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iTRAQ analysis of low-phytate mung bean sprouts treated with sodium citrate, sodium acetate and sodium tartrate

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ABSTRACT

The effects of sodium citrate (SC), sodium acetate (SA) and sodium tartrate (ST) spraying on mung bean germination were investigated. Exogenous SC, ST and SA treatments significantly reduced the phytic acid content and increased the antioxidant enzyme activities. In this study, an iTRAQ-based proteomic approach was employed to explore the proteomes of mung bean sprouts, and 81, 101 and 90 differentially expressed proteins were identified in 4-day-old SC-, SA- and ST-treated mung bean sprouts, with 38 proteins present in all samples. Functional classification analysis showed that most of the differentially expressed proteins in mung bean sprouts subjected to the three treatments were involved in carbohydrate and energy metabolism. The inhibitory effect of the SA treatment was probably due to impairments in protein biosynthesis, whereas enhanced energy metabolism, accelerated reserve hydrolysis and protein processing were very important strategies for growth stimulation in response to ST and SC treatments.

1. Introduction

Mung beans are consumed in large quantities throughout the world (Fery, 2002). During germination, the decomposition of mung bean reserves, such as proteins and polysaccharides, contributes to the formation of biofunctional compounds. Meanwhile, as an anti-nutrient, phytic acid is degraded during mung bean sprouting, thus increasing the nutritive values of the sprouts (Vayupharp, 2013). In addition, the process of sprouting is very simple and does not require sunlight nor soil and is not limited by seasonal factors (Jom, Frank, & Engel, 2011). In addition, germination has positive enhancement effects on antioxidant compounds in mung bean sprouts (Guo, Li, Tang, & Liu, 2012). However, the consumption of raw mung bean sprouts might constitute potential hazard with respect to microbial contamination (Mohle-Boetani et al., 2009). It has been reported that organic acid salts, such as sodium acetate and sodium citrate, could inhibit the growth of pathogenic microorganisms in the germination of zinnia (Szopińska, 2013) and lettuce (Arin & Balci, 2014). In addition, exogenous organic acid salt spraying might act as a type of stimulus to induce physiological changes and defence or stress-induced responses (Baenas, García-Viguera, & Moreno, 2014). Gebaly, Fatma, and Alia (2013) reported that spraying cotton plants with citrate could increase catalase and peroxidase activities in comparison with the control. Lipid oxidation in sliced salmon (Sallam, 2007) and rainbow trout (Haghparast, Kashiri, Shabanpour, & Pahlavani, 2010) was delayed by the application of sodium acetate and sodium citrate. On the other hand, citrate treatment could enhance phytase activity, thus promoting phytic acid degradation (Porres, Etcheverry, Miller, & Lei, 2001). However, the influence of exogenous organic acid salt spraying on phytic acid degradation in mung bean sprouts has not been reported. In addition, although the antimicrobial effects of these organic salts help prevent bacterial growth, few studies on the overall physiological profile and growth of mung bean sprouts in response to organic acid salt treatments are available.

A considerable number of studies have focused on the effects of germination time on the nutrients and anti-nutrients in mung bean sprouts (Jom et al., 2011; Vayupharp, 2013). Recently, comparative proteomic analyses were conducted on mung bean cotyledons at different germination times (Ghosh & Pal, 2012; Mubarak, 2005). Considering that proteomics approaches can provide greater molecular insights into the overall metabolism during germination, the proteomic profiles of mung bean sprouts treated with different organic acid salts were investigated in this study.

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In this study, we performed an iTRAQ-based quantitative proteomic analysis on seed germination and the growth of mung bean sprouts following sodium acetate (SA), sodium citrate (SC) and sodium tartrate (ST) spraying treatments. The objective was to reveal the response strategies and the adaptive mechanisms of mung bean sprouts in response to different organic acid salt treatments.

2. Materials and methods

2.1. Seed germination and exogenous organic acid salt treatments

The mung bean seeds (*Phaseolus radiatus* L. cv. Qindou 20) were germinated using the protocol reported by Jin et al. (2016) with minor modifications. Initially, the mung bean seeds were surface-sterilized by soaking them in 5% (v/v) sodium hypochlorite for 15 min, and then washed with distilled water until a neutral pH was achieved. After being steeped in distilled water for 4 h at 30 ± 1 °C, the soaked seeds were put into a sprouter (BX-801, Beixin Hardware Electrical Factory, Zhejiang, China) and germinated at 30 °C in the dark. The seeds were watered by an automatic spraying system that provided a 2 min spray every hour. Based on previous results (Supplementary Fig. 1), 0.6 mM solutions of SC, SA and ST (all from Sigma-Aldrich, St. Louis, USA) were freshly prepared as the spraying solutions and refreshed every 24 h. The control was watered with deionized water. The 4-day-old sprouts were rapidly and randomly sampled for the subsequent experiments using the proteomic approach.

2.2. Determination of the phytic acid content and phytase activity

The phytic acid content was determined using the procedure reported by Ma et al. (2005) with some modifications. Briefly, dried samples were extracted with 30 mL of 1.2% HCl and shaken vigorously for 2 h at 30 °C. The supernatant was centrifuged (5000*g* for 10 min) before filtration. A total of 10 mL filtered extract was diluted to 30 mL with distilled water after mixing with 1 mL 30 g/L NaOH and then passed through an anion resin column (AG1-X4 resin, 100–200 mesh, Alfa Aesar, Germany). The column was washed before use with 20 mL of 0.5 mol/L NaCl solution and deionized water till no Cl^- can be detected. The column was washed with 15 mL of distilled water and 20 mL of 0.05 mol/L NaCl solution after sample application. The eluate from the resin was eluted with 0.7 mol/L NaCl to 25 mL. Five millilitres of the collected eluate were mixed with 4.0 mL of Post-column reagent (0.03% $FeCl_3$ solution + 0.3% sulphosalicylic acid). After centrifugation at 3000*g* for 10 min, the absorbance of the supernatant was measured at 500 nm. The standard curve was prepared using phytic acid as the standard (Sigma, USA) in concentrations ranging from 0 to 100 mg/100 g.

