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Full Length Research Paper

Mevastatin-induced inhibition of cell growth in avocado suspension cultures and reversal by isoprenoid compounds

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Cell suspension cultures were established using soft, friable callus derived from nucellar tissue of 'Hass' avocado (*Persea americana* Mill.) seed from fruit harvested 190 days after full bloom. Cell cultures were maintained in liquid medium supplemented with naphthalene acetic acid (NAA), isopentenyl adenine (iP) and sucrose and sub-cultured at 14 day intervals. Growth was typically sigmoidal with a lag phase of 7 days followed by an exponential phase of approximately 14 days. Mevastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) was used to probe the contribution of metabolites of the isoprenoid pathway for avocado cell growth. Treatment with mevastatin inhibited cell growth and caused loss of cell viability. Inhibition of cell growth was transient and at all concentrations of mevastatin tested, recovery was evident within 17 days. The arrest of cell growth by 1 and 40 µmol/L mevastatin was negated when this inhibitor of HMGR was supplied in the presence of either mevalonolactone (MVL) or farnesyl diphosphate (FDP) . By comparison, co-treatment of cells supplied 1 µmol/L mevastatin with stigmasterol showed little or no response whereas at 40 µmol/L mevastatin, stigmasterol induced partial recovery of cell growth. The results indicate a requirement for mevalonic acid (MVA) and cytosolic isoprenoid biosynthesis, in particular FDP, for avocado cell growth and support the hypothesis that appearance of the small-fruit phenotype in 'Hass' is inextricably linked to activity of HMGR.

Key words: Avocado, cell suspensions, farnesyl diphosphate, HMGR, mevalonic acid, *Persea americana*.

INTRODUCTION

'Hass' avocado (*Persea americana* Mill.) which produces both normal and phenotypically small fruit, presents an ideal system in which to investigate the underlying mechanisms contributing to the metabolic control of fruit growth. Furthermore, unlike most other fruits, cell division in avocado continues throughout development albeit at a reduced rate as maturity is approached (Schroeder, 1953).

Unfortunately, detailed biochemical and molecular studies of fruit growth in subtropical tree crops such as avocado, are limited by seasonality and geographical location of the species. One way to overcome this limitation is to establish cell cultures that can be maintained in liquid suspension and which mimic the response of the parent tissue to chemical and environmental stimuli.

In attempting to elucidate the causal factors responsible for the appearance of the small-fruit variant in 'Hass' avocado, the following information has been obtained using intact fruit as the experimental system. Firstly, growth of the small-fruit variant is limited by cell number (Cowan et al., 1997), which might imply genotypic differences similar to

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those reported recently for melon (Higashi et al., 1999). Secondly, cessation of growth in the small-fruit variant is associated with early senescence and death of the seed coat and a reduction in symplastic solute transport (Moore-Gordon et al., 1998; Richings et al., 2000). Thirdly, the characteristics mentioned above are phenocopied when normal fruit are treated with either abscisic acid (ABA) or inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34). Interestingly, the small-fruit variant has elevated endogenous abscisic acid (ABA) and reduced microsomal HMGR activity (Cowan et al., 1997), characteristics that are simulated by exogenous glucose (Richings et al., 2000). Since ABA-induced retardation of growth, and inhibition of HMGR activity are negated by co-treatment with cytokinin, and to a lesser extent stigmasterol, it was proposed that appearance of the small-fruit phenotype might be the result of an altered cytokinin/ABA ratio which affected HMGR activity and phytosterol production during fruit growth (Cowan et al., 1997; Moore-Gordon et al., 1998; Cowan et al., 2001). The recent demonstration that cytokinin stimulates ABA catabolism in competent tissue provides partial support for this proposal (Cowan et al., 1999). In brief, it is hypothesized that increased ABA is central to the appearance of small 'Hass' fruit and that ABA influences at least four areas of cell function to depress cell cycle activity and fruit growth. These include: 1) the cytokinin/ABA ratio;

2) initiation of ABA signal transduction; 3) reduced symplastic solute transport; and, 4) retardation of cell division cycle activity. Clearly, the interactions alluded to do not occur in isolation and together they comprise a complex system, the underlying molecular mechanism of which has still to be elucidated.

