Biosci. Rep. (2010) / **30** / 33–40 (Printed in Great Britain) / doi 10.1042/BSR20080124

Original Paper

Bioscience Reports

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Lithospermic acid B is more responsive to silver ions (Ag^+) than rosmarinic acid in *Salvia miltiorrhiza* hairy root cultures

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Synopsis

LAB (lithospermic acid B) is a dimer of RA (rosmarinic acid) and has been suggested to be derived from RA, but the detailed biosynthesis process has not yet been identified. The accumulation of RA has been intensively investigated in the plant species of Boraginaceae and Lamiaceae. In the present study, we report that silver ions (Ag^+; 15 μM), an abiotic elicitor, did not stimulate RA accumulation but dramatically enhanced LAB from approx. 5.4% to 18.8% of dry weight in *Salvia miltiorrhiza* hairy root cultures, and the rise in LAB was found to be coincident with the decline of RA content at each time point after treatment. Meanwhile, a profiling analysis of genes and metabolites (intermediates) involved in the RA synthesis pathway was performed; the result indicated that several gene transcripts and metabolite accumulations show temporal changes in abundance consistent with LAB production. Thus a potential (putative) biosynthetic route from RA to LAB was presumed, which was suggested to be significantly activated by Ag^+ in *S. miltiorrhiza* hairy root cultures. Further intermediate monitoring and compound feeding experiments were performed to rank the strength of this hypothesis. Our study, for the first time, provides evidence that RA is a precursor leading to LAB synthesis.

Key words: biosynthesis pathway, Dan-shen, lithospermic acid B (LAB), rosmarinic acid (RA), silver ion (Ag^+), transcript and metabolite profiling.

INTRODUCTION

Salvia miltiorrhiza Bunge ('Dan-shen' in Chinese) is a commonly used traditional Chinese medicine for improving body function (e.g. promoting circulation and improving blood flow), as well as for the treatment of angina pectoris, myocardial infarction and other cardiac symptoms [1]. The active components of Dan-shen could be classified as lipid-soluble and water-soluble ones. The former are mainly tanshinones, whereas the latter include caffeic acid and related phenolic compounds such as RA (rosmarinic acid) and its derivative LAB (lithospermic acid B) [2]. The phenolic acids have attracted the attention of scientists in the last 20 years because of their notable pharmacological activities and the conventional use of herbs by decocting with water [3,4]. RA biosynthesis was first elucidated in suspension cultures of *Coleus blumei* (Lamiaceae), which involves both the phenylpropanoid pathway and the tyrosine-derived pathway (Figure 1) [5,6].

LAB, a dimer of RA, has been suggested to be derived from RA [7,8], but the detailed biosynthesis process is poorly understood and no information about the postulated LAB biosynthesis was hitherto available. Omoto et al. [9] measured LAB and RA in the roots of plants from 26 species in Lamiaceae and found that high levels of LAB existed only in the roots of *S. miltiorrhiza*, with approx. 5.1% of dw (dry weight). Thus *S. miltiorrhiza* is unique in accumulating LAB. Meanwhile, this is true in *S. miltiorrhiza* hairy root cultures, where the content of LAB was found to be much higher than that of RA [10]. Therefore it is proposed that the potential (putative) biosynthetic route leading to LAB is also particularly active in *S. miltiorrhiza*.

Hairy root cultures of *S. miltiorrhiza* have been suggested to be more stable and efficient than cell suspension cultures in active constituent accumulation [10]. Similar to that in cell suspension

Abbreviations used: C4H, cinnamic acid 4-hydroxylase; dw, dry weight; fw, fresh weight; ESI-MS^n, multi-stage electrospray ionization MS; HPPD, 4-hydroxyphenylpyruvate dioxygenase; HPPR, 4-hydroxyphenylpyruvate reductase; LAB, lithospermic acid B; PAL, phenylalanine ammonia-lyase; RA, rosmarinic acid; TAT, tyrosine aminotransferase; 4CL, hydroxycinnamate coenzyme A ligase.

