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Detection of genetically modified plants using LAMP (loop-mediated amplification) technologies

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Purpose. Analysis of the current state and experience on the loop-mediated amplification (LAMP) use to detect genetically modified plants. **Methods.** Literature search and analysis. **Results.** General information on the current state and use of the genetically modified plants is provided. Despite the wide distribution of genetically modified plants, the attitude towards them in society continues to remain somewhat wary. About 50 countries have introduced mandatory labeling of GM feed and products, provided that their content exceeds a certain threshold. In order to meet labeling requirements, effective and sensitive methods for detecting known genetic modifications in a variety of plant materials, food products and animal feed must be developed and standardized. The most common approaches to the detection of genetically modified organisms (GMOs) are the determination of specific proteins synthesized in transgenic plants and the detection of new introduced genes. Methods for the determination of GMOs based on the analysis of nucleic acids are more common, since such methods have greater sensitivity and specificity than the analysis of protein composition. Polymerase chain reaction (PCR) method is the main method of nucleic acid analysis, which is now wide used for the detection of GMOs. Loop-mediated amplification (LAMP), which can occur at a constant temperature and therefore does not require the use of expensive equipment may be an alternative to the PCR. Scientific articles about the use of the loop-mediated amplification (LAMP) for the detection of genetically modified plants were analyzed. Advantages and disadvantages of the polymerase chain reaction and loop-mediated amplification are compared. **Conclusions.** The main criteria for applying a method of GMO detection analysis are as follow: its sensitivity, time of reaction, availability and ease to use, cost of reagents and equipment, and the possibility for simultaneous detection of many samples.

Keywords: *genetically modified organisms; targets for detection; PCR; LAMP; detection limit.*

Introduction

Genetically modified (GM) plants, created using the recombinant DNA technologies, have been widely used all over the world since 1996. In 2019, 29 countries grew biotech crops on an area of 190.4 million hectares (Mha) [1]. The top producers of genetically modified plants are the United States (the total area occupied 71.5 Mha), Brazil (52.8 Mha), Argentina (24 Mha), Canada (12.5 Mha) and India (11, 9 Mha). In the global world market in 2019, the share of GM plants accounted for 79% from the area under all rapeseed crops in the world, 74% from the area under soybeans, 31% from the area under corn and 27% from the area under cotton [1]. The leader among biotech plants in terms of the total planted area was soybeans, which were cultivated on 91.9 Mha (while the area under crops in 2019 decreased by 4% compared to 2018), followed by corn (60.9 Mha), cotton (25.7 Mha), and rapeseed (10.1 Mha). The International Service for the Acquisition of Agri-biotech Applications (ISAAA) assumes that the level of use of the main GM crops has already reached its saturation [1]. It is likely that global producers of biotech plants will continue their further expansion into the seed market of the aforementioned crops using technologies other than recombinant DNA, like genome editing technologies. In addition to the above four main biotech crops (soybeans, corn, cotton and rapeseed), it is worth mentioning other genetically modified plants cultivated on an industrial scale – alfalfa (the area under crops in 2019 was 1.3 Mha), sugar beet (473 thousand hectares), sugar cane (20 thousand hectares), papaya (12 thousand hectares), sunflower (3.5 thousand hectares), potatoes (2.265 thousand hectares), eggplants (1.931

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thousand hectares), as well as zucchini, apples and pineapples, which were grown on areas less than 1 thousand hectares. In general, the ISAAA database contains information on more than 300 different plants with genetically modified events "that have been approved for commercialization/planting and importation (food and feed)" (https://www.isaaa.org/gmapprovaldatabase/default.asp).

When analyzing the list of authorized GM plants, it should be remembered that private and public research centers are actively working on the creation of new genetically engineered varieties for many plant species – rice, bananas, potatoes, wheat, mustard, chickpeas, peas, melons, flax, plums, etc., which will have various new beneficial properties and improved nutritional value. In addition to countries that grew GM plants, 42 countries around the world (26 EU member states, plus 16 other countries) also imported biotech plants for use as food, animal feed and for processing in 2019. Thus, in 2019, genetically modified plants were approved and used for various purposes in 71 countries of the world in total [1].