It is generally believed that developing seeds are the source of regulatory molecules that modulate cell division and fruit growth. As a first priority, therefore, we sought to establish a cell suspension culture from 'Hass' avocado seed with which to study more detailed aspects of avocado fruit growth. Additionally, an earlier report indicated that mevastatin-induced retardation of fruit growth arose as a result of a reduction in HMGR activity, which it was proposed, contributed to the arrest of cell division (Cowan et al., 1997). To confirm this proposal we investigated the effect of mevastatin on cell growth *in vitro* and, determined whether intermediates in the isoprenoid biosynthetic pathway could restore cell division *in vitro* as was recently shown for avocado fruit treated *in vivo* (Richings et al., 2000). The results are discussed in terms of the relationship between isoprenoid biosynthesis and cell division in avocado seed cell suspension cultures.

MATERIALS AND METHODS

Growth regulators, isoprenoid compounds and inhibitors

α-Naphthalene acetic acid (NAA), 6-(γ,γ-dimethylallylamino)-purine (isopentenyl adenine; iP), mevalonolactone (MVL), arnesyl diphosphate (FDP), fluorescein diacetate (FDA), stigmasterol and mevastatin (compactin) were purchased from Sigma Chemical Co., St Louis, MO, USA.

Plant material and callus induction

Phenotypically normal 'Hass' avocado fruit were harvested 190 days after full bloom during the linear phase of growth from 9 year-old trees grafted on clonal 'Duke 7' rootstock and growing in a commercial orchard on Bounty Farm in the KwaZulu-Natal midlands, South Africa. Fruit was washed with Extran (Merck, Darmstadt, Germany) and immersed in 3.5% sodium hypochlorite containing several drops of Tween 20 for 15 min, followed by 80% ethanol for 1 min, after which fruit was rinsed in three changes of ultra pure water. Seeds were dissected from surface-sterilized fruit and sectioned into explants of approximately 100 mg fresh weight. Explants were cultivated on 0.8% agar containing MSB media (Murashige and Skoog, 1962; Gamborg et al., 1968) supplemented with NAA (5 mg/L), iP (5 mg/L) and 3% sucrose and produced a substantial mass of proliferated cells. Culture vessels were incubated at room temperature and under continuous illumination from laboratory lights.

Cell suspension initiation and maintenance

Cell suspensions were initiated by transferring approximately 5 g 'wet' callus, derived from nucellar tissue of 'Hass' avocado seed, to 20 mL liquid MSB medium supplemented with NAA (5 mg/L), iP (1 mg/L) and 3% sucrose. Suspension cultures were maintained on a rotary shaker (120 rpm) in 250 mL Erlenmyer flasks at 27^{o}C under a 16 h light (35 µmol m^{-2} s^{-1}) / 8 h dark photoperiod and sub-cultured every 14 days. Cell aggregates were excluded during subculture by removal of 10 mL suspension, using a thin-bored pipette, and transfer to 10 mL fresh MSB medium at intervals of 14 days. Growth was monitored by measuring the change in cell number (see below).

Application of chemicals

Cell suspensions were initiated as described above. The effect of mevastatin on cell growth was determined by addition of this inhibitor in the range 0.01-40 µmol/L (prepared in dimethyl sulfoxide, DMSO) prior to the exponential phase of growth (ca. 10 days after culture initiation). At the intervals specified in Results, a 50 µL aliquot was removed from each replicate, pooled to create a treatment sample, stained with FDA (10 µg/mL) and examined using UV light (450-490 nm) through a Zeiss Axiophot microscope to qualitatively monitor cell viability. Cell number was monitored as described below. Each treatment was replicated five times and the experiment was repeated at least two-times.

For studies on the effect of mevastatin plus isoprenoid compounds on cell growth, MVL (6 mmol/L), FDP (10 µmol/L) and stigmasterol (10 µmol/L), prepared in DMSO, were added to cell cultures together with 1 µmol/L or 40 µmol/L mevastatin after 7 days of cell growth and changes in cell number monitored. Results are from at least two experiments in which the treatments were replicated 6 times.

