

Increase in Free and Bound Abscisic Acid during Natural and Ethylene-induced Senescence of Citrus Fruit Peel

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ABSTRACT

Free and bound abscisic acid and neutral growth inhibitors were detected in citrus fruit peel using two bioassays and gas-liquid chromatography. None of the inhibitor components seems to be directly associated with chloroplasts or chromoplasts in citrus fruit peel.

Green, nonsenescent fruits contain mostly neutral inhibitors and relatively low amounts of free and bound abscisic acid. Upon harvest and storage in ethylene, 50 microliters per liter, both free and bound abscisic acid accumulate rapidly, attaining within 24 hours a level of 1 microgram per gram. After 48 hours bound abscisic acid reaches a much higher level than free abscisic acid. Fruits allowed to senesce on the tree follow a similar course of abscisic acid accumulation, attaining finally a 10:1 ratio of bound to free abscisic acid.

MATERIALS AND METHODS

Shamouti orange (*Citrus sinensis* L. Osbeck) fruits were used, unless otherwise specified. Ethylene at 50 μ l/liter was supplied in some experiments to intact fruits in 13-liter jars with a continuous stream of humid air at the rate of 200 ml/min. The outer, colored peel layer (the flavedo) was removed with a carrot peeler and either extracted immediately or frozen in liquid air and stored at -20°C until extraction.

The procedures of extraction and partition are outlined in Figure 1. An ice-cold aqueous medium containing sucrose was used in extraction to avoid rupture of chloroplasts and chromoplasts. Centrifugation of fruit peel extracts yielded a dense floating pellet which contained the plastid material; the remnants of plastid particles were removed by a second centrifugation. The plastid-free supernatant was used for further steps of purification. The appearance of the colored plastid material as a floating pellet might be due to its low density in mature citrus fruits or to some association of plastid particles with essential oils which are abundant in citrus fruit peel.

The addition of methanol to the aqueous extract precipitated many substances, presumably proteins which are insoluble in organic solvents. The solvent partition was only slightly modified from previous studies (5, 7) except for the additional alkaline hydrolysis which liberates the bound ABA (14). Further purification was achieved through paper or thin layer chromatography (Table I).

Samples prepared for GLC² were chromatographed on 0.5 mm thick layers of Silica Gel HF₂₅₄ with toluene-ethyl acetate-acetic acid (15:3:2). The R_F zone corresponding to synthetic ABA was scraped off and eluted with methanol-diethyl ether (1:1) through a short column, and the eluate was rechromatographed on Silica Gel HF₂₅₄ with *n*-propanol-*n*-butanol-ammonia-water (6:2:2:1). The zone corresponding to synthetic ABA was scraped off and eluted as before.

The wheat coleoptile section elongation bioassay and the barley endosperm sugar release bioassay in the presence of 100 nM GA₃ were used for detection of biological activity of growth inhibitors as described previously (5, 6), using standard curves prepared with synthetic ABA for quantitative estimation.

Fractions for gas-liquid chromatography were esterified with diazomethane, dissolved in hexane, and injected into a Packard 7400 gas chromatograph using a spiral glass column 1.8 m long \times 3 mm internal diameter packed with 1.5% QF-1 on Gas-chrom Q, 60 to 80 mesh. The column temperature was 200 $^{\circ}\text{C}$, with injection and detector temperature of 250 $^{\circ}\text{C}$. An electron capture detector was used (22) with radioactive ⁶³Ni

The identification of ABA as a major component of the fraction previously known as the " β -inhibitor" has greatly encouraged the study of growth inhibitors and their regulatory role in higher plants. Certain plant tissues have been shown to contain, besides free ABA, a polar fraction identified as (+)-abscisyl- β -D-glucopyranoside (10, 14), sometimes referred to as "bound ABA" (21). The term bound ABA will be used also in the present study although it is quite clear that no real binding to a specific kind of macromolecule is involved. Neutral growth inhibitors which share some structural similarities with ABA and may have physiological significance also received closer attention recently (4, 23, 24).

Citrus fruits contain considerable amounts of growth inhibitors throughout their development (7, 11, 18). Using bioassay of crude extracts, we have shown previously that ABA-like growth inhibitors increase rapidly in citrus fruit peel after harvest (5). Similar accumulation of ABA and ABA-like components was reported for other fruits (3, 20, 21) and for leaf tissues approaching senescence (2, 12).

