of other changes in heterozygous mutant fruit was intermediate between normal and homozygous *Gr*.**

Specific mechanisms regulating the initiation and coordination of the biochemical and physiological changes associated with fruit ripening remain largely unknown (24). Recent attempts to elucidate these mechanisms have involved studies utilizing several mutants of tomato (*Lycopersicon esculentum*) in which the individual major fruit ripening parameters are altered either simultaneously or differentially.

Three mutations, never ripe (*Nr*), ripening inhibitor (*rin*), and nonripening (*nor*), which effect multiple aspects of the ripening process, have been characterized and described (27). In addition, many mutations which effect pigment changes alone (19), or are additional alleles of previously described mutations (18), are known.

The green ripe (*Gr*) mutant has not been examined with respect to its ripening behavior. It was reported in 1952 as a monogenic recessive mutation effecting chlorophyll retention in mature fruit (17). It arose as a somatic mutation and was isolated from a chimeric fruit showing red and green sectors. It is now known to be a dominant mutation, and genetic tests have clearly indicated that this mutation is not allelic with *Nr*, *rin*, or *nor* (Tigchelaar, unpublished). This study was undertaken to examine the effect of the *Gr* mutation on tomato ripening in comparison with normal ('Rutgers') tomato fruit.

Materials and Methods

Plant material. Plants of the mutant 'Green ripe' (*Gr*)[2] and 'Rutgers' (normal) were grown in the greenhouse at Purdue Univ. in 5 gallon plastic pots filled with a greenhouse potting mix. In several studies, heterozygous fruit of the F_1 hybrid ['Rutgers' x 'Green ripe' (+*Gr*)] also were included for comparison. Supplemental fluorescent lighting was used to provide a 16 hr photoperiod. Plants were pruned to a single stem, and

Received for publication 30 Jan. 1984. Purdue Agr. Expt. Sta. Journal No. 9779. This research represents a portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the PhD degree. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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[2]*Gr* stocks used were 4th backcross isogenic lines using Rutgers as the recurrent parent.

flowers were vibrated mechanically and tagged at anthesis. Not more than 3 fruit were allowed to develop per cluster. Since it was not possible to determine the initiation of ripening in *Gr* or *+Gr* fruit visually, we defined 'mature green' as those fruit which initiated an increase in ethylene evolution 3 to 5 days after harvest. This stage of maturity was achieved about 40 days after anthesis for fruit of normal, *Gr* and *+Gr*. Unless specified otherwise, all fruit utilized in this study were harvested at the 'mature green' stage of maturity and stored at 20°C.

Respiration and ethylene. Twenty-eight fruit of each genotype were harvested and surface sterilized by immersion for 1 min in a solution containing 0.5% NaOCl, 0.1% (w/v) Benlate, and Tween 20 (2 drops/100 ml). Individual weighed fruit were placed in one liter sterilized glass canning jars and maintained at 20 ± 1°C. Jars were ventilated with a continuous stream of CO_2-free humidified air at a flow rate of one liter ± 100 ml/hr. CO_2 determinations were made daily on a Beckman infrared gas analyzer (Model 315A). One milliliter samples of the effluent air stream were taken daily and analyzed for ethylene by gas chromatography (Hewlett-Packard model 5730A), and peak areas were determined with an integrator (Hewlett-Packard 33805). At specified intervals, 5 fruit were removed, ground together, and aliquots were frozen or lyophilized.

For determination of wound ethylene, 5 fruit at various stages of maturity were quartered, surface sterilized by immersion in 0.5% NaOCl, rinsed in sterile distilled water, and returned to the glass canning jars for measurement of CO_2 and ethylene (10). Respiration and ethylene measurements were made at 3 hr intervals for a total period of 24 hr as described earlier.

