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INHIBITION OF STEROL BIOSYNTHESIS DURING ELICITOR-INDUCED ACCUMULATION OF FURANOCOUMARINS IN PARSLEY CELL SUSPENSION CULTURES

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Key Word Index—*Petroselinum crispum*; Apiaceae; parsley; cell suspension cultures; sterol biosynthesis; furanocoumarins; squalene synthase.

Abstract—Addition of a cell wall extract from *Phytophthora megasperma* f. sp. *glycinea* to parsley cell suspension cultures was found to trigger a rapid and marked inhibition of sterol biosynthesis as demonstrated by feeding experiments with tracer amounts of radioactive sodium acetate over short periods of time. Such an inhibition was detected as soon as 7 hr after elicitor addition, i.e. just before the furanocoumarin accumulation in the culture medium. It resulted in a dramatic decrease in the radioactivity incorporated into the main Δ^5-sterols (24-methylcholesterol, sitosterol and stigmasterol) as well as into their precursors, squalene, 4,4-dimethyl- and 4α-methyl-sterols. In elicitor-treated cells, the bulk of the residual radioactivity was recovered into the 4,4-dimethylsterol pool, suggesting a block at the level of the C-4 demethylation of 24-methylenecycloartanol to give cycloeucalenol. The elicitor addition was also found to induce an important change in the sterol profile of parsely cells consisting of an 8-fold increase in the ratio of stigmasterol to sitosterol after 80 hr. Measurements of the squalene synthase activity in microsomal fractions from control and elicitor-treated cell cultures clearly showed a rapid decline of this enzyme activity in response to the elicitor addition, which might be responsible, at least in part, for the inhibition of the overall sterol pathway.

INTRODUCTION

The defensive responses of plants to pathogen attack consist of a vast array of reactions, including production of antimicrobial compounds known as phytoalexins [1]. In Solanaceae, the phytoalexins are sesquiterpenoids (e.g. capsidiol or rishitin), the first committed step on this pathway being the cyclization of *trans–trans* farnesyl diphosphate (FPP) into either 5-epi-aristolochene [2] or vetaspiradiene [3] by a sesquiterpenoid cyclase. The induction of this sesquiterpene cyclase and, hence, sesquiterpenoid synthesis has been correlated with the inhibition of the squalene synthase (SQS) (EC 2.5.1.21) and consequent decline in sterol biosynthesis [2, 4, 5]. Because of the possible competition between sesquiterpenoid and sterol pathways for the same substrate, FPP, the inhibition of SQS has been interpreted as a mechanism to channel the available FPP molecules into phytoalexin formation [2, 4, 5]. Treatment of cell suspension cultures of *Tabernaemontana divaricata* (Apocynaceae) with a *Candida albicans* elicitor preparation was shown to inhibit both growth and monoterpene indole alkaloid production within a few hours and to cause a rapid accumulation of pentacyclic triterpenoid phytoalexins (i.e. ursolic acid and derivatives) [6]. In this case, synthesis of phytosterols is inhibited at the level of the squalene 2,3-oxide:cycloartenol cyclase. In all these plant systems, the inhibition of sterol biosynthesis could appear as a means of diverting the biosynthetic flux of isoprenoids towards phytoalexin biosynthesis. Thus, it was of great interest to investigate whether such an inhibition of sterol synthesis triggered by an elicitor was a general event or a phenomenon limited to plants synthesizing isoprenoid phytoalexins. In order to gain more insight into relationships between accumulation of phytoalexins and the ability of plant cells to synthesize sterols, we decided to use parsley cell suspension cultures, which synthesize furanocoumarins in response to the addition of a cell-wall extract of the fungus *Phytophthora megasperma* f.sp. *glycinea* (*Pmg*) [7, 8]. The elicitor-active component has been shown to be a 42-kDa glycoprotein [9]. Parsley cell cultures were first challenged with the *Pmg* elicitor over different periods of time and then administered a tracer amount of radioactive sodium acetate 2 hr before harvesting cells. Results clearly show a rapid and marked inhibition of sterol biosynthesis upstream of squalene formation as well as a block of the C-4 demethylation of 24-methylenecycloartanol.

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Measurements of SQS and 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) (EC 1.1.1.34) activities in cell-free systems prepared at different times after elicitor addition were also performed.

