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Incorporation of all-*trans*-farnesol into sterols and ubiquinone in *Nicotiana tabacum* L. cv Bright Yellow-2 cell cultures

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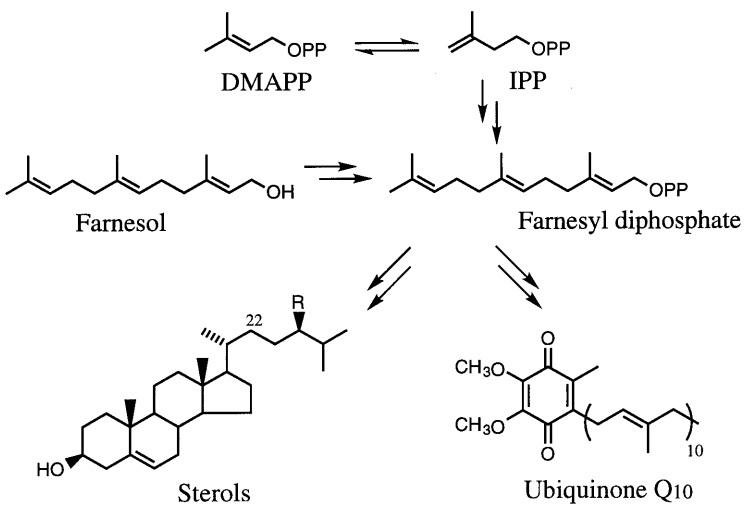
Abstract—Feeding of *E,E*-[1-^{3}H] farnesol to *Nicotiana tabacum* L. cv Bright Yellow-2 cells demonstrated large incorporation of the radioactivity into free sterols, the predominant end products of the mevalonate pathway. All the individual sterols, including cholesterol, were found to be labeled. Farnesol radioactivity was also detected in the prenyl side-chain of ubiquinone Q_{10}.

Isoprenoids arise from isopentenyl diphosphate (IPP). In higher plants, two distinct pathways have been shown to concomitantly operate in isoprenoid biosynthesis.[1] In plastids, IPP is used for the synthesis of carotenoids, mono- and diterpenes, or the prenyl chains of chlorophylls and plastoquinone. It arises from the condensation of pyruvate with glyceraldehyde-3-phosphate and involves the intermediate formation of 2-*C*-methyl-D-erythritol 4-phosphate.[1] In the cytosol, IPP is formed via the classical acetate/mevalonate (MVA) pathway,[2] giving rise to a completely different set of compounds such as sterols, polyprenols or prenylated proteins. In this pathway, farnesyl diphosphate (FPP) is a key intermediate, from which isoprene units are dispatched to the different classes of isoprenoids, probably within distinct metabolic channels.[3] Sterols represent the predominant end products, but what controls this multi-branched pathway in plant cells is still far from being understood.



Scheme 1. Biosynthesis of cytoplasmic isoprenoids from FPP. R=H, cholesterol; R=CH_3, 24-methylcholesterol; R=C_2H_5, sitosterol; R=C_2H_5, Δ^{22}, stigmasterol.

Keywords: farnesol; sterols; ubiquinone Q_{10}; tobacco; isoprenoid biosynthesis.

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Mammalian cells have been shown capable of using free farnesol to synthesize isoprenoids, especially cholesterol.[4] To be a substrate for squalene synthase, the first committed enzyme in sterol biosynthesis, farnesol has to be converted into FPP, a process which proceeds in two steps.[5] Inversely, farnesol can also arise from FPP by removal of the pyrophosphate group, a reaction catalyzed by a FPP-specific pyrophosphatase.[6] However, free farnesol probably does not accumulate inside the cells as it has been reported to induce toxic effects.[7] Recently, the same has been proved to hold true for plant cells, especially for tobacco (*Nicotiana tabacum* L. cv Bright Yellow-2) (TBY-2) cells.[8] By using the same biological system, we planned to extend this study by investigating whether exogenous farnesol could be incorporated into identified sterols. In contrast to animal cells, which contain only one major sterol, cholesterol, plant cells synthesize a complex array of sterols, in which sitosterol, stigmasterol and 24-methylcholesterol often predominate (Scheme 1).[9] We also wanted to check whether exogenous farnesol could be used for synthesis of the prenyl side-chain of ubiquinone Q_{10}, an important component of the electron transfer chain in mitochondria. The synthesis of this all-*trans*-polyprenol was reported to arise from the initial condensation of IPP with either FPP or geranylgeranyl diphosphate.[10] In TBY-2 cells, this synthesis has been shown to be clearly dependent on a cytosolic source of IPP,[11] but the utilization of other isoprenic units remained to be investigated.

