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Review

Crop Improvement: Comparison of Transgenesis and Gene Editing

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Abstract: The development and improvement of molecular biology methods have led to the creation of new technologies that make it possible to modify plant genomes by transferring and integrating into the genomes' heterologous genes from various expression systems (genetic engineering), as well as inducing knockouts of one or more target genes of interest (genomic editing). The development of genome-editing methods is a new milestone in the development of modern breeding methods and certainly relies on the knowledge and technologies developed for transgenesis. This review will discuss issues related to the advantages and disadvantages of both technologies for improving the economically valuable traits of important crops.

Keywords: GMO; transgenic plants; gene editing; CRISPR/Cas; gene-edited crops; plant breeding; crop improvement

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1. Introduction

Of all organisms, only plants and photosynthetic bacteria are able to use the energy of the sun to create organic substances from inorganic ones. The existence of the animal world is impossible without plants. Crops provide food, feed, fuel, and other consumable resources for human life, thereby contributing enormously to society. At the same time, in recent decades, humanity has faced unexpected difficulties that confront the world's agricultural productivity. The planet's population is growing steadily, climatic conditions are changing, and all these factors pose new challenges for plant breeders [1]. It is necessary to develop and apply new approaches to plant breeding. Traditional approaches to crop breeding are inefficient, time consuming, and complex, necessitating the development of more efficient breeding methods that must save time [2]. The most promising genome modification technologies today are transgenesis and genome editing. This review is devoted to examining the similarities and differences between these methods and the resulting advantages and disadvantages of both of them.

Until the beginning of the 21st century in modern agriculture, the main technologies for crop improvement were crossbreeding, mutation breeding, and transgenic breeding. Since the first gene-targeting experiment in tobacco (*Nicotiana tabacum*) protoplasts in 1988 [3] and the discovery that DNA double-strand breaks (DSBs) enhance gene-targeting efficiency in 1993 [4], scientists have sought to develop tools for the targeted editing of plant genomes. The increasing number of plant species with a fully deciphered genome every year, the development of genetic engineering methods, and the emergence of accessible libraries of plasmids allow scientists to change genes precisely, opening up new possibilities for crop improvement. The development of effective genome-editing technologies has provided researchers with powerful tools for decoding gene functions and enhancing plant traits through diverse biological systems involving zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced

short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas) systems. Gene-editing technologies enable the modification of a specific genomic site (e.g., gene knockout/single-base editing and guided editing) using artificially designed nucleases [5]. Consequently, CRISPR/Cas9 has gradually become the most widely used and advanced gene-editing system, opening up vast opportunities for improving crop plants and studying gene function [6]. However, the creation and use of transgenic plants is still an equally widely used technology. It was transgenic plants that made it possible to change intensive farming technologies, and the cultivation of transgenic plants led to a decrease in the amount of herbicides and pesticides used in agriculture [7]. Transgenic plants will certainly be created and grown in the future, since in some aspects, this technology has proven itself very well; moreover, transgenesis is often one of the stages in obtaining gene-edited plants. Transgenesis and editing have their own specific features; this review is devoted to the comparison of these two methods of changing the plant genome, with a special emphasis on changing agriculturally important traits in horticulture crops.

2. Improving Economically Valuable Traits in Crops

The purpose of crop quality improvement is to control and enhance the genetic characteristics of crops through breeding technology to boost the production performance of crops and to improve quality indicators such as palatability and nutrients. Genetic traits determined by hereditary information encoded in DNA and determining phenotypic traits are inherited from parents from generation to generation. At the same time, genetic information is constantly subject to changes due to the presence of spontaneous or induced mutations, errors that occur during replication, the activity of mobile elements, the processes of meiotic crossing over, and cross-fertilization. In addition, there are a number of pathogenic and symbiotic bacteria capable of transferring part of their DNA into the genome of a plant cell [8]. Bacteria thereby influence the metabolism of the plant cell, forcing it to produce the substances it needs. Thus, we can say that the plant genome is constantly being modified.

Improving the economically valuable traits of plants is also based on introducing various modifications to the genome of the plant cell. The history of plant mutagenesis dates back to 300 BC, as humans have used natural mutations generated in nature for selective breeding. Plant breeding involves systematic selection among the entire population of plants of samples bearing target properties. Thus, it is estimated that humans have been successfully breeding plants for over ten thousand years [9] when seeds of plants with favorable features were saved for the next plantation, a practice known as domestication. Among the various mutations that can either improve or worsen some plant characteristics, the breeder also selects the most interesting and important ones and uses them in his breeding work.

One of the most important achievements of the early to mid-20th century that should be considered is the development of methods for induced mutagenesis. A large number of varieties of cultivated plants grown today were obtained precisely as a result of induced mutations. To date, more than 3400 varieties obtained by mutagenesis have been registered, belonging to more than 200 different plant species (according to The Mutant Varieties Database, a joint initiative of the FAO/IAEA (International Atomic Energy Agency/Food and Agriculture Organization of the United Nations) https://nucleus.iaea.org/sites/mvd, (accessed on 29 November 2023). The most significant advances in plant breeding techniques have been achieved as knowledge and understanding of plants and their genetic structure have accumulated. The most important stage in plant breeding was the Green Revolution, which made it possible to dramatically increase the productivity of agricultural crops through the development of high-yielding varieties of cereals, particularly dwarf wheat and rice. Norman Borlaug, Nobel Prize laureate and father of the Green Revolution, emphasized that the key to the success of these semi-dwarf varieties was their wide adaptability, short plant height, high sensitivity to fertilizers, and resistance to disease, which ultimately made it possible to obtain more yield at a lower cost [9]. Induced mutagenesis

has been most widely used to modify the genome of cereals [10]. Among horticultural species, the greatest success of induced mutagenesis was achieved in ornamental plants, especially chrysanthemums and roses [11–13].

