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High Level Expression of Chorismate Pyruvate-Lyase (UbiC) and HMG-CoA Reductase in Hairy Root Cultures of *Lithospermum erythrorhizon*

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Shikonin, a red naphthoquinone pigment, is produced by cell cultures of *Lithospermum erythrorhizon* (Boraginaceae). It is biosynthetically derived from two key precursors, 4-hydroxybenzoate (4HB) and geranyldiphosphate (GPP). The bacterial *ubi*C gene, encoding chorismate pyruvate-lyase (CPL) which converts chorismate to 4-hydroxybenzoate, was expressed in *L. erythrorhizon* under the control of the strong $(ocs)_3$mas-promoter. This introduced an efficient biosynthetic pathway to 4HB, i.e. a one-step reaction from chorismate, in addition to the endogeneous multistep phenylpropanoid pathway. Feeding experiments with [1,7-$^{13}C_2$]shikimic acid showed that in the most active transgenic line, 73% of 4HB was synthesized via the genetically introduced pathway. However, there was no correlation between CPL activity and 4HB glucoside or shikonin accumulation in the transgenic lines. HMG-CoA reductase (HMGR) is involved in the biosynthesis of GPP in *L. erythrorhizon*. Two forms of HMGR1 of *Arabidopsis thaliana* were expressed in *Lithospermum* under control of the $(ocs)_3$mas promoter. Only moderate increases in enzyme activity were obtained with the complete enzyme, but high activity was achieved using the soluble cytosolic domain of HMGR1. Shikonin accumulation remained unchanged even upon high expression of soluble HMGR.

Keywords: Chorismate pyruvate-lyase — Genetic engineering — HMG-CoA reductase — *Lithospermum erythrorhizon* — Plant promoter — Shikonin.

Abbreviations: CPL, chorismate pyruvate-lyase; GBA, 3-geranyl-4-hydroxybenzoic acid; GPP, geranyldiphosphate; 4HB, 4-hydroxybenzoic acid; 4HBOG, 4HB glucoside; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

Introduction

The roots of *Lithospermum erythrorhizon* (Boraginaceae) have been used for centuries in Chinese and Japanese traditional medicine for the treatment of wounds, burns and skin disorders (Tsukada et al. 1983, Tang and Eisenbrand 1992). The roots contain red naphthoquinone pigments, i.e. shikonin derivatives (Tang and Eisenbrand 1992), which show antibacterial, anti-inflammatory and wound-healing properties (Tanaka and Odani 1972, Hayashi 1977). Shikonin presented the first example of the commercial production of a secondary metabolite using plant cell cultures (Tabata and Fujita 1985).

Shikonin is derived from two different pathways (Fig. 1). The aromatic precursor 4-hydroxybenzoic acid (4HB) is formed *via* the shikimate and the phenylpropanoid pathway. The reaction sequence to 4HB has been investigated in detail in this cell culture system, and shown to proceed by a mechanism analogous to the β-oxidation of fatty acids (Löscher and Heide 1994).

A simpler biosynthetic pathway to 4HB exists in *E. coli*, i.e. the direct conversion of chorismate to 4HB in a single reaction step (Fig. 1). This reaction is utilized in *E. coli* for the biosynthesis of ubiquinone (Siebert et al. 1994, Meganathan 2001). It is catalyzed by the enzyme chorismate pyruvate-lyase (CPL) encoded by the gene *ubi*C. Feeding experiments with isotope-labelled shikimate unequivocally established that this reaction is not utilized in *L. erythrorhizon* cell cultures (Heide et al. 1989).

The isoprenoid precursor of shikonin, geranyldiphosphate (GPP), is not derived from the methylerythritol-4-phosphate pathway, the source of most other monoterpenoid compounds in plants, but from the classical mevalonate pathway (Li et al. 1998). HMG-CoA reductase was shown to be tightly regulated in correlation with shikonin biosynthesis in these cultures (Lange et al. 1998, Gaisser and Heide 1996), making it a likely regulatory enzyme for the isoprenoid part of shikonin biosynthesis.

The 4HB geranyltransferase reaction (Heide and Tabata 1987, Mühlenweg et al. 1998) links the aromatic precursor 4HB to the isoprenoid precursor geranyldiphosphate (GPP) and yields 3-geranyl-4-hydroxybenzoate (GBA). GBA undergoes decarboxylation and oxidation to geranyl hydroquinone, which is subsequently hydroxylated (Yamamoto et al. 2000) prior to cyclization to the naphthalene ring system. Further oxidation

