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Research Progress of the Functional Nucleic Acid Techniques in GMO Detection

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Abstract

Functional nucleic acids are a kind of nucleic acid sequences with special functions, which can specifically bind with the target substances or catalyze the reactions. Many target, including mycotoxins, small RNA, heavy metal ions and DNA segment, can bind to particular selected oligonucleotides, and then realized the detection. The uses of functional nucleic acids to detect the genetically modified organism (GMO) have been pursued using different approaches. Meanwhile, the flanking sequence, which was the most specific target in the GMO detection, was also usually separated with the help of functional nucleic acid. During the detection, the functional nucleic acid provided superior sensitivity, specificity and success rate compared with the traditional methods. In this report, we described different functional nucleic acids used in the GMO detection, they were classified based on their structures, and some of them were developed in our lab. The principle, structural composition, advantage, and the comparisons of the functional nucleic acids were reported. Considering most of the functional nucleic acids are fluorescently-labeled, in order to reduce the cost, more and more functional nucleic acids without labeling are under research.

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Introduction

An increasing number of genetically modified organisms (GMOs), especially the GM crops, which are widely cultivated as sources of food and feed in many countries, have been developed using recombinant DNA technology [1]. Although the transgenic technology has been developed for many years, however, several controversial issues are being discussed in the whole world, including food safety, environmental risk, and ethical concerns [2]. Consequently, in order to protect consumers' freedom of choice, more than 40 countries have established series of laws and rules for GMO labeling. For instance, the threshold for labeling of GM material in a non-GM background is defined as 0.9% in the European Union, 3% in Korea, 5% in Japan, and zero in China [3]. Just because the different requirements among the countries, the quantitative detection and identification of the GMO are very important.

The methods, which are based on the polymerase chain reaction (PCR) amplification, are widely used in the detection of GMOs [4; 5]. As the increasing of the GM crops, the government request becomes much stricter, and the detection techniques are increasing correspondingly. Nucleic acids are always used as functional detection medium in the detection. Some small molecules, such as mycotoxins, small RNA, heavy metal ions, can bind to particular selected oligonucleotides, and then realized the detection procedure [6; 7]. The most significant part of oligonucleotides is called aptamer. Other functional nucleic acids, such as DNA probes and DNAzymes, are also viewed as applicable food safety detection media and signal indicators [8].

This article gives an overview of the functional nucleic acids, which were employed in the GMO detection methods, including the flanking sequence separation and the quantitative detection. The detection specificity, sensitivity and success rate can be obviously enhanced when applying the functional nucleic acid. Most of them are labeled with the fluorescence groups. The principle, structural composition, advantage, and the comparisons of the functional nucleic acids were reported. In order to decrease the cost and as the development of the biotechnology, more and more functional nucleic acids without labeling have been discovered, and the novel functional nucleic acid with new working principle must be developed and used in the GMO detection.

The Functional Nucleic Acids used in the Genome Walking Techniques

In the GMO detection, the most specific methods are designed based on detecting the flanking sequence. The flanking sequence is a conjunction region between the exogenous inserted gene and the endogenous gene. Due to the random insertion of the exogenous genes, and the detailed flanking sequence is usually unknown. Only after the genome walking, which is used to identify this unknown sequence flanking already known DNA regions in any given genome, the flanking sequence can be revealed [9].

The traditional genome walking methods, such as Inverse PCR (I-PCR) [10], Thermal Asymmetric InterLaced PCR (TAIL-PCR), Random priming PCR [11], are relatively earlier technologies, and only with the help of specific and random primers. As the novel genome walking methods developed, the functional nucleic acids were employed. According to the structural shape of the functional nucleic acid, they can be classified into two groups. The first category is the "Y" shape; the second is the loop shape.

The "Y" shape Functional Nucleic Acid Method

The A-T Linker

The A-T linker was reported in 2012, it is a double strands adaptor with a "Y" shape. The structure is shown in figure 1A. It is a double strands functional nucleic acid, containing the long- and short-strand primers [12]. The long-strand primer consists of 40-50 bp and has a high GC content at the 3′ end, which ensures its strong binding with the short-strand primer. The formation of hairpin structures should have low energy and avoided the formation at the 3′ end. Because they could interfere with the binding of the short-strand primer. The short-strand primer consists of 8-15 bp, and can generate a T overhang when binding with the long strand. Its 5′ end is phosphorylated, which can increase the efficiency of ligation with the digested fragments. Moreover, an amino C6 moiety was added at the 3′ end as an -NH_2 group, on one hand to prevent the extension of the short-strand primer, and on the other hand to avoid generating non-specific products. The two adaptor primers with high melting temperature were designed according to the sequence of the long-

strand adaptor.