RESULTS

Growth and phytoalexin production by parsley cell suspension cultures in response to elicitor addition

Growth characteristics of control and elicitor-treated parsley cell suspension cultures over a period of 100 hr were monitored by fresh weight (Fig. 1). The *Pmg* elicitor was added at time 0, i.e. 10 hr after subculturing cells (see Experimental). Whereas control cells grew rapidly and increased approximately three-fold in fresh weight within 100 hr, the biomass of elicitor-treated cells remained constant whatever the period of contact with the elicitor.

In Fig. 2 is shown the time-course accumulation of phytoalexins triggered by the addition of the elicitor. Parsley cells synthesize linear furanocoumarins, which were mostly recovered in the culture medium. The main compounds were identified by HPLC analysis as bergapten, xanthotoxol and xanthotoxin and found to accumulate with distinct time courses and rates. Bergapten accumulated first whereas xanthotoxol and xanthotoxin increased later, with kinetics in agreement with the precursor to product relationship between both compounds.

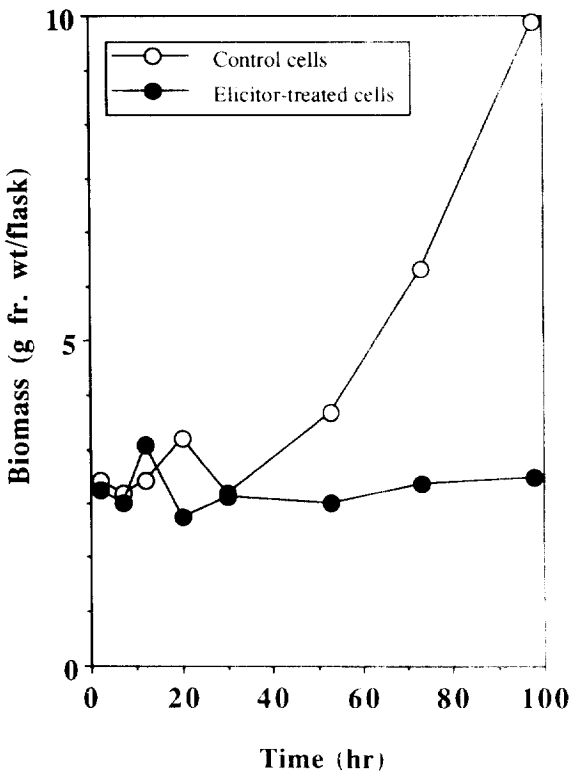

Fig. 1. Growth characteristics of control and elicitor-treated parsley cell suspension cultures. The elicitor was added at time 0.

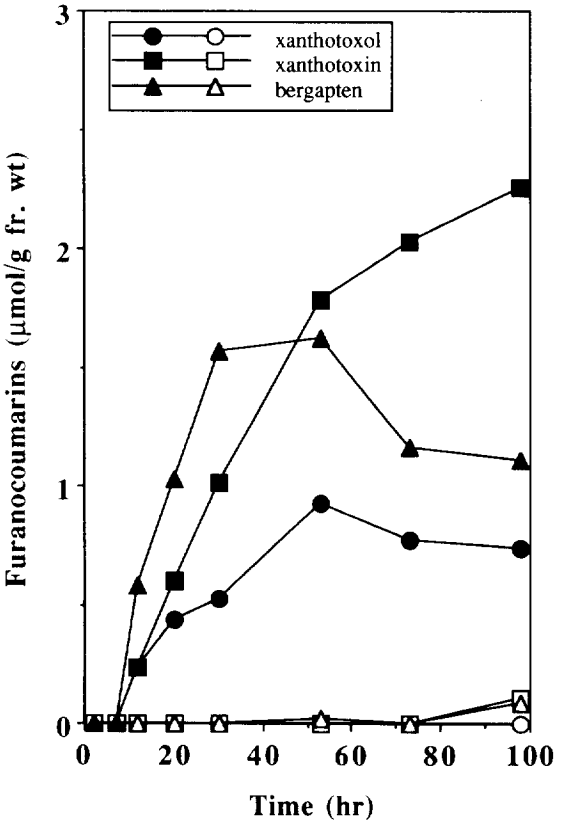

Fig. 2. Time course of furanocoumarin accumulation in the external medium by control (empty symbols) and elicitor-treated (closed symbols) parsley cell suspension cultures.

a reaction which is catalysed by xanthotoxol:*S*-adenosyl-L-methionine-*O*-methyltransferase [8]. The synthesis of coumarin derivatives was detected as soon as 7–10 hr after elicitor addition. Phytoalexins were either absent or barely detectable in control cells.