Determination of cell number

Cell number was determined by adding 100 µL chromic acid (8 % (w/v) chromic trioxide) to a 50 µL aliquot of cell suspension followed by incubation at 70^{o}C for 15 min. After dilution, 1 mL was transferred to a counting chamber (Sedgewick-Rafter Cell S50, ProSciTech, Thuringowa, Australia) and the number of cells in 20 random squares determined using a Zeiss KF2 light microscope. All values were corrected for dilution.

RESULTS

Properties and dynamics of the 'Hass' avocado cell suspension

Cultures selected for use in the present study were of nucellar tissue from embryo callus derived from seed of 'Hass' avocado fruit harvested 190 days after full bloom. For sustained cell division, an exogenous supply of NAA, iP and sucrose was necessary (data not shown). Cell suspension growth followed a sigmoidal curve with a lag phase of ca. 7 days and an exponential phase of ca. 12–15 days followed by a stationary phase (Figure 1).

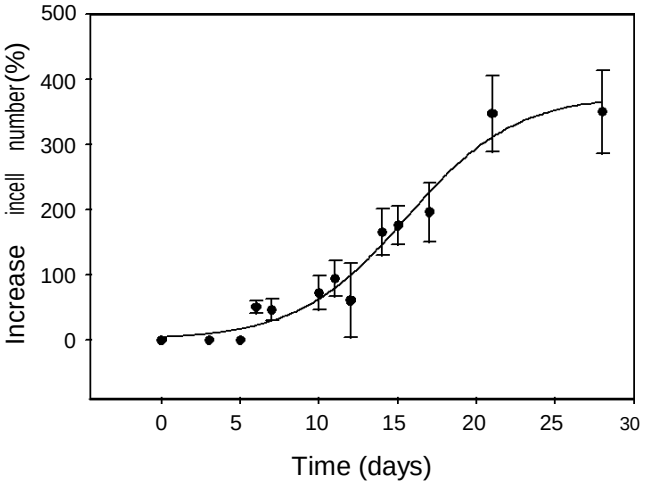

Figure 1. Population dynamics of cells derived from 'Hass' avocado seed callus incubated in MSB liquid medium containing NAA (5 mg/L) and iP (1 mg/L). Cells number and viability were determined at each time interval as described in Materials and Methods. Data were collected from nine replicates. The fitted curve is a three-order polynomial (sigmoidal, r^2=0.95) with a zero intercept.

To investigate the requirement for HMGR and hence cytosolic isoprenoid biosynthesis in avocado cell growth, the response of cell suspensions to increasing concentrations of mevastatin (a specific inhibitor of HMGR) was determined and the results are presented in Figure 2. Mevastatin inhibited cell growth within three days of application irrespective of concentration and appeared to do so in a dose-dependent manner. By day 14, cells treated with 0.01 µmol/L mevastatin had recovered and showed positive growth while by day 17, the 0.1 µmol/L and 1 µmol/L mevastatin-treated cells displayed positive growth. It is interesting that all treatments caused a severe decline in viable cell number in the 7-day period following application. Thereafter, mevastatin-treated cell cultures recovered, albeit at different rates. Only cells treated with 10 and 40 µmol/L mevastatin failed to show growth above the

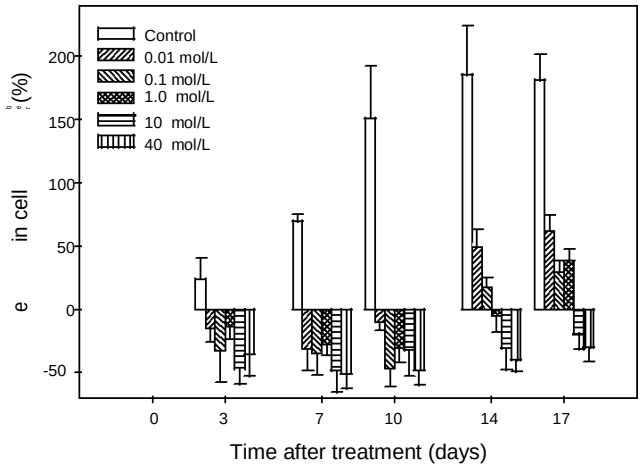

Figure 2. Growth response of 'Hass' avocado seed cell suspension cultures to increasing concentrations of mevastatin (0 - 40 µmol/L) added at the start of exponential growth (7 days after sub-culture initiation). Cell number was determined at the specified intervals and the response to mevastatin treatment expressed as percentage change in cell number. Data are the mean of five replicates from two independent experiments.

inoculation density. At all stages during the experiment, growth of untreated cells was significantly (P<0.05) greater than those supplied mevastatin.

