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Overexpression in *Catharanthus roseus* Hairy Roots of a Truncated Hamster 3-Hydroxy-3-Methylglutaryl-CoA Reductase Gene

Teresa Ayora-Talavera,[1,4] Joseph Chappell,[2] Edmundo Lozoya-Gloria,[3] and Victor M. Loyola-Vargas*,[1]

[1]Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43, No. 130 Col. Chuburna de Hidalgo, C.P. 97200, Mérida Yucatán, México, E-mail: vmloyola@cicy.mx; [2]Plant Physiology/Biochemistry/ Molecular Biology Program, Agronomy Department, University of Kentucky, Lexington, KY 40546-0091; [3]Departamento de Ingeniería Genética, CINVESTAV-IPN, Unidad Irapuato, Km 9.6 Libramiento Norte, Carretera Irapuato-León, Apdo. Postal 629, CP 36500, Irapuato, Gto., México; and [4]Departamento de Biotecnología y Bioingeniería, CINVESTAV-IPN, Unidad México, Avenida Instituto Politécnico Nacional 2508 Col. San Pedro Zacatenco, CP 07360, DF, México

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Abstract

Catharanthus roseus (L.) G. Don hairy roots harboring hamster 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (EC 1.1.1.88) cDNA without membrane-binding domain were evaluated by quantifying the levels of sterols and some indol-alkaloids. Clone 236, with the highest hybridization signal, had the lowest soluble and microsomal HMGR activity and produced more ajmalicine and catharanthine than the control but had reduced campesterol concentration. Clone 19, with low hybridization signal, had high soluble HMGR activity and produced high levels of campesterol and five to seven times more serpentine than the control but a low level of ajmalicine and no accumulation of catharanthine. These results suggest a possible role for HMGR in indole alkaloid biosynthesis and a possible cosuppression of both the endogenous and foreign HMGR genes in clone 236.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Alkaloids; *Catharanthus roseus*; hairy roots; 3-hydroxy-3-methylglutaryl-CoA reductase.

Introduction

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is one of the most important traditional medicinal plants in the world. It produces more than 200 indole alkaloids, some with pharmaceutical properties such as the antineoplasic compounds vinblastine and vincristine and the antihypertensive compounds ajmalicine and serpentine *(1)*. Their therapeutic use, commercial value, and low content in the plant have led to efforts to produce them by different in vitro culture methods. However, with the sole exception of shoot culture, which produces bisindolic alkaloids *(2)*, all other efforts to produce vinblastine and vincristine have been unsuccessful.

A better knowledge of the corresponding biosynthetic pathways would provide a greater opportunity to manipulate secondary metabolite production in in vitro cell cultures. The biosynthesis of monoterpene indole alkaloids begins with the synthesis of strictosidine, which is the universal precursor of these alkaloids, by strictosidine synthase using tryptamine and secologanin as substrates. Tryptamine is the result of decarboxylation of tryptophan by tryptophan decarboxylase. Secologanin, an isoprenoid glucoside, is synthesized from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate, the precursors of all terpenes.

Precursor feeding experiments suggest that secologanin plays an important role in the control of indole-alkaloid biosynthesis, at the level of the terpenoid pathway *(3–8)*. The overexpression of genes coding for tryptophan decarboxylase and strictosidine synthase in plants and tissue culture also supports the hypothesis that the availability of secologanin *(9,10)*, or perhaps a precursor, is an important limiting factor.

There are two possible pathways for IPP biosynthesis: the cytoplasmatic mevalonate pathway and the chloroplastic deoxyxylulose phosphate pathway (Fig. 1) *(11,12)*. Evidence is still contradictory about which route provides IPP for monoterpene biosynthesis. Contin et al. *(13)* propose that both pathways provide IPP for secologanine biosynthesis, either together or in parallel, depending on the particular physiologic state of the cells. Recently, Eichinger et al. *(14)* proposed the possibility of crosstalk between the two pathways with some precursors to form loganine. These hypotheses are reinforced by the finding of a putative IPP transporter in plastids *(15)* and the existence of a nongreen plastidic and a chloroplastic geranyl diphosphate synthase *(16)*.

