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REVIEW ON LEGAL, SOCIAL AND ECONOMIC ASPECTS OF THE NEW BREEDING TECHNIQUES

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Abstract

The paper aimed to present a review on the social and economic aspects of NBTs, the studies on two different species of plants, subjects of NBT's. The plants variants generated by NBTs are more readily accepted in the market and for crop improvement. In this article we will present briefly the benefits, application and expected developments, regulatory status of NBTs in and outside the EU. It was developed a system for the detection of a broad spectrum of GMOs for analysis of food/feed matrices by the characterization of transgene flanking regions and the typical combinations for transgene constructs. We will describe two different species of plants, subjects of NBTs: 1) Tomatoes for carotenoid sequestration mechanisms and the carotenoid biosynthesis. The carotenoid accumulation and changes in carotenoid profiles suggest that the plastid can adapt to changes in carotenoid content through plastid differentiation and preferential sequestration; 2) Edited maize genome by biolistic delivery of pre-assembled Cas9-gRNA ribonucleoproteins into maize embryo cells and regeneration of plants with both mutated and edited alleles. As a conclusion, CRISPR/Cas9 is the most used technology for genome editing due to its simplicity and efficiency. In this article, we aim to highlight the application of CRISPR/Cas9 technique system, like the powerful genome editing tool for crop improvement.

Key words*: CRISPR/Cas9, genome editing, crop improvement, NBTs.*

INTRODUCTION

In the next 37 years, by 2050, the world population is estimated to grow by about 200,000 net people per day to a total of 9.6 billion people (Populaire Reference Bureau, 2012).

Among the benefits of genetic engineering in agriculture are increasing crop yields, reducing costs for food or feed production, reducing the need for pesticides, increasing nutrient composition and food quality, resistance to pests and diseases, and the benefits of food intake for the growing population world.

In support of these goals, agricultural and food scientists have developed new plant breeding techniques (NBT) in recent years, including CRISPR/ Cas9, the simplest and most effective technique for crop improvement, known as genome editing. This is a technique that allows the plant genome to be precisely modified by removing unwanted genes or byspecifying which genes can get new functions (Wolt et al., 2016). The genome-generated varieties are similar to the naturally occurring variations. It is less time-consuming and easier to accept on the market.

MATERIALS AND METHODS

The research methodology used in this paper has the following main aspects:

- bibliographic study of the national and international literature;
- collecting the concrete information within the researched area;
- ordering, processing and presenting of results in synthetic form;
- analysis and interpretation of results, formulation of conclusions and recommenddations.

RESULTS AND DISCUSSIONS

The site-specific nucleoside-based genomic editing system can be classified into three categories: the zinc nucleus finger (ZFN), the transcriptional effector nucleases (TALEN), and the intermediate short-acting palindromic groups that have been associated with the

binding protein of Cas9 on specific DNA sequences (CRISPR/Cas9). The main differences between categories consist in their mechanism of inducing the double-catenary break and their efficiency in targeting the desired sequences.

In plants, the administration of Cas9-RNA complexes (Figure 1.) has been demonstrated through protoplasts of several species. Protoplasts are "bare" cells generated by the enzymatic removal of cell walls and have a unique property of cell wall reforming and regeneration in plants. Protoplasts have been successfully used to edit the genome in a variety of plants such as tobacco, salad, rice and some flowers.

For most monocotyledonous species such as corn, wheat, rice, barley and sorghum, plant regeneration in protoplasts is less effective.