Sterol composition of control parsley cell cultures

Sterol analysis of 6-day-old parsley cell suspension cultures revealed that sterols occurred as free forms (60%) as well as esterified (20%) and glucosylated conjugates (3 and 17%, for steryl glucosides and acyl steryl glucosides, respectively). The sterol composition of the unsaponifiable fraction (free and esterified sterols) is given in Table 1. Parsley cells were found to contain a mixture of 24-methylcholesterol, stigmasterol and sitosterol as the main compounds. Low amounts of cholesterol and isofucosterol as well as of some Δ^7-sterols such as Δ^7-campestenol, Δ^7-sitostenol and avenasaterol were also detected. The 4,4-dimethylsterols were represented by cycloartenol and 24-methylenecycloartanol, and the 4α-methylsterols, mainly by obtusifoliol, 24-methylenelophenol and 24-ethylidenelophenol. A separate analysis of the free and esterified sterol pools showed that these biosynthetic intermediates were essentially present as ester derivatives (75–80%) (data not shown).

Table 1. Sterol composition of 6 day-old parsley cells cultures

Sterols	Percentage of total sterols
4,4-dimethylsterols	
Cycloartenol	2.2
24-Methylene cycloartanol	2.3
4α-Methylsterols	
24-Methylene lophenol	2.2
Obtusifoliol	0.7
Cycloeucalenol	
24-Ethylidene lophenol	0.6
4-Demethylsterols	
Chotesterol	0.5
24-Methyl cholesterol	17
Stigmasterol	34
Δ^7-Campestenol	0.5
Sitosterol	36
Isofucosterol	2
Δ^7-Sitostenol	1.5
Avenasterol	1
Total sterols (mg g^{-1} dry wt)	3.15

Sterol biosynthetic capacity of control and elicitor-treated parsley cell cultures

To investigate whether the growth inhibition of cells triggered by elicitor addition could affect the ability of cells to synthesize sterols, cultures were challenged with the elicitor for periods of time ranging from 2 to 100 hr and fed with a tracer amount of [1-^{14}C]-labelled sodium acetate 2 hr before cell sampling. Sterols were extracted from each sample of cells and analysed as the sum of free and esterified forms as described in Experimental. The unsaponifiable fractions were separated into squalene, 4,4-dimethyl, 4α-methyl- and 4-demethyl-sterols. Because acetate is not only incorporated into sterols, but also into many other compounds like fatty acids, di- and tri-acylglycerols or phospholipids, an additional step of purification of these different classes of sterols was performed. Thus, except for squalene, acetate derivatives were prepared and purified by TLC before identification, quantification and radioactivity measurements.

In Fig. 3 is shown the comparative ability of control and elicitor-treated parsley cells to synthesize sterols. The height of each bar of histograms corresponds to the total radioactivity incorporated into sterols (4-demethyl-sterols) and their precursors (squalene, 4,4-dimethyl- and 4α-methyl-sterols). The sterol biosynthetic capacity of control cells displayed a maximum between 7 and 12 hr (i.e. 17–22 hr after cell subculturing) and declined thereafter (Fig. 3A). Whereas no difference was detected between control and elicitor-treated cells after only 2 hr of contact with the elicitor, a significant decrease in the radioactivity recovered in sterols was observed for cells treated for 7 hr, corresponding to a 47% reduction compared to control cells (Fig. 3B). After 12 hr of treatment,

