**ADVANCES IN BIOTECHNOLOGICAL TOOLS AND TECHNIQUES FOR CROP IMPROVEMENT**

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**Abstract**

Biotechnology is a collection of biological tools that were established through fundamental study and are currently used in research and crop improvement. Modern plant breeding uses biotechnological tools to genetically improve crops for desired traits and productivity. Most conventional breeding techniques are based on hybridization and selection of advanced breeding lines. The slow and challenging method of conventional breeding for genetic improvement of crops has limitation brought on by high heterozygosity, long juvenile phase and self-incompatibility. The two main techniques of introducing heterologous DNA into plants, direct transformation methods (such as Biolistic) and indirect approaches (such as Agrobacterium tumefacien). Plants are now genetically altered more precisely and quickly because of advances in biotechnological tools (NBTs) which include RNA interference (RNAi), cisgenesis/intragenesis, trans-grafting and genome editing tools like CRISPR/Cas9 and zinc-finger. New biotechnological tools (NBTs) consisting of genetic engineering strategies that can activate insertion of essential genes into the genome of various plant species, resulting in greater efficiency and reliable genetic improvement of clonal propagated plants, keeping stability of the essential traits of the clone.

**Keywords –** New Biotechnological Tools, RNA interference, CRISPR/CAS9, genome editing, zinc finger, cisgenesis/transgenesis

1. **Introduction**

Biotechnology refers to collection of techniques use to manipulate desired genetic material both within and between organisms. The term biotechnology is derived from the Greek word “bios’’ (everything relating to living systems) and “technikos’’ (containing human knowledge and skills). Plants have been subjected to genetic modification for more than three decades. Genetic engineering in plants has been in progress for more than three decades. Direct transformation methods (Biolistic) and oblique methods (Agrobacterium tumefaciens-mediated transformation) evolved many years ago, where the number one technique is of heterologous DNA creation into plants. All genetically modified flora commercially grown, along with woody fruit species, were produced using this sort of method. Often the capability to gain fruit tree plants with new inclinations or mutations via genetic engineering or via NBTs is predicated upon the existence of a well-established *in vitro* regeneration protocol, that is primarily based upon at the genotype and the plant tissue used. Although NBTs share the same objective as accurate, quick and efficient crop improvement but they each have a very different methodology and set of qualities. Furthermore, it's recommended from an agronomic factor of view to *in vitro* regenerate a brand-new fruit tree plant from mature tissues, because of the excessive heterozygosity, which signify the bulk of those species. The advent of recombinant DNA generation paved the manner for a tremendous capability within the area of plant biotechnology. In this experience relevant improvement had been revamped in the past for some difficultly to transform woody species, such as peach or grapevine genotypes, where protocols for the regeneration of adventitious shoots had been advanced from mature tissues. Introduction of one or greater new genes or regulatory elements using genetic engineering techniques, without delay manipulates the genome of an organism that allows you to explicit or silence unique traits. Transgenic processes having international effects are aimed for the manufacturing of plants with new resistance genes in opposition to pests and diseases, or herbicide tolerance and plants with suitable dispositions and dietary levels, which encompass the golden rice with an extended vitamins A.

1. **RNA Interference**

RNA interference is a revolutionary mechanism in which a short double-stranded RNA blocks the expression of gene by inducing the cytoplasmic destruction of a specific target mRNA sequence. The first transgenic plant to exhibit the RNAi was *Petunia hybrida*. Chalcone synthase gene (CHS A), which encodes essential enzymes in the anthocyanin biosynthesis pathway was introduced in Petunia to increase anthocyanin pigmentation. Due to unexpected silencing of endogenous homologous gene, transgenic plants instead of producing dark purple blossom produced white or chimeric flowers this phenomenon was known as ‘co-suppression’. RNAi is an inherently occurring biological process that controls gene expression prior to translation or "turns off" undesirable or damaging nucleic sequences. It has been demonstrated that RNAi uses dsRNAs as trigger molecules to find homologous mRNAs whose transcription is negatively controlled in both plants and animals.

Consequently, in fungi, insects, bacteria, viruses and plants RNA silencing has become the primary technique for gene targeting. Currently, numerous methods of gene silencing have been discovered in plants including PTGS, microRNA silencing and transcriptional gene silencing. All these processes need the presence of dsRNA molecules of various sizes, which are introduced into the plant cell by protein families, including Dicer or Dicer-like (DCL), Argonaute (AGO) and RNA-dependent RNA polymerases. Different model organisms have so far revealed numbers of short non-coding regulatory RNAs which include small vault RNA (svRNA), miRNA, siRNA, piRNA (PIWI interacting RNA), qiRNA (QDE-2-interacting RNA) all of which have various biochemical processes for their formation. The RNAi strategy has demonstrated its potential for crop improvement by helping to achieve desirable features by modifying genetic expression. So, the discovery of RNA interference resulted in creation of new techniques that has wide potential for use in genetic analysis, crop improvement and plant protection with better understanding of gene regulation, analysis and functions. Other benefits of RNAi technology include improved nutrition, morphology or increased secondary metabolite synthesis. In addition to controlling gene expression, RNAi also functions as a natural defence system against molecular parasites including jumping genes and viral genetic components that threaten genomic stability.

**Mechanism of RNA interference**

In plants, the mechanism of RNA silencing operates on at least three different levels: mRNA is cleaved by dsRNA-mediated cytoplasmic silencing, endogenous mRNAs are silenced by micro-RNAs (miRNAs), which suppress gene expression by base-pairing to certain mRNAs. This results in either RNA cleavage or preventing protein translation, known as post-transcriptional gene silencing (PTGS) and DNA sequence-specific methylation is linked to RNA silencing, which causes repression of transcription. Small interfering RNAs (siRNAs), which are 21 to 24 nucleotides long and staggered cut starts the overall process of RNA interference in plants which are produced intracellularly from lengthy internal or external dsRNA molecules by the activity of a ribonuclease III-type enzyme called Dicer. These siRNAs (21–24 nt) are subsequently added to the RNA–induced Silencing Complex (RISC), which also includes numerous other proteins like AGO. The double-stranded siRNA is unwound by the RISC which is ATP-activated. After an RNA helicase activity destroys the sense strand of the siRNA duplex, the antisense strand of the siRNA molecule is integrated into a nuclease-containing RISC complex. By using base-pairing interactions to target the homologous transcript, RISC with an antisense siRNA sequence either cleaves the mRNA or prevents translation, which inhibits protein production.



 **Figure 1. Mechanism of RNA interference**

**MicroRNAs (miRNAs)**

miRNAs are most prevalent and significant family of short regulatory RNAs. These are endogenous 23nt RNAs that are transcribed by RNA Polymerase II. They play a key role in crucial gene-regulatory processes by partnering with the mRNAs of protein- coding genes to control their suppression. A homolog of the dicer, DCL1 is required for the conversion of both pri-miRNA to pre-miRNA and pre-miRNA to mature miRNA in plants. The miRNA induced silencing complex (miRISC), which contains Argonaute and other effector protein is recruited after the production of mature miRNAs. By antisense base-pairing target mRNAs with miRNAs, the miRISC can result in miRNA-mediated gene silencing. A cell's state, cell type, developmental stage, target site etc. can all affect the silencing mechanisms in different ways.

