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Metabolic profiling of transgenic rice with *cryIAc* and *sck* genes: An evaluation of unintended effects at metabolic level by using GC-FID and GC–MS

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ABSTRACT

The *cryIAc* and *sck* genes were introduced to the rice for the purpose of improving the insect resistance. Metabolic profiles of wild and transgenic rice were compared to assess the unintended effects related to gene modification. Wild samples with different sowing dates or sites were also examined to determine the environmental effects on metabolites. The polar compounds of grains were extracted, trimethylsilylated and analyzed by gas chromatography-flame ionization detection (GC-FID). Partial least squares-discriminant analysis (PLS-DA) and principal component analysis (PCA) were applied to differentiate transgenic and wild rice grains. The significantly distinguishable metabolites were picked out, and then identified by gas chromatography–mass spectrometry (GC–MS). It was found that both the environment and gene manipulation had remarkable impacts on the contents of glycerol-3-phosphate, citric acid, linoleic acid, oleic acid, hexadecanoic acid, 2,3-dihydroxypropyl ester, sucrose, 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester and so on. Sucrose, mannitol and glutamic acid had a significant increase in transgenic grains in contrast to those in non-genetically modified (GM) rice.

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1. Introduction

The focused concern about genetically modified crops and products is whether biotechnology causes wide unexpected effects and how much the level is. There has been no confirmed proof about harmful effects related to genetically modified (GM) crops or products [1]. Anyhow, it is essential that genetic products should be subjected to detailed safety assessment including unintended effects before entering into market [2].

The concept of substantial equivalence was elaborated by the Organization for Economic Co-operation and Development (OECD) [3]. The core is the comparison of the GM crop or product with its closest traditional counterparts. Targeted analysis [4,5] of specific compounds especially the nutrients, anti-nutrients and toxicants is the currently accepted approach to assess compositional equivalence. But it is biased and probably omits some useful information due to gene modification, especially some unforeseen and unintended effects. Non-targeted approaches like genomics [6–9], proteomics [10–12] and metabolomics [13–15] have been developed to identify unpredicted changes of gene, protein and metabolite, and have become important tools complementary to safety assessment [16]. Several technologies including GC–MS [17–19], LC–MS [20–22], NMR [23–25], and FT-MS [26] have been established in metabolomic studies. Especially, GC–MS has long been used for metabolite profiling analysis of plant and microorganism with a relatively broad coverage of analytical compounds and easy peak identification. However, the responses from MS were strongly influenced by the ionization potential of particular metabolites. Compared to GC–MS in full scan monitoring mode, GC-FID is considered of higher sensitivity, more reliable repetition and wider dynamic range, thus, can be better adopted in quantitative analysis. Combining GC–MS and GC-FID to utilize both advantages is more preferable and has been applied to many types of samples [27,28].

Oryza sativa L. is a major food crop consumed by a half of the world's population. During the growth period, disease, insect pest and abiotic stress (drought, heat, cold, salt etc.) are main factors causing the yield reduction. To solve the above-mentioned problems, modern biotechnology is applied in rice breeding which introduces improved agronomic characteristics. Two kinds of insecticidal genes including *Bacillus thuringiensis* gene and cowpea trypsin inhibitor gene (Bt and CpTI genes) are widely used for the production of insect-resistant plants. Bt gene from *Bacillus thuringiensis* strains encoding toxin proteins (Cry or Cyt protein) has been introduced into crop plants for its activity against lepidoptera pests but not mammals [29–32]. Bt insecticidal protein has a rapid and strong effect but relatively narrow spectrum of insect-resistant and it is easy to induce insect tolerance. *Sck* gene,

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a modified cowpea trypsin inhibitor gene has enough resistance to lepidoptera and part of the coleopteran pests which is often used together with *cry* gene [33–35]. The double transgenic rice with *sck* and *cryIAc* genes combined different insect-resistant mechanism, exhibited wider anti-insect spectrum and higher resistance against the pests than the single transgenics [36]. Some work has been carried out to assess the changes of proteins, metabolites, genes and physicochemical properties of the GM rice [21,37–43]. Most of the studies on metabolism only paid attention to the key nutrients and anti-nutrients such as proximates, fiber compounds, total amino acids, total fatty acids, micronutrients, phytic acid, trypsin inhibitors, lectins and so on. Researches of the global, untargeted metabolite profile of transgenic rice were relatively few.

To investigate the unintended effects of transgenic rice expressing extraneous *sck* and *cryIAc* genes at metabolic level, we developed a metabolic profiling method based on GC-FID and multivariable analysis to find out the metabolic variation between GM and non-GM grains. Rices sowed at different dates and sites were also taken into account to evaluate environmental influence. The two effects were compared to determine whether the variation related to gene insertion exceeded the range of environmental influence. The compounds with significant differences in concentration were identified by GC–MS.

2. Experimental

2.1. Chemicals

Pyridine and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) used for silylation were purchased from Sigma–Aldrich (Beijing, China). Ultra-pure water from Milli-Q system (Millipore, MA) was used in the experiment.

Standards of amino acids, sugars, organic and fatty acids used for identification were purchased from Sigma–Aldrich (Beijing, China) and prepared in the mixed solvents of water and methanol (TEDIA, Fairfield, OH).

