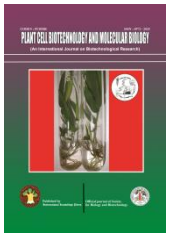

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BREEDIT: A Hybrid Approach to Advance Crop Breeding

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Ensuring food security in the face of a rapidly growing global population and adapting to climate change is one of the most paramount challenges for 21st-century agriculture. Although advanced technologies are being employed to tackle this issue, conventional breeding is reaching its limits in terms of achieving significant crop improvement. The advent of CRISPR/Cas9-based gene-editing technologies has revolutionized plant breeding, providing a powerful tool to accelerate genetic advancements. However, many essential traits are regulated by complex networks of multiple

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small-effect genes, making their improvement challenging. To overcome these obstacles, there is need of introducing the gene discovery platform *BREEDIT*, an innovative system that integrates multiplex genome editing with traditional breeding methodologies. By enabling the simultaneous editing of entire gene families, *BREEDIT* bridges the gap between classical breeding and advanced genetic engineering. This approach accelerates the development of improved crop varieties by generating high-throughput mutant lines with desirable traits, such as enhanced yield and resilience to biotic and abiotic stresses. The application of *BREEDIT* spans staple crops such as maize, wheat, barley, and rice, demonstrating its transformative potential for global food security and sustainable agriculture. This review explores the core principles, applications, benefits, challenges, and future prospects of *BREEDIT*, establishing it as a pivotal innovation in next-generation crop improvement strategies.

Keywords: CRISPR/Cas9; BREEDIT; multiplex editing; traditional breeding.

1. INTRODUCTION

The increasing global population, coupled with climate change, poses significant challenges to food production due to extreme temperatures, water scarcity, and shrinking arable land (Zhang and Cai, 2011; Long et al., 2015; Brás et al., 2021). Over the past century, conventional breeding has played a crucial role in adapting crops to local conditions while improving yields under stress (Nuccio et al., 2018; Snowdon et al., 2021). Genomics-assisted breeding has further advanced crop improvement by leveraging haplotype information in breeding programs (Bhat et al., 2021). However, traditional breeding approaches are nearing their limits as gene discovery and allele introgression cannot keep pace with stress-induced yield losses. Innovative strategies are essential to integrate traditional breeding with molecular biology to enhance complex traits like yield. Crop yield results from interactions between environmental factors and genetically regulated growth processes (Elias et al., 2016). Traits such as seedling vigor, root architecture, biomass allocation, and resource use efficiency significantly influence yield. While some traits, like disease resistance, are controlled by a few key genes (Poland and Rutkoski, 2016), most yield-related traits are governed by numerous small-effect genes (Mickelbart et al., 2015). Breeding focuses on recombining genetic material for better performance, while molecular biology explores gene function. Genetic variants associated with improved traits are often identified through phenotypic-genotypic correlations in multi-location field trials (Rasheed et al., 2017). Techniques like genome-wide association studies (GWAS) and genomic prediction have enhanced breeding accuracy and speed, but the underlying genes for many QTLs remain unidentified (Voss-Fels and Snowdon, 2016). Advances in integrating molecular -omics data with GWAS now allow the mapping of complex molecular networks (Baute et al., 2015, 2016; Xiao et al., 2016; Miculan et al., 2021).

Throughout agriculture's history, mankind have chosen plants with the best qualities that result in higher productivity or better adaptation to new environments. Conventional breeding is based on exploiting genetic diversity obtained by crossing varieties with desirable characters, then analyzing phenotypic variance among their offspring and selecting individuals with enhanced attributes. However, this is a complex process that involves numerous generations of crossing; it might take years to get a new variety, and the results are generally only minor improvements. Furthermore, several yield-related attributes are controlled by multiple minor genes with redundant functions in plant development, or by many diverse genes acting in the same pathway, each with a minor effect on the final phenotype (Mickelbart et al., 2015), implying that several chromosomal segments must be combined to attain significant improvements. Modern genomics methods promise to significantly increase the speed and precision of plant breeding (Van Vu et al. 2022), but further advances are required to fully reap their benefits (Gaillochet et al., 2021).

Model organisms such as *Arabidopsis thaliana* and rice have been instrumental in elucidating molecular pathways underlying traits like seed development, drought tolerance and plant architecture. Studies have shown that modifying specific regulatory genes can improve yield-related traits (Gonzalez et al., 2012; Vercruysse et al., 2020). However, high-throughput approaches by agro-biotech companies have often resulted in inconsistent or minor field-level improvements, emphasizing the challenge of

translating molecular insights into tangible crop gains (Paul et al., 2018; Simmons et al., 2021). Gene expressivity plays a crucial role in crop improvement, as genes operate within complex regulatory networks. While individual gene perturbations often have limited effects due to network buffering, combining multiple gene modifications can lead to additive or synergistic effects. For instance, combining growth-related genes in *Arabidopsis* and maize significantly enhanced leaf and seed size (Sun et al., 2017; Vanhaeren et al., 2017). However, identifying optimal gene combinations remains resource-intensive, especially for higher-order combinations. CRISPR/Cas9 technology offers a promising solution by enabling simultaneous editing of multiple genes, creating variability for complex traits. Successful applications of CRISPR in species like tomato, wheat, rice, and maize have demonstrated its potential to reshape plant architecture and improve traits (Meng et al., 2017; Rodríguez-Leal et al., 2017; Doll et al., 2019). Large-scale CRISPR screens in crops such as maize, soybean, and rice have further expanded its utility (Bai et al., 2020; Liu et al., 2020).