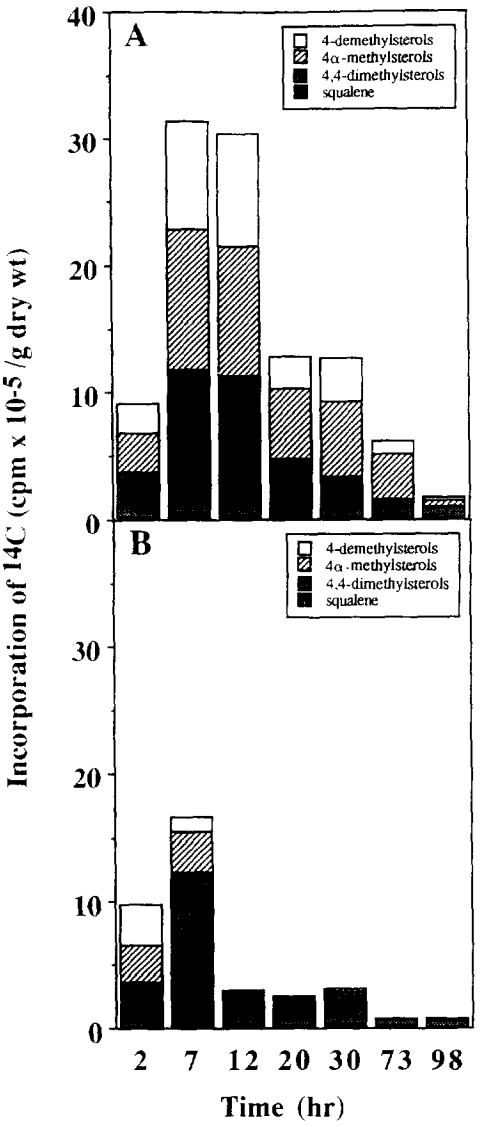

Fig. 3. Distribution of radioactivity incorporated from [1-^{14}C]-labelled sodium acetate after a 2 hour-pulse into squalene (■), 4,4-dimethyl-(▩), 4α-methyl-(▨) and 4-demethyl-sterols (□) by control (A) and elicitor-treated (B) parsley cell suspension cultures for periods of time ranging from 2 to 100 hr after elicitor addition.

this percentage of inhibition amounted to 90%. Such a dramatic decrease coincided with the onset of the increase in the furanocoumarins in the culture medium (Fig. 2). It should be pointed out that this decline in the capacity of cells to synthesize sterols probably did not result from a decrease in the uptake of the radiosubstrate by elicitor-treated cells, since after 12 hr of elicitation, only 18% of the radioactivity initially added was recovered in the external medium, a value slightly higher than that found for control cells at the same time (6%). However, for periods of time ranging from 70 to 100 hr, the uptake of acetate by elicitor-treated cells was severely decreased (data not shown).

Data from Fig. 3 also indicate that elicitor addition to parsley cells induced a strong modification of the distribution of the total radioactivity among squalene and the different classes of sterols. Thus, during the first 30 hr of incubation of control cells in water, the radiolabel from acetate incorporated into squalene, 4,4-dimethyl-, 4α-methyl- and 4-demethyl-sterols after the 2 hr-pulse represented 1–3, 25–39, 33–46 and 20–29%, respectively. In elicitor-treated cells, the bulk of the residual radioactivity was mainly recovered in 4,4-dimethylsterols. For instance, after 12 hr of contact with the elicitor, 79% of the total radioactivity was found to be associated with this fraction with 13% in squalene, 5% in 4α-methylsterols and 3% in 4-demethylsterols. Such a distribution remained almost unchanged for longer periods of elicitation.

The amount of individual compounds in each class of sterols was determined by GC analysis. Both control and elicitor-treated parsley cells exhibited similar amounts of 4-demethylsterols, expressed in mg g^{-1} of dry weight, for periods of time ranging from 2 to 53 hr. However, an important change in the sterol profile of this fraction was observed in the case of elicitor-treated cells. Whereas the relative percentage of 24-methylcholesterol remained quite constant (i.e. 16–19 and 12–15% of total free and esterified sterols in control and elicitor-treated cells, respectively), a progressive increase in the relative percentage of stigmasterol occurred concomitantly with a decrease in the relative amount of sitosterol, resulting in an eight-fold increase in the stigmasterol to sitosterol ratio after 80 hr (Fig. 4). Elicitor addition was also found to induce a progressive increase in the 4,4-dimethylsterol pool, which rose from a value of 0.14 to *ca* 1 mg g^{-1} of dry weight after 12 hr and then kept this high level for longer periods of contact with the elicitor. Such an accumulation of these sterol precursors concerned both cycloartenol and 24-methylenecycloartanol. However, for periods of time exceeding 30 hr, a slightly higher increase in the relative percentage of the second compound was noticed. In control cells, 4,4-dimethylsterols did not accumulate whatever the period of time. In both control and elicitor-treated cells, the amounts of 4α-methylsterols were found to be similar and not affected by the elicitation process.