2.2. Samples

The rice (*O. sativa* L.) for study was provided by the Key Laboratory of Agriculture Genetic Engineering, Fujian Academy of Agricultural Sciences (Fuzhou). The transgenic rice was developed by Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. M86 was used as the host for the *cryIAc* and *sck* genes. The marker-free transgenic samples (N6: N6080, N6130 and N6188) integrated with two insecticidal genes were grown side-by-side with the wild parent (M86-C) in the same period (sowed on Nov. 25, 2005). Additionally, there were other wild M86 samples from different sowing dates or sites including M86-D1 (sowed on Dec. 15, 2005), M86-D2 (sowed on Dec. 25, 2005), and M86-F (sowed on May 25, 2005). All samples but M86-F sowed in Fuzhou City, Fujian Province, China were planted in and obtained from the experimental field of Hainan Province, China. Detailed sample information was listed in Table 1.

Table 1
Sample information.

Sample	Description	Sowing time and site
M86-C	Wild comparator	25/11/2005, Hainan (18.33°N, 109.52°E)
M86-D1	M86 sowed at different time	15/12/2005, Hainan (18.33°N, 109.52°E)
M86-D2	M86 sowed at different time	25/12/2005, Hainan (18.33°N, 109.52°E)
M86-F	M86 planted at different time and site	25/05/2005, Fujian (26.1°N, 119.3°E)
N6*: N6080, N6130, N6188	M86 transformed with *cryIAc* and *sck* genes without the marker gene hpt	25/11/2005, Hainan (18.33°N, 109.52°E)

N6*: 3 lines of transgenic rice.

2.3. Sample preparation

Rice grains in the husks were stored at 4 °C until analysis. Brown rice after hulling were ground and passed through a 60-mesh sieve before extraction. About 300 mg powder was weighed into a 10-mL tube. 3 mL of methanol–water mixture (4:1, v/v) and 80 μL capric acid (0.3 mg/mL) internal standard (IS) were added for extraction. This was followed by vortex-mixing for 1 min. Sample was soaked for 30 min at room temperature and then sonicated for 40 min. After centrifugation at 12,000 × *g* for 10 min, 2 mL of supernatant was lyophilized in a Labconco Freezone 4.5 freeze dry system (Kansas, USA). The dried polar extract was trimethylsilylated using 90 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) and 80 μL of pyridine at 75 °C for 45 min to increase the volatility of polar compounds with functional groups, such as —OH, —SH, or —NH group.

2.4. Instrument analysis

1 μL of the derivatized sample was injected into an Agilent 6890 GC-FID for profiling analysis and a Shimadzu QP 2010 GC–MS (Kyoto, Japan) for identification of important compounds using a 10:1 split injection ratio. All samples were analyzed in a randomized order. To diminish the residue of several strongly retained substances, a blank run was inserted every two samples. A HP-5 fused silica capillary column of 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness (J&W Scientific, USA) was used. The initial oven temperature was 60 °C for 4 min, ramped to 170 °C at 8 °C/min, and then to a target temperature of 285 °C at 4 °C/min with duration of 15 min. The linear velocity of carrier gas (helium) was constant at 40 cm/s. The inlet temperature was 285 °C, and the FID was 300 °C with a data acquisition rate of 20 Hz. For QP 2010 GC–MS, the parameters of gas chromatography were the same as those of GC-6890. Mass spectra were acquired using full scan monitoring mode with a mass scan range of 44–600 *m*/*z* at a scan speed of 1000 u/s. The ionization mode was electron impact at 70 eV and a detector voltage was 0.9 kV. The temperatures of the ion source, transfer line and interface were maintained at 200 °C, 285 °C and 285 °C, respectively.

2.5. Data analysis

Peak alignment method was the same as our previous work [44], briefly to say, peaks with a signal to noise ratio (S/N) > 15 were extracted by Agilent ChemStation. Peak alignment was carried out by comparing retention times with those in a reference chromatogram which had the most peaks among all samples. The matching window of retention time was set to 0.1 min. The value of zero was given to the peaks with intensity lower than the detection limit in the corresponding time window. To minimize the number of missing values, peaks present in less than 80% samples were discarded from the total peak list [45]. In the end, all peak areas were normalized to the internal standard and then subjected to statistical analyses.

Multivariate statistics was performed using Soft Independent Modeling of Class Analogy (SIMCA)-P (version 11.0, Umetrics AB, Umea, Sweden). All variables were Pareto scaled prior to principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) for classification. Loading plot was employed to screen the notable metabolite differences in PCA. Variables located far from the origin contributed greatly to the discrimination and were extracted for further analysis. For PLS-DA [46], variables with