In this context, Christian Damian Lorenzo and colleagues (Lorenzo et al., 2022) introduce BREEDIT, a new tool that speeds up plant breeding by accelerating the discovery of genetic determinants that control agronomically important traits (e.g. yield and stress tolerance). The BREEDIT approach merges traditional breeding with advanced gene-editing technologies, offering a transformative solution for crop improvement. By integrating multiplex CRISPR editing with strategic crossing schemes, BREEDIT accelerates the identification and manipulation of genetic determinants that regulate complex traits. It enables the simultaneous knockout of multiple gene family members, making it possible to target networks of interacting genes for rapid, precise crop development. In maize, for instance, BREEDIT identified key gene knockouts linked to yield improvements within just two generations, demonstrating its efficiency and scalability. This approach bridges the gap between conventional breeding, which often requires years for modest improvements, and modern genomics tools, which, while accelerating plant breeding, still face limitations in fully exploiting complex gene networks. BREEDIT's ability to edit entire gene families at high throughput provides a powerful tool for accelerating the development of crops with enhanced yield, stress tolerance, and other desirable agronomic traits, positioning it as a key strategy for sustainable agriculture in the face of global challenges.

2. PRINCIPLES OF BREEDIT

BREEDIT merges the strengths of traditional breeding and CRISPR-mediated genome editing. This system focuses on editing multiple members of gene families that regulate specific agronomic traits, such as growth, stress tolerance, and yield optimization.

2.1 Multiplex Genome Editing

Multiplex genome editing refers to the simultaneous modification of multiple genes or genomic loci within an organism using advanced techniques like CRISPR/Cas9. This approach enables researchers to target several genes in a single experiment, increasing the efficiency and speed of genetic modifications. CRISPR-Cas based gene editing technologies allow the easy modification of two or more specific DNA loci in a genome with high precision. These tools have greatly increased the feasibility of introducing desired changes in specific but different genes, resulting in the development of new plant genotypes carrying multiple mutations in a single generation. It has emerged as a powerful tool for crop improvement, particularly in addressing complex traits controlled by multiple genes. In crop breeding, multiplex genome editing allows for the precise alteration of genes involved in critical traits such as yield, stress tolerance, disease resistance, and plant architecture. Targeting several genes at once, it can accelerate the development of crops with desirable traits, bypassing the lengthy process of traditional breeding, which often requires multiple generations to combine beneficial genetic variations. This technique is particularly useful for traits influenced by gene networks, where individual gene mutations may not have significant effects due to compensatory mechanisms in the plant's genetic network. By editing multiple genes simultaneously, multiplex genome editing can produce more pronounced and consistent phenotypic changes. Furthermore, it can help overcome redundancy in gene functions, where multiple genes with similar roles might mask the effects of a single mutation. By knocking out or modifying several related genes at once, it is possible to achieve substantial improvements in traits like growth, yield, and stress resistance.

