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Metabolic Control of Avocado Fruit Growth[1]

Isoprenoid Growth Regulators and the Reaction Catalyzed by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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The effect of isoprenoid growth regulators on avocado (*Persea americana* Mill. cv Hass) fruit growth and mesocarp 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity was investigated during the course of fruit ontogeny. Both normal and small-fruit phenotypes were used to probe the interaction between the end products of isoprenoid biosynthesis and the activity of HMGR in the metabolic control of avocado fruit growth. Kinetic analysis of the changes in both cell number and size revealed that growth was limited by cell number in phenotypically small fruit. In small fruit a 70% reduction in microsomal HMGR activity was associated with an increased mesocarp abscisic acid (ABA) concentration. Application of mevastatin, a competitive inhibitor of HMGR, reduced the growth of normal fruit and increased mesocarp ABA concentration. These effects were reversed by co-treatment of fruit with mevalonic acid lactone, isopentenyladenine, or *N*-(2-chloro-4-pyridyl)-*N*-phenylurea, but were not significantly affected by either gibberellic acid or stigmasterol. However, stigmasterol appeared to partially restore fruit growth when co-injected with mevastatin in either phase II or III of fruit growth. In vivo application of ABA reduced fruit growth and mesocarp HMGR activity and accelerated fruit abscission, effects that were reversed by co-treatment with isopentenyladenine. Together, these observations indicate that ABA accumulation down-regulates mesocarp HMGR activity and fruit growth, and that in situ cytokinin biosynthesis modulates these effects during phase I of fruit ontogeny, whereas both cytokinins and sterols seem to perform this function during the later phases.

HMGR catalyzes the irreversible conversion of HMG-CoA to MVA, the committed step in isoprenoid biosynthesis in all eukaryotic organisms (Goldstein and Brown, 1990). For plant growth and development, synthesis of isoprenoids is fundamental because the pathway supplies compounds that are essential for full morphogenic expression. This class of compounds is of structural significance, e.g. carotenoids and the side chain of chlorophylls and plastoquinone for photosynthesis, the side chain of ubiquinone for respiration, sterols for membrane structure, and phytoalexins for defense. The pathway also supplies several regulatory molecules, including ABA, brassinosteroids, GAs, and the side chain of CKs, which contribute to the control of both temporal and spatial events during higher plant ontogeny. Despite this, surprisingly little information is available concerning regulation of isoprenoid biosynthesis in plants and plant parts, particularly developing fruit. Whereas controversy still surrounds the subcellular site of MVA metabolism (Campos and Boronat, 1995; Chappell, 1995a, 1995b), it is generally agreed that reduction of HMG-CoA is potentially a major point of regulation of isoprenoid biosynthesis in plants (Bach, 1987; Gray, 1987; Gondet et al., 1992; Moore and Oishi, 1994; Chappell et al., 1995).

Using tomato as a model system, Narita and Gruissem (1989) demonstrated that HMGR expression and activity are required during early fruit development. Furthermore, these authors showed that in vivo inhibition of HMGR during early fruit development disrupted the process, whereas inhibition during the later expansion stage had no significant effect. Since ripening was apparently unaffected, it was concluded that inhibition of HMGR reduced the MVA pool required for phytosterol biosynthesis, that phytosterols were produced during early fruit development, and that fruit expansion and ripening were independent of HMGR activity.

Why phytosterols? In an attempt to address this question, Gillaspy et al. (1993) produced a comprehensive overview of the potential regulatory networks operating in the metabolic control of fruit development, including cell division, expansion, and differentiation. Although the arguments did little to cement a direct role for phytosterols in metabolic control of fruit growth and development, it was suggested that intermediates in isoprenoid biosynthesis (e.g. farnesyl diphosphate and geranylgeranyl diphosphate) could be important components in this program. Thus, it was concluded that cell proliferation during fruit ontogeny may be an ideal system with which to dissect the regulatory interactions

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Abbreviations: AMO 1618, 2′-isopropyl-4′-(trimethylammonium chloride)-5′ methyl phenyl piperidine-1′-carboxylate; ANOVA, analysis of variance; CK, cytokinin; CPPU, *N*-(2-chloro-4-pyridyl)-*N*-phenylurea; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; iP, 6-(γ,γ-dimethylallylamino)-purine; MVA, mevalonic acid; MVAL, mevalonic acid lactone.

between synthesis of isoprenoids and signal transduction pathways that lead to differentiation.

Development of avocado (*Persea americana* Mill.) fruit, like that of most fleshy fruits, follows a single sigmoid curve with a lag period of approximately 10 weeks (phase I) followed by a growth phase of about 30 weeks (phase II), dependent on cultivar and environment, and finally a mature phase (phase III) during which growth slows (Valmayor, 1967). Unlike most fruits, cell division in avocado mesocarp tissue is not restricted to phase I, but proceeds throughout ontogeny (Schroeder, 1953), albeit at a slower rate during the latter stages. Thus, avocado presents an ideal system with which to study the role of isoprenoids in the metabolic control of fruit growth from fruit set to maturity.

