Plant Physiol. (1976) 58, 377-379

Ethylene-induced Formation of ABA in Citrus Peel as Related to Chloroplast Transformations

Received for publication January 9, 1976 and in revised form April 23, 1976

HANANYA E. BRISKER,[1] ELIEZER E. GOLDSCHMIDT, AND RAPHAEL GOREN
Department of Horticulture, The Hebrew University of Jerusalem, Rehovot, Israel

ABSTRACT

When mature green harvested Shamouti oranges (*Citrus sinensis* L. Osbeck) were exposed to 35 μl/liter of ethylene, a 3-fold increase in free abscisic acid (ABA) of the flavedo could be detected after 12 hours and a 10-fold increase after 24 hours, while chlorophyll destruction did not exceed 20%. The increase in free ABA continued up to 24 hours and leveled off. Bound ABA accumulated strongly after 24 hours suggesting that excess of free ABA was being converted into the bound form. Similar increases in ABA upon exposure to ethylene occurred also in fully mature orange fruits which had already lost all of their chlorophyll, in white and green portions of the flavedo of variegated lemons, and in the colorless albedo of Eureka lemons.

The synthetic cytokinin benzyladenine which retards chlorophyll breakdown delayed the ethylene-induced accumulation of free and bound ABA. GA_3 had no effect on endogenous ABA levels.

Application of ^{14}C-2-mevalonolactone to fruits resulted in low but distinct labeling of R_F zones corresponding to ABA markers, in both free and bound acid fractions. However, there were no significant differences in patterns of incorporation between ethylene-treated and control fruits.

The results indicate that ABA accumulates in citrus peel upon exposure to ethylene irrespective of the type of plastids present in the tissue. The possible role of chloroplasts in ABA formation is discussed.

Ripening fruits and wilting leaves exhibit large and relatively rapid increases in ABA. Whereas wilting leaves show the most striking response (up to 40-fold increase in ABA within 0.5 hr [19]) ripening fruits were found to provide better experimental systems for biosynthetic studies (11). The extraction of labeled ABA from tissues pretreated with labeled mevalonate has been taken as evidence that the newly appeared ABA arises by synthesis rather than by release from "latent" forms of ABA (13). The chloroplast has been postulated as a major site for ABA biosynthesis (11, 13) which seems attractive in view of the role ascribed to chloroplasts in senescence (3). The flavedo (the outer colored portion of citrus fruit peel) has been shown to accumulate large amounts of both free and bound ABA during natural or ethylene-induced senescence (4, 5).

In the present investigation, we examined the relationship between ethylene-induced formation of ABA and the senescent degradation of chloroplasts in citrus peel tissues. Attempts to demonstrate ethylene-induced incorporation of labeled MVA[2] into ABA by senescing citrus flavedo will also be reported.

[1] Division of Fruit and Vegetable Storage, Agricultural Research Organization, Volcani Center, Rehovot, Israel.

[2] Abbreviations: MVA: mevalonic acid; BHT: 2,6-di-*tert*-butyl-4-methyl phenol.

MATERIALS AND METHODS

Citrus fruits in these experiments included oranges (*Citrus sinensis* L. Osbeck) cv. Shamouti and Valencia late, and lemon (*Citrus limon* L. Burm. f.) cv. Eureka and a variegated lemon clone. Ethylene at 35 μl/liter was supplied in 12-liter jars with a continuous stream of humid air at the rate of 200 ml/min. Ethylene concentration in the system was occasionally checked using a Packard 7400 gas chromatograph.

The outer colored peel layer (the flavedo) was removed with a carrot peeler or a scalpel and extracted immediately. Free and bound ABA was extracted, partitioned and purified according to Goldschmidt *et al.* (5), modified as follows. Solvents used for extraction contained 1 to 10 μg/ml 2,6-di-*tert*-butyl-4-methyl phenol (BHT) (13). Separation of the diisopropyl ether neutral fraction was omitted, and only the "free acid fraction" and the hydrolyzed "bound acid fraction" were further analyzed.

Samples prepared for GLC were chromatographed on silica gel GF 254 plates with benzene-ethyl acetate-acetic acid (50:5:2, v/v) according to Lenton *et al.* (8). R_F zones corresponding to synthetic ABA markers were eluted through short columns with methanol-diethyl ether (1:1, v/v) and methylated with diazomethane. The methylated material was rechromatographed on silica gel GF 254 plates with hexane-ethyl acetate (2:1, v/v) according to Milborrow (13). The R_F zone corresponding to methylated synthetic ABA marker was eluted as before, evaporated to dryness, dissolved in 1 ml of toluene, and injected into a Packard 7400 gas chromatograph as described by Goldschmidt *et al.* (5). The wheat coleoptile section bioassay (14) served for estimation of ABA in some experiments, as previously described (4, 5).