However, the methods of traditional selection and chemically/physically induced mutagenesis have a number of disadvantages. The use of traditional selection is a very long and labor-intensive process, and in addition, the researcher is limited by the set of genes that are present in the genome of a given species. As for mutagenesis, although it still plays a significant role, it produces random mutation events, is hazardous to humans, is not eco-friendly, and its dose rate differs for each genotype and requires standardization [14].

In the second half of the 20th century, with an increase in the quantity and quality of food consumption, a revolution in plant breeding occurred, the key achievements of which were achieved in the creation of hybrids and transgenesis [9]. Transgenesis and editing, which appeared later, make it possible to obtain targeted changes in the genome in a shorter time, without the series of backcrossing and lengthy selection of successful events, as well as with a more predictable result, without destroying the existing combinations of genes in a particular variety.

2.1. Modifying Plant Genomes Using Gene Engineering Technologies

As data accumulated on the organization of the gene and the functioning of genetic information in the cell, new technologies for genome modification began to appear. Transgenesis became the main one for a long time. Transgenesis changes the genetic information of a plant cell, resulting in a so-called genetically modified organism (GMO) that carries in its genome a fragment of foreign DNA that gives the plant new useful traits that cannot be obtained by conventional breeding methods. Transgenesis serves not only for the transfer of genes originating from any other organisms (bacteria, animals, viruses, other plants, etc.) into the plant genome but also as an improved method of induced mutagenesis and a tool for manipulating the level of expression of host cell genes (gene silencing) [14]. Transgenic crops are now widespread globally and are increasingly accepted as food and feed. The development of genetic engineering, the emergence of PCR, and the simplification and improvement of sequencing methods have contributed to the wide spread of transgenesis technologies in the world.

To create transgenic plants, mainly two methods are used—agrobacterium transfer carried out using the soil bacterium *Agrobacterium tumefaciens* and transfer using bioballistics [15,16]. The bacterium *A. tumefaciens* naturally infects the wound of a plant to develop crown gall disease. This is possible because Agrobacterium carries the tumor-inducing (Ti) plasmid, which has a virulence (vir) region and a T-DNA (transfer DNA), which actually transferred from the bacterium to the plant. During the process of transformation, multiple components of the Ti plasmid work together for the effective transfer of the gene of interest into the plant cells. Agrobacterium-mediated transformation in our days is a simple and inexpensive biological technique, which can be applied in plants as well. The transformation results in either a single or low copy number of T-DNA insertions, which prevent homology-dependent gene silencing or the rearrangement of inserted genes by recombination. This is advantageous over methods that insert target sequences in multiple copies. However, it can be applied successfully more toward dicot plants than that of monocots; monocots are generally hard to transform by this method [16–20]. There are a huge number of examples of the use of agrobacterial transformation for the delivery of transgenic constructs into the genome of horticultural species. To date, protocols have been developed for many species, including fruit trees and many others [21]; for species that have difficulties with in vitro cultivation, methods of tissue culture-independent agrobacterial transformation have been developed [22]. Some of them are shown in Table 1.

Table 1. A list of selected examples of transgenic and gene-edited horticulture crops.

Plant	Specific Trait	Target Gene	Transgenic	Gene-Edited	Used Transgenesis	Reference
Herbicide Resistant						
Savoy cabbage (*Brassica oleracea* var. *sabauda*)	phosphinothricin (L-PPT) resistant	*bar*	yes	no	Yes *A. tumefaciens*	[23]
Sweet potato (*Ipomoea batatas* L. Lam.)	phosphinothricin (L-PPT) resistant	*bar*	yes	no	Yes Bioballistic	[24]
Potato (*Solanum tuberosum*)	glyphosate tolerance	*EPSPS*	yes	no	Yes *A. tumefaciens*	[25]
Easter lily *(Lilium longiflorum* Thunb.)	phosphinothricin (L-PPT) resistant	*bar*	yes	no	Yes Bioballistic	[26]
Tomato (*Solanum lycopersicum* L.) Potato (*Solanum tuberosum*)	chlorsulfuron-tolerant plants	*SlALS1*, *SlALS2* *StALS1*, *StALS2*	no	Cas9 + Base editor	Yes *A. tumefaciens*	[27]
Watermelon (*Citrullus lanatus* (Thunb.))	chlorsulfuron-tolerant plants	*ALS*	no	Cas9 + Base editor	Yes *A. tumefaciens*	[28]
Cassava (*Manihot esculenta*)	glyphosate tolerance	*EPSPS*	no	Cas9, HDR editing	Yes *A. tumefaciens*	[29]
Lettuce (*Lactuca sativa* L.)	paraquat	uORF of *LsGGP1* and *LsGGP2*	no	Cas9	Yes *A. tumefaciens*	[30]
Pathogen Resistance						
Tomato (*Solanum lycopersicum* L.)	*tomato yellow leaf curl virus* inactivation	coat protein, replicase	yes	Cas9	Yes	[31]
Papaya (*Carica papaya* L.)	resistance to *papaya ringspot virus*	coat protein gene	yes	no	Yes *A. tumefaciens* Bioballistic	[32]
Tomato (*Solanum lycopersicum* L.)	resistance to larvae of *Helicoverpa armigera* and *Spodoptera litura*	*cry1Ab*	yes	no	Yes *A. tumefaciens*	[33]
Mustard, (*Brassica juncea* L.)	resistance to *fungal pathogens*	*NPR1*	yes	no	Yes *A. tumefaciens*	[34]
Chrysanthemum (*Chrysanthemum morifolium*)	resistance to *Spodoptera exigu*, *Aphis gossypii*	*CaXMT1*, *CaMXM1* *CaDXMT1*	yes	no	Yes *A. tumefaciens*	[35]