Measurements of HMGR and SQS activities in cell-free systems

Microsomal fractions were prepared from control and elicitor-treated cells at different times after elicitor addition and tested for both HMGR and SQS activities. Despite many attempts, the activity of HMGR in parsley membranes was always found to be below the level of detectability. In Fig. 5 is shown the time-course evolution of SQS specific activity in microsomes from control and elicitor-treated parsley cells. The SQS activity of control cells displayed a maximum after about 30 hr and declined thereafter. The addition of the elicitor was found to trigger a strong decrease in the SQS activity, corresponding to an inhibition of *ca* 90% as soon as 5 hr. This inhibition was still observed even after longer periods of contact with the elicitor.

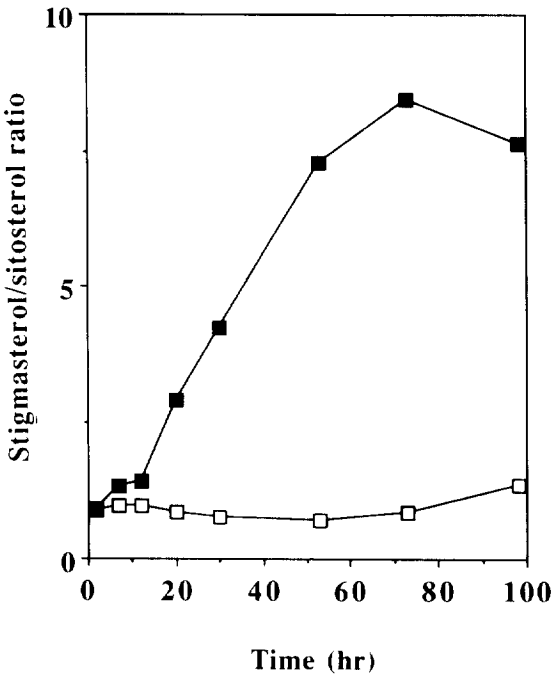

Fig. 4. Time course of changes in the stigmasterol to sitosterol ratio in control (□) and elicitor-treated (■) parsley cell suspension cultures.

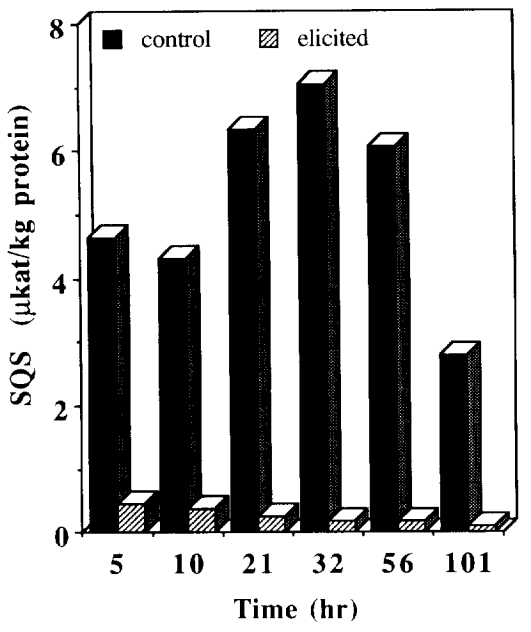

Fig. 5. SQS activities of cell-free systems isolated from control and elicitor-treated parsley cell suspension cultures at different periods of time after elicitor addition.

DISCUSSION

The present results clearly indicate that the addition of a *Pmg* elicitor to parsley cell suspension cultures induced an inhibition of cell growth as well as an accumulation of furanocoumarin phytoalexins in the culture medium. Such an inhibition of growth following treatment of cell suspension cultures from various plant families by an elicitor has been frequently reported [5, 6, 10, 11] and probably results, at least in part, from the accumulation of phytoalexins, which are toxic compounds also for plant cells. The parsley cell suspension cultures are shown to synthesize mainly bergapten, xanthotoxol and xanthotoxin, a few hours after elicitor addition. Our results concerning the nature of the furanocoumarins synthesized, the range as well as the pattern of their accumulation are fully in agreement with previous data [7, 8]. Isopimpinellin was not detected by UV analysis. However, under our HPLC analysis conditions, this coumarin derivative was found to have an elution time very close to that of xanthotoxin. Thus, the presence of a tiny amount of this compound cannot be excluded. Although the biosynthesis of furanocoumarins requires a prenylation step catalysed by the dimethylallyl diphosphate:umbelliferone dimethylallyl transferase [12], no incorporation of radioactivity from acetate into furanocoumarins after the 2 hr-pulse was observed. A similar result was reported in the case of *Ammi majus* (another member of Apiaceae) cell suspension cultures [11]. This enzymic step might take place within the plastid envelope rather than in the endoplasmic reticulum membranes as proposed for the constitutively expressed enzyme from *Ruta graveolens* [13].