Phytase was extracted using the method reported by Hegeman and Grabau (2001) with some modifications. Briefly, mung bean sprouts (1.0 g) were ground with 5 mL of pre-cooled buffer (50 mM sodium acetate, pH 5.5), and then the supernatant was used to measure the enzyme activity. Phytase activity was measured using the method reported by Heinonen and Lahti (1981). One phytase unit corresponded to 1 mmol of inorganic phosphorus formed per minute. The specific activity was expressed as units per gram of dry matter.

2.3. Measurement of sprout length and respiration

Twenty sprouts from each treatment group were sampled and their lengths were measured using a calliper. Respiration rates were determined using the method reported by Zheng et al. (2009). The CO_2 content during seed germination was measured using an infrared gas analyser LB-BZ (Qingdao Loobo Environmental Protection Technology Co., Ltd., China). The respiratory rate was expressed as mg CO_2/g DW h.

2.4. Measurement of lipid peroxidation and relative electrolyte leakage

Membrane lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced in the presence of 5% (w/v) trichloroacetic acid (TCA) (Dhindsa, Plumb-Dhindsa, & Thorpe, 1981). Briefly, the mung bean material (1.0 g) was homogenized in 5 mL of 5% (w/v) trichloroacetic acid. After centrifugation, 2 mL of the supernatant was mixed with 2 mL of 0.67% (w/v) thiobarbituric acid, and then the mixture was placed in a 100 °C water bath for 30 min. The absorbance was measured at 440 nm, 532 nm and 600 nm and the result was calculated using the following equation: MDA equivalents (μmol/l) = $[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{440}]$. The result was expressed as μmol g^{-1} DW.

The electrolyte leakage was determined using the method described by Li et al. (2014) on a DDS-370 electrical conductivity meter (Shanghai Electronics Scientific Instruments, China). Electrical conductivity was first determined in the 25 °C bath solution (C_1), and then the samples were heated at 100 °C for 30 min and conductivity was determined again in the bath solution (C_2). Relative ion leakage was expressed as a percentage of the total conductivity after heating at 100 °C (i.e., relative ion leakage % = $C_1/C_2 \times 100\%$).

2.5. Determination of the antioxidant enzyme activities

The germinated sprouts (0.5 g) were homogenized in a mortar with 5 mL of 50 mM phosphate buffer (pH 7.0) containing 1 mM EDTA and 2% PVP at 4 °C. After centrifugation at 12,000*g* for 20 min at 4 °C, the supernatant was used for the enzyme activity assays. The SOD, CAT and POD activities were determined using an SOD Assay Kit (A001, Nanjing Jiancheng Bioengineering Institute, China), a CAT Assay Kit (A007, Nanjing Jiancheng Bioengineering Institute, China), and a Plant POD Assay Kit (A084-3, Nanjing Jiancheng Bioengineering Institute, China), respectively. GPX was measured spectrophotometrically as the decrease in the A_{340} of NADPH according to the method reported by Drotar, Phelps, and Fall (1985). The reaction mixture consisted of 1.49 mL of phosphate buffer (50 mM, pH 7.0), 0.1 mL of 1 mM EDTA, 0.1 mL of 1 mM NaN_3, 0.1 mL of 1 mM glutathione (GSH), 0.1 mL of 0.2 mM NADPH, 0.01 mL of 0.25 mM H_2O_2, and 0.1 mL of supernatant in a final volume of 2.0 mL. The reaction was initiated by the addition of H_2O_2.

2.6. Determination of the reducing sugar, soluble protein and free amino acid contents

The reducing sugar contents were determined using the 3,5-dinitrosalicylic acid method (Miller, 1959), with some modifications. Glucose was used as a standard, which contains 1 g of 3,5-dinitrosalicylic acid, 0.2 g of phenol, 0.05 g of sodium sulphite, 1 g of NaOH and 20 g of potassium sodium tartrate in a sufficient amount of water to make 100 mL of reagent. Three millilitres of the reagent and the sample (1 mL) were mixed, heated in a boiling water bath for 15 min, and the absorbance was read at 550 nm.

Free amino acids were extracted with 5 mL of distilled water in 85 °C water bath for 30 min using the ninhydrin method and leucine as the standard (Moore & Stein, 1954). Approximately 0.2 g of mung bean sprouts were homogenized in 5 mL of phosphate buffer (pH 7.0), and then centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant was used to determine the soluble protein

content using the Bradford method (Bradford, 1976) and bovine serum albumin as the standard.

2.7. Protein extraction and iTRAQ labelling

Protein extracts were obtained using the P-PER™ Plant Protein Extraction Kit (89803, Thermo Fisher Scientific, CA, USA) according to the manufacturer's recommendations. The extracted protein samples (250 μg each) were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with sequencing-grade trypsin (V5111, Promega, CA, USA) using the Filtered aide sample preparation (FASP) method. After trypsin digestion, the peptides were dried by vacuum centrifugation, reconstituted in iTRAQ dissolution buffer, and labelled using iTRAQ 8-plex kits (AB Sciex Inc., MA, USA), according to the manufacturer's protocol. Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 70 μL of isopropanol. The control and SC, SA and ST spraying treatment samples were labelled with the 113, 114, 115 and 116 tags, respectively. Three biological replicates were performed. After labelling and quenching, the samples were combined and dried by vacuum centrifugation.

2.8. Fractionation by high pH RP chromatography

High pH RP chromatography was performed on an UltiMate™ 3000 HPLC Pump system (Thermo Fisher Scientific, CA, USA). The iTRAQ-labelled peptide mixtures were reconstituted with RP buffer A (98% H_2O and 2% ACN, pH 10.0) and loaded onto a 2.1 × 100 mm ACQUITY UPLC BEH C18 column containing 1.7-μm particles (Waters, MA, USA). The peptides were eluted with a gradient of 97% RP buffer A for 12 min and 3–98% RP buffer B (98% ACN and 2% H_2O, pH 10.0) for 40 min at a flow rate of 0.2 mL/min. The system was then maintained in 98% RP buffer B for 15 min before equilibrating with RP buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 12 fractions and vacuum-dried.

