DOI:10.1021/jf902676y

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Unintended Compositional Changes in Transgenic Rice Seeds (*Oryza sativa* L.) Studied by Spectral and Chromatographic Analysis Coupled with Chemometrics Methods

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Unintended compositional changes in transgenic rice seeds were studied by near-infrared reflectance, GC-MS, HPLC, and ICP-AES coupled with chemometrics strategies. Three kinds of transgenic rice with resistance to fungal diseases or insect pests were comparatively studied with the nontransgenic counterparts in terms of key nutrients such as protein, amino acids, fatty acids, vitamins, elements, and antinutrient phytic acid recommended by the Organization for Economic Co-operation and Development (OECD). The compositional profiles were discriminated by chemometrics methods, and the discriminatory compounds were protein, three amino acids, two fatty acids, two vitamins, and several elements. Significance of differences for these compounds was proved by analysis of variance, and the variation extent ranged from 20 to 74% for amino acids, from 19 to 38% for fatty acids, from 25 to 57% for vitamins, from 20 to 50% for elements, and 25% for protein, whereas phytic acid content did not change significantly. The unintended compositional alterations as well as unintended change of physical characteristic in transgenic rice compared with nontransgenic rice might be related to the genetic transformation, the effect of which needs to be elucidated by additional studies.

KEYWORDS: Transgenic rice seeds; *Oryza sativa* L.; compositional changes; discrimination; chemometrics methods

INTRODUCTION

Rice genetic transformation has made rapid strides in the past 10 years. A number of agronomically important traits including enhancement of stress tolerance (*1*), quality improvement (*2*), and nutrition value increases have been introduced to rice (*3*). Rice fungal diseases such as rice blast and sheath blight are common diseases occurring during rice growth and affect rice production heavily (*4*). The desirable resistance to rice blast was achieved by introducing four antifungal genes (*RCH10*, *RAC22*, *β-1,3-Glu*, and *B-RIP*) into rice (*Oryza sativa* L. ssp. *indica*) (*5*). To increase the rice resistance to sheath blight, two antifungal protein genes (*RC24* and *β-1,3-glu*) were introduced into rice (*O. sativa* L. ssp. *indica*) (*6*). Research on transgenic insect-resistant rice was also reported, with *cry1AC* and *sck* genes introduced into rice (*O. sativa* L. Minghui 86) (*7*). PCR, Southern blot, and Northern blot analyses have demonstrated that these foreign genes in transgenic rice were inherited and expressed steadily in the following generations. Field demonstration also indicated that they had increased resistance to fungal diseases or insect pests (*Chilo suppressalis* and *Cnaphalocrocis medinalis*) (*8*, *9*).

Despite being the most important crop in the world, the commercialization of genetically modified rice has lagged behind other cereals such as maize. The reason could be that rice is a staple food crop in the world: its safety must be evaluated strictly prior to availability in the market. To ensure the safety of transgenic plants, a substantial equivalence principle was developed by OECD (*10*) and further elaborated by FAO/WHO (*11*). Comparison of the chemical composition of the genetically modified plant to that of a traditionally obtained counterpart has been a key element in the evaluation of substantial equivalence.

Several comparative studies have been reported for nutritionally enhanced rice (*12*), herbicide-tolerant rice (*13*), and insect-resistant rice (*14*). These studies presented the mean and standard error of contents of every determined component; after one-to-one comparison, good correspondence was found between transgenic and nontransgenic rice. More unbiased profiling technologies are considered as emerging technologies that would extend the breadth of comparative analyses, reduce uncertainty, and identify the need for further risk assessment (*15*). Nuclear magnetic resonance (NMR), near-infrared reflectance (NIR), gas chromatography−mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC) were often exploited in profiling studies. Chemometrics methods were suitable for classifying the large data set from transgenic and nontransgenic samples, such as visual assessment of the score plot and loading plot provided by principal component analysis (PCA) and dendrogram provided by hierarchical cluster analysis (HCA) as well as partial least-squares discrimination analysis (PLS-DA).

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 Published on Web 01/05/2010

The profiling approaches have been shown to be effective in identifying and describing the differences and similarities between the compositions of transgenic and nontransgenic plants (*16*, *17*).

In this study, the compositional differences between three kinds of transgenic rice seeds and their respective counterparts were evaluated. First, NIR fingerprint technology was conducted to obtain the comprehensive composition characteristic followed by more detailed profiles of amino acids, fatty acids, vitamins, etc., where significant differences were suspected. Data analysis procedures PCA and PLS-DA were applied to find the compounds responsible for differences between transgenic and nontransgenic rice seeds. Finally, the differences of discriminatory compounds were assessed by analysis of variance (ANOVA), and the varied extent was calculated. It is hoped that this study could provide some reference value for safety evaluation of transgenic rice from a compositional aspect and also propose a comparison method between transgenic rice and nontransgenic counterparts based on compositional differences.

MATERIALS AND METHODS

Standards and Reagents. Phytic acid and α-tocopherol (vitamin E) were obtained from Sigma (St. Louis, MO). Amino acids were biochemical reagents obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Vitamins B were purchased from Yueshen Scientific Instrument Co., Ltd. (Guangzhou, China). Linoleic acid, oleic acid, and palmitic acid were obtained from Guangdong Institute for the Control of Pharmaceutical Product (Guangzhou, China). The stock solutions of elements were obtained from the National Bureau of Standards (Beijing, China). All chemicals were of standard reagent grade unless otherwise stated.

Rice Samples. Three kinds of transgenic rice and the respective counterparts were included in this study. For convenience, T1, T2, and T3 were used to stand for three kinds of transgenic rice and C1, C2, and C3 for their respective nontransgenic counterparts (**Figure 1**). The first kind of transgenic rice (*O. sativa* L. ssp. *indica*), T1, contained four antifungal genes (*RCH10*, *RAC22*, *β-Glu*, and *B-RIP*) using a particle bombardment transformation method (*5*). The second kind of transgenic rice (*O. sativa* L. ssp. *indica*), T2, contained a rice basic chitinase gene *RC24*, an alfalfa *β*-1,3-glucanase gene (*β-1,3-glu*), and *p35H*, containing a hygromycin phosphotransferase gene *hpt*. They were simultaneously bombarded into rice (*6*). The third kind of transgenic rice, T3 (*O. sativa* L. Kefeng No. 6), was insect-resistant. Rice *O. sativa* L. Minghui 86 was used as transgenic parental control and host for *sck* gene, a modified cowpea trypsin inhibitor gene, and a *cry1Ac* gene from *Bacillus thuringiensis* (*7*). The transgenic rice was grown side by side with the nontransgenic rice to eliminate any influence from the growing conditions. All of the third generation of transgenic rice seeds was selected in this study. After harvest, the rice was dehulled, and brown rice seed samples were ground in a cyclone mill with an 80 mesh sieve for the determination of their chemical components.

