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GC-MS-BASED METABOLOMICS ANALYSIS OF TRANSGENIC RICE WITH HUMAN SERUM ALBUMIN

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Abstract

This study was to analyze the difference of the metabolite profiles between non-transgenic (TP309-8) and human serum albumin (HSA) transgenic rice (TP309-HSA-8, TP309-HSA-9, corresponding to 8th and 9th generation) by gas chromatography-mass spectrometry followed by multivariate analyses methods including principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA). As a result, 12 differential metabolites were identified between TP309-HSA-8 and TP309-8, of which 6 were known compounds (trehalose, citric acid, valine, glycine, asparagine and pantothenic acid) and they were enriched in starch and sucrose metabolism, carbon fixation pathways in prokaryotes, valine, leucine and isoleucine degradation and biosynthesis, glycine, serine and threonine metabolism, and antidyslipidemic agents pathways, respectively. There were 4 different compounds between TP309-HSA-8 and TP309-HSA-9, including known compounds [asparagine and oleic acid (C18:1)]. However, no pathways were enriched for them. Our findings preliminarily reveal transgenic HSA may be beneficial for rice growth and providing more essential amino acid for human beings by altering the metabolite profiles.

Key words: Transgenic rice; Human serum albumin; Untargeted metabolic profiling; Gas chromatography-mass spectrometry; Pathway annotation.

Introduction

Human serum albumin (HSA) is characterized as a globular, soluble and un-glycosylated monomeric multidomain macromolecule, which comprises 60~65% of total plasma protein (Fanali *et al.*, 2012). HSA has a critical role in maintaining colloid osmotic pressure *in vivo* (Roopenian *et al.*, 2015). HSA has multiple ligand-binding capacities, suggesting it may play key roles in the transporting, distribution, and metabolism of many endogenous and exogenous compounds, such as fatty acids (Kragh-Hansen *et al.*, 2013). HSA also attracts the interest of pharmacologists as it can alter pharmacokinetic and pharmacodynamics properties of a variety of drugs and reduce their side effects (Yang *et al.*, 2014). Another striking function of HSA is acting as an antioxidant to scavenge excessive reactive oxygen species *in vivo* (Taverna *et al.*, 2013). Thus, HSA has been widely used in clinic for treatment of various diseases as well as for nanodelivery of drugs (Hastings & Wolf, 1992; Cai *et al.*, 2006).

At present, considerable efforts have been made to meet the market demand of HSA, approximately 500 tons/year worldwide. Purifying HSA from human plasma is a traditional approach, which is suffered by limited supply, high price and even fake HSA due to the shortage of human plasma (Turell *et al.*, 2014). To overcome this disadvantage, recombinant expression of HSA by a variety of systems, e.g. transgenic plants, can produce HSA in a cost-effective way (Sun *et al.*, 2011). Rice seeds have been successfully used as a bioreactor for producing *Oryza sativa* recombinant HSA in a large-scale (He *et al.*, 2011). The HSA transgenic rice is suggested as an alternative strategy for producing commercial HSA (Zhang *et al.*, 2013). However, untargeted metabolite profile in response to HSA insertion has not been studied. Further analyzing the change of metabolite profile in response to HSA insertion will help understand the effects of transgene on rice growth and metabolism, as well as the biosafety issue.

Metabolomics research can be performed by gas chromatography-mass spectrometry (GC-MS), a widely-applied approach for metabolite profile analysis based on its broad coverage of analytical compounds in plants and microorganisms (Koek *et al.*, 2011). To investigate the unintended metabolic profile of transgenic rice expressing extraneous HSA, the metabolic differences between transgenic and non-transgenic rice lines were compared by multivariable analyses of GC-MS data. The different metabolites were further subjected to pathway annotation to identify their biological relevance.

Materials and methods

Materials and sample preparation: Seeds of the 8th (TP309-HSA-8) and 9th (TP309-HSA-9) transgenic rice line, as well as those of the non-transgenic counterpart (TP309) were provided by the Engineering Research Center for Plant Biotechnology and Germplasm Utilization, College of Life Sciences, Wuhan University (Wuhan, China). The 8th and 9th transgenic rice lines were generated via backcrossing the transgenic rice line TP309-HSA with the non-transgenic TP309 continuously. All seeds were sown in an experimental field in Wuhan City, Hubei Province, China.