The preliminary observations on ABA-like growth inhibitors in senescent citrus peel have been refined and extended in the present study. Attempts were made to characterize and identify the major component of the inhibitor complex and to obtain quantitative estimates of the changes which occur during natural and ethylene-induced senescence.

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² Abbreviations: GLC: gas-liquid chromatography; MS: mass spectrometry.

Plant material homogenized in 5 volumes of cold 0.1M phosphate buffer pH 7.2, and 0.4M sucrose

+

Crude debris removed by filtration through glass wool

+

Centrifuged at 2500 rpm, supernatant then centrifuged at 10,000 rpm. Pellets discarded

+

Supernatant mixed with 4 volumes of methanol and left at 5 C overnight. Precipitates removed by filtration through glass wool

+

Methanolic extract washed 3x with petroleum ether (b.p. 40-60 C). Petroleum ether discarded

+

Methanolic extract evaporated *in vacuo* at 45 C, leaving water residue

+

pH adjusted to 7.2, washed 3x with diisopropyl ether (+ neutral fraction)

+

pH adjusted to 3.0, washed 3x with diethyl ether (+ free acid fraction)

+

pH elevated to 11.0 and held at 60 C for 1 hr. pH readjusted to 3.0. Washed 3x with diethyl ether (+ bound acid fraction). Residual aqueous phase discarded.

FIG. 1. Flow sheet showing procedure for extraction and separation of inhibitors.

Table I. R_F Values of Neutral Inhibitors, Acidic Inhibitors, and Synthetic ABA in Several Chromatographic Systems

Chromatographic System	R_F Values		
	Neutral inhibitors ¹	Acidic inhibitors ^{1, 2}	Synthetic <i>cis</i> , <i>trans</i> -ABA ³
Whatman 3MM paper, Isopropanol-ammonia-water (90:1:9)	0.80-0.95	0.50-0.60	0.55
Silica Gel HF ₂₅₄ , Toluene-ethyl acetate-acetic acid (15:3:2)	0.50-0.60	0.50-0.60	0.53
Silica Gel HF ₂₅₄ , <i>n</i> -Propanol- <i>n</i> -butanol-ammonia-water (6:2:2:1)	0.85-1.00	0.65-0.70	0.67
Silica Gel HF ₂₅₄ , Ethyl acetate-hexane (4:1)	0.55-0.65	0.05-0.15	0.10

¹ Located on chromatograms by bioassay.

² Inhibitors from the "free" and "bound" fractions behaved identically.

³ Located on chromatograms by ultraviolet scanner.

foil. N₂ carrier gas was used at a flow rate of 55 ml/min, column inlet pressure 22.5 psi. The electrometer range was 3×10^{-10} ampere. A pulsating voltage of 50 v amplitude, lasting 0.01 msec (Textronic pulse generator) was applied at 0.1-msec intervals to the detector, as described previously (16). One microliter of plant extract diluted 3 to 30 times was injected. A calibration curve for *cis*,*trans*-ABA methyl ester was constructed as previously described (16).

Synthetic 2-*cis*,4-*trans*-ABA was obtained as a gift from F. Hoffman-LaRoche and Co., Ltd., Basel, Switzerland.

RESULTS AND DISCUSSION

Intracellular Localization of Growth Inhibitors. Pigmented plant tissues are believed to be rich in growth inhibitors (9). In citrus fruits, the flavedo contains more inhibitors than other fruit tissues (7). ABA and certain neutral growth inhibitors also show some links with carotenoids which are components of chloroplasts and especially chromoplasts (23). The question was asked, therefore, if growth inhibitors could be directly associated with chloroplasts and chromoplasts in citrus fruit peel. A partial answer was obtained through careful separation and comparative bioassay of isolated plastid fractions and the supernatant. Figure 2 shows that most of the inhibitor occurs in the supernatant. This holds also for the yellow fruit which

contains a higher level of inhibitors. However, plastids may serve as a source of inhibitors or their precursors which then move into the cytoplasm.

Plastid pigments cause great difficulties in purification of extracts. The finding that the concentrations of inhibitors were low in plastid fractions permitted their removal as an initial step in our purification procedure (Fig. 1). This resulted in almost colorless fractions upon solvent partition.