NIR Spectral Analysis and Spectral Data Pretreatment. NIR spectroscopy analysis was carried out using a Foss NIR Systems 5000 monochromator (Silver Spring, MD). The powder was scanned in reflectance mode (1100−2500 nm). Reflectance data were stored as log (1/*R*), where *R* was the relative reflectance at 2 nm intervals (700 spectral data points). A tungsten−halogen lamp was used as light source, and a PbS detector was used to collect reflected light. For each sample (analyzed in triplicate), 32 scans were collected and averaged.

The preprocess and calculations were carried out using WinISI III software (InfraSoft International, Port Matilda, PA). Data preprocessing methods including standard normal variant transformation (SNV), first derivative, and second derivative were applied comparatively. SNV was a mathematical transformation method of the log (1/*R*) spectra used to remove slope variation and correct for scatter effects. Compared to SNV, first and second derivatives eliminated baseline drifts and enhanced small spectral difference. The derivative was taken at two gap data points. To avoid enhancing the noise, which was a consequence of derivative, spectra were first smoothed. This smoothing was done by using the Savitzky−Golay algorithm, which essentially performed a local polynomial regression on a series of values to determine the smoothed value for each point (*18*).

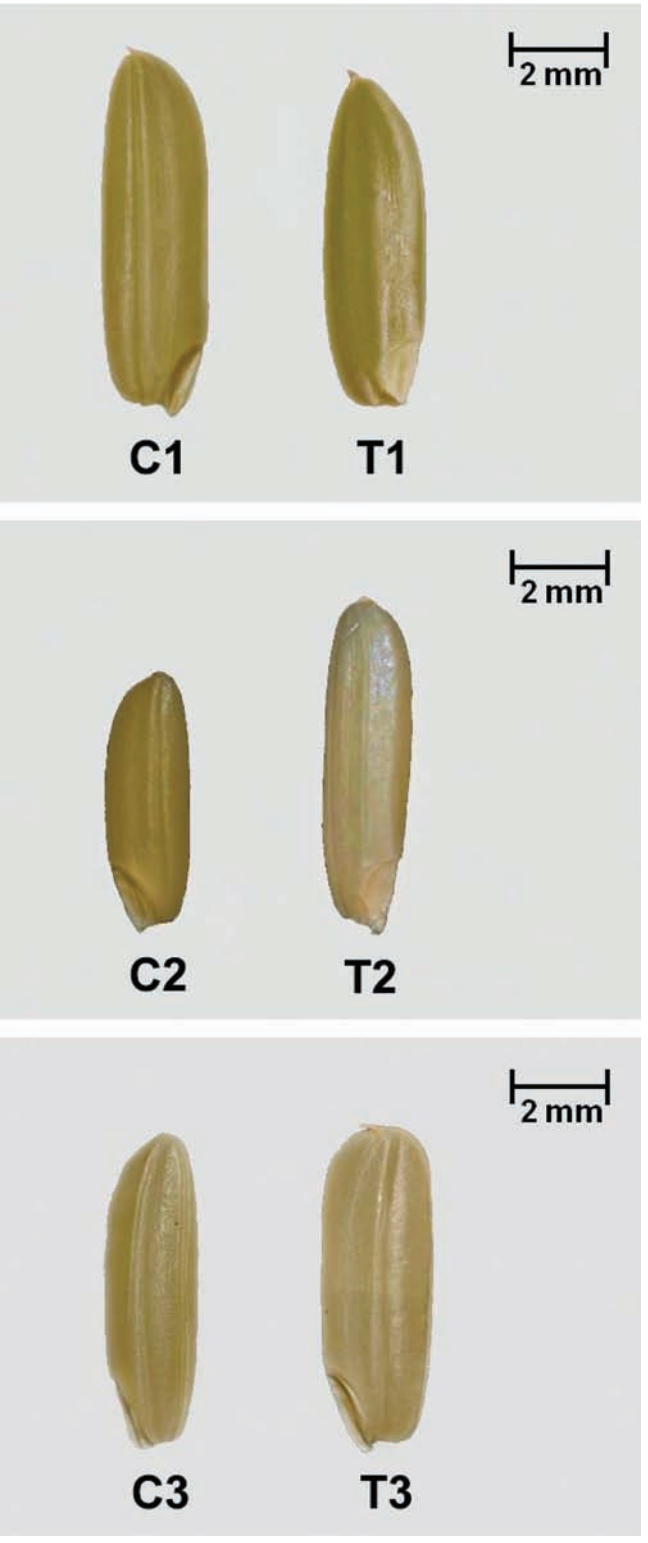

Figure 1. Comparative morphology between transgenic rice seeds (T1, T2, and T3) and their nontransgenic counterparts (C1, C2, and C3).

GC-MS Analysis. *Amino Acids*. Hydrolysis and derivatization of rice seed powder were performed in accordance with previously described procedures (*19*, *20*). Peptides and proteins were first completely hydrolyzed to yield free amino acids. Then after esterification and acylation, they were determined by GC-MS. Hydrolysis was performed with 0.5 g of rice seed powder in constant-boiling hydrochloride (mass fraction of 37%) under vacuum at 110 °C for 24 h. The hydrolyzed extract was made up to

25 mL, from which 0.05 mL was dried under a nitrogen flow and etherified with butyl acetate at 100 °C for 1 h, following acylation with trifluoroacetic acid for 20 min. After the sample had been dried, it was redissolved in 50 μL of ethyl acetate and 1 μL was injected. The chromatographic separation was carried out on a Shimadzu GC-MS-QP2010 system with a DB-5 coated fused silica capillary column (30 m × 0.32 mm, 0.25 μm film thickness) (J&W Scientific, Folsom, CA). One microliter of the derivative sample was injected into GC-MS using split mode (10:1). Ultrapure helium (constant flow, 1.5 mL min^{-1}) served as carrier gas with the purge flow of 3 mL min^{-1}. The injector temperature was 250 °C. The oven was programmed at the following rates. The initial temperature of the column was 100 °C (2 min hold) followed by a ramp of 10 °C/min to 140 °C (1 min hold), a second ramp of 10 °C/min to 170 °C (1 min hold), a third ramp of 15 °C/min to 185 °C (2 min hold), and finally a ramp to 230 °C at 15 °C/min (5 min hold). Mass conditions were as follows: electron impact ionization (EI); interface temperature, 250 °C; ion source temperature, 200 °C; detector voltage, 1 kV; solvent delay, 1.5 min. All data were obtained by collecting the full-scan mass spectra within the scan range of 30−600 amu. Amino acids were tentatively identified by comparing their mass spectra with those of the National Institute of Standards and Technology (NIST) library and further verified by comparing their mass spectra and retention times with those of authentic amino acids.

