RESEARCH ARTICLE

Effect of elicitors on the metabolites in the suspension cell culture of *Salvia miltiorrhiza* Bunge

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Abstract The effect of elicitors on the metabolites in the suspension cells of *Salvia miltiorrhiza* Bunge was elucidated by comparing and analyzing the metabolites of induced and uninduced suspension cells. The primary metabolites were detected by GC–MS. Twelve types of secondary metabolites, namely, shikimic acid, tanshinol, protocatechuic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone II A were detected by HPLC. Results indicated a total of 90 primary metabolites in the cells. These metabolites consisted of 49 kinds of sugars and their derivatives, 15 organic acids, 9 amino acids and their derivatives, 9 hydrocarbons, and 8 other substances. OPLS-DA results indicated five differential primary metabolites, namely, gluconic acid, mannopyranose, glucose, inositol, and ketoisovalerate, between the callus and suspension cells. SA significantly induced glucose metabolism in the *S. miltiorrhiza* suspension cells, and the mean contents of glucose, fructose, and mannose were significantly lower in the induced cells than in the control. However, SA, as a plant inducer, could significantly promote the increase in 10 secondary metabolites, except protocatechuic acid and tanshinone I, in the suspension cells at different times or degrees. The effect of NaCl on the *S. miltiorrhiza* cell mainly depended on the downstream pathway of glucose metabolism. Fructose and glucose were the decomposition products of sucrose, and glucose was processed through monosaccharide metabolism. Induction by NaCl resulted in significantly lower levels of these primary metabolites in the induced cells than in the control group. However, NaCl could significantly promote 10 secondary metabolites, except tanshinol and protocatechuic acid, at different times or degrees. After elicitation by AgNO$_3$, sucrose and proline were higher in the induced cells than in the control group. However, galactose and fructose were lower in the experimental cells than in the control. This phenomenon may have been caused by the induction of plant stress response by AgNO$_3$. AgNO$_3$ could significantly promote shikimic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA. This study will provide a reference for future studies on the effects of primary metabolites on the secondary metabolites and the potential relation of these metabolites to plant stress response in *S. miltiorrhiza*.

Abbreviations

SA	Salicylic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
6-BA	6-Benzylaminopurine
NAA	1-Naphthylacetic acid
PCA	Principal component analysis

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OPLS-DA	Orthogonal projection to latent structure with discriminant analysis
VIP	Variable importance in projection
PTFE	Poly tetra fluoroethylene

Introduction

Salvia miltiorrhiza Bunge, also known as Danshen in China, is a well-known medicinal plant, because its root is effective for relieving pain, removing blood stasis, blood circulation improvement, and treating cardiovascular and cerebrovascular diseases (China Pharmacopoeia 2005). Although *S. miltiorrhiza* and many medicinal plants are primarily cultivated in the field, plant tissue culture provide a more sustainable and well-controlled system for the efficient production of the desired bioactive compounds. Plant tissue and cell suspension cultures are the most useful and effective systems to study in vitro the biological significance of bioactive metabolites and explore effective measures to enhance the production of these herbs (Zhao et al. 2010). Tsutomu et al. (1983) induced several cell lines from plant seedlings and screened out a cell line that can produce significant amounts of cryptotanshinone and ferruginol, which are diterpenes. Wu et al. (2003) indicated that cryptotanshinone was significantly higher in the callus cultured in MS basal medium supplemented with 0.2 mg/L of BA than in the commercial crude drug (processed underground parts of *S. miltiorrhiza*).

Many studies have recently focused on treating *S. miltiorrhiza* tissue or cell suspension cultures with elicitors (Shi et al. 2007; Zhao et al. 2010; Dong et al. 2010). An elicitor is a special kind of trigger factor. This compound can open the metabolic process of enzymatic activity, which can increase the content of secondary metabolites and sometimes even induce new compounds. Elicitors can be classified as biological and abiotic elicitors, depending on their sources (Park et al. 2017). Dong et al. (2010) revealed that 6.25–22.25 mg/L salicylic acid (SA) could elicit the accumulation of phenolic compounds. Ag^+ and Co^{2+} may selectively promote the accumulation of tanshinone, while Fe^{2+} and Mn^{2+} can promote the accumulation of protocatechualdehyde (Zhao et al. 2010; Guo et al. 2005). Secondary metabolites are important sources for pharmaceuticals, food additives, flavors, and other industrial materials either as part of a final product or as a raw material (Zhao et al. 2005). Meanwhile, primary metabolites are also key components and play a crucial role in cell proliferation and differentiation (Cuadrosinostroza et al. 2016).

To our knowledge, the effect of elicitors on the primary metabolites in the tissue or suspension cell cultures of *S. miltiorrhiza* has not been reported. Thus, the differential primary metabolites between callus and suspension cells were investigated in this study. Moreover, the effects of elicitors, namely, SA, NaCl, and $AgNO_3$, on the primary and secondary metabolites in the cell suspension of *S. miltiorrhiza* were also investigated. This study will provide a reference for future studies on the effects of primary metabolites on secondary metabolites and the potential relation of these metabolites to plant stress response in *S. miltiorrhiza*.

Materials and methods

Plant material

Callus culture

Young leaves of *S. miltiorrhiza* were soaked in sterile distilled water for 10 min and then sterilized for 1 min with 75% ethanol. After rinsing with sterile distilled water, the leaves were soaked in 0.5% (m/v) NaCl solution for 5 min and rinsed five times with sterile distilled water. Then, the leaves were transferred to an MS basal medium (Murashige and Skoog 1962) supplemented with 0.5 mg/L 2,4-D, 1 mg/L kinetin, 30 g/L sucrose, 6 g/L gelrite, and 1 g/L acid hydrolyzed casein. The pH of the medium was adjusted to pH 6.0 with 1 mol/L NaOH before autoclaving. Each bottle of medium was placed with four explants. The callus grew after 20 days of culture at 27 ± 0.5 °C in the dark. Subculturing was performed five times at 20-day interval to maintain sufficient stock plants for experimentation. In the fifth-generation subculture, three bottles were randomly removed every 3 days for GC–MS detection. The calli were freeze-dried and ground to powder using a mortar and pestle. The powders were kept in a freezer at − 80 °C before use.

Suspension cell culture

Suspension cell cultures were initiated by subculturing five generations of stable callus (3.0 g each) into 100 mL of MS liquid media in 250 mL Erlenmeyer flasks. The media is the same as described above. The suspension cell culture was incubated at 27 ± 0.5 °C on a rotary shaker (Innova4900, NBS, USA, diameter 25 mm) at 120 rpm in the dark. This process was performed 5 times at 11-day intervals. In the fifth-generation subculture, 3 bottles were removed randomly every 3 days for GC–MS detection. The cells were freeze-dried and ground powder using a mortar and pestle. The powders were kept in a freezer at − 80 °C before use.

Suspension cell elicitation

The fifth-generation subcultured cells were transferred in MS liquid media (3 g of cells per bottle), and the cells after 18 days of culture were used for elicitation. The elicitors included 200 μmol/L SA, 50 mmol/L NaCl, and 60 μmol/L AgNO$_3$. The control group (CK) did not contain any elicitor. The subcultured cells were collected after 1, 3, 5, and 7 days of elicitation. The cells were freeze-dried and ground into powder using a mortar and pestle. Each treatment was repeated four times.