**Short-interfering RNAs (si RNAs)**

The process of RNAi is started by long dsRNA or short-hairpin RNA (shRNA) precursors that have a sequence homology to the target gene to be silenced. RNAi pathway is activated by entry of foreign entities like virus, genetic material like transposon or by introduction of transgene which provides the long dsRNA. The dsRNA is cleaved into siRNA which are short 5-phosphorylated dsRNAs (21–25 nt) with two nucleotide overhangs at the 3 ends, because of the recruitment of the dicer enzyme in the cell. The sense strand of siRNA is degraded because of siRNA-induced silencing complex (siRISC). The target mRNA is then cleaved in a sequence-specific manner as a result of the siRISC being integrated into the antisense strand of siRNA, which works in conjunction with AGO and other effector proteins. The RISC that has been activated can frequently take part in mRNA degradation and protein synthesis inhibition, resulting in PTGS.

1. **Trans Grafting Technique**

This method primarily uses grafting, a horticultural practise used for centuries to increase the quality and production of fruits. This process combines two autonomous genotypes that have been independently chosen for their fruiting traits and rooting capacity. The scion and the rootstock are grafted together to combine their superior features. The rootstock can change the phenotypic character of scion by altering its vigour and enhancing fruit set although the grafted tissues are linked, the rootstock and scion maintain their genetic integrity because their genetic matter do not mix. Trans grafting is a technique that combines conventional grafting techniques with plant genetic engineering.

 

 **Figure 2. Grafting**

A non-genetically modified scion is grafted onto a rootstock that has undergone genetic modification. The scion gains the advantages and characteristics that transgenes in the rootstock impart, but the finished goods, like fruits, do not carry the transgene and are therefore not genetically changed. The trans-grafting process is based on the transport of RNA molecules through the vascular system from the rootstock to the scion. Recent studies demonstrated that the phloem transmits few specific RNA molecules to regulate organ development. Over 15% of the transcripts in the functional studies of phloem are associated with signal transduction. RNAi can be used to create transgenic plants that are resistant to viruses if RNAi-based rootstocks can effectively transfer the silencing molecules to non-transformed scions. The systemic transmission of silencing signals via phloem and from cell to cell via plasmodesmata has been linked to microRNAs and transacting siRNAs. For the downward flow of photosynthetic products and the upward movement of water and mineral nutrients, as well as for the transmission of the RNAi silencing signal into the scion and the start of systemic silencing, compatibility is important between scion and rootstocks interaction.

1. **Cisgenesis/Intragenesis**

Considering public issues regarding protection against transgenic crops, cisgenesis and intragenesis have been advanced as new tools in crop amendment and plant breeding. The term “cisgenic plant” was introduced first, some years in the past and it refers to “a crop plant that has been genetically changed with one or more genes (containing introns and flanking areas inclusive of native promoter and terminator areas in an orientation) isolated from a crossable donor plant. Basically, it means that the genetically changed cisgenic crop carries genes preserving their natural genetic composition i.e., entire replica of a gene with all its regulatory elements. The supply of a cisgene is the identical plant species or a sexually well-suited species as used for traditional breeding. However, in contrast to conventional breeding, cisgenic include the gene or genes of interest and no undesired genetic elements. On the opposite hand, intragenesis refers to GMOs in which the delivered intragene additionally originates from the identical species or a crossable species, however in comparison to cisgenes, intragenes are hybrid genes, which could have genetic factors from specific genes and loci. As a result, the expression of a gene may be modified with the usage of various promoter or terminator regions.

Intragenesis lets to the development of new genetic combinations introducing variability for gene expression, the introduction of novel expression styles and therefore new GMOs with progressive properties. Based on using native genes in contrast with using hybrid genes, cisgenesis can be taken into consideration a whole lot nearer to conventional breeding than intragenesis. RNA interference (RNAi) strategies using DNA sequences are also taken into consideration as intragenesis. Another key distinction among cisgenesis and intragenesis, is concerning the T-DNA borders or different sequences finally transferred to the plant because of the Agrobacterium mediated transformation process, a subject which isn't always exempted from a diploma of controversy. While a few agree within the truth that intragenic and cisgenic must have generated the usage of border sequences originating from the crossable gene pool, others have proposed that the supply of border sequences isn't a prescribing factor; opening the opportunity to apply canonical T-DNA borders comparable to any GMO. Cisgenic and intragenic ought to additionally be lose from different non-plant sequences, together with vector spine and choice markers.

 Several techniques to both keep away or remove marker genes have been described, for instance, whilst the transformation performance is high, using certain markers may be avoided. Then converted lines are selected on via means of the presence of the specific gene sequence introduced, but this requires the assessment of several plants and is each highly priced and time consuming. To conquer this, marker deletion strategies primarily based upon precise recombination has been developed. In other cases, where marker selection genes are flanked with recombination sites (R/Rs system), and later converted plant have been selected, the recombination is induced with the resultant launch of the marker gene. Marker excision in addition was shown via molecular biology techniques. Cisgenic and intragenic apples in addition to intragenic strawberries were acquired using this marker-free technology.

 Co-transformation is other way to supply marker in unfastened plants. This technique is primarily based on the marker gene and the transgene of interest in present in exclusive positions of the plant’s genome, permitting the segregation of each gene in one of a kind progeny. Co-transformation may be executed using transformation vectors, one along with the gene of interest and one with the marker gene. Examples of this method are cisgenic barley plants and durum wheat. The medical community has interest in promoting less stringent rules for cisgenic/intragenic crops. For instance, the European Commission (EC) asked the European Food Safety Authority (EFSA) to decide the dangers of cisgenic/intragenic in comparison to transgenesis or traditional breeding. It was proposed that cisgenesis can have comparable hazards to breeding, while transgenesis and intragenesis are much less predictable. In the USA similar situation arose; The Environmental Protection Agency (EPA) is discussing the regulatory framework for those crops. Cisgenic and intragenic have been meant to facilitate the approval of the stringent rules of transgenic cultures, but alas the modern-day situation puts both technology in the identical class as transgenic crops.

 

 Limera C, et al., (2017)

 **Figure 3. Cisgenesis and Transgenesis**

**Application Of Cisgenesis and Intragenesis to Improve Traits in Crops**

Several traits were integrated which were applicable to plants by cisgenic or intragenic approaches. These species include potato, apple, strawberry, alfalfa, perennial ryegrass, poplar, barley and durum wheat. The first intragenic potato was evolved in order to provide high amylopectin content. The method employed was primarily based on silencing of the granule certain starch synthase gene (GBSS), which is liable for the synthesis of amylose in potato. The composition of starch in potato is an important trait, and presently it's hard to achieve the cultivated tetraploid potatoes with the preferred content material of amylose and amylopectin. Thus, techniques to silence both amylose and amylopectin artificial genes. The possibility of acquiring tetraploid cultivars that includes all the favoured tendencies present within the authentic cultivar. This potato was released in the field in 2007 by the agency AVEBE and incorporated T-DNA borders and a GBSS terminator from potato or a nopaline synthase gene terminator from *Agrobacterium tumefaciens* for law of gene expression.