Green lemon fruits were dipped twice for 30 min each time in 100 mg/liter solutions of BA and GA_3 dissolved in 5% ethanol + 0.02% Tween 20. The same solution without BA or GA_3 was applied to control fruits. Dipped fruits were dried in the air for few hr prior to exposure to ethylene.

DL-Mevalonic acid-2-^{14}C lactone (6.85 mCi/mmol; The Radiochemical Centre, Amersham) was dissolved in Tween 20-acetone-water (1:1:8, v/v) (13) and applied with a small camel-hair brush on an area of 3 × 5 cm of the peel of citrus fruits at a rate of 1 μCi in 0.1 ml/fruit, five fruits constituting a sample. The wetted area was covered with four layers of tissue paper soaked with distilled H_2O to keep the area moist during the experiment and to aid penetration of the label. The MVA-treated fruit was exposed to ethylene and air as described above. After 48 hr, the fruit was removed from the jars and wiped three times with methanol-soaked cotton. The patch of flavedo treated with ^{14}C-MVA was removed with a scalpel. Samples (about 10 g from five fruits) were extracted and purified as described above, the only modification being the use of a toluene-ethyl acetate-acetic acid (25:15:2, v/v) system in the first TLC step, to ensure sufficient separation between MVA and ABA. Radioactive scintillation counting was conducted using a Bray solution in a Packard

scintillation spectrometer equipped for external standardization.

RESULTS

The kinetics of ABA formation in mature green citrus fruits is outlined in Figure 1 in comparison with the senescent degradation of Chl. Fruit kept in air showed almost no change in either ABA or Chl contents within the 48 hr of the experiment. Exposure to ethylene caused abrupt changes in both ABA and Chl. Free ABA increased about 3-fold during the first 12 hr and attained a 10-fold level at 24 hr, with relatively slow increase continuing subsequently. The increase in bound ABA was relatively slow during the first 24 hr but rapid accumulation occurred between 24 and 48 hr. The level of Chl fell by about 20% during the first 24 hr and continued to fall linearly thereafter.

Accumulation of ABA upon exposure to ethylene has been demonstrated also with fully mature Valencia oranges which had lost all of their Chl (Table I). Such fruits, which had already accumulated much ABA in the course of their natural maturation, were nevertheless capable of further rise in both free and bound ABA upon exposure to ethylene.

The ethylene-induced rise in ABA is not confined to the flavedo but occurs also in the albedo (the white, pigmentless portion of citrus peel). Table I shows that levels of ABA in the albedo approach those of the flavedo, and that levels of ABA rose at a similar extent in both tissues upon treatment with ethylene.

The accumulation of ABA as related to the presence of chloroplasts was further examined in variegated lemon fruits whose flavedo consists of distinct green and albino strips. Electron microscopic observations (Zamski and Goldschmidt, unpublished) indicated the absence of typical chloroplasts from the albescent tissue, but revealed grana-less plastids containing few single thylakoids. Green and albescent sections contained equal amounts of free and bound ABA when exposed to air (Table I). Upon exposure to ethylene, the ABA content rose in albino sections even more than in green ones.

Benzyladenine, which interferes with ethylene-induced degreening of citrus fruit, has been shown previously to limit the accumulation of ABA-like growth inhibitors (4). Pretreatment with BA reduced the ethylene-induced formation of ABA (Table II), thus confirming previous observations. GA_3, which is known to antagonize the effect of ethylene on Chl destruction (3), did not interfere with ethylene-induced ABA formation. Similar results have been obtained with bioassay (Table III). Benzyladenine counteracts ethylene-induced ABA formation affecting mainly the accumulation of bound ABA.

In order to facilitate incorporation studies, attempts were made to use a flavedo-disc system which was expected to behave like intact fruit and accumulate ABA when exposed to ethylene. Detailed experiments showed that ethylene had no apparent effect on ABA levels in the disc system, probably due to wounding effects. Discs have been found to behave differently from intact fruit also with regard to other ethylene responses (16).