Primary metabolite extraction and analysis by GC–MS

The sample powder (0.1 g) was extracted with 4 mL of 70% aqueous methanol by sonication at room temperature (480 W, 40 kHz, 30 min, SZFEAT, China). The supernatants were collected by centrifugation at 10,000 rpm for 10 min, and the remaining solid residues were further extracted twice using the same procedure. The combined supernatant from the three extractions were freeze-dried, mixed with 100 μL of methoxamine pyridine solution (20 mg/L), and incubated at 37 °C for 90 min. Then, 100 μL of the derivatization reagent BSTFA (containing 1% TMCS) was added, and the solution was incubated at 70 °C for 60 min. Finally, the samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was transferred into a 1.5-mL sample vial for GC–MS analysis.

The sample (1 μL) was injected into the GC–MS (Agilent 7890A GC/5975C MS, Agilent, USA) system for analysis. An HP-5MS capillary column (30 m × 250 μm × 0.25 μm, Agilent, USA) and quadrupole detector were utilized. The temperature of the column was varied as follows: 70 °C, which was held for 3 min; increased to 140 °C at 10 °C/min; to 160 °C at 1 °C/min; and to 230 °C at 10 °C/min; maintained at 230 °C for 30 min; increased to 280 °C at 15 °C/min; and finally maintained at 280 °C for 10 min. The mass spectrometer conditions were as follows: EI ionization mode; electron energy, 70 eV; scan 15–800 m/z; full scan mode; ion source temperature, 250 °C; quadrupole temperature, 200 °C; and solvent delay, 5 min.

Secondary metabolite extraction and analysis by HPLC

Preparation of sample and standard solutions

The sample powder (0.1 g) was extracted with 15 mL of 70% aqueous methanol by sonication at room temperature (300 W, 30 kHz, 15 min). After concentration of the solution, it was diluted to a volume of 50 mL with deionized water. For HPLC, a moiety of the solution was filtered through a 0.22 μm PTFE membrane filter, and 10 μL of the filtrate was injected into the HPLC system (Agilent 1200, Agilent, USA).

Standard solutions of the secondary metabolites were prepared at seven concentration levels by diluting with methanol. The concentration ranges were as follows: shikimic acid, 0.5–50 μg/mL; tanshinol, 0.2–9 μg/mL; protocatechuic acid, 0.01–0.25 μg/mL; caffeic acid, 0.1–5 μg/mL; p-coumaric acid, 0.002–0.5 μg/mL; rosmarinic acid, 4.8–246 μg/mL; salvianolic acid B, 4.3–390 μg/mL; salvianolic acid A, 0.02–5.27 μg/mL; dihydrotanshinone, 0.21–20 μg/mL; cryptotanshinone, 1.1–20 μg/mL; tanshinone I, 0.37–5.86 μg/mL; and tanshinone IIA, 2–30 μg/mL.

Equipment and chromatographic conditions

All analyses were performed on the HPLC system equipped with a dual-pump solvent delivery system, an online degasser, an auto-sampler, a column temperature controller, and an ultraviolet detector. Chromatographic separation was conducted on the Agilent Poroshll 120 EC-C18 column (4.6 mm × 100 mm, 2.7 μm, Agilent, USA) coupled with an Agilent Eclipse XDB-C18 guard column (4.6 mm × 12.5 mm, 5 μm, Agilent, USA). The detection was performed at 215 nm, the flow rate was 1 mL/min, and the column temperature was maintained at 30 °C. The mobile phase was in gradient elution, which consisted of solvents A (0.1% aqueous phosphoric acid, v/v) and B (acetonitrile). The gradient program was optimized and conducted as follows: 0–27 min, 5–35% B; 27–32 min, 35–50% B; 32–52 min, 50–60% B; 52–56 min, 60–5% B; 56–60 min, 5% B.

Data analysis

GC/MSD ChemStation Software (Agilent) was used to collect data and generate the GC total ion chromatogram and mass spectra of metabolites. All detected peaks were identified by comparing the MS spectra with those available in the NIST mass spectral library (Wiley Registry, 2008 edition) and customized reference mass spectral libraries (Song et al. 2012). Only the retention time of compounds with a matching probability of more than 80% were examined, and m/z data pairs were used as identifier for each peak within each sample. The area normalization was used for quantitative analysis, and 5 μg/mL ribitol was used as internal standard (Chen et al. 2014).

The normalized data were exported to SIMCA-P V11.0 (Umetrics AB, Umea, Sweden) to perform principal component analysis (PCA) and orthogonal projection to latent structure with discriminant analysis (OPLS-DA) (Trygg

and Wold 2002). PCA was performed with variance scaling (UV), in which each variable was divided by the deviation of the variance. This process can eliminate the influence of the variable response to a certain extent. According to metabolomics, UV scaling is used to obtain more reliable and more intuitive results before sample classification. The quality of the model was described by cross-validation Q^2 and R^2X, and these variables represent the total explained variables for the X matrix. In the PCA model, $R^2X > 0.4$ indicates that the model is reliable, while in the OPLS-DA models, $Q^2 > 0.4$ indicates that the model is reliable. Differential metabolites were determined by combining the variable importance in the projection of the first preliminary component in the OPLS-DA model with p value (threshold 0.05) in Student's t test. The data of the secondary metabolites were analyzed by Microsoft Excel 2013.

Results

Comparison of primary metabolites in the callus and suspension cells

The primary metabolites in the callus and cell suspension of *S. miltiorrhiza* were detected by GC–MS. The metabolite qualitative results were obtained from the NIST database support. The results indicated that the callus and cell suspension had basically identical types and numbers of metabolites (Figure S1, Table S1). A total of 63 different metabolites were found in the calli. These metabolites included 36 kinds of sugars and their derivatives, 11 organic acids, 8 amino acids, 4 hydrocarbons, and 4 unknown substances. By contrast, 65 kinds of metabolites were found in the suspension cells. These metabolites included 37 kinds of sugars and their derivatives, 12 organic acids, 8 amino acids, 4 hydrocarbons, and 4 unknown substances. The uniformity in the metabolite levels of the calli and suspension cells also suggested that the metabolites were regulated by genes not by culture conditions and cell state.

The GC–MS data were subjected to PCA, which is an unsupervised clustering method that reduces the dimensionality of multivariate data while preserving most of the variance (Eriksson et al. 2001). Two principal components were obtained, and the cumulative R^2X and Q^2 were 0.835 and 0.722, respectively. These values indicated the reliability of the model (Fig. 1a). A clear separation could be observed in the PCA score plot during the prophase of growth, that is, at 0, 3, and 6 days, in the calli and suspension cells. However, on day 9, the calli and suspension cells began to aggregate, and these cells overlapped for the next 12–24 days and could not be distinguished. The

findings indicated that the different methods of cell culture of *S. miltiorrhiza* resulted in varied contents of metabolites in the early stages. However, with prolonged culture time, the two kinds of cells gradually entered from the active state division into a stable state, and the intracellular components converged.

OPLS-DA, which is a supervised method, was used in this study to obtain the differential metabolites between callus and suspension cells. One principal component and one orthogonal component were obtained in the OPLS-DA model. The cumulative R^2X, R^2Y, and Q^2 were 0.463, 0.697, and 0.607, respectively, indicating the reliability of the model (Fig. 1b). OPLS-DA and PCA analysis showed similar results. A clear separation could be observed during the prophase of growth, that is, at 0, 3, and 6 days, in calli and suspension cells. However, on day 9, the calli and suspension cells began to aggregate. In the following 12–24 days, the two types of cells overlapped and could not be distinguished. The differential metabolites are listed in Table 1. Five differential metabolites, namely, gluconic acid, mannopyranose, glucose, inositol, and ketoisovalerate, were found between the calli and suspension cells. Gluconic acid, inositol, and ketoisovalerate had higher mean concentrations in the suspension cells than in the calli. Meanwhile, mannopyranose and glucose had higher mean concentration in the calli (Table 1).