Despite the wide distribution of genetically modified plants, the attitude towards them in society continues to remain somewhat wary, which manifests in constant discussions regarding the possible risks to humans and animals from the use of GM plants and the products obtained with their use. About 50 countries have also introduced mandatory labeling of GM feed and products if their content exceeds a certain threshold value [2]. For example, the threshold for labeling GM products is 0.9% in the EU, 3% in South Korea, 5% in Japan [2–4]. It should be noted that GMO labeling in no way concerns the issue of their safety, but only informs the consumer about their presence.

Results

In order to meet labeling requirements, efficient and sensitive methods for detecting many known genetic modifications in a wide variety of plant materials, food products and animal feed must be developed and standardized. The most common methods for the determination of GMOs (genetically modified organisms) are based on the detection of specific proteins synthesized in transgenic plants due to the expression of new genes in their genome, and on the detection of the introduced genes themselves by analyzing certain nucleotide sequences of DNA isolated from a genetically modified organism. The developed enzyme immunoassay methods allow the determination of proteins encoded in transgenic plants by *cp4-epsps*, *cry1Ab*, *cry1Ac*, *cry2A*, *cry2Ab*, *cry3A*, *cry9C*, *nptII*, *pat*, *gox*, *cpti* genes [5]. Unfortunately, this inventory does not completely cover the entire list of GM plants currently presented on the market. Methods for the determination of GMOs based on the analysis of nucleic acids are the most common, since such methods are more sensitive and specific than the analysis of protein composition, and allow the detection of a significantly larger number of genetically modified plants. For the detection and monitoring of transgenic DNA, four main regions in the genome of a new organism are selected. These are specific nucleotide sequences characteristic of universal elements and marker genes that were inserted into the genetic cassette used for transformation; in fact, the newest introduced genes; genome section on the border between universal elements and introduced new genes; the boundary between the nucleotide sequence in the host genome and integrated new genes [6]. The use of methods for detection of these four regions in the genome makes it possible, respectively, to screen a particular sample for the presence of genetic modifications in it; determine the specific introduced gene; reveal the structure used for transformation; or identify a GM event. With the rapid emergence of new GM events, the most common methods of laboratory analysis are routine screening and determining the presence of a new gene or elements of the used construction. Since the 90s of the last century, most detecting laboratories began to actively use DNA-based polymerase chain reaction PCR) techniques. Since then, the polymerase chain reaction, through which a large number of copies of certain regions of the genome are possible to obtain, has become a routine method of molecular biology, biotechnology and genomics. PCR is widely used both in fundamental research and for solving various practical issues, including the detection of GMOs. Among the various PCR options used to detect genetically modified organisms, it is worth recalling the quantitative Polymerase Chain Reaction (qPCR), multiplex PCR, and digital drop PCR (ddPCR). Multiplex PCR attracts special attention, since it allows detecting several targets simultaneously. The publication [7] provides information on the use of multiplex PCR for the simultaneous detection from 4 to 9 different targets:

- 4-Plex PCR (taxon specific soybean lectin (*lec*) gene, maize zein gene, element-specific sequence of *35s* promoter of cauliflower mosaic virus and *nos* terminator (nopaline synthase gene terminator) are detected);

• 5-Plex PCR (taxon specific gene of the maize alcohol dehydrogenases (*adh*) and GM events GA21, MON810, NK603, Bt11 are detected);

• 6-Plex PCR (taxon specific *acp1* gene – cotton gene that encodes an *acyl* carrier protein) and GM events Bollgard, Bollgard II, RR, 3006-210-23, 281-24-231 are detected);

• 8-Plex PCR for the detection of 8 different GM events (Bt176, Bt11, HN1, RRS, T25, MON88913, MIR604, and MON1445)

• 9-Plex PCR, where taxon specific *hmg* gene (highly mobile maize protein gene) and GM – events T25, GA21, TC1507, MON863, MON810, NK603, Bt176, Bt11 are determined.