Incorporation studies were undertaken to test whether the ethylene-induced rise in ABA was due to *de novo* synthesis. Penetration of labeled mevalonolactone did not exceed 3%, and the rate of penetration was not affected by pretreatment of fruit surface with 1.5% dimethyl sulfoxide. However, due to the relatively high level of ^{14}C-MVA supplied, enough label penetrated the fruit to allow further analysis.

Accumulation of ABA in ethylene-treated fruits and distribution of label within fractions are shown in Table IV representing one out of several similar experiments. The marked rise in ABA in ethylene-treated fruits (5-fold for total ABA) was not accompanied by corresponding enrichment of label in the free and bound acid fractions.

Further purification of these fractions through TLC, methylation, and a second TLC step showed that considerable portions of the label migrated to R_Fs corresponding to ABA markers (Fig. 2). The absolute counts of these R_F zones were, nevertheless, quite low and there were no consistent differences between air and ethylene-treated fruits. Use of mevalonolactone-1-^{14}C gave essentially the same results. Similar experiments using ^{14}C- or ^{3}H-acetate did not result in augmented incorporation of label into fractions containing ABA.

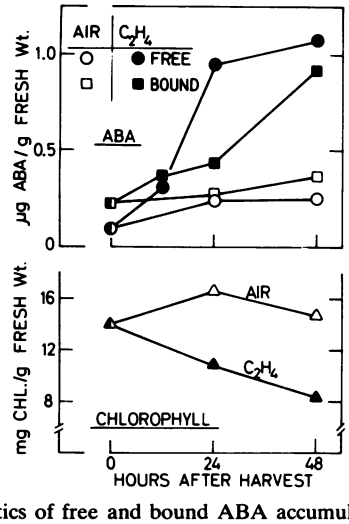

FIG. 1. Kinetics of free and bound ABA accumulation and chlorophyll degradation in flavedo of controls and ethylene-treated Shamouti oranges.

Table I. *Levels of Free and Bound ABA in Various Citrus Tissue after 48 to 72 Hr in Air or Ethylene, Measured by GLC*

Tissue	Treatment	ABA content Free	Bound	Total
		μg/g fresh wt		
Flavedo, mature yellow Valencia orange	air	0.12	0.66	0.78
	ethylene	0.67	1.55	2.22
Flavedo, green Eureka lemons	air	0.15	0.34	0.49
	ethylene	0.76	0.55	1.31
Albedo, green Eureka lemons	air	0.15	0.19	0.33
	ethylene	0.63	0.28	0.91
Flavedo, variegated lemons, green sections	air	0.34	0.74	1.08
	ethylene	0.52	0.87	1.39
Flavedo, variegated lemons, white sections	air	0.24	0.77	1.01
	ethylene	0.61	1.24	1.85

Table II. *Levels of Free and Bound ABA in Flavedo of Green Eureka Lemons Treated with Combinations of Ethylene and BA or GA, Measured by GLC*

Treatment	ABA/content Free	Bound	Total
	μg/g fresh wt		
air	0.16	0.34	0.50
air + BA	0.11	0.24	0.35
air + GA	0.05	0.18	0.23
ethylene	0.77	0.55	1.32
ethylene + BA	0.52	0.37	0.89
ethylene + GA	0.85	0.66	1.51

Table III. *Levels of Free and Bound ABA in Flavedo of Green Eureka Lemons Treated with Combinations of Ethylene and BA*

The fruit was treated at 0 to 72 hr, and the ABA was estimated by the wheat coleoptile section bioassay (14).

Treatment	ABA/content Free	Bound	Total
	μg/g fresh wt		
air 0 hr	0.39	0.31	0.70
air 24 hr	0.26	0.39	0.61
air 72 hr	0.28	0.26	0.54
ethylene 24 hr	0.52	0.52	1.04
ethylene 72 hr	0.61	0.78	1.39
ethylene + BA 24 hr	0.40	0.38	0.78
ethylene + BA 72 hr	0.66	0.48	1.14

Table IV. *Levels of ABA and Distribution of Radioactivity within Extracts of Fruits Treated with MVA-2-^{14}C Held in Air or Ethylene Atmospheres*

Averages from two replicate fruit samples. Numbers in parentheses refer to the total counts found in the 80% methanol extracts.

