中華農業研究 28(2) : 109~116 (1979)

Studies on fruit ripening

1. Comparisons of ripening parameters, peroxidase activity, and peroxidase zymograme of normal and *rin* mutant tomatoes [1]

Shue-cheng Lin[2]

Abstract Fruit of tomato (*Lycopersicum esculentum* Mill.) cv. Floradel and of an isogenic mutant *rin* harvested at mature-green stage were ripened at 21°C and were measured their activity and zymograme of peroxidase at designated color stages. Ripening of Floradel tomatoes were appeared to follow a pre-determined patterns ; peroxidase activity increased about three times when fruit color chranged from mature-green to breaker stage ; zymograme of peroxidase changed dramatically during fruit ripening. *Rin* mutant did not induce peroxidase activity, respiration, and ethylene evolution ; fruit color changed to yellow after 99 days ; zymograme of peroxidase were not changed ; isozyme *b* and *i* were not appeared in *rin* mutant when compared with Floradel cultivar.

Failure in ripening of *rin* mutant is postulated that lack of some isozymes of peroxidase resulting unability to receive activator(s) , then to activate peroxidase for ethylene synthesis.

Ripening plays important role in quality and subsequent storage life of fruits. Many fruits require ripening for optimum quality and usually senescence is occured soon thereafter. However, the mechanism of fruit ripening remains largely unknown. To reveal the cause of induction in fruit ripening is of interest.

Recently, a number of reports of naturally occurring mutations which affect various aspects of fruit ripening in tomatoes have published [14,18,22]. An isogenic mutant, *rin*, is particularly of interesting because the fruit do not undergo a number of the physiological changes associated with ripening in normal strains. This mutant is considered the best tool to study fruit ripening [5]. It has been reported that *rin* mutant do not ripen in terms of color change to red [21], little or no change in respiration and ethylene evolution [8], neither propylene nor ethylene exogenous applications induced normal ripening [8] and no response to exogenous abscisic acid and benzyladenine [16].

Ripening associated enzymes has been investigated in *rin* mutant with results of no

1. Contribution No. 878 from the Taiwan Agricultural Research Institute.

2. Senior Specialist. Department of Horticulture, TARI. Wufeng, Taichung Hsien, Taiwan 431, ROC.

polygalacturonase activity detected, a relative constant pectinesterase activity, and increasing Cx-cellulase activity during ripening [2]. Peroxidase has been fond essential in ethylene synthesis through a methionine-methional-ethylene pathway in tomatoes [24]. It supposed that characteristic changes of peroxidase can be involved in fruit ripening.

This research was done to compare changes in respiration, ethylene evolution, peroxidase activity and its zymograme between a normal strain and *rin* mutant tomatoes.

Materials and menthods

Sampling of fruit. Tomato flowers occupied the no. 2 and 3 positions of each cluster were tagged at anthesis and remaining flowers were removed to increase fruit set. Mature-green fruits with same growth age were harvested and sampling was completed within 24 hours after harvest. Only fruits that were blemish-free and uniform were selected for treatment.

Respiration, ethylene evolution, and color measerment. Respiration was determined by passing a known volume of air over a weighed amount of fruit enclosed in a wide-mouth gallon glass jar at 21°C. Evolved CO_2 from the respiring tomatoes was picked in the air stream and its concentration was analyzed with a Beckman Model 215. A infrared CO_2 analyzer and results were printed using a 24-point westronics recorder. Ethylene was measured by using a Hewlett Packard 5710 A gas chromatograph with a flame ionization detector. The column was 6' x 1/8" O. D. aluminum tubing packed with activated alumina. The flow rate of hydrogen and carrier gas (purified nitrogen)was 60 ml/mil ; compressed air was 300 ml/min. Oven temperature was maintained at 100°C and the injector temperature was 150°C. Color was based on an index with 0 (mature-green), 1 (breaker), 2 (turning), 3 (pink), 4 (red), and 5 (soft-ripe). *Rin* tomato color was classified into four grades as 1 (mature-green), 2 (green-yellow), 3 (yellow-green) and 4 (yellow). Each sample contained 20 tomatoes and total sample score was calculated as product of color index x number of fruit.