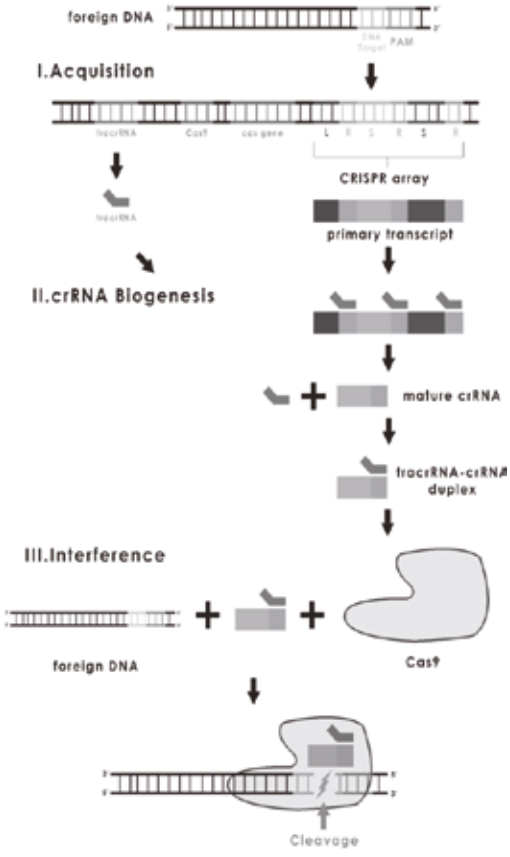

Figure 1. The CRISPR/Cas9 system involves three steps - acquisition, crRNA biogenesis and interference at DNA target cleavage
Source: www.intechopen.com

RNA-guided *Streptococcus pyogenes* Cas9 endonuclease was successfully used to modify the genome in several plant species. In most of the experiments, guideline RNA (gARN) as well as selectable marker genes and Cas9 were delivered to plant cells using either T DNA (*Agrobacterium tumefaciens* infection) or plasmid DNA (particle bombardment).

The supplied DNA integrates relatively easily into the target genome, but can lead to various side effects, such as gene disruption, plant mosaicism, and potential off-site cutting.

To alleviate potential negative effects, pre-integrated Cas9 nucleosus plants were generated and used to administer RNAs in the form of RNA molecules.

This approach requires time and resources for the development and characterization of pre-integrated lines (Abdallah et al., 2015).

New genome editing techniques may be accompanied by some unintended effects (cellular damage if repair mechanisms are imprecise, cleavage and mutation to similar unwanted genomic sites, mutations outside the target to genomic edited plants).

The genomic editing techniques, in general, show a much smaller number or a lack of unintentional mutations compared to organisms obtained by conventional reproductive techniques (Table 1.).

The absence of unintentional, potentially harmful effects can be verified with the whole genome sequencing (SAM, 2017).

The main modes of action in genome editing, as seen in Table 1, were:

- gene disturbance,
- genetic regulation,
- gene insertion.

Among the main attributes and expressed functions of the modified genes were:

- biosynthesis of important nutritional and health substances, as well carotene, inositol, tartaric acid, phytic acid, acetolactate, gibberellin,
- growth regulators,
- RNA regulation of biogenesis,
- initiating factor of translation,
- luorescence,
- cereal dormancy regulator,
- growth of root hair factors,
- auxin response factor.

Table 1. List of plants based on genome modification via CRISPR Cas9 system
Source: www.intechopen.com

Species name	Target gene	Gene function	Description	Action mode
Arabidopsis thaliana	BR11, JAZ1, GAI	growth regulators	displayed retarded growth	Gene disturbance
Brassica oleracea	BolC.GA 4.a	biosynthesis of gibberellin	displayed dwarf phenotype	Gene disturbance
Citrus sinensis	CsPDS	carotenoid biosynthesis	displayed albinism expression	Genetic regulation
Cucumis sativus	eIF4E	Initiating factor translation	developed resistance toward a broad range of virus	Gene disturbance
Glycine max	Bar, GmFE11 GmFE12	Growth of root hair factors	displayed higher root hair growth induction	Genetic regulation
Hordeum vulgare	HvPM19	Cereal dormancy regulator	displayed signs of dormancy	Gene disturbance
Marchantia polymorpha	ARF1	Auxin response factor	showed no response toward auxins	Gene disturbance
Medicago truncatula	GUS	Fluorescence	displayed no signs of staining	Gene disturbance
Nicotiana benthamiana	NbPDS	Carotenoid biosynthesis	displayed albinism expression	Gene insertion
Nicotiana tabacum	NtPDS	Carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Oryza sativa	OsPDS, OsMPK2 OsBAD2	Carotenoid biosynthesis, growth regulator	displayed albinism and dwarfism	Gene disturbance
Petunia hybrid	PDS	Carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Populus tomentosa	PtoPDS	Carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Solanum lycopersicum	SlAGO7	Involved in the RNA regulation of biogenesis	displayed needle-like or lacking lamina leaves	Gene disturbance
Solanum tuberosum	StALS1	Acetolactate biosynthesis	showed increased resistance on herbicides	Gene insertion
Sorghum bicolor	DsRED2	Fluorescence	showed signs of red fluorescence	Gene insertion
Triticum aestivum	TaINOX, TaPDS	Inositol and carotenoid biosynthesis	displayed albinism expression	Gene disturbance
Vitis vinifera	IdnDH	Tartaric biosynthesis	showed no signs of tartaric acid in their fruits	Gene disturbance
Zea mays	ZmIPK	Phytic acid biosynthetic pathway catalyst	showed reduction of phytic acid level	Gene disturbance