Components of the Inhibitor Moiety. The purification method (Fig. 1) was devised step by step, and each fraction was screened for biological activity using the wheat coleoptile and barley endosperm bioassays. The petroleum ether wash of the 80% methanol extract removed some colored materials but was practically devoid of inhibitory activity. The neutral fraction contained considerable amounts of growth inhibitors which were distinguishable from ABA (Table I). Neutral pH does not in itself prevent partition of ABA into organic fractions (4, 6). However, diisopropyl ether is far less polar than diethyl ether and does not seem to extract ABA from aqueous solutions (Zucconi and Goren, unpublished data). The neutral inhibitor was separated from ABA using thin layer chromatography with relatively nonpolar solvent systems, and some separation could be obtained even with paper chromatography (Table I). In its general behavior the neutral inhibitor resembled xanthoxin and other recently described nonpolar growth inhibitors (1, 4).

The free acidic inhibitor resembled ABA in its chromatographic behavior (Table I). In GLC (Fig. 3), a distinct peak with the same retention time as *cis*,*trans*-ABA appeared with little interfering substances. Upon ultraviolet irradiation *cis*,*trans*-ABA was converted into equal amounts of *cis*,*trans*- and *trans*,*trans*-ABA (19).

The water residue remaining after removal of the acid fraction contained considerable inhibitory activity (5, 7). Hydrolysis according to Milborrow (14) and repartitioning against diethyl ether at pH 3.0 yielded the bound fraction. The inhibitor found in this fraction also behaved like ABA in all chromatographic systems (Table I and Fig. 3). Upon our request the inhibitor was further purified and identified as ABA using GLC-MS and MS by N. Takahashi and I. Yamaguchi (unpublished data). The polar ABA complex in the water residue might then be the (+)-abscisyl- β -D-glucopyranoside which has already been identified in several plant tissues (10, 14, 21).

Quantitative Changes During Senescence. Figure 4 shows the quantitative changes of inhibitor components in mature green harvested fruit during the first 48 hr of ethylene treatment, as estimated by GLC and wheat coleoptile bioassay. The

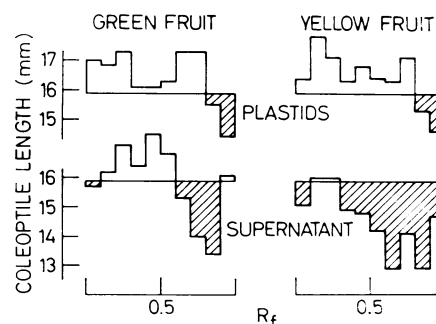


FIG. 2. Biological activity (wheat coleoptile bioassay) of 80% methanol extracts prepared from plastid fractions (2500 rpm pellet) and supernatant from mature green and yellow fruits, respectively. Whatman No. 3 MM paper chromatograms (350 mg of fresh material equivalent per strip) developed with isopropanol-ammonia-water (80:1:19).

pattern revealed by both methods is basically identical. Similar results (not reported in detail) were obtained also with the barley endosperm bioassay, although this bioassay tends to yield exaggerated values in terms of ABA equivalents.

The levels of both free and bound ABA were relatively low on the day of harvest. During the first 24 hr both ABA fractions increased dramatically, whereas the neutral inhibitor (which could be estimated only through bioassay) increased only slightly. The level of free ABA did not increase markedly during the second 24 hr. (There was some discrepancy between the different assays at this point: the barley endosperm system indicated no change during the second 24 hr.) Bound ABA continued to accumulate, attaining at 48 hr a much higher level than free ABA. The absolute concentrations of ABA detected in the citrus peel system (1-2 $\mu\text{g/g}$) are among the highest reported in the literature (13).