To address these questions, feeding experiments using *E,E*-[1-^{3}H] farnesol to TBY-2 cells were carried out.[12,13] Exogenous farnesol was found to be efficiently taken up by TBY-2 cells as only 16% of the initial radioactivity remained in the external medium after 24 h. Around 35% of the farnesol radioactivity were recovered in the hexane extract. Free sterols were analyzed as described.[14–16] TBY-2 cells were found to have a relatively high content in free sterols (2.75±0.15 mg/g dwt), mainly present as 4-demethylsterols (97%). Major compounds were the typical plant Δ^5-sterols: 24-methylcholesterol (31%), stigmasterol (31%) and sitosterol (15%), with significant amounts of 24-methylenecholesterol and isofucosterol as late biosynthetic intermediates (Table 1). Cholesterol represented 1%. Stanols (0.9% of total sterols) as well as Δ^8- and Δ^7-sterols (less than 0.3%) were also identified. The remaining compounds corresponded to early biosynthetic precursors such as 4,4-dimethyl- (cycloartenol and 24-methylene cycloartanol) and 4α-methylsterols.

Table 1. *E,E*-[1-^{3}H] farnesol incorporation into major free 4-demethylsterols from TBY-2 cells

	Moles%	Cpm/nmol
Cholesterol	1	1155
24-Methylenecholesterol	13	370
24-Methylcholesterol	33	635
Stigmasterol	29	390
Sitosterol	15	510
Isofucosterol	9	325

The different 4-demethylsterols as acetate derivatives were separated from each other by argentation TLC and reverse-phase HPLC. They were quantified by GC and their radioactivity determined by liquid scintillation counting. The relative sterol composition of 4-demethylsterols is given in moles%.

Free sterols were found to contain 60% of the radioactivity present in the hexane extract, with most of it incorporated into 4-demethylsterols, the end products. This fraction was resolved into individual compounds by reverse-phase HPLC and the specific radioactivity of each was determined after quantification by GC and liquid scintillation counting. All the components of the sterol mixture, including cholesterol, were labeled (Table 1), indicating that farnesol can be effectively used as a precursor for all sterols. The highest specific radioactivity was found to be associated with cholesterol, likely because of its low pool size in TBY-2 cells. These results extend the preliminary data from Thai et al.,[17] who previously observed an incorporation of farnesol into non identified digitonin-precipitable sterols in tobacco cells and demonstrated the occurrence in these cells of two distinct CTP-dependent kinases involved in the conversion of farnesol into FPP.

Ubiquinone Q_{10} was isolated from the same hexane extract as sterols.[18] This compound was further purified and identified by positive FAB-MS. The spectrum displayed the characteristic fragmentations corresponding to the reduced form of ubiquinone Q_{10}.[19] The de novo synthesized quinone was found to contain about 1% of the radioactivity of the hexane extract. To further check its radiochemical purity, this compound was analyzed by reverse-phase HPLC after addition of carrier and detection at 275 nm.[20] The peak detected at 275 nm corresponded to the standard of ubiquinone Q_{10} and was found to contain 80% of the injected radioactivity. Finally, another proof that farnesol radioactivity was incorporated into this compound came from recrystallizations to constant specific radioactivity, in the presence of a carrier.[21]

Taken together, these data provide clear evidence that exogenous farnesol can be utilized both for the synthesis of sterols and the prenyl side-chain of ubiquinone Q_{10} in TBY-2 cells as has been demonstrated for animal cells.[4] Further work is in progress to identify other isoprenoid derivatives toward which farnesol might be directed.