Table 1. *Cont.*

Plant	Specific Trait	Target Gene	Transgenic	Gene-Edited	Used Transgenesis	Reference
Cucumber (*Cucumis sativus* L.)	resistance to *cucumber vein yellowing virus*, *zucchini yellow mosaic virus*, *papaya ringspot mosaic virus-W*	*eIF4E*	yes	Cas9	Yes *A. tumefaciens*	[36]
Banana (*Musa* spp.)	inactivation of *banana streak virus*	ORFs of banana streak virus	yes	Cas9	Yes *A. tumefaciens*	[37]
Chilli pepper (*Capsicum annuum* L.)	resistance to *Colletotrichum truncatum*	*CaERF28*	no	Cas9	Yes *A. tumefaciens*	[38]
Grape (grape cultivar *Chardonnay*) Apple (apple cultivar *Golden delicious*)	resistance to powdery mildew and fire blight disease	*MLO-7 DIPM-1*, *DIPM-2*, *DIPM-4*	no	Cas9	No (RNP)	[39]
Abiotic Stress Resistance						
Apple (*Malus pumila Mill.*)	adaptation to cold and drought stress	*Osmyb4*	yes	no	Yes *A. tumefaciens*	[40]
Chilli pepper (*Capsicum annum.*)	improved salt tolerance	osmotin	yes	no	Yes *A. tumefaciens*	[41]
Grape (*Vitis vinifera* L.)	improved cold-resistance	*AtDREB1b*	yes	no	Yes *A. tumefaciens*	[42]
Grape (*Vitis vinifera* L.)	resistance to drought stress	*VaNCED1*	yes	no	Yes *A. tumefaciens*	[43]
Potato (*Solanum tuberosum*)	improved resistance to salt and drought stress	*SOD*, *APX*, *codA* under SWPA2 promoter	yes	no	Yes *A. tumefaciens*	[44]
Eggplant (*Solanum melongena* L.)	salinity tolerance	*TaNHX2*	yes	no	Yes *A. tumefaciens*	[45]
Tomato (*Solanum lycopersicum* L.)	improved salt tolerance	*SlABIG1*	no	Cas9	-	[46]
Potato (*Solanum tuberosum*)	resistance to abiotic stress and viruses	Coilin	no	Cas9	No, RNP Bioballistic Vacuum infiltration	[47]
Ethiopian mustard (*Brassica carinata*)	reduced root length under phosphorus stress	*BcFLA1*	-	Cas9	Yes *A. tumefaciens*	[48]

Table 1. *Cont.*

Plant	Specific Trait	Target Gene	Transgenic	Gene-Edited	Used Transgenesis	Reference
Lettuce (*Lactuca sativa* L.)	high temperature resistance	*LsNCED4*	yes	Cas9	Yes *A. tumefaciens*	[49]
Potato (*Solanum tuberosum*)	improved cold stress resistance	*VInv*	no	Cas9	No *A. tumefaciens* Transient expression	[50]
Enhanced Quality						
Tomato (*Solanum lycopersicum* L.)	enhanced fruit softening	*LeEXP1*	yes	no	Yes *A. tumefaciens*	[51]
Apple (*Malus domestica*)	non-browning	*PPO*	yes	no	Yes *A. tumefaciens*	[52]
Potato (*Solanum tuberosum*) Tomato (*Solanum lycopersicum* L.) Strawberry (*Fragaria vesca*)	higher vitamin C	*GGPorVTC2*	yes	no	Yes *A. tumefaciens*	[53]
Orchid (Oncidium Gower Ramsey)	early flowering	*OMADS1*	yes	no	Yes *A. tumefaciens*	[54]
Tomato (*Solanum lycopersicum* L.)	high ɣ-aminobutyric acid (GABA)	*SlGAD2* *SlGAD3*	-	Cas9	Yes *A. tumefaciens*	[55]
Tomato (*Solanum lycopersicum* L.)	high lycopene	*SGR1*, *LCY-E*, *Blc*, *LCY-B1*, *LCY-B2*	-	Cas9	Yes *A. tumefaciens*	[56]
Potato (*Solanum tuberosum*)	high amylopectin starch	*GBSS*	no	Cas9	No *A. tumefaciens* transient expression	[57]
Potato (*Solanum tuberosum*)	non-browning	*StPPO2*	no	Cas9	No, RNP, PEG transfection	[58]
Banana (Cavendish banana cultivar (cv.) *Grand Naine*)	β-carotene-enriched	*LCYε*	-	Cas9	Yes *A. tumefaciens*	[59]
Strawberry (*Fragaria vesca*)	improvement of sugar content	uORF of *FvebZIPs1*.1	-	Cas9 + Base editor	Yes *A. tumefaciens*	[60]
Watermellon (*Citrullus lanatus* (Thunb.))	albino phenotype	*CIPDS*	-	Cas9	Yes *A. tumefaciens*	[61]

Bioballistics is a physicochemical method that bombards microcarriers containing genes of interest at high speed on plant cell walls using the so-called gene gun [15,16]. Nanoparticles coated with DNA are accelerated with gas pressure and shot using a gene gun into plant tissue kept in a Petri dish. The gene construct can be a circular or linear

plasmid or a linear expression cassette. Factors that affect successful transformation include the size and density of the microcarriers, velocity of the microcarriers at the point of impact, nature of the plant tissue to be transformed, and suitable pre-culture or pretreatment of the target plant explants [16]. However, this technique requires expensive equipment. In addition, the transformation with the gene gun often gives rise to chimeric plants that consist of both transformed and non-transformed cells. If the reproductive cell line of such chimeras does not contain the target gene, then the next generation of such a plant would not be transformed. Transformation by this method can result in multiple copies of target DNA being inserted randomly anywhere in the plant genome. However, this method poses less physiological risk to the plant cell since there is no need for microbial intermediaries (Agrobacterium strains) and it requires less additional DNA. Moreover, it can adapt to both monocots and dicot plants. Examples of the successful use of bioballistics for the transformation of horticultural crops are also presented in Table 1.