Enzyme preparations and activity analysis. A representive sample of 2-3 fruits were cut into sections, the seeds and highly acid locular contents were removed. One hundred g of fruit wall tissue were then commixed in a waring blender with 25 ml of 0.1M, pH 6.0 potassium phosphate buffer containing 2mM EDTA, 0.5% (W/V) insolubilized polyvinylpyrolidone (PVP). The slurry was squeezed through 4-layer cheesecloth and centrifuged at 30,000 g for 20 min. The supernatant was partial purified with ammonium sulfate. Precipitated protein was collected by centrifugation when the fraction measured 60-80% saturation. This was redisolved in 10 ml of 0.1M, pH 6.0 potassium phosphate buffer and dialyzed against 50 ml of same buffer for 12 hrs. Changed twice during dialysis. This clear dialyzed enzyme solution was stored at 0°C and used to determine peroxidase and for disc gel electrophoresis. Protein content determined by the method of Lowry *et al.* [12]. Peroxidase activity was measured by following the change in absorbance at 460 μM due to the oxidation of o-dianisidine in the presence of H_2O_2 [1]

The electrophoresis procedure used was basically that of Davis [1] using a Buchler Polyanalyst Disc Electrophoresis apparatus and Beckman Spino Duostat power supply. The gel columns (5 mm I. D., 65 mm long) were composed of 2 sections, the upper 5 mm with a 5.0% spacer gel and the lower 60 mm with a 7.0% (W/V) running gel. The gel was in a low-conductivity TRIS,(tris-hydroxy-methylamino methane)-glycine-HCl, buffer of pH 8.9 in upper chamber and pH 8.6 in the lower chamber. The temperature of the buffer surrounding the gel columns was kept at 5°C. The end electrophoresis was completed for 2 hours. Gel staining was followed the method of Seevers *et al.* [9] .Bands were recorded by visual intensity.

Results

Mature-green Floradel fruits initiated the climacteric rise about 2 days after harvest and attained the climacteric peak on the 6th day, whereas *rin* fruits decreased in respiration rate for 4 days, then stabilized thereafter for the duration of the experiment. Ethylene evolution was directly related to respiration changes in Floradel fruit; however, *rin* tomatoes evolved a very low (almost zero) amount of ethylene during the experiment (Fig. 1).

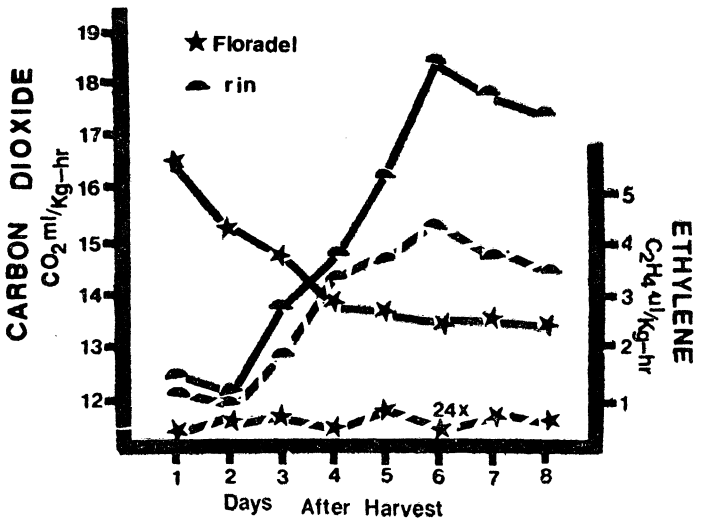

Fig. 1. Comparisons of respiration rate and ethylene evolution by rin and Floradel tomatoes at 21°C. Value of ethylene evolution of *rin* was magnified 24 folds.

Color of Floradel fruits remained green for 2 days, then changed to an advanced breaker stage after 4 days. The turning stage was attained after 6 days. *Rin* tomatoes Stayed at the green stage for about 15 days, then, changed to green-yellow stage after about 30 days, changed to yellow-green after 61 days, and turned to complete yellow after 99 days (Table 1).

Table 1. Days attained the designated color stages of Floradel and *rin* tomatoes at 21°C.

Floradel		*rin*	
Color Stage	Days from Harvest	Color Stage	Days from Harvest
Mature-green	2	Mature-green	15 ± 2[a]
Breaker	4	Green-yellow	30 ± 8
Turning	6	Yellow-green	61 ± 12
Pink	7	Yellow	99 ± 20
Red	10		

[a] Average of three replication with 5 fruits per sample, and standard deviation.