Other intragenic approaches have been addressed for potato processing qualities. For instance, enzymatic browning become dwindled through the silencing of a polyphenol oxidase gene (PPO), which catalyses the oxidation of cytoplasmatic polyphenols inflicting the precipitation of black melanin and compromising tuber growth throughout storage. Another essential potato trait is in relation to cold induction which results in sweetening, which due to excessive starch degradation prompted via means of low temperature at some point of storage. To avoid this, intragenic potatoes silenced in genes involved in starch degradation, water dikinase (R1) and amyloplast-targeted phosphorylase-L (PhL) have been constructed. Subsequently, the PPO, R1 and PhL genes have been silenced in potato, generating tubers with splendid properties. Induction of tolerance to the most critical potato disease, late blight caused by the oomycete *Phytophthora infestans*, is predominant challenge in potato research. Resistance genes (R-genes) found in wild potatoes were transferred into potato varieties by traditional breeding, but this is confined due to the variations within the ploidy tiers among potato species. For this reason, the Durable Resistance in opposition to *Phytophthora* (DuRPh) programming ends to introduce numerous R-genes from wild potatoes, with their regulatory sequences into cultivated potatoes. This cisgenic method additionally considered using marker-free technologies.

In case of fruit trees, the most crucial apple disease is scab, that's because of the ascomycete *Venturia inaequalis*. All cultivated apples are at risk of this pathogen, and currently the HcrVf2 gene within the scab resistance locus Vf became added in apple cv. Gala. The transferred gene incorporates its personal regulatory sequences i.e., promoter and terminator and in fact, claims to be the first record of a “proper cisgenic plant”. An intragenic method additionally aimed to set off scab resistance used the same HcrVf2 gene, but in this case, the resistance gene is managed with the aid of using the promoter and terminator of the small subunit of the apple rubisco gene and in addition no scab resistance in apples has been shown till now.

Modification of tree structure and growth rate is cumbersome and difficult for the woody plant industry. Based on this, a cisgenic method advanced in poplar seeks to address this issue. For this, genes coding for gibberellic acid biosynthesis enzymes collectively with the irregular sequences have been overexpressed in poplar trees, leading to growth in tree increase. Overexpression of catabolic genes in addition to negative regulators results in decreased tree size. Therefore, cisgenic or intragenic techniques might be beneficial for plants with lengthy lifetimes and excessive heterozygosity levels, in which conventional breeding methods may be very limited and tedious.

 Cisgenic barley became advent through numerous copies of the phytase gene. Phytase catalyses phosphate launched from phytic acid, making phosphate available to be absorbed by animals. This strategy has been proven to be promising in increasing phosphate bioavailability, and consequently ought to constitute an opportunity to keep away from the addition of microbially-derived phytase to feed, and to lessen the cutting-edge environmental pollutants derived from phosphate.

1. **Genome Editing**

 Genome editing tools have great potential to alternate the genomic construction of a genome at specific positions, by means of anticipated exactness. These tools have been apt competently for trait unearthing and for the generation of plants with high crop yields and resistance to biotic and abiotic stresses. Some of the most important genome editing tools are: Homologous Recombination (HR), Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), Pentatricopeptide Repeat Proteins (PPRs) and the CRISPR/Cas9 system. In addition to this gene editing also includes site-directed sequence editing and oligonucleotide-directed mutagenesis.

1. **Homologous Recombination**

 Chromosomal recombination is the most efficient genome engineering system that is naturally present within the cell. This system can be utilized for genome editing by the commencement of double-stranded breaks (DSB) in the chromosome. DSBs during cell division lead to meiotic recombination. The Homologous Recombination acquaint in a precise section is identified as a recombination hotspot. Because all chromosome endures at least one recombination event during meiosis, alleles generate new combinations. The association of alleles inherited from every parent determine aren't conserved, as alternated by the unconventional germ cells deliver chromosomes with new combinations of alleles of the genes. This rehashing of combinations of alleles is a rich foundation of assortment in a populace.

 

 **Figure 4. Homologous Recombination**

 Over a timespan, recombination disseminates alleles at one locus from alleles at a linked locus. A chromosome is not static over generations, nonetheless it is "fluid," having numerous dissimilar combinations of alleles. This leads to nonfunctional (much less functional) alleles to be vacated from a population. If recombination does no longer transpire, then one deleterious mutant allele could be the reason for the elimination of entire allele from the population. However, with recombination, the mutant allele can be separated from the alternative genes on that chromosome. Negative selection can eradicate flawed alleles of a gene from a population which affects the frequency of alleles, only of genes in close linkage to the mutant gene. Contrarywise, the infrequent useful alleles of genes can be verified in a population deprived of being irrevocably linked to any possible deleterious mutant alleles of adjacent genes. This retains the actual target magnitude for mutation near to that of a gene and not the whole chromosome.

**Mechanism of Recombination**

 Recombination befalls when a section of the paternal chromosome is bartered. For the homologous piece of DNA on the corresponding maternal chromosome (or vice versa). Perceptibly, this kind of DNA switch ought to be carried out cautiously and with equivalence, in order that the ensuing DNA does no longer gains or lose information. To make certain this precision in recombination, the non-sister homologous chromatids are held collectively through proteins in a synaptonemal complex (SC) during prophase I. This ladder-like complex starts evolving to shape within the zygotene degree of prophase I and completes in pachytene. The whole SC includes proteinaceous lateral elements (aka axial elements) that run alongside the chromatids and a short applicable element composed of fibrous proteins forming the rungs of the ladder perpendicular to the two lateral elements. Recombination may also arise without or with the formation of double strand breaks and in fact, can arise without. The formation of the synaptonemal complex, even though the SC possibly complements the performance of recombination. In maximum cases, recombination is preceded via means of the formation of recombination nodules, which might be protein complexes that shape at capability factors for recombination.

 The mechanism studied for meiotic recombination includes a double-stranded break of one of the chromosomes initiated with the aid of using the meiosis-precise endonuclease, Spo11.The 5’ ends of this reduce are degraded barely to shape 3’ single-stranded overhangs. These unpaired ends cause the formation of Holliday junctions (named after Robin Holliday) with a strand from other chromatid appearing as a template for synthesis of lacking part of the chromatids, which gives rise to sister chromatids that are "entangled" through one strand of DNA paired with a unique chromatid. This entanglement can be resolved without or with a crossover. The recombination is initiated in pachytene and completes in diplotene, currently synaptonemal complicated breaks down. As the chromatids starts to separate, chiasmata become obvious at several of the recombination sites. As prophase completes, the chiasmata resolve from the middle of the chromosomes towards the end.

**Recombination used as a restore mechanism!**

 Homologous recombination happens in meiotic cells. In most species, every chromosome will undergo at least one recombination event. However, the capacity to use one chromosome as a template for a damaged section also can be utilized in times of DNA damage, particularly DNA spine breaks. The potential of the cell to use homologous recombination to restore a damaged chromosome relies upon on whether it can ‘find’ a homologous series to apply as a template to re-synthesize a broken chromosome.

1. **Zinc Finger Nucleases**

 The category of targeting reagents that proved to be of maximum flexibility and effectiveness in recent decades is that of zinc-finger nucleases (ZFNs), that have separate DNA-binding and DNA-cleavage domains. These synthetic proteins originated under the attention of Chandrasegaran that the natural type IIS restrict enzyme, FokI, has bodily separable binding and cleavage activities. A customary ZFN monomer is made via means of fusing two domains: an artificially prepared Cys2-His2 zinc finger domain and the non-specific DNA cleavage domain of the FokI DNA limit enzyme. A standard zinc finger area is composed of three to four fingers, each able to spot an about 3-bp-lengthy sequence. This cautioned that many unique sequences can be attacked via making unique assemblies of ZFs. Dimerization of the FokI area is important for its enzymatic activity. Thus, digestion of DNA may be accomplished while ZFN monomers that bind to their respective DNA target sequences and align in reverse configuration. The two ZFN monomers are normally designed such that they will flank a 5- to 6-bp-long sequence within the DNA target sequence, permitting the FokI dimer to digest inside that spacer sequence.

