ポリメラーゼ連鎖反応を用いた遺伝子組換えトウモロコシBt11系統特異的定性検知法の非特異的増幅低減を目的とした改良

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Note

Improvement of Polymerase Chain Reaction-Based Bt11 Maize Detection Method by Reduction of Non-Specific Amplification

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The Bt11 maize-specific qualitative detection method based on polymerase chain reaction (PCR) in the JAS analytical test handbook has been widely used for administrative monitoring of GM crops and quality control of commercially distributed grains. In the present investigation, some apparently false-positive detections were observed in assays using the Bt11 maize-specific method, and these erroneous results were proved to have been caused by non-specific DNA amplification. We improved the detection method to reduce non-specific amplification by decreasing the concentration of magnesium ions in the PCR mixture. The subsequent evaluation of analytical performance demonstrated no marked difference between the currently used and the improved methods, except for the reduced non-specific amplification. We conclude that the currently used standard method should be replaced with the improved method for the reliable detection of Bt11 maize.

Key words: polymerase chain reaction (PCR); genetically modified organism (GMO); detection; non-specific amplification

Introduction

The production of genetically modified (GM) crops has expanded in many parts of the world, and large amounts of GM crops and their products have been imported into Japan[1)]. A system for enforcing the safety assessment of GM foods in Japan was introduced in 2001, and only authorized genetically modified organisms (GMOs) are allowed to be imported[2)]. Meanwhile, the utilization of GM foods was controversial among general consumers, and a food labeling system for GMOs was also implemented in 2001 under The Law Concerning Standardization and Proper Labeling of Agricultural and Forestry Products (The Japanese Agricultural Standards Law) to expand consumers' choices. Simultaneously, a labeling system was adopted under The Food Sanitation Law in order to publicize the fact that the product has undergone the safety assessment. According to the implementation of the labeling system, standard GMO detection methods for monitoring the labeling system were developed and published in the Japanese Agricultural Standard (JAS) analytical test handbook from the Food and Agricultural Materials Inspection Center (Saitama, Japan) and "Testing for Foods Produced by Recombinant DNA Techniques" from the Ministry of Health, Labour, and Welfare of Japan*1, *2 These detection methods have been widely used, not only for monitoring the food labeling system, but also for the quality control of food and feed materials, and commercially distributed seeds.

The Bt11 maize-specific qualitative detection method based on polymerase chain reaction (PCR) is one of the detection methods described in the JAS analytical test handbook. The specificity of PCR detection in a qualitative analytical method is critical, and may directly affect the testing results. However, we confirmed that the Bt11 maize detection method can generate false-positive results owing to non-specific DNA amplification. Thns, we attempted to improve the method by eliminating the potential for false-positives. Furthermore, we evaluated the performance of the improved method.

*1 Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products (2002). The Food and Agricultural Materials Inspection Center, Japan. http://www.famic.go.jp/technical_information/jashandbook/index.html

*2 Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001)

Materials and Methods

Cereal materials for analytical samples

We used Bt11, Event176, GA21, MON810, MON863, NK603, T25, and TC1507 maize as representative GM maize events. As a representative GM soy event, we used 40-3-2 soybean (Roundup Ready Soybean, RRS). F1 generation seeds of Bt11 and Event176, and ground F1 generation seeds of GA21 were kindly provided by Syngenta Seeds AG (Basel, Switzerland); F1 generation seeds of MON810, MON863, and NK603 were kindly provided by Monsanto Company (St. Louis, MO, USA); and F1 generation seeds of TC1507 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). F1 generation seeds of T25 and progeny seeds of RRS were imported directly from the USA. The following five seed samples of conventional dent corn were used as non-GM maize. Strike5512 maize was directly imported. Maize variety LG2265 was obtained in Japan. Maize varieties DK537 and RX740 were provided by Monsanto Co. QC9651 maize was obtained from Quality Technology International (Huntley, IL, USA). Dry soybean seeds harvested in Ohio in 1998 were also imported directly and used as a non-GM soy sample. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan and used as non-GM materials.