There was no difference in protein content due to either cultivear or stage of color development. Peroxidase total activity in Floradel tomato increased about 3 times from green to breaker stage, increased slight to the turning stage and then, decreased at the pink stage. However, peroxidase activity was not associated with color stage in *rin* tomatoes, although enzyme activity was slightly higher at mature-green stage. Peroxidase specific activity showed similar changes as total activity in Floradel tomato but there was no change in *rin* tomato (Table 2).

Table 2. Comparison of protein content and peroxidase activity of Floradel and *rin* tomato at designated stages of color development.

Color stages	Protein content	Total activity	Specific activity
	mg/g f. w.	units	units/mg protein
Floradel[a]			
Mature-green	0.0240±0.0028[c]	3.320±0.622	139.067±21.956
Breaker	0.0248±0.0026	8.913±1.536	355.055±51.187
Turning	0.0242±0.0040	8.756±1.292	368.153±77.193
Pink	0.0245±0.0038	7.469±1.188	316.169±74.057
rin[b]			
Mature-green	0.0286±0.0084	4.865±1.392	174. 81±10.952
Green-yellow	0.0241±0.012	4.530±1.084	186. 95±38.238
Yellow-green	0.0244±0.029	4.173±0.974	171. 24±11.103
Yellow	0.0246±0.049	4.579±1.074	185. 43±18.548

a. Average of 3 replications with 3 fruits per sample.

b. Average of 2 replications with 2 fruits per sample.

c. Standard deviation.

Tomato isoenzymes separated by electrophoresis appeared as a brown color in peroxidase when detected with o-dianisidine-H_2O_2. A total of nine isoenzymes were

detected in Floradel tomato and were coded from *a* to *i*. Their rate of mobility (≒R_f) value was 0.016, 0.05, 0.29. 0.49, 0.54, 0.63, 0.68, and 0.79 respectively, starting from anode to cathode. These isoenzymes were all anionic ; cationic isoenzyme was non-detectable (Fig. 2).

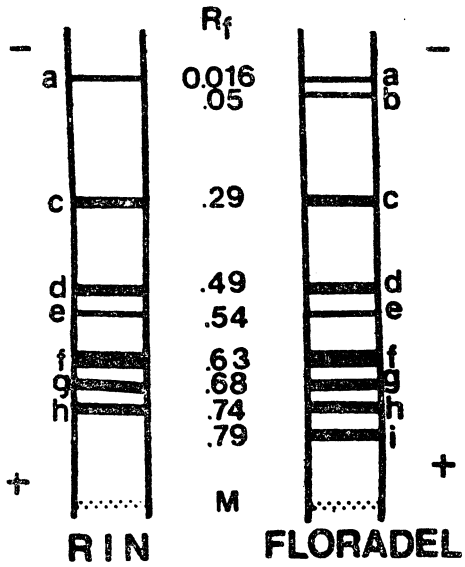

Fig. 2. Mobility rate (≒R_f) of anionic peroxidase zymozrame of *rin* and Floradel tomatoes after electrophoresis on polyacrylamide gels.

The zymograme of Floradel at the mature-green stage did not contain isoenzymes *b* and *c*. The former *b* appeared when fruit attained the turning stage, the latter *c* appeared when fruit reached the breaker stage. Isoenzyme *f* possessed strong color intensity and a wider band than *e* isoenzyme at the mature-green and breaker stages, whereas *e* isoenzyme was more intense than *f* isoenzyme at the turning and pink stage (Fig. 3). The zymograme of *rin* tumato did not contain *b* and *i* isoenzymes compared with Floradel tomato and there were no changes in isoenzyme pattern among treatments during fruit color development. Strong color intensity developed in *h* and *f* isoenzyme in all color stages of *rin* fruits (Fig. 3).