Comparison of primary metabolites in the suspension cells under treatment with different elicitors

Qualitative analysis of primary metabolites under treatment with different elicitors

The primary metabolites of *S. miltiorrhiza* suspension cells under different elicitor treatments were detected by GC–MS, and the qualitative results of these metabolites were obtained (Figure S2, Table S1). The results showed that the number and types of the primary metabolites were lower in the suspension cells treated with elicitors than in the control groups. A total of 45 kinds of primary metabolites in the suspension cells were elicited by SA. These primary metabolites included 29 kinds of sugars and their derivatives, 6 organic acids, 4 amino acids, 3 hydrocarbons, and 3 unknown substances. By contrast, 40 primary metabolites were detected in the suspension cells under NaCl induction. These primary metabolites included 24 kinds of sugars and their derivatives, 6 organic acids, 2 amino acids, 2 hydrocarbons, and 6 other substances. Meanwhile, 40 primary metabolites elicited by $AgNO_3$ were detected in the suspension cells. These metabolites included 21 kinds of sugars and their derivatives, 10 organic acids, 3 amino acids, 3 hydrocarbons, and 3 other substances.
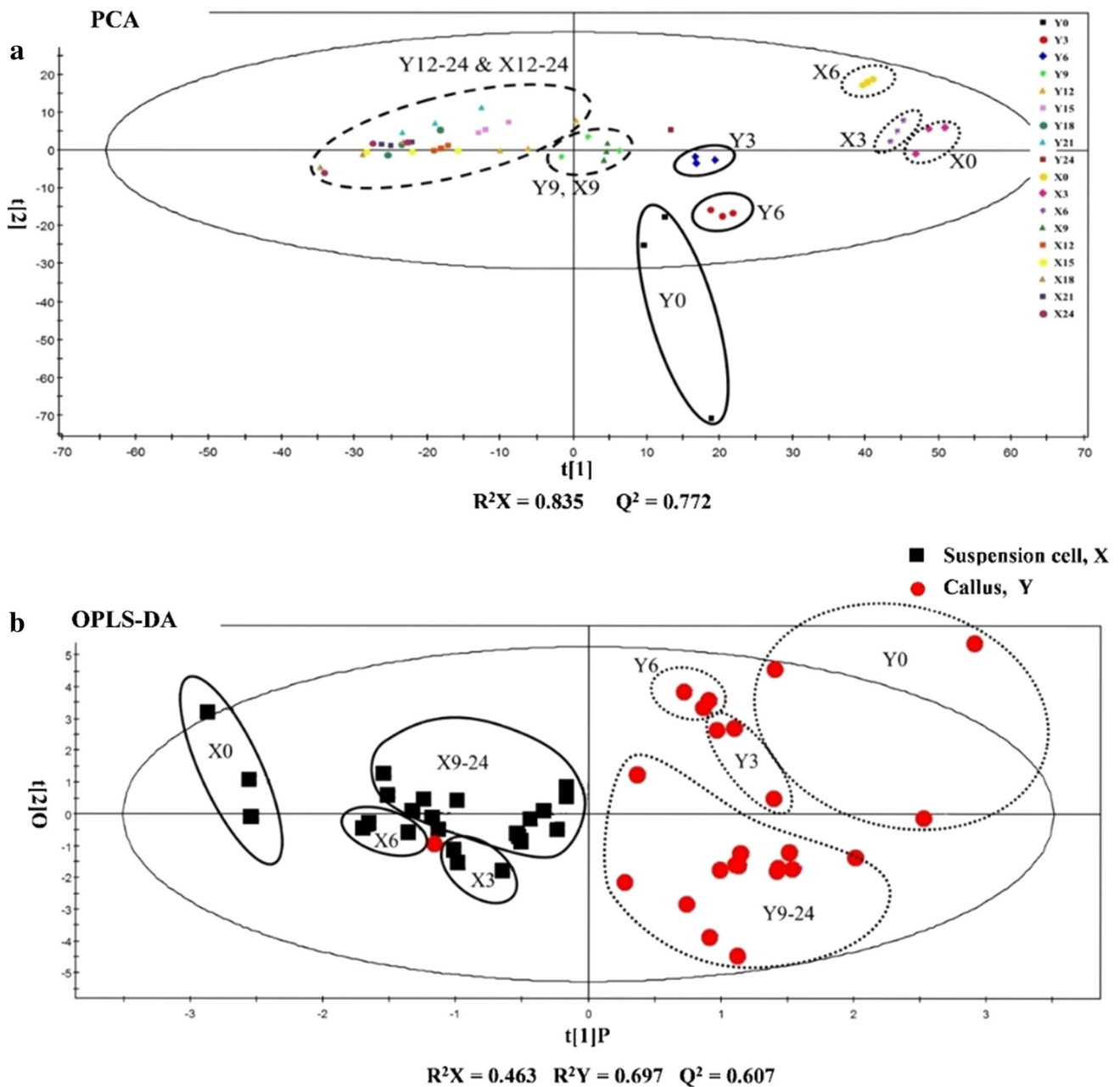
Fig. 1 PCA and OPLS-DA of primary metabolites in callus and suspension cell. **a** PCA analysis. **b** OPLS-DA analysis

Effect of SA on the suspension cells

The PCA analysis of metabolites in the suspension cells elicited by SA and the control group indicated two principal components. The cumulative R^2X and Q^2 were 0.576 and 0.0526, respectively. These results indicated that the model was reliable (Fig. 2a). The PCA score plot shows that the SA-induced cells and control group could be clearly distinguished but not in accordance with the culture time to perform further distinction. This phenomenon indicated that the SA-induced intracellular physiological activities and the product differed from those of the normal growth of the cells.

OPLS-DA was used to obtain the differential metabolites between the SA-induced cells and the control group. One principal component and one orthogonal component were obtained in the OPLS-DA model, and the cumulative R^2X, R^2Y, and Q^2 were 0.468, 0.636 and 0.47, respectively. These results indicated that the model was reliable (Fig. 2b). OPLS-DA and PCA showed that the sample could not be separated in accordance with the culture time. The differential metabolites are listed in Table 2. The

Table 1 Differential primary metabolites between callus and suspension cells

Metabolites	VIP	p	Fold change (X/Y)
Gluconic acid	1.83296	0.000	1.556
Mannopyranose	1.47404	0.002	− 3.061
Glucose	1.29874	0.007	− 2.296
Inositol	1.27919	0.008	1.987
Ketoisovalerate	1.11175	0.022	0.768

The value of the ratio of the mean difference in multiples of suspension cells (X) and callus (Y) (base 2), a positive sign indicates that the group with respect to the cell suspension callus set to rise, the negative sign indicates decline

differential metabolites were sugars and their derivatives, indicating that SA significantly induced glucose metabolism in *S. miltiorrhiza* suspension cells. The mean contents of glucose, fructose, and mannose were significantly lower in the cells after induction than in the control.

Effect of NaCl on the suspension cells

Four principal components were obtained after PCA of the metabolites in the suspension cells elicited by NaCl and the control group. The cumulative R^2X and Q^2 were 0.988 and 0.678, respectively (Fig. 3a). The PCA score plot shows that NaCl-induced cells and control group could be clearly separated. In the control cells, the 1st and 3rd day cells clustered together, while those on day 5 clustered together. The cells on day 7 also clustered together. However, the cells induced by NaCl could not be separated in accordance with the culture time. The results were similar to those induced by SA.