Additional information on some other developed variants of multiplex PCR for GM plants detecting can be found in the review [5]. Further improvement of the methods for the simultaneous analysis of several different DNA targets was obtained thanks to the development of the so-called MPIC technologies (multiplex Microdroplet PCR Implemented Capillary gel electrophoresis). This approach made it possible to detect simultaneously from 8 to 24 different GM events [8].

The main advantage of molecular detection methods based on the analysis of nucleic acids is their high specificity and sensitivity. Among the shortcomings of the methods, it is worth mentioning the need for sophisticated equipment that is not always available for laboratories with limited financial resources. The advantages and disadvantages of some methods for the determination of GMOs based on the use of the PCR are shown in Table 1.

The need for complex and expensive PCR equipment prompted the development of amplification methods that can be implemented at a constant temperature and, therefore, are an alternative to PCR. First of all, we are talking about isothermal amplification methods implemented at a constant temperature.

Table 1

Disadvantages and advantages of various PCR variants used to determine GMOs [9]

Method	Target for detection	Advantages	Disadvantages
Traditional PCR	DNA / RNA	Relatively cheap method	The analysis is time-consuming and requires the use of a thermal cycler
Nested PCR	DNA / RNA	Highly specific method of analysis	High cost, time-consuming analysis, requires the use of a thermal cycler
Real-time PCR	DNA / RNA	Allows to reveal the relative amount of analyzed DNA in the studied sample; no need for further separation of the obtained amplicons	Time-consuming analysis. Requires the use of highly purified genetic material, requires the use of a special amplifier
Digital drop PCR (ddPCR)	DNA / RNA	Highly sensitive method, not sensitive to impurities, allows to determine the absolute amount of DNA	Very high cost of analysis, requires the use of special equipment

Their main advantage is the absence of need for sophisticated equipment – you only need an ordinary laboratory thermostat, or a water bath. Among the methods of isothermal amplification of nucleic acids, it is worth mentioning strand-displacement amplification (SDA) [10], helicase-dependent amplification system (HAD) [11], rolling circle amplification (RCA) [12], loop-mediated amplification (LAMP), Self-sustained sequence replication (3SR) [13] and nucleic acid sequence-bases amplification (NASBA) [14] and which is a further improvement of the 3SR method, Q beta replicase amplification, etc.

Isothermal amplification methods can be classified into two groups depending on how the nucleic acid is denatured – using enzymes or spontaneously, thanks to specific primers or probes. The methods are simple in terms of their implementation, and some of them can be used even in the field, since the main difference between isothermal amplification methods and PCR is that the reaction can occur at a constant temperature. The main purpose of this article is to analyze the experience of using the LAMP reaction for detecting genetically modified plants.

Loop-mediated isothermal amplification method was first developed by Japanese scientists in 2000 [15] and is based on the unique feature of DNA polymerase from the bacterium *Bacillus stearothermophillus* (Geobacillus), which, in addition to DNA polymerase, also has a high revertase activity. Usually, the reaction itself is carried out at a temperature in the range of 55–65 °C. LAMP reaction was first described using 4 primers, however, later it was found that the use of an additional pair of primers for loop formation significantly increases the sensitivity of the method [16].

The LAMP study can be implemented rather quickly, since in this amplification reaction, unlike PCR, there are no separate stages of denaturation, hybridization and synthesis. At the same time, the loop-mediated amplification

can be divided into the following conditional stages – initiation, cyclic amplification and elongation. For loop-mediated amplification DNA polymerase that can replace the strand during synthesis (*B. stearothermophillus Bst* DNA polymerase), Forward Inner Primer (FIP) and Backward Inner Primer, (BIP) and external primers (F3, B3) that recognize 6 different regions on the target are needed (Figure 1). Two looping primers are needed to form a loop, and two pairs of stripping primers are needed to synthesize linear nucleic acid strands.