Fatty Acids. Fatty acids were assayed using AOAC official method 996.01. Rice seed powder (10 g) was placed in a Soxhlet extraction system and heated at 85 °C in a water bath for 8 h. Extraction solvent was petroleum ether (90−120 °C boiling point range). Then the extraction solvent was vaporized using a vacuum machine (Shenke Instrument, Shanghai, China) and redissolved in 10 mL of hexane and 2 mL of KOH in methanol solvent (0.5 mol/L). After 70 °C esterification for 30 min, distilled water (16 mL) was added to the vials; the upper layers of the extracts were withdrawn and vaporized under pure nitrogen stream and then redissolved in 1 mL of hexane for analysis by GC-MS. The GC-MS system was the same as that for amino acid analysis. One microliter of the derivative sample was injected into GC-MS using split mode (50:1). The injector temperature was 250 °C. The oven was programmed at the following rates. The initial temperature of the column was 100 °C followed by a ramp of 10 °C/min to 200 °C (2 min hold), a second ramp of 2 °C/min to 230 °C (2 min hold), and finally a ramp to 250 °C at 10 °C/min (5 min hold). Mass conditions were as follows: electron impact ionization (EI); interface temperature, 230 °C; ion source temperature, 250 °C; detector voltage, 1 kV; solvent delay, 3 min. All data were obtained by collecting the full-scan mass spectra within the scan range of 50−600 amu. Fatty acids were also tentatively indentified by NIST mass database, and then some selected fatty acids were further verified by authentic substances.

HPLC Analysis. *Vitamins B*. The analytical method of vitamins B was adapted from the literature (*21*). Rice seed powder of 3.0 g was extracted with potassium dihydrogen phosphate (0.05 mol/L) at 85 °C for 15 min by microwave-assisted extraction (CEM, USA). The mixture was centrifuged at 4000 rpm, and the upper layer was separated and made up to 25 mL. Analysis of vitamins B was carried out on an LC-2010C system (Shimadzu, Kyoto, Japan). The mobile phase consisted of eluent A (acetonitrile) and eluent B (potassium dihydrogen phosphate, 0.05 mol/L, pH 8.0). The Diamonsil C_{18} column (250 mm × 4.6 mm, i.d., 5 μm, Dikma, Beijing, China) was eluted with a linear gradient: 10% of solvent A (v/v) (0−7 min), 10−20% of solvent A (7−10 min), 20−28% of solvent A (10−20 min).

Vitamin E. The analytical method for vitamin E was adapted from that of Kurilich et al. (*22*). Twenty milliliters of ethanol containing pyrogallol (4.0%, m/v) was added to each rice seed powder (1.5 g) and saponified for 10 min with 50% potassium hydroxide by microwave-assisted extraction (MAS-I microwave oven, Sineo Microwave Chemistry Technology Co., China). The resulting solvents were extracted three times with 20 mL of ether, and the combined ether layers were washed by water to adjust the pH at 7.0. Then it was condensed in a rotor evaporator (Shenke Instrument, Shanghai, China). Residues following reduction were redissolved in ethanol and made up to 10 mL. Vitamin E analysis was carried out on an LC-2010C system (Shimadzu) and analyzed using an Inertsil ODS-P column (150 mm × 4.6 mm, 5 μm, i.d., 5 μm, Dikma) with a nonlinear gradient of 96% eluent A (methanol) and 4% eluent B (water). The analysis time was 20 min, and detection was done at 290 nm.

Phytic Acid. The analytical method for phytic acid was adapted from Lehrfeld (*23*). One gram of rice seed powder was extracted with 0.5 mol/L hydrochloric acid by microwave-assisted extraction for 10 min. The resulting extracts were centrifuged at 4000 rpm, and the upper layer was made up to 25 mL. All of the sample solutions were filtered by a 0.45 μm micropore filter before LC analysis. Detection of phytic acid was done under wavelength 254 nm on an LC-2010C system (Shimadzu). The Diamonsil C_{18} column (250 mm × 4.6 mm, i.d., 5 μm, Dikma) was eluted with a nonlinear gradient of 80% acetonitrile and 20% water over 25 min at a flow rate of 1.0 mL min^{-1}. Identifications of vitamins B, vitamin E, and phytic acid were based on retention time of known standards by HPLC.

ICP-AES analysis. The elements were analyzed using a method adapted from the literature (*24*). Half a gram of rice seed powder was digested with 5.0 mL of nitric acid and 2.5 mL of hydrogen peroxide for 6 min in a microwave system (Sineo Microwave Chemistry Technology Co.). Then the elements were determined by ICP-AES (Spectro, Kleve, Germany). The blank sample was prepared for corrections applied throughout the entire digestion step. Standard solution of each element was prepared for the calibration curves under the same condition. All glassware was washed with detergent, soaked for 24 h in 10% (v/v) nitric acid, rinsed with deionized distilled water, and dried before use for analysis.

Protein Analysis. Protein was analyzed using AOAC official method 2001.11. Protein was calculated from nitrogen content multiplied by the factor of 6.25.

Validation of Analytical Methods. The extraction conditions for the compositions above were optimized using an orthogonal test. Chromatographic analytical conditions were also optimized. The calibration curves (based on the integrated peak area) were calculated using five points at different concentrations, and each standard solution was injected three times. The precision, expressed by relative standard deviations (RSDs) of the concentration, was studied by extraction of the samples five times. The accuracy of the analytical method, achieved by recovery test, was conducted by spiking the rice samples with known concentrations of these compounds.

Data Analysis. Chemometrics analysis was performed based on the Unscrambler software (Camo ASA, Oslo, Norway). Data transformation in our study involved centering, scaling to unit variance, and log centering. PCA and PLS-DA were used to classify the transgenic and nontransgenic rices on the basis of the compositional profile differences (*25*). The output from PCA consisted of score plots to visualize the contrast between different samples and loading plots to explain the reason for cluster separation (*25*). PLS-DA was used in this study to establish calibration models for quantifying transgenic and nontransgenic rice. It is a partial least-squares application for the optimum separation of classes, and each sample was assigned a dummy variable of 1 or 0 as a reference value. It is an arbitrary number that indicated whether the sample belonged to a particular group or not (*26*). In this case, the NIR data from transgenic samples were assigned a numeric value of 1, and those of nontransgenic rice were assigned 0. The PLS-DA model was then developed by assigning the reference value for each sample. The transgenic sample would be classified correctly if the value was between 0.5 and 1.5, else the samples were classified incorrectly. It was a nontransgenic sample if the value was between −0.5 and 0.5.

The comparative assessment for target compound differences was made using ANOVA on Statistical Package for Social Sciences (SPSS) version 13.0 (USA). Statistically significant differences between mean values were deemed at $P < 0.05$, or else the differences will not be considered significant ($P > 0.05$).

RESULTS AND DISCUSSION

Physical Characteristics of Transgenic and Nontransgenic Rice Seeds. Physical characteristics are important factors influencing product characteristics (*27*). The outside appearances of three kinds of transgenic rice seeds and their respective counterparts are compared in **Figure 1**. The length, width, thickness, and weight of transgenic rice seeds were compared to those of the nontransgenic

counterparts (see Supporting Information). Among the three pairs of rice samples, the most marked difference of seed appearance was seen between seeds of C2 and T2, and the hardness of rice seed T2 decreased obviously compared to C2. The weight of rice seed T3 increased compared to C3. The minimum difference of seed appearance was seen between rice seeds C1 and T1.

