

ADVANCES IN BIOTECHNOLOGICAL TOOLS AND TECHNIQUES FOR CROP IMPROVEMENT

Abstract

Biotechnology encompasses a suite of biological tools that have arisen from fundamental research and are currently utilized in both research and crop improvement. Modern plant breeding heavily relies on biotechnological methods to genetically enhance crops for desired traits and increased productivity. Traditional breeding techniques primarily rely on hybridization and the selection of advanced breeding lines. However, these conventional methods face challenges such as long juvenile phases, high levels of heterozygosity, and self-incompatibility. The introduction of foreign DNA into plants, achieved through methods like Biolistic transformation or *Agrobacterium tumefaciens*-mediated transformation, has played a pivotal role in genetic modification. Recent biotechnological advancements, particularly New Biotechnological Tools (NBTs) like RNA interference (RNAi), cisgenesis/intragenesis, trans-grafting, and genome editing techniques such as CRISPR/Cas9 and zinc-finger technology, now enable more precise and efficient genetic alterations in plants. These NBTs use genetic engineering to insert essential genes into the genomes of various plant species, resulting in improved efficiency and reliable genetic enhancements in clonally propagated plants while maintaining the stability of essential traits.

Keywords: cisgenesis/transgenesis, CRISPR/CAS9, genome editing, New Biotechnological Tools (NBTs), RNA interference, zinc finger

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I. INTRODUCTION

Biotechnology comprises a range of methods for manipulating specific genetic material within and between organisms. The term "biotechnology" originates from the Greek words "bios" (related to living systems) and "technikos" (indicating human knowledge and skills). Genetic modification of plants has been ongoing for more than three decades, using techniques like direct transformation (Biolistic) and indirect methods (Agrobacterium tumefaciens-mediated transformation) to introduce foreign DNA into plants. Most commercially grown genetically modified plants, including woody fruit species, have been developed using these methods. Achieving new traits or mutations in fruit-bearing trees through genetic engineering or NBTs often relies on established in vitro regeneration procedures tailored to specific genotypes and plant tissues. While both NBTs and traditional methods share the goal of precise, rapid, and effective crop improvement, they employ distinct approaches and possess unique characteristics. From an agricultural perspective, it is often advisable to regenerate new fruit-bearing tree plants from mature tissues due to the high degree of heterozygosity in most of these species. The emergence of recombinant DNA technology has greatly expanded the possibilities of plant biotechnology, enabling advancements even in challenging woody species such as peach or grapevine through the development of protocols for regenerating new shoots from mature tissues. Genetic engineering techniques involve the direct introduction of one or more new genes or regulatory elements into an organism's genome, thereby controlling the expression of specific traits. Transgenic approaches have global implications, aiming to create plants with new resistance genes against pests and diseases, herbicide tolerance, desirable characteristics, and improved nutritional profiles, as exemplified by Golden Rice with increased vitamin A content.

II. RNA INTERFERENCE

RNA interference (RNAi) represents a revolutionary mechanism in which short double-stranded RNA molecules hinder the expression of genes by triggering the degradation of specific target mRNA sequences in the cytoplasm. The first instance of RNAi in transgenic plants was observed in *Petunia hybrida*, where the introduction of the chalcone synthase gene (CHS A) with the goal of enhancing anthocyanin pigmentation unexpectedly led to the suppression of endogenous homologous genes. This phenomenon, known as "co-suppression," resulted in transgenic plants producing white or chimeric flowers instead of dark purple blossoms. RNAi is an inherent biological process that regulates gene expression before translation or "silences" undesirable or harmful nucleic sequences. It has been demonstrated that RNAi employs double-stranded RNA (dsRNA) molecules as triggers to locate homologous mRNAs, thereby negatively regulating their transcription in both plants and animals. As a result, RNA silencing has become the predominant technique for gene targeting in fungi, insects, bacteria, viruses, and plants.

Currently, various gene silencing methods have been discovered in plants, including post-transcriptional gene silencing (PTGS), microRNA silencing, and transcriptional gene silencing. These processes necessitate the presence of dsRNA molecules of varying sizes, which are introduced into plant cells by protein families such as Dicer or Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerases. Various model organisms have unveiled a multitude of short non-coding regulatory RNAs, including small vault RNA

(svRNA), miRNA, siRNA, piRNA (PIWI interacting RNA), and qiRNA (QDE-2-interacting RNA), each with distinct biochemical processes governing their formation. The RNAi strategy has demonstrated its potential in crop improvement by enabling the attainment of desired traits through the modulation of genetic expression. Consequently, the discovery of RNA interference has given rise to novel techniques with broad applicability in genetic analysis, crop enhancement, and plant protection, fostering a deeper understanding of gene regulation, analysis, and functionality. Additionally, RNAi technology offers advantages such as improved nutrition, altered morphology, and enhanced synthesis of secondary metabolites. Beyond its role in controlling gene expression, RNAi serves as a natural defence mechanism against molecular parasites, including transposable elements and viral genetic components that pose threats to genomic stability.

- 1. Mechanism of RNA Interference:** In plants, RNA silencing operates at multiple levels: cytoplasmic silencing involves the cleavage of mRNA by dsRNA, endogenous mRNAs are silenced through microRNAs (miRNAs) that suppress gene expression via base-pairing with specific mRNAs, resulting in RNA cleavage or translational inhibition, known as post-transcriptional gene silencing (PTGS). Moreover, RNA silencing is linked to DNA sequence-specific methylation, leading to the repression of transcription. The initiation of RNA interference in plants primarily relies on small interfering RNAs (siRNAs), which are 21 to 24 nucleotides in length and commence the overall process. These siRNAs are generated intracellularly from extended internal or external dsRNA molecules, facilitated by the ribonuclease III-type enzyme known as Dicer. Subsequently, these siRNAs are incorporated into the RNA-induced Silencing Complex (RISC), comprising various proteins including Argonaute (AGO). Within the RISC, the double-stranded siRNA is unwound in an ATP-activated process. Following the destruction of the sense strand of the siRNA duplex by RNA helicase activity, the antisense strand is integrated into a nuclease-containing RISC complex. Utilizing base-pairing interactions to target homologous transcripts, RISC harbouring the antisense siRNA sequence either cleaves the mRNA or inhibits translation, ultimately suppressing protein production.
- 2. MicroRNAs (miRNAs):** MiRNAs constitute the most abundant and crucial category of short regulatory RNAs. These naturally occurring RNAs, which are 23 nucleotides long, are generated through transcription by RNA Polymerase II. They play a pivotal role in controlling gene expression by associating with the mRNAs of protein-coding genes to modulate their suppression. In the plant context, the conversion of both primary miRNA (pri-miRNA) to precursor miRNA (pre-miRNA) and pre-miRNA to mature miRNA relies on a plant homolog of dicer known as DCL1. After mature miRNAs are produced, the miRNA-induced silencing complex (miRISC), comprising Argonaute and other effector proteins, is recruited. Through complementary base-pairing with target mRNA molecules, the miRISC initiates gene silencing mediated by miRNAs. Several factors, including cell state, cell type, developmental stage, and target site, can influence the mechanisms of silencing in various ways.
- 3. Short-interfering RNAs (si RNAs):** The RNA interference (RNAi) process begins with long double-stranded RNA (dsRNA) or short-hairpin RNA (shRNA) precursors that share sequence similarity with the target gene to be silenced. RNAi is triggered when foreign entities like viruses, genetic material such as transposons, or transgenes that produce long dsRNA are introduced. The dsRNA is cleaved into siRNAs, which are short dsRNAs

measuring 21–25 nucleotides in length and featuring two-nucleotide overhangs at their 3' ends. This cleavage is facilitated by the cellular enzyme dicer. The sense strand of siRNA is degraded by the siRNA-induced silencing complex (siRISC). Subsequently, the target mRNA undergoes sequence-specific cleavage. This is accomplished due to the siRISC's integration with the antisense strand of siRNA, working in coordination with Argonaute (AGO) and other effector proteins. Activated RISCs frequently participate in mRNA degradation and inhibit protein synthesis, leading to post-transcriptional gene silencing (PTGS).

