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ANALYSIS OF GENOME EDITING APPLICATIONS IN THE CREATION OF NEW MAIZE GERMPLASM

Dorina BONEA

University of Craiova, Faculty of Agronomy, 19, Libertatii Street, Dolj County, Romania,
Phone/Fax: +40 251 418 475, E-mail: dorina.bonea@edu.ucv.ro

Corresponding author: dbonea88@gmail.com

Abstract

The main concern of today's scientists is the sustainable development of agriculture to achieve global food and nutrition security. Genome editing technology is recognized worldwide for its potential for sustainable agricultural intensification. In this paper, applications of genome editing in maize were analyzed and exemplified, based on statistical data collected from the EU-SAGE Database from January 1996 to July 2023. In addition, the existing regulations for this new technology were also discussed. The results showed that, so far, 51 applications of maize genome editing have been reported in the EU-SAGE database, and the CRISPR/Cas system was the most used genome editing tool with the potential to rapidly generate new genotypes with high yield, improved quality, and stresses resistance. Most applications were developed in China and the USA. Due to current GMO legislation and political indecision regarding the authorization of genome-edited products, the European Union is not yet able to use and cultivate these new improved genotypes.

Key words**:** *CRISPR/Cas, genome-edited products, improved traits, maize*

INTRODUCTION

The continuous growth of the global population, which is estimated to reach 10 billion people in 2050, requires a proportional increase in food production [16].

In order to more quickly obtain cultivars resistant to current threats and increase the amount of food, it is necessary to use efficient and safe molecular tools [19].

Maize *(Zea mays*) is one of the most important crops that provide a major source of food globally, having multiple uses (food for humans, fodder, raw material for various industries, biofuel) [7, 39].

The current climate changes threaten the global production of maize and other major agricultural crops. For example, in years of severe drought, Europe (the third largest maize-producing region in the world after the Americas and Asia) recorded a reduction in total maize production of 37.9 million tons in 2015, of 24.1 million tons in 2016 and 30.9 million tons in 2017 compared to 2021, and Americas recorded reductions of 70.4 million tons in 2015, of 15.9 million tons in 2016, of 13.9 million tons in 2017 compared to 2021 [10].

A recent IPCC report [18] shows that to reduce future losses in maize yield, improved agricultural practices by developing new cultivars with good genetic adaptation are needed. To overcome these challenges and improve its production and quality, scientists have used various new biotechnological tools.

Genome editing techniques comprise a set of tools developed to precisely modify genomes using variants of SDN (site-directed nuclease) technologies and ODM (oligonucleotide-directed mutagenesis). Thus, new SDN systems based on Zinc Finger Nucleases (ZFN), Meganucleases, and Transcription Activator-Like Effector Nucleases (TALEN) have been successfully used to generate targeted genomic changes in various crops [28]. However, these tools are considered very expensive and time- and labor-consuming. Relatively recently, a new SDN system, namely Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein (Cas) began to be used for the rapid improvement of agricultural crops, including for maize [2, 17].

Unlike transgenic plant modification that produces involuntary gene insertions, genome editing techniques produce well-defined mutations, and new genotypes can be used reliably without major concerns [34].

Transgenic crops and foods are a solution to the limitations of conventional breeding and ensuring food security, but they are strictly regulated regarding their use and marketing, and some international markets do not accept them at all [1]. According to [14], the changes generated by the application of genome editing tools are identical to those derived from conventional breeding or natural/induced mutations. As a result of the acceptance as non-GM of the cultivars developed by genome editing both in the countries that produce about 80% of the global crops, as well as in other countries, it is expected that this new technology will contribute to the democratization of agricultural biotechnology for the benefit of sustainable food production [16].

New germplasm developed through genome editing tools promise opportunities and benefits for farmers, consumers and society [32]. This paper presents information about genome editing applications in maize, one of the most important crops worldwide with implications for ensuring food security.

MATERIALS AND METHODS

This paper was carried out using statistical data collected from the EU-SAGE Database [9] for the period January 1996 to July 2023 and other sources.

The main aspects addressed were: the distribution of maize genome editing applications according to genome editing tools, SDN type, countries and different traits categories. The obtained results were synthetically illustrated in graphs and tables.