Samples were prepared as previously described (Wu *et al.*, 2012), with small modifications. Briefly, 100 rice grains from each sample were hulled and then ground at 4°C for 1 min using a mixer-mill (MM400, Retsch, Germany). Next, 100 mg rice powder was transferred to a 200 mL centrifuge tube, followed by addition of 1 mL of extraction buffer consisting of isopropanol, acetonitrile and water mixed at a volume ratio of 3:3:2. Then the samples in 200 mL centrifuge tube were subjected to vortex-mixing for 10 s, soaking for 15 min, and subsequent centrifuging at a speed

of 12,200 g for 2 min at 4°C. The supernatant (100 µL) were transferred into a glass insert vial (5182-0549, Aglient, USA), supplemented with myristic acid d_{27} (ISOTEC, USA) as an interior label and evaporated in an SpeedVac concentrator system (SPD111 V-230, Thermo Electron Corporation, Waltham, USA) for 6 h at 4°C. Finally, the dried extract was processed for derivatization, first with 10 µL of 40 mg/mL methoxamine hydrochloride (Sigma, Munich, Germany) in pyridine (Merck, Darmstadt, Germany) for 90 min at 30°C to protect the carbonyl group, and then with 90 µL of methyl-trimethyl-silyl-trifluoroacetamide (Macherey&Nagel, Düren, Germany) supplemented with 1% trimethylchlorosilane (Sigma-Aldrich ChemieGembH, Deisenhofen, Germany) for 30 min at 30°C.

GC-MS analysis: The derivatized samples were transferred to a clear glass autosampler vial (2 mL) with micro insert (Aglient, Santa Clara, USA) GC-MS with optimized parameters based on previous description (Lisec *et al.*, 2006). In brief, 1 µL of the derivatized sample was splitlessly injected into a Agilent GC-MS system consisting of autosampler (7683B), gas chromatograph (7890A) and quadrupole mass spectrometer (5975C) (Agilent, Atlanta, USA), with 16 biological replications for each rice line. The 7890A gas chromatograph was equipped with a 30 m capillary column Rtx-5Sil MS (0.25-mm in inner diameter) containing a 0.25 µm film and an integrated guard column (Restek Gm bH, Bad Homburg, Germany). Helium was used as the carrier gas at a constant linear velocity of 1.0 mL/min. The temperatures of injection, transfer line and ion source were respectively set as 230°C, 250°C and 200°C, respectively. The column temperature was initially maintained at 70°C for 5 min, then increased to 350°C at 5°C/min with the duration of 15 min and reduced to a final temperature of 330°C for 5 min. After a solvent delay of 9.5 min, MS detection was performed in an electron ionization mode (electron energy of 70 eV) and at 2 scans/s with a scanning range of 50–400 m/z. Myristic acid d_{27} (ISOTEC Inc., Des Plaines, IL, USA) was used as an internal quality standard.

Identification of metabolites: The GC-MS data were first subjected to pretreatments (i.e., normalization, baseline correction, and retention time alignment) using Automated Mass Spectrometry Deconvolution and Identification System (AMDIS) software (V2.66, National Institute of Standards and Technology, Gaithersburg, USA). For peak detecting and mass spectrum deconvolution, parameters were set as followings: retention time (0.15 min), math factor (80%), sensitivity (high) and adjacent peak (3). Peaks with signal to ration (S/N) ratio < 10 were removed from the peak list. Then, the obtained peaks were aligned with mass spectral libraries (NIST 08 and the Q-MSRI library) (Steinhauser *et al.*, 2004) by comparing retention time.

Data analysis: The obtained data in Elu and Fin format were submitted to Mass Profiler Professional (MPP) software V2.0 (Aglient Technologies, Palo Alto, USA) for unsupervised principal component analysis (PCA) (Abdi & Williams, 2010), hierarchical cluster analysis (HCA) (Almeida *et al.*, 2007) and supervised partial least squares-discriminant analysis (PLS-DA) (Boccard & Rutledge, 2013). Parameters of PLS-DA were set as follow: frequency filter (90%), abundance (300), ions (3), retention time (0.05 min) and match factor (0.3). Fold changes of metabolites among groups were also analyzed and visualized using volcano graphs. Subsequently, 2-tailed Student's *t*-test was used to determine the statistical significance of the metabolic changes with thresholds of $p<0.05$ or <0.01 using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Pathway annotation: The differential metabolites identified between transgenic and non-transgenic rice were further submitted to Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/kegg/pathway.html, last updated at April 6, 2015) (Qiu, 2013) for pathway annotation.