Preparation of test samples and DNA extraction

All dry seeds except for non-GM maize seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Idar-Oberstein, Germany). Ten kernels in each of 5 conventional maize samples were ground with a Multi-beads shocker (Yasui Kikai Co., Osaka, Japan). The ground materials were stored below −20℃ until DNA extraction. For maize, soy, wheat, barley, and rice, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany) as described previously[3]. The DNA concentrations of solutions were determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). DNA concentration was calculated by taking one optical density unit at 260 nm as equal to 50 ng/μL. All extracted DNAs were diluted to 10 ng/μL with sterile distilled water. For the evaluation of sensitivities of detection methods, a Bt11 maize genome sample (10 ng/μL) was mixed with a non-GM maize genome sample (10 ng/μL) to make three concentrations [0.2% (v/v), 0.1% (v/v), and 0.05% (v/v)].

PCR assay and electrophoresis conditions

We used two PCR assay methods described in the JAS analytical test handbook, i.e., detection methods for Bt 11 maize and starch synthase IIb (*SSIIb*) gene as the maize endogenous reference gene. Twenty-five microliters of reaction mixture contained 25 ng of genomic DNA, 200 μmol/L dNTPs, 0.625 units of AmpliTaq Gold Polymerase (Applied Biosystems, Inc., Foster City, CA, USA), 1.5 mmol/L $MgCl_2$, 2.5 μL of 10×PCR buffer II (Applied Biosystems, Inc.), and 0.5 μmol/L of a primer pair for the respective detection method. The primer pair of Bt11 3-5′ (5′-AAAAGACCACAACAAGCCGC-3′) and Bt11 3-3′ (5′-CAATGCGTTCTCCACCAAGTACT-3′), and the primer pair of *SSIIb* 3-5′ (5′-CCAATCCTTTGACATCTGCTCC-3′) and *SSIIb* 3-3′ (5′-GATCAGCTTTGGGTCCGGA-3′) were used for the Bt11 maize-specific and *SSIIb*-specific detection methods, respectively[3), 4)].

To improve the Bt11 maize-specific method, we modified the concentration of $MgCl_2$ to 1.2 mmol/L. The PCR amplification was carried out on an ABI PRISM 9700 (Applied Biosystems, Inc.) with thermal cycles consisting of 95℃ for 10 min for preincubation, 40 cycles of 95℃ for 30 sec for denaturation, 60℃ for 30 sec for annealing, and 72℃ for 30 sec for extension, and 72℃ for 7 min for final extension. For the experimental positive control in the PCR assays, GM maize detection positive control plasmid (Nippon Gene Co., Ltd., Tokyo, Japan) was used as template DNA. For the analysis of PCR products, agarose gel electrophoresis was carried out with 3% (w/v) LO3 agarose gel (Takara Bio, Inc., Otsu, Japan) in Tris-acetate-ethylenediaminetetraacetate (TAE) buffer with 0.5 μg/mL of ethidium bromide (Sigma Aldrich, St. Louis, MO, USA). Five microliters of each reaction mixture was mixed with 1 μL of 6×loading buffer (Nippon Gene Co., Ltd.), and the samples were subjected to electrophoresis at a constant voltage (100 V) for approximately 20 min in the TAE buffer. After the electrophoresis, the gel was photographed under UV radiation using a Densitograph system (ATTO, Tokyo, Japan).