Results of feeding experiments provide evidence that the addition of the *Pmg* elicitor to parsley cell suspension cultures triggers a rapid and marked inhibition of sterol biosynthesis as monitored by a general decrease in the radioactivity incorporated from [^{14}C]-acetate into squalene, 4,4-dimethyl-, 4α-methyl- and 4-demethyl-sterols. Such an inhibition of the sterol metabolic pathway upstream to squalene appears to occur just before the onset of phytoalexin accumulation; it accounted for *ca* 90% as soon as 12 hr after elicitation and was not reversed. At this time, parsley cells were found to still take up most of the radio-substrate. Thus, the inhibition of sterol biosynthesis cannot be attributed to a shortage of acetate, but rather to its rechannelling towards other metabolic pathways, for instance, toward phospholipids [11].

In elicitor-treated parsley cells, the distribution of the radiolabel among squalene and the various classes of sterols was found to be different from that obtained for control cells. Thus, 4,4-dimethylsterols, which do not accumulate in metabolically active control cells, accounted for the greatest proportion of the residual radioactivity incorporated into the sterol pathway as soon as 12 hr after elicitor addition (Fig. 3B). During the same period of time, GC analysis indicated a six to eight-fold increase in the pool of these sterol precursors. As the size of the 4α-methylsterol pool was found to be unchanged whatever the period of time and as radioactivity was no longer incorporated into these compounds, the hypothesis of a block at the level of the 4-demethylation step of 24-methylenecycloartanol to give cycloeucalenol can be proposed. It should also be recalled that our experimental protocol does not allow discrimination between free and esterified 4,4,-dimethylsterols. Thus, a large proportion of 4,4-dimethylsterol molecules accumulating in elicitor-treated cells might be present as esters. A significant increase in the levels of steryl esters in tobacco cell suspensions following treatment with cellulase was recently reported [14]. In our case, the occurrence of such an esterification process would allow the excess of these biosynthetic precursors to be diverted away from the pathway of free sterols that would no longer be required because of the arrest of cell division. The large increase in the pool of 4,4-dimethylsterols would cause the isotopic dilution of the newly synthesized molecules and thus might be responsible for the dramatic reduction in the amount of radioactivity associated with 4α-methyl- and 4-demethyl-sterols. The fact that no change in the relative proportions of cycloartenol and 24-methylenecycloartanol was found in elicitor-treated cells compared to control cells, at least for the first 50 hr, suggests that the enzyme involved in the C-24 methylation of cycloartenol remained functional.

GC analysis also gave evidence of profound changes in the relative composition of 4-demethylsterols during the elicitation process, consisting in a progressive increase in the stigmasterol to sitosterol ratio, which became eight-fold higher after 80 hr, with no change in the proportions of 24-methylcholesterol (Fig. 4). The stigmasterol to sitosterol ratio appears to be modified by a wide range of environmental stresses such as drought [15], ozone exposure [16], salinity [17, 18], chill sensitivity [19, 20] and wounding [21], but all the reported changes except those in one case [20] are of very slight magnitude. Although it has been claimed that sitosterol ans stigmasterol might have a common precursor [22], most of the biosynthetic studies suggest that stigmasterol derives from sitosterol. The C-22 desaturase involved into this step has not yet been characterized in plants. Our results indicate that this enzyme might be stimulated by the elicitation process. Previous work in the laboratory has shown that stigmasterol and sitosterol play different roles in the fluidity and permeability of soybean bilayers [23, 24]. Thus, such an enrichment in stigmasterol triggered by elicitor addition should have a profound impact on the properties of the plasma membrane of parsley cells.

Our results demonstrate a net slowing down of the metabolic flux from acetate to sterols, upstream to squalene formation, in parsley cell suspension cultures following elicitor addition, an event entirely similar to that occurring in tobacco [2, 5], potato [4] or *T. divaricata* [6]. Therefore, inhibition of sterol biosynthesis in response to the addition of a fungal elicitor corresponds to a general event, probably related to the growth arrest [25] and not restricted to plant cells synthesizing isoprenoid phytoalexins. Results recently reported by Fulton *et al.* [11] in the case of *A. majus* cells, which also produce

furanocoumarins as phytoalexins, are in agreement with this interpretation.