III. TRANS GRAFTING TECHNIQUE

The technique of trans-grafting primarily relies on grafting, a well-established practice in horticulture with a long history of improving the quality and yield of fruits. This approach involves combining two distinct genotypes, each selected for its specific fruiting characteristics and root capacity. By grafting together, the scion and rootstock, their superior attributes are merged. While the rootstock can influence the physical traits of the scion by enhancing its vigour and improving fruit production, both the rootstock and scion maintain their genetic integrity since their genetic material remains separate. Trans-grafting integrates traditional grafting methods with plant genetic engineering.

In this process, a scion that is not genetically modified is grafted onto a rootstock that has undergone genetic modification. The scion inherits the advantages and characteristics imparted by the transgenes present in the rootstock. However, the end products, such as fruits, do not carry the transgene and thus remain genetically unaltered. Trans-grafting relies on the transportation of RNA molecules through the vascular system from the rootstock to the scion. Recent research has demonstrated that the phloem, a plant's vascular tissue, transports specific RNA molecules to regulate the development of organs. More than 15% of phloem transcripts are associated with signal transduction. If RNAi-based rootstocks can effectively transfer silencing molecules to non-genetically modified scions, it may be feasible to create transgenic plants resistant to viruses. The systemic transmission of silencing signals through the phloem and cell-to-cell communication via plasmodesmata has been linked to microRNAs and transacting siRNAs. The compatibility between scion and rootstock interactions plays a crucial role in facilitating the downward movement of photosynthetic products, the upward flow of water and minerals, the transmission of RNAi silencing signals into the scion, and the initiation of systemic silencing.

IV. CISGENESIS/INTRAGENESIS

Considering public concerns regarding the safety of transgenic crops, cisgenesis and intragenesis have emerged as new tools in crop improvement and plant breeding. The term "cisgenic plant" refers to a crop plant genetically modified with one or more genes (including introns and flanking regions, along with native promoter and terminator sequences) obtained from a cross-compatible donor plant. Essentially, this means that cisgenic crops retain their natural genetic composition, including complete copies of genes with all their regulatory elements. The source of the cisgene is the same plant species or a sexually compatible species, similar to traditional breeding. Unlike conventional breeding, cisgenesis includes only the gene(s) of interest, without undesired genetic components. In contrast, intragenesis involves genetically modified organisms (GMOs) where the introduced intragene also

originates from the same species or a cross-compatible species, but intragenes are hybrid genes, potentially incorporating genetic elements from various genes and loci. As a result, intragenesis can introduce variability in gene expression, novel expression patterns, and create GMOs with innovative properties. Intragenesis, including RNA interference (RNAi) strategies employing DNA sequences, is also considered part of this category.

Intragenesis facilitates the creation of new genetic combinations, introducing variability in gene expression and enabling the development of novel expression patterns, resulting in the generation of innovative genetically modified organisms (GMOs). When comparing cisgenesis, which primarily uses native genes, with intragenesis, it becomes evident that cisgenesis is much closer to conventional breeding. Intragenesis also encompasses RNA interference (RNAi) strategies employing DNA sequences. Another significant distinction between cisgenesis and intragenesis revolves around the T-DNA borders or other sequences transferred to plants during the *Agrobacterium*-mediated transformation process, a subject that is not without controversy. While some argue that intragenic and cisgenic plants should be created using border sequences originating from the cross-compatible gene pool, others suggest that the source of border sequences should not be a limiting factor, potentially allowing the use of canonical T-DNA borders similar to those in any other GMO. It is essential for both cisgenic and intragenic approaches to be devoid of non-plant sequences, including vector backbones and selection markers.

Various techniques have been developed to either avoid or remove marker genes. For instance, in cases where transformation efficiency is high, specific markers can be omitted. Then, converted plant lines are selected based on the presence of the specific introduced gene sequence. However, this selection process requires assessing multiple plants, incurring significant costs and time consumption. To address this, marker deletion strategies based on precise recombination have been developed. In other scenarios, marker selection genes are flanked with recombination sites (R/Rs system). Subsequently, after converted plants have been selected, recombination is induced, leading to the removal of the marker gene. Marker excision has also been achieved using molecular biology techniques. This marker-free technology has been applied in the creation of cisgenic and intragenic apple and intragenic strawberry plants.

Co-transformation offers an alternative method to introduce markers into non-GMO plants. This approach involves placing the marker gene and the gene of interest in different locations within the plant's genetic makeup, allowing each gene to segregate in different offspring. Co-transformation can be achieved using transformation vectors, with one containing the gene of interest and the other containing the marker gene. Examples of this technique include cisgenic barley plants and durum wheat.

There is growing interest in advocating for more lenient regulations concerning cisgenic and intragenic crops within the scientific community. For instance, the European Commission (EC) has requested the European Food Safety Authority (EFSA) to assess the risks associated with cisgenic and intragenic crops in comparison to transgenesis or traditional breeding. It has been suggested that cisgenesis may entail risks similar to those of conventional breeding, while transgenesis and intragenesis could be less predictable. A similar situation is unfolding in the United States, where the Environmental Protection Agency (EPA) is deliberating on the regulatory framework for these crops. Despite the initial

intention of cisgenic and intragenic approaches to simplify the approval process compared to stringent regulations for transgenic crops, the current situation classifies both technologies in the same category as transgenic crops.

Application of Cisgenesis and Intragenesis to Improve Traits in Crops: The application of cisgenesis and intragenesis has been extended to enhance various traits in a variety of crops, including potato, apple, strawberry, alfalfa, perennial ryegrass, poplar, barley, and durum wheat. For instance, the first intragenic potato was developed to increase its amylopectin content, a crucial component of starch. This achievement involved silencing the granule-specific starch synthase gene (GBSS), responsible for amylose synthesis in potatoes. Creating tetraploid cultivars with desired amylose and amylopectin content has been a challenge, and techniques were developed to silence genes involved in both amylose and amylopectin synthesis, resulting in desirable potato varieties. These potatoes were introduced to the field in 2007 and included T-DNA borders and terminator sequences for gene expression regulation.

Intragenic approaches have also been employed to improve other potato traits. For example, silencing a polyphenol oxidase gene (PPO), responsible for enzymatic browning, reduced browning and improved tuber storage. Addressing the issue of cold-induced sweetening, which occurs due to starch degradation during low-temperature storage, involved silencing genes associated with starch degradation, such as water dikinase (R1) and amyloplast-targeted phosphorylase-L (PhL). These efforts resulted in potatoes with superior properties. Additionally, enhancing resistance to late blight, a significant potato disease caused by *Phytophthora infestans*, was a key focus. This was achieved by transferring resistance genes (R-genes) from wild potatoes into cultivated varieties using cisgenic techniques, along with marker-free technologies.

In the context of fruit trees, apple scab, caused by the ascomycete *Venturia inaequalis*, is a prominent disease. All cultivated apple varieties are susceptible to this pathogen. To combat scab, the HcrVf2 gene, found within the scab resistance locus Vf, was introduced into the Gala apple cultivar. This gene carried its regulatory sequences, including a promoter and terminator, making it the first reported "true cisgenic plant." Another intragenic approach aimed at inducing scab resistance using the same HcrVf2 gene, but controlled by the promoter and terminator of the apple rubisco gene's small subunit, has not demonstrated scab resistance in apples to date.

Modifying tree structure and growth rate poses significant challenges in the woody plant industry. To address this issue, a cisgenic approach in poplar was developed. In this approach, genes encoding gibberellic acid biosynthesis enzymes, along with irregular sequences, were overexpressed in poplar trees, resulting in increased tree growth. Conversely, overexpression of catabolic genes and negative regulators led to reduced tree size. Therefore, cisgenic and intragenic techniques hold promise for plants with long lifespans and high levels of heterozygosity, where conventional breeding methods are limited and laborious.

Cisgenic barley was developed by introducing multiple copies of the phytase gene. Phytase catalyses the release of phosphate from phytic acid, making phosphate more accessible for absorption by animals. This approach shows promise in increasing phosphate

bioavailability and may offer an alternative in reducing the pollutants derived from phosphates.