One principal component and one orthogonal component were obtained in the OPLS-DA model. The cumulative R^2X, R^2Y, and Q^2 were 0.826, 0.716, and 0.674, respectively (Fig. 3b). The results of OPLS-DA analysis were similar to those of PCA. The differential metabolites are listed in Table 3. The differential metabolites under the induction of NaCl were monosaccharides. This finding showed that the effect of NaCl on *S. miltiorrhiza* cell mainly depended on the downstream pathway of glucose metabolism. Fructose and glucose are the decomposition products of sucrose, and glucose is derived from monosaccharide metabolism. These three kinds of sugar were significantly lower in the cells after induction by NaCl than in the control group.

Effect of AgNO₃ on the suspension cells

Two principal components were obtained after the PCA of the metabolites in the suspension cells elicited by AgNO₃

and the control group. The cumulative R^2X and Q^2 were 0.708 and 0.221, respectively (Fig. 4a). The PCA score plot shows that AgNO₃-induced cells and control group could be clearly separated. The suspension cells of *S. miltiorrhiza* induced by AgNO₃ could be further differentiated according to culture time but not completely separated. This phenomenon indicated that the cells were consistent at the metabolite level at different induction times, and that the induction effect of AgNO₃ did not have a strong sustained effect.

One principal component and one orthogonal component were obtained in the OPLS-DA model. The cumulative R^2X, R^2Y, and Q^2 were 0.572, 0.703 and 0.567, respectively (Fig. 4b). The results of OPLS-DA analysis were similar to those of PCA. The differential metabolites are listed in Table 4. The differential metabolites contained sucrose, galactose, fructose, and proline. Sucrose is the only source of carbon and energy in the cell suspension culture. This sugar is also the most absorbed nutrient by cells. The results showed that AgNO₃ induction had an important impact on the physiological metabolism of cells. At the same time, the increase in proline content also indicated that the cells were affected by AgNO₃, and this effect was often negative.

Comparison of secondary metabolites in the suspension cells under treatment with different elicitors

Effect of SA on the suspension cells

The dynamic changes in the secondary metabolites in the suspension cells induced by SA are summarized in Fig. 5. The results showed that the contents of the 12 secondary metabolites changed in varying degrees with prolonged culture time. Shikimic acid, salvianolic acid A, and dihydrotanshinone showed similar variations, and these three substances were significantly higher on days 3 and 7 than in the control. Tanshinol content decreased continuously after SA treatment and was significantly lower than in the control. The dynamic changes in protocatechuic acid, caffeic acid, rosmarinic acid, salvianolic acid B, cryptotanshinone, and tanshinone IIA in the suspension cells after SA treatment showed an increasing trend. Cryptotanshinone content was 4.39 times higher than that of the control after SA induction. *p*-Coumaric acid and tanshinone I contents in the suspension cells decreased. The *p*-coumaric acid content was significantly lower on day 7 after SA treatment than in the control, while tanshinone I was higher than in the control.
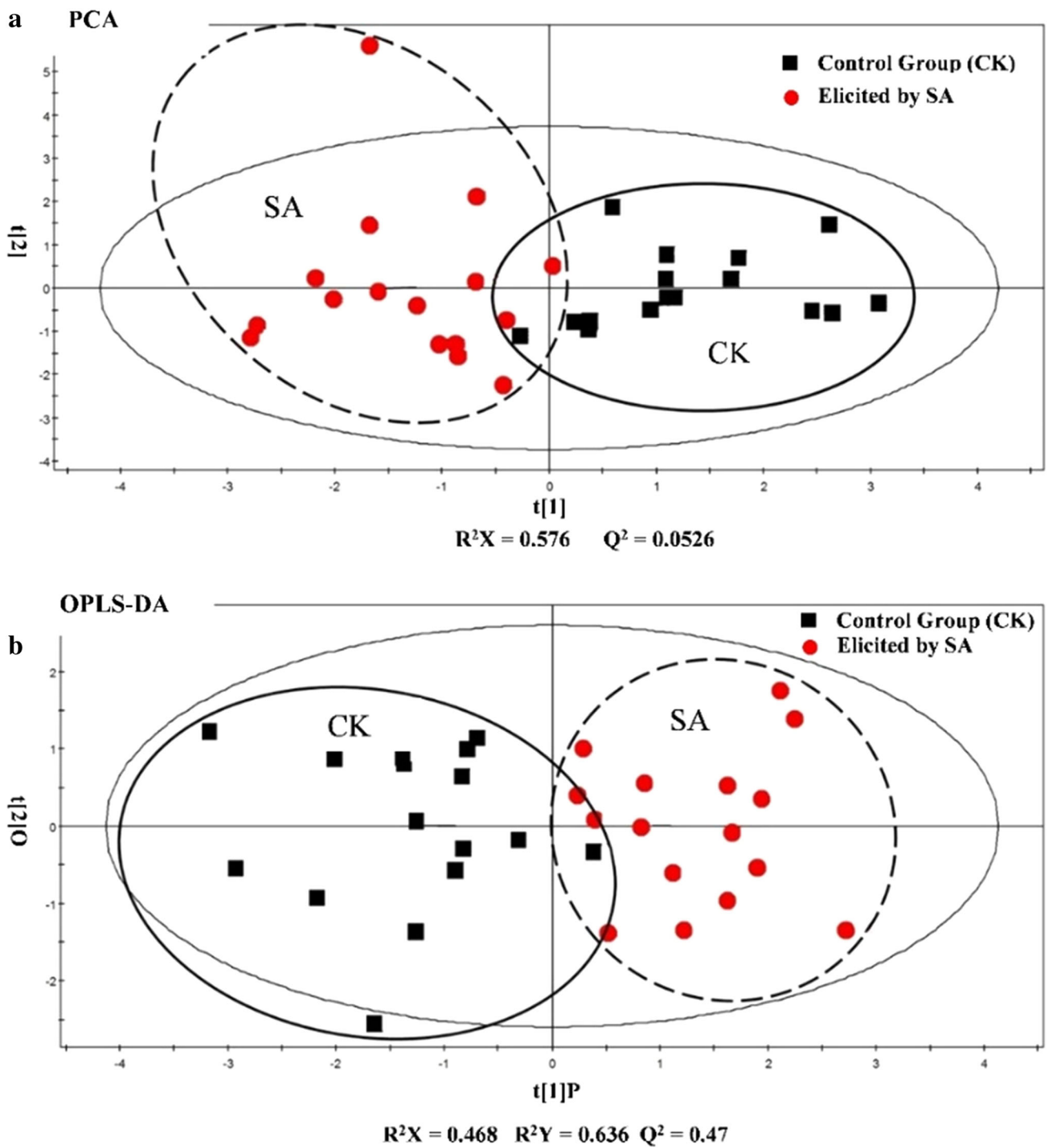
Fig. 2 PCA and OPLS-DA of primary metabolites in suspension cells elicited by SA. **a** PCA analysis. **b** OPLS-DA analysis

Effect of NaCl on the suspension cells

The contents of the 12 secondary metabolites in the suspension cells after NaCl treatment varied with prolonged culture time (Fig. 6). Shikimic acid, caffeic acid, and *p*-coumaric acid showed similar variations. These substances initially increased and then decreased. These substances were significantly higher in the suspension cells on day 3 than in the control but significantly lower on day 7 than in the control. Tanshinol and rosmarinic acid increased after NaCl treatment. Rosmarinic acid was 9.08 times higher in the suspension cells on day 7 after NaCl treatment than in the control. Protocatechuic acid, salvianolic acid B, and salvianolic acid A first decreased and then increased, while

Table 2 Differential primary metabolites in suspension cells elicited by SA and in CK

Metabolites	VIP	p	Fold change (SA/CK)
α-D-mannopyranoside	1.459	0.001	1.218
Glucose	1.412	0.001	− 0.312
Fructose	1.305	0.004	− 0.285
Mannose	1.215	0.007	− 0.569

Fold change values than SA induction and control of the means of the (base 2), a positive sign indicates that the group with respect to the cell suspension callus set to rise, the negative sign indicates decline

salvianolic acid B and salvianolic acid A were significantly higher in the suspension cells during the late stage of treatment (5–7 days) than in the control group. The other four secondary metabolites showed a generally decreasing trend. Dihydrotanshinone, cryptotanshinone, and tanshinone IIA were lower in the suspension cells after NaCl treatment compared with the control group.

