The integrated risk assessment of transgenic rice Oryza sativa: A comparative proteomics approach

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Abstract

The identification of unintended effects resulting from genetic modification processes is an important but difficult aspect of evaluations of the biosafety of genetically modified organisms (GMOs). Non-targeted techniques offer considerable potential for improving the detection of unintended effects of genetic modification in crop plants. In this study, total seed protein expression patterns of two strains of transgenic rice (Bt rice and PEPC rice) were examined using a comparative proteomics approach with two-dimensional electrophoresis (2-DE), and differences determined comparing to each line’s non GM counterpart. The results indicated that some of the seed proteins from the two transgenic rice lines differed in their relative intensities. Twenty eight proteins were successfully identified with MALDI-TOF-MS, five of which were well-characterized and these were discussed. In summary, transgenic rice were found to be different in their protein contents from their non-GM counterparts, which might raise concerns regarding their potential risks for human health and ecology.

Keywords:
Transgenic rice
Oryza sativa
Genetic modification
Proteomics
Biosafety

1. Introduction

Biosafety assessment of genetically modified organisms (GMOs) includes evaluating their expected and unintended effects (Conner & Jacobs, 1999, 2000; Pedersen, Eriksen, & Knudsen, 2001). In current commercial GMOs, insertion of exogenous DNA sequences into the plant genome is random, which may lead to physical damage of the plant genome, such as the inactivation of endogenous genes. In addition to the effects of single gene, the transgenes, their products, or the changed biochemical pathways may also interact with other genes or pathways. Globally, GM crops have been planted and used commercially for more than 10 years, but it remains very challenging for regulators to get information on unintended changes outside of a narrow range of agronomically-relevant metabolites that are profiled (GM Science Review Panels UK, 2004; Society of Toxicology, 2003).

Targeted analyses of specific compounds which make an important contribution to the nutritional value or safety of the crop species have traditionally been used to compare the compositions of GM and non-GM crops including unintended effects (Kok & Kuiper, 2003). In order to improve the capability for detecting unintended effects, non-targeted techniques, such as differential genome display and comparative proteomics, are increasingly being used in evaluations of the biosafety of GMOs (European Commission, 2003; FAO/WHO, 2000; National Research Council Safety of Genetically Engineered Foods, 2004). These techniques can simultaneously measure and compare thousands of plant constituents without any prior knowledge of the presence or concentrations of those constituents. Additionally, in the biosafety assessment of GMOs, these non-targeted methods are considered more effective than targeted methods with narrow ranges of analyzes to identify unintended effects, since they screen for effects in an unbiased manner and so extend the breadth of comparative analyses and reduce uncertainty. Jiao, Si, Li, Zhang, and Xu (2010) found several unintended compositional changes in GE rice using non target or screening methods of analysis, and suggested that these changes should be taken into account in subsequent studies on the risk of GM rice and recommended further testing to confirm the biosafety of GM rice.

Proteomics is a novel untargeted method applying to risk assessment to detect the changes of different protein expressing pattern for providing baseline data of GMOs (Heinemann, Kurenbach, & Quist, 2011). The aim of this study was to apply proteomics to identify any unintended effects caused by genetic engineering in two kinds of transgenic rice, which was achieved via comparison of transgenic rice seed samples and their parent rice lines analyzed using two-dimensional electrophoresis (2-DE).

2. Materials and methods

2.1. Rice seeds

The transgenic Bacillus thuringiensis (Bt) rice O. sativa, Bt Shan-you 63 (abbreviated below as Bt63) and its parental line Shanyou...
63 were provided by Nanjing Institute of Environmental Sciences (NIES), Ministry of Environmental Protection of China, Nanjing, China. The Bt63 rice line was derived from Minghui63 with the fusion gene cry1Ab/cry1Ac, while Shanyou 63 was generated by the same restorer without the bt gene (Tu et al., 2000). Transgenic phosphoenolpyruvate carboxylase (PEPC) rice and its control line Kitaake, which is the host rice line that received the pepc gene, were supplied by Jiangsu Academy of Agricultural Sciences, Nanjing, China.

2.2. Protein extraction from rice seeds

Three parallel samples of each of the four types of rice were prepared. The method employed for total protein extraction from rice seeds is described in reference (Xu, Li, Deng, Chong, Xue, & Wang, 2008). The sampling method was: the seeds (10 g) of each of the four rice varieties were weighted out, grounded to powder and the powder sample were divided into four parts. One was stored at −80 °C as backup, and the other three parallel samples were used in our experiment. The three samples were all mix ones to minimize the difference between individuals and heighten the aid of our research. Concentration of the total proteins in solution was quantified according to the Bradford method (Bradford, 1976). The protein solution was either used immediately in 2-DE or stored at −80 °C until use.