To determine the potential target enzyme(s) involved in such an inhibition, cell-free systems were prepared from control and elicitor-treated parsley cultures and assayed for HMGR and SQS activities. HMGR catalyses the first step committed in isoprenoid biosynthesis, by converting 3-hydroxy-3-methylglutaryl CoA into mevalonic acid, and is the rate limiting enzyme in overall sterol biosynthesis in mammalian [26] and probably in plant [27, 28] cells. It is well established that in higher plants distinct genes control the expression of HMGR activity [29, 30]. In potato, evidence has been presented for several HMGR isoforms and differential regulation of the members of this gene family [30]. In particular, expression of the constitutive isoform involved in the sterol and steroid glycoalkaloid pathways was found to be suppressed by treatment with the fungal elicitor arachidonic acid, which induces at the same time the synthesis of the isoform responsible for sesquiterpenoid formation. An inhibition of HMGR activity was also reported for soybean cell suspension cultures in response to the addition of *Pmg* elicitor [31], in agreement with our own results (unpublished data). The cell-free systems isolated from parsley cell suspension cultures were found to display no detectable HMGR activity, despite many attempts to improve the test (e.g. washing of microsomal fractions, activity assayed immediately after membrane isolation or induction of Dowex-1 in the homogenization buffer). Such results precluded us to draw any conclusion about a putative regulatory role of HMGR during the elicitation process.

SQS catalyses the first step specific to sterols, which consists in the condensation of two molecules of FPP to give squalene, and is located at the junction between a pathway of soluble metabolites (from mevalonic acid to FPP) and a pathway of highly hydrophobic compounds, the synthesis of which requires endoplasmic reticulum-bound enzymes [32]. Here is presented evidence that the addition of the *Pmg* elicitor to parsley cell suspension cultures triggers a rapid and marked decrease in SQS activity (Fig. 5). A 90% inhibition was observed as soon as 5 hr after elicitor addition, a value which might be responsible for the strong decrease in the radiolabel incorporated into squalene, sterols and their precursors (4,4-dimethyl- and 4α-methyl-sterols). Such an inhibition of SQS activity appears to be irreversible, but not complete, since, as already outlined, a synthesis of 4,4-dimethylsterols was still observed after elicitor addition. This reduction in SQS activity was also found to occur in elicitor-treated parsley cells grown in the presence of both 5μM mevinolin and 0.5 mM mevalonic acid (unpublished results), suggesting that a limited amount of mevalonic acid would not be responsible for the inhibition of the enzyme. In the case of *A. majus* cells, no inhibition of SQS activity by elicitor addition was observed. The rationale for such a discrepancy in the behavior of two cell suspension cultures belonging to the same plant family remains to be elucidated. The time course as well as the range of SQS inhibition reported in the present work do not differ from results reported for Solanaceae. The question now is to determine by which mechanism SQS activity is regulated in parsley cells. A direct interaction of the elicitor with the enzyme is very unlikely. Attempts to measure levels of HMGR and SQS transcripts in control and elicitor-treated parsley cells are in progress in the laboratory and are expected to shed more light on the regulation of the whole sterol biosynthetic pathway.

EXPERIMENTAL

Radiochemicals and chemicals. [1-^{3}H]FPP (555 GBq mmol^{-1}) and [1-^{14}C] labelled sodium acetate (2.22 GBq mmol^{-1}) were purchased from Isotopchim (France) and Amersham, respectively. FPP was a generous gift from Prof. B. Camara (Strasbourg, France). Xanthotoxin, bergapten and xanthotoxol were from Roth (Germany).

Cell cultures. Cell suspension cultures of parsley (*Petroselinum crispum* L.) were usually grown in 250-ml Erlenmeyer flasks containing 40 ml modified B_5-medium, at 26°, in the dark, with constant agitation (110 rpm) as described previously [33]. They were subcultured weekly by transfer of 4 ml suspension (*ca* 1 g fr. wt of cells) into 40 ml fresh medium. Growth was monitored by measuring both cell fr. wt and conductivity of the external medium.