Compositions Analysis of Transgenic and Nontransgenic Rice Seeds. NIR technology has been applied widely for rapid quantitative analysis of protein, fat, starch, and water content in rice seed samples. Thus, NIR was applied to study the overall compositional differences between transgenic and nontransgenic rice. The NIR spectrum for three kinds of transgenic rice and nontransgenic counterparts is displayed in **Figure 2A**. The absorption at 1200, 1460, and 1936 nm related to water content in rice seeds (*28*). The absorption at 2100 nm related to starch content in rice seeds (*29*). The absorption bands at 1726, 2306, and 2346 nm associated with fat content and the absorption at 2058 and 2174 nm are related to the peptide of the amide group and had high correlation with protein content in rice seeds (*30*). Discrimination between the third pair and the first two pairs of rice samples was observed, whereas transgenic rice and nontransgenic counterparts in each pair were not clear in NIR spectra. Therefore, it was necessary to conduct data analysis to obtain more knowledge about the potential differences.

In the following study, nutritional components including amino acids, fatty acids, vitamins, and elements and antinutrients were analyzed separately for three kinds of transgenic rice and their respective nontransgenic counterparts. The recoveries of the analytical methods for different compositions ranged from 81.5 to 109.3%, and the analysis precision was below 10.4%. The contents of 11 amino acids, 14 fatty acids, 19 elements, 5 vitamins, protein, and phytic acid in three kinds of transgenic rice seeds and the respective counterparts are provided in **Tables 1−3** (5 important element contents are listed in **Table 3**, the remaining 14 elements are listed in theSupporting Information). The reference value or reference range for the compounds in rice from the literature is also given. As seen in **Tables 1−3**, the values recorded were in agreement with the literature range provided by OECD (*31*). To find which compounds differentiated most between transgenic and nontransgenic rice seeds, PCA was conducted to discriminate different samples and distill the potential compounds that varied most between transgenic and nontransgenic rice.

PCA for the Compositional Differences. PCA of the NIR spectral data was performed after preprocessing, including the second derivative to reduce baseline variation and enhance the spectral features. After the application of PCA, a score plot was generated to visualize the results. If an unsupervised algorithm clusters samples close together, then they can be objectively considered to be similar, and if classes cannot easily be discriminated by supervised methods, then they are objectively similar (*15*). The results in **Figure 2B** show the distribution of the samples in the score plot with the first two principal components accounting for 75%. Each point represents a particular sample. First, rice samples of C3 and T3 were discriminated from C1 and T1 and from C2 and T2 along PC1, indicating that compositions such as fatty acids, amino acids, and protein might be quite different in these samples. Within each pair of transgenic and nontransgenic counterparts, the discriminations of C2 and T2 and of C3 and T3 were observed along PC2, which were more significant than C1 and T1.

The following study was carried out to develop a model based on the vibrational responses of chemical bonds to NIR radiation. Each model was established as follows: Each pair of transgenic

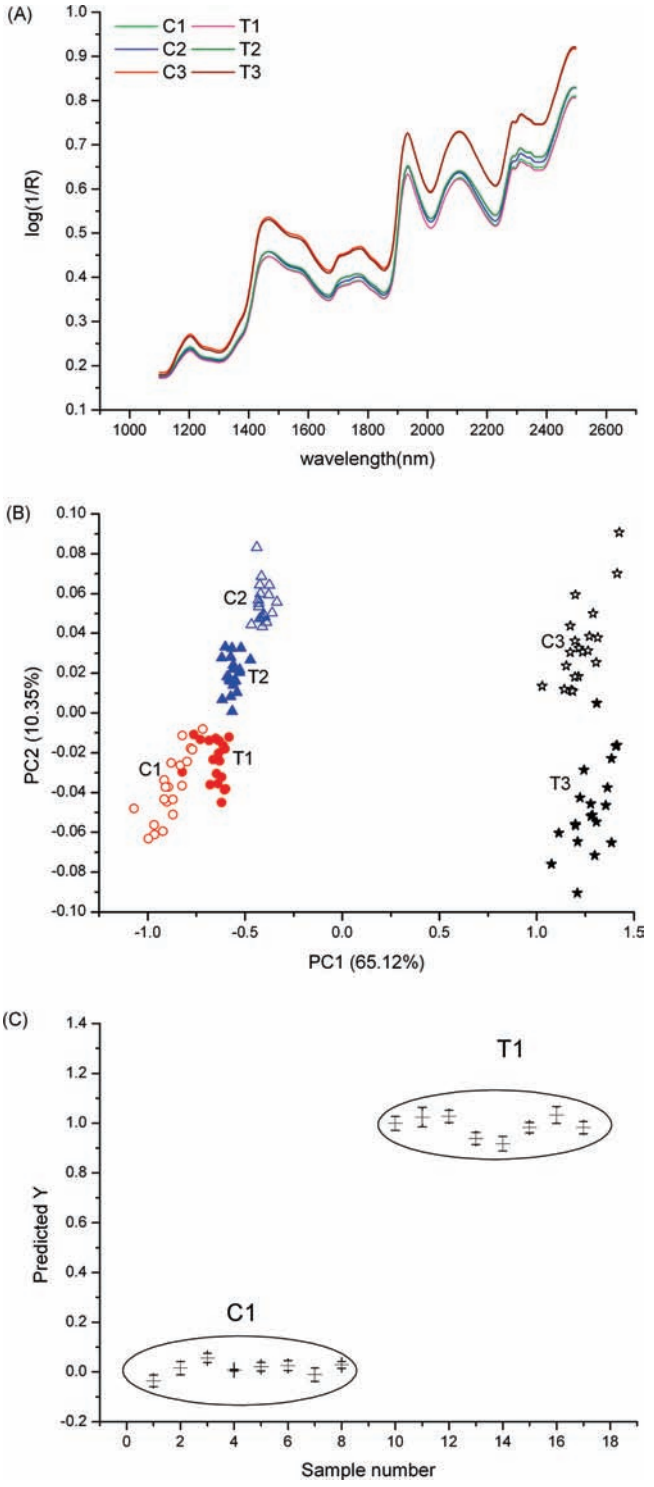

Figure 2. (**A**) NIR spectra for three pairs of transgenic rice and nontransgenic rice. (**B**) Score plot of PC1 and PC2 based on NIR spectrum of three pairs of transgenic rice and nontransgenic rice. (**C**) Prediction plot of transgenic rice T1 and nontransgenic rice C1 using PLS-DA model based on NIR spectra (samples 1−8 were nontransgenic rice, and samples 9−16 were transgenic rice).

and nontransgenic samples was divided into two sets. Twenty-four were assigned to training samples, and the remaining 16 test samples were predicted to see whether they would lie beside assigned values. It was expected to have ideal models with the lower root-mean-square error of calibration (RMSEC) and root-mean-square error of cross-validation (RMSECV) as well as the higher correlation coefficient of calibration and cross-validation,

