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Full Length Research Paper

A liquid chromatographic–tandem mass spectrometric method for the quantitation of eight components involved in lithospermic acid B biosynthesis pathway in *Salvia miltiorrhiza* hairy root cultures

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Elucidation of regulatory mechanisms of plant secondary metabolism requires quantitation of metabolic fluxes. A method of high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been developed for the trace analysis of eight components (rosmarinic acid, lithospermic acid B, L-phenylalanine, t-cinnamic acid,4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid) involved in lithospermic acid B biosynthesis pathway in *Salvia miltiorrhiza* hairy root cultures. The separation was performed by a ZORBAX SB-C_{18} column (3.5 µm, 2.1 x 150 mm, I.D. Agilent Corporation, MA, USA) and a C_{18} guard column (5 µm, 4.0 x 2.0 mm, Agilent Corporation, MA, USA) with an isocratic mobile phase consisting of acetonitrile-water (55:45, v/v) at a flow rate of 0.3 ml/min. The components were detected by an Agilent G6410A triple quadrupole LC/MS system equipped with a MassHunter interface. The components were detected using electrospray ionization (ESI) in negative-ion mode and quantified by multiple-reaction monitoring (MRM) mode using the transition mass of *m/z* 359→161, 717→519, 164→147, 147→103, 163→119, 180→119, 179→107 and 167→123 for rosmarinic acid, lithospermic acid B, L-phenylalanine, t-cinnamic acid, 4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid, respectively. The recovery of the method was in the range of 95.4 to 105.5%, and all the components showed good linearity (r＞0.995) in a relatively wide concentration range. This method has been successfully applied to the determination of the eight components in different elicitor (Methyl jasmonate and silver ions) treated *S. miltiorrhiza* hairy root cultures, for describing elicitor-based regulatory effect to lithospermic acid B metabolic fluxes.

Key words: *Salvia miltiorrhiza*, biosynthesis pathway, liquid chromatography–tandem mass spectrometry, phenolic acids.

INTRODUCTION

Salvia miltiorrhiza Bunge, called “Dan-Shen” in Chinese, is officially listed in the Chinese Pharmacopoeia and has been used for the treatment of disorders caused by poor blood supply such as coronary artery disease and angina pectoris (Zhou et al., 2005). According to the pharmacological investigations, the active components of *S. miltiorrhiza* are divided into two main groups: one is lipid-soluble tanshinones group, and the other is water-soluble phenolic acids group such as rosmarinic acid (RA) and its derivatives lithospermic acid B (LAB) (Chen et al., 2001).

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Recently, the research attention has been focused on the phenolic acids due to their pharmacological activities (Liu et al., 2006; Ma et al., 2006). In particular, compared with other Lamiaceae plants, *S. miltiorrhiza* has especially high level in accumulating LAB (Omoto et al., 1997), exhibiting the notable endothelium-dependent vasodilator and hypotensive effects (Kamata et al., 1993, 1994), which has been suggested to be derived from RA (Petersend et al., 2003; Tanaka et al., 1989). The biosynthetic pathway leading to RA is well defined, which involves both the phenylpropanoid pathway and the tyrosine-derived pathway (Figure 1) (Petersen et al., 1993, 1997). During the last decade, metabolic regulation by means of recombinant DNA technology or elicitor treatment has opened a new promising perspective for improved production in a plant or plant cell culture, which emphasizes metabolic pathway integration and relies on metabolic fluxes as determinants of cell physiology and measures of genetic control. Therefore, it is essential to develop analytical methods to quantitate related compounds involved in metabolic fluxes, for the purpose of systematic elucidation of metabolic regulation (Gregory et al., 1999; Namdeo et al., 2007). RA formation provides an excellent model to investigate regulatory mechanisms of secondary metabolism because two parallel and presumably concertedly regulated pathways are involved in its biosynthesis (Mizukami et al., 1993). However, there has been no report on the determination of metabolites involved in the specific metabolic flux, causing obstruction to further explore regulatory mechanisms of plant secondary metabolism.