2.3. 2-DE and staining

An aliquot (1 mg of proteins) of protein samples was diluted with rehydration buffer (6 M urea, 2 M thiourea, 0.5% CHAPS, 20 mM DTT, 0.5% immobilized pH gradient [IPG] buffer 3–10, and 0.002% bromphenol blue) to a final volume of 450 mL and loaded onto an IPG strip holder containing a 24-cm, pH 3–10 linear gradient IPG strip (GE Healthcare). The Ettan IPGphor isoelectric system (GE Healthcare) was used for the first dimension, and 2-DE was carried out in SDS–PAGE after balancing the IPG strips. After electrophoresis, Coomassie brilliant blue was used for staining. Gel images were analyzed with ImageMaster 2D version (GE Healthcare).

2.4. Protein identification with MALDI-TOF-MS and database searching

Differentially expressed protein spots were created for Ultraflex™ MALDI-TOF-MS/M5 mass spectrometer detection. Peptide mass fingerprinting (PMF) obtained with the MASCOT search engine (http://www.matrixscience.co.uk) in the NCBI database (http://www.ncbi.nlm.nih.gov/) were searched, using a tolerance of ±0.1 and one missed trypsin cut site.

3. Results

3.1. Comparative proteomic analysis of transgenic rice seeds and regular seeds

Fig. 1 shows the results of 2-DE of total proteins from the seeds of two lines of transgenic rice and those of their respective non-GM controls, following staining with Coomassie brilliant blue. Numbers of protein spots were detected from the sample of rice seeds, while the number and ratio of protein spots matching between GM rice and its control were counted. The numbers of proteins with different relative intensity varies were also calculated (Table 1).

3.2. Identification of proteins with significant differential expression

Protein spots showing a difference in relative intensity of ≥4-fold were selected for further analysis and were cut out of the gels and digested. These 77 protein spots were identified by mass spectrometry, and positive results were obtained for 28 samples, in which 13 protein spots were from transgenic Bt rice and its control, and 15 spots were from transgenic PEPC rice and its control. Excluding the proteins without clear functional description, there were five proteins or subunits with reliably known biological activity. Their basic characteristics are summarized in Table 2. The expressions of fructose-1,6-bisphosphate aldolase in Bt and PEPC rice were both up-regulated compared with each non-GM control. The expressions of 5-methyltetrahydropteroyltriglutamate-homocysteine methyl-transferase in transgenic Bt rice, Cyclin-dependent kinase B2-1 and Serpin-Z2B in transgenic PEPC rice were also up-regulated, while isocitrate lyase was downregulated in transgenic Bt rice.

4. Discussion

Although as a relatively new application of proteomics on risk assessment of GMOs, the integrating method has been widely used for analyses at the biochemical level of cells or tissues, including in the study of allelopathy and induced resistance in maize (Zolla, Rinalducci, Antonioli, & Righetti, 2008). Proteins can be described as the direct actors within cells, performing functional roles enabling cells and organisms to complete metabolism and other fundamental physiological functions. Theoretically, any change will be reflected at the level of protein expression, and proteomics methods offer the capacity to detect these changes. Hence, in the development of GMOs, modified organisms will respond to the changes made to their genomes, and such a response would logically occur via changes in its proteome. As a result, analyses of the proteome of GMOs compared to that of their non-GM lines should enable the determination of changes occurring as a result of the GM process, which is useful for studies on biosafety although they does not lead to final evidence of harm (Heinemann et al., 2011).

Our analyses of the identified proteins from transgenic Bt and transgenic PEPC rice showed that the expression of fructose-1,6-bisphosphate aldolase was up-regulated compared with in their respective non-GM controls. Fructose-1,6-bisphosphate aldolase, an important enzyme in the process of glycolytic/gluconeogenesis, can promote the synthesis of sugars and starch metabolism (Nakamura, Satoh, Hidaka, Kagaya, Ejiri, & Tsutsuji, 1996). Research and development of transgenic PEPC rice is aimed at transferring the photosynthetic characteristics of C4 plants into C3 plants, in an attempt to increase the photosynthetic rate efficiency of C3 plants. An increase in the expression of fructose-1,6-bisphosphate aldolase in transgenic PEPC rice may be an unintended response to this process.

The up-regulation of 5-methyltetrahydropteroyltriglutamate-homocysteine methyl-transferase in transgenic Bt rice was also confirmed in this study. Gene expressing 5-methyltetrahydropteroyltriglutamate-homocysteine methyl-transferase was located on the chromosomes 11 and 12 of rice, which were rich in disease resistance genes and recent gene duplications (The Rice Chromosomes 11 and 12 Sequencing Consortia, 2005). The detail function of this protein would be described after further biochemical researches.