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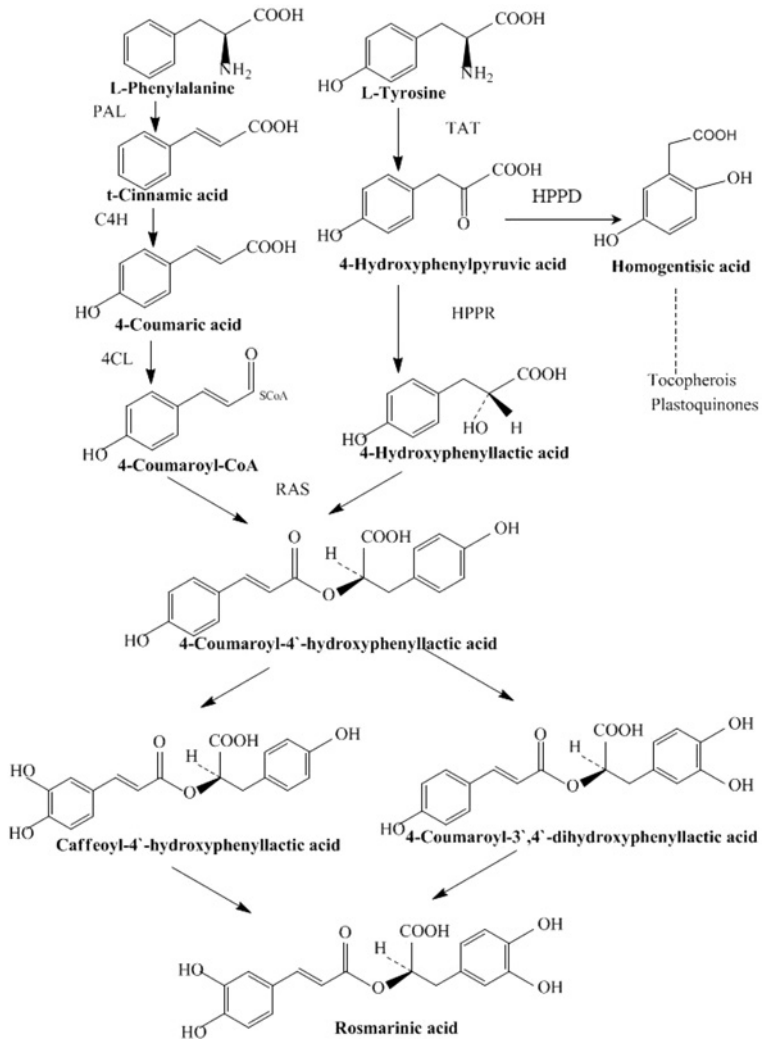

Figure 1 The metabolic pathway leading to RA

cultures, secondary metabolite accumulation in hairy root cultures can be induced and stimulated by elicitation or treatment of the culture with suitable biotic and abiotic elicitors. There have been reports indicating that the accumulation of RA and total phenolic acids, as well as tanshinones, could be stimulated by a yeast elicitor and silver ions (Ag^+) in *S. miltiorrhiza* hairy root cultures [2,11,12]. However, the effect of an elicitor on LAB, the unique constituent of *S. miltiorrhiza* hairy root cultures, has not been investigated so far.

In the present study, the accumulation of RA and LAB were simultaneously examined under Ag^+ stress, in *S. miltiorrhiza* hairy root cultures. Meanwhile, several related genes and metabolites (intermediates) involved in the RA synthesis pathway were also characterized in both control and Ag^+-treated hairy root cultures. The results were found to parallel the hypothesis that RA is a precursor leading to LAB synthesis. Thus a potential (putative) biosynthetic route from RA to LAB was presumed, which was intensively investigated by further intermediate monitoring and compound feeding experiments.

MATERIALS AND METHODS

Hairy root culture

The *S. miltiorrhiza* hairy root culture was derived after the infection of plantlets with an Ri (root inducing) T-DNA (transfer DNA)-bearing *Agrobacterium rhizogenes* bacterium (C58C1 strain). A stock culture of the hairy roots was maintained in a solid, hormone-free half-strength B5 medium containing 8 g/l agar and 30 g/l sucrose, at 25°C in the dark. All experiments in the present study were carried out in liquid shake-flask cultures in 200 ml Erlenmeyer flasks on an orbital shaker at 120 rev./min.

Each flask was filled with 40 ml of half-strength B5 medium and inoculated with 0.3 g of fresh roots from 3-week-old shake-flask cultures. Ag^+ treatment of the hairy root cultures was performed on day 18 post-inoculation and the hairy roots were harvested from the culture medium at selected times [0 day (before treatment), 6 day, 12 day, 18 day and 24 day]. Harvested hairy roots (0.1 g) were used for RNA isolation, and the others were filtrated, washed three times with distilled water, blotted dry by paper towels [yielding the fw (fresh weight)] and then dried at 45°C in an oven until constant dw. Untreated hairy roots were designated as the control. The experiment was performed in triplicate, and the results are means ± S.E.M.

Preparation of Ag^+ elicitor

Silver ions (Ag^+) were supplied to the culture at a final concentration of 15 μM, using a concentrated silver thiosulfate ($Ag_2S_2O_3$) solution prepared by mixing $AgNO_3$ and $Na_2S_2O_3$ at a molar ratio of 1:4 as described in [12].