Fraction	Air		Ethylene	
	Radioactivity %	ABA µg/g	Radioactivity %	ABA µg/g
Plastid pellet	18.5	-	19.3	-
Petrol ether	1.9	-	2.6	-
Diethyl ether, pH 3.0 ('free'acid fraction)	6.2	0.07	6.5	0.82
Diethyl ether, pH 3.0 following alkaline hydrolysis('bound'acid fraction)	3.8	0.25	6.4	0.71
Aqueous residue	69.6	-	65.2	-
Total	100 (303,548 dpm)	0.32	100 (187,970 dpm)	1.53

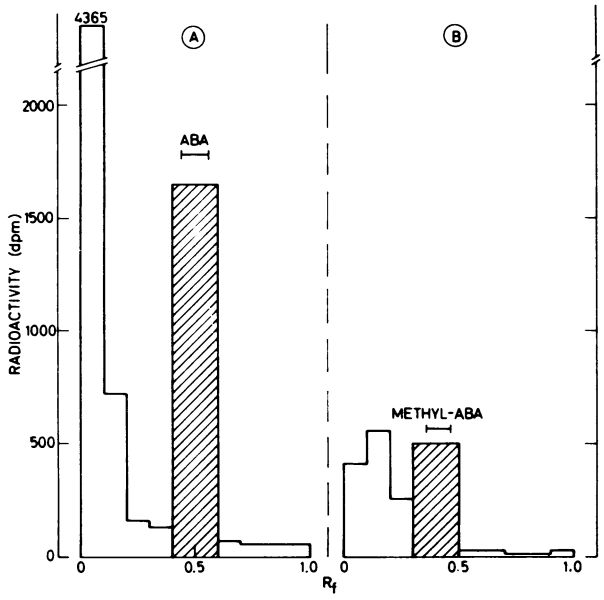

FIG. 2. Distribution of label from MVA-2-^{14}C along TLC chromatograms. A: An acid ether fraction developed with toluene-ethyl acetate-acetic acid (25:15:2, v/v); B: the R_F zone corresponding to ABA in the first run has been scraped, eluted, methylated and rechromatographed with hexane-ethyl acetate (2:1, v/v).

DISCUSSION

Rapid ABA formation has been shown to occur under the influence of ethylene in a variety of citrus tissues (Fig. 1 and Table I). The ethylene-induced formation of ABA in citrus peel tissues might be related to ethylene's role as a ripening hormone, and high levels of ABA have indeed been shown to be associated with maturation of numerous fruits (1, 2, 5, 17, 18). The inhibitory effect of BA on ethylene-induced ABA formation (Table II) indicates that the level of ABA might be controlled by some kind of balance between ethylene and other endogenous growth substances.

Our ^{14}C-MVA experiments indicate that some label was incorporated into ABA, but actual counts were low, and there was no clear difference between ethylene and control fruits. It is, therefore, difficult to draw meaningful conclusions with regard to the possibility of *de novo* synthesis of ABA via the MVA route in our system. Critical review of the literature shows that similar difficulties were encountered by other investigators. Reports by Noddle and Robinson (15), Milborrow and co-workers (11, 13) and by Loveys *et al.* (9) show that the number of counts found in ABA was low and accounted only for a small fraction of the tissue's ABA, so that true specific activities could not be calculated. In addition, Milborrow and Noddle (12) noted a large discrepancy between the ratios of MVA incorporation in turgid and wilted plants (2- to 9-fold in the latter) in comparison with the actual increases in ABA content (17- to 25-fold). Assuming that ABA is formed inside chloroplasts, Milborrow suggested that the poor incorporation of MVA into ABA could be related to permeability barriers of chloroplast membranes to mevalonate (1) as shown by Goodwin (6). Nevertheless, the studies of Hill *et al.* (7) indicated that plastids of ripening tomatoes were permeable to MVA which could be successfully incorporated into plastid terpenoids. In conclusion, it seems that we still lack an *in vivo* system which efficiently incorporates MVA into ABA.

It has been shown in the present study that citrus tissues containing chloroplasts (flavedo of green fruits), chromoplasts (flavedo of yellow fruits), abnormal albescent plastids (white sections of flavedo in variegated lemon fruits) or other unknown type of plastids (albedo; leukoplasts?) are capable of ABA formation in response to ethylene. It seems, therefore, that ABA formation is not dependent on the presence of chloroplasts, but may occur in other types of plastids as well, if indeed ABA synthesis takes place inside plastids. This conclusion is supported by observations that white portions of avocado mesocarp (11) and senescent rose petals (10) are also capable of synthesizing ABA.

Acknowledgment – The expert technical assistance of D. Galily is gratefully acknowledged.

