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Frontiers in global regulatory landscape of CRISPR-edited plants

Edited by
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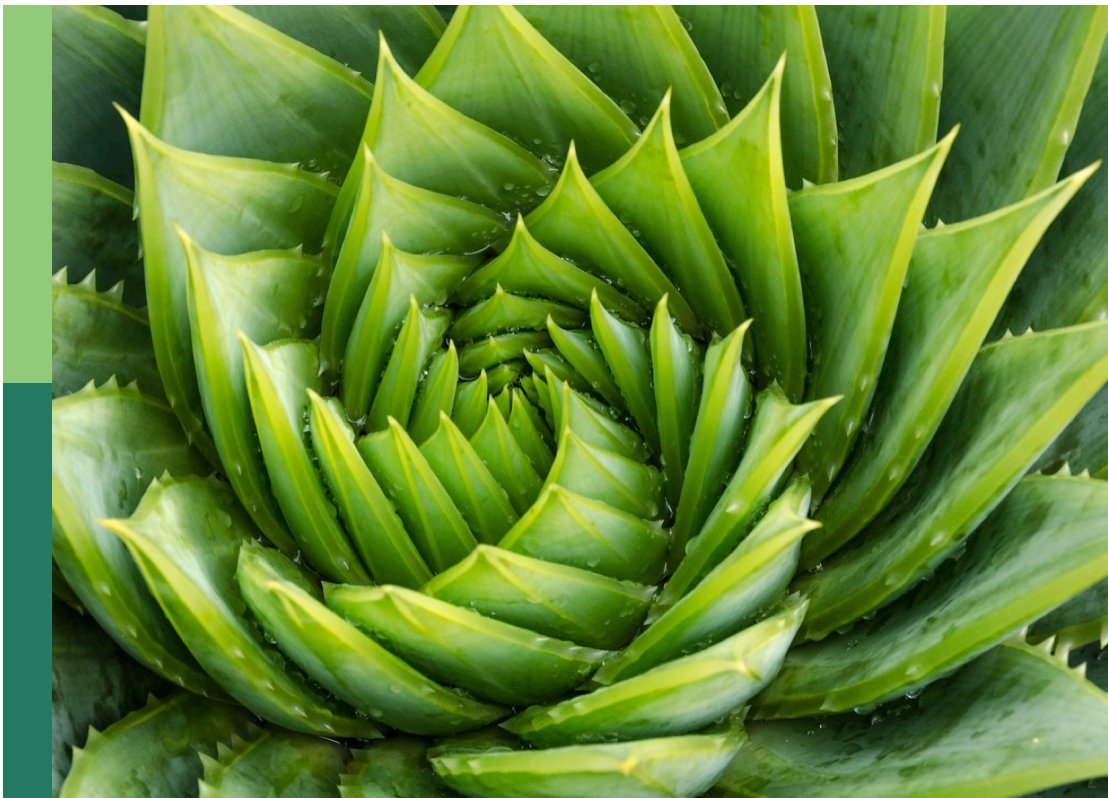

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Frontiers in global regulatory landscape of CRISPR-edited plants

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Editorial: Frontiers in global regulatory landscape of CRISPR-edited plants

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Editorial on the Research Topic
Frontiers in global regulatory landscape of CRISPR-edited plants

Emerging applications of CRISPR-Cas systems in plants

CRISPR systems have evolved rapidly for precise gene manipulation in diverse species, including bacteria, plants, animals, and humans. Ashraf et al. describe applications of CRISPR-Cas12a for improving resistance against cotton leaf curl virus diseases. The article demonstrates the successful editing of different genes of the virus using a multiplex CRISPR-Cas12 system to improve resistance against this virus disease. The findings could contribute to developing gene editing strategies for controlling plant virus diseases. Salvagnin et al. provide proof-of-concept gene editing in chicory using different delivery methods of CRISPR reagents. This study highlights the editing efficiency and off-target effects of different delivery methods. Pan et al. demonstrate the establishment of an efficient CRISPR-Cas system for gene editing in lettuce using intron-mediated enhancement (IME)-assisted 35S promoter to express Cas9 and gRNA in a single transcript. IME moderately enhanced expression of both Cas9 and gRNA and thus improved the efficiency of gene editing in lettuce. In addition, the developmental regulator gene GRF5 was also co-expressed with Cas9 and gRNA, which resulted in enhanced editing efficiency. The article demonstrates that both approaches resulted in high editing efficiency in lettuce and can be used to characterize genes in lettuce and other crops. Huffel et al. provide two

computational frameworks for an efficient and well-informed design of multiplex gene editing experiments in plants. Both approaches consider different design parameters, such as the number of genes, the number of gRNAs per target site per gene, and resulting editing efficiencies.

RNPs as CRISPR reagents: an effective strategy to generate transgene free CRISPR crops

Transgene-free CRISPR-edited crops are likely to be more acceptable from a regulatory and public perception standpoint. Kong et al. describe a more efficient positive screening method applicable to diverse plant species, denoted as the PARS (PAR-1 based) strategy for identification of transgene-free CRISPR edited plants using paraquat resistance (PAR-1). The authors demonstrate successful identification of transgene-free edited plants at target loci in the T1 generation. The authors suggest that the PAR-1 strategy could be used to identify transgene-free CRISPR-edited plants in many plant species. Salvagnin et al. compare three different delivery methods - agrobacterium-mediated transformation, transient transfection using plasmids, and RNPs for CRISPR-based gene editing in chicory (*Cichorium intybus* L.). The authors evaluate editing efficiency, off-targets and socioeconomic impact of these methods by editing the germacrene A synthase gene in chicory. The article demonstrates a high editing efficiency of genes with all these delivery methods. Although transient transfection is more convenient due to its simplicity, cost-effectiveness, fewer off-targets and more regenerants, the plasmid-based transient approach led to more plants with permanent integration of plasmid copies. In contrast, RNP transfection of protoplast does not introduce foreign DNA into the plant cell: the authors suggested that RNPs would improve future regulatory and consumer acceptance. Poddar et al. demonstrate applications of RNP-based CRISPR-Cas for editing disease susceptibility genes in wheat. The article demonstrates rapid screening of effective gRNAs using RNPs in wheat protoplast for gene editing in regenerable wheat immature embryos without using a selection marker. In addition, high temperature treatment enhanced gene editing both in protoplasts and immature embryos. The authors highlight the importance of their method for DNA-free gene editing in other crops.

Regulating CRISPR edited crops

The review of Mendelsohn et al. provides a perspective on regulatory oversight of plant-incorporated pesticides (PIPs) produced using conventional or biotechnological tools. The paper notes that PIPs made using conventional methods are not regulated and that the EPA recently revised their regulations to exempt PIPs developed by genome editing if they otherwise could be generated through conventional methods. Hoffman describes how the USDA revised regulations of gene-edited crops can contribute to achieve sustainable agriculture goals and address challenges associated with climate change. The paper identifies many examples of CRISPR-edited crops that may potentially contribute to these objectives, but most have not been commercialized under the legacy regulations and existing regulatory barriers. In 2020, the USDA revised its regulatory framework for gene-edited crops to make it more science-based, risk-proportionate, and streamlined. The author summarizes how each example might fare under the revised biotechnology regulations, expecting that many more innovations that could contribute to sustainable agriculture goals will be realized under the revised regulations. Ahmad et al. explore the ongoing debate on the regulatory and legal status of CRISPR-edited crops in different countries. The authors highlight the differences in the legal status of CRISPR-edited crops as GMOs or non-GMOs in different countries - the outcomes in the USA and EU will significantly impact future developments of CRISPR-Cas, public perception, and commercialization of edited crops. The authors suggest that the current division as GMOs or non-GMOs of CRISPR crops will persist until a universal, transparent, science-based and scalable regulatory system has been agreed. San-Epifanio et al. review the possible regulatory landscape for the approval of CRISPR edited crops in the EU. The paper highlights that if a proposed regulatory framework for gene-edited crops is approved in 2024, CRISPR-edited crops in the EU will be approved as either crops whose genome has been modified by mutagenesis, cis-genesis, intra-genesis or transgenesis, where regulation would vary depending on the category. However, if the proposed legislation is not approved, CRISPR-edited crops will be considered as GM crops. The authors suggest that although the legislation initiated in 2021 is not sufficient for CRISPR-edited crops, the current framework offers short-term benefits compared to its alternatives. Consequently, the member states should strive for substantial improvements in the legislation for CRISPR-editing and plant breeding in the EU. In an opinion, Forbes et al. explore the possibility of approving genetically modified late blight-resistant potato for commercial cultivation in Norway by emphasizing sustainable considerations during regulation of CRISPR-edited crops in the EU. The authors emphasize that approval would signify a major development in Norway's stance on GM crops with potential implications on food production and sustainable agriculture. The authors suggest that the EU could adopt a similar approach to address the challenges of sustainable agriculture. This article explores regulatory and public perception challenges of introducing GMOs in Norway. Tachikawa and Matsuo demonstrate that regulatory oversight of gene-edited organisms and products is not yet aligned. The international regulatory landscape is a mosaic, posing challenges for harmonization. The paper demonstrates two regulatory frameworks for gene-edited crops: i) a process-based system which considers gene-edited

crops as GMOs, but with simplified regulation, ii) a product-based system, which considers gene-edited crops as non-GMOs but needs confirmation. The article explores the reason behind the tendency of convergence between these two approaches to regulate CRISPR-edited crops, and examines the challenges and implications of these approaches in governance of the agriculture and food sector in the context of gene-editing.

Author contributions

AA: Writing – original draft. NH: Writing – original draft, Writing – review & editing. MJ: Writing – original draft, Writing – review & editing. BZ: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Covering the Combinatorial Design Space of Multiplex CRISPR/Cas Experiments in Plants

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Over the past years, CRISPR/Cas-mediated genome editing has revolutionized plant genetic studies and crop breeding. Specifically, due to its ability to simultaneously target multiple genes, the multiplex CRISPR/Cas system has emerged as a powerful technology for functional analysis of genetic pathways. As such, it holds great potential for application in plant systems to discover genetic interactions and to improve polygenic agronomic traits in crop breeding. However, optimal experimental design regarding *coverage* of the *combinatorial design space* in multiplex CRISPR/Cas screens remains largely unexplored. To contribute to well-informed experimental design of such screens in plants, we first establish a representation of the design space at different stages of a multiplex CRISPR/Cas experiment. We provide two independent computational approaches yielding insights into the *plant library size* guaranteeing full coverage of all relevant multiplex combinations of gene knockouts in a specific multiplex CRISPR/Cas screen. These frameworks take into account several design parameters (e.g., the number of target genes, the number of gRNAs designed per gene, and the number of elements in the combinatorial array) and efficiencies at subsequent stages of a multiplex CRISPR/Cas experiment (e.g., the distribution of gRNA/Cas delivery, gRNA-specific mutation efficiency, and knockout efficiency). With this work, we intend to raise awareness about the limitations regarding the number of target genes and order of genetic interaction that can be realistically analyzed in multiplex CRISPR/Cas experiments with a given number of plants. Finally, we establish guidelines for designing multiplex CRISPR/Cas experiments with an optimal coverage of the combinatorial design space at minimal plant library size.

Keywords: multiplex CRISPR/Cas screens, combinatorial gene knockout libraries, experimental design, plant genetic studies, crop breeding

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1. INTRODUCTION

Genetic mutagenesis is a widespread and powerful strategy for the functional characterization of genes in various biological processes. It provides a complementary approach to the mapping of genotype-phenotype relationships based on quantitative genetic analyses [such as genome-wide association studies (Brachi et al., 2011) and quantitative trait locus mapping (Mauricio, 2001)], gene regulatory network analyses through differential expression (Clifton et al., 2006), and gene expression perturbation through RNA interference (Travella et al., 2006).

Further, genetic mutagenesis may be used to test hypotheses of gene functional redundancy based on phylogenetic analyses of gene families (Zhang et al., 2018). Within this context, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system has paved the way for targeted genome editing in many different organisms, including plant species (Brooks et al., 2014; Zhang et al., 2014, 2016; Ma et al., 2016; Fellmann et al., 2017; De Bruyn et al., 2020; Liu Z. et al., 2020). The CRISPR/Cas system relies on the delivery of a specific guide RNA (gRNA) and the Cas nuclease into a target cell. After formation of the gRNA/Cas complex, the gRNA directs Cas to induce DNA cleavage in the genomic sequence homologous to the gRNA. In case the target sequence is located inside a conserved region, e.g., in highly homologous paralogous genes, multiple sites in the genome can be cleaved in parallel. Subsequently, each cleaved site can undergo error-prone DNA repair *via* the non-homologous end-joining pathway, creating a mutation. Mutations in the coding region of a gene might disrupt the open reading frame or one of the mRNA splice sites, leading to the formation of a mutated, truncated, or out-of-frame protein sequence that, in turn, can result in the knockout (KO) of gene function (Mali et al., 2013). If the gRNA was designed to target regulatory sequences of the gene, gene expression levels may be disrupted. Ultimately, examining the impact of these genetic perturbations on the plant phenotype can contribute to unraveling gene function and genetically improving agronomic traits in breeding materials.

The multiplex CRISPR/Cas system forms a relatively novel extension to the standard CRISPR/Cas system (Shen J. P. et al., 2017; Zhou et al., 2020), allowing for the simultaneous editing of multiple unique targets in a single plant cell (Zhang et al., 2016). The latter enables to identify genetic redundancy as well as genetic interactions, which contributes to elucidating the complex interplay of genes in metabolic and/or regulatory pathways. Thus, multiplex CRISPR/Cas genome editing speeds up the functional analysis of genetic pathways thanks to its ability to specifically target multiple genes simultaneously. To accomplish simultaneous editing activity, multiple gRNA/Cas complexes are co-expressed in each target cell. A first strategy to deliver multiple gRNA sequences per cell is to assemble multiplex gRNA/Cas constructs by cloning methods such as Golden Gate ligation (Engler et al., 2008; Ma et al., 2015) and Gibson Assembly (Jacobs et al., 2017), yielding binary vectors with arrays of gRNA expression cassettes for stable *Agrobacterium* transformation. Second, multiple vectors transiently expressing Cas and/or one or more gRNAs can be co-transfected into protoplasts *via* electroporation or polyethylene glycol-mediated transfection, followed by whole plant regeneration (Toda et al., 2019). Third, preassembled gRNA/Cas ribonucleoprotein complexes with mixtures of gRNAs can be delivered into plant cells *via* particle bombardment (Liang et al., 2018), polyethylene glycol-mediated transfection, or nanoparticles (Cunningham et al., 2018).

Each of the aforementioned gRNA delivery methods can be designed to introduce a specific number of gRNAs per target cell, hence enabling the study of a particular order of interaction among a set of target genes in a multiplex CRISPR/Cas system. For simplicity, we focus on one prototypical delivery method, namely the stable transformation of multiplex gRNA/Cas constructs containing an array of gRNA expression cassettes into target cells. This approach starts with the design of a pool of gRNA sequences targeting a set of target genes (**Figure 1A**), after which these gRNA sequences are randomly assembled into combinatorial gRNA/Cas constructs with a specific number of gRNA sequences per vector (**Figure 1B**). For instance, to study pairwise interactions of genes, combinatorial gRNA/Cas constructs with two gRNAs per vector can be produced, such that, after transformation, two gRNA/Cas complexes are generated per plant cell. Likewise, for investigating up to k-order genetic interactions, combinatorial gRNA/Cas constructs are designed so that k gRNA/Cas complexes are expressed in each target cell. Throughout this paper, the term *construct library* will refer to the collection of all combinatorial gRNA/Cas constructs that can be generated from the initial gRNA pool by randomly sampling k gRNAs (with replacement) into an expression array. After transformation of the combinatorial gRNA/Cas construct library into the target cells (one construct per independent cell), the corresponding combinatorial gRNA/Cas activity results in a collection of cells containing different combinations of gene knockouts. Following plant regeneration of a random selection of these mutated cells, a genetically diverse collection of mutated plants is obtained, which is referred to as the *plant library* (**Figure 1C**). All genotypes that can theoretically be present in the *plant library*, i.e., all possible combinations of k (or fewer) target gene knockouts, constitute the *plant design space*. We gradually build up and visualize this design space in Section 2.1. By subjecting the *plant library* to a phenotypic screen (e.g., one that examines variations in traits such as flowering time, leaf density, internode length, number of root nodules, maturity time, metabolic profile, plant height or drought tolerance), one can assess the effect of many combinations of gene knockouts on the plant phenotype in parallel. In this manner, a deeper understanding of the genetic interactions in a specific metabolic or regulatory pathway can be acquired. Recent studies illustrate how insights gained from these assays have contributed to improving several agronomic traits in crop breeding (Li et al., 2018; Zhang Y. et al., 2020).

As a multiplex CRISPR/Cas experiment is designed to examine a larger number of target genes (x) and order of genetic interaction (k), the number of possible k-combinations of gene knockouts that occur in the *plant design space*, denoted by $\binom{x}{k}$, expands quickly. Accordingly, there is a combinatorial explosion of the number of plants that need to be screened in order to study all k-order genetic interactions. The (combinatorial) *coverage* ($\gamma_{x,k}$) of a plant library is defined as the fraction of all $\binom{x}{k}$ gene knockout combinations that is contained at least once in this plant library. The plant library size required for full coverage refers to the minimal number of plants that needs to be included into a plant library to completely cover all $\binom{x}{k}$ gene knockout combinations in a multiplex CRISPR/Cas experiment (reaching $\gamma_{x,k} = 1$) and is denoted by $N_{x,k}$. Note that in our model representation the plant library is obtained by random selection and regeneration of mutated cells. Due to the stochastic nature of this sampling process, $N_{x,k}$ is a stochastic variable as

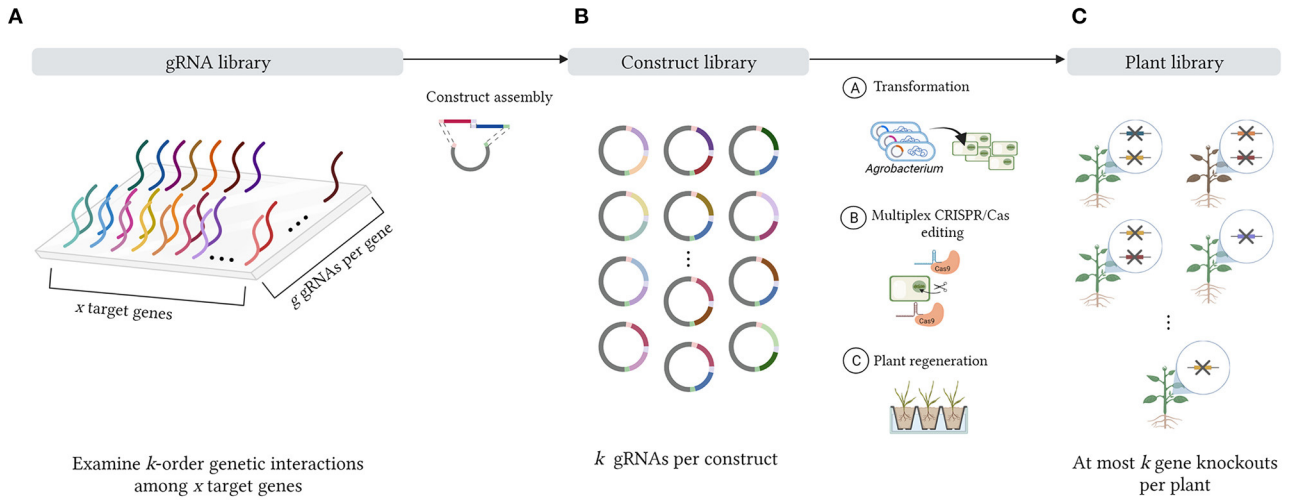

FIGURE 1 | Schematic representation of a multiplex CRISPR/Cas experiment. **(A)** First, x target genes are selected for CRISPR/Cas editing. A number of g gRNAs are designed for each target gene, forming an initial gRNA library. **(B)** During vector assembly, gRNA sequences from the gRNA pool are randomly combined into a combinatorial gRNA/Cas construct library with k gRNA sequences per vector to study the effects of k-combinations of gene knockouts, i.e., k-order genetic interactions among the set of x target genes. After transformation of the combinatorial gRNA/Cas constructs into the target cells, the gRNA/Cas complexes can be expressed, leading to genome editing of the associated target loci. **(C)** After plant regeneration, a diverse mutant plant collection is obtained with at most k gene knockouts per plant, forming the plant library.

well, its value varying with every execution of a specific multiplex CRISPR/Cas experiment. Quantification of the expected value and the standard deviation of $N_{x,k}$ for a given experiment is a main objective of this study. The central parameters and variables in our study are summarized in **Table 1**.

Gaining insight in $N_{x,k}$ for a multiplex CRISPR/Cas experiment is vital. Consider performing such an experiment to investigate all k-combinations of gene knockouts for a set of x target genes. Assume that the final plant library size N is too small to contain all $\binom{x}{k}$ gene knockout combinations ($N < N_{x,k}$), hence not reaching full coverage ($\gamma_{x,k} < 1$). Performing a phenotypic screen on this plant library might give rise to misleading conclusions in two ways. First, effective combinations of gene knockouts might be misclassified as not associated with the desired phenotype as a result of not being represented in the plant library, leading to false-negative results. Second, one cannot evaluate whether the absence of a particular combination of gene knockouts in the plant library is due to lethal effects or rather the result of an inadequate plant library size. Therefore, a main consideration when determining the size N of the plant library resulting from a multiplex CRISPR/Cas experiment is to account for coverage of all $\binom{x}{k}$ gene knockout combinations. However, optimal design of such experiments in plants has remained largely unexplored in this regard. Existing tools that assist in determining an appropriate sample size for multiplex CRISPR/Cas screens in mammalian cells are not applicable to screens in plants due to different experimental protocols. Additionally, focus on the exploration of the complete combinatorial design space is lacking in these studies (Nagy and Kampmann, 2017; Shen J. P. et al., 2017; Imkeller et al., 2020; Diehl et al., 2021).

In this study, first and foremost, we suggest two independent approaches for determining $N_{x,k}$ for multiplex CRISPR/Cas experiments in plants. The first approach makes use of computational simulations, reproducing subsequent stages of a multiplex CRISPR/Cas experiment *in silico*, to gain insight into $N_{x,k}$. The second approach employs the BioCCP framework, which was presented in our previous work (Van Huffel et al., 2022), to provide a quick approximation of $N_{x,k}$ and related statistics. BioCCP is a general framework based on the Coupon Collector Problem (CCP) studied in probability theory and statistics. The CCP allows one to determine minimal sample sizes for screening experiments in combinatorial biotechnology that guarantee full coverage of the design space. Apart from establishing the computation of $N_{x,k}$ using these frameworks, we illustrate how $N_{x,k}$ is impacted by some critical design parameters of a multiplex CRISPR/Cas experiment (e.g., the number of target genes, the number of elements in the combinatorial gRNA array, the relative abundances of gRNAs in the combinatorial gRNA/Cas construct library, the guide-specific genome editing efficiency and the global knockout efficiency). By means of a quantitative analysis, we demonstrate that a naive approach for experimental design might become prohibitively expensive, and that the maximal number of plants that can be genotyped and phenotyped in a multiplex CRISPR/Cas screen imposes limitations on the number of target genes and order of genetic interaction that can be investigated. Finally, we propose two main strategies (named the *Split–Select–Combine* strategy and the *Overshoot–Select–Purify* strategy) for designing multiplex CRISPR/Cas experiments with a minimal $N_{x,k}$, and establish additional guidelines for experimental design to optimize design space coverage at minimal plant library size.

TABLE 1 | Central design parameters and stochastic variables of this study.

Symbol	Short description
Design parameters	
x	The number of target genes in a multiplex CRISPR/Cas experiment
k	The order of genetic interaction among the target genes to be investigated
N	The number of plants in a plant library, also called the *plant library size*
$\binom{x}{k}$	The number of possible k-combinations of gene knockouts for x target genes
Stochastic variables	
$\gamma_{x,k}$	The fraction of all $\binom{x}{k}$ gene knockout combinations that is contained by a plant library, also called the *(combinatorial) coverage* of a plant library
$N_{x,k}$	The minimal plant library size required for full coverage ($\gamma_{x,k} = 1$)

2. RESULTS

2.1. Representation of the Design Space and Stochastic Sampling in a Multiplex CRISPR/Cas Experiment

In the following, we discuss the representation of the design space at different stages of a multiplex CRISPR/Cas experiment. Additionally, we clarify how experimental materials created in such an experiment can be interpreted as physical samples from a virtual design space. These ideas are depicted in **Figure 2**.

Consider a multiplex CRISPR/Cas experiment that aims to investigate pairwise interactions among a set of five target genes. Such an experiment departs from an initial pool of gRNA sequences that are specifically designed to target this set of genes (three gRNAs per gene). At this stage, the *design space* encompasses all possible gRNA sequences (**Figure 2A**). In the following, we regard a *library* as the collection of experimental units that represent material samples from the *design space*. Here, the *gRNA library* is the collection of gRNA sequences that are generated in the laboratory, as a physical equivalent of the virtual *gRNA design space*.

The combinatorial aspect of the multiplex CRISPR/Cas experiment arises as these gRNA sequences are randomly combined into gRNA/Cas constructs through vector assembly. In **Figure 2B**, each construct contains a random combination of two gRNA sequences. All possible combinations of two gRNAs that can occur in these constructs form the design space at the construct level, called the *construct design space* (**Figure 2B**). During a multiplex CRISPR/Cas experiment, gRNA/Cas constructs containing random combinations of gRNAs are randomly collected, forming a *construct library*. Importantly, not all constructs (i.e., gRNA combinations) are equally likely to be sampled, but occur with different relative abundances (due to biases during synthesis of gRNA sequences, quantification, and non-equal pooling during assembly of the constructs). Therefore, the sampling process takes place according to a sampling distribution, shaped by the probability of sampling each gRNA combination from the *construct design space*. We assume that the probability of encountering a specific pairwise combination of gRNA sequences in a gRNA/Cas construct equals the product of the probabilities of the individual gRNA sequences occurring in a construct, i.e., these occurrences are independent.

Continuing to the next stage of the experiment, the *construct library* is delivered into target plant cells by *Agrobacterium* transformation, assuming the integration of one construct per cell. In these target cells, expression of the gRNA/Cas complexes leads to genome editing with a gRNA-specific efficiency. A specific fraction of the induced mutations will lead to an effective knockout of gene function. The *design space* at this stage is the collection of all plant genotypes that can occur, referred to as the *plant design space* (**Figure 2C**). In the example of **Figure 2C**, we distinguish three classes of genotypes: those characterized by a pairwise combination of gene knockouts, a single knockout, or the absence of knockouts. Not every combination of knockouts has the same probability of being sampled from the design space, hence does not occur with the same frequency in the mutated *plant library*. The latter is caused by three factors: (1) the unequal relative abundances of gRNAs in the *construct library*, (2) the varying levels of genome editing activity across the set of gRNAs, and (3) the varying impact of a given mutation at a given location of the gene on the function and activity of the encoded protein (not every mutation results in an effective gene knockout). Random selection of mutated cells for plant regeneration in order to construct a *plant library* implies sampling combinations of gene knockouts according to a sampling distribution that integrates these three inefficiencies at the gRNA level for every target gene combination. Here, once more we apply an independence assumption, considering the probability of a pairwise combination of gene knockouts appearing in a plant cell to be equal to the product of the probabilities of occurrence of the individual gene knockouts in a plant cell.

In the remainder of this paper, we will focus on the exploration of the *plant design space*. The multiplex CRISPR/Cas experiment depicted in **Figure 2** aims to study all pairwise combinations of gene knockouts for five target genes. Here, the total number of possible pairwise combinations is denoted by $\binom{5}{2}$. The central study aim here is to quantify the number of plants that is to be randomly collected to encounter all $\binom{5}{2}$ possible combinations of gene knockouts in the *plant library*; this number is denoted by $N_{5,2}$. This way, the phenotypic effect of all 2-order genetic

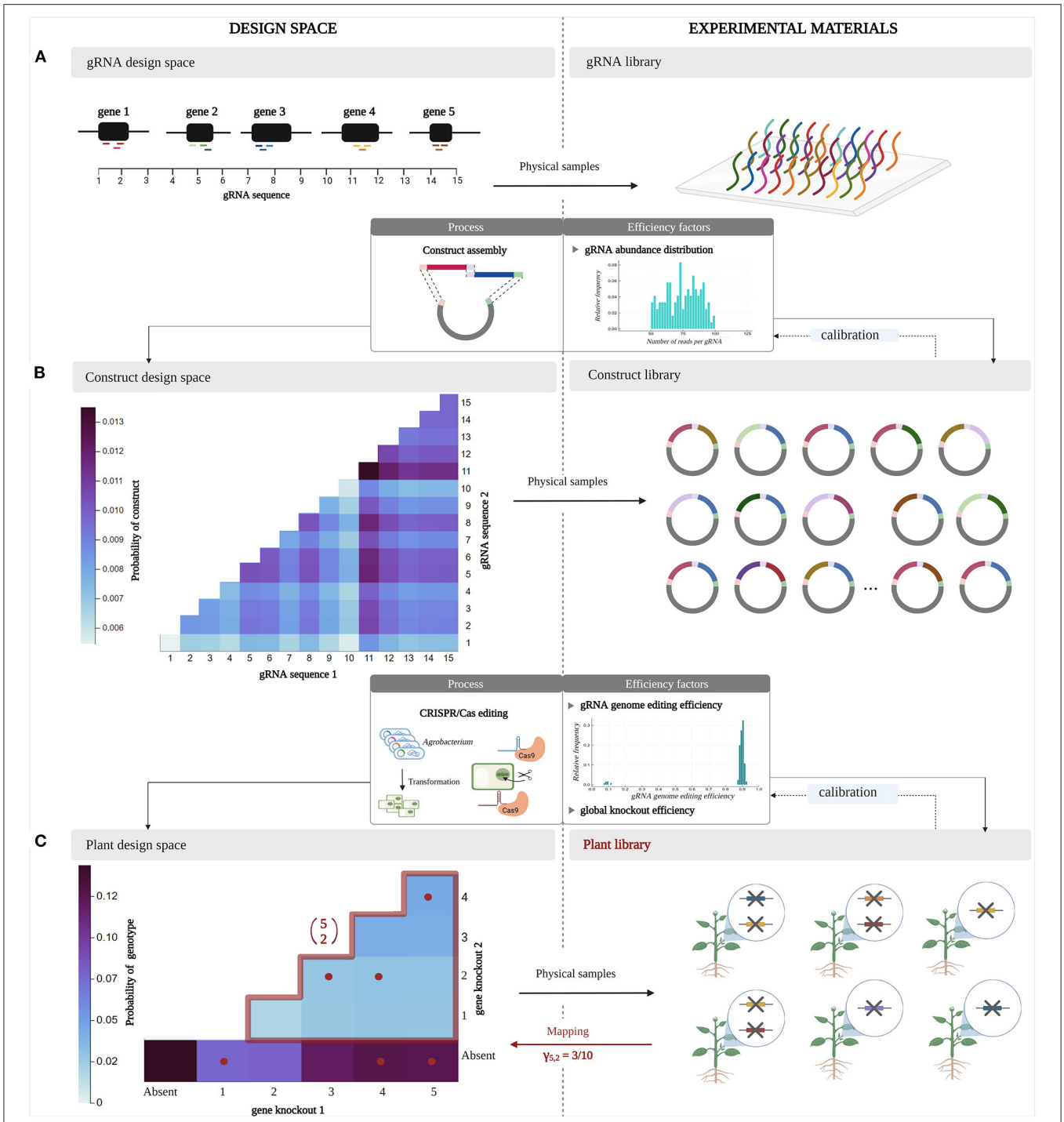

FIGURE 2 | Design space representation and parallel experimental materials at different stages in a multiplex CRISPR/Cas experiment. A *design space* at a certain point in the experiment is considered as a virtual space encompassing all possible designs, whereas a *library* represents a collection of physical samples from the design space. These material experimental units are obtained by random sampling of the design space according to a sampling distribution, which describes the probability of sampling each design and is determined by inefficiencies at different stages of the multiplex CRISPR/Cas protocol. **(A)** Here, an experiment targeting pairwise combinations of five target genes is considered. Three gRNAs are designed per gene. **(B)** In the construct library, two gRNAs are included per gRNA/Cas construct. **(C)** The resulting plant library contains up to two gene knockouts per plant.

interactions among the set of five target genes can be assessed. Recall that the *coverage* $\gamma_{5,2}$ is defined as the fraction of all $\binom{5}{2}$ pairwise gene knockout combinations that is included in a *plant library* of a given size. For the CRISPR/Cas experiment visualized in **Figure 2**, the gene knockout combinations present in the plant library are mapped back to the plant design space (**Figure 2C**). It can be seen that by randomly sampling six plants, in this case three out of the 10 possible pairwise combinations of gene knockouts are present, resulting in a coverage $\gamma_{5,2}$ of 0.3. The other three plants are not counted as they, by chance, only contain a single knockout and are considered a by-product in the plant library.

2.2. Computing the Plant Library Size That Guarantees Full Combinatorial Coverage During Screening

First, we outline a simulation-based approach for computing $N_{x,k}$ of a multiplex CRISPR/Cas screen in plants. More specifically, we describe how to statistically sample the design spaces described in Section 2.1, taking into account the efficiencies at subsequent stages of the experiment. Recall that $N_{x,k}$ of a multiplex CRISPR/Cas screen is defined as the minimal size of the plant library or number of plants to be screened in order to encounter all $\binom{x}{k}$ combinations of gene knockouts in the set of genotypes at least once, such that the effect of all relevant genetic interactions on the phenotype can be explored. As a basis for comparison, we also compute the minimal plant library size for full coverage of single gene knockouts in a (non-multiplex) CRISPR/Cas experiment, denoted by $N_{x,1}$, before considering coverage of pairwise and triple gene knockout combinations in multiplex CRISPR/Cas experiments. Secondly, an alternative approach of computing $N_{x,k}$ and some related statistics is presented through the use of our BioCCP.jl package (Van Huffel et al., 2022). Both approaches are available on GitHub (https://github.com/kirstvh/MultiplexCrisprDOE), enabling researchers to compute $N_{x,k}$ and related statistics for custom multiplex CRISPR/Cas experiments.

2.2.1. A Simulation-Based Approach to Compute the Expected Value and Standard Deviation

In the following paragraphs, we describe a simulation experiment to determine $N_{x,k}$ of a multiplex CRISPR/Cas screen that aims to study the k-order genetic interactions among a set of x target genes. In this simulation, we generate plant genotypes *in silico* by modeling the subsequent stages of a multiplex CRISPR/Cas experiment (Jacobs et al., 2017). The genotype of each plant is represented by its set of gene knockouts. We virtually collect a set of these plants by random sampling, while storing the relevant combinations of gene knockouts observed in each plant. The process of collecting plants is halted as all $\binom{x}{k}$ combinations of gene knockouts are represented, guaranteeing the study of all k-order genetic interactions. This experiment is repeated 500 times to obtain an estimate of the expected value ($\mathrm{E}[N_{x,k}]$) and associated standard deviation ($\sigma[N_{x,k}]$) of the number of plants required for full coverage.

Consider a multiplex CRISPR/Cas experiment targeting $x = 20$ genes. For each target gene, $g = 6$ different gRNAs are designed, which results in a pooled library with a total number of 120 gRNAs, reflecting the typical diversity feasible to clone in parallel *via* Golden Gate ligation and Gibson Assembly (Jacobs et al., 2017; Bai et al., 2020). Each gRNA is assumed to target only one locus in the genome. From this initial gRNA pool, multiplex gRNA/Cas constructs are assembled, sampling r gRNA sequences per construct. In this case, r equals k, since the goal is to study k-order genetic interactions among the target genes (**Table 2**). In an ideal gRNA/Cas construct library, all gRNAs are represented with the same frequencies. However, due to inaccuracies and technical constraints during gRNA synthesis, quantification and vector assembly steps, the abundance of gRNAs over the constructs is not uniform. We describe the gRNA abundance distribution in the construct library by the ratio of the frequency of the most abundant gRNA to the frequency of the least abundant gRNA, symbolized by ρ. More information on the construction of this distribution can be found in Section 4.1.1. The relative frequencies of the gRNAs are taken into account when generating the construct library *in silico*. **Figure 3A** depicts the gRNA abundance distribution (also called the frequency distribution) used for illustration throughout this study.

Following the simulation of vector assembly, the gRNA/Cas constructs are virtually transformed into plant cells, assuming one construct per plant cell. The corresponding k gRNA/Cas complexes are assumed to induce mutations in the associated target loci, potentially resulting in a successful gene knockout. Importantly, not every gRNA/Cas complex brings about a mutation and not every mutation results in a loss-of-function gene knockout. Hence, at most k gene knockouts per plant genotype can be achieved. In this context, a genome editing efficiency ϵ_{edit} specific to each gRNA and a global knockout efficiency ϵ_{KO} is introduced. The genome editing efficiency ϵ_{edit} of a gRNA indicates the relative frequency by which a gRNA accomplishes a genome edit in the target sequence. A distinction is made between a group of active gRNAs with a high average genome editing efficiency $\epsilon_{\mathrm{edit,act}} = 0.95$ and a group of inactive gRNAs with a low average genome editing efficiency $\epsilon_{\mathrm{edit,inact}} = 0.1$. The fraction f_{act} of all gRNAs that is assumed to be active, i.e., the fraction of gRNAs following a distribution with a high average genome editing efficiency, was set at 0.9 (**Table 2**). Given the fraction of active gRNAs and the average editing efficiency of active and inactive gRNAs, a probability distribution for the genome editing efficiency is constructed from which a genome editing efficiency is sampled for each gRNA. More information on the construction of this distribution can be found in Section 4.1.2. An example distribution is represented in **Figure 3B**. The global knockout efficiency ϵ_{KO} indicates the fraction of genome edits leading to a loss-of-function gene knockout. By default, the value of ϵ_{KO} is set to 0.8.

After executing the aforementioned stages, a plant genotype with a specific combination of (at most k) gene knockouts is obtained. Plants are collected until all $\binom{x}{k}$ combinations of gene knockouts are seen, i.e., $N_{20,k}$ is reached. Note that during this simulation, several (in)efficiencies are taken into account at subsequent steps of the experiment: (1) the imbalance in the abundances of gRNAs in the gRNA/Cas construct library, (2) the genome editing efficiency of each gRNA when inducing

TABLE 2 | Input parameters and outcome variables of the multiplex CRISPR/Cas simulation experiment.

Symbol	Short description	Default value
	Input parameters	
x	The number of target genes	20
g	The number of gRNAs designed per target gene	6
k	The order of genetic interaction to investigate	
	Single gene knockouts	1
	Pairwise combinations of gene knockouts	2
	Triple combinations of gene knockouts	3
r	The number of gRNAs per gRNA/Cas construct	k
ρ	The ratio of the frequency of the most abundant gRNA to the frequency of the least abundant gRNA in the gRNA/Cas construct library	2
f_{act}	The fraction of total number of gRNAs that are active	0.9
$\epsilon_{edit,act}$	The average genome editing efficiency for active gRNAs	0.95
$\epsilon_{edit,inact}$	The average genome editing efficiency for inactive gRNAs	0.1
ϵ_{KO}	The global knockout efficiency	0.8
	Outcome variables	
$E[N_{x,k}]$	The expected value of the plant library size for full coverage of all k-combinations of gene knockouts for a set of x target genes	
$\sigma[N_{x,k}]$	The standard deviation of the plant library size for full coverage of all k-combinations of gene knockouts for a set of x target genes	

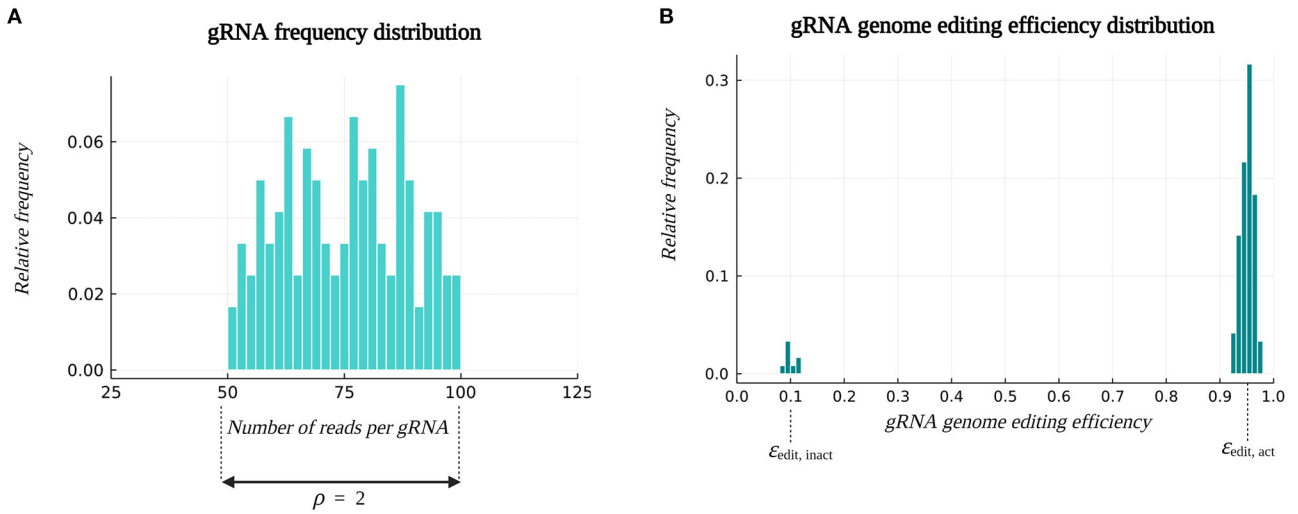

FIGURE 3 | Example of default distributions. **(A)** The gRNA frequency distribution in the construct library. Parameter ρ denotes the ratio of the frequency of the most abundant gRNA to the frequency of the least abundant gRNA, and is by default set at the value of 2. **(B)** The gRNA genome editing efficiency distribution. By default, 90% of the gRNAs are active, having an average genome editing efficiency $\epsilon_{edit,act}$ of 0.95, and 10% of the gRNAs are inactive, characterized by an average genome editing efficiency $\epsilon_{edit,inact}$ of 0.1. Information on the construction of these distributions and the sampling process can be found in Sections 4.1.1 and 4.1.2.

mutations in the plant genomes, and (3) the global knockout efficiency indicating the fraction of mutations leading to loss-of-function of the gene product. Section 2.2.3 explains how these efficiency parameters can be inferred from real experimental data.

Multiplex CRISPR/Cas simulation experiments were executed following the procedure described above in order to compute the expected value and standard deviation of $N_{20,1}$, $N_{20,2}$, and $N_{20,3}$. In these simulations, default settings of the input parameters were employed as specified in **Table 2**. The results are summarized in **Table 3**. Next, the influence of experimental parameters on $N_{x,1}$ and $N_{x,2}$ was investigated (**Figures 4**, **5**). For this purpose, we perform CRISPR/Cas simulation experiments, employing the default settings in **Table 2** for all parameters, except for the parameter under investigation.

TABLE 3 | Statistics of $N_{20,1}$, $N_{20,2}$, and $N_{20,3}$ resulting from the simulation- and BioCCP-based approaches for the multiplex CRISPR/Cas experiments as specified by the default parameters in **Table 2**.

	Simulation			BioCCP		
k	$\mathbf{E[N_{20,k}]}$	$\boldsymbol{\sigma[N_{20,k}]}$	**Runtime**	$\mathbf{E[N_{20,k}]}$	$\boldsymbol{\sigma[N_{20,k}]}$	**Runtime**
1	103 plants	35 plants	0.2752 s	104 plants	37 plants	0.0015 s
2	2,518 plants	581 plants	6.503 s	2,453 plants	580 plants	0.0272 s
3	32,154 plants	5,825 plants	302.9 s	31,348 plants	5,979 plants	1.839 s

Each simulation experiment is repeated 500 times.

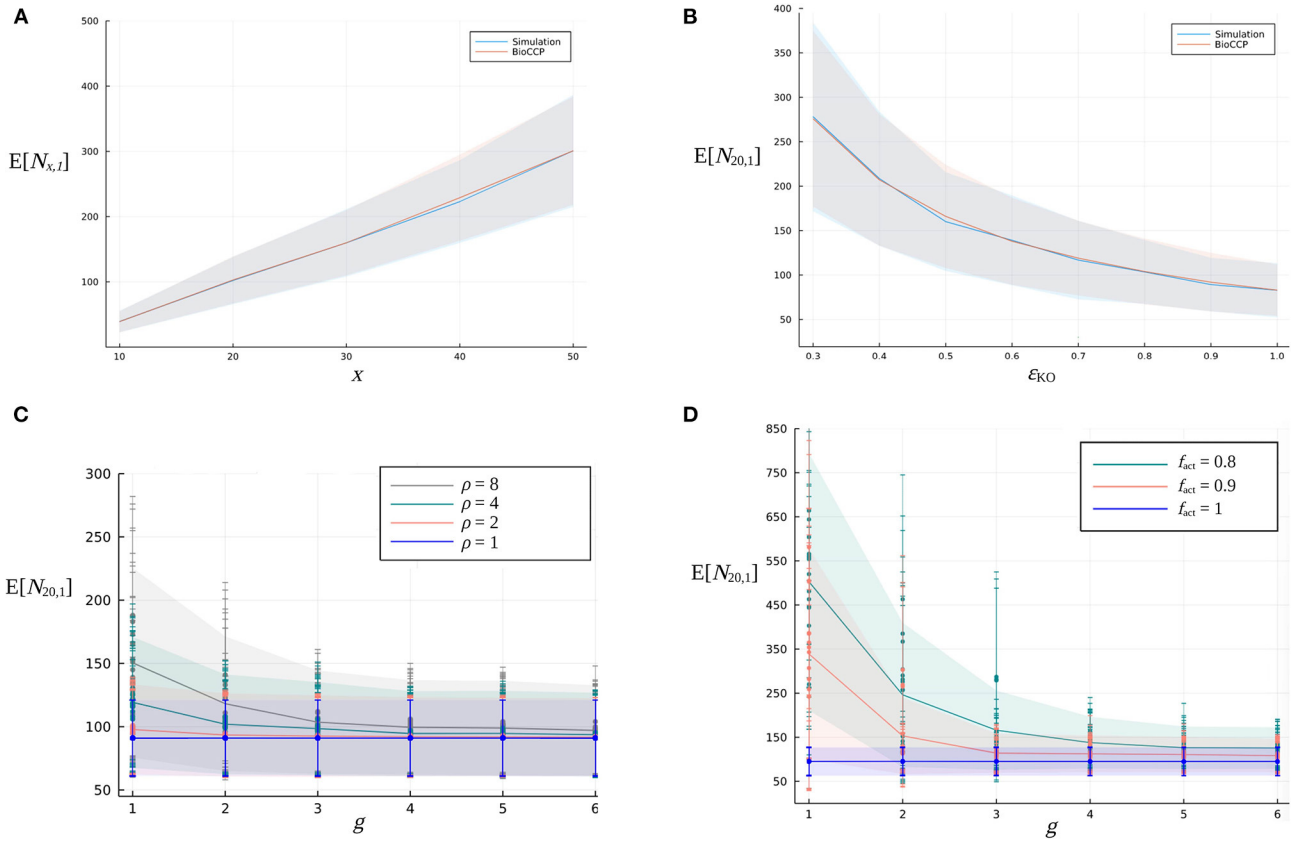

FIGURE 4 | Relation between the expected value of the minimal plant library size for full coverage of all single gene knockouts and design parameters of a multiplex CRISPR/Cas experiment. **(A)** Effect of an increasing number of x target genes on $E[N_{x,1}]$. **(B)** Effect of the global knockout efficiency ϵ_{KO} on $E[N_{20,1}]$. The blue curve indicates the simulation-based results, while the red curve indicates the BioCCP-based results. The width of the shaded area around these curves represents $\sigma[N_{20,1}]$. In each of the graphs, the parameter under investigation is varied while the values of the other parameters are fixed at the default values of the CRISPR/Cas experiment as specified in **Table 2**. **(C)** Effect of parameter ρ, a measure for the width of the gRNA frequency distribution, for an increasing number of g gRNAs per gene on $E[N_{20,1}]$. **(D)** Effect of the fraction f_{act} of active gRNAs for an increasing number of g gRNAs per gene on $E[N_{20,1}]$.

(i) Firstly, we vary the number of target genes x in the CRISPR/Cas experiment in the range $[10, 50]$. **Figure 4A** illustrates that, for this specific range of values for x, the expected value of $N_{x,1}$ increases in an approximately linear way. **Figure 5A** visualizes how $N_{x,2}$ escalates quickly with an increasing number of x target genes, due to the total number of $\binom{x}{2}$ pairwise combinations and the corresponding combinatorial plant design space expanding combinatorially with a larger x.

(ii) **Figures 4**, **5B** demonstrate the impact of the global knockout efficiency ϵ_{KO} on $N_{x,k}$. As expected, less effective gene editing inevitably demands a larger plant library size to completely cover all single gene knockouts ($N_{20,1}$) as well as pairwise combinations of gene knockouts ($N_{20,2}$).

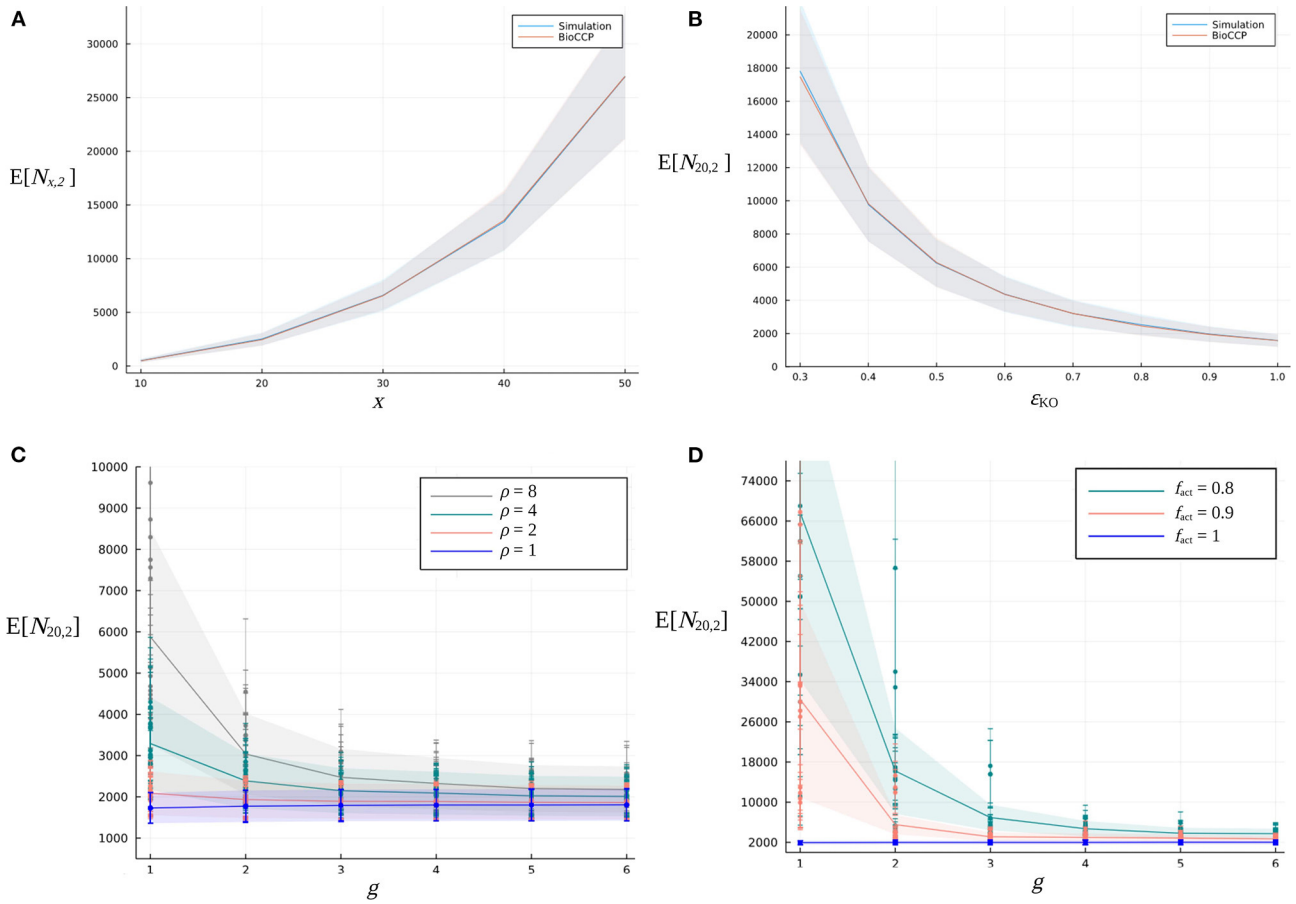

FIGURE 5 | Relation between the expected value of the plant library size for full coverage of pairwise combinations of gene knockouts and design parameters of a multiplex CRISPR/Cas experiment. **(A)** Effect of an increasing number of x target genes on E[$N_{x,2}$]. **(B)** Effect of the global knockout efficiency ϵ_{KO} on E[$N_{20,2}$]. The blue curve indicates the simulation-based results, while the red curve indicates the BioCCP-based results. The width of the shaded area around these curves represents σ[$N_{20,2}$]. In each of the graphs, the parameter under investigation is varied while the values of the other parameters are fixed at the default values of the CRISPR/Cas experiment as specified in **Table 2**. **(C)** Combined effect of parameter ρ of the gRNA frequency distribution for an increasing number of g gRNAs per gene on E[$N_{20,2}$]. **(D)** Effect of the fraction f_{act} of active gRNAs for an increasing number of g gRNAs per gene on E[$N_{20,2}$].

(iii) In **Figures 4**, **5C**, the combined effect of the parameter ρ of the gRNA frequency distribution and the number of gRNAs per gene on $N_{20,1}$ and $N_{20,2}$ is illustrated. For a fixed number of g gRNAs per gene, it is clear that a more uneven gRNA frequency distribution, indicated by a larger ratio ρ, substantially increases the plant library size $N_{x,k}$ for full coverage. Importantly, when more gRNAs are designed per gene, the increase of E[$N_{20,k}$] caused by a higher ρ gradually diminishes. The latter suggests that, in the case of a construct library with highly underrepresented gRNA sequences, the plant library size for full coverage might be reduced by including more gRNAs per gene in the experiment. In **Figures 4**, **5C**, the genome editing efficiency of all gRNAs is set at the ideal value of 1 (instead of sampling them from the genome editing efficiency distribution) in order to isolate the effect of sampling the gRNA relative frequencies from distributions with different ρ. Note that for each value of ρ, the set of gRNA relative frequencies was sampled multiple times from the same distribution, resulting in a variable outcome for E[$N_{20,k}$] at a specific ρ (represented by different data points in the graph).

(iv) In **Figures 4**, **5D**, the fraction f_{act} of active gRNAs is demonstrated to exhibit a similar type of relation to $N_{x,k}$. Here, several series of genome editing efficiencies were drawn from genome editing distributions characterized by a specific value of f_{act}. The expected value of $N_{20,k}$ of each corresponding multiplex CRISPR/Cas experiment is represented by a different data point in the graph. Clearly, at a fixed number of g gRNAs per gene, CRISPR/Cas experiments with a lower f_{act} require a higher number of plants for full coverage. Yet, this increasing effect on $N_{x,k}$ can again be mitigated by including a greater number of gRNAs per gene. Note that the experiments visualized in **Figures 4**, **5D** are executed by applying a uniform gRNA frequency distribution.

Figures 4, **5** demonstrate that $N_{20,2}$ is in general more sensitive to changes of the values of design parameters as compared to $N_{20,1}$. For graphs visualizing the impact of experimental design parameters on $N_{20,3}$, we refer to the **Supplementary Figure 1**.

2.2.2. BioCCP-Based Approach to Compute the Expected Value and Standard Deviation and Other Relevant Statistics

In previous work, we designed the BioCCP.jl package in the Julia Programming Language (Bezanson et al., 2017), providing tools for computing a minimal sample size that adequately covers the design space of combinatorial screening experiments in biotechnology (available at https://github.com/kirstvh/BioCCP.jl). This package reformulates the computation of a minimal sample size for covering the design space of a combinatorial library as a variant of the Coupon Collector Problem (CCP), a well-known problem in probability theory and statistics (Flajolet et al., 1992; Doumas and Papanicolaou, 2016). The standard formulation of the CCP describes a situation where there are n different types of "coupons" of which a collector tries to obtain a complete set (e.g., a set of stickers). Therefore, (s)he samples repeatedly with replacement one coupon at a time from a population (e.g., cereal boxes that each contain one random sticker). The goal then is to compute how many coupons should be drawn on average to complete the collection. This abstraction renders BioCCP fit for answering questions concerning minimal sample sizes for a wide range of combinatorial biotechnology experiments. Extrapolating to multiplex CRISPR/Cas experiments in plants, the relevant combinations of gene knockouts can be regarded as coupons, and the collector sampling from the population is the researcher performing a randomized screening experiment on a mutated plant library. For elaborate information about BioCCP, we refer to our recent paper (Van Huffel et al., 2022) and the accompanying tutorials.

2.2.2.1. Plant Library Size for Full Coverage

Here, we apply BioCCP to compute $\mathrm{E}[N_{x,k}]$ for a multiplex CRISPR/Cas experiment configured by the same experimental design settings as specified in **Table 2**. **Figures 4**, **5** demonstrate that the simulation-based results are closely approximated by the computations of BioCCP. Based on these results, we regard BioCCP as a suitable framework for gaining insight into $N_{x,k}$ for multiplex CRISPR/Cas screens in plants. **Table 3** compares the execution time to compute $\mathrm{E}[N_{x,k}]$ for the simulation-based and BioCCP-based approaches, demonstrating a speed-up by more than two orders of magnitude when using the BioCCP-based approach. In the following, we apply other functionalities of the BioCCP package to answer additional questions related to coverage of multiplex CRISPR/Cas screens. For more information about the relevant BioCCP functions and usage, please consult Section 4.

2.2.2.2. Probability of Full Coverage w.r.t. Plant Library Size

BioCCP provides the functionality to compute a so-called *success probability* of full coverage w.r.t. the sample size of a screening experiment. In the case of multiplex CRISPR/Cas screens, this measure indicates the probability of achieving full coverage of all k-combinations of gene knockouts for x target genes w.r.t. the number of plants analyzed in a randomized screening assay, and will be further denoted by $P_{x,k}$. More specifically, $P_{x,1}$ and $P_{x,2}$, respectively, represent the probability that all single gene knockouts and all pairwise combinations of knockouts are represented at least once in a CRISPR/Cas screen with a specified plant library size N. **Figure 6A** illustrates that, for a multiplex CRISPR/Cas screen targeting double gene knockouts described by the experimental design settings in **Table 2**, $P_{20,2}$ amounts to 0.95 when N is approximately equal to 3,560 plants. For an analogous screen targeting single gene knockouts, 170 plants need to be screened to obtain a probability $P_{20,1}$ of 0.95. **Supplementary Figure 2A** visualizes the probability of full combinatorial coverage w.r.t. plant library size for a multiplex CRISPR/Cas experiment targeting triple combinations of gene knockouts.

One can also bring forward a different interpretation of $P_{x,k}$ in the context of screens for lethal (combinations of) gene knockouts. In particular, when a specific gene knockout or combination of gene knockouts is not represented in a CRISPR/Cas screen with a plant library size corresponding to a probability $P_{x,k}$ of 0.95, one can conclude that there is a probability of 0.95 that its absence is caused by lethality rather than non-saturation of the plant design space. Regarding the multiplex CRISPR/Cas screen targeting double gene knockouts considered in **Figure 6A**, one can conclude with 0.95 confidence that a pairwise combination of knockouts is not present in a mutated plant collection consisting of 3,560 randomly selected plants due to a lethal effect, and not as a result of an insufficient plant library size. By all means, this statement is only valid given that all parameter values of the multiplex CRISPR/Cas experiment used to compute $P_{x,k}$ are properly calibrated (see Section 2.2.3).

2.2.2.3. Expected Combinatorial Coverage w.r.t. Plant Library Size

We can apply BioCCP to determine the fraction of the total number of $\binom{x}{k}$ gene knockout combinations in the plant design space that is expected to be covered w.r.t. the plant library size N of a (multiplex) CRISPR/Cas experiment. This way, one can get insight into the expected coverage ($\mathrm{E}[\gamma_{x,k}]$) obtained with a given number of plants. The curve $\mathrm{E}[\gamma_{20,2}]$ in **Figure 6B** represents the expected coverage of pairwise combinations of gene knockouts w.r.t. the plant library size of an experiment described by the parameters in **Table 2**. The plant library size at an expected coverage $\mathrm{E}[\gamma_{20,2}]$ of 0.95 can be considered as the number of plants guaranteeing that on average 95% of all $\binom{20}{2}$ pairwise combinations of knockouts will be observed at least once (**Figure 6B**). By way of comparison, **Figure 6B** also visualizes the relation between the expected value of the fraction of single gene knockouts that is observed in a CRISPR/Cas screen targeting single gene knockouts and the plant library size of the screen. Here, one can expect to cover on average 95% of all 20 single gene knockouts when including 80 plants by random selection in a plant library. For graphs visualizing the expected coverage w.r.t.

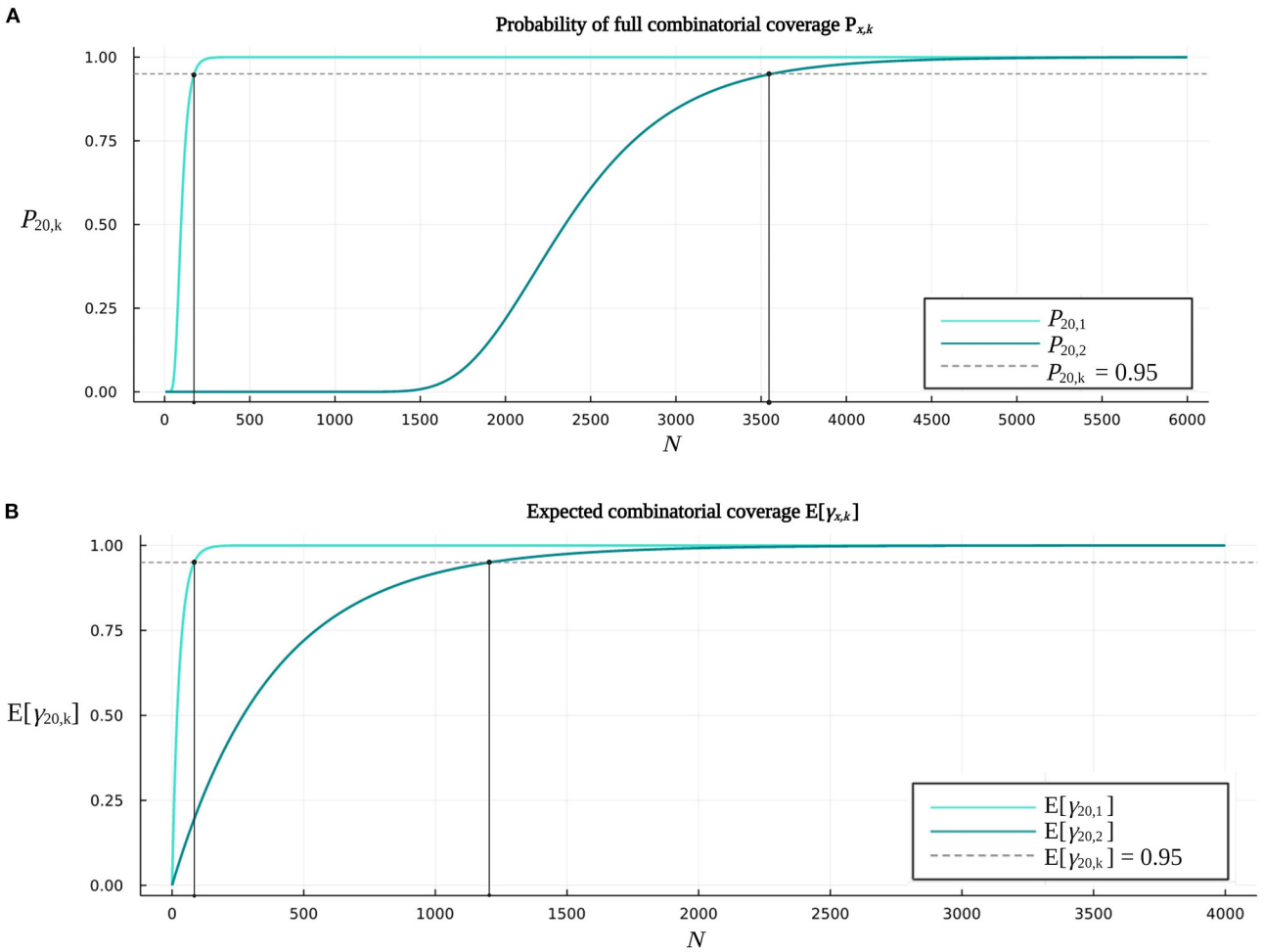

FIGURE 6 | Additional functionalities provided by BioCCP to gain insight into the coverage of multiplex CRISPR/Cas screens. **(A)** The probability of full coverage $P_{20,k}$ w.r.t. the plant library size N of a (multiplex) CRISPR/Cas screen. The graph $P_{20,1}$ indicates the probability of full coverage of all single gene knockouts for an increasing number of plants in the plant library. Parameter settings of the CRISPR/Cas screen are specified by **Table 2** ($k = 1$). The graph $P_{20,2}$ quantifies the probability of full coverage of all $\binom{20}{2}$ pairwise combinations of gene knockouts w.r.t. the plant library size. A multiplex CRISPR/Cas screen as specified by the parameter settings in **Table 2** is considered ($k = 2$). **(B)** Expected coverage of all single gene knockouts ($E[\gamma_{20,1}]$) and pairwise combinations of gene knockouts ($E[\gamma_{20,2}]$) w.r.t. the plant library size of a (multiplex) CRISPR/Cas screen. Parameters of the experiments correspond to the default settings as specified in **Table 2**.

plant library size for experiments targeting triple combinations of gene knockouts, please consult **Supplementary Figure 2B**.

2.2.3. Model Calibration

In previous sections, computing $N_{x,k}$ by means of the simulation- and BioCCP-based approaches was demonstrated for virtual CRISPR/Cas experiments. Hence, we adopted a hypothetical set of sensible parameter values in order to define the efficiency of processes at several stages of the experiment, such as the relative abundances of gRNAs in the construct library, the genome editing efficiencies of the gRNAs and the global knockout efficiency (**Table 2**). However, to effectively model the relation between the coverage and the plant library size of a concrete multiplex CRISPR/Cas experiment performed in the wet lab, one should calibrate the models with parameter values that approximate reality as closely as possible. For this purpose, a calibration round prior to carrying out a full-scale multiplex CRISPR/Cas experiment can be conducted. In this calibration stage, experimental data is gathered at different stages of the experiment to obtain more accurate estimates of experimental parameters.

The first type of calibration data can be generated at the level of the gRNA/Cas construct library, which is produced by vector assembly of the initial gRNA pool. At this stage, the gRNA expression cassettes in the bulk construct library can be amplified by PCR followed by next-generation sequencing, obtaining a number of reads per gRNA. As such, the relative abundances of gRNAs in the library or the empirical frequency distribution of gRNAs can be precisely determined and fed into the model. After delivery of the constructs into the target cells for the creation of the mutated plant library, a second data collection process can be executed to retrieve information about the genome editing

efficiencies of the individual gRNAs. More specifically, both the gRNAs stably integrated in the genome of the mutated lines and the mutations at the associated target sites can be detected by next-generation deep sequencing (Jacobs et al., 2017; Gaillochet et al., 2021; Schaumont et al., 2022). The rate at which a specific gRNA has successfully induced a mutation at the target site determines the genome editing efficiency of the gRNA (ϵ_{edit}). Lastly, the global knockout efficiency is the remaining parameter that needs to be assessed to improve the accuracy of the model. It would be of interest to develop a model that can *in silico* predict the disruption of protein function based on an observed mutated gene sequence. Such predictions could assist in identifying the fraction of mutations leading to an effective gene knockout, i.e., the empirically calibrated global knockout efficiency for a given set of gRNAs (ϵ_{KO}). Schaumont et al. (2022) are currently working toward implementing a method for high-throughput multiplex gRNA design, molecular characterization of induced mutations at hundreds of loci in parallel, and automated interpretation of functional consequences of mutations, all as part of the Stack Mapping Anchor Points (SMAP) package.

After executing this calibration round, the simulation-based and BioCCP-based approaches for computing $N_{x,k}$ can be updated by injecting the empirically measured efficiencies. As a result, one can obtain a more precise estimation of the expected minimal number of plants needed for full coverage, allowing for well-informed design of the final multiplex CRISPR/Cas experiment. For instance, it may require less effort to recalibrate a suboptimal gRNA frequency distribution in the laboratory, or to enrich the gRNA library for empirically proven active gRNAs (or to eliminate the inactive gRNAs), and repeat the construct library assembly rather than to scale up plant transformation to finally obtain a desired combinatorial coverage within a practically feasible plant library size.

2.3. Strategies for Increasing Coverage of Multiplex CRISPR/Cas Screens in Plants

As demonstrated by **Figures 4**, **5**, the design parameters of a multiplex CRISPR/Cas experiment greatly impact the minimal plant library size to achieve full coverage when exploring all k-combinations of knockouts for x target genes. By means of these findings, we define two experimental design strategies that reduce the number of plants that needs to be screened for studying all relevant genetic interactions: the *Split–Select–Combine* strategy and the *Overshoot–Select–Purify* strategy.

2.3.1. The *Split–Select–Combine* Strategy

The *Split–Select–Combine* strategy studies interactions among a set of target genes in multiple distinct subsets, hence shrinking the number of possible gene knockout combinations in the plant design space and in its turn the plant library size for full coverage. **Figure 7** gives a schematic overview of this approach. The *Split–Select–Combine* strategy starts with dividing a multiplex CRISPR/Cas experiment with a large number of x target genes into multiple screening experiments, each targeting a distinct gene subset of size x_{subset}, addressed as the *Split* phase (**Figure 7A**). Grouping genes is meaningful considering that prior knowledge indicates that genes within a subset are members of the same gene family or contribute to the same metabolic or regulatory pathway, and minimally interact with genes belonging to other groups. In this regard, insights from gene family protein sequence alignments and phylogenetic analyses, genome-wide association studies, quantitative trait locus analysis and/or co-expression networks can be valuable to guide the construction of effective gene subsets (Gaillochet et al., 2021). After grouping of the genes, gRNA sequences specifically targeting each gene subset are designed. Subsequently, for each gene subset a construct library is generated, with each construct containing k gRNA sequences in case of exploring k-order genetic interactions. After transformation of these construct libraries into target cells, plant libraries exhibiting genetic perturbations in a specific gene subset are obtained. During the *Select* phase, plants with an advantageous phenotype or genotype are collected. Subsequently, further supertransformations and/or crossings between these lines can be performed to stack mutations and explore genetic interactions between different gene groups (*Combine*).