In the present assay, we have developed and validated a rapid and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS) method to quantitatively determine eight metabolites involved in phenolic acids biosynthesis pathway from *S. miltiorrhiza* hairy roots, including RA, LAB, L-phenylalanine, t-cinnamic acid, 4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid (Figure 1). This method relies on high performance liquid chromatography (HPLC) with an Agilent ZORBAX SB-C_{18} column followed by negative electrospray ionisation-tandem mass spectrometry detection. A multiple-reaction monitoring (MRM) mode was utilized to optimize sensitivity and accuracy. The method is rapid, sensitive, and specific for quantifying these eight components in *S. miltiorrhiza* hairy roots, which will be a great motivation for investigating the secondary metabolics of phenolic acids in *S. miltiorrhiza*.

EXPERIMENTAL

Chemicals and reagents

RA, LAB, L-phenylalanine, t-cinnamic acid, 4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid were purchased from Sigma (St. Louis, MO, USA); Methanol and acetonitrile of HPLC grade were purchased from Merck Company (Darmstadt, Germany). HPLC grade ethanol was purchased from Tedia Company Inc. (Tedia Fairfield, OH, USA). Ultrapure water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). All other reagents were of analytical grade. All samples prepared for HPLC were filtered through a membrane filter (0.22 µm pore size) before use.

Instrumentation

All experiments were carried out on an Agilent 1200 series HPLC and interfaced to an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Corporation, MA, USA).

All data were acquired and analyzed using Agilent 6410 Quantitative Analysis version B.01.02 analyst data processing software (Agilent Corporation, MA, USA).

Chromatographic conditions

The separation was performed by a ZORBAX SB-C_{18} column (3.5 µm, 2.1 x 150 mm, I.D. Agilent Corporation, MA, USA) and a C_{18} guard column (5 µm, 4.0 x 2.0 mm, Agilent Corporation, MA, USA) with an isocratic mobile phase consisting of acetonitrile-water (55:45, v/v) at a flow rate of 0.3 ml/min. The column temperature was maintained at 30°C. The injection volume was 10 µl and the analysis time was 3.5 min per sample.

Mass spectrometric conditions

RA, LAB, L-phenylalanine, t-cinnamic acid, 4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid achieved using electrospray in the negative mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas and nebulizer pressure was set at 40 psi with a source temperature of 105°C. Desolvation gas (nitrogen) was heated to 350°C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high purity nitrogen was used as collision gas at a pressure of about 0.1 MPa. Quantitation was performed using multiple reaction monitoring (MRM) mode, Table 1 shows the optimized MRM parameters for detected components. The peak widths of precursor and product ions were maintained at 0.7 amu at half-height in the MRM mode.

Preparation of analytical samples

The seeds of *S. miltiorrhiza* were purchased from local market. The plant was grown in the herb garden of Second Military Medical University, Shanghai, China, and identified by Professor Hanming Zhang (School of Pharmacy, Second Military Medical University). The specimen (Voucher specimen No. 127) was deposited in the Department of Pharmacognosy, Second Military Medical University.

The *S. miltiorrhiza* hairy root culture was conducted as described by Yan et al. (2006). Methyl jasmonate (MeJA) and silver ions (Ag^+) elicitor preparation, treatment and treated hairy roots harvest were conducted as Xiao et al. (2009, 2010), respectively.

Harvested hairy roots were dried at 45°C in an oven until constant dry weight (DW).

The dried hairy root sample (50 mg) was ground into powder, comminuted (100 mesh) and extracted twice with 30% ethanol (25 ml) under sonication for 30 min, and then centrifuged at 4500 rpm for 5 min. The supernatant was diluted with distilled water to 50 ml total volume, and the extract solution was then filtered through a 0.22 µm organic membrane before analysis.