RNA isolation and real-time quantitative PCR analysis

Total RNAs from *S. miltiorrhiza* hairy roots were extracted at selected time points using TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions [13]. The quality and concentration of RNA and DNA samples were examined by ethidium-bromide-stained agarose-gel electrophoresis and spectrophotometer analysis. Total RNA was reversely transcribed by using AMV (avian myeloblastosis virus) reserve transcriptase (Takara) to generate cDNA. All the gene-specific primers were designed according to the conserved region of the corresponding sequences of *S. miltiorrhiza*. Part of the polyubiquitin gene was amplified with primers (5′-ACCCTCACGGGGAAGACCATC-3′ and 5′-ACCACGGAGACGGAGGACAAG-3′) as a control. The real-time quantitative PCR was performed according to the manufacturer's instructions (Takara) under the following conditions: 1 min pre-denaturation at 95°C, 1 cycle; 10 s denaturation at 95°C, 20 s annealing at a given temperature (set according to the character of the primers used as determined by Primer Primier 5.00 software); and 15 s for collecting fluorescence data at 72°C, 40 cycles. The products of real-time quantitative PCR were subjected to 1.5% agarose-gel electrophoresis and showed an equal-sized band as the predicted product PCR. Quantification of the gene expression was done with the comparative CT method. Each data point represents the average of three experiments.

Metabolite analysis

Compound extraction and analysis followed the methods described by Yan et al. [12] with minor modifications. The dried hairy root sample (50 mg) was ground into comminuted powder (100 mesh) and extracted twice with 30% ethanol (25 ml) under sonication for 30 min, and then centrifuged at 1000 ***g*** for 5 min. The supernatant was diluted with distilled water to 50 ml total volume, and the extract solution was then filtered through a 0.2 μm organic membrane before analysis. The metabolite content was determined by a triple-quadrupole mass spectrometer (Agilent 6410) equipped with a pump (Agilent 1200 G1311A) and an autosampler (G1329A). Chromatography separation was performed with a Thermo® C_{18} column (150 mm × 2.1 mm internal diameter; 3.5 μm particle size; Agilent) with the column temperature set as 30°C. The mobile phase consisting of acetonitrile/water (55:45, v/v) was used. The flow rate was set as 0.3 ml/min and the run time was 3.5 min. The MRM (multiple reaction monitoring) mode was used for the quantification, and the selected transitions of *m*/*z* were 359→161 for RA, 717→519 for LAB, 164→147 for L-phenylalanine, 147→102 for t-cinnamic acid, 163→119 for 4-coumaric acid, 180→119 for L-tyrosine, 179→107 for 4-hydroxyphenylpyruvic acid and 167→123 for homogentisic acid. All standards were purchased from Sigma–Aldrich.

Identification of putative intermediate by HPLC/ESI-MS^n (multi-stage electrospray ionization MS) (ion trap spectrometry)

Based on the above studies, a potential (putative) biosynthetic route from RA to LAB was presumed. Since the target intermediate involved in this route was commercially unavailable because of its unstable character, we identified it by a full scan of the compounds with HPLC/ESI-MS^n (ion trap spectrometry). An Agilent 1100 HPLC system was coupled with an LC/MSD Trap XCT ESI mass spectrometer, equipped with a quaternary pump, a vacuum degasser, an autosampler and a column heater–cooler (Agilent Technologies). The sample with maximum LAB content as determined previously was used for analyses; the sample preparation method and the chromatographic conditions were the same as those used for metabolite analysis as described above. The direct MS analysis was performed by using an LC/MSD trap XCT mass spectrometer (Agilent Technologies) equipped with an ESI source. The MS conditions were as follows: collision energy (Ampl), 0.6–1.0 V; collision gas, He; nebulizer/drying gas, N_2, 5 l/min; temperature, 325°C; pressure of nebulizer, 15 lbf/in^2 (1 lbf/in^2 = 6.9 kPa); high voltage, 3.5 kV; and scan range, 100–1000 units in positive and negative ion mode. Data acquisition was done by using Chemstation software (Agilent Technologies). In particular, the sample solution should be injected immediately after preparation because of the unstable character of the target compound.

Compound feeding studies in Ag^+-elicited and non-elicited cultures

RA was added to Ag^+-elicited and non-elicited cultures to examine the effect of RA addition on LAB accumulation under both conditions and to further investigate whether RA was a precursor leading to LAB synthesis. Ag^+ treatment was performed on day 18 post-inoculation as described above, and RA was added to Ag^+-elicited and non-elicited cultures on day 19. Cultures were harvested on day 33 post-inoculation, i.e. 15 days after Ag^+ treatment. Stock solution of RA was prepared in 30% ethanol and added to the medium to achieve a final concentration of 0.2 mM.