The avocado cv Hass produces a large number of phenotypically small fruit. Results from our recent investigations show that the incidence of the small-fruit variant correlates with sensitivity of cv Hass trees to abiotic/biotic pressure, and that environmental perturbations affect crop yield (i.e. fruit quality and quantity) seemingly through modulation of the CK:ABA ratio (C.S. Moore-Gordon, A.K. Cowan, and B.N. Wolstenholme, unpublished data). In short, we propose that a decline in the CK:ABA ratio lessens sink strength of developing organs by influencing HMGR and cell division cycle activity to reduce final fruit size. This hypothesis is supported by evidence that shows that ABA retards cell division cycle activity (Müller et al., 1994) and inhibits HMGR activity (Russell and Davidson, 1982; Moore and Oishi, 1994) in several higher plant tissue systems.

To examine the interrelationship between HMGR, isoprenoid growth regulators, and the small-fruit phenotype, we used mevastatin to specifically inhibit in vivo HMGR activity in normal avocado fruit during phases I, II, and III of the developmental program. Supplementation with products of the isoprenoid biosynthetic pathway and similarly derived plant hormones revealed that CKs were the most important limiting factors during Hass avocado fruit growth and development.

MATERIALS AND METHODS

Isotopes, Isoprenoid Compounds, and Inhibitors

DL-[3-^{14}C]HMG-CoA (58.0 mCi/mmol) was purchased from Amersham. Mevastatin (compactin), MVAL, ABA, GA_3, iP, CPPU, stigmasterol, and cholesterol were purchased from Sigma. AMO 1618 was purchased from Calbiochem.

Plant Material and Application of Chemicals

Experiments were conducted during the 1994–1995 and 1995–1996 seasons using 7-year-old trees of avocado (*Persea americana* Mill. cv Hass) propagated on clonal Duke 7 rootstocks in an orchard on the Everdon Estate in the KwaZulu-Natal midlands, South Africa.

For application of chemicals, compounds of interest were formulated in Tween 20:acetone:water (1:1:8, v/v) to a final concentration of 1 mg mL^{-1}, and 20 μL of each or combinations thereof were injected into the pedicels of individual fruits (eight fruits per treatment) using a 1-μL syringe (7105, Hamilton Co., Reno, NV) 55 d (phase I), 92 d (phase II), and 210 d (phase III) after full bloom, unless stated otherwise. Control fruit were treated with and without Tween 20:acetone:water (1:1:8, v/v). Following injection, the wound was covered with silicone grease, and fruit growth was monitored by measuring the increase in both the fruit length and diameter using digital calipers (Mitutoyo-500, Mitutoyo Corp., Tokyo, Japan) at the intervals specified in "Results." Since identical trends were observed for both the fruit length and fruit diameter, only results for percentage increase in fruit length are shown.

Estimation of Cell Size and Cell Number

Whole fruits (during phase I) and three 5-mm^3 tissue samples (during phases II and III), excised from three distinct zones (viz. a zone including the endocarp and seed coat, a zone including the exocarp, and a zone from mesocarp tissue midway between the exo- and endocarp, across the equatorial region of each of the three randomly selected fruit), were fixed in formalin:acetic acid:95% ethanol:water (2:1:10:7, v/v), dehydrated in a graded ethanol/*tert*-butanol series, and embedded in wax. Thin sections were prepared using a rotary microtome (Reichert, Vienna, Austria), dewaxed and stained with Safranin and Fast Green (Merck, Darmstadt, Germany), and examined using a light microscope (BH-2, Olympus). The number of cells present in a representative area of 90,000 μm^2 was determined. For cells at the borders, if greater than 50% of cell area was within the designated sample area, the cell was regarded as part of the sample. The number of cells per 90,000 μm^2 was used to estimate apparent cell size. To convert the number of cells in the sample area to the number of cells across the fruit, the following expression was used: $n = d\sqrt{x}$, where n is the number of cells across the fruit, d is the fruit diameter in millimeters at the equatorial region, and x is the number of cells in the sample area.

HMGR Assay

Freeze-dried mesocarp tissue was homogenized in an ice-cold 100 mM potassium phosphate buffer (pH 7.0) containing 4 mM $MgCl_2$ and 5 mM DTT, and the homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 10,000*g* for 15 min at 2°C. Microsomes were prepared by adding 8 mM $CaCl_2$ to the 10,000*g* supernatant and the membranes sedimented at 27,000*g* for 15 min at 2°C, as described by Cinti et al. (1972). The pellet was washed in 150 mM KCl and recentrifuged at 27,000*g* for 15 min at 2°C, and the microsomes were resuspended in a small volume of 100 mM potassium phosphate buffer (pH 7.0) containing 50 mM DTT. Approximately 100 μg of the microsomal protein (Bradford, 1976) was incubated in a total volume of 300 μL containing 5 mM NADPH and 1.72 nmol [3-^{14}C]HMG-CoA.

