Farnesol-Induced Cell Death and Stimulation of 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Activity in Tobacco cv Bright Yellow-2 Cells\textsuperscript{1,2}

Andréa Hemmerlin and Thomas J. Bach*

Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire des Plantes, Département Isoprénoïdes, Institut de Botanique, Université Louis Pasteur, 28 rue Goethe, F–67083 Strasbourg, France

Growth inhibition of tobacco (Nicotiana tabacum L. cv Bright Yellow-2) cells by mevinolin, a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) could be partially overcome by the addition of farnesol. However, farnesol alone inhibited cell division and growth as measured by determination of fresh weight increase. When 7-d-old tobacco cv Bright Yellow-2 cells were diluted 40-fold into fresh culture, the cells exhibited a dose-dependent sensitivity to farnesol, with 25 \( \mu \text{M} \) sufficient to cause 100\% cell death, as measured by different staining techniques, cytometry, and monitoring of fragmentation of genomic DNA. Cells were less sensitive to the effects of farnesol when diluted only 4-fold. Farnesol was absorbed by the cells, as examined by [1-\(^3\)H]farnesol uptake, with a greater relative enrichment by the more diluted cells. Both mevinolin and farnesol treatments stimulated apparent HMGR activity. The stimulation by farnesol was also reflected in corresponding changes in the steady-state levels of HMGR mRNA and enzyme protein with respect to HMGR gene expression and enzyme protein accumulation.

Isoprenoids constitute compounds involved in an array of fundamental biological processes, which occur at the level of individual cells, tissue, and intact organisms. In plants they are physiologically essential for a wide range of activities like photosynthesis, cell division, mitochondrial respiration, and growth control, as well as contributing to a bewildering array of isoprenoid secondary products that play a role in signaling between plants and in defense against pathogen attack. In animal cells, all isoprenoids are derived from mevalonic acid (MVA), which is a direct precursor of isopentenyl diphosphate, the “active isoprene unit” (Bach, 1995). In plants the situation seems to be more complicated in that a second pathway occurs, starting from pyruvate and glyceraldehyde 3-phosphate via 1-deoxy-d-xylulose 5-phosphate and 2-C-methyl-d-erythritol 4-phosphate (MEP), which can also give rise to isopentenyl diphosphate without proceeding through MVA. This MEP pathway was initially identified in bacteria (Rohmer et al., 1993, 1996). More recent experimental proof demonstrated the exclusive occurrence of this non-mevalonate pathway in green algae and in plastids of higher plants (Lichtenthaler et al., 1997; Rohmer, 1999a, 1999b, and refs. therein). Nevertheless, it is also clear that in higher plants several classes of isoprenoids, including sterols and farnesylated proteins as well as mitochondrial ubiquinone, are dependent on cytoplasmic generated MVA (Disch et al., 1998).

A key regulatory role in MVA biosynthesis and thus in the synthesis of cytoplasmic and mitochondrial isoprenoids is purported for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR). In animal cells only a single gene coding for HMGR seems to exist and the level of HMGR activity is subject to multiple control mechanisms (Goldstein and Brown, 1990). However, HMGR gene families have been described in plants, which are differentially regulated by various endogenous and exogenous stimuli (Enjuto et al., 1994; Stermer et al., 1994; Chappell, 1995; Dale et al., 1995; Weissborn et al., 1995; Learned, 1996; Korth et al., 1997). Inhibition of microsomal HMGR in vivo by mevinolin renders cells MVA auxotrophic. As this latter compound is a precursor of essential constituents, pleiotropic effects can be expected. Mevinolin or its analogs such as compactin act as efficient inhibitors of plant cell proliferation (Ryder and Goad, 1980; Ceccarelli and Lorenzi, 1984; Döll et al., 1984; Bach and Lichtenthaler, 1987; Hata et al., 1987; Crowell and Salaz, 1992; Randall et al., 1993; Morehead et al., 1995), and analogous to early observations made with various animal and human cell lines, specific effects on progression through a cell cycle of plant cells have been predicted (Bach, 1987). Using tobacco (Nicotiana tabacum L. cv Bright Yellow-2 [TBY-2]) cells (Nagata et al., 1992), we recently demonstrated that mevinolin treatment leads to an arrest of a majority of cells in late G1 phase (Hemmerlin and Bach, 1998). The arrest

\textsuperscript{1} A part of this study was made possible by a Ph.D. fellowship of the Ministère de l’Éducation Nationale, de l’Enseignement Supérieur et de la Recherche to A.H.

\textsuperscript{2} Parts of the results of this study were presented at the Annual Meeting of the American Society of Plant Physiologists, Vancouver, August 2–6, 1997.

* Corresponding author; e-mail Thomas.Bach@bota-ulp.unistraabg.fr; fax 33–3–88–84–84.
in G1 seems to be the consequence of a lack of some MVA-dependent signal produced during mitosis and apparently essential for passage of a check point control. Highly synchronized cells were only sensitive toward mevinolin in mitotic telophase, but not in G1 itself (Hemmerlin and Bach, 1998). Mevinolin caused also cell death in about 20% of unsynchronized TBY-2 cells (Hemmerlin and Bach, 1998).