V. GENOME EDITING

Genome editing tools have significant potential for precisely modifying the genetic structure of a genome at specific locations. These tools have been effective in revealing traits and developing crops with increased yields and resistance to both biological and environmental stresses. Key genome editing techniques include Homologous Recombination (HR), Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), Pentatricopeptide Repeat Proteins (PPRs), and the CRISPR/Cas9 system. Additionally, gene editing encompasses site-specific sequence alterations and oligonucleotide-directed mutagenesis.

1. Homologous Recombination: Homologous recombination is a highly efficient natural system for genome engineering occurring within cells. It can be utilized for genome editing by inducing double-stranded breaks (DSB) in chromosomes. DSBs during cell division lead to meiotic recombination. Specific regions of homologous recombination within a chromosome are known as recombination hotspots. Because every chromosome undergoes at least one recombination event during meiosis, alleles generate new combinations. The combinations of alleles inherited from each parent are not fixed and can vary, introducing diversity within a population.

Over time, recombination redistributes alleles from one locus to alleles at an adjacent locus. Chromosomes are not static across generations but rather "fluid," with various combinations of alleles. This process removes nonfunctional or less functional alleles from a population. Without recombination, a deleterious mutant allele could potentially eliminate an entire allele from the population. However, with recombination, the mutant allele can be separated from other genes on the same chromosome. Negative selection can then eliminate defective alleles

- **Mechanism of Recombination:** Recombination is a biological process where a segment of one chromosome exchanges places with a corresponding segment from the other parental chromosome, or vice versa. This genetic swapping must occur precisely to avoid any loss or gain of genetic material. To ensure this precision in recombination, proteins within a structure called the synaptonemal complex (SC) hold together non-sister homologous chromatids during prophase I of cell division. The formation of the SC begins during the zygotene stage of prophase I and is finalized in pachytene. The SC consists of proteinaceous lateral elements (axial elements) that run alongside the chromatids and a shorter central element made of fibrous proteins that form ladder-like rungs perpendicular to the lateral elements. Recombination can take place with or without double-strand breaks, although the SC likely enhances the efficiency of recombination. In most cases, recombination is preceded by the creation of protein complexes known as recombination nodules at potential recombination sites.

The mechanism behind meiotic recombination involves the initiation of a double-stranded break by a specialized enzyme called Spo11, which is active during

meiosis. The 5' ends of this break are slightly degraded, forming 3' single-stranded overhangs. These unpaired ends lead to the formation of structures called Holliday junctions, where a strand from another chromatid acts as a template to synthesize the missing portion of the chromatids. This results in intertwined sister chromatids connected by a single strand of DNA. This entanglement can be resolved either with or without crossover events. The process of recombination begins in pachytene and concludes in diplotene, coinciding with the dismantling of the synaptonemal complex. As prophase progresses, chiasmata become visible at multiple recombination sites, gradually resolving from the centre of the chromosomes toward the ends.

- **Recombination used as a Restore Mechanism:** Homologous recombination mainly occurs in meiotic cells. In most species, every chromosome undergoes at least one recombination event. However, the cell's ability to utilize one chromosome as a template to mend a damaged section can also be employed in cases of DNA damage, particularly DNA double-strand breaks (DSBs). The cell's capacity to employ homologous recombination for repairing a damaged chromosome depends on whether it can locate a matching sequence to serve as a template for the repair process.
2. **Zinc Finger Nucleases:** Zinc Finger Nucleases (ZFNs) are a highly adaptable and efficient class of targeting tools that have emerged recently. These synthetic proteins were developed based on the understanding that the natural type IIS restriction enzyme, FokI, possesses physically separate DNA-binding and DNA-cleavage domains. A typical ZFN monomer comprises two fused domains: an artificially designed Cys2-His2 zinc finger domain and the nonspecific DNA cleavage domain of the FokI DNA restriction enzyme. A standard zinc finger domain consists of three to four fingers, each capable of recognizing approximately 3-base pair sequences. This flexibility allows targeting many unique sequences by assembling different combinations of zinc fingers. FokI's enzymatic activity requires dimerization of its domain. Therefore, DNA cleavage occurs when two ZFN monomers, each binding to their respective target DNA sequences, align in a reverse configuration. Typically, the two ZFN monomers are designed to flank a 5- to 6-base pair sequence within the DNA target, allowing the FokI dimer to cleave within that spacer sequence.

Groundbreaking work by Dan Voytas established the utility of Zinc Finger Nucleases (ZFNs) for editing genes within plant cells. Experiments involving tobacco protoplasts demonstrated that ZFNs could significantly enhance gene targeting frequencies, up to a hundred times more than random integration. Additionally, ZFNs were used to induce mutations in an artificially introduced restriction site in the Arabidopsis genome through a process known as nonhomologous DNA end joining (NHEJ). Subsequent research showcased the potential of ZFNs to modify native genes in tobacco and maize by inducing DSB-mediated homologous recombination (HR). In tobacco, ZFNs were used to modify the SuRA and SuRB loci at the single-nucleotide level, resulting in resistance to various herbicides. In maize, ZFNs enabled the modification of the maize IPK1 gene, leading to herbicide-resistant phenotypes. ZFNs emerged as a pivotal tool for genome editing in plants. However, their prominence may have declined due to the development of more promising tools for inducing site-specific DNA double-strand breaks (DSBs).

One common issue associated with synthetic nucleases like ZFNs is their potential to cleave off-target sites in the genome, which may be similar but not identical to the intended target site. These "off-target effects" raise concerns as they can cause unexpected mutations, potentially resulting in undesired side effects. Some ZFNs have been found to negatively impact cell proliferation, indicating that they can create unintended DSBs at secondary sites. Moreover, the presence of multiple binding modules within the zinc finger binding arrays can affect each other, making the prediction and efficient design of new genomic sites less straightforward. While kits for constructing ZFNs are available, the process remains more time-consuming compared to other gene-editing tools like TALENs or the CRISPR/Cas system and is constrained by the limited suitability of target sequences.

Application of Zinc Finger Nucleases

- **Targeted Mutagenesis:** In the past, various techniques for random mutagenesis were utilized, including methods like radiation, EMS mutagenesis and T-DNA insertion. However, with the emergence of Zinc Finger Nucleases (ZFNs), it became possible to create precise gene knockouts by designing ZFNs that are specific to particular DNA sequences, inducing double-strand breaks (DSBs) at predetermined locations in the genome. This approach allowed for the generation of new genetic variations through targeted mutations, opening up new opportunities in plant biotechnology. The initial application of ZFNs in plants involved the deliberate mutation of a transgenic region in Arabidopsis. Constructs containing ZFNs, which could be induced by heat shock, along with the target DNA sequence, were introduced into Arabidopsis. This marked the first instance of ZFN-induced mutation in an endogenous gene in a crop plant, with a specific focus on exon 2 of the Maize IPK1 gene.

Targeted gene mutations have been successfully achieved in economically important plant species such as soybean, canola, rice, tomato, fruit trees, and hybrid poplar. These targeted mutations have the potential to introduce novel traits and characteristics into modified plants. ZFNs have proven to be versatile in generating gene mutations, utilizing different expression strategies (constitutive or inducible) and DNA delivery methods (both transient and stable). The mutations induced by ZFNs have been shown to be inheritable, with a high degree of accuracy, specifically targeting genes from multigene families, with minimal evidence of mutations occurring at non-target sites.

- **Gene Editing:** Using a ZFN in conjunction with a donor molecule, specific codon mutations were introduced into the SuR locus, resulting in the production of acetolactate synthase (ALS) mutants resistant to herbicides. Additionally, ZFNs were employed to induce mutations in the protoporphyrinogen oxidase (PPO) gene in Arabidopsis, demonstrating the feasibility of gene editing. In the realm of plant genetics, ZFNs have primarily been used to target loci that offer herbicide resistance as a selection marker for gene edits. However, there is room for improvement in the efficiency of this process, particularly for large-scale editing of alleles and the promotion of genetic diversity.