The function of the protein isocitrate lyase, downregulated in transgenic Bt rice, relates to the growth mechanism of plants growing in situations of stress. The ability of plants under stress to survive and develop successfully depends on the glyoxylate cycle, a branch of tricarboxylic acid cycle (TCA), to complete a certain de-
gree of metabolic activity. The down-regulation of this enzyme observed in transgenic rice (Table 2) may be indicative of a weakness of this line to stress (Diehl & McFadden, 1994; Rehman & McFadden, 1997; Reynolds & Smith, 1995).

Cyclin-dependent kinase B2-1, which was up-regulated in transgenic PEPC rice, usually appears in the active organizations of cells, and gibberellin and cytokinin can promote the expressing of the enzyme (Fabian, Lorbiecke, Umeda, & Sauter, 2000). Generally, the presence of the enzyme is related to the regulation of the G2/M phase in mitosis (Guo, Song, Wang, & Zhang, 2007; Itoh, Tanaka, Barrero, et al., 2007; Lee et al., 2003; Umeda, Umeda-Hara, Yamaguchi, Hashimoto, & Uchimiya, 1999). In transgenic PEPC rice, the increase also observed in the expression of the serine protease inhibitor Serpin-Z2B could suppress the digestion of serine protease. As a result, transgenic PEPC rice could be resistant to the feeding of Lepidoptera larvae because serine protease is one of the major digestive enzymes in their midgut (Lee & Anstee, 1995; Ortego, Novillo, & Castanera, 1996; Xu & Qin, 1994).

It is because that the other four proteins, besides the serine protease inhibitor Serpin-Z2B, were all the enzymes working in a certain biochemical pathway, and their function could be offset by other steps in the same pathway, which meant there were not any directly visible effects causing by those four proteins. The results about these four proteins reported in the literatures contained little function of the single protein in the pathways.

The scientific biosafety assessment of GMOs is currently receiving a lot of attention, and the identification and assessment of non-target effects is a key focus of these studies, although most studies

### Table 1
Comparison of isolated proteins between transgenic rice and their respective non-GM lines.

<table>
<thead>
<tr>
<th>Rice line</th>
<th>Number of proteins</th>
<th>Number of matching proteins</th>
<th>Number of proteins with significant difference in abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\geq 2$-fold</td>
</tr>
<tr>
<td>Bt63</td>
<td>312</td>
<td>276</td>
<td>169</td>
</tr>
<tr>
<td>Shanyou 63</td>
<td>303</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transgenic PEPC rice</td>
<td>355</td>
<td>346</td>
<td>121</td>
</tr>
<tr>
<td>Kitaake</td>
<td>353</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. 2-DE results of total proteins from transgenic rice and their controls. (A) Transgenic Bt rice; (B) Shanyou 63 rice; (C) detailed protein spots of transgenic Bt rice and (D) detailed protein spots of Shanyou 63 rice. The spots with the same annotations were expressed significantly differentially.
are still conducted at the ecological level. Although, in theory, the proteomics approach can be applied to the biosafety assessment of GMOs, in practice only a small amount of studies currently use proteomics to identify the candidate proteins involved in the non-target effects of GMOs in a controlled environment (Cellini et al., 2004; Ruebelt, Lipp, Reynolds, Astwood, Engel, & Jany, 2006). In the whole area of biosafety assessment, the application of these methods is limited because environmental factors must be considered to avoid false positive results. These uncertainties may indeed result in false positive results, but available results do indicate differences between GMOs and their non-GM controls. At the same time, differences in protein constituents caused by variation in environmental factors can be reduced to a minimum through careful experimental design, in particular concerning growth conditions, and protein extraction. In conclusion, this study demonstrated that a proteomics approach could contribute to the assessment of the biosafety of GMOs effectively and provide reference data for the overall biosafety of GM crops. According to the current results, transgenic rice lines were found to differ in their protein contents from their non-GM counterparts, which could raise concerns regarding their potential risks for human health and ecology. In addition, the results obtained highlight issues for further research on the unintended effects of GMOs. More rigorous requirements on risk assessment and more sophisticated methods for risk evaluation are needed to evaluate the potential biosafety risk of unintended “non-target” effects of GMOs.

Acknowledgement
This research was supported jointly by “985 Project” of Minzu University of China (MUC98507-08 and MUC98504-14), 111 Project B08044, the Fundamental Research Funds for the Central Universities, National Special Transgenic Project (2008ZX08012-005, 2011ZX08012-005) and Project 30771381 supported by NSFC.

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