A huge number of technologies have been developed to increase the efficiency of the transformation and regeneration of transgenic plants. These include various new delivery methods such as the use of nanoparticles, magnetofection, viruses, etc. [15,62], as well as the use of genes involved in embryogenesis or meristem maintenance, such as *BBM*, *GRF*, and *WUS2* [18,63,64].

As a whole, both transformation methods were successfully used for the transformation of many horticultural crops (Table 1). In general, traits affecting the production of the crop are preferred over the traits involved in the modification of the final product. In this case, we can distinguish two main categories of GM crop traits. Firstly, it is tolerance to the application of specific herbicides. The most commonly developed trait has been tolerance to glyphosate, followed by glufosinate (phosphinothricin). Such traits were successfully inserted in potato [25] and sweet potato [24], cabbage [23], ornamental plants [26], as well as in many cereal crops [7,65]. Since 2016, crops with additional tolerance to active ingredients like 2,4-D and dicamba have been introduced, mostly in North America [66]. In 2020, ~90% of all corn, cotton, and soybeans planted in the U.S. were GM variants tolerant to one or more herbicides [67]. Secondly, it is resistance to specific insect pests of eggplant, potato, tomato, broccoli, and other crops [68]. This GM insect resistance (other name "Bt" technology—from Bacillus thuringiensis) offers farmers resistance in the plants to major pests, such as stem and stalk borers, earworms, cutworms, and rootworms in maize, bollworm/budworm in cotton, caterpillars in soybeans, and the fruit and shoot borer in eggplant [69–71].

The size of the area occupied by GM varieties grown In the world in 2022 was estimated at 202.2 million hectares. They are mainly occupied by soybeans (98.9 million hectares), corn (66.2 million hectares), cotton (25.4 million hectares), and rapeseed (9.9 million hectares) [72]. Despite the fact that the same use of plants resistant to various pests made it possible to reduce the amount of pesticides used in the areas where these plants are grown by 7.2% [7], society is wary of transgenic plants. An analysis of 2 million cases mentioning GMOs in various social networks and web resources in 2019–2021 and an assessment of their emotional connotation showed that 54% of mentions can be classified as neutral, 32% as negative, and only 14% as positive [73]. GMO organisms are perceived ambiguously by society, which stems from the fact that obtaining state registration for a GMO variety in some countries is significantly difficult or completely impossible.

Despite all the successes of transgenesis, the impact on public opinion complicates the use of this technology for improving crops. But since the demand for the development of new varieties still exists, a new technology has emerged in response to this request—gene editing. Unlike transgenesis, the insertion of foreign genes into the genome using gene editing is significantly difficult but gene editing allows you to make small changes to the target DNA sequence, thereby performing site-specific mutagenesis. In the last ten years, gene editing has been gaining popularity as a safe and cheap technique allowing you to quickly develop a new variety that meets all regulatory requirements [74].

2.2. Genome-Editing Technologies

With the development of genetic engineering methods and the accumulation of data on plant genomes, gene-editing technologies began to develop—making it possible to perform site-specific changes in the target site of the genome. The first methods that appeared were zinc-finger nuclease (ZFN) and, later, transcription activator-like effector nucleases (TALEN). Both TALEN and ZFN are composed of repeated tandem sequences of DNA-binding domains and an attached Fok1 nuclease protein; such a recombinant protein can be targeted to recognize a target DNA sequence and, therefore, create double-strand breaks (DSBs) at the target site. For each target site, a new TALEN or ZFN protein must be prepared to recognize the target DNA sequence, which requires labor-intensive genetic engineering and is significantly limited by the widespread use of these gene-editing technologies [75,76]. However, there are examples of the successful use of ZFN to manipulate genes in tobacco, Arabidopsis, and maize [77–79], as well as for some horticulture species like tomato [80], potato [81], apple, and fig trees [82]. TALENs, which are easier to target to a specific DNA region because each TALEN domain recognizes one target nucleotide, as opposed to ZFN, where each domain recognizes a triplet of nucleotides, have been successfully used in horticultural crops. In potatoes, to change the functioning of the vacuolar invertase gene [83], in tomatoes, for targeted mutagenesis of the negative regulator of GA signaling gene PROCERA [84], and in cabbage, where the vernalization determinant allele of FRIGIDA was targeted [85]. However, the major drawback related to ZFNs and TALENs are their off-targeting effects, prolonged screening process, toxicity to the host cell, and complex genetic engineering procedures, limiting their applicability. The most widespread method of genome editing today is CRISPR technology; the first article on the successful application of this technology on plant cells was published in 2013 and the first edited plants were *Arabidopsis thaliana* and *Nicotiana benthamiana* [86]. Over the past ten years, a large number of examples of the use of CRISPR technology on various plant species, including horticultural species, have appeared; some of them, for example, tomatoes with a high GABA content [55], are already commercially available. This and other examples are presented in Table 1.