The A-T Linker can effectively avoid the self-ligation between the adaptors by appropriate modifications. Moreover, the use of a common adaptor for all restriction enzymes makes the method more flexible and cost saving. The novel A-T linker adaptor PCR method is versatile, with high specificity, flexibility, efficiency, and can be used in high-throughput work.

The 5′ End-Directed Adaptor

The 5′ end-directed aptamer was developed by Xu et al in 2013 [13]. The adaptor is double strands, which is partially (in 11 bp) reverse complemented with each other, and each strand is consist of 33 bp (figure 1B). When the fragments are ligated to the adaptors, the adaptor in the 5′ ends of the ligation products are different from those in the 3′ ends. Because the adaptors are not completely reverse complemented, the primers used for amplifying the ligation product are designed based on the sequences of the adaptors. The sequence of AP1 is the same with Aptamer-1, and the sequence of AP2 is reverse complemented with Aptamer -2. There would be four types of amplification styles during the PCR when different pairs of primers are used. After verification, the specificity of genome walking can be enhanced when we use AP1 with specific primer, which is also one of the advantages of this new directed -adaptor.

By using the 5′ end-directed adaptor, the self-ligation between the adaptors can be avoided effectively by a T overhang in the 3' end, and the PCR can be conducted through combining different adaptor primers with specific primers, hence the success rate of the reaction can be enhanced effectively.

The Loop Linker

Loop-linker PCR was first developed in 2012 to obtain the flanking sequences of GM crops [14] (figure 1C). Several genome walking methods including Vectorette PCR [15], boomerang PCR [16], panhandle PCR [17] and self-formed aptamer PCR [18] have adopted the loop-linker structure. In the loop-linker PCR, the loop-linker aptamer is a 52 bp single strand oligonucleotide with two complementary regions at its 5′ and 3′ ends. At the 5′ end of the sequence, an additional 5 bp oligonucleotide (5′ GATCT-3′ or 5′-AATTT-3′) is used to generate an enzymatic protruding end site and a thymine nick site when the adapter is ligated with restricted DNA. This ligation site includes *BamH*I (/GATC) or *EcoR*I (/AATT) protruding ends and one T that generates a nick when the adapter is ligated with restricted DNA. To ensure the stability of the stem-loop structure, complementary sequences of approximately 8-12 bp were designed. The annealing of the formed-adapter primer can generate either self-complementary or trans-complementary products. To guarantee the efficient ligation between the aptamer and digested DNA, the 5′ end of the aptamer primer is modified with a -PO_4 group.

The nick position in the adapter is beneficial to prevent adapter self-ligation and to form a site for the extension of *Taq* DNA polymerase. In the PCR process, the initial annealing step in the primary PCR is a key factor that determines the specificity and efficiency of this method. Through extension by *Taq* DNA polymerase, each ligation product (i.e., adapter-digested DNA-adapter) will form two extension products with panhandle structures. These structures are more stable than the primer-template hybrid, and will therefore the exponential amplification except from ligation products that contain the specific primer-binding site can be suppressed.

All the three functional nucleic acids can separate the flanking sequence efficient, when the GMO is generated by different ways, the copy number of the exogenous gene is different. When the exogenous gene inserted into the genome in single copy, the A-T Linker and Loop Linker can be used to obtain the flanking sequence. When the exogenous gene inserted into the genome more than one copy and randomly, the 5′ end-directed adaptor can get the multiple flanking sequences simultaneously.

The Functional Nucleic Acids Used in the GMO Detection Techniques

In order to fulfill the GMO labeling demands, the quantitative detection is very important. During the PCR detection methods, especially the quantitative detection, the functional nucleic acid is widely used. They were usually labeled with fluorescence group in their one or two ends, under the action of FRET (fluorescence resonance energy transfer), and the quantitative detection can be realized. According to the structure and constitute of the nucleic acid, the functional nucleic acids

were mainly classified into three categories, the single linear strand, double linear strand and hairpin structure.