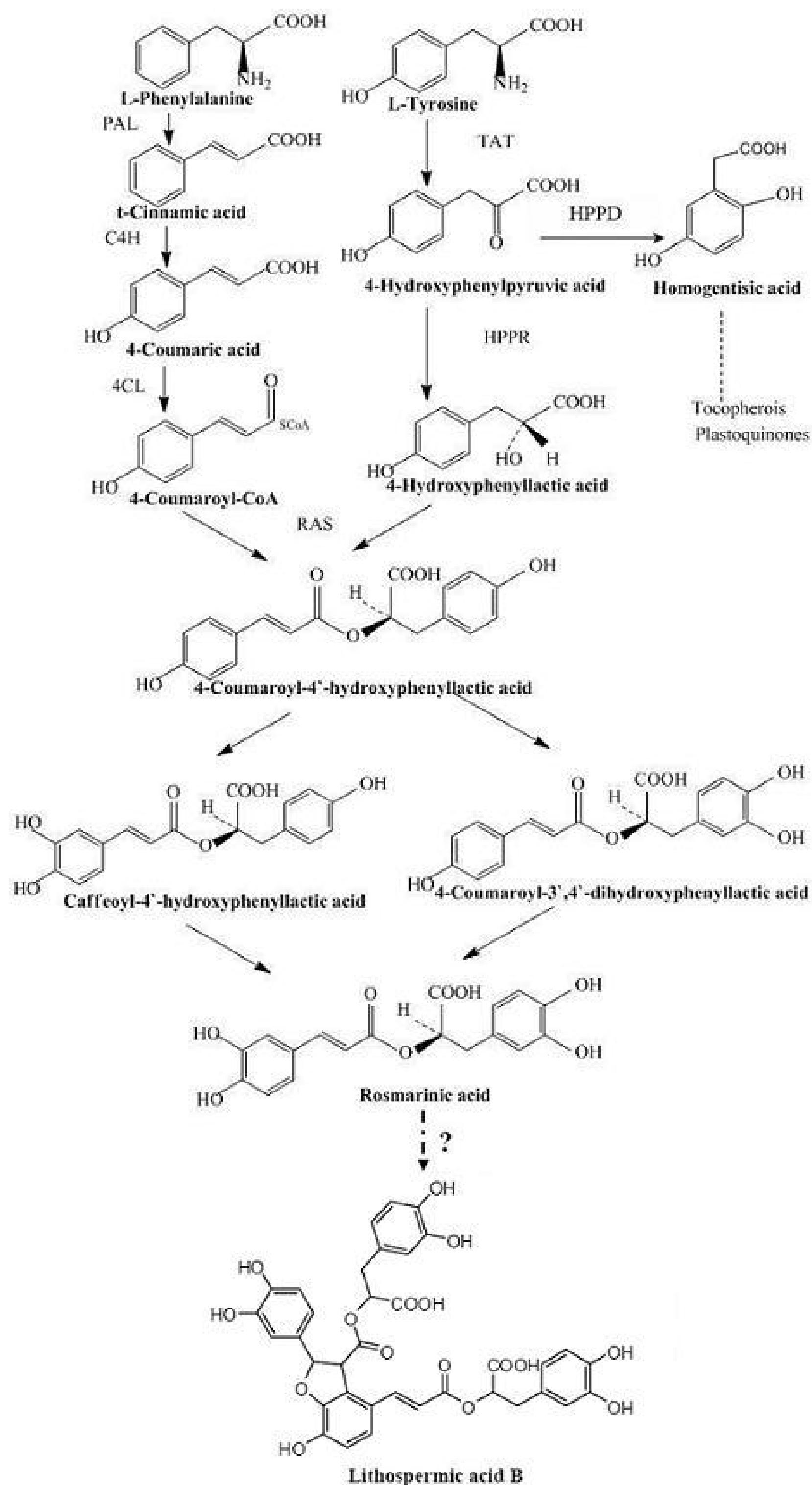

Figure 1. The metabolic pathway leading to RA.

Method validation

Calibration curves

Calibration curves of eight concentrations of RA, LAB, L-phenylalanine, t-cinnamic acid, 4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid ranging from 3000 to 30000, 1200 to 120000, 4 to 40, 2.5 to 25, 0.5 to 5, 3.5 to 35, 30 to 300 and 50 to 500 ng/ml, respectively, were assayed according to the conditions described thus;

Table 1. Optimized MRM parameters for RA, LAB, L-phenylalanine, t-cinnamic acid, 4-coumaric acid, L-tyrosine, 4-hydroxyphenylpyruvic acid and homogentisic acid.

Component	Precursor ion	Fragmentor energy(V)	Collision energy(eV)	Product ion
RA	359	120	14	161
LAB	717	150	12	519
L-phenylalanine	164	100	15	147
t-cinnamic acid	147	100	10	103
4-coumaric acid	163	100	12	119
L-tyrosine	180	100	18	119
4-hydroxyphenylpyruvic acid	179	70	8	107
homogentisic acid	167	100	8	123

Limits of detection (LOD) and quantification (LOQ)

The standard stock solutions were diluted with methanol to provide a series of solutions with the appropriate concentrations. The limit of detection and quantification under the chromatographic conditions were determined by measuring the signal-to-noise ratio for each component by injecting a series of solutions until the S/N ratio 3 for LOD and 10 for LOQ.

