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Review

Learning from transgenics: Advanced gene editing technologies should also bridge the gap with traditional genetic selection

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ABSTRACT

We highlight the importance of the mixed genetic approaches (classical and currents) to improve the social perception related to the GMOs acceptance. We pointed out that CRISPR/Cas9 events could carry DNA variability/rearrangements related to somaclonal variations or epigenetic changes that are independent from the editing *per se*. The transformation of single cells, followed by plant regeneration, is used to generate modified plants, transgenic or genome editing (CRISPR/Cas9). The incidence of undesirable somaclonal variations and/or epigenetic changes that might have occurred during *in vitro* multiplication and regeneration processes, must be carefully analyzed in replicates in field trials. One remarkable challenge is related to the time lapse that selects the modified elite genotypes, because these strategies may spend a variable amount of time before the results are commercialized, where in all the cases it should be take into account the genotype × environment interactions. Furthermore, this combination of techniques can create an encouraging bridge between the public opinion and the community of geneticists who are concerned with plant genetic improvement. In this context, either transgenesis or genomic editing strategies become complementary modern tools to facing the challenges of plant genetic improvement. Their applications will depend on case-by-case analysis, and when possible will necessary associate them to the schemes and bases of classic plant genetic improvement.

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Contents

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1. Introduction

Plants are continuously exposed to several stressors, both abiotic (heat, cold, drought, physical and chemical) [1] and biotic (bacteria, fungi, nematode, virus and insect). These stressors modulate the plant's response when facing changes in the environment [1]. In this sense, genetic improvement of plants has been a very old tool used by humanity that, in recent decades, has been influenced by the development of modern technologies based on the knowledge and manipulation of genomic DNA.

In this context, this scientific literature has introduced a new approach, the CRISPR/Cas9 system. It is a robust technology for targeting mutagenesis in plants. Targeted genome editing relies on the use of site-specific nucleases that create precise modification at specific DNA locations [2]. The correction of these changes occurs through the repair mechanisms of the host cell. Until now, three major programmable nucleases have been developed for genome editing. The first one is namely zinc finger nucleases (ZFNs) [3]. The second nuclease is called transcription activator-like effector nucleases (TALENs) [2,4,5] and the third is RNA-guided nucleases (RGNs) from the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein (Cas) system [6]. Among these, ZFNs and TALENs require two different artificial proteins, comprising a DNA binding protein domain and the C-terminal FokI nuclease domain for the targeted mutagenesis. It is successfully their use in plants. However, these methods require exclusive design and are time consuming. On the contrary, CRISPR/Cas9 has emerged as the most efficient tool chosen for genome modification, owing to its versatility, design flexibility and efficiency. In recent times, the literature describes several researches that satisfactorily use the CRISPR/Cas9 technology in many plant species [2].

However, to achieve the point mentioned above, an efficient genome editing using CRISPR/Cas9 system in plants is necessary for an effective delivery of Cas9/sgRNA complex into the target cells. The application of the CRISPR/Cas9 system also has some hurdles in its prospective use especially in crops with large, complex and polyploid genomes. However, unlike transgenic crops, which yield random insertions of target genes, genome-editing tools enable targeted gene insertion at a specified locus (knock-in), inactivation of desired genes from the genome (knockout), and genome modification (replacement). Moreover, genome edited crops differ from their transgenic counterparts because there is the possibility to realize the screening for genome editing in the T_1 generation thereby, selecting the modified crops that possess only the desired mutation and not the vector sequences. Due to this, genome-edited crops have escaped the regulations that suffer the transgenic crops. We are pointing out that the CRISPR/Cas9 system might be an efficient and sophisticated manner to support the principles of selection of elite individuals, at the same time that, modified elite genotypes should follow different selection steps (genotype × environment interactions) as a bridge with the traditional genetic improvement.