An important intermediate in the cytosolic, MVA-dependent isoprenoid pathway is trans, trans-farnesyl diporphosphate (FPP). FPP serves as a substrate for squalene synthase, in farnesylation of proteins catalyzed by protein farnesyltransferase (Parmryd et al., 1996; Qian et al., 1996; Randall and Crowell, 1997), and via generation of trans,trans,cis-geranylgeranyl diporphosphate, it is important for the formation of dolichyl phosphate, cofactor in protein glycosylation (Crick et al., 1997). Though the quantities of FPP needed for sterol biosynthesis are greater than those needed for protein prenylation or dolichol biosynthesis, the levels of FPP should be closely controlled. The mevinolin-induced block of TBY-2 cells in late G1 was overcome by addition of exogenous MVA (Hemmerlin and Bach, 1998). Thus we thought of exploring the potential of exogenous farnesol, which is absorbed better than FPP, to also release cells from growth inhibition (compare with Ghosh et al., 1997). It has been shown that in animal cells farnesol could internally arise from FPP by removal of the pyrophosphate group through action of an FPP-specific pyrophosphatase (Meigs and Simoni, 1997). Those and other observations prompted us to examine in greater detail the effect of exogenous farnesol on HMGR activity and on cell shape by using TBY-2 cells.

RESULTS
Effect of Farnesol on Mevinolin-Treated TBY-2 Cells

An important effect of mevinolin is the inhibition of cell cycle progression in TBY-2 cells (Hemmerlin and Bach, 1998). The aim of the experiment described in Table I was to determine whether inhibition of cell division by mevinolin could be reversed by farnesol, by direct metabolism of farnesol, or by other mechanisms. Farnesol could partially counteract the inhibition of cell division induced by 5 μM mevinolin (normalized to 100%) when added 24 h later, as indicated by an increase of fresh weight after farnesol treatment (Table I). However, this moderate stimulation leveled off rapidly at farnesol concentrations higher than 50 μM and was much less complete as compared to nearly complete reversion by exogenous MVA (Table I). However, greater than 1 mM MVA was necessary for complete reversion (Hemmerlin et al., 1999). It should be noted that cells grown in the complete absence of mevinolin enter the rapid growth phase much earlier and have a relatively higher weight after the culture period. Because MVA or farnesol was added 24 h after growth in presence of mevinolin, a full restoration would not be expected before reaching stationary phase. Consequently, we chose to set the 5 μM mevinolin treatment as representative of 100% growth instead of the fresh weight of untreated cells.

Growth Inhibition by Farnesol Treatment

A sharp threshold concentration of farnesol was capable of limiting growth in cells in stationary phase, when cells were diluted 41-fold into new modified Murashige and Skoog medium (Fig. 1A). Long-term treatment (7 d) by farnesol was toxic when added to the cells at a concentration greater than 12.5 μM, whereas lower concentrations of farnesol exerted no significant inhibitory effects. Short-term exposure (48 h) of less diluted cells (5-fold) to farnesol concentrations above 25 μM also inhibited growth (Fig. 1B). This latter experimental series suggested that a higher initial cell inoculum led to a lower efficiency to farnesol as a cytotoxic agent. This observation was confirmed by the data presented in Figure 2. With higher initial inoculum concentration, sensitivity to farnesol decreased accordingly. In cells diluted 41- and 17-fold, 25 μM farnesol induced complete inhibition, whereas cells diluted 9- and 5-fold were more tolerant.

The above results suggested that a closely defined intracellular concentration of farnesol was necessary to induce toxic effects. Furthermore, these results were the first indication that the cells might actively accumulate farnesol; a smaller initial number of cells would thus have greater potential to accumulate cytotoxic levels of farnesol. This notion was supported

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stimilation of Mevinolin-Inhibited Cell Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>298.0</td>
</tr>
<tr>
<td>5 μM Mevinolin**</td>
<td>100.0</td>
</tr>
<tr>
<td>5 μM Mevinolin, plus 10 μM farnesol***</td>
<td>118.3</td>
</tr>
<tr>
<td>5 μM Mevinolin, plus 50 μM farnesol***</td>
<td>123.4</td>
</tr>
<tr>
<td>5 μM Mevinolin, plus 75 μM farnesol***</td>
<td>124.7</td>
</tr>
<tr>
<td>5 μM Mevinolin, plus 0.5 mM MVA****</td>
<td>212.1</td>
</tr>
<tr>
<td>5 μM Mevinolin, plus 2.0 mM MVA****</td>
<td>273.4</td>
</tr>
</tbody>
</table>