Results

HSA insertion induced an opaque phenotype of rice seeds: Morphological features of seeds were visually compared between the non-transgenic rice TP309-8 and HSA-transgenic rice TP309-HSA-8. The grains of both the transgenic rice line TP309-HSA-8 and the non-transgenic TP309-8 rice line had a golden appearance and a similar size before hulling (Fig. 1A&B). After dehulling, the size of two rice lines had no significant difference. But the color of seeds derived from transgenic rice TP309-HSA-8 displayed more clearly than those from non-transgenic rice, which indicated seeds from transgenic rice were more opaque. We concluded that HSA insertion may be responsible for the change of color (Fig. 1C&D).

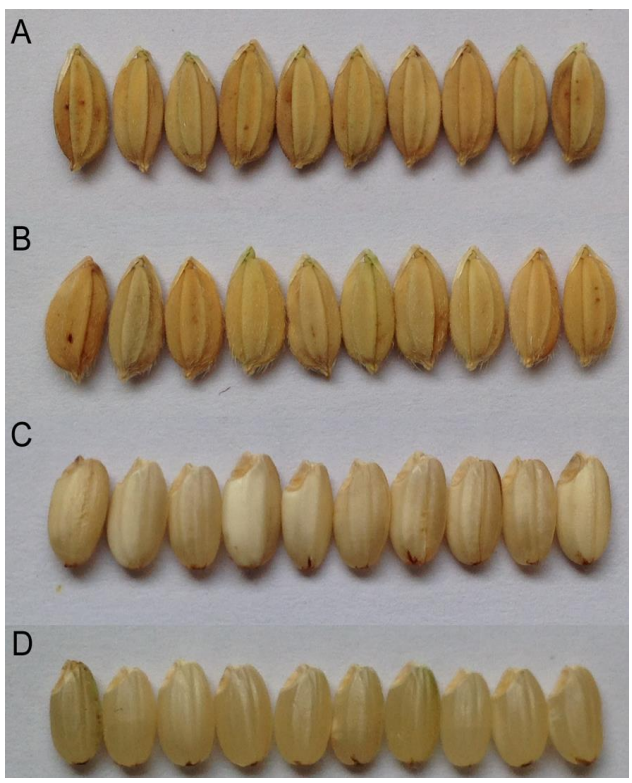

Fig. 1. Effects of HSA insertion on the appearance of different rice seeds.
A, TP309-HSA-8 before hulling; B, TP309-8 before hulling; C, TP309-HSA-8 after hulling; D, TP309-8 after hulling. HSA, human serum albumin; TP309-8, wild-type rice; TP309-HSA-8, the transgenic rice developed via backcrossing of the 8th generation to the recurrent parent TP309.

Table 1. Metabolites identified in transgenic rice seeds.