Elicitor treatment. The elicitor was prepared from hyphal cell walls of the fungus *Pmg* race 1 according to ref. [34]. Elicitation experiments were performed using 6-day-old cultures (log phase, conductivity of *ca* 2). An aliquot of suspension (2 g) was introduced into 40 ml fresh medium. After 10 hr, a sterile elicitor soln was added to each flask to a final concn of 50 μg ml^{-1} corresponding to 20 μg Glc eq. and 4.8 μg prot. ml^{-1}). After various periods of contact with the elicitor, cells were harvested by vacuum filtration, frozen in liquid N_2 and stored subsequently at −80° until further analysed. For feeding experiments, [1-^{14}C]-labelled NaOAc (0.185 MBq, 2.3 μM final concn) was added to each flask 2 hr before collecting cells. Cells and culture media were analysed for radioactivity incorporated into sterols and furanocoumarins.

Preparation of microsomal fractions. Frozen parsley cells were ground in a mortar in the presence of liquid N_2. The resulting fine powder was resuspended in a medium consisting of 0.1 M Na-Pi buffer (pH 7.4) containing 250 mM sucrose, 4 mM EDTA, 100 mM KF, 40 mM Na ascorbate and 0.2% (w/v) BSA (5 ml g^{-1} fr. wt of cells). The homogenate was filtered through a nylon blutex and the suspension was centrifuged at 10 000 *g* for 15 min. The resulting supernatant was centrifuged at 100 000 *g* for 60 min. The microsomal pellet was resuspended in 0.1 M Pi buffer (pH 7.5) containing 30% (w/w) glycerol and 1.5 mM 2-mercaptoethanol and stored at −80° at a concn near to 1 mg ml^{-1}. Protein content was determined according to Bradford [35], with BSA as standard.

Enzyme assays. For the squalene synthetase activity, parsley microsomes (*ca* 10 μg protein) were incubated in 0.1 M Tris–HCl (pH 7.4), 10 Mm $MgCl_2$, 10 mM DTT, 10 mM KF, 2.5 mM NADPH, 5 mM Chaps and 50 μM (7.4 kBq) [1–^{3}H]FPP in a total vol. of 100 μl. After incubation at 30° for 30 min, the reaction was stopped by addition of KOH–*iso*-PrOH containing 50 μg carrier squalene. Samples were agitated and centrifuged at 10000 *g* for 2 min. An aliquot of the organic phase was subjected to TLC with CH_2Cl_2 as solvent. After 10 min of migration, bands corresponding to squalene were scraped off and counted for radioactivity in 10 ml of scintillation cocktail. Specific activities were expressed in μkat of FPP consumed kg^{-1} protein. HMGR activity was measured as described in ref. [36].

Furanocoumarin analysis. Coumarin derivatives were extracted from culture media. An aliquot (10 ml) was centrifuged at 20000 *g* for 15 min and extracted (3 ×) with 30 ml $CHCl_3$. After filtration through Na_2SO_4, the organic phase was evapd. The residue was dissolved in 0.5 ml EtOH and analysed by HPLC on a 5-μm Partisil column (250 × 4.6 mM) under isocratic conditions with hexane–PrOH–MeOH, 47:4:1) at a flow rate of 1 ml min^{-1}. Detection was at 254 nm. Xanthotoxol, bergapten and xanthotoxin were eluted after 6, 7 and 8 min, respectively. Furanocoumarins were identified by their UV spectra. *Bergapten*, λ_{max} (EtOH) nm: 308, 267, 249, 241sh; *Xanthotoxin*, λ_{max} (EtOH) nm: 298, 262sh, 248; *Xanthotoxol*, λ_{max} (EtOH) nm: 306, 267, 261, 249; *Isopimpinellin*, λ_{max} (EtOH) nm: 310, 270, 249, 241. Quantification was made by comparison of peak areas with those obtained by direct injection of known amounts of authentic compounds.

Sterol analysis. Sterols were extracted from lyophilized parsley cells and analysed as the sum of free and esterified sterols as previously described [32]. Briefly, unsaponifiable lipids were dissolved in CH_2Cl_2 and subjected to TLC on 60 F_{254} silica gel (Merck) with CH_2Cl_2 as developing solvent (2 runs). Bands corresponding to squalene, 4,4-dimethyl-, 4α-methyl- and 4-demethyl-sterols were scraped off and compounds eluted with CH_2Cl_2. Sterols and their precursors (4,4-dimethyl- and 4α-methyl-sterols) were then acetylated and purified by TLC (one run in CH_2Cl_2). Radioactive products on plates were located using an LB 2820-1 Berthold automatic linear analyser. Steryl acetates were identified by GC-MS [37], quantified by GC with cholesterol as int. standard [32] and assayed for radioactivity by liquid scintillation spectrometry.

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