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Comparative analysis of the proteomic and nutritional composition of transgenic rice seeds with *Cry1ab/ac* genes and their non-transgenic counterparts

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ABSTRACT

Nutritional assessment of transgenic crops used for human food and animal feed is an important component of safety evaluations. Profiling techniques, such as proteomics, are currently used as complementary analytical tools to detect the unintended effects of transgenic. We analyzed the proteomic profiles and nutritional composition of transgenic rice seeds containing the Cry1Ab/Ac protein to assess the safety of these transgenic seeds. We focused primarily on the effects of genetic modification and growth environment. By comparing proteomic profiles, we found that 21 proteins were up- or down-regulated as a consequence of environmental influence (WT01 vs. WT02). Similarly, 20 to 22 protein levels were differentially modulated in transgenic rice seeds in comparison to their non-transgenic counterparts (T01 vs. WT01; T02 vs. WT02). These latter changes may be due to the influence of growth environment and the insertion of a single gene into the rice genome. Based on the nutrient composition analysis (proximates, amino acids, fatty acids, minerals, vitamins and anti-nutritive components), we conclude that the nutritional quality of the rice from the transgenic lines was equivalent to that of its non-transgenic counterparts and that the effect of growth environment on the rice was no less than that of the single gene insertion.

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1. Introduction

Rice (*Oryza sativa* L.) is an important, traditional crop and staple food that serves as a primary source of calories for half of the world's population (Li et al., 2008). However, many biotic and abiotic factors limit the production of rice, including insect pests, which annually

cause the destruction of 20–30% of the world's crops (Estruch et al., 1996). Transgenically modified crops are becoming an increasingly important feature of the agricultural landscape. *Bacillus thuringiensis* (Bt) is an already useful alternative or supplement to synthetic chemical pesticide applications in commercial agriculture, forest management and mosquito control. It is also a key source of genes for transgenic expression providing pest resistance in plants (Kuiper et al., 2001). *Cry1* is a group of relatively highly homologous genes that exhibit specific toxicity toward Lepidoptera. The Cry protein encoded by the *Cry1ab/ac* gene, a fusion of *cry1ab* (GenBank Accession No. X54939) and *cry1ac* (GenBank Accession No. Y09787) into a single gene, is highly toxic to *Chilo suppressalis*, *Scirpophaga incertulas* and *Cnaphalocrocis medinalis*, three important lepidopteran pests of rice (Xu et al., 2009).

Exogenous DNA sequences introduced into the plant genome in transgenically modified organisms may affect the regulation of other genes or biochemical pathways. A major concern of these genetic modifications is the possible occurrence of unintended effects; for example, the interruption of important open reading frames or regulatory sequences can result in modified metabolism, novel fusion proteins or other pleiotropic effects that may

Abbreviations: Bt, *Bacillus thuringiensis*; OECD, Organization for Economic Co-operation and Development; AOAC, American Organization of Analytical Chemists; FAO, Food and Agriculture Organization; WT, non-transgenic; T, transgenic; TCA, trichloroacetic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2-DE, two-dimensional gel electrophoresis; IEF, isoelectric focusing; IAA, iodoacetamide; CBB, Coomassie Brilliant Blue; MALDI-TOF/TOF-MS/MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; pI, isoelectric point; MW, molecular weight; ACN, acetonitrile; TFA, trifluoroacetic acid; HCl, hydrochloric acid; S.D., standard deviation; ICPOES, inductively coupled plasma optical emission spectrometry.

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compromise product safety (Chassy, 2010; Ruebelt et al., 2006a). Other concerns include intended and unintended toxic or nutritionally harmful side effects by new allergens or toxins. It is therefore necessary to assess the safety of transgenically modified crops to address both the intended and unintended effects of genetic modification (Herman et al., 2009; Lehesranta et al., 2005; Ruebelt et al., 2006b).

The increasing use of transgenic plants has led to a greater number of animal and human transgenic consumables, provoking strong demand for appropriate risk assessment for the safety of transgenic plants (Davies, 2010; Llorente et al., 2011). As a staple food crop throughout the world, safety concerns regarding transgenic rice have been raised by producers, consumers, and responsible health authorities. The concept of substantial equivalence has been used internationally for the risk assessment of transgenic foods. A primary focus of comparative assessment has been the thorough evaluation of crop composition (Herman et al., 2009). Typically, 50–100 or more individual components are determined for each crop variety; the selection of analytes follows the recommendations of OECD Consensus Documents that describe key nutrients, anti-nutrients and other compounds of biological interest for many crops. Nutrients, including proximates, amino acids, fatty acids, minerals and vitamins, are measured. The anti-nutritive components, such as phytic acid, lectin and trypsin inhibitors, are also examined (Li et al., 2008). This evaluation of crop composition is sometimes referred to as “targeted analysis”. It should be noted that the “targets” of the analysis typically comprise more than 95% of the crop composition (Herman et al., 2009). However, concerns are seldom reported regarding the analytical procedures used to detect the unintended effects of transgene. Current developments in non-targeted approaches, including the profiling techniques of genomics, proteomics, and metabolomics, have been presented with a critical discussion of their potential and their limitations (Barros et al., 2010; Chassy, 2010; Ruebelt et al., 2006c; Takahashi et al., 2005). In principle, proteomic technologies can allow the visualization of all of the changes that take place when the genetics, nutrition or environment of an organism is altered (Zolla et al., 2008). To date, proteomic technologies have not been formally applied to the food safety assessment of transgenic crops (Chassy, 2010; Khalf et al., 2010; Takahashi et al., 2005).