Pigments. Chlorophyll and carotenoid determinations were made on freeze-dried powders. Chlorophyll was determined as described by Arnon (1). One hundred mg of powdered fruit tissue was blended with 80% aqueous acetone (total volume 10 ml) in a polytron homogenizer for 60 sec, centrifuged at 3,000 × g for 5 min at 3°C, and the optical density was determined on aliquots of the supernatant in a Beckman DU-2 spectrophotometer.

Carotenoids were extracted from the lyophilized fruit with an acetone : hexane (4 : 5, v/v) mixture (19). Pigment profiles were obtained on a Beckman DU-8 spectrophotometer.

Extraction of enzymes. Polygalacturonase (PG) and pectinesterase (PE) were extracted from the frozen ground tomato fruit used for respiration and ethylene studies essentially as described by Pressey and Avants (23). Fruit tissue was blended with cold water (1 : 1, w/v) for one min and centrifuged at 5,000 × g for 10 min. The pellet was resuspended in one M NaCl and rehomogenized with a polytron homogenizer (Ultra Turrax, model SDT, Tekmar Co.) for 60 sec. The suspension was adjusted to pH 6.0 with 1.0 N NaOH and stirred for 3 hr at 3°C. Debris was removed by sedimentation at 5000 × g for 10 min. Partial purification was achieved by further fractionation with the addition of solid $(NH_4)_2SO_4$ to 80% saturation (56 g $(NH_4)_2SO_4$/100 ml). The precipate was collected by centrifugation at 20,000 × g for 15 min, resuspended in 0.15 M NaCl (pH 6.0), and dialized for 24 hr against 0.15 M NaCl, pH 6.0. After recentrifugation at 20,000 × g for 20 min, the supernatant served as a source of partially purified enzyme for determination of PG and PE activity. All activities are expressed on a mg protein basis. Protein was determined as described by Bradford (4) using bovine serum albumin as standard.

Assay of pectinesterase. Pectinesterase activity was determined in partially purified preparations as described by Rouse and Atkins (25) using 1% pectin (polygalacturonic acid methyl ester, Grade I, Sigma Chemical Co.) as substrate. PE activity was determined from the amount of 0.05 N NaOH required to maintain a substrate pH of 8.0 for 5 min at 25°C. Activity is expressed as milliequivalents of acid produced per min per mg protein.

Assay of Polygalacturonase. PG activity in partially purified extracts was assayed by measuring the formation of reducing groups at 25°C in reaction mixtures containing 0.25% polygalacturonic acid (Grade II, Sigma Chemical Co.), 0.15 M NaCl, in 0.05 M Na acetate buffer, pH 4.5 (22).

The relative proportions of heat stable polygalacturonase (PG I) to heat labile polygalacturonase (PG II) activity were estimated by heating an aliquot of enzyme extract at 65°C for 5 min in 0.05 M Na acetate buffer pH 4.5 (29). A preliminary time course study indicated that this treatment consistently inactivated in excess of 90% of the total PG activity present in extracts from ripe normal fruit, with less than 2% further loss of activity with an additional 5 min heating. Heat treated samples were cooled and immediately assayed.

Results

Fruit of the *Gr* mutant are climacteric and evolve increasing amounts of ethylene after harvest; however, the maximal rates of CO_2 and ethylene evolution are much lower than normal (Figs. 1 and 2). Carbon dioxide evolution from 'Rutgers' fruit rapidly increased about 250% during the ripening period of 4 to 6 days. In contrast, respiratory activity in *Gr* fruit declined for 8–10 days after harvest and then increased slowly over a 20 day period. In normal fruit, the respiratory climacteric and ethylene evolution were initiated more or less simultaneously, whereas in *Gr* fruit a detectable increase in C_2H_4 production preceeded the slow increase in respiratory activity by 4 to 8 days. Initial increases in C_2H_4 evolution occurred 4 to 8 days after harvest and continued to increase over a period of about 20 days in *Gr* fruit as compared to 3 to 5 days in normal fruit. Total amounts of ethylene evolved, from initiation to maximal rates of evolution, were about the same in *Gr* and normal fruit. The respiratory behavior of fruit heterozygous at the *Gr* locus more closely resembled the response of the homozygous mutant. While the maximal rates of CO_2 and C_2H_4 evolution from the heterozygote were intermediate to those of the other types, the time from the