The rationale behind the *Split–Select–Combine* strategy is to search for genetic interactions in multiple, distinct design spaces with reduced combinatorial complexity (*Split*), after which valuable genotypes (*Select*) can be crossed in a more focused design space (*Combine*). **Figure 7B** illustrates the impact of the *Split* phase on the plant design space of a multiplex CRISPR/Cas screen. Consider a multiplex CRISPR/Cas screen targeting a total number of $x = 20$ genes for studying pairwise interactions ($k = 2$). In this case, the plant design space contains all possible $\binom{20}{2}$ pairwise combinations of gene knockouts. On the contrary, the *Split–Select–Combine* strategy visualized in **Figure 7B** divides the target genes into two smaller subsets of size $x_{\text{subset}} = 10$ based on prior knowledge, and investigates pairwise interactions in these gene subsets separately. The plant design space now only comprises two smaller subspaces of the original design space, each containing a significantly smaller number $\binom{10}{2}$ of possible gene knockout combinations. It is intuitive that this will require a smaller number of plants to achieve full saturation. As such, this strategy can contribute to optimally exploiting a limited availability of plants in multiplex CRISPR/Cas screens.

We can quantify the reduction in the plant library size for full coverage by using the *Split – Select – Combine* strategy, in particular for the default multiplex CRISPR/Cas experiment described in **Table 2**. Consider a total number of 20 target genes that is split into two subsets of 10 genes. The minimal number of plants for reaching full coverage $\gamma_{10,2}$ in each subset of size $x_{\text{subset}} = 10$ is denoted by $N_{10,2}$. The variable $N_{(10,10),2}$ represents the minimal plant library size for full coverage of both subsets. For this *Split* scenario, **Figure 7C** demonstrates that the $\binom{10}{2}$ gene knockout combinations in one gene subset can be saturated by screening on average less than $\text{E}[N_{10,2}] \approx 400$ plants, resulting in a total minimal plant library size of $\text{E}[N_{(10,10),2}] \approx 800$ plants for entirely covering the plant design space in the *Split* scenario. When splitting the 20 target genes into four subsets of 5 genes, the minimal plant library size for full coverage of all pairwise interactions in all gene subsets even further decreases to $\text{E}[N_{(5,5,5,5),2}] \approx 300$ plants. In contrast, a pooled screen examining all the pairwise combinations of the 20 target genes requires on average more than $\text{E}[N_{20,2}] \approx 2,700$ plants to

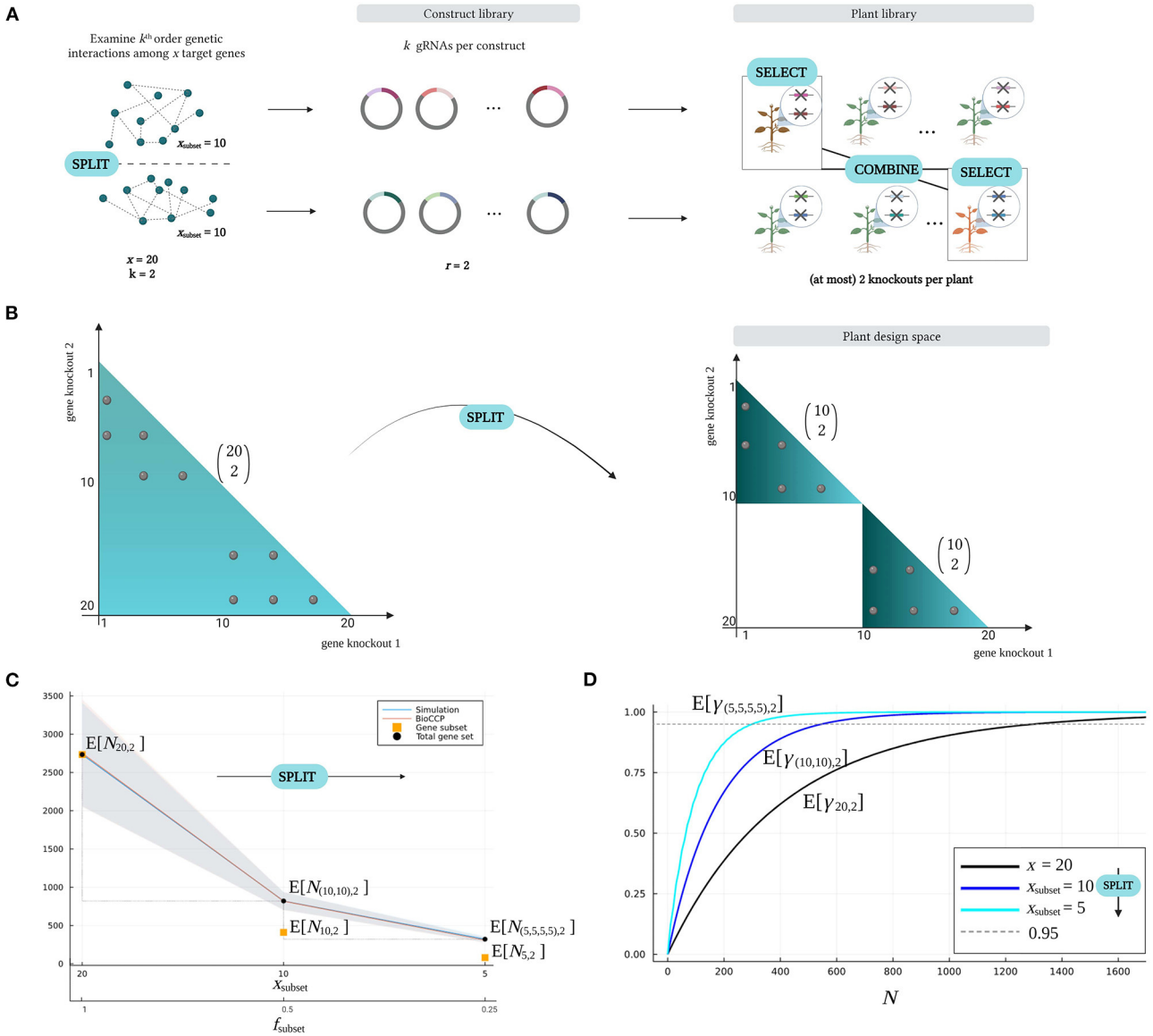

FIGURE 7 | The *Split–Select–Combine* strategy. **(A)** The *Split–Select–Combine* strategy starts with dividing a set of target genes into meaningful subgroups. Here, a total number of $x = 20$ genes is divided into two subsets, each of size $x_{subset} = 10$ genes (*Split*). To study genetic interactions within these subsets separately, a construct library is created for each subset. Each construct contains two random gRNAs to explore pairwise genetic interactions ($r = k = 2$). Performing a phenotypic screen on the corresponding plant libraries allows to select plants with an interesting phenotype (*Select*), which can be crossed to investigate genetic interactions between the gene subsets (*Combine*). **(B)** Studying 20 target genes in two subgroups of 10 genes (*Split*) creates a plant design space that consists of two subspaces of the original design space. **(C)** Impact on the plant library size for full coverage as a total set of $x = 20$ genes is grouped into subsets of size $x_{subset} = 10$ or size $x_{subset} = 5$ (*Split*). The fraction of the total number of genes that is present in the subset is denoted by f_{subset}. The plant library size for full coverage of all pairwise combinations of gene knockouts within the subsets separately is indicated as $N_{10,2}$ and $N_{5,2}$ (orange squares), respectively. The plant library size for full coverage of the entire gene set, is given by $N_{(10,10),2}$ and $N_{(5,5,5,5),2}$ (black dots). **(D)** Impact on the expected coverage w.r.t. plant library size as a total set of $x = 20$ genes is grouped into subsets of size $x_{subset} = 10$ or size $x_{subset} = 5$ (*Split*). Note that all parameters of the experiment (except for the number of target genes that is varied on the x-axis) are configured with the default settings in **Table 2**.

saturate all $\binom{20}{2}$ gene knockout combinations. Hence, the total plant library size that covers all genetic interactions in the gene subsets is substantially lower than the plant library size that saturates a single screen examining the pairwise combinations of all target genes. As previously illustrated in **Figure 5A**, this is due to the number of gene knockout combinations in the plant design space as well as the plant library size for full coverage increasing exponentially with a larger number of x target genes. **Figure 7D** illustrates that full coverage is reached at lower plant library size when genes are split in smaller subsets. For graphs visualizing the impact of the *Split–Select–Combine* strategy on the plant library size for full coverage and the expected coverage w.r.t.

plant library size for experiments targeting triple combinations of gene knockouts, we refer to **Supplementary Figure 3**.

2.3.2. The *Overshoot–Select–Purify* Strategy

Consider a multiplex CRISPR/Cas experiment investigating all k-order genetic interactions among a set of x target genes, hence all $\binom{x}{k}$ gene knockout combinations need to be observed at least once in the plant library. The *Overshoot–Select–Purify* strategy intends to explore more than one k-order genetic interaction per plant in the plant library, requiring a lower plant library size for full coverage of all $\binom{x}{k}$ gene knockout combinations. For this purpose, *Overshoot–Select–Purify* proposes to design a multiplex CRISPR/Cas experiment as illustrated in **Figure 8A**. After designing a collection of gRNAs specifically for the set of target genes, a construct library is generated by assembling more than k gRNAs per vector, which is addressed here as *Overshoot*. The generated plant library will contain plants with more gene knockouts than the order of genetic interaction under investigation (k). Therefore, from a geneticist's perspective, *Overshoot* implies studying multiple k-combinations of gene knockouts in a background of other knockouts. The latter is only valid assuming that perturbation of most target genes does not affect the phenotype of interest, rendering most background mutations neutral, and genetic interactions with an order higher than k are rare. For example, in **Figure 8A**, pairwise genetic interactions ($k = 2$) among a set of $x = 20$ target genes are investigated, and $r = 3$ gRNA sequences are assembled per gRNA/Cas construct in the construct library. The plants in the corresponding mutated library can contain up to three gene knockouts, which is considered equivalent to three specific *pairwise* combinations of gene knockouts in the *Overshoot* scenario. Upon detecting a plant with beneficial traits during the screening phase (*Select*), its genotype can be decomposed from higher-order combinations of gene knockouts through Mendelian segregation (*Purify*). In this manner, a genotype with the minimal combination of gene knockouts that is responsible for the phenotypic change can be isolated.

Figure 8B depicts the *Overshoot–Select–Purify* strategy at the level of the design space. Consider a multiplex CRISPR/Cas experiment in which up to three gene knockouts are induced per plant when investigating all pairwise genetic interactions among 20 target genes. Hence, each plant genotype in the plant library, indicated by a single point in the plant design space, is characterized by up to three gene knockouts. Here, a set of three knockouts in a plant is regarded as a "bag of three pairwise combinations" rather than a "single third-order genetic interaction." Note that we are ignoring that each pairwise combination of gene knockouts occurs in a background of one other knockout. This illustrates that each plant with three gene knockouts allows to study up to three pairwise gene knockout combinations. In this example, six plants comprise 18 pairwise gene knockout combinations as a result of *Overshoot*. Intuitively, since a given number of plants is able to cover multiple pairwise gene knockout combinations, the plant library size for full coverage $N_{x,k}$ is reduced.

The effect of *Overshoot* on the expected value of the plant library size for full coverage ($\mathrm{E}[N_{x,k}]$) is quantified in **Figure 8C**. As an example, consider an experiment to investigate all pairwise gene knockout combinations ($k = 2$) for a set of 20 target genes. **Figure 8C** visualizes the relation between the number of gRNAs per vector in the construct library (i.e., the degree of *Overshoot*) and the plant library size for full coverage ($N_{20,2}$). Suppose we create a construct library with three gRNAs per vector, then the expected value of the plant library size for full coverage ($\mathrm{E}[N_{20,2}]$) is ∼850 plants. In contrast, if only at most two genes per plant are knocked out, then the expected value of $N_{20,2}$ amounts to more than 2,500 plants. When knocking out over three genes per plant, $N_{20,2}$ decreases even more drastically (**Figure 8C**). **Figure 8D** illustrates that full coverage of all pairwise combinations of gene knockouts is reached at lower plant library size as a result of *Overshoot*. For graphs visualizing the impact of the *Overshoot–Select–Purify* strategy on the plant library size for full coverage and the expected coverage w.r.t. plant library size for experiments targeting triple combinations of gene knockouts, we refer to **Supplementary Figure 4**.

Lastly, **Figure 9** visualizes the impact of *Overshoot* on the distribution of the number of knockouts per plant in a library. Multiplex CRISPR/Cas experiments targeting double gene knockouts ($k = 2$) with varying global knockout efficiency ϵ_{KO} are considered. **Figure 9A** depicts the distribution of the number of knockouts per plant resulting from multiplex CRISPR/Cas experiments employing a standard approach, involving the generation of a construct library with the number of gRNAs per vector equal to the order of genetic interaction to investigate ($r = k = 2$). However, due to inefficiencies during CRISPR/Cas-mediated genome editing, a large fraction of the plants in the resulting plant library will carry a knockout in only one target gene, or might not possess a gene knockout at all. As these lower-order mutated lines do not enable the study of any pairwise combinations of gene knockouts, they do not contribute to covering relevant k-combinations of gene knockouts in the combinatorial design space. **Figure 9A** illustrates that these plants occupy a larger part of the plant library as the global knockout efficiency ϵ_{KO} decreases. On the contrary, in **Figure 9B**, an *Overshoot* scenario is depicted, in which six gRNAs are included per gRNA/Cas construct ($r = 6$), while targeting pairwise combinations of gene knockouts ($k = 2$). Here, all plants in the library that hold a number of gene knockouts that is equal to or greater than k, i.e., all double to sextuple knockout lines, are valuable for covering k-combinations of knockouts (possibly in a background of other knockouts, in this case providing multiple k-combinations per plant). This way, on the one hand, *Overshoot* is able to optimally exploit a set of plants for combinatorial coverage, compensating for inefficiencies during CRISPR/Cas-mediated gene perturbation. On the other hand, note that *Overshoot* is also able to leverage these inefficiencies as a tool to create diverse orders of combinations of gene knockouts in a single plant library.

Importantly, a large fraction of the mutated lines resulting from *Overshoot* contains combinations of gene knockouts of an order higher than the order of genetic interaction under investigation. These higher-order combinations potentially hold mutations additional to the minimal set of gene knockouts that is causative for the phenotype of interest. Therefore, after

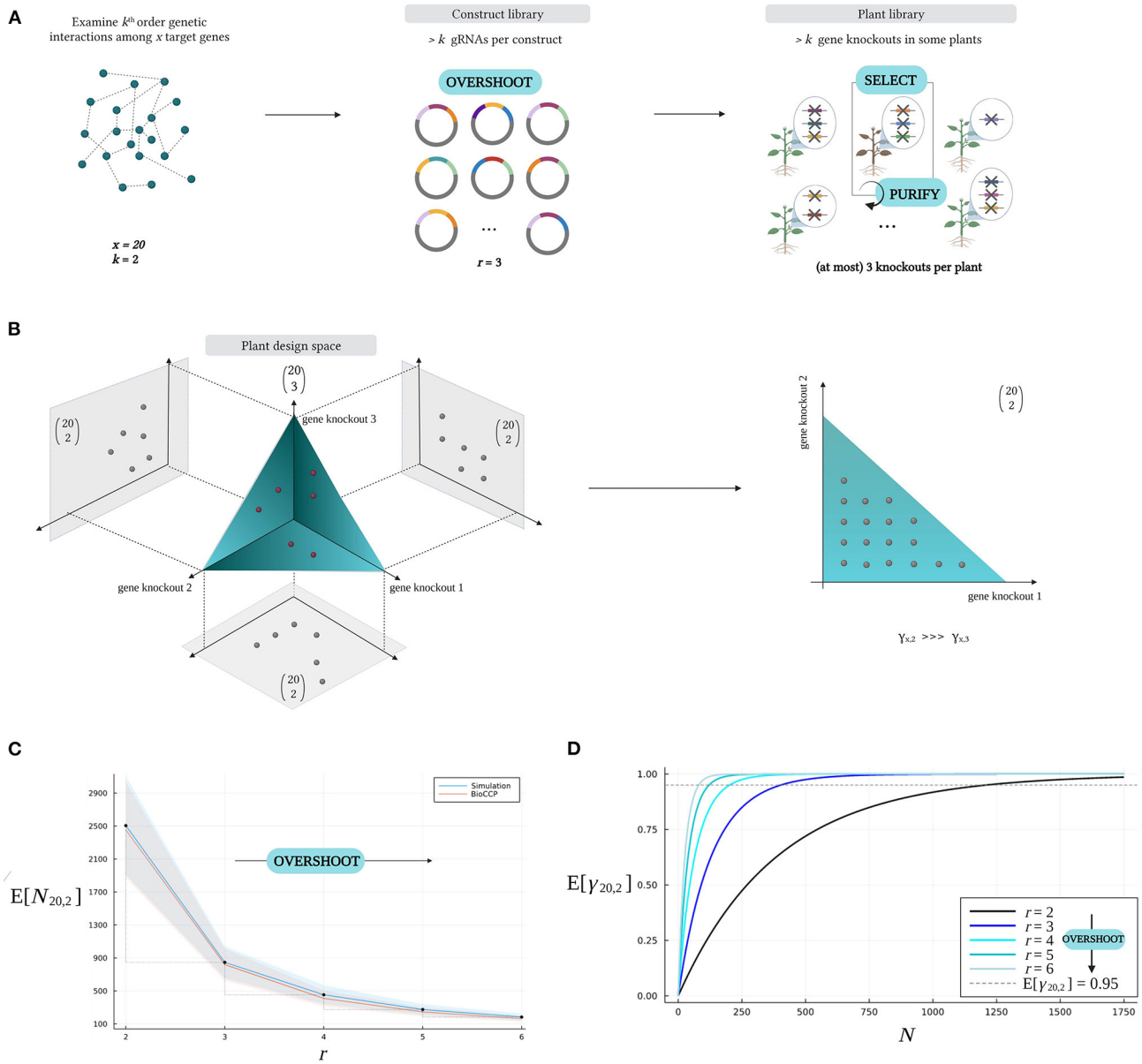

FIGURE 8 | The *Overshoot–Select–Purify* strategy. **(A)** For a multiplex CRISPR/Cas experiment investigating all k-combinations of gene knockouts for x target genes, *Overshoot–Select–Purify* starts with designing a construct library with more than k gRNA sequences per vector (*Overshoot*). Here, pairwise interactions ($k = 2$) are investigated among $x = 20$ target genes, and $r = 3$ gRNA sequences are included per vector. The resulting plant library will contain plants with up to three gene knockouts. Interesting plant phenotypes can be collected (*Select*), after which the minimal causative genotype can be purified by Mendelian segregation (*Purify*). **(B)** Impact of *Overshoot* on the plant design space. In this example, the plant library includes six plants with each three gene knockouts, which are mapped onto the plant design space (red dots). The bag of three gene knockouts in each of the six plants can be decomposed in three pairwise combinations of gene knockouts (gray dots), resulting in a total of 18 pairwise combinations of gene knockouts. A higher coverage $\gamma_{20,2}$ is reached per plant compared to a standard approach where $r = k$ (yielding at most one pairwise combination per plant). **(C)** Reduction of $N_{x,k}$ as a result of *Overshoot*. $N_{20,2}$ decreases as a higher number of r gRNAs are included per construct, exploring a larger fraction of all possible pairwise combinations of gene knockouts per plant. **(D)** Increased coverage $\gamma_{20,2}$ at fixed plant library size N as a result of *Overshoot*. In these graphs, r is varied, while the other parameters were configured with the default settings in **Table 2**.

the selection of individuals with relevant phenotypes from the mutated collection (*Select*), the *Purify* step involves the isolation of the effective combinations of gene knockouts through several backcrosses. Backcrossing implies subjecting lines to crossings with plants from a different genetic background in order to achieve a progeny in which the higher-order combinations of gene knockouts segregate into lower-order combinations. Then, individuals with interesting phenotypes are once again selected from the progeny, whereafter genotyping through massively parallel sequencing serves to identify the set of mutations that

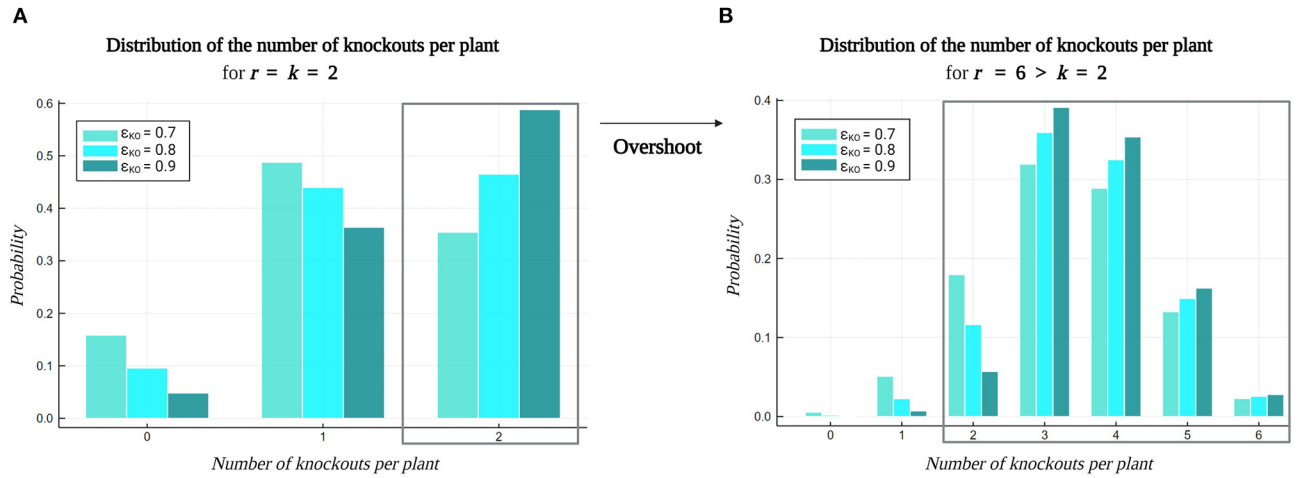

FIGURE 9 | Impact of the *Overshoot–Select–Purify* strategy on the distribution of the number of knockouts per plant. A multiplex CRISPR/Cas experiment investigating pairwise combinations ($k = 2$) of gene knockouts for a set of 20 target genes was considered (see default settings in **Table 2**). The probability of the number of gene knockouts per plant is dependent on the global gene knockout efficiency ϵ_{KO}. **(A)** The standard multiplex CRISPR/Cas approach incorporates $r = k$ gRNAs per vector in the construct library, producing a plant library containing wildtypes, single and double knockout lines, as a result of inefficiencies related to CRISPR/Cas-mediated genome editing. **(B)** Applying *Overshoot* ($r > k$) yields a diverse library of plants containing relevant combinations of gene knockouts of an order that ranges from k to r.

is associated with the observed phenotype. An iterative process of estimating genotype-phenotype associations and Mendelian segregation through several backcrosses may be necessary to arrive at the minimal causative set of gene knockouts. The total number of plants that needs to be generated, phenotyped, and genotyped during the *Purify* phase depends on the order of genetic interaction that is responsible for the desired phenotype. In fact, isolating a lower-order combination of gene knockouts from a higher-order mutational background implies substantially shrinking the initial search space, requiring an increased number of backcrosses. Therefore, the trade-off between the advantage of minimizing the scale of the initial CRISPR/Cas screen by *Overshoot* on the one hand, and the substantial amount of resources and efforts for purifying the genotypes afterwards on the other hand, is decisive for the ideal number of knockouts per plant. Note that the quantification of the number of plants that needs to be generated, phenotyped, and genotyped during *Purify* is left for future work. In addition, it should be emphasized that an increasing number of gRNAs per gRNA/Cas construct (more than six gRNAs per gRNA/Cas construct) may result in competition among the individual gRNAs for the common Cas nuclease core and hence reduced genome editing efficiencies (Stuttmann et al., 2021). This reduced efficiency imposes additional constraints on the degree of *Overshoot* that can be applied in a multiplex CRISPR/Cas experiment.

3. DISCUSSION

The development of multiplex CRISPR/Cas systems has advanced the study of genetic interplay in biological processes, by allowing for the targeted mutation of multiple genes simultaneously in a single cell or plant line. This technology has the potential to improve understanding about how synergistic, additive, and/or redundant gene function impacts complex agronomic traits in various plant species, and hence facilitate the development of optimal plant phenotypes. In this study, we focused on the application of the multiplex CRISPR/Cas system in plants to investigate all k-order genetic interactions among x target genes. To that end, it is of primary importance that the plant library contains all possible k-combinations of gene knockouts in the plant design space. Otherwise, one cannot distinguish whether a particular gene knockout combination is missing in the set of plants with a relevant phenotype due to an insufficient plant library size or whether a particular combination of gene knockouts is lethal. Moreover, when only a fraction of all relevant combinations of gene knockouts is represented in the multiplex CRISPR/Cas screen, effective genetic interactions and complex genotype-phenotype associations are discovered merely by chance. To avoid misleading conclusions, the design of such experiments must correct for full coverage of all $\binom{x}{k}$ combinations of gene knockouts in the design space. For this purpose, a sufficient number of plants needs to be included in the plant library by random sampling. Notably, current protocols for multiplex CRISPR/Cas screens in plants lack guidelines in this regard. In this study, the *plant library size* of a multiplex CRISPR/Cas experiment that achieves full coverage was referred to as $N_{x,k}$. The central objective of this study was to develop tools for quantifying the expected value and standard deviation of $N_{x,k}$. Note that we focus on a minimal plant library size guaranteeing the representation of all relevant knockout combinations at least once in the plant library. However, the latter forms a theoretical lower bound to the actual number of plants that is required for a researcher to effectively link gene knockout combinations to qualitative or quantitative phenotypic

effects. In practice, an even larger plant library size will have to be considered to distinguish true effects from phenotypic noise resulting from biological variation and false positive results due to off-target CRISPR/Cas editing activity or spontaneous mutations. In particular, to ensure a minimal level of statistical power, the plant library size for the reliable detection of effects will increase with a higher phenotypic variation among biological replicates.

To contribute to well-informed experimental design of multiplex CRISPR/Cas screens in plants, we provided two approaches to gain insights into the plant library size guaranteeing full coverage of all *k*-combinations of gene knockouts for *x* target genes. First, a simulation-based approach was presented, which repeatedly generates mutated plant lines *in silico* until all $\binom{x}{k}$ gene knockout combinations in the plant design space are fully covered. These simulations reflect subsequent stages throughout a multiplex CRISPR/Cas experiment, modeling several sampling processes starting from the assembly of gRNA/Cas constructs to the CRISPR/Cas-mediated genome editing of plant cells and the collection of a library of mutated plants. In order to closely resemble a realistic scenario, imbalances in the relative abundance of gRNAs and inefficiencies on the level of genome editing and the induction of loss-of-function mutations were taken into account. These parameters can be varied to assess their impact on the expected value and the standard deviation of the plant library size for full coverage. An advantage of the simulation-based approach is its transparency, providing a clear overview of the subsequent stages and associated efficiencies of a multiplex CRISPR/Cas experiment. As a consequence, information regarding the separate stages can be extracted for deeper understanding of, e.g., the composition of the construct library, the distribution of mutations after genome editing and the distribution of gene knockouts in the plant library for a specified set of experimental design parameters. Alternatively, we provided the comprehensive BioCCP-based approach, translating the computation of an adequate plant library size for multiplex CRISPR experiments into a variant of the Coupon Collector Problem (CCP). This higher level of abstraction allows for a quick estimate of the expected value and standard deviation of the minimal number of plants needed for full coverage, yielding immediate insights into the practical feasibility and potential cost and effort of genotyping and phenotyping of a specific multiplex CRISPR/Cas experiment. Both the simulation- and BioCCP-based approach can be easily repurposed for multiplex CRISPR/Cas experiments based on other gRNA delivery methods than *Agrobacterium* transformation of multiplex gRNA/Cas constructs, such as protoplast transfection or particle bombardment (Cunningham et al., 2018; Liang et al., 2018; Toda et al., 2019). Furthermore, they may be extended to account for additional processes and efficiencies associated with a CRISPR/Cas experiment (e.g., vector propagation in a bacterial host, plant cell transformation, and plant regeneration). It should be noted that the BioCCP-based approach offers an approximate solution, as a result of abstracting combinations of gene knockouts into independent modules and neglecting their dependence when multiple gene knockouts are present per plant. The simulation-based approach will deliver an accurate solution, given that the efficiency parameters are well-calibrated and that a sufficient number of repetitions is performed during the simulation. Notwithstanding, the BioCCP-based approach offers a more computationally friendly way for computing $N_{x,k}$, demonstrating speed improvements of more than two orders of magnitude. Therefore, the BioCCP package was employed to develop complementary tools for studying the coverage $\gamma_{x,k}$ of a plant library. As such, the BioCCP-based approach allows to compute the probability of full coverage $P_{x,k}$ w.r.t. the plant library size N for a specific experiment. Further, insights regarding the expected coverage $\mathrm{E}[\gamma_{x,k}]$ w.r.t. a given plant library size can be gained. This precise quantification of the representation of gene knockout combinations in function of plant library size has not yet been systematically addressed in published studies. Both measures facilitate a deeper understanding of adequate plant library sizes for multiplex CRISPR/Cas experiments.

Moreover, we illustrated the impact of several experimental design parameters on the expected value of $N_{x,k}$, improving understanding of how adjustments of design settings can contribute to minimizing the number of plants that should result from a multiplex CRISPR/Cas experiment to achieve full coverage. Importantly, an increasing number of target genes results in an explosion of the combinatorial plant design space and hence also brings about a rapid increase in the associated plant library size guaranteeing its full coverage (**Figure 5**). Equally important is the order of genetic interaction (*k*) one intends to study, since a combinatorial explosion of the design space occurs as higher-order interactions are to be investigated (**Table 3**). The latter clearly indicates that the number of target genes and order of genetic interaction investigated in an experiment when imposing full coverage is strongly constrained by the manageable number of plants for genotyping and phenotyping. Hence, designing a CRISPR/Cas experiment in a naive way might lead to an unfeasible plant library size for covering the combinatorial design space. Most current studies on multiplex CRISPR/experiments investigate genetic interactions among a relatively small pool of 5 to 15 target genes (Ma et al., 2015; Zhang et al., 2016; Jacobs et al., 2017; Shen L. et al., 2017; Miao et al., 2018; Li et al., 2019; Bai et al., 2020; Lin et al., 2020; Rojas-Murcia et al., 2020; Trogu et al., 2021), or intend to assess the individual phenotypic effects of a large number of 50–13,000 genes (Meng et al., 2017; Liu H.J. et al., 2020; Zhang N. et al., 2020; Chen et al., 2022). In these papers, a thorough assessment regarding the coverage of (combinations of) gene knockouts in the CRISPR/Cas screen to determine an adequate plant library size is lacking. We highlight that although the emergence of efficient multiplex CRISPR/Cas systems is rendering the generation of high-order mutant plant libraries technologically realizable (Shen L. et al., 2017; Miao et al., 2018; Stuttmann et al., 2021; Trogu et al., 2021), typical plant library sizes only allow for a limited number of gene knockout combinations to be properly investigated. Additionally, inefficiencies at different stages of the multiplex CRISPR/Cas protocol play a major role, as $N_{x,k}$ increases significantly with: (1) an unequal abundance distribution of

gRNA sequences in the gRNA/Cas construct library, (2) inferior genome editing efficiencies of gRNAs, and (3) a lower fraction of mutations leading to loss-of-function of the gene product, i.e., a reduced global knockout efficiency. These factors form potential bottlenecks in achieving full coverage ($\gamma_{x,k} = 1$) in multiplex CRISPR/Cas screens. It was shown that the effect of unequal abundances and inferior genome editing efficiencies of gRNAs on $N_{x,k}$ can be mitigated by designing more gRNAs per target gene. For the sake of future work in the multiplex CRISPR/Cas field, the quantitative analysis presented in this paper raises awareness about the limitations on the order of genetic interaction that can be investigated among a number of target genes, given that the feasible number of plants in a screening assay is confined and that inefficiencies at several stages of the multiplex CRISPR/Cas protocol are inevitable. Our model provides the opportunity to calibrate all stages of the experiment "on the fly" by injecting the empirically observed gRNA abundances and efficiency distributions into the model and updating all estimates accordingly. It should be acknowledged that there exist additional inefficiencies and bottlenecks in the multiplex CRISPR/Cas protocol that are not included in our models, which might further magnify $N_{x,k}$. For example, if heterozygous and homozygous mutations are considered as separate genotypic states, the combinatorial design space that is to be covered inflates even further.

An additional goal of this work was to suggest experimental design strategies to construct multiplex CRISPR/Cas screens in plants with a lower $N_{x,k}$. First, we proposed the *Split–Select–Combine* strategy, which groups target genes into meaningful subsets based on prior knowledge, resulting in multiple screens with a lower total combinatorial complexity compared to a single screen encompassing all target genes. As a result, full coverage can be reached at a reduced $N_{x,k}$. Second, the *Overshoot–Select–Purify* strategy was presented. Here, the number of knockouts induced per plant is larger than the order of genetic interaction under investigation (k) in order to study multiple k-order genetic interactions per plant. In this manner, one intends to initially span as much as possible relevant combinations of gene knockouts with a limited number of plants. Afterwards, relevant areas in the combinatorial design space can be more thoroughly explored by Mendelian segregation and the minimal causative genotype can be purified. Furthermore, it should be highlighted that, without altering the experimental design strategy, coverage of a multiplex CRISPR/Cas screen can be enhanced by minimizing inefficiencies at several stages of the experiment. For instance, as seen from **Figure 5C**, a more equal distribution of the relative frequencies of the gRNAs in the construct library might substantially lower $N_{x,k}$. Hence, there is a need for approaches to mitigate biases during the synthesis, quantification, and cloning of gRNA sequences that result in specific gRNAs to be over- or underrepresented in a construct library (Wegner et al., 2019; Imkeller et al., 2020). Further, $N_{x,k}$ can be reduced by optimizing the genome editing activities of gRNAs. The latter implies improvements in gRNA design to maximize the genome editing rate and minimize off-target activity, diminishing the occurrence of false negatives and false positives, respectively. Various web-based tools for gRNA design have been developed (Gerashchenkov et al., 2020). Furthermore, the gRNA pool may be enriched for biologically active gRNAs after initial *in vitro* or *in planta* high-throughput screens, and inactive gRNAs may be removed before construct library assembly. In addition, testing multiple nuclease orthologs of the Cas protein to identify highly efficient variants and the use of alternative promotors driving the expression of the Cas nuclease can boost genome editing efficiency (Bortesi et al., 2016; Najm et al., 2018; Hassan et al., 2021). Moreover, it is critical that the gRNAs are designed to specifically target functional protein domains and that the editing outcome results in loss-of-function of the protein, improving the global knockout efficiency. We anticipate that implementing these guidelines will greatly increase effective coverage of relevant gene knockout combinations in multiplex CRISPR/Cas screens in plants as well as contribute to the correct interpretation of these screening experiments.

4. MATERIALS AND METHODS

All code accompanying the simulation- and BioCCP-based approaches was run in Julia-Jupyter Notebook and is available at https://github.com/kirstvh/MultiplexCrisprDOE. Runtime experiments were performed on an Intel core i7 2.60 GHz processor machine with 32 Gbytes of RAM and a 64-bit operating system.

4.1. Simulation-Based Approach

4.1.1. gRNA Relative Frequency Distribution and *in silico* Vector Assembly

During the simulation procedure, each gRNA in the library is assigned a read number by random sampling from the gRNA frequency distribution. This distribution is characterized by a fixed ratio ρ of the frequency of the most abundant gRNA to the frequency of the least abundant gRNA. More specifically, the gRNA frequency distribution is defined as a double truncated normal distribution, with a lower bound of truncation (l) and upper bound of truncation (u) such that $\rho = \frac{l}{u}$. Note that only the ratio $\frac{l}{u}$, and not the exact value of l and u, is crucial, since downstream in the simulation a normalization step is performed on the gRNA reads in order to obtain a series of probabilities that add up to 1 (determining the sampling probability of each gRNA to be included in a vector of the construct library). The expectation of the normal distribution (μ) is set to $\frac{l+u}{2}$ and the standard deviation (σ) is set to $\frac{u-l}{2}$. In the default scenario, the following settings are applied: $l = 50$, $u = 100$, $\mu = 75$, $\sigma = 25$, resulting in $\rho = 2$. The histogram of the gRNA abundances is depicted in **Figure 3A**. Relative frequencies are calculated by normalizing the abundances to add up to 1. Each gRNA/Cas construct is assembled *in silico* by sampling k gRNAs according to a multinomial distribution with the probability of sampling each gRNA being equal to its relative frequency in the gRNA library.

4.1.2. gRNA Genome Editing Efficiency Distribution, Global Knockout Efficiency, and *in silico* Genome Editing

After generating a gRNA/Cas construct *in silico*, this vector is assumed to be transformed and consequently expressed in a target cell with 100% efficiency. The genome editing process is simulated as follows. A genome editing efficiency is attributed to each gRNA. By default, $f_{act} = 90\%$ of the gRNAs is assumed to be highly active. For these active gRNAs, the genome editing efficiency is obtained by sampling from a normal distribution with $\mu = 0.95$ (denoted as $\epsilon_{edit,act}$) and $\sigma = 0.01$. The remaining $(1 - f_{act}) = 10\%$ of all gRNAs is assigned a low activity, drawing a genome editing efficiency from a normal distribution with $\mu = 0.1$ (denoted as $\epsilon_{edit,inact}$) and $\sigma = 0.01$. This sampling procedure is equivalent to drawing genome editing efficiencies from a bimodal distribution (see also the CRISPR/Cas simulation study of Nagy and Kampmann, 2017). To model the induction of mutations in the target cell, for each gRNA present in the construct a value is drawn from a Bernoulli distribution with p equal to the genome editing efficiency ϵ_{edit} of the gRNA. This sample takes the value 1 with probability $p = \epsilon_{edit}$, simulating the effective induction of a mutation, and takes the value 0 with probability $1 - \epsilon_{edit}$, representing the absence of a mutation. After deciding on whether a gRNA has effectively induced a mutation, another Bernoulli distribution is used to model whether a mutation results in a loss-of-function gene knockout. Here, the Bernoulli parameter p is equal to global gene knockout efficiency ϵ_{KO}, which describes the fraction of gene edits resulting in a loss-of-function mutation and which is equal for all gRNAs. By default, ϵ_{KO} is set at a value of 0.8. The foregoing sampling process is executed for each gRNA of a virtually transformed construct in order to determine whether the gRNA has effectively knocked out the target gene in the plant cell. At the end, the corresponding plant is characterized by a specific set of gene knockouts.

4.1.3. Computation of the Expected Value and the Standard Deviation of the Plant Library Size for Full Coverage

Plants are virtually collected and the observed combinations of gene knockouts are stored. The repeated sampling of plant genotypes ends when all genetic interactions of interest have been targeted, i.e., full coverage of all $\binom{x}{k}$ gene knockout combinations in the combinatorial design space is achieved. The plant library size at which this goal is realized, is stored as the $N_{x,k}$ of the current trial of the multiplex CRISPR/Cas experiment. To obtain an expected value and standard deviation of the plant library size for full coverage, each specific multiplex CRISPR/Cas experiment is simulated 500 times.

Note that the relative abundances in the construct library and genome editing efficiencies of the gRNAs are randomly sampled from a distribution. The outcome of this stochastic process is dependent on the seed value that is used to initialize the pseudo-random number generator. Therefore, the series of relative abundances and genome editing efficiencies of the gRNAs, and hence the value of $E[N_{x,k}]$ and $\sigma[N_{x,k}]$, will vary with the chosen seed. Therefore, when investigating the influence of ρ of the gRNA frequency distribution and f_{act} of the genome editing efficiency distribution on $E[N_{x,k}]$ and $\sigma[N_{x,k}]$ in **Figures 4**, **5C,D**, $E[N_{x,k}]$ and $\sigma[N_{x,k}]$ are computed for several series of gRNA frequencies and genome editing efficiencies corresponding to a specific ρ and f_{act}, respectively. The different outcomes for $E[N_{x,k}]$ and $\sigma[N_{x,k}]$ are averaged.

4.2. BioCCP-Based Approach

BioCCP is a general framework focusing on determining sample sizes for screening experiments in combinatorial biotechnology that guarantee full coverage of the design space (Van Huffel et al., 2022). BioCCP requires the input of the total number of distinct modules in a design space, the number of modules per design and the probability distribution of the modules, describing the probability of being included in a design, in order to define the design space and compute its statistical properties (e.g., how many designs should be sampled on average to observe each module at least once, or what the expected coverage of all modules is w.r.t. a given sample size). In the following, we describe the translation of a CRISPR/Cas experiment into the BioCCP framework.

4.2.1. Definition of Inputs

The problem setting of calculating the expected value of the minimal plant library size for full coverage of all single gene knockouts ($E[N_{x,1}]$) in a CRISPR/Cas screen is translated into BioCCP terms as follows. Each plant design is regarded as an assembly of single gene knockouts, and the specific goal is to collect a set of plants that spans each possible gene knockout at least once. Therefore, the number of possible single gene knockouts (which is equal to the number of target genes) is fed into the BioCCP model as the number of distinct modules in the design space that needs to observed at least once. For an experiment targeting single gene knockouts, by default one gRNA is included per gRNA/Cas construct. Accordingly, the number of modules per design is set to the value of 1. The probability to encounter a knockout in a gene (module) is calculated by summing up the probabilities of the relevant gRNAs (the gRNAs specifically designed to target this gene) to induce a knockout in this gene, taking into account the relative frequencies of the relevant gRNAs in the construct library, the genome editing efficiencies of the gRNAs and the global gene knockout efficiency. The relative frequencies and genome editing efficiencies of the gRNAs are sampled according to the distributions described in Sections 4.1.1 and 4.1.2.

For examining the expected plant library size for full coverage of all k-combinations of gene knockouts ($E[N_{x,k}]$) in multiplex CRISPR/Cas screens ($k > 1$), abstraction into BioCCP terms implies the following. First, the number of k-combinations of gene knockouts is considered as the total number of modules to be collected. Secondly, the number of modules per design is set as the number of gRNA combinations per vector in the gRNA/Cas construct library. For instance, a construct library with six gRNA sequences per vector contains 15 pairwise combinations, corresponding to 15 modules per design. The probability to encounter a gene knockout combination (module) in a plant (design) is computed as follows. For each gene knockout combination, the corresponding combinations of

gRNAs are listed. The probability of encountering a combination of gRNAs in the construct library is calculated by multiplying the relative frequency of the individual gRNAs in the construct library and subsequent normalization of all probabilities to add up to one. Thereafter, the genome editing efficiency for each gRNA in the combination and the global knockout efficiency are incorporated to obtain the probability of all gRNA combinations to induce an effective combination of gene knockouts. Finally, the probability of encountering a specific k-combination of gene knockouts is obtained by summing up the probabilities of all corresponding gRNA combinations to induce this particular k-combination of gene knockouts in a plant.

4.2.2. Computation of the Expected Value and Standard Deviation of the Plant Library Size for Full Coverage and Other Relevant Statistics

After definition of the above-mentioned inputs, the BioCCP functions are employed to compute statistics related to the plant library size for full coverage ($N_{x,k}$). The functions `BioCCP.expectation_minsamplesize` and `BioCCP.std_minsamplesize` are used to compute respectively $\mathrm{E}[N_{x,k}]$ and $\sigma[N_{x,k}]$ of a given multiplex CRISPR/Cas experiment. Computation of the expected coverage ($\mathrm{E}[\gamma_{x,k}]$) w.r.t. the plant library size is carried out by applying the `BioCCP.expectation_fraction_collected` function. Finally, the computation of the probability of full coverage ($P_{x,k}$) involves employing the function `BioCCP.success_probability`.

Documentation describing the precise computation of these statistics is available at https://github.com/kirstvh/MultiplexCrisprDOE. This GitHub repository also provides customized functions for automatically converting the characteristics of a multiplex CRISPR/Cas experiment into BioCCP terms/inputs.

DATA AVAILABILITY STATEMENT

The data and source code generated for this study can be found in a GitHub repository (https://github.com/kirstvh/MultiplexCrisprDOE) under an MIT software license. A Galaxy version of the tool is available to be installed at the Galaxy ToolShed and to be used directly at usegalaxy.be.

AUTHOR CONTRIBUTIONS

KV, MS, TR, and BD conceptualized the study. KV wrote the source code for the computational frameworks and performed the simulation experiments. All authors contributed to writing the original draft of the manuscript and approved the submitted version.

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Establishment of an Efficient Genome Editing System in Lettuce Without Sacrificing Specificity

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The efficiency of the CRISPR/Cas9 genome editing system remains limited in many crops. Utilizing strong promoters to boost the expression level of *Cas9* are commonly used to improve the editing efficiency. However, these strategies also increase the risk of off-target mutation. Here, we developed a new strategy to utilize intron-mediated enhancement (IME)-assisted *35S* promoter to drive *Cas9* and sgRNA in a single transcript, which escalates the editing efficiency by moderately enhancing the expression of both *Cas9* and sgRNA. In addition, we developed another strategy to enrich cells highly expressing *Cas9*/sgRNA by co-expressing the developmental regulator gene *GRF5*, which has been proved to ameliorate the transformation efficiency, and the transgenic plants from these cells also exhibited enhanced editing efficiency. This system elevated the genome editing efficiency from 14–28% to 54–81% on three targets tested in lettuce (*Lactuca sativa*) without increasing the off-target editing efficiency. Thus, we established a new genome editing system with highly improved on-target editing efficiency and without obvious increasement in off-target effects, which can be used to characterize genes of interest in lettuce and other crops.

Keywords: genome editing, CRISPR/Cas9, intron-mediated enhancement, *GRF5*, lettuce

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INTRODUCTION

The CRISPR/Cas9 system is a powerful genome editing tool that has been widely used in the past decade (Gao, 2021). With the complementary base pairing mechanism, the Cas9 endonuclease is guided to the specific DNA sequence by the guide RNA (gRNA), and generates double-stranded DNA breaks (DSBs) at the desired loci. Predominantly, the DSBs are repaired by the error-prone non-homologous end joining (NHEJ) pathway, which introduces insertions/deletions (indels) that range from one to hundreds of base pairs, that could lead to site-specific genetic alterations (Gao, 2021; Hassan et al., 2021). Until today, this technology has been successfully used to generate mutant plants and for agronomic trait enhancement in many crops. Nevertheless, the editing efficiency remains quite limited in several vegetable and crop plants.

Boosting the expression of *Cas9* or sgRNA is the major method to improve the genome editing efficiency (Castel et al., 2019; Hassan et al., 2021). Several studies have utilized strong promoters, such as the *RPS5A* promoter (Tsutsui and Higashiyama, 2017; Castel et al., 2019; Ordon et al., 2020; Oh and Kim, 2021), the *UBQ10* promoter (Wang and Chen, 2019; Wolabu et al., 2020), and

the *MAS* promoter (An et al., 2021), to strengthen the expression level of *Cas9*, which leads to increasements in the genome editing efficiency. Also, the augmentation of sgRNA level, by using native U6/U3 promoters (Sun et al., 2015; Ren et al., 2021), or by using Pol II promoters such as the *ubiquitin* promoter (Ding et al., 2018), or the *cestrum yellow leaf curling virus* (*CmYLCV*) promoter (Cermak et al., 2017; Li et al., 2021), escalates genome editing efficiency. Also, the *Cas9* with multiple introns efficiently generates more mutants than the conventional *Cas9* (Grutzner et al., 2021). However, these strategies also increase the risk of off-target mutation, which might interfere phenotypic analysis of desired genes and more severely hinder deregulation and commercial release of genome-edited crops.

Intron-mediated enhancement (IME) is a well-known phenomenon to enhance homogeneous protein expression in plants and animals (Vain et al., 1996; Laxa, 2016). The introns located in the 5′-UTR region from several strong and constitutive genes, such the first intron of *UBQ10, ACTIN, TRP1* (Rose, 2004; Jeong et al., 2009), have been proved to greatly improve the expression of downstream gene. For example, the first intron of maize *ubiquitin 1* (*ZmUbi1*) located in the 5′-UTR region combines with CaMV *35S* promoter leads to a over 90-fold increasement of gene expression in maize and bluegrass (Vain et al., 1996). Many works have attempted to identify the key cis-elements in this process, but the detailed mechanism is still not clear, since it has been found that the sequence and splicing process are not the key feathers of these introns (Rose and Beliakoff, 2000; Rose, 2004; Back and Walther, 2021). Thus, it's promising to engineer these introns to enhance the strength of the promoters that drive the expression of the CRISPR/Cas9 system.

Several *DR* (*DEVELPMENTAL REGULATOR*) genes, such as the *WUS* (*WUSCHEL)*, *BBM* (*BABY BOOM*) and *GRFs* (*GROWTH-REGULATING FACTORs*), have been proved to improve the transformation efficiency (Lowe et al., 2016; Debernardi et al., 2020; Kong et al., 2020; Qiu et al., 2022). Ectopic expression of the *BBM* gene, which is originally identified in *Brassica napus*, has diverse functions in plant cell proliferation, growth and development (Jha and Kumar, 2018). The co-expression of *BBM* with *WUS* greatly boosts the transformation efficiency of several monocot species, including rice, maize and sorghum (Lowe et al., 2016). Several plant-specific GRF transcription factors have successfully elevated the regeneration and transformation efficiency of crop plants, such as soybean, canola, and sunflower (Kong et al., 2020; Pan et al., 2022). The overexpression of a chimeric protein consisting of the GRF4 and GRF-interacting factor 1 (GIF1) proteins reinforce the regeneration efficiency and regeneration speed in wheat, triticale, rice and watermelon (Debernardi et al., 2020; Feng et al., 2021; Qiu et al., 2022). However, the effect of these DRs on the genome editing efficiency in the regenerated plants has not been investigated.

Lettuce is one of the most popular vegetable crops that is cultivated worldwide (Su et al., 2020; Assefa et al., 2021). The substantial amounts of ascorbic acid, vitamin A, carotenoids, folate, and other primary and secondary metabolites are beneficial to human health (Assefa et al., 2021). However, the candidate genes behinds these traits are poorly investigated. The CRISPR/Cas9 genome editing system, which is powerful and promising in generating the desired mutants and in crop breeding, has been utilized in the studies of lettuce in recent years (Bertier et al., 2018; Zhang et al., 2018; Luo et al., 2021). However, an improved and efficient genome editing has not been established for lettuce and is urgently needed.

In this work, we established an intron-mediated enhancement-based strategy to increase the expression of *Cas9* and sgRNA, and also tested the effect of *GRF5* on lettuce (*Lactuca sativa*) transformation and genome editing. These two methods successfully boosted the genome editing efficiency from 14–28% to 54–81% on three targets tested in lettuce without increasing the off-target editing efficiency.

RESULTS

IME-Mediated Enhancement of Cas9 and sgRNA Expression

First, we decided to moderately enhance the expression of both *Cas9* and sgRNA through IME. Previous studies have successfully engineered the sgRNA expression cassette tRNA-sgRNA-tRNA into the first intron of *ZmUbi1*, thus the mature sgRNA can be generated by the endogenous tRNA-processing system (Xie et al., 2015; Zhong et al., 2020). We generated pZKD672 by inserting this engineered intron (**Supplementary Table 1**) into a *35S* promoter-driven Cas9 expression cassette (**Figure 1A**). In this way, Cas9 and sgRNA were driven by an IME-assisted *35S* promoter and co-expressed from a single transcriptional unit. We speculated that the expression levels of both *Cas9* and sgRNA could be improved. The pKSE401 vector, in which *Cas9* and sgRNA are respectively driven by *35S* promoter and *Arabidopsis U6-26* promoter (Xing et al., 2014), was used as the control.

To test our hypothesis, we utilized pKSE401 and pZDK672 to construct an sgRNA targeting *LsPDS* (*PHYTOENE DESATURASE*) in lettuce, and examined the expression of *Cas9* and sgRNA in lettuce protoplasts. Compared with those from pKSE401, transcript levels of *Cas9* and sgRNA from pZDK672 were increased by 0.74- and 1.42-fold, respectively (**Figures 1B,C**), proving the power of IME in moderately enhancing the expression of Cas9 and sgRNA.

IME Boosts the Genome Editing Efficiency in Transgenic Lettuce Plants

To investigate whether the pZDK672 could elevate the editing efficiency in stable transgenic plants, we selected *LsPDS* and two additional target genes, *LsBIN2* (*BR-INSENSITIVE 2*) and *LsGGP2* (*GDP-L-GALACTOSE PHOSPHORYLASE*) to generate stable transgenic lettuces through *Agrobacterium*-mediated transformation (Zhang et al., 2018). The average mutation efficiencies by the pZKD672 were 38.59, 41.85, and 24.23% for *LsPDS*, *LsGGP2*, and *LsBIN2* in 3 biological repeats, respectively, while only

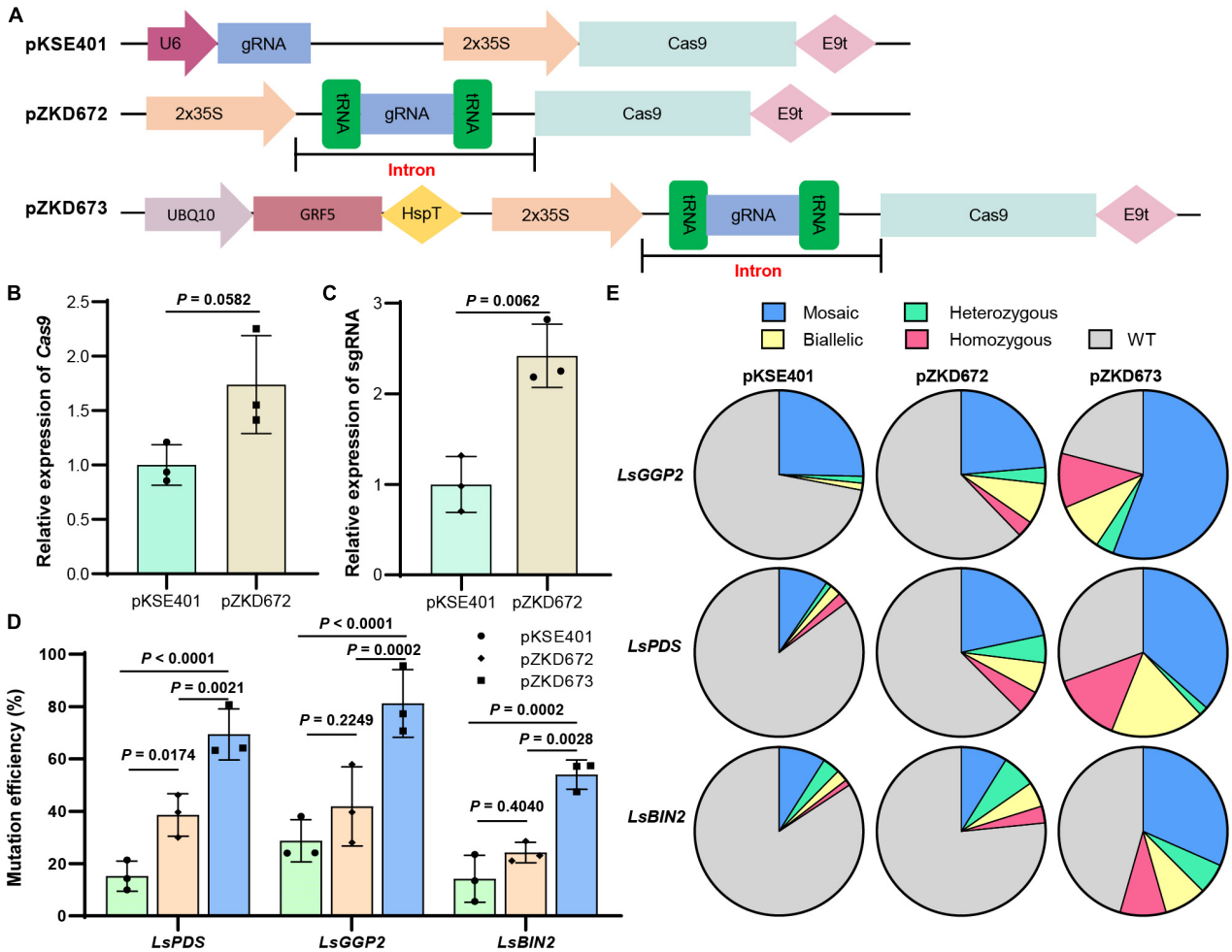

FIGURE 1 | Boost genome editing efficiency through intron-mediated enhancement and *GRF5* co-expression. **(A)** The schematic diagram of the vectors. In the conventional genome vector pKSE401, sgRNA is driven by the *U6* promoter, and *Cas9* is driven by the CaMV *35S* promoter. In pZKD672, engineered intron contain sgRNA was used to boost *Cas9* and sgRNA expression through the intron-mediated enhancement mechanisms. The endogenous tRNA-processing system was used to generate mature sgRNA. In pZKD673, the *GRF5* driven by the *UBQ10* promoter was added to the genome editing vector to facilitate the screening of plants with high expression levels. **(B,C)** The expression level of *Cas9* **(B)** and sgRNA **(C)** in lettuce protoplasts using the pKSE401 or the pZKD672 vector. *LsACT* was used as the internal control. The *P* value was calculated with paired two-tailed Student's *t*-test. **(D)** The mutation ratio of three tested target sites using the indicated vectors in transgenic lettuce plants in the T0 generation. The *P* value was calculated with Two-way ANOVA test. **(E)** The proportion of different mutation types among all the transgenic plants from three replicates.

15.24, 28.74, and 14.20% transgenic plants were mutated by pKSE401 (**Figure 1D** and **Supplementary Tables 2**, **3**), with 1.53-, 0.80-, and 0.71-fold increasement. It suggests that the moderate magnification in the expression of *Cas9* and sgRNA through IME resulted in a weak augmentation in editing efficiency.

Optimizing the Genome Editing Efficiency by the *GRF5* Co-expression

Next, we hoped to optimize pZKD672 to further elevating its editing efficiency. The plant genetic transformation often generates populations with diverse gene expression levels. In the plant genome editing processes, the cells with higher expression levels of *Cas9* and sgRNA, which leads to higher mutation rates, are the desired ones for regeneration. Direct enrichment of these cells or plants could also increase the mutation efficiency, such as the *GLABRA2* mutation-based visible selection (GBVS) system which adds the *GL2* target as a visible selection marker to identify plants with high mutation efficiency (Kong et al., 2021). However, mutation of a second gene might be a concern for crop breeding. This prompted us to explore novel strategies to enrich these cells. Several *DRs* have been proved to improve the transformation efficiency by promoting the somatic embryogenesis or regeneration rates (Lowe et al., 2016; Kong et al., 2020; Qiu et al., 2022). They have been widely used in plants recalcitrant to transformation (Kong et al., 2020; Pan et al., 2022; Qiu et al., 2022). However, the effect of these *DRs* on the editing efficiency of recipient plants during stable transformation had not yet been investigated. We surmised

that the expression level of *DRs* should be correlated with that of *Cas9* and sgRNA when they were constructed in a single T-DNA. The cells, highly expressing *DRs*, *Cas9* and sgRNA, could gain an advantage over other cells to redifferentiation, hence co-expression of DRs could elevate mutation rate during stable transformation. To verify our speculation, we added an *Arabidopsis GRF5* overexpression cassata (**Supplementary Table 1**) to pZKD672 to generate pZKD673 (**Figure 1A**). pZKD673 with corresponding target spacers were also transformed into lettuce. The mutation efficiencies of *LsPDS, LsGGP2*, and *LsBIN2* by pZKD673 are 69.38, 81.22, and 54.00% in three biological replicates, respectively, and exhibit 0.80-, 0.94-, and 1.23-fold increase, compared with 38.59, 41.85, and 24.23% by pZKD672, respectively (**Figure 1D** and **Supplementary Tables 2**, **3**). To further prove our postulate, we randomly selected about 24 transgenic plants for each vector and mixed them into 3 samples to check the expression level of *Cas9*. The result showed that the expression of Cas9 in pZKD673 transgenic plants is about 2.58-fold higher than the pZKD672 vector (**Supplementary Figure 1**). Our data indicate that co-expressing *GRF5* could improve the editing efficiency of pZKD672.

Among all the T0 transgenic plants, the ratio of null mutants (homozygous and biallelic) was also increased. For example, the amount of *lspds* null mutants was raised from 4.23% for pKSE401 to 10.53% for pZKD672 and 31.21% for pZKD673 (**Figure 1E** and **Supplementary Table 3**). These results demonstrated that our new vectors could generate more null mutants, which are suitable for phenotyping or breeding in the offspring, and are labor- and time-saving.

Off-Target Analysis of the New Genome Editing Vectors

Finally, to evaluate off-targeting efficiency, five predicted highly risky off-target sites for each target gene were identified through the CRISPOR program[1] (Haeussler et al., 2016), and 20 on-target mutant lines for each plasmid were examined (except for the *LsBIN2* by the pKSE401, with only 14 mutants obtained). No off-target mutation was detected, even at the off-target 1 (OT1) and OT2 of *LsGGP2*, and the OT1 of *LsBIN2*, which have 2 mismatches with the corresponding target sequence (**Table 1** and **Supplementary Table 4**). These results indicated our newly established systems do not increase off-target efficiency.

DISCUSSION

In this study, we established a new genome editing system for creating mutations with high frequency in lettuce. With an intron expressing the sgRNA, and GRF5-mediated enrichment, we dramatically boosted the mutation efficiency compared with the commonly used vector in transgenic lettuce plants.

[1]http://crispor.tefor.net/crispor.py

Successful engineering of introns to express sgRNA has been reported in other studies (Ding et al., 2018; Zhong et al., 2020). In these studies, the intron is inserted into the 5′-UTR or within the coding region of *Cas9*. All these experiments were conducted in rice, and compared to traditional genome editing vectors, these sgRNA containing introns didn't significantly improved the editing efficiency (Ding et al., 2018; Zhong et al., 2020). This is probably because that the rice *UBQ10* promoter or the maize *Ubi* promoter is used to drive Cas9. These Ubiquitin promoters itself contains the introns with IME. Therefore, additional adding of another IME introns probably doesn't make functions. In this work, the genome editing is conducted in a dicot plant lettuce, and the most widely used *35S* promoter is used to drive Cas9. It has been well proved that adding an IME intron could significantly boost the power of *35S* promoter (Vain et al., 1996; Laxa, 2016).Our result proved that this modified intron could indeed improve the activity of the *35S* promoter that expresses the *Cas9*. And it should be pointed out that the IME intron strategy might not be applicable to all the genome editing vectors, and not all the plant species.

In order to generate mature sgRNAs within the intron, the tRNA sequence was placed upstream and downstream of the spacer-sgRNA sequence. It has been well proved that these polycistronic gene can be processed by the endogenous tRNA-processing enzyme RNAse Z and RNAse P (Xie et al., 2015; Zhong et al., 2020). Successfully genome editing of endogenous targets in our experiments also confirmed these results. In addition to the tRNA-processing system, other sgRNA processing system, such as the dual HH-HDV ribozyme system (Gao and Zhao, 2014), or the sequence-specific RNase Csy4 (Przybilski et al., 2011), has also been used to express sgRNA in plants. In these experiments, the tRNA-processing system enables efficient sgRNA expression by the Pol II promoters, and efficient multiplex genome editing (Xie et al., 2015; Li et al., 2021). Also, the tRNA-processing system exhibited higher or comparable processing efficiency and mutation rates than the ribozyme system and the Csy4 system in these experiments (Tang et al., 2019; Hsieh-Feng and Yang, 2020; Zhong et al., 2020). Thus, our new vectors are promising in efficient multiplex genome editing in lettuce, and other dicot plants.

The power of DRs in genetic transformation has been observed in many plant species. With the assistance of DRs such as *GRF5*, efficient and genotype-independent transformation can be achieved without obvious growth abnormities, and this system has been used to generate mutants by the CRISPR/Cas9 genome editing system (Debernardi et al., 2020; Pan et al., 2022). In our previous experiments, we demonstrated that *GRF5* outperforms other DR genes, such as *GRF4-GIF1*, *BBM* and *WUS*, in the genetic transformation of watermelon (*Citrullus lanatus*) (Pan et al., 2022). However, the effect of GRF5 on the genome editing efficiency has not been investigated. In our work, we proved that overexpressing the *GRF5* gene could dramatically elevate the genome editing efficiency. We suspect that this is a transgenic enrichment effect: as the GRF5 and Cas9 are constructed in a single T-DNA, efficient expression of

TABLE 1 | The off-target analysis results.

Target	Off-Target sites	Sequence (5′–3′)	No. mismatch	CFD Score[a]	No. off-target mutant from 20 on-target mutant plants[b]		
					pKSE401	pZKD672	pZKD673
LsPDS	On-target	GGCCACCGAGTGACTCGATGTGG	0	1			
	OT1	GtCCACtagGTGACTCGATGAGG	4	0.34	0/20	0/20	0/20
	OT2	GGCtACCtAGTGACaCaATGTGG	4	0.27	0/20	0/20	0/20
	OT3	GGCCACttAGTGtCaCGATGTGG	4	0.08	0/20	0/20	0/20
	OT4	GGCCACttAGTGcCaCGATGTGG	4	0.06	0/20	0/20	0/20
	OT5	GGCCACCcAGgGcCTCGAgGGGG	4	0.01	0/20	0/20	0/20
LsGPP2	On-target	ACGACAAGTTGCAGACATCACGG	0	1			
	OT1	ACGAgAAGTTGaAGACATCAAGG	2	0.42	0/20	0/20	0/20
	OT2	ACGAgAAGTTGaAGACATCAAGG	2	0.42	0/20	0/20	0/20
	OT3	AtGAgtAGTTGCAGACATCATGG	3	0.39	0/20	0/20	0/20
	OT4	ACcAaAAGTTGCAGACATgAAGG	3	0.02	0/20	0/20	0/20
	OT5	tCGAgAAGTTaaAGACATCAAGG	4	0.42	0/20	0/20	0/20
LsBIN2	On-target	ATCACAGTGATGCTCGTCAAAGG	0	1			
	OT1	ATCACAGTGcgGCTCGTCAAGGG	2	0.4	0/14	0/20	0/20
	OT2	caCACAGTGATGtTCGTCAAGGG	3	0.5	0/14	0/20	0/20
	OT3	taCACAaTGtTGCTCGTCAACGG	4	0.74	0/14	0/20	0/20
	OT4	caCACAGTGATGtTCaTCAAAGG	4	0.5	0/14	0/20	0/20
	OT5	ATaACAaTGAaGCTCGTtAATGG	4	0.41	0/14	0/20	0/20

[a] *The CFD score indicates the potential of off-target editing (Haeussler et al., 2016).* [b] *Only 14 mutants were obtained by the pKSE401 vector at the LsBIN2 site.*

Cas9 should co-relate with efficient expression of DRs, which facilitates the regeneration process. Thus, most of the transgenic plants we obtained with the pZKD673 vectors should have higher mutation efficiency. Our observation that the expression level of *Cas9* is indeed higher in pZKD673 transgenic plants than the pZKD672 transgenic plants confirmed our hypothesis. Very recently, another group also observed the same mutation efficiency increasement by co-expressing *WUS* in sorghum (Che et al., 2022), but the detailed mechanism hasn't been revealed. These works revealed the power of DRs, not only on genotype-independent genetic transformation, but also in efficient genome editing.

With great improvement in the genome editing efficiency, we can easily obtain large number of mutants. And the efficiency amplification also leads to higher ratio of homozygous and biallelic mutants in the T0 generation. What's more, we didn't observe significant increasement in the off-target mutation efficiency. Thus, these homozygous and biallelic mutants could directly be used for phenotype analysis and functional verification, which could save plenty of time and efforts.

In summary, we utilized two novel strategies, IME-mediated the moderate enhancement of *Cas9*/sgRNA expression and *DR* gene-associated transgene enrichment, to establish a highly efficient plant genome editing system without obvious off-targeting increase. These strategies could also be applied in other genome editing tools, such as base-editors and the prime-editors, and other crop species, to boost the editing efficiency.

MATERIALS AND METHODS

Plant Materials

The *L. sativa* L. var. capitata 101 was bought from Jingyan Yinong (Beijing) Seed Sci-Tech Co., Ltd. Plants were grown under a photoperiod of 16 h light (150 μmol m^{-2} s^{-1}) and 8 h dark at 25°C.

Vector Construction

The PTG sequence and codon optimized GRF5 coding sequence were synthesized at Sangon Biotech. The conventional CRISPR/Cas9 vector pKSE401 (Xing et al., 2014) were used as the control. The pKSE401 vector was first digested by *Hin*dIII, and the 14.5 kb backbone were ligated by T4 DNA ligase. The product was then digested by *Xba*I, and Gibson assembled (Sangon Biotech) with the PTG product amplified with the primer pair PTG-F/PTG-R, generating the pZKD672 vector.

The Arabidopsis UBQ10 promoter, the Arabidopsis Hsp terminator and codon optimized GRF5 coding sequence were amplified with UBQ10p-F/UBQ10p-R, HspT-F/HspT-R and GRF5-F/GRF5-R, respectively. The PCR products were then used as the template and amplified with UBQ10p-F/HspT-R. The 2.75 kb produce were then Gibson assembled with *Hin*dIII digested pZKD672, generating the pZKD673 vector.

The pKSE401, pZKD672, and pZKD673 vector were digested with *Bsa*I, and ligated with annealed target oligos.

The primers were listed in **Supplementary Table 5**.

Protoplast Transfection and Analysis

The lettuce protoplast preparation and transfection were performed according to a previous established method (Woo et al., 2015) with some modifications. Briefly, the heart of *L. sativa* L. var. capitata L 101 was sliced with double sides razor blades. The leaves were then digested with 1% Cellulase R10, 0.25% Macerozyme R10, 0.4 M Mannitol, 20 mM KCl, 20 mM MES pH 5.7, 20mM KCl for about 4 h. the enzyme solution was filtered with Miracloth (CALBIOCHEM), and collected by Centrifuged for 1 min at 100 *g*. The protoplasts were washed twice by 10 mL W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7. Then the protoplasts were suspended by 10 mL W5 solution and stand on ice for 30 min. The supernatant was removed and the MMG solution (0.4 M Mannitol, 15 mM $MgCl_2$, 4 mM MES pH 5.7) was added to a final concentration of 2×10^4 to 2×10^5 cell/ml.

Ten microgram vector were mixed with 200 μL protoplast and mixed gently. Then 220 μL PEG/$CaCl_2$ solution (40% PEG4000, 0.2 M Mannitol, 100 mM $CaCl_2$) was added and mixed gently. The transfection was performed at room temperature for 15 min and stopped by adding 1.5 mL W5 solution and mixed gently. The protoplast was then washed by 1 mL W5 solution and risen in 200 μL. Then the protoplasts were kept at dark for 2 days at 24°C.