* TBY-2 cells were subcultured (20 mL added to 80 mL of new Murashige and Skoog medium, 5-fold dilution), followed by culture for 72 h in the absence of any product.
** Five-fold diluted cells were treated with 5 μM mevinolin for 72 h. This value corresponds to 100% (5.5 g fresh wt in absolute value; mean of three independent experiments).
*** Five-fold diluted cells were treated with 5 μM mevinolin for 24 h. After this period farnesol was added at the concentrations indicated and cells were incubated for another 48 h.
**** Cells were treated with 5 μM mevinolin as described above. After 24 h MVA was added at 0.5 and 2 mM, respectively, followed by further incubation for 48 h. After harvesting of cells, fresh wt was determined as parameter for cell growth.
by an incorporation experiment in which cells were fed low, non-toxic quantities of labeled \([1-^3H]\) farnesol (Fig. 3). The data shows that (a) farnesol indeed entered the cells (rate of incorporation up to 8%), and (b) diluted cells contained an average of 4.5-fold more intracellular farnesol as expressed in radioactivity per milliliter cell volume than cells from cultures with a higher initial cell density, when incubated in the presence of identical initial concentrations.

HMGR Activity in Farnesol-Treated TBY-2 Cells

Farnesol has been described as accelerating HMGR degradation when added to Chinese hamster ovary cells and might represent the non-sterol MVA derivative responsible for down-regulating HMGR activity in response to accumulation of certain pathway products (Correll et al., 1994; Meigs and Simoni, 1997). Unexpectedly, farnesol treatment exerted a stimulating effect on apparent microsomal HMGR activity in TBY-2 cells. This effect was concentration dependent (Fig. 4A). Addition of farnesol at the same concentrations to in vitro assays did not affect enzyme activity (data not shown), suggesting that the previously observed activation was not due to a direct interaction of the compound with the protein. When the concentrations of exogenously supplied farnesol exceeded the level of acute cytotoxicity, the stimulation of activity dropped as the cells died (see below). The induction of activity was also correlated with an increase in HMGR protein (Fig. 4B) and the steady-state mRNA level for at least one HMGR gene (Fig. 4C). It is interesting to note that farnesol at cytotoxic concentrations (100 \(\mu\)M) still strongly induced HMGR protein levels, whereas there was no...
such effect on apparent enzyme activity. We cannot exclude the possibility that farnesol could also block HMGR protein turnover and degradation in TBY-2 cells. HMGR protein from visibly dead cells might have been locked into an inactive conformation by interaction with compounds leaking out of vacuoles, as well as by the low pH. However, after denaturing electrophoresis and blot transfer, it would still present epitopes recognized by the antibody.

Microsomes were also isolated from farnesol-treated cells used for the experiments described in Table I and tested for HMGR activity. There was an apparent synergism between farnesol and mevinolin in stimulation of HMGR activity (Fig. 5). However, for determination of the full apparent activity it was necessary to wash the microsomes free of mevinolin. The inhibitor seems to bind tightly to HMGR protein, most likely in a 1:1 molar ratio, in view of $K_i$ values of mevinolin being in the range of $10^{-9} \text{ M}$, which is far below the concentrations used for treatment of cells.

Figure 4. Influence of farnesol treatment on apparent activity of microsome-bound HMGR. TBY-2 cells were treated for 48 h with different concentrations of farnesol before the isolation of membranes. A, Enzyme activity in TBY-2 cells treated by farnesol. Activity was always measured in the presence of 30 $\mu$M R,S-[3-14C]HMG-CoA (10-fold $K_m$) and of 30 $\mu$g of microsomal protein. Control, Untreated cells. 100% activity corresponds to 4.8 nmol mg$^{-1}$ h$^{-1}$ (80 pmol mg$^{-1}$ min$^{-1}$). Mean of three independent experiments. B, Western-blot analysis of HMGR protein. All lanes contained 15 $\mu$g of protein; an identical gel was run in parallel and used for silver staining to be sure that the intensity of individual protein bands was identical for each lane (not shown). C, Northern-blot analysis of HMGR RNA. All lanes contained 20 $\mu$g of total RNA. Intensity of major ribosomal RNA bands on the agarose gel was identical for each lane. *, Not determined.

Figure 5. Determination of HMGR activity in mevinolin- plus farnesol-treated cells. Microsomes were isolated from TBY-2 cells described in Table I and were used directly or buffer-washed before corresponding HMGR activity was measured. 100% activity (243.3 pmol mg$^{-1}$ min$^{-1}$) corresponds to that found with unwashed microsomes isolated from TBY-2 cells, which were treated with 5 $\mu$M mevinolin. Microsomes isolated from TBY-2 cells that were cultivated for 72 h in the absence of both mevinolin and farnesol showed an apparent HMGR activity of 126 pmol mg$^{-1}$ min$^{-1}$). The experiments were repeated three times, and sds are indicated.