Global acceptance of plant biotechnology

The genome editing with modified nucleases has evolved as a highly specific and efficient tool for crop improvement, with the potential to quickly generate new phenotypes.

Figure 2. Global scheme of biotechnology acceptance
Source: www.nbtplatform.org

It arise the question how genetically modified plants with the desired characteristics will be received by the public and regulated under GMO legislation.

According to a recent study comparing the views of researchers and citizens on a range of scientific, engineering and technology issues (Funk and Lee, 2015), 37% of the general public responded that genetically modified foods are safe for consumption and 88% of scientists interviewed recognize genetically modified foods as safe (Wolt et al., 2016).

With all the studies done so far, it is undeniable that the CRISPR Cas9 system is about to change the pace and course of the agricultural industry.

Judgment of the European Court of Justice (C528/16ECJ)

As stated in the European Law, the definition of GMO means an organism except human beings where the genetic material has been altered in a way that does not occur naturally by mating and / or natural recombination.

The European Commission has stressed that the decision to include or exclude a technique within the scope of Directives 2001/18 and 2009/41 EC depends only on the interpretation of the definition of genetically modified organisms and genetically modified micro-organisms and the conditions for exemption laid down in the two directives (Laaninen, 2016).

There are regulatory authorities such as the German Consumers Protection Association or known as the Verbraucherzentrale Bundesverband (VZBV) and Swedish scientists

who call for the exclusion of such a "genetic modification" from the GMO Regulation if such crops do not contain any "foreign DNA" (Ammann, 2016).
On 25 July 2018, the European Court of Justice delivered its judgment in Case C-528/16, holding that organisms obtained by mutagenesis must be considered as GMOs, binding on national courts. The judgment of the above-mentioned European Court of Justice says that organisms created by new gene editing techniques (such as CRISPR Cas9 and related methods) are GMOs - Directive 2001/18/EC. The Directive requires organisms produced by genome editing to be detected as such by testing laboratories. EURL-GMFF has developped a draft report in this area that is not public yet but discusses detection issues and suggests potential ways to detect these products.

Examples of legal status of new breeding techniques outside the EU
Argentina is one of the first countries in the world to establish a regulatory framework and to underline the legal heterogeneity that characterizes cultures derived from New Breeding Techniques in Resolution 173/2015. It establishes a case-by-case assessment if a product falls under the category of a GMO or not.
Brazil, in line with the new regulatory resolution 16 (NR 16) published on 16 January 2018, the Brazilian National Biosafety Technical Commission may exempt new products from the same assessment of the GMO regulation, which has an annex to the BNT procedures that can create a product that is not considered GMO.
The United States of America, through the United States Department of Agriculture (USDA), has confirmed that it does not intend to impose new or additional regulations on crops developed by new breeding techniques, such as gene editing. The agency says new breeding methods can introduce new plant features faster and more accurately, saving years or bringing farmers new varieties.