2. Delivery systems and host cell

The effective delivery of CRISPR/Cas cassette into plants is crucial for efficient genome modification; however, due to the presence of cell walls it becomes a challenge. The most applied methods for delivering vector(s) in plants include *Agrobacterium*-mediated delivery, *Agro*-infiltration, biolistic delivery, electroporation, virus-based delivery and PEG-mediated or direct delivery into protoplasts [7]. These methods vary according to plant species and the desired target. Although, all these methods have merits and demerits, yet having effective delivery systems is necessary to optimize the application of CRISPR/Cas9 for plant cell modifications. Improving the delivery systems will also help to increase the on-target efficiency by minimizing the off-target cleavage. Currently, most common applications of CRISPR/Cas9 in plants use the *Agrobacterium*-mediated transformation, which introduces T-DNA carrying both the Cas9 and sgRNA expression cassette directly into the plant genome by a type IV secretion mechanism [8]. As examples, the development of virus resistance in cucumber (*Cucumis sativus* L.) using Cas9/subgenomic RNA (sgRNA) was attained by co-cultivation of cotyledons with *Agrobacterium tumefaciens* EHA105 containing the CRISPR/Cas9 constructs (pRCS-35S:Cas9-AtU6:CECsgRNA1 or CECsgRNA2) [9]. In the same way, the CRISPR/Cas9 system was effective to generate watermelon plantlets with knockout mutations. In this case, cotyledons without embryo were co-cultivated with *A. tumefaciens* strain EHA105 that harbors the binary vector ClPDS, phytoene desaturase, to generate mutants with albino phenotype [10].

Remarkably in rice as in many other monocot plants, it is essential to highlight the use of calli as a starting material for *Agrobacterium* co-cultivation [11]. For example, the pC1300-Cas9 binary vector carrying eight sgRNAs was employed for genetic transformation of rice *via* *Agrobacterium*-mediation [12]. The method for generating transgenic rice (*cv*. Nipponbare) was according to previously described protocols, which use regenerable calli as explants for transformation [13]. Another example, Wang et al. [14] regenerated mutants of the elite indica rice line IR58025B (*Oryza sativa* var. Indica) using CRISPR/Cas9 technology. The transformation of rice calli of IR58025B occurred by *Agrobacterium* following the previously established protocols [15].

Moreover, it is necessary to mention that plant genomes used as receptors of CRISPR/Cas events can carry a pre-existing genetic variability, which is generally more frequent in the case of polyploids with more complex genome arrangements [16]. This fact must be evaluable to the technique choice of genome editing. Additionally, DNA rearrangements may occur in the host cells in response to environmental stresses, which are independent of the CRISPR/Cas modifications [17].

On the other hand, studies provide evidence that epigenetic changes can alter the pattern of inheritance of plant phenotypes [18]. Furthermore, there is a RNA-based system in plants involving small (s) RNAs that influence *de novo* establishment and maintenance of DNA methylation at many sites in plant genomes. A recent review reiterates the effects of stress and genome interactions in hybrid plants as well as systems involved in RNA-based mechanisms that can influence heritable phenotypes in plants [19].

2.1. Chloroplast transformation

Transplastomic plants show potential for metabolic engineering purposes because of the wide range of metabolic pathways located as well as the advantages in gene expression of plastids, in comparison with gene expression from the nucleus [20]. In plants originated from chloroplast transformation, theoretically it should only consider the genetic variability accumulated by the donor and the variability induced and/or evidenced by *in vitro* tissue culture. Nonetheless, plastid transformation is limited to a minor amount of plant species, being extremely difficult in monocotyledonous plants, [DOVV2] which groups several C4 species that demonstrate a high photosynthetic efficiency that reveals their commercial importance. Thus, developing efficient transformation protocols should be a crucial challenge in plastid biotechnology requiring efforts and long-term investments in both basic and applied research [21].

One obstacle to developing plastid transformation of commercial plants has been that their plant regeneration occurs from non-green embryonic cells (containing proplastids), rather than from leaf cells with functional chloroplasts [22]. Chloroplast transformation is potentially an ecologically viable alternative for plant genetic engineering. However, it is a constant that this method also involves the need for an efficient system of *in vitro* cell culture for the subsequent regeneration of whole plants. As examples, particle bombard, PEG treatment and microinjection have proven different levels of progress [23,24]; even though, searching for innovative methods of gene delivery into chloroplasts and the consequent plant regeneration events should be desirable before reaching further progress in this field.