As an important aspect of substantial equivalence, compositional analysis was used in this study to assess transgenic rice seeds based on nutritional composition and anti-nutrient content. Proteomics was used as a complementary analytical tool to assess the existing potential safety hazards caused by unintended effects. Because the rice seed proteome is highly dynamic, dependent on species variability and environmental influences, we analyzed the proteomic profiles of a transgenic rice variety (*cry1ab/ac*) and its isogenic controls (WT01 and WT02) in two different growth environments (T01 and T02).

2. Materials and methods

2.1. Rice sample

The rice lines used in this study were grown in the experimental field of the Institute of Subtropical Agriculture; WT01 and T01 were from the Chinese Academy of Sciences, Xuancheng City, Anhui Province, China (118.758816 East longitude, 30.940718 North latitude); and WT02 and T02 were from the Chinese Academy of Sciences, Jingzhou City, Hubei Province, China (112.239741 East longitude, 30.335165 North latitude). WT stands for “non-transgenic”, and T stands for “transgenic”. The study took place from May to September 2007. The transgenic rice line containing the *cry1ab/ac* gene and its isogenic controls were grown in adjoining fields under

the same environmental conditions and field management. Crops were harvested on September 26 in Jingzhou City, Hubei Province, and on September 16 in Xuancheng City, Anhui Province. Samples were collected, and the paddies were then hulled to obtain the rice samples. Samples were stored at 4 °C until use.

2.2. Protein extraction

We manually grinded approximately 0.8 g of each sample in liquid nitrogen and then resuspended the fine powder in cold acetone containing 10% TCA. For each rice line, three biological replicates were done and sampling randomly. After vortexing the samples for 10 min and centrifuging them at 200×g for 5 min, the supernatants were collected. The samples were kept at –20 °C for 3 h and then centrifuged. The pellets were then washed three times with acetone. After drying the pellets, the resulting powder was dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) IPG buffer (pH 3–10), 40 mM Tris–HCl (pH 8.8), 65 mM DTT, 1 mM PMSF, and 0.5% (v/v) protease inhibitor cocktail. The protein solution was sonicated intermittently for 100 s and then centrifuged to remove the insoluble material. The protein extraction was carried out in triplicate, and the protein concentrations were measured by Bradford assay.

2.3. SDS-PAGE and two-dimensional electrophoresis

Protein samples were mixed in 250 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) IPG buffer (pH 3–10) (GE Healthcare, USA), and 0.002% bromophenol blue). Samples were loaded onto IPG strips (13 cm, with a nonlinear range of IPG pH 3–10NL; GE Healthcare) after brief sonication and centrifugation. Electrofocusing was carried out in an IPGphor at 20 °C for 20 kV h following the manufacturer's instructions. Prior to the second dimensional electrophoresis (JY-SCZ6, gel size 180 × 185 mm) step, IPG strips were equilibrated by reduction with DTT and carboxymethylation with IAA. The equilibrated strips were run on 12.5% SDS polyacrylamide gels at 10 mA/gel for 1 h and 20 mA/gel until the dye front (bromophenol blue added in the agarose solution) reached the bottom end of the gel. Proteins were visualized with CBB R-250 after 1 h protein fixation in a solution made of 50% ethanol, 10% acetic acid and 40% water. Destaining was performed with a solution made of 30% ethanol, 8% acetic acid and 62% water for 2 h, followed by 5 washes with water.

Image digitization was carried out with an Image Scanner (GE Healthcare Bio-Sciences, USA) in transmission mode. The image files were subsequently analyzed with ImageMaster Platinum 2D software version 5.0 (GE Healthcare Bio-Sciences, USA), and spot intensity was normalized to the total spot value on a given gel with a multiplication factor of 100. For the identified spots, more than two-fold changes were observed, with a statistical significance of $p < 0.05$ using the Student's *t*-test.

2.4. In-gel tryptic digestion and protein identification by MS/MS

Protein spots with significant changes (at least two-fold) were carefully cut out from CBB R250-stained gels and subjected to in-gel trypsin digestion according to Sun et al. (2007), with minor modifications. Gel pieces were first discolored in 50% (v/v) acetonitrile (CAN) and 25 mM ammonium bicarbonate and then subjected to reduction and alkylation with 10 mM of DTT and 55 mM of iodoacetic acid, respectively. Following vacuum drying, the gel pieces were incubated for 16 h at 37 °C with sequencing-grade modified trypsin (Promega) at a final concentration of 0.01 mg/mL in 25 mM ammonium bicarbonate. Supernatants were

collected, vacuum-dried, and then redissolved in 50% ACN and 0.1% (v/v) trifluoroacetic acid (TFA) for MS analysis.