Precision and repeatability

The precision of the assay was determined from the standard samples by replicate analyses ($n = 6$). The repeatability of the samples were also assessed by repeated analyses ($n = 6$). The concentration of each sample was determined using the calibration curve prepared and analyzed on the same batch.

Recovery

The recovery tests for the LC-MS/MS method were carried out by adding the standards to the raw materials. Three initial concentrations were prepared to estimate the recovery. Then the materials mixed with standards were prepared using ultrasonic extraction according to the procedure described in “preparation of analytical samples”. The samples were then analyzed by LC-MS/MS according to the conditions described in “chromatographic and mass spectrometric conditions”.

Recovery (%) = (mean of measured concentration / spiked concentration) x 100

Stability

The stability of analytes in sample solutions was investigated. It was carried out by determining the NO.9 analytes same treated sample solutions after being stored at room temperature for different durations (0, 2, 4, 6 and 12 h).

RESULTS AND DISCUSSION

Optimization of chromatographic and MS/MS conditions

It is crucial to optimize the chromatographic conditions before applying MS-MS because impurities in the samples can greatly decrease the sensitivity of determination by affecting the ionization of the components of interest.

An isocratic mobile phase was implemented to optimize the HPLC separation for eight components. In the course of the experiments, several systems such as methanol-water, acetonitrile-water, and methanol- acetonitrile-water performed in different ratios were examined. Finally, in consideration of economy and simplicity, the mobile phase composition was simplified by using acetonitrile-water mixtures.

To determine eight components involved in phenolic acids biosynthesis pathway in *S. miltiorrhiza* hairy root cultures using the MRM mode, full scan and product ion spectra of eight components were investigated under the present HPLC conditions. The possibility of using positive or negative ion detection was first evaluated. It was found that negative mode could offer higher sensitivities than positive mode. Therefore, the ESI source in negative mode was chosen for eight components detection. To get the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energies and collision energies were optimized, and the MRM transition were chosen to be m/z 359→161 for RA, m/z 717→519 for LAB, m/z 164→147 for L-phenylalanine, m/z 147→103 for t-cinnamic acid, m/z 163→119 for 4-coumaric acid, m/z 180→119 for L-tyrosine, m/z 179→107 for 4-hydroxyphenylpyruvic acid and m/z 167→123 for homogentisic acid. Figure 2 shows the spectra of full scan product ion of precursor ions of the eight analytes.

Method validation

Assay selectivity

The LC-MS/MS method has high selectivity because only selected ions produced from selected precursor ions are monitored. Comparison of the chromatograms of the blank and the spiked standard substance (Figure 3) indicated no significant interference at the retention times of the analytes. The method was validated for parameters