 

 Limera C, et al., (2017)

#  **Figure 5. Zinc-Finger Nuclease (ZFN) structure and mechanism of inducing double strand breaks (DBSs) on its target**

 The studies organized under Dan Voytas pioneered use of ZFNs for gene editing in plant cells. Restoration of a defective preference marker gene in tobacco protoplasts, researchers confirmed that GT frequencies can be brought up to a hundred and one in contrast with random integration. Simultaneously, it was demonstrated that ZFNs may be used to mutate an artificially introduced restriction site in the *Arabidopsis* genome via nonhomologous DNA end joining (NHEJ). Effort and time were required to clarify the use of ZENs for engineering in plant genomes of non-transgenic sequences. In 2009, Dan Voytas’ group, along with scientists from Dow Chemicals, demonstrated independently that it was feasible to modify endogenous genes in tobacco and maize via means of DSB-prompted HR using the ZFNs. In tobacco, the SuRA and SuRB loci were modified at the Single-nucleotide degree through ZFN-mediated GT to exhibit resistance to diverse herbicides. An impressively exorbitant gene targeting (GT) frequency was reported with high percentage. In maize, ZFN-mediated GT of the maize IPK1 gene expression ended in an herbicide-resistant phenotype. Also, nine endogenous soybean (*Glycine max*) genes have now been mutated using ZFNs. ZFNs have consequently come out as an essential component type of genome editing in plants. Nevertheless, the dominance of ZFNs may also already be over due to the recent improvement of appealing equipment for site-specific DSB induction.

 A popular problem with synthetic nucleases is that they'll reduce additional sites within the genome which is probably similar but not identical to the target site. Such ‘off-goal effects’ are a reason of concern as unexpected mutations can be induced. The respective mutations might also bring about unwanted secondary effects. Indeed, it's been found that a few ZFNs have terrible outcomes on cell proliferation. This shows that nucleases cause undesirable DSBs at secondary sites. Further uncertainty stems from the reality that the numerous binding modules within the zinc-finger binding arrays affect each other. Therefore, production of domains for new genomic sites is not as predictable and efficient as expected. Although kits for constructing ZFNs are available, the creation of ZFNs nonetheless seems to be greatly time-consuming than that for TALENs or the CRISPR/Cas system and much less versatile, as ZFN layout is often confined via means of shortage of suitability of the target sequence.

**Application of Zinc Finger Nucleases**

1. **Targeted Mutagenesis**

Multiple strategies for random mutagenesis were utilized in the past (radiation, EMS mutagenesis, T-DNA insertion, to call a few). However, the appearance of ZFNs supplied the capacity to create centred knockouts by designing sequence unique ZFNs to create DSBs at pre-described genomic locations. Creating new alleles via targeted mutations unlocks an entire new place of potential benefit in plant biotechnology. The first utility of ZFNs in a plant involving targeted mutagenesis of a transgenic locus in *Arabidopsis*. Constructs containing heat shock inducible ZFN as along with target containing a ZFN recognition series have been delivered into *Arabidopsis*. The first example of ZFN-induced mutation of an endogenous gene in a crop plant was illustrated in maize (*Zea mays*) cells through expressing an engineered ZFN focused to exon 2 of the Maize inositol-1,3,4,5,6-pentakisphosphate 2-kinase (IPK1) gene.

Targeted mutations of endogenous genes within the cells of economically vital plant species consisting of soybean (*Glycine max*), Canola (*Brassica napus*), rice (*Oryza sativa*), tomato, fruit trees (apple and fig), and hybrid poplar have also been generated. Targeted gene mutations are very beneficial to allow new attributes/phenotypes within the altered plants. ZFNs were designed and implemented to modify endogenous genes, multigene families, and transgenic loci in plants, crops, and trees. ZFN has validated quite to be versatile in generating gene mutations, with a couple of expression techniques (constitutive or induced) in addition to a couple of techniques of DNA delivery (each brief and stable). The mutations created using ZFNs have been shown to be heritable, and the fidelity of the ZFNs has been high, with singular genes from multigene being projected with little proof of mutations at nontarget sites.

1. **Gene Editing**

While developing gene knockouts the usage of ZF generation is a useful way for developing loss of functional mutants, gene enhancing via alteration (converting one or greater bases in a gene sequence) gives infinite opportunities in plant biotechnology. Gene editing permits alteration of gene of interest to create a favoured phenotype. Gene editing occurs when, in addition to supplying a ZFN for focused DSB formation, a donor molecule containing the desired modification (edit) is also provided. When the donor molecule is used as a restorer template for the DSB, unique change on the targeted site may occur. One such instance of gene editing is the modification of the endogenous tobacco acetolactate synthase genes (ALS SuRA and SuRB) that's the goal enzyme for the Sulfonylurea and imidazolinone herbicides.

Using a ZFN and a donor molecule, specific codon mutations were brought about into the SuR locus, for this reason producing acetolactate synthase (ALS) mutants which have been herbicide resistant. Similarly, to mutagenesis the use of ZFNs designed to cleave the protoporphyrinogenoxidase (PPO) gene in Arabidopsis, led to the demonstration of gene editing. The edited PPO gene contained two separate mutations permitting the PPO enzyme to be insensitive to the butafenacil herbicide. In plants the application of ZFNs in gene editing has been constrained to the target loci only that provide herbicide choice for the gene edits. The efficiencies of the process need to be progressed for large-scale editing of alleles and creating genetic diversity.

1. **Targeted Gene Addition**

Targeted gene addition (gene targeting) can allow, for instance, the stacking of traits in a single locus. This form of trait stacking is favourable in the agricultural biotechnology industry as it might streamline the deployment of several traits when introduced throughout germplasms. Likewise, gene editing, gene targeting can also make use of a ZFN and a donor molecule; however, in contrast to editing, gene concentrated outcomes in massive addition of the donor on the site (as opposed to simply converting bases, bases are added).

In a tobacco test system, HR-based gene targeted was accomplished using a donor and ZFN delivered into protoplasts of transgenic tobacco strains containing nonfunctional reporter genes with an inner ZFN recognition site. Firstly, intrachromosomal recombination essential for reconstituted functional reporter gene was performed following DSBs induced by a ZFN. Secondly, induced DSBs upstream of previously integrated target locus containing Plant Biotechnology Applications of Zinc Finger Technology 301 intron and 3’fragment of the selectable marker was repaired by a donor DNA containing the 50 fragment of the selectable marker and an intron as homology to the target region. Homology directed repair (HDR) used to supply gene concentrated on target to reconstitution of selectable marker. Lastly, gene targeting into the endogenous tobacco endochitinase gene turned into confirmed the use of a construct having a ZFN and a selectable marker containing donor introduced into tobacco cells.

Resistant isolates acquired on choice have been PCR showed indicating HDR gene focused on to the native endochitinase gene. Targeted gene addition has been sought in plant biotechnology and has now been deployed in lots of forms. Gene concentrated on the usage of ZFNs has been established in transgenic and endogenous loci of crop plant life. Two exceptional forms of DNA repair (NHEJ and HDR) had been applied for focused insertion of transgene. As new advances are made within the field, inefficiencies in the procedure are being addressed. One of the precept applications of this technology is the stacking of biotech traits, which has been exemplified but not however commercialized. The value in agricultural biotechnology is enormous, specifically thinking about the scale of the attempt devoted to breeding and introgression of biotech developments in business agriculture.