Figure 6. Farnesol-induced cell death in TBY-2 suspension cells. A, Seven-day-old cells were subcultured (2–80 mL, dilution 41-fold) in the presence of different farnesol concentrations. After 5 d of culture the proportion of dead cells was determined. Cells were stained with fluorescein diacetate (specific for living cells) and propidium iodide (penetrates only into dead cells and stains nuclei orange-red). The percentage of dead cells corresponds to the proportion of red nuclei. Asterisks indicate values below 3% of dead cells, as was found in control cultures. B, Seven-day-old cells were subcultured by addition of 2, 5, 10, or 20 mL, respectively, to 80 mL of modified Murashige and Skoog medium (dilution factors of 41-, 17-, 9-, and 5-fold, respectively) containing 25 $\mu$M farnesol (final concentration). The percentage of dead cells was determined as in A.
Induction of Cell Death and of DNA Fragmentation

Farnesol induced cell death in TBY-2 cells at concentrations starting with 25 μM (Fig. 6A), apparently in an all-or-nothing response; a defined concentration of farnesol induced total cell death, whereas a slightly lower concentration had no such effect. A similar threshold reaction was observed when different cell dilutions were exposed to 25 μM farnesol (Fig. 6B). When 10 mL of 7-d-old cells was added to 80 mL of modified Murashige and Skoog medium (9-fold dilution) only 14% of cells died as compared with untreated controls (final farnesol concentration was always 25 μM). At a higher initial dilution, 100% of cells were dead as indicated by staining of nuclei with propidium iodide. We observed a slight increase in the percentage of dead cells at higher initial cell densities (Fig. 6B). This can easily be explained through the earlier attainment of stationary phase in cultures with a higher inoculum. After the culture period of 7 d, those cultures contain a higher proportion of old cells that eventually degenerate and die. The shape of TBY-2 cells changed dramatically upon farnesol treatment under non-lethal conditions (<25 μM), appearing cube-shaped, compact, and frequently stuck together in longer clusters (Fig. 7A). Control cells, in contrast, exhibited the typical, rod-shaped morphology, with cells arranged in small clusters, usually consisting of four cells (Fig. 7D). The appearance of small and condensed nuclei in farnesol-induced dead cells (Fig. 7, B and C) suggested that farnesol might exert an effect at the DNA level. Indeed, DNA extracted from farnesol-treated cells showed a ladder-like fragmentation pattern after agarose-gel separation (Fig. 8A), whereas mevinolin-induced DNA degradation lacked such a pattern (Hemmerlin et al., 1999). This DNA fragmentation was accompanied by an apparent decrease in the content of intact genomic DNA relative to control cells, based on the cytometrical DNA analysis (Fig. 8B).

Figure 7. Photographs of cells that were treated with 25 μM farnesol for 5 d. The culture was started by adding 10 mL of 7-d-old TBY-2 cells to 80 mL of new modified Murashige and Skoog medium. A through C, Same conditions showing differential effects on the induction of cell death and on morphological features. D, Control cells. Cells were doubly stained as described in Figure 6. The white bar in A corresponds to 100 μm in A and D, and to 50 μm in B and C. Under the conditions presented in A through C, 14% of total cells were dead and the total fresh weight of suspension cells was reduced from 23.5 (control) to 4.6 g. At the dilution of 2 and 5 mL of 7-d-old TBY-2 cells into 80 mL, 100% of cells were dead (only red nuclei were visible, data not shown).
DISCUSSION

As TBY-2 cells are very sensitive to various inhibitors, possibly due to an easy penetration of the rudimentary cell wall and of the plasmalemma, they were used as a model system to study the effect of farnesol on cell proliferation and death. Farnesol, a non-sterol isoprenoid compound, was identified as a putative regulator of HMGR activity and thereby cholesterologenesis in animal cells (Bradfute and Simon, 1994; Correll et al., 1994). In farnesol-treated TBY-2 cells, in contrast, there was no inhibition of apparent HMGR activity. On the contrary a drastic increase in activity was observed, paralleled by stimulation of HMGR transcription and translation. Since plant cells contain several isogenes encoding HMGR, and the transcription of one or more of them seems to be highly activated under stress conditions (Yang et al., 1991; Stermer et al., 1994), an apparent stimulation of HMGR activity could be due to a specific increase in transcription/translation of one isogene. Such an increase could conceivably mask negative effects of farnesol on other HMGR isogenes or on their products. Consequently, the inhibitory effects observed in animal cells would not be apparent in TBY-2 cells. To test such a hypothesis, it will be mandatory to use probes that specifically recognize the various isogenes and isozymes, a difficult task in view of sequence similarities between HMGR isogenes/proteins in tobacco (Crévenat, 1997).

We have demonstrated that TBY-2 cell proliferation was inhibited by increasing concentrations of farnesol and that HMGR was increasingly stimulated. Also, partial cell death in diluted TBY-2 cultures was possible only within a surprisingly narrow range of farnesol concentrations above which 100% cell death occurred. In light of these results it seems possible that farnesol-induced overstimulation of HMGR activity may stand at the threshold of processes leading to an induction of cell death. If so, intracellular inhibition of HMGR by mevinolin should diminish the toxic effect of farnesol. Hence, mevinolin, which alone induces cell cycle arrest in TBY-2 cells such that about 80% were blocked in late G1 phase, and about 20% in G2 (Hemmerlin and Bach, 1998), may have a protective role in the presence of farnesol. Indeed, cells cultured in the presence of mevinolin were less inhibited by farnesol. Not only were mevinolin-treated TBY-2 cells not inhibited by addition of exogenous farnesol, but cell growth was actually stimulated compared to cells that were cultured in the presence of mevinolin only.