ZFNs have also been used to induce mutations in the protoporphyrinogen oxidase (PPO) gene in *Arabidopsis*, demonstrating the feasibility of gene editing. In plants, ZFNs have primarily been applied to target loci that provide herbicide resistance as a selection marker for gene edits. However, there is room for improvement in the efficiency of the process to enable large-scale editing of alleles and the creation of genetic diversity.

- **Targeted Gene Addition:** Targeted gene addition, also known as gene targeting, enables the incorporation of multiple traits into a single genetic locus. This method is highly advantageous in agricultural biotechnology, as it simplifies the introduction of numerous desirable traits across different genetic backgrounds. Unlike gene editing, which involves modifying existing genetic material, gene targeting entails the substantial addition of donor DNA to the target site, rather than just modifying individual DNA bases.

In a tobacco model system, targeted gene addition was achieved by using homologous recombination (HR) along with a donor DNA and ZFNs, introduced into protoplasts of transgenic tobacco strains containing nonfunctional reporter genes with an internal ZFN recognition site. This process involved several steps: first, intrachromosomal recombination was initiated to restore a functional reporter gene after ZFN-induced double-strand breaks (DSBs). Second, DSBs were induced upstream of a previously integrated target locus containing an intron and the 3' fragment of a selectable marker, and these breaks were repaired using a donor DNA containing the 5' fragment of the selectable marker and an intron with homology to the target region. Homology-directed repair (HDR) facilitated gene targeting and the reconstitution of the selectable marker. Finally, gene targeting was confirmed in the endogenous tobacco endochitinase gene using a construct comprising a ZFN and a donor carrying a selectable marker introduced into tobacco cells.

Resistant isolates obtained through selection were verified through PCR, confirming HDR-mediated gene targeting of the native endochitinase gene. Targeted gene addition has been explored in various forms in plant biotechnology and has been implemented in both transgenic and endogenous loci of crop plants. It utilizes two different DNA repair mechanisms, nonhomologous end joining (NHEJ) and HDR, for the precise insertion of transgenes. As advancements continue to be made in this field, the inefficiencies in the procedure are being addressed. One primary application of this technology is the stacking of biotech traits, which has been demonstrated but is not yet commercially widespread. The potential value in agricultural biotechnology is immense, particularly considering the substantial efforts invested in breeding and introducing biotech innovations in commercial agriculture.

- **Gene Deletion:** A more advanced and precise approach for inactivating a gene is to completely eliminate it. Gene deletion is achieved by creating double-strand breaks (DSBs) on both sides of the gene sequence using ZFNs, resulting in the removal of the intervening segment. Furthermore, ZFN-induced DSBs near genomic regions with high homology can trigger homology-directed repair (HDR), leading to the deletion of the intervening DNA segment. Successful demonstrations of creating deletions in tandemly arrayed genes (TAGs) have been accomplished in *Arabidopsis* using ZFNs.

By employing ZFNs, seven genes from three different TAGs were effectively targeted for deletion. Deletion sizes ranged from 4.5 kb to 55 kb, with a frequency of approximately 1% in vegetative tissue, although germline transmission of these deletions was not observed. Deletions as large as approximately 9 Mb were achieved, albeit with lower frequency (0.046%). Additionally, ZFNs have been used to induce inversions and duplications within these gene clusters, showcasing their potential to generate novel genomic configurations through targeted DSBs involving chromosome rearrangements and chimeric genes. Whether deployed in endogenous or transgenic loci, ZFNs have successfully removed DNA segments, some as large as approximately 9 Mb. Building upon this concept, this approach holds promise for enhancing genetic diversity. The potential for genetic modification is significant, as deletions, inversions, and duplications have all been achieved through ZFN-induced DSBs.

- **Gene Regulation:** The ability to manipulate gene expression offers significant potential for both fundamental biology and applications in plant biotechnology. By combining knowledge about the interaction between zinc finger proteins (ZFPs) and DNA with information about transcriptional regulators and engineered DNA-binding domains, it becomes possible to create engineered zinc finger protein transcription factors (ZFP-TFs) capable of modulating gene expression. For instance, ZFP-TFs can be designed to map DNase I accessible regions within specific genes, such as the vascular endothelial growth factor A (VEGF-A) gene. The application of this technology in plants was initially demonstrated in *Arabidopsis*, focusing on the *APETALA3* (*AP3*) gene. ZFP-TFs were designed to target regions approximately 50 base pairs upstream of the TATA box. When introduced into leaf protoplasts, ZFPs fused with the VP16 activation domain resulted in increased expression of a transgenic reporter and the anticipated floral phenotypes in stable plants. ZFP-TFs achieved a remarkable 450-fold increase in reporter gene expression in tobacco plants. Tissue-specific activation was also observed in vascular tissue through the use of a ZFP-TF driven by a phloem-specific promoter. Additionally, ZFP-TF-mediated activation responded in a dose-dependent manner when chemically induced. Furthermore, transcription activation domains derived from plants were developed, enabling higher levels of gene expression compared to the previously used VP16 domain.

The application of ZFP-TFs has been exemplified in the crop *Arabidopsis*, where ZFP-TFs derived entirely from plant sequences demonstrated the ability to upregulate the expression of an endogenous gene throughout the entire plant. Seed-specific expression of ZFP-TFs led to increased seed α -tocopherol levels in various lines. The utility of engineered ZFP-TFs for both upregulating and downregulating the expression of endogenous plant genes has been demonstrated. Transgenic ZFP-TF lines with an activation domain showed increased lignin content, while lines with a repression domain exhibited reduced lignin content. Engineered ZFP-TFs have also been employed to modulate the expression of an endogenous gene in the crop plant canola (*Brassica napus*). Engineered ZFPs have proven highly effective in the regulation of plant genes, affecting both endogenous and transgenic genes in both model and crop plants. In many instances, this regulation of gene expression has resulted in significant phenotypic modifications. The use of ZFPs allows for the

modulation of gene expression across a wide range of phenotypes, some of which may not be achievable through knockout mutations created via mutagenesis. By utilizing tissue-specific promoters, it becomes possible to envision tissue-specific gene regulation, potentially offering a more refined array of possible phenotypic outcomes. This approach could be particularly advantageous for the regulation of genes that exhibit severe phenotypic consequences when constitutively altered.

- 3. TALENs (Transcription Activator-Like Effector Nucleases) Advancements:** Recent progress in the field of Transcription Activator-Like Effector Nucleases (TALENs) involves merging a non-specific DNA-cutting enzyme with a DNA-binding segment. This fusion enables the development of highly customizable TALENs capable of precisely targeting almost any DNA sequence. This capacity to efficiently manipulate gene sets using TALENs has significant implications for biological research and potential therapeutic strategies for genetic disorders. TALENs have emerged as a viable alternative to Zinc Finger Nucleases (ZFNs) for genome editing and generating precise DNA double-strand breaks (DSBs). TALENs share a structural similarity with ZFNs, consisting of a non-specific FokI nuclease linked to a customizable DNA-binding domain. This DNA-binding domain comprises well-conserved repeats derived from transcription activator-like effectors (TALEs), which are proteins secreted by *Xanthomonas* bacteria to regulate gene transcription in host plant cells.

In recent years, techniques and methods originally developed for ZFNs have been adapted for TALENs to manipulate native genes in a wide range of organisms, including yeast, fruit flies, roundworms, crickets, zebrafish, frogs, rats, pigs, cows, thale cress, rice, silkworms, and human somatic and pluripotent stem cells. While a direct comparison between ZFNs and TALENs hasn't been made, many studies have shown that TALENs and ZFNs exhibit similar efficiencies when targeting the same gene. Consequently, TALENs' design advantages, high cleavage activity, and virtually limitless targeting possibilities make them accessible even to researchers without specialized expertise. Furthermore, nuclease-mediated modifications of plants and agricultural animals have the potential to significantly reduce the time required to generate new varieties compared to traditional breeding methods. For instance, through micropropagation techniques, certain plant species can be modified at the cellular level and subsequently grown into mature plants. To date, TALENs have been employed to create gene knockouts in *Arabidopsis* and bestow resistance to *Xanthomonas* bacteria in rice by disrupting the target site of naturally occurring TALEs that contribute to pathogenicity. Gene-editing nucleases have also induced targeted NHEJ-induced indel mutations in pigs and cows, along with substantial deletions and inversions of sequences larger than 6 kb in length in pigs through the use of TALEN pairs targeting the same chromosome.