In the mevalonate pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (EC 1.1.1.88) catalyzes the reduction of HMG-CoA to mevalonate, an IPP precursor, and is considered a key enzyme in the synthesis of cytoplasmic isoprenoids in plants *(17)* such as fitosterols (campesterol, stigmasterol, and sitosterol) and prenyl chains for some proteins, sugars, and lipids. The enzyme activity is changed by different factors

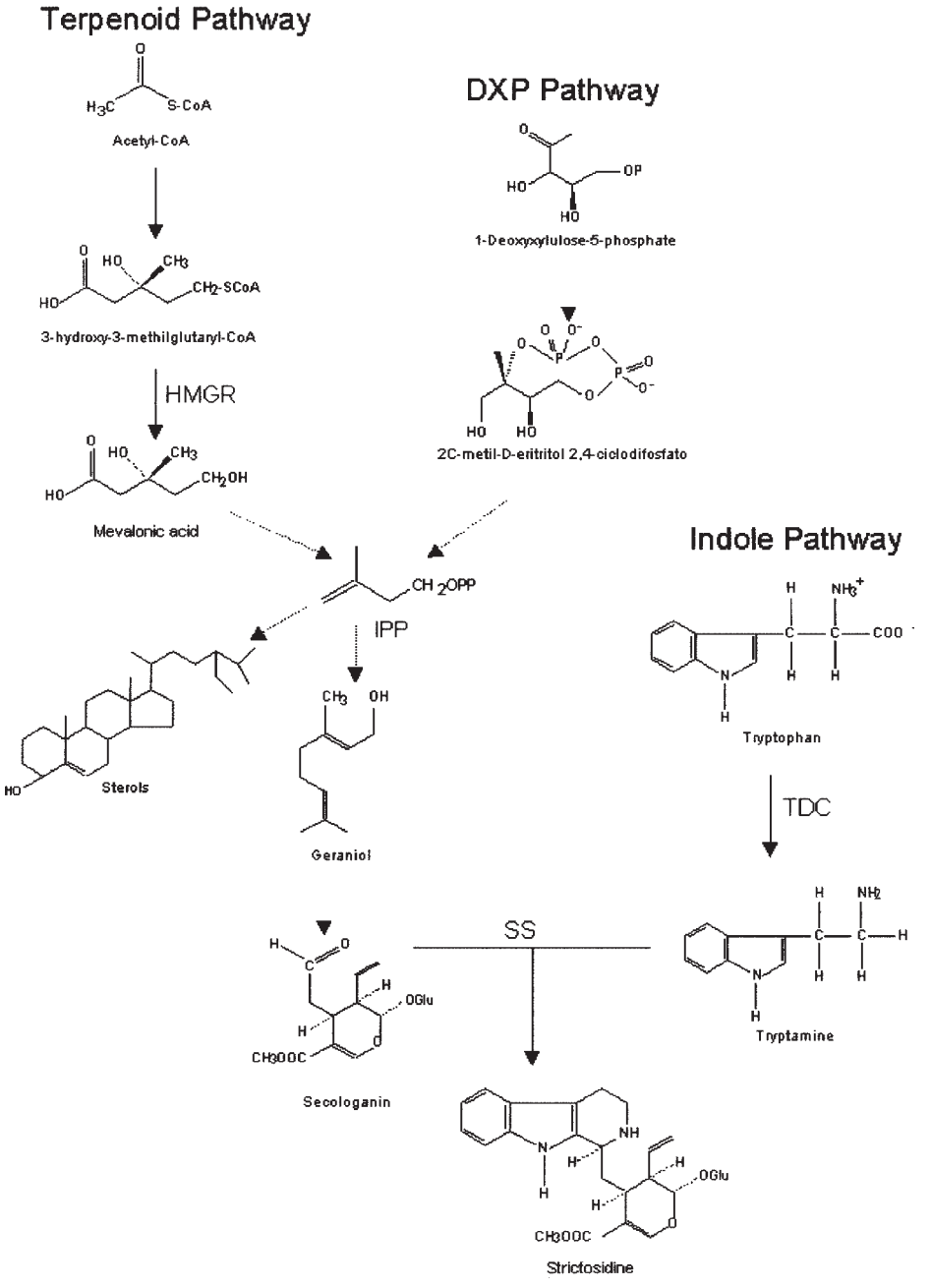

Fig. 1. Strictosidine biosynthetic pathways. Deoxyxylulose 5-phosphate (DXP) pathway.

such as light *(18–21)*, the developmental stage of the plant *(22–24)*, and the presence of elicitors *(25–31)*.

Assuming that mevalonic IPP is participating in the biosynthesis of secologanin precursors and that the availability of these is a constraint to

strictosidine production, HMGR deregulation should affect indol-alkaloid biosynthesis. To explore this hypothesis and to evaluate the role of HMGR in the biosynthesis of these compounds, we obtained *C. roseus* hairy roots transformed with a hamster gene coding for a soluble HMGR protein lacking the membrane-binding amino-terminal end.