Although cells treated with 0.01µmol/L mevastatin recovered fastest these did not necessarily display a growth response that was significantly greater (P<0.05) than that observed for cells treated with higher mevastatin concentrations (Figure 2). Figure 3 shows the results of FDA staining for cell viability in cultures 17 days after application of mevastatin. The micrographs give a clear indication of loss of cell viability in response to increasing concentrations of mevastatin. Generally, control cultures and, 0.01 and 0.1 µmol/L mevastatin- treated cultures contained a large proportion of viable cells (Figure 3A-C). At 1 µmol/L mevastatin, however, loss in cell integrity was clearly evident (Figure 3D). Analysis of FDA-stained cells from cultures supplied 10 and 40 µmol/L mevastatin revealed an almost total loss of viability (Figure 3E, F).

Effect of isoprenoid compounds on mevastatin-induced inhibition of cell growth

In order to investigate the effect of isoprenoid compounds on the growth of mevastatin-treated cells, two concentrations of mevastatin were selected; 1 µmol/L to minimize non-specific effects, and 40 µmol/L, for comparative purposes. Mevalonolactone, FDP and stigmasterol were formulated in solutions containing either 1 or 40 µmol/L mevastatin respectively. After addition to cell cultures, cell number and viability were determined

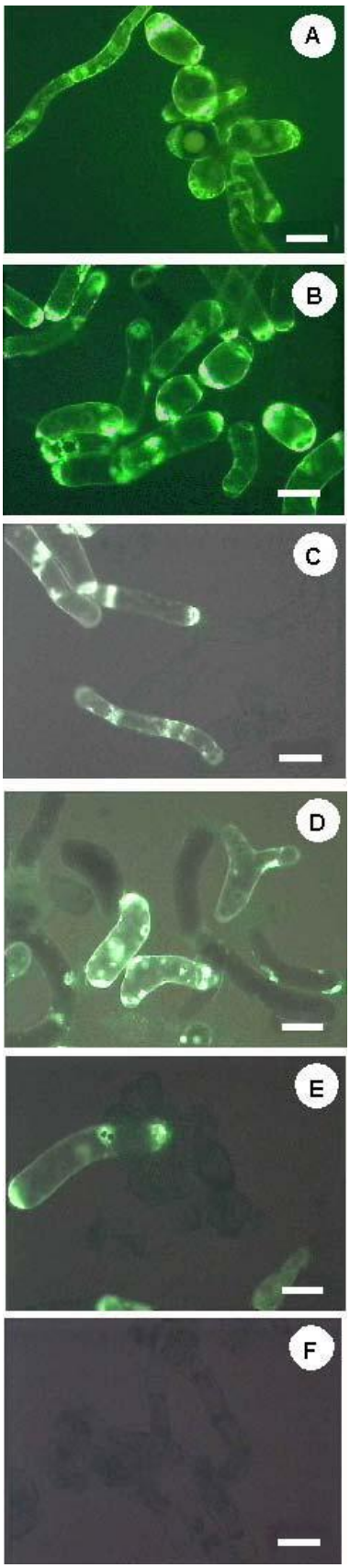

Figure 3. Determination of viability of mevastatin-treated cells. Aliquots of cells were harvested 17 days after treatment, stained with FDA (10 µg/mL) for 1 min, viewed under UV (450-490 nm) light (maximum exposure, 5 min) and fluorescence recorded digitally. Images were captured using a Panasonic WV-CP450 digital camera and AnalySIS version 3.0, image analysis software (Soft Imaging System, Munster, Germany). Note: differences in background are due to alterations in gain (increase) and saturation (decrease) to enhance contrast between fluorescing and non - or weakly fluorescing cells. Experimental details are as described for Fig. 2. (A) Control; (B) 0.01 µmol/L mevastatin; (C) 0.1 µmol/L mevastatin; (D) 1.0 µmol/L mevastatin; (E) 10 µmol/L mevastatin; (F) µmol/L mevastatin. Scale bar is 60 µm.

throughout the incubation period. For ease of presentation, only changes in cell number are shown (Fig. 4A, B).