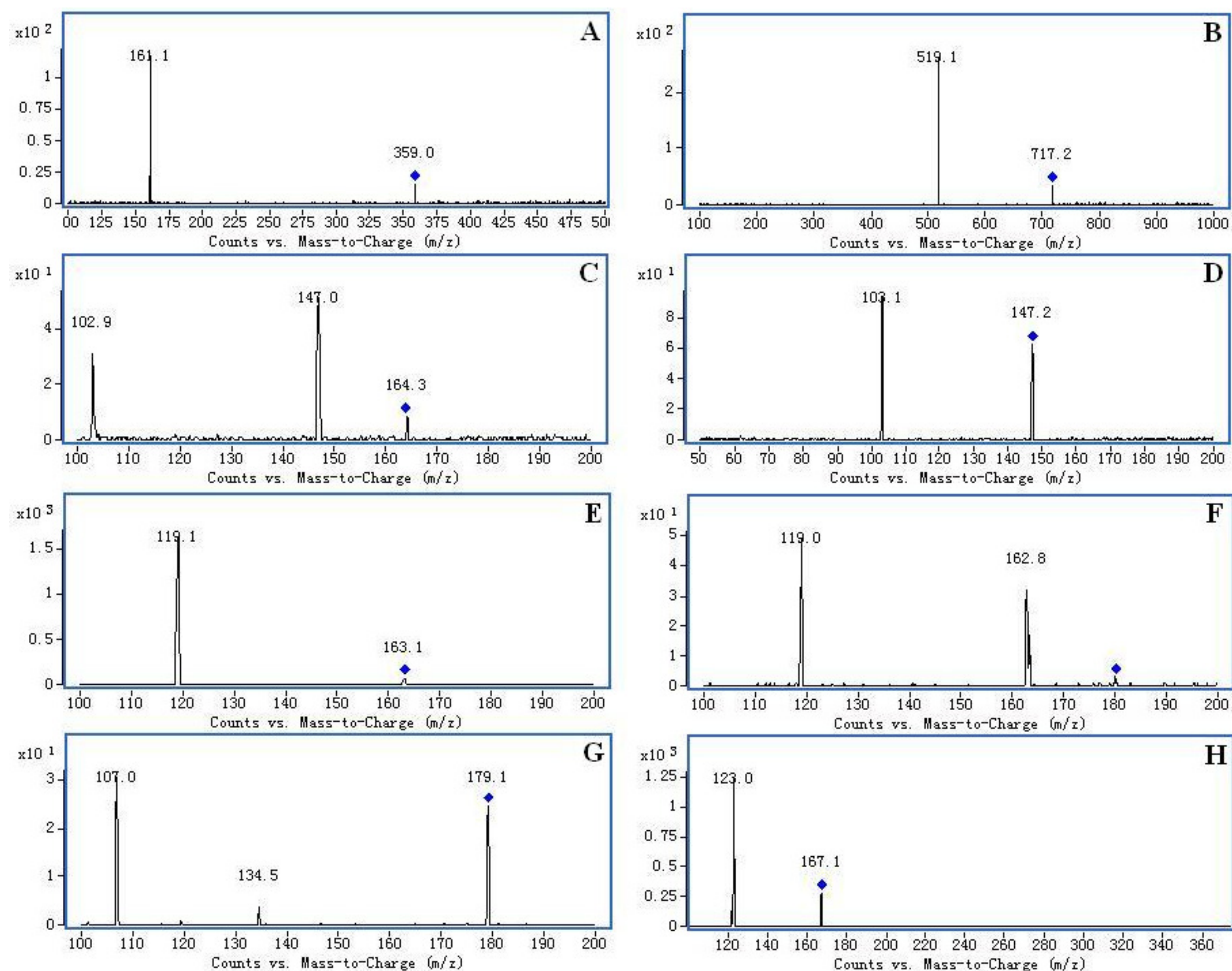

Figure 2. Mass spectrum and product ion spectrum of eight components. (A) RA, (B) LAB, (C) L-phenylalanine, (D) t-cinnamic acid, (E) 4-coumaric acid, (F) L-tyrosine, (G) 4-hydroxyphenyl pyruvic acid and (H) homogentisic acid.

such as linearity, precision, accuracy and stability following the international conference on harmonization guidelines.

Calibration curves

Linear regression analyses for the eight components were performed by using the external standard method. The peak area values were the average values of three replicate injections. The results of calibration are summarized in Table 2. All components showed good linearity ($r > 0.995$) in a relatively wide concentration range.

Limits of detection (LOD)

The LOD under the chromatographic conditions was determined by measuring the magnitude of analytical background by means of injecting blank samples, and calculating the signal-to-noise ratio for each component by injection series of solutions until the S/N ratio 3 for LOD, then 5 replicate injections of the solution gave the RSD less than 3%. LOD was reported in Table 2 for each component.

Precision and repeatability

The precision and repeatability data for each marker substance are listed in Table 3; relative standard deviations (RSD) of the precision and repeatability were 1.75 to 9.67% and 0.53 to 9.89%, respectively.

Recovery

Recovery was determined with the LC-MS/MS method. The average recoveries of standards spiked into *S. miltiorrhiza* hairy root were 95.43% for rosmarinic acid,