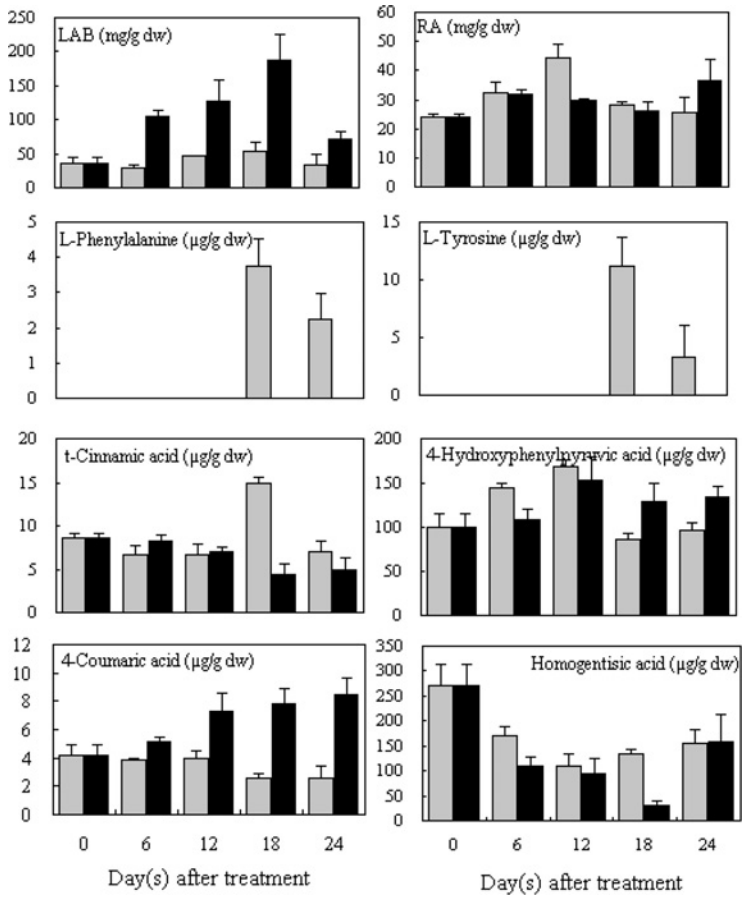

Figure 2 **Effects of Ag^+ (15 μM) on related metabolite accumulations during the *S. miltiorrhiza* hairy roots culture period**
The untreated control is shown in grey and the Ag^+-treated hairy roots culture is shown in black.

RESULTS AND DISCUSSION

Time courses of hairy root biomass growth after Ag^+ treatment

In shake-flask cultures, the biomass of control hairy roots exhibited a steady and linear increase between day 0 and 18 post-treatment and then a period of slow increase in the next 6 days. Correspondingly, their counterparts (Ag^+-treated) showed a steady and linear growth trend at the earlier biomass growth period (days 0–12), and were nearly constant afterwards (results not shown). In other words, the use of Ag^+ elicitor resulted in a decrease in biomass accumulation, especially in the later culture time, which indicated that elicitation switched the cells partially from primary metabolism to secondary metabolism. Furthermore, elicitation also reduced the water content of hairy roots as indicated by the decrease in fw/dw ratio (results not shown).

Ag^+-induced changes in transcript and metabolite accumulation profiles

Integration of transcript and metabolite profiles was intensively investigated in recent studies by using cell cultures of *Catharanthus roseus* [14] and *Papaver somniferum* [15]. As for *S. miltiorrhiza*, RA formation provides an excellent model to investigate the regulatory mechanisms of secondary metabolism because two parallel pathways, presumably regulated in concert, are involved in its biosynthesis [16]. In the present study, the effect of Ag^+ on RA and its derivative LAB was examined. Results showed that LAB was dramatically enhanced, with the maximum content (188 mg/g dw) observed on day 18 post-treatment, approx. 3.5-fold higher compared with the control on the same day, which was much higher than total phenolic content under various conditions from earlier observations [2,10,12]. However, RA accumulation was not induced after elicitation, the accumulation peak was observed on day 12 in control cultures, but there was no obvious up-regulation or down-regulation trend observed after treatment (Figure 2). This finding was somewhat inconsistent with the paper by Yan et al. [12], who observed that Ag^+ (15 μM) induced RA accumulation by approx. 1.4-fold on day 8 after treatment in 15834-induced *S. miltiorrhiza* hairy root cultures. The inconsistency may be because of a difference between the bacterial strain used for transformation and the root line analysed [17].

The profiles of gene transcript and metabolite accumulations indicated that both the phenylpropanoid pathway and the