No.	Metabolite name	Retention time/min	CAS No.
1.	Lactic acid	7.305	79-33-4
2.	Glycollic acid	7.4942	79-14-1
3.	Alanine	7.8249	56-41-7
4.	Leucine	8.5224	61-90-5
5.	Mimosine	8.9968	500-44-7
6.	Valine	9.2691	72-18-4
7.	Malonic acid	9.5324	141-82-2
8.	Urea	9.5964	57-13-6
9.	Benzoic acid	9.7475	65-85-0
10.	Serine	9.8156	56-45-1
11.	Glycerinum	10.0096	56-81-5
12.	Phosphoric acid	10.0124	7664-38-2
13.	Isoleucine	10.2905	443-79-8
14.	Threonine	10.3008	72-19-5
15.	Niacin	10.3597	59-67-6
16.	Proline	10.3706	147-85-3
17.	Glycine	10.4799	56-40-6
18.	Succinic acid	10.5594	29915-38-6
19.	Glyceric acid	10.7712	473-81-4
20.	Porphin	10.8215	681295-24-9
21.	Uracil	10.8661	66-22-8
22.	Fumaric acid	11.0229	17013-01-3
23.	Aspartic acid	11.9939	56-84-8
24.	Threose	12.3481	95-43-2
25.	Threitol	13.0662	6968-16-7
26.	Glutamic acid	13.2167	56-86-0
27.	Phenylalanine	13.545	63-91-2
28.	Lauric acid	14.7874	143-0707
29.	Ribose	14.8967	24259-59-4
30.	Arbaitol	15.3882	488-82-4
31.	Xylitol	15.3885	87-99-0
32.	Citric acid	16.5927	5949-29-1
33.	Tagatose	17.1466	87-81-0
34.	Allose	17.3491	579-36-2
35.	Glucose	17.4144	59-23-4
36.	Mannose	17.6192	87-78-5
37.	Lysine	17.6782	56-87-1
38.	Tyrosine	17.8384	60-18-4
39.	Pantothenic acid	18.3562	137-08-6
40.	Gluconic acid	18.4924	526-95-4
41.	Palmitic acid	18.9164	64519-82-0
42.	Allantoin	19.1470	97-59-6
43.	Ferulic acid	19.3364	1135-24-6
44.	Heptadecanoic acid	19.8285	506-12-7
45.	Linoleic acid	20.4459	60-33-3
46.	Tryptophan	20.4642	73-22-3
47.	Oleic acid	20.4928	112-80-1
48.	Stearic acid	20.7205	57-11-4
49.	Arachidic acid	22.3868	506-30-9
50.	Cellobiose	22.6877	528-50-7
51.	Phytosphingosine	23.8047	554-62-1
52.	Sucrose	23.9260	111-11-5
53.	Trehalose	24.7402	6138-23-4
54.	Stigmasterol	28.4532	110-15-6
55.	Raffinose	29.2360	10030-67-8
56.	Melezitose	29.4608	

Identification of metabolites in transgenic rice seeds: With reference to NIST08 and Fiehn databases, a total of 56 metabolites were identified in transgenic rice seeds, which mainly comprised amino acids, fatty acids, organic acids, sugars, and etc. (Table 1).

Metabolic profile comparison: As shown in PCA plot, metabolic profile of TP309-HSA-8 and TP309-8 was well separated, with X and Y axes accounting for 47.73% and 22.17% of the total variation, respectively (Fig. 2A). Consistent with PCA plot, PLS-DA analysis also showed different metabolic profiles between non-transgenic and transgenic seeds (Fig. 2B). However, clustering analysis couldn't separate the transgenic and non-transgenic rice seed samples into two groups definitely (Fig. 2C).

The metabolic profiles of the 8^{th} and 9^{th}generation transgenic rice lines were also compared. As shown in the PCA plot, X, Y and Z axes accounted for 65.07, 13.72 and 9.04 of the total variation, respectively (Fig. 3A). In addition, further PLS-DA analysis suggested different metabolic profiles between TP309-HSA-8 and TP309-HSA-9 rice samples (Fig. 3B). Furthermore, HCA plot also well separated TP309-HSA-8 samples from TP309-HSA-9 rice samples, which was consistent with the results of PCA and PLS-DA analyses (Fig. 3C).

Metabolite changes between the transgenic and non-transgenic rice seeds, and seeds of different generations: According to the fold change analyses, 12 differential metabolites were detected to be significantly changed between the transgenic and non-transgenic rice seeds ($p<0.05$), including 6 known compounds (trehalose, citric acid, valine, glycine, asparagine and pantothenic acid) and another 6 unidentified compounds (Table 2). Among the six identified compounds, valine had the largest fold change (FC) value, while pantothenic acid was the only one with a significantly lower level in the transgenic seeds ($p<0.05$) (Table 2). Meanwhile, 4 differential metabolites were detected between seeds of 8^{th} and 9^{th} generation transgenic rice, including asparagine, oleic acid (C18:1) and two unidentified compounds (Table 3).

Pathway annotation of the differential metabolites: Pathway annotation of the differential metabolites was performed to further understand their biological functions. As shown in Table 4, linestrehalose was involved in the starch and sucrose metabolism and ABC transporters pathway; Citric acid was involved in the carbon fixation pathways in prokaryotes; Valine appeared in the valine, leucine and isoleucine degradation and biosynthesis pathways; Glycine was in the glycine, serine and threonine metabolism pathway; while pantothenic acid was defined in the antidyslipidemic agents pathway. The asparagine and oleic acid (C18:1) were not enriched in any KEGG pathway.