In natural conditions, CRISPR/Cas9 plays a part in bacterial immunity, providing bacteria with the ability to recognize and cut the nucleic acids of bacteriophages and plasmids [87,88]. Modified versions of the CRISPR/Cas9 editing tools used in the laboratory are typically a complex consisting of two components: the Cas9 endonuclease protein and a single guide RNA (sgRNA) with 20-nucleotide homology to the target DNA region [89–91]. The Cas9 endonuclease binds to the protospacer adjacent motif (PAM) DNA sequence (for Cas9, the PAM site is NGG) and the sgRNA complementarily binds to the DNA sequence adjacent to the PAM site, and if the binding is successful, Cas9 carries out a DSB in the target site [90,92]. DSBs caused by the Cas9 endonuclease lead to the activation of DNA repair systems, which can take two pathways: the error-prone nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). Errors in the DNA repair system result in deletions, insertions, or substitutions of DNA at DSB sites, which in turn disrupt gene function or cause a reading frameshift, known as a gene mutation or knockout [89–91]. As a result of DSB repair via the NHEJ pathway, insertions/deletions (indels) of several bases are usually observed during genome editing. The use of the mechanism of HDR, in turn, makes it possible, using editing systems, to replace individual nucleotides in the DNA sequence and even obtain a site-specific insertion of a gene or group of genes.

At the moment, editing technologies have become so widely developed that they make it possible to influence any stage of the implementation of genetic information in a cell—at the level of transcription, translation, post-translational changes, the epigenetic level, etc. [93]. Over the past 10 years, a number of different CRISPR-based tools have been developed, allowing editing at almost any desired location in the genome. Some examples include DNA base editors [94,95], epigenetic modifiers [96,97], prime editors [95,98,99], and transcription regulators [97,100]. The fusion of various additional molecules with partially disrupted (nickase Cas9, nCas9) or nuclease-deficient (dead Cas9, dCas9) Cas9 has been

used as a vehicle to deliver the CRISPR fusion protein to the target genomic site [97,101]. RNA-targeting Cas proteins also enable a variety of RNA manipulations beyond simple RNA editing, such as RNA degradation, detection of ribonucleic acids and pathogens, single RNA base editing, and live imaging of RNA, which can be read in more detail in recently published reviews [93,102].

3. Advantages and Disadvantages of Two New Molecular Technologies for Modifying Plant Genomes

The examples presented in Table 1 show that, in most cases, transgenesis is one of the mandatory stages of gene editing. All previously developed methods of transgenesis remain relevant today both as an independent method and as a technology that has made it possible to quickly adapt genome-editing technology for plants. However, when editing tools are delivered to a plant cell in the form of genes encoding Cas9 and gRNA that are stably integrated into the genome, the editing site and the insertion site are physically separated; this makes it possible to subsequently remove the transgenic construct, leaving only the changes made to the target site in the genome. In this section, we would like to highlight the differences in the features of both methods. Different mechanisms underlie gene insertion using agrobacterial transformation and when DSBs created by editing tools are used for the targeted insertion of genes. The mechanisms of T-DNA-induced mutagenesis and site-specific mutagenesis differ. Different strategies should be chosen to remove unwanted DNA sequences in the genome depending on the method used to introduce changes to the genome. This section will explore the differences between transgenesis and gene editing in more detail.

3.1. Features of the Integration of Foreign Genes into the Plant Genome

Both transgenesis and editing can be used to insert genes into the plant genome. Moreover, during transgenesis, integration into the genome occurs with the help of DNA polymerase theta in a random place according to the theta-mediated end-joining mechanism, which is one of the forms of microhomology-mediated end-joining (MMEJ). During integration, small mutations may occur at the junction of T-DNA and plant DNA due to the insertion mechanism, which result in integrated T-DNA molecules lacking sequences near the 3′ end (left border region) [8]. The transgene may enter an area that can subsequently be inactivated; accordingly, the transferred transgene may lose expression, and the phenotype will be lost accordingly. Moreover, transgene silencing can be caused by the insertion of multiple copies of T-DNA, and, especially, inverted repeats are strongly correlated with gene silencing of the T-DNA-encoded sequences [103–105]. T-DNA integration is rarely a clean event. Deletions, insertions, filler DNA sequences, and chromosomal translocations frequently accompany T-DNA integration [8]. During Agrobacterial transfer, plasmid DNA sequences lying outside the T-DNA, up to the entire vector sequence, can be stably integrated into the genome; most often this is due to the mechanism of T-strand formation in the Agrobacterium cell and the omission of one or both edge vector repeats [106,107]. In most cases, vector sequences are inserted into the genome adjacent to one or another T-DNA boundary and directly connected with it, so that they are relatively easy to detect. However, there are examples of the insertion of backbone vector sequences into a random location in the genome, regardless of T-DNA, and identifying such insertions is a separate task [108].

During transgenesis, several copies of transgenes can also be integrated into the genome, including in different orientations. This may be due to the mechanisms of DNA insertion during transgenesis [8], as well as with the method of its delivery [15,16], which, as numerous examples show, can lead to undesirable consequences associated with the inactivation of transgenes, as well as instability in the insertion area [104,109].

Genome editing allows you to insert the necessary genes into a given region of the genome. With site-specific insertion using editing tools, we can obtain a single-copy insertion in the appropriate region with stable expression. In this type of transgene integration, editing tools are used to create a DNA DSB. Once a DSB is made, cell repair systems start

their work; they heal the DNA break according to either HDR or one of the variants of NHEJ mechanisms. The integration occurs according to the NHEJ or HDR mechanism, depending on the presence of homology regions surrounding the inserted DNA. Both mechanisms can lead to mutations and rearrangements at the ends of the inserted DNA region [110]. Moreover, while in experiments on animal cells, there are examples of highly efficient integration through the NHEJ mechanism [111], in the case of plants, the efficiency of integration through this mechanism is usually not so high [112]. So, most of the research on the creation of transgenic plants carrying a site-specific insertion of the target gene relies on integration using the HDR mechanism [113–115]. In knock-in genomic editing, the gene cassette for integration into the selected target region is flanked by sequences homologous to the insertion region mostly 400–1000 bp long. The formation of concatamers and multiple insertions is prevented by the presence of flanking sequences. The ability to select an integration area allows the researcher to preliminarily characterize several potential areas and select the most optimal one for integration.