We previously reported the development of real-time PCR array analysis as a comprehensive detection method for GM crops[5]. In this investigation, the method was utilized to confirm that the analytical samples of conventional maize were genuine non-GM maize. The assay was performed with a real-time PCR array plate including 31 targets, such as GM maize and soy events, recombinant DNA segments, and endogenous reference genes.[5]

Nucleotide sequence analysis

For the nucleotide sequence analyses of the non-specific amplification product, the products were cloned with pGEM-T vector (Promega, Madison, WI, USA) in *Escherichia coli* DH5α. Then, the cloned DNAs were analyzed by a DNA sequencing system, CEQ8000 (Beckman Coulter, Inc.) according to the manufacturer's protocol. The nucleotide sequences were subjected to a homology search using the Basic Local Alignment Search Tool (BLAST) system.*3

*3 Basic Local Alignment Search Tool at the National Center for Biotechnology Information website, http://blast.ncbi.nlm.nih.gov/Blast.cgi

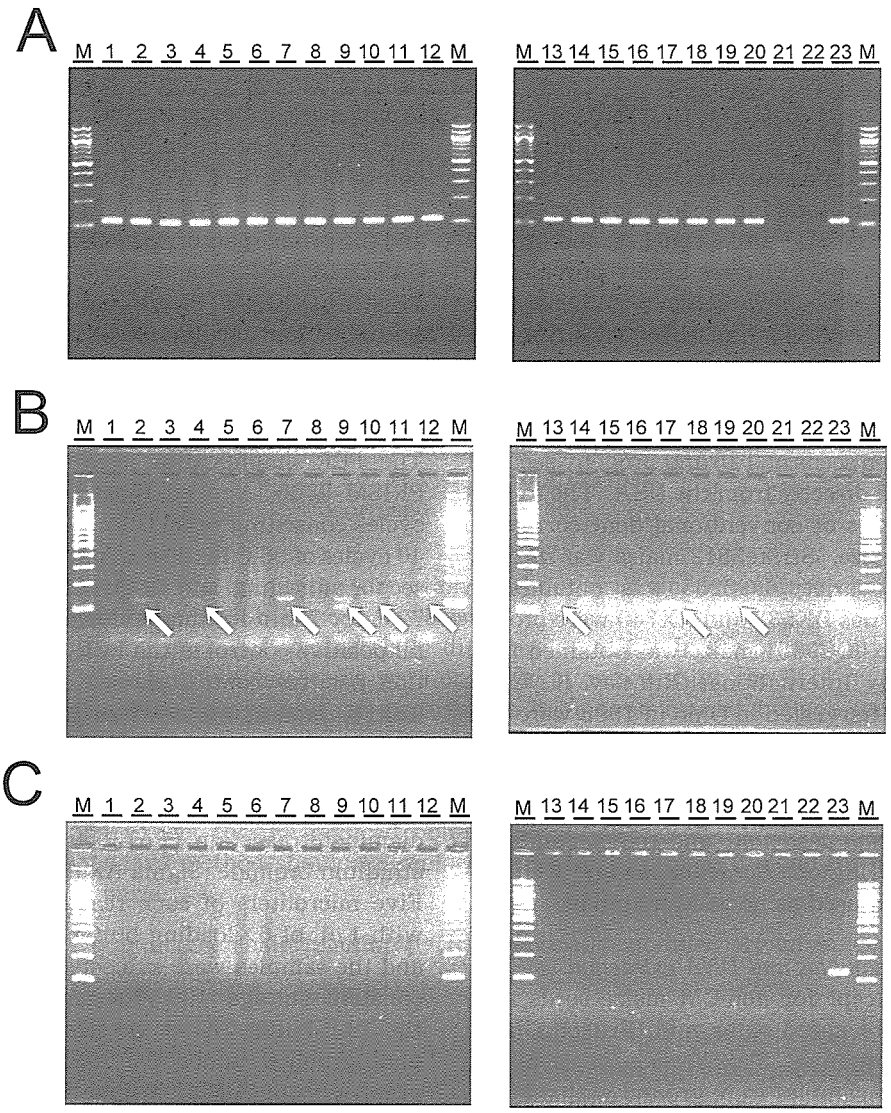

Fig. 1. The PCR assay results for the non-GM maize DNAs

A, the *SSIIb* assays; B, the assays with the currently used Bt11 maize-specific method; C, the assays with the improved Bt11 maize-specific method. Duplicate PCR assays of two DNA extracts were performed for each kind of maize materials. Lanes 1–4, the Strike5512 maize; Lanes 5–8, the LG2265 maize; Lanes 9–12, the DK537 maize; Lanes 13–16, the RX740 maize; Lanes 17–20, the QC9651 maize. In lane 21, no primer reaction for the negative control. In lane 22, no template reaction for the negative control. In lane 23, the reaction with positive control plasmid. In B and C, higher intensity of UV radiation was adopted to show the experimental results clearly. The arrows indicate non-specific amplification products.

Results