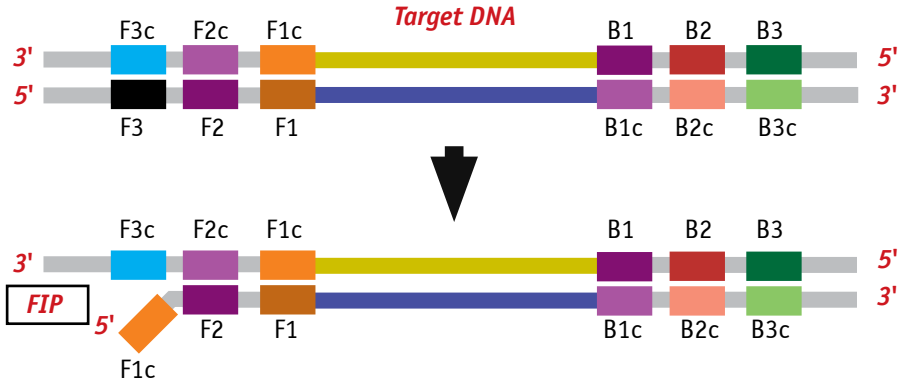

Fig. 1. Description of primers used in (LAMP)

Internal primers FIP and BIP correspond to regions F2 (B2) and F1c (B1c). External primers are designed for regions F3 and B3. Loop forming primers are designed for regions between F1c (B1c) and F2c (B2c) (http://www.premierbiosoft.com/tech_notes/Loop-Mediated-Isothermal-Amplification.html)

At the beginning of the reaction, the primers forming the loop hybridize with F2 or B2 regions in order to initiate the synthesis of complementary DNA strands (Fig. 2, stage 2). After that, primers necessary for the synthesis of linear strands of nucleic acids hybridize with loci F3 or B3, and amplification of complementary DNA strands begins, which further leads to the release of the synthesized chains of the molecule (stage 3).

At this stage, the single-stranded chain of the nucleic acid molecule already has a nucleotide sequence that allows the formation of a loop-like structure (stage 4). The F1 and B1 regions at the 5'-end act as primers for generating a double-stranded loop (step 5). The regions containing the loop (F1 and B1) are single stranded, so new primers that generate the loop can hybridize with these regions.

As a result, a new complementary DNA strand is formed (stage 6, stage 7 and stage 8). At the 5'-end, a loop is formed (step 9), similar to how it happened in step 4. The synthesis of the molecule from regions F2 and B2, and the synthesis, which is caused by primers that generate a structure in the form of a loop, occurs alternately (steps 9, 10 and 11), which leads to the formation of a large-size reaction product containing nucleotide sequences that correspond to the target (step 11).

Further improvement of LAMP method was aimed at the development of its variants, allowing the detection of RNA. As a result, conditions for performing LAMP along with reverse transcription, (RT-LAMP) were developed. Reverse transcriptase is added to the reaction mixture in order to provide reverse transcription, which is carried out with the participation of primers that form a loop and primers necessary for the synthesis of linear strands of nucleic acids. RT-LAMP is used primarily for the detection of RNA-containing viruses, while the «traditional» LAMP is successfully used to determine various DNA-containing pathogenic microorganisms (viruses, bacteria, fungi) and parasites, to determine the sex of embryos, in research on the study of cancer [18].

Using the LAMP method, a very small amount of target DNA can be detected; it is also important that the amplification reaction can also occur in the presence of foreign nucleic acids. These circumstances make the method quite suitable for detecting GMOs. As in the case of PCR, the use of LAMP for detecting GMOs can be implemented in several ways: routine screening to study the presence of GMOs due to the detection of universal and most common regulatory elements, such as the *35s* promoter and *nos* terminator, and the identification of a GM event, what requires specific primers.

The results of the detection of many transgenic plants by the method of loop-mediated isothermal amplification have been published in the scientific literature (Table 2).