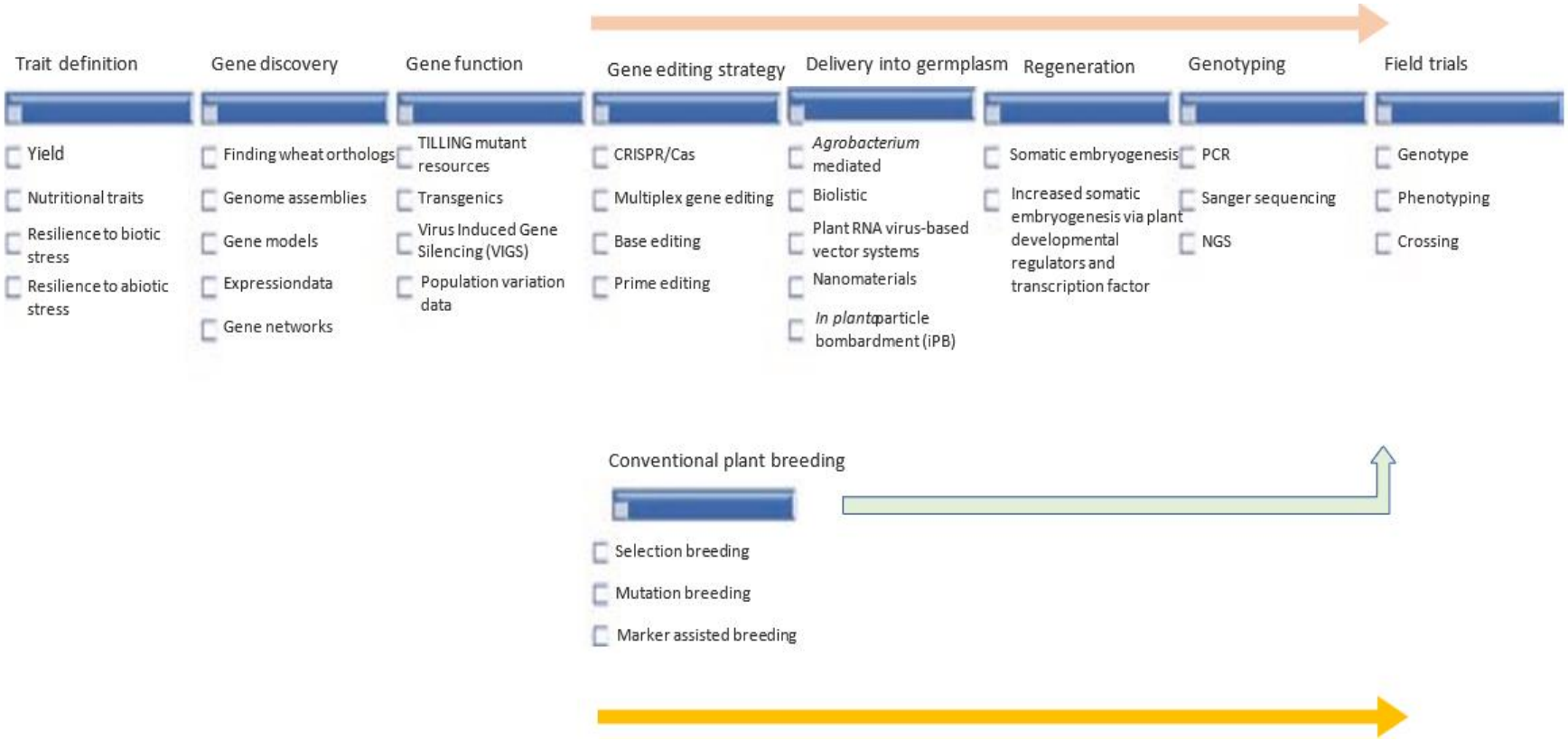

Fig. 1. Pathway for enhancing genetic traits through gene-editing and traditional breeding approaches

There are three primary approaches for generating multiple single-guide RNAs (sgRNAs) in gene-editing systems:

1. **Conventional Multiplex Strategy:** Individual sgRNAs are driven by separate U3 or U6 promoters.
2. **tRNA-Processing System:** This method utilizes the natural cleavage mechanism of transfer RNAs to produce multiple sgRNAs (Xie et al., 2015).
3. **Ribozyme-Processing System:** Self-cleaving ribozyme sequences are employed to release sgRNAs from a single transcript (Camerlengo et al., 2020).

The polycistronic tRNA-sgRNA system involves linking multiple sgRNAs using tRNA sequences, resulting in their transcription as a single RNA molecule. These tRNA sequences are subsequently recognized and cleaved by endogenous RNases, which process the transcript to release individual sgRNAs for functional use (Xie et al., 2015).

The ribozyme system utilizes a single transcript containing multiple sgRNAs, with each sgRNA flanked by self-cleaving ribozyme sequences, such as the hammerhead ribozyme (HH) and the hepatitis delta virus (HDV) ribozyme. These ribozymes autonomously cleave to release individual sgRNAs for subsequent activity (Zhou et al., 2019; Camerlengo et al., 2020). The cis-acting ribozymes cleave transcripts post-transcriptionally. A multiplex gene-editing strategy utilizing the tRNA-processing system has been applied in wheat to simultaneously target the TaGW2, TaLpx-1, and TaMLO genes (Zhou et al., 2021). A comparable tRNA-based approach was employed in a durum wheat cultivar (Gao et al., 2014) to edit the α-amylase/trypsin inhibitor subunits *WTAI-CM3* and *WTAI-CM16* in grains, aiming to reduce allergenic proteins. Similarly, (Abdallah et al., 2022) utilized this multiplex system to create *TaSal1* mutants, enhancing drought tolerance in wheat. Three primary multiplex CRISPR-Cas systems were evaluated for simultaneous gene editing at eight target sites in bread wheat (Li et al., 2021). Among these, the ribozyme and tRNA-based systems demonstrated greater efficiency in gene editing compared to the conventional multiplex system, where individual promoters drive separate sgRNAs (Li et al., 2021).

Within the BREEDIT framework, multiplex genome editing enables the precise targeting of multiple genes involved in complex traits. CRISPR/Cas9 technology is employed to edit several growth-related genes simultaneously, creating genetic variability for complex traits in a much shorter time frame. This is particularly effective for traits regulated by interacting gene networks or those exhibiting redundancy, such as yield-related traits. By enabling precise, high-throughput modification of multiple genes, BREEDIT accelerates crop productivity and resilience, addressing challenges like climate change and a growing global population. By targeting entire gene families, BREEDIT generates a wide spectrum of genetic knockouts (KOs).

2.2 Selection of Gene Family

The first stage of the BREEDIT method involves the selection of gene family members responsible for regulating a single trait. In this initial step, researchers identify and focus on genes that play a significant role in controlling a specific trait, such as negative growth regulators, which can impact plant growth and development (Lorenzo et al., 2023). By targeting a gene family rather than individual genes, the approach allows for more efficient manipulation of complex traits that are controlled by multiple genes within a family. This stage is critical for setting the foundation of the breeding process, ensuring that the genes selected are key drivers of the desired trait and paving the way for more targeted and effective gene-editing interventions in later stages of the BREEDIT framework.

A gene family refers to a group of related genes that share a common ancestor and are typically involved in similar biological functions (Lespinet et al., 2002). These genes arise through the duplication of an ancestral gene, followed by divergence over evolutionary time, which can result in slightly different functions or specialized roles within the organism. Gene families often regulate complex traits and processes in organisms, and the genes within these families can have varying degrees of redundancy or functional overlap. For example, in plants, a gene family might include genes involved in the regulation of growth, stress tolerance or disease resistance. These genes might be involved in the same metabolic pathways or play roles in similar cellular processes, but they may have subtle differences in their activity or expression patterns.