Effect of AgNO₃ on suspension cells

The contents of the 12 secondary metabolites in suspension cells after AgNO₃ treatment showed varying degrees with prolonged culture time (Fig. 7). The concentration of shikimic acid, caffeic acid, p-coumaric acid and tanshinone I decreased in the suspension cells treated with AgNO₃, and these substances were significantly lower than those of the control on day 7 after treatment. Shikimic acid decreased by 10.07 times lower than that of the control. Tanshinol and dihydrotanshinone showed similar variations in the suspension cells. These components decreased first and then increased after AgNO₃ treatment, and dihydrotanshinone was significantly higher than that of control on day 7 after treatment. Protocatechuic acid and cryptotanshinone increased after AgNO₃ treatment. Cryptotanshinone was 18.07 times higher than that of the control on day 7 after treatment. Rosmarinic acid and salvianolic acid A changed in the suspension cells after AgNO₃ treatment. Both metabolites increased first and then decreased. Rosmarinic acid was always lower in the treatment group than in the control group. Salvianolic acid A was higher in the treatment group than in the control group on days 1–5. Salvianolic acid B initially increased, then decreased, and finally stabilized.

Discussion

The primary metabolites in the calli, cell suspension, and elicited cell suspension of *S. miltiorrhiza* were detected by GC–MS. A total of 90 metabolites, including 49 kinds of sugars and their derivatives, 15 organic acids, 9 amino

acids and their derivatives, 9 hydrocarbons, and 8 other substances, were detected (Table S1). These results showed that metabolome analysis based on GC–MS detected mainly sugars, organic and amino acids, and other primary metabolites. These results were also observed in other plants (Tarpley et al. 2005; Desbrosses et al. 2005). However, the primary metabolites are the key components, and they play a crucial role in cell proliferation and differentiation (Cuadrosinostroza et al. 2016). Similar to the chaliana T87 cell line, the sugars and their derivatives in this study accounted for 54.44% of all metabolites (Fukusaki et al. 2006). This phenomenon may be due to the fact that the plant cells were independent of each other and could not differentiate into tissues and organs under the shear force of the liquid medium. Moreover, the cells were in heterotrophic state and had a short growth cycle. The physiological activities of the cells were reduced, except for the primary metabolic activities, such as glycolysis, carboxylic acid cycle, and amino acid metabolism. The biosynthesis of the other secondary metabolites was affected to varying degrees. This phenomenon also explains the lesser bioactive components in the cell cultures of *S. miltiorrhiza* than those grown in the field.

The end products of cellular regulatory processes result in metabolites, which increase or decrease their level depending on the enviromental changes and biological conditions (Fiehn 2002). In the induction of *S. miltiorrhiza* suspension cells, the elicitors SA, NaCl, and AgNO₃ could elicit differential metabolites. These metabolites could be used as biomarkers. *S. miltiorrhiza* suspension cells induced by different elicitors showed 12 kinds of differential primary metabolites, including 7 monosaccharides (fructose, galactose, mannose, glucose, mannopyranose, and gulose), 1 disaccharide (sucrose), 2 carbohydrate derivatives (gluconic acid and alpha-D-mannan glucoside), 1 amino acid (proline), and 1 amino acid metabolic intermediate (ketoisovaleric acid). The differential metabolites were mainly carbohydrates when the suspension cells were induced by elicitors. This phenomenon may be due to three factors. First, as one of the basic metabolism in plants, carbohydrate metabolism is involved in many important physiological processes, such as photosynthesis and respiration. Numerous studies have shown that carbohydrates could accumulate as osmotic adjustment substances under salt stress or induction. Therefore, the metabolism level of sugars in plants is also used to measure the effects of stress or induction on plants (Parida and Das 2005). Second, an increase in the carbohydrate content in plants influence the osmotic adjustment of cells. Many carbohydrates are the signal substances for plants to adapt to the environment (Shen et al. 1999). Finally, during suspension cell culture, sucrose is the only energy and carbon source for cell growth, that is, the metabolic activities of cells mainly
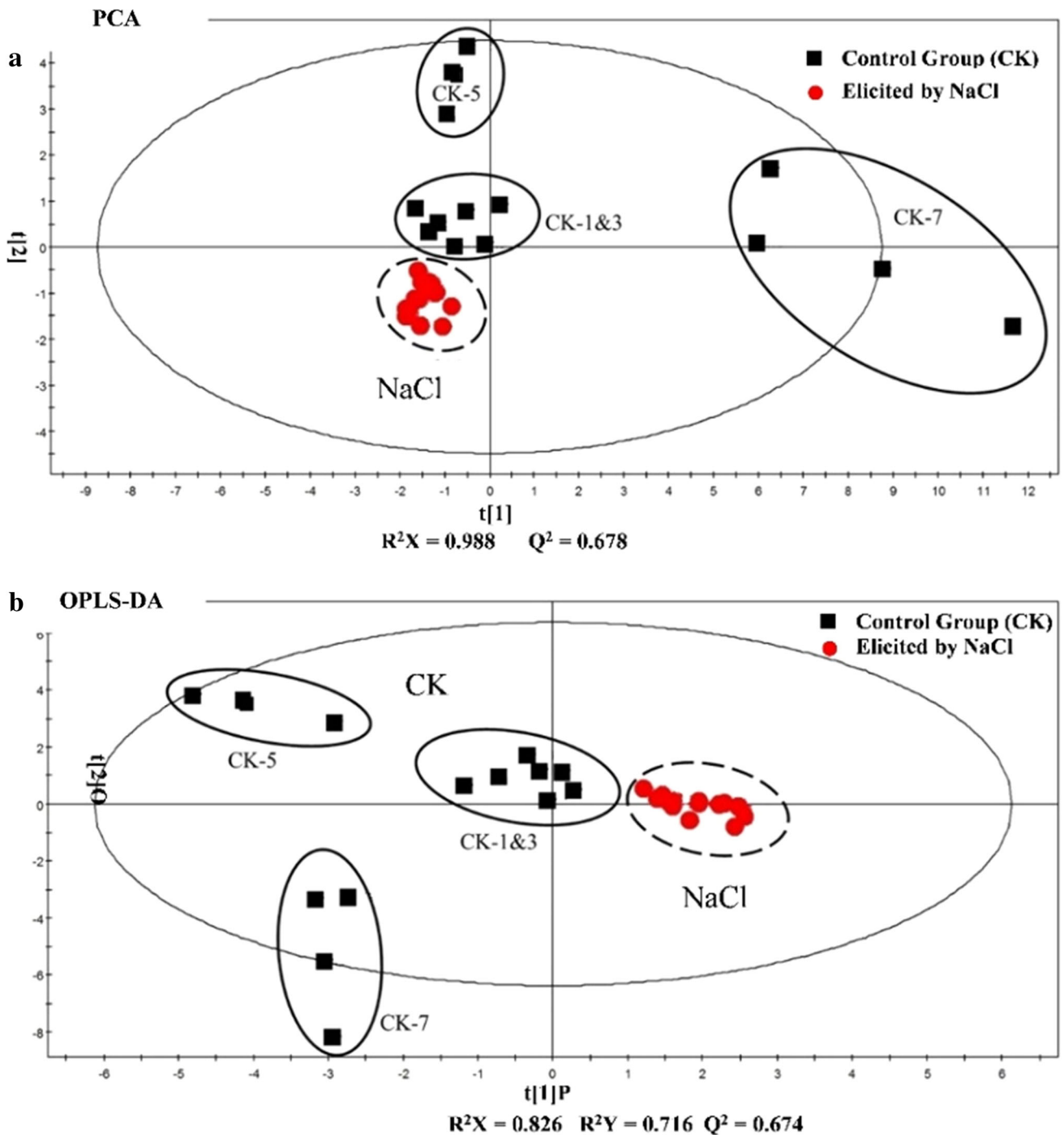
Fig. 3 PCA and OPLS-DA of primary metabolites in suspension cells elicited by NaCl. **a** PCA analysis. **b** OPLS-DA analysis

