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USE OF MOLECULAR MARKERS IN PLANT BIOENGINEERING

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Abstract

Genetic markers represent different forms of the same gene that control mutant phenotypic expression and allow individual quantification of genes. One of the basic criteria in the molecular markers usage is the molecular polymorphic capacity of plant genetic material. This paper aimed to distinguish and generalize some of the relevant results regarding the applications of different molecular markers in plants bioengineering. Thus, the paper describes the main categories of markers used in plant bioengineering, with topical examples of their utility in modern plant breeding programs. The used methods included searching of the various databases (Web of Science, Google Scholar, The Food and Agriculture Organization, The International Service for the Acquisition of Agri-biotech Applications) and identification some relevant results. From this point of view, the results indicate the immense opportunity of molecular markers in the individual study of plants, without conditioning the stage of their development. Some of the practical applications of molecular markers in plant genetic programming relate to the investigation of the diversity of genetic material; precise location of genes; germplasm certification, etc. Molecular markers applications generate an explosive growth of vital information for genetic research, the natural consequence being the rapid advancement of basic and applied knowledge. However, correct and objective information to the general public is an important step in making it easier to accept innovations in plant bioengineering and enables progress in agriculture.

Key words**:** *genetic markers, DNA, PCR, applications, polymorphisms*

INTRODUCTION

Plant bioengineering is an area of paramount importance, as it can provide solutions to many major global problems, one of which is ensuring global food security.
Genetic diversity is critical for a population to adapt to changing environments [5, 6, 31, 32].
The marker represents an identifiable DNA sequence that facilitates the study of the inherited transmission of a character or gene. The genetic markers are used to map the order (sequence) of genes along chromosomes and to track the hereditary transmission of certain genes. Genes closely related to the marker will generally be transmitted (inherited) with it. Markers should be easily identifiable at the phenotype level.
Molecular markers are genetic markers that can mark the presence of a certain gene in DNA. Genetic markers are mutant alleles that mark the presence of a gene at the individual level and control easily identifiable characters. They are of two types: (a) morphological markers, which control morphological characters (for example, the red colour of the maize grains is determined by the R^r gene or the R^{st} gene for variegated grains) and (b) biochemical markers, which control some biochemical properties.
The molecular markers are the most used due to some advantages: their unlimited number, the location in uncoded regions of the DNA as well as the fact that they are not influenced by the environmental conditions or the stage of plant development. The most important characteristics of molecular markers are the following: they can be obtained in unlimited numbers, from any tissue, at any stage of development; are independent of gene

expression and are not influenced by environmental conditions; are not subject to selection pressure; usually, it does not show non-allelic (epistatic) interactions and does not show pleiotropy; are simple transmitted via mendelian way, etc. Molecular markers can be dominants (when heterozygotes do not differ from homozygous) or co-dominants (when heterozygotes can be clearly distinguished from both homozygous parents).

Marker deletion via transposons is a process that allows certain genes to "jump" to a certain position in the plant's genome. The process is analogous to site-specific recombination, with transposons ("jumping genes") being used instead of recombinase and recognition sites. They contain a gene that encodes a special enzyme (transposase), which recognizes certain signals in DNA. The enzyme cleaves the DNA fragment flanked by these signals and integrates it randomly into the genome. The gene of interest or the marker gene can be placed in the "jumping" sequence, so that the two genes can be separated from each other after transposase activation [28].

The term *Free Marker Technology* refers to any technology used to remove selected marker genes from transformed cells, tissues, or plants. Such technologies are based on cotransformation, transposable elements, situs-specific recombination or intrachromosomal recombination.

Obtaining of transgenic plants without antibiotic resistance markers can be achieved by application one of two main strategies: (1) excision or segregation of marker genes from the host genome after regeneration of transgenic plants; (2) transformation without marker. The second strategy is based on the transformation of plant tissue explants or cells with a virulent strain of *Agrobacterium tumefaciens* and the selection of transformed cells or shoots after PCR analysis. This strategy has proven to have the advantage of improvement frequency of transformation of recalcitrant species [28].

Marker genes can be used to identify those cells into which the new gene has been stably integrated and expressed, provided that the marker gene for selection is located alongside the gene of interest in the pattern used for transformation. The most commonly used marker genes are genes for resistance to antibiotics or herbicides. All of these genes give genetically modified cells the ability to detoxify substances that would otherwise be fatal. For example, a gene for herbicide resistance confers tolerance to cells on that herbicide. After transformation, they come into contact with the substance encoded by the marker gene (which may be included in the culture medium). This is the time when only those plants that have the marker gene stably integrated and properly expressed in their cells will survive. Transgenic plants will be regenerated from these cells [28].