Materials and Methods

The *Agrobacterium rhizogenes* strain 1855FD was transformed with the pKYLX71-Δ227 plasmid holding an *Eco*RI/*Cla*I DNA fragment (35S CaMV-227 HMGR [–22 to 3388 bp of truncated hamster HMGR cDNA]-rbcss 3') *(32)* by electroporation *(33)*. Transformants were grown at 28°C in YEB semisolid medium with rifampicin (100 mg/L) and carbenicillin (100 mg/L), and selection was carried out using streptomycin (100 mg/L). Selected clones were verified by colony hybridization using the 35S CaMV promoter fragment labeled with Random Primer Extension (Gibco-BRL) as a probe, and by the soluble activity of HMGR *(32)*.

Plant Material

One-week-old *C. roseus* seedlings were incubated in liquid Gamborg's medium *(34)* in the presence of 535 μ*M* nattalen acetic acid for 24 h at 25°C in the dark. The leaves and stems were inoculated with *A. rhizogenes* 1855FD/pKYLX-Δ227. After 1 wk some roots appeared at the infection site. Roots of 2 to 3 cm in length were separated from the stems or leaves and cultivated in solid, hormone-free, half-strength salts Gamborg's medium, with complete vitamin concentration and sucrose (30 g/L), in the presence of claphoran (1 mg/mL), carbenicillin (1 mg/mL), and kanamycin (25 mg/L). Clean hairy roots were cultivated in the same liquid medium with kanamycin alone. Previously established hairy roots of the J1 line transformed with the same *Agrobacterium* strain were used to compare the behavior of the HMGR transformed clones *(35)*.

DNA Extraction and Southern Blotting

Plant DNA extraction was carried out according to Dellaporta et al. *(36)*. The plant DNA (15 μg) was digested with *Eco*RI and *Bam*HI, fractionated by electrophoresis, and blotted onto nylon membranes. The labeled 35S CaMV promoter fragment was used as a probe.

Biochemical Assays

The enzyme extraction and activity assays were carried out according to Moreno-Valenzuela et al. *(37)*. Protein concentration was determined according to Peterson *(38)* with bovine serum albumin (Sigma, St. Louis, MO) as the standard. Alkaloid extraction and serpentine quantification were carried out according to Monforte-González et al. *(39)*. The levels of catharanthine and ajmalicine were determined by high-performance liq-

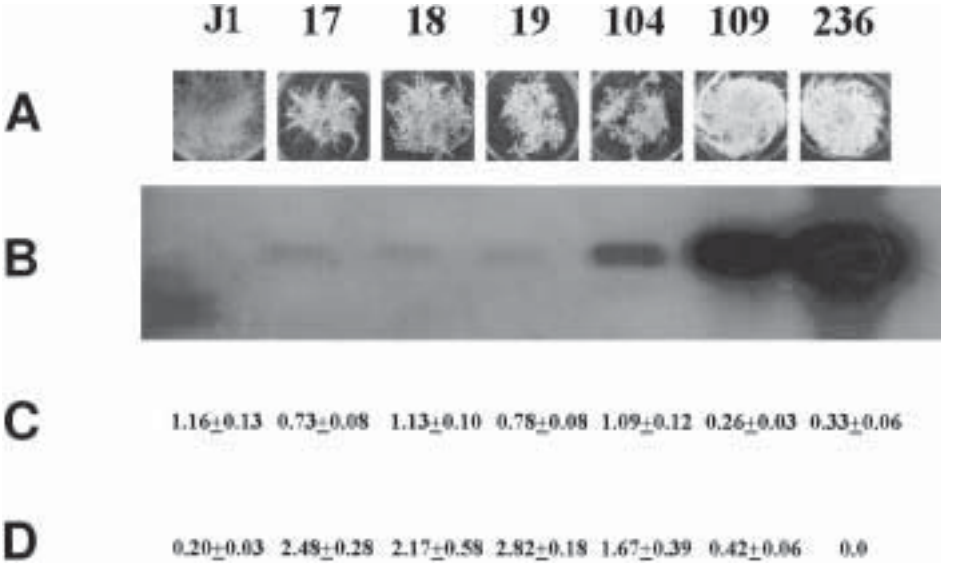

Fig. 2. **(A)** *C. roseus* hairy root cultures. J1 is the line of *C. roseus* transformed roots with the *A. rhizogenes* strain 1855, and 17, 18, 19, 104, 109, and 236 are the hairy root clones harboring pKYLXΔ227 plasmid; **(B)** Southern blot of hairy root cultures harboring pKYLXΔ227 plasmid using promoter 35S CaMV as probe; **(C)** microsomal HMGR specific activity (pkat·mg^{-1} protein); **(D)** soluble HMGR specific activity (pkat·mg^{-1} protein). All analyses are the average of three independent experiments.

uid chromatography *(37)*. Alkaloid identification was achieved by comparison against commercial standard (Sigma). Sterol extraction and gas chromatography analysis were performed according to Chappell et al. *(32)*.