Treatment of avocado cell suspensions with mevastatin (1 µmol/L) plus MVL or FDP negated the inhibitory effect of mevastatin on cell growth (Figure 4A). By comparison, stigmasterol had little or no effect on inhibition of cell growth induced by mevastatin at 1 µmol/L. The response of cell cultures treated with mevastatin (40 µmol/L) in the presence or absence of MVL (or FDP, or stigmasterol) is shown in Figure 4B. MVL, FDP, and stigmasterol negated the inhibitory effect of mevastatin on cell growth and the order of efficacy was MVL > FDP > stigmasterol.

DISCUSSION

The arrest of cell growth by inhibitors of HMGR activity is not novel and the results described for avocado cell suspensions in the present investigation confirm and extend what has previously been demonstrated using cell cultures from *Catharanthus roseus* (Imbault et al., 1996), *Lycopersicon esculentum* (Jelesko et al., 1999), *Nicotiana tabacum* (Crowell and Salaz, 1992; Morehead et al., 1995; Hemmerlin and Bach, 1998) and *Solanum xanthocarpum* (Josekutty, 1998). Although protoplasts have been isolated from nonmorphogenic avocado callus (Blickle et al., 1986), avocado mesocarp (Percival et al., 1991), and from embryogenic suspension cultures (Witjaksono et al., 1998) there are no reports on the development of cell suspension cultures from avocado seed tissue, and the effects of inhibitors of HMGR on avocado cell growth have never been documented. Furthermore, this study reports for the first time the ability of FDP to negate the arrest of plant cell growth by an inhibitor of HMGR.

FDA was used to demonstrate loss of cell viability in response to inhibition of HMGR by mevastatin. While cell growth and viability data corresponded, it is apparent that the increase in cell viability at 10 and 40 µmol/L mevastatin, as shown in Figure 2, was not reflected in the micrograph data as might have been expected. FDA is non-polar and permeates freely across the intact plasma membrane. Hydrolysis of FDA within the cell by non-specific esterases yields fluorescein which cannot cross the lipophilic plasma membrane. Fluorescein thus accumulates and fluoresces

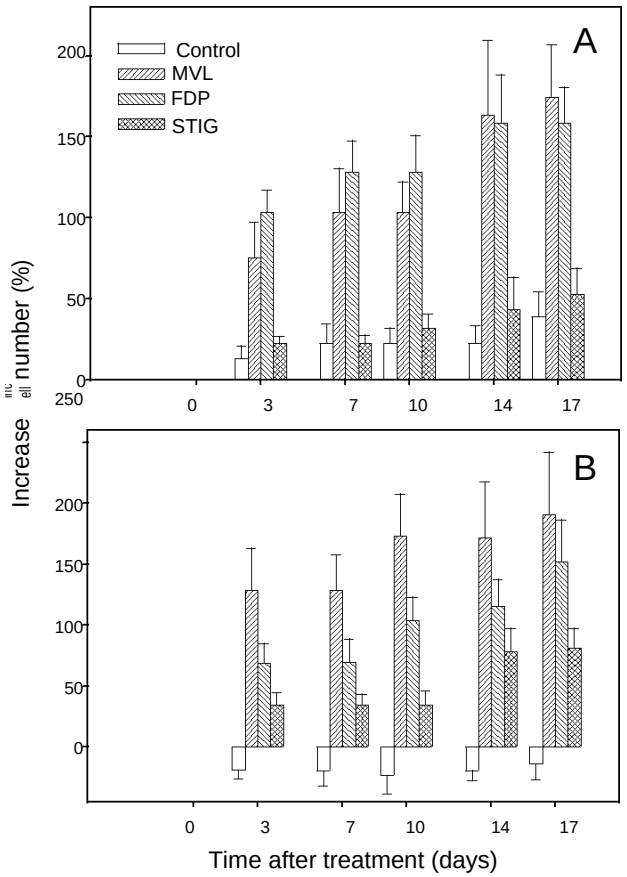

Figure 4. Response of 'Hass' avocado cell suspensions to co-treatment with either 1µmol/L (A) or 40 µmol/L (B) mevastatin with or without MVL (6 mmol/L), FDP (10 µmol/L) and stigmasterol (STIG, 10 µmol/L). Experiments were initiated 7 days after inoculation of cells into fresh medium. At the specified intervals, an aliquot was removed from each treatment and cell number and viability determined as described in Materials and Methods. Results are expressed as, change in cell number and are the mean of three replicates from at least two independent experiments.