Non-specific amplification in the currently used standard method

The DNA extraction of ground non-GM maize samples was carried out in duplicate. The DNA extracts were analyzed using the real-time PCR array and were confirmed to be non-GM maize DNAs. The DNA samples were assayed twice in parallel with the *SSIIb* detection method as an experimental control, and DNA amplifications with the expected size in all the reactions were observed in the electrophoresis analyses (Fig. 1A). Samples were then analyzed twice in parallel with the currently used standard Bt11 maize-specific detection method. Faint signals of DNA amplification products of a size similar to that of the original Bt11 maize-specific amplification were observed in some reactions (Fig. 1B).

Nucleotide sequence analyses of non-specific amplification

The nucleotide sequence of the non-specific amplification product from the Strike5512 maize sample was analyzed as described above. In the homology search using the BLAST system, the nucleotide sequence of the non-specific amplification matched a part of the conventional maize genome (GenBank Accession No. CG441379) with more than 90% homology in the internal region between the two primers. The nucleotide sequence alignment of the original Bt11 maize-specific

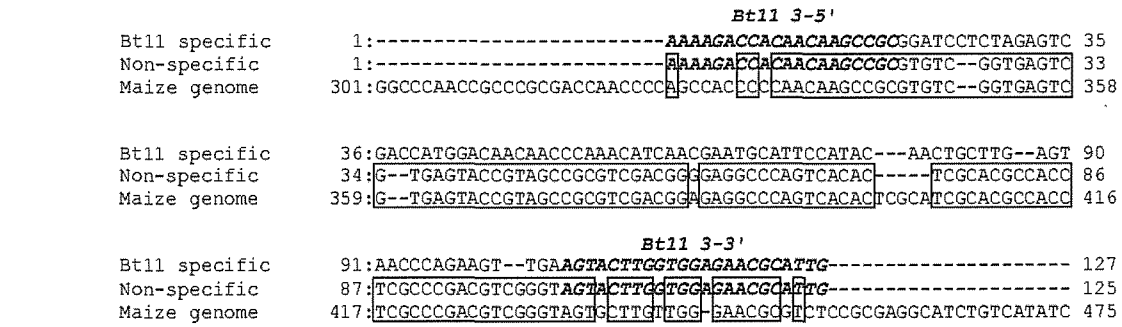

Fig. 2. The nucleotide sequence alignment of the original Bt11 maize-specific amplification product, the non-specific amplification product in the currently used detection method, and a part of a conventional maize genome obtained from the public database

The regions corresponding to the Bt11 primer pair in the the original Bt11 maize-specific amplification product and the non-specific amplification product are shown with bold and italicized face. The homologous nucleotides in the non-specific amplification product and the conventional maize genome are surrounded by boxes.