Table 1. Summary description of the achievements made using multiplex genome editing (MGE) to revolutionize plant biology and crop enhancement programs.

Application target genes	Crop	Aim	Ref
Plant biology Knock-out of multiple loci Two targets at ssDNA tomato yellow leaf curl virus (TYLCV) viruses	Tomato	Virus biological control in crops	Ali et al., (2015)
OsGW2, *OsGW5*, *OsTGW6*	Rice	Superior grain size	Xu et al., (2016)
RAS-PDS1, *RAS-PDS2*	Banana	Demo of gene knockout in banana	Kaur et al., (2018)
OsSWEET11, *OsSWEET14*	Rice	Wide range disease resistance	Xu et al., (2019)
TMS5, Pi21, *Xa13*	Rice	Hybrid rice production and disease resistance	Li S. et al., (2019)
Multiplex base alterations Three sgRNAs pointing *ALS* and *FTIP1e*	Rice	Herbicide tolerance	Shimatani et al., (2017)
OsDEP1-T1, *OsDEP1*-T2, *OsACC*-T1, *OsALS*-T1, *OsCDC48*-T3, *OsNRT1.1B*-T1.	Rice	Herbicide tolerance	Li C. et al., (2018)
Two marks *StGBSSI*	Potato	Diminished amylose biosynthesis in potato	Veillet et al., (2019)
ZmALS1 and *ZmALS2*	Maize	Herbicide resistance	Li Y. et al., (2019)
BnALS1, BnALS3	Rapeseed	Herbicide resistance	Wu et al., (2020)
Multiple spots at *OsALS1*	Rice	Facilitating the directed evolution of plant	Kuang et al., (2020)
Multiple spots at *OsACC*	Rice	Herbicide resistance	Liu et al., (2020)
Breeding diversity and crop domestication Two sites at locule number (*Lc*) QTL + eight gRNAs targeting the promoters of *SlCLV3*, *S* and *SP*.	Tomato	Wild species domestication	Rodríguez-Leal et al., (2017)
FRUIT WEIGHT 2.2, OVATE, *BETA CYCLASE*, *LYCOPENE*, *MULTIFLORA, FASCIATED/YABBY, SELF PRUNING*	Tomato	Domestication of *Solanum pimpinellifolium*	Zsögön et al., (2018)
GN1A, GS3, *GW2*,	Rice	Domestication of *Oryza glaberrima*	Lacchini et al., (2020)
Increasing crop yield potential *OsGW2*, *OsTGW6, OsGW5*	Rice	Larger grain size	Xu et al., (2016)
OsGS3, OsGn1a, OsGW2	Rice	Larger grain size	Zhou et al., (2019)
GS3, *GN1A, GW2*	Rice	Increasing grain size	Lacchini et al., (2020)

In crop breeding and genetic modification, targeting entire gene families rather than individual genes allows for more comprehensive manipulation of traits, especially when multiple genes contribute to the same characteristic. By modifying multiple members of a gene family simultaneously, breeders can achieve more pronounced and consistent changes in complex traits.

2.2.1 Identifying target traits

- **Define the Trait of Interest**: First, clearly define the trait you want to improve, such as yield, drought resistance, disease tolerance or plant architecture. This helps to narrow down the gene families involved.
- **Understand Trait Complexity**: Recognize that complex traits like yield or stress tolerance are often controlled by networks of genes. Thus, identifying the key gene family members is crucial for targeting the right ones.

Several techniques can be employed to effectively select gene family members for this purpose:

Genomic Data Mining: GWAS is a powerful tool used to associate specific genetic variations with phenotypic traits. By analyzing large populations and identifying loci that correlate with the desired traits, breeders can pinpoint gene families

involved in these traits. This method allows for the identification of gene family members that influence complex traits (Li et al., 2019).

RNA sequencing: RNA-Seq provides insights into gene expression patterns across various conditions (Wang et al., 2009). By comparing the expression of genes across different environments or stress treatments, researchers can identify gene families whose members are upregulated or downregulated in response to specific stimuli. This can help select genes involved in crucial biological processes such as growth regulation or stress responses.

Functional Genomics: Techniques like CRISPR/Cas9 can be used to knock out specific members of a gene family to evaluate their role in regulating a trait. This helps determine which genes within the family are critical for the desired trait (Chen et al., 2019). Knockdown or knockout of gene family members allows researchers to assess their impact on plant growth, stress tolerance, or other important traits. Overexpressing genes or silencing their expression can be used to study the effects of specific gene family members on complex traits. This can help identify which members contribute most significantly to a trait, such as yield or resistance to abiotic stress.

Phylogenetic Analysis: Phylogenetic analysis can be used to study the evolutionary history of gene families (Yang et al., 2019). By analyzing conserved regions and evolutionary relationships, breeders can identify functionally similar genes that belong to the same family. This analysis helps ensure that the selected gene family members are evolutionarily related and likely to have similar biological functions. Identifying gene duplication events within a gene family is crucial because these duplications may lead to the emergence of gene family members with redundant or specialized functions. By understanding the timing and functional divergence of these duplications, researchers can better select gene family members that regulate complex traits.

Network Analysis and Systems Biology: Gene family members often act in networks, with genes interacting to regulate a trait. Systems biology approaches can be used to construct gene regulatory networks that include gene families. By integrating genomics, transcriptomics, and proteomics data, these networks help identify critical gene family members and their interactions, which are involved in regulating complex traits. For instance, if a gene family regulates a pathway involved in stress tolerance, selecting the most influential members can help improve a crop's ability to withstand environmental stress.