when exposed to UV-light (Rotman and Papermaster, 1966; Larkin, 1976). Perturbations in membrane integrity, or the presence of conditions which damage the cell without disrupting its morphological integrity (such as aging or treatment with surface agents) result in reduced, or a lack of fluorescence (Rotman and Papermaster, 1966). The isoprenoid pathway is integral to cell survival and any disruption of this pathway may cause a cascade of events affecting many areas of cell function. Inhibition of HMGR may result in conditions where FDA is not metabolized and/or fluorescein not retained in cells. This might explain the lack of fluorescence observed in cells treated with high concentrations of mevastatin, which has been shown to cause cell death (Hemmerlin and Bach, 1998). Nevertheless, the above results do indicate that sub-lethal doses of mevastatin (0.01-10 µmol/L) retard cell growth while at higher concentrations (10-40 µmol/L) loss of cell viability and inhibition of growth occur.

Since MVA is the immediate product of the reaction inhibited by mevastatin, and is subsequently phosphorylated and decarboxylated to generate isopentenyl diphosphate, the precursor to all higher isoprenoids (Chappell, 1995), it is not surprising that addition of MVL reversed the effects of mevastatin. The identical response has been observed in other cell culture systems (Crowell and Salaz, 1992; Imbault et al., 1996; Morehead et al., 1995) and there are clear indications that MVA is required for cell cycle progression (Hemmerlin and Bach, 1998; Jelesko et al., 1999). By comparison, the reversal of mevastatin-induced inhibition of cell growth by FDP, which has not previously been reported, poses several questions particularly as FDP restores both growth and HMGR activity in seed of mevastatin-treated fruit (Richings et al., 2000).

FDP is not only a precursor to higher isoprenoids (e.g. sterols, sesquiterpenes) but also serves as a substrate for protein farnesylation. Furthermore, its immediate derivative, geranylgeranyl diphosphate (GGPP), can also act as a substrate for protein prenylation. Protein prenylation is a post-translational event, the products of which play key roles in signal transduction, cytoskeletal and nuclear architecture, membrane transport and progression through the cell cycle. Indeed, prenylated proteins have been documented in plants and the respective farnesyl- and geranylgeranyl-transferase activities described (Nambara and McCourt, 1999; Rodríguez-Concepción et al., 1999). Three different prenyltransferases are associated with protein prenylation in animals and yeasts – a single farnesyl transferase (Ftase) and two geranylgeranyl transferases (GGTase-I and II). In plants, the presence of Ftase and GGTase-I and GGTase-II (Rab-GGTase) has been confirmed (Randall et al., 1993; Schmitt et al., 1996; Yalovsky et al., 1996). While there is little information on plant GGTases, Ftase activity in plants has been well characterized. Studies by Qian et al. (1996) revealed that farnesylation of proteins is involved in the control of cell cycle activity. Thus, manumycin, an Ftase inhibitor completely blocked the entry of tobacco BY- 2 cells into mitosis. Similarly, inhibitors of both HMGR and Ftase impaired growth of cultured tobacco cells (Morehead et al., 1996). The process of protein prenylation is not considered to be regulatory because the covalent attachment of FDP or GGPP is non-reversible (Rodríguez-Concepción et al., 1999). It might, therefore, be reasonable to suggest that HMGR inhibition causes a reduction in available MVA for FDP synthesis leading to decreased protein farnesylation and, consequently, limited cell division. The addition of exogenous FDP reverses this affect by satisfying Ftase substrate demand.

Of course, FDP might be converted to GGPP, which is then used as the substrate for protein prenylation in avocado cells. This is particularly so given that exogenous application of GGPP negated mevastatin-induced inhibition of microsomal HMGR activity and avocado fruit growth (Richings et al., 2000). At least one report has shown that

GGPP is an essential metabolite of the mevalonate cascade in animals and is required for cell cycle progression (Tatsuno et al., 1997).