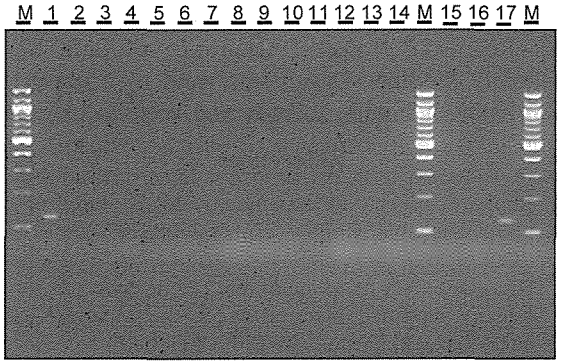

Fig. 3. The results of specificity evaluation for the improved Bt11 maize-specific method

Lanes 1–14, PCR results with each of the template DNAs extracted from Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507, non-GM maize, RRS, non-GM soy, non-GM wheat, non-GM barley, and non-GM rice; lane 15, no primer reaction for the negative control; lane 16, no template reaction for the negative control; lane 17, the reaction with positive control plasmid.

amplification product, the non-specific amplification product, and the part of the conventional maize genome obtained from the public nucleotide sequence database are shown in Fig. 2.

Improvement of the Bt11 maize-specific detection method

We modified the concentration of $MgCl_2$ in the PCR reaction mixture from 1.5 to 1.2 mmol/L and evaluated non-specific amplification with the same non-GM maize DNA samples that we had used for the standard method. Non-specific amplification was not observed in any of the reactions (Fig. 1C).

Evaluation of analytical performances

For the specificity evaluation of the improved method, we assayed representative GM maize and soy samples, the non-GM maize sample, the non-GM soy sample, the non-GM wheat sample, the non-GM barley sample, and the non-GM rice sample using the improved method (Fig. 3). The amplification product was observed only in the reaction with Bt11 maize DNA as a template, besides the positive control reaction. For the sensitivity evaluation of both the currently used and the improved methods, simulated DNA samples at various concentrations of Bt11 maize DNA [0.2% (v/v), 0.1% (v/v), and 0.05% (v/v)] were assayed 21 times. The results are shown in Table 1. All the assay results for 0.2% (v/v) and 0.1% (v/v) samples by the both methods were positive and the false-negative rates were 0% in the present examination. The assay results for 0.05% (v/v) samples included several false-negative results and the false-negative rates were 9.5% and 24% for the currently used and the improved methods, respectively.

Table 1. Sensitivity evaluation of the Bt11 maize-specific detection methods

Mixing level	The currently used method	The improved method
0.2% (v/v)	21 (0%)	21 (0%)
0.1% (v/v)	21 (0%)	21 (0%)
0.05% (v/v)	19 (9.5%)	16 (24%)

The numbers of table elements indicate the numbers of positive results in the total of 21 assays.
The percentages in parentheses indicate the false-negative rate in the total of 21 assays.

Discussion

Non-specific amplification was observed in testing with non-GM maize genome DNA as template DNA by the currently used Bt11 maize-specific detection method, as shown in Fig. 1B. The non-specific amplification products could not be distinguished on agarose gel electrophoresis assay, because the size of non-specific products was similar to that of the original products. This result suggested that the non-specific amplification may cause erroneous decisions about the presence or absence of Bt11 maize in analytical samples in practical testing. The nucleotide sequence analyses of the non-specific products and homology search in the

BLAST system proved that the non-specific amplification was caused by miss-annealing of the primer pair to conventional maize genomes. It was reported that the magnesium concentration in the PCR mixture affected primer annealing, strand dissociation temperatures of both the template and PCR products, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity[6]. We designed the improved detection method by decreasing the magnesium concentration and succeeded in eliminating non-specific amplification, as shown in Fig. 1C. The specificity evaluation demonstrated that the improved method was highly specific to Bt11 maize. In Europe, the European Network of GMO Laboratories validates analytical performances of official GMO detection methods as described in the report entitled "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"*4 in agreement with international standards, *i.e.*, the IUPAC Protocol for the Design, Conduct and Interpretation of Method Performance Studies[7] and International Standard ISO 5725*5, ISO 21571*6 and ISO 24276*7. According to the report, the limit of detection (LOD) is defined as the lowest amount or concentration of analyte at which the presence of the analyte should be detected at least 95% of the time, ensuring less than 5% false-negative results. For example, in the in-house validation of the European official Bt10 maize-detection method, simulated DNA-mixtures containing Bt10 maize DNA at low concentrations were assayed 21 times and the LOD was determined based on the examination result in accordance with the LOD definition.*8 We also performed LOD determination based on the results in Table 1 under the criteria, and the LODs of both the currently used and the improved detection methods were determined as 0.1% (v/v). The LOD determined in this investigation was representative of the performances of the methods. However, the LOD determined here may not directly reflect the actual LOD in practical testing, because we evaluated the LOD without taking into accout the bias derived from sample matrices and the dispertion of the target DNA's copy number occurring in the DNA extraction and dilution steps. We did, however, demonstrate that the sensitivity of the improved method was equivalent to that of the currently used method in this study. Thus, we concluded that the improved method has a lower incidence of false-positive detection owing to non-specific amplification, and would be more reliable than the currently used method in terms of analytical performance.