Comparative Genomics: Comparing the genomes of different species or varieties can help identify conserved gene families involved in similar traits. For example, studying the gene families that regulate drought tolerance in one species can help identify analogous genes in another species, facilitating the selection of gene family members for breeding. Using homology-based methods, researchers can identify genes in closely related species that regulate similar traits. This comparative approach can be valuable in selecting gene families with conserved functions across species.

Chromosomal Mapping: By constructing linkage maps based on genetic markers, researchers can map gene families to specific chromosomal regions. This enables breeders to select gene family members based on their position on the chromosome and their association with desirable traits.

2.3 Different Single-Guide RNAs and Primers Need to be Designed for Each Gene

In the BREEDIT method, after the initial selection of gene family members responsible for regulating a specific trait, the next critical step involves designing and selecting the appropriate single-guide RNAs (sgRNAs) for each target gene to enable precise genome editing (Lorenzo et al., 2023).

2.3.1 Designing sgRNAs and primers

The first step is to design single-guide RNAs (sgRNAs) that will guide the CRISPR/Cas9 system to the target regions within the genes selected in the previous step. The sgRNA is composed of a 20-nucleotide sequence that matches the genomic target site, adjacent to a protospacer adjacent motif sequence (NGG) (Hsu et al., 2014; Moreno-Mateos et al., 2015). This sequence is crucial for Cas9 to recognize and bind to the DNA for cleavage. The sgRNAs should specifically target the gene family members of interest without off-target effects. Computational tools like CRISPR design software (e.g., CRISPRscan, CHOPCHOP) can be used to identify optimal target sites with minimal off-target risks (Doench et al., 2016; Labun et al., 2019).

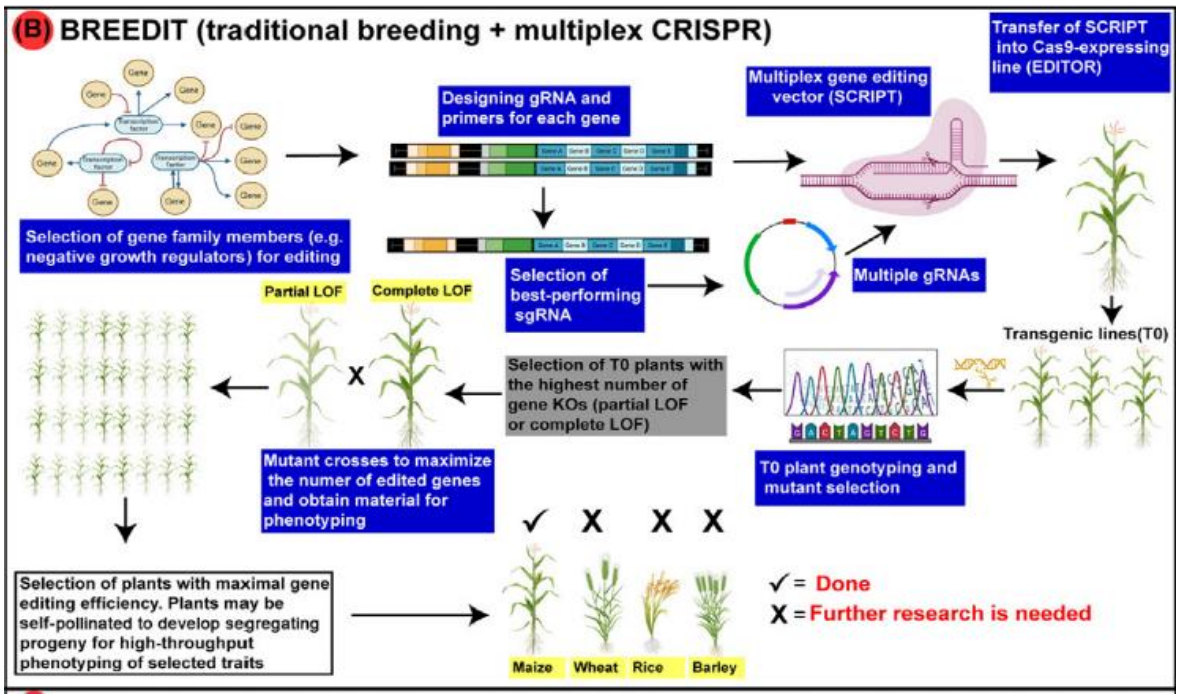

Fig. 2. BREEDIT: Quantitative Trait Improvement with Key Stages Highlighted (blue boxes)