- **Mechanism of TALENS:** Transcription Activator-Like Effector Nucleases (TALENs) are a sort of genome enhancing device that use two-components to introduce modifications in DNA sequences. The mechanism of TALENs involves the use of designer proteins called TALEs (Transcription Activator-Like Effectors) to guide the nuclease activity to specific DNA sequences. Here's a step-by-step explanation of how TALENs work:

- **TALE Protein Design:** The initial step in the TALEN mechanism involves the design and assembly of TALE proteins. TALEs are derived from bacteria, specifically the plant pathogen *Xanthomonas*. These bacteria naturally produce TALEs as part of their virulence machinery for manipulating host plant cells. Each TALE protein consists of repeating units, with each repeat capable of recognizing a specific DNA base pair. These repeats are customizable, and their sequence specificity can be engineered to target a particular DNA sequence. The key to TALEN specificity lies in a customizable region within each repeat called the "repeat-variable di-residue" (RVD), which can be tailored to recognize a specific DNA base: adenine (A), cytosine (C), guanine (G), or thymine (T).
- **DNA Binding:** Once TALE proteins are designed to recognize the desired DNA sequence, they are coupled with a nuclease enzyme, typically the FokI endonuclease. The FokI nuclease domain is inactive on its own but becomes active when two FokI domains come together as dimers. TALE proteins are designed in pairs, with each TALE targeting one strand of the DNA double helix. The TALEN pair is engineered to target adjacent sequences on complementary DNA strands. When the TALE proteins encounter their target DNA sequence, the RVDs within each TALE bind specifically to the corresponding DNA bases via hydrogen bonding. This binding is highly specific due to the customizable nature of the RVDs.
- **Nuclease Cleavage:** Once the TALE proteins are bound to their target DNA sequence, they facilitate the dimerization of the FokI nuclease domains. This dimerization activates the nuclease activity. The FokI endonuclease then cleaves the DNA at a specific location within the target site, resulting in a double-strand break (DSB).
- **DNA Repair:** After the DSB is generated, the cell's natural DNA repair mechanisms are activated to mend the break. Two primary DNA repair pathways are involved:
 - **Non-Homologous End Joining (NHEJ):** Often leading to small insertions or deletions (indels) at the DSB site, which can disrupt a gene's reading frame and result in gene inactivation.
 - **Homology-Directed Repair (HDR):** This pathway can be harnessed to introduce precise changes into the DNA by providing a DNA template with the desired sequence. This template can be used to insert or replace specific DNA sequences.

The overall outcome of the TALEN mechanism is the introduction of targeted genetic modifications at the desired genomic location. Researchers can utilize this mechanism to create gene knockouts, gene replacements, or other precise genetic alterations in a variety of organisms, including plants, animals, and microorganisms.

- **Application of Transcription Activator-Like Effector Nucleases (TALENs):** Transcription Activator-Like Effector Nucleases (TALENs) were applied in several programs in plant biology and biotechnology. TALENs are a type of genome editing tool that allows for precise modification of plant genomes. Here are some detailed applications of TALENs in plants:

- **Custom Trait Enhancement:** TALENs enable the targeted introduction of specific genetic modifications into crop plants, resulting in desirable traits.
 - **Disease Resistance:** TALENs have been used to impart resistance to plant pathogens such as bacteria, fungi, and viruses. This reduces the reliance on chemical pesticides and augments crop productivity.
 - **Pest Resistance:** TALENs facilitate the creation of crops that are impervious to insect pests, mitigating crop damage and decreasing the need for chemical insecticides.
 - **Herbicide Tolerance:** TALENs have been harnessed to develop plants capable of withstanding particular herbicides, allowing for more effective weed management without harming the crops.
 - **Nutritional Enhancement:** TALENs play a pivotal role in modifying the nutritional profile of crops. For example, researchers have employed TALENs to enhance essential nutrient levels, such as vitamins and minerals in plants, thereby addressing malnutrition concerns.

- **Boosting Stress Resilience**
 - **Abiotic Stress Resilience:** TALENs are instrumental in engineering plants with heightened resilience to environmental stressors like drought, salinity, and extreme temperatures. This adaptation is vital for aligning agriculture with changing climatic conditions.
 - **Biotic Stress Resistance:** TALENs are utilized to develop plants with augmented resistance to pathogens, encompassing viruses, bacteria, and fungi. This can lead to a reduction in the requirement for chemical pesticides and an increase in crop yields.

- **Functional Genomics**
 - **Studying Gene Functions:** TALENs empower researchers to disrupt or modify specific plant genes, facilitating the exploration of their roles in plant growth, development, and responses to environmental cues.

- **Advancing Plant Breeding**
 - **Accelerated Breeding:** TALENs expedite the breeding process by introducing precise genetic alterations into plants. This stands in contrast to traditional breeding methods, which can span multiple generations, thus achieving desired traits more rapidly.

- **Enhancing Quality**
 - **Fruit Ripening:** TALENs have been instrumental in modifying genes associated with fruit ripening, prolonging the shelf life of fruits and enhancing post-harvest handling.

- **Aroma and Flavour Enhancement:** TALENs can be deployed to modify genes responsible for the production of aroma compounds and flavours in fruits and vegetables, elevating their taste and market appeal.
- **Medicinal Plant Cultivation**
 - **Boosting Secondary Metabolites:** TALENs are employed to increase the production of secondary metabolites in medicinal plants. This has significant implications for improving the yield and potency of medicinal compounds utilized in pharmaceuticals and herbal remedies.
- **Environmental Conservation**
 - **Restoration Ecology:** TALENs play a role in conserving and restoring native plant species. They are employed to create genetically modified plants that exhibit greater resilience to environmental shifts and habitat restoration efforts.
- **Complex Biosynthesis Pathway Engineering**
 - **Simultaneous Modification:** TALENs are applied to simultaneously modify multiple genes within a biosynthetic pathway. This allows for the engineering of intricate traits, such as the production of biofuels or bio-based chemicals within plants.
- **Education and Research**
 - **Invaluable Tools:** TALENs serve as invaluable tools for both plant biology education and research, affording researchers and students the means to gain a deeper comprehension of plant genetics and genomics.
- **Examples of TALEN Applications in Plants**
 - **Soybeans:** In order to produce healthier soybean varieties with reduced polyunsaturated fats, TALENs were used to introduce multiple mutations into two genes responsible for fatty acid desaturation (FAD2-1A and FAD2-1B). These modifications led to soybeans with over 80% oleic acid and less than 4% linoleic acid content, resulting in high-quality high-oleic soybean oil marketed as Calyno by Calyxt, the first gene-edited plant product available in the U.S. market in 2019.
 - **Rice:** TALENs were employed to engineer resistance against bacterial blight in rice by editing the regulatory region of the OsSWEET14 gene, resulting in heritable disease resistance. Additionally, TALENs were used to transform non-aromatic rice varieties into aromatic ones by disrupting the OsBADH2 gene, leading to the production of the major fragrance compound, 2-acetyl-1-pyrroline.
 - **Potato:** TALENs were used to knock out the vacuolar invertase gene (Vlnv) in commercial potato varieties, resulting in potatoes with undetectable reducing sugars. This led to reduced acrylamide levels and improved chip color. TALENs were also utilized to reduce cholesterol and toxic steroidal glycoalkaloids in potatoes by targeting the SSR2 gene.