The position of other world stakeholders interested in new plant breeding techniques
Jan Plagge, President of IFOAM in the EU: "The European Court of Justice's confirmation that the new GMOs will be traceable and labelled is good news for organic farmers, farmers and processors, but also for all European producers and consumers, the freedom to avoid such genetically modified products and the protection of the environment against the potential risks of these new technologies."(IFOAM EU Press Release: New genetic engineering techniques will be regulated as EU-EU GMOs welcomes the ECJ decision, 2018).
Franziska Achterberg, EU Greenpeace Director for Food Policy: "The Court clarifies that plants and animals derived from genetic publishing are subject to the same safety and labelling requirements as other genetically modified organisms. These requirements exist to prevent harm and to inform consumers the release of these new GMOs into the environment without adequate safety measures is illegal and irresponsible, especially given that gene editing can lead to unintended side effects. The European Commission and European Governments need to ensure that all new GMOs are fully tested and labelled and that all field trials are brought into line with GMO standards.", she said. (EURACTIV: Industry shocked by the European Court's decision to put the genetically engineered technique in place with GM law, 2018).
USDA Secretary Perdue Statement on the ECJ judging genomic publishing: "We encourage the European Union to seek the contribution of the scientific and agricultural communities and its trading partners to determine the proper implementation of the decision of innovations in biotechnology, such as genome editing, include their benefits include healthier, high-quality foods at affordable prices. For farmers, they include improvements in productivity, plant and animal health and environmental sustainability."(USDA Press, Release No. 0155.18, 2018).

Socioeconomic aspects of new breeding techniques in plants
Based on the position expressed by various stakeholders, such as farmers' associations, researchers and plant breeders, the ECJ's decision is expected to have a profound impact on European agriculture, research and trade.

Benefits for plant growth:
• increased yield,
• reducing the use of natural resources,
• reducing dependence on chemical protection
• contribution to biodiversity,
• adapting to changing conditions.

Benefits for farmers
• Increased resistance to biotic and abiotic stress factors (reduced pesticide treatment).
• Reducing the impact on the environment.
• Improving land and investment efficiency.
• Improving crop efficiency and product diversity.
• Rapid adaptation to climate change.
• Longer preservation time, so less food waste.
• Development of the plant breeding sector (selection of edible species from wild populations, selection of plant species with desired characteristics) (Figure 3).

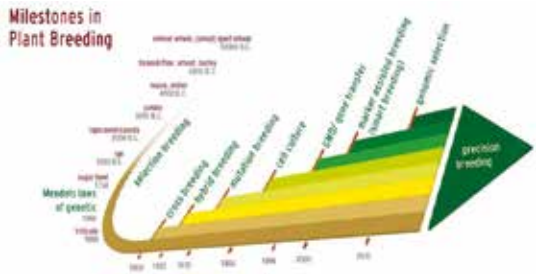

Figure 3. Highlights for plant breeding
Source: www.nbtplatform.org

Problem of detected genome edited
Modifications of the DNA sequence introduced by genomic editing methods are not presently identified, as compared to DNA sequence changes produced by natural processes or conventional mutagenesis. The exception is when genome editing is used to introduce more than two base pair changes into DNA at a single location, these being less likely to be natural or mutagenic.
If there is no information about the changes introduced (no known target), it is difficult to detect the changes. Detection could only be possible with a suitable reference genome for comparison (Lusser et al., 2011).

Ways suggested to detect the genome edited
• If a body with the genome edited has undergone a documentation process (example an authorization) detailing the modified DNA sequence of the gene region being edited and providing complete details of the organism itself (example variety), it would be possible To identify if an unknown sample corresponds to the edited variety. This clearly shows that the unknown sample originates from a gene with an edited genome, but it is not a direct proof that the sample was from an edited genome.
• If the suspect sample originates from a cultivated plant, the sample genome could be compared to a genomic reference database of non-genomic varieties of that plant to identify DNA sequence differences. Differences would be modifications by genome editing. This is a poor assumption because new mutations could have occurred (naturally or by mutagenesis) in any variety in each generation. The proposal to set up a database for genomic comparisons would be a very large economic effort. There are 14,442 varieties of wheat bread, Durham wheat, maize, soy beans, barley, rape, rape and potatoes registered in the EU, as shown by the European Commission's plant varieties database.
From Wikipedia, there are 7500 apple varieties and 10,000 varieties of tomatoes, without their varieties. This very costly way may be impossible to put into practice and would provide relative proofs.