At the time of harvest nonsenescent mature green fruit con-

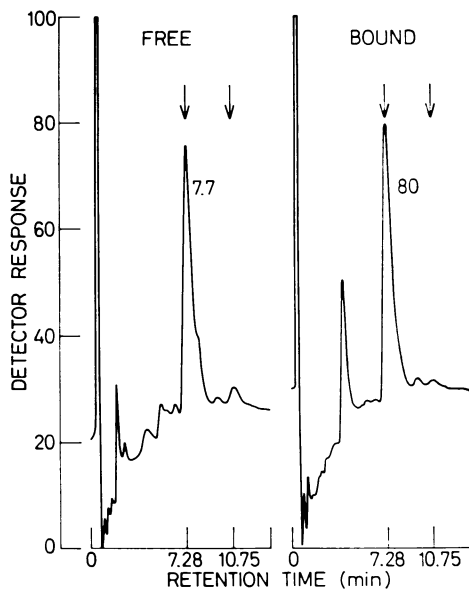


FIG. 3. GLC determinations of *cis,trans*-ABA in the free and bound inhibitor fractions from peel of fruit which senesced on the tree. Retention time for *cis,trans*- and *trans,trans*-ABA, indicated by arrows, is 7.28 and 10.75 min, respectively. The 1 μl of plant extract which was injected was equivalent to 10 mg of fresh fruit peel in the free fraction and to 1 mg in the bound fraction. Numbers at the peaks indicate the calculated amounts of *cis,trans*-ABA in $\mu\text{g/kg}$ fresh material.

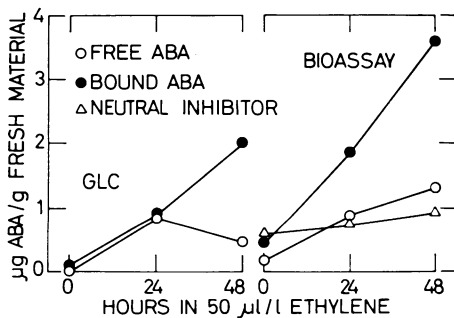


FIG. 4. Quantitative estimates of inhibitor components in peel of mature-green "Valencia" fruit, harvested and stored in 50 $\mu\text{l/l}$ ethylene. Each point represents an average from two samples of plant material which were purified for GLC. Each sample of plant material was analyzed at least in duplicate with GLC, and four replicates were bioassayed with wheat coleoptile sections.

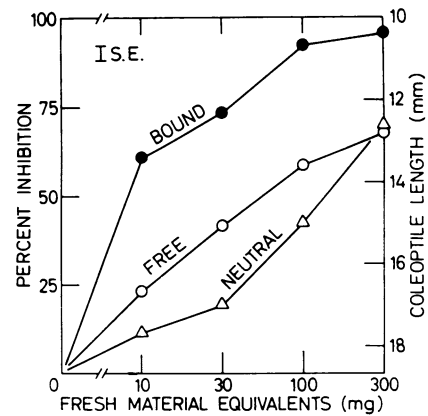


FIG. 5. Dilution experiment showing the relative concentrations of free and bound ABA and neutral inhibitors in peel of fruit which senesced on the tree. Each point represents an average of 5 replicates in the wheat coleoptile bioassay.

tained more of the neutral inhibitors than of ABA, in terms of biological activity; this seems to be true also for earlier stages of fruit development (7). However, citrus fruits which were allowed to senesce on the tree follow a course of ABA accumulation similar to that of green ethylene-treated fruit. GLC and bioassay data (Figs. 3 and 5, respectively) show that in fruits which were allowed to senesce on the tree the concentration of bound ABA is about 10-fold higher than that of free ABA, whereas the neutral inhibitor (Fig. 5) contributes slightly less than free ABA to the over-all inhibitor activity.

CONCLUSIONS

The inhibitor complex of citrus fruit peel consists of neutral inhibitors and of free and bound ABA, none of which seems to be directly associated with plastid fractions (Fig. 2). The apparent absence of inhibitors in chromoplasts may indicate that carotenoids do not serve as precursors of ABA, as proposed by Taylor (23). Nevertheless, the possibility for rapid turnover of plastid components and their excretion into the cytoplasm cannot be excluded.

Neutral inhibitors are responsible for most of the inhibitory activity prior to the onset of senescence (Fig. 4). The situation changes abruptly during ethylene-induced or natural senescence. Both free and bound ABA rise markedly, attaining finally much higher levels than the neutral inhibitors. Net conversion of the bound form to free ABA does not seem to account for these changes (see Fig. 4), as concluded also by Milborrow (15) and Rudnicki and Pieniazek (21).

The relevance of ABA accumulation to citrus peel senescence is further emphasized by the finding that *N*₆-benzylaminopurine, which retards senescence, also prevents the sudden rise in growth inhibitors (5).

Sudden increases in endogenous ABA have been demonstrated also for several stress phenomena (8, 16, 17, 25). However, the hypothesis that accumulation of ABA enables the plant to resist stress conditions (8, 17) cannot be easily extended to explain the rise of ABA in senescent tissues. The accumulation of ABA might indeed reflect the tissue's response to senescence-inducing stimuli, but, at the same time, it might also serve as a trigger for more advanced stages of senescence.

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