2.9. MS/MS analysis using an LTQ-Orbitrap XL

Each fraction was reconstituted in buffer A (2% ACN and 0.1% FA) and centrifuged at 20,000*g* for 10 min. Using an auto sampler, 20 μL of supernatant was loaded onto a 2 cm C18 trap column (inner diameter 200 μm) on a nano HPLC. The peptides were eluted onto a resolving 10 cm analytical C18 column (inner diameter 75 μm) that was assembled in-house. The samples were loaded at 4 μL/min for 8 min and eluted with an 88 min gradient at 400 nL/min from 3 to 28% B (98% ACN, 0.1% FA), 20 min from 28 to 55% B, followed by a 5 min linear gradient to 98% B, maintenance at 98% B for 20 min, and finally a return to 3% B over 1 min. The peptides were subjected to nano electrospray ionization followed by tandem mass spectrometry (MS/MS) in an LTQ-Orb iTRAQ XL (Thermo Fisher Scientific, CA, USA) coupled online to the HPLC. Intact peptides were detected in the Orbitrap with a resolution of 60,000. Peptides were selected for MS/MS using the high-energy collision dissociation (HCD) operating mode with a normalized collision energy setting of 40.0; ion fragments were detected in the orbitrap at a resolution of 7500. A data-dependent procedure that alternated between one MS scan followed by 5 MS/MS scans was applied for the 5 most abundant precursor ions above a threshold ion count of 5000 in the MS survey scan, with duration of 60 s. The electrospray voltage applied was 2.4 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the orbitrap. The AGC target for the full MS was 1e6 and 1e5 for MS2. For the MS scans, the m/z scan range was 350–1800 Da. For the MS2 scans, the m/z scan range was 100–1800.

2.10. Database search and iTRAQ quantification

The samples were analysed by nano-LC-MS/MS. The peak lists were generated with Proteome Discoverer 1.3 (Thermo Fisher Scientific, CA, USA). The proteins were identified using the SEQUEST search engine by searching against the NCBI *Glycine max* database (Apr 20th, 2015). For protein identification, a mass tolerance of 10 ppm was permitted for the intact peptide masses and 0.02 Da for the fragmented ions, with an allowance for one missed cleavage in the trypsin digests. Oxidation at methionine was set as the potential variable modifications, whereas carbamidomethylation at cysteine was set as the fixed modification. The charge states of the peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in SEQUEST by choosing the decoy checkbox in which a random sequence of the database is generated and tested for the raw spectra, as well as the real database. Only peptides identified at the 99% confidence interval by a SEQUEST probability analysis were counted as identified and each identified protein included at least one unique peptide to reduce the probability of false identification. Only proteins that were identified in all three independent experiments were considered. In this study, a protein was considered differentially abundant when it had a fold change of >1.5.

2.11. Bioinformatic analysis of proteins

Functional annotations were generated with the Blast2GO software (http://www.geneontology.org) using the non-redundant protein database and Clusters of Orthologous Groups of Proteins System (COG) software (http://www.ncbi.nlm.nih.gov/COG/). We used the KEGG databases (http://www.genome.jp/kegg/pathway.html) to take advantage of the current knowledge of biochemical pathways and other types of molecular interactions.

2.12. Statistical analysis

The experiments were performed with three biological replicates. The results were presented as the means ± SD. Statistical analyses of the data were performed using ANOVA tests. The means were separated by the least significant difference (LSD) test at the $p < 0.05$ level.

3. Results and analysis

3.1. Changes in the phytic acid content and phytase activity

During germination, there was a decrease in phytic acid content accompanied by an increase in phytase activity (Fig. 1). The phytic acid content was decreased by 55.4%, 57.1% and 65.6% after the ST, SA and SC spraying treatments, respectively (Fig. 1A). Increased phytase activity was also observed in response to these three treatments on the 4th day of germination, although the stimulatory effect was not observed on the 2nd day for the ST and SA treatments (Fig. 1B).

3.2. Growth performance, respiratory rate, MDA content and electrical conductivity in germinated mung bean sprouts

Compared with the control, the exogenous ST and SC treatments significantly increased the length of the mung bean sprouts on the 4th day of germination ($p < 0.05$) (Fig. 2A). The respiratory rate of mung bean was also increased by the ST and SC treatments (Fig. 2B) ($p < 0.05$). The highest MDA content was observed after the SA spraying treatment ($p < 0.05$), followed by the control and ST and SC spraying treatments, with no significant difference

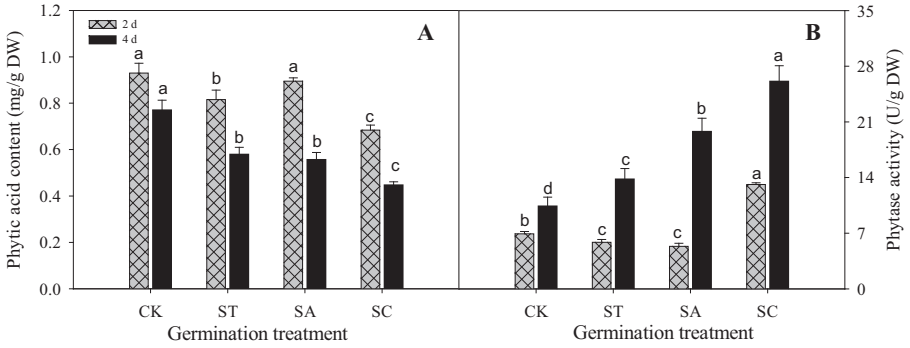

Fig. 1. Changes in the phytic acid content (A) and phytase activity (B) of mung bean sprouts in response to ST, SA and SC treatments. Different letters indicate significant differences among the samples on each day ($p < 0.05$). ST, SA and SC represent sodium tartrate, sodium acetate and sodium citrate, respectively. CK is sprayed with distilled water.