Editing corn genome through ribonucleo-protein complexes
The biolistic introduction of Cas9-gARN ribonucleoproteins pre-assembled as ribonu-cleoprotein complexes into maize embryo cells by particle bombardment and plant regene-ration with mutant alleles and edited was successfully performed, using this method, DNA-tagged DNA mutagenesis is also obtained in maize (Zhang et al., 2016).
Four genomic regions, liguleless1 (LIG), acetolactate synthase (ALS2), and two male fertility genes (MS26 and MS45) were targeted by pre-assembled Cas9 protein pre-assembled with in vitro transcribed gRNAs. Cas9-gRNA complexes were delivered to corn embryonic cells on gold particles (0.6 μm) using a helium gene gun. Total genomic DNA was extracted and fragments surrounding the target sequences were amplified by PCR and analysed by

sequencing (Table 2). Mutations were found at all target sites where the Cas9-gARN complexes were provided (Svitashev et al., 2016).

Table 2. Mutations in corn embryonic cells
Source: www.ncbi.nlm.nih.gov

Target site	Target site sequence with PAM	Cas9 (%)	DNA (%)	Cas9 g ARN (%)
LIG	GCGTACGCGTACGTGTGAGG	0.004	0.56	0.57
ALS2	GCTGCTCGATTCCGTCCCCATGG	0.020	0.51	0.45
MS26	GCACGTACGTCACCATCCCGCCGG	0.004	0.43	0.21
MS45	GGCCGAGGTCGACTACCGGCCGG	0.002	0.34	0.69

To measure the frequency of plant-level mutations, 60 bombarded embryos were placed on growth medium and 36 segments of herbicide-resistant callus segments, which were tested for mutations, were regenerated. Of the 36 events, 17 (47%) contained mutant alleles and 19 (53%) had only wild type alleles. The ability to provide Cas9-gARN complexes on gold particles in corn cells combined with the high frequency of mutant plant recovery without selection makes this approach practical for genome editing in cultured species. The results obtained on maize provide new opportunities for advancement of agricultural reproductive practices for any species of plants subject to biolytic delivery (Svitashev et al., 2016).

Differential accumulation of carotene in tomatoes by chromoplasts

Carotenoids are high-value compounds for the food industry. The global market for these substances will grow to $ 1.95 billion by 2025, based on an annual growth rate of 5.1% (Accuray Research LLP, 2017).

Tomato (*Solanum lycopersicum*) is the plant model for carotenoid-related studies because the fruits contain high levels. The study of a tomato line specifically designed for a higher capacity of carotenoid accumulation via the PSY1 gene (psy1 - the carotenoid biosynthesis enzyme) has led to the observation of chloroplast differentiation in chromoplasts in immature fruits.

The PSYsense overexpression line has a phenotype in which the associated carotenes accumulate at the onset of fruit growth, resulting in a pink-orange colour of the mature green fruit (Fraser et al., 2007).

Because of their unique characteristics, carotenoids can function as modulators of membrane structures, a hypothesis that has been tested in bilateral lipid models mixed with different carotenoids *in vitro*.

The way in which carotenoids are captured (Figure 4) in the membrane depends on the trans or cis configuration and leads to different ways of membrane integration (Widomska et al., 2009).

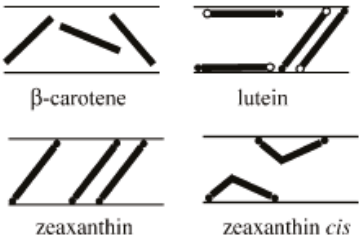

Figure 4. Carotenoid lipid interactions
Source: www.pure.royalholloway.ac.uk

DNA for the coding sequences of the genes of interest was obtained from *Escherichia coli* DH5a genomic DNA samples and *Rhesus capsicum annuum* (sweet pepper) RNA samples for plastid extraction (Cheng and Jiang, 2006).