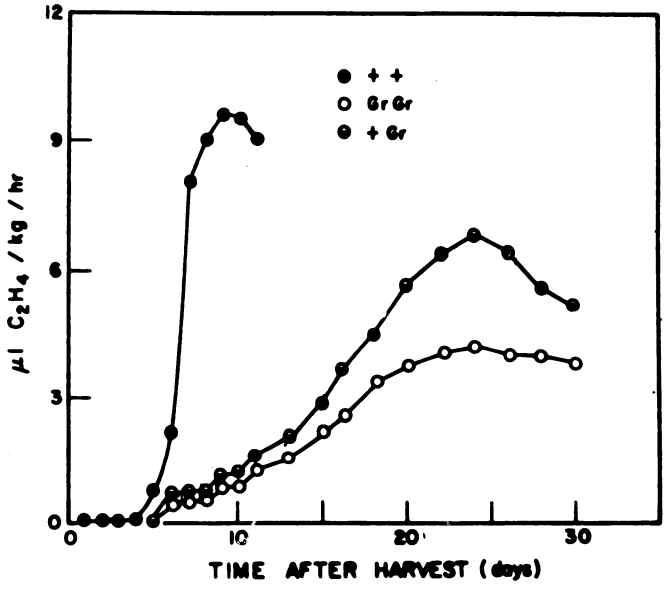

Fig. 1. Ethylene production of normal, *Gr*, and *+Gr* fruit.

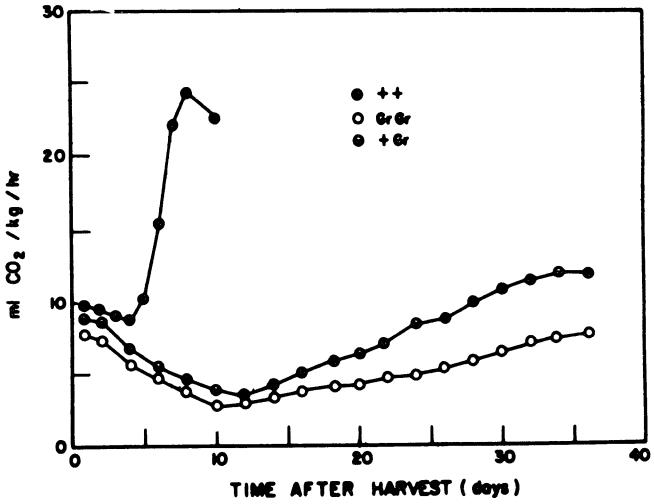

Fig. 2. Respiratory rates of normal (+), *Gr*, and +*Gr* fruit.

initial increase to maximal rates of evolution was similar to the homozygous mutant. Also, the onset of the respiratory climacteric in heterozygous fruit lagged behind a detectable increase in ethylene evolution by 4 to 8 days.

The presence of a factor affecting autocatalytic production of ethylene was examined by observing the capability of these fruit to evolve wound ethylene (by quartering whole fruit) at various stages during ripening (Fig. 3). Mature green *Gr* fruit produced only a small amount of ethylene 24 hr after cutting. As ethylene evolution accelerated in intact *Gr* fruit, so did the capability of these fruit to evolve increased amounts of C_2H_4 after wounding. Similar effects of wounding on pericarp disks from normal tomatoes have been observed (16). Ethylene evolution from wounded fruit was monitored over a period of 24 hr and was still increasing after this time in all fruits examined.