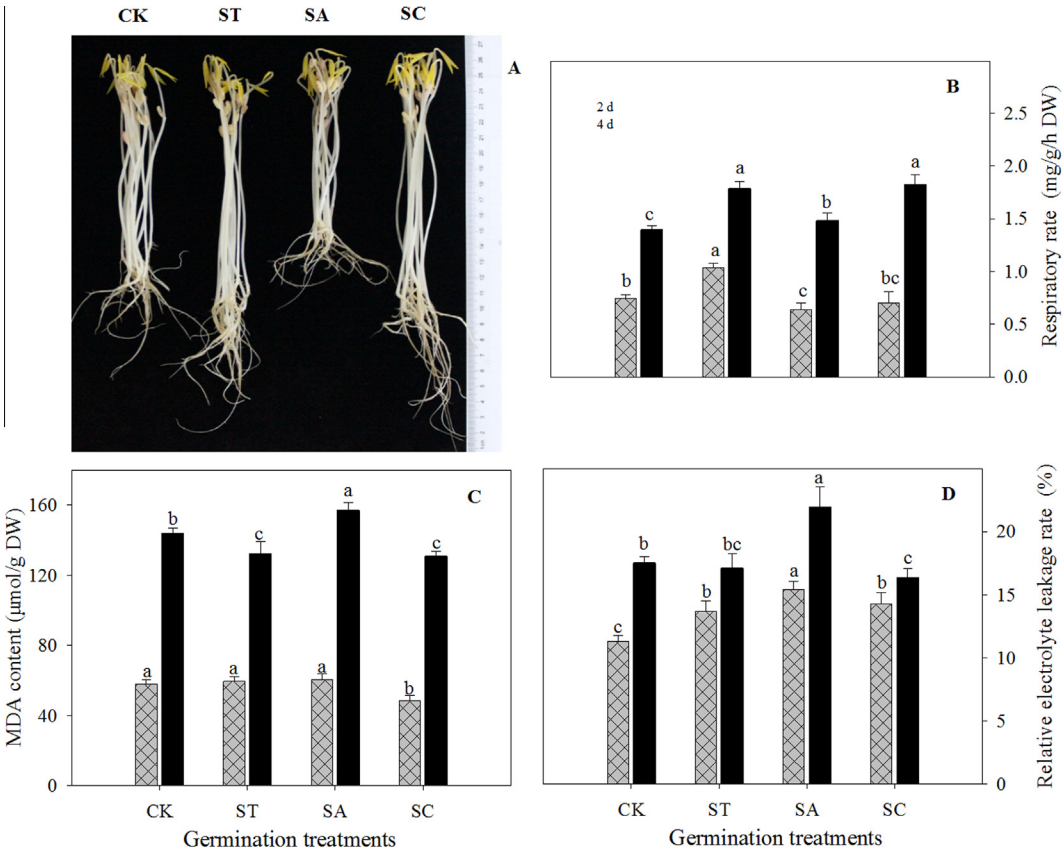

Fig. 2. Changes in the patterns of growth performance (A), respiratory rate (B), MDA content (C) and cellular membrane damage (D) of mung bean sprouts in response to ST, SA and SC treatments. Figure A showed the growth performance on the 4th day of germination. Different letters indicate significant differences among the samples on each day ($p < 0.05$).

between the ST and SC spraying treatments (Fig. 2C). In terms of the electrolyte leakage rate, it is noted that SA spraying produced the highest relative electrolyte leakage rate, whereas ST and SC spraying showed significantly reduced relative electrolyte leakage rates compared with the control ($p < 0.05$), indicating that SA spraying may cause membrane injury to some extent (Fig. 2D).

3.3. Changes of storage substances in mung bean sprouts

As shown in Fig. 3, the soluble protein content was initially increased and then decreased during the 4-day germination, showing the maximum value on the 3rd day ($p < 0.05$). These three spraying treatments increased the soluble protein contents in mung bean sprouts during the first two days of germination, with SA producing the lowest soluble protein content on the 4th day of germination (Fig. 3A). A corresponding trend was also observed in the free amino acid content (Fig. 3B); the highest free amino acid content was observed after SA spraying, followed by ST, SC and the control, with no significant difference in SC and ST. In contrast, a consistent increase in the reducing sugar content was observed during the 4-day germination (Fig. 3C). These three spraying treatments increased the reducing sugar content compared with the control, with the exception of the 4th day. A significant decrease in the reducing sugar content was observed after SA spraying, but no significant difference was observed between ST, SC and the control on the 4th day.

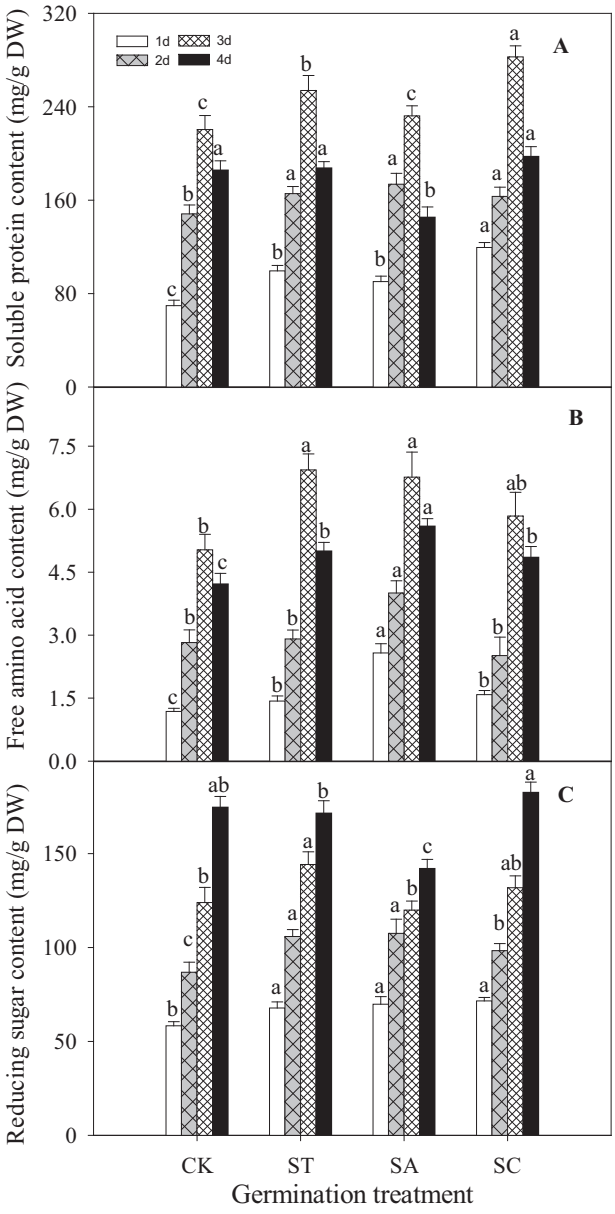

Fig. 3. Levels of storage materials, including soluble protein (A), free amino acids (B) and reducing sugars (C), in mung bean sprouts subjected to the ST, SA and SC treatments. Different letters indicate significant differences among the samples on each day ($p < 0.05$).