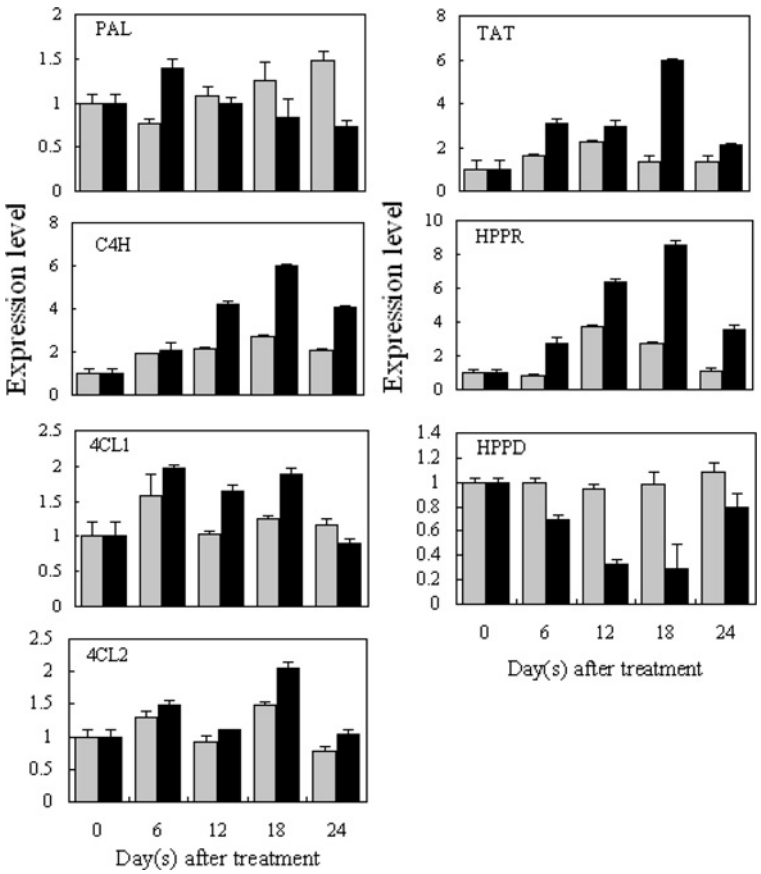

Figure 3 **Effects of Ag^+ (15 μM) on related gene transcripts during the *S. miltiorrhiza* hairy roots culture period**
The untreated control is shown in grey and the Ag^+-treated hairy root culture is shown in black.

tyrosine-derived pathway were apparently activated after Ag^+ treatment. At the transcript level, TAT (tyrosine aminotransferase), HPPR (4-hydroxyphenylpyruvate reductase) and C4H (cinnamic acid 4-hydroxylase) were dramatically stimulated (with the maximum level observed on day 18) by 4.6-, 3.1- and 2.2-fold compared with the control respectively. 4CL1 and 4CL2 (hydroxycinnamate coenzyme A ligases 1 and 2) were also induced compared with the control, but only slightly. For PAL (phenylalanine ammonia-lyase), its transcript level was enhanced within a short time after treatment and increased to the greatest extent on day 6, followed by a decrease. In contrast, HPPD (4-hydroxyphenylpyruvate dioxygenase) was dramatically down-regulated at an earlier time point (with the minimum level 3.4-fold lower than the control observed on day 18), followed by a rapid increase (Figure 3). At the metabolite level, RA and LAB were the most abundant compounds as described above. Furthermore, the change in their accumulation was found to be coincident with each other after Ag^+ treatment. For example, the LAB content of Ag^+-treated hairy root cultures on day 18 was higher than that on day 12, and was much higher than that on day 6, whereas in the case of RA, Ag^+-elicited content on day 18 was lower than that on day 12, and much lower than that on day 6. In other words, the increase in LAB content was coincident with the decrease in RA content during the elicitor-treated culture period. Homogentisic acid accumulation was down-regulated by the Ag^+ elicitor at the earlier time point (with the minimum level 4.5-fold lower than the control observed on day 18) and then increased rapidly to the same level as that of the untreated control. Meanwhile, homogentisic acid content under Ag^+ stress was found to parallel the changes of RA and was also coincident with LAB accumulation. For t-cinnamic acid, an accumulation peak was observed on day 18 in untreated control cultures, whereas in Ag^+-treated cultures, its value on the same day was dramatically decreased to the lowest level ($\sim$ 3.4 times lower than the control). In contrast, 4-coumaric acid was gradually induced by the Ag^+ elicitor with prolongation of the treatment duration. 4-Hydroxyphenylpyruvic acid accumulation was slightly down-regulated by Ag^+ at an earlier stage of the culture period and was slightly up-regulated later. In addition, L-tyrosine and L-phenylalanine were found in the control only at a later culture period, with very low content (Figure 2).