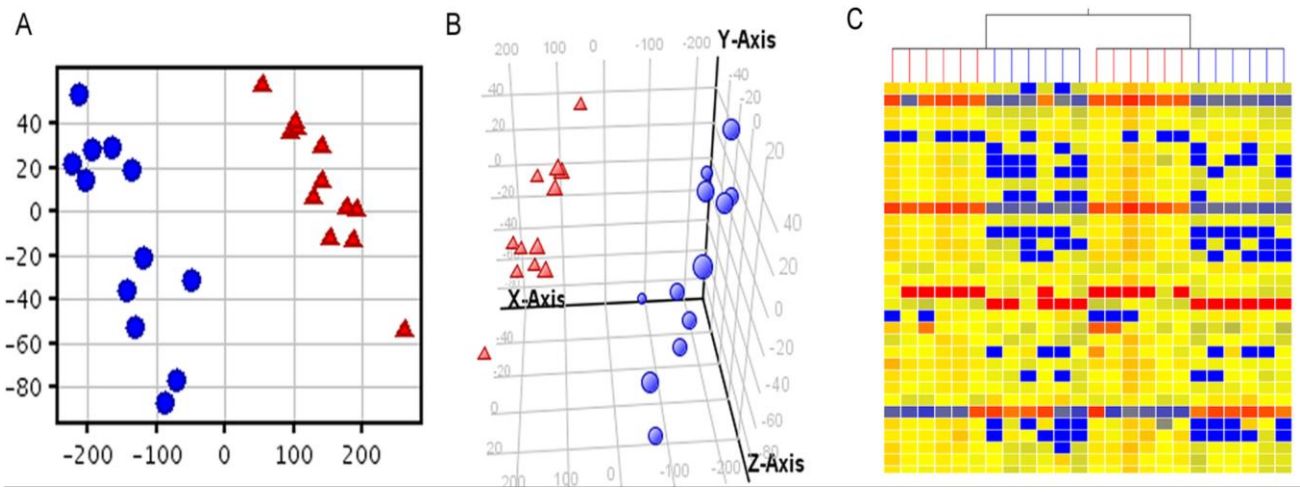

Fig. 2. Effects of HSA insertion on the metabolic profiles of TP309 rice.
A, Principal component analysis between TP309-9 (blue circles) and TP309-HSA-8 (red triangles) metabolites; B, Partial least squares-discriminate analysis between TP309-8 (blue) and TP309-HSA-8 (red) metabolites; **C**, Hierarchical cluster analysis of TP309-8 and TP309-HSA-8 based on the identified metabolites, in which the red line represents TP309-HSA-8 while the right blue line represents TP309-8. HSA, human serum albumin; TP309-8, wild-type rice; TP309-HSA-8, the transgenic rice developed via backcrossing of the 8th generation to the recurrent parent TP309.