In principle farnesol could overcome inhibition by mevinolin through a bypass of the biosynthetic block after being converted into FPP. A similar effect of farnesol has been observed in Helianthus tuberosus explants (Ceccarelli and Lorenzi, 1984). Growth inhibition by the mevinolin analog compactin was overcome in these explants by supplying them either with 2.2 mM MVA, or by 0.1 to 0.5 mM farnesol, thus suggesting the requirement of one or more, not-precisely-known non-sterol MVA derivatives (Ceccarelli and Lorenzi, 1984). Similar concentrations of MVA were needed to restore root elongation growth of radish seedlings grown in the presence of mevinolin (Bach and Lichtenthaler, 1983) or to overcome mevinolin-induced cell cycle arrest in TBY-2 cells.
(Hemmerlin and Bach, 1998). However, our experiments suggest also that farnesol, as such, may not be directly responsible for reversal of the mevinolin-induced growth inhibition, but may have a regulatory effect on an early step of isoprenoid biosynthesis, i.e. at the HMGR level. Mevinolin (or analogs) may become less efficient over time as the cells are induced to reverse the diminution of enzyme activity by farnesol-stimulated synthesis of the target enzyme HMGR. Such an increase in activity, due to a concomitant increase in immunoreactive protein, was observed in microsomes from farnesol-treated TBY-2 cells. When TBY-2 cells were cultured in the presence of 5 μM mevinolin, microsomes had to be carefully buffer-washed for correct measurement of apparent HMGR activity, as residual inhibitor appeared otherwise to remain bound to microsomes. Intracellular HMGR activity might remain suppressed by the inhibitor, and only after an extended period of mevinolin treatment, which by itself triggers overproduction of HMGR (Hemmerlin, 1997), might partial alleviation of inhibition by mevinolin occur. Some tendency of mevinolin to tightly bind to HMGR was already observed during attempts to purify the enzyme from radish seedlings, after preceding stimulation of HMGR accumulation by mevinolin treatment (Bach et al., 1986). It is possible that a limited, farnesol-induced overproduction of HMGR (of an isoform that remains to be identified), may lead to a better tolerance of TBY-2 cells against mevinolin. On the other hand, it seems that the presence of mevinolin inside the cells efficiently blocked the effect of farnesol-induced HMGR activity and thus protected the cells.

Farnesol alone, which apparently is actively taken up, exerted an inhibitory effect on growth of TBY-2 cells. Similar observations had been made with pancreatic tumor cells (Burke et al., 1997). It has been demonstrated that in animal cells, farnesylated proteins such as p21ras play an important role in cell division (Casey et al., 1989). At first sight it might be reasonable to assume that farnesol in the cell could act as a competitor of protein farnesylation and thus inhibit cell division. However, the potential to inhibit protein farnesyl transferase alone cannot explain its effects. It has been reported that farnesol-derived inhibitors and farnesol itself are much less efficient than specific protein farnesyl transferase inhibitors such as chaetomellic acid (Singh et al., 1993; Cassidy and Poulter, 1996; Ratemi et al., 1996), with the Ki values needed for enzyme inhibition in vitro being clearly above those concentrations of farnesol adequate for cell death induction when applied to TBY-2 cells in vivo.

What, then, could be another explanation for farnesol inhibition of cell division? It has been shown that in mammalian cells, farnesol is derived from turnover of farnesylated proteins. For example, farnesyl-Cys, a product of proteolysis of farnesylated proteins, is degraded to Cys and farnesol by action of a prenyl-Cys lyase (Zhang et al., 1997). Farnesol could also be produced from FPP via a specific allyl pyrophosphatase (Bansal and Vaidya, 1994). A recycling system for the farnesol appears to be present in mammalian cells, as rat liver contains two distinguishable enzymes: an ATP-dependent farnesol kinase associated with the inner, lumenal surface of microsomal vesicles, and a CTP-dependent farnesyl phosphate kinase, which is apparently localized to the outer, cytoplasmic surface of microsomal vesicles (Bentinger et al., 1998). Given the potential toxicity of farnesol, such a recycling system may represent some sort of detoxification mechanism.

A similar farnesol recycling/detoxification system may be present in plants, as tobacco cells have recently been shown to utilize [3H]farnesol for the biosynthesis of digitonin-precipitable sterols and sesquiterpenoids (Thai et al., 1999). This is in agreement with our unpublished observations that indicate that in TBY-2 cells, as in mammalian cells (Crick et al., 1995), radiolabeled farnesol is readily incorporated into major end products of the MVA pathway. Thai et al. (1999) also demonstrated that tobacco microsomes contain efficient enzyme systems capable of double-phosphorylating the free alcohol. Therefore plant cells have mechanisms for detoxifying farnesol by directing it into metabolic channels, i.e. for sterol biosynthesis (Chappell, 1995), which, however, may have a limited capacity. It appears that these mechanisms work sufficiently well up to a certain concentration for farnesol, but apparently fail to protect cells when this threshold value is exceeded. If this metabolic capacity for FPP utilization is exceeded, the result would possibly be its accumulation.