The development of site-specific insertion tools has led to the emergence of the term "genomic safe harbors". Genomic safe harbors are regions of the genome that can maintain transgene expression without disrupting the function of host cells [116,117]. This approach makes it possible to carry out knock-in genome editing without a loss or reduction in other yield indicators of the improved variety. Based on works with human cells [118,119], an approach has been developed to identify such genome regions and there are examples of its successful application in practice to change the genome of crop plants [120,121]. The optimized CRISPR/Cas9 editing method made it possible to obtain a targeted insertion of a 5.2 kb carotenoid biosynthesis cassette at two genomic safe harbors in rice. As a result, marker-free rice plants with high carotenoid content in the seeds and no detectable penalty in morphology or yield were obtained [120]. Similar work using a modified prime editing system in combination with recombinases made it possible to obtain precise large DNA insertions of up to 11.1 kilobases into the rice genome. A site-specific insertion region led to the development of blast resistance in these rice plants [121]. Thus, using the example of genomic safe harbors, we see a successful combination of the advantages of editing and transgenesis. However, the mechanism of agrobacterium transformation is more well studied. Generating transgenic plants using Agrobacterium transfer is a much faster, simpler, and cheaper method than inserting genes using editing tools. The ease of obtaining transgenic plants makes it possible to obtain a set of transformants in a limited time and select among them the most successful events of insertion of the target gene. Accordingly, the use of editing tools for site-specific transgenesis has not yet become a widely used method.

3.2. Ability to Cause Mutations in the Genome of a Modified Plant

The methods of molecular biology make it possible to purposefully modify certain genes of interest in order to improve some economically valuable traits of important agricultural crops. The development and improvement of these methods allow the identification of the mechanisms underlying the responses regulated by a large number of plant genes [102]. Transgenesis is still one of the main research methods in plant genetics. Mutations induced by the random integration of foreign genes (transgenes) during Agrobacterial transformation or bioballistics into the regions of the plant's genes are a convenient tool for the identification of those genes, whose normal functioning has been disrupted by this insertion [8]. In plants with mutant phenotype, a fragment of exogenous DNA acts as a marker that allows for the cloning of DNA regions adjacent to the insertion.

A wide range of mutations has been obtained by integrating exogenous DNA fragments into the *A. thaliana* genome using genetic transformations. The genomes of these mutants are subject to further investigation by the cloning of their large extended DNA regions adjacent to the insertion, followed by their sequencing to identify various disorders: translocations, inversions, deletions, and others [104,122–124]. In some cases, these insertions are accompanied by intra- and interchromosomal rearrangements, as well as by the inactivation of foreign DNA integration regions and changes in chromatin marks.

In the work of Jupe and colleagues, four T-DNA mutants from the SALK collection [125] were randomly selected and de novo sequenced using Oxford Nanopore Technologies MinION reads to very high contiguity [104]. This study showed the presence of long T-DNA insertions up to 236 kb, along with long-molecule evidence for genome structural rearrangements including chromosomal translocations and induced epigenomic variation. The phenotyping of the mutant line SALK_008491 created by T-DNA and known as *nhd1-1*, which should presumably have the knocked out gene of plastid Na+/H+ antiporter, showed that in a fluctuating light regime, its growth rate decreased, and this is accompanied by photosystem II (PSII) damage [124]. A more intense study of the nucleotide sequence in the insertion region of the SALK_008491 line permits authors to reveal a 14 kb deletion affecting five genes upstream of the *NHD1* locus on chromosome 3 (Chr3). In addition to *NHD1*, the stromal NAD-dependent D-3-phosphoglycerate dehydrogenase 3 (*PGDH3*) locus was knocked out. Thus, just the loss of two plastid proteins, PGDH3 and NHD1, increased the sensitivity of the studied line to dynamic light stress. Thus, despite the perspective of the application of the insertional mutations, which are able to lead to changes in phenotypic traits, this approach possesses some limitations associated with multiple chromatin rearrangements. This prompted a reconsideration of the relevance of using such mutations to identify the functions of genes of interest, since the manifestation of a mutation caused by the insertion of foreign DNA into the region of the gene under study and causing the loss of its function (knockout) can be masked by other insertions in regions remote from the region of the target gene. The development of new genome-editing tools using Cas nucleases makes it possible to specifically make changes only in the target gene and obtain a new series of knockouts for genes of interest.

Mutations arising from the simplest version of editing lead to minimal changes in the genome, since a deletion/insertion of just one nucleotide is sufficient to shift the reading frame. Moreover, in some cases, even replacing a single nucleotide is sufficient to change the function of a gene, without changing the length of the nucleotide sequence [94,95]. The combination of accumulated knowledge in the field of molecular biology and the ability to make site-specific changes makes it possible to specifically influence not only the protein-coding part of the gene, but also the regulatory regions—the promoter region of the gene, binding sites for transcription factors, and enhancer regions. A mutant form of Cas9 lacking the ability to introduce double-strand breaks can cause epigenetic changes in the genome without stimulating any changes in DNA. In the same way, nucleases that edit RNA do not cause mutations at the DNA level, but when editing a particular RNA at the phenotypic level, they produce the effect of mutation [93].

Editing, like transgenesis, has side effects; that is, in both cases, unplanned events can occur. With genome editing, a side effect is off-target editing; this is the introduction of mutations to off-target sites [126–128]. Off-target sites have a number of common characteristics—from one to four different nucleotides from the target site and differences in the PAM site. Accordingly, if you know the full genome sequence of the edited species, you can find and take into account off-target sites and then detect potential mutations. In addition, both in the case of transgenesis and editing, a number of methods have already been developed to reduce the likelihood of unwanted events [126–128].