The protoplasts were collected and total RNA were extracted with the Ultrapure RNA Kit (CWbio). The RNA was reverse transcript with the FastQuant RT Kit (With gDNase) (Tiangen) with some modification: 0.5 μL 10 μM qRT-LsPDS-R primer was added to a final 20 μL reverse transcription mixture. The quantitative real-time PCR were performed with CFX Opus real-time PCR system (BioRad) with the Talent qPCR PreMix (SYBR Green) (Tiangen). The corresponding primers were listed in supplementary **Table 5**.

Lettuce Transfection

The protocol for lettuce transfection was previously described (Zhang et al., 2018). In brief, surface sterilized lettuce seeds were placed on MS medium and incubated under a photoperiod of 16 h light (150 μmol m^{-2} s^{-1}) and 8 h dark at 25°C. The cotyledons were excised from germinated seedlings and incubated for 10 min with the *Agrobacterium* (EHA105) suspension carrying the desired construct. The treated explants were placed on MS co-cultivation medium (MS supplemented with 30 g L^{-1} sucrose, 0.8% plant agar, 0.1 mg L^{-1} α-naphthalaneacetic acid, and 0.5 mg L^{-1} 6-benzylaminopurine) and incubated at 25°C in dark for 48 h.

Afterward, explants were transferred to MS selection medium (MS supplemented with 30 g L^{-1} sucrose, 0.8% plant agar, 0.1 mg L^{-1} α-naphthalaneacetic acid, 0.5 mg L^{-1} 6-benzylaminopurine, 40 mg/l kanamycin monosulfate, and 250 mg L^{-1} carbenicilin), and incubated under a 16 h light/8 h dark cycle at 25°C. After about 25 days, regenerated shoots were excised and transferred to MS rooting medium (1/2 MS supplemented with 15 g L^{-1} sucrose, 20 mg L^{-1} kanamycin monosulfate, and 250 mg L^{-1} carbenicilin) for root induction. The plantlets with well-developed shoot and root were transferred to soil and further examined.

Analysis of the Genome Editing Efficiency and Potential Off-Target Edits

The genomic DNA of regenerated lettuce plants was extracted with the CTAB method. Positive transgenic plants were examined with the Cas9-check-F2/Cas9-Check-R2 primer pair. The target regions were amplified with corresponding primer pairs, and the analyzed with Sanger sequencing. The sequencing chromatogram were decoded with the TIDE program[2] (Brinkman et al., 2014).

The potential off-target editing sites were chosen through the CFD score in the CRISPOR program[3](Haeussler et al., 2016). For each vector, 20 mutant plants were randomly chosen and the target regions were amplified with the corresponding primer pairs. The PCR products were analyzed with Sanger sequencing.

The primers were listed in **Supplementary Table 5**.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/**Supplementary Material,** further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

HZ conceived the study and agreed to serve as the author responsible for contact and ensures communication. HZ and DL supervised the research. WP and XL performed all experiments and analyzed the data with help from HZ and DL. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.930592/full#supplementary-material

[2]http://shinyapps.datacurators.nl/tide/

[3]http://crispor.tefor.net/crispor.py

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USDA's revised biotechnology regulation's contribution to increasing agricultural sustainability and responding to climate change

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Biotechnology can provide a valuable tool to meet UN Sustainable Development Goals and U.S. initiatives to find climate solutions and improve agricultural sustainability. The literature contains hundreds of examples of crops that may serve this purpose, yet most remain un-launched due to high regulatory barriers. Recently the USDA revised its biotechnology regulations to make them more risk-proportionate, science-based, and streamlined. Here, we review some of the promising leads that may enable agriculture to contribute to UN sustainability goals. We further describe and discuss how the revised biotechnology regulation would hypothetically apply to these cases.

KEYWORDS

genome editing, regulatory policy, genetic engineering, plant biotechnology, environmental protection

Introduction

The UN Sustainable Development Goals (SDG) are critically important for humanity and the planet (United Nations, 2015). Agricultural innovation can positively contribute to many of these goals such as ending hunger, promoting sustainable agriculture or clean energy, to name a few (Secretary-General, 2019). Through Executive Order #14008 (Executive Office of the President, 2021), the Biden Administration prioritized building a modern sustainable infrastructure and an equitable clean energy future demonstrating a commitment to many of these same goals.

Biotechnology has contributed to agricultural sustainability through traits that reduce over the top insecticide application. (Brookes and Barfoot, 2017). Regulatory barriers have limited both diverse trait development and developers who use biotechnology

(Bradford et al., 2005; Hoffman, 2021). In May 2020, the U.S. Department of Agriculture (USDA) issued revised biotechnology regulations that offer a more risk-proportionate approach and are expected to spur innovation while ensuring products are safe for agriculture and the environment. Below, we review key changes in the revised regulations, illustrate their application using examples of promising leads in the literature, and show how they are likely to spur trait development that hold promise for improving agricultural sustainability and addressing some climate challenges.

Changes in USDA's revised biotech regulation

Under the revised regulations, and using Plant Protection Act authority, USDA considers whether an organism developed using genetic engineering poses an increased plant pest risk relative to a suitable comparator (USDA-APHIS, 2020). Several key changes contribute to more risk-proportionate regulation for plants created using genetic engineering. First, the revised regulations establish three exemptions for certain modifications a plant developed using genetic engineering may contain.[1]

These exemptions are based on types of modifications that commonly occur during conventional breeding (USDA-APHIS, 2020; Hoffman, 2021). Basing the exemptions on specifically described modifications, rather than a risk assessment, allows developers to objectively assess whether their plants meet the criteria exemption.

Second, the revised regulations establish an exemption for a plant-trait-mechanism of action (MOA) combination that USDA previously reviewed and determined not to pose a plant pest risk. This provision eliminates the burden of unnecessary re-reviews of plants whose risks were already considered.[2]

Third, the revised regulations establish a risk-based approach called regulatory status review (RSR) to determine whether a non-exempt plant is regulated. A key difference between RSR and the previous petition process is that RSR uses problem formulation and risk assessment to evaluate the characteristics and risk of the plant prior to a formal determination to continue to exercise oversight (Hoffman, 2021). Under the revised regulation, a developer can elect to undergo the RSR process prior to field testing. USDA expects that the RSR process will be an effective means to winnow the potentially riskier plants developed using genetic engineering from the less risky ones without imposing substantial regulatory burdens on the less risky ones (Hoffman, 2021). Among other things, USDA considers whether a genetic modification to a plant will increase the likelihood of harm to non-target species beneficial to agriculture or will increase the distribution or exacerbate the impact of plant pests that may be associated with that plant. USDA will undertake an initial review to efficiently distinguish plants developed using genetic engineering that do not pose plausible pathways to increased plant pest risk from those that do and, thus, require further evaluation. This initial review, which is based on a description of the plant, the trait, and the MOA, does not initially require field data. When USDA finds a plant does not pose plausible pathways to increased plant pest risk during the initial review phase, developers can attain regulatory certainty early in product development, which helps developers raise venture capital to see a product through to launch.[3]

Biotechnology crop development under the legacy regulation

In 2008-2012, the mean cost for discovery, development, and authorization of a new crop created using genetic engineering was $136 M, where regulatory requirement costs averaged $35.1M, and the mean duration to bring a crop to market was 13.1 years (McDougall, 2011). The high-cost developments and long duration reduce return on investment. Consequently, the technology has principally been used on large acreage crops (corn, soybean, cotton, alfalfa, sugarbeet, potato, and canola) engineered with relatively few traits. Of the 136 petitions granted non-regulated status by the USDA, 109 were from those 7 major crops, and 80 had no traits other than either herbicide or insect resistance[4]. Under the legacy regulations, not all crops created with genetic engineering fell under the regulations. In 2010, USDA instituted a process known as "Am I Regulated" (AIR process), which provided a voluntary mechanism for developers to obtain USDA's opinion about whether a plant was subject to

1 The SECURE rule (15) exempts plants containing a single modification where1) "the genetic modification is a change resulting from cellular repair of a targeted DNA break in the absence of an externally provided repair template; or2) the genetic modification is a targeted single base pair substitution; or3) the genetic modification introduces a gene known to occur in the plant's gene pool or makes changes in a targeted sequence to correspond to a known allele of such a gene or to a known structural variation present in the gene pool.

2 Developers can request a confirmation from APHIS that a modified plant qualifies for an exemption and is not subject to the regulations in 7 CFR part 340. USDA APHIS | Confirmation Letters accessed 09.27.22

3 USDA APHIS | Plant-Trait-Mechanism of Action (MOA) combinations that have been determined by APHIS not to require regulation under 7 CFR part 340.accessed 09.27.22. USDA APHIS | Confirmation Letters accessed 09.27.22

4 USDA APHIS | Petitions for Determination of Nonregulated Status accessed 09.27.22

regulation[5]. During the last 10 years (2011-2020), the number of organizations using the AIR process increased nearly 4 fold relative to the petition process, while the number of different crops considered increased greater than 5 fold (Hoffman, 2021), suggesting the legacy regulation created a significant barrier to market development of any crop that fell under the regulations.

We expect that the new more risk-appropriate regulation will lead to the commercialization of additional crops and traits. Current literature shows hundreds of proofs of concept for traits with potential to meet SDGs. The examples we provide: exclude plants currently subject to regulation as most have associated Confidential Business Information claims; only include crop plants tested either in the field or greenhouse; and include one representative example when the same plant-trait-MOA was discussed in more than one paper. For each example, we considered whether the plant would likely qualify for an exemption (Table 1) or likely be evaluated under the RSR process (Table 2) based on the information in the paper (Supplementary Table 1 provides additional information on the MOA). Importantly, these considerations are meant to give an approximation of traits relevant to sustainability goals that are ripe for development; they are not and should not be construed as regulatory decisions since we may be missing key details.

Traits that may promote sustainable agriculture and/or mitigate adverse impacts of climate change

Producing more with fewer resources

Producing more food with fewer resources directly supports SDGs associated with reducing poverty, ending hunger, climate action, and conservation (SDGs #1, #2, #6, #13, and #15).

Knockout (KO) strategies in corn, rice, soybean, tomato, and wheat have been used to delete quantitative trait loci (QTL) negatively associated with yield (Zhou et al., 2019), a subfamily of ABA receptors (Miao et al., 2018), or genes that change plant architecture to allow denser planting (Tian et al., 2019), changes in spike inflorescence architecture (Wang et al., 2022), simultaneous increases in panicle number and tiller number (Song et al., 2022; Huang et al., 2018), timing of flowering (Cai et al., 2020), or result in more fruit and less shoot per plant (Rodríguez-Leal et al., 2017). Such plants are likely exempt from regulation and have been shown to increase yield in initial studies without increasing inputs.

USDA would evaluate crops developed with transgenic modifications under the RSR process, as in the case of tobacco engineered for increased production through reduced photorespiration (Cavanagh et al., 2022) or acceleration of the relaxation of non-photochemical quenching during sun-shade transitions (De Souza et al., 2022). Likewise, KOs of all homoeologous alleles in polyploids that increase production would be evaluated under the RSR process, as in the case of canola (Yang et al., 2018; Karunarathna et al., 2020; Zheng et al., 2020) and tef (Beyene et al., 2022). Diploid rice with a KO of 3 different cytochrome P-450 genes to increase production (Usman et al., 2020b) may qualify for exemption depending on whether the individually exempt traits are stacked by breeding (which could be exempt) or molecularly (which would require RSR). Overexpression of a transcription factor in rice was shown to increase both yield and resistance to blight by Xanthomonas (Liu et al., 2019).

Reduced postharvest losses

The Food and Agriculture Organization (FAO) estimates that 14% of the world's food is lost from production before reaching the retail level (FAO, 2019). Reducing postharvest losses could increase food availability and could free land for conservation (SDGs #12, #2, #15). Developers have used genome editing to increase a tomato's shelf life by either KO of pectate lyase (Uluisik et al., 2016), polygalacturonse (Nie et al., 2022) or by recreating the allele of a known delayed fruit deterioration mutation (Yu et al., 2017). Each of these tomato varieties would likely qualify for exemption.

Increased disease tolerance

FAO estimates that annually 20-40 percent of global crop production is lost to pests (FAO, 2019). Increasing disease tolerance could result in increased food and could reduce the volume of crop protection chemicals applied for disease control supporting SDGs related to protecting human health, the land, and water related ecosystems (SDGs #2, #3, #15, #6).

Pathogens often exploit susceptible plant genes to facilitate their infection (Van Schie and Takken, 2014). KOs have been used on a variety of susceptible genes to increase disease tolerance in apple (Pompili et al., 2020), barley (Hoffie et al., 2022), canola with the susceptibility gene only in the A genome (Pröbsting et al., 2020), cassava (Gomez et al., 2019), corn (Liu et al., 2022), cucumber (Chandrasekaran et al., 2016), rice (Zhou et al., 2018), tomato (Nekrasov et al., 2017), and watermelon (Zhang et al., 2020a) (Table 1). Increased resistance to wheat stem rust Ug99 was conferred into wheat (*T. aestivum*) by introducing resistance genes from either einkorn wheat (*T. monococcum*) (Chen et al., 2018) or durham wheat *T. turgidum* (Zhang et al., 2017b), both of which are in the wheat gene pool. These examples and others in banana, rice and tomato (Tripathi et al. 2019; Macovei et al., 2018; Ortigosa et al., 2019; Zhou et al 2022b) would likely be exempt

5 USDA APHIS | Regulated Article Letters of Inquiry accessed 09.27.22

TABLE 1 Crops that may contribute to sustainable agriculture that could potentially qualify for USDA regulatory exemption.

Trait	Distinct MOAs	Crops	SDG
Better suitability for urban agriculture	1	tomato	2, 11, 15
breeding innovation	8	alfalfa, cabbage, corn, cucumber, potato, sorghum, tomato, wheat	1, 2, 9
domestication	2	canola, ground cherry	1, 2, 3, 9, 11, 15
improved nutrition	10	Cassava, pennycress, rice (4), soybean, tomato(3)	2, 3, 15
increased disease tolerance	14	apple, banana, barley, canola, cassava, corn, cucumber, rice (3), tomato, watermelon, wheat (2)	1, 2, 13, 15
increased tolerance of abiotic stress	4	rice (4)	1, 2, 13, 15
increased yield	11	corn, rice (7), soybean, tomato, wheat	1, 2, 11, 15
reduced postharvest losses	3	tomato (3)	1, 2, 11, 12, 15
increased yield and increased tolerance to abiotic stress	2	rice (2)	2, 13, 15
improved nutrition increased disease tolerance	1	soybean	2, 3, 15
increased yield and reduce fertilizer requirement	1	rice	2, 13, 14, 15
increased yield, better suitability for urban agriculture	5	Tomato (4), ground cherry	2, 3, 11, 13, 15
increased yield and increased tolerance to abiotic stress and reduced fertilizer requirement	1	rice	2, 13, 15
Total	63		

Table 1 represents a tally of all the plant-trait MOAs listed in Supplementary Table 1 that could potentially qualify for exemption under USDA's revised biotechnology regulation. Plant-Trait-MOAs for representative cases from the literature where crops could contribute to UN sustainable goal are listed in Supplementary Table 1. In each case, a high-level category corresponding to column 1 of this table was assigned, as was the SDGs that could be impacted by the launch and adoption of that crop. Column 2 lists the distinct number of MOAs responsible for the phenotype categorized in column 1. The UN sustainability goals listed in the table are as follows:
Sustainability Development Goals (SDG)
#1 ending poverty
#2 ending hunger
#3 good health
#9 industry innovation and infrastructure
#11 resilient and sustainable cities
#12 sustainable consumption and production
#13 climate action
#14 life below water
#15 life on land
Currently the exemptions under the revised regulation allow a single modification to a pair of homologous chromosomes per life cycle. Multiple modifications based on stacking traits through conventional breeding qualify for the exemption, but molecular stacks do not. Therefore, a single change to all homoeologous alleles in a polyploid presently do not qualify for the exemption.[6]
It should be noted that there are opportunities to expand the exemptions when new information emerges demonstrating such modifications are possible by conventional breeding.[7]

In other instances, developers have used strategies that involve multiple modifications or the use of transgenes to increase disease resistance, which USDA would likely evaluate under the RSR process. KOs of susceptibility genes in canola (Sun et al., 2018), citrus (Peng et al., 2017), cotton (Zhang et al., 2018b) and wheat (Zhang et al., 2017c; Koller et al., 2019; Li et al., 2022b) conferred resistance to various diseases. Multiple promoter deletions in several sugar transport genes conferred broad spectrum resistance in rice to several races of bacterial blight (Oliva et al., 2019). With respect to transgenes, broad resistance to three rice diseases was accomplished by transcriptional and translational controlled expression of the *Arabidopsis* NPR1 gene (Xu et al., 2017). Expression of transgenes to confer host induced gene silencing has shown promise in controlling fungi, nematodes (Kong et al., 2022), sap sucking and chewing insects, and viruses (Koch and Wassenegger, 2021) and Crispr-Cas lines targeting virus coat proteins have conferred resistance to viruses (Tashkandi et al. 2018).

Increased tolerance of abiotic stress

Climate change is expected to have a net negative impact on agricultural productivity (Raza et al., 2019). Traits that could

6 q-a-confirmation-process.pdf (usda.gov) p.3-4.

7 CFR part 340.1(b)(4)

TABLE 2 Crops that could contribute to sustainable agriculture that would undergo regulatory status review.

Trait	Distinct MOAs for RSR	Crops	SDG
breeding innovation	2	canola, rice	1, 2, 9, 15
domestication	3	canola, kiwi, wild tomato	1, 2, 3, 15
improved nutrition	18	banana, camelina, canola (4), cotton (3), potato (2), sorghum sugarcane tomato (3), wheat (2)	2, 3, 14, 15
increased pest tolerance	11	canola, citrus, cotton, rice (2), soybean, tomato (2), wheat (3)	1, 2, 13, 15
increased tolerance of abiotic stress	19	banana, barley, canola, corn (2), poplar, rice (5) soybean, tobacco (2), tomato, wheat (4)	1, 2, 13, 15
increased yield	6	canola (3), soybean, tef, tobacco	1, 2, 13, 15
reduced fertilizer requirement	2	rice, tomato	1, 2, 6, 13, 14, 15
improved nutrition, reduced postharvest loss	1	tomato	1,2,3,11,12,15
improved nutrition and net zero aviation fuel	1	pennycress	3, 6, 7, 13, 14, 15
increased yield and increased quality	1	rice	1, 2, 3, 15
Increased yield and increased disease tolerance	1	rice	1, 2, 15
increased yield and increased tolerance to abiotic stress	14	corn (2), cotton (2), rice (7), potato, wheat (2)	1, 2, 13, 15
increased yield and reduce fertilizer requirement	2	rice, wheat	1, 2, 6, 13, 14, 15
Total	80		

Table 2 represents a tally of all the plant-trait MOAs listed in Supplementary Table 1 that would likely be evaluated by the RSR process under USDA's revised biotechnology regulation at 7 CFR part 340. Plant-Trait-MOAs for representative cases from the literature where crops could contribute to UN sustainability goals are listed in Supplementary Table 1. In each case, a high-level category corresponding to column 1 of this table was assigned as was the SDGs that could be impacted by the launch and adoption of that crop. Column 2 lists the distinct number of MOAs responsible for the phenotype categorized in column 1. The UN sustainability goals listed in the table are as follows:
Sustainability Development Goals (SDG)
#1 ending poverty
#2 ending hunger
#3 good health
#6 clean water
#7 affordable clean energy
#9 industry innovation and infrastructure
#13 climate action
#14 life below water
#15 life on land

help crops adapt to climate change include tolerance to heat, drought, and salinity.

Table 1 lists 4 cases for increased abiotic tolerance, all in rice, that would likely qualify for an exemption based on partial deletions (Liu et al., 2020a; Santosh Kumar et al., 2020) or KO (Zhang et al., 2019) of endogenous transcription factors or addition of an allele of Sub1, a transcription factor within the rice gene pool that confers tolerance to flooding (Xu et al., 2006). KO of an ABA receptor increased both yield and tolerance to drought (Usman et al., 2020a) and a triple KO of a putative auxin transport protein, a QTL negatively associated with yield, and a MYB30 transcription factor increased both yield and cold tolerance (Zeng et al., 2019).

Tolerance to environmental stress has been conferred by overexpression of transcription factors that turn on genes in the stress response (Casaretto et al., 2016). Other strategies with successful proof of concept include reducing stomatal opening (Głowacka et al., 2018), increasing specific protein turnover (Alfatih et al., 2020), overexpressing glutaredoxins (Sprague et al., 2022), and modulating stress related signaling (Zang et al., 2018). In all these cases, crops yield better than the comparator under abiotic stress. In some cases, the modified plants yield better than controls even in the absence of stress (Beznec et al., 2021; Esmaeili et al., 2021; Yu et al., 2021; Sprague et al., 2022). Examples likely requiring an RSR are included in Table 2 representing 27 cases from banana (Sreedharan et al., 2013), barley (Hughes et al., 2017), canola (Wu et al., 2020), corn (Shi et al., 2017; Nuccio et al., 2015), cotton (Mishra et al. 2017), poplar (Li et al., 2018b), potato (Yu et al., 2021), rice (Shim et al., 2018; El-Esawi and Alayafi 2019; Liu et al. 2020b; Caine et al., 2019; Zhang et al., 2018a; Jiang et al., 2019; Park et al., 2020; Joshi et al., 2019; Selvaraj et al. 2017; Selvaraj et al., 2020, Usman et al., 2020a), soybean (Ribichich et al., 2020), tobacco (Sharma et al., 2019), tomato (Yin et al. 2018), and

wheat (El-Esawi et al., 2019; Zhang et al., 2017a; Mega et al., 2019; Zhou et al. 2022a; González et al., 2019).

Reduced fertilizer requirement

Fertilizer costs are among the most expensive inputs for a farmer (Langemeier et al., 2019) and they represent one of the largest energy expenditures for agriculture (Amenumey and Capel, 2014). Agriculture nutrient runoff is a significant contributor to impairment in assessed rivers and streams (US-EPA, 2018), and the single largest source of nutrient pollution to the Gulf of Mexico's "dead zone" (Ribaudo et al., 2011). Unabsorbed nitrogen by crops leads to increased production of nitrous oxide, a greenhouse gas which is 300 times more potent than carbon dioxide (Sisharmini et al., 2019). Crops that require less fertilizer input could contribute to clean water and streams, climate action, life below water, life on land, and to ending poverty by reducing fertilizer costs (SDGs #6, #13, #15, #1). Expression of rice GR4 transcription factor from its own promoter results in increases in nitrogen use efficiency (NUE) and yield (Li et al., 2018c). Rice with C-terminal indels in the gene lonely guy (OsLOG5), which catalyzes the formation of active cytokinin from inactive forms, led to increased yields under well-watered, drought, normal nitrogen and low nitrogen (Wang et al., 2020). Both may qualify for exemption.

Examples likely to require RSR evaluation include increased NUE in rice by expression of a cucumber alanine aminotransferase under the control of a rice root specific promoter (Sisharmini et al., 2019), increased NUE in rice through co-overexpression of the rice nitrate transporter (OsNRT2.3a) and its partner protein (NAR2.1a) under the control of the CaMV35S promoter (Chen et al., 2020), increased tolerance to potassium deficiency in rice by overexpression of a rice peroxiredoxin gene (Mao et al., 2018), and increased tolerance to phosphate deficiency in tomato by expression of choline oxidase from the bacteria, *Arthrobacter globiformis* (Li et al., 2019).

Improved nutrition

Increasing the nutritional value of crops is consistent with good health and well-being (SDG #3) and has been accomplished through KO strategies likely qualifying for exemption in diploids and RSR in polyploids. Healthier fatty acids have been made in oil crops (eg. (Haun et al., 2014; Jarvis et.al, 2021; Okuzaki et al. 2018; Jiang et al., 2017). Other improvements in nutrition include low reducing sugars (potato) (Clasen et al., 2016), high amylose (rice) (Sun et al., 2017), high gamma aminobutyric acid (tomato) (Nonaka et al., 2017), increased vitamin D3 (tomato) (Li et al., 2022a), increased ascorbic acid (tomato) (Do et al., 2022; Zheng et al., 2022), high isoflavone (soybean) (Zhang et al., 2020b), high carotenoid (banana, rice, and tomato) (Paul et al., 2017; Li et al., 2018d), increased protein (canola) (Xie et al., 2020; Zhai et al., 2020), high anthocyanin (tomato) (Butelli et al., 2008), and high iron (Wheat) (Connorton et al., 2017).

In some cases, the crop was made healthier by reducing an anti-nutrient. KO of key transporter proteins resulted in plants that had low uptake of cadmium (Tang et al., 2017) or cesium (Nieves-Cordones et al., 2017). Cotton seed was engineered to be gossypol free thereby creating a new food source by making the meal and oil suitable for human and animal consumption (Sunilkumar et al., 2006; Janga et al., 2019; Li et al., 2021). Other toxic substances eliminated or reduced from crops include steroidal glycosides in potato (Nakayasu et al., 2018), erucic acid in pennycress (Mcginn et al., 2019), lignin in sugarcane (Kannan et al., 2018), kafirin (a difficult protein to digest) in sorghum (Li et al., 2018a), reduced phytic acid in canola (which increases the bioavailability of phosphate in feed) (Sashidhar et al., 2020), reduced cyanide in cassava (Juma et al., 2022), and reduced amylose (rice) and reduced gluten wheat which is of benefit to some on restricted diets (Sánchez-León et al., 2018; Yunyan et al., 2019).

Domestication

Domestication of wild crops through centuries of breeding and selection has inadvertently reduced genetic diversity (Smýkal et al., 2018), limiting traits beneficial for sustainable agriculture. With an understanding of domestication traits, genome editing can rapidly improve agronomic performance of wild relatives by reuniting lost but desirable traits, such as stress tolerance, with agronomically valuable characteristics (Zsögön et al., 2018). For example, in a wild relative of tomato, an initial attempt has been made to make a new tomato variety by introducing six domestication traits that resulted in improvements in fruit number, size, shape, nutrient content and plant architecture (Zsögön et al., 2018). Similar examples are seen in alternative crops such as pennycress (Mcginn et al., 2019), ground cherry (Lemmon et al., 2018) and kiwi (Varkonyi-Gasic et al., 2019), and in standard crops such as canola and tomato (Braatz et al., 2017; Zhai et al., 2019; Kwon et al., 2020). The KO strategies used in (Lemmon et al., 2018; Zhai et al., 2019; Kwon et al., 2020) might qualify for exemption while the RSR process is more likely for the others named above.

Breeding innovations

Hybrid corn seed is almost exclusively grown in the U.S. because of its dramatic increases in yield and vigor (Nielsen, 2020). Widespread use of hybrid seed technology occurred first in corn because the crop's separate male and female flowers simplifies the hybridization procedure. In crops where hybrid seed is not economically viable because flowers have both male and female organs (perfect flowers), yield gains have typically languished relative to hybrid seed crops (Perez-Prat and Van Lookeren Campagne, 2002). In crops with perfect flowers, KO strategies have been used to introduce male sterility and/or eliminate self-incompatibility (Li et al., 2016; Li et al., 2017; Ye et al., 2018; Ma et al., 2019; Okada et al., 2019; Dai et al., 2022; Ye et al., 2022; Chen et al., 2019; Cigan et al., 2017) to enable efficient hybrid seed production. All female plants were produced in cucumber by KO of a gene required for carpel development (Hu et al., 2017) all of these would likely qualify for exemption. Potato, normally a tetraploid, is being reinvented into a diploid inbred line-based crop that will help achieve yield and vigor gains seen in other hybrid crops (Jansky et al., 2016; Hosaka and Sanetomo, 2020). Recently, apomixis was engineered in rice (Khanday et al., 2019). This trait will enable hybrid seeds to be propagated clonally thereby dramatically reducing the cost of hybrid seed. It involves the KO of three genes and the expression of a normally pollen-specific gene in the egg cell so it would be evaluated under the RSR process. These outcomes contribute to industry innovations (SDG #9) and are expected to facilitate the development of new crops with increased yields and tolerance to abiotic and biotic stresses consistent with numerous other SDGs.

Urban agriculture

Urban vertical farming has been touted as a means to increase agricultural sustainability through demonstrated increases in agricultural productivity, food safety, biosecurity and reduced inputs (water, fertilizer, and pesticides), land use, and transportation costs (SDG #11) relative to outdoor agriculture (Benke and Tomkins, 2017) (SDGS #2, #3, #6, #11, #13-15). Using controlled environments with recycling of nutrients and water, renewable energy, and automation, vertical farming may also offer solutions to climate change and labor shortages that have plagued outdoor agriculture.

Genome editing has been used to create tomato varieties more suitable to controlled environment production (Klap et al., 2017; Soyk et al., 2017; Ueta et al., 2017; Tomlinson et al., 2019; Kwon et al., 2020). The tomato varieties described in the urban agriculture section would likely be eligible for exemption provided the traits were combined by breeding.

Discussion

In this paper, we provide over 140 examples of crops that were created with biotechnology that could contribute to UN sustainability goals. This survey represents just a fraction of the traits being developed to improve sustainability and/or other purposes. Furthermore, traits that show promise in one species frequently prove to be valuable in improving closely related species. Considering that there are hundreds of crops related to those in Tables 1 and 2 (Khoshbakht and Hammer, 2008), it implies that tens of thousands of new crop varieties can be created based on the examples listed in Tables 1 and 2 alone. We estimate that over 60 of the described crop varieties would likely qualify for exemption from USDA oversight. For the remaining crops that would likely be evaluated through the RSR process, the regulatory pathway will be more risk-proportionate, science-based, product-based and streamlined compared to the former petition process (Hoffman, 2021). We already see academics interested in developing products that could successfully navigate the revised regulations. For example, although the Martin lab published the successful creation of a high anthocyanin tomato in 2008 (Butelli et al., 2008), they did not seek regulatory approval in the United States until April 2021 when the RSR process became first available for tomatoes and they received regulatory clearance September 2022.

Based on the large number of promising crop-trait-MOA combinations that have been discovered and the more streamlined, risk-proportionate, and science-based oversight in USDA's revised regulation, we fully expect to see diverse developers advance more traits that may help promote sustainability. Whether they are ultimately commercialized may depend on overcoming other obstacles including requirements from other regulatory authorities, social understanding and acceptance, and on their economic viability. Both United States Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) continue to play a role in the approval of biotechnology crops in the United States and are actively considering how to streamline their regulations regarding genome editing (OSTP, 2017). EPA has proposed an exemption for certain plant incorporated protectants created through biotechnology that could have otherwise been created through conventional breeding (US-EPA, 2012), while FDA has produced a plant and animal biotechnology innovation action plan and intends to update existing procedures for voluntary premarket consultations (US-Food and Drug Administration, 2018). Several countries (Brazil, Columbia, Argentina, Chile, Israel, Australia, and Japan) do not regulate some genome edited crops lacking foreign DNA as Genetically Modified Organisms (GMOs) and several other countries are considering adopting a similar approach

(Schmidt et al., 2020). As the number of like-minded countries grow, regulatory obstacles are expected to diminish.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1055529/full#supplementary-material

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A simple and efficient strategy to produce transgene-free gene edited plants in one generation using paraquat resistant 1 as a selection marker

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Introduction: DNA integration is a key factor limiting the marketing of CRISPR/Cas9-mediated gene edited crops. Several strategies have been established to obtain transgene-free gene edited plants; however, these strategies are usually time-consuming, technically difficult, providing low mutagenesis efficiency, and/or including a narrow host range.

Method: To overcome such issues, we established a paraquat resistant 1 (PAR1)-based positive screening (PARS) strategy, which achieved efficient screening of transgene-free gene edited plants.

Results: With PARS, the screening efficiency of mutant increased by 2.81-fold on average, and approximately 10% of T1 plants selected via PARS were transgenefree. Moreover, heritable transgene-free mutations at target loci were identified in the T1 generation.

Discussion: Based on the previous reports and our data, we know that paraquat is toxic to all green plants, PAR1 is conserved among all plant species tested, and the transient expression of Cas9 editor can produce transgene-free gene edited plants. Thus, we assume that the PARS strategy established here has the potential to be widely used to screen transgene-free mutants in various crops using diverse CRISPR/Cas9 delivery approaches.

KEYWORDS

transgene-free gene edited plant, paraquat resistant 1, selection marker, CRISPR/Cas9, *Agrobacterium*-mediated transformation

Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) system has been applied to multiple plant species for gene editing to facilitate studies on gene function and crop improvement (Chen et al., 2019; Gaillochet et al., 2021; Gao, 2021). However, the integration of the CRISPR/Cas9

construct can lead to phenotype instability, internal gene expression disturbance, and concerns related to genetically modified organism-associated legislation (Jones, 2015; Kim and Kim, 2016; Turnbull et al., 2021). Therefore, transgene-free gene edited plants is ideal for gene functional studies and agricultural applications. Two strategies are typically applied to produce transgene-free gene edited plants: (1) elimination of the integrated CRISPR/Cas9 construct *via* genetic segregation and (2) transient expression of the Cas9 editor (Gu et al., 2021). The first strategy has been used extensively. Moreover, it is suitable for most species that can be transformed with *Agrobacterium*. However, this strategy requires the selection of transgene-free gene edited plants from the progenies of transgenic plants, which is laborious and unfeasible in vegetatively propagated plants, such as potato or trees, with a long juvenile period (Gao et al., 2016; Lu et al., 2017; He et al., 2018; He et al., 2019; Stuttmann et al., 2021). The second strategy delivers DNA, *in vitro*-transcribed RNA, or preassembled CRISPR/Cas9 ribonucleoproteins to protoplasts, zygotes, and embryo cells *via* particle bombardment or polyethylene glycol Ca^{2+} (Svitashev et al., 2016; Zhang et al., 2016a; Liang et al., 2017; Park and Choe 2019). These approaches are mostly technically difficult and inefficient. Therefore, an inexpensive, convenient, and highly efficient approach is required for producing transgene-free gene edited plants.

Agrobacterium-mediated transformation is a low-cost and simple method to deliver Cas9 editors in plant cells (Hwang et al., 2017). More importantly, transgene-free gene edited plants have been detected in regenerated seedlings from cells inoculated with *Agrobacterium* carrying the CRISPR/Cas9 construct without selection (Chen et al., 2018). However, the efficiency of this strategy is relatively low, as most of the regenerated seedlings are unmutated. Thus, a screening marker is required to enrich the transgene-free gene edited plants. Previous studies have shown that some exogenous and endogenous genes can be used as co-editing markers to improve screening efficiency. For example, the hygromycin resistance gene (HygR) in construct and GLABRA2 (*GL2*) in genome (Xu et al., 2020; Kong et al., 2021). Whereas, these markers are not conducive to the screening of transgene-free gene edited plants. We assume some genes that provide plants with herbicide or antibiotic resistance when mutated, e.g., acetolactate synthase (*ALS*) and multiple antibiotic resistance 1 (*MAR1*), can be used as markers for this purpose (Aufsatz et al., 2009; Zhang et al., 2019; Rinne et al., 2021). However, *ALS* can only serve as a selection marker for base editors because some point mutation forms of *ALS*, rather than its knockouts, provide herbicide resistance to plants (Yu and Powles, 2014). Null mutation of *MAR1* results in plant resistance to several aminoglycoside antibiotics, including kanamycin, streptomycin, gentamicin, etc. (Rinne et al., 2021). Among these antibiotics, kanamycin has been applied to select transgenic plants in multiple plants but works inefficiently in some species, such as tomato (Honda et al., 2021). Paraquat resistant 1 (*PAR1*), a gene that encodes a putative L-type amino acid transporter protein localized to the Golgi apparatus, was screened from an *Arabidopsis* ethyl methanesulfonate (EMS) mutant library, and its paraquat resistance phenotype was further confirmed by analyzing its T-DNA mutants, indicating that the *par1* loss-of-function mutant could be selected through paraquat treatment. Importantly, paraquat is nonselective herbicide for green plants (Nazish et al., 2022) and *par1* mutants exhibit no obvious developmental defects in *Arabidopsis* (Li et al., 2013). However, it is not clear whether *PAR1* can be used as a screening marker for CRISPR/Cas9.

Here, we created *par1* mutants in *Arabidopsis* using the CRISPR/Cas9 system, which revealed that abolishing the function of PAR1 exhibited a strong paraquat-resistant phenotype without growth penalties under both normal growth and several stress conditions. We also confirmed that *PAR1* can be used as a coediting marker to enrich mutants of target loci. Furthermore, we found that transgene-free gene edited plants could be easily detected in the T1 generation using *PAR1* as a selection marker. Given that PAR1 is a conserved protein in all plant species tested, we proposed that this *PAR1*-based positive screening (hereafter, referred to as PARS) strategy may be applicable for various plant species and multiple CRISPR/Cas9 delivery approaches.

Materials and methods

Plant growth conditions, transformation, and selection

Arabidopsis thaliana accession Col-0 was used as the wild type. Seeds were surface sterilized and plated on 1/2 Murashige and Skoog (MS) medium containing 2.2% (w/v) MS basal salts, 1.5% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) and 0.8% (w/v) agar or in soil with a 16 h light/8 h dark photoperiod at 22 °C. *Agrobacterium*-mediated transformation of plants was performed by floral dip (Clough and Bent, 1998). To select transgenic plants, T0 seeds were surface sterilized and sown on 1/2 MS medium containing 25 mg/L hygromycin. While for transgene-free gene edited plants selection, T0 seeds were sown on 1/2 MS medium containing 1 μM paraquat. After 14 days of growth on sterile agar plates, resistant seedlings were transferred to soil. To further confirm the T1 edited plants without Cas9, their seeds were sown on 1/2 MS medium supplemented with 25 mg/L hygromycin for two weeks

Construction of CRISPR/Cas9 vectors

Four single guide RNAs (sgRNAs), i.e., $sgRNA_{par1}1$–4, targeting *PAR1* were designed using the online predictor tool CCTop (https://cctop.cos.uni-heidelberg.de/), and the efficacy

score and mismatch positions were used as evaluation criteria. Additionally, sgRNAs targeting jasmonate-ZIM-domain protein 1 (*JAZ1*) and gibberellic acid insensitive (*GAI*) were used as previously described (Kong et al., 2021). Each oligonucleotide pair coding for the designed sgRNAs was annealed to form double-stranded DNA (dsDNA). The sequences of the synthesized DNA oligonucleotides and all primers used in this study are listed in Supplementary Table S1.

To construct pHEE401E (Wang et al., 2015) vectors targeting four different sites of *PAR1* and one site of *JAZ1* or *GAI*, the dsDNA was fused to the Bs*a*I-digested vector pHEE401E using T4 DNA ligase (EL0011, Thermo Scientific™). To construct a new vector named pPARS, which targets *PAR1* as a selection marker, the DNA sequence of $sgRNA_{par1}3$ was introduced into the *PAR1*-2Tar-R primer when the primer was synthesized. The primer pair PAR1-2Tar-F/R (Supplementary Table S1) was then used to amplify a sequence containing the U6 terminator-U6 promoter-$sgRNA_{par1}3$ element using the pCBC-DT1T2 plasmid as a template (Xing et al., 2014). Through homologous recombination using ClonExpress® II One Step Cloning Kit (C112, Vazyme Biotech co., ltd.), this element was ligated into the pHEE401E binary vector digested by *Bsa*I. Additionally, a *Bsa*I restriction site was added to the PAR1-2Tar-F/R primers; hence, the final vector retained the *Bsa*I cleavage site, allowing the insertion of sgRNAs for the target loci of interest. DNA sequence for pPARS plasmid was shown in Supplementary Data Set S1. To produce the pPARS constructs targeting *JAZ1* or *GAI*, their dsDNAs were introduced into the *Bsa*I sites of pPARS vectors. *Agrobacterium tumefaciens* strain GV3101 was transformed with the final binary vectors for floral dip.

Genotyping of the transgenic plants

Genomic DNA was extracted from 4-week-old plant leaves using DNA extraction buffer [50 mM Tris–HCl (pH 7.5), 300 mM NaCl, and 300 mM sucrose]. To confirm the integration of T-DNA or CRISPR/Cas9-introduced mutations of the target gene, gene-specific primers (Supplementary Table S1) were used for PCR amplifications with *EasyTaq*® DNA Polymerase (AP111; TransGen Biotech Co., Ltd.) under the following cycling conditions: initial denaturation at 95°C for 5 min; 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 60 s; and final extension at 72°C for 5 min. The PCR products were visualized on a 1% agarose gel, and samples with no Cas9-specific band were considered transgene-free gene edited plants. Additionally, Sanger sequencing was performed to identify mutations in the target region. All sequencing data were analyzed using online software (http://shinyapps.datacurators.nl/tide/ and https://ice.synthego.com/#/).

Biomass and flowering time analysis

To detect the fresh weight of Col-0 and *par1*, plants were sown in soil and grew in chamber with a 12 h light/12 h dark photoperiod at 22°C. After three and a half weeks, plant weight was determined. For the determination of flowering time, Col-0 and *par1* were grown in soil at 22°C under 16-h light/8-h dark cycles. Flowering time was estimated by counting the number of rosette leaves according to the visible flower buds at the center of the rosette and the days from sowing to flowering.

Root growth assay

To analyze whether *PAR1* affects plants to cope with multiple stresses, WT and *par1* were sown on 1/2 MS medium vertically with or without 200 mM mannitol, 150 mM NaCl or 8 mM $NaHCO_3$, respectively, and growing under 12 h light/12 h dark photoperiod at 22°C for 12 d. To detect the response of WT and *par1* to low temperature, WT and *par1* were sown on 1/2 MS medium vertically and growing under 12 h light/12 h dark photoperiod at 12°C for 12 d. To evaluate whether *PAR1* mutation affects the functional analysis of target genes, *Arabidopsis* seeds of WT, T1 seeds of *par1* #10 and *jaz1 par1* #17 were surface sterilized and sown on 1/2 MS medium vertically with or without 50 μM methyl-jasmonate (JA) for 10 d, following which primary root length was measured using the ImageJ software (https://imagej.nih.gov/).

Sequence alignment and phylogenetic analyses

Amino acid sequences of PAR1 in different species were obtained from NCBI (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed using the ClustalW algorithm with 1,000 bootstrapping trials and the phylogenetic analysis was conducted using the neighbor-joining method in MEGA 7.0.14 software with 1000 bootstrap value.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 8.0. The statistical significance levels (value of $p<0.05$) was determined by Student's t-test or one-way ANOVA.

Accession numbers

PAR1: *At1g31830*; *JAZ1*: *At1g19180*; *GAI*: *At1g14920*.

Results

Design of the PARS strategy

As a screening marker of the CRISPR/Cas9 system, its mutants should be easily screened out under certain conditions and should not have obvious fitness costs for subsequent application. Using a literature search, we confirmed that *PAR1* met these necessary criteria. PAR1 is a Golgi-localized transporter that may be responsible for the uptake of paraquat into chloroplasts to generate toxic reactive oxygen species (Li et al., 2013). Knockout of *PAR1* resulted in a paraquat-resistant phenotype and moreover showed no growth defect, which perfectly fit our requirements. Thus, we designed the PARS screening strategy to efficiently select transgene-free gene edited plants based on *PAR1* mutation. First, the expression cassette of *PAR1* sgRNA driven by the *AtU6* promoter was cloned into pHEE401E to construct the pPARS vector (Figure 1A), which has an additional sgRNA expression cassette for the target site and a Cas9 expression cassette driven by the enhancer of egg-cell specific promoter *EC1.2* fused to the *EC1.1* promoter, thus reducing the ratio of chimeric mutants (Wang et al., 2015). The sgRNA for the target locus was then introduced into pPARS, and the final construct was used to transform *Arabidopsis* plants *via* floral dip (Figure 1B). The seeds of T0 plants were collected and sown on 1/2 MS plates supplemented with paraquat to screen *par1* mutants. Additionally, DNA was extracted from the *par1* mutants to determine whether they were transgene-free mutants *via* PCR with Cas9 primers. Finally, the transgene-free T1 plants were sequenced to determine whether mutations occurred at target loci (Figure 1B).

PAR1 is a suitable marker for the selection of CRISPR/Cas9-created mutants

To select a suitable sgRNA for *PAR1*, we designed four sgRNAs for this gene. As shown in Supplementary Figure S1, sgRNA$_{par1}$1–4 targeted 79, 118, 313, and 905 bp downstream of the translation start site ATG. We obtained ≥ 30 T1 plants for each sgRNA$_{par1,}$ and all T1 plants of the four sgRNA$_{par1}$s were sequenced. The mutation rates of the four target sites were 46.67%, 13.64%, 43.33%, and 15.91%, respectively, and sgRNA$_{par1}$3 exhibited the highest homozygous and biallelic mutation efficiency, i.e., 20% (6/30) and 10% (3/30) of the T1 plants were homozygous and biallelic, respectively (Figure 2A and Supplementary Table S2). Therefore, sgRNA$_{par1}$3 was used to construct pPARS. A homozygous *par1* mutant created using sgRNA$_{par1}$3 was selected to determine whether the *par1* mutant can be easily screening out using paraquat and has no growth penalty as reported (Li et al., 2013). To determine the feasibility of screening *par1* mutants using paraquat, we mixed 25 *par1* homozygous seeds with 0.2 g of WT seeds and selected them using 1 μM paraquat according to a previously published method (Li et al., 2013) All plants screened using 1 μM paraquat were *par1* mutants (Figure 2B and Supplementary Figure S2), indicating the screening with 1 μM paraquat is very efficient. To determine whether the *par1* mutants exhibit growth defects, we compared the growth statuses of three- and six-week-old WT and *par1* mutant plants. As shown in Figures 2C, D, the growth of the *par1* mutant and WT did not differ significantly at these stages. We also measured the biomass, flowering time, and seed weight per plant of the WT and *par1* mutant and found no significant difference between them (Figures 2E, F and Supplementary Figure S3). Importantly, mutating *PAR1* exerted no negative effects on plant growth under multiple stress conditions (Supplementary Figure S3). Collectively, these results suggest that *PAR1* is a suitable marker for selecting gene edited CRISPR/Cas9-created mutants.

Transgene-free *PAR1*-edited plants can be screened from T1 plants

As we know, transiently expressing Cas9 and sgRNA can create transgene-free gene edited plants (Zhang et al., 2016a), and furthermore, transgene-free gene edited plants were obtained in plants that were regenerated from calli transiently expressing Cas9 from T-DNA delivered by *Agrobacterium* without selection (Chen et al., 2018). We assumed that the transgene-free *par1* mutant could be detected in T1 plants if we selected T0 seeds by paraquat. For this purpose, we equally divided T0 seeds (1.2 g) obtained from the same transformation event (with sgRNA$_{par1}$3) into two groups, sowing these seeds on 1/2 MS plates supplemented with either hygromycin or paraquat. In total, 217 and 60 positive seedlings were observed on hygromycin and paraquat plates, respectively (Figure 3A). We sequenced all positive seedlings and found that 63 of 217 hygromycin-resistant plants were *par1* mutants, with 26.98% (17), 12.70% (8), 53.97% (34), and 6.35% (4) being homozygous, biallelic, heterozygous, and chimeric, respectively (Supplementary Figure S4 and Supplementary Table S2). All 60 paraquat-resistant seedlings were *par1* mutants, with 43.33% homozygotes (26), 31.67% biallelic mutants (19), 20.00% heterozygotes (12), and 5.00% chimeras (3) (Figure 3B and Supplementary Table S2). We were surprised to detect heterozygotes in the paraquat plates because the *par1* mutation is recessive according to a previous report (Li et al., 2013). However, the ratio of homozygotes and biallelic mutants was higher and that of heterozygotes was lower in paraquat-resistant plants than in hygromycin-resistant *par1* mutants, indicating that the screening is efficient. Given that increasing the concentration of paraquat in selection medium is lethal to

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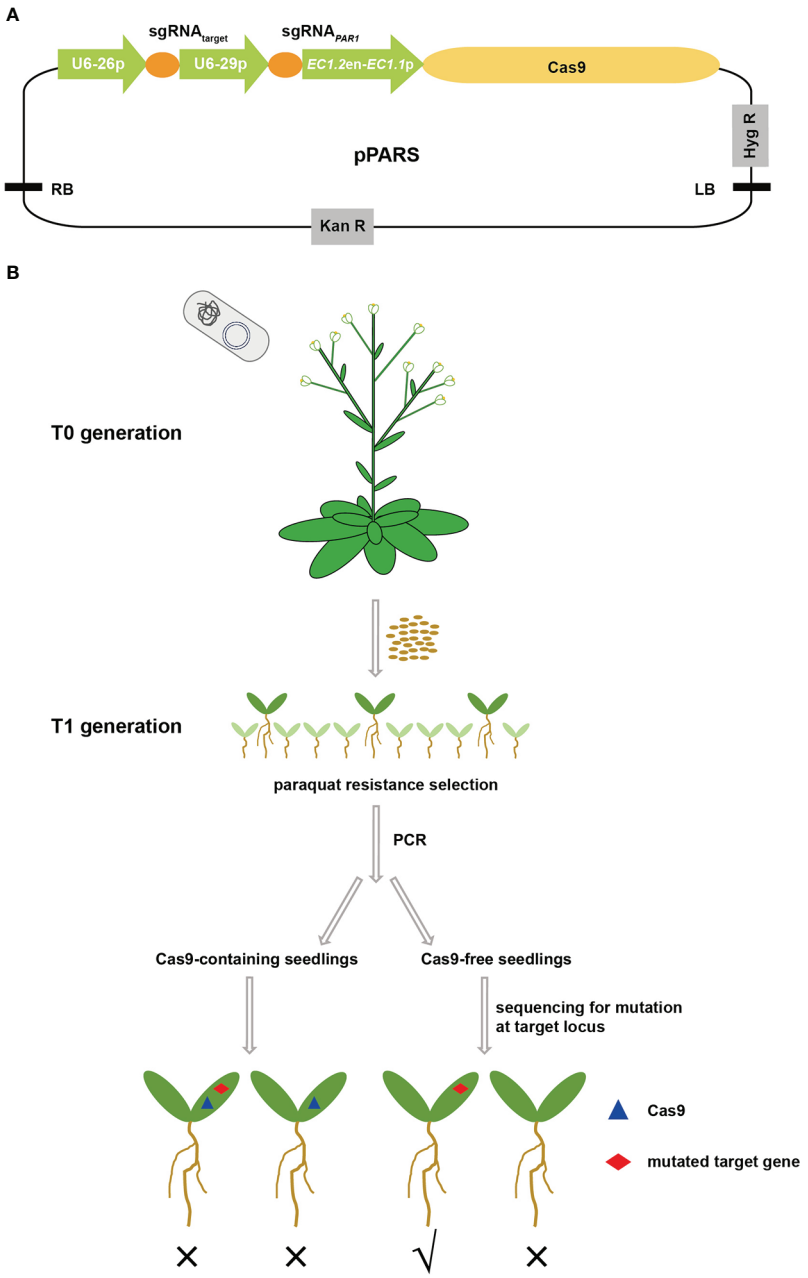

FIGURE 1
Schematic showing the PARS strategy to screen transgene-free gene edited plants. **(A)** The structure of the pPARS plasmid. The expression of sgRNA$_{PAR1}$ and sgRNA$_{target}$ is driven by the U6-29 and U6-26 promoters, respectively. The Cas9 expression cassette includes the *EC1* promoter (*EC1.2* enhancer plus *EC1.1* promoter) and the CDS of *Cas9*. Hyg R, hygromycin resistance gene; Kan R, kanamycin resistance gene; RB and LB, T-DNA right and left borders, respectively. **(B)** Outline of the PARS strategy. pPARS constructed with sgRNA$_{target}$ was transformed into Col-0 plants by floral dip. T0 seeds were collected and sown on paraquat-containing plates to select paraquat-resistant plants, which are shown as large green seedlings. The DNA of paraquat-resistant T1 seedlings was extracted, and PCR was performed using Cas9-specific primers to select transgene-free plants. The Cas9-containing seedlings were discarded, and Cas9-free seedlings were further sequenced for mutation at the target locus to obtain transgene-free gene edited plants. The blue triangle on the seedling indicates insertion of Cas9, and the red diamond indicates mutation at the target site. Portions of the images were modified from the Microsoft PowerPoint clip art database.

>20% of *par1* homozygotes, we continued to use 1 μM paraquat for the following experiments (Li et al., 2013).

The above results indicate that more *par1* homozygous and biallelic mutants were obtained from paraquat plates than from hygromycin plates with the same amount of T0 seeds; therefore, some *par1* mutants selected on paraquat plates were likely transgene-free. To confirm this hypothesis, Cas9-specific primers were used to validate the integration of T-DNA in paraquat-selected

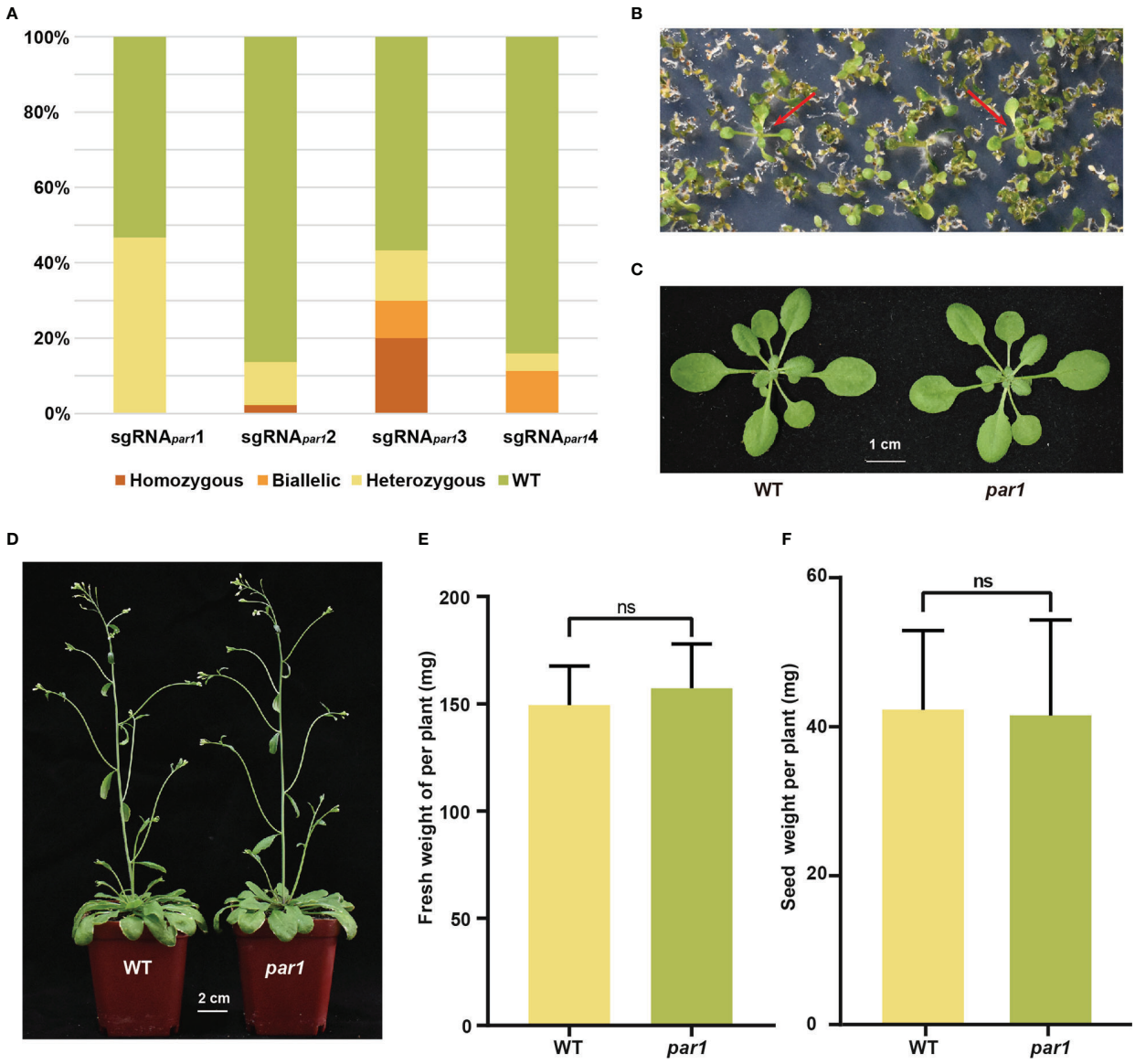

FIGURE 2
PAR1 is a suitable selection marker for transgene-free gene edited plants. **(A)** Percentages of WT and different types of mutations at four sgRNA$_{PAR1}$ target loci among their T1 plants. **(B)** Selection of the *par1* mutant on 1/2 MS medium supplemented with 1 μM paraquat. The large and green seedlings indicated by red arrows are paraquat-resistant seedlings. **(C)** Representative three-week-old WT and *par1* mutant plants. **(D)** Representative six-week-old WT and *par1* mutant plants. **(E)** The fresh weight per plant of WT and *par1* mutant (n = 20) growing in soil with a 12 h light/12 h dark photoperiod at 22°C for three and a half weeks. **(F)** Seed weight per plant for WT and the *par1* mutant (n = 30). Data are shown as mean ± SD. ns, no significant difference.

plants. We found that 11.67% (7/60) of paraquat-resistant plants were transgene-free (Figure 3C and Supplementary Figure S5A), and all of them were *par1* homozygous or biallelic mutants (Supplementary Figure S5B). Subsequently, we sowed the T1 seeds of these plants on hygromycin plates and found that they were all hygromycin-sensitive, supporting the finding that these lines were transgene-free (Figure 3D). Overall, these results indicate that *PAR1* can be used as a screening marker to obtain transgene-free gene edited plants using the CRISPR/Cas9 system.

PARS strategy enhances screening efficiency at target sites

As co-editing is a common phenomenon in multiplex gene edited plants, adding selection markers has been shown to enrich the mutants of target sites created using the CRISPR/Cas9 system (Zhang et al., 2016b; Wang et al., 2019; Kong et al., 2021). We assumed that the *PAR1* locus could also serve as a selection marker

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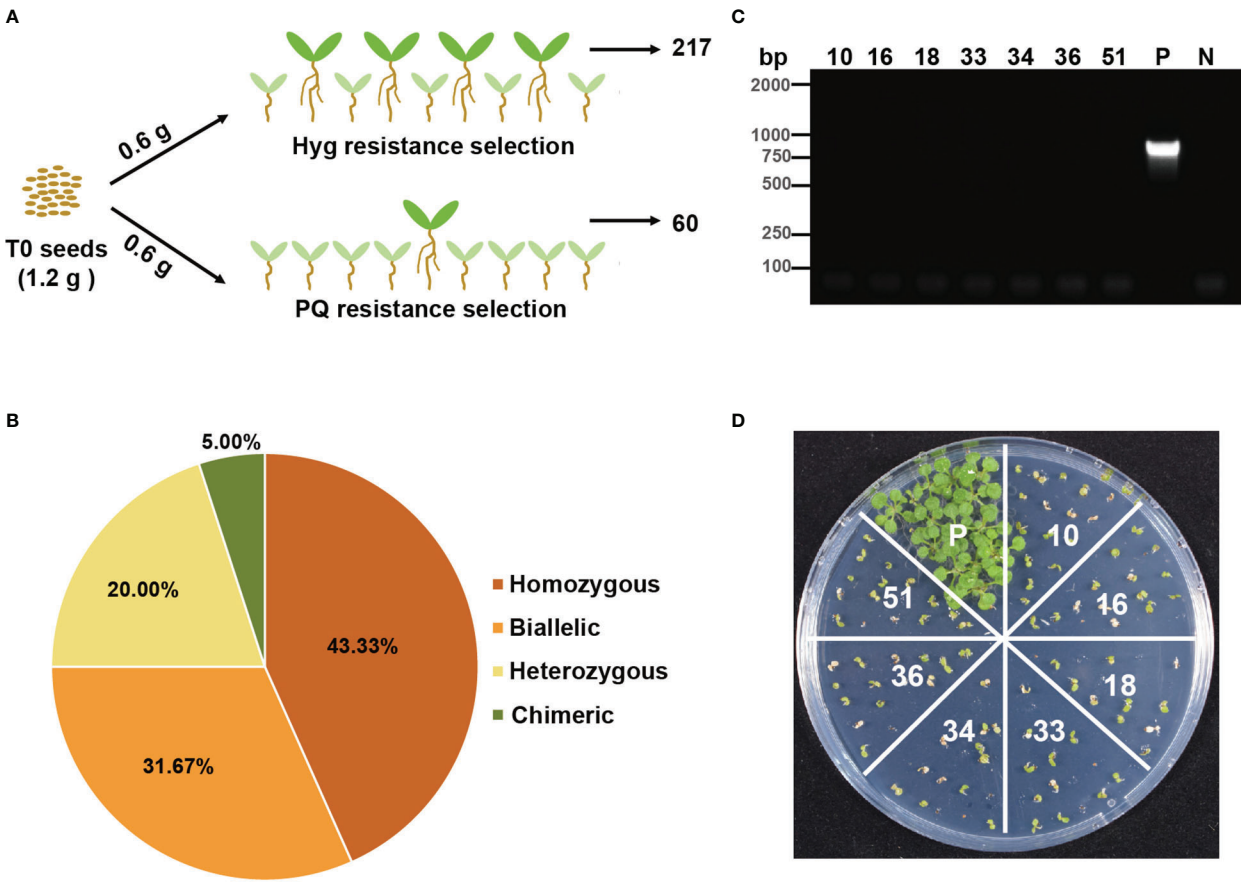

FIGURE 3
Transgene-free *par1* mutants could be detected when T0 seeds were selected by paraquat. **(A)** Outline of selecting equal amount of T0 seeds transformed with sgRNA*par1* by hygromycin and paraquat. **(B)** Percentages of different types of mutations at the *PAR1* locus among all T1 plants selected on paraquat plates. **(C)** The PCR results of transgene-free T1 plants amplified with *Cas9*-specific primers. P: positive control (PCR with DNA extracted from a transgenic plant containing Cas9). N: no sample control (PCR with no DNA sample). **(D)** The progenies of transgene-free *par1* mutants are sensitive to hygromycin. Seeds of these transgene-free T1 plants were sown on 1/2 MS plates supplemented with 25 mg/L hygromycin for two weeks.

to enhance the screening efficiency. Therefore, we constructed two sgRNAs targeting *JAZ1* and *GAI* on pPARS, and the final constructs were transformed into *Arabidopsis*. A third of the T0 seeds were selected on 1/2 MS supplemented with hygromycin, whereas two-thirds of these seeds were selected on paraquat plates. In total, 57 hygromycin-positive seedlings and 25 paraquat-positive seedlings were detected for *JAZ1*, and all seedlings were sequenced to identify the mutation form at the target. We found that the screening efficiency increased from 12.28% to 72.00% (Figure 4A, Supplementary Figure S6A and Supplementary Table S2). Furthermore, 56.00% of paraquat-positive plants were homozygous or biallelic *jaz1* mutants compared with only 5.27% of hygromycin-positive plants (Figure 4A, Supplementary Figure S6A and Supplementary Table S2). For the *GAI* target, we identified 70 hygromycin-positive seedlings with 12 *gai* mutants (17.14%) and 25 paraquat-positive seedlings with 8 *gai* mutants (32.00%) (Figure 4B, Supplementary Figure S6B and Supplementary Table S2). Among the selected seedlings, 4.29% (3) and 28.00% (7) were homozygous or biallelic mutants in hygromycin-positive and paraquat-positive seedlings, respectively (Figure 4B, Supplementary Figure S6B and Supplementary Table S2). Taken together, these findings confirm that PARS facilitates the mutant screening at target loci.

To rule out the effect of expressing two sgRNAs on editing efficiency, we constructed sgRNAs of *JAZ1* and *GAI* to pHEE401E and found that the mutation frequencies were 30.14% and 10.64%, respectively (Figures 4C, D). The homozygous/biallelic ratio of T1 seedlings was 21.32% for *JAZ1* and 2.12% for *GAI* (Figures 4C, D and Supplementary Table S2). Even though the mutation efficiency was affected by coexpressing sgRNA$_{par1}$3, PARS still facilitates mutant screening at target loci, especially the screening of homozygous and biallelic mutants. Based on our results, PARS increased screening efficiency by 2.03-fold for *JAZ1* and 3.58-fold for *GAI* compared with the original vector pHEE401E (Figure 4 and Supplementary Table S2).

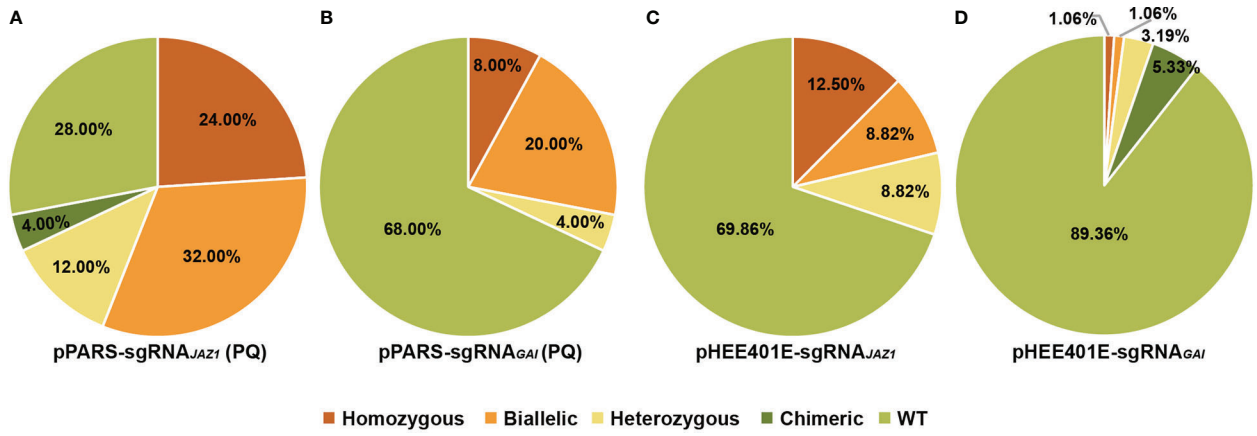

FIGURE 4
Mutation type and frequency at target sites with or without PARS. **(A)** Percentages of WT and different types of mutations at *JAZ1* loci selected using PARS on paraquat (PQ) plates. **(B)** Percentages of WT and different types of mutations at *GAI* loci selected using PARS on paraquat (PQ) plates. **(C)** Percentages of WT and different types of mutations at *JAZ1* loci using the original system (pHEE401E). **(D)** Percentages of WT and different types of mutations at *GAI* loci using the original system (pHEE401E).

Transgene-free gene edited plants with modifications at target sites can be obtained using PARS

To determine whether transgene-free plants with a modification at the target locus can be obtained using PARS, we retransformed the construct of pPARS-sgRNA$_{JAZ1}$ into 24 of *Arabidopsis* plants to obtain more T0 seeds, after which we selected all T0 seeds on 1/2 MS plates supplemented with 1 μM paraquat. In total, 106 paraquat-resistant seedlings were obtained for sgRNA$_{JAZ1}$, 10 of which were transgene-free, as identified *via* PCR with Cas9-specific primers (Figure 5A and Supplementary Figure S7A). We sequenced these 10 transgene-free seedlings and found 8 biallelic mutants and 2 heterozygotes for the *JAZ1* locus (Figure 5B). For example, #17, a biallelic mutant for *JAZ1*, had a 1 bp insertion in one allele and a 1 bp deletion in the other allele (Figure 5B). The T1 seeds of all lines were hypersensitive to hygromycin, confirming that they were transgene-free mutants (Supplementary Figure S7B). JAZ1 belongs to a member of the transcriptional repressor family involved in jasmonic acid (JA) signaling, and the *JAZ1* sgRNA, designed to target the coding region of the JA-associated domain, generates mutants with compromised sensitivity to exogenous JA (Thines et al., 2007). Therefore, we sought to determine whether the *PAR1* mutation impedes the functional study of JAZ1. Accordingly, we sowed WT, *par1*, and *jaz1 par1* seeds on 1/2 MS plates supplemented with 50 μM MeJA, and observed no significant difference between the WT and *par1* mutant indicating PAR1 mutation does not change plant response to JA (Figure 5C, D). The *jaz1* mutant phenotype was further investigated in the *par1* background. Consistent with the results of a previous study (Kong et al., 2021), JA resistance was increased in the T2 seedlings of *jaz1* #17 compared with that in control plants (Figures 5C, D). Collectively, these results indicate that the *PAR1* mutation does not affect the functional study of target genes.

We also showed that transgene-free *gai* mutant could be identified *via* PARS using pPARS-sgRNA$_{GAI}$. Among all 33 paraquat-positive T1 plants for *GAI*, three transgene-free plants were detected (Figure 5E and Supplementary Figure S8A). Additionally, one transgene-free *plant* (line 21) was identified as *gai* heterozygous (Figure 5F). Furthermore, its progenies were hygromycin-sensitive (Supplementary Figure S8B). We believe that screening more T0 seeds on paraquat plates would lead to the identification of homozygous/biallelic mutants for *GAI*. These results support our claim that PARS is an appropriate strategy for obtaining transgene-free gene edited plants *via* the CRISPR/Cas9 system. ALS has been used as selection marker to obtain the transgene-free base edited plants (Zhang et al., 2019). As CRISPR-STOP could result in gene silence through base editor-created null mutations (Kuscu et al., 2017), we assume transgene-free base edited mutants could also be screened using PARS with PAR1 sgRNAs to generate stop codons.