Recent examples of applications to improve the main traits in maize were presented.

Also, the existing regulations in different countries for these new technologies were summarized and discussed.

RESULTS AND DISCUSSIONS

The distribution of genome editing applications in maize

So far, genome editing has been applied to more than 63 crops from 25 countries [5].

As shown in Figure 1, between January 1996 and July 2023, a number of 757 applications were reported, the largest shares in the structure of the studied crops occupying rice (32%), tomatoes (14%), maize (7 %) soybean (6%) and wheat (6%). Maize registered 51 applications.

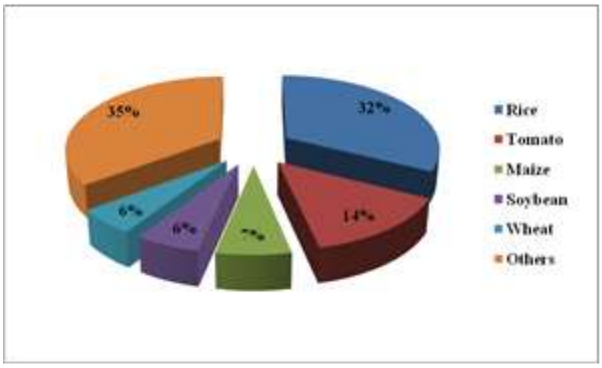

Fig. 1. The distribution of genomic editing applications by studied crops

Source: Own design and processing based on the data from [9].

Regarding genome editing tools, the use of the CRISPR/Cas system was reported in 38 applications out of a total of 51 maize applications, being the most used tool in the creation of new improved genotypes, compared to ZFN and other tools (Figure 2).

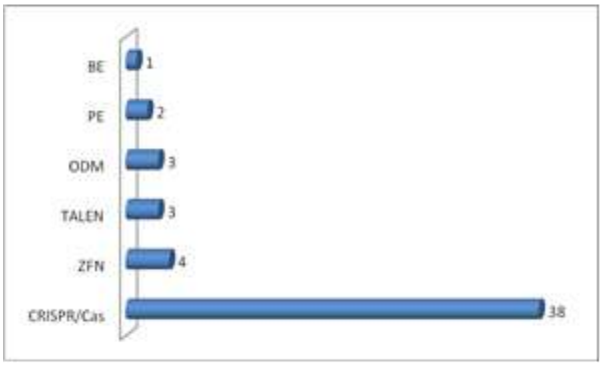

Fig. 2. The distribution of genomic editing applications in maize by genome editing tools

Legend: BE = Base Editing; PE = Primary Editing; ODM = Oligonucleotide-Directed Mutagenesis; TALEN= Transcription Activator-Like Effector Nucleases; ZFN = Zinc Finger Nucleases; CRISPR/Cas = Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein (Cas)

Source: Own design and processing based on the data from [9].

The CRISPR system was discovered in 1987 by Ishino and his colleagues when they were studying the iap gene in the *E. coli* genome [34]. The working principle of the innovative CRISPR/Cas9 tool was elucidated in 2012 [20], and since 2013 it has been applied in several fields, including crops both to increase the efficiency of gene knockout and to enable multiple genes knockouts [41].

ZFNs (Zinc-Finger-directed nuclease) were discovered in 1996 by [21], and in 2009 they were successfully used by researchers from the USA in maize to reduce phytate [35].

TALENs (Transcription Activator-Like Effector Nucleases) were discovered in 1989 in a bacterium called *Xanthomonas* [3], and in 2013 it was used in maize to reduce phytic acid (PA) synthesis in seeds, this acid being considered an anti-nutritional factor because it chelates the micronutrients in food thus preventing their absorption [25].

The efficiency of genome editing mediated by chimeric oligonucleotides (ODM) in maize was reported in a 1999 study by [42].

Since 2020, base editors (BE) and primary editors (PE) based on dCas9 and nCas9 have been frequently used in plant genome editing, being 10 to 100 times more efficient than homology-directed repair (HDR) [41].