Work with mevalonate kinase purified from Catharanthus roseus demonstrates one of the possible side effects of FPP accumulation: It has been shown that the enzyme was competitively inhibited by FPP with respect to ATP with a Ki of about 0.1 μM, a concentration more than 3 orders of magnitude lower than the Km toward ATP (Schulte, 1998). Such a direct effect on MVA kinase could lead to an efficient blockage of MVA conversion and thus of the entire cytosolic pathway, including the formation of a putative end product responsible for feedback regulation. If this product, which has to be synthesized de novo, is lacking, overstimulation (of one or more isoforms) of HMGR may result, and this deregulation could finally lead to the formation of some signal and/or toxic compounds capable of inducing cell death. Whether parallel feedback regulation occurs at preceding enzymatic steps catalyzed by HMG-CoA synthase and acetoacetyl-CoA thiolase, as in mammalian cells (Honda et al., 1998), remains to be more closely investigated with plant cells. However, mevinolin-induced increase in acetoacetyl-CoA thiolase mRNA has been observed in radish seedlings (Vollack and Bach, 1996).
Inhibition of cell growth and induction of cell death may also be due, in part, to inhibition of tumor-like cell proliferation. Farnesol was described as having an antitumor effect (Adany et al., 1994; Haug et al., 1994; Burke et al., 1997), and in TBY-2 cells, the negative effects of farnesol-stimulated HMGR activity on cell division may be evidence of this principle. When TBY-2 cells, which have been described as the analog of tumor-derived “HeLa cells in plant science” (Nagata et al., 1992), were transformed with the gene encoding HMGR1 of Arabidopsis, we never obtained dividing cells, although the efficiency of transformation was high (Hemmerlin, 1997). This lack of cell division in the presence of overexpressed HMGR appears similar to the reversion of the tumoral phenotype of human lung adenocarcinoma A549 cells that was achieved by expression of HMGR cDNA (Seronie-Vivien et al., 1995).

In accordance with the antitumor principle, possibly, is the apoptotic effect of farnesol. Induction of cell death by exogenous farnesol in TBY-2 cells was correlated with genomic DNA fragmentation typical of programmed cell death (apoptosis), as in animal cells (Haug et al., 1994). Farnesol-induced apoptosis in mammalian cells, i.e. in human acute leukemia CEM-C1 cells, appears to result from a reduced rate of formation of diacetylglycerol and phosphololine, the products of phosphatidyl choline (PC) degradation. This indicates an inhibition of PC-specific phospholipase C, followed by inhibition of phospholipase D, resulting in a decrease in the synthesis of PC-derived phosphatidylethanol (Voziyan et al., 1995). Similarly, the primary role of farnesol for the above-mentioned A549 cells may be inhibition of PC biosynthesis (Miquel et al., 1998). These and other observations suggest the involvement of cellular signal transduction pathways in the inhibition of cell proliferation and induction of apoptosis by farnesol.

In the yeast Saccharomyces cerevisiae, farnesol induced growth inhibition by acceleration of mitochondrial production of reactive oxygen species, possibly via interference with a phosphatidylinositol type of signal (Machida et al., 1998). Moreover, the binding of farnesol or of its metabolites to members of the zinc-finger containing superfamily of nuclear hormone receptors (Forman et al., 1995) may also apply to the situation in TBY-2 cells. Such receptors are capable of altering the transcription initiation rate through binding to elements in the promoter region of target genes, as has been shown to occur in mammalian cells (Meier, 1997). Those aspects clearly await further studies, especially since some very basic reactions following farnesol treatment, i.e. induction of HMGR in TBY-2 cells versus induction of its degradation in animal cells, also exhibit fundamental differences, although the ultimate effect, induction of apoptosis, appears to be similar.

In conclusion, it seems conceivable to ascribe a dual role to farnesol in TBY-2 cells: one that may be based on interaction with a signaling cascade, and a second one that may involve blockage of an essential route responsible for the formation of a final product of the multi-branched cytosolic pathway. This latter compound, which remains to be identified, may be essential for feedback regulation of HMGR.

MATERIALS AND METHODS

Materials
A suspension culture of tobacco (Nicotiana tabacum L. cv Bright Yellow-2) cells, originally derived from young plants was provided by Prof. T. Nagata (University of Tokyo). Mevinolin was a kind gift from Drs. M. Greenspan and A.W. Alberts (Merck Research Laboratories, Rahway, New Jersey). Before use, the lactone of mevinolin was converted to the open-acid form according to the protocol described by Kita et al. (1980). All trans-farnesol and MVA lactone were from Fluka (Buchs, Switzerland), R,S-HMG-CoA, NADPH, dihydroethrythritol, and bovine serum albumin were purchased from Sigma (St. Louis). Other sources of biochemicals and radiochemicals were reported previously (Vollack et al., 1994; Hemmerlin and Bach, 1998).