PARS has the potential to be applicable to multiple crop species

To determine whether the PARS strategy has the potential to be used for obtaining transgene-free gene edited crop plants, we investigated the conservation of PAR1 in different crop species. The *Arabidopsis* PAR1 amino acid sequence showed high similarity with its homologous proteins from *Nicotiana*

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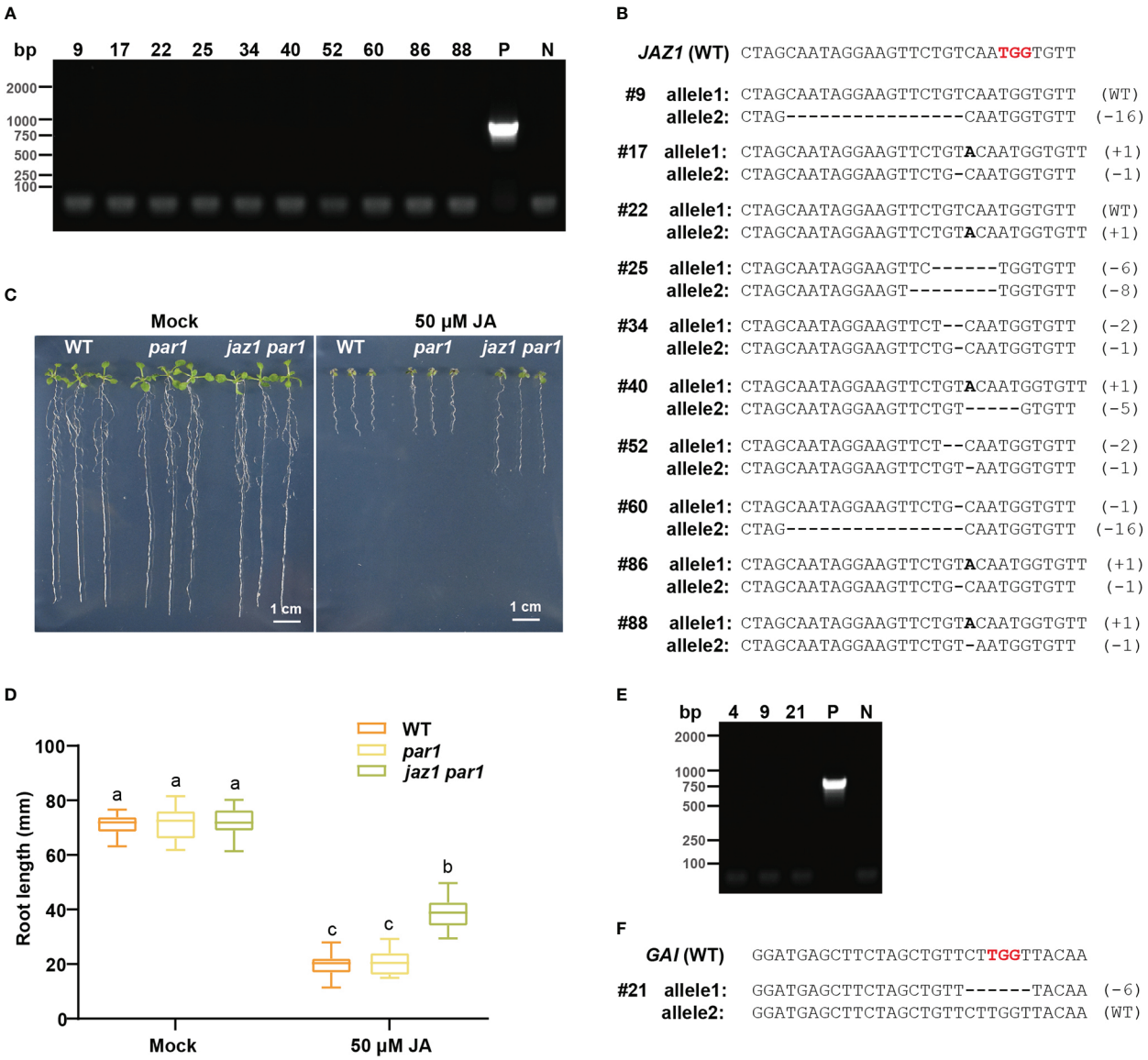

FIGURE 5
Transgene-free gene edited plants could be created by PARS strategy. **(A)** The PCR results of transgene-free T1 plants of pPARS-sgRNA$_{JAZ1}$ amplified with *Cas*9-specific primers. P: positive control (PCR with DNA extracted from a transgenic plant containing Cas9). N: no sample control (PCR with no DNA sample). **(B)** The DNA sequences of the transgene-free *jaz1* mutants. The reference sequence of WT is shown on the top. The PAM sequence is shown in red. These indels in mutant alleles are depicted by a dash (deletion) or bold letter (insertion). **(C)** Representative seedlings of WT, *par1* mutant and *jaz1 par1* double mutant grown on 1/2 MS plates with or without 50 μM JA. **(D)** The analysis of primary root length of WT, *par1* mutant and *jaz1 par1* double mutant grown on 1/2 MS plates with or without 50 μM JA. **(E)** The PCR results of transgene-free pPARS-sgRNA$_{GAI}$ T1 plants amplified with *Cas*9-specific primers. P: positive control (PCR with DNA extracted from a transgenic plant containing Cas9). N: no sample control (PCR with no DNA sample). **(F)** The DNA sequences of the transgene-free *gai* mutant. The reference sequence of WT is shown on the top. The PAM sequence is shown in red. The deletions in mutant allele are depicted by dashes.

tabacum, *Solanum lycopersicum*, *Glycine max*, *Triticum aestivum*, *Zea mays*, *Sorghum bicolor*, and *Oryza sativa* (Supplementary Figure S9). To further investigate the relationship between the PAR1 protein and its homologs in different species, a phylogenetic tree was generated with the neighbor - joining likelihood method using MEGA 7.0.14, which further confirmed PAR1 is a conserved protein (Figure 6). Since multiple PAR1 homologs were identified in each crop, e.g., three *PAR1*-like genes were observed in tomato, the *PAR1* homolog that drives the transport of paraquat in the corresponding plant must be identified to enable the selection of transgene-free gene edited plants using PARS. Additionally, whether eliminating the

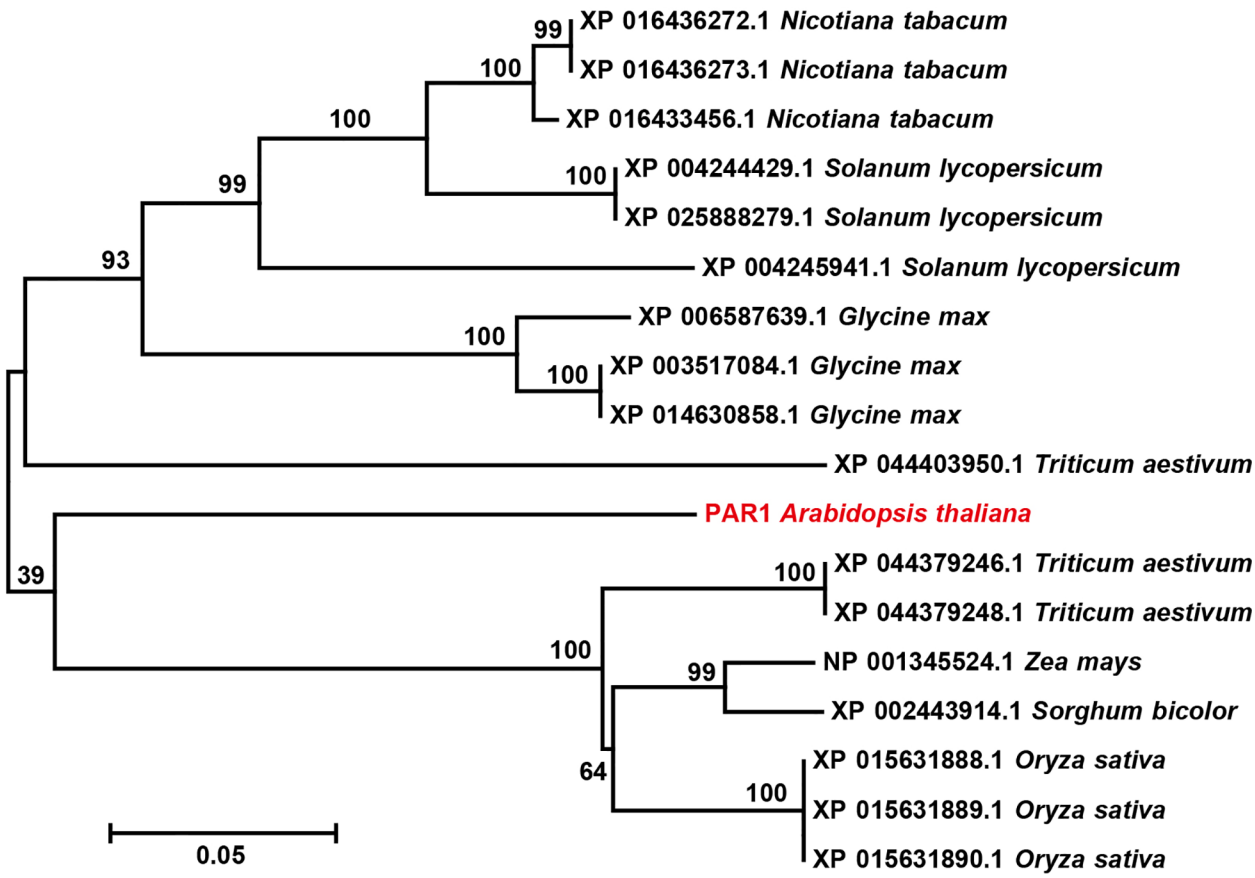

FIGURE 6
The phylogenetic analysis of PAR1 and PAR1 homologs in several crops. *Arabidopsis* PAR1is highlighted in red. The numbers above the branches indicate bootstrap values. The bar indicates a genetic distance of 0.05.

function of PAR1 homologs will harms crop development also needs to be considered before applying PARS in the corresponding crop.

Discussion

To avoid the insertion of foreign DNA, multiple strategies have been established to transiently express genome editors in plants (Zhang et al., 2016a; Liang et al., 2017; Chen et al., 2018). However, most of these strategies are generally accompanied by low mutant frequency, as no selection marker can be used to exclude the unmodified seedlings. Thus, there is an urgent need for a method to easily screen gene edited plants out from all regenerated ones. The PARS strategy we developed in this study meets this need well. We believe *paraquat resistant 1* (*PAR1*) is a perfect selection marker to screen transgene-free gene edited plants based on the following two reasons. First, the loss-of-function mutant of *PAR1* could be easily screened out through paraquat treatment and no obvious developmental defects was detected in this study (Figure 2). Second, paraquat is a nonselective herbicide for green plants, and the *PAR1* gene is conserved in different plants, suggesting that this strategy has the potential to be adopted to other crops (Figure 6) (Nazish et al., 2022). We used *Agrobacterium* transformation, the most widely used approach of CRISPR/Cas9 delivery for plants, as an example to verify our hypothesis in this study. We believe PARS is also applicable to gene editing achieved with other transformation methods, such as particle bombardment. nanomaterials scenario and RNA- or RNP-mediated gene editing.

This study revealed that *PAR1* can serve as a selection marker to enrich screening efficiency at target loci (Figure 4). Because coediting frequently occurs when multiple sgRNAs are delivered in the same plant cells (Zhang et al., 2016b; Wang et al., 2019), multiple coselection strategies have been established to enrich edited plants at target loci (Zhang et al., 2019; Xu et al., 2020; Kong et al., 2021). For example, base editing on P174 of wheat ALS enriches the mutation frequency of the target by several fold (Zhang et al., 2019), and GLABRA2 mutation-based visible selection increased the screening efficiency by 2.58- to 7.50-fold in *Arabidopsis* (Kong et al., 2021). In the PARS system established here, the screening efficiency was increased on average by 2.81-fold (Figure 4). Moreover, 28% of *gai* mutants

and 56% of *jaz1* mutants among T1 plants were homozygous/biallelic (Figure 4). Importantly, mutating *PAR1* had no obvious effect on the functional study of the *jaz1* mutant (Figure 5). We assume that transgene-free gene editing events mediated by Cas9, such as fragment deletion and insertion and fragment replacement, could also be enriched and screened using PARS. Moreover, PARS could also facilitate isolating transgene-free gene editing events mediated by base editor thorough creating stop codons in *PAR1* (Kuscu et al., 2017).

Paraquat, the third most commonly used herbicide in the world, is nonselective and has a broad spectrum (Nazish et al., 2022). Other than the *PAR1* mutation, paraquat tolerance 15-D (*PQT15-D*) is the dominant mutation that confers high resistance to paraquat (Xia et al., 2021). The overexpression of paraquat resistance genes, such as *Ochrobactrum anthropi pqrA* and *EiKCS*, also confers paraquat resistance in plants (Jo et al., 2004; Luo et al., 2022). Thus, paraquat could serve as a potential alternative for selecting transgenic plants, especially when the target plants are tolerant to conventional antibiotics such as kanamycin and hygromycin. To the best of our knowledge, no transgene-free paraquat-resistant crops have been developed to date owing to the lack of knowledge on the mechanisms underlying paraquat resistance (Nazish et al., 2022). If the functional PAR1 and PQT15 homologs that transport paraquat were identified in different crops, PAR1 and PQT15 would likely be the best editing targets for producing paraquat-resistant crops. The transgene-free gene edited plants obtained through the PARS strategy will also offer the possibility of crops with herbicide resistance, which is a major requirement in modern agriculture (Zhu et al., 2020).

The application of PARS should be extended to other gene editing methods, including zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Zhang et al., 2017; Shamshirgaran et al., 2022). As PAR1 is conserved among different plants tested (Figure 6), we propose that the PARS system has the potential to be widely used in the gene editing of various plant species using multiple gene editing systems.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

HZ and LL conceived this research. XK performed all the experiments with the help of WP and TZ. LL and XK wrote the draft. All authors read and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Optimization of highly efficient exogenous-DNA-free Cas9-ribonucleoprotein mediated gene editing in disease susceptibility loci in wheat (*Triticum aestivum L.*)

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The advancement of precision engineering for crop trait improvement is important in the face of rapid population growth, climate change, and disease. To this end, targeted double-stranded break technology using RNA-guided Cas9 has been adopted widely for genome editing in plants. *Agrobacterium* or particle bombardment-based delivery of plasmids encoding Cas9 and guide RNA (gRNA) is common, but requires optimization of expression and often results in random integration of plasmid DNA into the plant genome. Recent advances have described gene editing by the delivery of Cas9 and gRNA as pre-assembled ribonucleoproteins (RNPs) into various plant tissues, but with moderate efficiency in resulting regenerated plants. In this report we describe significant improvements to Cas9-RNP mediated gene editing in wheat. We demonstrate that Cas9-RNP assays in protoplasts are a fast and effective tool for rational selection of optimal gRNAs for gene editing in regenerable immature embryos (IEs), and that high temperature treatment enhances gene editing rates in both tissue types. We also show that Cas9-mediated editing persists for at least 14 days in gold particle bombarded wheat IEs. The regenerated edited wheat plants in this work are recovered at high rates in the absence of exogenous DNA and selection. With this method, we produce knockouts of a set of three homoeologous genes and two pathogenic effector susceptibility genes, engineering insensitivity to corresponding

necrotrophic effectors produced by *Parastagonospora nodorum*. The establishment of highly efficient, exogenous DNA-free gene editing technology holds promise for accelerated trait diversity production in an expansive array of crops.

KEYWORDS

wheat, gene editing (CRISPR-Cas9), ribonucleoprotein (RNP), DNA-free, transgene-free, temperature treatment, protoplasts, immature embryo, necrotrophic effector sensitivity genes

Introduction

Amidst a rapidly growing population and threats posed by climate change and disease, there exists a need for the advancement of crop biotechnology to increase the speed and precision of crop varietal development. Cas9 has emerged as a plant gene editing tool of choice for its accuracy and programmability to engineer allelic diversity for beneficial traits to support global food security. Guided by RNA, Cas9 efficiently makes sequence-specific double-stranded breaks in genomic DNA (Jinek et al., 2012). The host's double-stranded break repair mechanisms are then elicited. Non-homologous end joining (NHEJ), the predominant and often error prone pathway in plants, can lead to insertions or deletions (indels) at the Cas9 cut site upon repair (Puchta, 2004). Exploitation of this system allows for targeted knockout of endogenous genes.

Cas9 and guide RNA (gRNA) encoding plasmid DNA systems have been developed and delivered to plant and major crop species including *Arabidopsis* (Li et al., 2013), potato (Butler et al., 2015; Wang et al., 2015), tomato (Brooks et al., 2014; Lor et al., 2014), soybean (Jacobs et al., 2015), maize (Svitashev et al., 2015; Char et al., 2017), barley (Lawrenson et al., 2015; Garcia-Gimenez et al., 2020), rice (Feng et al., 2013), and wheat (Wang et al., 2014) by *Agrobacterium tumefaciens* or particle bombardment. These methods rely on random integration of Cas9-gRNA cassettes into the genome, and optimization of expression for each plant system. As a result, the gene editing process is encumbered by variables such as promoter and terminator choice when cloning constructs and copy number and integration location of transgenes upon transformation. Additionally, gene editing by these methods raise transgenic regulatory concerns. Regulation aside, transgenes can often be segregated away through breeding, but the process is laborious, time consuming, and particularly difficult for plants with complex genomes. Moreover, crops with lengthy generation times or those that are vegetatively propagated, such as cassava and banana, cannot be bred to segregate transgenes. There have been reports in which plant gene editing has been achieved by transient expression of Cas9 and gRNA (Zhang et al., 2016; Hamada et al., 2018), however full experimental control over the fate of transgene integration and tracking has not been achieved. For these reasons, there is a clear need for advances in exogenous DNA-free genome editing technology.

The direct delivery of preassembled Cas9-gRNA ribonucleoproteins (RNPs) is one such technology and has been demonstrated in various plant protoplast systems to induce targeted mutations (Woo et al., 2015; Malnoy et al., 2016; Brandt et al., 2020; Poddar et al., 2020; Sant'Ana et al., 2020). Some have produced edited plants arising from the transfected single cells. However regeneration of wheat and other crop plant protoplasts is not feasible with current methods. Cas9-RNP based editing of maize (Svitashev et al., 2016), rice (Banakar et al., 2019), and wheat (Liang et al., 2017) regenerable embryos by biolistics has also been reported. Gold particles coated with Cas9-RNPs are bombarded with high pressure into immature embryos (IEs) that are ultimately regenerated into plants through tissue culture. Co-delivery of DNA vectors with selective markers or helper genes along with Cas9-RNPs have been utilized to improve editing efficiency (Svitashev et al., 2016; Banakar et al., 2019). In the absence of selection, however, editing rates have generally been low.

The use of Cas9-RNPs to generate edited plants provides unique benefits. Because the gene editing reagents are delivered as pre-assembled complexes, researchers do not need to optimize DNA vectors, the host plant tissue does not bear the burden of transcribing or translating Cas9 or gRNA, and breeding for segregation is unnecessary due to the absence of transgenes. Additionally, the Cas9-RNPs, which exist in a finite amount in the target tissue, are ultimately degraded by endogenous proteases and nucleases. However, there remains room to improve the editing pipeline and increase efficiency.

Low rates of Cas9 mediated editing in plant tissue may indicate that the endonuclease is not reaching its full potential due to suboptimal environmental conditions. For example, studies across organisms including *Arabidopsis*, citrus (LeBlanc et al., 2017), and wheat (Milner et al., 2020) have shown that Cas9 generates more targeted indels at elevated temperatures.

Here, we present advances in Cas9-RNP based gene editing in the global food crop, wheat (*Triticum aestivum*). To determine if temperature can be harnessed to enhance Cas9-RNP mediated editing, we explore the effects of heat treatment on transfected wheat protoplasts and IEs. We examine the relationship of editing efficiency between non-regenerable protoplasts and regenerable IEs and monitor the rate of editing over time. We demonstrate that treatment at elevated temperatures increases gene editing efficiency in both tissue systems and find that the RNP transfection technique of gold particle bombardment results in sustained editing of tissue at least 14 days after bombardment. We also find that editing rates in protoplasts correlate linearly with editing rates in IEs. Therefore, rapid *in vivo* protoplast assays can be instituted as a standard gene editing pipeline step to select the most effective gRNAs for IE gene editing and regeneration. Lastly, we regenerate wheat plants edited *via* Cas9-RNP biolistic transfection. As a proof of method, we simultaneously target three wheat homoeologous orthologs of a rice gene, *Pi21* (Os04g0401000), and successfully generate lines with knockouts in all copies. We also target wheat genes *Tsn1* (GU259619) and *Snn5*, producing lines that are insensitive to the *Parastagonospora nodorum* pathogenic effectors SnToxA and SnTox5 and establish DNA and selection-free Cas9-RNP mediated editing as an efficient and feasible technique for generating targeted gene knockouts in wheat.

Results

Cas9-RNP transfection and the effect of temperature in wheat protoplast gene editing

We first quantified cell viability after heat treatment of non-transfected protoplasts to determine the feasibility of testing higher temperatures for wheat protoplast gene editing. Protoplasts were isolated from partially etiolated wheat seedlings and incubated at 25°C, 30°C, or 37°C for 16 hours followed by 25°C for 8 hours. During the 24-hour period, the protoplasts were monitored for viability every 8 hours using Evans blue staining and microscopy. Viability of protoplasts treated at 37°C decreased markedly compared to those treated at 25°C and 30°C (Figure S1) and suffered from media evaporation. It was therefore concluded that the protoplast gene editing pipeline was not amenable to a 37°C heat treatment.

Five single guide RNAs (sgRNAs), Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were selected and commercially synthesized for this study. To assess the efficacy of the sgRNAs *in vivo*, and to determine the effect of temperature on wheat protoplast gene editing, Cas9-RNPs were assembled and transfected into wheat mesophyll protoplasts. Purified Cas9 with a C-terminal double nuclear-localization tag was complexed with sgRNA. The resulting sgRNA-Cas9 RNPs were transfected into wheat protoplasts using polyethylene glycol (PEG). Transfected protoplasts were treated at 25°C or 30°C and harvested for genotypic analysis after 24 hours. Editing rates at the target loci were determined by amplicon next-generation sequencing (NGS). With incubation at 25°C and 30°C, average editing rates ranged from 2.5-50% and 5.8-62% respectively. Despite this variability between different sgRNA-Cas9 RNPs, editing efficiency was consistently higher in protoplasts treated at 30°C compared to 25°C for any given sgRNA (Figure 1). To determine statistical significance, unpaired two tailed t-tests were performed, comparing 30°C treatment data to 25°C treatment data for each target. All comparisons were found to be statistically significant, with p-values ranging from 0.0393 to 0.0001. Taken together, the data suggest that a higher temperature treatment is advantageous to RNP-mediated gene editing in wheat protoplasts.

Biolistic Cas9-RNP delivery and the effect of temperature in wheat immature embryo gene editing

To determine if a high temperature treatment similarly improves Cas9-RNP based editing in wheat IEs as it does in protoplasts, RNPs were transfected into IEs by particle bombardment. The experimental pipeline is summarized in Figure 2A. Single guide RNA and Cas9 were complexed *in vitro*, adsorbed onto 0.6 µm gold particles, and biolistically delivered with a helium-pressured particle gun. For each sgRNA and temperature being tested, 30 IEs were bombarded and incubated at 26°C, 30°C, or 37°C for 16 hours. They were then maintained at 26°C on callus-induction media before inducing regeneration at around 63 days post-bombardment (dpb). Plasmid DNA was not co-delivered with any of the Cas9-RNPs, and callus induction and regeneration were performed under selection-free conditions. From each set of 30 RNP-transfected embryos, ten were randomly harvested and pooled for genomic analysis at 14 dpb and again at 48 dpb. The remaining ten embryos were kept for regeneration into M_0 plants. All independent shoots were isolated and treated as individual M_0 plants. Plants were transplanted from tissue culture media to soil approximately 100 dpb. Each resulting M_0 plant was independently genotyped, and the percent tissue edited rate was calculated as the percentage of mutant alleles among total alleles in the M_0 plant pool. The percentage of plants edited was also calculated as a percentage of the number of plants with any edit among the number of total M_0 plants regenerated. The data generated and analyzed are from 15 independent biolistic transfections of 30 IEs each. All genomic analysis was done by amplicon NGS.

Elevated temperature treatment of both 30°C and 37°C led to higher percentages of edited tissue compared to 26°C for all five sgRNA-Cas9 RNPs across all timepoints (Figure 2B). Tissue

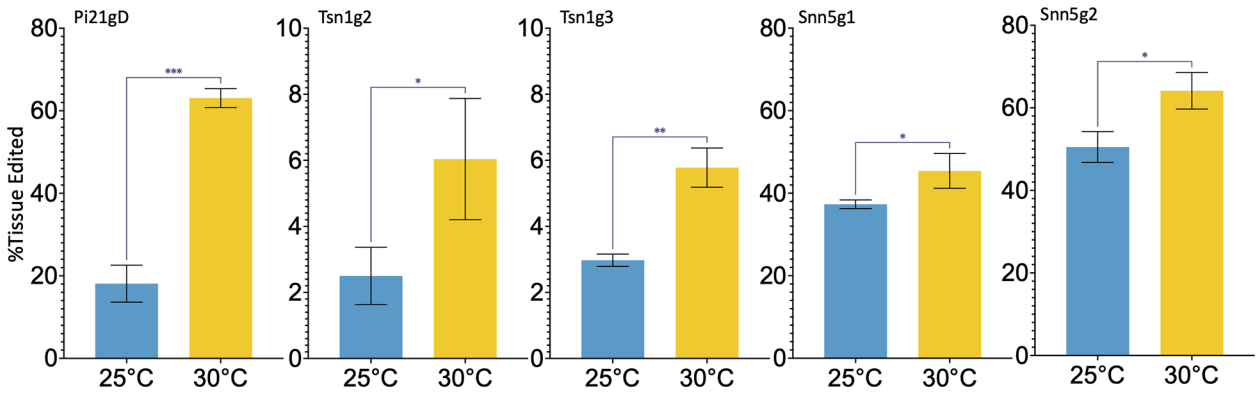

FIGURE 1
Targeted editing efficiency of gRNA-Cas9 ribonucleoproteins with different temperature treatments in wheat protoplasts. Five gRNAs, Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were tested and transfected independently into protoplasts. N=3. P-values from left to right are, p=0.0001, p=0.0393, p=0.0015, p=0.0152, p=0.0319. A p-value less than 0.05 is flagged with one star (*), less than 0.01 is flagged with 2 stars (**), and less than 0.001 is flagged with three stars (***). Error bars indicate SD.

editing rates were higher at 48 dpb than at 14 dpb and editing rates in the M_0 regenerant tissue pool were comparable to those at 48 dpb. From the ten embryos per treatment allowed to regenerate, 10-40 M_0 plants were produced. Plants with wild type, heterozygous, biallelic, and homozygous mutations at the target loci were obtained. Editing efficiency in the M_0 regenerants is summarized in Table 1 and genotypes of each individual edited M_0 regenerant are described in Table S3. Across all targets, 11.8-50% of regenerated plants that underwent temperature treatment of 30°C or 37°C were edited.

Cas9-RNP mediated editing is sustained over time

Notably, gene editing rates were more than doubled, regardless of temperature treatment, in tissue assayed at 48 dpb compared to 14 dpb (Figure 2B). To further investigate the difference in editing rates over time, the number of unique mutant alleles was determined at the 14 and 48 dpb timepoints. With minimal exception, there were more unique mutant alleles at 48 dpb compared to 14 dpb (Figures 3A, S2).

An additional 50 IEs were bombarded with Snn5g1-Cas9 RNP to determine the length of time that Cas9 remains present in biolistically transfected tissue. Western blot analysis was performed with 10-embryo tissue samples taken 0, 2, 7, and 14 dpb. Given the finite amount of Cas9 protein delivered by RNP bombardment and rapid cell division and growth in each IE over time, we normalized the experiment by volume extracted from total tissue originating from ten IEs at any given timepoint, rather than total protein extracted. Cas9 was detected in tissue from all four timepoints with decreasing band intensity over time (Figure 3B). Cas9 was not detected in embryos that were not subjected to bombardment of Cas9-RNPs. Due to the large mass of tissue from exponential growth of callus from IEs, it was not feasible to extract protein from and perform Western blot analysis on ten-embryo 48 dpb samples. Taken together, these results suggest that Cas9 mediated editing activity is sustained over the course of at least 14 days after biolistic delivery of Cas9-RNPs into immature wheat embryos. When using this method, the degradation of Cas9 protein in the target tissue is not as rapid as previously hypothesized (Kim et al., 2014), and evaluation of editing efficiency should occur 14 to 48 dpb for increased accuracy.

Relative editing rates in protoplasts correlate linearly with editing rates in M_0 regenerants from bombarded immature embryos

The different sgRNA-Cas9 RNPs used in this study conferred different levels of efficacy in both PEG transfected protoplasts and biolistically transfected embryos. To determine whether the editing rates in the two tissue systems correlated with one another, each sgRNA-Cas9 RNP's average editing efficiency in 30°C treated protoplasts was plotted against its editing efficiency in 48 dpb 30°C treated bombarded IEs as well as the M_0 30°C treated regenerant tissue pool. A linear regression model was applied to the data, revealing a positive linear correlation with $R^2 = 0.744$ and $R^2 = 0.994$, respectively (Figure 4). Though a survey of a greater number of sgRNAs would strengthen this association, the present data suggest that editing efficiency in protoplasts can be predictive of editing efficiency in IEs. Given the positive correlation between RNP-mediated editing rates in protoplasts and in biolistically transfected IEs, it can be beneficial to first rapidly score the efficiency of various gRNA candidates in protoplasts to optimize for the highest rate of edited regenerant tissue.

TABLE 1 Summary of editing outcomes in Pi21, Tsn1, and Snn5 targeted M0 plants.

					Genome A		Genome B		Genome D			
	Temperature Treatment	Immature embryos regenerated	Total M0 Plants	Edited Plants	Heterozygous	Biallelic or Homozygous	Heterozygous	Biallelic or Homozygous	Heterozygous	Biallelic or Homozygous	% Tissue Edited	% Plants Edited
Pi21gD	26°C	10	4	1	0	1	1	0	1	0	16.7	25
	30°C	10	4	2	0	1	0	1	1	1	29.2	50
	37°C	10	5	2	0	1	1	1	1	1	26.7	40
	Temperature Treatment		**Immature embryos regenerated**		**Total M0 Plants**		**Edited Plants**	**Heterozygous**		**Biallelic or Homozygous**	**% Tissue Edited**	**% Plants Edited**
Tsn1g2	26°C		10		20		1	1		0	2.5	5.0
	30°C		10		22		3	1		2	11.4	13.6
	37°C		10		40		7	6		1	10.0	17.5
Tsn1g3	26°C		10		32		4	4		0	6.3	12.5
	30°C		10		17		2	0		2	11.8	11.8
	37°C		10		20		3	2		1	10.0	15.0
Snn5g1	26°C		10		19		5	5		0	13.2	26.3
	30°C		10		16		6	2		4	31.3	37.5
	37°C		10		10		3	2		1	20.0	30.0
Snn5g2	26°C		10		10		1	1		0	5.0	10.0
	30°C		10		14		4	1		3	25.0	28.6
	37°C		10		18		5	3		2	19.4	27.8

Data for Pi21 is broken down by subgenome. Tsn1 and Snn5 are only present on subgenome B. "% Tissue Edited" indicates the percentage of edited alleles among the total alleles analyzed from the M0 pools. "% Plants Edited" indicates the percentage of plants with any level of editing among the total plants analyzed from the M0 pools.

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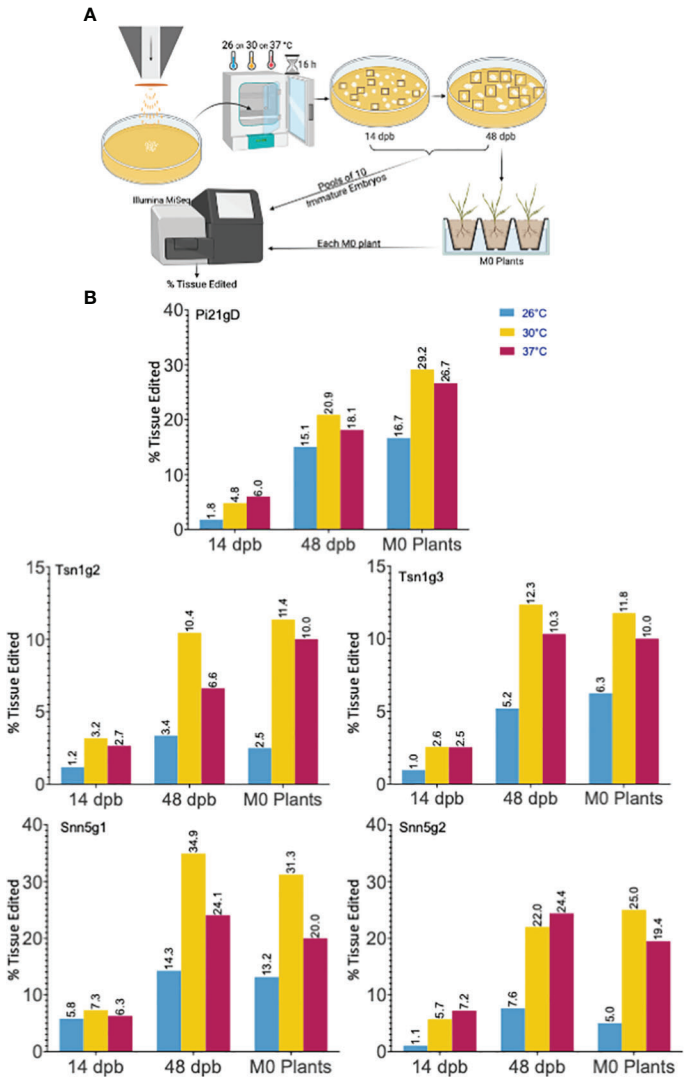

FIGURE 2
Cas9-RNP particle bombardment and temperature treatment of wheat immature embryos (IEs). **(A)** A schematic of the particle bombardment and editing efficiency assay pipeline. **(B)** Targeted editing efficiency of gRNA-Cas9 ribonucleoproteins with different temperature treatments in IEs across time points. Five gRNAs, Pi21gD, Tsn1g2, Tsn1g3, Snn5g1, and Snn5g2 were bombarded independently into IEs. Tissue pools at 14 dpb and 48 dpb consisted of 10 randomly chosen initially bombarded IEs. Editing efficiency for M_0 plants is based on aggregate data from all independently genotyped M_0 plants that emerged from 10 randomly chosen initially bombarded IEs. Percent tissue edited is defined as the percentage of tissue with insertions or deletions within 2 bp of the target cleavage site out of the total tissue pool.

Cas9-RNP mediated knockout of three homoeologous genes

Through a BLAST search of the International Wheat Genome Sequencing Consortium (IWGSC) *Triticum aestivum* genome assembly *via* EnsemblPlants, putative wheat orthologs for the rice gene *Pi21* (Os04g0401000) were identified in the three wheat sub-genomes as TraesCS2A03G0723000.1 (*TaPi21A)*, TraesCS2B03G0799000LC.1 (*TaPi21B*), and TraesCS2D03G0666600LC.1 (*TaPi21D*). For this study, a gRNA, Pi21gD, was designed to simultaneously target all three of these homoeologous genes. Across all treatments, 5 edited plants were regenerated. Two of these contained biallelic or homozygous mutations in all three homoeologous genes.

Cas9-RNP mediated knockout of *Parastagonospora nodorum* necrotrophic effector sensitivity genes

The wheat genes *Tsn1* and *Snn5* recognize necrotrophic effectors produced by *Parastagonospora nodorum*, and each exist

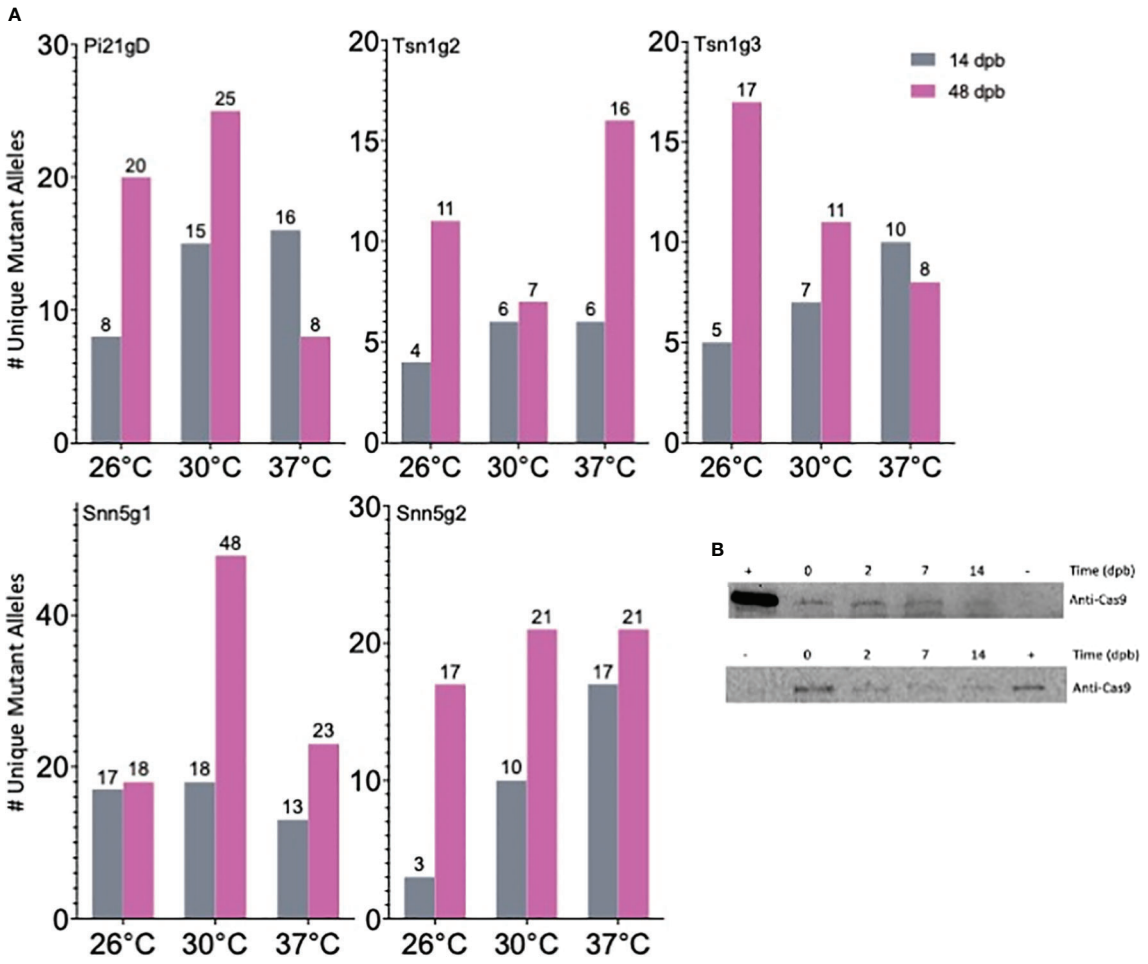

FIGURE 3
Cas9-RNP mediated editing in gold particle bombarded immature embryos IEs is sustained over time. **(A)** Quantification of the number of unique mutant alleles detected *via* deep sequencing. **(B)** Western blot detection of Cas9 in 10-IE bombarded samples taken 0, 2, 7, and 14 dpb with anti-Cas9 antibody. The top and bottom blot represent 2 independent sets of 10 IEs. + = 9 ng (top) and 3 ng (bottom) Cas9; - = IEs that were not bombarded with Cas9-RNP; loading volume of 25 µl (top) and 40 µl (bottom) total soluble protein extract per IE sample.

as single copy genes on the B genome of allohexaploid wheat. In this study, 20 M_0 *Tsn1* edited plants were produced from 30 transfected embryos maintained for regeneration. Of those, 14 had heterozygous mutations and 6 had biallelic or homozygous mutations. Fully expanded secondary leaves of a subset of M_0 *Tsn1* edited plants, M_0 *Tsn1* WT plants, and untransfected WT Fielder were infiltrated with SnToxA expressed in *Pichia pastoris*. After 72 hours, M_0 heterozygotes, M_0 WT, and Fielder plants had necrotic lesions extending from the site of infiltration. Meanwhile, M_0 plants with biallelic or homozygous mutations exhibited no necrosis (Figure 5).

Similarly, a total of 24 M_0 *Snn5* edited plants were produced from 30 transfected embryos maintained for regeneration. Of those, 14 had heterozygous mutations and ten had biallelic or homozygous mutations. Fully expanded secondary leaves of a subset of M_0 *Snn5* edited plants, M_0 *Snn5* WT plants, and untransfected WT Fielder were infiltrated with SnTox5 containing culture filtrates. After 72 hours, M_0 heterozygotes with in-frame deletions, M_0 WT, and Fielder plants exhibited necrotic lesions. Results for M_0 heterozygotes, however, displayed a mixture of phenotypes ranging from sensitive to insensitive. Two heterozygous plants with an in-frame deletion on one allele appeared insensitive to SnTox5. Notably, all plants with biallelic or homozygous mutations leading to premature termination were insensitive to SnTox5 (Figure 6).

These results demonstrate that loss-of-function mutations can be introduced to both copies of a gene within the M_0 generation, leading to insensitivity to agronomically relevant necrotrophic fungal effectors. M_0 heterozygotes and biallelic plants can be self-fertilized to establish lines with homozygous deleterious mutations in the susceptibility genes. All mutations were found to be inherited in M_1 generations. The biolistic

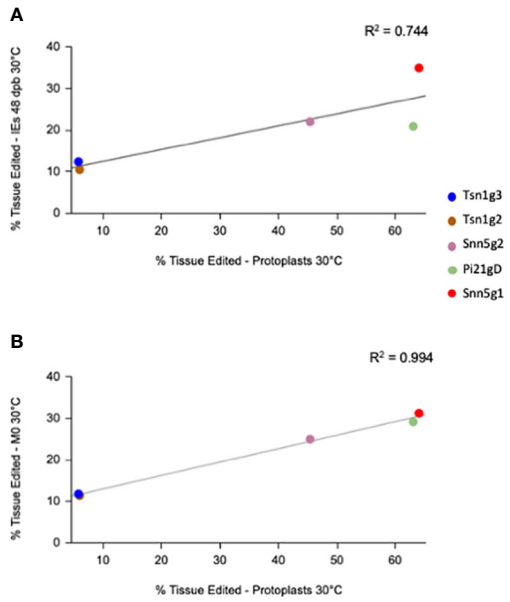

FIGURE 4
Correlation plot between targeted editing efficiency of gRNA-Cas9 RNPs in protoplasts and immature embryos (IEs) at **(A)** 48 dpb and in **(B)** M_0 plants treated at 30°C.

method with 30°C or 37°C heat treatment is highly efficient, and edited plants can be identified from a small number of regenerants without the use of selection in tissue culture.

Discussion

CRISPR-based RNPs have been used for editing in various plant species and tissue types (Zhang et al., 2021). In this work, we improve upon exogenous DNA-free Cas9-RNP technology for genome editing in wheat. We establish heat treatment as a parameter to increase the rate of editing *in vivo*, show that particle bombardment-based editing is sustained over more than 14 days, and demonstrate that results from protoplast assays can be utilized as a proxy for predicting editing rates in regenerable tissue and as a tool to rank gRNA efficacy. By delivering gene editing reagents as protein-RNA complexes, several complications associated with *Agrobacterium tumefaciens* and biolistic DNA vector delivery are avoided.

Cas9 from *Streptococcus pyogenes*, a bacterium that grows optimally at 37°C (Zhou and Li, 2015), has been shown to exhibit increased cleavage activity at 37° compared to 22°C *in vitro* (LeBlanc et al., 2017). Plant protoplast and IE transfections and regeneration are typically performed at ambient

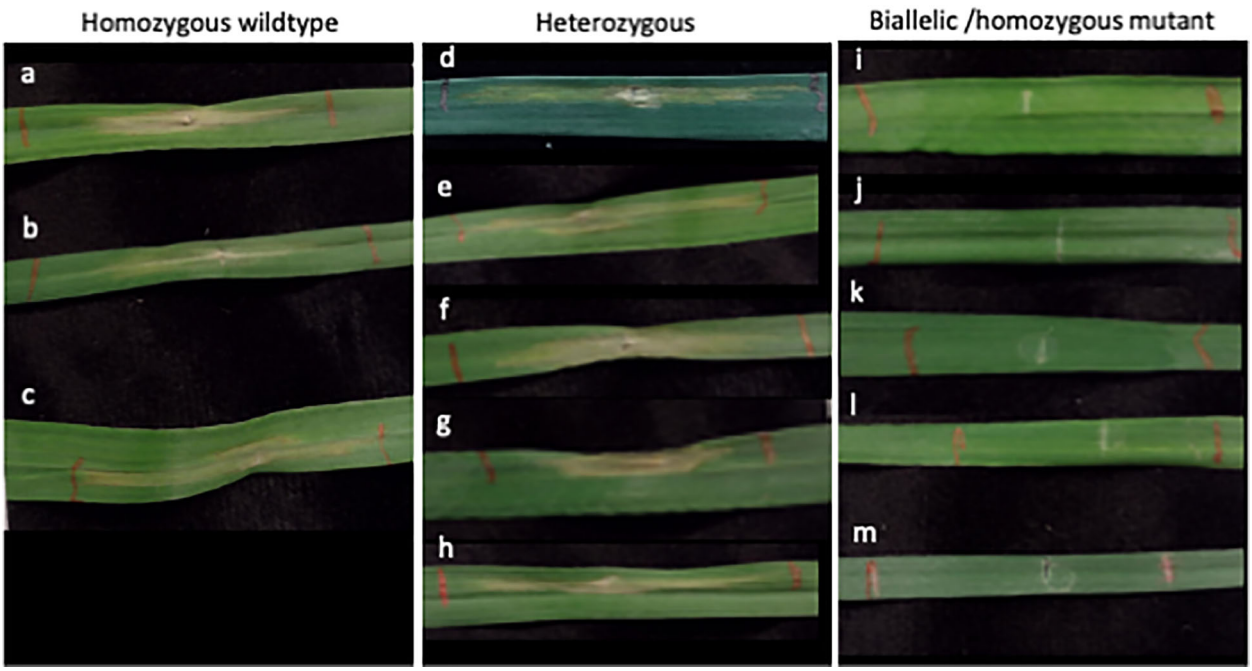

FIGURE 5
SnToxA assay in *Tsn1* targeted M_0 regenerants. **(A)** Fielder control grown from seed. **(B–M)** Independent M_0 regenerants with **(B, C)** homozygous wildtype; **(D–H)** heterozygous **(D)** -2; **(E)** -5; **(F)** -31; **(G)** -1; **(H)** +1; and **(I–M)** biallelic or homozygous mutant **(I)** -2, -5; **(J)** -2, -2, **(K)** -1, -1; **(L)** -2, -2; **(M)** -1, -1 genotypes. Mutation notation is as follows: a positive number, +, indicates the number of bases inserted, a negative number, -, indicates the number of bases deleted.

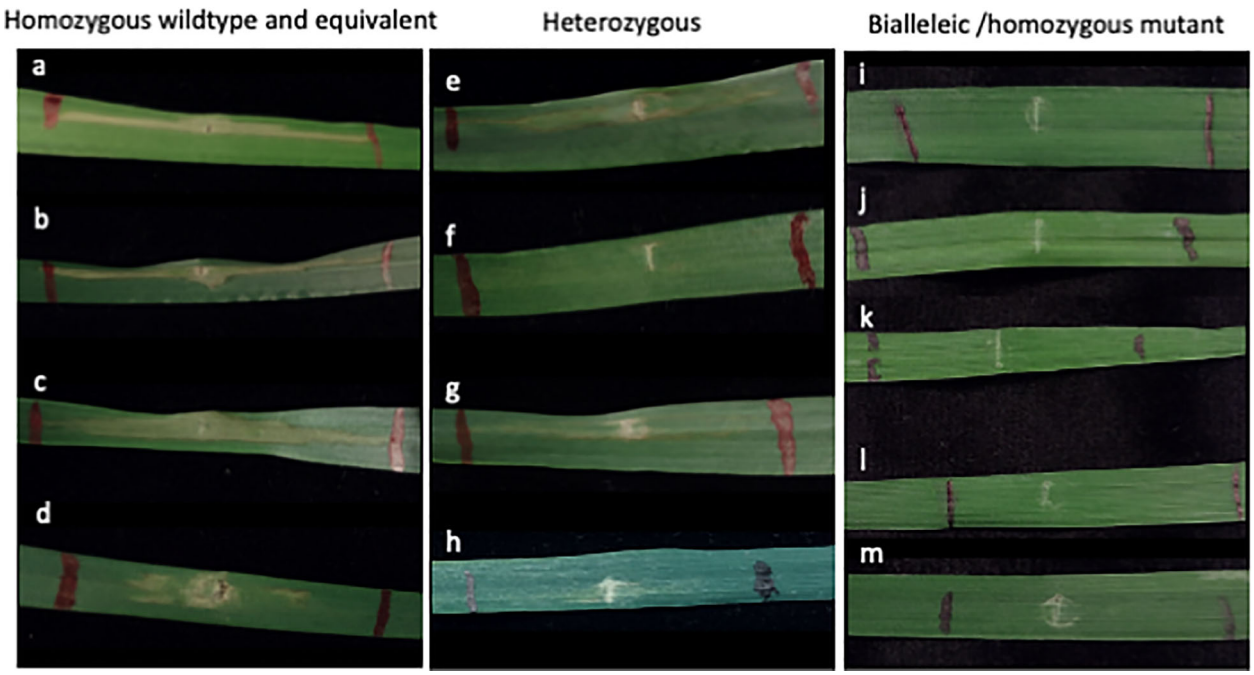

FIGURE 6
SnTox5 assay in *Snn5* targeted M_0 regenerants. **(A)** Fielder control grown from seed. **(B–M)** Independent M_0 regenerants with **(B)** homozygous wildtype; **(C, D)** heterozygous in-frame mutant: **(C)** -3; **(D)** -6; **(E–H)** heterozygous mutant: **(E)** -5; **(F)** +20; **(G)** +2-1; **(H)** -4; **(I–M)** biallelic or homozygous mutant: **(I)** -11, -4; **(J)** -8, -2; **(K)** -10, -10; **(L)** +1, -2; **(M)** -5, -1 genotypes. Mutation notation is as follows: a positive number, +, indicates the number of bases inserted, a negative number, -, indicates the number of bases deleted.

temperatures (25°C and 26°C respectively). Although modulation of temperature has not been previously performed in protoplast gene editing experiments, an increase in temperature for DNA-based plant gene editing studies have resulted in higher targeted mutation frequencies (LeBlanc et al., 2017; Malzahn et al., 2019). The application of temperature treatment to increase Cas9-RNP mediated editing efficiency in any plant tissue system has not previously been demonstrated. Here, we found that 16 hours of exposure of Cas9-RNP transfected protoplasts to 30°C markedly increased indel formation at the Cas9 cut site (Figure 1). Similarly, 16 hours of exposure of Cas9-RNP bombarded IEs to 30°C or 37°C resulted in increased targeted indel formation. In IEs assayed at 48 dpb we achieved editing rates of 10.4-34.9% with 30°C treatment, 6.63-24.39% with 37°C treatment, and just 3.36-14.25% with standard 26°C incubation (Figure 2B). Although both increased temperature treatments improved editing efficiency, the 30°C incubation outperformed the 37°C incubation, indicating that the higher temperature is valuable but not optimal within wheat tissue. For this reason, follow-up work investigating temperature treatments between 30°C and 37°C may be beneficial to determine the highest possible editing rates. Interestingly, the benefit of increased temperature treatment was consistent between the two target tissues and across the five different target sites tested. In our work, there were no discernable defects in regenerability for IEs treated at a higher temperature compared to the standard 26°C. We detected no positive or negative correlation between temperature treatment and the number of M_0 plants recovered.

Two reports have described the biolistic delivery of Cas9-RNPs into wheat and maize embryo cells in the absence of DNA and selection (Svitashev et al., 2016; Liang et al., 2017). Both achieved moderate targeted mutagenesis frequencies in the regenerated plants ranging from 1.3-4.7% and 2.4-9.7% respectively and did not utilize increased temperature treatment. This is comparable to, albeit slightly lower than the 2.5-16.7% editing rates achieved in the present study in regenerated M_0 plants originating from IEs maintained at 26°C without high temperature treatment after transfection (Figure 2B). Differences in targets, methodology, and reagents can account for variant rates of editing. As seen in this work, there exists inherent variability in efficacy across different sgRNA-Cas9 RNPs (Figures 1, 2B). The previously published studies, as well as the current utilized Cas9 protein with two C-terminal SV40 nuclear localization sequences as described by Lin et al., 2014. However, while we used commercially synthesized modified sgRNAs, Liang et al., 2017 and Svitashev et al., 2016 both produced the sgRNAs for their study with T7 *in vitro* transcription. Methodologies for Cas9-RNP assembly and delivery also varied. Liang et al., 2017

outlines a method in which 2 µg Cas9 is complexed with 2 µg sgRNA and mixed directly with 5 µl gold particles for a single bombardment shot into wheat immature embryos. The concentration for the gold particles, the number of shots used per transfection, and the rupture pressure are not described. Svitashev et al., 2016 outlines a method in which 7 µg Cas9 is complexed with 3 µg sgRNA and adsorbed to 50 µl gold particles in a water suspension of 10 mg ml^{-1} using a cationic lipid polymer for five bombardment shots into maize immature embryos using 425 pounds per square inch pressure. The number of shots used per transfection are not described. The current study complexes 6.4 µg Cas9 with 3.23 µg sgRNA and adsorbs the RNP to 10 µl gold particles in a water suspension of 10 mg ml^{-1} using a cationic lipid polymer for a single bombardment shot. Each transfection is composed of two bombardment shots. It is worth noting that no studies to our knowledge have been carried out to quantify the amount of Cas9-RNP that is truly adsorbed to gold particles by these methods. Such data would be valuable to avoid underloading or overloading gold particle preparations with Cas9-RNPs.

We noted that the previous exogenous DNA-free Cas9-RNP studies each assayed for editing efficiency in the IEs 2 dpb and universally achieved <1% targeted editing. In contrast, the editing efficiencies in regenerated plant tissue were substantially higher, ranging from 1.3-4.7% (Liang et al., 2017) and 2.4-9.7% (Svitashev et al., 2016). To investigate this discrepancy between timepoints, we monitored editing efficiency at 14 dpb, 48 dpb, and in the M_0 regenerants in our study. Irrespective of temperature treatment or gRNA sequence, editing frequencies at 48 dpb were considerably higher than at 14 dpb (Figure 2B). Percentage of tissue edited in the M_0 plant pool was comparable to that at 48 dpb. The observed difference in editing efficiency between earlier timepoints and regenerated M_0 plants was consistent with previous reports (Svitashev et al., 2016; Liang et al., 2017).

In mammalian cells, Cas9 was shown to be undetectable 48-72 hours after Cas9-RNP transfection by nucleofection (Kim et al., 2014). For this reason, it has been thought that enzymatic degradation of Cas9-RNPs *in vivo* is rapid and that editing must occur within the first few days of transfection. In the present study, if Cas9-RNPs were fully degraded from the tissue prior to the 14 dpb timepoint, all gene editing would have had to occur before 14 dpb. Consequently, approximately the same number of unique alleles would have been expected to be detected at both 14 dpb and 48 dpb if proliferation of edited and unedited cells occurs at the same rate. On the contrary, consistently higher rates of mutagenesis as well as a greater number of unique alleles at the later timepoints were observed at 48 dpb (Figures 2B, 3A, S2), suggesting that Cas9 may somehow be stabilized for at least 14 days and gradually released within the wheat IEs after biolistic delivery for sustained editing over time. As further evidence in support of this hypothesis, Cas9 protein was detected in 10-embryo tissue samples taken 2, 7, and 14 dpb (Figure 3B). Taken together, these results indicate that Cas9 is maintained in tissue at least 14 dpb and facilitates sustained and gradual editing of tissue over time when delivered as Cas9-RNP *via* gold particle bombardment. The data offer a plausible explanation for the discrepancy between editing rates at early versus late timepoints in both the current study and studies by Svitashev et al., 2016 and Liang et al., 2017. Further biochemical exploration is necessary to understand the mechanism of this Cas9 stabilization and persistent editing.

Numerous plant protoplast systems have been used for targeted mutagenesis using Cas9-RNPs (Woo et al., 2015; Malnoy et al., 2016; Shan et al., 2019; Brandt et al., 2020; Poddar et al., 2020; Sant'Ana et al., 2020; Yu et al., 2020). Although the method is useful for producing Cas9-RNP edited plants for protoplasts that are amenable to regeneration, most crop plants cannot easily be regenerated in this manner. Though wheat protoplasts are recalcitrant to regeneration through existing methodology, protoplasts in the current study prove to be a beneficial screening system. Cas9-RNP mediated editing rates in protoplasts correlated linearly with editing rates in IEs. Because biolistic Cas9-RNP transfection of IEs requires significant time, energy, resources, and commitment, a means for rational selection of gRNA sequences for optimal editing efficiency is preferred. It is noteworthy that there were major differences in mutation rates for the 5 gRNAs used in this study. Unfortunately, existing predictive software to select gRNAs often do not translate upon experimentation. A current method for ranking gRNAs is through an *in vitro* cleavage assay (Karmakar et al., 2021). While this is practical for selecting gRNAs before delivery into plant cells, it does not account for complexities within the genome that ultimately can affect gene editing rates *in vivo*. Therefore, when attempting to select the best gRNA to produce the highest rate of stable editing in regenerable IEs, transient protoplast assays can serve as a rapid pipeline to rank gRNAs and forecast editing rates in Cas9-RNP bombarded regenerable tissue.

The calculation of editing efficiency in M_0 regenerants has the potential to be confusing. To be explicit in our analysis, we present editing rates of regenerants in two ways. The percentage of total edited alleles in the M_0 regenerant pool is indicated as "% Tissue edited", while "% Plants edited" is the percentage of total edited plants among all the M_0 plants (Figures 2B, 4, Table 1). The former is meant to compare overall editing efficiency more fairly across tissue types and timepoints, taking biallelism, homozygosity, and heterozygosity of regenerated plants into consideration. The latter value is more relevant for evaluating the method's ability to produce individual plants with gene edits but should be interpreted while considering the complexity of regeneration and the fact that only a subset of the originally edited tissue ultimately gives rise to an edited plant. Improvements in control of regeneration will be beneficial to the field.

One major application of Cas9 mediated gene editing is towards the creation of targeted genetic edits for plant disease

resistance, and a variety of studies have made progress in this realm (Karmakar et al., 2022). The gene *Pi21* was first characterized in rice (*Oryza sativa*) as a negative regulator of resistance for blast disease (Fukuoka et al., 2009). We identified putative orthologs in wheat that consisted of three homoeologous genes. The functionality of wheat *Pi21* has not been formally assessed but may potentially play a role in disease susceptibility. Wheat *Pi21* was selected as a target to demonstrate the DNA-free Cas9-RNP gene editing method in a gene present in all three diploid subgenomes (AABBDD). Pi21gD was designed to simultaneously target all six alleles. Despite the genetic complexity, we were able to regenerate plants with biallelic or homozygous mutations across all three subgenomes for a full variety of genotypes including two with biallelic or homozygous triple mutant edits within the M_0 generation (Table S3).

The wheat genes *Tsn1* and *Snn5* recognize the *Parastagonospora nodorum* pathogenic effectors SnToxA and SnTox5, respectively (Faris et al., 2010; Kariyawasam et al., 2021). *Tsn1* is a gene with resistance gene-like features including protein kinase, nucleotide binding, and leucine-rich repeats, and the ToxA necrotrophic effector is produced by at least three economically important fungal pathogens of wheat (Friesen and Faris, 2021). *Snn5* belongs to a different class and contains protein kinase and major sperm protein domains (details regarding the cloning and characterization of *Snn5* will be published in the future; K.L.D. Running and J.D. Faris, personal communication), but like *Tsn1*, it functions as a target for a necrotrophic effector leading to disease susceptibility (Kariyawasam et al., 2021). Therefore, *Tsn1* and *Snn5* are practical targets for disruption *via* DNA-free gene editing. Using DNA-free biolistic delivery of Cas9-RNPs, we successfully generated plants with heterozygous, biallelic, and homozygous mutations within the M_0 generation from a mere ten IEs per treatment. Biallelic and homozygous mutants of *Tsn1* and *Snn5* were demonstrated to be insensitive to SnToxA and SnTox5, respectively, demonstrating effective bioengineering of decreased susceptibility to an agronomically relevant pathogen. Due to the high rate of editing, particularly using Snn5g1 and Snn5g2 with 30°C and 37°C heat treatment, screening of M_0 plants for edits was fully feasible. Contrary to previous reports, a selection scheme can reasonably be foregone with Cas9-RNP mediated editing so long as gRNAs are pre-tested in protoplasts and deemed to be highly effective.

In summary, heat treatment enhancement of Cas9-RNP mediated wheat editing combined with a protoplast-based approach to select optimal gRNAs, and findings that editing is sustained for more than 2 weeks advances this exogenous DNA and selection-free gene editing approach in crops. Given the persistence of Cas9 in bombarded tissue, additional work with increased length or punctuated exposure to heat, beyond 16 hours, throughout callus induction may further augment the benefit of heat treatment. The success of this method in targeting single loci warrants exploration of furthering the technique to multiplexing. In addition to knocking out genes, editing *via* Cas9-RNPs can conceivably be applied to generating allelic series by targeting non-coding genomic regions such as promoters (Rodríguez-Leal et al., 2017). The method may also be used toward advancing homology directed repair (HDR) outcomes in plant tissue *via* co-delivery with template DNA. The presented advancement to this technology can be applied to numerous crops that are amenable to particle bombardment and encourages the establishment of tissue culture and regeneration protocols in crop species that are vegetatively propagated.

Materials and methods

Plant material

The allohexaploid wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD genomes) cultivar Fielder was used for this study. Plants were grown at 24°C, 16-hour days and 15°C, 8-hour nights under light intensity of 130 µmol $m^{-2}s^{-1}$. Immature seeds containing IEs, sized 1.7-2.2 mm were harvested from wheat spikes 10-13 days after flowering, surface sterilized in 20% (v/v) bleach with one drop of Tween 20 and triple rinsed with sterile water, followed by extraction of the IEs.

Cas9-gRNA RNP assembly

Cas9 protein with a C-terminal double nuclear-localization tag (QB3 Macrolab, University of California, Berkeley) and sgRNAs with modifications of 2'-O-Methyl at 3 first and last bases, and 3' phosphorothioate bonds between first 3 and last 2 bases (Synthego, Menlo Park, CA) were complexed *in vitro* to form Cas9-gRNA RNPs.

For each protoplast transfection, a 25 µl reaction was assembled. Thoroughly mixed were 10 µg sgRNA, 2.5 µl 10X NEBuffer 3.1 (New England Biolabs, Ipswich, MA), and nuclease-free water. Then, in a drop-wise manner, 10 µg Cas9 was added slowly with constant mixing, followed by 20 min incubation at 37°C.

For each IE biolistic transfection, a 40 ul reaction was assembled. Thoroughly mixed were 6.4 µg sgRNA, 4 µl 10X NEBuffer 3.1, and nuclease-free water. Then, in a drop-wise manner, 12.8 µg Cas9 was added slowly with constant mixing, followed by 20 min incubation at 37°C.

The resultant RNP mixtures were stored on ice until transfection.

Protoplast isolation and transfection

Partially etiolated seedlings were used as donor tissue for protoplast isolation. Seeds were surface sterilized in 20% (v/v)

bleach and rinsed in sterile water. Seedlings were grown under sterile conditions on wet filter paper in the dark for 12-14 days at 25°C with exposure to ambient light for 6 hours every 5 days. Wheat protoplasts were isolated from the donor tissue using a previously described method (Shan et al., 2014). An example image of protoplasts is provided in Figure S3. For each transfection 25 µl of Cas9-gRNA RNP mixture, as defined above, were added to 5 X 10^5 protoplasts. PEG-meditated transfection was performed as described in the literature (Shan et al., 2014). Protoplasts were harvested 24 hours post-transfection for analysis.

Gold particle preparation for bombardment

Cas9-RNPs were precipitated onto 0.6 µm gold particles (#1652262, Bio-Rad, Hercules, CA) using the cationic lipid polymer TransIT-2020 (Mirus, Madison, WI) as previously described (Svitashev et al., 2016), with modifications. Briefly, for each 30-IE transfection, 40 µl Cas9-RNP mixture, as described above, was mixed gently with 20 µl sterile gold particles (10 µg $µl^{-1}$ water suspension) and 1 µl TransIT-2020 and incubated on ice for 20 min. The Cas9-RNP coated gold particles were pelleted in a mini microcentrifuge at 2,000*g* for 30 s. The supernatant was removed, and the gold particles were resuspended in 20 µl of sterile water by brief sonication. The coated gold particles were immediately applied to 2 macrocarriers (10 µl each) by spotting numerous small drops and allowed to air dry in a laminar flow hood. For a single transfection, each 30-IE set was bombarded twice using the 2 prepared macrocarriers.

Immature embryo bombardment and regeneration

Immature embryos were placed on DBC3 media (Cho et al., 1998), scutellum side up and incubated overnight at 26°C prior to biolistic transfection. Four hours prior to bombardment, IEs were placed on 55 mm filter paper in the center of DBC3 osmoticum media containing 0.2 M mannitol and 0.2 M sorbitol (Cho et al., 2000). Using two prepared microcarriers holding Cas9-RNP coated gold microparticles, IEs were shot twice using the PDS-100/He gene gun (Bio-Rad, Hercules, CA) with rupture pressure of 1100 psi. The bombarded IEs were transferred from the filter paper directly to the media below and incubated at 26°C, 30°C, or 37°C for 16 hours. IEs were transferred to standard DBC3 media in dim light (10 µmol m^{-2} s^{-1}) at 26°C for 9 weeks and subcultured every 3 weeks. Callus tissue originating from each IE was transferred to DBC6 media for regeneration and subcultured every 3 weeks (Cho et al., 2015). Resultant shoots, which emerged 90-120 dpb were transferred to rooting media and incubated in high light (90 µmol m^{-2} s^{-1}) at 26°C and grown to 4-6 inches before being transplanted to soil.

Amplicon next generation sequencing analysis

To determine mutation rates by amplicon sequencing, PCR was performed with target-specific primers (3 S1), amplifying approximately 225 bp around the cut site using Phusion High Fidelity (New England Biolabs, Ipswich, MA) polymerase. Primers contained a 5′-stub compatible with Illumina NGS library preparation. PCR products were ligated to Illumina TruSeq adaptors and purified. Libraries were prepared using a NEBNext kit (Illumina, San Diego, CA) according to the manufacturer's guidelines. Samples were deep sequenced on an Illumina iSeq at 200 bp paired-end reads to a depth of approximately 10,000 reads per sample. Cortado (https://github.com/staciawyman/cortado) was used to analyze editing outcomes. Briefly, reads were adapter trimmed then merged using overlap to single reads. These joined reads were then aligned to the target reference sequence. Editing rates are calculated by counting any reads with an insertion or deletion overlapping the cut site or occurring within a 3 bp window on either side of the cut site. SNPs occurring within the window around the cut site are not counted. Total edited reads are then divided by the total number of aligned reads to derive percent edited.

Western blot

Total plant tissue originating from 10 IEs at different timepoints were frozen in LN2, ground to a fine powder by mortar and pestle, and resuspended in 200 µl 2x Laemmli Sample Buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol. Samples were boiled for 5 min, and the total soluble protein extracts (25 µl or 40 µl per well) were separated on 4-20% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad, Hercules, CA) and subsequently transferred to a 0.45 µm nitrocellulose membrane (GVS, Sanford, ME). For detection of Cas9 protein, anti-CRISPR/Cas9 C-terminal mouse monoclonal antibody (SAB4200751; Sigma-Aldrich, St. Louis, MO) and ProSignal Dura ECL Reagent (Genesee Scientific, San Diego, CA) were used. PageRuler Plus Prestained Protein Ladder (10–250 kDa, Thermo Fisher, Waltham, MA) was used as a molecular weight marker, and Cas9 protein with a C-terminal double nuclear-localization tag (QB3 Macrolab, University of California, Berkeley) was used as a positive control.

Production of SnToxA

SnToxA was expressed in the *Pichia pastoris* yeast strain X33 (Liu et al., 2009) and cultured in yeast peptone dextrose broth (10 g yeast extract, 20 g peptone, 100 ml 20% dextrose in 900 ml

distilled water) for 48 hours at 30°C. Culture filtrate was harvested and filtered through a 0.45 µm HVLP filter membrane (Merk Millipore Ltd., Cork, Ireland) and dialyzed overnight against water using 3.5 kDa molecular weight cut off Snake Skin dialysis tubing (Thermo Scientific, IL, USA). Dialyzed filtrate was loaded onto a HiPrep SP XL 16/10 cation exchange column (GE Healthcare Piscataway, NJ). Unbound protein was washed off the column using a 20 mM sodium acetate (pH 5.0) buffer prior to a gradient elution of SnToxA using a buffer consisting of 300 mM sodium chloride and 20 mM sodium acetate (pH 5.0). Fractions that contained SnToxA were collected and frozen prior to lyophilizing to increase the concentration of SnToxA. Lyophilized samples were dissolved in a buffer consisting of 5 mM MOPS sodium salt (Alfa Aesar, MA, USA) and water, prior to infiltration into the plants.

Production of SnTox5

P. nodorum strain Sn79+Tox5-3, generated by transforming *SnTox5* in to the avirulent *P. nodorum* strain Sn79-1087 (Kariyawasam et al., 2021), was used to prepare the culture filtrates containing SnTox5 as previously described (Friesen and Faris, 2011) with minor modifications. In brief, Sn79+Tox5-3 was grown on V8-potato dextrose agar medium till spores were released from pycnidia. The plates were flooded with 10 ml of sterile distilled water, and 500 µl of spore suspension was used to inoculate 60 ml of liquid Fries medium (5 g ammonium tartrate, 1 g ammonium nitrate, 0.5 g magnesium sulfate, 1.3 g potassium phosphate [dibasic], 3.41 g potassium phosphate [monobasic], 30 g sucrose, 1 g yeast extract in 1000 ml of distilled water). Cultures were grown on an orbital shaker at 100 rpm for a week prior to two weeks of stationary growth under dark conditions at room temperature. Culture filtrates were filtered through a layer of Miracloth (EMD Millipore Corp, MA, USA) and were concentrated 5-fold using Amicon Ultracel – 3K centrifugal filters (Merk Millipore Ltd., Cork, Ireland). Culture filtrates were diluted in a 1:1 ratio with sterile water prior to infiltration into the plants.

Necrotrophic effector infiltrations

Infiltrations with SnToxA and SnTox5 containing culture filtrates were conducted as previously described (Friesen and Faris, 2011). Three infiltrations were performed per plant, and sensitivity was evaluated on a binary scale at 3 days post infiltration.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

SP conceived and designed the experiments, analyzed the data, prepared the tables and figures, and wrote the manuscript with input from all co-authors. SP and JT performed the experiments. KR, GK, JF, TF, and M-JC provided critical reagents, information, and discussion. JC and BS supervised the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1084700/full#supplementary-material

SUPPLEMENTARY TABLE 1
gRNA target sequences.

SUPPLEMENTARY TABLE 2
Primers used to amplify the target region for amplicon next generation sequencing. Nucleotides shown in capital letters are the 5′-stub compatible with Illumina NGS library preparation.

SUPPLEMENTARY TABLE 3
Genotypes of all edited M_0 plants obtained. + indicates the number of base pairs inserted, - indicates the number of base pairs deleted.

SUPPLEMENTARY FIGURE 1
Protoplast viability curve. N=3. Error bars indicate SEM.

SUPPLEMENTARY FIGURE 2
Example of the difference in the number of unique mutant alleles between 14 dpb and 48 dpb. Provided are the detected alleles in immature embryos bombarded with Tsn1g2-Cas9 RNPs and treated at 37°C. The vertical bold dashed line represents the Cas9 cleavage site. Mutant alleles are marked with *. Wild type alleles are marked as WT. Dashes indicate base pair deletions, red boxes indicate base pair insertions, and bold letters indicate base pair substitutions.

SUPPLEMENTARY FIGURE 3
Wheat protoplasts isolated from leaf tissue.