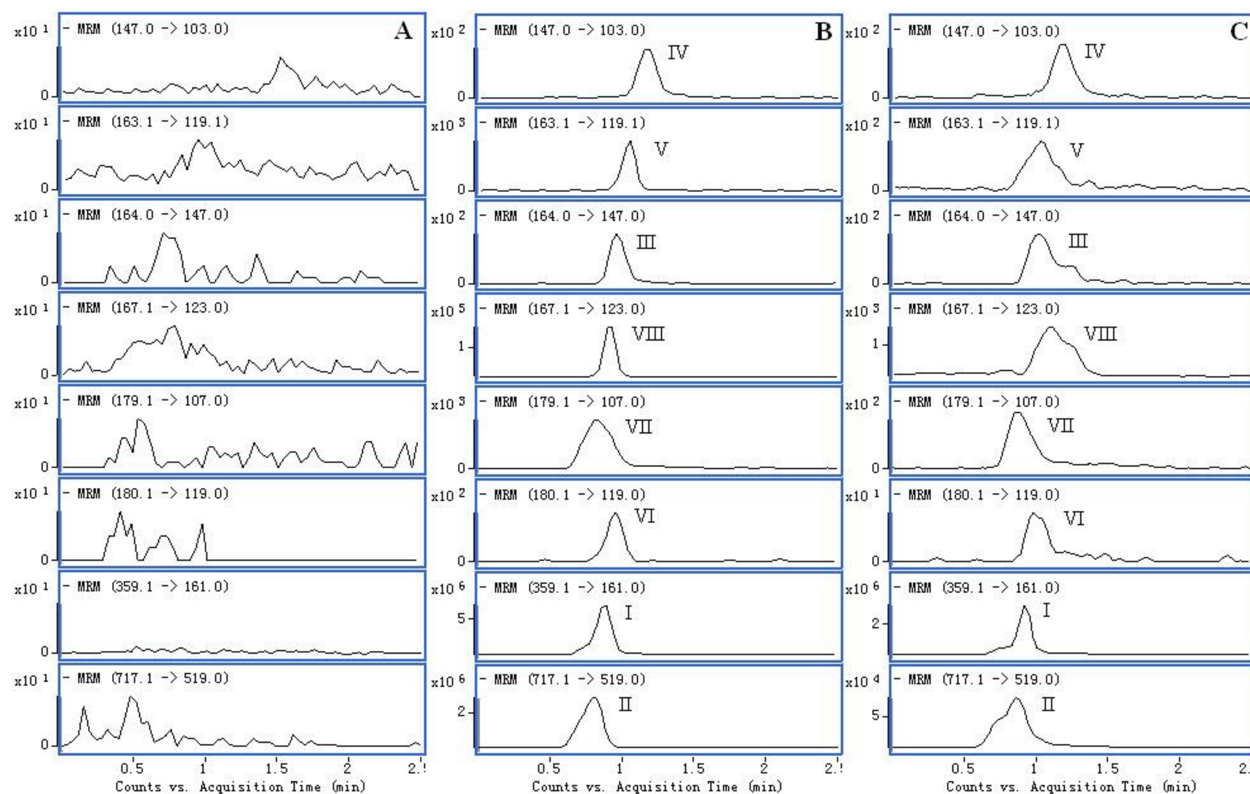

Figure 3. Representative MRM chromatograms of RA (I), LAB (II), L-phenylalanine (Ⅲ), t-cinnamic acid (Ⅳ), 4-coumaric acid (V), L-tyrosine (VI), 4-hydroxyphenylpyruvic acid (VII) and homogentisic acid(VIII). (A) a blank sample, (B) a blank sample spiked with eight components and (C) the NO.9 sample.

Table 2. Calibration analysis of eight components involved in phenolic acids biosynthesis pathway in *S. miltiorrhiza* hairy root cultures.

Component	Linearity range (ng/ml)	Calibration equation[a]	LOD[b] (ng/ml)	Correlation factor (R^2)
RA	3000.0~30000.0	1697.90*C* + 2 X 10^6	0.37	0.9968
LAB	1200.0~120000.0	654.04*C* - 1 X 10^6	9.37	0.9965
L-phenylalanine	4.0~40.0	131.91*C* - 11.15	0.50	0.9951
t-cinnamic acid	2.5~25.0	334.76*C* - 394.87	0.31	0.9988
4-coumaric acid	0.5~5.0	2152.69*C* + 233.40	0.03	0.9991
L-tyrosine	3.5~35.0	49.46*C* + 10.49	1.75	0.9974
4-hydroxyphenylpyruvic acid	30.0~300.0	29.49*C* + 589.69	1.87	0.9913
homogentisic acid	50.0~500.0	486.17*C* + 2186.9	0.78	0.9990

C: the concentration of eight components standard (ng/mL),[a]Five data points (*n*=5), [b]LOD = limit of detection.