- **Maize:** TALENs were employed in maize to create stable, heritable mutations at the glossy2 (gl2) locus, leading to transgenic lines with a glossy phenotype.
 - **Wheat:** Researchers used TALENs and CRISPR-Cas9 to engineer resistance to powdery mildew in wheat by targeting the MILDEW-RESISTANCE LOCUS (mlo) gene. TALEN-induced mutations in all six sets of mlo genes resulted in wheat plants with nearly complete resistance to powdery mildew.
- **Other TALEN Applications**
 - **Sugarcane:** TALENs can be used to target lignin biosynthetic genes in sugarcane, resulting in mutants with reduced lignin content, improving saccharification efficiency for bioethanol production.
 - **Algae:** TALENs were employed to edit genes in algae, specifically *Nannochloropsis* species, to enhance lipid production for biodiesel.
 - **Yeast, Insects, and Mammals:** TALENs have been successfully used to modify genes in various organisms beyond plants and algae.
 - **Mitochondrial DNA Editing:** TALE-based editors were used to precisely edit mitochondrial DNA, offering potential applications in treating mitochondrial-associated diseases.

TALENs have gained recognition for their significance in crop improvement and other organisms, being named the "Method of the Year" by Nature Methods in 2011 and receiving accolades in agricultural biotechnology.

4. **Pentatricopeptide Repeat Proteins (PPRs):** Pentatricopeptide repeat (PPR) proteins have gained prominence due to their abundant presence and vital roles in angiosperm species. They were discovered through genome sequencing analysis in *Arabidopsis thaliana* just two decades ago. PPR proteins share similarities in amino acid composition and structure with tetratricopeptide repeat (TPR) proteins, which usually mediate protein-protein interactions. Both PPR and TPR proteins are encoded by nuclear genes and characterized by multiple repeating units. PPR proteins belong to the α -solenoid RNA-binding proteins (RBPs) superfamily and play various roles in messenger RNA (mRNA) life cycles.

It's important to note that PPR proteins also have associations with tandem repeat (TR) proteins, including armadillo (ARM), leucine-rich repeats (LRRs), tetratricopeptide, ankyrin (ANK), and WD40 proteins. These TR proteins have been studied extensively in plants, suggesting potential roles for PPR proteins in stress responses and developmental processes.

Mutations in genes encoding PPR proteins often result in defects related to chloroplast development, pigmentation, embryo growth, and seed growth. These defects typically occur due to the absence of one or more mitochondrial or chloroplast gene products crucial for organelle development. However, there are exceptions, such as *gun1* and defectively organized tributaries 4, which are involved in multiple developmental and stress-related signals in young seedlings and mature leaves. Some photosynthesis-defective mutants can survive during the seedling stage until their seed reserves are depleted. Therefore, embryo-lethal phenotypes resulting from plastid defects may

primarily arise from dysfunctional plastid translation mechanisms, preventing the formation of key components required for normal chloroplast development and photosynthesis. The similarity in phenotypes resulting from disruptions in individual PPR proteins has made it challenging to study their functions.

- **Redefining PPR Proteins' and Cataloguing of Motifs:** Based on the arrangement of motifs in its members, the PPR protein family is further divided into subgroups known as PLS and P. P-class PPR proteins typically contain between 2 and over 30 loosely conserved PPR (P) motifs, each consisting of 35 amino acids. Some members of the P subfamily also include an additional small MutS-related (SMR) domain following a series of P-class PPR motifs, leading to their classification as the PPR-SMR subgroup. In contrast, members of the PLS subfamily generally possess an arrangement of triplets, including the standard P motif, L motif, and S motif. Both S and L motifs are associated with the PPR motif but vary in length, with S motifs spanning 31 amino acids and L motifs spanning 35 or 36 amino acids. PLS subfamily PPR proteins can be further categorized based on the presence of associated domains located downstream of the PPR motifs, including E, E+, and DYW domains, which may occur in various combinations or independently. The classification of PPR proteins has evolved as more species are sequenced and new PPR proteins are identified. A comprehensive genomic analysis of 41 terrestrial plant species with evolutionary distinctions has resulted in the redefinition of PPR motifs. For example, the P motif is further divided into P1 and P2 based on differences in the first helix. Additionally, the PLS motif, consisting of L1 and L2 motifs with 35 and 36 amino acids, respectively, is distinguished by variations in the second helix. Similarly, the S motif is subdivided into the standard 31-amino acid S1 motif and the 32-amino acid S2 motif. An additional S-like motif (31 amino acids) known as SS is frequently found adjacent to other S motifs. Meanwhile, the E, E+, and DYW motifs have also been redefined, introducing new 34-amino acid motifs, E1 and E2. As a result, the PLS subfamily has been reorganized into six subcategories, in addition to a mixed P subfamily.
- **PPR Proteins' Role in Chloroplast Function:** Functional chloroplasts are crucial for photosynthesis, providing an autonomous microenvironment and essential proteins for plant autotrophy. They are vital for plant survival and development. PPR proteins are closely linked to chloroplasts because they influence plastid gene expression, thereby impacting chloroplast development and maintenance. Consequently, defects in PPR proteins can lead to chloroplast dysfunction and result in similar phenotypic outcomes. However, various PPR proteins exhibit distinct functions and behaviours.
- **Mechanism of PPRs:** The mechanism of action of Pentatricopeptide Repeat Proteins (PPRs) relies on their ability to bind specific RNA sequences, primarily in post-transcriptional processes within organelle gene expression, especially in chloroplasts and mitochondria. PPRs consist of multiple repeats of the pentatricopeptide motif, forming a helical structure crucial for recognizing specific RNA sequences. Here is a detailed explanation of the mechanism of PPR proteins:

➤ **RNA Binding and Recognition**

- PPR proteins comprise repeating units, each consisting of approximately 35 amino acids with a conserved pentatricopeptide repeat motif. These repetitions form a binding surface with a helical structure.
- Each PPR repeat interacts specifically with individual RNA bases or small RNA base groups. The precision of this interaction arises from the specific arrangement of amino acids within the PPR motif.
- PPR proteins possess diverse combinations and quantities of repeats, enabling them to identify particular RNA sequences.

➤ **Target RNA Recognition**

- PPR proteins identify and bind to precise RNA sequences within organelle transcripts. These RNA sequences often contain information for post-transcriptional processing events, such as RNA editing or splicing.
- The binding of PPR proteins to their target RNA sequences is extraordinarily precise, allowing them to distinguish between different RNA molecules within the cell.

➤ **RNA Editing**

- One of the most renowned functions of PPR proteins is their involvement in RNA editing, a process that alters specific nucleotides within RNA molecules, particularly in chloroplasts and mitochondria.
- PPR proteins function as guides for editing enzymes by binding to the target RNA sequences requiring editing. These binding recruits editing enzymes to these sites.
- Editing enzymes subsequently catalyse the transformation of specific nucleotides, e.g., converting cytosine (C) to uridine (U) within the RNA sequence, leading to modifications in the RNA molecule's sequence.

➤ **RNA Splicing and Other Functions**

- In addition to RNA editing, PPR proteins may participate in various post-transcriptional processes, including RNA splicing and stabilization.
- By binding to particular sites within the RNA sequence, PPR proteins can either facilitate or inhibit splicing events.
- They can also stabilize RNA molecules, guarding them against degradation.

➤ **Gene Expression Regulation**

- PPR proteins have a pivotal role in regulating gene expression in organelles like chloroplasts and mitochondria. They ensure the correct translation of organelle genes by modifying RNA transcripts as necessary.
- Depending on the specific PPR protein and the context, they can either promote or inhibit gene expression by influencing post-transcriptional processes of organelle transcripts.

In summary, PPR proteins are RNA-binding molecules characterized by a unique repeat motif, allowing them to recognize and interact with precise RNA sequences. Their primary function revolves around post-transcriptional processes, including RNA editing, splicing, and stabilization, predominantly within organelles such as chloroplasts and mitochondria. By binding to target RNA sequences, PPR

proteins facilitate precise modifications and the regulation of organelle gene expression.