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Divergence and convergence in international regulatory policies regarding genome-edited food: How to find a middle ground

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Regulations for organisms and products to which genome-editing technologies are applied are increasing in diversity, with the path-dependent effect of previous regulations for genetically modified organisms. Regulations for genome-editing technologies are a patchwork of international regulations that are difficult to harmonize. However, if the approaches are arranged in chronological order and the overall trend is examined, the regulation of genome-edited organisms and GM food products has recently been trending toward a middle ground which can be characterized as "limited convergence." There is a trend toward the adoption of two approaches: one that considers GMOs but tries to apply simplified regulations and another that excludes them from the scope of regulations as non-GMOs but requires confirmation. In this paper, we discuss why there is a tendency toward convergence of these two approaches and examine the challenges and implications of these two approaches for the governance of the agricultural and food sectors.

KEYWORDS

genome-editing, regulation, policy convergence, divergence, trade

1 Introduction

Regulations on living organisms and food products to which genome-editing technology is applied are being considered in various countries based on the current regulations on genetically modified organisms (hereafter GMOs). An overview of existing regulations (Menz et al., 2020; Entine et al., 2021; Turnbull et al., 2021; Jones et al., 2022) shows that only a patchwork of regulations exists instead of an international regulatory harmonization.

Regulatory review of genome-editing technology originally began with a review of existing regulations on GMOs in each country. This kind of review aimed to determine whether regulatory gaps existed between the new breeding techniques and existing regulations on GMOs. For example, in the European Union (EU), which was the earliest

to examine the regulatory issues of new breeding techniques, including genome-editing technologies, the New Techniques Working Group was established within the European Commission in 2007 to identify and examine the characteristics of each technology and the regulations.

In identifying these regulatory gaps, each country is considering how to regulate organisms derived from genome-editing technologies, and some countries have responded by developing guidelines that partially revise or supplement existing laws regulating GMOs. International differences in the regulation of GMOs led to further differences in the regulation of organisms derived from genome-editing technology. This can be understood as path dependency in the sense that GMO policy has influenced subsequent policy on genome-editing technologies. As a result, international regulatory harmonization has become very difficult if not impossible. However, while regulatory approaches vary internationally, they are not randomly divergent. Rather, they converge in particular ways and within particular limits.

This study examines the regulatory considerations of various countries[1], and confirms that a certain common direction, "limited convergence," can be found in diversity. Then we discuss the regulatory context and reasons for this convergence. This study also discusses the socioeconomic implications of the lack of complete regulatory convergence, especially in the context of research and development, marketization, and trade. Genome-editing technologies are often discussed in three categories: SDN-1 (Site-Directed Nuclease 1), SDN-2, and SDN-3 (Podevin et al., 2013)[2]. SDN-3 is usually treated as subject to GMO regulations because it introduces foreign genes. Since the judgment on the treatment of SDN-1 and SDN-2 is different among countries, unless otherwise specified, the following discussion of genome-editing technologies will exclude SDN-3. The following section presents a framework for categorizing regulatory approaches in each country and then briefly reviews the regulatory situations of each country. Subsequently, the background to the convergence is examined as a cross-sectional discussion. Since complete convergence is unlikely to be reached, the socioeconomic implications of this situation are discussed, and conclusions are drawn.

2 Regulatory approaches and overview of each nation

Although many countries in the world are still considering the regulation of genome-edited organisms, some countries and regions have already established their own policies. At this point, regulatory approaches in each country can be broadly divided into the following four approaches (Table 1 and Box).

The first position is to subject the genome-edited organisms to genetic modification regulations, which involves two approaches: (1) applying the GMO regulation as it is (Approach 1) and (2) applying a slightly simplified regulation (Approach 2). The former approach includes the EU and New Zealand Environmental Protection Agency, which considers genome-edited organisms to be the subjects to the same regulations as GMOs. The latter includes China and the United Kingdom (under discussion[3]), which are trying to adopt a framework that allows the authorization of genome-edited organisms through a simplified procedure in safety assessment while including them in the regulatory framework for GMOs.

The second position differs from the above position in that it excludes genome-edited organisms from the scope of GMO regulations. There are two approaches to this position: The first approach requires prior confirmation (Approach 3), while the second does not (Approach 4). The former approach, which includes Argentina and Japan, requires prior confirmation from the government, and if no problems are found after confirmation, the product is put on the market. Typically, if the developer notifies the government and the government (or the risk assessment agency) determines that it is not a genetically modified organism, it can be used commercially without any of the requirements stipulated for GMOs. The latter includes the US Department of Agriculture (hereafter USDA) and the Australian Office of Gene Technology Regulator (hereafter OGTR), where developers can make their own decisions and market products that are determined to be exempt from regulation without prior confirmation from the regulatory agency (allowing self-determination by developers).

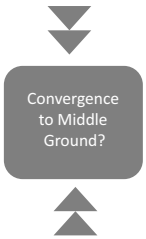
Approaches 1 through 4 can be categorized as a sequence from the most to the least stringent in terms of regulation of organisms subject to genome-editing technologies. The difference between the above-mentioned two positions dictates whether the regulation of GMOs is focused on the technology applied (process-based regulation) or on the characteristics of the organisms produced (product-based regulation). Based on this repeatedly argued dichotomy, we note that a further difference is emerging within each position.

[1] This paper discusses 10 countries/regions, ranging from Europe (EU) to North America (US) to South America (Argentina) to Oceania (Australia and New Zealand) to Asia (Japan, China, India, and the Philippines). These countries and regions were selected because they are considered suggestive of the diversity of rules surrounding genome-editing technology at this point in time. Other countries are also mentioned in Table 2 to present the status of rulemaking.

[2] According to Podevin et al. (2013: 376-77), three types SDNs are explained as follows. "The SDN-1 techniques use SDNs to generate site-specific random mutations; in most cases via a single DSB [double-strand breaks] that plants repair mainly by nonhomologous end joining (NHEJ)." "The SDN-2 techniques are based on the use of an externally supplied DNA template for the repair that proceeds by homologous recombination (HR)." "[T]he SDN-3 techniques aim to introduce long DNA fragments (e.g., transgenes) at a predefined locus using a donor DNA [...] in combination with an SDN. This facilitates the targeted integration of DNA [...]."

[3] In the UK, a new category of organism called "precision bred organisms" is being proposed which "will be subject to a new regulatory framework as defined in the Genetic Technology Bill. This framework will contain aspects of both the GMO legislative framework, which will remain in place for all other organisms made using modern biotechnology, and the framework as it applies to traditionally bred organisms. As such, the level of regulatory scrutiny is somewhere between that of GMOs and traditionally bred organisms." (UK Parliament Impact Assessment 2022, p.20) https://publications.parliament.uk/pa/bills/cbill/58-03/0011/ImpactAssessmentGeneticTechnology(PrecisionBreeding)Bill.pdf

TABLE 1 Four Approaches of Regulation of Genome-Edited Products.

Approaches	How the product is treated under the regulation: GMO or non-GMO	Applied Regulatory Oversight	Country or authority	
Approach 1	GMO	GMO Regulation as it is	EU, NZ (EPA)	
Approach 2	GMO	Simplified GMO regulations	UK*, FSANZ*, China	
Approach 3	non-GMO	Exempted but with confirmation by regulatory authority	Argentina and South America, Japan, India, Philippines	
Approach 4	non-GMO	Confirmation not required by regulatory authority	US (USDA), Australia (OGTR)	

An asterisk (*) indicates that it is under consideration. Since products using SDN-3 is subject to GMO regulations, SDN-3 is excluded from this Table.

BOX Explanation of the Four Approaches

Approach 1: GMO regulations are applied as it is to genome-edited products. As a result, prior safety assessment and approval by the government are required.
Approach 2: Simplified GMO regulations will be applied to genome-edited products. As a result, simplified safety review and approval procedures will be applied.
Approach 3: Genome-edited products are exempt from GMO regulations. However, confirmation by the government is required before placing on the market.
Approach 4: Genome-edited products are exempt from GMO regulations. Prior confirmation is not required by the government.

The following section provides an overview of the regulations of each country and describes their positions based on the above classification of approaches. Table 2 shows when each country decided on their regulations based on such classification.

2.1 The United States

The USDA, the Food and Drug Administration (hereafter FDA), and the United States Environmental Protection Agency (hereafter USEPA) have been regulating GMOs, and updates have been provided to the Coordinated Framework for Regulation of Biotechnology since 1986[4]. Each of the three agencies has been responsible for regulating transgenic organisms from its own perspective. The Office of Science and Technology Policy (OSTP) in the Executive Office of the President has played an important role in allocating and coordinating the roles of the three agencies. The decision to make genetically modified fish subject to FDA regulation is one example. Furthermore, the OSTP had urged the agencies to modernize their regulations to accommodate future biotechnology producsts. In this context, the USDA issued a notice in the Federal Register on May 18, 2020, regarding a major revision (the Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient (SECURE) biotechnology regulations) to the biotech regulations (7 CFR 340)[5]. The SECURE rule clarifies the regulatory policy for plants with genome-editing applications. The USEPA also published a proposed review of the current Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) at the end of August 2020, and the FDA also collected comments in January 2017. Below is a summary of the regulatory policies of each ministry and agency.

2.1.1 United States Department of Agriculture

The USDA-APHIS (Animal and Plant Health Inspection Service) has been regulating transgenic crops based on the Plant Protection Act from the viewpoint of regulating plant pests. In pre-SECURE regulations, developers sought safety review from the USDA under two procedures; notification and permit; those that were not found to pose a plant pest risk by the USDA were deregulated[6]. While building on previous regulatory experience, the revision of regulations was much needed to accommodate new technologies and new products. Against this backdrop, the USDA revised its federal regulation as the SECURE rule in May 2020, and through this update, the USDA has clarified its regulatory authority over plants derived from genome-editing technologies. They are now exempted from the regulation if any one of the following occur:

(1) The genetic modification is a change resulting from the cellular repair of a targeted DNA break in the absence of an externally provided repair template.
(2) The genetic modification is a targeted single base pair substitution.

[4] The Coordinated Framework has been updated in 1992 and 2017.

[5] 85 Fed. Reg. 29790 (May 18, 2020)

[6] In addition, state governments also have authority for oversight of transgenic crops and collaborate with federal agencies. In the case of state government oversight, interests exist not only in health and the environment but also from an economic perspective (Taylor et al. 2004).

TABLE 2 Time Series of Regulatory Decisions of Genome-Edited Products in Each Country.

Approach	2014	2015	2016	2017	2018	2019	2020	2021	2022
Approach 1	New Zealand (EPA)				EU				
Approach 2								Australia & New Zealand (FSANZ)*	China (MARA), UK (Defra)*
Approach 3		Argentina		Chile Israel	Brazil	Japan			Canada (Health Canada), India, Philippines
Approach 4						Australia (OGTR)	US (USDA)		

An asterisk (*) indicates that it is under consideration.

(3) The genetic modification introduces a gene known to occur in the plant's gene pool, or makes changes in a targeted sequence that correspond to a known allele of such a gene or a known structural variation present in the gene pool.

(4) The Administrator may propose to exempt plants with additional modifications, based on what could be achieved through conventional breeding.

As stated above, while template-based genome-editing technologies are subject to regulation, point mutations (to which oligonucleotide-directed mutagenesis (ODM) may fall under) are exempted from the regulation, unlike Australia and the Japanese Ministry of Environment. It is also important to note that the SECURE rule does not require an application to the USDA for a genetically modified (hereafter GM) crop that has been previously reviewed by the USDA, as long as a plant- trait- mechanism of action combination is the same. The limited scope of the regulation allows developers to self-determine[7] and commercially grow GM crops that the USDA has previously screened and genome-edited crops as stipulated above[8]. The USDA allowed such self-determination to redirect the administrative resources to newly developed biotech products (NASEM, 2017) in the future.

2.1.2 United States Environmental Protection Agency

The USEPA, under the FIFRA, has regulatory authority over pesticide ingredients produced in plants or plant-incorporated protectants (PIPs). This includes pesticide components such as cry1A, which is produced in Bt maize and other crops. In August 2020, the EPA released its proposed revisions to the FIFRA, which would exempt from the registration of PIPs, previously only allowed in conventionally bred plants, to plants derived from new technologies such as genome-editing technologies[9]. In other words, the proposal is to treat Bt crops produced by genome editing in the same way as crops obtained by conventional breeding. The USEPA also proposed that the Federal Food, Drug, and Cosmetic Act (FFDCA) allowable limits for PIPs be exempted for above-mentioned case. A final decision will be made based on public comment.

The proposed revisions would implement the Executive Order 13874, 84 Fed. Reg. 27899, "Modernizing the Regulatory Framework for Agricultural Biotechnology Products," issued on June 11, 2019. This revision would advance the exemption from regulation for low-risk products, as required by the Executive Order.

2.1.3 Food and Drug Administration

Under the "Statement of Policy: Foods Derived from New Plant Varieties" issued in 1992, the FDA accepts voluntary consultations from companies regarding GM foods and oversee safety on a case-by-case basis. This voluntary consultation system provides a comprehensive framework that can include new technologies such as genome-editing technologies in the sense that it covers "new plant varieties." Therefore, the FDA did not introduce any new regulations; thus, the existing approach is still being used for genome-edited food.

The FDA regulates GM animals as well as GM foods under the FFDCA. GM animals are regulated as part of the animal drug regulations under the above law and are handled by the Office of Veterinary Medicine within the FDA. For animals derived from genome-editing technologies, the Draft Guidance for Industry on Intentional Modification of DNA in Animals (GFI #187) was published in January 2017. In contrast to the treatment of food products, this policy treats animals derived from genome-editing technologies as equivalent to GM animals and places them under strict regulation. The FDA's policy has caused concern among companies and developers. In this context, in January 2021, the United States Department of Health and Human Services (the FDA's headquarter) signed a memorandum of understanding with the USDA to consider changing the jurisdiction from the FDA to the USDA concerning livestock animals. However, while these developments are occurring, the FDA has decided to "not regulate" genome-edited slick-haired cattle through an exercise of enforcement

[7] Whether to allow developers to self-determine is one of the key issues in the SECURE regulations, and its pros and cons are discussed in detail in the Federal Register (85 Fed Reg 29798, May 18, 2020).

[8] However, developers can request a confirmation from the USDA through the "Confirmation Request Process" that their product is not subject to regulation. The result, that is, the confirmation letter, will be posted on the USDA website. Since US companies have product liability for food, they are also likely to be eager to obtain safety confirmation from the government.

[9] 85 Fed. Reg. 64308 (October 9, 2020)

discretion in March 2022[10]. The FDA has the option of taking this action on a case-by-case basis when an application is filed with the FDA and when there is little or no concern about trait alteration or food safety[11].

Based on the above, the US regulation of genome-edited organisms falls under Approach 4 for USDA (crop) and EPA (under review), Approach 3 for FDA (food), and Approach 1 for FDA (animals) (under review). Different policies are applied to different subject areas in the United States due to each institutional jurisdiction. The existence of different policies for each item in the United States influence decisions within research and development and also has a significant impact on trade. These points are discussed later.

2.2 European Union

In the EU, the Environmental Release Directive was revised in 2001 (Directive 2001/18/EC). The revised directive incorporated the precautionary principle, reflecting public concerns about food safety and new science/technology at the time, such as the bovine spongiform encephalopathy (BSE) scandal. In this directive, GMOs are defined as those to which specific technologies (those listed in Annex 1A, Part 1) were applied: "genetically modified organism (GMO) means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (Article 2). Among mutagenesis technologies, those that do not use recombinant nucleic acid molecules or GMOs were categorized as GM techniques but were excluded from the Directive (Annex 1B). This was because they "have conventionally been used in a number of applications and have a long safety record" ("whereas clause" (17) of the Directive). In 2015, the Directive was further amended to permit EU member states to prohibit the cultivation of GM crops in their territory based on environmental or agricultural policy objectives. Against this backdrop, new breeding techniques have been attracting attention.

Since around 2007, the EU has been considering how to deal with new breeding techniques, which are difficult to position under the existing GM regulations. The European Commission established the New Breeding Techniques Working Group to examine the regulatory status of new technologies, including other technologies that already existed at that time. The European Joint Research Center also examined eight new technologies (e.g., zinc finger nucleases and reverse breeding) and their relationship to the regulations (Lusser et al., 2011). As the results of these studies were disseminated, industry associations, such as EuropaBio, the European Seed Association, and anti-GM campaign groups tried to express their own views to influence the EU policy.

Although various positions on the potential of the new breeding technology and its regulatory status were discussed, several years passed without a clear regulatory policy from the European Commission. In the meantime, environmental groups filed a lawsuit against the French government over the legal status of mutation breeding. This then led to the problem of the legal status of genome-editing technology, and the French court asked the Court of Justice of the European Union (CJEU) for a legal interpretation of mutagenesis technology, including genome-editing technology. As a result, in July 2018, the CJEU rendered a decision regarding the legal status of products derived from mutagenesis under the Environmental Release Directive (Court of Justice of the European Union, 2018). In conclusion, it was held that organisms derived from mutagenesis are, in principle, GMOs and are subject to the legal obligations of the Directive. However, those with a long history of safe use (e.g., radiation breeding) were excluded from the scope of the regulation in accordance with the provisions of Annex 1B of the Directive. In other words, organisms derived from genome-editing technologies without a long history of safe use were deemed to be GMOs and subject to the regulation under the EU's Environmental Release Directive.

The ruling of the CJEU had a significant impact on European stakeholders, as it affected the position of genome-editing technologies in the EU as a whole. In response to this ruling, the European Commission was instructed to gather information regarding new genomic techniques (e.g., regulatory status in member states, detection techniques, risk assessment, market trends, ethical considerations, etc.) from various EU institutions. The results were compiled in April 2021. In September 2021, the Commission then presented a roadmap for the future for public comment to consider a legal framework for targeted mutagenesis and cisgenesis in plants. The draft regulation is expected to be published in 2023. The United Kingdom, which has left the EU, has begun to consider its own regulations as the Precision Breeding Bill.

Based on the above, the European Union's position at this point represents Approach 1.

2.3 Argentina and other South American countries

South American countries, especially Argentina, Brazil, Chile, Paraguay, and Colombia, are clarifying their regulatory positions and starting commercialization from the standpoint of promoting genome-editing technology (Kuiken and Kuzma, 2021). The following discussion is limited to Argentina as a representative country[12].

In May 2015, Argentina's Ministry of Agriculture, Livestock, and Fisheries (MAGyP) established a "prior consultation procedure" for

[10] https://www.fda.gov/news-events/press-announcements/fda-makes-low-risk-determination-marketing-products-genome-edited-beef-cattle-after-safety-review

[11] With regard to transgenic Glofish, the FDA also exercised enforcement discretions on the grounds that they would pose low risk. However, such exercise of discretionary authority is a decision that is made only after an application for approval has been filed, and there is little predictability on the part of the applicant.

[12] As Kuiken and Kuzma, 2021 point out, Argentina was the first country in the South American region to adopt the rule, and other countries, such as Brazil, Chile, Colombia, Paraguay, Honduras, and Guatemala have followed suit. Therefore, the Argentine example is used here to represent it.

crops derived from new breeding techniques such as genome-editing technologies (Decision 173/15). This decision was the first of its kind in the world and was subsequently followed by other South American countries. In the pre-consultation process, the product is examined to determine whether "novel combination of genetic material" (foreign genes) remain in the genome (Whelan and Lema, 2015). The Ministry of Agriculture, Livestock and Fisheries accepts the preliminary consultation and then asks the Committee on Biosafety (CONABIA) to review it and decide whether it should be subject to regulation. This includes instances of transient introduction; if the foreign gene does not remain in the final organism, it will not be subject to regulation.

However, even if the crop is treated as a non-GMO, the relevant government department will consider additional measures if the novelty of the crop exists and such measures are deemed necessary. This Argentine approach of making regulatory decisions based on the presence or absence of foreign genes is also being adopted by neighboring countries. The results of this prior consultation are not made public[13]. This is because it is believed that public disclosure would distinguish certain technologies from conventional breeding and could lead to discriminatory treatment.

From the above, the current position of Argentina and other South American countries represents Approach 3, although there are some minor differences within the region.

2.4 Oceania

2.4.1 Australia

In Australia, the environmental safety of GMOs is regulated by the OGTR under the Gene Technology Act of 2000 and the Gene Technology Regulations enacted in 2001. The OGTR oversees the implementation of regulations.

In light of the emergence of new breeding techniques such as genome-editing technologies, the OGTR, after several years of study, revised their regulations on April 4, 2019, that is, the Gene Technology Amendment Regulations 2019. The revision clarifies that genome-editing technologies that fall under SDN-1 are exempted from the current regulation, while genome-editing technologies that use artificially created templates outside the cell (SDN-2) are subject to the regulation[14].

The revisions made in 2019 include the following points:

1) Revisions to Schedule 1A (technologies that are not gene technologies) to clarify the conditions under which RNA transfection is not considered a gene technology.

2) Revisions to Schedule 1 (organisms that are not GMOs) to add six items, including the case without templates and matters related to null segregants.

3) Establishment of Schedule 1B (technologies that are gene technologies) to clarify technologies that use ODM and templates.

Although the revisions exclude some genome-editing technologies (SDN-1) from the scope of the regulation, its content can be considered to be identical as the USDA's regulations and the Japanese Ministry of Environment's policy (see below) in terms of making genome-edited organisms that use templates being subject to regulation. In addition, the OGTR rules share with the USDA rules in that no confirmation is required for exemptions from the regulations and developers can self-determine.

2.4.2 New Zealand

In New Zealand, genome-edited organisms are subject to GMO regulation, and like in the European Union, this was triggered by a court case. In April 2013, the New Zealand Environmental Protection Agency (hereafter NZEPA) ruled that trees produced with ZFN-1 (Zinc Finger Nucleases) and TALEN (Transcription Activator-Like Effector Nucleases) were not subject to regulation under the country's GMO control law, the Hazardous Substances and New Organisms Act (HSNO Act). However, environmental NGOs appealed this administrative decision and filed a lawsuit. Then, in May 2014, the High Court ruled, and the arguments of the plaintiff NGOs were accepted ((New Zealand High Court Wellington Registry, 2014)), stating that ZFN-1 and TALEN should not be excluded from HSNO Act. According to the High Court, these genome-editing technologies were considered novel and not scientifically well-established. Therefore, in view of the precautionary approach on which the HSNO Act relies, the judge ruled it as inappropriate to exclude these techniques from the HSNO Act.

In response to the ruling, the NZEPA revised the relevant statutes to explicitly state that mutagenesis techniques utilized before July 29, 1998 (the effective date of the HSNO Act) would be treated as non-GMOs. In other words, those created using mutagenesis technologies, including genome-editing technologies, developed after July 1998 are now subject to regulation as GMOs.

2.4.3 Food Standards Agency of Australia and New Zealand

While environmental safety is regulated by Australia's OGTR and the NZEPA, food regulations are regulated by the Food Standards Agency of Australia and New Zealand (FSANZ), which has been established jointly by the two countries. In particular, the revision of Food Standards, which define GM foods, is an issue. In February 2018, the FSANZ collected opinions on the revision, compiled the results in December 2019, and published a draft proposal (P1055) of the Food Standards in October 2021[15]. In the proposal, FSANZ indicated to expand the process-based definition to capture all methods for genetic modification including genome-editing. However, at the same time, FSANZ is proposing to revise the definition for 'food produced using gene technology' to exempt certain products based on product-based criteria. Criteria includes food from which foreign genes have been removed, food with characteristics that can be produced by conventional breeding, and

[13] In Brazil, however, limited information of notification to the government will be published in the Federal Gazette, according to the Normative Resolution No.16 (January 15, 2018) by the National Biosafety Technical Commission (CTNbio).

[14] Another type, SDN-3, is treated as a genetically modified organism not only in Australia but also internationally because of the introduction of a foreign gene into its DNA.

[15] https://www.foodstandards.gov.au/code/proposals/Documents/P1055%201st%20Call%20for%20Submissions.pdf

processed food that does not contain foreign genes or new proteins. Food that does not meet these exclusion criteria will be subject to a prior safety review. A final decision is expected to be made after further review.

From the above, Australia's OGTR represents Approach 4, NZEPA represents Approach 1, and FSANZ (under review) represents Approach 2.

2.5 Asian countries

After 2019, Asian countries have also been actively considering regulations. The following sections discuss Japan, China, India, and the Philippines as countries that have clarified their regulatory policies.

2.5.1 Japan

In Japan, the Ministry of the Environment (MOE) decided in February 2019 and the Ministry of Health, Labor and Welfare of Japan (MHLW) in September of the same year on the policy for handling genome-edited organisms from the context of environmental safety and food safety, respectively (Matsuo and Tachikawa, 2022). The MOE's decision was a Director-General's notice, while the MHLW's was a decision by a counselor, and the handling policy was determined by administrative decision without revision of laws and regulations. The policy of both ministries state that genome-edited organisms are exempted from the regulation of GMOs, but there are some differences between the policies of the two ministries.

According to the MOE, those not containing foreign genes are exempted from the regulation, but those created using templates such as SDN-2 are subject to the regulation. On the other hand, according to the MHLW, those with "a risk that could occur even with conventional breeding techniques" were exempted from the regulation. Therefore, based on the MHLW, SDN-1 is not regulated, and SDN-2 is judged on a case-by-case basis. In Japan, even when exempt from regulations, confirmation by each administrative body is required and labeling is encouraged by the government.

The Japanese regulations do not distinguish between plant, animal, and microorganism, and the rules have been clarified for all types of uses, such as cultivation, food use, and feed use.

2.5.2 China

In January 2022, the Ministry of Agriculture and Rural Affiars (MARA) of the People's Republic of China published the "Guidelines for Safety Evaluation of Gene-Edited Plants for Agricultural Use (trial)"[16]. If the risk is found to be low, a small-scale intermediate test would be conducted, and the results are submitted to apply for a safety certificate for commercial production. The above guidelines have been adopted by the Chinese government.

Therefore, the above guidelines attempt to promote research and development and commercial use by maintaining China's existing GM regulations and including plants derived from genome-editing technologies. This introduces a simplified procedure (i.e., a safety certificate can be applied for after a small-scale test). While the basic legal regime for GMOs in China has been maintained, China has also been actively revising its regulations on GM crops in recent years and trying to promote the use of life sciences[17].

2.5.3 India

On March 30, 2022, the Ministry of Environment, Forest, and Climate Change of India issued an office memorandum[18] and decided that plants produced under SDN-1 and SDN-2 that do not contain foreign genes are not subject to GM regulations. In other words, the Ministry has indicated that Articles 7 to 11 (import/export, manufacturing/processing, environmental release, food use, etc.) of the GM Regulations (Regulations for the Manufacture, Use/Import/Export and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells, 1989) are exempted for those plants.

In May 2022, the Department of Biotechnology, Ministry of Science and Technology also released the "Guidelines for Risk Assessment of Genome-Edited Plants"[19] to provide information on where applicants can submit notifications and detailed application procedures.

2.5.4 Philippines

The Philippines has been conducting technical and regulatory studies on new plant breeding technologies since 2016. In particular, since June 2019, a decision has been made by the Philippine government to develop a government policy under the Department of Agriculture, and guidelines on plant breeding innovations (PBI) have been considered. In May 2022, the Philippine Department of Agriculture issued Memorandum Circular No. 8 based on the above considerations and published the rules and procedures for the marketing of products based on PBI[20]. Products that do not contain exogenous genes (new combinations of genetic material) were exempted from the regulations. In particular, developers were to provide information and follow procedures to the Bureau of Plant Industry of the Department of Agriculture. If the organism is exempted from the GM regulations (JDC1), a certificate is issued to the developer, and the information excluding confidential information is published on the website.

[16] Please refer to the USDA-FAS website as follows. https://www.fas.usda.gov/data/china-mara-issues-first-ever-gene-editing-guidelines

[17] For example, the Chinese government recently revised the biosafety assessment from being on a "crop variety and event" basis to solely on an "event" basis. English explanations can be found in the following report. https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agriculture%20GMOs%20Safety%20Assessment%20Administrative%20Measures%20Finalized_Beijing_China%20-%20People%27s%20Republic%20of_01-24-2022.pdf

[18] Office Memorandum F. No. C-12013/3/2020-CS-III, https://dbtindia.gov.in/sites/default/files/Final_%2011052022_Annexure-I%2C%20Genome_Edited_Plants_2022_Hyperlink.pdf

[19] https://dbtindia.gov.in/sites/default/files/Final_%2011052022_Annexure-I%2C%20Genome_Edited_Plants_2022_Hyperlink.pdf

[20] https://www.da.gov.ph/wp-content/uploads/2022/06/mc08_s2022_Revised.pdf

Therefore, it is clear that in Asia, China reflects Approach 2 and Japan, India, and the Philippines have reflected Approach 3. What approach other Asian countries will adopt still needs to be examined.

3 Discussion: Cross-regulatory considerations

Various approaches have been adopted by multiple countries. However, if the approaches are arranged in chronological order and the overall trend is examined (Table 2), it can be seen that the regulation of genome-edited organisms has recently been trending toward a middle ground. In other words, there seems to be a trend toward the adoption of two approaches: one that regards them as GMOs but tries to apply simplified regulations (Approach 2) and one that excludes them from the scope of regulations as non-GMOs but requires confirmation (Approach 3). These two approaches are an attempt to take a middle ground between applying strict GMO regulations and excluding GMOs from the regulations as equivalent to conventional breeding. The following sections discuss why there is a tendency at this stage to converge on these two approaches and examine the challenges and implications of these approaches for the governance of the agricultural and food sectors with the application of genome-editing.

3.1 Why convergence is emerging

Although further considering the regulatory situation in each country is essential, hypothesizing a couple of points regarding why convergence is currently occurring is possible[21]. This study will discuss (1) the background to the limited number of jurisdictions adopting Approaches 1 and 4 and (2) the background to the convergence toward a middle ground approach.

The EU and New Zealand are the only jurisdictions that are currently adopting the same regulations to organisms subject to genome-editing technologies as to genetic modification (Approach 1)[22]. However, the current policies of the EU and New Zealand were decided through court processes rather than voluntarily adopted by their respective governments. Furthermore, the laws and regulations referred to by the judges in the EU and New Zealand were all enacted around the year 2000 (the Environmental Release Directive 2001/18/EC in the EU and the HSNO Act 1996 in New Zealand.), when concerns about science, technology, and the precautionary principles were being emphasized in the wake of the BSE incident in Europe. At this point, it is unknown whether there will be more countries adopting Approach 1 in the future. However, given the high expectations of industry and governments for genome-editing technologies, it is not highly likely that such an approach will be widely adopted.

Second, contrary to the above, only the Australian OGTR and the USDA adopt Approach 4 and allow the use of genome-edited organisms without confirmation from the government. However, both of these agencies authorize organisms only for environmental release, and another government agency will be involved in the regulation of food use, such as FDA and FSANZ. The Australian OGTR found it difficult to introduce a notification system because "organisms modified using SDN-1 […] do not pose risks that warrant regulating these organisms as GMOs" and regard them out of regulatory scope (OGTR, 2018, p.25). Conversely, the USDA attempted to strike a balance with genome-edited organisms by excluding GM crops that met certain conditions outside the scope of the regulation *via* the recently mentioned regulatory revision. Therefore, the number of countries adopting Approach 1 and 4 has been limited to date.

Considering the above, we would like to discuss the underlying factors of the convergence phenomenon currently occurring. Policy convergence and policy transfer have been discussed in policy studies (Bennett, 1991; Vogel and Kagan, 2004; Holzinger and Knill, 2005) regarding the underlying factors of international policies moving toward the same content and direction. In the context of globalization, policy harmonization has been promoted under the leadership of international organizations (e.g., the World Trade Organization) and/or leading nations in various fields (e.g., the US and the EU).

To date, however, there has been no clear policy coordination effort on how to regulate genome-edited organisms in international organizations such as the Cartagena Protocol of Biosafety (CPB), the Codex Alimentarius Commission (Codex), and the Organization for Economic Co-operation and Development (OECD)[23]. Policy formation has been conducted in the absence of clear rules, with each country accumulating its own domestic considerations and information gathering. In this sense, a phenomenon that could be discussed by policy convergence theory, rather than the policy transfer theory, which focuses is on the process by which a particular policy model diffuses (Knill, 2005), has emerged. In the theory of policy convergence, the focus is on the effects of the commonality of the situation in which countries find themselves,

[21] Regarding whether regulatory convergence is occurring, this paper focuses mainly on (1) whether the product is categorized as a GMO or (2) whether notification is required. It does not focus on other aspects, such as the meaning of SDN-2 (Jones et al., 2022) or whether notification results should be made public. In this sense, there are some aspects that are not fully converged in the strict sense of the word.

[22] South Africa has decided in October 2021 to follow the same policy as the EU regarding new breeding techniques. This is presumably because the definition of GMO in South Africa is largely in line with the EU's Directive, making it difficult to introduce different regulations. For more information, please refer to the following website. https://acbio.org.za/gm-biosafety/battle-regulation-new-breeding-techniques-south-africa/#:~:text=In%20October%202021%2C%20the%20South,of%20new%20genetic%20engineering%20technologies

[23] Opportunities have been taken to exchange information, such as the workshop or conference held in February 2014, June 2018, but there has been no significant activity toward policy harmonization among member countries. For example, the role played by the OECD in the process of discussion for unconfined use of GM organisms (such as the publication of the so-called Blue Book in 1986) has not been fulfilled this time.

resulting in policy convergence. Therefore, it is appropriate to focus on this theory in this study[24].

The policy convergence theory proposes that there are two types of factors that facilitate convergence: (1) causal mechanisms and (2) facilitating factors (Knill, 2005). The former includes independent problem-solving, international harmonization, regulatory competition, and transnational communication. The latter includes cultural, institutional, and socioeconomic similarity. Based on the findings of these previous studies, there are three relevant points to consider regarding the convergence of regulations on genome-edited organisms.

First, while there are high expectations for the potential of genome-editing technologies, countries are not only engaged in research and development but also regulatory competition (Knill, 2005). Several countries have developed strategies and policy documents that seek to increase industrial competitiveness while maximizing the use of genome-editing technologies (e.g., the US Agricultural Innovation Strategy and Japan's Biotechnology Strategy). These expectations for new technologies (Borup et al., 2006; Yamaguchi and Fukushima, 2019) are likely to increase the tendency to adopt a more technology-promoting orientation due to the international presence of regulatory competition. In this situation, Approach 1 will be less likely to be adopted.

Second, and related to the above, it is conceivable that countries have rushed to enact rules through administrative procedures alone rather than by revising basic laws due to the extreme regulatory competition. When a political review process is introduced, seeking a compromise that transcends political positions is essential, and a variety of options will be explored. Opportunities for participation in policy formation processes from civil society organizations will also increase, and involvement from groups that have demonstrated opposition to GMOs may adopt Approach 1. Which approach will be chosen will be unpredictable. To avoid such risks, convergence may have resulted from the fact that most countries have tried adopting regulations through administrative procedures rather than legal revisions. This point is also related to the next aspect of institutional similarity.

Third, the process of determining regulations for the handling of genome-edited organisms has been handled by the administrative bodies in charge of regulating GMOs in each country. These administrative agencies have overseen food safety, environmental safety, and other regulatory enforcement, as well as external information gathering through regulating GMOs. In the domestic context, these agencies have consultative processes with relevant experts and stakeholders regarding safety and proper handling and have continued to exchange information with various international forums such as the CPB, and the Codex, and the OECD. These administrative procedures would be considered to have enhanced their institutional similarity as far as scientific information and stakeholder opinion is collected as typical procedures for authorization[25]. In addition, since the emergence of new breeding technologies, including genome-editing technologies, this topic has been discussed at various international meetings, such as the OECD and the APEC, regarding their opportunities and challenges. In this context, the institutional similarities and transnational communication highlighted by Knill (2005) may have encouraged policy convergence regarding genome-edited organisms.

Table 3 summarizes the above points as the main actors involved in the formation of regulations.

3.2 Limited negative responses

Why has the approach of revising the law not been taken? This may be related to the fact that there have not been much negative responses against genome-edited food. Two factors may be involved in this situation: (1) social recognition of genome-edited food has been suppressed by various factors and (2) the EU has imposed regulations same as those for GMOs.

First, the lack of major negative responses of genome-edited food is related to the fact that genome-editing has been used for multiple purposes, in particular, for medical applications and, as a result, has attracted much attention in the medical field. For example, while the birth of genome-edited babies in China (Wang et al., 2019) led to an instant increase in social recognition of this technology, its application to agriculture and food products has not attracted comparable social attention (Shew et al., 2018; Gatica-Arias et al., 2019; McFadden et al., 2021). In addition, in countries where commercial release has already begun, the following measures are also presumably linked to the result that awareness among general consumers is not high (Kato-Nitta et al., 2021). In other words, it is difficult for general consumers to be aware of the results of the confirmation of genome-edited food because they are not disclosed, as is the case in Argentina. In countries where information is disclosed, such has in the United States and Japan, the food is distributed for commercial use or direct to consumers (D2C) and not for general market distribution, and this discouraged the expression of concern by general consumers or retailers. These factors may explain the lack of public awareness of genome-edited food.

Second, the lack of negative responses may be related to the actions of civil society organizations in the EU, which have been very vocal in their opposition to GM food. In other words, it may be related to the fact that the Europe Union currently regulates foods with genome-editing as GM food. In this context, the movement within the EU has not gained much momentum. A significant resistance to GMOs has occurred in the EU since 1996, when GM soy was criticized for being imported without labeling (Schurman and Munro, 2006). Unlike in the United States, the public protest movement in the EU is characterized by its high visibility and open

[24] In other words, it is difficult to analyze the policy regarding genome-editing technology as a mere transfer or diffusion of policy because the policies regarding GMOs in each country differ in their starting points. However, if a particular regulatory model is diffused in a region with similar GMO policies as a starting point (e.g., South America), it may be possible to use policy transfer theory to analyze the diffusion of the model.

[25] This point may be explained in terms of organizational isomorphism, as discussed by DiMaggio and Powell (1991). It is thought that administrative organizations in charge of regulating genetic modification originally had organizational isomorphism across countries, and that the probability of forming similar rules increased as a result of these administrative organizations considering rules without political processes.

TABLE 3 Actors Involved with Introduction of Regulatory Measures related to Genome-Edited Organisms.

Approach	Country	Decisions/Measures	Administrative Process	Parliamentary Process	Court Ruling
1	EU/France	Court of Justice of the European Union (2018)	●		●
1	New Zealand	High Court Decision (2015)	●		●
2	China	Guidelines for Safety Evaluation of Gene-Edited Plants for Agricultural Use (Trial) (2022)	●		
2	Australia&NZ (FSANZ)	Foods Standard Codes: Proposal P1055 (2021)	●		
2	UK	(Draft) Precision Breeding Bill	●	●	
3	Argentina	Resolution No. 173/2015 (2015)	●		
3	Japan	Decision of MOE and MHLW (2019)	●		
3	India	MOEFCC Office Memorandum (2022)	●		
3	Philippines	MOA Memorandum Circular No. 8 (2022)	●		
4	US(USDA)	SECURE rule (2020)	●		
4	Australia (OGTR)	Gene Technology Amendment Regulations 2019 (2019)	●	●(1)	

(1): In Australia, changes to the Gene Technology Regulations are tabled in Parliament for 15 days and if no objections are raised, the changes are implemented as proposed.

political campaigns (Bernauer and Meins, 2003)[26]. If there is a move to revise the regulations in the EU in the future, a wave of criticisms regarding genome-editing originating in the EU may occur[27].

3.3 Challenges posed by the two approaches

As far as the countries considered in this paper are concerned, the regulation of genome-editing is positioned by most countries to be somewhere between the situation of no regulation at all and the situation of strict regulation equivalent to that of GM. However, in a strict sense, policy convergence has not yet been fully reached. The difference in regulatory status between GM (Approach 2) and non-GM (Approach 3) results in significant differences, especially when ex-post facto regulations such as GM labeling, traceability, crop registration, and license renewal are applied. On the other hand, if these ex-post facto regulations are exempted, there may not be a significant difference between GM (Approach 2) and non-GM (Approach 3). What are the implications of such regulatory measures for the introduction of genome-edited foods into society? Although there are only a limited number of cases in the market so far, it would be beneficial for countries currently considering regulations to consider these issues and implications.

3.3.1 Issues in research and development

In the research and development stage, which is the stage prior to receiving confirmation from the regulatory authorities, it is expected that even if an organism is ultimately exempted from the regulation as an organism derived from genome-editing technologies, it will be treated as a GMO subject to biosafety regulations. However, if there are significant differences between Approach 2 and 3 in the subsequent safety review procedures, there may be substantial differences in research and development. As a result, there would be a significant disparity in the application process for developers. In Argentina, where Approach 3 has been adopted, the percentage of domestic companies developing genome-edited products has increased compared to that of GMOs (Whelan et al., 2020). Depending on the size of the burden of safety review procedures, there could be a significant disparity in the number and types of products developed. If the burden is lessened, it is possible that a greater variety of research and development processes could be pursued with more diverse development goals.

3.3.2 Issues in marketization

In the case of Approach 1 and Approach 2, GM labeling regime raises concerns from consumers, which will make the commercialization of the product difficult. There is a possibility that genome-editing will be

[26] Conversely, social recognition of such movements is limited in the US, as social movement organizations that oppose to genetic modification tend to pursue legal battles. This kind of difference between the EU and the US regarding the form of protest movement can be explained by each jurisdiction's preference of rulemaking, as discussed by Bradford (2020, 41): "The EU's tendency to respond to various regulatory risks with stringent standards partially stems from its reliance on ex ante government regulation as opposed to ex post enforcement by private litigants." This contrast might be rooted in the different legal traditions between civil law and common law. Because of this backdrop, even if commercialization of genome-edited products in the US results in filing lawsuits against industry, this kind of social movement tactic tends to be less recognized by the general public.

[27] Public trust has been pointed out as a factor influencing European NGOs' criticism of GMOs (Aerni and Bernauer, 2006). The discourse that European NGOs have won public trust by criticizing GMOs, serving their corporate interests, has influenced NGOs abroad and resulted in a polarization of opinion for and against GMOs. If EU policy shifts and NGOs develop this discourse on genome-edited products, the impact may spread abroad. In this case, divergence, not convergence, could occur.

applied only to products that are not subject to mandatory labeling or non-food uses (e.g., flowers).

On the other hand, if genome-edited organisms are exempted from the GM regulation as in the case of Approach 3 and Approach 4, they will not be labeled, which will encourage their use in a variety of products. Since labeling is not required for such products, the number of products with genome-editing is expected to steadily increase.

Existing studies indicate that consumers have the same concerns about genome-editing technology as they do about transgenic technology, while there are different concerns regarding the purpose of the technology's application and the organisms (plants or animals) to which it is applied (Kato-Nitta et al., 2021; Busch et al., 2022). Regarding concerns about the technology itself, some argue that there is no need to distinguish between genome-editing technology and genetic modification technology (Mikami and Tachikawa, 2019). From this standpoint, criticism may be directed at the invisible distribution of products based on genome-editing within markets. In this context, there is a possibility that distribution through commercial use or D2C, rather than general market distribution, will continue in the future.

3.3.3 Issues concerning trade

The approaches that different countries adopt will also pose significant challenges for trade. There are several challenges, but this study focuses on three points as follows.

The first is the asymmetry problem that arises when trading partners take different approaches. Based on Table 4, when genome-edited food is exported from countries that adopt Approach 3 (confirmation is required) but do not disclose notification results (e.g., Argentina) or from countries that adopt Approach 4 (companies are allowed to make their own decisions) to countries that adopt Approach 1 or 2, if the exporters do not actively provide information, concerns regarding GM food being distributed under cover ("hidden GMOs") may spread which disrupts the market (Bertheau, 2021). Some consumer groups are demanding that developers of genome-edited products should develop tracking methods and ensure consumers' right to choose[28]. In short, the issues of information disclosure and transparency from exporter to importer are important. In light of the major opposition which happened in Europe over the importation of GM soybeans in 1996, it is undeniable that the same thing could occur with genome-edited products, and negative reactions could grow in the future.

Exports from countries adopting Approaches 1 or 2 to countries adopting Approaches 3 or 4 may be avoided by consumers in the importing country because the genome-edited product is designated as a GMO and might be labeled as such in the exporting country. As a result, from the exporter's point of view, this would create a non-tariff barrier problem.

Second, since there will be limits to how individual countries can deal with such situations, international frameworks or coordinated responses are necessary. Although it would be desirable to have a database registration of products and an identification code for each product, such as an equivalent to the Biosafety Clearing House and unique identifiers in GMOs, it would be difficult to achieve.[29] Efforts to form international governance for genome-edited organisms are not discussed frequently at the CPB, the Codex and the OECD, and it would be difficult to formulate international rules to avoid confusion over such imports and exports in the short term. As Jasanoff and Hurlbut (2018) pointed out, in addition to issues related to trade, international discussion on ethical and other issues related to genome-editing technologies is crucial. The pressing future task is to establish a framework, such as global observatory, to facilitate international dialogue on various issues.

Third, as a kind of international response to the above, the same policy could be introduced in regions with close trade interdependence. For example, since Argentina decided on its policy in 2015, Brazil, Chile, and Colombia have introduced almost identical rules (Kuiken and Kuzma, 2021). All these South American countries adopt Approach 3 and other countries in this region would follow suit. In addition to the South American countries, the United States, Australia, and others have also published a communication in the World Trade Organization (WTO), claiming that differences in the rules surrounding genome-editing technologies could impede trade and innovation (WTO Committee on Sanitary and Phytosanitary Measures, 2018).

Table 4 shows expected response of importing country in different situations.

[28] For example, GM Watch's argument can be found at the following website. https://www.gmwatch.org/en/106-news/latest-news/20090-gene-editing-just-label-it

[29] The development of detection techniques also faces difficulties in reality (European Network of GMO Laboratories, 2019).

TABLE 4 Expected Response of Importing Country in Different Situations.

		Importing Country			
		Approach 1	Approach 2	Approach 3	Approach 4
Exporting Country	Approach 1	Under GMO regulations	Under GMO regulations	Consumer Negative Responses	Consumer Negative Responses
	Approach 2	Under GMO regulations	Under GMO regulations	Consumer Negative Responses	Consumer Negative Responses
	Approach 3	Advance Notice/Pre-market Authorization	Advance Notice/Pre-market Authorization	Advance Notice/Pre-market Authorization	Conventional Trade
	Approach 4	"Hidden GMO"	"Hidden GMO"	Advance Notice/Pre-market Authorization	Conventional Trade

If a country with Approach 3 does not disclose genome- editing information to traders, similar responses in the case of Approach 4 would happen.

4 Conclusion

Scientific factors alone cannot explain the emergence of the abovementioned convergence phenomenon in the formation of rules concerning genome-edited organisms. This study identifies four approaches to the regulatory consideration of genome-edited organisms in various countries and demonstrates that these approaches are converging into two main approaches. The study then discusses the factors that have led to this convergence, using the concepts of regulatory competition and organizational similarity, and the underlying factors of this convergence, focusing on the fact that there have been no major negative responses to genome-editing technology. Even though genome-editing technology has attracted widespread attention as a game changer in the life sciences, it is important to point out that no country has revised its basic laws on genetic modification. Therefore, convergence in a strict sense, has not been reached. Furthermore, this study discusses issues regarding the challenges posed by the situation of two separate approaches, particularly from the perspective of trade and other issues. Further convergence would require a revision of the basic legislation, and there is no guarantee that the initially intended objectives would be achieved by inviting a political process. In this sense, further convergence is difficult to foresee in the short term.

In the international perspective, many countries have not yet completed their policy-making processes. As these countries move forward, patterns other than those discussed in this paper may emerge, or further convergence may occur. Of particular importance is the possibility that the policy convergence observed in this study may be reversed as a result of a major civil society resistance against genome-editing technologies triggered by trade disruption or other factors. In this sense, the discussion in this paper needs to be further explored. In addition, this study did not examine in detail who participated in the policy-making process in the administrative bodies. Depending on who participates, the detailed design of the rules (e.g., disclosure of notified information) and the time it takes to formulate the rules may vary. If a trade issue arises and becomes the cause of a dispute in the World Trade Organization, the dynamics may take on a different dimension than the policy convergence observed in this paper. Policy competition can lead to convergence to a certain extent, but, as pointed out above, it is far from convergence at the level of basic GMO legislation. To get there, dynamics at a different level from policy competition might be needed. This issue, however, is beyond the scope of this paper. Nevertheless, we need to continue to monitor policy trends closely.

Author contributions

MT and MM discussed the study design and conducted research reviews and interviews. MT drafted the manuscript based on the study and MM edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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U.S. EPA oversight of pesticide traits in genetically modified plants and recent biotechnology innovation efforts

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Before pesticides can be sold in the United States, the United States Environmental Protection Agency (EPA) must evaluate them thoroughly to ensure that they meet U.S. federal pesticide registration standards for human health and the environment. EPA considers pesticidal substances produced and used in plants as pesticides and defined them in the regulations as "plant-incorporated protectants" (PIPs). PIPs that are created through conventional breeding are exempted from registration requirements, while those created through biotechnology require individual assessments and approval by EPA before they can be distributed or used. This currently includes PIPs that are identical to those that could be moved through conventional breeding but are created through biotechnology (e.g., through genome editing or *via* precision breeding techniques). EPA proposed an exemption in October 2020 to allow certain PIPs created through biotechnology to be exempt from EPA requirements for pesticides where those PIPs: 1) pose no greater risk than PIPs that EPA has already exempted, and 2) could have otherwise been created through conventional breeding.

KEYWORDS

genome editing, plant biotechnology, plant protection, pesticide, regulatory policy, environmental protection

Introduction

The U.S. Federal government issued its Coordinated Framework for Biotechnology Products in 1986 and updated this framework in 1992 and 2017. The framework describes a comprehensive regulatory policy for ensuring the safety of biotechnology products and the allocation and coordination of oversight responsibilities under the relevant statutes and among the U.S. Federal agencies. Under the Coordinated Framework, EPA regulates PIPs as pesticides.

Before pesticides can be sold in the United States, the United States Environmental Protection Agency (EPA) must evaluate them thoroughly to ensure that they meet U.S. federal pesticide registration standards to protect human health and the environment. EPA grants a "registration" or license that permits a pesticide's distribution, sale, and use only after the company meets scientific and regulatory requirements. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), EPA evaluates pesticides including PIPs for their effects on the environment and human health and regulates their development, sale, distribution, and use. Under the Federal Food, Drug and Cosmetic Act (FFDCA), EPA evaluates PIPs that are proposed for use in food or feed. In its assessment the Agency considers all anticipated dietary exposures, as well as residential and other outdoor uses.

EPA considers pesticidal substances produced and used in plants to be pesticides and defines them as "plant-incorporated protectants" (PIPs) at Title 40 of the Code of Federal Regulations (40 CFR) § 174.3. PIPs include not only the pesticidal substances produced by plants but also the genetic material necessary for the plant to produce that substance. For example, a gene for a specific pesticidal protein, such as the Cry1Ab protein derived from the soil microorganism *Bacillus thuringiensis*, is introduced into the genome of a plant. The plant then produces from that gene the pesticidal protein that protects the plant from plant-feeding pests. Here, both the Cry1Ab protein and its genetic material in the plant are regulated by EPA as a pesticide.

EPA's 2001 exemption for PIPs moved through conventional breeding does not include PIPs developed through biotechnology

Plants naturally produce substances that have pesticidal properties. When EPA developed its regulations for PIPs (Federal Register, 2001), it determined that these pesticidal substances naturally produced by plants are PIPs when intended for pesticide use in the plant. However, EPA did not intend to regulate PIPs that naturally occur in plants, which had long been selected for in conventional plant breeding. Thus, when EPA promulgated its regulations for genetically engineered PIPs in 2001, it also published exemptions for PIPs created from sexually compatible plants moved through conventional breeding (40 CFR 174.25, 174.705, and 174.508) with the exception that adverse effects reporting requirements still apply (40 CFR 174.71). These exemptions reflect the history of safe use of PIPs in conventional breeding.

Because the "conventional breeding" definition that formed the basis of the 2001 exemptions specifically excludes PIPs developed through biotechnology (40 CFR 174.3), PIPs that are identical to those that could be moved through conventional breeding but are created through biotechnology currently must be registered. This includes those PIPs that are created through gene editing. When the 2001 rule was promulgated, precision breeding techniques such as genome editing were unavailable and EPA determined that additional criteria needed to be developed that would allow the Agency to include PIPs developed through biotechnology that are found in sexually compatible plants in the exemption. Thus, EPA issued a supplemental proposal entitled: "Plant- Incorporated Protectants (PIPs); Exemption for Those Derived Through Genetic Engineering From Sexually Compatible Plants." This proposed rule was ultimately withdrawn in 2018 (Federal Register, 2018) because the Agency determined that to exempt PIPs created through genetic engineering from sexually compatible plants, exemption criteria needed to be developed to reflect advances in genetics and molecular biology since the 2001 proposal. Consequently, EPA indicated that to pursue a future exemption, the Agency would issue a new proposed rule based on the types of products possible to create with the current state of scientific advances rather than issue a final rule based on previous proposals.

Biotechnology innovation efforts within the United States Government and at EPA

EPA indicated in the "National Strategy for Modernizing the Regulatory System for Biotechnology Products" (OSTP, 2016) that it intended to clarify its approach to pesticidal products derived from genome editing.

On October 29, 2018, FDA issued its "Plant and Animal Biotechnology Innovation Action Plan" where it indicated FDA's intent to develop guidance for industry on how current FDA regulatory policy for foods derived from new plant varieties applies to foods produced using genome editing.

On June 11, 2019, Executive Order 13874 on "Modernizing the Regulatory Framework for Agricultural Biotechnology Products" was issued. Section 4(b) of that Executive Order directed the U.S. Department of Agriculture (USDA), EPA, and the Food and Drug Administration (FDA) "to the extent consistent with law and the principles set forth in section 3" of the order to "use existing statutory authority, as appropriate, to exempt low-risk products of agricultural biotechnology from undue regulation."

Subsequently on May 18, 2020, USDA revised its plant pest biotechnology regulations at 7 CFR part 340. In that rule, USDA amended its regulations in response to advances in genetic engineering as well as USDA's understanding of the associated plant pest risk posed by genetically engineered organisms.

EPA then proposed a rule on October 9, 2020 to exempt certain PIPs based on sexually compatible plants created through biotechnology. It should be noted that EPA and USDA use the term "conventional breeding" in the context of their own regulations.

On September 12, 2022, Executive Order 14081 on Biotechnology and Biomanufacturing was issued. Under this new Executive Order, EPA, USDA and FDA are working to improve the clarity and efficiency of regulatory processes for biotechnology products. EPA is currently working to finalize its proposed exemption for certain PIPs.

EPA's proposed exemption for PIPs based on sexually compatible plants created through biotechnology

Advances in genome editing (e.g., the CRISPR-Cas nuclease system, meganucleases, zinc-finger nucleases, and transcription activator-like effector nucleases) allow for targeted, rapid, and precise changes to chromosomes of living cells (NASEM, 2017). These technologies allow editing of the genome in a way that the resulting genes can be indistinguishable from those found in a plant created through conventional breeding.

EPA's proposed rule reflects these scientific advances and would allow certain PIPs created through biotechnology to be exempt under the pesticide licensing and use law (FIFRA) and the law used to regulate pesticide residues in food and feed (FFDCA), in cases where those PIPs: 1) pose no greater risk than PIPs that EPA has already exempted, and 2) could have otherwise been created through conventional breeding. To further describe the types of PIPs that would meet these criteria, the Agency proposed new definitions to limit the pesticidal substances that would fit under the exemption to those found in plants that are sexually compatible with the recipient plant, i.e., definitions for "native gene" and "native allele." "Native allele" is proposed to mean a variant of a native gene that is identified in the genetic diversity of plants sexually compatible with the recipient plant. "Native gene" is proposed to mean a gene that is identified in the recipient plant or plants sexually compatible with the recipient plant; and has never been derived from a source that is not sexually compatible with the source plant." Through these definitions, the proposal also excludes use of transgenes that could be moved between sexually compatible plants through conventional breeding. For example, a Cry1Ab protein from *B. thuringiensis* that was engineered into a source plant would not qualify as a native gene to be used in a recipient plant since *B. thuringiensis* and the recipient plant are not sexually compatible. By limiting the pesticidal substances to only those that are found in plants sexually compatible with the recipient plant, EPA can rely on the history of safe use associated with conventional breeding to conclude negligible risk of novel exposures or hazards.

The proposal allows developers to modify an existing gene to create a "native allele" or insert a "native gene." This allows for modifications within the coding region of an existing native gene in a plant to create a native allele, and insertion of a native gene into non-genic regions of the genome.

The proposal also allows developers to make modifications in the expression level of an existing native gene and for the reduction or elimination of a substance that is itself not pesticidal, but its absence has a pesticidal effect.

Lastly, the proposal included a process to determine the eligibility for exemption: 1) a developer may submit either a self-determination letter, and/or 2) request EPA confirmation that their PIP meets the criteria for exemption.

What will the final exemption for PIPs based on sexually compatible plants created through biotechnology look like and when will it be final?

EPA received a total of 8,120 comments in response to its proposed rule. Of those, 28 were unique and one of those unique comments was supported by 8,093 co-signers. Many commenters supported EPA's effort to exempt certain PIPs that are created through newer biotechnology techniques. However, commenters across industry, trade, and academia felt that the proposed exemptions could be broadened. Some commenters found the proposal to be too permissive and recommended specific modifications.

EPA is taking these comments into consideration and is in the process of developing a final rule to exempt PIPs based on sexually compatible plants created through biotechnology.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Will genetically modified late blight resistant potatoes be the first GM crops to be approved for commercial growing in Norway?

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Last decade's advances in biotechnology, with the introduction of CRISPR, have challenged the regulatory framework for competent authorities all over the world. Hence, regulatory issues related to gene editing are currently high on the agenda both in the EU and in the European Economic Area (EEA) Agreement country of Norway, particularly with regards to sustainable agriculture. During the negotiations on the EEA Agreement, Norway was allowed to retain three extra aims in the Gene Technology Act: "That the production and use of GMO happens in an ethical way, is beneficial to society and is in accordance with the principle of sustainable development". We argue the case that taking sustainability into the decisions on regulating gene edited products could be easier in Norway than in the EU because of these extra aims. Late blight is our chosen example, as a devastating disease in potato that is controlled in Norway primarily by high levels of fungicide use. Also, many of these fungicides are being banned due to negative environmental and health effects. The costs of controlling late blight in Norway were calculated in 2006, and since then there have been new cultivars developed, inflation and an outbreak of war in Europe increasing farm input costs. A genetically modified (GM) cisgenic late blight resistant (LBR) potato presents a possible solution that could reduce fungicide use, but this could still be controversial. This paper aims to discuss the advantages and disadvantages of approving the commercial use of a GM LBR potato cultivar in Norway and compare these against currently used late blight management methods and conventional potato resistance breeding. We argue that a possible route for future regulatory framework could build upon the proposal by the Norwegian Biotechnology Advisory Board from 2019, also taking sustainability goals into account. This could favour a positive response from the Competent Authorities without breeching the European Economic Area (EEA) Agreement. Perhaps the EU could adopt a similar approach to fulfil their obligations towards a more sustainable agriculture?

KEYWORDS

***Solanum tuberosum*, *Phytophora infestans* resistance, financial impact, regulatory framework, sustainability, preparedness, CRISPR, Norwegian Gene Technology Act**

1 Background

Today, most nations face food security as being vulnerable, and Norway is potentially the most vulnerable nation concerning food security in Europe. Only 3% of Norwegian land is used for growing food, and of that only 30% is used for grain and 1.4% for potatoes (Flaten and Hisano, 2007; Lombnæs et al., 2011). Over half of the calories consumed in Norway and around 25% of potatoes are imported (Svennerud, 2021; Angelsen and Rebnes, 2022), making Norway highly dependent on the global food supply chain. In the event of a global food crisis, such as drought, war or a severe pandemic, major food exporting countries may significantly reduce export of basic food products, as we saw recently with Ukraine after the Russian invasion (Glauben et al., 2022), and in severe cases Norway could struggle to feed its population.

Potatoes are the fourth most important crop in the world after corn, rice and wheat, and they are an important source of nutrition both globally and in Norway (Lombnæs et al., 2011). The potato plays an important role in sustainably maintaining Norwegian food security, especially under a crisis scenario (Flaten and Hisano, 2007), as potato production is more land and fertiliser efficient per hectare than grain production, potatoes can be produced over the whole of Norway, and tubers are full of important minerals and vitamins and can be stored for long periods of time (Devaux et al., 2014; Store Norske Leksikon, 2021).

The oomycete pathogen *Phytophtora infestans* causes the disease late blight in potato, that threatens potato harvests globally (Hijmans et al., 2000). It is the most significant potato disease in Norway (Sæthre et al., 2006), resulting in high levels of fungicide application with negative effects on human health and the environment. It produces zoospores and sporangia that can travel large distances, as well as overwintering oospores that can survive in soils up to 5 years and act as primary inoculum, making crop rotation as a control less effective (Sæthre et al., 2006). With wetter summers and warmer winters, the effects of climate change in Norway are expected to create more favourable conditions for the spread and infectiousness of *P. infestans* on potato (Cooke et al., 2011).

In 2006, late blight in potato was estimated to cost 55-65 million NOK annually, with fungicides costing farmers on average 22.9 million NOK, application costs 25.6 million NOK, yield losses 5 to 14 million NOK and inspection, research, and advisory services 3.3 million NOK annually (Sæthre et al., 2006). We have recalculated the cost of late blight in Norway to be 105 million NOK before the Ukraine war in 2021, and 125 million NOK after in 2022, considering increased input prices due to inflation and the Ukraine war, as well as the use of modern cultivars, and including VAT of 25% (Supplementary Table 1). In addition to this, there can be yield losses from late blight, though these are harder to calculate.

Several popular Norwegian potato varieties, such as 'Mandel', are heavily susceptible to late blight, so can only be grown at high altitudes and in Northern Norway where conditions are too harsh for late blight to survive (Roer, 1987; Sæthre et al., 2006). Many of these varieties contain desired traits and have commercial value, so by creating resistant cultivars, they could also be grown in low lying areas with less use of fungicides and with better soil conditions (H A Krogsti, personal conversation, 14 Mar 2022).

Genetic modification (GM) and gene editing (GEd) methods have both been proposed as methods of developing new late blight resistant (LBR) potato cultivars, with an estimated potential to reduce fungicide inputs by over 80% (Kessel et al., 2018). However, the use of GM technology in agriculture is highly controversial in Europe, while China has recently approved another eight new GM crops (ISAAA, 2023).

2 Current control methods for *Phytophtora infestans*

Norwegian potato fields were sprayed with fungicide on average 5.6 times a year before 2006 (Sæthre et al., 2006). However, in recent years, this has increased to 8-9 times in a year of heavy infections, even up to 16 times a year, due to more aggressive *P. infestans* strains (B Glorvigen, personal conversation, 15th October 2022). Using fungicides can have severe negative economic, environmental and health effects. Controlling late blight in potatoes can be as much as 25% to 30% of Norway's entire fungicide usage. Hence, these effects are significant (M Alsheikh, personal conversation, 4th April, 2022). For example, the commonly used fungicide ingredient cymoxanil is suspected to cause birth defects, may cause organ damage over long-term exposure, and is moderately toxic to mammals, honeybees, aquatic organisms and earthworms (Lewis et al., 2016; Plantevernguiden, 2020). Zorvec-Endavia contains the bioactive compound Benthiavalicarb isopentenyl, which has shown carcinogenic potential in two different species (Alvarez et al., 2021). All fungicides used for late blight control have the warning symbol for toxic aquatic effects with long lasting effects (Plantevernguiden, 2020).

Because of these concerns, former common effective fungicides have been banned by the EU and therefore also in Norway (EU regulation, 2020; Saha et al., 2022). As more data is collected on the harmful environmental and health effects of fungicides, pressure is increasing on the EU to continue to ban fungicides, potentially threatening farmer's ability to chemically control late blight in the EU and Norway (European Regualtion, 2020; Forbond, 2021).

P. infestans is notorious for its large genetic variation and ability to constantly mutate and develop resistance to fungicides due to an ability for both asexual and sexual reproduction (Haverkort et al., 2016). Strains with resistance to some fungicide active compounds such as propamocarb, have been found (Lehtinen et al., 2007). In our increasingly globalised world, these mutations spread quickly, making fungicide resistance to *P. infestans* a growing threat to potato production globally.

Plant breeding for LBR is a potential way of reducing need for fungicides against late blight. However, it has many challenges due to the potato's complex genome, that the potato reproduces

primarily by vegetative reproduction making it difficult to cross, and that there is low genetic diversity (Gálvez et al., 2017). Potato is also very susceptible to inbreeding depression (Zhang et al., 2019). Introgression of resistance genes without unwanted effects on the potato genome is difficult due to linkage drag, in addition to the fact that potato is tetraploid, whereas many of its wild relatives containing resistance genes are diploid. Some highly resistant varieties resulting from conventional plant breeding such as 'Sarpo Mira' do exist, however these are poorly suited to the Norwegian market and growing conditions (Kim et al., 2012; Gillund et al., 2016; Colon et al., 1995).

3 Genetic modification for late blight resistance

Genetic modification presents an alternative to plant breeding without many of the abovementioned issues. Resistance (R) genes from other potato cultivars and wild relatives has to have inserted using traditional GM techniques (Zhu et al., 2012; Witek et al., 2016). In addition, it has been demonstrated that silencing certain susceptibility (S) genes for late blight can increase resistance, however more field trials are necessary to further determine how S gene silencing could affect other crop traits (Sun et al., 2016; Kieu et al., 2021). Multiple R and silenced S genes can be 'stacked' in a cultivar to increase the strength and long-term viability of resistance to the pathogen, known as pyramiding (Kim et al., 2012; Sliwka et al., 2012).

One concern regarding GM crops is that introduced genes will escape to wild relatives and have negative ecosystem effects (Quist, 2007). However, *S. tuberosum* is not sexually compatible with either of the two common *Solanum* wild relative species that grow in western Europe: black nightshade (*S.nigrum*) and bittersweet (*S. dulcamara*). This has been demonstrated in studies by Eijlander and Stiekema (1994) and McPartlan and Dale (1994).

Another concern is that cisgenes could spread to other potato growing areas. However, regulations on physical distance and growing intervals between GM and non-GM potato crops and disinfection of machinery can significantly reduce this risk (VKM, 2006; Anastassiadou et al., 2020).

4 GM terms and the regulatory framework

The EU defines a GMO as an organism in which "the method of altering genetic material is done in a way that is not natural mating and/or recombination", and because of this, gene edited organisms using techniques such as CRISPR are regulated as GMO by the EU (Turnbull et al., 2021). However, a proposal that GEd techniques including CRISPR could be regulated separately from GM has been suggested by the Norwegian Biotechnology Advisory Board to the Norwegian government in 2018 (Turnbull et al., 2021). A governemental Committee is currently reviewing the legislation in Norway. As Norway is a member of the European Economic Area (EEA), it follows most EU rules and regulations, and therefore the definitions of GMO as given by EU's Deliberate Release Directive of 2001 (European Directive 2001; Turnbull et al., 2021). On the other hand, Brexit has lead to a revision of the rules for deliberate release of certain GEd higher plants in England, if the traditional plant breeding techniques (e.g. mutation breeding) could have obtained the same result (UK Practical Law, 2022).

Therefore, following the discovery and use of new breeding techniques (NBTs) including gene editing techniques, there is now a degree of uncertainty regarding what should be legally defined as GMO and if other classifications are necessary (Eckerstorfer et al., 2019). Gene editing is a novel technique involving site directed nucleases (SDN) to make precise incisions or insert DNA sequences at the target DNA area (Turnbull et al., 2021).

In Norway, the use of GM or GEd methods such as CRISPR in agriculture is essentially limited to research use and no GM food crops are grown commercially (Turnbull et al., 2021). However, this may be subject to change as political and consumer pressure to sustainably increase crop yields and adapt to climate change grows, while scientific understanding of gene technologies and their implications advances rapidly (Hjelkrem et al., 2021; Turnbull et al., 2021).

The three additional aims of the Norwegian Gene Technology Act (Norwegian Government, 1993): "That the production and use of GMO happens in an ethical way, is beneficial to society and is in accordance with the principle of sustainable development", make Norway one of the most restrictive countries in the world for approval of GM crops. However, by demonstrating that the potential GM crop can satisfy all these points, it is more likely that consumers, farmers and industry will support the decision. With the example of the LBR GM potato, this was shown in a workshop by Gillund et al. (2016), and a study by Bioteknologirådet (2020) that showed over 70% of respondents being positive about GM if it would reduce fungicide use and yield losses and thus make agriculture more sustainable.

5 Conclusion

In conclusion, there is a strong case for that a LBR GM potato could be the first GM crop to be approved for commercial growing in Norway. Increasing resistance to fungicides, the banning of fungicides by the EU (and Norway), climate change and an increasing focus on Norwegian food self-sufficiency, all create urgent demand for potato cultivars with long lasting and significant LBR. GM and GEd techniques can be used to create potatoes with high levels of LBR in relatively short timeframes that would not otherwise be possible through conventional plant breeding, and therefore present an important potential tool in maintaining food security in Norway in an uncertain future.

It is important that the decision for GM LBR approval in Norway is made based on rational arguments and scientific understanding of

its consequences, weighed against the disadvantages of the current control methods and the limitations of potato breeding, and the Norwegian three-part approval system is arguably well adapted to this. Measures can be taken to mitigate the concerns of a GM LBR potato, and arguably the risks of continuing to not use GM in Norway outweighs the risks of allowing it.

Author contributions

The first author (EF) has done the calculations, under the guidance of the second (AW-V) and the third author (TH-E). All authors contributed to the article and to the writing, and approved the submitted version.

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Supplementary material

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Possible *EU futures* for CRISPR-edited plants: Little margin for optimism?

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This article addresses the scenarios that may be encountered by the first application for pre-market approval of a CRISPR-edited plant in the EU. Two alternative scenarios are considered in the short and medium term. One of these possible EU futures depends on the final drafting and approval of EU legislation on certain New Genomic Techniques, which was started in 2021 and is due to be quite advanced before the next European Parliament elections in 2024. Since the proposed legislation excludes plants with foreign DNA, two different approval processes for CRISPR-edited plants will coexist if the legislation enters into force: one for plants whose genome has been altered, resulting in mutagenesis, cisgenesis and intragenesis; and the second for plants whose alterations result in transgenesis in general. In the event that this legislative process does not succeed, CRISPR-edited plants in the EU could face a regulatory scenario whose foundations were laid in the 1990s: the regulatory framework that applies to GM crops, food and feed. In this review, an *ad hoc* analytical framework has been built that considers in depth the two possible futures for CRISPR-edited plants in the EU. This framework emphasises the way in which the European Union and the Member States (MS), with their respective national interests, have historically shaped the regulatory framework for plant breeding in the EU. On the basis of the analyses carried out on the two possible futures for CRISPR-edited plants and of their potential with respect to plant breeding, the main conclusions are the following. Firstly, that the regulatory review that started in 2021 is not in itself "good enough" for plant breeding and CRISPR-edited plants. Secondly, that compared to its alternative, the regulatory review currently underway contains at least some promising improvements in the short term. Hence, thirdly, in addition to adopting the current regulation, the MS need to continue to work towards a substantial improvement in the legal status of plant breeding in the EU in the medium term.