The effect of fruit age on the timing of the initiation and maximal rates of C_2H_4 and CO_2 evolution was examined in the

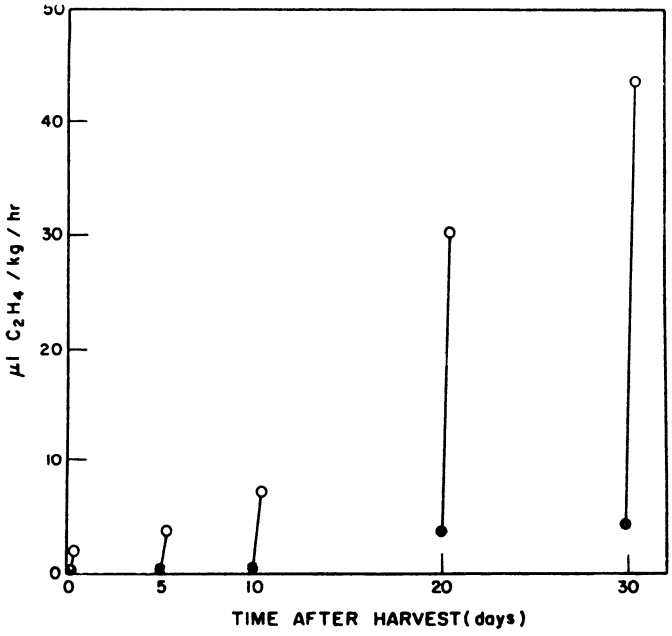

Fig. 3. Ethylene production of intact (●) and wounded (○) *Gr* tomato fruit. At various times after harvest the intact fruit were quartered; wound ethylene from these measured 24 hr later.

event that some unidentified compound was accumulating in, or being translocated into the fruit tissue, thus affecting the rate at which these processes occur (6). It has been previously shown that fruit age at harvest affects only the timing of the initiation of ripening and not the magnitude or the rate at which changes occur. We found this effect to be the case in *Gr* fruit harvested 30 and 40 days postanthesis (65% and 90% maturity, respectively) (Fig. 4). Initiation of ripening, as determined by the occurrence of the respiratory climacteric and increased levels of ethylene evolution, was delayed in the immature fruit (15 days postharvest) to a greater extent than in 40-day old fruit (5 to 7 days postharvest). The rate at which these ripening parameters changed after initiation was similar in both 30- and 40-day-old fruit. The maximal rates of CO_2 and C_2H_4 evolution also were similar.

Chlorophyll degradation proceeded rapidly in normal fruit in contrast to *Gr* or +*Gr* fruit in which chlorophyll degradation proceeded slowly. Extracts from mature green normal fruit contained an average of 7.8 mg chlorophyll/g fresh weight, which dropped to below 1% of this original value 10 days after the initial increase in ethylene evolution. Forty-day postanthesis *Gr* fruit initially contained less chlorophyll (4.4 mg/g fresh weight) than normal. Additionally, chlorophyll loss proceeded over a period of about 40 days in *Gr* fruit. The 1st visually detectable signs of chlorophyll degradation in *Gr* fruit coincided approximately with initiation of ethylene evolution. Chlorophyll degradation in *Gr* and +*Gr* fruit was irregular and resulted in blotchy fruit. Initial chlorophyll values and the rate of degradation in +*Gr* fruit were similar to those in the homozygous mutant.

Wavelength scans of acetone/hexane extracts of pericarp tissue from ripe normal fruit and 60 day postharvest *Gr* and +*Gr* fruit dramatized the effect of this mutation on carotenoid synthesis and accumulation (Fig. 5). Total carotenoids were greatly reduced in 60 day postharvest *Gr* fruit in comparison with normal. Homozygous mutant fruit occasionally showed a small blush of red pigmentation in the centermost region of the columella portion of the fruit, and also in the outermost cell layer(s) of the pericarp. Heterozygous *Gr* fruit had accumulated more carotenoids by 60 days postharvest than *Gr* fruit; however, these levels were well below those present in normal fruit. Differences in pigment profiles between extracts from homozygous and heterozygous *Gr* fruit seemed to be primarily quantitative in nature, although a slight shoulder at 502 nm suggested the presence of additional lycopene in +*Gr* extracts.