3.4. Changes in antioxidant enzyme activities

As shown in Fig. 4, significant increases in the GPX, POD, CAT and SOD activities were observed on the 4th day of germination compared with the 2nd day ($p < 0.05$). The ST, SA and SC treatments resulted in similar patterns of POD, CAT and SOD activities. All treatments reduced the GPX activity on the 4th day of germination, with SA spraying showing the lowest activity among all of the treatments (Fig. 4A). However, the highest CAT activity was observed in mung bean sprouts treated with SA spraying (Fig. 4C), followed by SC, ST and the control. Meanwhile, the highest POD and SOD activities were also observed after SA treatment, which were 1.84- and 1.59-fold higher than the control, respectively (Fig. 4B and D) ($p < 0.05$).

3.5. Differentially expressed proteins detected by iTRAQ

The numbers of differentially expressed proteins and their overlap in response to the different organic acid salt treatments were illustrated in a Venn diagram analysis (Fig. 5A). Only proteins that were identified in all three independent experiments were considered. A protein was considered differentially expressed when the protein had both a fold-change of more than 1.5 and a P-value of less than 0.05. Based on the two criteria, we identified a total of 143 differentially expressed proteins. There were 81 differentially expressed proteins in SC treatment, 101 proteins in SA treatment, 90 proteins in ST treatment, and 38 proteins in all of the samples (Fig. 5A). These 38 common differentially expressed proteins were mainly involved in carbohydrate and energy metabolism, cell wall and cytoskeleton metabolism, and protein metabolism, and included glucan *endo*-1,3-β-glucosidase, phosphoenolpyruvate carboxykinase, malate dehydrogenase, α-xylosidase and phenylalanine ammonialyase. In particular, some functionally important proteins were phosphorylated, suggesting their key roles in sprout development (Supplementary Tables 1 and 2).

According to the molecular functions listed on the UniProt and Gene Ontology website, the iTRAQ-quantified proteins in SC treatment group were functionally classified into 12 functional classes, i.e., carbohydrate and energy (30.7%), protein metabolism (13.6%), biological regulation and signal transduction (11.1%), cell wall and cytoskeleton metabolism (8.7%), inositol phosphate (3.7%), lipid metabolism (2.5%), cell transport (3.7%), nucleic acid (6.2%), secondary metabolism (8.6%), response to stress (4.9%), etc. (Fig. 5B). A similar trend was observed in the SA and ST treatment groups, with the carbohydrate and energy metabolism comprising the highest percentage of differentially expressed proteins in both treatment groups (Fig. 5C and D).

Fifty-three proteins were up-regulated and 28 proteins were down-regulated in response to SC treatment (Fig. 5E). Fifty-two proteins were up-regulated and 49 proteins were down-regulated in response to SA treatment (Fig. 5F). Fifty-eight proteins were up-regulated and 32 proteins were down-regulated in response to ST treatment (Fig. 5G). The responses of the proteins to the different organic acid salts were substantially different. In mung bean sprouts, only 8 (21.1%) of the down-regulated proteins and 30 (79.9%) of the up-regulated proteins were commonly regulated by the three different treatments and involved in the same biological process (Supplementary Table 2). Therefore, the results showed that the effects of these three treatments on mung bean sprouts have some commonalities but also have additional, specific characteristics, including exchangeable sodium and organic acid anion reactions in a complex network involving multiple physiological and metabolic pathways. The list of peaks used to identify the proteins is shown in the Supplementary Table 3.

4. Discussion

Our results showed that mung bean sprout growth was significantly increased by the SC and ST treatments and was accompanied by a higher respiratory rate, whereas the SA treatment inhibited mung bean growth on the 4th day. The slightly higher MDA content and electrical conductivity in SA treatment indicated that excess free radicals were generated (Li, Zhang, Jiang, & Liu, 2013). In response to the SA-induced stress, significant increases in the antioxidant enzymes activities of CAT, POD, SOD were detected, which was further confirmed by the increased abundance of POD 52-like (GI: 734397902) and CAT-4 (GI: 17865456) in our iTRAQ analysis (Supplementary Table 2). In addition, SA treatment significantly increased the expression of isoflavone synthase (GI: 170783768) and isoflavone 2′-hydroxylase (GI: 571487382) compared with the control, which might indicate that isoflavone accumulated in response to SA treatment. The accumulation of isoflavones might play an essential role in scavenging reactive oxygen species, which might occur in response to the high MDA