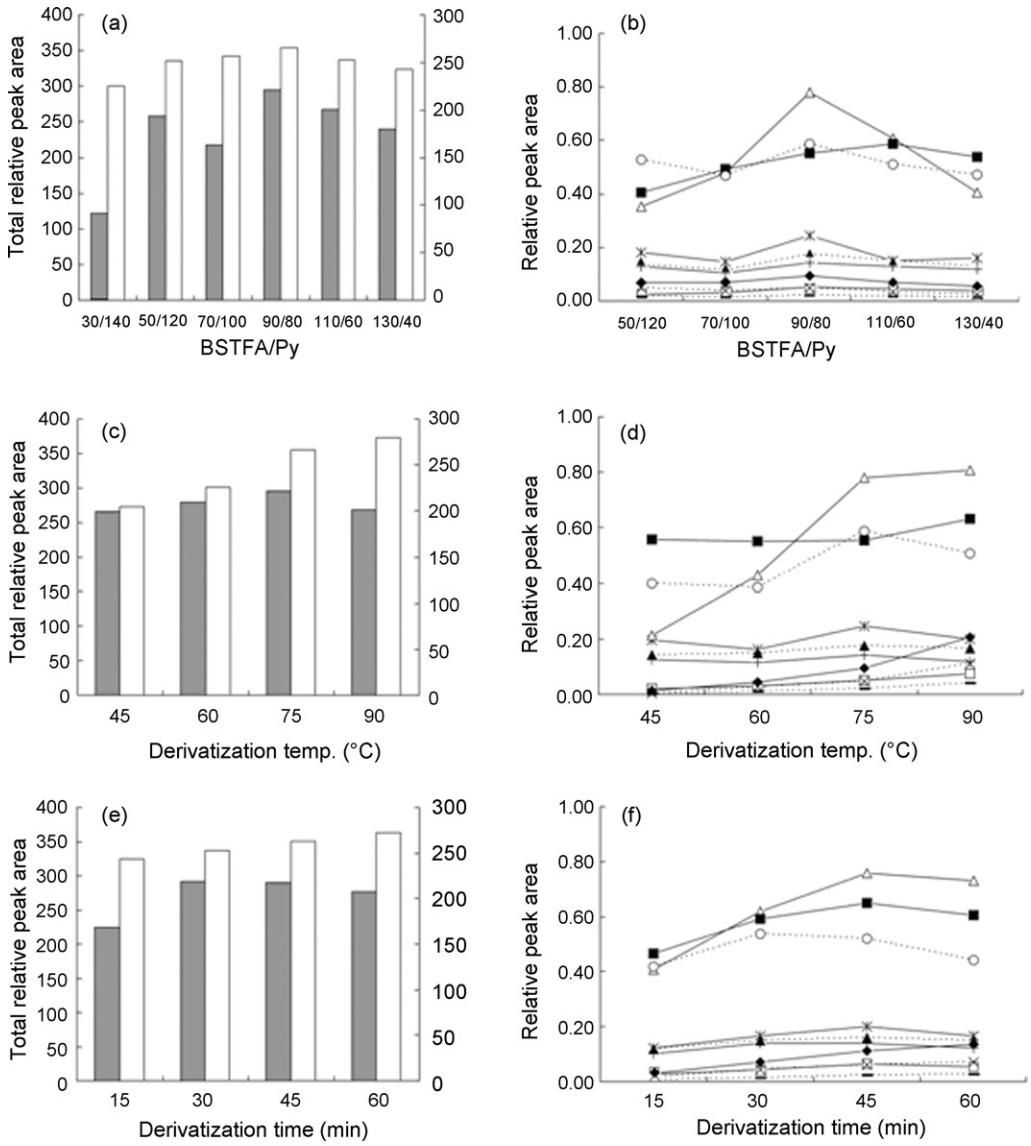

Fig. 1. Effect of silylation conditions on the number of peaks, total relative peak area and the peak areas of ten randomly selected peaks (corrected by IS). (a) and (b) BSTFA/pyridine ratio (v/v, μL); (c) and (d) derivatization temperature; (e) and (f) duration time for silylation. ■: Total peak area (corrected by IS). □: Number of peaks detected. Symbols and peak retention time (min): (···-···) 7.77; (—□—) 8.40; (—♦—) 8.95; (···×···) 12.76; (—×—) 16.73; (—△—) 17.42; (. . .▲. . .) 19.90; (—■—) 27.68; (—+—) 32.49; (···◯···) 36.36.

VIP (Variable Importance in the Projection) > 1 which played important roles in the classification were picked out, and the Jack-knifing confidence interval was taken into account [47]. In the meantime, the PLS-DA S-plot reflecting both the covariance and correlation information were used to reduce the risk of false positives [48]. Subsequently, independent *t*-test was applied to exclude the variables without significant differences ($p > 0.05$) between the control and compared groups (SPSS 13.0). Compounds finally selected were identified via GC–MS by comparing their mass spectra to the NIST (National Institute of Standards and Technology, USA) library and standard compounds.

3. Results and discussion

3.1. Optimization of derivatization conditions

The derivatization and instrument analysis conditions were optimized using a wild sample (M86). Several factors which have strong effects on derivatization efficiency were investigated, including the temperature, time and ratio of derivatization reagent to solvent. The final method parameters were chosen according to both the total peak areas (divided by internal standard area) and the number of peaks detected. The relative peak areas to IS for 10 randomly selected peaks were given in Fig. 1 which displayed the change tendency of individual peaks along with varied derivatization conditions.