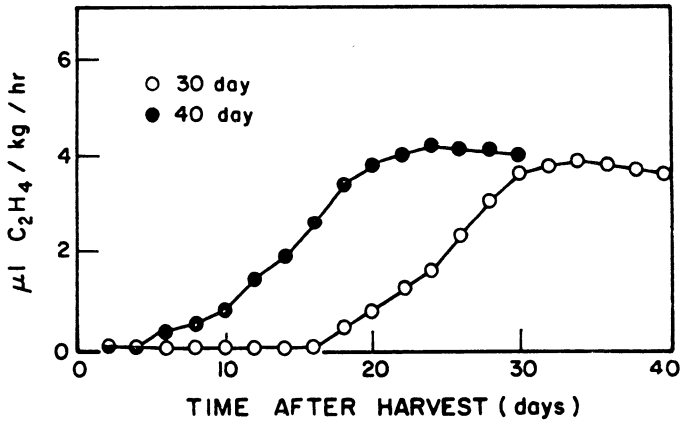

Fig. 4. Etheylene evolution from *Gr* fruit harvested at 30 and 40 days postanthesis.

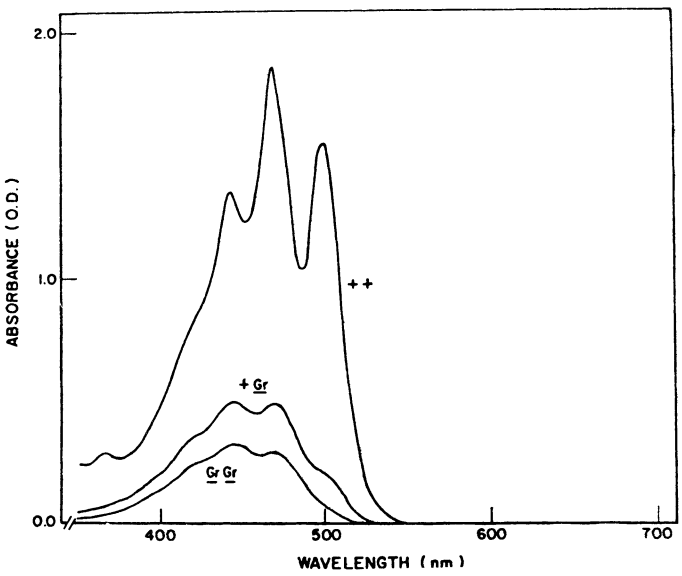

Fig. 5. Wavelength scans of pigment extracts from 15 day postharvest + + and 60 day postharvest *Gr* and + *Gr* fruit.

Changes in levels of pectinesterase (PE) activity in all genotypes appeared to be loosely correlated with ripening (Table 1). PE activity in extracts of *Gr* fruit declined slightly after harvest, increased over a period of 30 days, and then declined again slightly. PE in control fruit followed a similar trend. Levels of PE from + *Gr* fruit were intermediate to those of the parental genotypes.

Total PG activity in partially purified extracts from normal fruit increased dramatically during the ripening period (Fig. 6). Heat treating a partially purified protein extract at 65°C for 5 min (29) decreased enzyme activity dramatically in extracts from 'Rutgers' fruit. The percentage of total activity lost in heat treated extracts increased as fruit ripened. Heat stable PG activity reached a low level (1 μmol reducing groups/hr). Heat labile activity, by subtraction, accounted for 90% to 95% of the total activity present in the late stages of ripening (25 μmol reducing groups/hr).