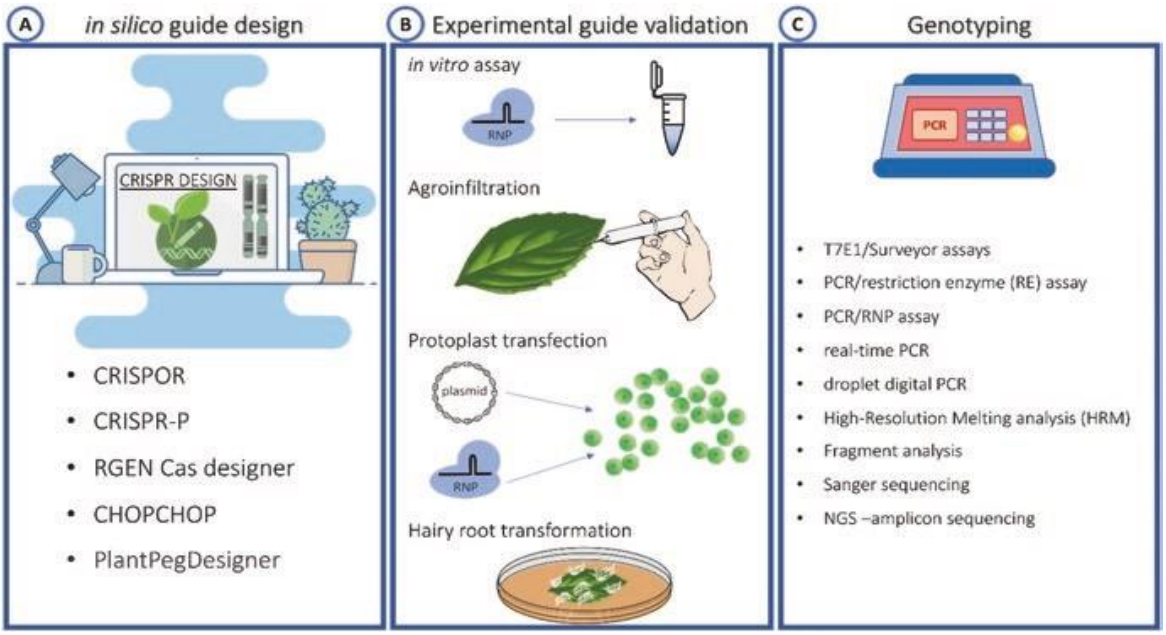

Fig. 3. Outline of CRISPR-mediated genome editing experiment design

(A) Schematic representation of the initial stage in CRISPR-mediated genome editing experiments highlights the utilization of bioinformatic tools for designing precise and efficient guide RNAs tailored to plant genomes. The guides that fulfill the required specificity and efficiency parameters are subsequently subjected to experimental validation, as illustrated. (B) Experimental validation techniques for the selected guide RNAs include an in vitro cleavage assay, which serves as a pre-screening tool to narrow down the number of guides for in planta validation. Depending on the plant species and the required genotyping depth, various methods such as agroinfiltration, protoplast transformation, or hairy root induction can be utilized. (C) Genotyping methods employed in genome editing experiments can vary depending on the specific needs of the study. Different techniques are available, allowing for selection based on factors such as precision, throughput, and the characteristics of the plant species involved.

The sgRNAs need to be highly efficient in inducing double-strand breaks at the target sites, which is crucial for successful editing. In parallel with the sgRNA design, primers are designed for PCR amplification of the target regions in the gene family. These primers will be used to validate the editing results after the CRISPR/Cas9 system has been introduced into the plant cells. Primers are designed to amplify the region of interest both before and after editing to assess the efficiency of gene modification.

2.3.2 Selection of optimal sgRNAs

Once a range of potential sgRNAs is designed for each target gene, they need to be tested for their efficiency and specificity. This is usually done through *in vitro* assays or through computational predictions based on sequence alignments and accessibility of the target region. *In vitro* validation: Before proceeding with cloning, the sgRNAs may be synthesized and tested in a model system or cell culture to confirm their ability to target the intended gene with high efficiency. Select Best Performing sgRNAs: Based on the validation results, the best performing sgRNAs those that produce the highest levels of target-specific genome editing with minimal off-target effects are selected for the next step.

2.3.3 Cloning sgRNAs into a multiplex gene editing vector

After selecting the optimal sgRNAs, they need to be cloned into a multiplex gene editing vector. A multiplex vector is designed to simultaneously express multiple sgRNAs in a single transformation event, allowing for the editing of several genes at once. Here's how it works:

- **Vector Construction**: The sgRNAs are inserted into a plasmid vector, which is designed to carry multiple sgRNAs. This vector also typically contains a Cas9 gene (or other genome-editing enzyme) that will perform the editing at the target sites. The vector should also include regulatory elements to ensure that the Cas9 protein and the sgRNAs are expressed effectively in the plant cells. Additionally, it's crucial to consider the vector backbone to ensure compatibility with the host plant species.
- **Efficient Delivery**: The vector containing the sgRNAs is then introduced into plant cells using a transformation technique like Agrobacterium-mediated transformation or biolistic particle delivery (gene gun). These methods allow for the integration of the vector into the plant genome or its transient expression, depending on the approach used. It's important to choose an appropriate delivery method based on the plant species and the desired outcome (stable or transient expression).
- **Design for Simultaneous Targeting**: In the case of multiplex editing, the vector is specifically constructed to enable simultaneous targeting of multiple gene family members. The number of sgRNAs cloned into the vector depends on the number of genes in the family that are to be edited. A vector could carry multiple sgRNAs, each targeting a different gene or locus within the gene family, allowing for efficient modification of several genes at once. It's also beneficial to consider the efficiency and specificity of each sgRNA to ensure effective editing across all target sites.

Subsequently, sgRNAs (SCRIPTS) are transformed into a Cas9-expressing plant line (EDITOR) to generate the T0 generation. This process involves introducing the multiplexed sgRNAs, along with the Cas9 gene, into the plant cells, which will then enable the precise editing of the targeted. The Cas9 protein, guided by the sgRNAs, will induce double-strand breaks at the specified genomic locations, leading to the desired genetic modifications.