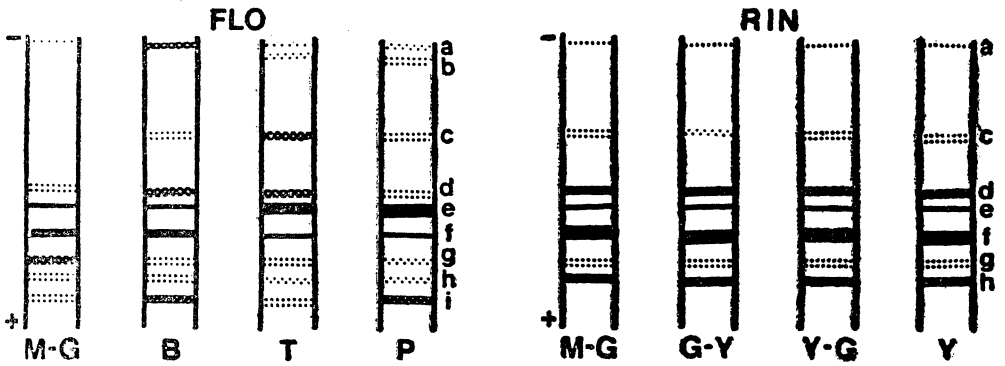

Fig. 3. Zymograme of peroxidase on polyacrylamide gel from Floradel and *rin* tomato at designated stages of color development.

Discussion

Ethylene has been demonstrated as an essential hormone in initiating fruit ripening [3]. L-methionine is the only general accepted prccussor of ethylene in the tissue of higher plant [22]. Three enzymes are included in this methionine-methional-ethylene pathway and peroxidase probably one of the most limiting enzyme in ethylene synthesis [13,24]. From results, peroxidase was not induced in activity in *rin* mutant compared with normal strain which has a significant increase when fruits changed from mature-green to breaker stage (table 2). Unchanged activity in peroxidase of *rin* mutant seemed not due to the substrate limiting factor, since methionine level showed no different between *rin* tomato and normal strain [7]. It may be derived from peroxidase *per se*. Results indicated that there were lack of isozyme *b* and *i* in *rin* tomato compared with normal cultivar (fig. 2). A possible explaination could be that the unchanged activity in peroxidase of *rin* is due to lack of isozymes resulting unability to receive enzyme activators. This is supported that the *rin* may contribute some proteins which play a role as ripening inhibitors [15]. From stock-scion interaction of normal and *rin* mutant experiment, it has been suggested that the inability of *rin* fruits to ripen normally stem from a non-translocatable ripening inhibitor [17]. A further supposition is made that the RNAs for synthesis peroxidase activators are derived from the mutated allele gene [9] by either in negative control [10] or in positive control [6] on the process of transcription.

Zymograme of peroxidase had a great changes in activity in the course of ripening according the designated color stages in normal tomato (fig. 3). Isozyme *b* was appeared only in the pink stage, it is considered a result of physiological expression when fruit get in senecence. The zymograme showed kind of a co-operative expression among isozymes and could not give a clear cut concept in each isozyme during fruit ripening. This is no doubt due to that different isozyme pattern appeared in different varieties and developmental stage [20]. It is also of difficulty to try to explain the interactions among isozymes since the activity of each isozyme expressed in intensity by artificial dye staining would have some variation because of not all isozymes were equally reactive to any one substrate [11]. The appearence or disppearance of a specific isozyme during fruit ripening does not a prior reflect gene action in the sense of transcription. However, it does reflect the expression of genetic information and the regulation of such differential gene activity can be at a number of control points.

Literature Cited

1. Anonymous. 1972. The Worthington enzyme manual. Worthington Biochem. Corp. Freehold, New Jersey. 216 pp.
2. Buescher, R. W. and E. C. Tigchelaar. 1975. Pectinesterase, polygalacturonase, Cx-cellulase activaties and softening of the *rin* tomato mutant. Hort. Science 10 : 624-625.
3. Burg, S . P. and E. A. Burg. 1965. Ethylene action and the ripening of fruits. Science 148 : 1190-1196.
4. Davis, B. J. 1964. Disc electrophoresis II. Method and application to human serum proteins. Ann.