MALDI-TOF/TOF-MS/MS experiments were carried out according to Sun et al. (2007), with minor modifications. GPS Explorer™ software version 3.6 (Applied Biosystems) was used to create and search files with the MASCOT search program for peptide and protein identification. The NCBI Greenplant Database 2009 was used for the search and was restricted to tryptic peptides. Carboxymethyl and oxidation were selected as variable modifications. One missed cleavage was allowed. Precursor error tolerance was set to ~0.2 Da, and MS/MS fragment error tolerance was set to ~0.3 Da. All of the proteins identified had protein scores greater than 61 and individual ion scores greater than 21, with expected *p* values less than 0.05. All of the MS/MS spectra were further validated manually.

2.5. Compositional analysis

All compositional analyses were performed at the Supervision, Inspection and Testing Center for Agricultural Products Quality, Ministry of Agriculture (Beijing, China). For compositional analyses, four random samples were selected for each type of rice; the content of each component was expressed as the mean ± standard deviation (S.D.).

2.5.1. Main nutrients

Moisture content was determined by the gravimetric measurement of weight loss after drying the samples in a hot air oven at 100 ± 5 °C until the samples reached a constant weight (Chinese standard GB/T 5009.3-2003). The Kjeldahl method was used to determine total nitrogen content. Protein levels were calculated by multiplying the nitrogen content value by a factor of 6.25 (Chinese standard GB/T 5009.5-2003). Crude fat was analyzed by the Soxhlet extraction method (Chinese standard GB/T 5009.6-2003). Total dietary fiber was analyzed according to the AOAC method 991.43 (AOAC, 1995). Ash content was determined by gravimetric measurement of the sample residue after ignition in an oven at 550–600 °C until it reached a constant weight (Chinese standard GB/T 5009.4-2003).

2.5.2. Amino acids, fatty acids, vitamins and minerals

For amino acid analysis, the sulfur-containing amino acids cysteine and methionine were oxidized by performic acid and then hydrolyzed with hydrochloric acid (HCl). Directly after protein hydrolysis with HCl, the remaining 15 types of amino acids were analyzed with an automatic amino acid analyzer (Chinese standard GB/T 5009.124-2003).

Individual fatty acids were determined by gas-liquid chromatography following the Chinese standard GB9695.2-88 (Chinese standard GB/T 9695.2-88).

Thiamin and riboflavin content was determined by fluorometric methods, following the Chinese standards GB/T 5009.84-2003 and GB/T 5009.85-2003 (Chinese standard GB/T 5009.84-2003; Chinese standard GB/T 5009.85-2003), respectively. Vitamin E levels were determined by high-performance liquid chromatography, following the method of Chinese standard GB/T 5009.82-2003 (Chinese standard GB/T 5009.82-2003); the total amount of vitamin E is the sum of $\alpha + \gamma + \delta$ vitamin E. Levels of phosphorus, calcium, potassium, magnesium, zinc, iron, copper and manganese were determined by inductively coupled plasma optical emission spectrometry (ICPOES), according to AOAC method 999.11 (AOAC, 2000a).

2.5.3. Anti-nutrients

Phytic acid was analyzed by ion exchange chromatography using AOAC 986.11 (AOAC, 2000b). Trypsin inhibitor activity was

determined on alkali solvent-extracted rice flour using a modification of the American Association of Cereal Chemists Method 71-10 (AACC, 1983).

2.6. Statistical analysis

The obtained data were analyzed with the proof of safety approach (Hothorn and Oberdoerfer, 2006), a modified form of the common *t*-test. The means of the non-transgenic group were first calculated for each nutritional component, and equivalence boundaries were then set to 20% of the means, according to Li et al. (2008). The values were considered to be equivalent in these studies if the 90% confidence interval of the difference was within 20% of the mean value of the respective reference treatment (non-transgenic plants). The levels of nutritional components in this study were also compared with that of traditional, domestic rice, as described in the literature. The statistical analysis was carried out with Microsoft Office Excel 2007.

3. Results

3.1. Proteomic analysis

When evaluating the unintended effects of genetic modification, the natural variability that exists between rice and its environment must be considered (Barros et al., 2010). We therefore compared the proteomic profiles of rice samples that have the same genome (WT01 and WT02). This enabled us to eliminate any natural variation and to determine the environmental effects on seed proteomic profiles. On the other hand, any detectable differences in protein expression levels between the WT01 and T01 seeds (and also between the WT02 and T02 seeds), which originated from plants grown in the same environment, can be strictly assigned to the genomic alteration. Finally, by comparing the T01 and T02 proteomic profiles, it was also possible to evaluate how transgenic plants with one extra gene insertion responded to different environmental conditions in comparison to their non-transgenic counterparts. A scheme of the workflow is similar with Zolla et al. (2008).

A total of 900 spots were obtained in each sample using ImageMaster Platinum 2D software version 5.0 (GE Healthcare, USA). In general, the proteomic profiles were similar between the transgenic and non-transgenic rice samples planted in two different places; the total number of proteins in the four samples were nearly the same. Most of the proteins had molecular weights between 14 and 94 kDa. In each sample, albumins and globulins were mostly distributed in the pH range of 4–7 (Fig. 1).