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12. TBY-2 cells were cultivated in a modified Murashige & Skoog medium and subcultured weekly as reported.[13] Feeding experiments were conducted by diluting 20 ml of 8-day-old TBY-2 cells (approximately 7 g of fresh weight) with 80 ml of fresh medium. Then, cells were allowed to grow aerobically for 24 h at 28°C in the dark in the presence of *E,E*-[1-^{3}H] farnesol (25 μC_i, 4.5 nM). Cells were harvested by filtration. Freeze-dried material was ground and extracted by refluxing two times with dichloromethane/methanol (2:1, v/v) for 2 h. Extracts were combined, dried under reduced pressure and thoroughly washed at room temperature with hexane to recover free sterols and ubiquinone.
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14. The hexane extract was applied on silica gel plates and developed with dichloromethane (2 runs). The radioactivity was detected on TLC plates by using a linear radioactivity analyzer Berthold LB 2820-1. Sterols and their precursors, 4,4-dimethyl- and 4α-methylsterols were recovered, then acetylated as previously described.[15] After purification, steryl acetates were quantified and analyzed by a GC equipped with a flame ionization detector and a DB-1 capillary column (30 m×0.25 mm i.d.), with hydrogen as the carrier gas. The temperature program was: from 60 to 230°C at 30°C min^{-1}, then from 230 to 280°C at 2°C min^{-1}, and hold at 280°C for 5 min. Cholesterol was used as an internal standard. The 4-demethylsterol acetates were applied on silica plates impregnated with 10% $AgNO_3$ and chromatographed with ethanol-free chloroform as solvent. Five fractions corresponding to 24-methylene cholesterol (R_f 0.11) (I), isofucosterol (R_f 0.19) (II), stigmasterol (R_f 0.44) (III), the mixture of cholesterol, 24-methylcholesterol and sitosterol (R_f 0.51) (IV) and a mixture of stanols, Δ^8- and Δ^7-sterols (R_f 0.58) (V). Constituents of fraction IV were separated from each other by reverse-phase HPLC using a C_{18} ODS Ultrasphere 5 μm-column (4.6×250 mm) and a mixture of methanol–H_2O (99.7:0.3, v/v) as solvent at a flow rate of 1 ml min^{-1}. OD was monitored at 210 nm. The identity of each peak was checked by GC and quantified and its radioactivity measured by liquid scintillation counting. All sterols were identified by GC–MS (electron impact ionization: 70 eV).[16]
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18. Ubiquinone Q_{10} was recovered from the hexane extract after TLC as a radioactive band with a R_f similar to that of a standard (R_f 0.47, one run in dichloromethane). The band corresponding to ubiquinone was further purified by TLC using cyclohexane–dichloromethane (1:1, v/v) as the solvent (R_f 0.18), resulting in elimination of about half of the radioactivity. The identity of ubiquinone was confirmed by positive FAB-MS (direct inlet). The spectrum displayed the following fragmentations: m/z: 864 ubiquinol (M^+, 9%), 235 (A^+, 20%) and 197 (B^+, 100%).[19]
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20. After addition of carrier, ubiquinone was analyzed by reverse-phase HPLC on a C-18 Beckman ODS Ultrasphere 5 μm-column (4.6×250 mm) using a linear gradient of 2-propanol in methanol from 15:85 (v/v) to 40:60 (v/v) as the solvent at a flow rate of 1 ml min^{-1}. OD was monitored at 275 nm. Elution time for ubiquinone Q_{10} was 38 min.
21. The de novo labeled ubiquinone Q_{10} was mixed with carrier (30 mg) and dissolved in a few drops of dichloromethane. Then, methanol was added (3 ml) and heated at 60°C until a clear liquid was obtained. The solution was then kept at room temperature for 20 min and at 4°C for 15 min. The microcrystals were recovered by filtration, dried under reduced pressure, and weighed. Four successive recrystallizations were performed. Initial specific radioactivity: 3590 cpm/mg; after the 1st, 2nd, 3rd and 4th recrystallization, values were: 2830, 2550, 2825 and 2350 cpm/mg, respectively.