Reactions were initiated by addition of the substrate and allowed to proceed for 45 min at 30°C. At the end of the incubation period, reactions were terminated by the addition of 2 μL of MVAL (100 mg mL^{-1}) and 20 μL of HCl (6

N) followed by vortexing, and the MVA was lactonized at room temperature for 15 min. Particulate material was removed by centrifugation and the supernatant analyzed for [^{14}C]MVA. Using a modification of the method described by Chappell et al. (1995), 700 μL of 0.5 M potassium phosphate (pH 6.0) followed by 1 mL of ethyl acetate was added to the supernatant. After thorough mixing and centrifugation, radioactivity in the ethyl-acetate phase was determined by liquid scintillation spectrometry. Alternatively, the ethyl-acetate fraction was applied to thin layers of silica gel (GF_{254}) and plates developed to 15 cm in chloroform:acetone (2:1, v/v), and radioactivity in the MVAL-containing zone (R_F 0.65) was determined by liquid scintillation spectrometry. Assays were performed in triplicate, with less than 10% variation between samples and the two methods of analysis.

Determination of ABA Content

For analysis of ABA, aliquots of freeze-dried mesocarp tissue were homogenized in ice-cold methanol:ethyl acetate (50:50, v/v), containing a known amount of radiolabeled ABA (to correct for losses) and diethyldithiocarbamate (200 mg L^{-1}) as an antioxidant, in the presence of insoluble PVP (10%, w/w) and extracted for 24 h in darkness at −20°C. The homogenate was centrifuged and the pellet extracted with further methanol:ethyl acetate (50:50, v/v). The combined supernatants were reduced in vacuo, and the residue was resuspended in 0.5 M potassium phosphate buffer (pH 8.5) and partitioned three times against equal volumes of diethyl ether to remove neutral and basic impurities. The pH of the aqueous phase was adjusted to 2.5 and ABA partitioned into diethyl ether (repeated three times). Purified ABA-containing samples were analyzed by reversed-phase HPLC. Chromatography was carried out on a 5-μm C_{18} column (250 × 4.6 mm i.d., ODS 2, Spherisorb, Phase Separations, Inc., Clwyd, UK) eluted with a linear gradient of 0 to 100% methanol in 1% aqueous acetic acid over 60 min at a flow rate of 1.0 mL min^{-1}. ABA was quantified at 254 nm by peak integration following calibration with authentic standards using a programmable UV-visible light detector (model 990, Waters).

Data Analysis

Treatment effects on fruit growth and differences in cell size and number were analyzed using Genstat (Rothamsted Experimental Station, UK), compared by ANOVA and *F* tests used to determine the level of significance ($P < 0.01$). All other data are the mean of at least four independent measurements and were either compared by ANOVA and SE (difference) generated ($P < 0.05$) or presented as the mean ± SE for a treatment.

RESULTS

Inhibition of Avocado Fruit by Mevastatin and Effect of Sterols

Injection of mevastatin, a competitive inhibitor of HMGR, through the pedicel during either phase I or phase II retarded avocado fruit growth and development by 60% (Fig. 1). In both experiments, mevastatin-induced retardation of fruit growth was reversed by co-injection with MVAL, resulting in recovery of the normal phenotype. Sterols reduced avocado fruit growth when applied either in phase I or phase II (Fig. 1, A and B). A combination of cholesterol and stigmasterol, administered during phase I, also reduced fruit growth and eventually arrested the process (Fig. 1A), causing 50% fruit abscission 70 d after treatment. Although stigmasterol retarded avocado fruit growth to the same extent when applied in phase II, it partially reversed the inhibitory effect of mevastatin (Fig. 1B).

Cell Number: The Limiting Factor in Avocado Fruit Growth

To determine whether cell size and/or cell number was limiting during development of phenotypically small cv Hass avocado fruit, measurements of cell number and cell size were taken throughout the course of this program. The data were computed using a general logistic curve and an ANOVA performed on each parameter in the nonlinear regression. The resultant trends are shown in Figure 2. The mean equatorial mesocarp cell number was significantly higher in control fruit (Fig. 2A), whereas there was no significant difference between mean mesocarp cell size of the small and control fruit (Fig. 2B).

