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Degradation and detection of transgenic *Bacillus thuringiensis* DNA and proteins in flour of three genetically modified rice events submitted to a set of thermal processes

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ABSTRACT

This study aimed to investigate the degradation of three transgenic *Bacillus thuringiensis (Bt)* genes (*Cry1Ab*, *Cry1Ac*, and *Cry1Ab/Ac*) and the corresponding encoded *Bt* proteins in KMD1, KF6, and TT51-1 rice powder, respectively, following autoclaving, cooking, baking, or microwaving. Exogenous *Bt* genes were more stable than the endogenous *sucrose phosphate synthase* (*SPS*) gene, and short DNA fragments were detected more frequently than long DNA fragments in both the *Bt* and *SPS* genes. Autoclaving, cooking (boiling in water, 30 min), and baking (200 °C, 30 min) induced the most severe Bt protein degradation effects, and Cry1Ab protein was more stable than Cry1Ac and Cry1Ab/Ac protein, which was further confirmed by baking samples at 180 °C for different periods of time. Microwaving induced mild degradation of the *Bt* and *SPS* genes, and Bt proteins, whereas baking (180 °C, 15 min), cooking and autoclaving led to further degradation, and baking (200 °C, 30 min) induced the most severe degradation. The findings of the study indicated that degradation of the *Bt* genes and proteins somewhat correlated with the treatment intensity. Polymerase chain reaction, enzyme-linked immunosorbent assay, and lateral flow tests were used to detect the corresponding transgenic components. Strategies for detecting transgenic ingredients in highly processed foods are discussed.

1. Introduction

Rice is one of the most important crops worldwide, and China, accounting for approximately 20% of the world's rice paddies and 30% of the world's rice production, is the largest producer and consumer in the world (FAO, 2012). Advances in biotechnology and the increased demand for rice production led to the development of genetically modified (GM) technology to confer herbicide tolerance and pathogen or insect resistance characteristics in rice (Cao et al., 2004; Shu et al., 2000; Song et al., 1995). In China, at least 100 transgenic lines have been developed over the past 20 years (Bajaj and Mohanty, 2005). In 2009, the Chinese government made a landmark decision to issue biosafety certificates for nationally developed *Bacillus thuringiensis* (Bt) rice strains (James, 2010). Despite the great efforts made in the development of transgenic rice lines, there has been no large-scale commercial cultivation. At present, only a small number of transgenic rice varieties have been approved for release in the US, such as LL 62, LL 06, and LL 601 (Babekova et al., 2009), as well as the semicommercial cultivation of a Bt rice in Iran (James, 2007). Global expansion of areas sown with transgenic crops increases the likelihood of contaminating non-transgenic varieties with GM products. Examples include the accidental presence of herbicide-tolerant rice in Europe and TT51-1 rice on the Chinese market (Paine et al., 2005; Potrykus et al., 1995).

According to the regulations of China, the EU, as well as many other countries, food or feed containing GM material cannot be sold in the market unless the associated GM material has been authorized. In other side, although many GM crops have been authorized for commercialization in many countries, fear associated with the biosafety and environmental risks, along with various ethical concerns. So, detection and labeling of GM foods are required in more than 30 countries or regions (Yang et al., 2005). Concerning the labeling threshold for food and feed, however, there is no consistency as this is only in place in some but not all countries. For instance, the labeling threshold is defined as 0.9% in EU (European Parliament, 2003), 3% in Korea (Ministry of Agriculture and Forestry

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of Korea, 2000), and 5% in Japan (Bean, 2002). The labeling of GM foods is not compulsory in the United States and Canada (Matsuoka, 2001). To control the genetically modified organism (GMO) labeling system or living modified organism management for GM crops, main two types of methods were developed to detect GMO, namely, protein- and DNA-based methods (Holst, 2009; Miraglia et al., 2004). However, the applicability of protein- or DNA-based methods for GMO detection depends on the quality and quantity of the protein or DNA. Important food processing conditions, for example temperature, may lead to degradation of the protein and DNA, rendering the two methods impossible or GMO detection unreliable.

Food processing leads to serious degradation of DNA and proteins in food. Many studies of DNA and protein degradation in GM food during processing have been conducted. For example, Chen et al. (2007) studied the effect of processing procedures during soymilk preparation on fragment sizes of the endogenous gene *lectin* and the exogenous gene *epsps* in Roundup Ready Soybean. Vijayakumar et al. (2009) assessed the effects of high temperature and high pressure on the detection of gene sequences in MON-810 maize and Roundup Ready Soybean. The results indicated that high temperature and/or pressure lead to serious DNA degradation and significantly reduce the level of detectable DNA. Xiao et al. (2012) used polymerase chain reaction (PCR) and Western blotting methods to evaluate the degradation of the CP4-EPSPS gene and protein in various bean foodstuffs, such as dried bean curd crust and deep-fried bean curd, and reported inconsistent results between the two detection techniques. Previous studies focused mainly on the degradation of exogenous genes in transgenic soybean, transgenic corn, and transgenic maize (Gryson, 2010), and little information is available on exogenous *Bt* genes or Bt proteins in transgenic rice. Since 1987, *Bt* genes have been introduced into crops to combat crop damage (Barton et al., 1987; Vaeck et al., 1987). The rapid incorporation of the *Bt* genes in crops led to its emergence as one of the most widely grown GM crops worldwide (James, 2014). By 2014, the global area planted with commercial transgenic crops reached 181 million hectares, of which Bt crops accounted for 15%, or ~27.4 million hectares (James, 2014). The most commonly used *Bt* genes in transgenic crops, including rice, are *Cry1Ab*, *Cry1Ac* and the fusion gene *CryAc/Cry1Ab* in China (Tang et al., 2006). The study of dynamic situations of the Bt genes and proteins under thermal processing will not only provide insight into the degradation of the exogenous Bt genes and proteins, but it will also provide information for detecting and monitoring such GM ingredients in the food chain.