An elegant and unique (to our knowledge) research comparing both chloroplast transformation and nuclear transformation has been published in tobacco as a model plant. In this case, Yeast trehalose phosphate synthase (TPS1) gene was introduced into the tobacco chloroplast (particle bombardment) or nuclear genomes (*A. tumefaciens* strain LBA4404) to study resultant phenotypes. The expression analysis has demonstrated that the chloroplast transformant expressed 169-fold more TPS1 transcript than the best surviving nuclear transgenic plant, showing 15–25-fold higher accumulation of trehalose. Additionally, nuclear transgenic plants that showed even small amounts of trehalose accumulation, displayed stunted phenotype, sterility and other pleiotropic effects, whereas chloroplast transgenic plants showed normal growth and no pleiotropic effects [25].

The production of biomolecules for industrial or pharmaceutical [26] uses in bioreactors is an estimated alternative to scale the transformation of chloroplasts to a commercial stage, which may avoid the regeneration of whole functional plants and therefore diminishing any probable effect of somaclonal and/or epigenetic variants [21]. Temporary immersion bioreactors have been established as an alternative for biomass production from transplastomic plants with severe mutant phenotypes that are hard to grow in *ex vitro* environments [27]. Bioreactors could represent a practical option for high-value products (*e.g.* phytopharmaceuticals) which additionally might avoid the excess of regulation of transgenic plants cultivated for human consume. In this way, the standardization of *in vitro* autotrophic or mixotrophic plant cultures should also increase the spectrum and quality of the biosynthesized phytomolecules [28].

Finally, the development of a tissue culture-free procedure for plastid transformation would make the transplastomic technology available to a spectrum of applications and considering only the accumulated genetic variability of the donor genotype [16]. As an example, manipulations of the tobacco plastid genome in greenhouse-grown plants, especially the post-transformation removal of marker genes by site-specific recombination have been reported using phage-derived recombinases targeted to plastids. Despite this, the result reveals that at least some secondary manipulations of the plastid DNA could be possible in plants, an accurate tissue culture-independent method for primary manipulation of the plastid genome remains a reserved goal to achieve [29].

3. Genome edited and genetic engineered crops

Although both genome editing and genetic engineering use plant transformation, they are strikingly different in several ways. Genome engineering/editing enables researchers for precise gene targeting while genetic engineering always leads to random insertions of transgenes. Using engineered nucleases, it is possible to stack multiple genes in a simpler and efficient manner. Unlike genetic engineering, precise base editing/substitution is possible using genome editing. With the use of ribonucleoproteins, it is possible to obtain genome edited free of foreign DNA that are identical to their un-transformed counterparts. However, this is impossible with traditional transgenic technology. Genome editing offers uniform gene expression throughout the plant genome and even in polyploidy plants with complex genomes whereas it is not feasible with genetic engineering. It is possible to obtain genome edited lines devoid of foreign gene sequences in subsequent generations by segregation, while transgenic approach generates plants containing foreign genes that are stably inherited, unless a marker free approach is utilized. Altogether, genome editing using CRISPR/Cas9 enables rapid, efficient and versatile gene targeting and outwits transgenic approach in both functional genomics and crop improvement research. Moreover, they may not be regulated as GM crops since no foreign gene is present in such genome edited events unlike genetically modified crops.

3.1. Regulating crops with edited genomes

Currently, the genome editing (GE) poses various advantages over transgenic crops; however, how these crops will be regulated worldwide is not yet clear. In general, two approaches to regulation exist: a) process based regulation (Ex. European Union, Brazil) that considers the techniques used to develop new crops and b) product based regulation (Ex. United States, Canada) that focuses mainly on the final product and the risks it poses. Since the genetic changes introduced by GE tools are precise, researchers advocate a product based regulation for the genome edited crops. Huang et al. have recommended five step preliminary principles for the regulation of genome edited crops which would benefit mankind, if adopted [30]. Stringent regulation will in turn affect the development costs and delay the commercialization of GE crops. Moreover, public acceptance and consumption of genome engineered crops also play a critical role. Thus, it is necessary for the scientific community to convey the benefits and significance of genome editing technologies to the public in a convincing manner to make them embrace this novel advancement of crop improvement. Owing to the potentials of this approach, regulatory authorities through the world are carrying out considerable progress in order to devise a sensible and pragmatic regulation for the genome edited crops. With limited agricultural land and fast expanding population, there arises an urgent necessity to warrant global food security for which genome editing systems would represent a very promising solution for the coming years.