KEYWORDS

European integration, EU regulatory framework for plant breeding, EU legislation on new genomic techniques, premarket approval of CRISPR-edited plants, EU status of CRISPR-edited plants

1 Introduction

The Scottish journalist Allan Little is often quoted as saying that in Europe '*history is the unseen guest at every table*' (Little, 2012), irrespective of the agenda. This has been the case since the period immediately following the end of Second World War, when countries that had been using coal and steel to make weapons to kill each other (see list 1) tried to build the European Coal and Steel Community. One of the main objectives of that initiative was clear: "*the solidarity in production thus established will make it plain that any war between France and Germany becomes not merely unthinkable, but materially impossible*" (Schuman, 1950). Of course, preventing war is not the main element that holds the EU together nowadays, but for the countries of the region there is still something inevitable about EU membership. From a geostrategic point of view, countries of the region cannot afford to remain outside the EU. Although it has not been easy, integration offers numerous synergies and economies of scale to MS, as well as a much greater political reach than they would achieve individually. Therefore, France has described the EU as a power multiplier or *multiplicateur de la puissance* (Verluise, 2017).

However, this is not synonymous with the fact that all Member States (hereinafter MS) on all occasions and in all contexts participate in the EU with the same level of conviction and commitment. At least in the short term, some countries tend to accept decisions that entail sacrificing some of their more immediate interests, negotiating trade-offs in areas that have little or nothing to do with each other. The EU is at essence a common market, although this is not its exclusive function. Once the 27 heads of governments of the MS and the European Commission (hereinafter the EC) have established a possible 'common interest' in the Council of the EU or *Consilium* (hereinafter EU Council), the EC is responsible for fulfilling commitments and their legislative implementation, whether in the sphere of the Community institutions or in other international scenarios, with a view towards the global market (Bradford, 2020). The role of the European Court of Justice (hereinafter ECJ), is no less relevant: it is the ultimate guarantor of these Community commitments, supervising the correct implementation and application of EU Law within the constitutional framework defined by the EU Treaties.

Seventy-five years after the Declaration of Rome that gave birth to this unique union, the EU is the product of many concrete achievements and numerous lessons learned from complex situations (such as the introduction of a single currency, the failed attempt to draft a federal constitution or, recently, Brexit). The EU's governance model combines federal, quasi-federal and confederal elements, and it accommodates the institutional and political diversity of the MS while avoiding disruption of the integration process. This results in a complex multi-scale federalism (Filibi, 2013). Admittedly, from time to time analysts speak of integration fatigue, but the EU continues to expand into new areas and generate innovative policies (Lasa López, 2011; Bradford, 2020) and is radically redefining the classical concept of state sovereignty (Uncetabarrenechea and Filibi, 2022). However, history is always present, so MS are frequently forced to rewrite new consensuses on the palimpsest of older agreements, whether they were good or bad, and on a background of prior crises and disagreements.

The need to be mindful of this institutional peculiarity of the European Union, as well as the presence of historical precedents, becomes especially relevant when the object of analysis is a political and regulatory scenario as dysfunctional as the one experienced by European plant biotechnology. Although negotiation of EU regulations for CRISPR-edited plants for the 2020s seems superficially to be a technical issue, there are significant historical roots. It is important to make this context visible. It is possible to group these issues into three main blocks:

a) The first block of issues arises from the peculiarity of integration of agriculture into the EU. The six funding MS (see below list 1) agreed that agriculture could not be left out of the European project, even if there was still no clear consensus on how to realise its integration (McMahon, 2007). Among other things, the MS debated at the time the favouring of national production over third counties and mechanisms to stabilise prices (Tracy, 1993). Nowadays, related concerns are still alive in the European arena, embodied in expressions such as 'preservation of independence in food access', as declared by French ministers after the COVID-19 crisis. Those are some of the factors that explain why successive versions of the Common Agricultural Policy (CAP), which is updated every 5 years, have been so contentious. However, in addition to them, we have to consider that the CAP accounted for 66% of the Community Budget in the early 1980s and currently absorbs a third of the bloc's entire budget. This underlying background is reflected in many decisions that intersect with agriculture. For example, in 2015, when the approval of the agricultural GMO opt-out was being debated (EP Legal Observatory, 2015), the Committee of the Regions warned that the attitudes of some MS in the GMO decision-making process were not so much related to GMOs as to the positions they advocated on the CAP (CoR, 2015).

b) Another block of issues concerning negotiations on CRISPR-edited plants is the chronic disagreement of MS on plant biotechnology in particular. The EU was one of the first regions in the world to adopt a GMO regulation in the 1990s (Escajedo San-Epifanio, 2008; 2010), but its implementation has been very disappointing. In an official document, the EC acknowledged that decision-making in this area has turned out to be "largely the exception to the usual functioning of the EU comitology procedure as a whole" (EC, 2015). In an attempt to address the situation, the European Parliament (hereinafter EP) voted separately in 2015 for the recognition of an opt-out prerogative for GMO cultivation on the one hand, and an opt-out prerogative for GM food and feed trade on the other hand (EC, 2015). The former was adopted as Directive 2015/412; the latter was rejected by a strong majority of the members of the EP (Escajedo San-

Epifanio, 2017). The disagreement, however, is not only related "*to issues associated with the safety of GMOs for health or the environment*" (EC, 2015), but is also the result of the intersection of competing views about the management of domestic agricultural interests, the allocation of funds related to agricultural and plant science research, commercial interests, and the management of plant variety rights (80% of them dominated by just three countries: the Netherlands, France and Germany). Moreover, it should be noted that far from the improvement in the first model of GMO decision-making processes they agreed upon in the 1990s, the obsession of some MS to maintain some political room for manoeuvre has incorporated later reforms that would allow de facto blockages of GM crop authorisations (see below 3.2).

c) A third and final set of concerns is the limited way in which mechanisms of democratic political confrontation have been used in the EU to manage the ideological diversity around GMOs. On the one hand, MS have not reflected together on some forms of anti-GMO attitudes and actions that took place in the late 1990s and the 2000s. All democracies face situations of political conflict where (major) effort is needed to redirect political confrontation over conflicting ideas into the legitimate, constitutionally established spaces and channels set up for this purpose. While political disagreement is considered essential and natural to all democracies, violent expressions of disagreement are to be avoided. Nevertheless, the latter was not factored in when the EU faced anti-GMO attitudes and actions in the late 1990s and 2000s. Indeed, some anti-GMO activists in France, the UK, Germany and Switzerland carried out almost synchronously violent actions against crop-research fields whose institutions had been complying rigorously with regulatory standards (Kuntz, 2012; 2020). Further, governments and courts failed to protect those who were complying with the law. Moreover, a debate on ideas has systematically been avoided and some political scientists have found it striking the way that some politicians adopt parts of anti-GMO discourse and suggest that it is a common sentiment of the majority of European public opinion (Shurman and Munro, 2006; Hayes, 2007; Seifert, 2017; Seifert, 2020). As is often the case, excerpts from political opinion lose meaning when placed out of context. Some forms of rejection to GMOs were and continue to be linked to a deep and legitimate rejection of the dominant model of globalisation and its overriding environmental ethics. Yet, without delving into these positions as a whole, some governments have found it easy to adopt and exploit specific allegations of the anti-GMO movement. Finally, the literature also draws attention to the lack of EC reaction to MS that were notoriously non-compliant with the GMO Directive 90/220/EEC (Lee, 2008) and the permissiveness of the EC in face of the *de facto moratorium* (see details below). The perception of disagreement and ambiguity grew even stronger in 1999 when the EU Council, with many other issues on the table, chose to relegate the status of GM crops, food and feed pending a stronger consensus. Selecting the most urgent matters and postponing the work of negotiating others is not unusual in the EU. More than 20 years have passed since the adoption of Directive 2001/18/EC, and in that time the EU has expanded from 15 MS (see list 3) to 27 MS, even with the loss of the UK. However, the difficulty of adequately confronting political tensions remains unresolved, although some studies suggest that public opinion is evolving (Evanega et al., 2022).

According to the EC, the issues raised by MS around GMOs "*reflect national concerns which do not only relate to issues associated with the safety of GMOs for health or the environment*" (EC, 2015) and now that we are facing the regulatory future for CRISPR-edited plants, it is reasonable to ask whether MS are really in a position to commit to a "sufficiently good" legislation. As Robert Schuman said, a united Europe is not built all at once, but "*through concrete achievements that generate de facto solidarity*" (Schuman, 1950). Each collective achievement relies on the expression of a strong political will by the MS (EC, 1993). What kind of political will have the MS expressed regarding CRISPR-edited plants and what achievements might the EU build from it? Capturing and interpreting that political will is one of the objectives of this research. At least two EU futures may emerge for CRISPR-edited plants in the short term (see 2). The first of these futures is dependent on the approval of an *ad hoc regulation* (2.2). The second future for CRISPR-edited plants involves accommodating CRISPR-edited plants within the regulatory framework that was created in 1990 and reformed in 2001 for GM crops, food and feed (2.3). Both future scenarios - including some possible derivations thereof - are discussed in depth in this paper from political and legal perspectives (see sections 3 and 4), with consideration of how they are affected by "historical issues" (see a, b, c above). Methodologically, the research undertaken has involved tracing three decades of EU regulatory and non-regulatory decisions, as well as failed attempts at regulation and disagreements about plant biotechnology. This work has been carried out with a close eye on the institutional particularities of the EU from the origins of the Common Agricultural Policy to the present day, both in its structural and dynamic dimensions, i.e. the geopolitical and historical evolutionary dimensions.

2 Two possible near-term regulatory futures for CRISPR-edited plants in the EU

In 2019, the EU Council launched a process aimed at providing a "*clearer, more evidence-based, applicable, proportionate and sufficiently flexible regulatory framework*" for plants obtained by means of New Genomic Techniques (NGTs), in order to "*cope with advances in science and technology in this area*" (Council Decision 2019/1904). The EC commissioned a study on how this task should

be undertaken, with April 2021 as a deadline for submission. Given that 2024 is the date set for the next European Parliament elections (and thus the appointment of a new EC with a new political agenda), it was estimated that if the deadlines were met, a revision draft of the regulation is estimated to be ready within this parliamentary term (EC, 2022). The EC met the deadlines and in Autumn 2021 opened a time period to develop specific legislation for NGT-derived plants (2.1). Depending on whether the envisaged reform is achieved, CRISPR-edited plants will have one of the following two futures in the EU (2.2. and 2.3).

2.1 New times are here

In their document "Neue Zeiten, Neue Antworten" – New Times, New Answers- the German Greens (Bündnis 90/Die Grünen, 2018), in contrast to earlier times, reflected on the advisability of analysing whether certain new biotechnologies could help to meet the challenges posed by climate change. Similarly, many other political, scientific and/or governmental voices at European or national levels have recently alluded to the contributions that new genomic techniques could make to the ambitious sustainability goals of the European Green Deal (COM 2018/640), the Farm to Fork Strategy (COM 2020/381) and the Long-Term vision for rural areas (COM 2021/345). In addition, some governments are investing in scientific research programmes in the belief that an agreement will be reached sooner rather than later.

Nonetheless, a political context favourable to an agreement does not always guarantee a good consensus. The starting point of the EU's plant biotechnology policy was a period with many factors that were conducive to a good agreement. It was the late 1980s and the MS of the (then) EEC (see list 2) were still in the process of completing their internal market. Among the concerns on the table, the White Paper entitled *Completing the internal market* (EC, 1985) stressed the necessity of leaving behind the economic crisis of 1973. Integration required, among other things, a closer approximation of strategic regulations, more agility and efficiency in decision-making processes and a strengthening of the socio-economic cohesion of the region. A goal of strengthening the scientific foundations of the economic community also took on special relevance (EC, 1985). This period of integration coincided with the socio-political emergence of biotechnology. The European institutions launched at that time specific research programs to stimulate the development of biotechnology, as manifested in the Biotechnology Action Program (1985-1989) and later initiatives (e.g. BRIDGE).

As described by the OECD, since 1980 biotechnology has evolved "*from a scientific curiosity towards commercial applications*" (OECD, 1999) and, as a consequence, regulation at the national and international levels of some of its aspects was considered imperative. In the European region, the MS of the then EEC took an important decision at that time because there was a lack of national-level policies on the use of biotechnology, so they embarked together on this new and common regulatory path. Three decades later, we can affirm that the result of that decision has both light and shadows, depending on the biotechnology sector in question. This common path has yielded very good results in some biotechnology sectors -especially the health sector-, but European agri-biotechnologies collapsed due to a regulatory framework with many shortcomings (Escajedo San-Epifanio, 2017).

It is difficult to know whether the outcome would have been different for agri-biotechnologies if the MS had agreed on harmonisation in two stages: a first stage of national regulatory frameworks in the 1990s, followed by a second stage as a prelude to harmonisation at the EU level. We are not aware of any discussion at that time on the possibility of each MS regulating agri-biotechnologies separately, as an alternative to an EU framework. However, it is known that between 2010 and 2015 the possibility of some devolution of agricultural competence with respect to GMOs was discussed (Escajedo San-Epifanio, 2017). Although it did not go as far as a devolution, Directive 2015/412/EC, which recognises an opt-out prerogative for MS (see Box 1), can be read along these lines. Given the difficulty of reaching tangible agreements on GMOs, and as a more efficient alternative to an opt-out prerogative, some experts discussed why it would have been preferable to regulate an opt-in prerogative rather than an opt-out prerogative (Escajedo San-Epifanio et al., 2019; Eriksson et al., 2020; 2021). That is, some kind of mechanism that would protect the rights of those MS that wish to use specific GMOs once those GMO crops had been favourably assessed at the EU level.

The current situation is not comparable to that of the 1980s: The 27 MS know that all possible futures for CRISPR-edited plants should be *EU futures*; a devolution of powers to each of the MS seems to be unthinkable. Additionally, the foundation stone of any regulatory future for CRISPR-edited plants must be placed now,

BOX 1 Evolution of MS' policy discretion in GMO decision-making (1990-2015).

The decision to create a common regulatory framework for GMOs emerged from consensus by the MS in the late 1980s. At that time, there were no previous state frameworks in the MS on this issue, so harmonisation was not necessary. The immediate task was the creation ex novo of a minimum set of rules on the commercial and agricultural use of GMOs, which was expected to move towards further convergence in the future. The tendency to start by agreeing on minimum standards and gradually move towards greater policy convergence has been a constant in many areas of European integration. This is nothing new. But it is striking that, in the specific case of GMOs, there has been a drift over time towards a less united EU. Thus, while scientific assessment and decision-making have been progressively centralised, at the same time there has been a progressive increase in the margin of political discretion surrounding decision-making within the MS.

In the 1990s, the first regulatory framework for GMO crops opted for a system characterised by two elements: decentralisation of scientific assessment complemented by mutual recognition, and final administrative decision-making at the EU level with little room for individual political decision making. Since 2001, important changes have taken place in this respect. On the one hand, there has been centralisation of scientific assessment in the European Food Safety Authority (EFSA), created in 2002. On the other hand, two important actions of political discretion have been granted to the MS -see list 3-: a veto power incorporated in Directive 2001/18/EC, and an opt-out prerogative enacted in Directive 2015/412 (Escajedo San-Epifanio, 2017; González-Vaqué, 2017). These margins of discretion have important implications for the decision-making system (see Box 4) and its blocking facility (3.3.1).

before the 2024 elections to the EP. At this point in time in early 2023, there are only two possible options: the approval of some legal text in the context of the legislative initiative on NGTs (2.2) or the postponement of the decision to the next parliamentary period, which would place CRISPR-edited plants in a *by-default* legal situation until at least 2025 or 2026 (2.3). Let us now compare the main elements of these two possible EU futures concerning CRISPR-edited plants in the EU, leaving the more detailed development of both for Sections 3 and 4.

2.2 First possible future: Specific legislation for plants obtained by means of CRISPR?

As indicated in the introduction, the most promising future for CRISPR-edited plants is linked to the legislative process launched by the EC in Autumn 2021. That is the so-called initiative for the creation of legislation for plants produced by certain new genomic techniques, including their food and feed products (EC, 2022). This initiative has already passed the institutional and stakeholder feedback period, as well as the public consultation period.

Since the end of the consultations, there have been three EU actions that point to the possibility of the new regulation being adopted. The first action was the publication of the Factual Summary Report, which indicated that the results of the consultation had been "quite favourable" to the legislative amendment. Secondly, the preparatory work being carried out by EFSA on possible risk assessment criteria for plants derived from targeted mutagenesis and cisgenesis was made public in October 2022 (EFSA, 2022). Finally, as recently as November 2022, the preparation of this legislation on NGTs appeared on the list of tasks that the current EC, led by Ursula von der Leyen, plans to complete before the next European elections.

In section 4 we will examine in more detail what is currently known about the legislation that is in the pipeline. Prior to that, however, let us look at what the future might hold for CRISPR-edited plants if the legislative process does not succeed (2.3).

2.3 Second possible future for CRISPR-edited plants: A future by default

Rational choice theory highlighted at least two major explanatory factors that are often overlooked in political analysis (Huntington, 1997): first, that politicians are endlessly opportunistic; and second, that all decisions take place in some type of institutional setting. These factors must also be weighed when the choice made by policy-makers is a decision "to do nothing". In particular circumstances, doing nothing could be considered a good option or at least an option (Cantekin, 2016), and there are many cases in which policymakers opt for this choice if they consider that for some reason a dispute may be intractable. Applied to the case of CRISPR-edited plants, a hypothetical failure of the EU to reach a consensus in 2023-2024 on plants derived from certain NTGs cannot be interpreted as a lack of awareness of the potential of these techniques for current European plant breeding. To some extent, the legislative failure would be an unfortunate consequence of MS being unable to take on the effort of adopting the new legislation.

What would be the situation for CRISPR-edited plants if the legislation currently being drafted is not adopted? Applying Article 2.2 of Directive 2001/18/EC, CRISPR techniques give rise to GMOs (see details in 2.3.1). Therefore, by default, the same legal status applies to them as to transgenic plants with regard to market access or food use, irrespective of whether the CRISPR technique has led to mutagenesis, cisgenesis, intragenesis or transgenesis. Consequently, the default pre-market approval that will remain for CRISPR-edited plants is composed of two elements:

- As far as agricultural use is concerned, Directive 2001/18/EC on the deliberate release of GMOs would apply.
- In the case that CRISPR-edited plants or products obtained from them were to be used as food or feed, Regulations 1829 and 1830/2003 would also apply. These regulations establish the premarket approval procedure for food uses of GMOs, as well as their labelling and traceability requirements.

Regarding the possible application of this regulatory framework to CRISPR-edited plants, we must make two observations: 1) the particular political use that this regulation makes of some scientific concepts (2.3.1); 2) the veiled criticism that the EU Council has made of this regulation, indicating in 2019 (see above) that NGTs need a *more proportionate, applicable and science-based regulation* (2.3.2).

2.3.1 An observation on the dividing lines used by this old regulatory framework

The establishment of any regulation usually has among its components a section delimiting the scope of the regulation. This is a material decision about the substance of a regulation, but it also needs to be expressed in the text. In the particular case of the EU's GMO regulatory framework of the 1990s and the 2000s, delimitation was necessary for determining which GM crops and food fall under the EU's legal capacity and which do not. In order to carry out this delimitation in a very synthetic way, the MS visualised an imaginary subdivision of plant varieties, granting them a different legal status depending on the technique used to obtain them. Accordingly, as we will see below, the EU included a description of dividing lines that separate some subgroups from others as part of the Directive on the deliberate release of GMOs (Directive 220/90/EEC). Let us see how this was done in the legal texts (listed in Box 1) applicable to this case.

As far as GM crops are concerned, Directive 220/90/EEC was created primarily with consideration of techniques involving the use of rDNAs, but in the absence of more specific prior legislation, the following was done: firstly, the concept of GMOs in general was defined in the text of the Directive; and secondly, it was specified to which GMOs the legislation applied and which were exempted. Thus, a GMO was defined as any "*organism, with the exception of human beings, in which the genetic material has been altered in a*

way that does not occur naturally by mating and/or natural recombination" (art.2.2.). Additionally, a list was included in the annexes defining which techniques were exempted from the provisions of the Directive even though such techniques give rise to GMOs (e.g. classical mutagenesis). Directive 2001/18/EC maintained this approach for the delimitation of GMOs that were subject to and exempted from its application. Consequently, this defined three types of pre-market approval applicable to crops, depending on whether a new plant variety can firstly, be considered GMO or not; and secondly, whether the breeding technique used is an exempted technique or a technique under Directive 2001/18/EC (see summary in Box 2).

In other words, a rigorous scientific assessment could conclude that the risk associated with a particular GM food or feed would be equivalent to that of a conventional counterpart (e.g. a food or feed derived from a mutant plant). However, GM food and feed were removed from the novel food category in the 2000s, when specific regulations for genetically modified food and feed were enacted (see above Box 1). As some parts of the literature argue, from that point onwards, GM crops, food and feed were given a legal status in the EU that recognises them as distinct from their parent crops (Davison and Ammann, 2017). See summary in Box 2.

2.3.2 A "not sufficiently evidence-based nor proportionate" regulation

The default position of GMO rules applying to CRISPR-edited plants is not good news. As widely recognised, decision-making on GMOs has been stalled for decades in the EU and it is highly doubtful whether any operator would venture to process a pre-market approval application for CRISPR-crops under the current regulatory scenario. At most, stakeholders would only consider submitting applications for authorisation of CRISPR feed crops that possess high commercial interest, but little else would be judged viable. This situation is similar to the experience with GMOs. According to data published by the EC, the absence of GM crops and food in the EU contrasts with the situation for GM feed: soybeans used in animal feed come from countries where more than 90% of soybean production is GM (COM 2015, 176 final). European feed producers cannot compete on a level playing field.

Projecting this situation to the case at hand, this second possible future would be an EU without CRISPR crops and food, but with CRISPR feed imported from third countries and presumably difficulties in correctly identifying them as such (see below). This does not seem to be the situation desired by MS. Indeed, when in the aforementioned 2019 decision the EU Council called on the EC for "more evidence-based, enforceable and proportionate" regulation (Council, 2019), a veiled criticism of the current regulatory framework was revealed. Although not stated directly, the EU Council's opinion is in line with those expressed by major European scientific institutions, such as ALLEA/EUSAGE, EASAC, Leopoldina or COSCE. All of them suggested that the emergence of NGTs seems a good opportunity to comprehensively review (and improve) the pre-existing plant breeding framework.

The reference to scientific evidence and the idea of proportionality point to the legal need to properly situate NGT-derived plants in relation to the whole range of breeding techniques. A holistic regulatory framework, i.e. one that does not pre-classify risks according to the techniques used (see 2.3.1), would allow MS to control more effectively and proportionately the actual risks of each plant whose genome has been modified. Thus, the report prepared for the STOA (Custers and Dima, 2022: 13) states that new genome editing techniques are "highly precise", and that in relative terms their risks and uncertainties are "lower than the risks and uncertainties of conventional random mutagenesis, which makes use of radiation or chemicals to induce genetic changes" (Custers and Dima, 2022: 16). However, the current regulation of GMOs, which by default includes CRISPR-engineered plants, does not analyse the risks in this holistic framework. On the contrary, it pre-classifies potential risks according to the technique used to obtain the modified plant and, consequently, a plant obtained by random mutagenesis is considered to be of lower risk than a plant modified by CRISPR, regardless of the specific modification that has been carried out. In fact, only the latter technique is currently subject to authorisation requirements prior to its deliberate release for cultivation (see Box 3).

We agree with those who have taken a position on the desirability of a holistic review of the regulatory framework for plant breeding (see above), although such a legislative option is not currently on the table. In other words, if the legislative process described in point 2.2 does not succeed, the default future for CRISPR-edited plants will be exactly the same as the one in which transgenic plants have existed since the adoption of Directive 2001/

BOX 2 Classification of plant varieties according to the premarket approval process foreseen for crops, and the food and feed derived from them in the EU.

Applying the provisions of Directive 2001/18/EC and other rules on pre-market approval of new plant varieties and their food uses, we can distinguish three groups of plant varieties:

1) Plant varieties that cannot be considered GMOs according to Directive 2001/18/EC, and to which, consequently, Directive 2001/18/EC does not apply. The Regulation. EU 2015/2283 on novel food would only apply in the case that their derived products can be considered novel food.

2) Plant varieties that are GMOs but do not require premarket approval due to the techniques used for their breeding as defined by Directive 2001/18/EC. Their use as food and feed may require authorisation in the context of the EU 2015/2283 Regulation on novel food.

3) Plant varieties that are GMOs and whose premarket approval is subject to the above-mentioned Directive 2001/18/EC. Where applicable, food and feed obtained from these varieties require authorisation under Regulation 1829/2003, as well as compliance with the labelling and traceability rules provided for in Regulation 1830/2003.

BOX 3 The advantages of a holistic encompassing regulatory framework: the example of random and targeted mutagenesis.

Gamma rays are the most widely used mutagenic radiation in plant mutation breeding (Li et al., 2019), and ethyl methanesulfonate or EMA is among the most widely used chemical mutagens. These are two examples of classical mutagenesis. The first results of mutation induction in crop plants were published in the late 1920s and their use became widespread from the 1960s onwards. At the time of GMO regulation in the 1990s, the MS declared the following: first, that mutants obtained by radiation or chemical mutagens are GMOs; second, that pre-market approval is not required for their use in agriculture because they were considered to have a sufficient history of safe use. In contrast to what has been agreed at the EU level, French national legislation states, that random mutagenesis "*does not give rise to GMOs*" (art. L-531, Code de l'environnement). Today, it is possible that in some cases food and feed derived from mutant plants may require authorisation under the Novel Food Regulation 2015 as novel food; but these products are not subject to traceability or labelling procedures comparable to those that apply to GM food and feed (see Box 2). Nevertheless, in this future-by-default, a CRISPR-mutated plant would be treated as a transgenic.

This situation does not seem consistent with the comparative sequencing results of conventionally mutated and CRISPR-mutated plants. Today's technology, which did not exist in the 1960s, allows us to test case-by-case impacts on genomes, and it would make sense to have a regulatory framework in which risk assessment focuses on the actual changes made to genomes, rather than being biased or pre-classified by groups of techniques.

18/EC. However, the implementation of this rule will not be easy. Under current regulations, pre-market approval for agricultural or food use of genome-edited plants sourced from the EU will go through the processes mentioned in Box 2. But in the case of genome-edited plants and their products arriving from third countries, the EU is likely to encounter problems in enforcing the labelling and traceability requirements that Regulation 1830/2003 imposes on genetically modified food and feed (Van der Meer et. al., 2023). The information necessary to implement or develop an appropriate detection method for product identification may not be readily available to EU authorities (Ribarits et al., 2021).

3 The second possible future for CRISPR-edited plants: "frozen" since 2001

With regard to this regulatory framework-by-default for CRISPR-edited plants, it is also important to note that the legislative approach employed seemed to have "frozen" the framework at the time when Directive 2001/18/EC entered into force. Such a 'freeze' would imply, as AG Bobek describes (AG Bobek, 2018) in his opinion on case C-528/16, that twenty years later we would be required to interpret Directive 2001/18/EC on the basis of the *'factual or social circumstances prevailing when that rule was adopted'*. AG Bobek, as detailed in Box 4, argued why such a frozen interpretation should not be accepted. However, the ECJ in its judgment in this case C-528/16, held that in the specific case of GMOs there are reasons that justify limiting the interpretation to the date of adoption of the Directive, which is an exception to the established path of European case law.

At the end of the 1980s, the then MS were facing the socio-economic effects of the 1973 crisis and the previously mentioned White Paper of 1985 encouraged them to speed up the process of completing the internal market. Since strengthening the scientific and technological bases of the economic community was identified as one of the pillars of this process, it was unthinkable that biotechnology policy would remain excluded from this new European era. But building a good regulatory framework for agri-biotechnologies was not an easy task. Moreover, the EU did not yet have a sufficiently solid institutional structure or experience. By contrast, in the US, representatives from more than 18 federal agencies and executive offices spent two years working on the design of a specific and coordinated regulatory framework for agri-biotechnologies. In 1986, the federal government published the Coordinated Framework for the Regulation of Biotechnology (Farquhar and Meyer, 2007), which, as far as crops, food and feed are concerned, has relied on the coordinated action of the FDA, USDA and EPA (US Government, 1986).

BOX 4 A scientific-technical regulatory framework that became 'frozen' in 2001.

In the mid-2010s there was some debate in the EU, given that the wording of Directive 2001/18/EC indicates that "mutagenesis", without further precision, gives rise to GMOs that are exempt from the application of the Directive (see Box 2). A court case initiated in France led to a request to the ECJ to clarify whether targeted mutagenesis should (or should not) be considered as an exempted technique, in application of the literal provision of the Directive. AG Bobek, in his non-binding conclusion (AG Bobek, 2018), and the ECJ (ECJ, 2018) adopted a different position on the issue (Casacuberta & Puigdomènech, 2018).

AG Bobek said that while the EU does not have to act on all matters for which it has competence, once the MS decide to legislate on something, there is a constitutional obligation to keep that legislation up to date. Given that some aspects of Directive 2001/18/EC were reformed in 2008 and 2015, AG Bobek said there seems to be no basis for considering that what was not reformed in 2008 and 2015 was frozen in 2001. He also expressed his opinion on the legal argument that New Plant Breeding Techniques resulting in mutagenesis should be understood to be covered by the exemption that the Directive has provided with regard to mutagenesis, as long as they do not involve the use of rDNA. Consistent with that understanding, the AG interpreted the exemption that Directive 2001/18/EC applies to "mutagenesis techniques not involving the use of rDNA" (see Box 2) as follows. Any plant obtained by mutagenesis, whether classical or directed mutagenesis, shall be understood to be covered by the regulatory exemption as long as it does not involve the use of rDNA (Bobek, 2018: para 60 and 62; Purnhagen & Wesseler, 2018: 18).

The ECJ, however, made a particular use of self-restraint, as it has been especially criticised for its poor scientific basis (Casacuberta and Puigdomènech, 2018; Escajedo San-Epifanio, 2022). According to the court, mutant plants had been declared exempt from the GMO Directive in 2001 because of their long safety record, and after that date there had been no declaration on plants obtained by directed mutagenesis. For that reason, and applying the precautionary principle, the court considered that it was more appropriate to apply the transgenic regime to edited genome plants than to other mutant plants. However, this understanding ignores the fact that targeted mutagenesis did exist in 2015, the date of the last reform of the Directive. At the same time, it seems that the court recognizes the concept of "long history of safety" as a new criterion or dividing line; that is, as an additional criterion to the dividing lines that the Directive uses to distinguish between GMOs exempted by and subject to its regulatory regime. It so happens, however, that this term (long safety use) does not appear in the text. Moreover, there is no record that the MS reached any explicit agreement (regulatory or political) during the drafting of the standard that specified what is to be understood by the term "long safety record".

The US will not need to pre-classify plant breeding techniques in the same terms as the EEC (vid supra), because the competence over plant breeding, whether GMO or non-GM crops, will be the responsibility of a single institutional structure as a wholeIn the EU, on the other hand, a whole narrative will be built on the aforementioned dividing lines. In essence, the dividing lines that were created to establish who has the legislative capacity for each plant variety (see above) will condition the future of the regulatory framework. This is because the dividing lines embodied two immovable premises. The first premise is that plant breeding techniques, including those yet to come, have been ordered chronologically, and from the simplicity of the discourse it seems that any new plant breeding technology is bound to have more impact and potentially more risk than the pre-existing ones. The second premise, in line with this, is that the European regulatory framework does not need a clause or mechanism to revise its approach in the light of scientific and technological developments.

In an interesting non-binding opinion on case C-528/16, AG Bobek (see Box 4) explained in 2018 that the principles of European Community law prevented the GMO regulation from being considered frozen in 2001. It is also interesting to note that the discussion on whether and to what extent NBTs result in GMOs (or a similar regulatory term) has been ongoing since the mid-2000s in many jurisdictions around the world, as detailed in the 2021 paper by Van der Meer et al. However, the ECJ in its judgment on case C-528/16 ruled the opposite way. The ruling did not explicitly refer to whether the interpretation of Directive 2001/18/EC should be understood as "frozen" at its date of creation. Nevertheless, the ECJ did advocate an originalist interpretation or an interpretation in line with the time of the creation of the legislation. Namely, the ECJ stated that the consensus of the MS on certain elements of Directive 2001/18/EC had not been renewed since its drafting and, on that basis, argued that the precautionary principle recommends an interpretation of the Directive in line with its date of creationSee it detailed in Box 4.

3.1 The *evolution* of this frozen regulatory framework

Freezing, as is well known, is a preservation technique that, among other things, protects certain objects from the effects of the passage of time. Symbolically, it is used in this case to refer to the regulatory framework for GMOs, because the framework seems to have remained impervious to the passage of time. Why then are we now talking about the evolution of this regulatory framework? It is the case that while the content of the framework will not be revised except in specific areas, the practical application or, as it were, the way the regulatory framework functions will be affected by the passage of time.

The evolution of this fragmented regulatory model over time, together with some problematic elements it already contained from the beginning, will significantly affect the development of agri-biotechnologies in the EU, and because of the freeze, will be an obstacle to adequately accommodate the legal treatment of new plant breeding techniques (Abbot, 2015; Callaway, 2018). Former EU Ombudsman Diamandouros (Diamandouros, 2018) explained in 2018 that although the matter to be regulated was the use of a scientific technological advance, some other policy issues made the regulation of GMOs a "deeply political matter" that needed to be addressed at political level and could not be exclusively confined to an administrative ruling. This is not so much an attribute of the technical content, but is related to the unsatisfactory European decision-making structure for plant biotechnology and the fact that this structure has been in place since the 1990s (Daviter, 2012).

We argue that there are at least three deep policy issues, all of them strategic, that had to be addressed at the political level in the late 1980s, when the EEC was designing what would become Directive 90/220/EEC on the deliberate release of GMOs: the choice of the type of regulatory framework; the specification of GMOs under and exempted from Directive 2001/18/EC; and the decision-making model for authorisation.

The decisions on the regulatory approach (by sectoral or horizontal uses), as well as the delimitation of the EU's sphere of competence (see 2.3.1) were also decisions with an important political dimension. It is understood that we face a political use of scientific terms beyond the technical delimitation. The dividing line, described in detail in 2.3.1, determined in the early 1990s which GMOs were to be "EEC business", regulated on the basis of a common consensus, and which were not. And the very existence of this dividing line, which was kept in Directive 2001/18/EC, would also have two important consequences. First, any decision on the dividing line (to leave a future GMO technique to one side or the other) would inevitably have a political component associated with it. In other words, effectively it would mean determining the pre-market approval of a technique, but also defining which level of decision-making (Community or national) would be responsible for taking decisions. The second consequence would be a tendency to reinforce this dividing line, given that the MS would only maintain an important sovereign decision-making capacity over GMOs exempted from the Directive on deliberate release (currently Directive 2001/18/EC).

These decisions predictably led to another question: whether the EEC would use a product-based or process-based evaluation process (Sprink et al., 2016). Since the EEC would not have a holistic competence on plant breeding in general or even on GMOs as a whole (transgenic or not), a product-based evaluation model was very difficult to organise institutionally. Not impossible, but difficult. Nevertheless, it is not conclusively clear that Directive 2001/18/EC opted for a purely process- based regulation (Tagliabue, 2017). In fact, it has been discussed among different institutions and in the literature whether the interpretation and application has been focussed on the resulting GMO organisms, the techniques used to obtain them or a combination of both (Van der Meer et al., 2023).

Let us now look at a key element of the regulatory framework that the EU applies to GMOs under the Directive: the decision-making process.

3.2 The decision-making model or *Achilles' heel* of the EU regulatory framework to be applied by default to CRISPR-edited plants

The decision-making process on the premarket approval of GM crops food and feed has always been the Achilles' heel of the EU regulatory framework for GMOs. A two-fold need for authorisation decisions had to be addressed when the EEC first regulated the deliberate release of GMOs in the 1990's. Firstly, there was a need to articulate at the European level some way to address the scientific assessment of risks associated with GM food and feed. Unlike the FDA in the US, created in 1906, The European Food Safety Authority (EFSA) did not exist in the 90s. Secondly, an appropriate panel or committee had to be selected or established to make the final decision on the authorization of a GMO in the light of the scientific assessment.

The initially established model provided, firstly, for a scientific evaluation that could be carried out in any MS under common criteria and, secondly, for final decision-making involving all the MS at the level of the EEC (see Box 1). The model initially established provided for a scientific evaluation that could be carried out in any MS under common criteria, and subsequently a final decision-making process involving all the MS at the EEC level. Several factors, however, mostly of a political nature, hindered the proper functioning of this scheme from the outset (see Box 1). Between 1998 and 2004, no applications for authorisation of GMOs under Directive 90/220/EC reached the end of the decision-making process (Lee, 2008, 2-3), and some of the 15 MS (see list 3) introduced measures into their national legal systems that aimed to prohibit or hinder national market access for GMOs that had already been authorised at the EU level.

This situation was called "*de facto moratorium*" (Liebermann and Gray, 2006) and was formalised in 1999, when the MS expressed that since a major revision of the regulatory framework was underway - leading to the creation of Directive 2001/18/EC - it did not make sense to continue making authorisation decisions. Nevertheless, the new Directive and the creation of EFSA (in 2002) were not sufficient to unblock the situation. According to the new regulation, when EFSA issues an opinion in favour of the authorisation of a GM crop, the EC prepares a favourable draft decision. The MS then meet and analyse the draft; a favourable draft means that the application meets all the requirements set out in the regulations. The MS have then the political possibility to vote for or against the draft. However, in the event that the MS do not issue any decision, after a period of time, the regulations stated that the EC must formalise the draft decision. Nevertheless, in the few applications that were subsequently processed after 2002, the MS systematically opted for an extraordinary combination of decisions. A non-decision was agreed in the committee (see Box 1), but then external pressure was exerted on the EC not to formalise the draft for authorisation. One of the most striking cases was the one that reached the ECJ as case T-164/10, Pioneer Hi Breed Int. (Escajedo San-Epifanio, 2017). In view of the EFSA report, the Commission prepared an authorisation draft in which the MS expressed a non-decision. Subsequently, both the MS and the European Parliament pressured the Commission, recommending it not to formalise the authorisation of the crop. Without mentioning the attitude of the MS and the Parliament's resolution, the CJEU judgment in the Pioneer case condemned the Commission for its inaction, reminding it that it was obliged to comply with its obligations under Directive 2001/18/EC.

Uncomfortable with this situation, the Barroso Commission initiated a process to increase the discretion of the MS in exchange for the decision to be taken on a more scientific basis - see above Box 1 -; an arrangement that, as we have already seen, has been widely criticised (Paskalev, 2012). This gave rise to the opt-out prerogative, which allows any MS to partially withdraw from something that other MS have decided at the EU level. While there have been major disagreements in other EU areas, there is no other area in the EU where a similar prerogative is allowed (Salvi, 2016; Poli, 2015) and it has been considered that the *opt out* seems to be more of a trade-off and temporary solution than a long-term solution (Escajedo San-Epifanio, 2017).

4 The first (and only truly) possible *EU future* for CRISPR-edited plants: A new regulation

Let us consider the information available to date on the legislation that is being drafted on plants obtained by means of certain NGTs. The main novelty is the creation of a fourth subgroup of plants within the pre-market approval categories of crops, food and feed seen in Box 2 (see Box 5).

Seen from the political perspective and the EC's effort to generate a consensus among the MS on plants obtained through NGTs, the decision to reference its legal framework with respect to GM plants and those obtained through classical mutagenesis has a certain logic. When complex issues intersect, it is common for international agreements to apply what is known as the technique of compartmentalisation and specialisation of decisions. Narrowing and specifying the margins of agreements tends to allow existing

BOX 5 Placing plants obtained by means of NGTs between GMOs exempted from the Directive and GMOs "under Directive".

In addition to the status applicable to conventional plants (see Box 2), three different statuses for genetically modified plants will coexist in the EU if the current legislation is approved: (1) plants modified by techniques included in the list of exempted techniques in Directive 2001/18/EC; (2) plants obtained by techniques that remain under Directive 2001/18/EC; (3) and plants that, if applicable, would fall under the new legislation on plants obtained by means of certain NGTs.

Regarding food and feed: (a) the GM food and feed regulations would apply to the agri-food use of plants in group 2; (b) Regulation 2015/2883 on novel food would apply to exempted plants (group 1) if they give rise to novel foods; and (c) it remains to be seen what new regulations are envisaged for group 3. Some of the elements of the Roadmap and the fact that DG Health is in charge of proposing the Draft (EC, 2021 IIA) suggest that there will be a specific regulation on the food safety assessment of food and feed derived from plants obtained by NGTs.

consensus to flourish, thus avoiding the lack of consensus on related issues. In this sense, compartmentalisation can be positive, but it may also have disadvantages, which, as will be seen below, seem to have occurred in this case.

Before going into this, it is worth referring to the legislative instrument and the DG in charge of developing it. The regulation of GM plants is currently contained in different instruments, most notably Directive 2001/18/EC and Regulations 1829 and 1830 of 2003. The Directives allow MS a certain margin of self-regulation when transposing them into their respective legal systems, but this is not the case with the Regulations, which are applied homogeneously throughout the EU. At the current stage of the development of legislation on plants obtained *via* NGTs, it is unknown whether plants obtained through certain NGTs would be regulated by a Directive or a Regulation, but everything suggests that the legislative instrument will be only one of these two possible instruments. In any case, considering that food is involved, it is likely to be a Regulation. The political leadership in drafting the legislation seems to have been entrusted to DG Health, without prejudice to the possibility of coordinating with other bodies on certain elements.

Let us now see how the decision has been compartmentalised and specified in this case. Within the roadmap (EC, 2021, IIA), plants derived from certain NGTS have already been described as requiring regulation with reference to existing legislation (i.e. Directive 2001/18/EC and Regulations 1829 and 1830/2003). Since this reference regulatory framework established groups of organisms, drawing dividing lines according to their breeding processes (2.3.1), it was foreseeable that the technique would also serve as a reference for the delimitation between this prior regulation and the one applicable to NGTS. And, of course, some reference should be made to previous EU experience in assessing: 1) genetically modified plants (as mutants are not assessed *stricto sensu* at the EU level); 2) risks associated with food and feed derived from genetically modified plants (referred to in the legislation as genetically modified food and feed); and 3) risks of food and feed derived from mutants falling within the scope of the novel food legislation (see Box 2).

However, the extent to which these inescapable references should or should not limit the freedom of legislators to define an appropriate *ad hoc* framework for GM-derived plants is open to debate. The MS and stakeholders are currently reflecting on this issue. Two things seem to have been neglected from the outset: 1) the scientific-technical assessment of plants derived from certain NTNs would not be the same as that of mutated plants, because only food risks are assessed for the latter and not agricultural risks (see Box 2); 2) nor would the assessment of crops, food and feed obtained in this way have the status of transgenic. But this way of thinking, which has always been referenced with regard to mutant and transgenic plants, is guiding the legislative process towards the common assumption that the associated risks of an edited plant must necessarily be presumed to be higher than those of a mutant plant and lower than those of a transgenic plant.

Along these lines, the EFSA published a document in October 2022 entitled "*Criteria for risk assessment of plants produced by targeted mutagenesis, cisgenesis and intragenesis*", at the request of the EC (EFSA, 2022). In this proposal, it is assumed that "*where the history of safe use of genomically edited plants cannot be sufficiently demonstrated, the function and structure of the introduced allele should be carefully assessed*" (EFSA, 2022), in an approach that suggests the following elements. Firstly, the way in which the regulation establishes the concept of "history of safe use" (hereafter HoSU) will be key, not only in its definition, but especially in terms of allowing the evolution of scientific knowledge to be a reference for adjusting its concrete application. Otherwise, this regulation will remain the same as the GMO regulations: frozen in relation to the time of its formulation. Secondly, it seems that in the case of plants without HoSU, there is a tendency to apply a risk assessment system very similar to the one established for transgenics in Directive 2001/18/EC. This is despite the fact that both the report submitted to the STOA in 2022 (Custers and Dima, 2022) and EFSA itself (EFSA, 2022) have acknowledged that, in some cases, plants obtained by directed mutagenesis or cisgenesis do not pose new risks compared to those produced by mutant plants or even those obtained by conventional breeding techniques.

There are some inconsistencies associated with the above-mentioned EU Council objective of providing a "*clearer, evidence-based, enforceable, proportionate and sufficiently flexible regulatory framework*" for plants obtained by new genomic techniques, in order to "*cope with advances in science and technology in this field*" (Council Decision 2019/1904).

It seems that we are unlikely to achieve these objectives in this way. In terms of clarity, it does not seem a good decision to place such importance in a concept like HoSU, which is a legally indeterminate concept. Nor does it seem to achieve the objective of proportionality, given the difference between plants obtained through NGTs on the one hand, and mutants and conventionally bred plants on the other. For the latter, HoSU is not required to decide on their agricultural use, and at the same time, *ad hoc* assessment will only be used in relation to their food use if it is considered that substantial equivalence cannot be determined with respect to the homologous (conventional or classical mutants). However, contrary to the scientific opinions provided by the EFSA on NGT-derived plants, the possibility that some of them can be considered as equivalent to conventional or mutant plants in terms of agricultural or food use seems to be generally excluded.

These difficulties, however, are not only due to the criteria proposed for scientific assessment, but are the consequence of very different governance models for the risks associated with crops, food and feed in the EU and the US. The current institutional model in the EU is far from providing the regulatory flexibility that exists in the US through the FDA, USDA and environmental authorities, acting separately and/or jointly.

5 Conclusion: A milestone in the history of EU plant breeding is approaching, but it is not the end of the journey

The years 2021 to 2024 will be reported in the political history of European plant breeding as the first time the EU tried to provide CRISPR-edited plants with a regulatory framework, but it will not be the last. Of the two possible futures that have been described, only one of them is somewhat promising for CRISPR-edited plants, but only somewhat. In our opinion, a legislation whose risk assessment gives more weight to obtained products and their specific uses than to processes alone would be closer to the objectives expressed by the EU Council, namely, to achieve a "*clearer, evidence-based, enforceable, proportionate and sufficiently flexible regulatory framework*".

Nevertheless, the legislation in the pipeline *remains promising* because it seems that the EU has come to this point with some lessons learned. The GMO regulatory space has proven to be dysfunctional in many respects and the MS today continue to express conflicting views. History, as an invisible guest, is very much present in this scenario, and as explained above, a holistic review of all rules applying to plant breeding would have been unthinkable. As an alternative, the EC has made a very important effort to put on the table what should be on the table and exclude what should not. The goal has been to avoid competing interests that, while part of the long journey from farm to fork, can only be extrinsically related to plant breeding decisions. Seen in comparison to how the regulatory framework for GMOs has evolved, the merit of the EC in finding its own space for genome-edited plants should not be minimised.

This short-term future is, however, only promising to a certain extent, because in our opinion the proposed legislation will not be the best possible, even if it is passed. In terms of comparison with the other possible futures (or future *by default*, see above 2 and 3) it is certainly an acceptable option. But it should not be overlooked that in itself, the legislation underway pays the price of being overly referenced to or constrained by the influence of the GMO regulatory framework. This being the case, it is virtually impossible to shield the regulation of genome edited plants from the deep issues that have surrounded GMOs (see 1, especially a, b and c). This is particularly evident in the approach to risk assessment (see 5), although it is likely to be felt in other aspects of regulation.

The best approach for this specific legislation would have been to act as if there were a holistic framework for plant breeding and as if we had to fit genome-edited plants into that puzzle. In that way, flaws in other pieces of the puzzle, in particular transgenics, would not have unduly influenced this legislative development.

In any case, with the end of the current parliamentary term imminent, it is unlikely that the approach will be redirected. We must therefore start working now for the post-2024 future. Let us hope that the MS do not close this legislation but that work continues in the medium term to ensure a regulatory framework for European plant breeding that, while adequately protecting the environment and human health, offers plant breeders legal certainty to remain and develop in the EU.

6 Legal provisions cited in the text

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Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed, OJ L 268, 18.10.2003, p. 1–23.

Regulation (EC) No 1830/2003 of the European Parliament and of the Council concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified: Official Journal L 268, 18/10/2003.

Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods: OJ L 327, 11.12.2015, p. 1–22.

7 EU MS lists, by date

List 1. EEC founding Members. France, West Germany, Italy, the Netherlands, Belgium and Luxembourg.

List 2. EEC members, late 1980s: Belgium, Denmark, France, Germany, Greece, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain and United Kingdom.

List 3. EU members 2001. Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, Netherlands, Portugal, Spain, Sweden, and the United Kingdom.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

LE, IF, AL, and JU conceived and designed the study and the structure of the manuscript, as well as the basis for each of its sections. LE wrote the first draft of the manuscript. LE, IF, AL, and JU wrote sections of the advanced manuscript. PP reviewed in particular the technical sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A comparison of three different delivery methods for achieving CRISPR/Cas9 mediated genome editing in *Cichorium intybus* L.

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Root chicory (*Cichorium intybus* L. var. *sativum*) is used to extract inulin, a fructose polymer used as a natural sweetener and prebiotic. However, bitter tasting sesquiterpene lactones, giving chicory its known flavour, need to be removed during inulin extraction. To avoid this extraction and associated costs, recently chicory variants with a lower sesquiterpene lactone content were created by inactivating the four copies of the germacrene A synthase gene (*CiGAS-S1, -S2, -S3, -L*) which encode the enzyme initiating bitter sesquiterpene lactone biosynthesis in chicory. In this study, different delivery methods for CRISPR/Cas9 reagents have been compared regarding their efficiency to induce mutations in the CiGAS genes, the frequency of off-target mutations as well as their environmental and economic impacts. CRISPR/Cas9 reagents were delivered by Agrobacterium-mediated stable transformation or transient delivery by plasmid or preassembled ribonucleic complexes (RNPs) using the same sgRNA. All methods used lead to a high number of INDEL mutations within the *CiGAS*-S1 and *CiGAS*-S2 genes, which match the used sgRNA perfectly; additionally, the *CiGAS*-S3 and *CiGAS*-L genes, which have a single mismatch with the sgRNA, were mutated but with a lower mutation efficiency. While using both RNPs and plasmids delivery resulted in biallelic, heterozygous or homozygous mutations, plasmid delivery resulted in 30% of unwanted integration of plasmid fragments in the genome. Plants transformed *via* Agrobacteria often showed chimerism and a mixture of *CiGAS* genotypes. This genetic mosaic becomes more diverse when plants were grown over a prolonged period. While the genotype of the on-targets varied between the transient and stable delivery methods, no off-target activity in six identified potential off-targets with two to four mismatches was found. The environmental impacts (greenhouse gas (GHG) emissions and primary energy demand) of the methods are highly dependent on their individual electricity demand. From an economic view - like for most research and development

activities - employment and value-added multiplier effects are high; particularly when compared to industrial or manufacturing processes. Considering all aspects, we conclude that using RNPs is the most suitable method for genome editing in chicory since it led to a high efficiency of editing, no off-target mutations, non-transgenic plants with no risk of unwanted integration of plasmid DNA and without needed segregation of transgenes.

KEYWORDS

chicory, genome editing, CRISPR/Cas9, RNPs, protoplasts, germacrene A synthase, socio-economic impacts, environmental impacts

1 Introduction

Chicory (*Cichorium intybus* L.) is a horticultural plant belonging to the family Asteraceae whose cultivars are classically divided in three main groups: leaf chicory, witloof and root chicory (Raulier et al., 2016). Perhaps the most characteristic feature of the plant is its bitterness which is caused by the presence of sesquiterpene lactones, a class of compounds that most likely evolved as a defense mechanism against herbivores (Huber et al., 2016; Padilla-Gonzalez et al., 2016). All the bitter sesquiterpenes lactones (STLs) in chicory (lactucin, lactucopicrin and 8-deoxylactucin and their oxalate derivatives) belong to the class of guaianolides and ultimately derive from germacrene A (de Kraker et al., 1998; Chadwick et al., 2013). While the presence of bitter-tasting compounds is a desirable trait in leaf chicory (e.g. Italian radicchio), these compounds need to be removed during processing in root chicory (*C. intybus* var. *sativum*) because the plant is cultivated for the industrial purpose of extracting inulin, a fructose polymer used as a natural sweetener and a prebiotic (Van Laere and Van den Ende, 2002). Despite the natural variation that exists in the STLs content in the chicory germplasm (Ferioli et al., 2015), to our knowledge there is no industrial chicory variety reported having a null content of bitter tasting compounds. Hence, there is the need for the development of new varieties with a low or null content of STLs, as it would simplify the inulin purification process, making it also more sustainable.

Unfortunately, chicory is characterized by a strong sporophytic self-incompatibility, so it is very hard to obtain highly homozygous or inbred parental lines and produce new hybrids *via* classical breeding strategies (Barcaccia et al., 2016).

In this regard, New Genomic Techniques (NGTs) like CRISPR/Cas9 based genome editing have the potential to accelerate the breeding process, ensuring the preservation of elite varieties genotypes while still causing targeted genetic modifications, thus avoiding time consuming crossings (Zhang et al., 2019). In particular, the DNA-free approach (Woo et al., 2015) with the use of ribonucleoproteins (RNPs) looks promising for its potential higher acceptance from the public when compared to genetically modified organisms (Entine et al., 2021), although the public opinion seems to be very influenced by local groups of interest. Recently, the feasibility of genome editing in *C. intybus* was proved by using both protoplast cell culture and classic Agrobacterium-mediated transformation (Bernard et al., 2019; De Bruyn et al., 2020; Cankar et al., 2021). However, these reports used different varieties, different target sequences, and most importantly different delivery methods for CRISPR/Cas9 machinery (plasmid DNA, T-DNA, RNPs), making it difficult to compare the efficiencies and to understand which approach is the most suitable to be applied to chicory breeding. In this work the genes encoding germacrene A synthase (GAS) were edited using the CRISPR/Cas9 approach. The encoded GAS enzymes catalyze the first step in the STL biosynthesis, the conversion of farnesyl pyrophosphate to germacrene A (Bouwmeester et al., 2002). Germacrene A is next oxygenated by cytochrome P450 enzymes germacrene A oxidase (GAO) and costunolide synthase (COS) to form costunolide (Liu et al., 2011). Another key step to convert costunolide to the guaianolide precursor kauniolide in chicory was recently characterized, and it involves the action of three kauniolide synthases (KLS) (Cankar et al., 2022).

CiGAS gene family is an interesting target to study differences in editing characteristics of different methods, because there are four members (CiGAS-S1, -S2, -S3 and -L) that share the same intron/exon structure and have similarities but also small differences in the coding sequence (Bogdanović et al., 2019). In particular, CiGAS-S1 and -S2 share 98% sequence identity between their coding sequences, while CiGAS-S3 has a little lower identity, close to 90% (Bogdanović et al., 2019). CiGAS-L is more divergent, with 72% of identity at the aminoacidic level (Bouwmeester et al., 2002). Mutation of the CiGAS genes was described using plasmid-based and RNP-based transient delivery methods in root chicory (De Bruyn et al., 2020; Cankar et al., 2021), and both methods resulted in the successful elimination of STLs in chicory roots.

In this work, we systematically investigated the suitability of three different delivery methods (*A. tumefaciens*, plasmid and RNPs, Figure 1) using the same target sequence in the same variety (clone "Orchies 37"). For this we first sequenced and *de-novo* assembled the genome of this specific clone and used plants that were generated previously by Cankar et al. (2021) and additionally generated new edited plant lines. With the aim to define the best approach specifically for chicory breeding, we considered aspects related to the delivery, the editing efficiency, but also the off-target rates and the environmental impact to produce the new gene-edited varieties. This was performed by a

life cycle assessment focussing on greenhouse gas emissions and primary energy demand. Additionally, an economic comparison was done between the stable transformation and the RNP-based method, analyzing costs and broader economic impacts by applying a multi-regional input output model to quantify impacts on value added and job creation.

2 Materials and methods

2.1 Genome sequencing and assembly

1.2 g young leaves from one Chicory plant (clone "Orchies 37") grown at Wageningen University and Research were used for high molecular weight DNA extraction using a standard CTAB-based procedure. The extracted DNA was used for Pacbio SMRT bell library preparation without any further initial DNA shearing using SMRT bell template prep kit 2.0 (Pacbio). The resulting library, with a peak insert size of approximately 41 Kb was used for subsequent template DNA polymerase complexing using Binding kit 3.0 and finally loaded for sequencing on a Pacbio Sequel system using 8 SMRT cells. For all SMRT cells data was collected using a 10-hour movie time per cell. The total Pacbio sequence data (~55 Gb) was combined with previously generated data from the same clone (~30.4 Gb). Combined data was used for a *de-novo* assembly by the Assembler Flye v2.5 running on the *in-house* HPC. The obtained assembly was polished twice, first with Racon v 1.3.3 using the reads derived from the 55Gb dataset and then using Arrow v 2.3.3.

In order to further improve the *de-novo* assembly, optical genome mapping data was applied. For this, young leaves from the same Chicory plant were harvested. These leaves were processed to isolate nuclei and finally ultra-high molecular weight DNA molecules following Bionano Prep Plant Tissue DNA Isolation Base Protocol Revision D. This DNA was used for further fluorescent labelling (DLE-1) and staining according to manufacturer's protocol (Bionano Genomics). Labelled DNA was loaded on a single flowcell of one Saphyr chip (G1.2) and analysed on a Bionano Genomics Saphyr platform. Genome map data was used for *de-novo* assembly Using Bionano Solve v3.3 resulting in a consensus map (cmap). Finally, a hybrid assembly approach using Bionano Solve 3.4.1, Bionano Access 1.4.3 software was done to improve the polished Flye Pacbio based assembly. Therefore, the *de-novo* genome map assembly was used for scaffolding purpose and underlying genome map molecules were used for error corrections in conflict regions. Genome coverage analysis was performed by mapping raw Pacbio reads against the assembly with minimap2 v2.17-r941. Heterozygosity was identified by the presence of two peaks at approximately 20x and 40x coverage. There was an extra peak at 200x coverage that most likely contains the chloroplast genome. Next, we performed purging of haplotigs to generate a haploid assembly. The quality of this purged assembly was assessed by BUSCO analysis performed with eudicots_odb10 reference set containing a total of 2,326 BUSCO groups. By combining the sequence-based assembly and the genome mapping data a hybrid assembly "CHIC 2.0" was created consisting of 332 scaffolds with a total size of 1.76 Gb.

2.2 Single guide RNA design

A sgRNA was designed to introduce a frameshift mutation before the sequence coding for the "DDXXD" motif, a key feature of all terpene synthases (Chen et al., 2011) and located in Exon 4. Therefore, the four exon 4 of CiGAS-S1, -S2, -S3 and -L were aligned with ClustalW (Supplementary Figure 6) and the target GGTACTCTATCCCTTATGTA was chosen for being specific of CiGAS-S1 and CiGAS-S2 while having one mismatch in CiGAS-S3 and CiGAS-L, although at different positions.

2.3 Isolation and transfection of chicory protoplasts

Protoplast isolation, transfection and culture was performed as previously described (Yoo et al., 2007; Deryckere et al., 2012; Cankar et al., 2021) with some modifications. Briefly, *in vitro* shoot cultures of *C. intybus* var. *sativum* (clone "Orchies 37") were maintained on MS20 medium (MS salt including vitamins (Duchefa), sucrose 20 g/L, pH 5.7) with 0.7% agar in high plastic jars at 16/8 h photoperiod under 100 µmol m^{-2} s^{-1} PPFD at 24°C. Four young leaves were harvested, rapidly sliced parallel to their long axis and placed in a Petri dish containing 13 ml of P0 liquid medium + 1% w/v Cellulase Onozuka R-10 + 0.3% w/v Macerozyme Onozuka R10. Digestion was carried out at room temperature for 16 h under gentle tilt-shaking, in the dark. The protoplasts were filtered through a 60 µm nylon sieve and then harvested by centrifugation for 4 minutes at 80 x g without brake. Protoplasts were then resuspended in 2 ml of W5 buffer (Yoo et al., 2007) then added to a tube containing 21% w/v sucrose solution: this was then centrifuged for 4 minutes at 90 x g at room temperature. Live protoplasts were then harvested from the interphase, transferred to a fresh tube, and washed with 11 ml of W5. The protoplasts were centrifuged again (4 minutes at 90 x g) and gently resuspended in MMG buffer (Yoo et al., 2007) at a density of 10^6 protoplasts ml^{-1} (checked with a hemocytometer (Biosigma)). 20 µg of Cas9 (Thermofisher) + 20 µg of sgRNA (Thermofisher or Synthego) + 8 µl NEBuffer 3 (NEB) or alternatively 25 µg of plasmid - were mixed with 2.5 x 10^5 protoplasts and an isovolume of PEG solution (400 g/l polyethylene glycol 4000 (Sigma-Aldrich); 0.2 M mannitol; 0.1 M $CaCl_2$) was then added by gentle pipetting. The transfection was allowed to take place for 5 minutes at room temperature followed by the addition of 12 ml of WI buffer (Yoo et al., 2007). The protoplasts were harvested by centrifugation for 4 minutes at 80 x g.

2.4 Plant regeneration from protoplasts

For regeneration both WT and transfected protoplasts were gently resuspended in WI buffer at 0.2 x 10^6 cells ml^{-1}. An equal volume of alginate solution (1.6% w/v sodium alginate; 0.5 M mannitol) was added and gently mixed, and 1 ml of the mixture was layered on a Ca-agar (50 mM $CaCl_2$; 0.4 M mannitol; 1.4% agar) plate (5 cm dish, Thermofisher), forming a disk. The alginate

was allowed to polymerize for 40 minutes and the disk was then transferred to another 5 cm Petri dish containing 5 ml of MC1 medium (Deryckere et al., 2012). After 7 days of culture in the dark at 24°C the medium was replaced with 5 ml of MC2 medium (Deryckere et al., 2012) and the disk was cultured for further 14 days changing the medium another time at day 14 after the embedding. The disk was then cut into strips and transferred to a 9 cm Petri dish with solid B5 medium (Gamborg's B5 salts including vitamins, mannitol 36 g/L, sucrose 10 g/L, glutamine 750 mg/L, Low Melting Agarose PPC (Duchefa) 0.8% (w/v), NAA 0.5 mg/L, BAP 0.5 mg/L, pH 5.75). These were incubated at 24°C in dim light (20 µmol m^{-2} s^{-1} PPFD) for two weeks to form microcalli. For each experiment approximately 200 microcalli were picked with fine tweezers and transferred to solid MS10 plates (MS salt including vitamins, sucrose 10g/L, Low Melting Agarose PPC (Duchefa) 0.8%/w/v), IAA 0.25 mg/L, BAP 0.25 mg/L, pH 5.7) and incubated at 24°C under low light (60 µmol m^{-2} s^{-1} PPFD) until green calli were formed (3 weeks). The green calli were transferred to solid MC3 medium (Deryckere et al., 2012) under full light until shoots were visible. The developing shoots (20-60 per experiment) were then moved and rooted on MS20 medium (pictures of the whole regeneration process are visible in Supplementary Figure 5, upper part).

2.5 Stable transformation

C. intybus clone "Orchies 37" plants were grown under sterile conditions on MS medium containing 30 g/L sucrose and 8 g/L micro agar (pH 5.8) at 25°C, with a 16 h/8 h (light/dark) photoperiod of white fluorescent light at 80 µmol m-2 s-1. *A. tumefaciens* AGL0 strain carrying the proper binary vector (Supplementary Figures 3, 4) was grown overnight at 28°C at 250 rpm in LB medium supplemented with 50 mg/L kanamycine, 50 mg/L rifampicin and 100 µM acetosyringone. The bacterial cells were pelleted and resuspended in MS30T medium (4.4 g/L MS medium, 30 g/L sucrose, 500 mg/L tryptone, pH5.8) supplemented with 100 µM acetosyringone at optical density (OD_{600}) of 0.3. Leaf explants of approximately 0.5 cm^2 from 4 to 6 weeks old plants were immersed for 15 minutes in the bacterial suspension, placed on co-cultivation medium (4.4 g/L MS medium, 30 g/L sucrose, 500 mg/L tryptone, 1 mg/L BAP, 0.1 mg/L IAA, 100 µM acetosyringone, 8% micro agar, pH 5.8) and incubated for 2 days at 25°C under 16/8 h (light/dark) photoperiod with white fluorescent light at 30 µmol m-2 s-1. Next, explants were rinsed with MS30T medium containing 500 mg/L cefotaxime and transferred to regeneration medium (4.4 g/L MS medium, 30 g/L sucrose, 500 mg/L tryptone, 8% micro agar, pH5.8) supplemented with 1 mg/L BAP, 0.1 mg/L IAA, 100 mg/L kanamycin, 250 mg/L cefotaxime and 50 mg/L vancomycin. Explants were incubated for 7 days at 25°C under 16/8 h (light/dark) photoperiod with white fluorescent light at 60 µmol m-2 s-1. Next, the explants were transferred to the regeneration medium containing 1 mg/L kinetin, 0.4 mg/L IAA, 100 mg/L kanamycin, 250 mg/L cefotaxime and 50 mg/L vancomycin and the medium was frequently refreshed to avoid Agrobacterium outgrowth. After 6 weeks kinetin and IAA were omitted from regeneration medium. From two months on shoots were collected and placed on rooting medium (4.4 g/L MS medium, 20 g/L sucrose, 8% micro agar, 50 mg/L kanamycin pH5.8). Pictures of the whole regeneration process are visible in Supplementary Figure 1, lower part.

2.6 Genotyping of the plants and PCR to detect the presence of Cas9 sequence

Genomic DNA was isolated from young leaves using the NucleoSpin Plant II kit (Machery-Nagel) according to the manufacturer's instruction. The exon 4 of CiGAS-S1 and CiGAS-S2 containing the target site was amplified with specific primers (Supplementary Table 2) and overhang Illumina adapters to generate the Illumina library amplicons, which were sequenced on an Illumina MiSeq (PE300) platform (MiSeq ControlSoftware 2.0.5 and Real-Time Analysis Software 1.16.18) as reported by (Quail et al., 2012). The CRISPResso2 pipeline (https://crispresso.pinellolab.partners.org/submission; (Clement et al., 2019)) was used to process the raw paired-end reads and to visualize the mutations profiles.

To detect T-DNA integration in the case of stable transformation, or plasmid integration in the case of transient plasmid delivery, a PCR was performed using genomic DNA as template (100 ng) and the primer pair Cas9wt for (CTTCAGAAAGGACTTCCAATTC) and Cas9wt rev (ATGATCAAGTCCTTCTTCACTT), using PCRBIO Taq Mix Red (PcrBiosystems) according to manufacturer's instructions. A single specific amplicon of 693 bp was obtained in the case of positive signal.

2.7 Off-target analysis

Sequencing primers have been created using Primer3web (version 4.1.0.) (Untergasser et al., 2012), and the reverse primers have been tagged by five nucleotides, in order to pool the amplicons coming from different plants in the flow cell and separate them later in the analysis. Possible hybridisation between tag-combinations within the sample pools has been checked using the open-source software package "edittag" (Faircloth and Glenn, 2012). The short *Ci*GAS genes S1 and S2 have been amplified by nested PCR or from genomic DNA directly, whereas S3 and the long *Ci*GAS-L gene were amplified directly *via* PCR. Amplification took place in a mixture containing 0.5 U Phusion™ High-Fidelity DNA Polymerase; 0.2 µM dNTPs; 0.4 µM forward and tagged reverse primer in 10 µL 5x High-Fidelity buffer with added ddH_2O up to 50 µL. Initial heating was performed at 98°C for 30 sec., followed by dehybridisation at 98°C for 30 sec., annealing at 60°C for 30 sec., and elongation at 72°C for 30 sec., all but the initial heating was repeated 30 times, followed by a final elongation at 72°C for four minutes. The PCR mixture was kept at 4°C until verification of the correct amplification took place on a 2% TAE agarose gel, containing 0.005% Midori green Advance (NIPPON Genetics EUROPE GmbH). Then 45 µL of the PCR mixture was purified by column (Thermo Fisher Scientfic) and its DNA content measured *via*

NanoDrop (Thermo Fisher Scientific) and normalized to 20 ng/µL. The samples have been pooled by four plants each by combining 10 µL of four single samples and send to Genewiz/Azenta (Leipzig, Germany) for Amplicon-EZ (150-500 bp). The raw data was analyzed using the Galaxy JKI server. Paired reads were adjusted to the correct orientation using the forward primer. The quality trimming was done by Trim Galore! (Galaxy Version 0.6.3.), with the Phred Quality Score set between 30 to 35 and the minimum length set to 50. After trimming, the data set was split according to their tags using the Sabre tool (version 1.000). The tool usearch (v11.0.667_i86linux32) (Edgar et al., 2011) was then used to merge the two read pairs into one sequence. When merging forward and reverse read, up to five mismatches were allowed due to large overlaps between the reads. The merged datasets have been transformed into FASTA files, dereplicated and counted using vsearch (version 2.8.3) (Rognes et al., 2016). Then, only one representant of the identical sequence was mapped against the wild type sequences *via* BWA-MEM (Li and Durbin, 2010). The result was sorted according to the number of identical sequences. The output was aligned in the CLC Main Workbench 22.0 (QIAGEN Aarhus A/S). Spiked fastq files including six artificial mutated reads have been use as a positive control to verify the thoroughness of the workflow for finding off-target events. Additionally, sequences showing a variant in comparison to the wild type that were supported by at least 20 reads have been examined. For this purpose, individual Phred Quality Scores of the merged reads were collected and visualized as boxplot and median per position. If a drop coincides with a variant position, this variant was flagged as questionable.