The use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant achievements of molecular genetics.

The advantages of markers in selection, for studies of diversity and in the context of marker-assisted selection have been highlighted in many results [2, 4, 18, 20, 22, 24, 25].

The possible advantages of markers implementing in the genetic improvement of plants were suggested a long time ago but their real potential was developed after 1970, with the expansion of new techniques for testing variability at the DNA level. It can be appreciated that the family of molecular markers was founded by the development of RFLP technology in the 1980s, first for human genetics and later for plants. From this point of view, they began to be used successfully to establish genetic diversity and distance, based on DNA polymorphism and especially for the accurate detection and identification of genes of interest [11].

Combining classical plant improvement techniques with those of molecular biology through the prism of molecular markers (MAS technology) is one of the most important methods in modern agriculture. For the study of a large number of plants, the cost-benefit ratio must also be evaluated. From this point of view, the costs of making molecular methods are very rarely compared and can vary considerably, depending on the

availability of automated equipment and technical experience [9].

Molecular techniques for detecting variation at the DNA level require the use of a wide range of molecular markers: RFLP (*Restriction Fragment Length Polymorphism*), RAPD (*Randomly Amplified Polymorphic DNA*), AFLP (*Amplified Fragment Length Polymorphism*), SNPs (*Single Nucleotide Polymorphisms*) and so on. All of them differ from each other in several respects: development costs, polymorphism level, automation, recognition of a certain sequence, etc. [1, 11].

However, it is very important to know exactly each class of molecular markers and the information level of each in order to identify those that correspond optimally to the proposed purpose.

The paper describes the main categories of markers used in plant bioengineering, with topical examples of their utility in modern plant breeding programs.

There are two main classes of molecular markers, namely: (a) traditional markers and (b) markers based on the in vitro amplification reaction of DNA by PCR (Polymerase Chain Reaction) technology. The first category includes protein markers, RFLP markers and markers resulting from DNA sequencing. On the other hand, the category of PCR-based molecular markers includes RAPD, micro and minisatellite markers, AFLP, SSCP (Single-strand Conformation Polymorphism), ASAP (Allele specific Associated Primers) and EST (Expressed Sequence Tag) markers.

In another classification, there are markers that allow the detection of monolocus polymorphism (RFLP, PCR) and markers that allow the detection of polylocus polymorphism, i.e., at different genes or different chromosomes (RAPD).

MATERIALS AND METHODS

The topics followed in this research were: an overview of the global bioengineering situation, in terms of areas and the main transgenic crops which can support a rapidly growing world population; the advantages of the molecular markers compared to traditional phenotypic ones; DNA marker applications for improvement in various plant species; some practical applications of different molecular markers (RFLP, RAPD, STS, SSR, AFLP, etc.) and some briefly new issues about Vertebrate Genome Project (VGP).

The used methods included searching of the various databases and hand searching of the specialized literature with the latest publications in the field and identification of some relevant results. The main databases were Web of Science and Google Scholar as well as FAO (The Food and Agriculture Organization) and ISAAA (The International Service for the Acquisition of Agri-biotech Applications). Some relevant information was transposed in the form of adapted figures and tables.

RESULTS AND DISCUSSIONS

As the global population explosion progresses, more food, energy and goods are needed. The problem is that of limited natural resources, which forces us to produce more with less, to ensure global food security but in conditions of environmental protection. Although a controversial topic, plant bioengineering has led to significant improvements in crop yields, which support a rapidly growing world population. In the future, genomic screening will provide an even more complete picture of all organisms and will most likely find the solution of the many of the serious problems which humanity encounters.

The United States is a world leader in terms of cultivated area with genetically modified plants. Thus, 38% of the genetically modified global agricultural production is in the USA. Over 90% of the five major crops grown in the United States are GMO. These crops are: corn, rapeseed, soybeans, cotton and sugar beet. Most processed foods in the United States contain ingredients from genetically modified crops, and the first GM foods were approved for consumption in 1994 [10].

In addition to the above-mentioned genetic bioengineering crops, the United States also cultivates herbicide-tolerant alfalfa, virus-resistant pumpkin and papaya hybrids,

mechanical damage-resistant potatoes, and browning, which produce low levels of acrylamide when found in high temperatures, late blight resistant potatoes and more suitable for storage. Browning-resistant apple varieties as well as browning-resistant mushrooms are also grown. Pink pineapple, created by Del Monte Fresh Produce, has recently appeared on US markets, containing low levels of enzymes that turn lycopene (pink pigment) into beta-carotene (yellow pigment) [10].

Therefore, USA remained as the top producer of biotech crops globally [17], which planted 71.5 million hectares in 2019. Brazil landed on the second spot, with 52.8 million hectares (Table 1).