3.2. Environmental effects

Rice seeds in the WT01 and WT02 samples were harvested from different locations, and their proteomic profiles were compared. The differential expressed spots are highlighted with arrow symbols, and the protein numbers are shown in Fig. 1. Because there were no genomic differences between these two samples, the differential spot intensities should be due to the different environmental conditions. Both qualitative (newly induced or totally repressed spots) and quantitative (increased or decreased by at least two-fold) changes were analyzed. ImageMaster software analysis revealed that a total of 21 protein spots were significantly different between rice samples WT01 and WT02. Specifically, 9 spots were down-regulated (WT01 to WT02 ratio less than 0.5), and 10 spots were up-regulated (ratio greater than 2). Two spots were completely repressed. Because these two samples were

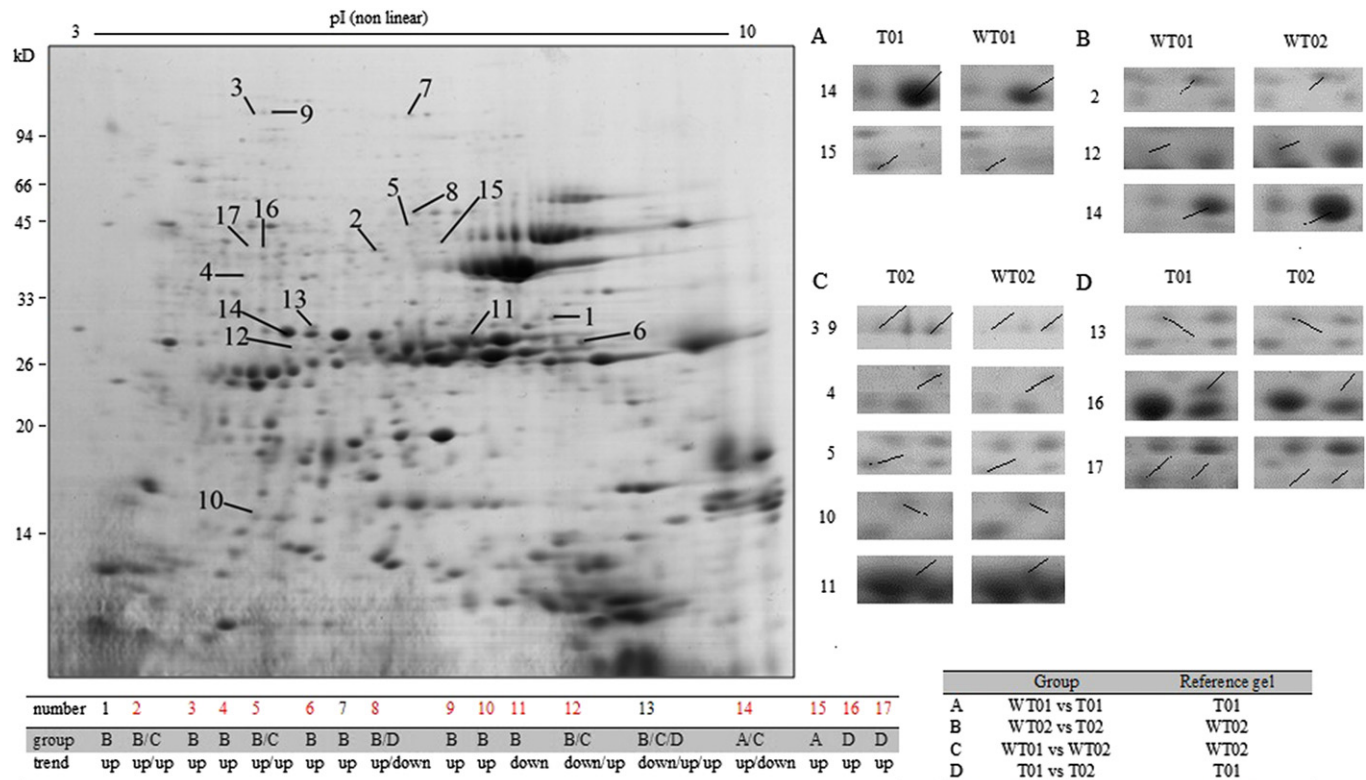


Fig. 1. Comparison of rice seed proteomes by 2-DE. Total protein (400 µg) was separated on 2D gels and stained with CBB R-250. Nonlinear IPGs pH 3–10 and 12.5% T polyacrylamide gels, were used for the first and second dimension, respectively. Four group comparisons were carried out, and protein spots that showed more than a two-fold difference in abundances were monitored. Arrows indicate proteins that are differentially expressed between the transgenic rice seeds and their non-transgenic counterparts. Down indicates the down-regulated proteins; up indicates up-regulated proteins. The spot numbering scheme was based on what is described in Table 1. WT, non-transgenic; T, transgenic.

grown in different places, this comparison reveals the effects of the growth environment on the seed protein composition.