3.2. Limitations of genome editing

Although genome editing using CRISPR/Cas9 has several advantages, there are certain limitations. Firstly, the off target effects pose a major concern. The CRISPR-Cas9 double stranded binding activity is modulated only by a single 20 nucleotide gRNA and is tolerant to some base pair mismatches between gRNA and the target DNA. Therefore, there is a chance that it could cut the genomic locations only partially complementary to the gRNA. However, recent reports prove that this can be overcome by careful selection of gRNA, using short gRNA and by direct employment of ribonucleoproteins [31,32].

Secondly, Kosicki et al. [33] reported that the alterations generated by CRISPR–Cas9 cannot only be limited to the vicinity of the target site but in distal off-target sequences as well. They found significant on-target mutagenesis, such as genomic rearrangements and large deletion in a human differentiated cell line, in mouse embryonic stem cells and mouse hematopoietic progenitors using the CRISPR/Cas9 tool, suggesting that its specificity can be lost in some cases.

Third, although the genome edited events are considered as non-transgenic, these events are modifications that initially occur in one host cell genome, which could carry DNA variability/rearrangements related to somaclonal variations or epigenetic changes that are independent of the editing *per se*. Since the beginning of the transgenesis development in plants, the team of Professor Francesco Sala in Italy was a pioneer in the prediction and studies of the presence of genomic changes in transgenic plants, demonstrating that these genomic modifications can occur and be verified independently from the genetic transformation event. This phenomenon has been evidenced in different hosts such as:

rice protoplasts [34]; transgenic rice obtained by calli electroporation and particle bombardment [35] or *Agrobacterium* infection [36]; poplar transformed with the *Bt* toxin gene [37]; and sugarcane plants transformed by electroporation or *Agrobacterium* infection of calli [38, 39], where in all cases, the results demonstrated their independence of the transgenic events. Occurrence of this pre-existing genetic variability must imply the evaluation of putative phenotypic changes in field conditions, *i.e.* in the *genotype* × *environment* interactions to verify the maintenance of the phenotypic traits of the donor genotype/variety [38]. Somaclonal variation is evidenced as qualitative and quantitative phenotypic mutation, sequence change, and gene activation and silencing. In this way, the DNA methylation patterns are highly variable among tissue-culture regenerated plants and their progeny, providing substantiation that DNA modifications are less stable in tissue culture than in seed-grown plants [40].

An illustrative example is seen in the case of the transgenic sugarcane transformed with the *cryIA(b)* gene from *Bacillus thuringiensis* which showed different degrees of resistance to borer (*Diatraea saccharalis* Fab.) attack in field trials [38]. A population of 42 genetically distinct transgenic sugarcane clones was compared with 21 control clones regenerated from dedifferentiated culture without transformation (C1-control) and with 41 plants that were clonally propagated in the field (C2-control). Results of the field trials evidenced both different expression of the resistance trait and, occurrence of limited but consistent morphological, physiological and phytopathological variation between clones. In parallel, DNA changes were verified and a total of 51 polymorphic DNA bands (out of the 1237 analyzed bands) were identified by extensive AFLP and RAMP analysis, thus showing rare, but consistent genomic changes in the transgenic plants, as compared with C1- and C2-control plants. Polymorphic bands found both in C1-control plants and in the transgenic ones can be interpreted as the sum of genomic variation preexisting in the original cultivar (C2-control) with that induced during the dedifferentiated cell culture stage. This may be the result of pre-existing genomic variation in plants taken for the original explants or of variation originated in cell culture, although epigenetic effects triggered by cell culture cannot be ruled out [38]. This experience with transgenic plants resistant to insects, offers to the scientific community a lesson that any technology for genetic modification should be considered only as a tool for genetic improvement. Field experiments must be essential to demonstrate and verify the occurrence of possible phenotypic changes (both dependent and independent to genetic modification) through the *genotype* × *environment* interaction in different years (Fig. 1).