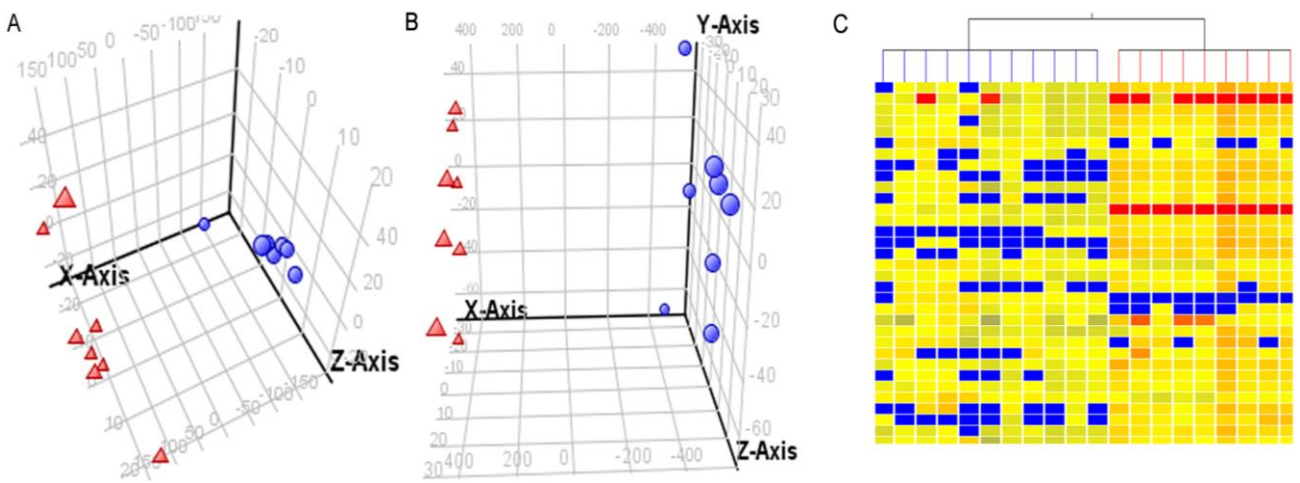

Fig. 3. Effects of backcrossing generation on metabolite profiles.
A, Principal component analysis between TP309-HSA-9 (blue circles) and TP309-HSA-8 (red triangles) metabolites; B, Partial least squares-discriminate analysis between TP309-HSA-9 (blue circles) and TP309-HSA-8 (red triangles) metabolites; C, Hierarchical cluster analysis of TP309-HSA-9 and TP309-HSA-8 based on the identified metabolites, in which the red line represents TP309-HSA-8 while the right blue line represents TP309-HSA-9. HSA, human serum albumin; TP309-HSA-9, the transgenic rice developed via backcrossing of the 9th generation to the recurrent parent TP309; TP309-HSA-8, the transgenic rice developed via backcrossing of the 8th generation to the recurrent parent TP309.

Table 2. Differential metabolites between HSA-transgenic and non-transgenic rice seeds (p<0.05).

Retention time (min)	Metabolite name	CAS No.	Fold change	Change tendency
9.20044	Valine	72-18-4	6.606358	Up
16.52989	Citric acid	5949-29-1	4.002328	Up
14.88629	Aspartic acid	70-47-3	3.119312	Up
18.28924	Pantothenic acid	137-08-6	2.742271	Down
20.40121	Trehalose	73-22-3	2.612823	Up
10.41067	Glycine		2.468308	Up
19.5066	Unknown		3.814303	Up
18.11752	Unknown		3.081557	Up
24.03593	Unknown		3.06135	Up
14.32267	Unknown		2.511684	Up
20.40115	Unknown		2.301549	Up
29.9295	Unknown		2.237321	Up

Note: HSA, human serum albumin

Discussion

HSA has important physiological functions as it has multi-binding potential features (Kragh-Hansen *et al*., 2013). Recombinant HSA produced by transgenic rice in a large-scale was suggested to use for production of commercial HSA to meet market demand (He *et al*., 2011). In this study, we showed that HSA insertion introduced an opaque appearance in the seeds compared to the wild-type counterpart. Based on our GC-MS data, multivariate statistical analysis revealed that metabolic profiles were different between the transgenic and the non-transgenic seeds, as well as between transgenic seeds of different generations.

Metabolomics study allows researchers to analyze metabolites in biological samples in both qualitative and quantitative manners (Want *et al*., 2007). In our study, 12

compounds had significantly different levels between the non-transgenic and transgenic rice seeds, including trehalose, citric acid, valine, glycine, asparagine, pantothenic acid and 6 unknown compounds based on GC-MS data. Our data suggested that introduction of HSA to rice genome may alter the metabolic profiles in the transgenic rice. This finding agreed with previous findings that genetic transformation or foreign gene introduction might introduce unintended compositional or physiological changes. Introduction of foreign genes may interfere with the original enzymes, thus leading to the accumulation or reduction of compounds in the host cells (Jiao *et al.*, 2010; Wu *et al.*, 2012). Therefore, we speculated that introduction of HSA might interact with some unknown enzymes leading to the increase or decrease of the 12 compounds. However, we could not exclude the possibility that the metabolite alterations might also be the result of environmental conditions change (Zhou *et al.*, 2009).