Table 1. Top 10 countries in terms of Global Area of Biotech Crops in 2018 and 2019 (million hectares)

Rank	Country	2018	2019
1	USA	75	71.5
2	Brazil	51.3	52.8
3	Argentina	23.9	24
4	Canada	12.7	12.5
5	India	11.6	11.9
6	Paraguay	3.8	4.1
7	China	2.9	3.2
8	South Africa	2.7	2.7
9	Pakistan	2.8	2.5
10	Bolivia	1.3	1.4

Source: Own calculation based on [17].

From the initial planting of 1.7 million hectares in 1996 when the first biotech crop was commercialized, the 2019 planting indicates a major increase (Figure 1).

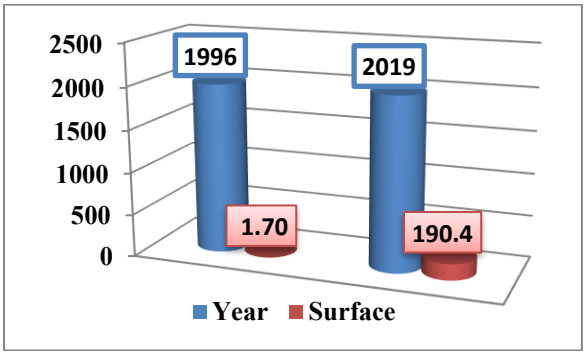

Fig. 1. Global area of Biotech crops, 1996 to 2019 (million hectares)
Source: Own design and calculation based on [17].

About future prospects, scientists all over the world are combining their efforts to develop new biotech crops and traits that will be beneficial to farmers and consumers [17].

The use of molecular markers in plant bioengineering is one of the high-performance research technologies. Molecular markers are of great importance in assessing the hereditary composition of the body and are the main driving forces of improvement, which are based on morphological characteristics, which largely depend on environmental conditions.

Some DNA marker applications for improvement in various plant species are highlighted in Table 2.

Compared to traditional phenotypic markers, the molecular ones have a number of advantages, such as the ability to improve the efficiency of plant breeding in general and the selection through molecular markers linked to the research character, in particular.

One of the major benefits of molecular markers is that they can predict the plants performance according to specific traits without lengthy and laborious tests in the greenhouse or field. From this point of view, the most important applications of molecular markers in plant bioengineering are represented by genetic fingerprinting and mapping, marker-assisted selection, backcross acceleration and detection of diversity and genetic differences between different populations. Variations within the DNA of genes or gene loci that correlate with different phenotypes of the plant can be used as molecular markers; however, in addition to these, there are other categories of molecular markers: hybridization-based and PCR-based molecular markers. The different types of molecular markers can be characterized by several methods for highlighting the polymorphisms present in the DNA sequence. DNA sequencing provides accurate and reproducible data that can be applied to a wide range of variations by selecting target regions in the genome, according to the intended purpose.

DNA sequencing has taken on an unprecedented scale since the advent of the PCR technique, which has made it possible to amplify orthologous regions of DNA from any organism of interest with astonishing speed [13]. There are currently universal PCR primer sets that allow the amplification and

subsequent sequencing of certain DNA regions in almost any plant of interest [27].
RFLP markers are molecular markers based on the hybridization difference between a cloned or PCR-derived DNA fragment, with DNA fragments from the sample to be analysed, which were obtained after restriction enzyme digestion. The marker is specific to a single restriction enzyme.

Table 2. Some examples of DNA marker applications

Application	Marker	Plant species	Ref.
Genetic diversity, DNA fingerprint and germplasm conservation	DArT; ISSR and RAPD; CDDP; RAPD and ISSR	*Zea mays; Ricinus communis; Musa L; Gloriosa superba; Oryza sativa L.*	[3, 19, 16, 30, 23]
Marker-assisted selection	SSR; SRAP	*Manihot esculenta; Camellia oleifera;*	[26, 12]
Association mapping	SSR	*Chickpea*	[19]
Hybrid identification	SSR; EST and SSR	*Citrus aurantifolia and Citrus limon); Elymus sibiricus*	[14, 34]

Source: Own calculation based on [1].

RFLP markers have some advantages that give them the priority to use compared to RAPD markers: they are codominant and are not affected by environmental conditions; any DNA source can be used for analysis; they can also be used in populations where the phenotypic effects are not obvious [33].
Although RFLP markers have a codominant phenotype and are virtually unlimited in number, they have been overcome by the advent of other simpler, cheaper, and much faster technologies, such as PCR technique.
However, it should be mentioned the role that RFLP technology has played since its discovery, by contributing to the development of knowledge on the detection of specific nucleic acid sequences, genetic fingerprinting, the characterization of genetic diversity or breeding patterns in plant and animal populations, etc.
In plants, two sources of DNA clones are used for RFLP mapping: complementary DNA clones and genomic clones, derived from the complementary DNA restriction [29].
A restriction fragment length polymorphism is said to occur when the length of a detected fragment varies between individuals, indicating non-identical sequence homologies. Each fragment length is considered an allele, whether it actually contains a coding region or not, and can be used in subsequent genetic analysis (Figure 2).