Table 1. Concentrations (Grams per 100 g) of Amino Acids in Six Kinds of Rice Seeds[a] ($n = 5$)

	pair 1		pair 2		pair 3		
amino acid	C1	T1	C2	T2	C3	T3	ref range[a]
alanine	0.55 ± 0.06	0.73 ± 0.05	0.57 ± 0.03	0.50 ± 0.14	0.58 ± 0.04	0.35 ± 0.02	0.47
glycine	0.51 ± 0.03	0.29 ± 0.02	0.44 ± 0.03	0.34 ± 0.03	0.47 ± 0.02	0.13 ± 0.01	0.39−0.69
threonine	0.42 ± 0.04	0.37 ± 0.03	0.38 ± 0.04	0.45 ± 0.05	0.39 ± 0.02	0.34 ± 0.02	0.26−0.35
valine	0.62 ± 0.07	0.52 ± 0.03	0.57 ± 0.05	0.68 ± 0.04	0.59 ± 0.06	0.46 ± 0.03	0.44−0.58
leucine	0.91 ± 0.05	0.94 ± 0.07	0.84 ± 0.06	0.78 ± 0.05	0.86 ± 0.07	0.78 ± 0.05	0.60−0.68
isoleucine	0.43 ± 0.03	0.28 ± 0.03	0.39 ± 0.03	0.42 ± 0.03	0.41 ± 0.03	0.31 ± 0.02	0.30−0.43
proline	0.54 ± 0.04	0.49 ± 0.04	0.49 ± 0.03	0.41 ± 0.04	0.51 ± 0.05	0.53 ± 0.04	0.37
aspartic acid	0.97 ± 0.08	0.82 ± 0.07	0.89 ± 0.08	0.77 ± 0.05	0.92 ± 0.06	0.83 ± 0.07	0.81
phenylalanine	0.50 ± 0.05	0.38 ± 0.03	0.41 ± 0.05	0.35 ± 0.03	0.55 ± 0.04	0.51 ± 0.04	0.34−0.42
tyrosine	0.43 ± 0.07	0.62 ± 0.09	0.39 ± 0.06	0.58 ± 0.04	0.86 ± 0.08	0.51 ± 0.06	0.26−0.71
glutamic acid	1.51 ± 0.09	1.48 ± 0.12	1.44 ± 0.11	1.27 ± 0.13	1.64 ± 0.24	1.27 ± 0.08	1.59

[a] Source: OECD (2004).

Table 2. Relative Contents of Fatty Acids in Six Kinds of Rice Seeds[a] ($n = 5$)

	pair 1		pair 2		pair 3	
compound	C1	T1	C2	T2	C3	T3
tetradecanoic acid (C14:0)	0.30 ± 0.02	0.31 ± 0.03	0.30 ± 0.04	0.33 ± 0.03	0.30 ± 0.02	0.26 ± 0.03
heptadecanoic acid (C17:0)	0.27 ± 0.03	0.37 ± 0.04	0.46 ± 0.03	0.31 ± 0.04	0.33 ± 0.04	0.30 ± 0.02
hexadecanoic acid (C16:0)	17.77 ± 0.95	16.68 ± 1.17	16.41 ± 1.09	17.79 ± 1.55	16.27 ± 1.25	13.81 ± 0.99
10-undecenoic acid (C11:1)	0.28 ± 0.03	0.29 ± 0.03	0.35 ± 0.03	0.47 ± 0.05	0.36 ± 0.04	0.44 ± 0.03
9,12-octadecadienoic acid (C18:2)	30.23 ± 1.36	33.00 ± 2.07	29.28 ± 1.99	29.88 ± 2.32	29.11 ± 2.06	30.09 ± 2.08
9-octadecenoic acid (C18:1)	46.13 ± 2.18	43.05 ± 2.56	48.07 ± 3.64	45.70 ± 4.06	48.94 ± 3.30	48.90 ± 2.33
10-octadecenoic acid (C18:1)	0.42 ± 0.02	1.03 ± 0.09	1.08 ± 0.08	1.13 ± 0.10	1.31 ± 0.11	1.36 ± 0.14
octadecanoic acid (C18:0)	0.52 ± 0.04	0.53 ± 0.04	0.52 ± 0.03	0.56 ± 0.06	0.51 ± 0.04	0.43 ± 0.03
ethanol, 2-(9,12-octadecadienyloxy)- (C18:2)	0.55 ± 0.05	0.57 ± 0.06	0.51 ± 0.05	0.52 ± 0.05	0.50 ± 0.05	0.52 ± 0.04
9-octadecenoic acid (C18:1)	0.23 ± 0.02	0.36 ± 0.04	0.38 ± 0.04	0.40 ± 0.03	0.46 ± 0.03	0.48 ± 0.03
eicosatrienoic acid (C20:3)	1.01 ± 0.08	1.43 ± 0.13	0.63 ± 0.06	0.86 ± 0.07	0.74 ± 0.06	1.13 ± 0.09
octadecatrienoic acid (C18:3)	1.15 ± 0.10	1.20 ± 0.10	1.40 ± 0.09	1.54 ± 0.13	1.27 ± 0.08	1.49 ± 0.11
docosanoic acid (C22:0)	0.56 ± 0.04	0.66 ± 0.07	0.60 ± 0.07	0.56 ± 0.06	0.34 ± 0.02	0.70 ± 0.05
tetracosanoic acid (C24:0)	0.52 ± 0.05	0.55 ± 0.04	0.84 ± 0.05	0.86 ± 0.07	0.49 ± 0.03	0.86 ± 0.06

[a] Relative contents of fatty acids (%) = (peak area of fatty acid/total peak area of all fatty acids) × 100. Comparable information is not available for fatty acids of rice seeds.

Table 3. Concentrations of Elements, Vitamins, Crude Protein, and Phytic Acid in Six Kinds of Rice Seeds ($n = 5$)

	pair 1		pair 2		pair 3		
composition	C1	T1	C2	T2	C3	T3	ref range[a]
elements							
P (mg/g)	1.87 ± 0.11	2.01 ± 0.26	1.84 ± 0.09	2.02 ± 0.02	2.89 ± 0.07	2.97 ± 0.04	2.0−5.0
K (mg/g)	2.64 ± 0.14	3.20 ± 0.23	2.43 ± 0.08	3.28 ± 0.14	2.34 ± 0.19	2.57 ± 0.05	0.7−3.2
Fe (mg/100 g)	1.08 ± 0.11	1.31 ± 0.08	0.56 ± 0.05	0.71 ± 0.04	0.82 ± 0.06	0.42 ± 0.02	0.2−6.0
Na (mg/100 g)	7.34 ± 0.67	7.68 ± 0.74	7.71 ± 0.81	7.96 ± 0.36	6.83 ± 0.28	7.06 ± 0.26	2.0−40
Zn (mg/100 g)	2.95 ± 0.25	3.23 ± 0.41	3.04 ± 0.21	3.61 ± 0.04	3.44 ± 0.16	3.65 ± 0.07	0.7−3.3
vitamins (mg/100 g)							
vitamin B_1	0.37 ± 0.03	0.46 ± 0.04	0.34 ± 0.03	0.32 ± 0.02	0.37 ± 0.04	0.29 ± 0.01	0.14−0.38
vitamin B_2	0.09 ± 0.05	0.10 ± 0.02	0.07 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.02	0.04−0.13
vitamin B_3	4.32 ± 0.32	3.74 ± 0.29	4.05 ± 0.28	3.41 ± 0.16	4.78 ± 0.24	3.14 ± 0.22	1.46−6.50
vitamin B_6	0.70 ± 0.02	0.82 ± 0.03	0.74 ± 0.05	0.63 ± 0.01	0.72 ± 0.02	0.53 ± 0.20	0.5−0.9
vitamin E	2.14 ± 0.12	0.92 ± 0.01	1.34 ± 0.03	1.68 ± 0.04	1.56 ± 0.11	1.60 ± 0.09	0.67−3.47
crude protein (g/100 g)	10.21 ± 0.63	11.37 ± 0.82	9.38 ± 0.91	7.03 ± 0.69	9.68 ± 0.53	8.68 ± 0.27	6.7−8.9
phytic acid (g/100 g)	0.26 ± 0.02	0.25 ± 0.02	0.28 ± 0.02	0.26 ± 0.03	0.29 ± 0.02	0.30 ± 0.03	0.1−0.3