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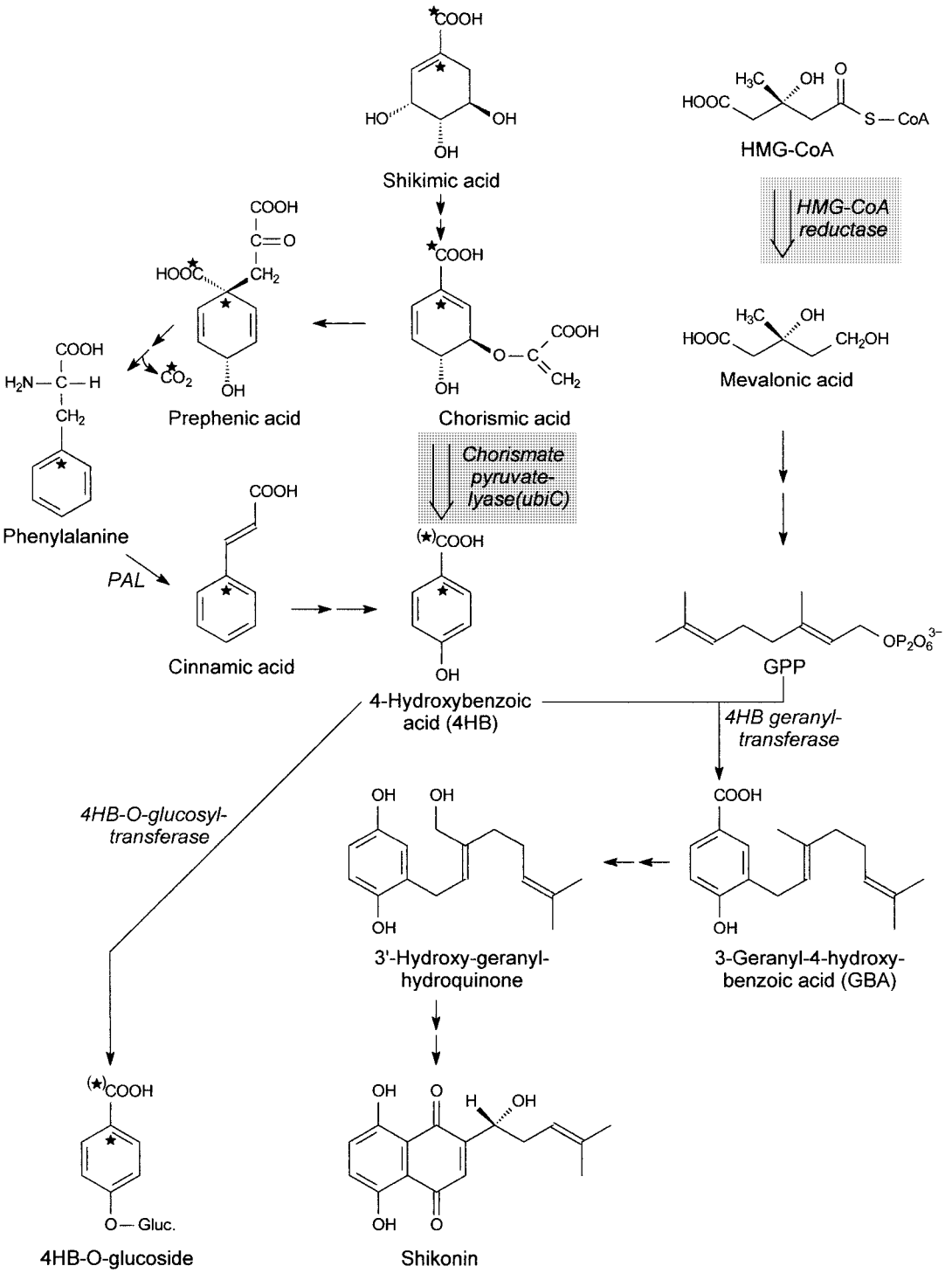

Fig. 1 Biosynthetic pathway to shikonin and 4HB glucoside in *L. erythrorhizon*. Expression of chorismate pyruvate-lyase introduces a new, shortened route from chorismic acid to 4-hydroxybenzoic acid. Labelled atoms in the feeding experiment with [1,7-$^{13}C_2$]shikimic acid are indicated by asterisks. PAL, phenylalanine ammonia-lyase; GPP, geranyldiphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

reactions lead to shikonin. Most of the reaction steps between GBA and shikonin have not been clarified yet, and neither the enzymes nor the genes for these reactions have been identified. Apparently, these reactions steps take place in vesicles formed from the endoplasmic reticulum, and shikonin is eventually secreted out of the cell (Tsukada and Tabata 1984).

Shikonin biosynthesis presents an interesting model system for experiments on the genetic engineering of secondary metabolism, due to its role as the first plant cell culture used for industrial production of a plant secondary metabolite, and due to its complex biosynthetic pathway combining both aromatic and isoprenoid precursors.

In a previous study (Sommer et al. 1999), an upregulation of the supply of the aromatic precursor 4HB in *Lithospermum* cultures was attempted by the expression of the bacterial gene *ubi*C, encoding chorismate pyruvate-lyase (CPL). The gene was fused to a transit peptide for targeting of the protein to the plastids and placed under the control of the CaMV 35S promoter. Expression of this construct led to a formation of 4HB via the CPL reaction. When the native phenylpropanoid pathway to 4HB was blocked using an inhibitor of phenylalanine ammonia-lyase, shikonin was still formed in the transgenic cultures, proving that 4HB formed by CPL was utilized for shikonin biosynthesis. However, the amount of 4HB formed via the genetically introduced CPL reaction was rather low: only 20% of the 4HB formed in these transgenic cultures was derived from the newly introduced CPL pathway, whereas 80% was still supplied from the genuine phenylpropanoid pathway.

Therefore, in the present study, we placed the same *ubi*C construct (*ubi*C fused to a transit peptide for plastidic targeting) under control of one of the strongest plant promoters known at present, i.e. the $(ocs)_3$mas promoter developed by Ni et al. (1995). The $(ocs)_3$mas-promoter consists of a trimer of the octopine synthase upstream activating sequence fused to a mannopine synthase activator/promoter region of the Ti-plasmid of *Agrobacterium tumefaciens.* In comparison to the CaMV 35S promoter, the $(ocs)_3$mas promoter has been reported to achieve a 156-fold higher expression, based on the relative activity of GUS expressed from the two promoters in tobacco.