2.4 Transformation of sgRNAs and Cas9 into the Plant Line (EDITOR)

The vector containing the sgRNAs (SCRIPTS) and Cas9 gene is introduced into the EDITOR plant line using a transformation method like Agrobacterium-mediated transformation or gene gun. The plant line used here, referred to as the EDITOR, is genetically engineered to express Cas9, the genome-editing enzyme, in a controlled manner (Lorenzo et al., 2023). Once inside the plant cells, the Cas9 protein is expressed and is guided by the sgRNAs to target specific regions of the plant's genome, inducing the desired mutations or edits.

2.4.1 Generation of the T0 generation

After successful transformation, the T0 generation refers to the first generation of plants that have been transformed with the edited

constructs (sgRNAs and Cas9). These plants will carry the edited genome, but the genetic changes have not yet been stabilized in subsequent generations. The T0 plants are grown and assessed for editing efficiency, and the desired edits are confirmed.

2.5 Crossing Schemes

In the BREEDIT approach, crossing schemes such as selfing and backcrossing are critical for stabilizing the edited traits and ensuring the inheritance of desirable genetic modifications across generations (Lorenzo et al., 2023). These classical breeding methods are integrated with multiplex genome editing (such as CRISPR/Cas9) to accelerate the development of improved crop varieties. The following outlines how selfing and backcrossing are utilized in BREEDIT:

2.5.1 Selfing in BREEDIT

Selfing, or self-pollination, is a breeding technique where an individual plant is crossed with itself to produce offspring. After applying multiplex genome editing to a crop, selfing is employed to produce homozygous plants, where the genetic edits are consistently passed down through generations. Since multiplex genome editing can generate heterozygous plants with modifications in multiple genes, selfing ensures that the edited traits are fixed in the plant's genome over time, allowing for more stable and predictable outcomes. Selfing helps in stabilizing edited traits by ensuring that the targeted genetic modifications are expressed uniformly in successive generations. This process is essential for establishing reliable phenotypic improvements, particularly for traits like yield or stress tolerance that are regulated by multiple genes. Selfing allows for the identification and tracking of the effects of genetic modifications across generations. This can be useful for monitoring the stability of edited traits, assessing gene interactions, and verifying whether the targeted traits are consistently inherited.

2.5.2 Backcrossing in BREEDIT

Backcrossing involves crossing an edited plant with a parent plant (often the recurrent parent) to integrate the desired genetic modification while maintaining the original desirable traits of the parent. One of the main goals of BREEDIT is to introduce multiplex genome-edited traits into elite breeding lines or commercially valuable varieties. By backcrossing, the edited plant is crossed with the recurrent parent, which may be a high-yielding or stress-tolerant variety. Successive backcrossing generations allow for the integration of the edited traits while maintaining the majority of the genetic background of the recurrent parent, ensuring that other desirable traits of the parent are retained. During backcrossing, plants that do not inherit the targeted gene edits or carry unwanted alleles are discarded. This helps to quickly purify the genetic background of the progeny while retaining the beneficial genetic edits. Backcrossing can thus refine the genetic makeup of the plant, making it more uniform and genetically stable. While conventional breeding may require multiple backcrossing generations to fix a trait in the genome, combining multiplex genome editing with backcrossing significantly reduces the time needed to incorporate complex traits into elite varieties. The precise nature of CRISPR/Cas9 editing accelerates the process of creating plants with the desired combination of edited and native genes.

By combining selfing and backcrossing with multiplex genome editing, BREEDIT accelerates the development of improved crop varieties, providing a more precise and efficient pathway for crop improvement than traditional breeding methods alone. This integration allows breeders to rapidly harness the potential of advanced molecular biology tools while maintaining the genetic diversity and stability needed for successful crop production. Mutant populations are strategically crossed to combine edited genes and evaluate their phenotypic effects. This allows researchers to assess additive or synergistic interactions among gene edits.

High-Throughput Phenotyping and Genotyping: Advanced phenotyping tools are used to measure yield-related traits, such as leaf size, seed weight, root architecture, and overall biomass. Genetic analyses are performed to link observed phenotypes with specific gene edits, enabling the identification of promising candidates for breeding.

2.6 High-Throughput Phenotyping and Genotyping in BREEDIT

In the BREEDIT approach, high-throughput phenotyping and genotyping are key components that significantly enhance the efficiency of crop improvement by enabling precise, large-scale assessments of both genetic makeup and

phenotypic performance (Lorenzo et al., 2023). These technologies work in tandem with multiplex genome editing to facilitate rapid and accurate selection of elite plants with desirable traits.

2.6.1 High-throughput phenotyping

Phenotyping involves the measurement of observable traits, such as growth, yield, stress tolerance, and disease resistance, which are influenced by both genetic factors and environmental conditions. High-throughput phenotyping (HTP) in BREEDIT enables the rapid collection of large amounts of phenotypic data, essential for evaluating the effects of multiplex genome editing on multiple traits. Technologies like unmanned aerial vehicles (UAVs), drones, and robotic platforms are employed to capture phenotypic data on large numbers of plants. Sensors and imaging tools (such as multispectral, hyperspectral, and infrared cameras) can monitor a wide range of traits, including plant height, leaf area, root architecture, chlorophyll content, biomass accumulation, and drought response (Furbank et al., 2011). This allows breeders to evaluate a vast number of plants in a short time, significantly accelerating the phenotypic evaluation process. The high-throughput phenotyping platform is essential for evaluating the phenotypic effects of genetic modifications made through multiplex genome editing. BREEDIT uses the data to identify plants that exhibit the desired traits—such as improved yield or enhanced resistance to environmental stress. These plants are then selected for further breeding, speeding up the process of identifying elite genotypes. In addition to assessing general growth traits, HTP can also assess how plants perform under stress conditions, such as drought, heat, or salinity. BREEDIT can leverage this data to identify edits that enhance stress tolerance, a crucial factor in addressing challenges posed by climate change.