[a] Source: OECD (2004).

r_c and r_{cv}, respectively. **Figure 2C** presents prediction results showing that transgenic sample T1 was classified correctly as they were all lying around 1.0 and those for nontransgenic sample C1 were lying around 0 with deviation of < 0.04. It was similar for rice seed samples C2/T2 and C3/T3 (see Supporting Information). This suggested that PLS-DA models successfully discriminated the transgenic samples from their counterparts because of their difference in chemical components. Through NIR fingerprinting allied to chemometric study, we made a conclusion that the compositional differences existed between transgenic and nontransgenic rice seeds. In the following study detailed profiles for each class of compositions were subjected to PCA to find the differences.

The amino acid compositions in all rice samples were scaled and subjected to PCA. As seen in the PCA scores plots (**Figure 3A**), C1 and T1 and C2 and T2 discriminated mainly along PC2, whereas C3 and T3 discriminated along PC1. The PCA-derived loading plots complement score plots. The loading

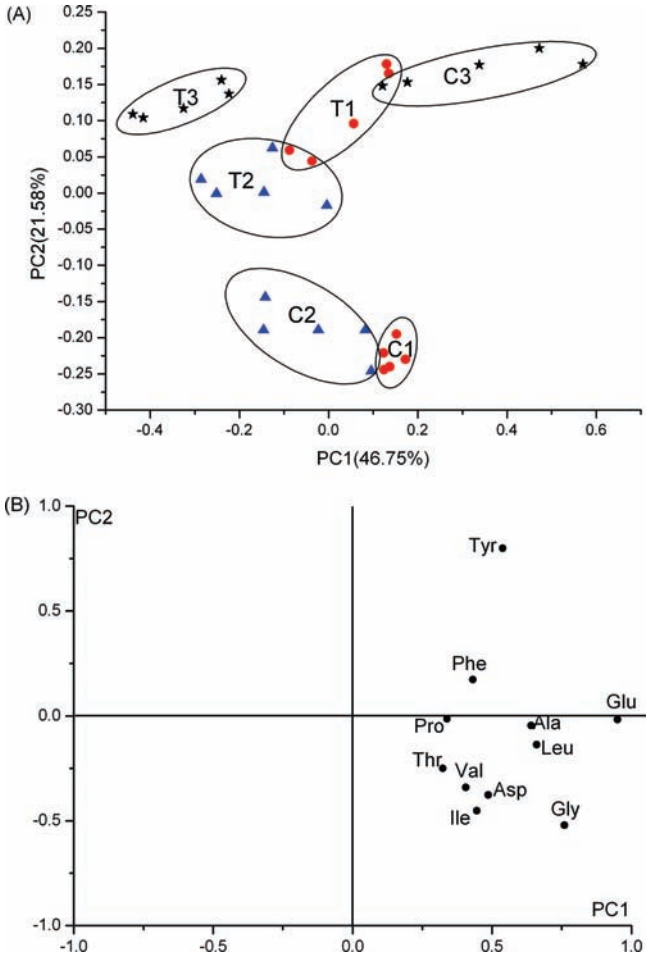

Figure 3. Score plot of PC1 and PC2 (**A**) and loading plot (**B**) for amino acid compositions from three pairs of transgenic rice and nontransgenic rice by PCA.

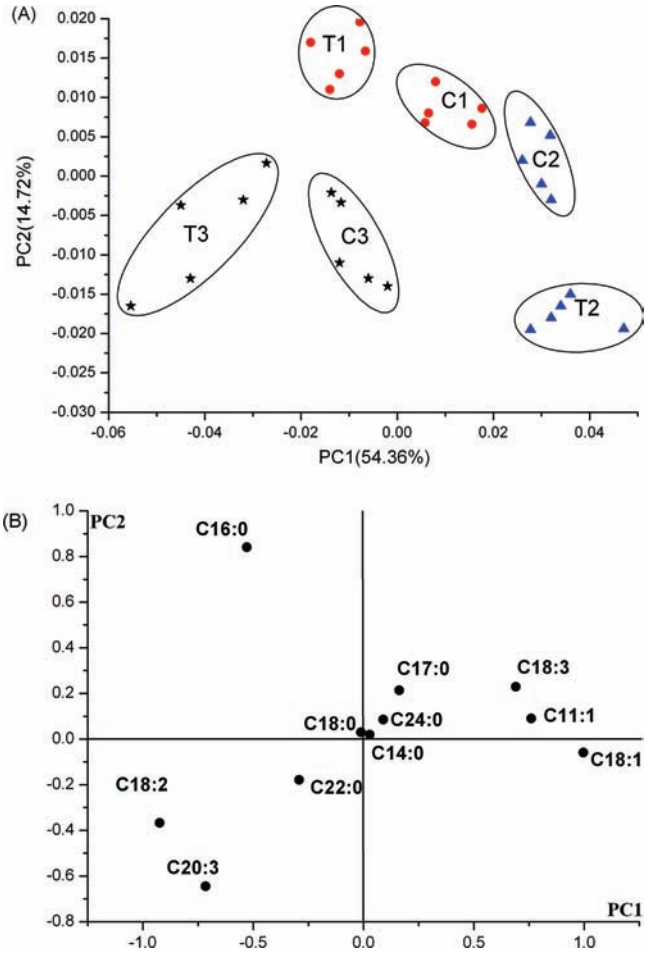

Figure 4. Score plot of PC1 and PC2 (**A**) and loading plot (**B**) for fatty acid compositions from three pairs of transgenic rice and nontransgenic rice by PCA.

plot showed that glutamic acid and alanine had an extreme position to the right of the plot along PC1, tyrosine and glycine were on a far position along PC2 as well as on PC1 (**Figure 3B**). PCA suggested that tyrosine and glycine contents varied in comparison of C1 and T1 and C2 and T2, whereas alanine, glutamic acid, glycine, and tyrosine might vary between C3 and T3.