Another possible reason for the FDP-induced elevated growth response of avocado cells may be that factors limiting for cell division are derived from FDP. In the cultures used, cytokinin was not limiting since the media contained iP. Therefore, cytokinin-regulated complexes in the cell division cycle were presumably active. Responses of cells to the addition of different concentrations of mevastatin, however, revealed a transient reduction in cell division before recovery. If iP was present, what caused this reduction in cell growth? Sterols are derivatives of FDP and synthesized from squalene, which reverses mevinolin (a mevastatin analogue) -induced inhibition of cell growth (Josekutty, 1998). Cell division activity requires a significant level of sterol biosynthesis (Chappell, 1995). Narita and Gruissem (1989) found that early fruit development in tomato was sensitive to mevinolin whereas carotenoid biosynthesis continued. In explaining this, it was suggested that HMGR activity might be limiting only for sterol biosynthesis, which would result in less cell division and reduced fruit development (Gillaspy et al., 1993). Furthermore, Cowan et al. (1997) showed that mevastatin-treated avocado fruit accumulated ABA. It is well established that high levels of ABA disrupt membrane lipid integrity and that phytosterols inhibit this action (Stillwell et al., 1989). While ABA content was not monitored following addition of mevastatin to avocado cell cultures, it is conceivable that a rise in ABA with a concomitant reduction in sterol biosynthesis, due to the inhibition of HMGR, would disrupt membrane integrity and result in decreased cell division and even cell death (Hemmerlin and Bach, 1998). The reestablishment of growth after addition of FDP to mevastatin-treated cells could be explained in part, by restoration of sterol biosynthesis and membrane integrity.

In conclusion, we believe that the further elucidation of spatial and temporal regulatory components involved in mediating cell division in the avocado seed suspension culture system may lead to a greater understanding of the biochemical and molecular processes involved in fruit growth and the physiological problems associated with this developmental programme. Furthermore, our results support the notion that intermediates in the isoprenoid biosynthetic pathway serve as end products essential for normal cell growth and function (Masferrer et al., 2002). It must however, be noted that growth of the 'Hass' small-fruit phenotype is limited by cell division in the mesocarp (Schroeder, 1953; Cowan et al., 1997). Whether cell suspensions from avocado mesocarp tissue respond similarly to those of seed tissue is currently under investigation.

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REFERENCES

Chappell J (1995). The biochemistry and molecular biology of isoprenoid metabolism. Plant Physiol. 107:1-6.

Blickle W, Muhlbach HP, Sanger HL (1986). Conditions for the isolation of protoplasts from callus cultures of avocado (Persea *americana*) (abstract). Int. Cong. Plant Tissue Cell Cult. V, Minneapolis, p 357.

Cowan AK, Cairns ALP, Bartels-Rahm B (1999). Regulation of abscisic acid metabolism: towards a metabolic basis for abscisic acid-cytokinin antagonism. J. Expt. Bot. 50:595-603.

Cowan AK, Cripps RF, Richings EW, Taylor NJ (2001). Fruit size: towards an understanding of the metabolic control of fruit growth using avocado as a model system. Physiol. Plant. 111:127-136.

Cowan AK, Moore-Gordon CS, Bertling I, Wolstenholme BN (1997). Metabolic control of avocado fruit growth: isoprenoid growth regulators and the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol. 114:511-518.

Crowell DN, Salaz MS (1992). Inhibition of cultured tobacco cells at low concentrations of lovastatin is reversed by cytokinin. Plant Physiol. 100:2090-2095.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Expt. Cell Res. 50:151-158.

Gillaspy G, Ben-David H, Gruissem W (1993). Fruits: a developmental perspective. Plant Cell 5:1439-1451.

Hemmerlin A, Bach TJ (1998). Effects of mevinolin on cell cycle progression and viability of tobacco BY-2 cells. Plant J. 14:65-74.

Higashi K, Hosoya K, Ezura H (1999). Histological analysis of fruit development between two melon (*Cucumis melo* L. reticulatus) genotypes setting a different size of fruit. J. Expt. Bot. 50:1593-1597.