This last aspect leads to an increase, often significant depending on the species, of the selection time for the elite modified individuals and their subsequent consolidation (recommendation) as new commercial varieties. Since the beginning of the genetic engineering development, at least in the case of plants, the technology, to which many biotechnologists continue to embrace, was compared with traditional genetic improvement promising a significant reduction in the time to obtain new modified genotypes, therefore greater efficiency in the selection of improved varieties. It is our intention with this article, that the scientists and promoters of the present technology of genomic modification be encouraged to the use of CRISPR/Cas9 system, even recognizing its effective advantages from the point of view of genomics and molecular biology. Additionally, they should distinguish the selection periods in the field, although they could be reduced, this potential time frame should not be shown as an advantage associated with the technology. This fact would avoid the possible rejection of public opinion and the community of geneticists who are concerned with non-traditional genetic improvement.

4. Epigenetic approach

The definition of the "epigenetics" term has changed slightly in the recent years. Today, the epigenetic concept states that it is an inheritable phenotypic alteration not caused by modification in the DNA sequence [41,42]. To date, several studies report the importance of epigenetic alterations in control of plant development and plasticity of phenotypes facing the environment [43]. The epigenomic approach is the better way to understand how environment and genotype produce the phenotypic features. It is known that epigenetic modifications are now recognized as an organism's response mechanism to environment alterations as well [44]. Currently, the researchers are concerned about improving crops without lacking genetic variability, as this is often seen among many technologies broadly employed in agriculture.

One the most desirable explanations is how epigenetic changes are heritable and how they stabilize in the genome. Understanding the molecular mechanisms of epigenetic inheritance has grown consistently in the last period, and it is now possible to profile the epigenome at high resolution. Epigenetic information plays a critical

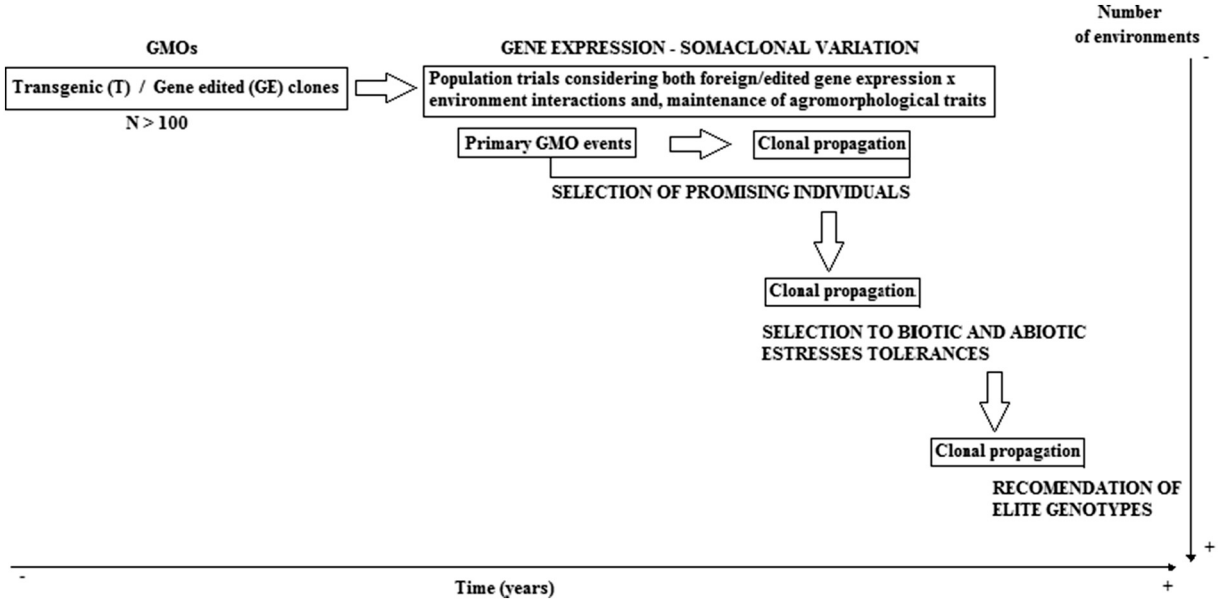

Fig. 1. General proposal of a selection scheme of elite clones of genetically modified plants considering the evaluation of the interaction genotype × environment (G × E) in parallel to the possible occurrence of somaclonal variation.