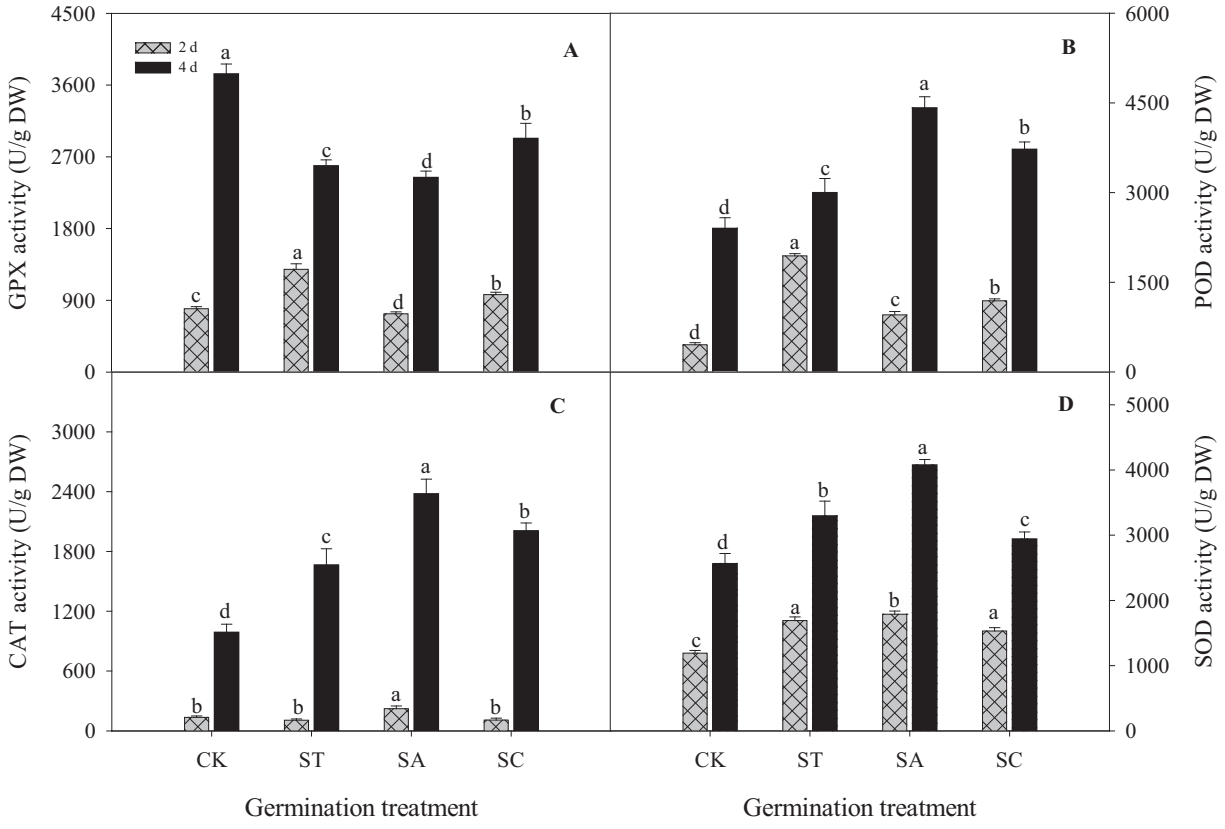

Fig. 4. Changes in the activities of the antioxidant enzymes GPX (A), POD (B), CAT (C) and SOD (D) in mung bean sprouts in response to SC, SA and ST treatments. Different letters indicate significant differences among the samples on each day ($p < 0.05$).

content induced by SA (Fig. 2C). However, the exogenous SC and ST treatments also produced higher antioxidant enzymes activities, which agreed with the existing results showing that SC and ST could decrease the formation of free radicals and increase the antioxidant activities in refrigerated sliced salmon (Sallam, 2007).

Meanwhile, phytic acid degradation was promoted by these three organic acid salt treatments due to the increased phytase activity. Moreover, increased expression of inositol-phosphate phosphatase (GI: 734423661) and purple acid phosphatase (GI: 734348105 and 351720816) were observed in SC-, SA- and ST-treated mung bean sprouts (Supplementary Tables 1 and 2). These findings suggested that these three treatments might be feasible ways to stimulate phytic acid degradation and antioxidant enzyme activities. However, the underlying molecular mechanisms by which the organic acid salts mediate mung bean metabolism needs to be discussed further.

4.1. Stimulation of the respiratory electron transport chain

In our studies, the expression of the identified enzymes associated with energy metabolism was increased by these exogenous organic acid salt treatments. ATP synthase β-subunit, mitochondrial (GI: 356536246), NADH dehydrogenase (ubiquinone) 1 α-subcomplex subunit 5 (GI: 351726335), and F-type H^+-transporting ATPase γ-subunit (GI: 351723485) belong to the oxidative phosphorylation process, in which ATP is formed as a result of the transfer of electrons from NADH or $FADH_2$ to O_2. The expression of these enzymes was increased by the SC, ST and SA treatments, except for F-type H^+-transporting ATPase γ-subunit in the ST treatment group (Supplementary Table 2), indicating that ATP synthesis was promoted by these exogenous organic acid salt treatments. In addition, cytochrome *b* 559 subunit alpha (GI: 94730464) and malate dehydrogenase (GI: 169977) were up-regulated by the SC and SA treatments, whereas increased ferredoxin-NADP reductase expression (GI: 356538289) was induced by the SA and ST treatments; all of these up-regulated enzymes were involved in the respiratory electron transport chain. This result indicated that these three organic acid salts promoted the activity of the respiratory electron transport chain, thus increasing sprout respiration. This result was also supported by the data that mung bean sprouts treated with these three organic acid salts had higher respiratory rates (Fig. 2B).

4.2. Enhanced carbohydrate degradation

The common differentially expressed proteins in the sprouts treated with these three organic acid salts were mainly up-regulated proteins that are involved in starch and non-starch polysaccharides hydrolysis, glycolysis, tricarboxylic acid cycle and other processes, including the α-1,4-glucan phosphorylase L (GI: 356551144) and L2 isozymes (GI: 571563869), α-glucan phosphorylase (GI: 356527232), glucan *endo*-1,3-β-glucosidase (GI: 356569016), α-xylosidase 1-like (GI: 356495935), and malate dehydrogenase (GI: 169977) (Supplementary Table 2). This result indicated that these organic acid salts might promote carbohydrate degradation. Phosphoenolpyruvate carboxykinase (PEPCK) (GI: 356496541) catalyses a key step in the conversion of fats to sugars during the germination of fat-storing seeds, and its activity is closely paralleled with the gluconeogenic flux (Bryce & Hill, 1993). This enzyme replenished oxaloacetate in the tricarboxylic acid cycle when operating in the reverse direction, and this step is catalysed by another enzyme - phosphoenolpyruvate carboxylase (PEPC) (GI: 1705587). In our results, the PEPCK (GI: 356496541) levels were reduced by the three organic acid salt treatments