Results and Discussion

Culture Establishment and HMGR Activity

Six kanamycin-resistant hairy root clones (17, 18, 19, 104, 109, and 236) showing different morphology were selected (Fig. 2A). Genomic integration of the 35S CaMV promoter fragment was demonstrated by Southern blot analysis (Fig. 2B). Hybridization signals differed widely from very dim to very strong, and these results were inversely proportional to the amount of soluble HMGR activity (Fig. 2D). Some of the transformed roots were thick, brown, nonhairy, and poor growing (clones 17, 18, and 19), but their soluble HMGR enzyme activity was up to 14-fold higher in comparison to the J1 line. The very low soluble activity of HMGR in the J1 line could be the result of microsomal contamination of the 100,000*g* supernatant.

Clones showing the strongest hybridization signal (104, 109, and 236) were white and the thinnest roots grew well, and their soluble HMGR activities were lower than the other clones. We were unable to detect any soluble HMGR enzyme activity in clone 236 even when taking microsomal contamination into consideration. The microsomal enzyme activity was

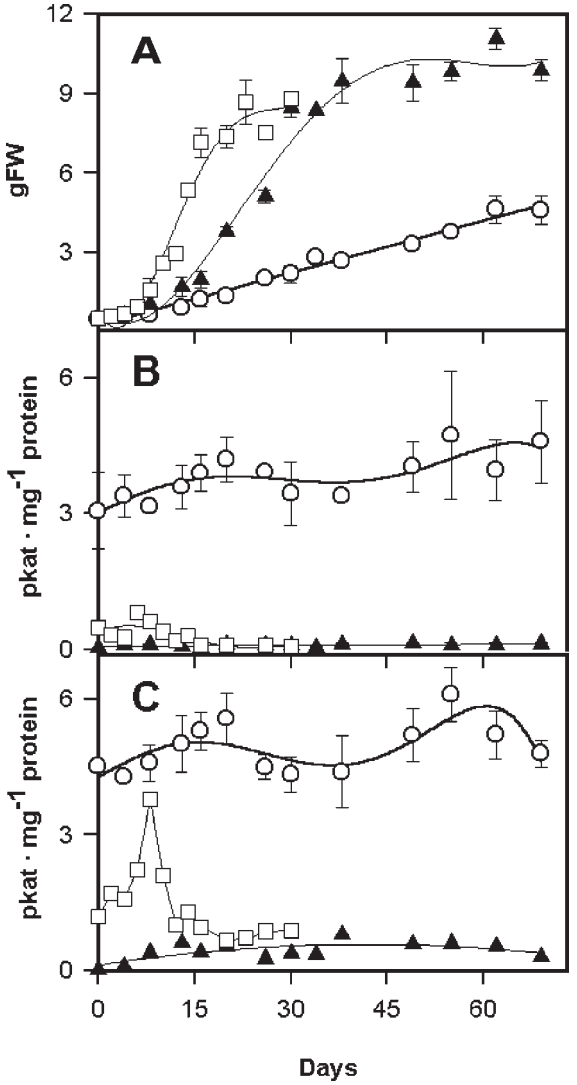

Fig. 3. **(A)** Growth (grams of fresh weight, gFW), **(B)** soluble HMGR activity (pkat·mg^{-1} protein), and **(C)** total HMGR activity (pkat·mg^{-1} protein) of clone 19 (○), clone 236 (▲), and J1 line (□). All analyses are the average of three independent experiments.

almost the same in the J1 line and clones 17, 18, 19, and 104, but lower in the clones 109 and 236. The most contrasting clones showing soluble HMGR enzyme activity (19 and 236) were selected to investigate alkaloid production and sterol content.