3.3. Effects of single gene insertion

By comparing the proteomic profiles of samples WT01 and T01 to samples WT02 and T02, we could compare how two groups with different genomic states responded to the same environmental conditions. Analysis of the WT01 and T01 samples showed approximately 20 protein spots with a greater than two-fold difference in abundance. Specifically comparing T01 relative to WT01, 8 spots were down-regulated, and 12 spots were up-regulated. In the samples from the other location (WT02 and T02), a total of 22 spots were found to be differentially expressed, with 4 spots totally repressed, 15 spots up-regulated, and 3 spots down-regulated in T02 relative to WT02.

3.4. Combined effects of genetic manipulation and environment

Samples T01 and T02 have the same genomic set containing a gene insertion but were harvested from different locations. We expected that differences in protein profiles could be due to the combined action of the environment and the genomic alteration. However, the combination of the two factors may not necessarily lead to an arithmetic sum of the differences caused by the single factor because each protein could be regulated in either a similar or an opposite way by the environment and gene insertion (Zolla et al., 2008). In the case of the transgenic samples T01 and T02, a total of 23 spots were differentially expressed, with 2 spots totally

repressed, 12 spots up-regulated and 9 spots down-regulated in sample T02 relative to sample T01.

3.5. Protein identification

The identities of the successfully identified proteins are listed in Table 1. The information provided here includes the various identification parameters (Mascot score and matched peptides/peptide count) and the indication of the protein GO annotations (biological process and molecular function). Interestingly, many differentially expressed proteins, including granule-bound starch synthase 1, ADP-glucose pyrophosphorylase and others, have been shown to be involved in the metabolism pathway of starch synthesis, glycolysis, gluconeogenesis and the glyoxylate cycle. Moreover, a number of seed storage proteins, such as globulins and a putative alternatively spliced globulin protein, exhibited molecular masses that were significantly lower than those calculated according to the database reported sequence (see Table 1, Spot ID numbers 12, 13, 16, 17). Finally, neither new nor chimeric proteins, such as allergens or toxic proteins, were revealed. Interestingly, none of the targeted Cry1ab/ac protein products could be identified in our 2-DE. However, a similar observation was made for Bt protein in transgenic maize, which also failed to be detected (Ruebelt et al., 2006a). The extraction procedure is used in the extraction of total protein, by which the content of Bt protein is lower than the limit of detection (LOD). According to Xu et al. (2005), the purified PAT was from the various tissues of GM maize (Bt11 and Bt176), and ELISA procedures cannot measure the PAT protein accurately.

Table 1
MS/MS Identification of the Differentially Expressed Proteins in Comparison.

Spot ID	Ratio	NCBI accession no. (gi:)	Protein name	Mascot score	Matched peptides/pep. count	MW (kDa) theor./exp.	pI theor./exp.	Function
2	3.1 B 2.1C	gi 169761	ADP glucose pyrophosphorylase	304	7/13	53.5/41	5.65/5.55	ADP-glucose pyrophosphorylase is involved in the biosynthesis of glycogen or starch
3	2.9 B	gi 115463815	Os05g0405000 [<i>Oryza sativa</i> (japonica cultivar-group)]	213	5/24	102.7/102	5.98/4.78	Pyruvate phosphorylated kinase; glyoxylate cycle
4	2.8 B	gi 115456908	Os04g0117800 [<i>Oryza sativa</i> (japonica cultivar-group)]	84	2/6	36.4/36	5.37/4.68	Hypothetical protein; Amidase family protein, catalyzes the formation of pyroglutamic acid
5	2.2 B 2.0 C	gi 2833382	Granule-bound starch synthase 1 [<i>Oryza sativa</i> (japonica cultivar-group)]	97	2/16	66.4/45	8.47/5.95	Transferase activity, transferring other glycosyl groups
6	2.5 B	gi 115447137	Os02g0601300 [<i>Oryza sativa</i> (japonica cultivar-group)]	90	3/11	36.5/29	7.68/7.89	Hypothetical protein; Glycolysis/Gluconeogenesis
8	2.0 B –2.2D	gi 115462001	Os05g0140800 [<i>Oryza sativa</i> (japonica cultivar-group)]	94	3/8	39.0/50	9.04/6.02	Unknown
9	2.7 B	gi 115463815	Os05g0405000 [<i>Oryza sativa</i> (japonica cultivar-group)]	197	5/25	102.7/102	5.98/5.06	Pyruvate phosphate dikinase; glyoxylate cycle
10	2.6 B	gi 115455865	Os03g0793700 [<i>Oryza sativa</i> (japonica cultivar-group)]	167	4/10	52.1/17	6.78/4.78	Hypothetical protein;
11	–3.6B	gi 115477633	Os08g0545200 [<i>Oryza sativa</i> (japonica cultivar-group)]	108	2/8	39.3/29	6.03/6.31	Hypothetical protein; Alcohol dehydrogenase superfamily
12	–3.0B 2.0 C	gi 41469581	Putative globulin (with alternative splicing) [<i>Oryza sativa</i> (japonica cultivar-group)]	140	5/7	63.4/28	8.35/5.15	Nutrient reservoir activity
13	–3.0B 2.0 C 2.7 D	gi 34495244	Globulin-like protein [<i>Oryza sativa</i> (japonica cultivar-group)]	66	3/10	52.0/30	6.78/5.36	Nutrient reservoir activity
14	2.6 A –2.5C	gi 115474621	Os08g0127900 [<i>Oryza sativa</i> (japonica cultivar-group)]	141	5/13	58.0/29	8.72/5.09	Nutrient reservoir activity
16	2.2 D	gi 41469581	Putative globulin (with alternative splicing) [<i>Oryza sativa</i> (japonica cultivar-group)]	199	7/13	63.4/42	8.35/4.97	Nutrient reservoir activity
17	2.7 D	gi 41469581	Putative globulin (with alternative splicing) [<i>Oryza sativa</i> (japonica cultivar-group)]	346	8/21	63.4/42	8.35/4.88	Nutrient reservoir activity