Maximal levels of total PG activity in *Gr* reached only 3% to 5% of that present in normal but also increased with fruit age (Fig. 7). Heat treating extracts from *Gr* fruit in the same manner had only a small effect of the total PG activity. Activity decreased from about 0.9 μmol reducing groups/hr to about 0.8 μmol reducing groups/hr. Heat labile activity (by subtraction) represented about 10% of the total activity present in 60 day postharvest *Gr* fruit. Total PG activity in heterozygous fruit

Table 1. PE activity (± SE) in partially purified enzyme extracts from + +, + *Gr* and *GrGr* tomato fruit at various times after harvest.

+ +		+ *Gr*		*GrGr*	
Days[z]	PE[y]	Days	PE	Days	PE
0	6.91 ± 1.6	0	10.9 ± 1.4	0	11.14 ± 1.1
3	6.76 ± 0.9	10	7.46 ± 1.8	10	8.42 ± 1.3
6	7.45 ± 1.1	20	9.14 ± 1.1	20	14.01 ± 1.7
9	7.08 ± 1.1	30	12.01 ± 1.6	30	14.06 ± 1.5
12	7.56 ± 1.0	45	12.0 ± 1.4	45	14.11 ± 1.1
15	7.44 ± 1.0	60	13.11 ± 1.2	60	9.86 ± 1.2

[z]Postharvest.
[y]m eq./hour.

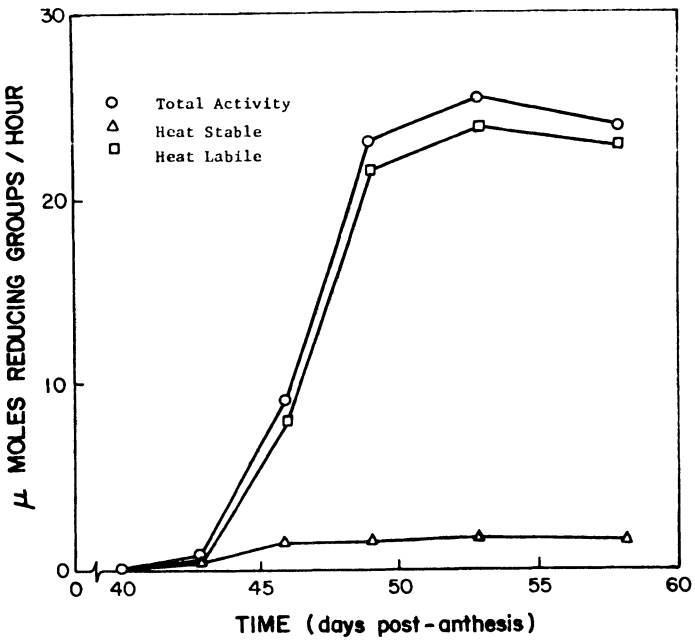

Fig. 6. Total, heat stable, and heat labile PG activity in normal fruit at various stages of maturity.

sampled at mature green, 30 and 60 days postharvest was nearly identical to that present in *Gr* at similar stages of maturity (data not shown). Additionally, it was found that the proportion of PG activity lost upon heating at 65°C for 5 min (10% to 15%) also was the same as for *Gr* fruit.

Discussion

The green ripe mutation affects the magnitude, timing, and the degree of synchronization of several events which occur during tomato ripening. Homozygous *Gr* fruit are climacteric and evolve increasing amounts of ethylene through the period following harvest. Although the percentage increases in CO_2 evolution from the preclimacteric minimum to maximal rates are about the same for both mutant and normal fruit, the time course for these events to occur is significantly altered by the *Gr* mutation. Peak levels of CO_2 and ethylene were achieved 15 to 25 days after the initial increase in *Gr* as opposed to 3 to 5 days in fruit of the normally ripening 'Rutgers'. The initiation of ethylene evolution from *Gr* fruit clearly preceeds any de-

