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Method article

Development and application of a general plasmid reference material for GMO screening

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ABSTRACT

The use of analytical controls is essential when performing GMO detection through screening tests. Additionally, the presence of taxon-specific sequences is analyzed mostly for quality control during GMO detection. In this study, 11 commonly used genetic elements involving three promoters (P-35S, P-FMV35S and P-NOS), four marker genes (*Bar*, *NPTII*, *HPT* and *Pmi*), and four terminators (T-NOS, T-35S, T-g7 and T-e9), together with the reference gene fragments from six major crops of maize, soybean, rapeseed, rice, cotton and wheat, were co-integrated into the same single plasmid to construct a general reference plasmid pBI121-Screening. The suitability test of pBI121-Screening plasmid as reference material indicated that the non-target sequence on the pBI121-Screening plasmid did not affect the PCR amplification efficiencies of screening methods and taxon-specific methods. The sensitivity of screening and taxon-specific assays ranged from 5 to 10 copies of pBI121-Screening plasmid, meeting the sensitivity requirement of GMO detection. The construction of pBI121-Screening solves the lack of a general positive control for screening tests, thereby reducing the workload and cost of preparing a plurality of the positive control.

1. Introduction

The performance of GMO detection is the prerequisite to implement GMO (genetically modified organisms) regulations. The scheme of GMO testing usually starts from the screening and ends in the event-specific quantification (Zel et al., 2012). The screening methods target a part of insert DNA sequence that is present in many GMOs, such as the sequences of promoters, terminators, maker genes or construct-specific sequences (Marmiroli et al., 2008; Holst-Jensen et al., 2003). GMO screening enables us to preliminarily determine the presence or absence of GMO ingredients in the testing samples. The event-specific methods target the nucleotide sequence at the junction between the host genome and the insert DNA, allowing unique identification of the individual GMO in the test samples (Holst-Jensen et al., 2003). With the rapid commercialization of GM crops, numerous GMOs entered into the market. In 2014, 357 GM crop varieties from 27 different species were commercialized worldwide (James, 2014), and numerous new events are in the pipeline for commercialization in the coming years. Due to the large number of GMOs on the market, a screening approach based on PCR techniques has become an integrated part of GMO detection (Holst-Jensen, 2007). Appropriate selection of screening methods can contribute to reduce the number of identification tests, reducing the workload and cost of analysis in subsequent steps. In order to help with the efficient selection of screening methods, the matrix approach with a reference table of the presence/absence of the corresponding elements in known GMOs was developed and recognized as the most appropriate approach, such as the developed GMOtrack or GMOseek tools (Kralj Novak et al., 2009; Block et al., 2013). The European Union (EU) built the JRC GMO-matrix to help the GMO testing laboratories to evaluate if their GMO screening strategies are appropriate (http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/) (Angers-Loustau et al., 2014).

Screening as well as event-specific detection require reference materials (RMs) for positive controls (Trapmann et al., 2002). RM for DNA-based methods is a material containing the analyte and can be categorized into powdered material, genomic DNA extracted from matrix material, or an artificial plasmid containing the specific nucleotide sequence. Currently, the production agencies of GMO RMs worldwide mainly include the Institute for Reference Materials and Measurement (IRMM) in the EU and the American Oil Chemists' Society (AOCS) in the USA. IRMM produces two types of RMs intended for detection of GMOs: flour and plasmid RMs (http://irmm.jrc.ec.europa.eu/Pages/rmcatalogue.aspx). While for that same purpose, AOCS produces seed, seed powder and genomic DNA RMs. (http://secure.aocs.org/crm/index.cfm). The GMO RMs produced by both the AOCS and IRMM

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were primarily designed to meet the requirements for event-specific detection.

At the GMO screening phase, the combination of multiple genetic elements was proposed as a universal screening approach, the principle being to cover as many GMOs as possible in order to improve detection efficiency and cost-effectiveness (Querci et al., 2010). With the increasing diversity of GMOs on the market, the selection of an appropriate screening strategy is becoming more complex. On the other hand, a GM event usually involves a limited number of genetic elements. Therefore, several transgenic events are mixed together for use in the quality control of the screening method. The use of a mixture of several GM materials as a positive control has been found to have several deficiencies, such as the unavailability of part elements (*HPT* gene) in authorized GM events, inconsistent copy number for different targets, different DNA extraction efficiencies for different GM events, and so on (Li et al., 2015). An additional drawback of these mixtures when they are made out of flour-based RM is the contamination at trace level with other GM events (Block et al., 2013). Combined with the above considerations, it is necessary and urgent to develop a general GM screening RM that can cope with different screening strategies and make GMO screening more efficient.

Plasmid DNA samples harboring targets of interest have been successfully used as alternative standards for GMO tests in place of matrix RMs since 2001 (Taverniers et al., 2001). Many plasmid references carrying a single target, double targets or multiple targets have been developed and described (Taverniers et al., 2004; Taverniers et al., 2005; Wang et al., 2011). The commutability analysis revealed that the matrix-matching error between genomic DNA and plasmid DNA would result in quantitative error of GM DNA using plasmids as a calibrator (Zhang et al., 2014). Still, the plasmids can provide convenient and reliable positive controls for qualitative detection of GMOs. The plasmids have several advantages as RMs compared to genomic DNA RMs such as not being dependent on plant materials, easily aggregating multiple targets, having cost-efficient production and rapid propagation. The plasmid standards can be used as “golden standards” (Taverniers et al., 2004). To solve the lack of RM for screening, we cloned the commonly used screening elements from transgenic materials and reference genes of major crops in order to co-integrate the screening targets and crop reference genes into the same one vector to construct a general plasmid RM for screening different GM crops. The development of the reference plasmid would provide a general positive control that could fit for high-throughput, efficient, cost-effective, and fast analytical tools to track GMOs.