In additional experiments, we placed two different HMG-CoA reductase cDNA sequences under the control of the $(ocs)_3$mas promoter: a complete cDNA encoding for both the membrane anchor of HMGR and the cytosolic domain (which contains the catalytic site), and a shortened version encoding only for the cytosolic domain. Both constructs were expressed in *Lithospermum* in order to determine which was more suitable to upregulate HMGR activity in this system.

Results

ubiC vector construction and transformation of L. erythrorhizon

The *ubi*C gene of *E. coli*, encoding chorismate pyruvate-lyase, was fused to the chloroplast transit peptide of the small subunit of Rubisco (Pichersky et al. 1986) and placed under the control of the constitutive $(ocs)_3$mas promoter to give p$(ocs)_3$mas-TP*ubi*C. This binary vector was introduced into *Agrobacterium rhizogenes*, and its integrity was confirmed by Southern analysis and PCR prior to plant transformation (data not shown). After transformation of shoot cultures of *L. erythrorhizon* (Shimomura et al. 1991, Yazaki et al. 1998), developing root tips were grown under continuous selection pressure by kanamycin, yielding 53 independent hairy root lines.

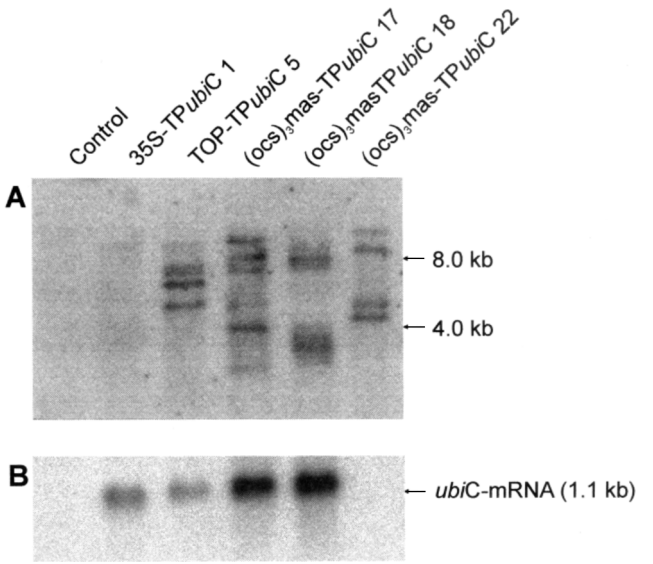

Fig. 2 Southern hybridization (A) and Northern hybridization (B) of hairy root cultures of *L. erythrorhizon* transformed with *ubi*C under the control of different promoters. Genomic DNA (10 μg) was digested by *Hin*dIII and separated by 0.7% agarose gel electrophoresis. Total RNA (10 μg) was separated on an 1% agarose gel. Hybridization was performed with a [^{32}P]-labelled *ubi*C probe.

Southern and Northern analysis of the transformed hairy root cultures

The presence of the *ubi*C gene in the transgenic cultures was demonstrated by PCR with specific primers (data not shown). Southern blotting analysis (Fig. 2A) confirmed integration of *ubi*C into the genomic DNA. Several copies were detected in each transgenic line, whereas in the control no hybridization signal was obtained with *ubi*C.

In order to assess the *ubi*C mRNA levels, a Northern blotting analysis of 4-day-old hairy root cultures was performed. *ubi*C mRNA was detected at the expected size of 1.1 kb. Fig. 2B shows the two most active and one of the least active lines transformed with $(ocs)_3$mas-TP*ubi*C, compared with the two most active lines transformed with 35S-TP*ubi*C and TOP-TP*ubi*C, respectively, obtained in our previous study (Sommer et al. 1998); the TOP promoter is a derivative of the 35S promoter (Gatz et al. 1992). Expression from the $(ocs)_3$mas promoter was approximately 4–5 times higher than from the 35S promoter, and eight times higher than from the TOP promoter.

Determination of chorismate pyruvate-lyase activity

The activities of chorismate pyruvate-lyase (CPL) were determined in control lines and different *ubi*C-transformed lines of hairy root suspension cultures (Fig. 3). In controls, no CPL activity was detectable (<1.4 pkat (mg protein)$^{-1}$). In 21 of

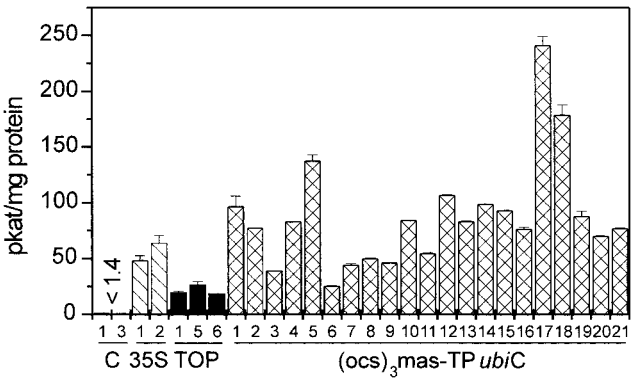

Fig. 3 Chorismate pyruvate-lyase activities in hairy root cultures transformed with *ubi*C under the control of the $(ocs)_3$mas promoter. The most active 21 lines are shown out of a total 53 independent transgenes. For comparison, the activities of the most active lines obtained with the CaMV 35S promoter and the similar TOP promoter are shown, as well as controls transformed with *A. rhizogenes* without binary vector. Bars indicate mean values ± SD ($n = 4$). C, control lines; 35S, 35S-TP*ubi*C; TOP, TOP-TP*ubi*C.