These two clones were cultivated for 69 d (Fig. 3), and the J1 line was cultured for 30 d. The growth rates of 0.086 g/d (clone 19) and 0.353 g/d (clone 236) were 16 and 4 times slower than the J1 line (Fig. 3A). The soluble enzyme activity was higher in clone 19 throughout the culture, but almost

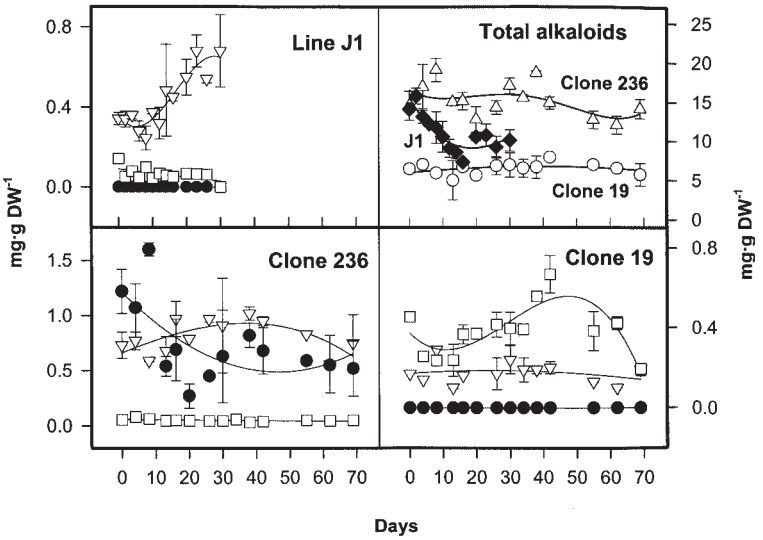

Fig. 4. Ajmalicine (▽), catharanthine (●), and serpentine (□) production by *C. roseus* clones during a culture cycle. All analyses are the average of three independent experiments. (DW = dry weight)

undetectable in clone 236 and the J1 line (Fig. 3B). The total HMGR enzyme activity was also higher in clone 19 during the whole culture, and lower in clone 236 with respect to the J1 line. This line showed a peak of activity around d 8 (Fig. 3C). The absence of soluble HMGR in clone 236 strongly suggests a cosuppression phenomenon of both the endogenous and foreign HMGR genes *(40–43)*.

Alkaloid and Sterol Production in Transformed Hairy Roots

The individual alkaloid production was very different between clones. Line J1, which does not have soluble HMGR, normally produces high levels of ajmalicine, with a maximum of 0.7 mg/g dry wt around d 28; very low amounts of serpentine; and no catharanthine (Figs. 2D and 3B). Clone 236 also had no soluble HMGR but produced 1.5 mg/g dry wt of catharanthine around d 5 and 0.85 mg/g dry wt around d 35; 0.9 mg/g dry wt of ajmalicine almost constantly; and very low levels of serpentine throughout the culture (Fig. 4). By contrast, clone 19, having the higher levels of soluble HMGR, produced 0.65 mg/g dry wt of serpentine at d 40, about five to seven times more than clone 236; low levels of ajmalicine; and no catharanthine throughout the culture. The total alkaloid production in clone 19 only reached an average of 7 mg/g dry wt during the culture, while clone 236 had an average of 15 mg/g dry wt. The J1 line showed alkaloid content between that of clones 236 and 19 (Fig. 4).

Campesterol, sitosterol, and stigmasterol are the main sterols in *C. roseus* hairy roots, and their accumulation was dependent on the developmental stage of the culture (Fig. 5). In the J1 line, there was an increase in these three

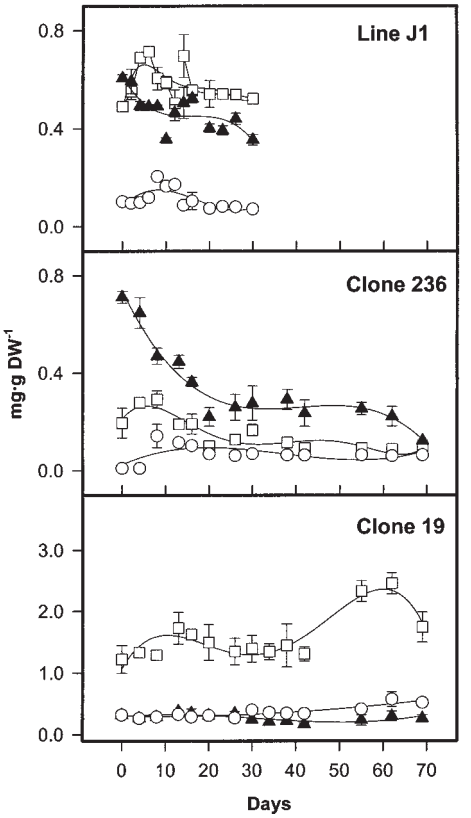

Fig. 5. Campesterol (□), stigmasterol (▲), and sitosterol (○) production by *C. roseus* clones during a culture cycle. All analyses are the average of three independent experiments. (DW = dry weight)