2. Materials and methods

2.1. Materials

Two frequently used binary vectors pCAMBIA1300 (Cambia, Brisbane, Australia) and pBI121 (Invitrogen, Carlsbad, CA, USA), were collected to clone screening targets and construct reference plasmids. To test the suitability of reference plasmids as positive controls for GMO screening, the following GMO RMs were purchased from the IRMM (Geel, Belgium) and AOCS (Champaign–Urbana, IL, USA), including the transgenic soybean A5547-127 (DNA, AOCS0707-C3), GTS-40-3-2 (10% seed powder, ERM-BF410gk), MON89788 (DNA, AOCS0906-B); transgenic rapeseed GT73 (seed, AOCS0304-B), Topas 19/2 (DNA, AOCS0711-D2), T45 (DNA, AOCS0208-A5), RF1 (DNA, AOCS0711-B); transgenic maize Bt176 (5% seed powder, ERM-BF411f), MON863 (9.85% seed powder, ERM-BF416d), MON89034 (seed powder, AOCS0906-E), MIR162 (seed powder, AOCS1208-A); transgenic cotton MON1445 (seed powder, AOCS0804-B), MON15985 (seed powder, AOCS0804-D), MON88913 (seed powder, AOCS0906-D), LLcotton25 (DNA, AOCS0306-E2); transgenic rice LLRICE62 (DNA, AOCS0306-I4). In addition, our laboratory collected three transgenic rice varieties TT51-1, KMD and KF6, and two conventional wheat varieties (Zhongmai 895, Xinmai 21).

2.2. DNA extraction

A QIAfilter™ Plasmid Giga Kit (QIAGEN, Crawley, UK) was used to extract plasmid DNA. The genomic DNA samples from the seeds or seed powders were extracted and purified with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of both DNA and plasmid was evaluated by examining the ratios of OD260/OD280 and OD260/230 using a NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA), and the DNA concentrations were measured using the Picogreen dye method with a VersaFluor™ Fluorometer System (Bio-Rad, Hercules, CA, USA) and a Quanti-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) (Li et al., 2014). The concentration of each DNA solution was adjusted to 20 ng/μL for further use.

2.3. Construction of the reference plasmid

A plasmid pBI121-ELEMENTS carrying 11 screening targets had been constructed to develop the screening-used matrix RM with the binary vector pBI121 as backbone (Li et al., 2015). In this study, the plasmid pBI121-ELEMENTS was used as an intermediate vector to construct a general reference plasmid through further inserting the reference gene fragments from major crops. The selected reference fragments were firstly assembled into a large fusion fragment, and then synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). The synthesized fragment was maintained by cloning into the vector pBluescript II SK.

In the process of constructing a complicated plasmid, we usually encounter the difficulty of no appropriate restriction sites. Hence, the digestion strategy was utilized to produce the appropriate overhangs with the assistance of T4 DNA polymerase (Zhang et al., 2006). T4 DNA polymerase possesses both polymerase and 3′ → 5′ exonuclease activities, if no dNTP is supplemented to the reaction system the polymerase ability would be prevented, and the 3′ → 5′ exonuclease activities would be activated (Stocki and Nonay, 1995). Adding one specific dNTP to the reaction system would enable the 3′ → 5′ exonuclease reaction to terminate at the corresponding nucleotide, thus generating suitable cohesive ends. The plasmid pBI121-ELEMENTS was cleaved using the endonuclease *Pme*I to generate a blunt end fragment, which was firstly treated with T4 DNA polymerase supplemented with 1 mM dCTP for 20 min at 12 °C to form overhangs, then treated in the presence of 1 mM dGTP at the same reaction conditions to further extend the sticky ends (Fig. 1). The primers EF and ER, containing adapter sequences identical to terminal sequences of linear vectors by *Pme*I at 5′ ends, were designed using the software Primer Premier 5.0 to amplify the fusion reference fragment (Table 1). In order to generate compatible overhangs with the sticky ends of the intermediate vector, the amplified fusion reference fragment was treated with T4 DNA polymerase successively supplemented with 1 mM dCTP and 1 mM dGTP (Fig. 1). The digested vector fragment and fusion reference fragment were put together to ligate with each other using the T4 ligase. The constructed plasmid was sequenced by Shanghai Sangon.

2.4. Primers and probes

The used primers and TaqMan® probes in this study were taken from the validated GMO detection standards or previously published papers. The nucleotide sequences of the primers and probes were identical to those provided in the original publications. All TaqMan® fluorescent probes were labeled with a reporter dye 6-carboxy-fluorescein (FAM) at the 5′ ends and with a quencher dye Black Hole Quencher 1 (BHQ1) at the 3′ ends. All primers and fluorescent probes were synthesized by Shanghai Sangon Biological Engineering Technology and

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