the $(ocs)_3$mas-TP*ubi*C lines, high CPL activities (>25 pkat (mg protein)$^{-1}$) were detected, whereas in the other 32 of these lines, activities ranged between 0 and 15 pkat (mg protein)$^{-1}$. In the most active lines, i.e. lines 17 and 18, the CPL activity was approximately 210 pkat (mg protein)$^{-1}$, i.e. 3.3-fold higher than in the most active 35S-TP*ubi*C line, and 8-fold higher than in the most active TOP-TP*ubi*C line. The observed enzyme activities correlated well with the mRNA expression levels. The strong variation of enzyme activities between the lines transformed with $(ocs)_3$mas-TP*ubi*C may be attributed to an integration of the *ubi*C construct at different positions in the genome.

Contribution of the CPL reaction to the overall 4HB glucoside formation

In *ubi*C-transformed lines, two pathways can contribute to the formation of 4HB (Fig. 1): the native shikimate/phenylpropanoid pathway and the newly introduced CPL reaction. In order to ascertain the contribution of the CPL reaction to 4HB formation, a feeding experiment was carried out using [1,7-$^{13}C_2$]-labelled shikimic acid.

As shown in Fig. 1, the carboxyl group of shikimate is lost in the phenylpropanoid pathway, but is retained in the direct conversion of chorismate to 4HB in the CPL reaction. By feeding of [1,7-$^{13}C_2$]shikimic acid, which carries an isotope label in the carboxyl group (C-7) and a reference label in the ring (C-1), the relative contribution of the two pathways can be assessed. The incorporation of label into C-7 of 4HB is caused by the CPL pathway alone, while incorporation into C-1 of 4HB is caused by both the CPL and the phenylpropanoid pathway. A comparison of the incorporation of ^{13}C label at positions C-1 and C-7 by ^{13}C-NMR therefore measures the contribution of both pathways to the formation of 4HB. Incorporation of ^{13}C at position C-1 alone will lead to an increase of the singlet signal of this carbon. Incorporation of ^{13}C at both C-1 and C-7 will lead to new doublet signals for both carbons, due to the effect of ^{13}C–^{13}C coupling.

From the integrals of the ^{13}C signals, the enrichments of ^{13}C at position 1 and 7 were calculated at 25.6% and 18.7%, respectively (Fig. 4), indicating that 73% of the 4HB glucoside

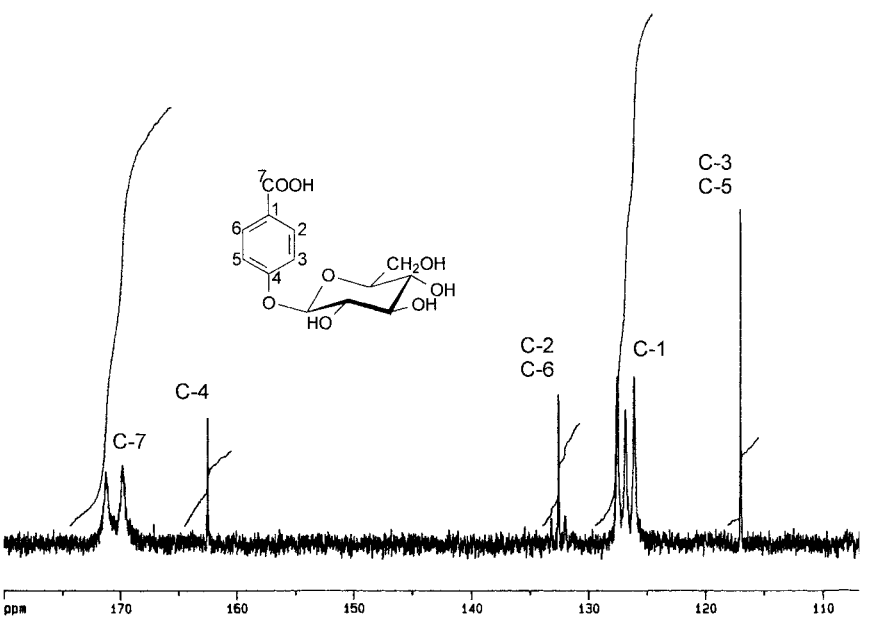

Fig. 4 ^{13}C-NMR spectrum of 4HB glucoside isolated from a $(ocs)_3$mas-TP*ubi*C transformed hairy root culture line 17 after feeding of [1,7-$^{13}C_2$]shikimic acid. The signals of the six glucose carbons appear at 60 to 105 ppm (not shown).

Table 1 Accumulation of shikonin and 4HB glucoside in control and *ubi*C-transformed hairy root cultures of *L. erythrorhizon*

	M9 medium		MSV medium
	Shikonin (μmol (g DW)$^{-1}$)	4HBOG (μmol (g DW)$^{-1}$)	4HBOG (μmol (g DW)$^{-1}$
Control (3 lines)	74.4 ± 64.7	9.7 ± 0.6	18.4 ± 3.5
35S-TP*ubi*C (3 lines)	72.2 ± 14.1	13.7 ± 7.3	33.0 ± 20.5
TOP-TP*ubi*C (4 lines)	82.9 ± 4.3	11.9 ± 5.5	27.3 ± 4.4
$(ocs)_3$mas-TP*ubi*C (21 lines)	107.0 ± 31.0	12.1 ± 4.8	25.4 ± 8.5

No detectable amount of shikonin is formed in MSV medium. Data shown are mean ± SD.

accumulated in line 17 had been formed via the genetically introduced CPL pathway. Only 27% were produced via the native phenylpropanoid pathway. The high overall incorporation demonstrated that shikimate was very efficiently taken up from the medium and transported into the plastids.