role in gene regulation, response to environment and natural variation of gene expression levels. Owing to these central roles, there is a high potential for epigenetics to play a role in crop improvement strategies including the selection of favorable epigenetic states, creation of novel epialleles and regulation of transgene expression [45].

Epigenetic variations are also involved in controlling plant development and they participate in shaping phenotypic plasticity in relation to the environment. Today, epigenetic diversity appears as a novel source of phenotypic variations to improve adaptation to adverse environments and ensure high crop yields. In this sense, the characterization of the stability and heritability of presumptive epigenetic variations is required for genetic improvement strategies [43].

Nowadays there are two flanks of study epigenetics: one, which is induced through the process of plant development and could be influenced by environmental conditions (mitotic inheritance; facultative heterochromatin) and, other that is obtained from transgenerational maternal memory (meiotic inheritance; constitutive heterochromatin). It is likely that these two forms of epigenetic memory will be distinct from one another [36]. In this context, it is expected that in the case of the cells and tissues that harbor the genetic modifications, through both transgenesis and genomic editing, the epigenetic changes that potentially occur should be explained mainly by the mitotic inheritance, not ruling out the possibility of epigenetic changes that could be caused by meiosis inheritance. In general, these genomic variations also include those known as pre-existing variability, which are more frequent in plants of high genetic complexity, such as the polyploids in their different classifications [46]. In this way, we support the premise that epigenetic regulation could influence the transgene (or genome editing modifications) behavior and might be used to develop novel epialleles to be used in breeding projects [45].

The alteration and mitotic memory of gene expression states are critical to plant development, where transcription factors are required for proper differentiation of plant cell types. However, the epigenetic memory related with both differentiation and gene expression could be often reached through chromatin modifications. These chromatin modifications can reset after every mitotic generation and there are papers showing evidence that the remodeling of chromatin contributes to plant development [47]. An explanation is that chromatin changes could control plant morphology and responses to the environment. The comprehension of these mechanisms may lead to a control over phenotypic traits, as well as, be particularly relevant for understanding the long time memory of environments originated in perennial species or in producing elite clones for plant species of asexual propagation [45].

4.1. Studies of epigenetic events in plants

In vitro cell cultures and plant regeneration are procedures that occur in an artificial environment, which are optimized in a *case by case* manner to achieve the highest efficiency and productivity of the plant's physiological processes. Adaptation to the *in vitro* environment is a challenge, or a stress, for plant cells and tissues that come from explants isolated from natural environments. Several studies affirm that environmental variables, such as temperature, light, hypoxia, drought, salt stress, and pathogen response demonstrate correlation to changes in epigenetic profile, chromatin modifications, or DNA methylation [48].

Changes in DNA methylation patterns and gene expression might be related with biotic stress [49] and environmental stress [50]. In this context, Lira-Medeiros et al. [51] reported changes in DNA methylation patterns in response to environmental conditions among mangrove (*Laguncularia racemosa*) individuals from two nearby habitats (at a riverside or near a salt marsh). These changes correlated with the morphological variation between individuals, while the differentiation of population structures among habitats was linked, mainly, to the epigenetic modifications.

In the case of salt stress, phylogenetic analysis using MSAP (methylation-sensitive amplified polymorphism) showed epigenetic variation within and between alfalfa (*Medicago* spp.) landraces under different saline conditions. Authors concluded that salinity amplified DNA methylation changes, particularly in plants exposed to the highest level of NaCl [52]. Similar results were found in date palm (*Phoenix dactylifera* L.) roots, which could be considered as a salt-tolerant plant species. The authors remark that the methylome and transcriptome relationships vary based on the methylated sequence context, the methylated region within the gene, the protein-coding ability of the gene and the salinity treatment [53].