Cell Culture
TBY-2 cells were cultured in a modified Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands). The concentration of KH₂PO₄ was increased to 540 mg/L. Other additives were as described by Nagata et al. (1992), except for the addition of 100 mg/L of myoinositol. Cell cultures (82 mL in 250-mL Erlenmeyer flasks) were kept in the dark at 26°C and shaken at 174 rpm. Cells were subcultured weekly (2 mL per 80 mL of new medium). Inhibitor solutions were filtered sterilized before addition. Results were compared to those obtained with cultures containing the same concentrations of solvents, as required. Cell growth was quantified by determination of fresh weight after collecting cells by suction filtration.

Farnesol Incorporation
Seven-day-old cells, diluted 41- or 5-fold into new Murashige and Skoog medium (Duchefa, Fullerton, CA). were incubated with 122 nCi mL⁻¹ (2.22 nM, final concentration) of [1-²H]farnesol (55 Ci mmol⁻¹, Isotopchim, Ganagobie-Peyruis, France) for 48 h. Cells were recovered by filtration and washed three times with PBS (phosphate-buffered saline) buffer (Sigma). After determination of total cell volume, cells were frozen (10 min at ~80°C), then transferred into boiling water for 5 min. This procedure was repeated. Cell juice was recovered after centrifugation (30 min, 10,000g). Radioactivity was determined (scintillation counter, automated external standard mode, Tri Carb 4000, Packard, Downers Grove, IL) both in cell juice and in cell suspension medium using suitable aliquots dissolved in a water-compatible scintillation cocktail (ReadyGel, Beckman Instruments, Fullerton, CA).
Cell Biology Techniques

For fluorescence microscopy, nDNA was stained with the aid of Hoechst H33258 as previously described (Hemmerlin and Bach, 1998). Viability of cells (Huang et al., 1986) was tested by addition of propidium iodide (600 μg mL⁻¹, final concentration in double-distilled water, which penetrates only dead cells and leads to a red staining of nuclei) and addition of fluorescein diacetate (100 μg mL⁻¹ in acetone, specific to living cells and leading to a yellow-green staining of the cytoplasm). Cells were incubated for 10 min at room temperature, prior to fluorescence microscopy.

Cytometric measurements were carried out according to the protocol of Hemmerlin and Bach (1998) with the aid of a Systeme d’Analyses Microscopiques à Balayage Automatique computerized image analysis system (Alcatel, Meulin, France), with nuclei previously stained by a Feulin reaction (Gabe, 1968).

Preparation of Microsomes, Protein, and HMGR Assay

Cells were frozen and kept at −80°C, followed by powdering in a mortar in the presence of liquid N₂. The powder was suspended in 12.5 mL g⁻¹ fresh weight of a 4°C cold phosphate buffer system A (0.2 M K₂HPO₄, pH 7.5, 0.35 M sorbitol, 10 mM Na₂ EDTA, and 5 mM MgCl₂), to which 20 mM dithioerythritol and 4 g 100 mL⁻¹ insoluble polyvinylpyrrolidone (Sigma) were freshly added. The homogenate was filtered through nylon gauze (50 μm) and centrifuged at 3,000 rpm for 5 min at 4°C; rotor JA-20, RC-5 superspeed centrifuge, Beckman). The pellet containing cell debris and polyvinylpyrrolidone particles was removed, and the supernatant was again centrifuged at 8,000 rpm (at 16,000g for 40 min at 4°C). The supernatant was centrifuged at 105,000g at 4°C for 1 h. The pellet (P105,000), considered as a microsomal fraction, was redissolved in the same buffer system and stored at −80°C. When microsomes had to be washed free of mevinolin, they were resuspended in 20 mL of the same buffer system and centrifuged again at 105,000g as described above. Protein content was quantified by a Lowry method (Bensa- doun and Weinstein, 1976) with some modifications (Bach et al., 1986). Bovine serum albumin was used as a standard. HMGR activity was determined as described by Bach et al. (1986), in the presence of an optimum protein concentration (30 μg), and in the presence of 30 μM (10 × Km) \( R,S-[3^{14}C]\) HMG-CoA (0.025 μCi = 55, 500 dpm). Incubation time was chosen such that substrate conversion did not exceed 25%.

Western-Blot Analysis of SDS-PAGE Separated Proteins

For western blotting, 15 μg of microsomes were solubilized in loading buffer containing 1% (w/v) SDS and separated in a 12% (w/v) acrylamide/0.1% (w/v) SDS gel (1 mm), using a minigel system (Hoefer Scientific Instruments, San Francisco). Proteins were electroblotted onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) as described by Towbin et al. (1979), and immunostained as described by Vollack et al. (1994), with polyclonal rabbit antibodies raised against the soluble domain of radish HMGR2 overexpressed in Escherichia coli, as described for radish isozyme HMGR1 (Ferrer et al., 1990). The antibodies apparently recognize all isofoms of plant HMGR (Vollack et al., 1994; Hemmerlin, 1997).