The effect of BSTFA to pyridine (Py) ratio on derivatization efficiency was studied. When the volume ratio was changed to about 1:1, peak number and total peak area achieved optimum combination (Fig. 1a). Similarly, most of the relative peak areas for 10 randomly selected peaks reached the maximum at this point (Fig. 1b). The derivatization at the ratio of 30:140 was incomplete because of very small relative peak area. Typical gas chromatograms corresponding to 30/140, 90/80, 130/40 of BSTFA to pyridine are given in Fig. 2.

Changing the derivatization temperatures from 45 °C to 75 °C markedly improved peak responses (Fig. 1c and d). Unstable metabolites might degrade when the derivatization temperature

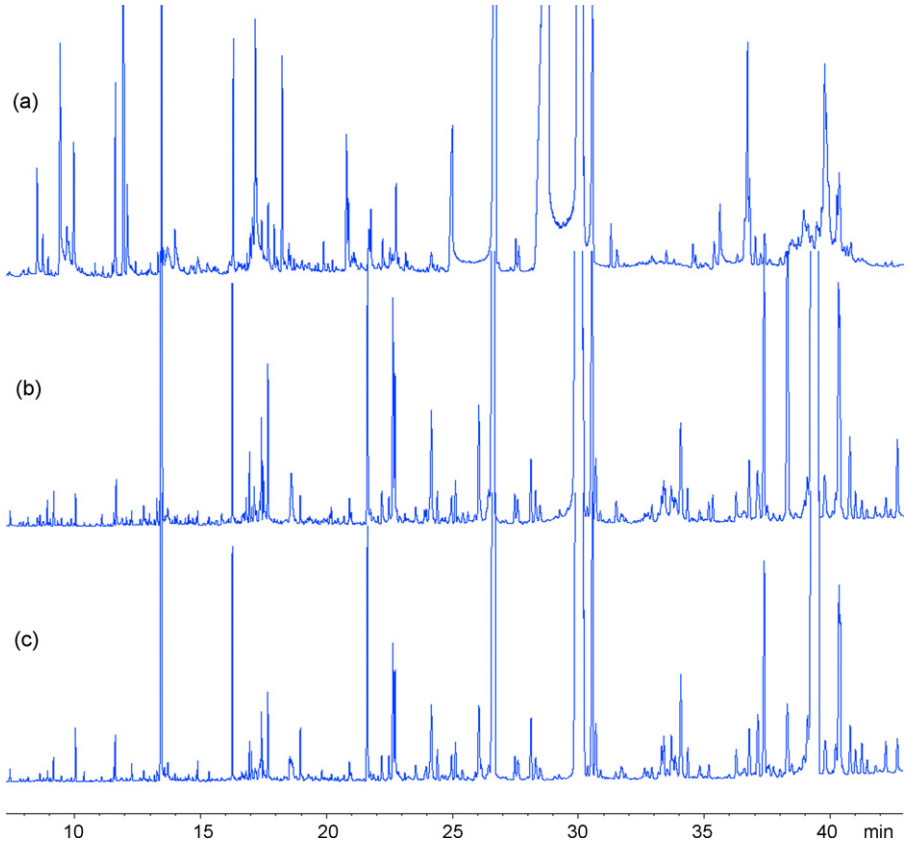

Fig. 2. Typical gas chromatograms obtained from different BSTFA/pyridine ratios (v/v, μL): (a) 30/140; (b) 90/80; and (c) 130/40.

was too high, so 75 °C was more suitable than 90 °C to prevent thermal decomposition.

Duration time for derivatization also caused changes in both peak number and peak area (Fig. 1e and f). The peak number and area showed no further increase when the reaction time was longer than 45 min. To shorten time, 45 min was selected for derivatization.

The stability of sample after silylation was tested for the batch analysis. A derivatized sample was placed on the autosampler and injected every 12 h during 72 h period. It can be observed that the total peak area only had a little change from 0 h to 24 h but significantly decreased after 36 h, therefore, the derivatized samples should be analyzed within 24 h. The relative standard derivations (RSDs) of relative peak areas of 10 random peaks to IS were lower than 11% at the end of 24 h besides the peak at 27.68 min with a 15.29% RSD. (Table 2).

Instrument precision experiment was performed using M86 sample. One M86 rice extract was prepared as described in Section 2 and analyzed 5 times continuously. 20 peaks were picked out at random, the RSDs of these peak areas (corrected with IS) were calculated, it is observed from Table 3 that the RSDs < 15%.

Finally, the reproducibility of sample preparation was explored under the defined conditions. Five replicated M86 samples were prepared and analyzed. The RSDs of relative peak areas of 20 peaks to IS varied from 1% to 15% (Table 3). For matabolomics study, until now there is no official guidance for method analytical characteristics, but the "Guide for Industry: Bioanalytical Method Validation" (http://www.fda.gov/cder/guidance/4252fnl.pdf) can be used for reference, based on this guidance, we can know our method for rice metabolites was reliable for further analysis.

3.2. Multivariate analysis of metabolic profiling

33 samples were pretreated and analyzed as mentioned in Sections 2.3 and 2.4. There were five samples from M86-D1, M86-D2 and M86-F, respectively, as well as nine M86-C samples and nine

Table 2
Results of stability experiment.