1. **Gene Deletion**

A more thorough and elegant technique to eliminate a gene’s characteristic could be to delete it entirely. Gene deletion may be performed via developing a ZFN-mediated DSB on each facet of the sequence and for that reason disposing of the intervening segment. In addition, ZFN-triggered DSB made adjoining to genomic areas of excessive homology can cause HDR ensuing deletion of the intervening section of DNA.

The use of ZFNs to create deletions in tandemly arrayed genes (TAG) has been confirmed in Arabidopsis. Using ZFNs, seven genes for 3 unique TAGs had been effectively centred for deletion. The length of deletions ranged from 4.5 kb to 55 kb with a frequency of ~1% in vegetative tissue, however germline transmission of these deletions was overlooked. Deletions as large as ~nine Mb had been acquired however with less frequency (0.046%). In addition to deletions, ZFN-brought about inversions and duplications of those gene clusters have additionally been obtained, thus displaying the capacity to create novel genomic areas through centred DSBs having chromosome rearrangements and/or chimeric genes. Whether deployed in local or transgenic loci, ZFNs have eliminated segments of DNA (with sizes as big as ~nine Mb). Depending upon this concept, this will help in developing genetic diversity. This opportunity for genetic alteration is profound about the deletions, inversion, and duplications have all been confirmed due to ZFN-induced DSBs.

1. **Gene Regulation**

 The functionality to alter gene expression gives capacity for primary biology and plant biotechnology applications. By combining facts of the interface of the ZF and DNA with information about transcription regulators and engineered DNA-binding domains, all the necessary elements to create engineered zinc finger protein transcription factors (ZFP-TFs) to alter gene expression. For example, ZFP-TFs had been designed to map DNase I reachable areas inside the vascular endothelial growth aspect A (VEGF-A) gene. The utility of this in plants initiated with an outlook on Arabidopsis APETALA3 (AP3) gene. ZFP-TFs were designed to target ~50 bp upstream of the TATA box. When delivered into leaf protoplasts, ZFPs fused to the VP16 activation area ended in each multiplied expression of a transgenic reporter and floral phenotypes consistent with expectation in stable plants. ZFP-TFs has proven a remarkable 450-fold boom in reporter gene expression in tobacco plants. In addition, tissue-specific activation has additionally been proven in vascular tissue the use of a ZFP-TF expression by the aid of using phloem-unique promoter. Additionally, ZFP-TF introduced activation responded in dose-dependent way while being induced chemically. Furthermore, transcription activation domain names derived from plants had been created, which allowed higher activation of expression in comparison to the VP16 domain previously used.

 The application of ZFP-TFs has been exemplified in the crop Arabidopsis, where ZFP-TFs absolutely derived from plant sequences exhibited the functionality to upregulate expression of an endogenous gene in an entire plant. Also, seed-particular expression of ZFP-TFs led to extended seed α-tocopherol for numerous lines. The utility of engineered ZFP-TFs to upregulate and downregulate expression of an endogenous plant gene was exhibited. Transgenic ZFP-TF traces with an activation area confirmed elevated lignin content, even as transgenic ZFP-TF traces with a repression area confirmed reduced lignin content. Engineered ZFP-TFs have additionally been used for the expression of an endogenous gene within the crop plant canola (Brassica napus). Engineered ZFPs have exquisite application in regulation of plant genes. Regulation of endogenous genes in addition to transgenes has been proven in each model and crop plants. In many times this expression regulation has caused amendment of phenotypes. The use of ZFPs permits expression modulation in quite a few phenotypes, which won't be viable with the aid of using knockout created through mutagenesis. Through the usage of tissue-precise promoters, possible envision of tissue-precise gene regulation which could offer for a more suitable realm of viable phenotypes. This technique could be particularly beneficial for regulation of genes that have severe phenotypes which when altered constitutively.

1. **Transcription Activator-Like Effector Nucleases (TALENs)**

The newly advanced transcription activator-like effector nucleases (TALENs) incorporate a non-particular DNA-cleaving nuclease fused to a DNA-binding vicinity that can results in easily engineered TALENs which can target essentially any sequence. The functionality to quickly and effectively regulate any gene collection the usage of TALENs guarantees to have profound influences on organic studies and to yield ability healing techniques for genetic diseases. Recently, transcription activator-like effector nucleases (TALENs) have unexpectedly emerged as an opportunity over ZFNs for genome modifying and introducing targeted DSBs. TALENs are just like ZFNs and incorporate a non-unique FokI nuclease fused to a customizable DNA-binding place. This DNA-binding area consists of distinctly conserved repeats derived from transcription activator-like effectors (TALEs) which may be proteins secreted by *Xanthomonas* bacteria to alter transcription of genes in host plant cells.

 

 S. Becker and J. Boch (2021)

 **Figure 6. TALE And TALEN Tools**

 Over the past few years, leveraging technology and methodologies formerly evolved for the usage of ZFNs, numerous agencies have used TALENs to regulate endogenous genes in yeast, fruit fly, roundworm, crickets, zebrafish, frog, rat, pig, cow, thale cress, rice, silkworm, and human somatic and pluripotent stem cells. Although ZFNs and TALENs have not been compared, many researchers have proven that TALENs and ZFNs have similar efficiencies while being focused to the identical gene. Thus, the benefit of design, excessive prices of cleavage activity, and the essentially infinitely focused on range of TALENs cause them to be the uses via non-expert researchers. Nuclease-mediated modifying of plant life and agricultural animals can also additionally significantly lower the time required to generate new accessions of each species relative to standard breeding strategies. For example, the use of micropropagation strategies, some plant species can be modified on the cellular degree and then grown into mature plants. To date, TALENs had been used to make knockout mutations in Arabidopsis and to introduce resistance to infection, via Xanthomonas microorganism in rice by disrupting the site of target of naturally present TALEs that make a contribution to pathogenicity. Gene-enhancing nucleases have additionally been used to make centred NHEJ-brought about indel mutations in pigs and cows. In addition, big deletions and inversions of sequence bigger than 6 kb in period were induced in pigs through concentrated on TALEN pairs to the similar 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:

 

 **Figure 7. TALENs**

* TALE Protein Design:

The first step in the TALEN mechanism is the design and construction of TALE proteins. TALEs are derived from bacteria, specifically the plant pathogen Xanthomonas. These bacteria produce TALEs as part of their virulence machinery to manipulate host plant cells.

Each TALE protein consists of a repeating unit, where each repeat can recognize a specific DNA base pair. These repeats are customizable, and their sequence specificity can be engineered to target a particular DNA sequence of interest. The key to TALEN specificity is the presence of a customizable region within each repeat known as the "repeat-variable di-residue" (RVD), which can be designed to recognize a specific DNA base: adenine (A), cytosine (C), guanine (G), or thymine (T).

* DNA Binding:

Once the TALE proteins are designed to recognize the desired DNA sequence, they are linked to 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 recognizing 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 in each TALE bind specifically to the corresponding DNA bases through 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 bring the FokI nuclease domains into proximity by dimerizing. This dimerization of the FokI domains activates the nuclease activity.

The FokI endonuclease cleaves the DNA at a specific location within the target site, generating a double-strand break (DSB).