The Single Linear Strand Functional Nucleic Acids

Taqman Probe

Taqman probe is the most widely used functional nucleic acid in the quantitative detection (figure 2A), and it has been reported in the detection of the GM maize, soybean, cotton and so on [19-22]. The reporter group, such as FAM and VIC, is labeled at one end of the probe, and the quenching group, such as TAMRA, is tagged at the other end. When the probe preserves its integrity, the quenching group absorbs the emission fluorescence of the reporter group. That is why when the probe hybridizes with the template, no fluorescence signal can be detected. With the amplification starts, the probe is hydrolyzed by the *Taq* DNA polymerase, the reporter and quenching groups are separated far away from each other, the reporter energy cannot be absorbed; and then one fluorescent signal is released. Therefore, both the fluorescent signal and the target fragment have an exponential increase with the PCR cycle number, and the quantification realized.

Taqman MGB Probe

Taqman MGB probe is an improved probe based on the Taqman probe (figure 2B), and the Minor Groove Binder molecule (MGB) is add on its 3′ end. The MGB molecule can combine with the minor groove of the double-stranded DNA specifically. It can significantly enhance the Tm value of the probe, so that it can distinguish the mismatch of one base. Even if one base was mismatched, no signal could be detected. Moreover, the labeled quenching group is a non-fluorescent group, during the reaction, there is no background fluorescence.

The Taqman MGB probe has many advantages compared with the Taqman probe, such as better quenching effect, labeling flexibility, improved signal-to-noise ratio of the fluorescent signal and higher annealing temperature. Due to its supersensitive specificity, it increases the recognition rate of SNP and has much more applications [23].

Self-Quenched Probe with Single Fluorophore

In the self-quenched probe, only one fluorescent group is involved. Due to the guanosine was reported to have a significant effect of fluorescence; hence, it is selected as the other "fluorescent group". The proximity of guanosine in the complementary strand with the fluorescent group upon hybridization can decrease the fluorescence intensity. And this kind of "self-quenched" probe is also called base-quenched probe (figure 2C) [24; 25]. Through labeling with a single fluorescent group at the 5′ end of the oligonucleotides, the fluorescence signal can be decreased upon hybridization, which may provide a basis for a cost-effective DNA quantitative detection method. However, the amount of signal generated by this decrease is small in comparison to the signal generated by FRET-labeled probes, which might affect the sensitivity of the detection [26].