Imbault N, Thiersault M, Duperon P, Benabdelmouna A, Doireau P (1996). Pravastatin: a tool for investigating the availability of mevalonate metabolites for primary and secondary metabolism in *Catharanthus roseus* cell suspensions. Physiol. Plant. 98:803-809.

Jelesko JG, Jenkins SM, Rodríguez-Concepción M, Gruissem W (1999). Regulation of tomato HMG1 during cell proliferation and growth. Planta 208:310-318.

Josekutty PC (1998). Inhibition of plant growth by mevinolin and reversal of this inhibition by isoprenoids. South Afr. J. Bot. 64:18-24.

Larkin PJ (1976). Purification and viability determinations of plant protoplasts. Planta 128:213-216.

Masferrer A, Arró M, Manzano D, Schaller H, Fernández-Busquets X, Moncaleán P, Fernández B, Cunillera N, Boronat A, Ferrer A (2002). Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic *Arabidopsis* induces a cell death/senescence-like response and reduced cytokinin levels. Plant J. 30:123-132.

Moore-Gordon CS, Cowan AK, Bertling I, Botha CEJ, Cross RHM (1998). Symplastic solute transport and avocado fruit development: a decline in cytokinin/ABA ratio is related to the appearance of the Hass small fruit variant. Plant Cell Physiol. 39:1027-1038.

Morehead TA, Biermann BJ, Crowell DN, Randall SK (1995). Changes in protein isoprenylation during the growth of suspension-cultured tobacco cells. Plant Physiol. 109:277-284.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nambara E, McCourt P (1999). Protein farnesylation in plants: a greasy tale. Curr. Opin. Plant Biol. 2:388-392.

Narita JO, Gruissem W (1989). Tomato hydroxymethylglutaryl-CoA reductase is required early in fruit development but not during ripening. Plant Cell 1:181-190.

Qian D, Zhou D, Ju R, Cramer CL, Yang Z (1996). Protein farnesyltransferase in plants: molecular characterization and involvement in cell cycle control. Plant Cell 8:2381-2394.

Percival FW, Cass LG, Bozak C, Christoffersen R (1991). Avocado fruit protoplasts: a cellular model system for ripening studies. Plant Cell Rep. 10:512-516.

Randall SK, Marshall MS, Crowell DN (1993). Protein isoprenylation in

suspension cultured tobacco cells. Plant Cell 5:433-442.
Richings EW, Cripps RF, Cowan AK (2000). Factors affecting Hass avocado fruit size: carbohydrate, abscisic acid and isoprenoid metabolism in normal and phenotypically small fruit. Physiol. Plant. 109:81-89.
Rodríguez-Concepción M, Yalovsky S, Gruissem W (1999). Protein prenylation in plants: old friends and new targets. Plant Mol. Biol. 39:865-870.
Rotman B, Papermaster BW (1966). Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad. Sci. USA 55:134-141.
Schroeder CA (1953). Growth and development of the 'Fuerte' avocado fruit. Proc. Amer. Soc. Hort. Sci. 68:253-258.
Schmitt D, Callan K, Gruissem W (1996). Molecular and biochemical characterization of tomato farnesyl-protein transferase. Plant Physiol. 112:767-777.
Stillwell W, Bringle B, Hester P, Wassall SR (1989). Interaction of abscisic acid with phospholipid membranes. Biochemistry 28:2798-2804.
Tatsuno I, Tanaka T, Oeda T, Yasuda T, Kitagawa M, Saito Y, Hirai A (1997). Geranylgeranyl-pyrophosphate, a metabolite of mevalonate, regulates cell cycle progression and DNA synthesis in human lymphocytes. Biochem. Biophys. Res. Comm. 241:376-382.
Witjaksono Litz RE, Grosser JW (1998). Isolation, culture and regeneration of avocado (*Persea americana* Mill.) protoplasts. Plant Cell Rep. 18:235-242.
Yalovsky S, Loraine AE, Gruissem W (1996). Specific prenylation of tomato Rab proteins by geranylgeranyl type-II transferase requires a conserved cysteine-cysteine motif. Plant Physiol. 110:1349-1359.