* DNA Repair:

After the DSB is created, the cell's natural DNA repair machinery is activated to repair the break. There are two primary DNA repair pathways:

* Non-Homologous End Joining (NHEJ): This pathway often results in small insertions or deletions (indels) at the site of the DSB, which can disrupt the reading frame of a gene and lead to 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 net result of the TALEN mechanism is the introduction of targeted genetic modifications at the desired genomic location. Researchers can exploit 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:

* Crop Improvement:
* Trait Modification: TALENs can be used to introduce specific genetic changes into crop plants to confer desirable traits, such as:
* Disease Resistance: TALENs have been employed to engineer resistance to plant pathogens like bacteria, fungi, and viruses. This reduces the need for chemical pesticides and enhances crop yields.
* Pest Resistance: TALENs can create crops that are resistant to insect pests, reducing crop damage and the use of chemical insecticides.
* Herbicide Tolerance: TALENs can be used to develop plants that can tolerate specific herbicides, allowing for more effective weed control without harming the crop.
* Nutritional Enhancement: TALENs can be used to modify the nutritional content of crops. For instance, researchers have used TALENs to increase the levels of essential nutrients like vitamins or minerals in plants, addressing malnutrition issues.
* Stress Tolerance:
* Abiotic Stress Resistance: TALENs can be used to engineer plants for improved tolerance to environmental stresses such as drought, salinity, or extreme temperatures. This is crucial for adapting agriculture to changing climatic conditions.
* Biotic Stress Resistance: TALENs can be employed to develop plants with enhanced resistance to pathogens, including viruses, bacteria, and fungi. This can reduce the need for chemical pesticides and increase crop yields.
* Functional Genomics:
* Gene Function Studies: TALENs enable researchers to disrupt or modify specific plant genes to study their functions. This helps in understanding the roles of individual genes in plant growth, development, and responses to environmental stimuli.
* Plant Breeding:
* Accelerated Breeding: TALENs can be used to speed up the breeding process by introducing specific genetic changes into plants. Traditional breeding methods can take many generations, while TALEN-assisted breeding can achieve desired traits more rapidly.
* Quality Improvement:
* Fruit Ripening: TALENs have been used to modify genes involved in fruit ripening, which can extend the shelf life of fruits and improve post-harvest handling. Aroma and Flavour Enhancement: TALENs can be employed to modify genes responsible for the production of aroma compounds and flavours in fruits and vegetables, enhancing their taste and marketability.
* Medicinal Plant Production:
* Secondary Metabolite Enhancement: TALENs can be used to increase the production of secondary metabolites in medicinal plants. This is important for improving the yield and potency of medicinal compounds used in pharmaceuticals and herbal remedies.
* Environmental Conservation:
* Restoration Ecology: TALENs can assist in the conservation and restoration of native plant species. They can be used to create genetically modified plants that are more resilient to environmental changes and habitat restoration efforts.
* Biosynthesis Pathway Engineering: TALENs can be applied to modify multiple genes involved in a biosynthetic pathway simultaneously, allowing for the engineering of complex traits such as the production of biofuels or bio-based chemicals in plants.
* Education and Research: TALENs are valuable tools for plant biology education and research. They provide a means for researchers and students to gain a deeper understanding of plant genetics and genomics.

It's important to mention that while TALENs have been used for genome editing in plants, newer genome editing technologies like CRISPR-Cas9 have become more popular due to their ease of use and versatility. However, TALENs still find applications in specific cases where their precision and reduced off-target effects are advantageous.

1. **Pentatricopeptide Repeat Proteins (PPRs)**

The pentatricopeptide repeat (PPR) proteins is widely known due to its ample participants and crucial functions in angiosperm species, having been located simply twenty years in the past in a genome sequencing evaluation of Arabidopsis thaliana. The amino acid composition and shape of PPR proteins are much like the ones of tetratricopeptide repeat (TPR) proteins, which generally mediate the interplay amongst proteins; both were encoded via nuclear genes and characterized via means of a tandem of more than one repeating units. PPR proteins belong to the α-solenoid RNA-binding proteins (RBPs) superfamily, and those RBPsare said to alter all steps of the existence cycle of messenger RNA (mRNA).Nonetheless, PPR proteins also are carefully associated with tandem repeat (TR) proteins, which incorporates armadillo (ARM), leucine-wealthy repeats (LRRs), tetratricopeptide, ankyrin(ANK), and WD40 proteins, whose capabilities were appreciably studied in plants, suggesting an ability function of PPR proteins at some point of pressure and improvement processes.

 

 T.K. Mohanta, et al., (2017)

**Figure 8. RNA editing by pentatricopeptide repeat proteins (PPR). PPR proteins with a nuclease or a cytidine deaminase domain trigger RNA modiﬁcations**

Mutations in PPR-encoding genes are constantly observed through defects in chloroplast biogenesis, pigmentation, and embryo and seed development. The corresponding phenotypes affiliated with PPR genes disruption likely get up from the lack of one to numerous mitochondrial or chloroplast gene products that are necessary for organelle development; of course, there are a few exceptions, such as gun1 and defectively organized tributaries 4, which can be integrated in more than one developmental and stress-associated indicators in younger seedlings and the leaves of adult Nevertheless, dozens of photosynthesis faulty mutants can continue to exist in the course of the seedling level till their seed reserves are depleted. Therefore, the embryo-deadly phenotypes due to plastid defects might also additionally normally be dysfunctional plastid translation tool that ultimately prevents the biogenesis of numerous key elements required for everyday chloroplast improvement and photosynthesis. Although there's extra proof to signify that PPR proteins take part mainly in organellar RNA processing, only some PPR proteins had been functionally characterised in comparison to the enormous majority of PPR members. This is specially attributed to the phenotypes of PPR genes disruption being very comparable amongst character PPR proteins, which makes gene function studies challenging.

**Redefinition and Classification of PPR Proteins’ Motifs**

Based on its members’ motif structure, the PPR relatives are in addition labelled into subfamilies, PLS and P. The P-category PPR proteins constantly incorporate from 2 to over 30 loosely conserved 35 amino acid PPR (P) motifs. In addition, some P subfamily participants incorporate a further small MutS-related (SMR) domain following an array of P-class PPR motifs, so those are categorized as PPR-SMR subgroup. Similar to SMR, there had been described numerous subgroups in step with their catalytic C-terminal domains, for example, TGM, TGM CCCH-zinc finger, LAGLIDADG, Mitochondrial RNA polymerase, and PRORP. In contrast, the PLS-elegance subfamily contributors generally encompass an array of triplets, namely, the canonical P motif, L motif, and S motif; each S and L motifs are associated with the PPR motif however with a variable duration of 31 amino acids (aa) and 35 or 36 aa, respectively. The PLS subfamily PPR proteins may be classified primarily based on connected domain names found downstream of the PPR motifs: E, E+, and DYW, which arise in aggregate or alone. The category of the PPR proteins has passed through revision as extra species are sequenced and new PPR proteins are discovered. Based on a genomic evaluation and evaluation of forty-one terrestrial plant species with evolutionary differences, the PPR motifs have been redefined as follows: the P motif is similarly divided into P1 and P2, in line with a distinction within the first helix. Then, the PLS motif, the L1 and L2 motifs, respectively, which includes 35 aa and 36 aa may be distinguished, with the aid of using a distinction within the 2nd helix. Similarly, the S motif is likewise divided into the normal 31-aa S1 motif and the 32-aa S2 motif. Besides the S1 and S2 motifs, a singular S-like motif (31 aa) named SS has been recognized this is normally juxtaposed to different S motifs. Meanwhile, the E, E+, and DYW motifs have been redefined, and new 34-aa motifs, E1 and E2, have been proposed. In this way, the PLS subfamily became re-divided into six subgroups and a blended P subfamily.