LITERATURE CITED

1. COOMBE, B. G. AND C. R. HALE. 1973. The hormone content of ripening grape berries and the effect of growth substance treatments. Plant Physiol. 51: 629-634.
2. DORFLING, K. 1970. Quantitative Veränderungen des Abscisinsäuregehaltes während der Fruchtentwicklung von *Solanum lycopersicum* L. Planta 93: 233-242.
3. GOLDSCHMIDT, E. E. 1974. Hormonal and molecular regulation of chloroplast senescence in citrus peel. *In:* Plant Growth Substances 1973. Hirokawa Publishing Co., Tokyo. pp. 1027-1033.
4. GOLDSCHMIDT, E. E., S. K. EILATI, AND R. GOREN. 1972. Increase in ABA-like substances during ripening and senescence of citrus fruits. *In:* D. J. Carr, ed., Plant Growth Substances 1970. Springer Verlag, Berlin. pp. 758-766.
5. GOLDSCHMIDT, E. E., R. GOREN, Z. EVEN-CHEN, AND S. BITTNER. 1973. Increase in free and bound abscisic acid during natural and ethylene-induced senescence of citrus fruit peel. Plant Physiol. 51: 879-882.
6. GOODWIN, T. W. 1958. Studies in carotenogenesis-25. The incorporation of $^{14}CO_2$, [2-^{14}C]acetate and [2-^{14}C]mevalonate into β-carotene by illuminated maize seedlings. Biochem. J. 70: 612-617.
7. HILL, H. H., S. P. J. SHAH, AND L. J. ROGERS. 1970. Incorporation of [2-^{14}C]glyoxylate, [2-^{14}C]acetate and [2-^{14}C]mevalonic acid into terpenoids during ripening of tomato fruit. Phytochemistry 9: 749-755.
8. LENTON, J. R., V. M. PERRY, AND P. F. SAUNDERS. 1971. The identification and quantitative analysis of abscisic acid in plant extracts by gas-liquid chromatography. Planta 96: 271-280.
9. LOVEYS, B. R., C. J. BRIEN, AND P. E. KRIEDEMANN. 1975. Biosynthesis of abscisic acid under osmotic stress: studies based on a dual labelling technique. Physiol. Plant. 33: 166-170.
10. MAYAK, S. AND A. H. HALEVY. 1972. Interrelationships of ethylene and abscisic acid in the control of rose petal senescence. Plant Physiol. 50: 341-346.
11. MILBORROW, B. V. 1974. Biosynthesis of abscisic acid by a cell-free system. Phytochemistry 13: 131-136.
12. MILBORROW, B. V. AND R. C. NODDLE. 1970. Conversion of 5(1.2-epoxy 2,6,6,-trimethylcyclohexyl)-3-methylpenta-*cis*-2-*trans*-4,dienoic acid into abscisic acid in plants. Biochem. J. 119: 727-734.
13. MILBORROW, B. V. AND D. R. ROBINSON. 1973. Factors affecting the biosynthesis of abscisic acid. J. Exp. Bot. 24: 537-548.
14. NITSCH, J. P. AND C. NITSCH. 1956. Studies on the growth of coleoptile and first internode sections. A new, sensitive, straight growth test for auxins. Plant Physiol. 31: 94-111.
15. NODDLE, R. C. AND D. R. ROBINSON. 1969. Biosynthesis of abscisic acid: incorporation of radioactivity from [2-^{14}C]mevalonic acid by intact fruit. Biochem. J. 112: 547-548.
16. RIOV, J., S. P. MONSELISE, AND R. S. KAHAN. 1969. Ethylene-controlled induction of phenylalanine ammonia-lyase in citrus fruit peel. Plant Physiol. 44: 631-635.
17. RUDNICKI, R., J. MACHNIK, AND J. PIENIAZEK. 1968. Accumulation of abscisic acid during ripening of pears (Clapp's Favourite) in various storage conditions. Bull. Acad. Pol. Sci. 16: 509-512.
18. RUDNICKI, R., J. PIENIZAEK, AND N. PIENIAZEK. 196 . Abscisin II in strawberry plants at two different stages of growth. Bull. Acad. Pol. Sci. 16: 127-130.
19. WRIGHT, S. T. C. AND R. W. P. HIRON. 1972. The accumulation of abscisic acid in plants during wilting and other stress conditions. *In:* D J. Carr, ed., Plant Growth Substances 1970. Springer Verlag, Berlin. pp. 291-298.