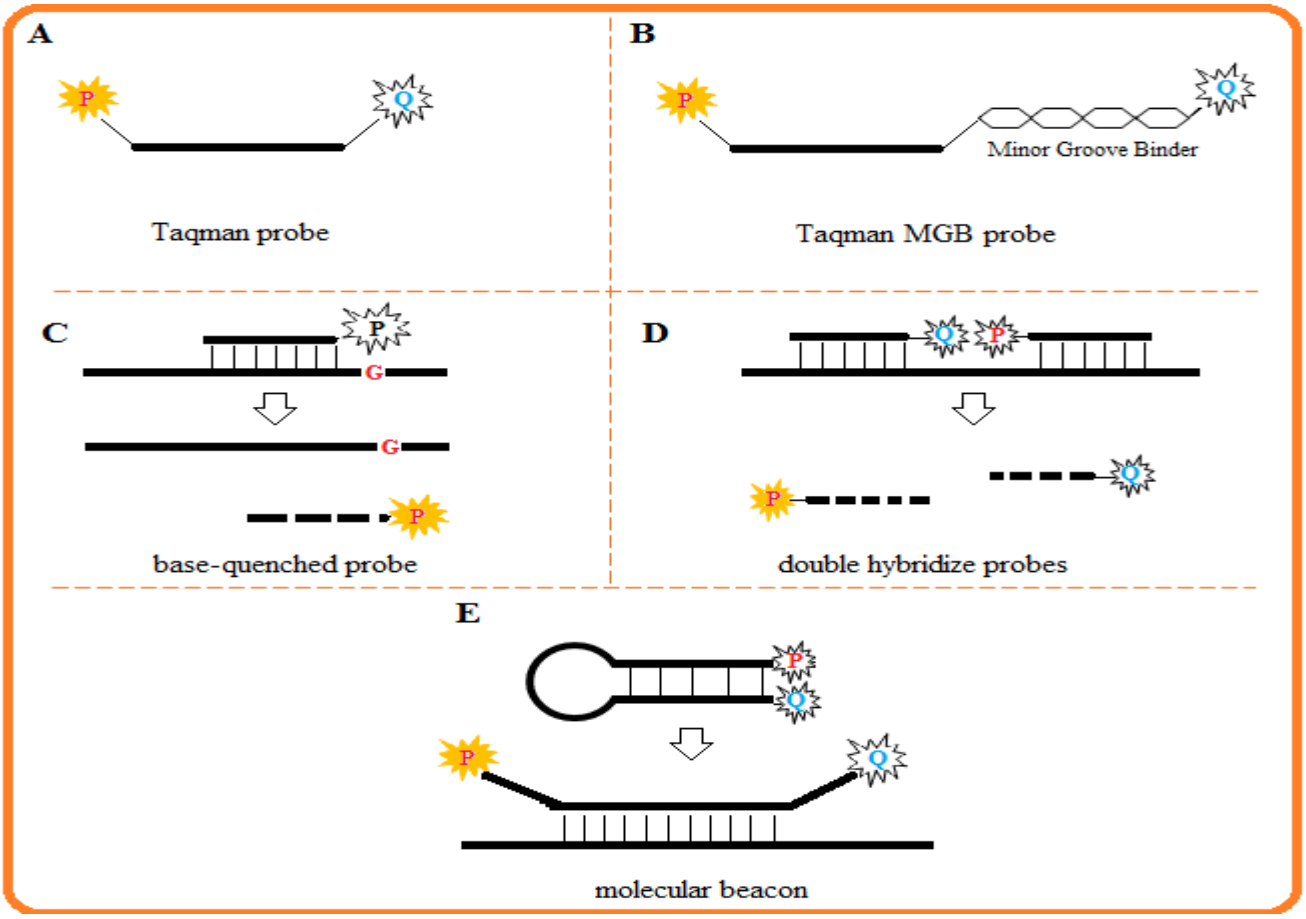
In the subsequent study, the fluorescent primer is designed to be "self-quenched" until it is incorporated into a double-stranded PCR product, whereupon its fluorescence increases, i.e., is "dequenched". The fluorescent primer is called Light Upon eXtension (LUX) primer, which is also self-quenched probe [27; 28]. In the quantitative PCR reaction, the counterpart primer used in conjunction with the fluorescent primer is a standard, unlabeled oligonucleotide. LUX primer is designed based on studies that demonstrate the effects of the primary and secondary structure of oligonucleotides on the emission properties of a conjugated fluorescent group. The design factors are largely based on the necessity of having guanosine bases in the primary sequence nearby the conjugated fluorescent group. The fluorescent group may be various commonly used dyes such as FAM and JOE. Furthermore, the LUX primers have a non sequence -specific 5′ tail that is complementary to the 3′ end of the primer. The 5′ tail enables the primer to assume a hairpin conformation at temperatures below the melting point of the hairpin, and it also effects the fluorescence of the LUX primer.

The Double Strands Functional Nucleic Acids

The double strands functional nucleic acid usually contains two DNA strands, the reporter group and quenching group are separately labeled each strand. The most commonly is the double hybridize probes.

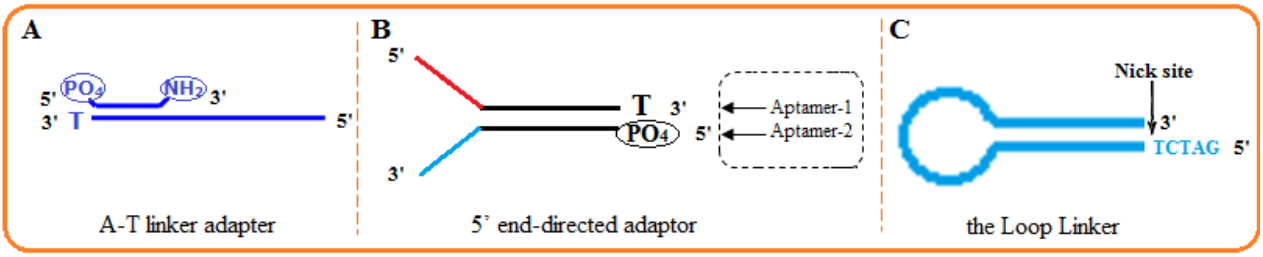
The double hybridize probes were developed by Roche Company, which was also rely on the FRET [29]. The hybridize probes contain two single strands and two fluorescence groups. The reporter and quenching probes

Figure 1. The Illustrations of the functional nucleic acids used in the genome walking



A, A-T Linker adapter; B, 5' end-directed adapter; C, Loop Linker.