**The Function of PPR Proteins within the Chloroplast**

Functional chloroplasts are the principal sites of photosynthesis, presenting each impartial area environment and the important proteins for its success operation, hence building the plant’s photoautotrophic condition. Plastid gene expression is necessary for the improvement of chloroplasts and their renovation of ordinary functions, and PPR proteins can have an effect on it considerably. Thus, it isn't unexpected that defects in PPR proteins’ capabilities can yield comparable phenotypes related to chloroplast dysfunction. However, there are mentioned variations among the numerous PPR proteins.

**Mechanism of PPRs**

The mechanism of action of Pentatricopeptide Repeat Proteins (PPRs) involves their role in binding to specific RNA sequences and participating in post-transcriptional processes, especially in the context of organelle gene expression, such as in plant mitochondria and chloroplasts. PPRs contain multiple repeats of a pentatricopeptide motif, which forms a helical structure and allows them to recognize specific RNA sequences. Here's a more detailed explanation of the mechanism of PPR proteins:

1. RNA Binding and Recognition:
* PPR proteins consist of a series of repeating units, each containing about 35 amino acids with a conserved pentatricopeptide repeat motif. These repeats create a helical structure that forms a binding surface.
* Each PPR repeat interacts specifically with one RNA base or a small group of RNA bases. The specificity of this interaction arises from the precise arrangement of amino acids within the PPR motif.
* The PPR proteins have different combinations and numbers of repeats, allowing them to recognize specific RNA sequences.
1. Target RNA Recognition:
* PPR proteins recognize and bind to specific RNA sequences in the 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 highly specific, enabling them to distinguish between different RNA molecules in the cell.
1. RNA Editing:
* One of the most well-known functions of PPR proteins is their involvement in RNA editing. RNA editing involves the conversion of specific nucleotides within RNA molecules. In plants, this often occurs in chloroplasts and mitochondria.
* PPR proteins act as guides for editing enzymes by binding to the target RNA sequences that require editing. The binding of PPRs recruits editing enzymes to these sites.
* The editing enzymes then catalyse the conversion of specific nucleotides, such as cytosine (C) to uridine (U), within the RNA sequence, leading to changes in the RNA molecule's sequence.
1. RNA Splicing and Other Processes:
* In addition to RNA editing, PPR proteins can be involved in other post-transcriptional processes, including RNA splicing and stabilization.
* PPR proteins can promote or inhibit splicing events by binding to specific sites in the RNA sequence.
* They can also stabilize RNA molecules, protecting them from degradation.
1. Gene Expression Regulation:
* PPR proteins play a critical role in regulating gene expression in organelles like chloroplasts and mitochondria. They help ensure the correct translation of organelle genes by modifying RNA transcripts as needed.
* Depending on the specific PPR protein and the context, they can either promote or inhibit gene expression by influencing the post-transcriptional processing of organelle transcripts.

In summary, PPR proteins are RNA-binding proteins with a unique repeat motif that allows them to recognize and interact with specific RNA sequences. Their primary function is to participate in post-transcriptional processes, including RNA editing, splicing, and stabilization, in organelles like chloroplasts and mitochondria. By binding to target RNA sequences, PPR proteins facilitate the precise modification and regulation of organelle gene expression.

**Application of Pentatricopeptide Repeat Proteins**

 Pentatricopeptide Repeat Proteins (PPRs) play crucial roles in plant biology, especially in regulating gene expression within plant organelles like chloroplasts and mitochondria. Their ability to recognize and bind to specific RNA sequences makes them valuable tools for various applications in plant research and biotechnology. Here are some applications of PPR proteins in plants:

1. Crop Improvement:
* Mitochondrial and Chloroplast Gene Expression: PPR proteins can be manipulated to enhance or fine-tune the expression of specific genes in chloroplasts and mitochondria. This can lead to improvements in photosynthesis, respiration, and overall plant growth.
* Disease Resistance: By modifying the expression of genes involved in plant-pathogen interactions within organelles, PPR proteins can be used to engineer plants with enhanced resistance to diseases.
1. Stress Tolerance:
* Abiotic Stress Tolerance: PPR proteins can be employed to modify organelle gene expression in response to environmental stresses such as drought, salinity, or temperature extremes. This can improve plant resilience to adverse conditions.
1. Chloroplast Engineering:
* Enhanced Photosynthesis: PPR proteins can be used to manipulate chloroplast gene expression to enhance photosynthetic efficiency, potentially increasing crop yields.
1. Seed Viability and Germination:
* PPR proteins can play a role in regulating gene expression in mitochondria and chloroplasts during seed development and germination. Modulating these processes can affect seed quality and germination rates.
1. Medicinal Plant Production:
* PPR proteins can be utilized to enhance the production of secondary metabolites in medicinal plants, leading to higher yields of bioactive compounds used in pharmaceuticals and herbal remedies.
1. Biosynthesis Pathway Engineering:
* By controlling organelle gene expression, PPR proteins can be employed to manipulate biosynthetic pathways, potentially increasing the production of biofuels or bio-based chemicals in plants.
1. Research Tools:
* PPR proteins can serve as valuable tools for researchers studying organelle gene expression and regulation. They can be used to investigate the roles of specific genes in plant development, physiology, and stress responses.
1. Gene Editing Platforms:
* PPR proteins can be used as components in gene editing systems to achieve specific changes in organelle genomes. By targeting and modifying organelle genes, researchers can study gene function and potentially correct genetic defects.
1. Development of Transgenic Plants:
* PPR proteins can be incorporated into the development of transgenic plants with altered organelle gene expression patterns, leading to novel phenotypes or improved agronomic traits.
1. Plant Conservation and Restoration:
* PPR proteins can potentially be used to help conserve and restore endangered plant species by ensuring the proper functioning of organelles, which is essential for plant health and survival.

While PPR proteins offer significant potential for improving plant traits and addressing agricultural and environmental challenges, it's important to note that their manipulation requires a deep understanding of organelle gene regulation and specificity to avoid unintended consequences. Additionally, advancements in genome editing techniques, such as CRISPR-Cas9, have expanded the toolbox for precise gene regulation in plants, complementing the capabilities of PPR proteins.

1. **CRISPR/Cas9**

CRISPR-Cas9 is a revolutionary gene-editing technology that has garnered significant attention in the fields of genetics, molecular biology, and biotechnology. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats. These are short, repetitive DNA sequences found in the genomes of bacteria and archaea. CRISPR regions contain sequences of genetic material from past viral infections, which the organisms have stored as a form of immune memory. Cas9 is an enzyme derived from bacteria, particularly from the species Streptococcus pyogenes. Cas9 is a protein that can act as molecular scissors, cutting DNA at specific locations when guided by a molecule called RNA. RNA molecules are used to guide the Cas9 enzyme to the specific DNA sequence that needs to be edited. These RNA molecules are designed to match the target DNA sequence precisely.