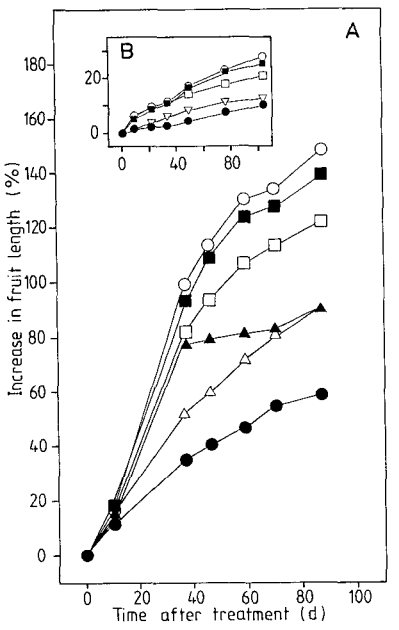

Figure 1. Effect of mevastatin, stigmasterol, and cholesterol on cv Hass avocado fruit growth. Compounds of interest were applied during the 1994–1995 season in 20 μL of Tween 20:acetone:water (1:1:8, v/v) via the pedicel at concentrations of 1 μg μL^{-1} either 55 d (A, phase I) or 92 d (B, phase II) after full bloom, and growth was monitored as percentage increase in fruit length. Each value represents the mean of eight determinations. SE (difference) in A ≤ 9.0; SE (difference) in B ≤ 0.9. ○, Control; ●, mevastatin; ■, mevastatin plus MVAL; ▽, mevastatin plus stigmasterol; □, stigmasterol; △, cholesterol; and ▲, stigmasterol plus cholesterol.

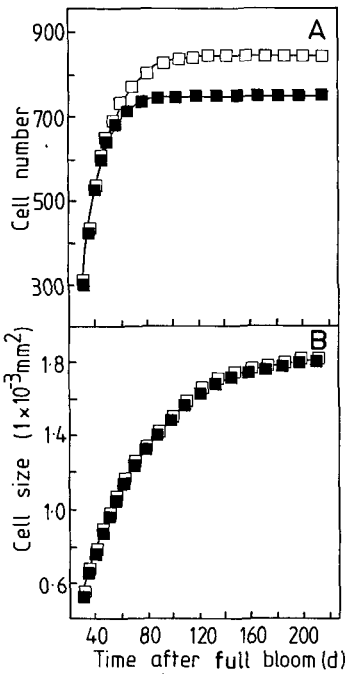

Figure 2. Estimated change in mean equatorial mesocarp cell number and cell size of small (■) and large (□) cv Hass avocado fruit throughout development. A, Cell number was estimated as described in "Materials and Methods" and regression lines for large and small fruit are represented as $y = 833.9 - 2560.5(0.9466)x$ and $y = 746.9 - 5576.3(0.9222)x$, respectively. B, For average cell size, regression lines for large and small fruit were $y = 1854.5 - 2330.0(0.9808)x$ and $y = 1852.9 - 2390.8(0.9806)x$, respectively. Regression lines were calculated from 54 measurements per treatment at each time interval.

Effect of Plant Growth Regulators on Mevastatin-Induced Inhibition of Fruit Growth

Results presented in Figure 3A show that mevastatin-induced retardation of cv Hass avocado fruit growth during phase I (55 d after full bloom) could be completely reversed by co-injection with MVAL, iP, or the cytokinin analog CPPU. GA_3 and stigmasterol, by comparison, had little or no effect. As shown in Figure 3B, CK, stigmasterol, or GA_3 did not markedly influence the "normal" course of cv Hass avocado fruit development when applied during phase I, although toward the conclusion of this growth period both GA_3- and stigmasterol-treated fruit showed a slowing of growth relative to the control. Likewise, AMO 1618, a purported inhibitor of kaurene synthase activity (Dennis et al., 1965) and sterol biosynthesis (Douglas and Paleg, 1972) did not markedly affect fruit growth, although it did cause growth to slow toward the end of the experimental period. Exogenously applied ABA, however, reduced fruit growth substantially and caused 90% fruit abscission within 50 d of application. Co-injection with iP reversed the growth-retarding effect of ABA (Fig. 3B) and reduced the incidence of fruit abscission to that observed in control treatments (Fig. 4).

During phase II (146 d after full bloom), treatment of fruit with iP countered the growth-retarding effect of mevastatin (Fig. 3C). Isopentenyladenine alone, however, had little or no effect on fruit growth during this phase.

In phase III (210 d after full bloom) mevastatin treatment reduced growth by 50% (Fig. 5A), whereas treatment with iP did not markedly affect this process (Fig. 5B). Surprisingly, only iP completely reversed the growth-retarding effect of mevastatin, although co-injection of mevastatin with either MVAL or stigmasterol reduced the effect of this inhibitor (Fig. 5A). ABA reduced avocado fruit growth by 50%, and this effect was reversed in fruits co-treated with iP.

Microsomal HMGR Activity of Mevastatin-Treated and Nontreated Avocado Fruit

In an attempt to further elucidate the proposed link between the CKs, the sterols, the small-fruit phenotype, and the synthesis of MVA, HMGR activity in small fruit and fruits treated with or without mevastatin in phases I, II, and III was determined and the results are presented in Figure 6.

During the course of cv Hass avocado fruit development, activity of microsomal HMGR remained unchanged (Fig. 6A). Although a similar trend was observed for small fruit, specific activity of microsomal HMGR was approximately 30% that of untreated and control fruit of a comparable age (Fig. 6C). Fruit pretreated with mevastatin in phase I, II, or III showed a substantial reduction in HMGR activity (Fig. 6D), with levels similar to those observed for small fruit.