*4 European Network of GMO Laboratories. Definition of Minimum Performance Requirements for analytical methods of GMO Testing, The website of Community Reference Laboratory for GM Food & Feed, http://gmo-crl.jrc.ec.europa.eu/default.htm.

*5 International Standard 5725, Accuracy (trueness and precision) of measurement methods and results. 1994.

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LC/ESI-MS/MSによる畜水産物中の大環状ラクトン寄生虫駆除剤の一斉分析法（報文）

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食衛誌 **51**(1), 1～9 (2010)

LC/ESI-MS/MSを用いた畜水産食品中のアバメクチン，8,9-*Z*-アバメクチン，エマメクチン，8,9-*Z*-エマメクチン，イベルメクチン，エプリノメクチン，ドラメクチン，モキシデクチン（8種類）の残留一斉分析法を開発した．畜水産試料（10種類）にアセトン-0.5%アンモニア水溶液を加え，ホモジナイズ後，イソオクタンにより抽出し，*n*-ヘキサンで脱脂を行った後，LC/ESI-MS/MS測定を実施した．定量法には，絶対検量線を用いて，検出限界0.02～1.5 ng/mLおよび定量限界は0.1～5 ng/mLであった．イオン化効果の影響を検討した結果，牛肝臓において，30%以上の抑制効果となったため，希釈法により，解決した．いずれの試料において，平均回収率70.8～117.1%の範囲であり，併行精度および室内精度において，15%以下であった．

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定量NMRを用いたコチニール色素中のカルミン酸の絶対定量（報文）

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井原俊英　吉田雄一　久保田領志　田原麻衣子　清水久美子
伊藤澄夫　山崎　壮　河村葉子　西村哲治

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天然着色剤として広く用いられているコチニール色素の主成分であるカルミン酸の絶対定量に定量NMR(qNMR)を応用した．各社より試薬カルミン酸が販売されているが，その純度が正確に値付けられていないため，この市販試薬を定量用標準品の代用品としてHPLCにより定量した場合，信頼性の高い分析値は得られない．そこで，我々は国際単位系(SI)にトレーサブルな絶対定量法の1つとして，NMRスペクトル上に観察されるシグナル強度がその核のモル量に正比例することを原理としたqNMRを開発している．qNMRによるカルミン酸の絶対定量を以下のように行った．認証標準物質フタル酸水素カリウムを用い，qNMR標準液中の基準物質2-ジメチル-2-シラペンタン-5-スルフォン酸-d_6ナトリウム塩(DSS-d_6)の濃度校正し，カルミン酸のフェニルプロトンとDSS-d_6のメチル基に由来する9つのプロトンとのシグナル強度比より，コチニール色素製品および試薬製品中のカルミン酸の含量（純度）をカルミン酸カリウム塩3水和物として絶対定量した．その結果，コチニール色素製品および市販試薬中に4.6～30.5%および25.3～92.9%含有されていることを明らかとした．qNMRによる絶対定量は，1測定当たりの所要時間が約10分と極めて迅速であり，測定対象の化合物と同一の定量用標準品を必要としないだけでなく，SIにトレーサブルな分析値を導く点が優れている．また，qNMRは，あらゆる有機化合物の絶対定量にも応用可能であり，分析値の信頼性向上のための重要な1つのツールとしてなりうるものと思われる．