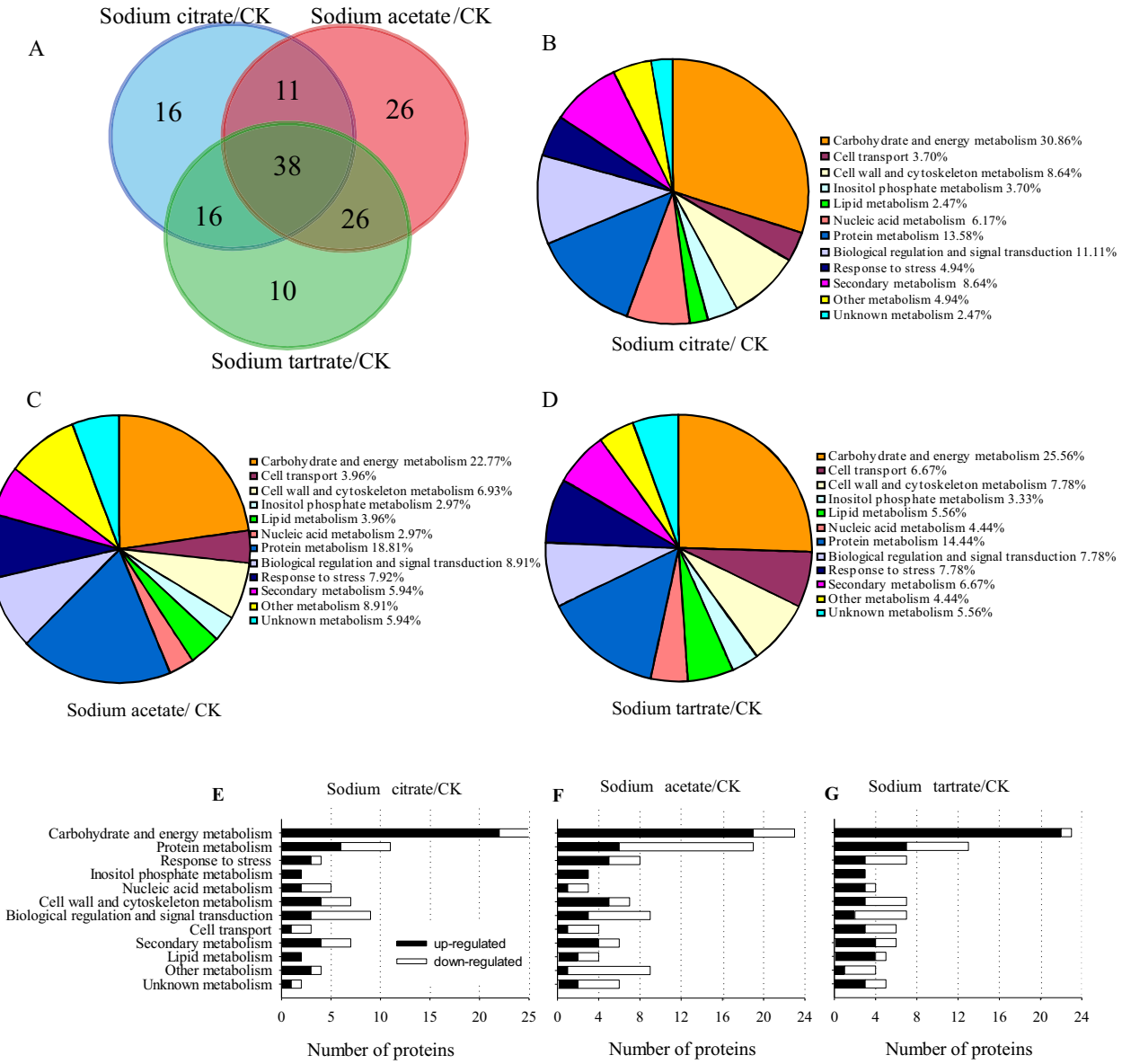

Fig. 5. Venn diagrams of the differentially expressed proteins (A) and functional classifications of these proteins in mung bean sprouts treated with sodium citrate (B and E), sodium acetate (C and F) and sodium tartrate (D and G). The numbers of differentially expressed protein spots in response to a given treatment are shown in different segments.

compared with the control, whereas PEPC (GI: 1705587) showed the opposite trend, suggesting that the conversion of lipids to sugars was inhibited by the organic acid salt treatments on the 4th day of germination. The inhibition might have resulted from a sufficient sugar supply for mung bean growth. On one hand, these organic acid salts might have acted as a carbon source to sustain the sprout growth (Casati, Drincovich, Edwards, & Andreo, 1999). On the other hand, the up-regulation of α-glucan phosphorylase (GI: 356527232) and the α-1,4-glucan phosphorylase L (GI: 356551144) and L-2 isozymes (GI: 571563869), could have resulted in a significant increase in the reducing sugar content (Fig. 3C). Yang et al. (2007) also determined that most TCA cycle and glycolysis-related proteins were up-regulated, such as UDP-glucose dehydrogenase, fructokinase, phosphoglucomutase, and pyruvate decarboxylase. The accumulation of succinyl-CoA ligase and cytoplasmic malate dehydrogenase had been observed in rice embryos during germination (Kim et al., 2009), suggesting that carbohydrate catabolism was promoted during early germination. In our study, these three organic acid salt treatments all accelerated the carbohydrate hydrolysis, which was responsible for sustaining post-germinative growth.

It was noted that most of the differentially expressed proteins associated with carbohydrate metabolism were up-regulated in the SC-treated mung bean sprouts, except for acid beta-fructofuranosidase-like (GI: 356540502), which catalyses the conversion of sucrose to fructose and glucose. The difference in the expression of this protein might be the result of the feedback inhibition due to the high reducing sugars content (Fig. 3C), which was consistent with the results reported by Zhang and Wang (2002). Increased expression of probable beta-D-xylosidase 2 (GI: 356556038) was also observed in response to ST spraying. Meanwhile, pyruvate kinase isozyme A (GI: 356535329) was up-regulated in response to the SC and SA treatments compared with the control, suggesting that glycolysis metabolism was increased.

4.3. Stimulation of cell wall extension and lignin synthesis

Fibre growth is a key developmental process, which undergoes four major discrete developmental stages (differentiation, elongation/primary cell wall synthesis, secondary cell wall synthesis, and maturity) and eventually produces a thick cell wall consisting of more than 94% cellulose (Kim & Triplett, 2001). Expansin proteins (EXPS) loosen the cell walls and modulate cell and plant growth. In the present study, the expression of several enzymes involved in cell wall extension and lignin synthesis (expansin, GI: 330858331 and annexin with 2 isoforms, GI: 356548893 and 356548895) was increased, indicating that the three organic acid salt treatments stimulated lignin synthesis and thus affected radical extension and sprout elongation to some extent (Daines, Minocha, & Kudakasseril, 1983).