An optimized vector used for overexpression of bacterial carotenoid genes crtZ and crtW was used as a source for promoter and terminator parts of the constructs created (Misawa and Shimada, 1998).

Qualitative confirmation for expression of the coding sequences was performed by PCR method.

The selected lines were mutant lines disrupted in the overexpressed carotenoid biosynthesis of the psy1, crt-iso, LCY-b and crt1 genes (Ailsa Craig). Sub-chromoplastic fractions show the accumulation of specific carotenoids, which may have changes at the grip sites. An important modifier of the grip preference is the "cis" or "trans" (Widomska et al., 2009).

These structural changes specifically modify the membrane integration of carotenoids (Gruszecki and Strzalka, 2005).

The significantly higher capacity of chromoplast accumulation allows them to function as a storage reservoir for carotenoids compared to chloroplasts (reviewed by Egea et al., 2010).

The increase in carotenoid content occurs simultaneously with chloroplast differentiation

in chromoplast, because the chromoplastic structures are found in the immature fruits of the PSY-1 sense line (Fraser et al., 2007).

Figure 5. Different accumulation of carotenoids
Source: www.pure.royalholloway.ac.uk

Early activation of the PSY1 enzyme results in the accumulation of carotenoids and the differentiation of chloroplasts in chromoplasts in immature fruits that have the green crown, but the tissues are orange pink.

Was observed two-fold increase in total carotenoid content (lycopene and β-carotene) for the PSY-1 sense line compared to control. This growth is explained by the occurrence of phytotecine (6.4 μg), phytophluene (4.3 μg), z-carotene (4.7 μg) and lycopene (2.0 μg) and lutein growth (1.5 times) carotene and xanthophils (1.5 times). (Nogueira et al., 2013).

Over-expression of psy1 in the PSY-1 sense line gives a similar response to immature fruits. Chromoplastic differentiation in tomato fruits occurs at the onset of maturation and regulates carotenoid biosynthesis at transcriptional level (Llorente et al., 2016; Toledo-Ortiz et al., 2014).

CONCLUSIONS

There are still many uncertainties about the use of plant genome editing. Therefore, in-depth studies are needed to ensure that these new plant breeding technologies will have zero risks, while maximizing benefits. The idea of editing the genome could also raise ethical questions from the public, and they should be approached appropriately by scientists who are well-trained in genome engineering. Educational discussions or workshops on genomic editing should also be offered to non-scientists to ensure that the benefits of this technology are well understood by consumers, the true beneficiaries of research and innovation in the agro-food industry.

More regulation will be needed to apply new plant breeding techniques to ensure that they are used responsibly without slowing down development and research. Generally, new mutagenesis techniques are faster and cheaper than conventional reproductive techniques. There are already several plants generated with the new mutagenesis techniques that are near or in the phase of field testing or marketing.

By editing the genome, mutations can be targeted. Depending on the technique, non-specific mutations (allele insertion) or specific mutations (oligonucleotide genetic mutation, targeted insertion) can be rapidly introduced into a desired gene, creating a desired donor allele. In polyploid plant species (wheat), all homologous copies of a gene may be targeted at the same time, so that traits that may be obtained with great difficulty through tradetional mutagenesis can be obtained.

Mutagenesis would allow farmers to combat the pests of plants resistance of pesticides, increase yields and improve the nutritional content of crops, and allow Europe to remain competitive with other major agricultural powers, such as the US and Brazil.

The results obtained in plants such as corn and tomatoes open new opportunities to develop new reproductive practices in a wide variety of cultured species. The ability to provide Cas9-gARN complexes on gold particles in corn cells combined with the high frequency of mutant plant recovery without selection makes this approach practical for genome editing in cultured species. Tomato mutant lines appear due to overactive or inactive key steps in carotenoid synthesis, and the significantly higher capacity of chromoplast accumulation allows them to function as a reservoir for carotenoid storage.

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