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ポリメラーゼ連鎖反応を用いた遺伝子組換えトウモロコシBt11系統特異的定性検知法の非特異的増幅低減を目的とした改良（ノート・英文）

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食衛誌 **51**(1), 32～36 (2010)

JAS分析試験ハンドブック記載の遺伝子組換えトウモロコシBt11系統特異的定性検知法は行政モニタリング検査および市場に流通する穀物の品質管理に幅広く利用されている．当該検知法を用いた検査において偽陽性検出が生じる可能性が確認されたため，分析法の改良を行った．改良分析法において非特異的増幅は確認されず，一方，反応特異性および検出感度について現行分析法との間に顕著な差異は確認されなかった．

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一日当たり350 g以上の野菜を使った食事中の硝酸塩および亜硝酸塩の含有量調査（報文）

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食衛誌 **51**(1), 10～18 (2010)

一日当たり350 g以上の野菜を使った食事中の硝酸塩および亜硝酸塩の含有量を調査した．野菜使用量は女子大学生の作成した献立A（20日分）で350±2 g，料理本を参考に作成した献立B（20日分）は457±77 gであった．硝酸塩含有量は，献立Aで321.0±139.3 mg（範囲104.2～636.9 mg），献立Bは245.7±90.7 mg（範囲140.1～507.3 mg）で，ADIを超えたものは，献立Aは16試料，献立Bで13試料あった．亜硝酸塩含有量は，献立Aで1.2±0.3 mg（範囲0.7～1.8 mg）で，いずれもADI以下であったのに対し，献立Bは2.8±0.9 mg（範囲1.3～4.6 mg）であり，6試料がADIを超えていた．さらに，女子大学生対象の野菜の調理法に関する認知度調査では，硝酸塩の多い野菜は，80%以上が「ゆでたり煮たりして食べる野菜」として認識していた．

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プロテインチップを用いた腸管出血性大腸菌O-157 : H7由来ベロ毒素の高感度検出（ノート）

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腸管出血性大腸菌O-157 : H7が産生するベロ毒素には免疫学的性状および物理化学的性状が異なるが，互いに60%のアミノ酸相同性を有するtype 1とtype 2が存在する．Type 1は志賀赤痢菌が産生する毒素と同じアミノ酸配列を有することから志賀毒素や志賀様毒素とも呼ばれ，感染すると水様便，血便，発熱，腹痛などの症状を引き起こす．現在，ベロ毒素はELISAやPCRなどで検出されるが，高感度検出において困難を伴う場合がある．本研究ではProteoChipを用いてベロ毒素タイプ1･2混合物を市販製品よりも少ない量の試薬で，より低濃度の検出限界を得ることに成功した．

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加工モデル実験によるコメ内在性DNAが検出されなかったビーフンに関する一考察（調査・資料）

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コメ加工品の遺伝子組換え食品検査において，PCR検査でDNAが抽出されていることを確認するための内在性DNAが検出されなかった検体（ビーフン）があった．そこで，コーンスターチおよびコメを原材料とするモデル加工実験（コメ粉含有率0, 2, 5, 10%）を行い，コメ粉含有量と加工度が内在性DNA検出へ与える影響を調べた．加工モデル実験試料はコメ粉とコーンスターチを水とともに混合し，加熱，蒸気および加圧の各加工処理により作製した．加熱および蒸気処理ではコメ粉含有率2%で，加熱加圧処理ではコメ粉含有率10%でコメ内在性DNAが検知された．100%コメ粉を用いた加工モデル実験試料から，加熱加圧処理によってDNAが著明に分解することが示された．

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