Spot ID corresponds to those in Fig. 1. Ratio, the average change in abundance; A, B, C, D in ratio column, the comparison group; Protein name, matched protein description and the species of the matched protein; NCBI accession, accession number from the NCBI database of matched proteins; Theo. Mr (kDa)/pI, the theoretical molecular mass and isoelectric point based on the amino acid sequence of the identified protein; Expt. Mr (kDa)/pI, experimental molecular mass and isoelectric point estimated from the 2D gels; Mascot score, score obtained from Mascot for each match proteins; Function, the function of identified proteins.

3.6. Composition analysis

Because of the natural variation that exists within different seed lines, the transgenic rice seed (T) data were compared with that of their isogenic (WT) controls (Table 2). The statistical analysis used in this study was a modification of the *t*-test (Altman and Bland, 2004). Instead of the *t*-test, the proof of safety approach was used to test whether the transgenic seed differences exceed the range of normal variation of their non-transgenic counterparts.

3.6.1. Main nutritional compositions

The nutritional compositions of the transgenic (T01 and T02) and the non-transgenic (WT01 and WT02) rice samples are shown in Table 2. Although carbohydrate fraction was not analyzed, total carbohydrate was calculated by the following formula: % carbohydrates = 100 – (% protein + % fat + % ash). The protein, fiber, ash, carbohydrate and moisture contents were similar among these four samples. Although the lipid content in rice harvested at Xuancheng (WT01 and T01) was slightly lower than that at Jingzhou (WT02 and T02), there were no significant differences between the transgenic and non-transgenic counterparts.

3.6.2. Amino acids

The levels of 17 amino acids were determined with an automatic amino acid analyzer. For a convenient comparison with the reference range, the percent fraction of a particular amino acid relative to the total protein was also calculated. The results in Table 2 show

that the amino acid profiles of the four rice samples were very similar in ratio (% to protein), except for significant differences in levels of glutamic acid. The ratio of glutamic acid in the transgenic rice was lower than in the non-transgenic counterparts. The ratio (% to protein) of arginine, cysteine, serine, and threonine was slightly lower than the reference range, primarily because of the high content of the total protein in the T01 rice. However, no statistically significant differences were observed.

3.6.3. Fatty acids

The primary fatty acids in rice samples are oleic acid (C18:1) and linolenic (C18:2). Evaluation of the fatty acid profiles of transgenic rice and the isogenic controls showed no statistically significant differences in the levels of myristic acid (14:0), palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), linolenic (C18:2) or arachidic acid (C20:0) (Table 2). The oleic acid (C18:1) and gamma linoleic (C18:3) contents of the WT01 and T01 samples were slightly lower than in WT02 and T02.

3.6.4. Micronutrients

The micronutrients contained in the rice samples, including three vitamins and eight elements, showed no significant differences between the transgenic and non-transgenic counterparts (Table 2). The levels of vitamin B1 and vitamin B2 in the WT01 and T01 rice samples were slightly higher than those in the WT02 and T02 rice samples; these differences are considered biologically relevant.

Table 2
Compositional analysis in transgenic rice seeds and their non-transgenic counterparts.