Integrating transcript and metabolite profiles, we found that: for the phenylpropanoid pathway, the elicitor-induced transient and rapid increase in C4H transcripts was coincident with

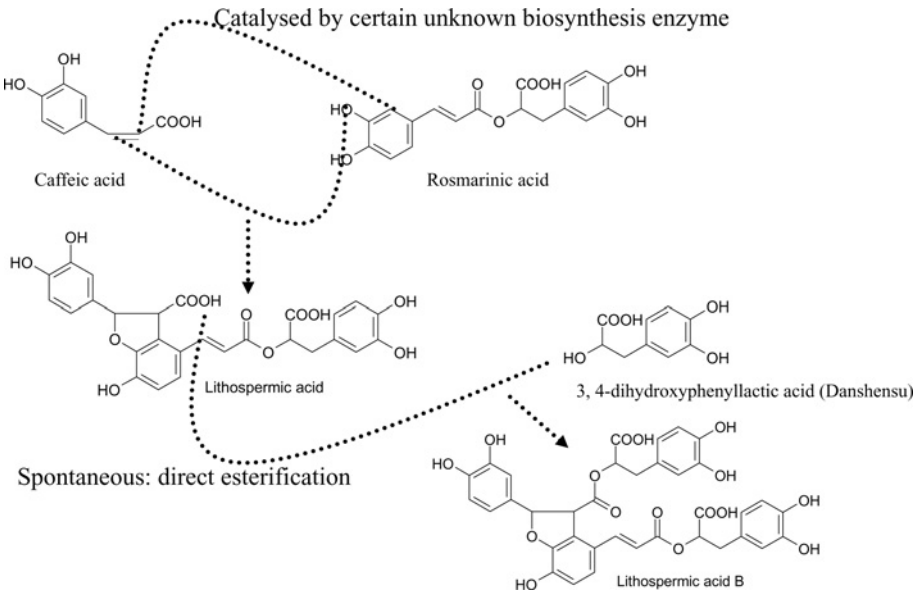

Figure 4 The potential (putative) biosynthesis process from RA to LAB

the decrease in t-cinnamic acid and elevation in 4-coumaric acid accumulation. For the tyrosine-derived pathway, HPPD, a side-branch gene that was heavily down-regulated by the Ag^+ elicitor, perfectly paralleled the decreased homogentisic acid content in treated cultures. However, the effect of Ag^+ on 4-hydroxyphenylpyruvic acid accumulation was not so obvious; this could be explained as the co-function of TAT, HPPR and HPPD transcript changes, since 4-hydroxyphenylpyruvic acid functions not only as a catalysate of TAT, but also as a co-substrate of HPPR and HPPD. The present study integrated comprehensive transcript and metabolite profiles of RA synthesis for the first time, and the observations on Ag^+-induced PAL and TAT transcription paralleled the paper by Yan et al. [12], who observed a high increase in TAT but a decrease in PAL activity after Ag^+ treatment.

The most striking observation was that the results of the above studies were found to parallel the hypothesis that RA was a precursor leading to LAB synthesis. First, the activation of both the phenylpropanoid pathway and the tyrosine-derived pathway did not make contributions to RA synthesis; on the contrary, its derivative LAB was dramatically stimulated after elicitation. Secondly, the elicitor-induced limitation on the side-branch gene HPPD transcript as well as homogentisic acid accumulation did not result in more RA production, but an increase in LAB was observed. Thirdly and most importantly, the elicitor-induced LAB increase was found to correspond well to the decrease in RA accumulation. Thus it was reasonable to believe that LAB was derived from RA, and the whole biosynthesis pathway including the unknown synthesis process between RA and LAB was significantly activated when exposed to Ag^+, finally leading to the accumulation of the ultimate secondary metabolite LAB. Therefore a potential (putative) biosynthetic route from RA to LAB was presumed: RA and caffeic acid were catalysed by a certain enzyme to synthesize an intermediate and then made to react with 3,4-dihydroxyphenyllactic acid (Danshensu) to synthesize LAB via a spontaneous esterification process (Figure 4).

Evidence for the LAB biosynthesis hypothesis provided by an intermediate monitoring and compound feeding experiment

To search for evidence of the potential (putative) biosynthetic route from RA to LAB, we performed an intermediate monitoring and compound feeding experiment. The target intermediate, named lithospermic acid [18], was detected by compound full scanning by using HPLC/ESI-MS^n (ion trap spectrometry), with a retention time of approx. 1.5 min and the $[M\text{-}1]^-$ peak observed at m/z 537 (Figure 5), and its MS^n fragmentations were shown to be identical with those reported by Zhu et al. [18]. In addition, we found that this target compound gradually degraded with the prolongation of treatment duration, which also agreed with the observation that it was only a temporal intermediate leading to LAB synthesis.

Precursor feeding studies have been frequently used to determine the limiting branch in the biosynthesis of important metabolites [19–22]. In the present study, we used the compound feeding experiment to examine whether a given compound was the potential precursor for leading to specific metabolites. As shown in Figure 6, addition of Ag^+ to the hairy root cultures dramatically enhanced LAB production (156 mg/g) but reduced RA, when harvested on day 15 after Ag^+ treatment, which was consistent with the observation described in the previous section.