Until now, the use of directed base editing (BE) of a target cytosine to thymine (C to T) has been reported in a single case in maize for generated sulfonylurea herbicide-resistant, but it is believed that the improvement of this technique will become a basic tool in precision crop breeding [22].

There are three categories of genetic changes generated by genome editing, namely three types of site-directed nuclease (SDN) 1/2/3, and according to the European Commission, SDN1 genetic changes are called "targeted mutagenesis", being considered similar to changes that they can appear spontaneously or due to conventional breeding [5].

Figure 3 shows that the highest percentage of maize applications was based on genetic changes belonging to the SDN1 type (93%).

The highest number of maize applications was reported in China (26) and USA (21), but research on maize genome editing also appeared in Europe (Figure 4).

For maize, the most important group of applications addresses yield and growth of plant (25%), industrial utilization (25%), food/feed quality (20%) and herbicide tolerance (18%) (Table 1).

All these improvements in targeted traits are related to the climatic, economic and agronomic challenges faced by farmers.

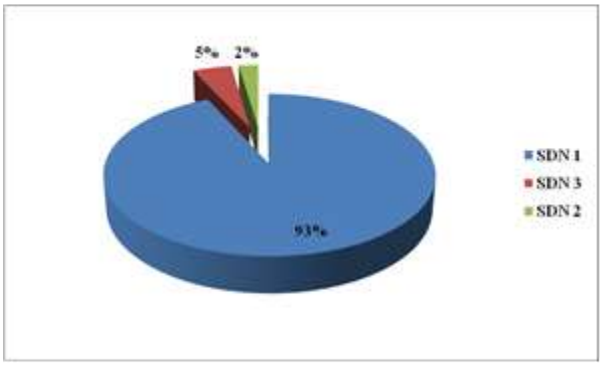

Fig. 3. The distribution of genomic editing applications in maize by SDN type
Source: Own design and processing based on the data from [9].

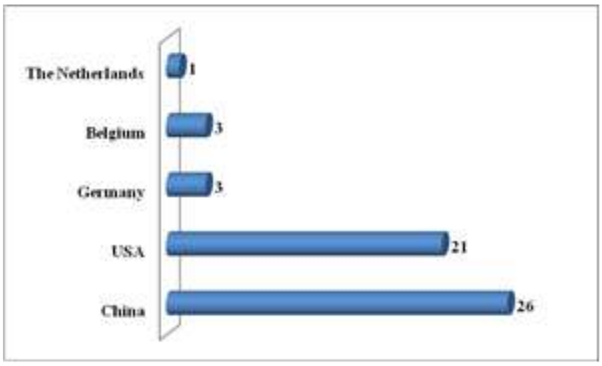

Fig. 4. The distribution of genomic editing applications in maize by countries
Source: Own design and processing based on the data from [9].

Improvement of grain yield

Improving crop yield is a major objective of breeding programs.The main traits related to yield are number, weight and grain size.

Recent research has shown the importance of new genomic techniques/tools in increasing the yield of maize and other crops.

For example, by using CRISPR/Cas9 technology, [26] created weak promoter alleles of CLE genes with increased grains per ear and maize yield.

Tassel branch number is another important agronomic trait for seed production because it provides pollen for hybridization. Over the course of modern breeding, male inflorescence (tassel) size and branch number have been reduced [12].

Table 1. The distribution of genome editing applications in maize by different traits

Categories of traits	Modifications	No.	%
Traits related to increased plant yield and growth	Improved field performance and increased plant yield due to architectural changes	13	25
Traits related to industrial utilization	Generating male sterility lines (MLS); enhanced haploid induction; trait stacking.	13	25
Traits related to improved food/feed quality	Sweeter kernels; reduced phytic acid (PA) synthesis in seeds; aromatic maize, modified composition, etc	10	20
Traits related to herbicide tolerance	Herbicide resistance	9	18
Traits related to biotic stress tolerance	Fungal resistance; visual detection of maize chlorotic mottle virus (MCMV); resistance to ear rot caused by *Fusarium verticillioides*	4	8
Traits related to abiotic stress tolerance	Drought tolerance	2	4

Source: Own design and processing based on the data from [9].