**How CRISPR-Cas9 Works:**

* Design: Researchers design a piece of RNA called guide RNA (gRNA) that matches the DNA sequence they want to edit. This gRNA is specific to the gene or region they want to modify.
* Delivery: The gRNA is combined with the Cas9 protein and introduced into the cells of interest. This can be done using various methods, such as viral vectors or nanoparticles.
* Recognition: The gRNA binds to the complementary DNA sequence within the cell's genome. This binding triggers the Cas9 enzyme to cut the DNA at that precise location.
* Repair: When the DNA is cut, the cell's natural repair machinery kicks in main two types of DNA repair pathways:
* Non-Homologous End Joining (NHEJ): This pathway often introduces small errors or mutations when the DNA is repaired, which can disrupt the function of the target gene.
* Homology-Directed Repair (HDR): This pathway can be used to introduce precise changes into the DNA by providing a DNA template with the desired sequence

 

  **Figure 9. Working of CRISPR/CAS9**

It is a more controlled way of editing genes. Researchers can use CRISPR-Cas9 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 ease and precision of CRISPR-Cas9 technology have made it a powerful tool for studying gene function, developing potential gene therapies, and conducting genetic engineering in a wide range of organisms, including plants, animals, and humans. However, ethical and safety concerns have accompanied its rapid development, particularly in the context of human genome editing, and careful regulation and ethical considerations are essential when using CRISPR-Cas9 in research and applications.

**Applications of CRISPR/Cas9 genome editing in plants**

Certain phenotypes or tendencies which can be expressed through plants, or in this example crops, may be tweaked and changed via the manipulation in their genes. In doing so, the predicted final results could provide more advantageous model of the crop, which may be useful to the overall populace from sure aspects. The precision of CRISPR/Cas9 era guarantees an exceedingly dependable technique in genome modifying that doesn't randomly produce unexpected changes somewhere within the genome. The efforts in seeking to follow CRISPR/Cas9 genome enhancing in plant had been huge for the reason than the discovery of the technology.

Prior to utilizing the genome editing technology to crops, a lot of research was focused completely on *Arabidopsis thaliana* as a model plant organism because of its convenience and usefulness in genetic experiments. For example, *A. Thaliana* turned into a plant imposing a sequential transformation method, which progressed CRISPR gene targeting. The performance of pKAMA-ITACHI Red vector in CRISPR/Cas9 become additionally first investigated in *A. Thaliana* while an observation regarding genes inclusive of PDS3, AG, and DUO1, was carried out in 2017. After the preliminary validation on *A. Thaliana*, the potentials of the era are being similarly explored in different plant species.

 Currently, CRISPR/Cas9 genome editing has been proven to achieve success on a variety of influential plants which include maize, wheat and apples, with a pretty excessive transformation efficiency. The sequencing of novel plant genomes had widened the programs of CRISPR/Cas9 genome enhancing in sorting out higher variety of genes in various plant species. CRISPR/Cas9 was known to knock out the phytoene desaturase gene in muskmelon (CmPDS), that's the primary report mentioning the use of CRISPR/Cas9 genome editing on the species. The identical PDS gene additionally effective in knocking out to provide an albino phenotype in CRISPR/Cas9 genome modifying pioneering research on watermelon and apples. However, the price of inheritance with the aid of using the following generations of transgenics couldn't be investigated through PDS gene knockout because the albino variants had low *in vitro* survival rates; hence, some other genes must be targeted to determine the rate of inheritance of mutations in the plant species.

 Targeted mutagenesis in sweet orange was achieved in 2014, was a novel tool for delivering the CRISPR/Cas9 reagents became advanced for citrus by the Xcc-facilitated agroinfiltration and inserted using Xanthomonas citri subsp. Citri (Xcc) to contaminate the citrus plant. Knockout of the CsWRKY22 gene in Wanjincheng orange, the use of CRISPR/Cas9 genome modifying exhibited more advantageous resistance closer to citrus canker, an adverse ailment in citrus resulting from Xcc, thereby similarly setting up the efficacy of CRISPR/Cas9 era in citrus. Similar enhancement of resistance was located into apples wherein the CRISPR/Cas9-mediated gene knockout ofMdDIPM4 conferred improved resistance to *Erwinia amylovora*, a bacterium that causes blight in apples.

**Challenges faced in the application of CRSPR/CAS9**

While CRISPR-Cas9 technology holds immense promise for improving crop agriculture, there are several challenges and considerations that must be addressed when applying it in plant research and breeding:

* Off-Target Effects: One of the primary concerns is the potential for CRISPR-Cas9 to induce unintended mutations at sites similar to the target sequence. Researchers must carefully design guide RNAs to minimize off-target effects and use validation techniques to confirm the specificity of editing.
* Regulatory Approval: The regulatory landscape for genetically modified organisms (GMOs) varies from country to country. Obtaining regulatory approval for CRISPR-edited crops can be a lengthy and expensive process, leading to delays in commercialization.
* Ethical and Social Acceptance: The use of gene-editing technologies in agriculture can raise ethical and social concerns. Public perception, consumer acceptance, and ethical considerations related to environmental impacts must be carefully addressed.
* Intellectual Property Issues: Patents related to CRISPR-Cas9 technology have been a subject of legal disputes, potentially limiting access to the technology and complicating its application in plant research.
* Delivery Methods: Effective delivery of CRISPR components into plant cells can be challenging. Different plant species may require different methods, and optimizing the delivery process is critical for successful editing.
* Polyploidy and Complex Genomes: Many important crop plants, like wheat and cotton, have complex genomes with multiple copies of genes (polyploidy). Editing such genomes can be more challenging than editing diploid genomes, as all copies may need to be targeted simultaneously.
* Gene Redundancy: Some plant genes have redundant functions, meaning that the loss of one gene may not result in a noticeable phenotype. Editing these genes may require targeting multiple homologous genes, which can be technically challenging.
* Tissue Regeneration and Transformation: After editing, plant cells need to be regenerated into whole plants through tissue culture and transformation processes. Not all plant species or varieties respond well to these procedures.
* Offspring Variability: In some cases, the edited trait may not be stably inherited by plant progeny. Researchers need to ensure that the desired traits are passed on reliably to the next generation.
* Long-Term Effects on Ecosystems: Altering plant traits through gene editing may have unintended consequences on ecosystems and interactions with other organisms, such as pollinators and pests. Assessing these potential long-term effects is crucial.
* Biodiversity Concerns: There is a concern that widespread adoption of CRISPR-edited crops could lead to reduced genetic diversity within plant populations, potentially making them more vulnerable to pests and diseases.
* Global Collaboration: To address challenges and share best practices, international collaboration and data sharing are essential. However, intellectual property issues and regulations can sometimes hinder such collaboration.
1. **Concluding Remarks**

As our knowledge and understanding of plant genomes expands, greater opportunities exist for the application of biotechnology to unravel the complex interaction of genes and phenotypes. Biotechnological approaches have rapidly evolved by incorporating new and useful tools for successful crop improvement initiatives. These methods allow the quick and more effective development of desired crop cultivars with desired features to boost agricultural output and feed the world's rapidly growing population. The genome editing method like CRISPR/Cas9 has shown significant potential for quickly tackling new problems in agriculture. RNAi does not introduce any new proteins into the plant so, there are no novel allergenicity concerns. NBTs like cisgenesis and intragenesis on the other hand may raise fewer biosafety concerns and should be viewed as more like conventional breeding techniques. Thus, the new biotechnological tools are driving breakthroughs in medicine, agriculture, environmental science, and beyond. They are paving the way for innovative solutions to complex problems and shaping the future of biotechnology as we continue to explore the vast potential of the biological world. As the field evolves, one can expect even more exciting developments in the years to come, pushing the boundaries of what is possible through biotechnology.

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