4.4. Increased phenolic biosynthesis

Phenylalanine ammonialyase (PAL) is considered the key enzyme in phenolic biosynthesis. It catalyses the reductive amination of L-phenylalanine to form transcinnamic acid, which is the first step in the biosynthesis of plant phenylpropanoid compounds. In this study, the expression of several enzymes involved in phenolic synthesis (caffeic acid 3-O-methyltransferase-like, GI: 356518669 and phenylalanine ammonialyase class 2-like, GI: 356548301) was increased in response to the three organic acid salt treatments. However, anthocyanidin 3-O-glucosyltransferase-like (GI: 356576401) was down-regulated in response to SC treatment, indicating that glycosylation modification was affected by SC treatment.

4.5. Opposite response of protein metabolism

The differentially expressed ribosomal proteins, which are known to play an essential role in ribosome assembly and protein translation, were mainly down-regulated in response to the SA spraying treatment, indicating that SA treatment impaired protein biosynthesis. However, the ribosomal proteins were insignificantly affected by the SC and ST spraying treatments (Supplementary Table 2). These results were consistent with our observation that the SA-treated sprouts had the lowest total soluble protein content (Fig. 3A). The inhibition of protein synthesis by the SA treatment can also be confirmed by the decrease in the expression of eukaryotic translation initiation factor 5A3 (GI: 351721220), which is involved in the initiation phase of eukaryotic translation. Furthermore, the nascent polypeptide-associated complex (NAC) (GI: 356509137) is considered to be involved in protein transport for the appropriate targeting of ribosome-nascent polypeptide complexes (Rospert, Dubaquie, & Gautschi, 2002). Proteins containing a pentatricopeptide repeat (PPR) (GI: 356512038) may act as sequence-specific adaptors for a variety of other RNA-associated proteins (Diniz et al., 2010). As shown in Supplementary Table 2, the NAC and PPR-like superfamily proteins were down-regulated in mung bean sprouts following the SA spraying treatment. These results further showed that SA spraying impaired protein biosynthesis in mung bean sprouts.

On the other hand, SC treatment significantly increased the levels of proteasome subunit beta type and several other proteases (also termed proteinase and peptidase-like serine carboxypeptidase-like 51, GI: 356550133, putative serine protease EDA2, GI: 363814290) (Supplementary Table 2) that played important roles in protein processing, particularly in modifying proteins and remodelling polypeptides during germination (Lepelley et al., 2012). This result indicates that protein modification and processing might be enhanced in SC-treated mung bean sprouts. Hence, the proteins possessing enzymatic activity that were up-regulated by SC spraying ensured the specific functions of the proteins.

Post-translational modifications (PTMs), such as glycosylation, phosphorylation and ubiquitination, are reported to play important roles in the regulation of abiotic stress responses. Our results showed that ST spraying increased levels of proteins involved in phosphorylation (such as receptor-like protein kinase FERONIA-like, GI: 571496773, probable serine/threonine protein phosphatase 2A regulatory subunit, GI: 571569749 and calcium-dependent protein kinase isoform, GI: 356521991) in the sprouts, whereas SC spraying also increased the level of receptor-like protein kinase FERONIA-like. However, the SC and SA spraying treatments decreased the sprout levels of ubiquitination (ubiquitin-conjugating enzyme E2 5-like isoform X2, GI: 571525370 and SKP1-like protein 1A, GI: 356496612, respectively) (Supplementary Table 2). This result shows that the PTMs of some proteins might be impaired in response to SA treatment. However, the sprout levels of phosphotyrosine protein phosphatase-like (GI: 571495059) were increased in response to the SA spraying treatment.

4.6. Opposite response of biological regulation and signal transduction

Ca^{2+} may act as a messenger in signal transduction in plants and it depends on a sensor to convey changes in its concentration. The main groups of proteins that bind Ca^{2+} and confer Ca^{2+}-mediated responses are EF-hand proteins and C2-domain proteins (Reddy & Reddy, 2004). In this study, the differentially expressed proteins related to Ca^{2+} sensors, such as calcium-dependent protein kinase 11 (GI: 257638616), were down-regulated by these organic acid salt treatments. We also found that calcium-binding EF-hand proteins, calcium-dependent protein kinase SK5 (GI: 356508898) and calmodulin (GI: 255644599) were down-regulated by the SA treatment. Because calcium-dependent protein kinase is primarily involved in signalling cascades involved in osmotic, developmental and nutritional changes (Valmonte, Arthur, Higgins, & MacDiarmid, 2014), it was reasonable to conclude that Ca^{2+}-related proteins may be involved in the responses of mung bean sprouts to organic acid salt treatments.

During the germination process, storage reserves, particularly starches, proteins and triglycerides, are broken down in the seed. These reserves are mobilized during germination and seedling growth to supply the energy and metabolic intermediates needed by the seedling prior to the establishment of photosynthetic autotrophism (Wilson, Rightmire, Chen, & Tan-Wilson, 1986). β-conglycinin (GI: 356535993), a main type of seed storage protein, accumulates during seed development and is hydrolysed after germination to provide a carbon and nitrogen source for the developing seedling (Angelovici, Fait, Fernie, & Galili, 2011). However, β-conglycinin expression showed different trends in response to the organic acid salt treatments. Compared with the control, the β-conglycinin levels were significantly increased by the SA treatment ($p < 0.05$), but its levels were significantly reduced by the SC treatment, with no significant difference between the ST spraying treatment and the control. This result indicated that SC spraying treatment stimulated β-conglycinin degradation during storage protein mobilization, whereas the degradation of β-conglycinin was inhibited by the SA treatment. The increased degradation of β-conglycinin ensures that a sufficient amount of amino acids will be available for new protein synthesis, and the low levels of β-conglycinin in mung bean sprouts subjected to the SC treatment were general responses that increased plant growth.

5. Conclusions

The exogenous SC, ST, SA treatments significantly affected mung bean sprout growth, whereas the SC and ST treatments stimulated growth and the SA treatment slightly inhibited growth. The

sprouts subjected to the SC, ST and SA treatments exhibited higher phytic acid degradation compared with the control. The up-regulation of phytase might due to the increased expression of carbohydrate-degrading enzymes and ATP-synthesising enzymes, which resulted in an increase in phosphate consumption. Moreover, the inhibitory effect of the SA treatment is probably due to impaired protein biosynthesis.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.029.

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