Analytes	WT01	WT02	T01	T02	Reference range
Nutrients g/100 g (n = 4)					
Moisture (fw)	10.4 ± 0.3	11.4 ± 0.3	10.5 ± 0.3	10.7 ± 0.2	8.3–13.7
Protein (dw)	10.3 ± 0.2	10.8 ± 0.3	11.8 ± 0.2	11.2 ± 0.3	8.2–13.0
Lipid (dw)	0.90 ± 0.05	1.31 ± 0.06	0.82 ± 0.03	1.40 ± 0.06	0.7–1.6
Ash (dw)	3.96 ± 0.06	3.84 ± 0.03	3.82 ± 0.04	3.8 ± 0.04	3.1–4.8
Fiber (dw)	0.79 ± 0.04	0.72 ± 0.06	0.70 ± 0.03	0.66 ± 0.06	0.6–0.9
Carbohydrates ^a (dw)	75.1 ± 0.3	73.0 ± 0.2	74.2 ± 0.2	72.6 ± 0.2	58.4–90.1
Amino acids^b (% of protein) (n = 4)					
Alanine	6.60 ± 0.10	7.78 ± 0.10	5.76 ± 0.08	6.61 ± 0.10	5.3–9.3
Arginine	10.87 ± 0.18	10.0 ± 0.09	7.80 ± 0.10	11.25 ± 0.17	8.0–13.0
Aspartic acid	10.87 ± 0.11	12.41 ± 0.10	10.25 ± 0.13	11.07 ± 0.10	8.7–14.9
Cysteine	1.58 ± 0.07	1.83 ± 0.13	1.19 ± 0.15	1.63 ± 0.09	1.3–2.2
Glutamic acid	15.92 ± 0.33	20.19 ± 0.42	12.37 ± 0.44	16.79 ± 0.23	12.7–24.2
Glycine	5.44 ± 0.08	5.74 ± 0.14	5.42 ± 0.17	5.36 ± 0.14	4.4–6.9
Histidine	3.37 ± 0.12	3.70 ± 0.21	3.75 ± 0.08	3.75 ± 0.10	2.7–4.4
Isoleucine	3.33 ± 0.08	3.52 ± 0.15	3.61 ± 0.14	3.14 ± 0.09	2.7–4.2
Leucine	8.35 ± 0.09	9.59 ± 0.10	7.12 ± 0.38	8.04 ± 0.22	6.7–11.5
Lysine	3.88 ± 0.21	4.54 ± 0.14	3.56 ± 0.26	3.93 ± 0.09	3.1–5.4
Methionine	1.78 ± 0.14	1.65 ± 0.08	1.76 ± 0.12	1.63 ± 0.16	1.3–2.1
Phenylalanine	6.41 ± 0.30	6.48 ± 0.22	5.51 ± 0.41	5.89 ± 0.29	5.1–7.8
Proline	4.72 ± 0.19	4.96 ± 0.07	4.12 ± 0.36	4.50 ± 0.22	3.8–6.0
Serine	5.24 ± 0.08	6.39 ± 0.19	4.07 ± 0.34	5.54 ± 0.11	4.3–7.7
Threonine	4.08 ± 0.10	4.81 ± 0.27	3.22 ± 0.28	4.11 ± 0.15	3.3–5.8
Tyrosine	4.08 ± 0.26	4.30 ± 0.11	4.39 ± 0.16	4.68 ± 0.13	3.3–5.2
Valine	5.27 ± 0.33	5.74 ± 0.03	5.22 ± 0.26	5.11 ± 0.12	4.2–6.9
Fatty acids g/100 g dw (n = 4)					
C14:0	0.42 ± 0.08	0.36 ± 0.04	0.38 ± 0.04	0.36 ± 0.03	
C16:0	16.62 ± 1.24	12.34 ± 1.31	14.65 ± 0.66	14.95 ± 1.22	
C16:1	0.32 ± 0.02	0.3 ± 0.04	0.28 ± 0.03	0.34 ± 0.02	
C18:0	2.04 ± 0.04	1.62 ± 0.04	1.84 ± 0.07	1.82 ± 0.04	
C18:1	41.55 ± 2.38	44.82 ± 2.46	42.64 ± 1.42	43.5 ± 0.33	
C18:2	36.09 ± 0.23	37.32 ± 0.27	36.97 ± 0.13	35.7 ± 0.28	
C18:3	1.29 ± 0.03	1.56 ± 0.05	1.34 ± 0.01	1.52 ± 0.03	
Micronutrients dw (n = 4)					
Vitamins E (ug/g)	9.7 ± 2.46	9.9 ± 2.20	10.5 ± 1.19	9.6 ± 2.52	9.0–25
Vitamins B1 (ug/g)	3.1 ± 0.05	2.39 ± 0.15	3.24 ± 0.08	2.48 ± 0.04	2.9–6.1
Vitamins B2 (ug/g)	1.18 ± 0.03	0.911 ± 0.03	1.29 ± 0.03	0.938 ± 0.02	0.4–1.4
Phosphorus (mg/g)	4.19 ± 0.08	4.44 ± 0.03	3.52 ± 0.10	4.48 ± 0.04	2.0–5.0
Potassium (mg/g)	1.36 ± 0.05	1.45 ± 0.03	1.53 ± 0.07	1.39 ± 0.06	0.7–3.2
Calcium (mg/g)	0.924 ± 0.03	0.986 ± 0.04	1.1 ± 0.01	0.972 ± 0.08	0.7–1.2
Magnesium (mg/g)	1.48 ± 0.05	1.76 ± 0.03	1.36 ± 0.06	1.67 ± 0.03	0.2–1.7
Manganese (ug/g)	37.7 ± 1.08	33.6 ± 0.66	36.9 ± 0.58	30.6 ± 0.94	2–42
Copper (ug/g)	3.74 ± 0.08	2.46 ± 0.05	3.59 ± 0.05	2.13 ± 0.03	1.0–7.0
Zinc (ug/g)	20.8 ± 0.29	18.8 ± 0.52	23.4 ± 0.59	17.6 ± 1.22	7–33
Iron (ug/g)	19.7 ± 0.66	17.8 ± 0.58	18.2 ± 0.46	16 ± 0.79	2–60
Anti-nutrients dw (n = 4)					
Trypsin inhibition (TIU/mg)	<0.1	<0.1	<0.1	<0.1	<0.1
Phytic acid (%)	0.23 ± 0.02	0.22 ± 0.01	0.20 ± 0.01	0.22 ± 0.01	Not available
Lectin (HU/mg)	No activity	No activity	No activity	No activity	Not available

fw: fresh weight; dw: dry weight.