2.8 Environmental assessment

Life Cycle assessment (LCA) defined in the International Standards ISO 14040 is a method to compile and assess the input and output flows as well as the potential environmental impacts of a product system during the various stages of its life cycle. The stages include extraction of raw materials, manufacturing, distribution, product use, recycling and final disposal (from cradle to grave/gate) (ISO 14040). A "cradle to gate" LCA was applied, and the impact focused on was greenhouse gas (GHG) emissions and primary energy demand. The functional unit is one experiment consisting of three cycles to gain one prototype of the Chicory variant with the desired characteristics. For the assessment the operational phase with all the needed inputs of the molecular breeding steps is included in the system boundary, the construction and end of life of the laboratories or machinery are not included. For the assessment of the contribution of the GHG emissions, the global warming potential on 100-year time horizon (GWP 100) was used. The GHG - CO_2, CH_4, N_2O - were expressed in terms of equivalent amount of CO_2 (CO_2-eq). Therefore, the CO_2-eq factors are taken from (Myhre et al., 2013) using the factors including climate carbon feedback. Direct and indirect emissions are included in the assessment. The cumulated primary energy demand includes the

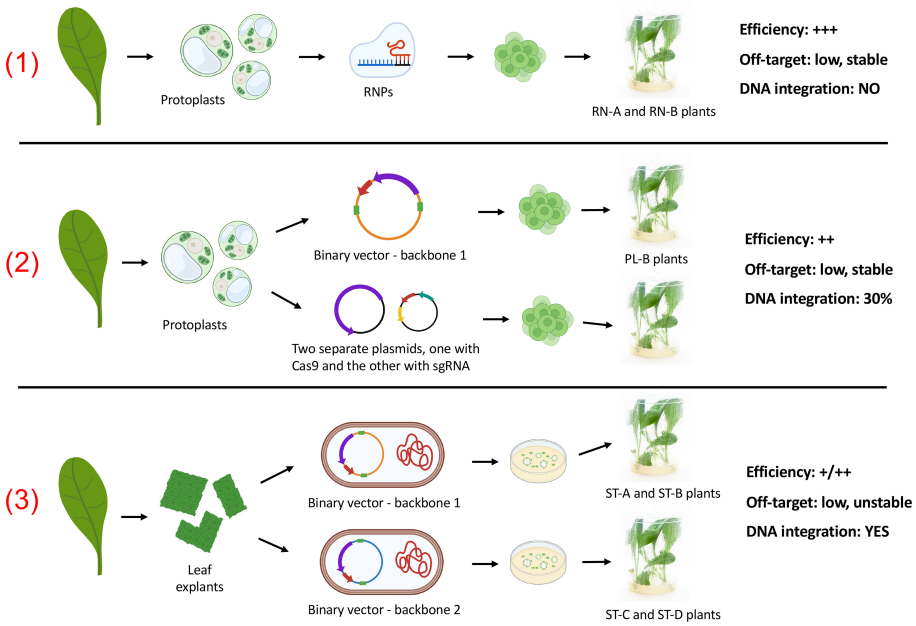

FIGURE 1
Scheme of the three CRISPR/Cas9 delivery methods as they are referred to in the main text and summary of their main outcome. The nomenclature adopted for the resulting plants is shown. Method 1: Stable integration of T-DNA containing CRISPR/Cas9 components, using two binary vectors with a different backbone. Method 2: transient expression of CRISPR/Cas9 components after protoplast transfection with one or two plasmids. Method 3: transient transfection of protoplasts directly with RNPs (DNA-free method). The purple arrow in the plasmids indicates Cas9 gene, the red arrow indicates the common guide RNA, and the green blocks indicate the Right and Left Borders (to highlight which vectors were binary and which were not).

total energy demand (fossil, renewable and other primary energy demand) of all process steps of the life cycle analysis. Two types of data are used in the LCA calculation - namely foreground and background data. Foreground data was mainly based on information collected from the laboratory work (mainly materials and energy demand for the molecular breeding technologies), while background data for materials, fuels and transport was mainly gathered from the database Ecoinvent 3.7.1 (Wernet et al., 2016). Information on the electricity mix for the EU28 mix is drawn from European Commission (2020).

2.9 Economic assessment

Multi-regional input-output (MRIO) analysis is conducted to quantify costs and broader economic impacts of the breeding methods. MRIO analysis is based on Leontief (Leontief, 1970) and relies on a set of linear equations. Starting from the basic balance of the input-output table, a series of equations can be deduced for the economic impact calculations. The total output can be expressed as Eq. 1 (in order to enhance readability and clarity for the reader we abstract from a sector disaggregation):

$$\begin{bmatrix} X_1 \\ X_2 \\ \vdots \\ X_R \end{bmatrix} = \begin{bmatrix} A_{11} & A_{12} & \cdots & A_{1R} \\ A_{21} & A_{21} & \cdots & A_{2R} \\ \vdots & \vdots & \ddots & \vdots \\ A_{R1} & A_{R2} & \cdots & A_{RR} \end{bmatrix} * \begin{bmatrix} X_1 \\ X_2 \\ \vdots \\ X_R \end{bmatrix} + \begin{bmatrix} Y_1 \\ Y_2 \\ \vdots \\ Y_R \end{bmatrix} \quad (1)$$

where X_i denotes the column vector of total output by economic sector in country i ($i,j \in R$), A is a coefficient matrix describing input per output ratios in the production of these sectors with A_{ij} denoting inputs from sectors in country i required to produce one unit of output from each sector in country j. Y_i is a column vector of total final demand for the output of country i. This equation can be transformed into $X=(I\text{-}A)^{-1}\ Y$, where $(I\text{-}A)^{-1}$ describes the inverse Leontief matrix, which captures all the direct and indirect links between the different economic sectors as well as regions and thus enumerates the total impacts across global supply chains. The Leontief matrix can then be extended by a row-vector of economic, social and environmental coefficients q (so-called satellite accounts) in order to illustrate how these indicators are distributed over sectors and countries. Thereby $q=[q^1,q^2, ,q^R]$ is a row vector of the respective coefficients by sector and region denoting the physical (e.g. number of jobs, land in m^2, water in m^3) or monetary (labor and value-added in USD) units per unit of output. This row vector of coefficients times the total output needed to meet any final demand yields embodied economic, social and environmental indicators, Q, in final demand ($Q=q\ *\ (I\text{-}A)^{-1}\ Y$; Q is a R x R matrix) (Gasim, 2015). The MRIO model is calibrated to the EXIOBASE data (Wood et al., 2015; Stadler et al., 2018) of 2019. The model comprises 163 industries and covers 44 countries and five rest of the world regions by continents. Multipliers were defined as values that quantify the economic impacts derived from a perturbation on the system. These include the direct consequence caused by the initial effects as well as the indirect ripples of the total effects on the economy (Miller and Blair, 2009). The multiplier effect was calculated as the ratio between the total economic impact estimated and the amount of money directly spent in the development of both methods in terms of goods and services.

3 Results

The target sequence to design the sgRNA common to all three delivery methods was intentionally chosen with perfect match in CiGAS-S1 and CiGAS-S2 and one mismatch in CiGAS-S3 and CiGAS-L (Figure 2): in this way, despite their role in germacrene A biosynthesis, CiGAS-S3 and CiGAS-L can also be considered as off-targets due to the singe mismatch. Notably, the position of the single mismatch differs in the two guides, being very close to PAM in CiGAS-S3 and in the middle of the sequence in CiGAS-L.

3.1 Genome assembly

In total 86 Gigabases of Pacbio long read sequencing data was produced and used for initial assembly, polishing and purging. The resulting purged assembly encompasses a total size of approximately 1249 Megabases consisting of 8773 contigs, with a N50 contig size of 469 Kb and L50 of 741 fragments. Additional purged haplotigs contained 303 Mb spanning sequences consisting of 8340 contigs. BUSCO analysis of the assembly before purging showed 94.7% BUSCO score for the initial assembly, consisting of 1418 complete single copy BUSCO and 785 complete and duplicated BUSCOs. In contrast, the purged assembly showed 94% BUSCO score, consisting of 1,823 complete single copy BUSCO and 364 complete and duplicated BUSCOs. The purging of the assembly significantly reduced the assembly size as well the BUSCO duplication rates of the initial assembly, but not the total BUSCO score suggesting. This, together with the comparison to the public *C. intybus* assembly GCA_023525715.1 (Supplementary Figure 2) suggest that the purged assembly is a good representation of the haploid genome of Chicory clone "Orchies 37". *De-novo* assembly of the genome mapping resulted in a total

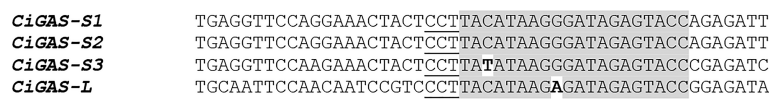

FIGURE 2
Alignments of the genomic sequences of the four CiGAS genes at the target site in exon 4. The target region of the sgRNA is highlighted in grey. The PAM is underlined, and the mismatches on CiGAS-S3 and CiGAS-L are in bold.

assembly consensus map of approximately 2.5 Gb representing the diploid genome. The subsequent hybrid and final assembly CHIC v 2.0 consisting of 332 scaffolds with a N50 length of 9.81 Mb and a total size of 1.76 Gb. All statistics of the different genome assembly steps and final genome assembly are provided in supplementary data (Supplementary Table 4 and Supplementary Figures 1, 2).

3.2 Method1: transient RNPs delivery and plant regeneration

For the DNA-free method, two types of chemically synthetized sgRNA molecules were used: 1) normal and 2) with both ends containing three phosphorothioate linkages and 2'-O-methyl RNA modifications. The last type is referred to as protected sgRNA and it is known to resist to endogenous nucleases, thus having the potential to increase its lifetime in the plant cell and its editing efficiency (O'Reilly et al., 2019; Allen et al., 2021). The mutation efficiency was first determined in a transient assay in a protoplast population, harvesting the genomic DNA 48 hours after transfection with 20 µg of Cas9 and sgRNA and then using amplicons on CiGAS-S1 and S2 genes for Illumina sequencing. The percentage of mutated reads on the total was of 52% for CiGAS-S1 and 54% for CiGAS-S2 (Supplementary Figure 7) with the normal sgRNA, and 38% for both genes with the modified version. In a parallel experiment, protoplasts were not sacrificed for the sequencing and were cultivated *in vitro* until plants were regenerated: these plants were genotyped as well by deep sequencing of the CiGAS genes. In total, 31 plants from the transformation with the normal sgRNA and 18 plants from the transformation with the modified guide were analysed (Table 1). It is important to note that since Chicory is a diploid species, in case of a monoallelic mutation (indel) it is expected that on the total amount of the Illumina reads, half is wild type, and the other half is coherently mutated. In the case of biallelic mutants, no wild type reads should be observed, with a unique mutation profile in the case of homozygosity or two equally represented in the case of heterozygosity. The absence of other genotypes in the sequenced sample indicates that the plant is not chimeric and that it derived from a single cell: indeed, this was the case for nearly all the plants generated by the RNP approach, and the deviating sequence reads with a very low frequency (i.e. less than 0.01%) of alternative

TABLE 1 CiGAS genotype of the plants regenerated from protoplasts transfected with RNPs.

Plant #	Modified sgRNA	CiGAS-S1	CiGAS-S2	CiGAS-S3	CiGAS-L
RN-A1	Yes	WT/WT	WT/WT	WT/WT	WT/WT
RN-A2	Yes	WT/WT	WT/WT	WT/WT	WT/WT
RN-A3	Yes	WT/WT	WT/WT	WT/WT	WT/WT
RN-A4	Yes	-5/-11	-9/-9	-7/ins.	WT/WT
RN-A5	Yes	-12/-9	WT/WT	WT/WT	WT/WT
RN-A6	Yes	-19/-4	WT/WT	-4/WT	WT/WT
RN-A7	Yes	-4/-17	-11/-11	-2/-7	WT/WT
RN-A8	Yes	-14/-7	-13/-10	-2/WT	WT/WT
RN-A9	Yes	-9/-9	-10/WT	-4/-4	WT/WT
RN-A10	Yes	-3/-5	-6/-3	-7/WT	WT/WT
RN-A11	Yes	-7/-7	-5/-5	-2/-2	WT/WT
RN-A12	Yes	-4/-7	+1/-7	-7/-16	WT/WT
RN-A13	Yes	-11/-11	-12/-6	-7/-9	WT/WT
RN-A14	Yes	-7/-7	-9/-7	-3/-3	-11/WT
RN-A15	Yes	WT/-16	WT/WT	WT/-5	WT/WT
RN-A16	Yes	-2/-11	-2/-11	-7/-9	-7/-16
RN-A17	Yes	-6/-5	-10/-6	-11/+1	-8/-9
RN-A18	Yes	-12/-12	-7/-7	-18/-11	-7/-6
RN-B1	No	-3/-5	-16	-9	WT/WT
RN-B2	No	-3/-9	-6/-7	-11/-13	WT/WT
RN-B3	No	-3/-9	-6/-7	-11/-13	WT/WT

(Continued)

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TABLE 1 Continued

Plant #	Modified sgRNA	CiGAS-S1	CiGAS-S2	CiGAS-S3	CiGAS-L
RN-B4	No	-3/-5	-16	-9	WT/WT
RN-B5	No	-3	+1	-3/-5	WT/WT
RN-B6	No	-11	-4/-1	+1	WT/WT
RN-B7	No	-3/-9	-6/-7	-11/-13	WT/WT
RN-B8	No	-3/-9	-6/-7	-11/-13	WT/WT
RN-B9	No	-3/-5	-16	-9	WT/WT
RN-B10	No	-3/-5	-16	-9	WT/WT
RN-B11	No	-3/-5	-16	-9	WT/WT
RN-B12	No	-16/-3	-11/-6	+1/WT	WT/WT
RN-B13	No	-16/-3	-11/-6	+1/WT	WT/WT
RN-B14	No	-11/-6	-14/-7	-9/WT	WT/WT
RN-B15	No	-11/-6	-14/-7	-9/WT	WT/WT
RN-B16	No	-9/-3	-6/-7	-13/-11	WT/WT
RN-B17	No	-9/-3	-6/-7	-13/-11	WT/WT
RN-B18	No	-3	-16/-7	-3	-9/WT
RN-B19	No	-5/-3	-16	-9	WT/WT
RN-B20	No	-5/-3	-16	-9	WT/WT
RN-B21	No	WT/WT	WT/WT	-8	-7/WT
RN-B22	No	-9	-4/-11	-11/-16	WT/WT
RN-B23	No	+1/-3	-4/-4	-6/-3	-7/WT
RN-B24	No	-3	+1	-3/-5	WT/WT
RN-B25	No	WT/WT	WT/WT	WT/WT	WT/WT
RN-B26	No	-7/-9	-2	-6	WT/WT
RN-B27	No	-9/-7	-9/-1	-14/-15	WT/WT
RN-B28	No	WT/WT	-3	WT/WT	-9/WT
RN-B29	No	-12/-3	-3	-3	WT/WT
RN-B30	No	-4/-11	-16/+1	-3/-26	WT/WT
RN-B31	No	-4/-5	-5/-3	-2/-4	WT/WT

Light grey colour indicates cases where no editing occurred. Medium grey colour indicates out of frame biallelic or homozygous mutations resulting in inactivation of the enzyme. No colour indicates either plants with in-frame deletions or plants in which the presence of one WT allele was detected.

genotypes could be explained by technical sequencing errors. Overall, the frequency of mutated alleles in CiGAS-S1 and -S2 which perfectly match with the guide RNA was 68% in the case of modified sgRNA and 92% in the case of the standard guide RNA, with 42% and 49% of true biallelic or monoallelic loss-of-function mutants (knockouts), respectively. In the case of CiGAS-S3 and CIGAS-L (only one mismatch with the guide) the frequency was lower compared to CiGAS-S1 and CiGAS-S2, with 47% of edited alleles (22% loss-of-function) in the case of the standard guide, and 43% of edited alleles (31% loss-of-function) in the case of the modified one. The position of the mismatch differed between CiGAS-S3 and -L (Supplementary Figure 1): surprisingly, the highest editing rate (87% of the alleles with standard guide, 66% with modified) was obtained in CiGAS-S3 where the mismatch is in the seed region, while in CiGAS-L, where the mismatch is in the middle of the target sequence, only 6.5% of the alleles were edited (21% with modified guide RNA).

3.3 Method 2: transient plasmid delivery and plant regeneration

For the DNA-based transient expression, two systems were used where sgRNA and Cas9 cassettes were in the same construct (single plasmid approach) or expressed from two independent plasmids (double plasmid approach). In the first strategy the same binary

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vector used in Method 3 for *Agrobacterium*-mediated transformation was exploited: the plasmid (Supplementary Figure 4) was 16.7 kb in size and contained in the T-DNA part the sgRNA under the control of an extended *A. thaliana* U6 promoter and a SpCas9 ORF with N- and C-terminal NLS (SV40) under the control of *A. thaliana* ubiquitin 10 promoter. In the second strategy, three guides hybridising to the same target sequence as the guide used in Method 1 was used, which was adjusted to have no mismatches to the different CiGAS genes. The guides were expressed under the control of the *A. thaliana* minimal U6 promoter and cloned into pMK-RQ vector (GeneArt). For expression of the *Sp*Cas9 protein, a coding sequence codon-optimized for *A. thaliana* with a C terminus NLS was used under the control of the parsley constitutive ubiquitin promoter. Protoplasts transfection efficiency was assessed in a transient assay in a protoplast population, using 20 µg of a plasmid bearing a fluorescent reporter cassette: fluorescent signal was detected in 58 ± 6% of the cells after 24 hours. As with method 1, the editing efficiency was evaluated through deep Illumina sequencing on CiGAS amplicons: in total, we analyzed 7 plants from the transient transformation with the binary vector and 9 plants from the transformation with the double plasmid system (Table 2). Overall, the frequency of mutated alleles in CiGAS-S1 and -S2 was 53% in the case of the single plasmid and 50% in the case of the double plasmid, with 14% and 39% of loss-of-function alleles, respectively. The single plasmid strategy in CiGAS-S3 and -L mirrored the RNP approach: both targets had the same mismatch in the same position, and in fact the editing results turned out to be similar, although at lower frequencies compared to RNPs. In particular, CiGAS-S3 maintained a high frequency of mutation (78% with 50% of loss-of-function) while CiGAS-L showed almost no editing (7%). The case of the double plasmid delivery (where the three guides were used with no mismatch in any of the genes) gave a very consistent output, with an average of 60% of edited alleles in each locus, whose majority were loss-of-function. An event known to occur when performing a transient plasmid delivery is the potential integration of fragments of plasmid DNA into the genome of the plant, which then results being transgenic and thus with heavier regulatory implications in many countries (Entine et al., 2021). Although the frequency might vary between species and it is generally considered to be low, however it was recently reported in Chicory to occur relatively frequently (Bernard et al., 2019). To assess the level of plasmid integration in the plants we performed a PCR on the SpCas9 ORF, which was present in 30% of the lines, proving that significant foreign DNA integration had indeed occurred.

3.4 Method 3: Stable T-DNA integration and plant regeneration from leaf explants

For the stable integration, two different binary vector backbones were used: one derived from pICSL4723 (Weber et al., 2011) was called backbone 1 (Supplementary Figure 3), and another one derived from pPZP (Karimi et al., 2002) called backbone 2 (Supplementary Figure 4). Four independent transformations were performed, and a fraction of all the regenerants (Cas9-positive by PCR) from all four regeneration experiments were

TABLE 2 CiGAS genotype of the plants regenerated from protoplasts transfected with plasmid.

Plant #	plasmid	CiGAS-S1	CiGAS-S2	CiGAS-S3	CiGAS-L
PL-A1	Double	WT/WT	WT/WT	WT/WT	WT/WT
PL-A2	Double	WT/WT	WT/WT	WT/WT	WT/WT
PL-A3	Double	WT/WT	WT/WT	WT/WT	WT/WT
PL-A4	Double	WT/WT	WT/WT	WT/WT	WT/WT
PL-A5	Double	-9/-12	WT/WT	-2/-9	-6/-6
PL-A6	Double	-1/-1	-4/-4	-2/-11	-1/-5
PL-A7	Double	-3/-3	-2/-2	-4/-4	-5/-5
PL-A8	Double	-13/-13	-11/-11	-7/-7	-2/-2
PL-A9	Double	-7/-8	-4/-4	-9/-7	-4/-5
PL-B1	Single	-20/WT	-9/-9	-10/-10	WT/WT
PL-B2	Single	WT/WT	WT/WT	-4	WT/WT
PL-B3	Single	-9	-7/complex	-5/-8	WT/WT
PL-B4	Single	WT/-9	-8/WT	WT/-5	WT/WT
PL-B5	Single	WT/WT	WT/WT	-13/WT	WT/WT
PL-B6	Single	WT/-14	-8/WT	-9/WT	WT/-8
PL-B7	Single	-9/-5	-7/-4	-9/-4	WT/WT

Light grey colour indicates cases where no editing occurred. Medium grey colour indicates out of frame biallelic or homozygous mutations resulting in inactivation of the enzyme. No colour indicates either plants with in-frame deletions or plants in which the presence of one WT allele was detected.

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screened by deep amplicon sequencing of all CiGAS genes as in the previous methods (Table 3). Chimerism was common in all of the different transformations, indicated by the fact that more than two alleles were detectable for each locus in most of the cases. When only two alleles were present, they were rarely in a 50:50 ratio. Since transgenic lines have stably integrated CAS9 gene, ongoing Cas9 activity is to be expected during the whole lifetime of the plants, possibly causing a change in the mutation profile of *CiGAS* genes during plant development. Therefore, three independent transgenic lines were selected and sampled at two time points: 5 months after the transformation and 23 months later after continuous *in vitro* propagation. The amount of wild type reads in the sequencing detectable at the beginning decreased (Figure 3) and the amount of new occurring indel mutations increased in all plants indeed indicating that the genes coding for Cas9 and the sgRNA had not been silenced in the timeframe considered, producing active CRISPR complexes that could bind to the targets and achieve new mutations.

TABLE 3 CiGAS genotypes of transgenic plants 5 months after agrobacterium-mediated transformation.

Plant #	Trasf.	Plasmid	CiGAS-S1	CiGAS-S2	CiGAS-S3	CiGAS-L
ST-A1	1	Backbone1	+1/-214/-6	-21/-7/WT	-4/+1/WT	WT
ST-A2	1	Backbone1	WT/+1/-7	WT/-8/-6	WT/+1/-9	WT
ST-A3	1	Backbone1	WT/-5	WT	WT	WT
ST-B1	2	Backbone1	-7/-6/+1/*	-14/+1/-3	-14/+1/WT	WT
ST-B2	2	Backbone1	+1/WT	-16/-11/+1	-16/-7/-4	WT
ST-B3	2	Backbone1	-4/-10/+1	+1/-4	WT/+1/-2	WT
ST-B4	2	Backbone1	-20/-4/+1	+6/+17	-2/+1	WT
ST-B5	2	Backbone1	WT/-11/-5	-9/-88/WT	-21/WT/-11	WT
ST-B6	2	Backbone1	+1/-4	-2/+7/+1	WT/-6/+1	WT
ST-B7	2	Backbone1	-20/-4	-3/-10	-6/-20	WT
ST-B8	2	Backbone1	-11/-15/+1	+1/-6/-7	-52/-7/-10	WT
ST-B9	2	Backbone1	-7/+1	-6/-9	-2/WT/+1	WT
ST-B10	2	Backbone1	+1/-1	-9/+1	+1/-2/-6	WT
ST-C1	3	Backbone2	WT	WT	WT	WT
ST-C2	3	Backbone2	-7/-5	-9/-7/-6	+1/-11	WT
ST-C3	3	Backbone2	-7/-5	-9/-6/+1	-11/+1	WT
ST-C4	3	Backbone2	-7/-5	-9/WT/-6	+1/-11	WT
ST-C5	3	Backbone2	-7/WT/-6	-6/-2	WT/-6/+1	WT
ST-C6	3	Backbone2	-7/-6	-11/-2/WT	WT/+1/-9	WT
ST-C7	3	Backbone2	WT	WT	WT	WT
ST-C8	3	Backbone2	-27/WT	-27/WT	WT/-6	WT
ST-C9	3	Backbone2	WT	WT/-2	WT	WT
ST-C10	3	Backbone2	-4/WT/-11	-6/+1	WT/+1	WT
ST-C11	3	Backbone2	-4/WT/-5	-6/+1	WT/+1	WT
ST-D1	4	Backbone1	-7/WT/+1	-6/-7/WT	WT/-2/+1	WT
ST-D2	4	Backbone1	-6/WT/-7	WT/-26/-11	WT/-5/+1	WT
ST-D3	4	Backbone1	+1/WT/-7	-6/+1/WT	WT/+1/-9	WT
ST-D4	4	Backbone1	WT/+1/-9	WT/+1/-6	-11/WT/-3	WT
ST-D5	4	Backbone1	WT/+1/-3	-2/WT/+1	-11/WT/-6	WT
ST-D6	4	Backbone1	WT/-4/+1	WT/+1/-4	WT/-9/-7	WT

The mutations are shown in decreasing order or reads count. For visual purpose, the threshold for a genotype to be displayed in the table was set to 3% of total reads, with the only exception of the locus CiGAS-S1 of line ST-B1 (indicated with *). Light grey colour indicates cases where no editing occurred. Medium grey colour indicates complete loss-of-function. No colour indicates editing which cannot be considered for sure loss-of-function because the indel occurred in multiples of 3bp, or because WT reads are still detectable.

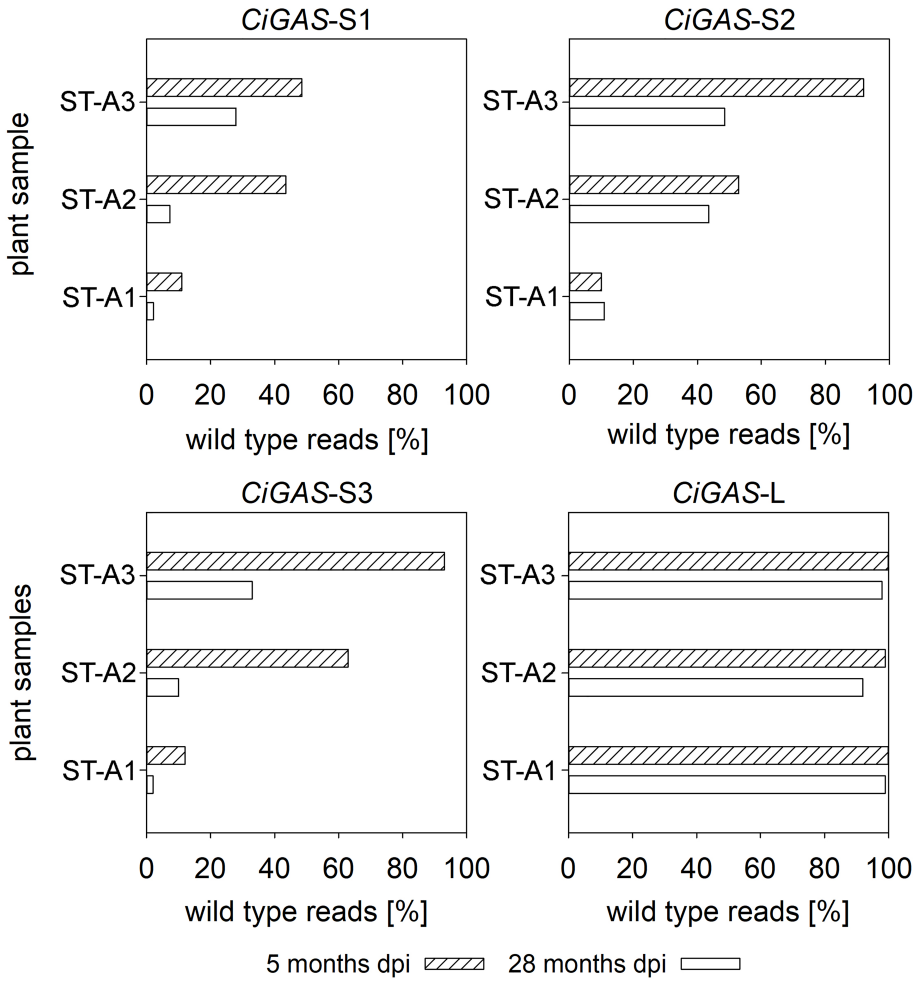

FIGURE 3
Change of wild type reads in transgenic chicory plants over two years. DNA was isolated five months and 28 months after transformation. All three plants show a decrease in their percentage of wild type sequences in NGS approach.

3.5 Off-target analysis

Even though the mutation frequency in *CiGAS*-S3 and -L was lower compared to *CiGAS*-S1 and CiGAS-S2, genome editing in these genes was detected despite single mismatches between the target sequence and the sgRNA. Therefore the improved genome CHIC2.0 was screened by the prediction algorithms CCTop (Stemmer et al., 2015) and CRISPOR (Haeussler et al., 2016) to identify possible off-target sites. While other online tools are available, CCTop and CRISPOR allow working with unpublished genome data on local server. A systematic review found possible off-target activity with four or less mismatches between sgRNA and genomic sequences (Modrzejewski et al., 2020). The screening for off-targets with up to four mismatches between the sgRNA used to mutate the four *Ci*GAS genes and the CHIC2.0 genome identified 18 potential off-targets (Supplementary Table 1). Of those off-targets, two mismatched the sgRNA in two bases, one in three bases and the other putative off-targets sides showed four mismatches to the sgRNA. Those sequences have been annotated by GeMoMa (Gene Model Mapper) (Keilwagen et al., 2016) using RNA-Seq data from Chicory as well as the annotated genomes from *Arabidopsis thaliana* L., soybean (*Glycine max* (L.) Merr.), sunflower (*Helianthus annus* L.) and lettuce (*Lactuca sativa* L.) to find homologies for gene prediction. With high variations within intragenic regions (Intrieri et al., 2007), the off-target search focused on deep analysis of five putative off-targets found in genomic regions, which are more conserved and could result in changes of the plants' phenotype. Additionally, one putative off-target side showed only two mismatches to the target sequence and was therefore included in the deep analysis. Possible off-target activity within those six regions were analysed by deep sequencing in 13 plants treated with RNPs, nine plants transfected *via* plasmid and 18 plants stably transformed by Agrobacterium. In none of these 40 plant lines mutations were detected in these potential off-target sites (Table 4). Even the prolonged exposure over two years to continuously expressed CRISPR/Cas9 within the stably transformed plants showed no off-targets mutations. To assess whether any of the 18 identified potential off-targets is prone to being mutated by the CRISPR/Cas9 delivery, one of the RNP-transfected plants with high mutation efficiency of the target site

TABLE 4 Total number of plants analysed for at least six possible off-targets.

Method	Number of plants	Modified sgRNA	mutated off-targets	Transfection/Transformation efficiency	Editing efficiency
RNP	13	no/yes	0*	n.d.***	80%
Plasmid	9	No	0	n.d.***	51.5%
Stable	18	No	0*	60%****	90%**

*one RNP treated and two transgenic plants were checked in all 18 possible off-target sides.
**being all the plants chimeric, this was calculated as percentage of loci where edited reads could be detected.
***in the case of transient delivery, the transfection efficiency was not determined, but it is at least equal to the editing efficiency.
****calculated as the number of Cas9 positive plants (detected by PCR) on the total number of regenerants.
Given are the methods of transformation and whether modified or unmodified sgRNA was used. For comparison, also the overall efficiencies of editing, transformation and transfection are reported.

was deep sequenced, as well as two stably transformed plants. Also here, no difference between transformed plants and wild type controls could be detected.

3.6 Environmental impacts of the molecular breeding approaches

For the environmental assessment the two molecular breeding technologies showing the largest differences in the frequency of introduction of loss-of-function mutations were used: the RNPs delivery (Method 1) and the stable transformation (Method 3) were analyzed and compared. To assess the environmental impacts the method of an attributional "Life Cycle Assessment (LCA)" was applied according to ISO 14040 considering one experiment with the need of three cycles to gain one prototype of the Chicory variant with the desired genotype to put on the market. All the material and energy demands of the molecular breeding technologies were included in the assessment and translated to GHG emissions and primary energy demand. To cover the electricity demand, the GHG intensity of a European electricity mix was applied. For the molecular breeding technologies an average electricity demand of lighting of 2.56 kWh per day was assumed. In general, only minor differences between the two technologies were found when comparing the greenhouse gas (GHG) emission (Figure 4) and the cumulated primary energy demand. The GHG emissions of the stable transformation method is estimated between 487 and 703 kg CO_2eq per experiment (from here on/e), and for the RNP delivery method between 492 and 710 kg CO_2 eq/e. If we are comparing these values with GHG emissions of a passenger car, we could drive approx. 2,200 to 3,100 passenger car-km/e, assuming 226 g CO_2eq/passenger car-km. If renewable energy would be used to cover the electricity demand the GHG emissions can be reduced significantly by 84% to 88% to approx. 59 to 105 kg CO_2eq/e in the case of stable transformation and to approx. 65 to 112 kg CO_2eq/e in the case of RNP delivery. The cumulated primary energy demand of stable transformation is estimated between approx. 2,900 and 4,250 kWh/e, of the RNP delivery between approx. 2,940 and 4,300 kWh/e assuming an EU 28 electricity mix to cover the electricity demand. If renewable energy is used to cover the electricity demand the cumulated primary energy demand can be reduced significantly by 41% to 44%. The electricity demand accounts for the biggest share of contributions to the GHG emissions and the primary energy demand followed by the contribution of plastic (Figure 4). In fact, with the EU28 electricity mix approx. 95% of the GHG emissions derives from the electricity demand. This value can be reduced to about 66% in the case of stable transformation and to 62% in the case of RNP delivery when using renewable electricity. The share of electricity to the primary energy demand can be lowered from approx. 94% assuming the EU28 electricity mix to a share of 90% in the case of stable transformation and 87% in the case of RNP delivery assuming a renewable electricity mix. Compared to the GHG emissions and primary energy demand of the molecular breeding technologies, the environmental impacts of the cultivation of Chicory variants and the processing to inulin (Hingsamer et al., 2022) are higher as these are reflecting an industrial value chain and a yearly production.

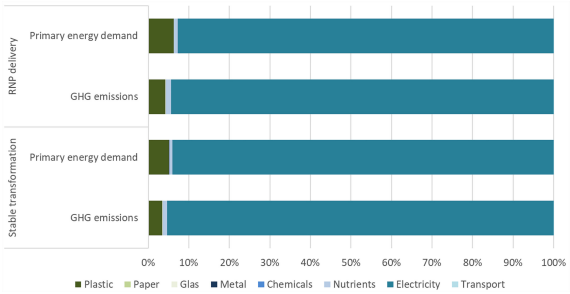

FIGURE 4
Shares of environmental impacts by auxiliaries for stable transformation and RNP delivery.

3.7 Economic impacts of the molecular breeding approaches

Additionally to the environmental assessment, direct and indirect economic impacts were calculated for the two breeding methods, RNP delivery (Method 1) and stable transformation (Method 3). In general, total costs for one experiment with three cycles were similar for both methods (16.7 thousand EUR per experiment for the RNP delivery and 18.4 thousand EUR per experiment for the stable transformation), with most of them concerning direct value-added (depreciation of capital and wages) while costs for material inputs with 20% for stable transformation and 32% for RNP delivery are small. In both methods around a third of total costs denote wages for scientists and other laboratory employees. In terms of material costs, electrical machinery, rubber, plastics and chemicals are the main inputs and cost categories (details are reported in Supplementary Table 3). Due to the similar height of costs, the total economic impacts do not differ much in height (Table 5) but in distribution across industries. In total stable transformation yields 41 thousand EUR of production of goods and services as well as 28 thousand EUR of direct and indirect value-added, while RNP delivery generates 43 thousand EUR of production of goods and services and 25 thousand EUR of direct and indirect value-added. Regarding employment effects, we find that the analyzed methods create around one job (number of persons) in the whole economy. In order to study in detail the nature of the impacts, final demand multipliers were also calculated (Table 5). Output multiplier with 4.1 and 4.2 respectively is similar in both methods, while the generated value-added is slightly higher in the stable transformation method. In particular, for 1 Euro in the development of the stable transformation 2.8 Euro value-added are generated, while the RNP delivery yields 2.4 Euro of value-added. This means, due to its higher labor intensity and slightly higher costs for one experiment the stable transformation has a higher value-added than RNP delivery in the application of the methods. Both methods show a high intensity of research and development intense activities. Due to their high labor intensity, higher employment and value-added multiplier effects compared to industrial or manufacturing processes. For instance, the job and value-added multiplier of the development of genome-editing methods are clearly higher than the respective multiplier of the industrial value chain processes based on the Chicory variants (Hingsamer et al., 2022). However, no conclusions can be drawn from the differences in value-added-multipliers about the efficiency of the methods, since the evaluation is aimed at the costs of experiments carried out.

4 Discussion

Three different CRISPR/Cas9 delivery methods were evaluated for their ability to introduce mutations into the Chicory genome: the use of a common guide RNA ensured that a comparison of all the methods could be made by looking at the efficiencies of the on-target and off-target mutations. The target chosen for the experiments was the small gene family of GAS genes in root Chicory, which is composed by four members (CiGAS-S1, -S2, -S3 and -L). Two of these genes matched perfectly with the designed guide RNA (CiGAS-S1 and -S2) while the other two can be considered as off-targets as they have each one, though different mismatch. Among the list of off-targets (Supplementary Table 1) they were the only ones with a single mismatch. With this particular experimental design it was possible not only to compare the different methods, but also the test the specificity in knocking out specific members of a gene family where the shared sequence homology is high (Bogdanović et al., 2019). Overall, all the methods were successful in the creation of many loss-of-function mutant lines in CiGAS-S1 and -S2, with no detectable off-target mutations in regions with two or more mismatches with the guide RNA. The single mismatch in CiGAS-S3 and -L did lead to editing although surprisingly, the mutation rate was high in -S3 (comparable to S1/S2) where the mismatch is close to the PAM, while it was low in -L where it stands in the middle of the sequence. These findings were consistent in all the three methods, contradict the assumption that mismatches in the seed region of the guide RNA have a higher impact on the editing efficiency (Modrzejewski et al., 2020). The first striking difference observed was between the transient (method 1 and 2) and stable (method 3) delivery: in the first case the genotypes were always well classifiable as mono- or bi-allelic (and in this case hetero or homozygous) (Tables 1, 2), while in the second case the mutation profile was always chimeric to some extent

TABLE 5 Economic effects of the two genome editing methods.

	Stable transformation	RNP delivery
Total impact on		
Production of Good & Services (EUR)	40,900	43,300
Value added (EUR)	27,600	25,100
Jobs (number of persons)	1.14	1.08
Final demand multiplier:		
Output multiplier	4.1	4.2
Value added multiplier	2.8	2.4
Job multiplier	0.072	0.067

Production output, value-added and job generated of the global economy in order to satisfy the final demand of all goods and services generated in the development of both genome-editing methods are reported.

(Table 3). Chimerism is deduced by the presence of wild type reads in the sequencing (indicating the presence of either untransformed cells, or cells in which Cas9 did not achieve any editing) or by the presence of more than two editing profiles in a locus. The lack of uniformity in the genotype of the plant may be a problem in case of vegetative propagation and can be fixed in principle only by other cycles of *in vitro* regeneration, or by reproduction through the germline (in Chicory complicated due to its self-incompatibility): both methods imply extra time, work, and costs. Another drawback of the stable transformation is the continuous expression of Cas9, which creates the possibility for new editing events during the lifetime of the plant, both in the wild type on-targets (therefore with a probable increase of chimerism) and in the off-targets. In fact, this was indeed detected in three independent lines that were sequenced 2 years apart (Figure 3). This timeframe was quite long and not likely to occur in a breeding programme that involves crossings, but it is representative of what may happen in the plant during the months necessary for the vegetative growth before the flowering period in the case a cross is needed to segregate away the T-DNA and erase chimerism. Hence, in chicory the methods based on transient delivery to protoplasts seemed more convenient as exposure to the editing complex can be controlled and because, although less simple to perform compared to the transformation with Agrobacterium, they lacked chimerism, did not have prolonged off-target activity and generally gave a higher number of regenerants, which are already a new variety that do not need further crossings to be used in the field. Indeed, both protoplast-based methods produced high numbers of plants with null alleles, but the transient plasmid approach (Method 2) led to a high percentage (a third) of plants with integrated plasmid DNA copies. It is relevant to note that this phenomenon might have been underestimated, since it is based on the detection by PCR of the Cas9 sequence, but not of other sequences in the plasmid. Considering that the design of primer pairs covering the whole plasmid is quite inconvenient, and assuming the possibility of plasmid rearrangements, the only way to prove absence of foreign DNA integration under Method 2 would be whole genome sequencing of the plants obtained after regeneration. Therefore, the data about plasmid DNA integration with this method should be taken as a lower estimate. Method 1 did not have this issue, because it was a DNA-free approach, where different guide RNAs could be easily produced or synthetized *in vitro*, making it very flexible for potentially tackling many genes. This last method may also be advantageous in the future: since no foreign DNA is introduced into the plant, mutants produced in this way might be subjected to a faster commercialization due to a simpler regulatory framework in some countries (Entine et al., 2021). Indeed, a crucial aspect to consider when choosing a method to create new varieties is the regulatory framework in the country in which the product is planned to be released. In Europe the above-mentioned techniques currently lead to plants considered as Genetically Modified Organisms (GMOs), no matter if foreign DNA is integrated into the genome or not. However, in other countries (Dima and Inzé, 2021) Method 1 could never result in a GMO, a status that plants obtain from Method 2 would need to be checked for plasmid integration and method 3 could achieve only by out-crossing the transgenes and proving thus the absence of exogenous DNA. From the environmental and economic point of view, the two methods examined show similar impacts but with difference distribution across sectors and therefore income distribution. The environmental impacts of molecular breeding are highly dependent on the electricity demand. Therefore, the use of electricity from renewable energy sources is a key factor for the contribution to decarbonization. Although total impacts on value-added and production of goods and services are modest, higher employment and value-added multiplier effects are created compared to common industrial or manufacturing processes. This traces back mainly due to the high labor intensity of R&D activities. Based on these considerations, from the comparison it can be concluded that the DNA-free approach from protoplasts is to be preferred, with the Agrobacterium-based method to be used only when other transformation methods cannot be implemented and/or plant regeneration from protoplasts cannot be achieved.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JAPIVL000000000.

Author contributions

DB and KC conceived the research. KC, TS, PB, SM and MM designed the experiments. US, KU, PB, RS, MB, ST, KC, ES, RN and JH performed the experiments, collected the samples and analyzed data. MH, VK and MK performed the economic and environmental impact analysis. ES and RN sequenced and assembled the CHIC 2.0 genome. The manuscript was written by US, KU, MH, VK and revised by US, KU, PB, MB, KC, ES, RN, MH, VK, MK, DB, SM and MM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors PB and RS were employed by the company Keygene N.V.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1111110/full#supplementary-material

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LbCas12a mediated suppression of *Cotton leaf curl Multan virus*

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Begomoviruses are contagious and severely affect commercially important fiber and food crops. *Cotton leaf curl Multan virus* (CLCuMuV) is one of the most dominant specie of *Begomovirus* and a major constraint on cotton yield in Pakistan. Currently, the field of plant genome editing is being revolutionized by the CRISPR/Cas system applications such as base editing, prime editing and CRISPR based gene drives. CRISPR/Cas9 system has successfully been used against biotic and abiotic plant stresses with proof-of-concept studies in both model and crop plants. CRISPR/Cas12 and CRISPR/Cas13 have recently been applied in plant sciences for basic and applied research. In this study, we used a novel approach, multiplexed crRNA-based Cas12a toolbox to target the different ORFs of the CLCuMuV genome at multiple sites simultaneously. This method successfully eliminated the symptoms of CLCuMuV in *Nicotiana benthamiana* and *Nicotiana tabacum*. Three individual crRNAs were designed from the CLCuMuV genome, targeting the specific sites of four different ORFs (C1, V1 and overlapping region of C2 and C3). The Cas12a-based construct Cas12a-MV was designed through Golden Gate three-way cloning for precise editing of CLCuMuV genome. Cas12a-MV construct was confirmed through whole genome sequencing using the primers Ubi-intron-F1 and M13-R1. Transient assays were performed in 4 weeks old *Nicotiana benthamiana* plants, through the agroinfiltration method. Sanger sequencing indicated that the Cas12a-MV constructs made a considerable mutations at the target sites of the viral genome. In addition, TIDE analysis of Sanger sequencing results showed the editing efficiency of crRNA1 (21.7%), crRNA2 (24.9%) and crRNA3 (55.6%). Furthermore, the Cas12a-MV construct was stably transformed into *Nicotiana tabacum* through the leaf disc method to evaluate the potential of transgenic plants against CLCuMuV. For transgene analysis, the DNA of transgenic plants of *Nicotiana tabacum* was subjected to PCR to amplify Cas12a genes with specific primers. Infectious clones were agro-inoculated in transgenic and non-transgenic plants (control) for the infectivity assay. The transgenic plants containing Cas12a-MV showed rare symptoms and remained healthy compared to control plants with severe symptoms. The transgenic plants containing Cas12a-MV showed a significant reduction in virus accumulation (0.05) as compared to control plants (1.0). The results demonstrated the potential use of the multiplex LbCas12a system to develop virus resistance in model and crop plants against begomoviruses.

KEYWORDS

agroinfiltration, *Cotton leaf curl virus*, CRISPR/Cas12, plant virus, tissue culture, transgenic plant, virus inhibition

1 Introduction

Agriculture is the world's most important source of revenue and a vital way to improve food security (Dethier and Effenberger, 2012). Improving agricultural productivity through better seeds, irrigation, and other technologies such as precision agriculture and biotechnology can help increase crop yield and reduce the number of people, suffering from hunger and malnutrition. Plant viruses significantly impact food security by causing extensive crop losses, reducing crop yields and crop quality and nutritional value (Makkouk, 2020).

Plant viruses are infectious agents containing genetic material (DNA or RNA) enclosed in a protein coat. They infect and replicate within the cells of plants, causing a wide range of symptoms such as stunted growth, leaf curling and reduced crop yield. Some plant viruses are transmitted by insect vectors, such as aphids, while others may spread through contaminated seeds or mechanical means (Matthews, 2012). Many plant viruses do not have an effective treatment options once infected and control measures are typically focused on preventing infection through crop rotation, sanitation and developing disease resistant plant varieties (Bragard et al., 2013; Panattoni et al., 2013; Sumner, 2018).

There are several families of plant viruses, each with its unique characteristics and replication methods. The most prominent family of pathogenic plant viruses is *Geminivirdae*. Geminiviruses infect economically important crops, ornamental plants, food and weeds, consequently causing extensive yield losses in temperate, subtropical and tropical areas (Zerbini et al., 2017; Al Shihi, 2019). The leading genus of the family *Geminivirdae* is *Begomovirus*, which are characterized with single-stranded DNA (ssDNA) viruses that may be monopartite (2.7 kb) or bipartite (5.4 kb) (Ramesh et al., 2019). *Begomovirus* (genus) currently contains 500 species, including *Cotton leaf curl virus* (CLCuV), *Tomato yellow leaf curl virus* (TYLCV), *Chili leaf curl virus* (ChiLCV), *Bean yellow mosaic virus* (BYMV) and *Cassava mosaic virus* (CMV). All these viruses cause significant economic losses in various countries by infecting the cash crops such as cotton, tomato, cassava and bean (Kil et al., 2016; Kothandaraman et al., 2016; Zubair et al., 2017b; Thakur et al., 2018). CLCuV is the top-ranked endemic virus specie of *Begomovirus* that infects the cotton crop in Pakistan, India and Africa (Sattar et al., 2013). CLCuV is one of the plant pathogenic virus species that is considered as a primary biotic constraint of cotton yield.

Cotton is an important commercial crop, cultivated annually in many parts of the world. More than 80 countries are cultivating cotton crop, with the major growers are the USA, Pakistan, China, Uzbekistan, and India. The most utilized part of the cotton plant is the cotton bud which acts as an essential raw material to produce many products such as fiber, medicinal products, edible oil, livestock feed and paper (Shan et al., 2014). Cotton is considered as cash crop in Pakistan and the country has consistently ranked among the top cotton producers in the world. The economy of Pakistan gets a substantial boost from its export (6^{th} position in the world ranking as an exporter of cotton). While Pakistan has been a major player in cotton production, it is worth noting that pest attack, drought, and the CLCuV cause a significant loss of yield every year. Over the past three decades, the yield of cotton has been reduced by 30–35% due to CLCuV, which resulted in direct economic consequences for Pakistan (Farooq et al., 2014; Hameed et al., 2014; Rosen et al., 2015; Rehman et al., 2019). The characteristic symptoms of cotton curl leaf disease include vein darkening, leaf curling, enation and vein swelling (Bananej et al., 2016). The most devastating species of this virus is the *Cotton leaf curl Multan virus* (CLCuMuV), a major source of yield reduction in Pakistan and India since the 1980s (Zubair et al., 2017a). CLCuV enters the host plant cell, un-coat and transmits its genome (ssDNA) into the host cell nucleus. Then, the RF gene transforms ssDNA into dsDNA as a replicative form. Bidirectional transcription produces the viral mRNA with the help of host RNA polymerase and viral protein is subsequently translated from viral mRNA. The ssDNA viral genome of CLCuV is replicated and transferred into neighboring cells by plasmodesmata (Yin et al., 2019). A phloem-feeder whitefly (*Bemisia tabaci*) is the main vector that transmits CLCuV from one place to another (Rosen et al., 2015).

Genome editing is a promising tool for overcoming diseases caused through plant viruses by introducing precise changes into the plant genome that confer resistance to the virus. Advancements in genome engineering methods have enabled scientists to introduce the precise gene modifications, i.e., delete, add or replace the genes on the specific target sites in the genomes. These precise genome alterations are preferable substitutes to conventional transgenic approaches (Gao, 2021) to fulfil the early promises of genetic engineering. There are several genome editing tools available, including transcription activator like effector nucleases (TALENs), gene silencing (RNAi), zinc finger nucleases (ZFNs) and CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats) for installing precise modifications in the genome (Chen et al., 2014; Cheng et al., 2015). Although, all these techniques have their own limitations, such as complex designing and off-target effects (Gaj et al., 2013) but they have succesffuly used to achieve gene editing in bacteria, plants and animals.

CRISPR/Cas system is the most powerful and emerging genome editing technology that is simple, cheap, efficient, and easy to use (Caplan et al., 2015). CRISPR/Cas is a revolutionary technology with enormous applications in bacteria, plants, animals and the medical science as a therapeutic and diagnostic tool (Fellmann et al., 2017; Zhang et al., 2019). Recently, researchers uncovered a new CRISPR-like system in eukaryotes such as OMEGA (Obligate Mobile Element Guided Activity) and Fanzor, that may have broad spectrum applications in eukaryotic gene editing with better efficiency (Altae-Tran et al., 2021; Saito et al., 2023). CRISPR/Cas toolbox have been successfully used to engineer plants with improved biotic and abiotic stresses, enhanced yield and nutritional quality, and herbicide tolerance (Zafar et al., 2020). Similarly, CRISPR system has been effectively employed to achieve different genetic modifications such as gene knockdown, knockout, knock-in, transcriptional regulation through CRISPRi and CRISPRa, and site specific base editing (Malzahn et al., 2017; Yu et al., 2020). It is a straightforward technique and simple techniques,

to introduce new traits in plants, without inserting any foreign gene, to generate transgene free CRISPR edited crops with better public acceptance (Ma et al., 2016).

CRISPR/Cas offers a promising approach for controlling plant viruses and producing disease resistant plants to overcome challenges of food security and zero hunger. It is an exciting and rapidly evolving field that holds a great promise for the future of agriculture, health, and sustainable environment. Researchers have demonstrated CRISPR/Cas9 applications, especially multiplex genome editing to introduce precise changes in the plant genomes that prevented the virus from replicating or spreading (Uniyal et al., 2019). Similarly, CRISPR/Cas13 may provide an alternate and better strategy for diagnosis and control of RNA viruses. Therefore, CRISPR/Cas technology offers an efficient and powerful approach to reduce the viral effect of begomoviruses in crop plants (Zaidi et al., 2016). In addition, CRISPR/Cas holds a great potential to enhance the expression of plant defense genes to make the plant more resistant to viral infection (Ali et al., 2015; Baltes et al., 2015; Chaparro-Garcia et al., 2015). For example, CRISPR/Cas was successfully used to improve resistance against several plant viruses including *Tobacco mosaic virus* (Zhang et al., 2018), *Cucumber mosaic virus* (Tzean et al., 2019) and *Potato virus* Y (Hameed et al., 2019). These studies demonstrated the enormous potential of CRISPR/Cas for controlling plant viruses and enhance the productivity of crops.

CRISPR/Cas12 (previously known as Cpf1), belongs to the Class II, type V CRISPR/Cas system, charcaterized from *Acidaminococcus* and *Lachnospiraceae bacterium* (Bernabé-Orts et al., 2019). Cas12 is an RNA-guided nuclease to create sequence specific DSB in the genome, thus allowing targeted DNA modifications. Like other CRISPR/Cas systems, CRISPR/Cas12 can be programmed to target specific DNA sequences and install precise modifications in the genome (Swarts, 2019). CRISPR/Cas12a is an emerging, versatile and powerful genome editing tool for agricultural advancement (Bandyopadhyay et al., 2020).

Compared with CRISPR/Cas9, CRISPR/Cas12 holds several advantages, such as smaller size of Cas12, which allows an efficient delivery into plant cells and its ability to produce sticky-ends (staggered cut), which can simplify the process of integrating edited DNA into the plant genome. In addition, Cas12 requires a T-rich PAM as compared with G-rich PAM in CRISPR/Cas9. T-rich PAM may facilitate the targeting of non-coding regions in the genome though CRISPR/Cas12. These features makes CRISPR/Cas12, a promising tool for genome editing especially in agriculture, health and metabolic engineering. Different subtypes of CRISPR/Cas12 have been used for genome manipulation in living cells (Gosavi et al., 2020). The versatility of CRISPR/Cas12a in plant genome editing has been demonstrated in various studies, where it has been used to edit genes involved in a range of traits including disease resistance (Mishra et al., 2021), abiotic stress tolerance (Rahman et al., 2022) and improved yield. All these applications of CRISPR/Cas12 in plant genome editing demonstrate the great potential of CRISPR/Cas technology, to develop climate resilient and disease resistant crops with better adoptability to climate change and meet the challenges of food security and sustainable development.

2 Materials and methods

2.1 Virus analysis

DNA sequences of 92 variants of CLCuMuV for period 2019–2021, were collected from the NCBI gene bank and uploaded to the Geneious Prime software. Multiple alignment (Geneious alignment) of 92 variants (period 2019–2021) of the CLCuMuV species was conducted on Geneious Prime software to check the similarity among all these variants. A phylogenetic tree was constructed to evaluate the geographic distribution of these variants on Geneious Prime by using the Geneious Tree builder method. According to geographical distribution, 27 variants of CLCuMuV (present in Pakistan) were selected for analysis.

2.2 Designing of crRNAs

Specific sites of open reading frames (ORFs) of CLCuMuV were identified as target sites on Geneious Prime software. crRNAs were designed for CRISPR/Cas12a (LbCpf1) against target sites in the viral genome. CRISPR/Cas12a requires a PAM sequence (TTTN) to target specific regions, therefore, TTTN (N = ATGC) was selected as PAM site for designing crRNA (Doench et al., 2016). Multiple sequences (CRISPR sites) were analyzed on Oligoanalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer, last access: 11-07-2023) and Cas-OFFinder (http://www.rgenome.net/cas-offinder/, last access: 11-07-2023) to select the best crRNAs. OligoAnalyzer is a web tool used to determine the physical characteristics of oligonucleotides. The OligoAnalyzer tool is available online from IDT (Integrated DNA Technologies), a company specializing in oligonucleotide synthesis and related products. The tool allows input of the nucleotide sequence and generates various parameters, such as melting temperature (Tm), GC content and potential hairpin or dimer formation (Jameel et al., 2022). The selected crRNAs were uploaded on the OligoAnalyzer for the analysis of secondary structure, hairpin, self-dimerization and heterodimerization. For this study, the off-target effects of the selected crRNAs were evaluated on Cas-OFFinder software (Bae et al., 2014). Off-target effects for *Nicotiana benthamiana*, *Nicotiana tabacum* and cotton were analyzed. All the properties including Tm, GC content, self-dimerization and heterodimerization were also double-checked on AmplifX software (Franz et al., 2017).

2.3 LbCas12a based construct

All vectors required to make the construct were obtained from Addgene (www.addgene.org, last access: 11-07-2023) (Zhang and Qi, 2021). The details of plasmids involved in the designing of the T-DNA vector (CRISPR/Cas12a multiplex vector (Cas12a-MV) containing Cas12 and multiple crRNAs) are given in Table 1. The T-DNA vector was constructed by the Golden Gate three-way cloning method. Firstly, duplexed oligonucleotides (selected crRNAs sequences) named crRNA1, crRNA2 and crRNA3 were phosphorylated, annealed and cloned into the linearized crRNA

TABLE 1 List of plasmids required to generate Cas12a construct.

Plasmids	Plasmid number	Plasmid name	Purpose	Size	Bacterial selection	Plant selection
crRNA cloning vectors	138096	pYPQ131-STU-Lb	Golden Gate cloning vector for 1st crRNA	3181 bp	Tetracyclin	–
	138099	pYPQ132-STU-Lb	Golden Gate cloning vector for 2nd crRNA	3181 bp	Tetracyclin	–
	138102	pYPQ133-STU-Lb	Golden Gate cloning vector for 3rd crRNA	3181 bp	Tetracyclin	–
Recipient vector	138107	pYPQ143-ZmUbi	Golden Gate recipient; used for the assembly of three crRNAs	5310	Spectinomycin	–
Cas12a entry vector	86210	pYPQ230 (Lb editing)	Cas12a Gateway entry plasmid	6939	Spectinomycin	–
Destination vector	86198	pYPQ202	T-DNA entry plasmid	12807	Kanamycin and Chloramphenicol	Hygromycin

expression plasmids such as pYPQ131-STU-Lb (expression vector for crRNA1), pYPQ132-STU-Lb (expression vector for crRNA2) and pYPQ133-STU-Lb (expression vector for crRNA3) at the *Esp3*I restriction site to construct crRNA cassettes. The crRNA cassettes were assembled with respective recipient plasmid (pYPQ143-ZmUbi) to construct crRNA entry vectors through the Golden Gate cloning method. Finally, the crRNA entry vector (cloned pYPQ143), Cas12a entry vector (pYPQ230) and destination vector (pYPQ202) were assembled through a three-way Gateway LR reaction. Flow sheet describing the steps to construct a T-DNA vector (Cas12a-MV) is given in Figure 1. The *EcoR*1 restriction enzyme and whole-genome sequencing were used to confirm the successful cloning. Table S1 shows the list of primers, used for WGS sequencing of Cas12a-MV.

2.4 Plant material and transformation

Nicotiana benthamiana was used as a model plant to test the potential of the multiplex Cas12a-MV construct. The transformation of plants through agro-infiltration was performed at the School of Plant Sciences, University of Arizona, USA. Seeds of *N. benthamiana* were grown in small pots, containing soil mixed with peat moss at an optimum temperature (28°C) and photoperiods (8 hours dark and 16 hours light photoperiod). After three weeks of transplanting, all the plants reached to an optimal developmental stage indicated by 2–3 fully developed true leaves, with no visible flower buds. These plants were used for infectivity analysis through agroinfiltration (Mubarik et al., 2019). The infectious clones, including *Cotton Leaf Curl Multan Virus-Rajasthan* (CLCuMuV-Ra) and *Cotton leaf curl Multan betasatellite* (CLCuMuB), were used to test the potential of Cas12a-MV construct. Betasatellite infectious clone was used to induce and develop symptoms of CLCuMuV in the plants. Agrobacterium-mediated transient transformation (agroinfiltration) was performed to express the Cas12a-MV construct with CLCuMuV infectious clone in *N. benthamiana*. A total of 36 plants were divided into three groups A, B and C, according to the given treatments. The description of different groups of plants, used in this study is given in Table 2.

2.5 Confirmation of viral gene in plants

Genomic DNA of virus was isolated through the CTAB method from all plants after 15 days post-inoculation (dpi) (Healey et al., 2014). PCR was performed using specific primers (according to the viral genome) to check the presence of infectious clones in all plants. The list of primers used in this study to amplify viral DNA from the infectious clones is given in Table S2.

2.6 Mutation detection

Sanger sequencing was utilized to detect mutation in the CLCuMuV genome isolated from the infected plants. The genomic DNA was isolated from *N. benthamiana* plant leaves after 15 dpi. The target sites were amplified with specific primers through PCR. Purified PCR products were subjected to Sanger sequencing. List of primers used for Sanger sequencing in this study is given in Table S3. The resulting traces from Sanger sequencing were uploaded on TIDE software (http://shinyapps.datacurators.nl/tide/, last access: 13-07-2023) and compared with the reference sequence (CLCuMuV genomic sequence) to detect the mutations in the targeted regions. TIDE software uses an R sequencer to analyze the sequencing data. The R Sequencer uses the R programming language, which provides a wide range of statistical and bioinformatics packages for data analysis (Brinkman and van Steensel, 2019). This tool is specific for Cas9 editing but some default settings also make it appropriate for Cas12a editing.

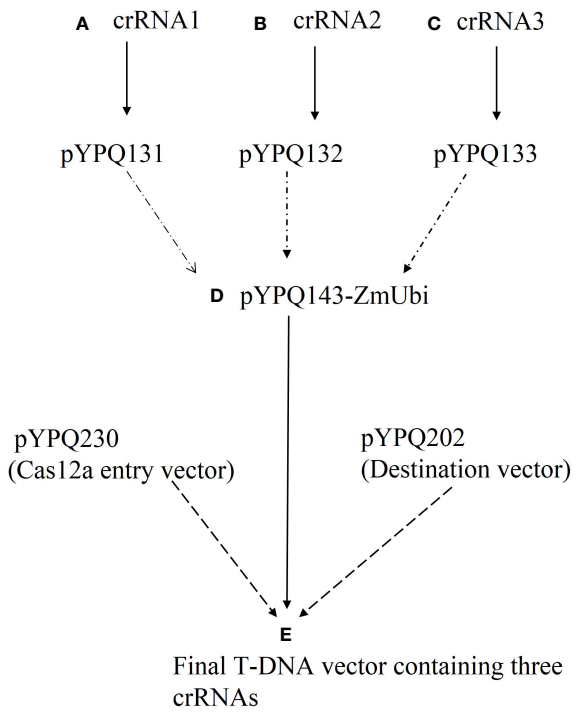

FIGURE 1
Flow sheet of vector construction with three crRNAs for multiplexed genome editing through CRISPR/Cas12a. **(A)** 1st crRNA was cloned into crRNA cloning vector pYPQ131. **(B)** 2nd crRNA was cloned into crRNA cloning vector pYPQ132. **(C)** 3rd crRNA was cloned into crRNA cloning vector pYPQ133. **(D)** All cloning vectors were assembled in the recipient vector (pYPQ143) using the Golden Gate assembly method. **(E)** Cas12a entry vector (pYPQ230), destination vector (pYPQ202) and crRNA recipient vector (pYPQ143-ZmUbi-pT) were assembled using a three-way Gateway LR reaction to form the final T-DNA vector.

2.7 Stable transformation of *Nicotiana* plants with Cas12a construct

Transgenic *Nicotiana tabacum* plants were generated through the leaf disc method (*Agrobacterium*-mediated). The leaves of 4-week-old, wild-type *Nicotiana* plants were picked and surface sterilized with 5% bleach and 0.1% Tween-20 for 5 minutes. In the next step, leaves were washed with sterilized water three times. Leaf discs of 1 cm square in size, were sliced from sterilized leaves and incubated with agrobacterium containing Cas12a-MV construct with an OD 0.8, for 8 minutes at room temperature. Then agrobacterium infected leaf discs were placed on a co-cultivation medium (3% sucrose, 1x MS powder, 1% agar, 2mg/L kinetin, 2mg/L IAA and 200µM acetosyringone) and incubated in the dark for two days at 22°C. After two days, leaf discs were shifted to an induction medium (3% sucrose, 1x MS powder, 1% agar, 2mg/L kinetin, 2mg/L IAA, 200mg/L timentin and 25mg/L hygromycin) and incubated at 28°C for the development of the callus. After a week, the explants were shifted to fresh induction medium until the shooting was initiated. Shoots of 2–3 cm in length were cut from the explant and transferred to the root induction medium (3% sucrose, 1x MS powder, 1% agar, 0.2mg/L IBA, 200mg/L timentin and 25mg/L hygromycin). After three weeks, plants with roots were shifted from rooting medium to peat moss and kept in a plastic box to maintain moisture. Two weeks later, the transgenic plants were shifted to the growth chamber and seeds were collected from mature plants after 4–6 weeks.

2.8 Expression analysis of Cas12a and gRNAs in transgenic plants

The transgenic *Nicotiana tabacum* plants were screened through PCR to check the presence of Cas12a. For this purpose, genomic DNA was isolated through the CTAB method from the leaves of *N. tabacum* transgenic plants. Cas12a gene specific primers were used to check the integration of Cas12a in transgenic plants. For expression analysis, total RNA was isolated from putative transgenic plants and used for cDNA synthesis. Real-time PCR was performed to quantify the expression of Cas12a and multiple crRNAs in transgenic plants (Ma et al., 2015).

2.9 Virus infectivity assay of transgenic plants

Wild-type and transgenic *Nicotiana* plants (expressing Cas12a and crRNAs) were agro-inoculated with CLCuMuV infectious clones to induce and develop the symptoms of the virus. The virus accumulation in transgenic plants of *N. tabacum* was checked through qPCR and compared with control plants (infiltrated with infectious clones) (Mubarik et al., 2019).

3 Results

3.1 Target selection

Genomic sequences of CLCuMuV for period 2019–2021 were retrieved from NCBI and analyzed to select the target sites using Geneious Prime software. According to geographical distribution, 27 variants of CLCuMuV were selected with Pakistan origin. The phylogenetic tree of 27 variants is shown in Figure 2A. All details of 27 variants including isolate name, accession number, size and

TABLE 2 Groups of plants according to treatment.

Group	Treatment	No. of plants
Group-A	Negative control (plants containing just inoculation buffer)	12
Group-B	Plants co-infiltered with Cas12a-MV vector and infectious clones	12
Group-C	Positive control (plants inoculated with infectious clones only)	12

origin are given in Table S4. The potential consensus sequences in the coding regions were selected as target sites, from 27 species of CLCuMuV, to produce broad-spectrum resistance against virus (Figure 2B). Three crRNAs were selected, targeting the C1 region (Rep), overlapping region of C2 (TrAP) and C3 (Ren Protein), and V1 (CP) to inhibit the replication of CLCuMuV (Figure 2C). The crRNAs were selected based on different properties including secondary structure, hairpin structure, self-dimerization and heterodimerization. Off-target effects were checked against cotton and *Nicotiana benthamiana*. The detail of selected crRNAs is summarized in Table S5.

3.2 Plasmid construction

We designed three crRNAs through Geneious Prime software, targeting the four coding regions (C1, C2, C3 and V1) to impede the replication of CLCuMuV. The crRNAs expression cassette and LbCas12a expression cassette were combined into a T-DNA vector (destination vector) through Golden Gate three-way cloning. In this study, two Pol-II promoters including AtUbi10 and pZmUbi were used to drive expression of Cas12a and tandem HH-crRNA-HDV arrays respectively. crRNAs were flanked with hammerhead (HH) ribozyme RNA and hepatitis delta virus (HDV) ribozyme RNA for precise processing of crRNA. This type of expression is considered as best to derive crRNAs and Cas12a (Figure 3A). The cloning vectors including pYPQ131, pYPQ132 and pYPQ133 were digested with the *Esp3*1 enzyme and then crRNA1, crRNA2 and crRNA3 were ligated in them respectively. Next, those multiple cloning vectors were assembled with the recipient vector pYPQ143 by T4 DNA ligase. In the final step, the Gateway LR reaction was done by the assembly of cloned recipient vector, Cas12a entry vector (pYPQ202) and destination vector (pYPQ230) to construct the final T-DNA vector (Cas12a-based construct). The Cas12a-based construct was first confirmed through restriction enzyme *EcoR*1 and resolving its product on agarose gel which showed eight bands with different sizes, as shown in Figure 3B. Whole genome sequencing was performed to confirm the cloning of crRNA and Cas12a in the final construct. The specific part of WGS consisting of multiple crRNAs is shown in Figure 3C.

3.3 Infectivity assay

N. benthamiana plants were divided into three groups named as group A, group B and group C. Group A plants were infiltrated with inoculation buffer only and showed no symptoms (Figure 4A). Group-B plants were co-infiltrated with infectious clones of CLCuMuV and Cas12a-MV. The appearance of leaf curl symptoms in systematic leaves of group B plants was delayed by 10–12 days as compared with the control plants (Figure 4B). The symptoms of the disease were mild, and plants recovered from the

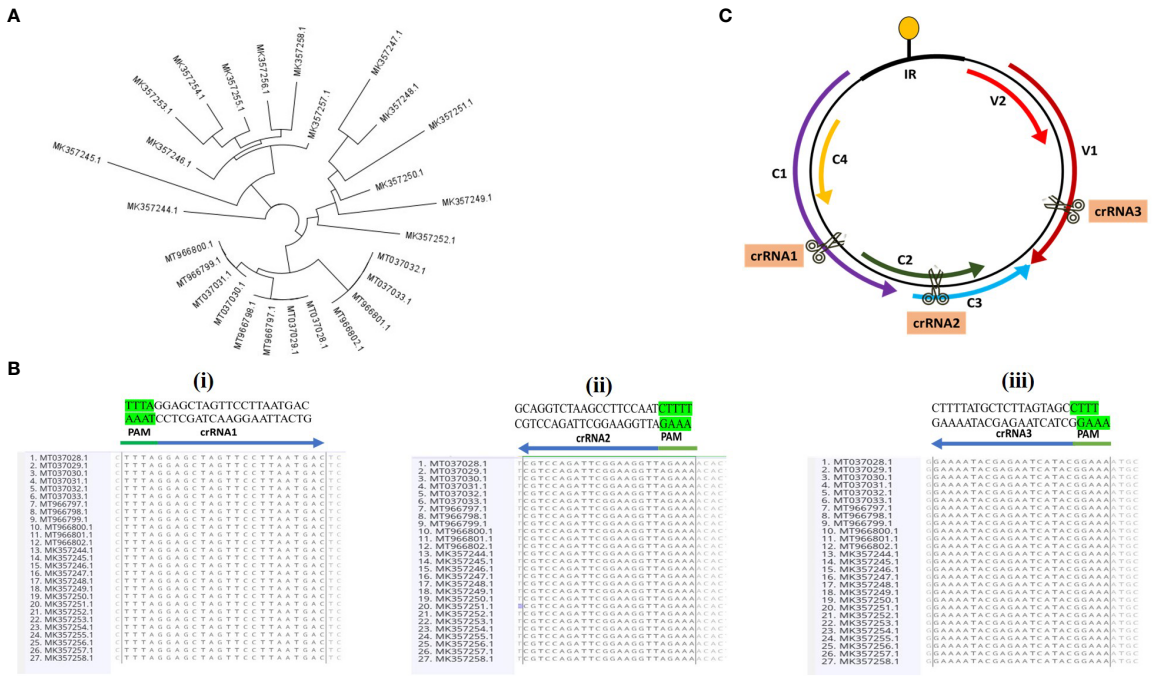

FIGURE 2
(A) The circular phylogenetic layout was derived from the alignments of 27 full-length sequences of CLCuMuV and conducted on Geneious Prime software. It shows the relationship among the different strains of CLCuMuV. The branches of the tree are arranged in a circle around a central point (showing the same ancestor), with the groups of strains placed at the tips of the branches. The length of the branches and the distance between the strains represent the evolutionary distances or differences among them. **(B)** All the nucleotide sequences were retrieved from the NCBI Gene Bank and aligned through Geneious alignment in Geneious Prime software. All the selected crRNAs sequences are shown, and the PAM sequence is highlighted with green color. **(C)** Schematic diagram of the CLCuMuV genome with the target sites. Arrows represent the ORFs (C1, C2, C3, C4, V1 and V2). crRNA1 targets the specific site of C1 and crRNA2 targets the overlapping region of C2 and C3. crRNA3 targets the specific site of V1.