97.32% for lithospermic acid B, 99.79% for L-phenylalanine, 103.42% for t-cinnamic acid, 98.99% for 4-coumaric acid, 104.52% for L-tyrosine, 105.47% for 4-hydroxyphenylpyruvic acid and 98.31% for homogentisic acid, respectively.

Stability

The stability of analytes in sample solutions was investigated. It was carried out by determining the NO.9 analytes in same treated sample solutions after being

Table 3. Results of precision and repeatability analysis for eight components (n=6).

Component	Concentration (ng/ml)	Precision		Repeatability (NO.9)	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
RA	12000.0	11907.00 ± 444.78	3.74	13673.99 ± 386.56	2.83
LAB	30000.0	31250.40 ± 772.31	2.47	1833.43 ± 9.65	0.53
L-phenylalanine	16.0	17.15 ± 0.95	5.52	33.61 ± 2.54	7.57
t-cinnamic acid	10.0	10.12 ± 0.18	1.75	7.35 ± 0.60	8.21
4-coumaric acid	2.0	2.01 ± 0.09	4.28	3.15 ± 0.26	8.17
L-tyrosine	14.0	14.31 ± 1.38	9.67	34.62 ± 3.42	9.89
4-hydroxyphenylpyruvic acid	120.0	122.56 ±5.26	4.29	247.07 ± 8.53	3.45
homogentisic acid	200.0	196.98 ± 4.43	2.25	394.35 ± 8.56	2.17

Table 4. MeJA-induced changes in the eight metabolites accumulation in *S. miltiorrhiza* hairy roots culture.

Compounds	Treatment	Time after treatment				
		0 day	3 days	6 days	9 days	12 d
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RA	CK	24.21±0.68	28.66±2.56	32.46±3.38	42.65±3.65	44.35±4.81
(mg/g DW)	MeJA	24.21±0.68	35.16±1.56	60.16±4.08	42.65±3.56	37.70±4.34
LAB	CK	35.33±9.44	32.58±6.56	29.40±4.53	34.56±3.67	46.69±1.05
(mg/g DW)	MeJA	35.33±9.44	80.15±10.25	192.61±18.22	100.65±8.24	26.21±8.67
homogentisic acid	CK	269.71±43.00	236.36±10.69	169.17±19.07	118.36±8.62	110.02±24.18
(µg /g DW)	MeJA	269.71±43.00	200.60±12.36	171.58±16.65	222.20±10.70	234.04±18.10
L-phenylalanine	CK	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
(µg/g DW)	MeJA	0.00±0.00	0.00±0.00	1.40±0.41	1.93±0.15	2.18±0.43
L-tyrosine	CK	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
(µg/g DW)	MeJA	0.00±0.00	0.00±0.00	0.00±0.00	1.25±0.25	3.23±0.42
t-cinnamic acid	CK	8.57±0.50	7.63±1.36	6.63±1.04	6.59±0.60	6.58±1.39
(µg/g DW)	MeJA	8.57±0.50	6.57±0.36	2.35±0.20	4.10±0.26	4.32±0.37
4-coumaric acid	CK	4.15±0.73	4.57±0.63	4.83±0.06	4.04±0.69	3.99±0.48
(µg/g DW)	MeJA	4.15±0.73	3.30±0.63	2.88±0.33	2.87±0.15	2.87±0.29
4-hydroxyphenylpyruvic	CK	100.81±13.92	125.00±4.50	144.48±5.68	158.00±6.00	168.81±6.99
acid (µg/g DW)	MeJA	100.81±13.92	136.00±15.60	166.81±38.38	144.00±3.60	144.23±9.16

DW: dry weight.

stored at room temperature at different time (0, 2, 4, 6 and 12 h). The NO.9 sample solutions were found to be stable within 12 h (RSD<5%). The stabilities of components in standard solution were also evaluated. It was showed that they were stable in methanol at 4℃ for at least 10 days.