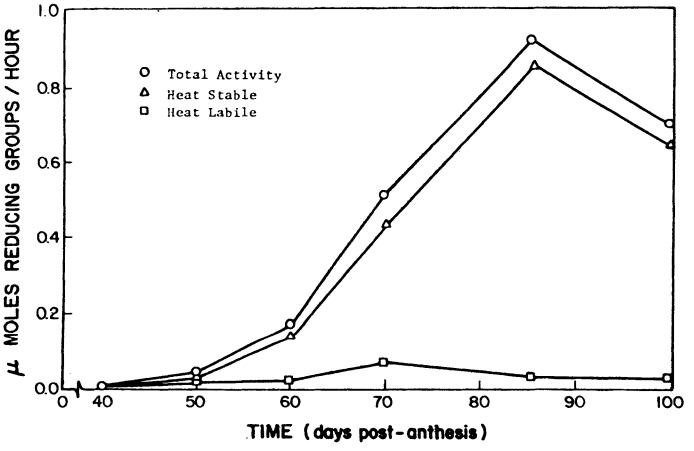

Fig. 7. Total, heat stable, and heat labile PG activity in *Gr* fruit at various stages of maturity.

tectable increase in respiratory activity and maximal rates are achieved 5 to 10 days prior to peak levels of CO_2. This increase in ethylene evolution prior to increased respiratory activity in *Gr* fruit is reminiscent of the ripening behavior of honeydew melons in which the rise in ethylene occurs over a prolonged period of time until a threshold level of C_2H_4 sufficient to trigger the climacteric is achieved (14).

The principle effect of the *Gr* mutation on respiratory activity and ethylene evolution seems to be on the rate at which these processes proceed once initiated, rather than on their time of initiation. The literature contains few reports on factors which regulate the rate of fruit ripening after initiation, although a role for growth regulating compounds has been proposed (2, 6, 7). We found that the rate of increase of ethylene evolution in intact *Gr* fruit is much slower than normal. Hoffman and Yang (14) have suggested that autocatalytic production of ethylene may be due to the inactivation of a repressor allowing the synthesis of 1-aminocyclopropane-1-carboxylate (ACC). Perhaps the *Gr* mutation is affecting the sensitivity of this repressor, reducing its susceptibility to destruction or inactivation.

Restraints to ethylene synthesis in preclimacteric cantaloupe and other fruit may be relieved by wounding, a treatment which also can induce ethylene formation from nonclimacteric tissues (14, 24). In this study, the responsiveness to wounding of *Gr* fruit increased with age. Changes in the responsiveness to wounding as tissues mature are apparently due to the relaxation of restrictions on the induction of ACC synthase and possibly the enzyme catalyzing the conversion of ACC to ethylene (EFE) (14, 16). The rapid response of *Gr* fruit to wounding, which increased in magnitude as fruit matured, is typical of normally ripening tomatoes (16).

Fruit age at harvest generally has no effect on either the magnitude or the rate of occurrence of the respiratory rise in climacteric fruit (3). It has been reported, however, that honeydew melons (21) and fruit of the wild tomato species *L. hirsutum* (9) evolve reduced levels of CO_2 and C_2H_4 if harvested immature, but that the magnitude of the rise increases as fruit are harvested at increasingly later stages of maturity. We found peak rates of CO_2 and C_2H_4 evolution in *Gr* to be independent of fruit age when harvested at 30 and 40 days postanthesis.

The most visible changes associated with fruit ripening of tomato are the loss of chlorophyll and the synthesis and accumulation of carotenoids, principally lycopene and β-carotene. Chlorophyll degradation normally occurs over a period of 3 to 10 days in tomato or over much longer periods of time in citrus (24). Chlorophyll loss proceeded very slowly in *Gr* fruit, and was occasionally incomplete in fruit 60 days after harvest when held at 20°C. A visually discernable loss of chlorophyll seemed to coincide with and parallel the onset and increase of ethylene evolution in contrast to fruit of the wild species *L. hirsutum* which remain green long after a burst of ethylene evolution (9), and fruit of the tomato mutant, leutescent, in which rapid chlorophyll loss in immature fruit (15) preceeds other ripening changes.