Peak no.	tr (min)	RSD[1] (%)	RSD[2] (%)	RSD[3] (%)
16	7.77	10.25	10.51	14.01
20	8.40	1.13	9.14	12.67
25	8.95	5.52	5.83	9.17
44	12.76	1.69	10.65	20.04
73	16.73	0.25	0.98	0.96
79	17.42	0.68	6.48	7.59
94	19.90	5.58	4.85	4.23
131	27.68	15.29	12.46	11.45
150	32.49	2.93	3.85	5.39
163	36.36	2.50	4.21	5.73

RSD[1] (%), RSD[2] (%) and RSD[3] (%) are RSD values in 24 h, 36 h and 48 h, respectively. tr: retention time.

Table 3
Results of precision and reproducibility experiments.

tr (min)	RSD[a] (%)	RSD[b] (%)	tr (min)	RSD[a] (%)	RSD[b] (%)
5.18	0.89	6.55	24.02	0.46	7.00
6.72	2.62	14.09	25.66	0.33	4.26
8.95	1.43	5.17	30.13	0.43	4.25
11.40	1.86	4.10	33.65	2.21	2.91
13.19	0.26	5.09	36.96	1.97	4.57
16.73	0.94	1.28	37.06	6.82	4.03
17.20	12.06	2.72	40.36	1.09	9.46
17.42	9.26	5.49	45.68	0.99	3.46
22.40	0.26	8.37	48.67	3.28	3.86
23.78	0.47	3.44	49.42	0.80	5.38

RSD[a] and RSD[b] are RSDs of peak areas for five successive injections and five replicate samples, respectively. tr: retention time.

GM samples (three for each line). Before pattern recognition, peaks were extracted and preprocessed (see Section 2.5) then a total peak list with 203 peaks was acquired. Finally, all peak areas were calibrated with the internal standard for the following statistical analyses.

3.2.1. Principal component analysis

3.2.1.1. Effect of different sowing dates and sites on metabolic profiling. To evaluate the influence of gene modification and environment change, PCA models for independent trial groups were constructed.

Seeds of M86 were sowed over a period of one month from November to December in the same location. In the score plot (Fig. 3a), three separated clusters corresponding to three respective seeding dates are observed. M86-C and M86-D1 located closer to each other, but M86-D2 at a greater distance which revealed that the metabolic differentiation was enhanced along with the delay of planting date. To find out the variables contributing to the classification, a loading plot (Fig. 3b) was applied as described in Section 2.5, and 12 variables were found with the most crucial influence on classification (Table 4). The ratios obtained by comparing the mean peak areas of M86-D1or M86-D2 to those of M86-C (Table 4) were used to measure the change levels of the selected 12 peaks. The value >1 meant the metabolites displayed increased concentrations and vice versa. It was observed that majority of these metabolites were present at higher levels in M86-D1 and M86-D2 than in M86-C. Especially, trehalose, sucrose, citric acid, oleic acid and glycerol-3-phosphate increased over 2-fold in M86-D2.

M86-F with different sowing site and date was compared to M86-C. A good separation was shown (Fig. 3c) between M86-F on one side and M86-C on the other side which suggested that the effect of sowing time and location on the seed metabolism was remarkable. The first principal component (PC1) explained 44% of total variability, and the second principal component (PC2) explained 31%. By using the same method as above (Fig. 3d), totally 14 peaks with evident change were selected after *t*-test, eight of them were confirmed with standard samples (Table 4). The metabolites with significant difference involved citric acid, malic acid, gluconic acid, sorbitol, trehalose, asparagines and glycerol-3-phosphate on the negative side which implied higher levels in M86-F, and linoleic acid and oleic acid on the positive side which meant a decreased level in M86-F.

3.2.1.2. Effect of gene modification on metabolic profiling. The unintended effects for insertion of *sck* and *cryIAc* genes were inspected using only GMs (N6) and wild comparator (M86-C). N6 were clearly separated from M86-C with PC1 explaining 83.6% original variances (Fig. 3e). Examination of the loading plot (Fig. 3f) describing the discrepancy of metabolic profiling