Determination of shikonin and 4HB glucoside content

The 21 $(ocs)_3$mas-TP*ubi*C hairy root lines with active expression of CPL, as well as 35S-TP*ubi*C, TOP-TP*ubi*C and control lines, were cultivated in the shikonin production medium M9 for 14 d and assessed for shikonin and 4HB glucoside accumulation (Table 1). The average shikonin content of the $(ocs)_3$mas-TP*ubi*C lines was 107 μmol (g DW)$^{-1}$, whereas the average content of the controls was 74 μmol (g DW)$^{-1}$. Although these data suggest, on first sight, an increase of shikonin concentration by *ubi*C expression, there was no correlation between CPL activity and shikonin concentration in the 21 investigated $(ocs)_3$mas-TP*ubi*C lines (r^2 = 0.01). Therefore, we conclude that TP*ubi*C expression did not cause an overall increase of shikonin accumulation.

The accumulation of 4HB glucosides was further investigated in MSV medium, i.e. in the absence of any shikonin formation (Table 1). Despite the high CPL activities in the $(ocs)_3$mas-TP*ubi*C lines, the content of 4HB glucoside was only moderately higher than in the controls and not higher than in the TOP-TP*ubi*C and the 35S-TP*ubi*C lines. Again, there was no correlation between CPL activity and 4HB glucoside accumulation. We therefore conclude that TP*ubi*C expression does not increase 4HB glucoside accumulation, and that the observed differences between different lines are due to somaclonal variation.

A possible reason for unchanged 4HB glucoside accumulation even after high expression of CPL might have been a limitation of the amount of chorismate in the cells. In order to test this hypothesis, a supplementation of the transgenic cultures with shikimate pathway intermediates was attempted. Chorismate itself is unstable and decomposes non-enzymatically to 4HB (Siebert et al. 1994). Therefore we added shikimate (172 μmol flask^{-1}) to the medium of the $(ocs)_3$mas-TP*ubi*C hairy root line 17.

After 10 days of culture, the amount of 4HB glucoside was only moderately increased in the cultures with shikimate addition (5.0±0.4 μmol 4HB glucoside flask^{-1}) compared to the culture without shikimate addition (3.5±1.5 μmol 4HB glucoside flask^{-1}).

Transformation with HMGR1S of Arabidopsis thaliana

Overexpression of HMGR has been used previously in order to upregulate the formation of isoprenoids (especially sterols) in plants (Schaller et al. 1995, Chappell et al. 1995). Therefore, we attempted an overexpression of HMGR in *L. erythrorhizon* cultures.

In the first experiment, we used a cDNA construct designated HMGR1S, coding for the smaller isoform of HMGR1 of *A. thaliana*. This protein consists of a short N-terminal region, two trans-membrane domains, a short linker region and a C-terminal catalytic domain (Caelles et al. 1989). The HMGR1S sequence was ligated into vector pATC940 (see Materials and Methods section), thereby placing the gene under control of the very strong $(ocs)_3$mas promoter (Ni et al. 1995). *A. rhizogenes*-mediated transformation of shoot cultures of *L. erythrorhizon* resulted in 25 kanamycin-resistant hairy root lines transformed with $(ocs)_3$mas-HMGR1S.

Since we were interested in the correlation between HMGR1S expression and shikonin biosynthesis, we determined the productivity of all 25 lines for shikonin and its pathway intermediate, 3-geranyl-4-hydroxybenzoic acid (GBA). As observed previously (Sommer et al. 1999, Boehm et al. 2000), there was considerable somaclonal variation of the contents among the lines. On average, the transgenic lines produced 40 μmol (g DW)$^{-1}$ shikonin derivatives (SD: 21 μmol (g DW)$^{-1}$), compared to an average of 46 μmol (g DW^{-1} in control cultures without HMGR expression. Likewise, there was no increase in the average concentration of GBA in the $(ocs)_3$mas-HMGR1S lines.

Seven HMGR-transformed lines, representing high, average, and low producers of shikonin, were chosen for Northern Blot experiments. Clear signals at the expected size of HMGR-mRNA were detected in five of these lines, but only a faint signal appeared in the control (data not shown). The probe in these Northern hybridization experiments was derived from *Arabidopsis* HMGR gene, therefore a quantitative comparison

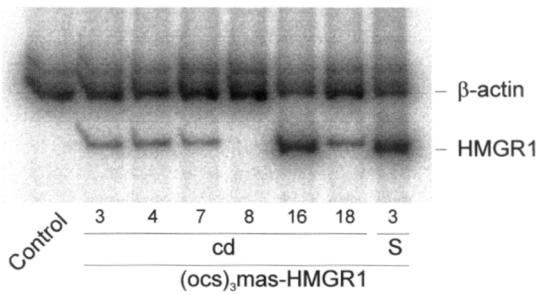

Fig. 5 Expression of HMGR1cd and HMGR1S from *A. thaliana* in hairy root cultures of *L. erythrorhizon*. mRNA was detected using RT-PCR with β-actin as an internal control.

of HMGR expression between transgenes and controls is not possible. There was no correlation between HMGR1S expression and the accumulated amounts of shikonin or GBA.