The *Gr* mutation also greatly attenuates the accumulation of carotenoids in mutant fruit. This effect is similar to that of the *rin* mutation which inhibits accumulation of lycopene and β-carotene and in which phytoene becomes the predominant carotenoid (26). Very old *nor* fruit show an attenuated but otherwise normal carotenoid profile (20), while *Nr* accumulates β-carotene (27). Coloration of *Gr* fruit was similar to that of the cultivar 'Alcobaca' (18) but a small blush of lycopene was present in the central portion of the columella and the outer layers of the pericarp. Heterozygous *Gr* fruit achieved a greater degree of coloration than homozygous fruit when harvested at 40 days postanthesis and held for 60 days at 20°C. This difference was more visually striking when fruit remained on the plant.

Fruit softening proceeded very slowly and was noticeably attenuated when *Gr* fruit were allowed to ripen at 20°C. This lack of softening could be attributed to very low levels of polygalacturonase in mutant fruit (13). Severe attenuation of the enzyme polygalacturonase is a common feature of all tomato ripening mutants (27). PG activity is virtually absent in *rin* mutant fruit (11), present only in trace amounts in *nor* (20, 28), and is less than 5% of normal in *Nr* (12). Like *Nr*, the *Gr* mutant seems dominant in its effects on total PG activity. Heterozygous *Gr* fruit left on the plant for 100 days postanthesis seemed to soften to a greater extent than homozygous mutant fruit, but these fruit were always cracked and unfit for sampling. There was no apparent difference in the degree of softening between 60 day postharvest *Gr* and $+Gr$ fruit when ripened at 20°C. PG activities in both genotypes were almost identical. Heat treatment of partially purified protein extracts from *Gr* and $+Gr$ fruit at 65° for 5 min resulted in a 10% to 15% decrease in PG activity. This treatment totally inactivates PG II, but only a small percentage of PG I (29). Thus, it seems that both *Gr* and $+Gr$ fruit contain predominantly PG I. Total PG activity in extracts from mixtures of mutant and normal fruit was almost proportional to the percentage of normal fruit in the initial homogenate (data not shown). However, the presence of a factor in *Gr* fruit conferring stability to the heat labile form of this enzyme cannot be discounted. It is interesting to note that the inhibitory effects of gibberellic acid on ripening (7) allowed for a gradual increase in PG activity to 4% of that present in control fruit (2), the approximate percentage of PG I activity present in ripe fruit, suggesting an isoenzyme specific effect for this hormone.

The presence of only a single isoenzyme of PG (PG I) was reported in *Nr* (29) and 'Longkeeper' (5, 8) tomato fruit. PG II likewise has been reported as being conspiciously absent from cell wall associated proteins of the *rin* mutant (30). Although $+Gr$ fruit had greater maximal rates of C_2H_4 and CO_2 evolution and to a lesser extent increased accumulation of carotenoids when compared to *Gr* fruits, total PG activity, lycopene formation, and the time course in which these changes occurred, were unaffected. Recent evidence (5, 30) suggests that polygalacturonase is not a principle factor in the initiation of ethylene evolution and other ripening events; however, the results presented here and elsewhere (5) do not discount the possibility of a catalytic role for this enzyme or for factors effecting the form of this enzyme.

These observations of the *Gr* mutant lead us to suggest that ripening is initiated normally in *Gr* fruit. Those events which normally occur early in the ripening process—C_2H_4 evolution, the appearance of a heat stable PG (29), the initiation of chlorophyll degradation, and an increase in respiration—do occur. Ripening then appears to be inhibited or ceases to be stimulated. Those processes which may be thought to occur later in the ripening process are greatly attenuated or absent, including lycopene accumulation, the appearance of heat labile PG (29) and the rapid rate of increase in CO_2 and C_2H_4 evolution. Owing to the severely protracted ripening pattern of *Gr* fruit, we feel that this mutation may be of use in detailed time course or cause vs. effect studies on fruit ripening.

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