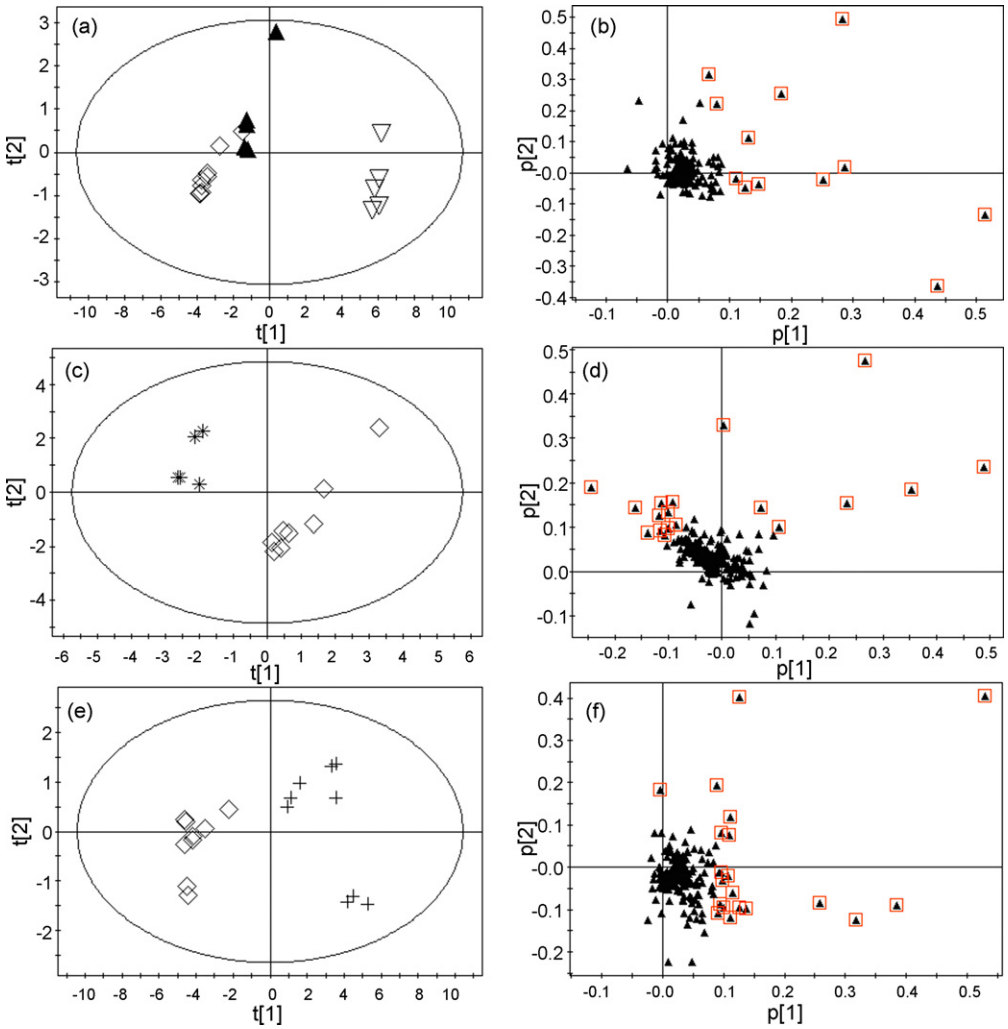

Fig. 3. PCA score plot (a) and loading plot (b) of M86-C, M86-D1 and M86-D2 (R2X = 0.875); PCA score plot (c) and loading plot (d) of M86-C and M86-F (R2X = 0.755); PCA score plot (e) and loading plot (f) of M86-C and N6 (R2X = 0.89). Symbols in score plots ◊: M86-C. +: N6. ▲: M86-D1.▽: M86-D2. *: M86-F. Red squares in loading plots symbolized that the selected variables were significantly different between groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 4
Metabolites responsible for separation.

Classification	tr (min)	Compound	Formula	Variance trend	
				M86-D1	M86-D2
M86-D1 and M86-D2 vs. M86-C	21.28	Glycerol-3-phosphate*	$C_3H_9O_6P$	↑, 1.4	↑, 2.7
	22.27	Citric acid	$C_6H_8O_7$	↑, 1.6	↑, 2.5
	29.57	Linoleic acid	$C_{18}H_{32}O_2$	–	↑, 1.3
	29.64	Oleic acid	$C_{18}H_{34}O_2$	↑, 1.3	↑, 2.2
	36.96	Hexadecanoic acid, 2,3-dihydroxypropyl ester*	$C_{19}H_{38}O_4$	↑, 1.2	↑, 1.3
	38.88	Sucrose	$C_{12}H_{22}O_{11}$	↑, 1.3	↑, 2.4
	39.29	UN	UN	↑, 1.5	↑, 4.0
	39.88	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester*	$C_{21}H_{40}O_4$	↑, 1.3	↑, 1.8
	40.56	Trehalose	$C_{12}H_{22}O_{11}$	↑, 40.7	↑, 392.9
	44.90	UN	UN	↑, 4.1	↑, 3.4
	46.88	UN	UN	↑, 1.3	↑, 1.4
	52.86	UN	UN	↑, 1.6	↑, 3.5
M86-F vs. M86-C	16.73	Malic acid	$C_4H_6O_5$	↑, 2.6	
	17.42	UN	UN	↑, 1.7	
	18.33	UN	UN	↑, 2.4	
	19.56	L-Asparagine	$C_4H_8N_2O_3$	↑, 3.1	
	21.28	Glycerol-3-phosphate*	$C_3H_9O_6P$	↑, 1.4	
	22.27	Citric acid	$C_6H_8O_7$	↑, 1.7	
	24.71	D-Sorbitol	$C_6H_{14}O_6$	↑, 7.8	
	26.01	D-Gluconic acid	$C_6H_{12}O_7$	↑, 2.8	
	29.57	Linoleic acid	$C_{18}H_{32}O_2$	↓, 0.9	
	29.64	Oleic acid	$C_{18}H_{34}O_2$	↓, 0.9	
	33.65	UN	UN	↑, 2.0	
	40.56	Trehalose	$C_{12}H_{22}O_{11}$	↑, 11.3	
	44.90	UN	UN	↑, 3.3	
	52.86	UN	UN	↓, 0.6	
N6 vs. M86-C	5.41	UN	UN	↑, 3.6	
	17.42	UN	UN	↑, 2.2	
	18.69	L-Glutamic acid	$C_5H_9NO_4$	↑, 3.4	
	21.28	Glycerol-3-phosphate*	$C_3H_9O_6P$	↑, 2.1	
	22.27	Citric acid	$C_6H_8O_7$	↑, 2.4	
	24.55	D-Mannitol	$C_6H_{14}O_6$	↑, 3.8	
	29.57	Linoleic acid	$C_{18}H_{32}O_2$	↑, 1.4	
	29.64	Oleic acid	$C_{18}H_{34}O_2$	↑, 2.1	
	33.65	UN	UN	↑, 1.8	
	36.96	Hexadecanoic acid, 2,3-dihydroxypropyl ester*	$C_{19}H_{38}O_4$	↑, 1.4	
	37.81	UN	UN	↑, 2.4	
	38.88	Sucrose	$C_{12}H_{22}O_{11}$	↑, 3.3	
	39.29	UN	UN	↑, 2.5	
	39.88	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester*	$C_{21}H_{40}O_4$	↑, 1.4	
	46.88	UN	UN	↑, 1.2	
	49.42	UN	UN	↑, 1.5	
	52.86	UN	UN	↑, 3.2	