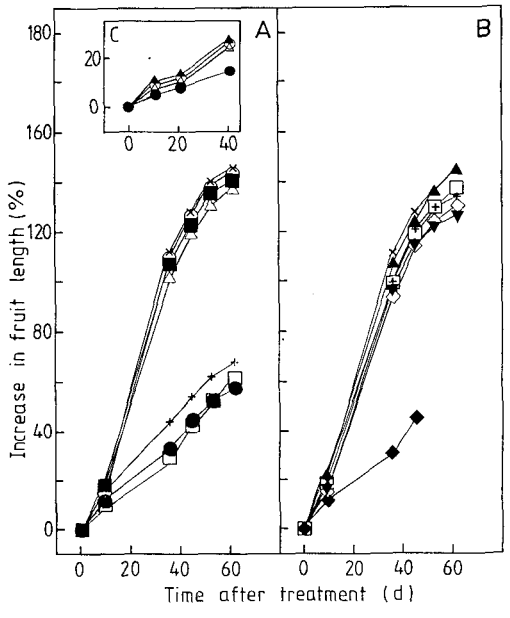

Figure 3. Influence of isoprenoid growth regulators on mevastatin-induced retardation of cv Hass avocado fruit growth. A and C, Mevastatin-treated. B, Control. Compounds of interest were applied in phase I (A and B, 55 d after full bloom) and phase II (C, 146 d after full bloom) of the 1995–1996 season, and fruit growth was measured as described in Figure 1. Each value represents the mean of eight determinations. SE (difference) in A ≤ 6.0; SE (difference) in B ≤ 6.0; SE (difference) in C ≤ 3.5. ○, Control; ●, mevastatin; □, stigmasterol; +, GA_3; ×, CPPU; ■, MVAL; △, iP plus mevastatin; ▲, iP; ◆, ABA; ▼, AMO 1618; ◇, ABA plus iP.

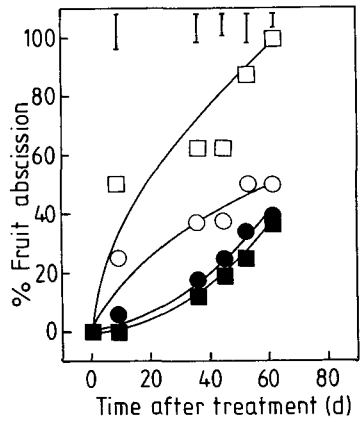

Figure 4. Comparison of the effect of ABA, ABA plus iP, and all other treatments on cv Hass avocado fruit abscission following pedicel injection of compounds during phase I of development. Experimental conditions were as described for Figure 3. ○, ABA plus iP; □, ABA; ●, all other treatments; ■, control.

Likewise, ABA treatment of fruit in phase III reduced in vivo HMGR activity by 70% to 1.41 ± 0.27 nmol h^{-1} mg^{-1} protein. Unfortunately, insufficient samples due to fruit abscission precluded a comprehensive assessment of the effect of ABA on in vivo HMGR activity during avocado fruit development. Even so, co-injection of ABA with iP during phase III partially restored HMGR activity (2.15 ± 0.24 versus 6.35 ± 0.92 nmol h^{-1} mg^{-1} protein in untreated fruit). HMGR activity was unaffected in fruits co-injected

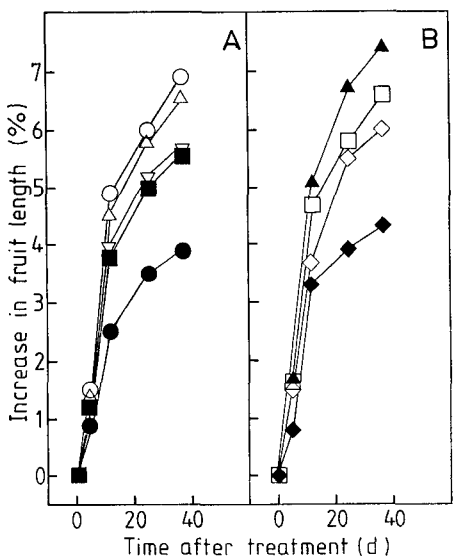

Figure 5. Effect of iP, MVAL, and stigmasterol on growth of mevastatin-treated fruit (A) and effect of iP, ABA, and stigmasterol on growth of control fruit (B) during phase III. Chemicals were applied in 20 μL of Tween 20:acetone:water (1:1:8, v/v) via the pedicel 210 d after full bloom (phase III) during the 1995–1996 season at concentrations of 1 μg μL^{-1}, and fruit growth was monitored as described in "Materials and Methods." Determinations are the mean of eight fruits per treatment. SE (difference) ≤ 1.4. ○, Control; ●, mevastatin; ■, mevastatin plus MVAL; ▽, mevastatin plus stigmasterol; △, mevastatin plus iP; ◆, ABA; ◇, ABA plus iP; □, stigmasterol; ▲, iP.