provide glycolysis with energy and metabolic products for mitosis and normal life. Wang et al. (2012) indicated that the change in phenolic acid content in roots was consistent with the variation in reducing sugar (mainly glucose and fructose) after SA induction. However, in the present study, glucose and fructose were significantly reduced after induction of SA, compared with the control, but the content of phenolic acid increased. This result was not consistent with that obtained by Wang et al. The difference may have been caused by the distinct materials used in the studies. Wang et al. used *S. miltiorrhiza* cultivated in the field, but we used suspension cells of *S. miltiorrhiza* in this study.

Before AgNO₃ induction, the concentration of AgNO₃ was first screened, and the concentration ensured the

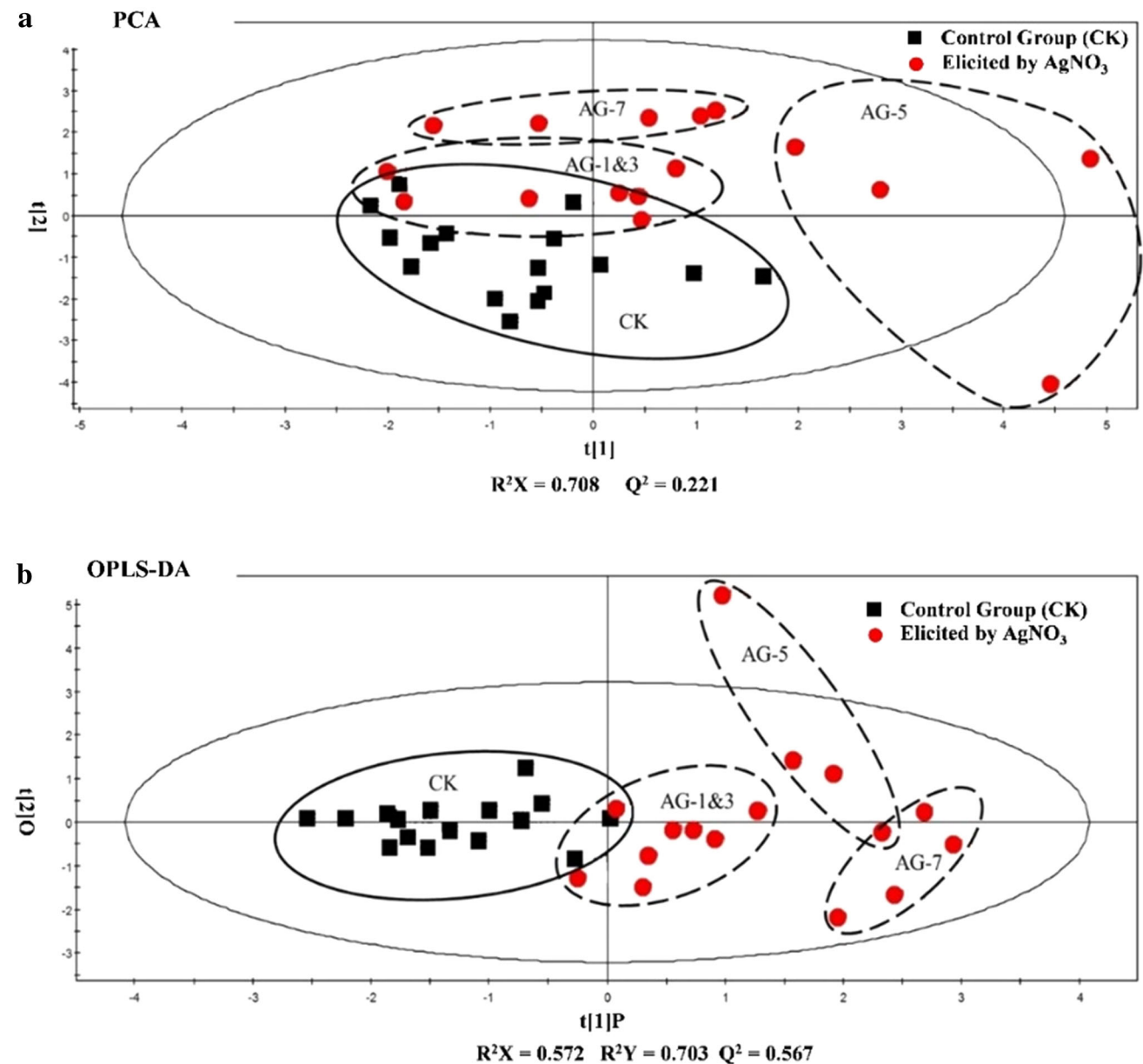**Table 3** Differential primary metabolites in suspension cells elicited by NaCl and in CK

Metabolites	VIP	p	Fold change (NaCl/CK)
Mannose	1.178	0.01	− 3.083
Glucose	1.131	0.02	− 3.065
Gulose	1.042	0.03	− 2.053

Fold change values than the NaCl-induced and control of the means of the (base 2), a positive sign indicates that the group with respect to the cell suspension callus set to rise, the negative sign indicates decline

normal growth and proliferation of the suspension cells. Sucrose and proline significantly increased in the suspension cells after AgNO₃ induction. Previous study showed that sucrose plays an important role in maintaining the membrane phospholipids in the liquid-crystalline phase and preventing the structural changes of soluble proteins during plant adaptation to stress (Kerepesi and Galiba 2000). Proline, which is a soluble osmotic adjustment substance, also contributes to the stability of cell membrane under stress (Mansour 1998). The results showed that, under AgNO₃, the suspension cell could adapt to the stress environment by regulating the primary metabolites (mainly sucrose and proline), which facilitate normal cell growth and proliferation.