Recently, [15] showed that loss of *ZmPAT7* function induced significant increases in tassel branch number.

Doubled haploid technology based on haploid induction in vivo is frequently used in maize breeding for the development of homozygous lines in 2-3 generations [33].

For example, [24] reported that the CRISPR/Cas9 system generated mutant of *Zea mays PHOSPHOLIPASE D3 (ZmPLD3)* could increase haploid induction in maize, and [33] showed that the generation of mutants for the *ZmPLA1* gene in the maize inbred line LM13 by CRISPR/Cas9 technology can increase the rate of haploid induction. Using CRISPR/Cas9 technology, [23] created double knockout mutants of *ZmPHYC1* and *ZmPHYC2* that showed a moderate early flowering phenotype under long-day conditions, providing valuable target genes to breeding high-density tolerant maize genotypes. Also, [13] created CRISPR-waxy hybrids that are agronomically superior to introgressed hybrids, producing on average 5.5 bushels per acre more.

Improvement of quality

The physical characteristics (color, size, texture, etc.) as well as the content in bioactive substances (flavonoids, carotenoids, etc.) and specific nutrients (protein, lipids, starch, etc.) influence the quality of the crops. Sweet and waxy maize has recently recorded increasing demands from the consumer market [36].

By using CRISPR/Cas tool and editing the *ZmSH2* and *ZmWAXY1* genes, [6] produced sweet and waxy compound maize.

Also, by simultaneously editing the genome of the two BADH2 genes (*ZmBADH2a* and *ZmBADH2b*), [38] generated a double mutant capable of accumulating between 0.028 and 0.723 mg/kg 2AP (2-acetyl-1-pyrroline), which is the first aromatic maize in the world.

Improvement of resistance to biotic stress

Bacterial, fungal and viral diseases can significantly reduce the yield and quality of maize crops if pesticides are not applied. These pesticides are expensive and harmful to the environment and biodiversity. It is estimated that fungal diseases and lepidopteran pests can cause damage of more than 20% or 30% of maize yield [39].

Fusarium ssp. causes fusarium ear rot in maize, a dangerous fungal disease that leads to significant yield reductions but also to contamination with mycotoxins that endanger animal and human health. It can also cause fusarium head blight in wheat for maize-wheat rotation systems [27].

Research into the applications of new genomic techniques to improve resistance to bacteria, fungi and viruses is numerous.

For example, [27] showed that the targeted generation of null mutants in *ZmFER1* in maize by using the CRISPR/Cas9 system could confer resistance to *Fusarium verticillioides*. Another recent application of CRISPR/Cas 9 system in maize aimed at the generation of mutations with the loss of function of the LOX3 gene, the mutant plants manifesting resistance to *Ustilago maydis* which causes galls on all aerial parts of the plant [31].

Improvement of tolerance to abiotic stress

Efforts to improve crop yield and quality are hampered by numerous abiotic stresses (drought, heat, salinity, soil pollution).

It is estimated that the annual yield loss due to drought is over 20% of the maize area, and due to high temperatures the global yield loss is 7.4% for every 1°C increase [39].

Research carried out in maize by editing the *ZmNUDX2* and *ZmNUDX8* genes mediated by the CRISPR/Cas9 system showed an improvement in drought tolerance [30]. Also, a mutation of the *ZmLBD5*, which is involved in the regulation of growth and response to drought by affecting the synthesis of gibberellin (GA) and abscisic acid (ABA), can improve the drought resistance of maize [11].

Improvement of herbicide tolerance

Yield and plant growth are often limited by weed competition. Weed management is based on the use of herbicides, but their overuse and the lack of new active ingredients has led to the growth of resistant weeds. An economic and ecological approach to combating them is the use of cultivars resistant to herbicides. Recent research has shown that the use of new genome editing techniques can accelerate the development o genotypes tolerant to various herbicides.

For example, the CRISPR/Cas9 system was used to generate a homozygous *ZmALS1* mutation or a *ZmALS1* and *ZmALS2* double mutation in maize that produce plants tolerant to chlorsulfuron, a sulfonylurea herbicide commonly used in agriculture [22].