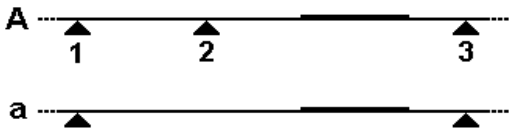

Fig. 2. The mechanism by which the size of a particular restriction fragment can be variable
Source: [29].

In recent years, many of the DNA fragments used in the RFLP technique have been sequenced and therefore, it is now possible that, based on sequence similarity, specific primers can be synthesized, which can be amplified by the PCR technique.
These markers are called STS (Sequence tagged sites) and are often used in plant bioengineering to identify and select genes of interest but only if they are associated with distinct expressions.
With the advent of PCR technology, a new generation of automated markers has appeared, almost infinite in number and relatively fast to test. Consequently, there is a wide range of molecular techniques, based either on the use of restriction enzymes or on target site PCR technology or both. Thus, SSR, AFLP and RAPD are some of the widely used PCR-based markers.
The advantages of the PCR technique in plant bioengineering are enormous. Depending on the purpose, any type of primer can be chosen, even non-specific ones. All types of primers used in PCR technique can be used in different combinations, and their potential for use in various experimental purposes is practically unlimited.
RAPD markers are generated by PCR via a single primer. They detect the polymorphism of DNA nucleotide sequences using a single primer with the arbitrary nucleotide sequence.

The RAPD technique is used in many genetic analyses. It is a less laborious method and allows obtaining an overview of the polymorphism at the level of a genome, faster and cheaper than other techniques, such as RFLP.

Microsatellites or simple repetitive sequences are present in all organisms, including plants. They are a major source of genetic variability, useful in plant bioengineering. They are considered neutral selective markers because they are not located inside or near the coding sequences and therefore cannot cause a gene to be disrupted and are not subject to selective pressure from a neighbouring gene [15, 21].

Metabolic markers are an alternative to the controversial genes for antibiotic resistance. They allow plants to be grown on unfamiliar culture media or to produces metabolic products that allow only transgenic to grow. Once the transgenic cells have been identified, the marker genes are no longer needed. This is why the most effective ways to achieve transfer without marker genes, or to remove them after transformation, are sought [8].

In 2018 have been marks 20 years since the inception of the National Plant Genome Initiative (NPGI), which is dedicated to advancing crop improvement through genome sciences. Many examples of the involvement of genomics in increasing of genetic progress to plants are presented in the literature, such as barley (in Germany) or pearl millet (in France). In fact, the pearl millet was re-sequenced from almost 1000 varieties in order to establish with certainty the evolution of this highly topical plant in terms of high tolerance to drought [7].

An example of a bold and current global initiative is the Vertebrate Genome Project (VGP), from which is expected to achieve all four stages of vertebrate genome sequencing, progressively, from each all order, genera and species of vertebrates [7].

The practical applications of molecular markers can be structured as follows: use in linkage analysis and genetic mapping; use in phylogeny and evolution studies; germplasm diversity analysis; variety genotyping; the study of hybridization and introgression; use in taxonomy and systematics, etc. However, the analysis of the plant genome via molecular markers has generated an enormous amount of extremely useful information in the scientific community in the field and beyond.

CONCLUSIONS

The use of molecular markers in plant bioengineering is one of the high-performance research technologies, which allows a maximum resolution for the identification of different genetic variations. This technology generates an explosive growth of vital information for genetic research, the natural consequence being the rapid advancement of basic and applied knowledge.

The genetic basis of molecular labelling methods is DNA, which allows the labelling of any genomic region. The use of molecular markers is suitable for the analysis of any tissue and organ, regardless of the stage of development of the organism. Compared to traditional phenotypic markers, the molecular ones have a number of advantages, such as the ability to improve the efficiency of plant breeding in general and the selection through molecular markers linked to the research character, in particular. In addition to these important advantages, molecular markers offer many other opportunities: accurate identification of gene locations; detecting morphologically invisible mutations, but of major importance for plant improvement; usefulness in genetic and phylogenetic analysis, etc.

The implementation of high performance technologies has already made it possible to complete the genome sequencing of several plants and animals. Plant bioengineering promises finding of big solutions for a small planet.

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