Addition of RA to non-induced cultures led to a slight increase in LAB, indicating that RA supply indeed makes contributions

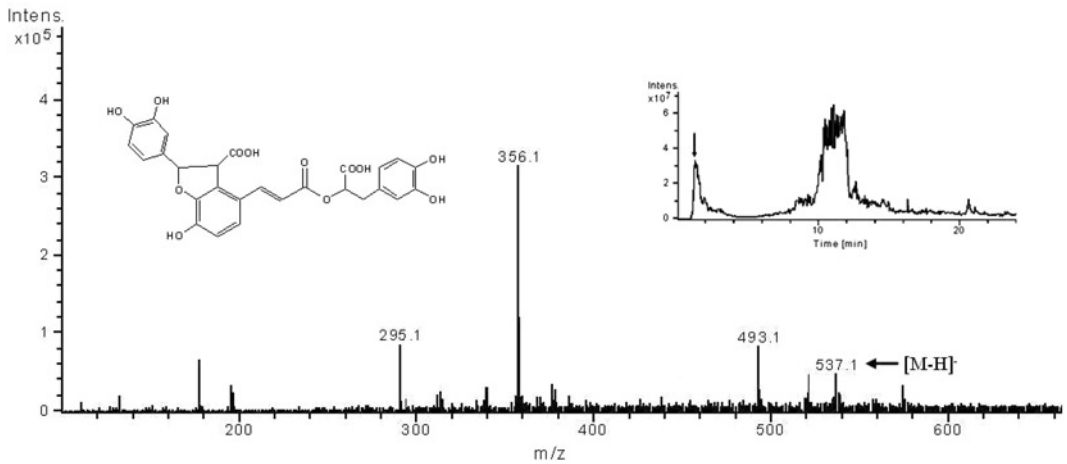

Figure 5 Chromatogram and negative ion ESI-MS^n spectra of putative target intermediate (lithospermic acid)

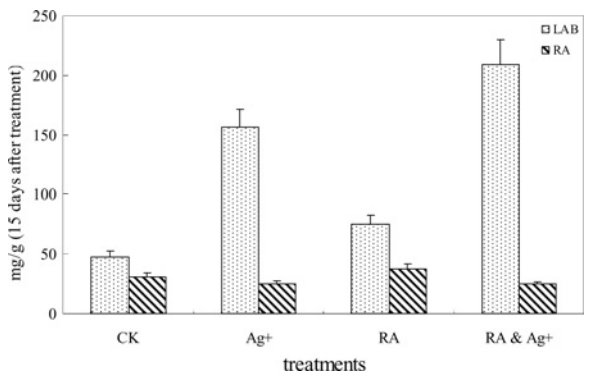

Figure 6 Effect of feeding Ag^+-elicited and non-elicited cultures with RA (0.2 mM) on LAB and RA production
Hairy root cultures were elicited with 15 μM Ag^+, fed with RA on the following day and harvested on day 15.

to LAB production. Meanwhile, more RA accumulation was observed, suggesting that some enzymatic steps downstream were limiting, resulting in its accumulation. However, this limitation was overcome in Ag^+-induced cultures where RA feeding dramatically enhanced LAB production (209 mg/g) by 280% compared with non-elicited RA-fed cultures, i.e. 134% compared with Ag^+-elicited non-fed cultures. In addition, RA content was found to decline to a lower level than that of non-fed cultures (Figure 6). The above finding suggests that after activation of certain enzymatic steps by Ag^+, when RA supply was large enough, RA transformed to LAB to a greater degree, thus resulting in the decrease in RA. To sum up, this study strengthened the proposed statement that LAB was derived from RA, and the potential synthesis process was perfectly activated by the Ag^+ elicitor. However, this remains to be investigated by further studies such as isolation of the valid enzyme.

In conclusion, the combined gene transcripts, metabolite profile, putative intermediate monitoring, as well as RA feeding studies, in *S. miltiorrhiza* hairy root cultures suggested that there existed a potential biosynthetic route from RA to LAB, which was extremely active when exposed to Ag^+. The present study, for the first time, provides evidence for the hypothesis that RA is a precursor leading to LAB synthesis.

FUNDING

This work was financially supported by the National Natural Science Foundation of China [30600807]; the Modernization of Traditional Chinese Medicine Foundation [08DZ1971502]; and the Western Development Cooperation Foundation, Shanghai Science and Technology Committee [084358014].

REFERENCES

1 Zhou, L. M., Zuo, Z. and Moses, S. (2005) Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. J. Clin. Pharmacol. 45, 1345–1359
2 Chen, H., Chen, F., Chiu, F. C. and Lo, C. M. (2001) The effect of yeast elicitor on the growth and secondary metabolism of hairy root cultures of *Salvia miltiorrhiza*. Enzyme Microb. Technol. 28, 100–105
3 Liu, A. H., Li, L., Xu, M., Lin, Y. H., Guo, H. Z. and Guo, D. A. (2006) Simultaneous quantification of six major phenolic acids in the roots of *Salvia miltiorrhiza* and four related traditional Chinese medicinal preparations by HPLC–DAD method. J. Pharm. Biomed. Anal. 41, 48–56
4 Ma, L. J., Zhang, X. Z., Guo, H. and Gan, Y. R. (2006) Determination of four water-soluble compounds in *Salvia miltiorrhiza* Bunge by high-performance liquid chromatography with a coulometric electrode array system. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 833, 260–263