In the present study, three lines of insect-resistant transgenic rice, KMD1, KF6, and TT51-1, harboring *Cry1Ab*, *Cry1Ac*, or the fused *Cry1Ac/Cry1Ab* genes, respectively, were selected for studies on the effect of thermal processing on the degradation and detection of these *Bt* genes and proteins in rice. At present, *Cry1Ab*, *Cry1Ac*, and *Cry1Ac/Cry1Ab* are the most commonly used *Bt* genes in transgenic crops, especially in China (Tang et al., 2006). Based on previous studies, temperature plays an essential role in DNA and protein degradation in the processing of transgenic crops (Gryson, 2010; Tian et al., 2014). To mimic the temperature effects on the degradation of Bt genes and proteins, different thermal treatments, including autoclaving, cooking, baking, and microwaving, were used. To eliminate matrix effects, KMD1, KF6, and TT51-1 rice seed powders were directly subjected to these thermal treatments without other components. Finally, conventional PCR methods, combined with methods based on detection of proteins by immunoassays such as enzyme-linked immunosorbent assay (ELISA) and lateral flow tests (LFT), were used to determine the degradation or detection of Bt genes and proteins.

2. Materials and methods

2.1. Materials

KMD1, KF6, and TT51-1 seeds were kindly provided by the Center of Science and Technology Development, Ministry of Agriculture of the People's Republic of China (Beijing, China). The insect-resistant transgenic rice lines KMD1, KF6, and TT51-1 contained a *Cry1Ab*, *Cry1Ac*, and *Cry1Ab/Ac* gene, respectively. Detailed information regarding the genetic constructs of TT51-1, KMD1, and KF6 is provided in Fig. S1.

2.2. Sample preparation

The seeds of each transgenic rice line were first ground to a fine powder with a DFT-100 mill (Linda machinery co., LTD, Zhejiang, China), and the resulting powder was fractionated using 0.4-mm standard sieves. The collected powder was then dried for 48 h at 55 °C to equalize the moisture content. The various thermal treatment conditions and the processing details are described in Table 1.

2.3. DNA extraction

DNA was extracted from seed flour material using a kit (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany). The quantity and purity of the extracted DNA were determined by the ratio of absorbance between 260 nm and 280 nm using a spectrophotometer Ultrospec 1100 pro (GE Healthcare, USA), and integrity was further characterized by agarose gel electrophoresis.

2.4. PCR primers

To evaluate the degradation of *Bt* genes in the three transgenic rice lines following thermal processing, distinct length primer pairs focusing on different regions were designed according the sequence of each *Bt* gene (Fig. 1). Sequence alignment of *Cry1Ab* (HQ154128), *Cry1Ac* (KF630361), and *Cry1Ab/Ac* (EU880444) showed that *Cry1Ab* has 86.6% and 85.3% sequence homology with *Cry1Ac* and *Cry1Ab/Ac*, respectively, and *Cry1Ac* maintains 98.3% similarity with *Cry1Ab/Ac*. Partial sequence alignment of the three *Bt* genes is presented in Fig. S2. A pair of primers (Bt-301F/Bt-301R) according to the Chinese National Standard (Jin et al., 2007) was used to detect the *Bt* genes. The rice gene *sucrose phosphate synthase* (*SPS*) is a suitable endogenous reference gene for GM rice detection, and primers for the *SPS* genes were designed as reported previously (Jiang et al., 2009). The design strategy for primers for the SPS and *Bt* genes is shown in Fig. 1. All primers were designed by Primer Premier 5.0 (Premier, Canada), and produced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China; Table 2).

2.5. PCR conditions

PCR amplification was performed in a 30-uL volume with 10 × PCR buffer, 200 μM dNTP, 0.4 μM of each primer, 1.25 U Taq DNA polymerase (TaKaRa Biotechnology Co.), and 50–100 ng DNA template. The amplification programs for each primer set are listed in Table 3. Each sample was extracted in triplicates and the PCR results of each extract were verified by three times and the number of positive amplifications for each assay statistically analyzed.

2.6. ELISA

The Bt-ELISA Kit (PSP 06200, Agdia Inc., Elkhart, IN, USA) was used to detect the *Bt* proteins. The detection process was performed