The minimum sensitivity of the LAMP method in the experiments of various authors, which are referenced in Table 2, was extremely

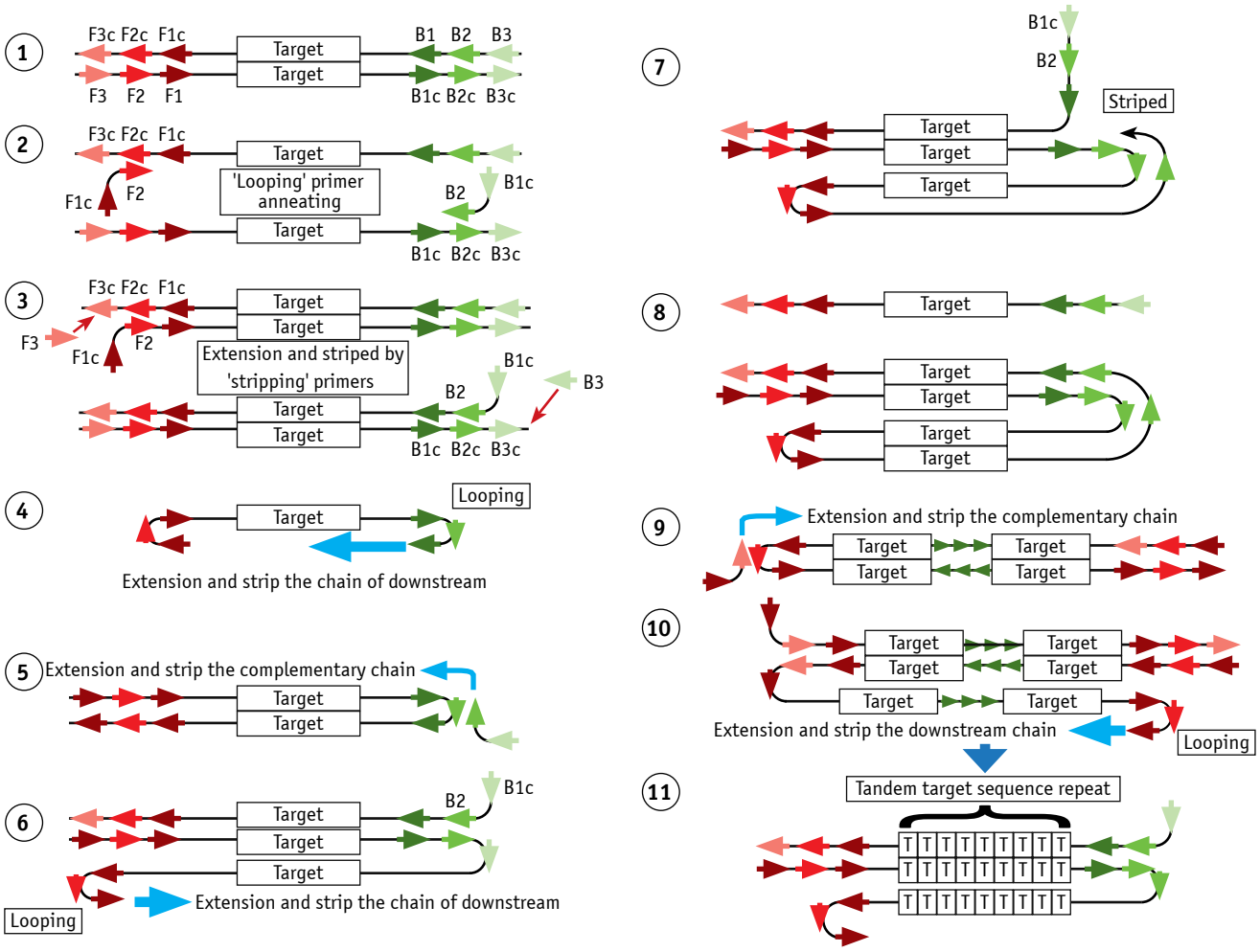

Fig 2. Scheme of loop-mediated DNA amplification [17]