↑ or ↓: higher or lower level in the compared group than that in M86-C group, respectively; the following value represents the peak area ratio of the compared group to M86-C group (with mean value of each group). tr: retention time; —: no significant difference existed between M86-C and the compared groups; UN: unidentified; *: identified only by NIST library (similarity >90%).

showed that mainly 17 variables with significant concentration changes were responsible for the separation (Table 4). 9 of 17 were identified, including carbohydrates (sucrose and mannitol increased 3.3- and 3.8-fold, respectively), organic acid (citric acid increased 2.4-fold), amino acid (glutamic acid increased 3.4-fold), fatty acid (linoleic acid, oleic acid increased 1.4- and 2.1-fold, respectively) and monoglyceride (glycerol-3-phosphate, 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester and hexadecanoic acid, 2,3-dihydroxypropyl ester increased 2.1-, 1.4-, 1.4-fold, respectively).

3.2.2. Partial least squares-discriminant analysis

To further display the effects of gene modification, the samples were generally divided into two classes—wild samples and GM samples which were put together for PLS-DA analysis. The score plot showed general separation using PC1 and PC2 (Fig. 4a). GM group was far from wild group which was dispersed to form several clusters corresponding to distinct sowing dates and sites, and M86-D2 in the remote region from other wild samples indicated that the sowing time also caused considerable changes of metabolites. All wild samples except M86-D2 were located in the positive side of the PC1 axis, while the GM samples appeared in the negative. GM group also had slight differences from non-GMs on PC2 which could be observed in the score plot (Fig. 4a). Additionally, three lines of N6 were widely distributed which hinted the discrepancies among lines were rather great. Based on VIP and S-plot (Fig. 4b), 15 compounds playing key roles in classification were found (Table 5) including sucrose, trehalose, mannitol, glutamic acid, citric acid, linoleic acid, oleic acid, hexadecanoic acid, 2,3-dihydroxypropyl ester, glycerol-3-phosphate, 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester and five unidentified compounds. To estimate whether the changes of above compounds in GMs were acceptable, the variation degree of each important metabolite was obtained by comparing the mean level of GM lines to that of non-GMs (Table 5). In addition to trehalose, other compounds in GMs had some degree of higher contents. It was particularly worth noting that sucrose, mannitol, glutamic acid and some unknown compounds had increments of more than 2-fold.

It has been known that metabolic phenotype differences result from gene insertion, growing conditions and so on. Changing

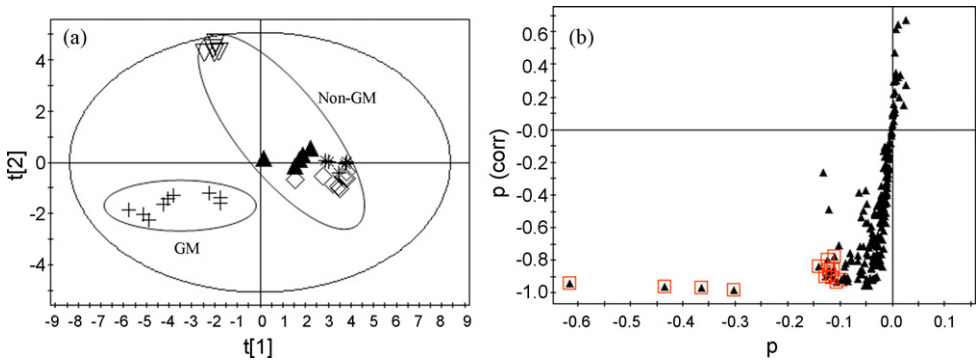

Fig. 4. Multivariate analysis of wild rice and GM rice. (a) PLS-DA score plot for PC1 and PC2 (R2Y = 0.98, Q2 = 0.969). (b) Corresponding S-plot. ◊: M86-C. +: N6.▲: M86-D1.▽: M86-D2. *: M86-F. Red squares in S-plot symbolized the selected variables which have significant difference between GM and non-GM groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 5
Metabolites responsible for separation of GM and non-GM groups.