In addition to primary metabolites, 12 secondary metabolites, namely, shikimic acid, tanshinol,

Fig. 4 PCA and OPLS-DA of primary metabolites in suspension cells elicited by AgNO₃. **a** PCA analysis. **b** OPLS-DA analysis

Table 4 Differential primary metabolites in suspension cells elicited by AgNO₃ and in CK

| Metabolites	VIP	p	Fold change (AgNO₃/CK)
Galactose	1.478	0.000	− 0.523
Sucrose	1.396	0.000	1.359
Fructose	1.346	0.000	− 0.480
Proline	1.019	0.006	1.843

Fold change values of AgNO₃ on induction and control of the ratio of the mean (base 2), a positive sign indicates that the group with respect to the cell suspension callus set to rise, the negative sign indicates decline

protocatechuic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA were also detected by HPLC in the elicited suspension cell of *S. miltiorrhiza*. SA, as a plant inducer,

has successfully increased the content of the secondary metabolites in many plant cell cultures, such as *S. miltiorrhiza* (Yan et al. 2006). The results of this study indicated that SA could significantly promote the increase of 10 secondary metabolites, except protocatechuic acid and tanshinone I, in the suspension cells at different times with varying degrees. Therefore, adding the appropriate concentration of SA to the cell culture of *S. miltiorrhiza* could increase phenolic acids and tanshinones. NaCl, as a common plant osmotic stress substance, has been extensively studied (Bekheet et al. 2006). However, few studies has been conducted on the improvement of secondary metabolites of plants using NaCl as elicitors. In this paper, the results showed that NaCl could significantly promote 10 secondary metabolites, except tanshinol and protocatechuic acid, at different times and with varying degrees. Although NaCl did not participate in intracellular
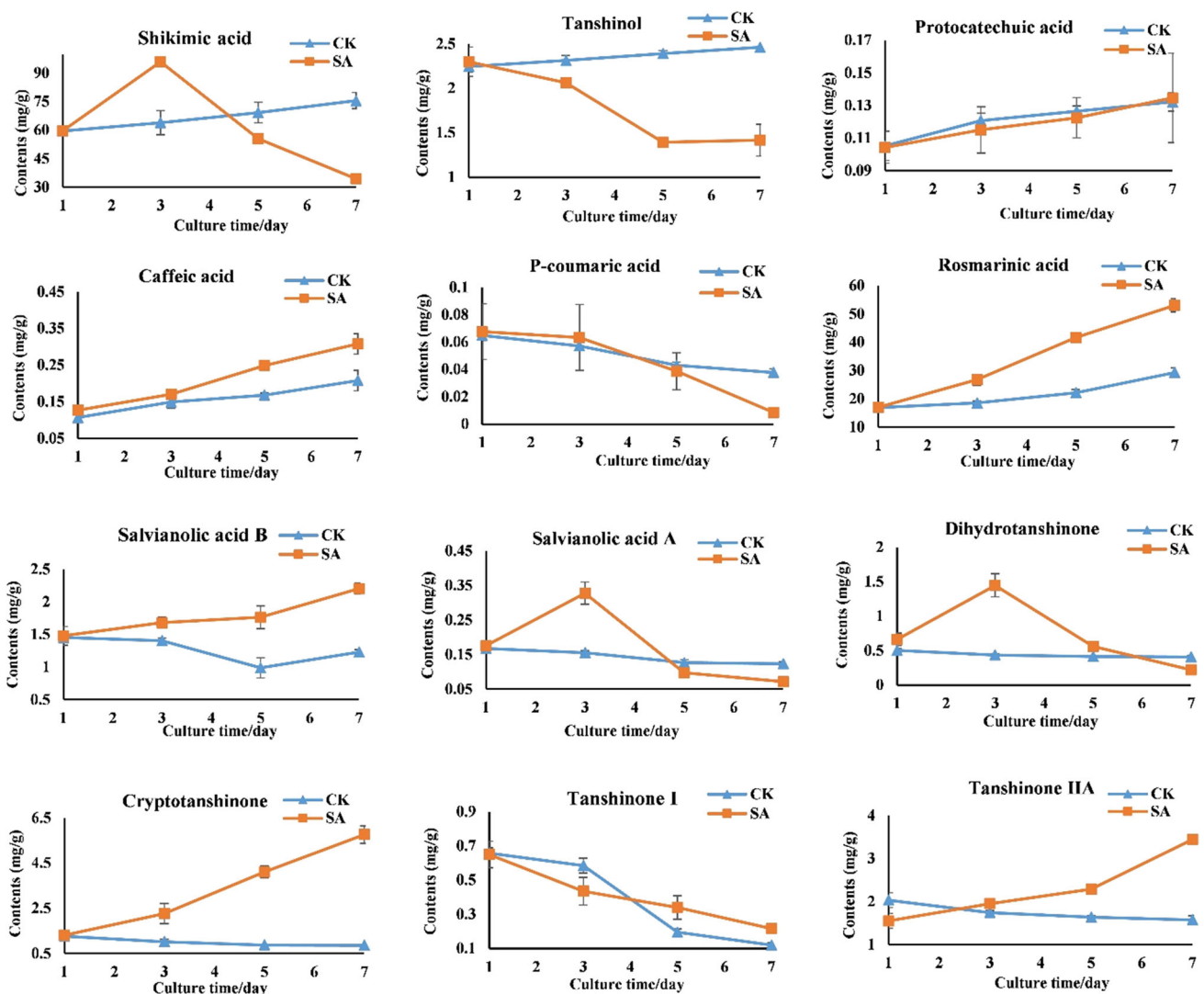
Fig. 5 Effects of SA elicitor on the 12 secondary metabolites of the *S. miltiorrhiza* suspension cells. Each value is the mean of triplicate (± SD)
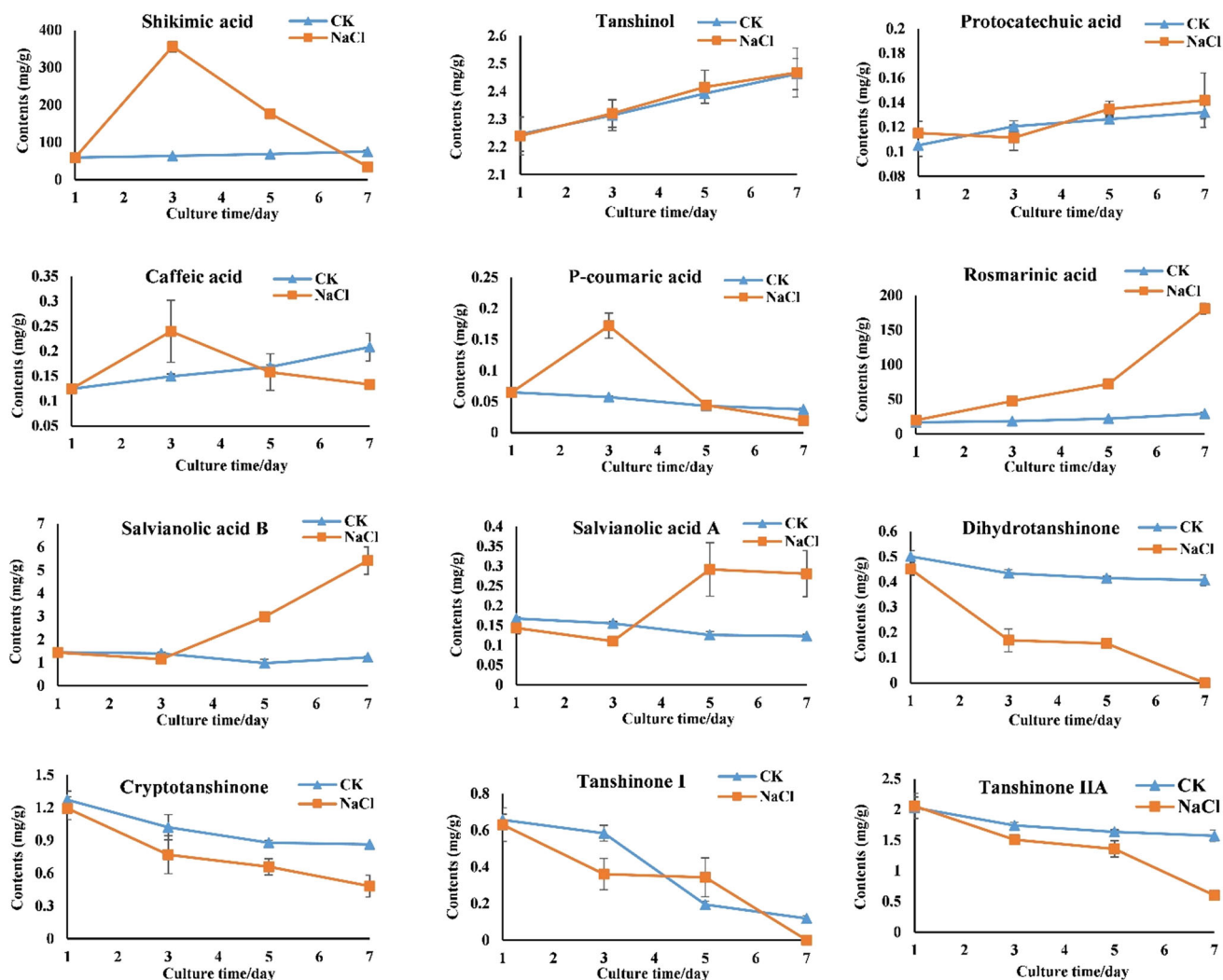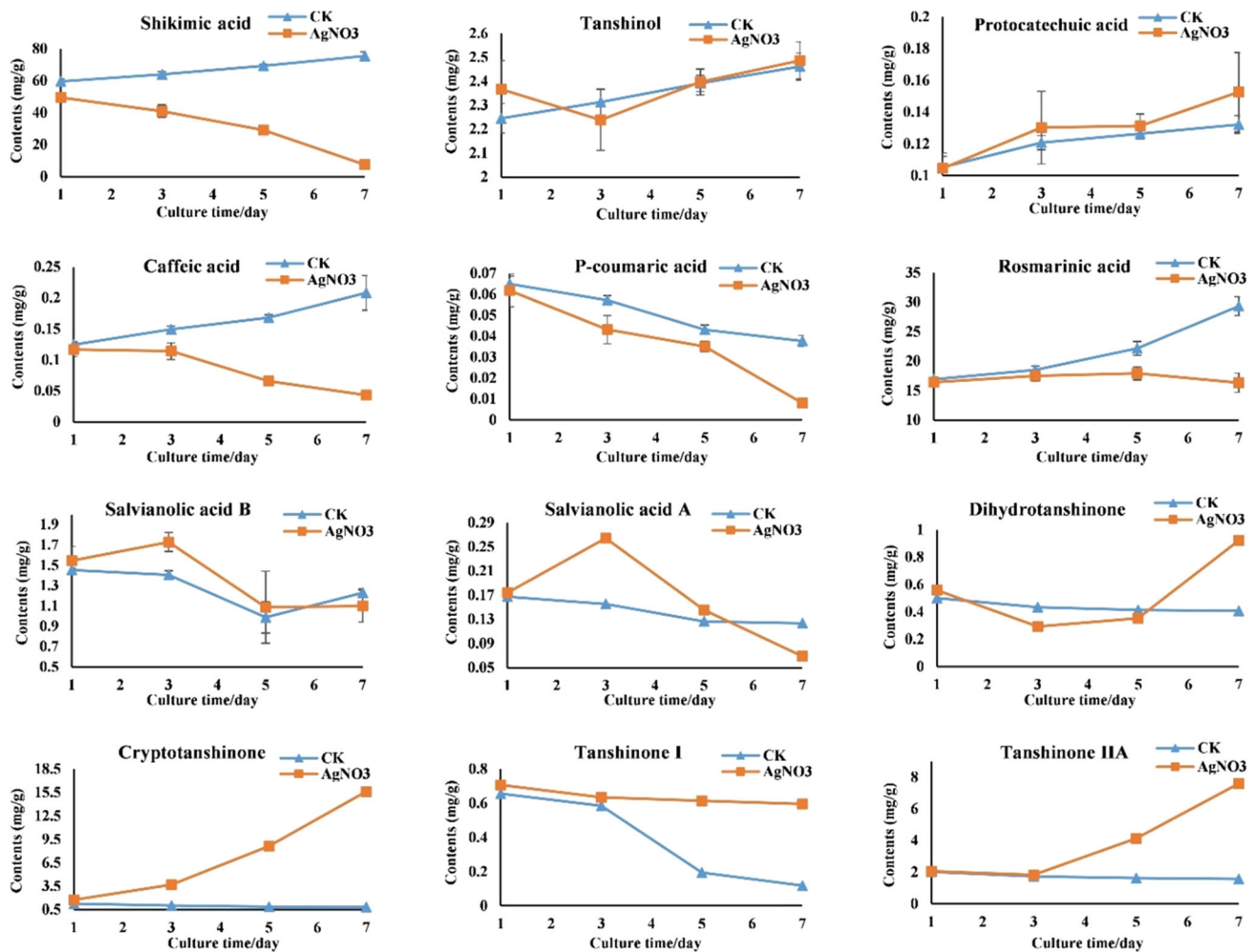
Fig. 6 Effects of NaCl elicitor on the 12 secondary metabolites of the *S. miltiorrhiza* suspension cells. Each value is the mean of triplicate (± SD)