- **Applications of Pentatricopeptide Repeat Proteins (PPRs) in Plants:** PPR proteins play pivotal roles in plant biology, especially in governing gene expression within organelles like chloroplasts and mitochondria. Their capacity to recognize and bind specific RNA sequences renders them invaluable tools for various applications in plant research and biotechnology:
 - **Crop Enhancement**
 - **Modulation of Mitochondrial and Chloroplast Gene Expression:** PPR proteins can be manipulated to enhance or fine-tune the expression of specific genes in chloroplasts and mitochondria, leading to improvements in photosynthesis, respiration, and overall plant growth.
 - **Disease Resistance:** By modifying gene expression related to plant-pathogen interactions within organelles, PPR proteins can be employed to engineer disease-resistant plants.
 - **Enhancing Stress Tolerance**
 - **Abiotic Stress Resistance:** PPR proteins can be used to modify organelle gene expression in response to environmental stresses such as drought, salinity, or extreme temperatures, bolstering plant resilience to adverse conditions.
 - **Chloroplast Optimization**
 - **Enhanced Photosynthesis:** PPR proteins can be deployed to manipulate chloroplast gene expression, increasing photosynthetic efficiency and potentially boosting crop yields.
 - **Viability and Germination of the Seed**
 - PPR proteins can influence gene expression in mitochondria and chloroplasts during seed development and germination, affecting seed quality and germination rates.
 - **Medicinal Plant Production**
 - PPR proteins can be harnessed to augment the production of secondary metabolites in medicinal plants, elevating the yield of bioactive compounds used in pharmaceuticals and herbal remedies.
 - **Biosynthesis Pathway Engineering**
 - By controlling organelle gene expression, PPR proteins can be employed to manipulate biosynthetic pathways, potentially enhancing the production of biofuels or bio-based chemicals in plants.
 - **Research Aids**
 - PPR proteins serve as valuable tools for researchers examining organelle gene expression and regulation. They facilitate investigations into the roles of specific genes in plant development, physiology, and stress responses.

➤ **Gene Editing Platforms**

- PPR proteins can be integrated into gene editing systems, facilitating precise changes in organelle genomes. This approach enables researchers to study gene function and potentially rectify genetic anomalies.

➤ **Development of Transgenic Plants**

- Incorporating PPR proteins into the development of transgenic plants can result in altered organelle gene expression patterns, yielding novel phenotypes or improved agronomic characteristics.

➤ **Plant Conservation and Restoration**

- PPR proteins hold potential in conserving and restoring endangered plant species. They ensure the proper function of organelles, vital for plant health and survival.

It is important to note that harnessing PPR proteins for manipulation requires a profound understanding of organelle gene regulation and specificity to prevent unintended consequences. Additionally, advancements in genome editing techniques, such as CRISPR-Cas9, complement PPR proteins' capabilities, expanding the toolbox for precise gene regulation in plants.

5. CRISPR/Cas9: CRISPR-Cas9, a groundbreaking gene-editing technology, has gained substantial recognition in the realms of genetics, molecular biology, and biotechnology. The acronym CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats, representing repetitive DNA sequences inherent in bacteria and archaea genomes. These CRISPR regions contain genetic material sequences from previous viral encounters, functioning as a form of immune memory. Cas9, an enzyme derived from bacteria, particularly *Streptococcus pyogenes*, serves as a molecular scalpel, precisely cleaving DNA under the guidance of RNA molecules. These RNA molecules, called guide RNA (gRNA), are tailored to match the target DNA sequence accurately.

- **How CRISPR-Cas9 Works**

- **Design:** Researchers craft a specific RNA called guide RNA (gRNA) that corresponds to the DNA sequence they intend to modify. This gRNA is custom-tailored to the gene or region targeted for alteration.
- **Delivery:** The gRNA combines with the Cas9 protein and is introduced into the cells of interest, utilizing various methods like viral vectors or nanoparticles.
- **Recognition:** Within the cell's genome, the gRNA aligns with the complementary DNA sequence, triggering the Cas9 enzyme to precisely cut the DNA at that location.
- **Repair:** Upon DNA cleavage, the cell's natural repair machinery engages in two primary DNA repair pathways:
 - **Non-Homologous End Joining (NHEJ):** Often leading to minor errors or mutations in the repaired DNA, potentially disrupting the target gene's function.
 - **Homology-Directed Repair (HDR):** Enables precise changes in the DNA by providing a DNA template with the desired sequence.

CRISPR-Cas9 offers a controlled approach to gene editing, allowing researchers to achieve various genetic modifications, including gene knockout (disrupting a gene's function), gene correction (repairing a faulty gene), and gene insertion (adding new genetic material). The precision and ease of CRISPR-Cas9 have positioned it as a potent tool for exploring gene function, developing potential gene therapies, and performing genetic engineering across a wide spectrum of organisms, encompassing plants, animals, and humans. However, the rapid advancement of CRISPR-Cas9 has also raised ethical and safety concerns, particularly in the context of human genome editing. Hence, its application in research and practical use necessitates meticulous regulation and ethical deliberation.

- **Applications of CRISPR/Cas9 Genome Editing in Plants:** The applications of CRISPR/Cas9 genome editing in plants offer the potential to modify certain plant traits or characteristics, particularly in crops, by manipulating their genetic makeup. This genetic manipulation aims to produce improved crop varieties that can benefit the overall population in various ways. The precision of CRISPR/Cas9 technology ensures a highly reliable method for genome editing, avoiding random and unintended changes elsewhere in the genome. The efforts to apply CRISPR/Cas9 genome editing in plants have been substantial since the technology's discovery.

Before applying genome editing techniques to crops, extensive research primarily focused on *Arabidopsis thaliana* as a model plant organism due to its convenience and utility in genetic experiments. For instance, *Arabidopsis thaliana* served as a platform for refining CRISPR gene targeting techniques, including the evaluation of the pKAMA-ITACHI Red vector's performance in CRISPR/Cas9. Notable experiments in 2017 involved genes such as PDS3, AG, and DUO1, further advancing the understanding of CRISPR/Cas9 in this model plant. Following the initial validation in *Arabidopsis thaliana*, researchers have continued to explore the capabilities of CRISPR/Cas9 technology in various other plant species.

Presently, CRISPR/Cas9 genome editing has demonstrated its effectiveness in a wide range of significant plant species, including maize, wheat, and apples, achieving a notably high transformation efficiency. The sequencing of novel plant genomes has expanded the scope of CRISPR/Cas9 genome editing, enabling the examination of a more extensive array of genes in various plant species. A significant breakthrough in CRISPR/Cas9 genome editing was the successful knockout of the phytoene desaturase gene in muskmelon (CmPDS), marking the first instance of CRISPR/Cas9 genome editing in this species. The same PDS gene was also utilized to induce an albino phenotype in pioneering CRISPR/Cas9 genome editing research conducted on watermelon and apples. However, studying the inheritance rate of mutations in subsequent generations of transgenics through PDS gene knockout was hindered because albino variants exhibited low in vitro survival rates. Consequently, other genes needed to be targeted to assess the inheritance rate of mutations in these plant species.

In 2014, targeted mutagenesis was achieved in sweet orange, introducing a novel method for delivering CRISPR/Cas9 reagents. This method was developed for citrus using *Xcc*-facilitated agroinfiltration and inserted via *Xanthomonas citri* subsp.

Citri (Xcc) to infect citrus plants. The knockout of the CsWRKY22 gene in Wanjincheng orange through CRISPR/Cas9 genome editing demonstrated enhanced resistance against citrus canker, a detrimental citrus disease caused by Xcc, further validating the efficacy of CRISPR/Cas9 technology in citrus. Similarly, improvements in resistance were observed in apples through CRISPR/Cas9-mediated gene knockout of MdDIPM4, providing enhanced resistance against *Erwinia amylovora*, a bacterium responsible for apple blight.