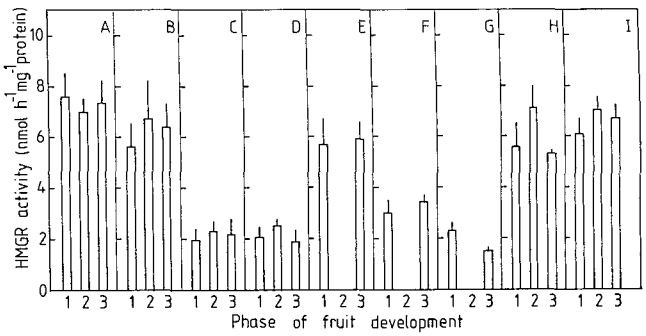

Figure 6. HMGR enzyme activity in developing cv Hass avocado fruit and fruit pretreated with mevastatin and/or iP and/or stigmasterol. Batches of fruit (eight per treatment) were injected with 20 μL of mevastatin and/or MVAL and/or iP and/or stigmasterol (all 1 μg μL^{-1}) 55 d (phase I), 146 d (phase II), and 210 d (phase III) after full bloom, and the fruit were harvested 40 d later. HMGR activity was determined in Ca^{2+}-sedimented microsomal membranes derived from freeze-dried mesocarp tissue, as described in "Materials and Methods." Each value is the mean ± SE of three to six determinations. A, Untreated; B, control; C, small fruit; D, mevastatin; E, mevastatin plus MVAL; F, stigmasterol; G, mevastatin plus stigmasterol; H, iP; I, mevastatin plus iP.

with MVAL and mevastatin (Fig. 6E), whereas stigmasterol was inhibitory and exacerbated the effect of mevastatin on enzyme activity (Fig. 6, F and G). Treatment of fruit with iP did not affect HMGR activity significantly during the course of fruit development (Fig. 6H). At all stages of fruit growth iP treatment reversed the inhibitory effect of mevastatin (Fig. 6I).

Effect of Mevastatin on Avocado Mesocarp ABA Content

Analysis of ABA in mesocarp from small fruit and fruit pretreated with or without mevastatin and/or MVAL, iP, or stigmasterol revealed the trends shown in Table I. ABA concentration declined over the normal course of avocado fruit growth and development. By comparison, mesocarp ABA content of small fruit increased, and at all stages of growth small fruit contained substantially more ABA than fruit from the control treatments. Mevastatin treatment significantly enhanced ABA concentration at all stages of fruit growth, whereas co-injection of this inhibitor with either MVAL or iP reversed the effect. MVAL resulted in a return to basal ABA concentration at all stages of fruit growth. In contrast, exogenous application of iP reduced basal ABA content by >50% during the early stage (phase I) of fruit growth, but was only 50% as effective as MVAL during the later stages (phases II and III) of this process. Stigmasterol reduced mevastatin-induced ABA accumulation by 30% in fruits treated in phase I, and by more than 50% when co-injected with mevastatin in phase III.

DISCUSSION

The occurrence of a substantial percentage of phenotypically small cv Hass fruit is common to all avocado-producing regions. Even healthy trees in well-managed orchards produce a significant number of small fruit, usu-

Table I. *ABA concentration of mesocarp tissue from developing small fruit and fruit pretreated with mevastatin, MVAL, stigmasterol, and iP*

Batches of fruit (eight fruit per treatment) were injected via the pedicel with 20-μL solutions of Tween 20:acetone:water (1:1:8, v/v) containing mevastatin, mevastatin plus MVAL, mevastatin plus stigmasterol, and mevastatin plus iP (all 1 $\mu g\ \mu l^{-1}$) 55 d (phase I), 146 d (phase II), and 210 d (phase III) after full bloom. Fruits were harvested between 50 and 100 d after application of chemicals and ABA content was determined as described in "Materials and Methods." Data are the mean of at least three determinations ($LSD_{(5\%)}$ = 10.9).

Treatment	Time after Full Bloom		
	163 d	216 d	290 d
	μg^{-1} dry wt (%)[a]		
Control	29.3 (100)a[b]	12.2 (100)a	10.9 (100)a
Small fruit	63.6 (217)d	81.8 (670)d	75.5 (693)c
Mevastatin	66.9 (228)d	39.0 (320)c	67.3 (617)c
Mevastatin + MVAL	33.0 (113)b	16.1 (132)a,b	11.1 (102)a
Mevastatin + iP	14.1 (48)a	26.4 (216)b	24.9 (228)b
Mevastatin + stigmasterol	44.6 (152)c	ND[c]	31.5 (289)b

[a] Percent relative to control. [b] At each time interval, values followed by different letters are significantly different ($P \leq 0.05$). [c] ND, Not determined.

ally characterized by early seed coat senescence and cessation of fruit growth 50 to 60 d after full bloom. We hoped to exploit this phenotypic variant and gain insight into the interaction between isoprenoid growth regulators, phytosterols, and HMGR activity in the metabolic control of avocado fruit growth and development.