Table 2

Examples of using LAMP for detecting various GM plants

GM culture	Target for detection	Literature
Cotton plant, GM events MON531, MON15985	*sad1* gene, *35s* promoter, FMV promoter, *aadA* gene, *nptII* gene, *uid* gene	[19–21]
Corn, GM events MON810, NK603, Bt11, DAS-59122-7, T25, BT176, TC1507, MON863, MON89034, MIR604	*35s* promoter, *cp4epsps* gene, *pat* gene, *Mannose-6-phosphate isomerase* gene, nucleotide sequence between inserted *cry1Ab* and *cry1Ac* genes, *cry2Ab* gene, *cry3A* gene, construct-specific sequence, phytase gene	[22–27]
Potatoes GM event EH92-527-1	GM event – specific sequence	[28]
Cane sugar	*bar* gene, *cry1Ac* gene	[29]
Soy, GM events GTS 40-3-2, MON89788, DP305423 × GTS 40-3-2 s	GM event – specific sequence	[30, 31]
Oilseed rape, GM event RF3	GM event – specific sequence	[32]
Wheat, GM events B73-6-1, MS8	GM event – specific sequence	[32, 33]
Rice, GM events TT51-1 KMD1, KF6, T1C-19 10	GM event – specific sequence	[34, 35]

high and made it possible to determine from 10 to 4 copies of the target gene.

The study [32] described the structure of primers and reaction conditions for the LAMP detection of universal elements (using the example of a reference material of transgenic soybean with resistance to Roundup (Roundup ready soybean), which is often used to create genetic constructs, such as the cauliflower mosaic virus (CaMV) *35S* promoter, as well as the promoter and terminator of the nopaline synthase gene from *Agrobacterium* spp. The authors also showed the possibility of detecting transgenic MS8 and RF3 lines of oilseed rape (*Brassica napus* L.) using primers with affinity to a GM event of a specific locus. It was found that the sensitivity limit of the LAMP method for determining both the *nos* terminator and the

35s promoter was 0.01%, what is well below the certain limit for labeling (0.9%), declared in the European Union regulations.

In publication [36], commercialized GM plants were analyzed and it was shown that the sensitivity of the LAMP method is 10 haploid genome equivalents (HGE) for the *fmv35s* promoter, *cry1Ac* gene, and *pat* gene. At the same time, when detecting the *35s* promoter of the cauliflower mosaic virus, *bar* gene, *nos* terminator, *cp4-epsps* and *nptII* genes, the sensitivity limit of the method corresponded to 5 HGE. The authors successfully confirmed the potential of the LAMP method for screening studies using samples of commercialized varieties of GM rapeseed, soybeans, and corn.

In the paper [34], the possibility of visual detection of amplicons synthesized in loop-mediated isothermal amplification reaction was shown. For this, intercalating dyes were added to the reaction mixture, such as the asymmetric cyanine dye SYBR green, or HNB (hydroxynaphthol blue), which, by binding to the fragments of the double DNA strand synthesized in the reaction, can change their color. This approach makes electrophoretic analysis of amplification products unnecessary and significantly reduces the time for research. In the cited work, the entire experiment lasted only about one hour, and in some publications cited above in Table 2, the LAMP reaction time was even less than one hour. The authors applied the developed technique to detect GM events in rice KMD1, TT51-1, and KF6. The obtained results allowed us to conclude that the LAMP method was more sensitive and specific in comparison with traditional PCR and can be used even in the field [34].

The authors of the article [28] published the results of studies where the *bar* gene in transgenic sugar cane was determined by LAMP and traditional PCR methods. It was shown that the use of LAMP made it possible to detect the transgene in 100 cases out of 100 studied samples (100%) and in 97 cases out of 100 studied samples (97%) in the case of PCR.

Taking into account the high sensitivity of the loop-mediated isothermal amplification method, it is logical to compare its advantages and disadvantages with the polymerase chain reaction method (Table 3).