RESULTS ANALYSIS

The LC-MS/MS method was used to characterize the eight metabolites involved in lithospermic acid B biosynthesis pathway, in both control and elicitor-treated (MeJA and Ag^+) hairy root cultures. For MeJA treatment, L-tyrosine and L-phenylalanine were found only in elicitor-treated samples in a later culture period, with very low content, and another six showed changes in abundance in response to elicitor at various levels. RA and LAB were the most abundant compounds, both of which were significantly ($P < 0.01$) induced by MeJA, the maximum contents (60.2 and 192 mg/g DW, about 1.9 and 6.6-fold of control samples on the same day, respectively) were observed on day 6 post-inoculation, followed by a gradual decline. Effect of MeJA on homogentisic acid was prominent only at a later stage of the elicitation (day 9 after treatment) when the homogentisic acid content in the control went on decreasing whereas that in the elicited samples began to increase ($P < 0.01$). In addition, MeJA had a negative effect ($P < 0.01$) on t-cinnamic acid and 4-coumaric acid accumulation, but more obvious in t-cinnamic acid, with the minimum level of 2.8-fold lower than the control observed on day 6. However, there was no significant ($P > 0.05$) change observed in 4-hydroxyphenylpyruvic acid accumulation after MeJA treatment (Table 4). For Ag^+

Table 5. Effects of Ag^+ (15 µM) on related metabolites accumulations.

Compounds	Treatment	Time after treatment 0 day Mean ± SD	6 days Mean ± SD	12 days Mean ± SD	18 days Mean ± SD	24 days Mean ± SD
RA	CK	24.21±0.68	32.46±3.38	44.35±4.81	28.32±0.95	25.31±5.36
(mg/g DW)	Ag^+	24.21±0.68	31.67±1.95	29.56±0.85	26.34±2.94	36.31±7.39
LAB	CK	35.33±9.44	29.40±4.53	46.69±1.05	53.85±13.95	32.64±17.33
(mg/g DW)	Ag^+	35.33±9.44	104.95±9.43	126.58±31.00	187.91±37.53	71.41±10.82
homogentisic acid	CK	269.71±43.00	169.17±19.07	110.02±24.18	134.26±8.17	154.78±26.64
(µg /g DW)	Ag^+	269.71±43.00	109.73±17.10	94.78±29.25	30.18±10.63	158.40±55.68
L-phenylalanine	CK	0.00±0.00	0.00±0.00	0.00±0.00	3.76±0.74	2.25±0.73
(µg/g DW)	Ag^+	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
L-tyrosine	CK	0.00±0.00	0.00±0.00	0.00±0.00	11.25±2.41	3.27±2.83
(µg/g DW)	Ag^+	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
t-cinnamic acid	CK	8.57±0.50	6.63±1.04	6.58±1.39	14.89±0.67	7.10±1.11
(µg/g DW)	Ag^+	8.57±0.50	8.21±0.76	6.96±0.55	4.35±1.24	4.92±1.37
4-coumaric acid	CK	4.15±0.73	4.83±0.06	3.99±0.48	2.59±0.31	2.62±0.84
(µg/g DW)	Ag^+	4.15±0.73	5.14±0.37	7.25±1.37	7.85±1.05	8.42±1.23
4-hydroxyphenylpyruvic	CK	100.81±13.92	144.48±5.68	168.81±6.99	86.43±6.07	96.13±8.98
acid (µg/g DW)	Ag^+	100.81±13.92	108.63±12.09	153.52±25.62	128.45±21.18	134.09±11.75

treatment, L-tyrosine and L-phenylalanine were found in the control only at a later culture period, with very low content. LAB was dramatically enhanced by Ag^+, 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, However, RA accumulation was not induced after elicitation, the accumulation peak was observed on day 12 in control cultures. 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. 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 (~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 (Table 5). It is clear that different elicitor treatments caused different change in the abundance of metabolites involved in lithospermic acid B biosynthesis pathway, and this result can be integrated with data of elicitor-induced changes in related transcripts or enzyme-activity profile, thus enabling construction of gene (or enzyme)-to-metabolite networks for secondary metabolism, help to prompt the investigaton regulatory mechanisms of phenolic acids biosynthesis in *S. miltiorrhiza*.

Conclusion

For the unique pharmacological properties of *S. miltiorrhiza*, the successful quantitation of phenolic acids metabolic fluxes in this study, can be integrated from metabolomics and other functional genomics platforms, thus enabling large-scale and parallel interrogation of cell states under different stages of development and defining environmental conditions to uncover novel interactions among various pathways, ultimately to obtain a global picture of plant cellular responses.

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