In other related research, apomictic dandelion (*Taraxacum officinale* Weber ex F.H.Wigg., 1780) plants have undergone modification to different environmental stresses, while the apomictic offspring were maintained under an unstressed environment. These plants had their genomes analyzed by methylation-sensitive amplified fragment length polymorphism (MS-AFLPs) markers to determine genome-wide methylation changes generated by stress. Additionally, the same technique was employed to evaluate the heritability of DNA methylation changes. The results showed that the stresses, mainly chemical induction of herbivore and pathogen defenses, generated differences in the methylation patterns, which were transmitted to offspring. The authors conclude that stresses originated a number of epigenetic changes between treatment and controls, increasing thus, the epigenetic variation among plants within treatments [54].

In poplar, a model forestry plant, the hypothesis responses to an environmental stress, specifically drought, might be conditional to the environmental history was confirmed in three commercial *Populus* spp. hybrids from two different locations. Variation in genome-wide DNA methylation corresponds to the transcriptome level tendency. Whereby, the clones with the most variable transcriptomes and the longest time under field stress demonstrated the most marked differences in the degree of DNA methylation [55]. In a related research, single-base-resolution methylomes of *Populus trichocarpa* Torr. & A. Gray ex. Hook. displayed the relationship between DNA methylation and drought stress. The authors showed that methylation in 100 bp upstream of the transcriptional start site (TSS) repressed gene expression, while methylations in 100–2000 bp upstream of TSS and within the gene body were associated with gene expression [56]. As another example, the Methylation Sensitive Amplification Polymorphism (MSAP) technique was used to evaluate the patterns and temporal changes of DNA methylation in leaves of AL35 poplar plants, pre-inoculated or not with either *Glomus mosseae* or *Glomus intraradices*, grown on unpolluted soil or on a Cu- and Zn-polluted one. Results showed extensive alterations (hypomethylation) occurred after 6 months in mycorrhizal plants grown in the presence of heavy metals. Seven MSAP fragments were analyzed for their transcript levels by means of qRT-PCR. Gene expression varied in treated samples relative to controls in response to heavy metals and/or mycorrhiza inoculation; in particular, the gene transcripts involved in RNA processing, cell wall and amino acid metabolism were upregulated in the presence of mycorrhiza with or without heavy metals [57].

Nevertheless, one of the first evidences for transgenerational responses related to environmental stress have been provided from studies of tissue-culture induced variation [40]. Epigenetic mechanisms play a role in somaclonal variation that include cytological abnormalities, qualitative and quantitative phenotypic mutation, sequence changes, gene activation and silencing, activation of transposable elements and retrotransposons, as well as a high change frequency of DNA methylation pattern. DNA methylation patterns have been demonstrated as highly variable among tissue-culture regenerated plants and their progenies, which provides evidence that DNA modifications become less stable in tissue culture than in seed-derived plants [30]. This issue should be a significant influence on the selection of promising transgenic plants.

Epigenetic variants could show a steady throughput for more than hundreds of years, though, some reports demonstrate that it might revert irregularly [58]. In this sense, the epigenetic approach could

permit the selection (natural or artificial) of potential promising phenotypes in order to increase the frequency of one epigenetic variant, but considering that those variants do not necessarily contribute to trait fixation [59]. In general, it is significant to consider the putative role of epigenetic changes and somaclonal variation contributing to select promising phenotypic traits, even using the modern genome manipulation techniques.

Still today, it is critical to understand the stability of epigenetic variation [45] to establish whether this variation can potentially be considered in plant genetic improvement programs. We encourage biotechnologists and researchers to consider this source of phenotypic variability when selecting the elite clones originated from both genome editing and transgene insertion events.

As plant transformation is used to generate genome-edited plants, the non-occurrence of undesirable somaclonal variations and/or epigenetic changes that might have occurred during *in vitro* regeneration, should be carefully analyzed in field trials replicated in time and space, an issue that could constitute an essential bridge with the traditional plant improvement.