HMGR enzyme activity was not significantly increased in most of the lines with HMGR1S expression. The most active line showed 13.3±1.6 pkat (mg protein)$^{-1}$ in the microsomal fraction, compared to an average of 7.9±2.0 pkat (mg protein)$^{-1}$ in the control cultures. Our results therefore suggested that expression of HMGR1S, even under control of a very strong promoter, was not effective in order to achieve high HMGR activities in *L. erythrorhizon*, and did not lead to higher secondary metabolite formation.

Transformation with HMGR1cd of A. thaliana

The catalytic activity of HMGR resides in the C-terminal cytosolic domain of the protein. Expression of this domain in *E. coli* resulted in the formation of active HMGR (Dale et al. 1995), and likewise expression of the catalytic domain of a hamster HMGR has been used successfully to upregulate sterol formation in tobacco plants (Chappell et al. 1995).

Therefore, we decided to express the cDNA clone HMGR1cd in *Lithospermum* cultures, which is a truncated form of HMGR1S and encodes the catalytic domain and six amino acids of the linker region of HMGR1 of *A. thaliana*; it lacks, however, the sequence for the two transmembrane domains and the N-terminal region of HMGR1 (Dale et al. 1995). The gene for HMGR1cd was cloned into pATC940 (see Method section) and transformed in *L. erythrorhizon* by *A. rhizogenes*-mediated transformation. 20 kanamycin-resistant hairy root lines, representing independent transformants, were obtained. The accumulation of shikonin and GBA in these lines was assessed. Again, there was considerable variation of the shikonin content among the lines (average: 46 μmol (g DW)$^{-1}$; SD: 27 μmol (g DW)$^{-1}$).

Six HMGR1cd-transformed lines, representing high, average and low producers of shikonin and GBA, were examined by Northern Blot (data not shown) and by the more sensitive RT-PCR (Fig. 5). Five of the lines gave clear signals of HMGR mRNA, while line 16 showed the highest HMGR mRNA levels.

When the enzyme activity was measured, only line 16 showed a strong increase over the controls. Total HMGR activity in this line amounted to 35 pkat (g DW)$^{-1}$, 30-fold higher than in the controls. As expected, this increase was due to a soluble form of HMGR, found in the supernatant of a 100,000×*g* centrifugation. The activity of the microsomal fraction, in contrast, was not significantly increased (line 16, 9.1 pkat (mg protein)$^{-1}$; control, 7.9 pkat (mg protein)$^{-1}$). Therefore, expression of the cytosolic domain of *A. thaliana* HMGR1 is capable of increasing the total HMGR enzyme activity in *L. erythrorhizon*. However, again there was no correlation between HMGR expression and shikonin or GBA accumulation. The highest expressing line (line 16) was surpassed in shikonin and GBA accumulation by several other lines which showed only average HMGR expression.

Discussion

In the present study, we have expressed two biosynthetic enzymes, chorismate pyruvate-lyase and 3-hydroxy-3-methylglutaryl-CoA reductase, in *Lithospermum* root cultures in order to upregulate the supply of the aromatic and the isoprenoid precursor of shikonin. Both genes were placed under the control of the very strong $(ocs)_3$mas promoter (Ni et al. 1995).

Expression of the bacterial gene *ubi*C, encoding CPL, introduces a new, single-step pathway from chorismate to 4-hydroxybenzoate in *Lithospermum*, in addition to the native shikimate / phenylpropanoid pathway, which requires at least 10 steps from chorismate to 4HB (Fig. 1). Since the native pathway is highly active in these cultures, expression of CPL from the CaMV 35S promoter in a previous study (Sommer et al. 1999) could only make a moderate contribution to the overall metabolic flux: 20% of 4HB were supplied via the CPL reaction, whereas 80% were still supplied via the conventional phenylpropanoid route. This picture changed dramatically when, in the present study, the strong $(ocs)_3$mas promoter was used. A feeding experiment with a double isotope-labelled shikimic acid unequivocally proved that 73% of 4HB was now formed via the newly introduced CPL pathway. Therefore, a profound change in the pathway leading to 4HB has been achieved, and the expression of this *ubi*C construct appears to provide a convenient method to upregulate the availability of 4HB in these cultures.

However, the high expression of CPL did not lead to an increased accumulation of shikonin. This may not be surprising, since the rate of shikonin formation may also depend on the availability of GPP and on the activity of enzymes for the later steps of shikonin biosynthesis.

We did expect, however, that the high expression of *ubi*C would increase the accumulation of 4HB glucoside, especially in the growth medium MSV where 4HB cannot be converted to shikonin. To our surprise, however, 4HB glucoside levels were not changed in the *ubi*C transgenes in comparison to the control cultures. This may be explained in several different ways.

An obvious explanation would be a limitation in the amount of chorismate available in the cell. Both the newly introduced CPL pathway and the native shikimate / phenylpropanoid pathway utilize chorismate as substrate (Fig. 1). If chorismate is consumed by the introduced CPL reaction, it may not be available for the complementary phenylpropanoid pathway. However, expression of the same $(ocs)_3$mas-TP*ubi*C construct in tobacco and potato led to a massive accumulation of 4HB glucosides (4–5% of dry weight) (Köhle et al., manuscript in press), whereas in *Lithospermum* the pre-transformation level of 0.5% of dry weight was not significantly elevated. At least in tobacco and potato, therefore, the flux to chorismate can be very efficiently upregulated when chorismate is consumed for the formation of 4HB.