Improvement of traits to industrial utilization

For hybrid seed production, creating male-sterile genotypes is very important, saving labor and time.

As a result, researchers have used CRISPR/Cas9 tool to develop new crop genotypes, including genotypes of maize.

For example, [4] constructed a CRISPR/Cas9 vector that targeted the MS8 gene mutation in maize. The mutations generated showed male-sterile phenotypes that were stably inherited in subsequent generations. [29] targeted the editing of the gene *Zm00001d043909 (ZmCals12) in* maize. In addition to male sterility, the obtained maize ms39 mutant also showed plant dwarfing.

Combining haploid technology with CRISPR/Cas system has successfully generated various haploid inducers for industrial use. Thus, mutations of *ZmPLD3* [24] and *ZmDMP* [40] led to an increase in the rate of haploid induction.

Regulatory status of genome-edited products

The main limitations of genome editing products are legal regulation and consumer acceptance.

To legally classify SDN genome editing applications, the SDN 1/2/3 terminology was used. According to [28], these three types of SDNs are explained as follows:

- SDN 1: the induction of single point mutations or InDels;
- SDN 2: short insertions or editing of a few base pairs by an external DNA template sequence
- SDN 3: the insertion long DNA fragments (e.g. transgenes, cisgenes).

The scientific community considers that the mutations generated by SDN 1 and SDN 2 do not differ from those induced by conventional breeding or natural mutations, therefore they should not be subject to the existing regulation for GMOs. In general, SDN-3s are subject to GMO regulations because they introduce foreign genes [28]. However, opinions differ from country to country. For the main countries that regulate genome-edited products developed by SDN 1 and SDN 2, [37] described two general positions each with two approaches (Table 2).

The USA has different regulatory approaches depending on the use of genome editing for crops, animals or food. Also, in Australia genome editing techniques that are considered SDN 1 are exempt from regulation, while SDN 2 are subject to regulation. It is noted that most countries apply approaches 2 and 3.

Table 2. Position and approaches for regulation of genome-edited products by countries

Position	Approaches	Countries
I. GMO regulations are applied as it is to genome-edited products	Approach 1: Applying GMO regulations as they are, requiring prior government safety assessment and approval.	EU, New Zealand, USA - for animals through the Food and Drug Administration (under review).
	Approach 2: Simplified GMO regulations	Australia (SDN 1), China, India, New Zealand
II. Genome-edited products are exempt from GMO regulations	Approach 3: Requires confirmation by the government before placing the product on the market	Argentina, Brazil, Chile, Colombia, Paraguay, Philippine, USA - for food through the Food and Drug Administration
	Approach 4: Prior confirmation is not required by the government	Australia (SDN 2), USA - to regulate crops through the USDA and Environmental Protection Agency (under review).

Source: [37].

In European Union, in 2018, the Court of Justice (CJEU) has clarified that those organisms from new genome editing techniques fall within the scope of EU GMO legislation. Before 2018, they were not subject to GMO legislation, with member states free to create their own policies. However, in 2021 the European Commission published a study on the status of new genomic techniques under EU legislation, and based on the results of this study, initiated a political action involving an impact assessment, including a public consultation through which a proposal for a new regulation on plants produced by certain new genomic techniques. The proposal, adopted on 5 July 2023, is part of a package of legislative proposals to support EU farm-to-food and biodiversity strategies [8].

CONCLUSIONS

Consumers are the final recipients of crops and foods obtained through new genomic techniques, which is why they need to have up-to-date information on the progress of genome editing applications and their safety. Increasing consumer confidence and resolving political issues will enable new genomic techniques to contribute more to agricultural sustainability.

So far, 51 genome editing applications in maize have been reported in the EU-Sage database. Most genome editing applications (38) have been carried out using the CRISPR/Cas system, and China is the world leader in these research and applications.

These applications promise opportunities and benefits not only for consumers (due to improved nutritional value and food safety) but also for farmers (due to increased resistance to drought, heat, diseases, pests, and weeds) as well as for society (due to the protection of biodiversity in agricultural systems)

Due to current GMO legislation and political indecision regarding the authorization of genome-edited products, the European Union cannot yet use and cultivate these new genotypes.

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