3.3. Residual Genetic Engineering Tools in the Plant Genome and Their Preservation in Generations of Transgenic and Edited Plants

Due to the public's wary attitude towards GMOs, researchers are almost always faced with the task of creating plants that do not carry any additional nucleotide sequences other than those needed for the target function. In the case of transgenesis, we are talking about the removal of selective marker genes and their control elements. Today there are several ways to remove unnecessary genes and obtain marker-free transgenic plants. One strategy is to use a site-specific recombinase-mediated marker excision system, such as Cre/lox [129–131] or FLP/FRT [132,133]. Another method involves various manipulations with T-DNA—the use of two T-DNA, when the target gene and the marker gene are

not physically connected in the genome, which allows them to be separated in the next generation and obtain a marker-free plant [134]. The use of complex T-DNA containing several T-DNA repeats, which actually divide the T-DNA into parts when inserted, leads to the marker gene being inherited independently of the target gene [135]. A method that has gained great popularity recently is the creation of cisgenic or intragenic plants, that is, those that, although obtained by genetic engineering, carry only sequences taken from themselves or from closely related species [136,137].

For gene editing, the issue of removing unnecessary sequences is less acute [138]. First, even with stable insertion of a construct carrying editing tools, the insertion site and the editing site are separated in the genome, are inherited independently, and can be separated by segregation in subsequent generations. Secondly, for editing, you can use not only genetic constructs but also RNP (ribonucleoprotein) complexes—when editing tools are delivered to the plant cell in the form of ready-made endonuclease and gRNA tools, they carry out editing and then degrade; accordingly, no additional genetic information enters the cell [39,47,58]. In addition, to remove nuclease and gRNA genes, you can use the same methods that were developed for creating marker-free transgenic plants. To date, quite a lot of edited plants have already been obtained that do not carry any additional genetic sequences in their genome [112,120,139–141]. As can be seen from the examples presented in Table 1, in most cases, when using editing to introduce point mutations, researchers try to remove genes encoding editing tools from the genome and obtain non-transgenic plants.

4. T-DNA Mutagenesis and Editing in Solving Fundamental Problems of Gene Functioning

In just over 20 years since the publication of the first sequenced plant genome, about 800 plant species have already been sequenced [142]. A huge amount of data has been accumulated that needs understanding and verification. Gene knockout is considered to be a major component of the functional genomics toolbox and is aimed at revealing the function of genes discovered through large-scale sequencing programs. And it was the T-DNA sequence-indexed mutant collections containing insertional mutants for most *A. thaliana* genes that primarily played an important role in plant biology research. By providing a large source of mutant alleles for the in vivo characterization of gene function, this resource has been leveraged thousands of times to study a wide range of problems in plant biology. Mutagenesis has been a central tool for studying the genetics underlying biological traits, as phenotypic analysis of mutants provides a direct method to measure a gene's contributions to biochemical, cellular, tissue, and organismal properties. The insertional indexing of large populations of T-DNA-mutated lines has been used to achieve mutant allele coverage for the majority of Arabidopsis genes. These lines contain in total over 260,000 individual mutant lines and represent potential disruption mutants for most *A. thaliana* genes. To date, these mutants are presented by four broad collections of T-DNA insertions (SALK, SAIL, GABI-Kat, and WISC) [125,143–145].

Unfortunately, such extensive collections of mutant lines exist only for Arabidopsis, and the data obtained on Arabidopsis cannot always be directly transferred to other plant species. However, modern methods of site-specific mutagenesis make it possible to specifically obtain a mutation of a gene of interest without a long search for the desired mutant among an extensive collection. Compared to T-DNA mutagenesis, CRISPR/Cas-caused gene knockout has some advantages. One is the specificity of CRISPR/Cas-based gene editing; it can be targeted on an individual gene without other side effects. In the same way, overexpression or ectopic expression of a particular gene can be obtained by careful site-specific insertion of the target gene into a safe region of the genome (safe harbor) without additional rearrangements and mutations caused by the random insertion of a transgene [115,120]. Editing also allows, using point mutations or targeted replacement of a gene region, the restoration of the functioning of a silent gene or the change in its functioning without completely disrupting the function. Editing tools influence the gene functioning by changing the level or timing of its expression [93], which greatly facilitates

the study of such genes, causing mutations that are lethal to plants. In addition, multiplex editing, that is, editing several sites simultaneously, can facilitate the study of complex multigene traits [146–149].

More and more evidence shows that epigenetics plays an equally important role in controlling plant traits. It is epigenetics that can control whether traits encoded in genes appear. Thus, epigenetic study has become one of the hottest research topics in the last couple of years. There are many layers for epigenetics to regulate gene expression, which majorly contain DNA methylation, histone modification, and non-coding RNAs. Transgenesis technologies have made a great contribution to the study and better understanding of epigenetic effects and silencing mechanisms. Transgenesis has also been successfully used for more than thirty years to induce the silencing of certain genes [150,151]. Gene-editing tools also influence gene function at the epigenetic level by changing the methylation or acetylation status of DNA and histones [152,153].

Both small RNAs, particularly miRNAs, and lncRNAs play a major role in the regulation of gene expression at all stages of cell life [154]. Unlike protein-coding genes in which deletion and/or addition of several nucleotides will result in base frameshift and further cause gene silencing, CRISPR/Cas-caused deletion or addition do not change too much on the non-coding RNA and particularly do not strongly affect mature miRNA biogenesis, which is the functional part of miRNA genes [155]. However, in the last few years, significant progress can be noted in the field of RNA editing; software resources have been developed for selecting editing tools [156], and a separate class of editing tools has appeared (Cas13a, b, d), capable of changing the RNA sequence [157,158]. Thus, editing certainly has the potential to surpass the time-consumption and regulatory limitations associated with conventional breeding techniques.