secondary metabolism, nitric oxide increased sharply in the cells after NaCl stress. Nitric oxide is an important signaling molecule that is widely present in plant cells to trigger plant defense response (Fatma et al. 2016). This phenomenon may be one of the reasons for NaCl to cause increase in the content of secondary metabolites in the suspension cells of *S. miltiorrhiza*. Previous studies showed that AgNO$_3$ promoted the key enzymes of DXS and HMGR pathway in the terpenoid biosynthesis of *S. miltiorrhiza* and thus increased the accumulation of tanshinones in *S. miltiorrhiza* hairy roots (Ge and Wu 2005). AgNO$_3$ could significantly promote shikimic acid, caffeic acid, *p*-coumaric acid, rosmarinic acid, salvianolic acid B, salvianolic acid A, dihydrotanshinone, cryptotanshinone, tanshinone I, and tanshinone IIA. However, the mechanism of AgNO$_3$, which is a physiological toxic heavy metal ion

pair, in improving the secondary metabolites of plants is still unclear. Therefore, we will further study the mechanism of AgNO$_3$ in the secondary metabolite synthesis pathway of *S. miltiorrhiza*.

Conclusion

The elicitors SA, NaCl, and AgNO$_3$ significantly affected the quantity and quality of the primary metabolites in the calli and suspension cells of *S. miltiorrhiza*. This study will provide a reference for future studies on the potential relation of these primary metabolites to plant stress response in *S. miltiorrhiza*. In addition, the contents of the secondary metabolites in the suspension cells of *S. miltiorrhiza* were also affected by these elicitors. Therefore, the

Fig. 7 Effects of AgNO₃ elicitor on the 12 secondary metabolites of the *S. miltiorrhiza* suspension cells. Each value is the mean of triplicate (± SD)

secondary metabolites can be increased by adding the suitable elicitors in the suspension cell cultures according to the experimental purpose.

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