- **Challenges faced in the application of CRISPR/CAS9:** Although CRISPR-Cas9 holds immense promise for crop improvement, several challenges and considerations must be addressed when employing it in plant research and breeding:
 - **Off-Target Effects:** Concerns centre around CRISPR-Cas9's potential to induce unintended mutations at sites resembling the target sequence, necessitating precise guide RNA design and validation techniques.
 - **Regulatory Approval:** Varying regulations governing genetically modified organisms (GMOs) worldwide require time-consuming and costly regulatory approval processes, delaying commercialization.
 - **Ethical and Social Acceptance:** Ethical, social, and environmental concerns associated with gene-editing technologies must be thoughtfully addressed to garner public acceptance.
 - **Intellectual Property Issues:** Legal disputes concerning CRISPR-Cas9-related patents may limit accessibility and complicate its application.
 - **Delivery Methods:** Efficiently delivering CRISPR components into plant cells remains a challenge, requiring optimization tailored to different plant species.
 - **Polyploidy and Complex Genomes:** Complex genomes with multiple gene copies (polyploidy) in major crops pose editing challenges, necessitating simultaneous targeting of all copies.
 - **Gene Redundancy:** Some plant genes have redundant functions, demanding the targeting of multiple homologous genes for effective editing.
 - **Tissue Regeneration and Transformation:** Successful editing necessitates the regeneration of plant cells into whole plants, a process that varies across plant species.
 - **Offspring Variability:** Ensuring the stable inheritance of edited traits by plant progeny is crucial.
 - **Long-Term Ecosystem Effects:** Editing plant traits may have unanticipated consequences on ecosystems and interactions with other organisms, necessitating assessment.
 - **Biodiversity Concerns:** Proliferation of CRISPR-edited crops may reduce genetic diversity within plant populations, potentially affecting resilience to pests and diseases.
 - **Global Collaboration:** Collaboration and data sharing among researchers globally are essential but can be hindered by intellectual property issues and regulations.

VI. CONCLUDING REMARKS

As our comprehension of plant genomes expands, it opens up greater opportunities for the application of biotechnology to decipher the intricate interplay between genes and traits. Biotechnological approaches have swiftly advanced, incorporating novel and valuable tools to enhance the effectiveness of endeavours aimed at improving crops. These methods facilitate the rapid development of desired crop varieties with specific attributes, thus contributing to increased agricultural productivity and addressing the needs of the world's rapidly growing population.

Genome editing techniques like CRISPR/Cas9 have displayed substantial potential for promptly addressing emerging challenges in agriculture. In contrast, RNA interference (RNAi) does not introduce new proteins into plants, mitigating concerns related to novel allergenicity. New Breeding Techniques (NBTs) such as cisgenesis and intragenesis, on the other hand, may raise fewer biosafety apprehensions and could be likened to conventional breeding methods.

Consequently, these novel biotechnological tools are propelling groundbreaking advancements across various fields, including medicine, agriculture, and environmental science, amongst others. They are leading the way in devising innovative solutions for intricate problems, effectively shaping the trajectory of biotechnology's future as we continue to explore the boundless potential of the biological realm. As the field evolves, we can anticipate even more thrilling developments in the years ahead, pushing the frontiers of what can be achieved through biotechnology.

REFERENCES

- [1] A. Kamthan, A. Chaudhuri, M Kamthan and A Datta A (2015) Small RNAs in plants: recent development and application for crop improvement, *Front. Plant Sci.* 6.
- [2] A.J. Wood, T.W. Lo, B. Zeitler, C.S. Pickle, E.J. Ralston, A.H. Lee, R. Amora, J.C. Miller, E. Leung, X. Meng and L. Zhang, (2011) Targeted genome editing across species using ZFNs and TALENs. *Science*, 333, pp. 307-307.
- [3] B. Mezzetti, G. Smagghe, S. Arpaia, O. Christiaens, A. Dietz-Pfeilstetter, H. Jones, K. Kostov, S. Sabbadini, H.G. Opsahl-Sorteberg, V. Ventura and C.N.T. Taning (2020) RNAi: What is its position in agriculture? *Journal of Pest Science*, 93, pp.1125-1130.
- [4] C Espinoza, R Schlechter, D Herrera, E Torres, A Serrano, C Medina and P Arce-Johnson (2013) Cisgenesis and intragenesis: new tools for improving crops, *Biol Res*, 46, pp. 323-31.
- [5] C. Limera, S. Sabbadini, JB Sweet and B Mezzetti (2017) New Biotechnological Tools for the Genetic Improvement of Major Woody Fruit Species, *Front. Plant Sci.* 8.
- [6] C. Petri and L. Burgos (2005) Transformation of fruit trees, Useful breeding tool or continued future prospect? *Transgenic Res*, 14, pp. 15–26.
- [7] D. Carroll, (2011) Genome engineering with zinc-finger nucleases, *Genetics*, 188, pp. 773-782.
- [8] D. Reyon, S.Q. Tsai, C. Khayter, J.A. Foden, J.D. Sander and J.K. Joung (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nature biotechnology*, 30, pp. 460-465.
- [9] J.K. Joung and J.D. Sander (2013) TALENs: a widely applicable technology for targeted genome editing. *Nature reviews Molecular cell biology*, 14, pp. 49-55.
- [10] JF Petolino (2015) Genome editing in plants via designed zinc finger nucleases, *In Vitro Cell Dev Biol Plant*, 51, pp. 1-8.
- [11] L. Bortesi, R. Fischer R. (2015) The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv*, 33, pp. 41–52.
- [12] M. Bibikova, K. Beumer, J.K. Trautman, D. Carroll (2003) Enhancing gene targeting with designed zinc finger nucleases, *Science*, 300, pp. 764.

- [13] M.K. Rai and N.S. Shekhawat (2014). Recent advances in genetic engineering for improvement of fruit crops, *Plant Cell Tissue Organ Cult*, 116, pp. 1–15.
- [14] N Podevin, Y. Devos, H.V. Davies and K.M. Nielsen (2012) Transgenic or not? No simple answer! New biotechnology-based plant breeding techniques and the regulatory landscape. *EMBO Rep.* 13, pp. 1057–1061.
- [15] P. Adhikari, M. Poudel (2020) CRISPR-Cas9 in agriculture: Approaches, applications, future perspectives, and associated challenges. *Malays. J. Halal Res.* 3, pp. 6–16.
- [16] Plant Breeding Innovation: TALENs Transcription Activator-Like Effector Nucleases Pocket Ks are Pockets of Knowledge, packaged information on crop biotechnology products and related issues available at your fingertips. They are produced by the Global Knowledge Centre on Crop Biotechnology (<http://www.isaaa.org/kc>). 2020.
- [17] S Novak (2018) Plant Biotechnology Applications of Zinc Finger Technology, *Transgenic Plants*, pp. 295–310.
- [18] S Satyajit, S Ambarish, Vidyarthi and D Prasad (2014) RNA interference: concept to reality in crop improvement, *Planta*, 239, pp. 543-564.
- [19] S. Becker and J. Boch (2021) TALE and TALEN genome editing technologies, *Gene and Genome Editing*, 2, p.100007.
- [20] T. Sprink, D. Eriksson, J. Schiemann and F. Hartung (2016) Regulatory hurdles for genome editing: process-vs. product-based approaches in different regulatory contexts, *Plant Cell Rep*, 35, pp. 1493–1506.
- [21] T.H. Park, V. Vleeshouwers, E. Jacobsen, E. Van Der Vossen and RGF. Visse (2009) Molecular breeding for resistance to *Phytophthora infestans* (Mont.) de Bary in potato (*Solanum tuberosum* L.): a perspective of cisgenesis, *Plant Breeding*, 128, pp. 109-11.
- [22] T.K. Mohanta, T. Bashir, A. Hashem, E.F. Abd_Allah and H. Bae (2017) Genome editing tools in plants. *Genes*, 8, p.399.
- [23] U.B. Jagtap, RG Gurav, and VA Bapat (2011) Role of RNA interference in plant improvement, *Naturwissenschaften*, 98, pp. 473-492.
- [24] W.C. Gan, A.P.K. Ling (2022) CRISPR/Cas9 in plant biotechnology: applications and challenges. *Bio Technologia*, 103, pp. 81-93.
- [25] X Wang, Y An, P Xu and J Xiao (2021) Functioning of PPR Proteins in Organelle RNA Metabolism and Chloroplast Biogenesis, *Front. Plant Sci*, 12.