^a Carbohydrates levels of dry weight were estimated by the formulation: %carbohydrates = 100 – (% protein + % fat + % ash).^b Sample contains 14% of moisture. No reference range of moisture available. Source: OECD.

3.6.5. Anti-nutrients

Next, levels of anti-nutrients contained in rice grain, such as phytic acid, lectin and trypsin inhibitors, which have been suggested to be measured in the OECD consensus documents, were compared between the transgenic and non-transgenic counterparts (Table 2). The inhibitory activities of trypsin inhibitors and the level of phytic acid were very low in both groups. Lectin activity was undetected.

4. Discussion

The nutrients and anti-nutritive components examined in this study demonstrated that the nutritional quality of transgenic rice was substantially equivalent to that of the non-transgenic counterparts. We note that similar results have also been reported for other transgenic rice lines (Han et al., 2005; Oberdoerfer et al.,

2005). We observed many vertical and horizontal position shifts when comparing the proteomic profiles. A number of seed storage proteins, such as globulins and putative globulin proteins, exhibited truncated forms with molecular weights significantly lower than the native ones. A possible explanation for this observation is that frequent mutations lead to changes in protein molecular weight (Zolla et al., 2008). However, the above results would not be observed in a traditional substantial equivalence assessment. The storage proteins are abundant in seeds and include albumins, prolamin, glutelins and globulins, all of which play important roles in determining the nutrition value of rice seeds (Wang et al., 2010). The truncated forms of storage proteins might affect the nutrition value or digestion and absorption in the intestinal tract of humans and animals in an unknown way. In agreement with our data, Zolla et al. (2008) found frequent molecular weight changes in the major storage proteins of transgenic maize lines.

In this study, a significant number of proteins were shown to be differentially regulated by both environment and genetic modification. The levels of approximately 21 proteins were differentially modulated as a consequence of environmental influence (WT01 vs. WT02), whereas 20 and 22 differentially expressed proteins were found in transgenic seeds in comparison to their non-transgenic lines (T01 vs. WT01; T02 vs. WT02). These differentially expressed protein spots were related to starch synthesis, glycolysis, gluconeogenesis and the glyoxylate cycle. However, no significant differences were observed between the different genomic and environmental groups. Therefore, we consider that the differences in protein spots that exist between transgenic and non-transgenic lines fall within the range of natural variation. Interestingly, MS/MS identification of modulated spots revealed that the proteins belong to several different metabolic pathways. No new or toxic proteins unique to individual transgenic lines were observed. The expression of the target *Bt* gene is below the limit of detection (LOD) of the method employed here. This is expected because proteomics is a useful method for comprehensive analyses but not if the level of a target protein is extremely low. In the work of Corpillo et al. (2004), the product of their marker gene (*np11l*) was only detectable with a highly sensitive immuno-detection technique.

We conclude that there is little difference in the proteomic profiles of the transgenic rice seeds and their non-transgenic counterparts. This also includes the different environment group comparisons, which indicate that both single gene insertion and slightly different growth environments have only a moderate effect on the rice proteome. Many environmental elements can seriously affect the growth of plants, including soil, climate and stress (insect pests and weeds); these factors then determine the quality and nutrition of cereal (Barros et al., 2010). The transgenic rice and the non-transgenic counterparts were planted in the same place under the same environmental conditions, such as climate, soil and moisture. However, because transgenic rice with the *Cry1ab/ac* gene is highly toxic to three important lepidopteran pests, it can grow in an ideal environment that is slightly different from the non-transgenic counterpart. In other words, the transgenic rice has slightly different growth conditions, even though they are planted in the same location. The differences that result from genetic modification can also be influenced by environment variation. To be more precise, the effects of environmental growth conditions on the rice proteome are not significantly different than effects caused by a single gene insertion (Barros et al., 2010). Single gene insertions may affect only a limited number of proteins; in contrast, a greater number of potential protein changes may be expected as a result of significant environmental changes.

Based on the nutritional composition and proteomic profile analyses of the transgenic rice seeds containing *Cry1Ab/Ac* protein, the levels of nutrients and anti-nutritive components were comparable to the non-transgenic counterparts. Since all significantly difference mean values for transgenic rice were within rice reference ranges (OECD), these differences were not considered biologically relevant. Moreover, protein spots with more than two-fold differences in abundances were monitored; however, the differentially expressed proteins identified were storage proteins or proteins involved in basic metabolism, not allergen or toxic proteins. The environmental effects were also not significantly different from the transgenic effects. These results thus confirm that the nutritional quality of transgenic rice seed was substantially equivalent to that of non-transgenic counterparts and that no detrimental changes exist related to potential unintended effects of the transgene were observed as a result of the insertion of *Cry1Ab/Ac* gene to improve insect control. Further, the method of our

research could provide a more comprehensive analysis for safety evaluation of genetically modified crops.

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