2.6.2 High-throughput genotyping

Genotyping refers to the analysis of the genetic makeup of an individual to identify specific genetic variants, alleles, or mutations. High-throughput genotyping (HTG) in BREEDIT is used to quickly and accurately assess genetic diversity, validate genetic edits, and track inheritance patterns of edited traits across generations. After growing the T0 plants, the next step is to genotype them to determine whether the targeted genetic modifications have been successfully incorporated into the plant's genome. After the T0 generation is genotyped, the plants with the highest editing efficiency—those that exhibit partial or complete loss-of-function mutations in the target genes—are identified. These plants are considered the best candidates for further breeding. Complete loss-of-function (LOF) mutations refer to cases where the gene is entirely knocked out, resulting in a total loss of its function. Partial LOF refers to mutations that reduce the gene's function but do not eliminate it completely. Genotyping involves extracting DNA from the T0 plants and performing PCR amplification and sequencing of the target gene regions. This helps confirm whether the desired edits (such as insertions, deletions, or mutations) are present (Lorenzo et al., 2023).

HTG technologies, such as next-generation sequencing (NGS) and SNP arrays, allow for comprehensive genetic screening of large plant populations. These technologies identify genetic variations and track the edited genes across breeding populations. By sequencing the genomes of large numbers of plants, breeders can efficiently identify plants that carry the desired multiplex edits, such as those conferring improved yield or stress tolerance. One of the primary applications of HTG in BREEDIT is the validation of gene edits made via multiplex CRISPR/Cas9. After performing genome editing, HTG technologies help confirm the presence, absence, and specificity of the targeted genetic modifications in the plants. This ensures that only the desired changes have been made without off-target effects. HTG enables the identification of QTLs (genetic regions linked to specific traits) in the context of complex traits like yield or drought resistance. By correlating genotypic data with phenotypic data obtained from high-throughput phenotyping, breeders can identify genetic loci associated with these traits, which can be targeted in future breeding programs. HTG allows breeders to track the inheritance of both the edited genes and native traits from the recurrent parent in backcrossing programs. Through marker-assisted selection (MAS), plants that carry the desired genetic edits can be identified early in the breeding cycle, accelerating the introgression of beneficial traits into elite varieties (Collard et al., 2008). HTG facilitates screening of large populations for favourable alleles and gene edits, enabling breeders to rapidly select individuals that exhibit the best combination of genetic traits. This significantly shortens breeding cycles and improves the accuracy of selection.

The integration of high-throughput phenotyping and genotyping in BREEDIT creates a powerful feedback loop for accelerating crop improvement (Tardieu et al., 2017).

3. CONCLUSION

BREEDIT represents a paradigm shift in modern crop improvement by seamlessly integrating the precision of CRISPR/Cas9-based multiplex genome editing with the efficiency of traditional breeding techniques. By enabling the simultaneous modification of multiple genes and leveraging advanced phenotyping and genotyping platforms, it accelerates the development of resilient, high-performing crop varieties with enhanced yield, stress tolerance, and disease resistance. Its ability to address complex traits controlled by interconnected genetic networks and to stabilize these improvements through selfing and backcrossing makes BREEDIT a scalable and robust solution. As global challenges like climate change, food insecurity, and declining arable land intensify, BREEDIT stands at the forefront of sustainable agricultural innovation, providing a powerful tool to secure global food systems and ensure agricultural resilience in the 21st century and beyond.

FUTURE PROSPECTS OF BREEDIT

The BREEDIT framework holds immense potential to revolutionize plant breeding by integrating advanced genome-editing tools with traditional breeding approaches. Its future prospects are vast, addressing critical challenges in global agriculture and enabling unprecedented crop improvement. Here are some of the key areas where BREEDIT could make a significant impact:

- BREEDIT can be leveraged to develop crops that withstand extreme environmental stresses such as drought, heat, salinity, and flooding. By targeting gene families associated with abiotic stress tolerance, this system offers a precise way to adapt crops to changing climates.
- By editing multiple genes simultaneously, BREEDIT provides an efficient pathway for addressing polygenic traits like yield, disease resistance, and quality, enabling more comprehensive crop improvement. Future innovations could make the process even faster, reducing the time from laboratory to field application.
- *De Novo Domestication* can aid in the domestication of wild relatives of crops by targeting domestication-related gene families, creating new crops with desirable agronomic traits.
- Combining BREEDIT with precision agriculture tools like sensors, drones, and AI-driven analytics could enable real-time phenotypic assessments and targeted interventions for improved breeding efficiency.
- Continued refinement of multiplex genome-editing techniques will help overcome challenges like functional redundancy within gene families, enabling more precise manipulation of gene networks, could be integrated with synthetic biology approaches to engineer entirely new biological pathways, creating crops with traits not found in nature, such as biofortification or enhanced photosynthetic efficiency.
- BREEDIT could lead to crops with enhanced pest and disease resistance, reducing the need for chemical inputs and fostering sustainable agricultural practices.
- BREEDIT offers opportunities for collaborative research programs that bring together plant breeders, geneticists, data scientists, and policymakers to tackle global agricultural challenges.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that no generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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