A feedback regulation by 4HB on the native phenylpropanoid pathway in *Lithospermum* may downregulate this pathway once 4HB is supplied by the CPL reaction. However, even if the contribution of phenylpropanoid pathway to 4HB biosynthesis is reduced to zero, the CPL reaction alone may still be expected to produce higher amounts of 4HB than observed in our present study (Köhle et al., manuscript in press). As mentioned, tobacco cell suspension cultures and potato shoot cultures transformed with the same *ubi*C constructs produced 4–5% 4HB glucosides per dry weight.

Another possible explanation for our results is that expression of CPL in fact did strongly increase the rate of 4HB formation, but that a concomitant increase of catabolic reactions prevented the accumulation of the 4HB glucoside. Such a catabolic pathway may not operate in tobacco and potato, which do not normally form 4HB as a secondary metabolite.

The second enzyme overexpressed in the present study was HMGR. It has been unequivocally established that the isoprenoid moiety of shikonin is derived from the mevalonate pathway, not from the MEP pathway (Li et al. 1998). In contrast to monoterpene-forming plants, the GPP synthase of *Lithospermum* is localized in the cytosol, and therefore GPP for shikonin formation is derived from the cytosolic pool of IPP (Sommer et al. 1995). HMGR was identified as a strongly regulated enzyme of this pathway (Gaisser and Heide 1996; Lange et al. 1998). Upregulation of HMGR may therefore be considered an important part in an overall strategy to upregulate shikonin formation, and in fact it has been used to upregulate the formation of other plant isoprenoids, especially sterols (Schaller et al. 1995, Chappell et al. 1995).

We first expressed the complete HMGR1 protein of *A. thaliana*, including its membrane anchor, using the cDNA sequence HMGR1S placed under control of the $(ocs)_3$mas promoter. However, only a moderate increase of HMGR activity was achieved by expression of this construct, i.e. approximately 40% in the most active line. Also in a previous study, expression of HMGR1S under control of the CaMV 35S promoter in *A. thaliana* had resulted in a rather moderate (2- to 3-fold) increase of HMGR activity (Re et al. 1995), despite a 40-fold increase of HMGR mRNA.

Therefore, we subsequently expressed the cDNA sequence HMGR1cd, encoding the soluble cytosolic domain of HMGR without a membrane anchor, and observed a 30-fold increase of activity in the most active line in comparison to the control. As expected, this activity was localized in the soluble fraction, not in the microsomal fraction.

Since the mRNA levels in the most active lines expressing HMGR1S and HMGR1cd were nearly equal, the observed difference in enzyme activities was not due to different transcription rates or different mRNA stability. Rather, it may have been due to different translation efficiency (since both clones have different translation starts), different protein stability, or factors relating to protein folding and membrane insertion of the membrane-anchored enzyme. Also, an inactivation of HMGR by specific protein kinases regulating HMGR activity may be involved (Dale et al. 1995). Despite high HMGR activity, no significant change in the accumulation of shikonin or its pathway intermediate, GBA was observed in the most active HMGR1cd-transformed lines. Since HMG-CoA is a soluble substrate in the cytosol, we expect that the active, soluble HMGR did in fact convert this substrate to mevalonate. However, other regulatory mechanisms may have limited the conversion of mevalonate to shikonin.

Shikonin biosynthesis combines the aromatic and the isoprenoid pathways. Our study showed that an upregulation of one of these two pathways alone by expression of CPL or of HMGR was insufficient to increase shikonin formation. The simultaneous overexpression of several pathway enzymes may be necessary in order to influence the accumulation of this secondary metabolite. One of the candidates for such experiments is the 4HB geranyltransferase reaction which has recently been successfully upregulated by expression of a bacterial prenyltransferase (Boehm et al. 2000).

Materials and Methods

Chemicals

[α-^{32}P]dCTP and 3-hydroxy-3-methyl-[3-^{14}C]glutaryl-CoA were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Chorismate as the barium salt, 3-hydroxy-3-metylglutaryl-CoA and R,S-[5-^{3}H]mevalonolactone were obtained from Sigma-Aldrich (Deisenhofen, Germany). [1,7-$^{13}C_2$]shikimic acid was synthesized as described by Cho et al. (1992). 3,5-Dihydroxybenzoic acid was obtained from Merck (Darmstadt, Germany). T4-DNA ligase and the random primed DNA labeling kit were purchased from Roche (Mannheim, Germany). PCR primers were synthesized by MWG Biotech (Ebersberg, Germany).

Bacteria and plant material

A. rhizogenes 15834 was purchased from ATCC (Rockville, MD, U.S.A.). *E. coli* XL1 Blue was obtained from Stratagene (Amsterdam, The Netherlands). Shoot cultures of *L. erythrorhizon* were grown on WP medium (Lloyd and McCown 1980) or MSV medium (Murashige and Skoogs with vitamins) (Murashige and Skoog 1962).

Hairy roots of *L. erythrorhizon* were held in MSV medium (solid or liquid) in the dark at 25°C. Suspension cultures were agitated at 50 rpm. For production of shikonin, roots were transferred in M9

medium (Fujita et al. 1981) and cultivated for 3 weeks under the same conditions.

Plasmids, DNA manipulation and plant transformation

pATC940 was obtained from the Biotechnology Research and Development Corporation (Peoria, ID, U.S.A.).