Introduction of HSA increased trehalose, citric acid, valine, asparagine and glycine, but decreased pantothenic acid level in transgenic rice seeds. Of those compounds, trehalose was related to starch and sucrose metabolism, whose pretreatment can protect against oxidative damage by reducing reactive oxy gen species (ROS) accumulation (Mostofa *et al.*, 2015). In plants, trehalose plays a key role in response to cold and salinity and in maintaining the sucrose and starch amounts (Lunn *et al.*, 2014). The increased trehalose induced by HSA insertion might be beneficial for rice growth by enhancing resistance to multiple abiotic stresses and increasing the sucrose and starch amounts in rice. Besides, as carbon fixation pathway is responsible for transforming CO_2 into organic compounds (Bar-Even *et al.*, 2010), the increase of citric acid associated with carbon fixation pathway in prokaryotes in this study might suggest an enhanced carbon fixation efficiency in transgenic rice. Also, three amino acids were identified to be increased in transgenic rice, among which valine was related to Valine, leucine and isoleucine degradation or biosynthesis pathways and glycine was associated with Glycine, serine and threonine metabolism. The valine, threonine, leucine and isoleucine were human essential amino acids (Wu, 2013), so the increase of valine and glycine may make transgenic rice produce more essential amino acid for human beings via changing amino acid metabolisms. Additionally, pantothenic acid was related to Antiyslipidemic agents, which is the precursor of 4'- phosphopantetheine moiety for coenzyme A essential for tricarboxylic acid (TCA) cycle (Chakauya *et al.*, 2008). Therefore, the decrease of pantothenic acid suggested that the TCA rate might have changed. In generally, the altered metabolites in transgenic rice did not suggest obviously harmful effects for human health but imply certain beneficial effects by increasing resistance to stimuli and amino acid production. However, the speculation needs further validation.

Meanwhile, 4 compounds, including asparagine, oleic acid (C18:1) and two unidentified compounds were found to have differential levels between TP309-HSA-8 and TP309-HSA-9 rice seeds. Backcrossing with the non-transgenic parental counterpart is advantageous for rapid elimination of unintended variations in transgenic plants (Zhou *et al.*, 2012). In backcrossing, a transgenic line with desirable traits is recurrently backcrossed to another non-transgenic line. In this study, HSA-transgenic rice seeds were obtained by recurrent backcrossing of the offspring containing exogenous HSA to the parental TP309, including TP309-HSA-8 and TP309-HSA-9. Although PCA or PLS-DA model indicated a separation between TP309-HSA-8 and TP309-HSA-9 samples, there were only 4 compounds contributing to the classification. This result illustrated that TP309-HSA-8 was a relatively stable rice line without excessive metabolite difference with the TP309-HSA-9.

Table 3. Differential metabolites between 8th generation and 9th generation transgenic rice seeds (p<0.05).

Retention time(min)	Metabolite	CAS No.	Fold change	Change tendency
14.88182	Asparagine	70-47-3	3.305816	Down
20.42081	Oleic acid	112-80-1	2.086181	Up
23.93386	Unknown		24.38897	Up
18.1178	Unknown		3.210776	Up

Table 4. Pathway annotation of the identified differential metabolites.

Differential metabolites	Pathway	Image_linked_help
Trehalose	map00500: Starch and sucrose metabolism	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map00500&keyword=trehalose
	map02010: ABC transporters	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map02010&keyword=trehalose
Citric acid	map00720: Carbon fixation pathways in prokaryotes	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map00720&keyword=citric%20acid
Valine	map00280: Valine, leucine and isoleucine degradation	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map00280&keyword=valine
	map00290: Valine, leucine and isoleucine biosynthesis	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map00290&keyword=valine
Glycine	map00260: Glycine, serine and threonine metabolism	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map00260&keyword=glycine
Asparagine	None	
Pantothenic acid	map07052: Antidyslipidemic agents	http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map07052&keyword=pantothenic%20acid

Conclusion

Our findings preliminarily reveal transgenic HSA may promote the accumulation of trehalose, citric acid, valine, glycine and asparagine metabolites in rice. These metabolites may be beneficial for rice growth and providing more essential amino acid for human beings. Since identifying the compositional alternations in transgenic crops is an important step for biosafety assessment (Reynolds *et al.*, 2005), this study might provide some useful information for risk assessment of HSA-transgenic rice in future researches.

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