The peak areas of the fatty acids were log-centered as input data for PCA (**Figure 4A**). Transgenic and nontransgenic rices were in relatively close position to each other, indicating that the fatty acids did not change significantly after genetic modification. PCA loading plot revealed that oleic acid (C18:1) and linoleic acid (C18:2) contributed mainly to discrimination by PC1, whereas palmitic acid (C16:0) contributed mainly to discrimination by PC2 (**Figure 4B**). The discrimination of C1 and T1 and of C3 and T3 was along PC1, indicating that oleic acid (C18:1) and linoleic acid (C18:2) contents might change. C2 and T2 were separated along PC2, so palmitic acid (C16:0) content might change.

The element concentrations were also scaled before being subjected to PCA. As seen in **Figure 5A**, variation between different kinds of rice samples was observed. The elements of C1 and T1, C2 and T2, and C3 and T3 discriminated along both PC1 and PC2. The loading plot showed that several variables (Mg, Mo, Co, Ni, Fe, V, Cu, K) had an extreme position along PC1, and Se, Zn, and Ca were on the far position plot along PC2. The objects lying to the extreme position contributed most to the compositional differences. This showed that the contents of the elements contributing to PC1 and PC2 varied between C1 and T1, between C2 and T2, and between C3 and T3.

The data set of vitamins B and E compositions were combined together. Before being subjected to PCA, the data were first scaled. As illustrated in **Figure 6A**, C1 and T1 and C2 and T2 were discriminated by PC2, whereas C3 and T3 were discriminated mainly by PC1. The loading plot of PCA indicated that vitamin B_3 had great influence on the discrimination of samples by PC1, whereas vitamin E had great influence on the discrimination of samples by PC2 (**Figure 6B**). Thus, vitamin E content might change between C1 and T1 and between C2 and T2, whereas vitamin B_3 content might change between C3 and T3.

Contents Change of Discriminatory Compounds. The amino acids that contributed most to the compositional differences between transgenic and nontransgenic rice seeds were selected, and the differences were corroborated with the one-way ANOVA indicating that the changes were statistically significant. The results showed that tyrosine content increased at about 43% in transgenic rice seeds T1 compared to C1, whereas glycine content decreased at 44%; the rest of the amino acid contents remained similar. Tyrosine increased at 49%, whereas glycine decreased at 23% in T2 compared to C2. The contents of alanine, glycine, and tyrosine decreased at 40−74% in transgenic rice seed T3 compared to C3. Rice has nutritionally a more complete balance of amino acids compared to other cereal grains; some amino acids contents in the transgenic rice decreased significantly compared to the nontransgenic rice, which might indicate that the nutrition value of transgenic rice has decreased.

Palmitic acid, oleic acid, and linoleic acid were the most discriminatory fatty acids between transgenic and nontransgenic

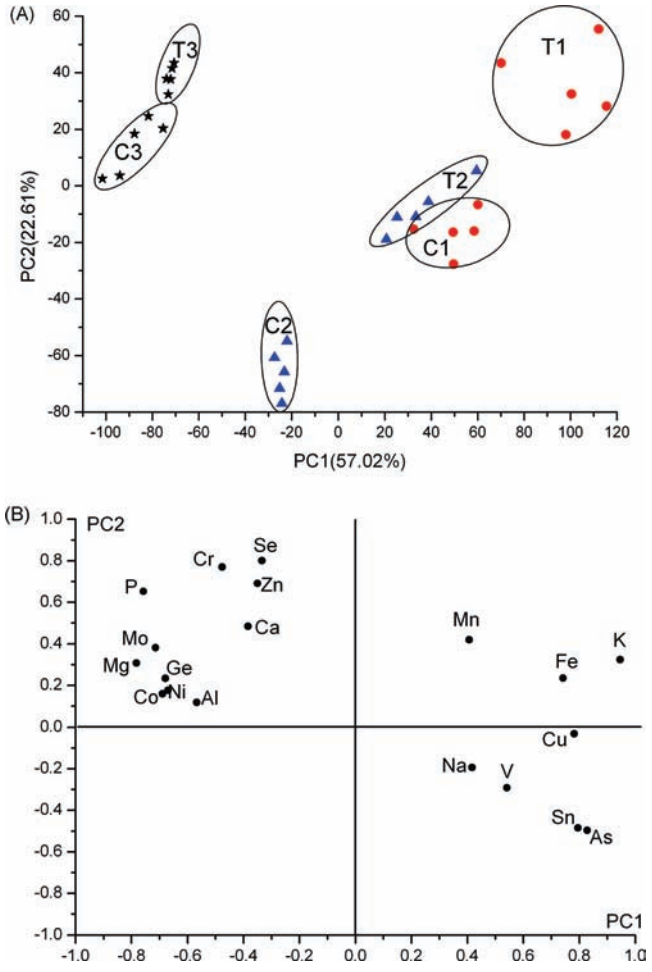

Figure 5. Score plot of PC1 and PC2 (**A**) and loading plot (**B**) for elemental compositions from three pairs of transgenic rice and nontransgenic rice by PCA.

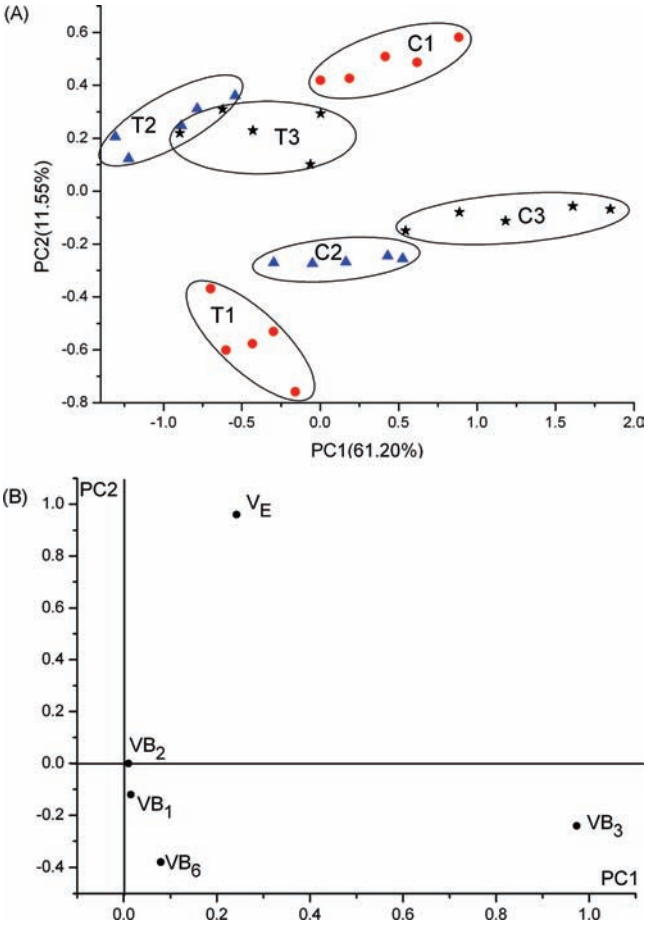

Figure 6. Score plot of PC1 and PC2 (**A**) and loading plot (**B**) for vitamin compositions from three pairs of transgenic rice and nontransgenic rice by PCA. VB_1, vitamin B_1; VB_2, vitamin B_2; VB_3, vitamin B_3; VB_6, vitamin B_6; V_E, vitamin E.

rice seeds revealed by PCA. They were also the dominant fatty acids determined in rice seeds. ANOVA showed that their contents changed significantly between three kinds of transgenic rice seeds and the respective nontransgenic counterparts. In transgenic rice seed T1, linoleic acid and oleic acid increased at 29 and 38%, respectively, compared to C1. In transgenic rice seeds T2, palmitic acid content decreased at 21% compared to C2. The amounts of oleic acid and linoleic acid in T3 increased at 19 and 25% in comparison to C3. The fatty acid composition in rice is dependent on the growing season and ecogeographical varieties. In terms of the two key fatty acids linoleic acid and oleic acid, their contents both increased in the transgenic rice seeds of T1 and T3, which meant that the transgenic effect was positive on the fatty acid compositions; nevertheless, this phenomenon was not observed in transgenic rice seeds T2.