N. Y. Acad. Sci. 121 : 404-427.

5. Dostal, H. C., W. G. McGlasson and E. C. Tigchelaar. 1974. Ripening mutants--a tool to study fruit ripening. Plant Physiol. 53 : s-61.
6. Englesberg, E. and G. Wilcox. 1974. Regulation: positive control. Ann. Rev. Genetica 8 : 219-242.
7. Gonzalez, A., P. E. Brecht and C. C. Rehkugler. 1976. Free methionine levels in *rin* and normal isogenic tomato fruits ripened in the field or in storage. Plant Physiol. 58 : 648-650.
8. Herner, R. C. and K. C. Sink, Jr. 1973. Ethylene production and respiratory behavior of the *rin* tomato mutant. Plant Physiol. 52 : 38-42.
9. Hobson, G. E. 1967. The effects of alleles of the *Never ripe* locus on the ripening of tomato fruits. Phytochemistry 6 : 1337-1341.
10. Jacob, R. and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of protein. J. Molec. Biol. 3 : 318-356.
11. Liu, E. 1971. The isozymic forms of peroxidase found in the horseradish plant. Ph. D. Dissertation, Michigan State Univ.
12. Lowry, O. H., N. J. Roobrounh, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol . Chem. 193 : 265-275.
13. Mapson, L. W. and D. A. Wardale. 1971. Enzymes involved in the synthesis of ethylene from methionine or its derivatives in tomatoes. Phytochemistry 10 : 29-39.
14. McGlasson, W. B., H. C. Dostal and E. C. Tigchelaar. 1973. Comparison of propylene-induced responses of immature fruit of normal and *rin* mutant tomatoes. Plant Physiol. 53 : 218 -222.
15. Mizrahi, Y., H. C. Dostal and J. H. Cherry. 1976. Protein differences between fruit of *rin*, a non-ripening tomato mutant, and a normal variety. Planta 130 : 223-224.
16. Mizrahi, Y., H. C. Dostal, W. B. McGlasson and J. H. Cherry. 1975. Effects of abscisic acid and benzyladenine on fruits of normal and *rin* mutant tomatoes. Plant Physiol. 56 : 544-546.
17. Mizrahi, Y., H. C. Dostal, W. B. McGlasson and J. H. Cherry. 1975. Stock-scion interactions of normal and fruit ripening mutants *rin* and *nor* in tomato. Physiol. Plant. 35 : 232-235.
18. Poovaiah, G. W., Y. Mizrahi, H. C. Dostal, J. H. Cherry and A. C. Leopold. 1975. Water pemeability during tomato fruit development in normal and *rin* non-ripening muatnt. Plant Physiol. 56 : 813-815.
19. Seevers, P. M., J. M. Daly and F. F. Catedral. 1971. The role of peroxidase isozymes in resistance to wheat stem rust disease. Plant Physiol. 48 : 353-360.
20. Shannon, L. M. 1969. Plant isoenzyme. Ann. Rev. Plant Physiol. 19 : 187-210.
21. Sink, K. C, Jr.. R. C. Herner and L. L. Knowlton. 1974. Chlorophyll and carotenoids of the *rin* tomato mutant. Can. J. Bot. 52 : 1675-1660.
22. Tigchelarr, E. C., M. L. Tomas, A. E. Kerr and R. J. Barmay. 1973. A new fruit-ripening mutant, Non-ripening (*nor*). Tomato Genet. Coop. 23 : 33-34.
23. Varner, J. E. and D. T. Ho. 1976. Hormones. In J. Bonner and J E. Varner (eds.), 'Plant Biochemistry', 3rd edition, Chapter 22 : 714-763. Academic Press, New York.
24. Yang, S. F. 1967. Biosynthesis of ethylene : Ethylene formation from methional by horseradish peroxidase. Arch. Biochem. Biophys. 122 : 481-487.

果實後熟生理之研究

1. 正常後熟蕃茄與不後熟 *rin* 突變品種對其後熟性狀暨過氧化酶活性及其異性酶圖之差異比較[1]

林 學 正[2]

中文摘要

正常後熟蕃茄品種 Floradel 與不後熟單因子突變種 *rin* 之果實於綠熟期 (Green-mature stage) 採收，置於 21°C 下後熟。觀察其呼吸率，乙烯放出率，果色及過氧化酶活性與其異性酶圖 (Zymograme) 的變化。Floradel 果實後熟正常，具有更年期高峰 (Climacteric rise) 現象，過氧化酶活性於後熟初期驟升至原來的三倍，異性酶圖有顯著變化且其異性酶數略有增減。*Rin* 果實無法後熟，其呼吸率與乙烯放出率甚低且無顯著變化，果色經久藏漸轉成黃色。在 Floradel 品種出現被命名爲 *b* 及 *i* 的異性酶並不被發現存在於 *rin* 品種中。*Rin* 品種不後熟的原因被假定爲該突變基因在染色體傳訊 (Transcription) 時爲一調節基因 (Regulatory gene)，以控制構造基因 (Structure gene) 生成 activator(s) 來活化過氧酶，但該酶因缺少部份異性酶而無法被活化，導致無法製造乙烯來引發果實後熟。

1. 臺灣省農業試驗所 研究報告 第 878 號。

2. 本所園藝系技正。臺灣省 臺中縣 霧峰鄉。