4.2. Improving crops using the epigenetic inheritance

In accordance with Gallusci et al. [43], as well as other studies, desirable agronomic features can be obtained through the epigenetic variations in crops. One alternative to achieve this goal is carrying out experiments with model plants. Currently, many technologies are employed to investigate the role of epigenetic alterations in crop improvement, as already explored in this review: CRISPR/Cas9, zinc finger nucleases (ZFNs), transcription activator like-nucleases (TALENs), epigenetic recombinant inbred lines (epiRILs) and RNA-directed DNA methylation with virus-derived vector. The strategy mentioned does not need plant transformation to generate the target methylation. However, the pattern of epigenetic inheritance must be studied more accurately.

One alternative to understand how the epigenetic alterations are transmitted to the successive generations, is utilizing the global methylation of genome (non-targeted epigenetic methylation) employing drugs that inhibit the production of the DNA methylases. This technique can produce hypomethylated genomes. Akimoto et al. [60] evaluated the effects of this approach in rice plants and could follow the transmission of certain characteristics acquired in this study for nine generations, proving that epigenetic changes can be inheritable. Another important approach to observe the inheritance of epigenetic changes is submitting the crop to stressed environments (*in vitro*) and monitor the alterations acquired in control crop and stressed crop [43].

Today, with methylome and transcriptome databases available in large scale, it is also possible to evaluate, through statistical modeling (bioinformatics approach), some inheritance patterns of the epigenetic changes in plants. Additionally, it is equally possible to predict the linkage of desirable phenotypes to these epigenetic alterations, aiming thus in the crops' improvement. For example, in *Arabidopsis thaliana*, using DNA methylation information to perform a statistical model, Hu et al. [61] predicted that approximately 65% of the variance in plant height is linked to the methylation patterns. These results also suggest that methylation information can play a crucial role in complex traits, during the moment that the epigenetic alterations are under study.

As explained, several techniques associated among them (genome editing, genetic engineering, traditional tools of genetic improvement and bioinformatic approaches) can provide a good scheme for crop improvement to be utilized in the agriculture field. The use of combined approaches can increase the quality of the study and reduce the elaboration time of the obtained data. This possibility of associate techniques to improve crops must be carefully evaluated by researchers of the area, taking into account case by case and the complexity of the studied organisms.

5. Conclusion and future perspectives

The modern genome editing technology should have an exponential growth in its applications that aim to identify the role of genes in which their functions are not yet recognized. Similar behavior should occur regarding the number of plant species that are modified by this technology. This increase could exceed, in many cases, the applications of genetic engineering by transgenesis, techniques that should not be exclusive and should be applied following case-by-case studies. Nevertheless, these technologies should be employed by biotechnologists and geneticists as tools for traditional breeding.

Since cell transformation and plant regeneration are used to generate genome edited (CRISPR/Cas9) plants, the non-occurrence of undesirable somaclonal variations and/or epigenetic changes that might have occurred during *in vitro* multiplication and regeneration processes should be carefully analyzed in replicates during field trials (time and space). We consider it as a lesson from the beginning of the transgenesis era, the fact of having minimized the indispensable requirement of field experiments and their potential selection stages of elite individuals and stable varieties. The transgenesis also allows finding out not only the expression levels of the recombinant gene, but also their correspondence with other phenotypic characters, many of these are of economic importance, with respect to the genomic background of the donor. The recognition, at this stage, by biotechnologists will undoubtedly establish a bridge with the traditional genetic improvement from these initial stages of verification of the expected efficiency in field trials of the genome editing crops. This requirement does not necessarily have to be indispensable for those cases in which the organism modified by CRISPR/Cas9 is used only at scales *in vitro*, or in controlled environments, for example in bioreactors to produce specific proteins or metabolites.

Every new technological tool to be recognized by society must explicitly propose advantages and/or novelties with respect to its precedents. However, it is also mandatory to consider and anticipate, if not all, the greatest possible number of disadvantages and risks. The precise and anticipated balance between the advantages and expectations *versus* the scientific analysis of the possible risks and disadvantages will ensure that genome editing technology does not cross the uncertainties, as well happed with transgenic plants, and that the application of this technology for crop improvement can be accepted worldwide.

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Conflict of interest

The authors declare that they have no conflict of interest.

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