compounds from 1.2 at the beginning up to 1.6 mg/g dry wt at the end of the culture. The amount of these three compounds increased in clone 19 from 1.85 up to 3.32 mg/g dry wt. By contrast, clone 236 had a decrease in these sterols from 1.02 to 0.36 mg/g dry wt. This fact contrasts with tobacco, transformed with the same construction used in the present work, in which the accumulation of the normal end-product sterols was not influenced as much by the upregulation of HMGR activity *(32)*. Schaller et al. *(44)* inserted HMG1 gene from *Hevea brasiliensis* into tobacco and found an increase in the level of total sterols up to sixfold and no difference in levels of the end products.

The analysis of the individual behavior of the sterols in *C. roseus* hairy roots showed that campesterol was the major component in lines J1 and clone 19, with an average of 42.3 and 69.3% of the total sterols, respectively, during the cell culture, whereas in clone 236, stigmasterol was the major component, with an average of 41.9%. Sitosterol was very similar for clones 19 and 236 (12.6 and 14%, respectively), while in the J1 line the average was 7.3% throughout the culture cycle.

Multiple HMGR genes, each with unique roles, have been reported in some plants such as potato HMG1 (expressed by wounding) *(44)* or tomato HMG2 (expressed by elicitors) *(46)*. In *C. roseus* only one gene, *HMG*1, has been found *(47)*, and there are no reports that relate it to a specific function. There will probably be more HMGR genes in this plant, so the insertion of the hamster truncated HMGR in clone 236 suggests *HMG*1 silencing or different endogenous reductase, or both. This is related to the biosynthesis of stigmasterol decreasing its levels and using the carbon skeletons to produce alkaloids, especially ajmalicine and catharanthine, by G3P/pyruvate or HMGR isoenzyme, or both. Another possibility is the use of these carbon skeletons in the biosynthesis of mevalonic derivatives such as prenyl chains essentially in cell function. There are some reports that suggest the key regulatory role of protein prenylation and its participation in cellular signaling, membrane trafficking *(48)*, and regulation of transcription factors *(49)*.

There are many reports in which the use of stress factors improved secondary metabolite production *(50)*. The high serpentine production of clone 19 could be related to stress response. This clone had high levels of soluble HMGR activity and produced high levels of campesterol and probably other mevalonic compounds, so there is a high concentration of mevalonic solutes inside the cell that can induce osmotic stress. Zhao et al. *(50)* reported the stimulation of higher alkaloid production in *C. roseus* cell cultures in the presence of osmotic shock. Godoy-Hernández and Loyola-Vargas *(51)* tested osmotic stress but in combination with fungal elicitors, and the alkaloid production increased 300% with respect to nontreated *C. roseus* cell cultures.

Sterols are membrane components and regulate membrane fluidity and permeability. They can also participate in the control of membrane-associated metabolic processes *(52)*. Recent evidence in maize roots *(53)* suggests that plant sterol modulates the activity of the plasma membrane H^+-ATPase. The high concentration of campesterol in clone 19 membranes could be changing their fluidity and modifying the membrane enzymes, mainly the tonoplast peroxidase that converts ajmalicine to serpentine *(54)*.

To summarize, our experiments showed that transgenic *C. roseus* hairy roots with higher levels of soluble HMGR (clone 19) have a lower alkaloid and higher sterol content. By contrast, the clone with nonsoluble HMGR activity (236) had a higher alkaloid and lower sterol content. Since the increase in the cytosolic form of the enzyme is able to enhance the production of sterols in transgenic hairy roots, it is possible that the carbon skeleton is the limiting factor in alkaloid biosynthesis.

Alternatively, but more speculatively, it may be that the hamster reductase becomes associated with a metabolic channel dedicated to sterols, mainly campesterol, but not with channels dedicated to alkaloid biosynthesis *(55)*. However, there could be a crosstalk between the two pathways, as has been pointed out recently by Eichinger et al. *(14)*. This could be the reason that we found small amounts of alkaloids in

clone 19. Another alternative could be the fact that secologanin, the terpenoid component of the *C. roseus* indole alkaloids, can be synthesized by the DXP pathway *(13,14)*.

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