Results from the present investigation provide convincing evidence for involvement of sterols, CKs, ABA, and HMGR in the metabolic control of avocado fruit growth. In this regard, several interesting observations were made. First, growth of phenotypically small cv Hass fruit was limited by cell number, and not by cell size, and these fruit showed reduced HMGR activity and elevated endogenous ABA during each phase of development. Second, in vivo inhibition of HMGR by mevastatin resulted in reduced fruit growth and increased fruit ABA concentration, irrespective of time of application after fruit set. Third, phytosterols did not appear limiting in phase I of cv Hass fruit growth. During phases II and III, however, stigmasterol reversed, albeit partially, the growth-retarding effect of mevastatin. Fourth, retardation of avocado fruit growth by mevastatin was reversed by co-injection with iP and the CK analog CPPU, in addition to MVAL. Only MVAL completely reversed mevastatin-induced inhibition of HMGR activity. MVAL was also more effective at reversing the mevastatin-induced increase in ABA than was iP, particularly during the later stages of fruit growth. This observation might account for the inability of iP to completely reverse ABA inhibition of HMGR in phase III. Retardation of fruit growth by exogenous ABA, and ABA induction of fruit abscission were reversed following co-injection with iP. Together, these findings support our proposed interaction between CKs, ABA, sterols, and HMGR, in which an increase in endogenous ABA causes down-regulation of HMGR enzyme activity and fruit growth, typified by the occurrence of phenotypically small fruit.

Several reports have implicated phytosterols in the control of fruit development (Narita and Gruissem, 1989; Gillaspy et al., 1993), and at least one study has suggested an essential role for stigmasterol in the support of plant cell division (Haughan et al., 1987). Although deprivation of MVA and sterols is reported to increase HMGR half-life, high levels of sterol enhance the rate of HMGR degradation (Correll and Edwards, 1994). Thus, it was not unexpected that treatment with stigmasterol (and/or cholesterol) would reduce in vivo HMGR activity and fruit growth.

Co-injection of mevastatin with stigmasterol, however, caused fruit to respond differently in phases I, II, and III. In phase I stigmasterol reduced fruit growth and accelerated abscission, whereas fruit treated in phase II showed partial recovery from mevastatin-induced inhibition of growth, and rates of abscission closely resembled those of control treatments. In phase III, however, stigmasterol alone did not affect fruit growth, but reversed the growth-retarding effect of mevastatin to the same extent as MVAL. Even so, stigmasterol did not reverse mevastatin-induced inhibition of HMGR, presumably due to mevastatin-induced ABA accumulation. Likewise, the ABA content of phenotypically small fruit resembled that of mevastatin-treated fruit and HMGR activity was substantially reduced.

Earlier studies on regulation of higher plant cytosolic HMGR suggested hormonal mediation of enzyme activity (Russell and Davidson, 1982). The authors demonstrated in vivo ABA, stigmasterol, and cholesterol inhibition of enzyme activity. When added to reaction mixtures in vitro, however, these products of isoprenoid biosynthesis had no effect on enzyme activity. It was therefore concluded that hormonal control was not allosteric, but was exerted via some unknown phosphorylation system. Similar conclusions were reached in studies on the effect of endogenous ABA on HMGR activity during seed maturation. *Vivipary* mutants of maize, which are defective in ABA biosynthesis, and the *Vp1* mutant, which is defective in an ABA response element, all show enhanced HMGR activity relative to wild-type siblings (Moore and Oishi, 1994). Since the *Vp1* gene product is involved in ABA signal transduction during seed development, it was proposed that HMGR activity during seed maturation is regulated via a *Vp1*-dependent signal transduction pathway that is affected by reduced ABA.

Mevastatin-induced ABA accumulation in avocado mesocarp was both surprising and interesting. First, this observation supports plastid-localized ABA synthesis (Zeevaart and Creelman, 1988), since mevastatin and its structural analogs are unable to inhibit chloroplast isoprenoid synthesis (Bach and Lichtenthaler, 1983; Bach, 1987). Second, the existence of an alternative pathway that does not involve MVA synthesis, similar to that proposed recently by Schwender et al. (1996), cannot be ignored. In this pathway, isopentenyl diphosphate is formed intrachloroplastically via condensation of pyruvate and glyceraldehyde phosphate. Third, the result might suggest that a product(s) of cytosolic isoprenoid biosynthesis is responsible for regulating ABA formation in or by chloroplasts. Two possible candidates include CKs and phytosterols.