tr (min)	VIP	Compound	Formula	*p*	Variance trend
5.41	1.43	UN	UN	<0.001	↑, 2.4
18.69	1.06	L-Glutamic acid	$C_5H_9NO_4$	<0.001	↑, 2.1
21.28	1.42	Glycerol-3-phosphate*	$C_3H_9O_6P$	<0.01	↑, 1.4
22.27	1.20	Citric acid	$C_6H_8O_7$	<0.001	↑, 1.5
24.55	1.41	D-Mannitol	$C_6H_{14}O_6$	<0.001	↑, 3.0
29.57	4.84	Linoleic acid	$C_{18}H_{32}O_2$	<0.001	↑, 1.3
29.64	3.64	Oleic acid	$C_{18}H_{34}O_2$	<0.001	↑, 1.6
36.96	1.53	Hexadecanoic acid, 2,3-dihydroxypropyl ester*	$C_{19}H_{38}O_4$	<0.001	↑, 1.3
37.81	1.68	UN	UN	<0.001	↑, 2.2
38.88	3.72	Sucrose	$C_{12}H_{22}O_{11}$	<0.001	↑, 2.4
39.29	1.21	UN	UN	<0.01	↑, 1.4
39.88	1.32	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester*	$C_{21}H_{40}O_4$	<0.01	↑, 1.2
40.56	6.36	Trehalose	$C_{12}H_{22}O_{11}$	<0.01	↓, 0.1
49.42	1.57	UN	UN	<0.001	↑, 1.4
52.86	6.44	UN	UN	<0.001	↑, 2.0

↑ or ↓: higher or lower level in GM group than that in the wild group, respectively; the following value means the peak area ratio of GM group to wild group. tr: retention time; UN: unidentified; *: identified only by NIST library (similarity >90%); *p*: *p*-value in independent *t*-test.

planting time and site both lead to environmental variation and somewhat affect the growth of plants. For compounds helpful in classification (Tables 4 and 5), carbohydrates including trehalose, sucrose, sorbitol, mannitol in grains were revealed by multivariate analysis as discriminatory metabolites. The level of trehalose changed significantly in non-transgenic samples as growth conditions varied, especially in M86 planted at different time. It enhanced with the delay of sowing date by 40-fold in M86-D1 and 400-fold in M86-D2 approximately. However, in GM samples, trehalose remained fairly stable compared to M86 grown side by side. We extrapolated that the content of trehalose might closely correlate with culture conditions and further investigation can be expected with respect to the mechanism for the quantity raise. The highest accumulation of sucrose was from GM samples which changed considerably 3.3-fold vs. M86-C and it was also strongly affected by growing date which rose 2.4 times in M86-D2. As for monosaccharide and sugar alcohol, the amount of mannitol upgraded by 3.8-fold after gene modification but slightly influenced by environment. M86-F group planted at Fuzhou was characterized by much higher level of sorbitol which was 8-fold vs. M86-C. Change of amino acids such as glutamic acid and asparagine also occurred. The concentration of glutamic acid was tripled in GM samples, and the increment was notably beyond the environmental influence. Moreover, 3.1-fold asparagine was observed in M86-F which was another feature for samples from Fuzhou. Palmitic acid, linoleic acid, oleic acid and octadecanoic acid were always the main fatty acid in grains. In M86-C, M86-F and M86-D1, these fatty acids are maintained at constant levels. However, both M86-D2 and GM samples contained more than twice oleic acid than that of M86-C, and the former appeared to be with the most abundant content. Three monoglycerides, glycerol-3-phosphate, hexadecanoic acid, 2,3-dihydroxypropyl ester and 9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester in GM samples did not show huge changes. GM samples contained about 2.4-fold citric acid in comparison with M86-C, and the variation was almost equal to that of M86-D2 which reached over 2.5-fold. In addition, over 2.5-fold increase of both malic acid and gluconic acid were indicated in M86-F. To sum up, the differences of above outlined metabolites in transgenic rice were within the same changing range as those in wild rice under various growth conditions except sucrose, mannitol, and glutamic acid.

4. Conclusions

The metabolic differences induced by genetic manipulation and environment were evaluated in the study by using GC-FID and GC–MS. Multivariate analyses (PCA and PLS-DA) were employed to visualize and analyze the metabolite information. It was indicated that growing conditions and gene modification induced similar influence on most of metabolites. For example, concentrations of glycerol-3-phosphate, citric acid, oleic acid and sucrose increased considerably in both M86-D2 and GM samples. Rice planted at different times was characterized with various amount of trehalose, and increases of malic acid, asparagine, sorbitol, gluconic acid was the major metabolic modifications caused by planting site alteration. In addition, sucrose, mannitol and glutamic acid were widely affected by gene insertion. There are lots of work remaining to be done in the future to get more information for safety assessment of

transgenic rice, the integration of metabolomics with proteomics and genomics will provide more comprehensive insight.

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