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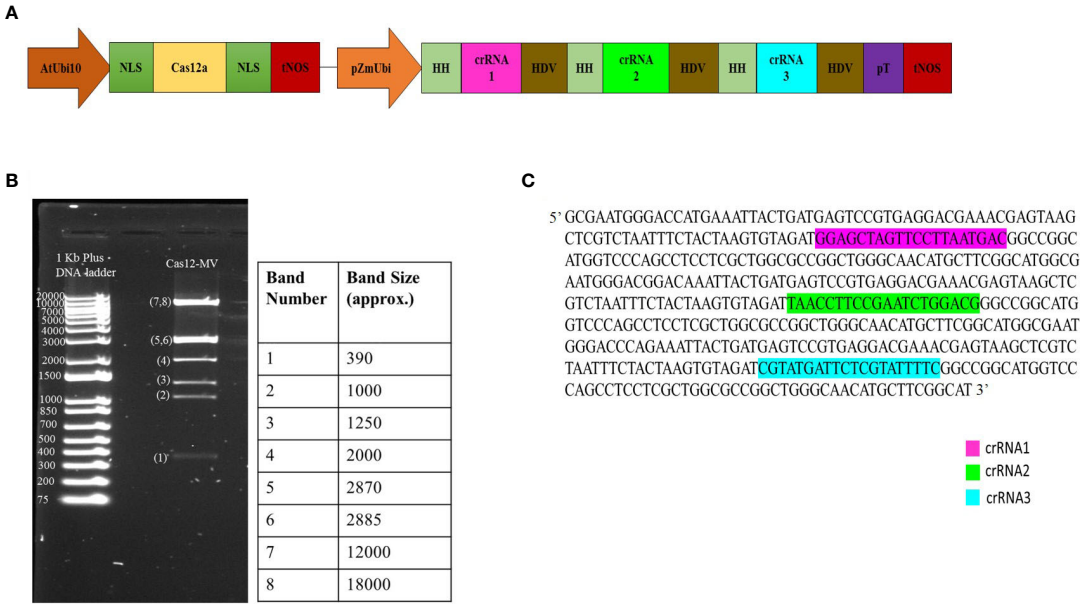

Band Number	Band Size (approx.)
1	390
2	1000
3	1250
4	2000
5	2870
6	2885
7	12000
8	18000

FIGURE 3
(A) Schematic diagram of multiplexed LbCas12a expression cassette with three crRNAs. AtUbi10, *Arabidopsis ubiquitin* 10 promoter; NLS, nuclear localization signal; tNOS, nopaline synthase terminator; pZmUbi, *Zea mays ubiquitin* promoter; HH, hammerhead; HDV, hepatitis delta virus; pT, terminator. **(B)** The construct was confirmed through the restriction enzyme digestion method using *EcoR*I. The product was run on 1.2% agarose gel. 1Kb plus DNA ladder was used (band sizes are mentioned in the table). **(C)** WGS result of the cloned construct of Cas12a-MV verified the presence of all three selected crRNAs (crRNA1, crRNA2 and crRNA3) in the construct.

disease after 30 days post-infection. Group C plants were kept as positive control and infiltrated only with infectious clones (CLCuMuV). The leaf curling symptoms appeared severe in the group C plants (Figure 4C). Table 3 shows the evaluation of the Cas12a constructs for the suppression of CLCuMuV.

3.4 Mutation detection and TIDE analysis

The selected crRNAs in this study, targeted the specific coding sequences of the CLCuMuV genome to reduce its viral effect. Sanger sequencing confirmed the mutations produced at the DSB sites by multiplex CRISPR/Cas12a system. Sanger sequencing is considered as a reliable method for DNA sequencing and detect Cas footprints because it has high accuracy and reproducibility. Comparison of mutant traces and control sequence is shown in Figure 5. Recently, an algorithm of TIDE is created to evaluate the Sanger sequencing traces. The trace sequences were analyzed through the TIDE program to access the targeting efficiency of selected crRNAs. This method gives indel spectrum by comparing and decomposing Sanger traces generated from PCR products of edited templates and wild type. TIDE analysis showed the editing efficiency of crRNA1 (21.7%), crRNA2 (24.9%) and crRNA3 (55.6%) (Figure 5).

3.5 Confirmation of viral gene in plants and virus accumulation determination

The presence of infectious clones in all plants was checked by PCR. Viral DNA was isolated from all infiltrated plants through the CTAB method and subjected to PCR using the virus specific primers CLVP1_500F and CLVP2_1500R. PCR confirmation of infectious clones in all infiltrated plants through PCR has been shown in Figure 6A. Amplified products of 1450bp with virus specific primers (CLVP1_500F and CLVP2_1500R) were resolved on 1% agarose gel and 1KB plus ladder was used as a marker. The virus accumulation in infected plants was determined by qPCR. The virus accumulation in *N. benthamiana* plants at 15dpi is shown in Figure 6B, which indicates the low virus accumulation (0.27) in plants containing Cas12a-MV as compared to the control plants (0.99), infiltrated with infectious clones.

3.6 Development of transgenic plant

Once Cas12a-MV construct was evaluated in *Nicotiana benthamiana* plants through transient transformation, the same construct was used for stable transformation in *Nicotiana tabacum* plants. About four-week-old plant leaf discs were infected with the agrobacterium containing Cas12a-MV construct (Figure 7A). The infected explants were placed on co-cultivation media and incubated at 22°C for two days to improve the transformation efficiency (Figure 7B). Explants (leaf discs) were placed on induction media and incubated at 28°C. Callus emerged from leaf discs after 1–2 weeks of transformation, as shown in Figures 7C, D. The transgenic callus was sub-cultured onto a freshly prepared induction medium after 7 days of interval, to avoid contamination (Figure 7E). Putative shoots were emerged from transgenic callus after four weeks (Figure 7F). Shoots of approximately 2–3 cm in length are shown in Figures 7G, H. Fully emerged shoots of 2–3 cm

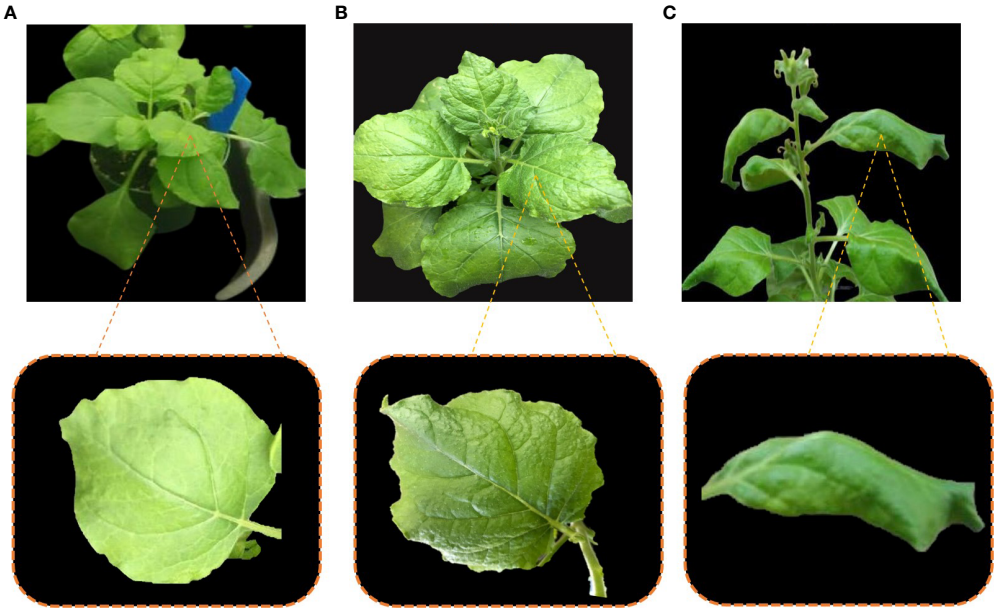

FIGURE 4
Plants showing susceptibility and resistance against CLCuMuV. **(A)** Plant A is a non-inoculated plant that showed no signs and symptoms of CLCuMuV. **(B)** Plant B is co-infiltrated with the Cas12a-MV construct and infectious clones of CLCuMuV, showed mild symptoms and resistance against CLCuMuV. **(C)** Plant C was inoculated with only infectious clones (CLCuMuV) and showed severe CLCuMuV symptoms.

in size, were shifted to the rooting medium. Roots were developed in all transferred shoots (Figure 7I). All rooted plants were transferred to soil-filled small pots for acclimatization and kept under controlled conditions (Figure 7J). Three weeks old plants were moved to a greenhouse to collect seeds (Figure 7K).

3.7 Expression analysis of transgenic *Nicotiana* plants

Transgenic *N. tabacum* plants were selected for transgene analyses. Genomic DNA was isolated from the leaves of transgenic *N. tabacum* plants through the CTAB method and subjected to PCR with specific primers of Cas12a. All plants showed amplification of the Cas12a gene, with amplicon of 1000bp in size, as shown in Figure 8A. The total RNA was isolated from all transgenic plants to evaluate the expression level of Cas12a and crRNAs through qPCR. The expression level of Cas12a (0.48) and all three crRNA1 (0.39), crRNA2 (0.39) and crRNA3 (0.38) are shown in Figure 8B. The actin gene of the *Nicotiana* plant was used as a control.

3.8 Transgenic plants with LbCas12a confer virus resistance

The transgenic plants expressing the Cas12a-MV construct containing crRNAs showed rare symptoms after 7–8 dpi as shown in Figure 9A. The non-transgenic control plants showed leaf curl disease symptoms within 7–8 dpi as shown in Figure 9B. Virus accumulation in control (wild type inoculated with the infectious clone) and transgenic plants was determined through qPCR. It was observed that transgenic plants of *N. tabacum* (0.05) showed low virus accumulation compared to control plants (1.0), as shown in Figure 9C.

4 Discussion

4.1 Plant viruses and control strategies

Plant viruses infect most of the cash crops and seriously threaten food security in various countries. Chemical approach has been used to protect crops against insects, pests, fungal and

TABLE 3 Evaluation of multiplex CRISPR/Cas12a construct to suppress the CLCuMuV in plants.

Groups	No. of plants	Severity of disease	Symptoms percentage	Recovery of the plant after 30 dpi
A	12	No	0%	–
B	12	Mild	20%	Yes
C	12	Severe	100%	No

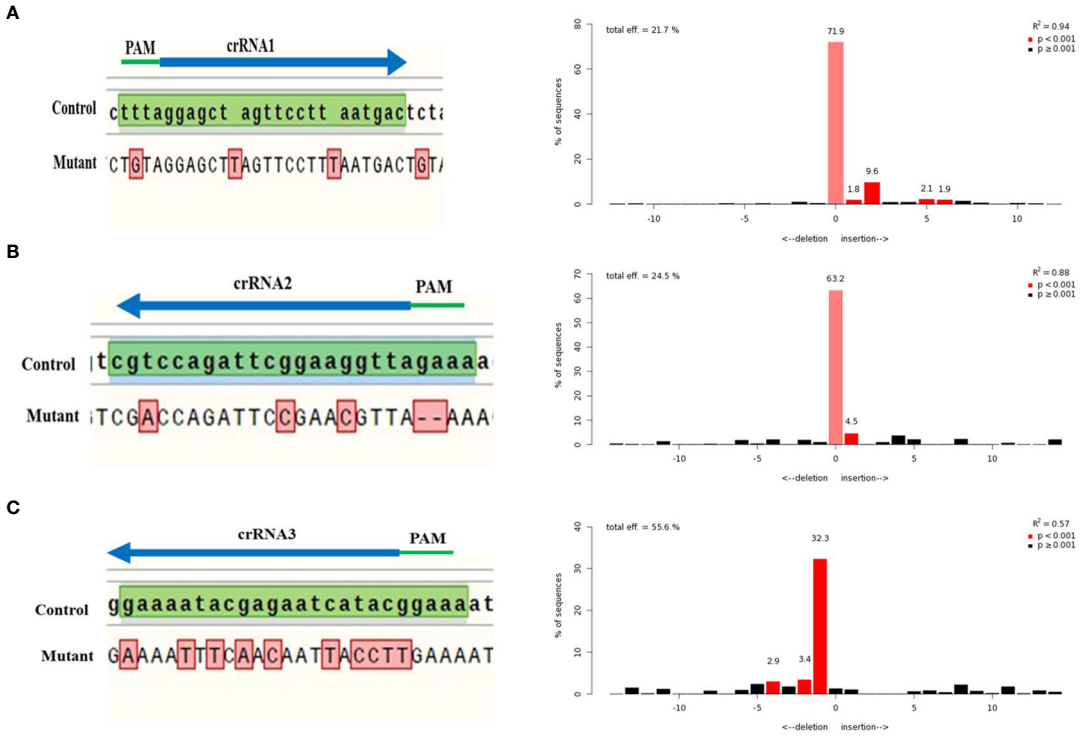

FIGURE 5
TIDE analysis of CLCuMuV (specific regions) targeted with selected crRNAs. The wild-type sequence of CLCuMuV was used as a control and compared with the targeted mutant sequences. **(A)** crRNA1 targets the C1 region and total editing efficiency of crRNA1 is 21.7%. **(B)** crRNA2 targets the C2 and C3 regions and total editing efficiency of crRNA2 is 24.5%. **(C)** crRNA3 targets the V1 region and total editing efficiency of crRNA1 is 55.6%.

bacterial infections, but these chemicals are ineffective against plant viruses (Bragard et al., 2013). Begomoviruses are a group of single-stranded DNA viruses that are known to infect plants mainly cotton, causing severe yield losses and economic consequences especially for the textile industry and farmers. CLCuV is the most devastating species of *Begomovirus* which has been responsible for the significant loss of cotton yield in Pakistan since the 1980s. There has been significant research on CLCuV, focused on understanding the biology of the virus and its interactions with the host plant, as well as the development of methods for controlling infections.

Many strategies such as crop rotation, chemical control and biotechnological methods have been used to control plant viruses and the most powerful one is pathogen-derived resistance (PDR). PDR is the process in which viral sequences are inserted in plant

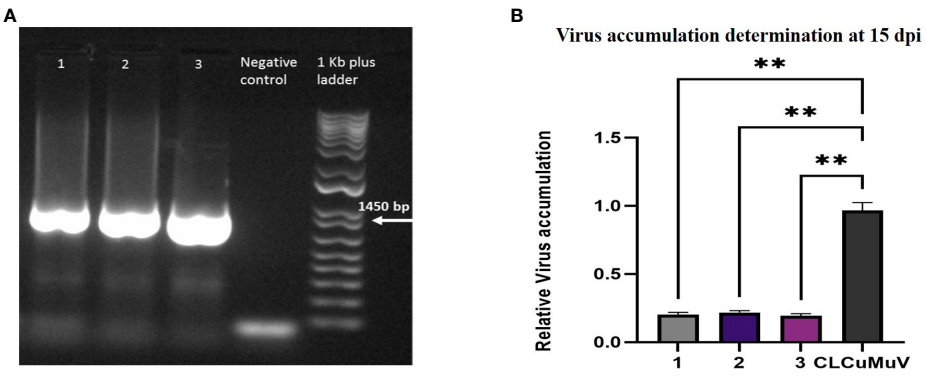

FIGURE 6
Confirmation of viral gene and virus accumulation determination in plants. **(A)** Lane 1–3 shows the presence of viral gene in plants infiltrated with Cas12a-MV and infectious clones. The master mix was used as a negative control. A 1KB Plus ladder was used. Primers were amplified at 1450 bp. **(B)** This graph represents the relative accumulation of CLCuMuV in *Nicotiana* plants at 15 dpi. Each bar (1–3) has three replicates of plants co-infiltered with Cas12a-MVconstruct and infectious clone (CLCuMuV). The plants showed low virus accumulation compared with the control (infectious clone). ** P<0.05 in ANOVA, which shows significant difference.

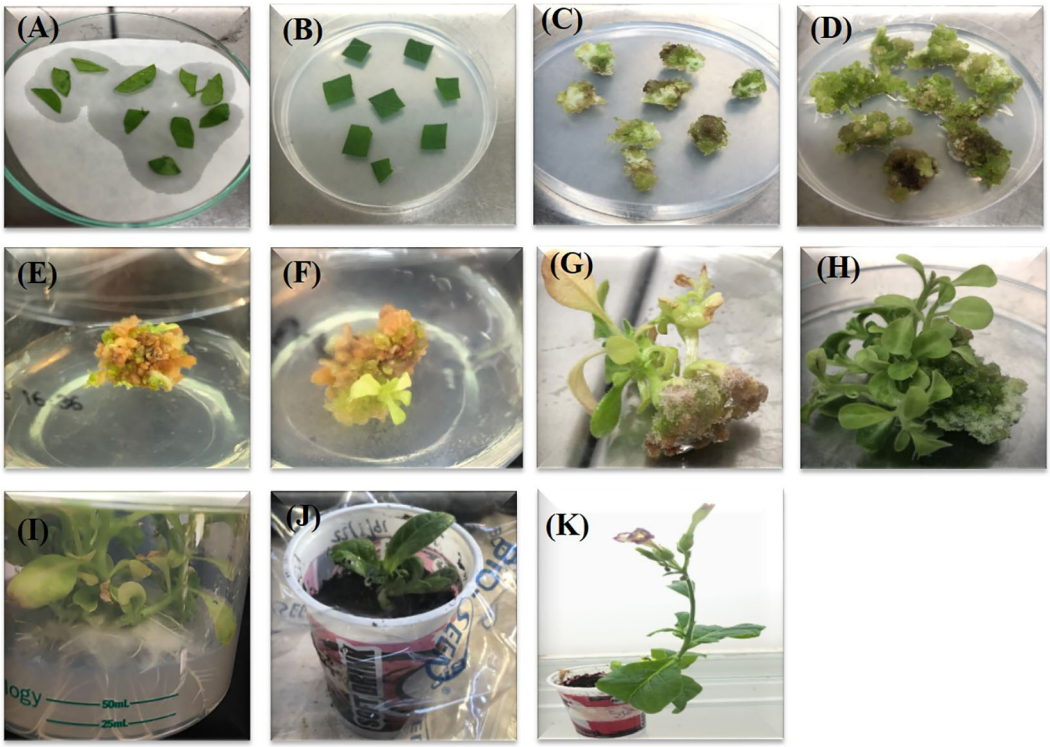

FIGURE 7
Different stages of *in-vitro* plant regeneration from leaf discs. **(A)** The inoculation (Agrobacterium suspension containing Cas12a-MV construct) of leaf discs (explant). **(B)** Shifting of inoculated explant to induction medium. **(C, D)** Transgenic Calli appeared within two weeks of transformation. **(E)** Regeneration of shoots from callus. **(F)** Initiation of shooting. **(G, H)** Shoots ready to shift on rooting media. **(I)** The rooting of putative transgenic shoots. **(J)** Transgenic plant under acclimatization conditions. **(K)** Transgenic plant.

cells to produce resistance against virus. RNAi-mediated resistance in plants against plant viruses, is the type of PDR that has been widely used against RNA viruses, but this approach is ineffective for DNA viruses including begomoviruses. Sera, 2005 used artificial zinc finger protein to target the most conserved region (IR) of *Beet severe curly top virus* (BSCTV) and successfully suppressed the replication of BSCTV. Chen et al. (2014) evaluated the effect of zinc finger nucleases by targeting the *rep* gene of the *Tomato yellow leaf curl China virus* (TYLCCNV) and successfully achieved the mutations in the viral genome of TYLCCNV. Cheng et al. (2015) have demonstrated TALENs to engineer resistance against DNA viruses including TYLCCNV. Although ZFNs and TALEN have been demonstrated for developing virus resistance in plants, however, these genome editing tools have some limitations such as low efficiency, complex designing, expensive, laborious, targeting only a single site at a time and difficult to multiplex.

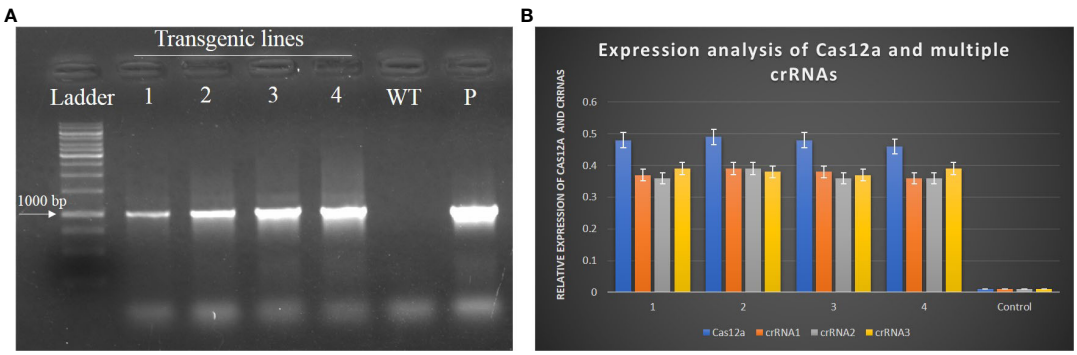

FIGURE 8
Evaluation of transgenic plants to check the expression of Cas12a and crRNAs. **(A)** PCR confirmed the presence of Cas12a in transgenic plants of *N. tabacum*. The size of the amplicon was 1KB. The purified vector was taken as the positive control and the DNA of wild-type plant was taken as the negative control. **(B)** Expression of Cas12a and multiple crRNAs was quantified through qPCR. All transgenic plants showed significant expression as compared to the control.

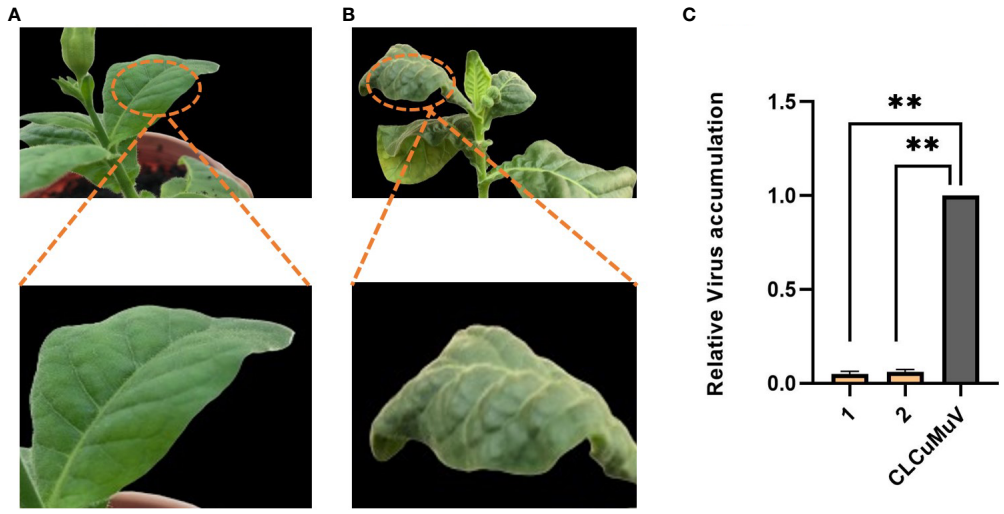

FIGURE 9
Evaluation of transgenic and wild-type plants of *N. tabacum* for CLCuMuV symptoms development. **(A)** The transgenic plant of *N. tabcum* was infiltrated with an infectious clone (CLCuMuV) and showed mild symptoms at early stages and no symptoms were observed at later stages and the plant became healthy. **(B)** Wild type plant of *N. tabacum* infiltrated with an infectious clone which showed severe symptoms. **(C)** This graph represents the relative accumulation of virus in transgenic plants of *N. tabacum* compared to control plants (only inoculated with CLCuMuV). Each bar (1–2) is the replicate of two transgenic plants. The transgenic plants showed the most promising results as they showed less virus accumulation (0.05) than the control plants (1.0) and remained healthy. ** P<0.05 in ANOVA, which shows significant difference.

4.2 Potential applications of CRISPR/Cas system against plant viruses

CRISPR/Cas toolbox is a more robust alternative to generate resistance against plant viruses (Cao et al., 2020). CRISPR/Cas technology holds an excellent potential for improving crop productivity by developing resistance against biotic and abiotic stresses thus contributing to food security. Early gene editing studies with CRISPR/Cas9 were especially focused on agricultural improvement (Zhang et al., 2021). For example, CRISPR/Cas9 system has been successfully used to improve yield, disease resistance (Zhu et al., 2020), complex trait improvement and altering plant architecture (Bao et al., 2019). Different studies have specifically demonstrated the potential CRISPR/Cas9 toolbox to engineer resistance against plant viruses. Example includes, Tashkandi et al. (2018) used the CRISP/Cas9 system against the TYLCV in *benthimiana* and achieved the reduction in virus accumulation. Similarly, Liu et al. (2022) created the mutation in the *ZmGDIα* gene through Cas9 to increase the resistance against MRDD in maize plants. Multiplex CRISPR/Cas system has been suggested as a promising approach for controlling CLCuV and other plant viruses, as it allows researchers to target multiple viral genes simultaneously thus increasing the effectiveness of the treatment and reducing the risk of the viral escape. Multiplexing with LbCas12a offers a promising approach to target several genes simultaneously in the targeted organism.

In this study, we suppressed the replication of CLCuMuV through a multiplex Cas12a-based system in model plants named *Nicotiana benthamiana* and *Nicotiana tabacum*. Recent studies have demonstrated that targeting a viral genome at a single gene is not enough to limit viral replication and generates viral escape. McCarty et al. (2020) revealed that the potential solution for viral escape is targeting two or more genes simultaneously with multiple guide RNAs. In multiplexing, Cas nuclease and multiple guide RNAs are expressed simultaneously to target multiple genes (or regulatory sequences) to reduce the chances of viral escape. Kurata et al. (2018) demonstrated that the multiplex approach using more than one sgRNA to target multiple genes was more effective than the conventional single gene targeting methodology with CRISPR/Cas system.

Similarly, in the present study, LbCas12a nuclease and multiple crRNAs were expressed in the host plant, targeting the multiple genes of the viral genome. LbCas12a has been recommended for the editing of plants by Zhang and Qi (2021), who described it as an efficient system for gene modifications in rice. The authors also demonstrated the usefulness of the Cas12a system in generating plant mutants. In a similar research, Bernabé-Orts et al. (2019) used the Cas12a-based constructs for successful editing of the genomes of *Arabidopsis*, *benthimiana*, and *Lycopersicum*. The versatility of the Cas12a system has made it one of the favorite genome editing tools in life sciences with a broad range of applications. For example, Zhang et al. (2021) used the similar strategy to construct the multiplex Cas12a-based constructs through Golden Gateway cloning to target 16 sites simultaneously in rice. The authors achieved high editing efficiencies in rice with this multiplex CRISPR/Cas12a approach. All these findings demonstrate the excellent potential of multiplex Cas12a editing system in targeting multiple genes of plant genome simultaneously. In this study, we

also successfully used the multiplex Cas12a vector to suppress the viral disease in model plants.

4.3 Sequence alignment

CRISPR/Cas system offers an excellent flexibility in terms of target selection. CLCuV is known for its high genetic variability, with numerous strains and variants circulating in different regions. By carefully selecting target sequences, conserved among different begomovirus strains, we can develop gRNAs with broad-spectrum activity against multiple virus variants. This would enable us to program the CRISPR/Cas-based strategies for an effective control of begomoviruses infections across diverse geographic locations. In this study, we aimed to investigate the geographic distribution of variants of the CLCuMuV species. To accomplish this, we collected genomic sequences of CLCuMuV variants, spanning the years 2019 to 2021 from the NCBI gene bank, and subsequently analyzed it on the Geneious Prime software. Multiple alignments of 92 CLCuMuV variants were performed using the Geneious Prime software to assess their similarity. The multiple alignments allowed us to compare the nucleotide sequences of all these variants and identify their conserved regions or potential variations present in their genomes. This analysis provided a valuable information regarding the genetic diversity within the CLCuMuV species. To evaluate the geographic distribution of the CLCuMuV variants, a phylogenetic tree was constructed using the Geneious Tree builder method within the Geneious Prime software. The phylogenetic tree provided insights into the evolutionary relationships and geographic clustering of these different CLCuMuV variants, enabling us to understand the spread and distribution patterns of this virus. We selected 27 CLCuMuV variants in Pakistan based on the geographic distribution analysis. Multiple alignments of these selected variants were conducted using Geneious Prime software to analyze their sequence conservation and variations. Comparative studies revealed interesting patterns between the selected CLCuMuV variants and those from other geographic regions. By comparing the genetic sequences and phylogenetic relationships, we observed similarities and differences among the CLCuMuV variants from Pakistan and other parts of the world. These findings revealed the relatedness and potential cross-contamination of CLCuMuV variants across different regions.

4.4 Designing of crRNAs for Cas12a: geneious prime software and other tools

One of the key advantages of the CRISPR/Cas system is its efficiency and specificity in targeting and modifying viral genomes. Naturally, Cas effectors, like Cas9 and Cas12, perform RNA-dependent DNA cleavage (Saito et al., 2023), which has been engineered for site specific genome editing in eukaryotes as well. A distinct advantage of RNA-guided systems is that it allows an effector to target multiple substrates by simply reprogramming the RNA guide (Altae-Tran et al., 2021) to different targets in the genome. By designing crRNAs complementary to the specific regions of the CLCuMuV genome, we induced targeted mutations in the viral genome, which resulted in the disruption of different viral genes, rendering the CLCuMuV unable to replicate and reduce the viral load in host plants. Geneious Prime software was used to design potential crRNAs (Doench et al., 2016). This software uses Python and R algorithms to find the CRISPR sites. Many other gRNA designing tools, including CHPCHOP, CRISPOR and CRISPR directs, have been used for designing gRNA, but these tools are only specified for Cas9 gRNA designing. Different Cas effectors and guide RNAs (gRNAs) design tools along with on target and off target parameters have been summarized by Li et al. (2022). However, Geneious Prime is a comprehensive bioinformatics software that provides a complete range to design gRNAs for the CRISPR/Cas9 and CRISPR/Cas12 systems. Geneious Prime is considered an excellent tool for gRNA design due to its fast and accurate algorithms, customizable parameters, and integration with other tools.

4.5 Cas12a-MV-mediated genome editing of CLCuMuV: comparative studies and insights

Our Cas12a-MV multiplex approach showed an efficient editing at specific sites of coding regions in CLCuMuV genome, leading to the suppression in the replication of CLCuMuV and viral load in the infiltrated plants. In this study, we found low virus accumulation in infiltrated plants with Cas12-MV construct, compared to control plants, infiltrated with infectious clones only. Real-time PCR was used to check the virus accumulation in infiltrated and control plants. The ct values obtained through real-time PCR were analyzed through one-way ANOVA. Figure 6B showed that the plants co-infiltrated with Cas12a-MV and infectious clones demonstrated less virus accumulation than control plants, which showed high virus accumulation. Binyameen et al. (2021) have also demonstrated a similar method that showed efficient suppression of CLCuKoV by simultaneously targeting multiple viral genes by multiplexed CRISPR/Cas9 thus reducing virus accumulation in host plants. In another study, Mubarik et al. (2021) also determined the virus accumulation in multiplex CRISPR/Cas9 transformed plants through real-time PCR and observed a 60–70% reduction in virus accumulation of CLCuKoV in transgenic plants. In another study, Khan et al. (2020) have demonstrated the reduced virus accumulation by 40–80% in *benthimiana* plants transformed with CRISPR/Cas9 against CLCuKoV. Similarly, Yin et al. (2019) used the CRISPR/Cas9 system to target and disrupt a specific region of the *rep* gene of CLCuMuV, which is known to be involved in the replication of the virus. By disrupting the *rep* gene, the researchers ultimately prevented the virus replication in transgenic *N. benthamiana* plants, conferring resistance to the virus. The researchers demonstrated that the edited

plants were healthy and showed no disease symptoms compared with control plants.

In the present study, Cas12a-MV vector was stably transformed in *Nicotiana tabacum* plants to analyze the virus accumulation in transgenic *N. tabacum* plants. The virus accumulation in transgenic plants was determined by real-time PCR and compared with non-transgenic plants. Virus accumulation is an important parameter to assess the efficacy of the CRISPR/Cas system. The transgenic plants containing Cas12a-MV constructs showed lower virus accumulation compared with control plants. Severe symptoms were appeared in control plants, but transgenic plants remained healthy. Yin et al. (2019) also stably transformed the Cas9-based multiplex vector in *benthimiana* against CLCuMuV and achieved the resistance to viral infection, evaluated through Southern blotting in transgenic plants. In contrast, we have used real-time PCR to determine the virus accumulation. Real-time PCR is a highly sensitive and specific method that allows real-time detection and quantification of a particular sequence of DNA. It is a fast and an efficient technique that requires minimal sample preparation and can detect low levels of target DNA with high precision. On the other hand, Southern blotting is a time-consuming and labor-intensive technique that requires a relatively large amount of DNA and may not be as sensitive as real-time PCR.

4.6 Future prospects

CRISPR/Cas technology and its recent developments provide a rapid and efficient solution for controlling begomoviruses infections in crops. As this study was performed in model plants, further experiments are required to translate these results in cotton. The prospects for CRISPR/Cas technology against begomoviruses are promising, but it is not free of challenges and limitations. One of the critical challenges that need to be addressed is the durability of the resistance to begomoviruses. Viruses are known to evolve rapidly and can develop resistance to control measures. The efficacy of CRISPR/Cas-mediated resistance would also require continuous monitoring, and multiple approaches such as CRISPRi and CRISPR/Cas14, with new gRNAs targeting coding as well as non-coding regions in virus could be helpful to limit virus escape. Another challenge is, an efficient delivery of the CRISPR/Cas reagents into plant cells. New delivery methods with improved efficiency and specificity of different CRISPR reagents especially in cotton, are critical for the practical applications of CRISPR/Cas technology to improve cotton against viral and other stresses. In conclusion, the prospects of CRISPR/Cas technology against begomoviruses are positive, but further research is required to realize its full potential and translate results with model systems, in commercial crops. In addition, regulatory outcomes of CRISPR edited crops in different countries will also determine the future of these crops and their public acceptance.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

AA and SK designed the study. SA performed the experiments and wrote the manuscript. JB provided the experimental protocols and proofread the manuscript. AJ and BS revised the manuscript and gave suggestions to improve the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1233295/full#supplementary-material

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GMOs or non-GMOs? The CRISPR Conundrum

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CRISPR-Cas9, the "genetic scissors", is being presaged as a revolutionary technology, having tremendous potential to create designer crops by introducing precise and targeted modifications in the genome to achieve global food security in the face of climate change and increasing population. Traditional genetic engineering relies on random and unpredictable insertion of isolated genes or foreign DNA elements into the plant genome. However, CRISPR-Cas based gene editing does not necessarily involve inserting a foreign DNA element into the plant genome from different species but introducing new traits by precisely altering the existing genes. CRISPR edited crops are touching markets, however, the world community is divided over whether these crops should be considered genetically modified (GM) or non-GM. Classification of CRISPR edited crops, especially transgene free crops as traditional GM crops, will significantly affect their future and public acceptance in some regions. Therefore, the future of the CRISPR edited crops is depending upon their regulation as GM or non-GMs, and their public perception. Here we briefly discuss how CRISPR edited crops are different from traditional genetically modified crops. In addition, we discuss different CRISPR reagents and their delivery tools to produce transgene-free CRISPR edited crops. Moreover, we also summarize the regulatory classification of CRISPR modifications and how different countries are regulating CRISPR edited crops. We summarize that the controversy of CRISPR-edited plants as GM or non-GM will continue until a universal, transparent, and scalable regulatory framework for CRISPR-edited plants will be introduced worldwide, with increased public awareness by involving all stakeholders.

KEYWORDS

CRISPR-Cas, gene editing, GMOs, GM regulations, transgenic plants

1 Introduction

Extreme weather patterns and climate variability have a negative impact on global food security for the growing world population. We must find new solutions and discover new technologies to meet the promises of food and nutritional security at the global level. CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/Cas associated

protein 9), a gene editing tool, has been predicted as a revolutionary discovery of the 21st century to reshape the genomic landscape of not only bacteria, but also animals and plants to achieve our goals in food security, therapeutics, and human health. Therefore, this technique grabbed the attention of scientists and private companies to engineer agricultural crops with climate resilience, disease resistance, and better nutritional profile. Similarly, CRISPR-Cas technology has been adopted universally for translational applications in human health, therapeutics, and product development. CRISPR-Cas as a gene editing tool uses endonuclease (known as Cas) recruited by a 20 bp guide RNA (gRNA) to introduce double-stranded breaks (DSBs) at a precise target sequence (complementary to gRNA), which results in specific and targeted genetic modification during DNA repair mechanisms (Figure 1; Hou et al., 2023). CRISPR has become a gold standard to create novel genetic variations by installing precise DNA modifications to introduce new and improved traits in animals and plants (Zaidi et al., 2019).

Applications of the CRISPR-Cas system in model and crop plants have become a routine to address emerging problems of insect/pests, disease resistance, and heat and drought tolerance in plants (He and Zhao, 2020). CRISPR-Cas has enabled precise changes in the genome, in a way never possible with traditional transgenic approaches. The technique is overcoming barriers and has the potential to fulfill the early promises of genetic engineering such as plants with higher yield, better biotic and abiotic resistance, disease and pest resistance, less water requirement, and more nutritious food (Bailey-Serres et al., 2019). CRISPR-Cas based techniques have been successfully used for proof-of-concept studies in model and crop plants for better yield and quality, herbicide resistance, and environment sustainability. Compared to traditional genetic engineering, where genetic modifications in the host genome were always random, CRISPR-Cas based modifications are precise, predictable, inheritable, and sometimes without introducing any external gene sequence in the host-genome. However, critics argue that both CRISPR technology and classic genetic engineering use the same transformation method (Agrobacterium and biolistic) to introduce genetic modifications and marker genes for the selection of transformants, which has created a debate among the scientific community if CRISPR edited crops should be considered genetically modified (GM) or not. However, marker genes could be removed from the CRISPR edited crops by crossing or transgene killer technology (Yubing et al., 2019). Similarly, off targets are also one of the potential concerns associated with CRISPR, especially in therapeutics and human health applications.

While CRISPR holds an incredible potential to rewrite the genomic landscape of agricultural crops, how CRISPR edited crops will be regulated will determine the future of this revolutionary technology. Regulation of CRISPR edited crops has lagged the pace of development and regulatory authorities are facing challenges in keeping with complexities and risk

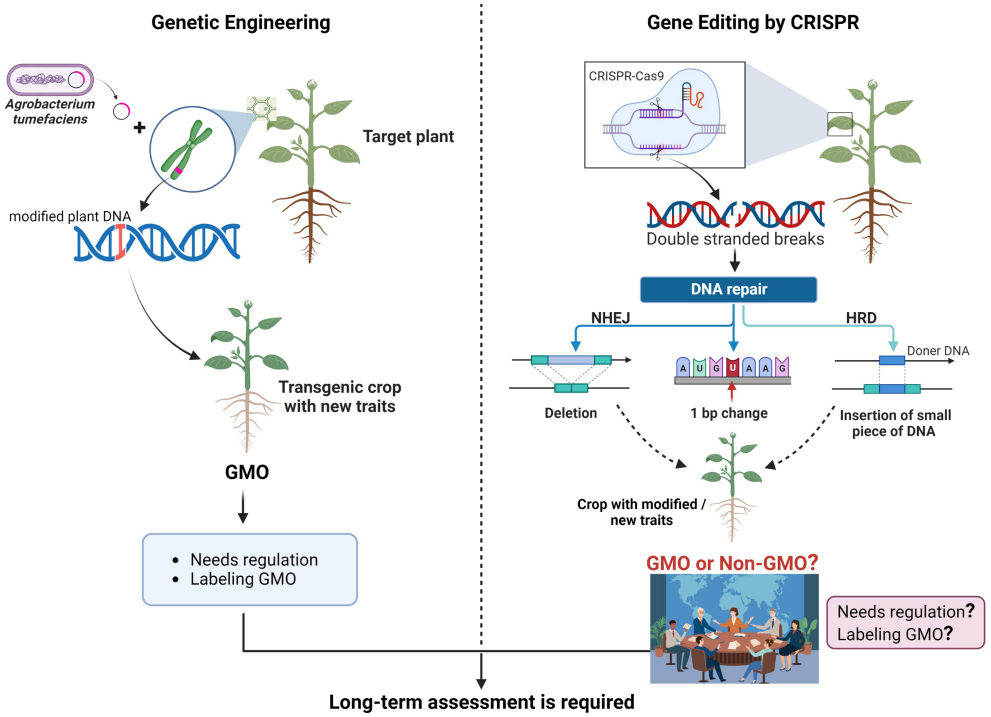

FIGURE 1
Comparison of traditional genetic engineering and CRISPR-Cas editing technique. Genetic engineering is used to introduce new traits in crops through the insertion of a gene from different species and produces GMOs which need strict regulations and product labeling (left side). CRISPR-Cas9 introduces a DSB in the target DNA and let the cell repairs itself to introduce small changes such as indels, which can be used to improve gene function(s). A small piece of DNA could also be introduced from the same species, or an existing gene could be modified without inserting any foreign DNA in the plant genome, to introduce a new trait in plants using CRISPR-Cas9 (right side).

evaluation. The pace of regulation is slower than the rate of scientific advancement in many jurisdictions, leading to the perception of gridlock in the system. The world community is divided over the policies, legal status, and regulatory requirements of the CRISPR edited crops. For example, the US and EU (European Union) have different opinions on the regulatory status of these crops. Nonetheless, the United States Department of Agriculture (USDA) decided to deregulate CRISPR edited crops, especially SDN1 (Site-Directed Nuclease 1) and SDN2 modified crops, because they do not contain any foreign DNA (transgene) and the modifications did not involve any pesticidal properties. USDA determined that CRISPR editing is equivalent to conventional breeding in some instances, thus does not require strict GMO (Genetically Modified Organism) regulations. For example, CRISPR-edited mushrooms developed by Yinong Yang at Pennsylvania State University in 2015 were approved by-passing the strict regulations of GMOs (Waltz, 2016a). As SDN1 and SDN2 modified plants are indistinguishable from conventionally bred plants, so SDN1 and SDN2 modifications are considered as non-GM plants by different countries such as the US, Argentina, and Brazil. On the other hand, European Union (EU) regulations follow a more precautionary approach and consider all plants, modified through either gene editing or genetic engineering, as GM even if they are free of any transgene. This decision may have serious implications on research, development, and commercialization of CRISPR technology in Europe (Hjort et al., 2021). Compared with SDN1 and SDN2 crops, SDN3 crops are always considered as GM and must pass through strict GMO regulations and risk assessment in many countries of the world. So far, the regulation of CRISPR edited crops varies significantly among different countries and this mosaicism in the regulation is partly due to the different definitions of genetic modifications and genetically modified organisms (GMOs) by various regulatory authorities and the world community (Wolt et al., 2016a). The lack of adequate regulatory and legal guidance on CRISPR edited crops has led to a debate on classification and legal status, particularly concerning whether these crops should be considered as GM or non-GM. In addition, the distinction between SDN1 and SDN2 modifications and point mutations created through spontaneous mutations or conventional methods is also an important part of the debate. Whether SDN1 and SDN2 modification and point mutations should be considered different from conventional GM crops or from each other, is an important discussion in the scientific community, regulatory authorities, and different stakeholders. It is also worth noting that none of the countries have designed a *de-novo* regulation of CRISPR edited crops, but the current regulations for CRISPR edited crops are based on the already existing regulatory framework of GMOs in different countries (Turnbull et al., 2021). Therefore, it is important to explore the current definition of genetically modified (GM) and gene edited (GE) plants to carefully determine the difference between GM and non-GM crops. A conducive, universal, and transparent regulatory framework, along with better social and consumer acceptance of CRISPR edited crops could have positive impacts on food security, environmental sustainability, and faster crop development.

2 Genetically modified or non-genetically modified?

Genetic modification is a broader term that involves different methods, such as traditional breeding and modern gene editing methods, to modify genetic composition of plants or animals to achieve the desired traits. Genetic engineering is a specific type of genetic modification that involves deliberate manipulation of an organism's genome through biotechnology techniques such as genetic engineering and gene editing. Transgenic plants are always produced through genetic engineering approaches by artificially inserting an exogenous DNA stretch into a plant genome, usually from an unrelated species to achieve a desired trait. For example, *Bt* gene(s) from *Bacillus thuringiensis* was transferred in different crops such as cotton and corn by genetic engineering, to improve these crops against insect attacks (Sarker et al., 2019; Figure 1). These plants, which undergo artificial DNA modifications to confer desired traits are called as genetically modified (GM) plants. Generally, scientists and regulatory authorities consider a plant as GM, if there has been a transfer of gene(s) in its genome, from distantly related species such as bacteria, insects, or animals, using biotechnology techniques (Rani and Usha, 2013). Conventional breeding and genetic engineering involve random and uncontrolled mutagenesis for introducing genetic changes to achieve desired traits in crops.

GM organisms have been used for various applications in basic and applied research. However, genetically modified crops and their respective food products have negative perceptions among the scientific community and the public due to their potential health concerns and lateral gene flow to non-target organisms, which could raise unknown environmental issues (Funk and Kennedy, 2016). In addition, the use of antibiotic resistant marker genes for the selection of transformants was also a concern in their public acceptance. Since the first release of GM crops in 1994, more than 70 countries have adopted GM crops for cultivating or importing to date (Srivastava et al., 2011; Borém et al., 2014; Wunderlich and Gatto, 2015; Turnbull et al., 2021; ISAAA Inc,). During 2020, 94% of soybean crops in the USA were GM having herbicide tolerance, while other major GM crops cultivated in the USA were cotton and corn (Edwards, 2020; Kumar et al., 2020; Brookes, 2022).

Despite the success of GM crops in improving agricultural productivity and addressing several other challenges, developers and agriculture companies are struggling for their better public acceptance and global commercialization because of their possible impact on the environment and human health (Raman, 2017; Ahmad et al., 2021). Majority of the transgenic crops contain genes from unrelated species, transferred through Agrobacterium, to improve crops against insects or to withstand herbicides. These crops could induce pest resistance by releasing toxins in soil and destroying crop biodiversity, thus could have an adverse environmental impact. GM crops having bacterial or insect genes have raised health concerns such as allergic reactions which have been reported in humans in different countries (Zhang et al., 2016). It is important to note that while there have been reports of allergic reactions and health concerns, there is lack of scientific evidences linking these concerns directly to consumption of GM crops (Dunn

et al., 2017). Scientific studies have not supported these claims about GM crops and regulatory agencies such as FDA (Food and Drug Administration), EFSA (European Food Safety Authority) and WHO (World Health Organization) conduct extensive regulatory and safety assessments of GM crops before their approval for commercial use. Therefore, GM crops approved by these regulatory agencies for commercial use are considered safe for human consumption.

GM crops containing DNA from other species must undergo a lengthy regulation and approval process (Figure 1; Halford, 2019). Therefore, multinational companies are exploring alternative biotechnological methods to improve crops without involving transgene transformation from unrelated species. For example, topical application of dsRNA has been used as a potential insecticidal approach for insect control in crops (Lu et al., 2023). Meanwhile, during the past decade, modern gene editing techniques involving various nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas9 using engineered nucleases emerged as new tools for site-specific DNA modifications in the genome (Li et al., 2020a). Although, ZFNs and TALENs have been used for targeted gene editing in plants, the CRISPR-Cas9 has emerged as a more powerful and versatile tool due to its simplicity, efficiency, and modularity. The detailed mechanism of action, applications, and limitations of different genome-editing techniques have been reported elsewhere (Munawar and Ahmad, 2022).

It is noteworthy that among different gene editing tools, CRISPR-Cas9 gained rapid popularity in crop improvement programs because of its simple design, less time consumption, cost-effectiveness, good reproducibility, high efficiency, precise targeting, and diverse applications. As shown in Figure 1, CRISPR-Cas9 simply introduces DSBs at the target site in the genome. DSBs in the genome provoke natural DNA repair systems such as non-homologous end joining (NHEJ), or homology-directed repair (HRD) in the cell, consequently introducing indels or precise insertions, respectively, at the target site in the host genome (Lin et al., 2014). While, CRISPR-Cas9 has been extensively used in plant gene editing, with remarkable efficiency and precision, many other CRISPR-Cas systems are also available, each with its unique properties and applications. These alternate CRISPR-Cas systems offer different advantages and capabilities that can be used for specific purposes and applications. For example, CRISPR-Cas12a recognizes a T-rich PAM and produces staggered ends at the DSB site. While Cas9 and Cas12 have been used specifically for DNA editing, CRISPR-Cas13 is an RNA editing system which can modulate expression at RNA level, without introducing any permanent change in the genome. Similarly, CRISPR-Cas14 offers a unique advantage in targeting ssDNA (single stranded DNA) instead of the usual dsDNA targeted by Cas9 and Cas12. CasX and CasY are relatively new members of the CRISPR-Cas family, which are being characterized for their potential applications in gene editing. CRISPR mediated base editing (BE) systems allow precise change from one nucleotide (A, T, G, C) into another, converting one DNA base pair into another, without causing DSB in the DNA. For example, CRISPR based adenine base editor (ABE) and cytosine base editor (CBE) can result in the conversion of adenine (A) to inosine (I) and cytosine (C) to uracil (U) respectively. The cellular repair system then converts inosine to guanine (G) and uracil to thymine (T), thus resulting in targeted and precise changes to the individual DNA bases in the genome. CRISPR based BE systems have shown a great promise to install precise modifications in the genome of crops to develop new crop varieties with improved traits (Gaudelli et al., 2017; Molla and Yang, 2019). CRISPR mediated prime editing (PE) is another innovative technique that allows precise insertion, deletion, or substitutions at the target site in the genome without causing DSBs. PE offers a greater control over genetic modifications, allowing researchers to make specific changes by directly writing new DNA sequences in the genome. It is a rapidly advancing field of research and its applications are being explored in crops for introducing new and desired traits (Anzalone et al., 2020; Li et al., 2020b). Very recently, CRISPR-like systems, such as OMEGA and Fanzor, have been identified in eukaryotes which may further improve gene editing with reduced off targets and improved efficiency (Altae-Tran et al., 2021; Saito et al., 2023). Desired traits in crops can be achieved through CRISPR-Cas by utilizing nature generated genetic variations present in the genomes of non-modified plants. For example, SDN1 and SDN2 genome edited plants which can be generated through targeted modifications of the plant's own genes without permanently integrating DNA in the plant genome may arise from spontaneous mutations or can be achieved through classical breeding. So, CRISPR edited SDN1 and SDN2 plants are generally characterized as non-GM, because they are not based on introducing new genes in the host plant to obtain desirable traits, thus making them more acceptable as compared to the plants generated through conventional genetic engineering (Abdallah et al., 2015; Jones et al., 2022). Compared to GM crops, non-GM crops have certain benefits associated with those such as faster development, precise modifications in the genome, absence of transgenes, predictable outcomes, and reduced regulatory challenges. In contrast, SDN3 crops are produced by providing a donor template containing large DNA fragment such as transgene or cis-gene and are regulated under strict GMO regulations (Georges and Ray, 2017).

Regardless of the rapid development of CRISPR-Cas technology and its potential applications in editing the genomes of model and crop plants since 2013 (Upadhyay et al., 2013), only a few CRISPR edited crops have reached the market so far (Hazman, 2022). Although CRISPR-Cas has been presented as a precise genome editing technique, off-targets remain a potential concern in the scientific community, especially in therapeutics, human health, and product development (Cribbs and Perera, 2017; Omodamilola and Ibrahim, 2018). However, off target effects can be mitigated by several approaches ranging from careful gRNA design to modifications in experimental strategies and different molecular diagnostic tools to detect and quantify off targets. In plants, off targets are not a major concern, because any off-target mutations are likely to be segregated out during subsequent breeding and selection steps (Yee, 2016). Several researchers have generated CRISPR edited plants without any off targets (Nekrasov et al., 2017). Scientists have also expressed concerns about CRISPR

based gene drives due to their potential environmental impact as these will be difficult to control once released into the environment (Mueller, 2019). However, CRISPR/Cas based gene editing is continuously evolving with new tools, having diverse functions which could be helpful in addressing these challenges in the future (Zong et al., 2022). Nevertheless, the main challenges to bring CRISPR edited crops in the market are consumer acceptance, a universal regulatory system, transparent policies, and public awareness about gene edited plants.

Both CRISPR mediated gene editing and traditional genetic engineering lead to genetic modifications, however, CRISPR based modifications are very precise, predictable, free of transgene, and sometimes as small as a single base pair (bp) editing in the entire genome. In addition, unlike transgenic methods where foreign genetic elements are always present such as marker genes (Zhang et al., 2020), CRISPR-Cas editing does not necessarily introduce foreign DNA elements in the host genome, but it depends upon the type of CRISPR-Cas reagents (Cas9 and sgRNA) and their delivery methods. The use of Cas9/sgRNA plasmid DNA for CRISPR-Cas gene editing is an efficient and simple method, nevertheless, it is not free of limitations (Liu et al., 2017; Eoh and Gu, 2019). The large size of plasmids (9–19 kb) and their permanent integration in the host genome may result in continuous expression, leading to higher off-target effects. Using *in-vitro* transcribed mRNA offers several advantages such as transient expression, reduced off-targets, and less risk of integration in the genome (Li et al., 2014; Jiang et al., 2017; Glass et al., 2018; Son and Park, 2022). But the poor stability of mRNA and reduced efficiency of gene editing with mRNA are the major limitations of this approach. Ribonucleoprotein (RNP) based CRISPR-Cas system has also been used for CRISPR applications in plants, which results in obtaining transgene-free plants, which are not considered GM according to one concept (Távora et al., 2022). For example, Cas9 protein and sgRNA as mRNA were delivered through lipofection in plants to obtain transgene free CRISPR edited plants (Zhang S. et al., 2021). The use of RNP does not introduce any foreign DNA sequence permanently into the plant genome and reduces off-target effects (Liu et al., 2017; Eoh and Gu, 2019). However, as the biotechnology process is used to install new modifications in the genome of the host plant, and the genome is "modified" it may be considered GM according to another concept (Kim and Kim, 2016).

Depending on the repair outcomes of DSBs, CRISPR mediated modifications are classified into three main categories: site-directed nuclease types 1, 2, and 3, known as SDN1, SDN2, and SDN3, respectively (Wolt et al., 2016b). In SDN1, DSBs are repaired through NHEJ repair system which introduces indels (adds or deletes nucleotides) without using any repair template. In SDN2, a microhomologies-mediated repair template is used to add, delete, or replace very few (2-10) specific nucleotides at the target site (Xue and Greene, 2021). The resultant plants in both SDN1 and SDN2 are indistinguishable from conventionally bred plants, and thus could be considered non-GM. Therefore, most countries like the US, Japan, India, Australia, and Ecuador consider SDN1 and SDN2 modified plants safe and do not regulate them under conventional GM regulations (Tachikawa and Matsuo, 2023). In SDN3, a repair template through homologous recombination is used to insert a gene segment or whole gene at the targeted site, resulting in transgenic or cis-genic plants, consequently, triggering regulatory oversight depending on the nature and origin of the introduced DNA segment (Friedrichs et al., 2019). CRISPR modifications are classified as SDN1, SDN2, and SDN3 based on the repair mechanism of DSB, and different countries have developed their regulatory framework to distinguish between these modifications. The legal and regulatory status of these crops vary from country to country, depending upon the definition of GMOs, existing regulations for GMOs, and the specific techniques used. For example, the US, Argentina, and Japan consider SDN1 and SDN2 edited plants as non-GM and deregulate them, however SDN3 edited plants are considered on case-by-case basis. In contrast, EU, and New Zealand, which use process-based triggers for regulating GM crops, determine CRISPR edited crops are same as conventional genetically modified plants, having transgenes. Therefore, every country has a distinct regulatory framework for CRISPR modified crops to address GM and GE controversies (Turnbull et al., 2021). In the following section, we have discussed the regulatory oversight of CRISPR edited crops in different countries.

3 Current global regulations of CRISPR edited crops

The world community has a strong division over regulatory triggers for GM crops (Sprink et al., 2016). There are two main regulatory triggers for the regulation of GM crops in the world: a product-based system and a process-based, while some countries are following a mixture of these two approaches, tailored to their needs (Sprink et al., 2016). The USA follows product-based regulations for GM crops, whereas the EU regulatory system is based on the method by which a product is made, without considering the traits expressed in the product (Jones, 2015; Wolt et al., 2016b; Jones et al., 2022). Compared with a process-based trigger, the product-based trigger is considered more straightforward, aligned with WTO, and reliable because any risk posed by the modified plant will be arising from the product itself but not from the method or technique used to generate it (Dederer and Hamburger, 2019). While Canadian regulation for genetically modified crops is based on plants with novel traits (PNTs). A novel trait in plants could be introduced through conventional breeding, genetic engineering, or gene editing.

CRISPR crops are touching global markets and some of those, especially SDN1 and SDN2 crops, have been approved by the US, Argentina, Japan, and Brazil (means that these crops are no longer considered regulated under the Plant Protection Act and can be marketed without the same level of regulatory oversight as traditional GM crops) bypassing the strict regulatory framework of conventional GMOs (Stoye, 2016; Unglesbee, 2016; Grossman, 2019; Menz et al., 2020). Deregulation of SDN1 and SDN2 crops in several countries in the world may accelerate the development of new crops with improved traits. However, SDN3 crops are still considered as GMOs and regulated under the conventional GM framework. Although CRISPR edited crops, especially SDN1 and SDN2, could be free of any transgene or foreign genetic elements (promoter or terminator), the debate persists on how to regulate

those crops and what precautionary measures are required before these crops appear in the market. Nonetheless, the difference in the scientific and legal communities on regulatory triggers of GM crops is also hindering the legislation for gene edited crops in other countries like Australia and European countries that insist to regulate GE crops like GMOs (Hamburger, 2019).

GMOs pass through strict regulations in many countries of the world, especially in the European countries due to the presence of foreign gene(s) and their potential risks to human health (Zhang et al., 2016). Public trust could be built only by providing clear and reliable scientific and legal information about the CRISPR-Cas technique and its possible impact in comparison to transgenic GM crops (Kato-Nitta et al., 2019). The current regulations for GM and GE crops and the responsible agencies for these regulations in different countries are shown in Table 1.

In the USA, USDA regulates GM and CRISPR edited crops if those contain a foreign DNA sequence from other species (Entine et al., 2021). As described earlier, the USA follows the product-based regulation of GMOs without any concern about the method used, focusing only on the traits expressed (McHughen, 2016; Sprink et al., 2016; Smyth, 2017). USDA statement about the regulation of GMOs "Under its biotechnology regulations, USDA does not currently regulate, or have any plans to regulate plants that could otherwise have been developed through traditional breeding techniques as long as they are developed without the use of a plant pest as the donor or vector and they are not themselves plant pests" (Grossman, 2019). Under this definition, base editing, deletion, and insertion from related species would not be regulated as GM by USDA. Therefore, several CRISPR-edited crops have been deregulated and approved for commercialization, bypassing the existing strict regulations of GMOs (Grossman, 2019). USDA in 2018 declared that it "does not currently regulate or have any plans to regulate" CRISPR-edited crops (Duensing et al., 2018). Although USDA is deregulating CRISPR-edited plants, experts still suggest that they need to consider regulatory, governance, and ethical oversight of CRISPR edited crops (Cotter and Perls, 2018). In addition, CRISPR based gene drives, multiplex gene editing crops, and crops with permanently integrated markers and Cas9 gene should pass through strict regulations (Arora and Narula, 2017). Similarly, in Israel, National Committee for Transgenic Plants

TABLE 1 Genome editing regulations in different countries and their regulatory agencies.

Country	Regulatory Agency	Genome Editing Regulations	SDN1	SDN2	SDN3	Approved Crops	References
United States	USDA, APHIS, FDA, EPA	New SECURE Rules (2020) Coordinated Framework for the Regulation of Biotechnology; Plant Protection Act; National Environmental Policy Act; Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA	Deregulated	Deregulated	Case-by-case different	Maize, Tomato, Soybean, Mushroom, Flax	Wolt and Wolf, 2018; Ahmad et al., 2021; Turnbull et al., 2021
Argentina	Argentine Biosafety Commission (CONABIA)	Resolution No. 173/15 (2015)	Deregulated	Deregulated	Deregulated (if not transgenic)	–	Lema, 2019; Whelan et al., 2020
Australia	Food Standards Australia New Zealand (FSANZ)	Gene Technology Amendment (Measures No. 1) to regulations (2019)	Deregulated	Regulated	Regulated	–	Turnbull et al., 2021
New Zealand	Environmental Protection Authority (EPA), Food Standards Australia New Zealand (FSANZ)	Hazardous Substances and New Organisms (HSNO) Act 1996	Regulated	Regulated	Regulated	–	Turnbull et al., 2021
Japan	The Ministry of Agriculture, Forestry and Fisheries (MAFF), the Ministry of Health, Labour and Welfare (MHLW), the Ministry of Environment (MOE)	Handling Procedures MHLW: Food Hygiene Handling. Procedures for Food and Additives Derived from Genome Editing (2019); Notification by MOE: Handling of organisms obtained through the use of genome editing technology that do not fall under "genetically modified organisms" as defined in the Cartagena Act(2019)	Deregulated	Deregulated	Regulated	Tomato	Igarashi and Hatta, 2018; Menz et al., 2020
Brazil	National Technical Commission for Biosafety (CTNBio)	Normative Resolution No. 16 (2018)	Deregulated	Deregulated	Deregulated (if not transgenic)	–	Gatica-Arias, 2020
Canada	Canadian Food Inspection Agency (CFIA)	Food and Drug Regulations (Division 28 of Part B) Directive 94-08 (CEPA) Seeds Act; Part V of the Seeds Regulations	Case-by-case (by novelty)	Case-by-case (by novelty)	Case-by-case (by novelty)	–	McHughen, 2016; Smyth, 2017

(Continued)

TABLE 1 Continued

Country	Regulatory Agency	Genome Editing Regulations	SDN1	SDN2	SDN3	Approved Crops	References
		Directive 95-03, Guidelines for the Assessment of Novel Feeds: Plant Sources Health. Canada's Guidelines for the Safety Assessment of Novel Foods Volume II					
EU		Directive 18/2001/EC (2001) after court decision in case C-528/16	Regulated	Regulated	Regulated	–	Menz et al., 2020
Israel	The National Committee for Transgenic Plants	Seed regulations 5765– 2005 (Genetically Modified Plants and Organisms) (2005) after decision of the National Committee for Transgenic plants (2017)	Deregulated	Deregulated	Transgenic: Regulated Cisgenic: Deregulated	–	Turnbull et al., 2021
Colombia	Colombian Agricultural Institute (ICA)	Resolution No. 00029299 (2019)	Case-by-case	Case-by-case	Deregulated (if not transgenic)	–	Turnbull et al., 2021
Honduras		Agreement SENASA 008-2019 (2019)	Case-by-case	Case-by-case	Deregulated (if not transgenic)	–	Gatica-Arias, 2020
Chile	Ministry of Agriculture's Agricultural and Livestock Services (SAG)	Introduction of methodological procedure (2017)	Deregulated	Deregulated	Deregulated (if not transgenic)	–	Turnbull et al., 2021
China	Ministry of Agriculture and Rural Affairs (MARA), National Biosafety Committee (NBC),	Regulations on Administration of Agricultural Genetically Modified Organisms Safety	Under development	Under development	Under development	Soyabean	Cao, 2018; Chen and Dai, 2020
India	Indian Ministry of Science and Technology (2020), Genetic Engineering Appraisal Committee (GEAC)	Draft Document on Genome Edited Organisms: Regulatory Framework and Guidelines for Risk Assessment (2020)	Under development	Under development	Under development	–	Turnbull et al., 2021
Pakistan	National biosafety committee		Under development	Under development	Under development	–	Babar et al., 2020

decided not to regulate GE crops, however, product developers must demonstrate that no foreign DNA has been inserted in the plant genome (Menz et al., 2020). Canadian regulatory system is based on plants with novel traits (PNTs) and remained unchanged with the emergence of gene edited crops (Smyth, 2017). PNTs is a flexible and product-oriented system in which plant products are subjected to regulation depending upon the novelty of the trait, irrespective of their production method. However, under PNTs regulation, all products are evaluated for their allergenicity and toxicity (Eckerstorfer et al., 2019). Argentina follows a flexible regulatory system based on the presence or absence of a transgene. If a transgene or a new combination of genetic material is present, it will be considered GMO, while if no transgene is used, the product will be considered non-GMO. Similarly, if a transgene was used but has been removed from the final product through crossing, the product would be considered non-GMO (Entine et al., 2021). In contrast to the USA, where genetic material determines the status of a plant as GMO, the EU defines GMO as any organism created through genetic modification technology (McHughen, 2016). In addition, labeling of products as GM foods is mandatory in the EU and any food must also be labeled as GMO if the source ingredients are attained from GMO, even if no GMO is present in the final product (Castellari et al., 2018). Based upon these regulations, the European Court of Justice (ECJ) ruled out that CRISPR edited plants would be considered GMOs and would pass through existing process-based regulations for GMOs (Hamburger, 2019). Similarly, the Australian government has decided to regulate the gene edited crops with foreign DNA integrated into the genome as GMO (especially constructed by SDN2 and SDN3), however, gene edited products with no foreign DNA present in them (constructed by SDN1) could be considered safe and exempted from regulations (Zhang Y. et al., 2021). So far, China has no formal regulation for gene edited crops, but the country has invested heavily in gene editing technology, showing its intention to develop its own gene edited crops (Hundleby and Harwood, 2022). While Chinese authorities are monitoring

carefully how the USA is regulating gene edited crops, it could be expected that China would have flexible regulations for gene edited crops. So, none of the countries have developed an entirely new regulatory framework for gene edited crops, but most of these frameworks are based on existing GMO regulations. Therefore, with the current lack of adequate legal guidance and a universal scalable regulatory system for CRISPR edited crops, the situation will remain uncertain about the regulatory status of gene edited crops (Pixley et al., 2022). Although the deregulation of CRISPR edited crops and self-determination of exemption status (SECURE rule) by USDA has accelerated trait improvement through this cutting-edge technology both in the public and private sectors, opponents are also concerned that companies may mislead regulatory authorities and market their GM crops through this exemption from regulatory oversight. In addition, unintentional modifications may pass unnoticed by this self-determining exemption, consequently posing risks. Moreover, self-determination of exemption by the GM crops developers would have a significant impact on consumers, especially regarding the safety of food products.

In our opinion, the rapid rise in CRISPR-Cas technology and its ability to redesign the genomic landscape for crop improvement needs a clear, universal, and scalable regulatory framework to accommodate future developments in CRISPR such as synthetic biology applications, multiplex gene edited crops and gene drives in crops. In addition, the regulatory and legal status of point mutations and base edited CRISPR crops (free of any transgene), must be defined to establish a clear and consistent regulatory framework for CRISPR edited crops. In addition, it will help researchers, developers and farmers to understand their requirements for commercialization and consumer acceptance of their crops. Point mutations in the crop genome that improve food quality could be exempted from strict GM regulations. For example, the deletion of a few base pairs from polyphenol oxidase (PPO) gene reduces 30% activity of the enzyme resulting in brown-resistant mushrooms (Waltz, 2016a). Similarly, waxy corn having high amylopectin was produced by knocking out *Wx1* gene (Waltz, 2016b). *Camelina sativa* (false flax) was also modified with improved omega-3 oil content (Waltz, 2018). All these studies highlight the potential of CRISPR-Cas technology to produce transgene free crops with small modifications in their genome to improve the existing traits as well as introducing new traits in the crops to meet the challenges of food security. The production of nicotine-free non-transgenic tobacco using CRISPR-Cas9 could be utilized to facilitate people in their efforts to reduce their nicotine addiction (Schachtsiek and Stehle, 2019). It is worth mentioning here that a strong link between specialized scientists, the public, and legislation authorities is required to assist policymakers in developing unambiguous and transparent regulations for GE crops and make edited crops reliable and acceptable for consumers. Nonetheless, the long-term effects of GM and GE crops should be evaluated before bringing these crops to the commercial market.

4 Conclusion

Although CRISPR-Cas technology holds an incredible potential for developing new crops with improved traits, however, several challenges persist, such as efficient delivery of CRISPR reagents, consumer acceptance, intellectual property rights, trait stacking and combinatorial editing, and different jurisdictions of CRISPR edited plants, to fully realize the potential of this revolutionary technology. Moreover, the difficulty in detection and traceability of CRISPR edited SDN1 and SDN2 crops is an important consideration in regulation, labelling, and commercialization of these crops. With the rapid rise in CRISPR technology, the old paradigms and regulatory frameworks of conventional GMOs should be reevaluated to accommodate new developments such as transgene free CRISPR edited crops with precise and point mutations. Thus, it is important to enhance international coordination among all stakeholders including scientists, policy makers, regulatory authorities, politicians, farmers, industry representative, and public to revisit the regulatory framework. All stakeholders must be engaged for globally harmonized definitions and regulatory policies for precise genetic modifications and increased public awareness to address the unique challenges about regulation of gene edited crops. It will be worth observing the European Commission's anticipated new policies in the coming years. EU policies about GE crops will have a high impact on R&D and innovation of technology. Development of a universal, transparent, scalable, and mutually agreed-upon regulation for gene edited plants, holds a tremendous potential to address the global challenges related to food security, sustainable agriculture, and the growing world population. At the same time, ethical responsibilities like self-determination of exemption of CRISPR modified crops by farmers and agricultural companies should be controlled by strict monitoring. The long-term effect of GE crops should not be ignored for a healthier and sustainable environment.

Author contributions

NM and AJ conceived the idea. AA wrote the manuscript. NM finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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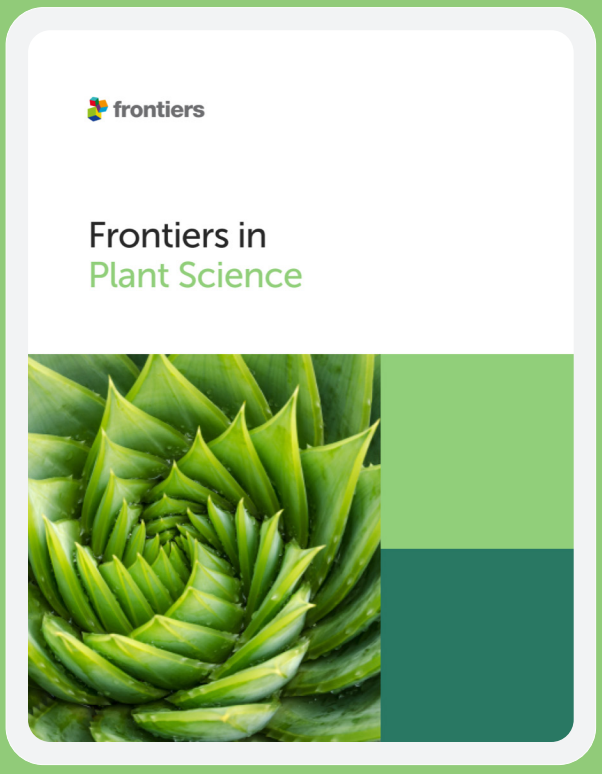

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