Isopentenyladenine reversed the inhibitory effects of mevastatin at all stages of avocado fruit development. Similarly, inhibition of tobacco cell growth by lovastatin (a mevastatin analog) was reversed by CKs (Crowell and Salaz, 1992). Furthermore, iP and its hydroxylated derivative zeatin replaced the essential role of MVA in initiating DNA replication in the cell cycle (Siperstein, 1984). Since CK biosynthesis is purported to involve prenylation of the purine moiety catalyzed by isopentenyl transferase, a process in which dimethylallylpyrophosphate is added to AMP at position N^6 (Binns, 1994), the above observations might suggest that inhibition of HMGR limits the MVA pool available for synthesis of dimethylallylpyrophosphate (isomerization of isopentenyl diphosphate) and, hence, in situ CK biosynthesis. Similar conclusions were reached by Crowell and Salaz (1992), who suggested that CK biosynthesis is more sensitive to HMGR inhibition than biosynthesis of other essential isoprenoids. Further support for this proposal comes from the observation that CPPU, a CK analog, was as effective as iP at overriding the inhibitory effect of mevastatin on avocado fruit growth.

Isopentenyladenine also reversed the inhibitory effects of ABA. The role of ABA in plant stress responses and its ability to retard developmental processes (Zeevaart and Creelman, 1988) suggest that it is a likely candidate to influence fruit growth under adverse conditions and thereby contribute to down-regulation of fruit development and emergence of small-fruit phenotypes. However, ABA concentration is high and declines during the normal course of fruit growth (Table I). Thus, an alternative interpretation might be related to CK homeostasis, which is purportedly regulated by a substrate-inducible (specifically iP) oxidase (Motyka et al., 1996). High concentrations of ABA during the early phase of fruit ontogeny may therefore be necessary to modulate CK synthesis, possibly at the level of HMGR, and hence cell proliferation.

Several studies have intimated a cell-cycle-regulating function for ABA because exogenous ABA inhibits nucleic acid and protein synthesis (Owen and Napier, 1988). Meyers et al. (1990) showed that exogenously applied ABA consistently inhibited cell division in cultures of maize kernels. More recently, Müller et al. (1994) obtained evidence to suggest that ABA functions to reduce cell-division cycle activity by retarding completion of the cell cycle. In addition, water deficit in developing endosperm of maize has also been reported to inhibit cell division (Artlip et al., 1995). Stress-induced accumulation of ABA might therefore be expected to exert an effect on fruit growth during the early stages, when cell-division cycle activity is at a maximum.

Avocado fruit enlargement in phases II and III is correlated with both cell division and expansion, developmental processes that require a significant level of sterol biosynthesis (Narita and Gruissem, 1989; Chappell, 1995a). It is well established that ABA increases permeability of lipid membranes (Stillwell and Hester, 1984; Bach, 1986; Stillwell et al., 1989; Purohit et al., 1992; Bürner et al., 1993) and that phytosterols inhibit these ABA-induced perturbations (Stillwell et al., 1990). ABA also appears to inhibit HMGR activity (Russell and Davidson, 1982; Moore and Oishi, 1994). In light of these observations it is tempting to suggest that stress-induced initiation of the ABA signal transduction pathway depresses HMGR activity, limiting synthesis of both CKs and phytosterols to reduce cell-division cycle activity in affected cv Hass fruits. Furthermore, accumulation of stress-induced ABA during fruit growth might be sufficient to induce "lipid melting" in affected membranes (e.g. seed coat), causing onset of senescence and cessation of fruit development, processes that would be reversed in the presence of sufficient sterol. This proposal is supported by the observation that fruit treated with stigmasterol in the presence of mevastatin, an inhibitor of HMGR that induces ABA accumulation, show a decline in endogenous ABA concentration and partial recovery of growth.

In conclusion, possible sources that may contribute to elevated fruit ABA concentration and, hence, reduced fruit growth in cv Hass avocado include the xylem/phloem continuum (i.e. stress-induced root- and leaf-derived ABA) and the developing fruit itself (i.e. in situ ABA biosynthesis). We have recently demonstrated more efficient incorporation of label from [1-^{14}C]Glc into ABA than from [2-^{14}C]MVAL in mesocarp of cv Hass avocado (J.C.G. Maurel and A.K. Cowan, unpublished data), which might indicate a novel source of carbon for ABA biosynthesis. In addition to reducing HMGR activity and fruit growth, in vivo application of ABA via the pedicel inhibits seed coat and mesocarp cell-cell communication (A.K. Cowan, C.E.J. Botha, R.H.M. Cross, C.S. Moore-Gordon, and I. Bertling, unpublished data). Whether this effect of ABA is linked to down-regulation of HMGR directly or indirectly remains to be investigated. The recent demonstration that plant HMGR kinase is related to Suc nonfermenting-1 protein kinase (Barker et al., 1996), a gene essential for release from Glc repression, suggests that isoprenoid metabolism, carbohydrate status, and fruit growth are indeed interrelated processes that could contribute to development of small-fruit phenotypes. This is particularly so given that ABA stimulates acid invertase activity (Ackerson, 1985) and transgenic tomato fruit, expressing a constitutive antisense acid invertase gene, show increased Suc concentration and decreased fruit size (Klann et al., 1996).

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