5 Petersen, M. (1997) Cytochrome P450-dependent hydroxylation in the biosynthesis of rosmarinic acid in *Coleus*. Photochemistry 45, 1165–1172
6 Petersen, M., Häusler, E., Karwatzki, B. and Meinhard, J. (1993) Proposed biosynthetic pathway for rosmarinic acid in cell cultures of *Coleus blumei*. Planta 189, 10–14
7 Petersen, M. and Simmonds, M. S. (2003) Rosmarinic acid. Phytochemistry 62, 121–125
8 Tanaka, T., Morimoto, S., Nonaka, G., Nishioka, I., Yokozawa, T., Chung, H. Y. and Oura, H. (1989) Magnesium and ammonium-potassium lithospermates B, the active principles having a uremia-preventive effect from *Salvia miltiorrhiza*. Chem. Pharm. Bull. 37, 340–344
9 Omoto, T., Murakami, Y., Shimomura, K., Yoshihira, K., Mori, K., Nakashima, T., Tanaka, M. and Ishimaru, K. (1997) Caffeic acid derivatives in Lamiaceae and Boraginaceae plants. Japan. J. Food Chem. 4, 11–16
10 Chen, H., Chen, F., Zhang, Y. L. and Song, J. Y. (1999) Production of lithospermic acid B and rosmarinic acid in hairy root cultures of *Salvia miltiorrhiza*. J. Ind. Microbiol. Biotechnol. 22, 133–138
11 Ge, X. C. and Wu, J. Y. (2005) Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag^{+} and yeast elicitor. Plant Sci. 168, 487–491
12 Yan, Q., Shi, M., Ng, J. and Wu, J. Y. (2006) Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. Plant Sci. 170, 853–858
13 Jaakola, L., Pirttila, A. M., Halonen, M. and Hohtola, A. (2001) Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biotechnol. 19, 201–203
14 Rischer, H., Oresic, M., Seppanen-Laakso, T., Katajamaa, M., Lammertyn, F., Ardiles-Diaz, W., Van, Montagu, M. C., Inze, D., Oksman-Caldentey, K. M. and Goossens, A. (2006) Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. Proc. Natl. Acad. Sci. U.S.A. 103, 5614–5619
15 Zulak, K. G., Cornish, A., Daskalchuk, T. E., Deyholos, M. K., Goodenowe, D. B., Gordon, P. M., Klassen, D., Pelcher, L. E., Sensen, C. W. and Facchini, P. J. (2007) Gene transcript and metabolite profiling of elicitor-induced *opium poppy* cell cultures reveals the coordinate regulation of primary and secondary metabolism. Planta 225, 1085–1106
16 Mizukami, H., Tabira, Y. and Ellis, B. E. (1993) Methyl jasmonate-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. Plant Cell Rep. 12, 706–709
17 Grzegorczyk, I., Krolicka, A. and Wysokinska, H. (2006) Establishment of *Salvia officinalis* L. hairy root cultures for the production of rosmarinic acid. Z. Naturforsch. 61, 351–356
18 Zhu, Z. Y., Zhang, H., Zhao, L., Dong, X., Li, X., Chai, Y. F. and Zhang, G. Q. (2007) Rapid separation and identification of phenolic and diterpenoid constituents from Radix *Salvia miltiorrhizae* by high-performance liquid chromatography diode-array detection, electrospray ionization time-of-flight mass spectrometry and electrospray ionization quadrupole ion trap mass spectrometry. Rapid Commun. Mass Spectrom. 21, 1855–1865
19 Lee-Parsons, C. W. T. and Royce, A. J. (2006) Precursor limitations in methyl jasmonate-induced *Catharanthus roseus* cell cultures. Plant Cell Rep. 25, 607–612
20 Morgan, J. A. and Shanks, J. V. (2000) Determination of metabolic rate-limitations by precursor feeding in *Catharanthus roseus* hairy root cultures. J. Biotechnol. 79, 137–145
21 Whitmer, S., van der Heijden, R. and Verpoorte, R. (2002) Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase over-expressing transgenic cell line T22 of *atharanthus roseus*. J. Biotechnol. 96, 193–203
22 Whitmer, S., van der Heijden, R. and Verpoorte, R. (2002) Effect of precursor feeding on alkaloid accumulation by a strictosidine synthase over-expressing transgenic cell line S1 of *Catharanthus roseus*. Plant Cell Tissue Organ Cult. 69, 85–93

Received 5 September 2008/3 February 2009; accepted 11 February 2009
Published as Immediate Publication 11 February 2009, doi 10.1042/BSR20080124