Figure 2. The Illustrations of the functional nucleic acids used in the GMO detection.



A, Taqman probe; B, Taqman MGB probe; C, based-quenched probe; D, double hybridize probe; E, molecular beacon.

are separately labeled on the 5′ and 3′ ends of the two probes (figure 2D). These probes can both hybridize with one template in the adjacent region (1-5 bp), and the FRET can happen during the hybridization. As the amplification, the probes can be hydrolyzed one by one, and then the fluorescent signal releases.

Although this method has high quenching efficiency, however, the amplification efficiency is relatively low comparing with the Taqman probe and Taqman MGB probe. In addition, two long probes are involved, which makes the cost relatively high.

The Hairpin Structure Functional Nucleic Acids

Molecular beacon is a loop-stem oligonucleotide probe with most typical hairpin structure, which is labeled with a pair of fluorescence groups [30]. The sequence on the loop (15-30 bp) can hybridize with the target sequence complementary. The reporter and quenching groups are labeled on its 3′ and 5′ end separately (figure 2E). The stem part (5-7 bp) cannot match the target. When the molecular beacon hybridizes with the target in the reaction system, the hairpin structure is no longer existed, and then leading to the restoration of fluorescence [31].

When the molecular beacon developed in the initial stage, it has been used in many kinds of detection, however, all of them only been used in the homogeneous liquid solution, which limited the applications of molecular beacon in in vivo biomedical studies and in DNA biosensor development. Hence, a biotinylated molecular beacon was developed, which structure was the same with that of the tradition one, only the biotin was label on the stem part [32].

Since the molecular beacon has been developed, it has been widely used in the detection method. As the presence of the isothermal detection, it was employed to enhance the specificity and sensitivity [33; 34]. Meanwhile, due to the advantages of molecular beacon compared with other functional nucleic acid, more and more study related to improve the modification and the length of the molecular beacon have been conducting in many research groups.

Locked Nucleic Acid

Locked nucleic acid (LNA) is a nucleic acid analogue. It cAN be used in any DNA strand or oligonucleotide, so it was not included in any categories of the functional nucleic acid. The ribose ring of the LNA is constrained by a methylene linkage between the 2′-oxygen and the 4′-carbon. It contains one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an mimicking sugar conformation [35; 36].

LNA oligonucleotides display unprecedented hybridization affinity toward complementary single-stranded RNA and complementary single- or double-stranded DNA. Structural studies have shown that LNA oligonucleotides induce A-type (RNA-like) duplex conformations. The wide applicability of LNA oligonucleotides for gene silencing and their use for research and diagnostic purposes are documented in a number of recent reports, including the GMO detection[37; 38].

When the LNA is applied in the conventional TaqMan probe, the affinity between the probe and the target sequence can be significantly enhance. Meanwhile, the Tm value of the probe is increased, which makes the length of the probe much shorter, and much suitable for detecting the short DNA segments.

Discussion

Functional nucleic acids are a kind of nucleic acid

sequences with special functions, which can specifically bind target substances or catalyze reaction. Besides the inherent characteristics of the nucleic acids, functional nucleic acids have many advantages compared with the traditional detection methods (especially the methods with the help of the antibody), such as easy to obtain and modify, various kinds of identifiable targets, high affinity, greater specificity and stability, and so on. Based on their special characteristics, they have demonstrated enormous potentials in many fields, such as biological analysis, food safety testing (GMO detection is one of the important aspect), environmental monitoring, and pharmaceutical testing [39-43]. During the GMO detection, most of the existed functional nucleic acids have their own advangates, but they are usually modified with fluorophores, through observing or quantifying the fluorescence signal, the detection is realized. However, the fluorophores are costly, even has the potential to impact the specific identification. Hence, the label-free functional nucleic acid has been a hot point for research, especially the G-quadruplexes, which can realized the self-reporting [44-46]. We believe that more and more functional nucleic acids which novel structure and principle will be established, and will have broad applicability for rapid, easy, quantitative, and ultrasensitive, analysis of any DNA.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Abbreviations

GMO: Genetically Modified Organism

PCR: Polymerase Chain Reaction)

I-PCR: Inverse PCR

TAIL-PCR: Thermal Asymmetric InterLaced PCR

FRET: Forster Resonance Energy Transfer

MGB: Minor Groove Binder

Tm: Melting Temperature

SNP: Single Nucleotide Polymorphisms

LUX: Light Upon eXtension

LNA: Locked Nucleic Acid

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