Table 3

Comparative analysis of PCR and LAMP methods [37]

Method	Time for analysis	Method for detecting reaction products	Use of UV radiation	The need for detection equipment	Cost of analysis	User friendliness	The need for a high degree of DNA purification
PCR	3 h	Gel electrophoresis	Yes	Yes	High	High	Yes
LAMP	60 min	Visual analysis, or gel electrophoresis	No or Yes	No or Yes	Low	Very high	No

Attempts to improve the conditions for carrying out loop-mediated isothermal amplification continue constantly. Thus, an attempt was described to use, instead of the rather valuable Bst DNA polymerase from *B. stearothermophillus*, another *Bsm* DNA polymerase, which has similar properties, but is cheaper and more accessible to the user [38]. An example of creating a consumable device, which is able to maintain a constant temperature for a certain time, has been published, which allows further use of LAMP as an express method of analysis in the field [39]. Attempts to create biosensors based on the use of LAMP [40] and the search for new stable dyes for detecting nucleic acid fragments synthesized in the reaction are also continuing [24].

It should be noted that the improvement of existing and development of new methods for GMO detection concerns not only loop-mediated isothermal amplification. One can recall the introduction of capillary electrophoresis for amplicon analysis; the use of "peptide nucleic acids" (PNAs are synthetic homologues of nucleic acids that contain standard DNA nucleotides, but in this case the polyamide chain is replaced by repetitive units of *N*-(2-aminoethyl) glycine to which the nucleotide pairs are attached via a methyl carbonyl linker. The neutral chain in this form does not have the ability to repel when hybridized. This explains that PNA can bind to DNA or RNA with a high degree of specificity); development of various biosensors; application of microarray technology and the development of DNA chips; use of new genome sequencing (NGS) technologies for detecting GMOs, etc. [5, 8, 41].

Conclusion

Summarizing, we can say that the main criterion for the application of a particular method of GMO detection analysis is, first of all, its sensitivity, time of reaction, availability and ease of implementation, the cost of reagents and equipment, as well as the possibility for simultaneous detection of many samples. LAMP technologies fully meet many of these requirements.

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Сорочинський Б. В. Детектування генетично модифікованих рослин з використанням технологій LAMP (реакція ампліфікації, що опосередкована через петлю). *Plant Varieties Studying and Protection*. 2021. Т. 17, № 1. С. 51–59. https://doi.org/10.21498/2518-1017.17.1.2021.228209

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Мета. Проаналізувати світовий досвід застосування реакції ампліфікації, що опосередкована через петлю (LAMP), для детектування генетично модифікованих рослин. **Результати.** Наведено загальну інформацію щодо сучасного стан і поширення генетично модифікованих рослин. Попри значне поширення генетично модифікованих рослин, ставлення до них у суспільстві й досі залишається дещо настороженим. Приблизно 50 країн запровадили обов'язкове маркування ГМ кормів та продуктів за умови, що їхній уміст перевищує певне порогове значення. Для того, щоб виконати вимоги до маркування, потрібно розробити та стандартизувати ефективні й чутливі методи визначення відомих генетичних модифікацій у різноманітній рослинній сировині, харчовій продукції та кормах для тварин. Найпоширенішими підходами до детектування генетично модифікованих організмів (ГМО) є визначення специфічних білків, що синтезуються у трансгенних рослинах, та детектування нових привнесених генів. Методи визначення ГМО, засновані на аналізі нуклеїнових кислот, є поширенішими, оскільки мають більшу чутливість та специфічність порівняно з аналізом білкового складу. Основним методом аналізу нуклеїнових кислот, що зараз використовується для детектування ГМО, є метод полімеразної ланцюгової реакції (ПЛР). Альтернативою методу ПЛР убачається реакція ампліфікації, що опосередкована через петлю (LAMP), яка може відбуватися за постійної температури й тому не потребує використання коштовного обладнання. Проаналізовано наукові публікації, що стосуються використання реакції LAMP для детектування генетично модифікованих рослин. Описано переваги та недоліки методів полімеразної ланцюгової реакції та ампліфікації, що опосередкована через петлю. **Висновки.** Основним критерієм для застосування того чи іншого методу аналізу ГМО є, насамперед, його чутливість, тривалість реакції, доступність та простота виконання, вартість реагентів і обладнання, а також можливість здійснювати одночасне детектування якомога більшої кількості зразків.

Ключові слова: *генетично-модифіковані організми; мішені для детектування; ПЛР; LAMP; межа чутливості.*

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