The elements that varied between transgenic rice and nontransgenic rice in PCA model were also analyzed by ANOVA; some elements were proved to be significantly different. This showed that in transgenic rice seed T1, the concentrations of elements K and Fe increased at about 21%, whereas the concentrations of elements Se, Mo, and V decreased at 20−32%. The difference of C2 and T2 was that the concentrations of Fe, K, Ca, and V increased at about 28−47%, whereas Mg content decreased at about half in T2. The difference of elemental compositions in C3 and T3 was that the concentration of Ca increased at 27%, whereas the concentrations of Cu, Co, Ni, and Fe decreased at about 36−50% in transgenic rice seed T3. Trace elements, whether essential or nonessential, above threshold concentration levels can cause morphological abnormalities, reduce growth, and increase mortality and mutagenic effects in human. The element levels that have varied in transgenic rice seeds might be influenced by soil condition and the capacity of rice to absorb them from soil (*32*). Because transgenic rice and nontransgenic counterparts were grown side by side, the latter might be the reason for the varied concentration; the capacity to absorb elements from soil might be influenced by genetic modification (*33*). Alarming phenomena occurred in rice seeds of T2 and T3, in which Mg and Fe contents decreased to half that in nontransgenic rice seeds C2 and C3, although still in the reference range value reported for rice (*31*).

For the vitamin compositions, vitamin B_3 and vitamin E were discriminatory compounds indicated by PCA model. Their contents in three kinds of transgenic rice seeds and nontransgenic counterparts also showed significant difference by ANOVA. Rice seeds of T1 contained decreased vitamin E at 57% compared to nontransgenic rice seeds C1 and transgenic seeds T2 contained increased contents of vitamin E at 25% compared to C2. The transgenic rice seed T3, contained decreased vitamin B_3 at 34% compared to the sample C3. The results showed that vitamin compositions varied after genetic modification. Because they are micronutrients, even small differences may be important in human health considerations. Therefore, the results indicate an alarming transgenic effect on the nutritional value of rice.

Comparison of Protein and Phytic Acid Contents. The contents of protein in the rice seeds of this study were analyzed and agreed

well with reference values (**Table 3**). This showed that the protein content in rice seed T2 decreased at 25% compared to C2 samples. In comparisons between C1 and T1 and between C3 and T3, the variation was not significant by ANOVA. It was reported that the appearance quality traits of rice are correlated to the protein content (*34*). The grain appearance of sample T2 changed greatly to C2; at the same time, protein content was also decreased significantly. Protein is one of the key nutrients in rice; the decreased protein content in T2 samples indicated the nutrient value had reduced after genetic modification.

Phytic acid has been related to human health as an antinutrient. Antinutrients are substances that inhibit or block important pathways in human metabolism or impair digestion. Phytic acid limits the bioavailability of minerals such as iron, zinc, calcium, and selenium by formation of indigestible chelates (*35*). In this study, phytic acid was analyzed by HPLC; the content in all samples seeds was in agreement with the literature (*36*) (see **Table 3**). Rice seeds of C3 and T3 contained generally higher concentrations of phytic acid than the other samples. However, the differences between the transgenic samples were not significantly different from the nontransgenic ones on the basis of the ANOVA, so we can infer that the genetic modification did not have an alarming effect on the antinutrients of rice.

The physical characteristic and chemical compositions were compared above between three pairs of transgenic and nontransgenic counterparts. The outside appearance of transgenic rice seeds changed to different extents compared to the respective counterparts; besides, the contents of some amino acids, fatty acids, vitamins, elements, and crude protein also changed significantly. Glycine, tyrosine, linoleic acid, oleic acid, vitamin E, and certain element levels varied at 20−57% in transgenic rice seed T1 compared to C1; contents of protein, tyrosine, palmitic acid, vitamin E, and certain elements varied at 21−49% in transgenic rice seed T2 compared to C2. With respect to the different levels of protein, amino acids, fatty acids, vitamins, and elements presented above, one possible explanation is that overexpression of the common *β-1,3-Glu* gene in T1 and T2 may alter cellular metabolism, and the unbalanced biochemical state may be misinterpreted by host cells as a pathogen infection, resulting in constitutive activation of pathogen response signals (*37*). Most amino acids, vitamin B_3, and certain elements decreased in T3, ranging from 27 to 74%; the reason might be that the introduced *cry1AC*/*sck* gene or its product may interfere with the metabolic pathway through the interaction with enzymes on the metabolic pathway, which would bring about an accumulation or disappearance of metabolites in the host cells.

Conclusion. As part of the safety assessment, compositional analysis of transgenic rice with resistance to fungal diseases and insect pests was carried out to determine whether the insertion of transgenes caused compositional changes in transgenic rice. NIR spectroscopy, GC-MS, and HPLC analysis combined with multivariate analysis proved to be a very powerful tool for the discrimination of transgenic rice and the nontransgenic counterparts. The results showed that some unintended compositional changes occurred in transgenic rice: nutrients such as protein, three amino acids, two fatty acids, two vitamins, and several elements varied to different extents in transgenic rice, whereas the antinutrient phytic acid did not change significantly. According to the substantial equivalence principle, these changed components in transgenic rice are required to be studied intensively in further research to evaluate the unintended effect of foreign genes to metabolic pathways. The significant decreases of vitamin E content in transgenic rice seed T1, protein content in transgenic rice seed T2, and amino acid contents in transgenic rice T3 provided alarming information with regard to the nutritional value of transgenic rice. Therefore, when the risk of transgenic rice is assessed in subsequent studies, these changed compositions must be taken into account. The unintended compositional changes detected in our study laid a good foundation for further safety assessment of transgenic rice. To confirm the biosafety of transgenic rice, more detailed nutritional and toxicological tests should be carried out.

ACKNOWLEDGMENT

We are grateful to Pingjian Deng for kindly providing transgenic insect-resistant rice (*Oryza sativa* L. Kefeng No. 6) and the nontransgenic counterparts.

Supporting Information Available: Figures and contents of compositions in three pairs of transgenic and the respective nontransgenic rice samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review July 31, 2009. Revised manuscript received December 15, 2009. Accepted December 21, 2009. We thank the National Natural Science Foundation of China (No. 20575081).