Northern-Blot Analysis

RNA was isolated according to the protocol described by Goodall et al. (1990). The final RNA pellet was dissolved in 100 μL of formamide. RNA was denatured in the presence of 50% (w/v) formamide, 20 mM MOPS (3-[N-morpholino]-propanesulfonic acid), 8 mM sodium acetate, 1 mM EDTA (pH 8), and 9% (w/v) formaldehyde, for 15 min at 60°C. RNA was electrophoresed on a 1.2% (w/v) agarose gel as described by Lehach et al. (1977) and transferred onto a nylon membrane (Amersham) by capillary blotting (Sambrook et al., 1989).

Northern blots were hybridized with a PCR fragment containing part of the open reading frame of a conserved region of tobacco HMGR isozymes (obtained from Prof. J. Chappell, University of Kentucky). DNA probes were labeled by random priming with a mixture of [³²P]dATP and [³²P]dCTP (Sambrook et al., 1989). Hybridization was performed at 42°C in presence of 50% (w/v) formaldehyde (Sambrook et al., 1989). Blots were washed with 2× SSC and 1% (w/v) SDS at 42°C, then with 0.1× SSC and 1% (w/v) SDS, followed by autoradiography at −80°C.

DNA Isolation and Analysis

RNA was isolated from TBY-2 cells by the method of Dellaporta et al. (1983), and then electrophoresed in 1.8% (w/v) agarose gels using a Tris (tris[hydroxymethyl]-aminomethane)-borate buffer system (Sambrook et al., 1989).

ACKNOWLEDGMENTS

We wish to thank Drs. A.W. Alberts and M. Greenspan for a kind gift of mevinolin. We are indebted to Dr. A. Ferrer (Barcelona) for a sample of antiserum against radish HMGR2 and to Dr. J-P. Ghnassia (Centre Paul Strauss, Strasbourg, France) for letting us use his instrumentation. We are grateful to Dr. M-A. Hartmann for stimulating discussions and for help in northern-blot assays. We thank Dr. Isabelle A. Kagan for helpful discussions and for critically reading the English manuscript.

Received January 24, 2000; accepted February 21, 2000.

LITERATURE CITED


Farnesol-Induced Cell Death in Tobacco cv Bright Yellow-2 Cells

Copyright © 2000 American Society of Plant Biologists. All rights reserved.

Bach TJ, Lichtenthaler HK (1983) Inhibition by mevinolin of plant growth, sterol formation and pigment accumulation. Physiol Plant 59: 50–60


Crévenot P (1997) Clonage de la mutation responsable de la surproduction de stérols chez un mutant de tabac (Nico-
tiana tabacum). Thèse de Doctorat, Université Louis Pasteur, Strasbourg, France


Crick DC, Andres DA, Waechter CJ (1997) Novel salvage pathway utilizing farnesol and geranylgeraniol for pro-

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


Disch A, Hemmerlin A, Bach TJ, Rohmer M (1998) Mevalonate-derived isopentenyl diposphates is the bio-
synthetic precursor of ubiquinone prenyl side-chain in tobacco BY-2 cells. Biochem J 331: 615–621

Döll M, Schindler S, Lichtenthaler HK, Bach TJ (1984) Differential inhibition by mevinolin of prenyllipid accumula-
tion in cell suspension cultures of Silybum marianum L. In P-A Siegenthaler, W Eichenberger, eds, Structure, Function, and Metabolism of Plant Lipids. Elsevier, Am-
sterdam, pp. 227–230

Enjuto M, Balcells L, Campos N, Caelles C, Arró M, Boronat A (1994) Arabidopsis thaliana contains two differen-
tially expressed 3-hydroxy-3-methylglutaryl coen-
yzme A reductase genes which encode microsomal forms of the enzyme. Proc Natl Acad Sci USA 91: 927–931


Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlm-

Gabe M (1968) Techniques Histologiques. Masson, Paris, France

Ghosh PM, Mott GE, Ghosh-Choudhury N, Radnik RA, Stapleton ML, Ghidoni JJ, Krebs FJ (1997) Lovasta-
atin induces apoptosis by inhibiting mitotic and postmitotic events in cultured mesangial cells. Biochim Bio-
phys Acta 1359: 13–24

Goldstein JL, Brown MS (1990) Regulation of the mevalo-


Hata S, Shirata K, Takagishi H, Kouchi H (1987) Accu-

Haug JS, Goldner CM, Yazlovitskaya EM, Voziyan PA, Melnykovych G (1994) Directed cell killing (apoptosis) in human lymphoblastoid cells incubated in the presence of farnesol: effect of phosphatidylcholine. Biochim Bio-
phys Acta 1223: 133–140
Hemmerlin A (1997) Etude du rôle de molécules d’origine isoprénnique (dérivées du mévalonate) dans la régulation du cycle cellulaire d’une suspension de cellules de *Nicotiana tabacum* Bright Yellow 2 (TBY-2). Thèse de Doctorat, Université Louis Pasteur, Strasbourg, France


Farnesol-Induced Cell Death in Tobacco cv Bright Yellow-2 Cells