5. Genome-Editing Methods for Improving Economically Valuable Plant Traits

Genome editing has a lot of applications, but its main advantage, as far as crop plants are concerned, is that with the help of gene editing, it is possible to obtain precise, targeted changes that do not affect the valuable traits of the variety and do not violate the genome architecture that has developed during breeding. In recent years, many review papers have been written on the use of gene-editing technologies to change economically valuable crop plant traits [1,93,159,160]. Therefore, in this section, we will try to emphasize the main directions of these works and those changes that were difficult to achieve using transgenesis.

Plant resistance to biotic stress caused by viruses, bacteria, nematodes, and fungi can be improved by the CRISPR/Cas9 tool. There are many examples of the effective improvement of resistance to bacterial and fungal diseases [159]. CRISPR/Cas technology has also been employed to create genome-editing plants with high resistance to viruses. Moreover, in this case, gene editing is used to change the plant's own genes, for example, genes encoding receptors that allow the virus to enter the plant cell [6]. Also, by modifying endogenous viruses, they lose the ability to infect as a result of editing the open reading frame, which prevents proper transcription or/and translation into functional viral proteins, as was performed on bananas [37], and directly as an immunity factor to cut the DNA of viruses entering the cell, as was performed on tomatoes [31]. The field of application of CRISPR/Cas9-mediated gene editing aimed at improving the abiotic stress tolerance of crops is developing very quickly [102]. Lots of structural and regulatory genes, including non-coding RNAs, are involved in plant response to different environmental stresses. All these genes can be the target for improving crop tolerance to abiotic stresses by using traditional transgenic technology and the currently advanced genome-editing tools. Some examples of the use of gene editing to improve the resistance of horticultural plants to pathogens and abiotic stress are presented in Table 1. However, since stress resistance is most often a multigenic trait, progress in this area has not been as rapid [6]. Editing tools are actively used to create herbicide-resistant plants. The most common target in this type

of work is the acetolactate synthase (*ALS*) gene. Point mutations in the *ALS* gene make plants resistant to sulfonylurea and imidazolinone [27,28,161].

Gene editing has been successfully used to improve the commercial and nutritional properties of horticulture plants and other crops and to increase their productivity (Table 1) [1,5,160,162]. There are examples of increased yields in cereal crops; in this case, the effect is on genes encoding traits including larger grain size, improved grain weight, and number per panicle [6]. As for improving commercial qualities, one of the main targets was the polyphenol oxidase (*PPO*) gene, which is responsible for browning. There are examples of *PPO* gene knockout in potatoes and eggplants [58,163]. Improving the nutritional qualities of plants is one of the most important tasks facing breeders. Gene-editing technologies are used to change almost any parameters—the content of proteins, fats, carbohydrates, macro- and micronutrients, the composition of fats and starches, etc. [1]. Gene-edited tomatoes with a high content of GABA and soybean oil with a reduced content of oleic acid have already entered the market; non-browning lettuce and mustard greens with reduced pungency are being prepared to enter the market [1].

To date, gene-editing technologies have been developed to circumvent the limitations associated with the lack of sexual reproduction or the lack of developed protocol for in vitro cultivation or regeneration for a particular elite variety. Editing tools are actively used to improve traditional breeding methods [93,161]. Using gene editing, it is possible to induce the induction of haploids, which allows you to quickly fix the mutated gene in the genotype and reduce the time otherwise spent on backcrossing. The induction of male sterility makes it easier to maintain hybrid varieties, and there are examples of such work in cereal crops [93,161]. Editing self-incompatibility genes is an extremely urgent task for breeders, and there are examples of successful work on potatoes and rapeseed [161]. In addition, the active development of gene-editing technologies has led to the emergence of methods for changing the genomes of elite cultivars and inbred lines used in commercial breeding that are recalcitrant to common transformation methods. Since these recalcitrant elite cultivars cannot be directly transformed with the relevant constructs, one proposed method calls upon crossing elite cultivars with other varieties that are more amenable to transformation with the constructs needed for CRISPR/Cas9-mediated genome editing, in sum, using one plant as a delivery vehicle. Another method developed for editing commercial wheat varieties is called iPB and its essence is the delivery of gold particles coated with CRISPR/Cas9 system into the shoot apical meristem of imbibed seeds [164].

In fact, CRISPR/Cas9 genome-editing technology has transformed all aspects of plant biology, including functional analysis, generation of mutant genes library, and crop improvement. The use of this modern technology to improve crops provides ample opportunities to confront existing challenges, like climate change and population growth, and to overcome the threat to food security in the future.

6. Conclusions

Transgenic plants have certainly opened a new era in agriculture and plant growing, but the prevailing public opinion and concern regarding GMOs have meant that the spread of this technology has slowed down significantly, and in some countries, it has even stopped. Transgenesis makes it possible to give plants properties that are difficult to achieve through editing; first of all, we are talking about the transfer of genes from other kingdoms, for example, the creation of plants resistant to pests by adding the Bt toxin gene to their genome. However, in many cases, editing can achieve the same result as transgenesis but with less impact on the genome of the plant cell and often at lower costs. In addition, completing all regulatory requirements today for edited plants takes less time and requires less cost than for transgenic plants [165]. Among the new methods of plant breeding, cisgenic, intragenic, and gene-edited plants are now coming to the fore, but, of course, such rapid development of plant genome-editing technologies would be impossible without the knowledge and technologies accumulated using transgenesis technologies.

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