Construction of the binary vectors 35S-TP*ubi*C and TOP-TP*ubi*C were described by Siebert et al. (1996) and Sommer et al. (1998), respectively. The DNA fragment TP*ubi*C was obtained from pTP0-*ubi*C (Siebert et al. 1996) by restriction with *Xba*I and ligated into the *Xba*I site of pATC940, directly downstream of the strong $(ocs)_3$mas-promoter (Ni et al. 1995) to give p$(ocs)_3$mas-TP*ubi*C.

The HMGR1S fragment was obtained from λcAT12 (Caelles et al. 1989) as a 2.29 kb *Eco*RI fragment and cloned into the *Eco*RI site of pBlueskript SK(+). The resulting vector was digested with *Hinc*II and *Pst*I, and the HMGR1S fragment was ligated into pUC19, digested with the same enzymes, to give pUC1N. HMGR1S was excised by restriction with *Kpn*I and *Pst*I and subcloned into pBluescript SK(–). From the resulting vector, the gene was excised with *Xba*I and cloned into the *Xba*I site of the binary vector pATC940 to give p$(ocs)_3$mas-HMGR1S.

The HMGR1cd fragment was obtained from pCD1 (Dale et al. 1995) by digestion with *Nde*I and *Hin*dIII. The fragment was treated with Klenow polymerase and ligated in the *Sma*I site of pBluescript SK(–). The gene was excised with *Xba*I and introduced into pATC940 as described above to give p$(ocs)_3$mas-HMGR1cd.

A. rhizogenes was transformed by electroporation (Mattanovich et al. 1989). The integrity of binary plasmid in *A. rhizogenes* was confirmed by PCR and Southern analysis prior to plant transformation. The transformation of shoots of *L. erythrorhizon* was performed according to Shimomura et al. (1991) with some modifications (Yazaki et al. 1998).

Southern and Northern analysis

Isolation of genomic DNA was carried out using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA (10–20 μg) was digested by *Hin*dIII, separated on an 0.7% agarose gel and transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by capillary blotting. A PCR product was obtained from pTP0-*ubi*C as described previously (Siebert et al. 1996), labelled with [α-^{32}P]dCTP, and used as probe. Hybridization was performed as described by Sambrook and Russell (2001).

RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Ten μg RNA was separated on a 1% agarose gel with 7% formaldehyde and transferred to Hybond-N^+ membranes.

Labelling and hybridization were performed as described above.

RT-PCR expression analysis

RT-PCR expression analysis was performed as described by Takakura et al. (2000). RNA was isolated with RNeasy Plant Mini Kit of Qiagen (Hilden, Germany). One μg RNA in a total volume of 20 μl was used for each RT reaction. The product was used as template for PCR.

The primers 5′-GAAGACGAGGAGATTGTGAA-3′ and 5′-ACTCAAGAACATTCTGCACA-3′ were specific for the DNA sequence of the catalytic domain of HMG-CoA reductase 1 from *A. thaliana*. As control, primers specific to the β-actin gene (5′-CAACTGGGACGAYATGGAGA-3′ (Y = C or T) and 5′-GATCCACATCTGCTGGAAGG-3′) were used. The annealing temperature was set at 55°C and the PCR was carried out with 25 cycles. The PCR products were separated on a 6% polyacrylamide gel.

Measurement of chorismate pyruvate-lyase (CPL) activity

Protein extracts were prepared and chorismate pyruvate-lyase activity was measured as described by Siebert et al. (1996). Protein concentrations were determined according to Bradford (1976), with bovine serum albumin as standard.

Measurement of 3-hydroxy-3-methylglutaryl-CoA reductase activity

Protein extraction and membrane isolation were carried out as described by Bach et al. (1986). HMGR activity was determined by a liquid scintillation method described by Feyereisen and Farnsworth (1987).

^{13}C labelling experiment and NMR analysis

Forty-four mg of [1,7-$^{13}C_2$] shikimic acid was added to a 4-day-old hairy root culture of $(ocs)_3$mas-TP*ubi*C line 17 in 75 ml MSV medium. The roots were cultivated for ten days and harvested. 4HB glucoside was purified as described by Siebert et al. (1996) and subjected to ^{13}C-NMR analysis. The spectrum was taken with an AC250 Spectrometer (Bruker, 62.5 MHz) in CD_3OD.

Determination of 4HB glucoside content

Hairy roots were pulverized in liquid nitrogen, of which 400 mg of the cell powder were extracted at room temperature with 800 μl methanol for 2 h and centrifuged. An aliquot of the resulting supernatant was evaporated to dryness, redissolved in methanol containing 1 mM 3,5-dihydroxybenzoic acid as internal standard and analyzed by HPLC. The HPLC system consisted of a Multospher 120 RP 18–5 column (CS Chromatographie Service, Langerwehe, Germany) with an linear gradient from 10 to 50% solvent B (CH_3OH–H_2O–HCOOH, 50 : 49 : 1) relative to solvent A (H_2O–HCOOH, 99 : 1). The flow rate was 1 ml/min, and 4HBOG was detected at 254 nm (retention time 6.7 min). The identity of 4HBOG was confirmed by preparative isolation of this peak and ^{13}C NMR analysis (see above). For quantification, a calibration curve with authentic 4HBOG was used.

Determination of GBA and shikonin content

GBA and total shikonin were measured according to Boehm et al. (2000). A linear gradient from 80% to 100% methanol in water containing 1% formic acid was used for determination of GBA by HPLC. Total shikonin was determined photometrically as described by Sommer et al. (1999).

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