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APPLICATION OF PRECISE GENOME EDITING IN PLANTS

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CONFLICTS OF INTEREST

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ABSTRACT

Genome engineering, the ability to manipulate and alter DNA sequences in living cells is entering to its golden age. This is due to the advent of quickly advancing techniques that enable to engineer the genome with significant impact. Many of the plants, yeast strains and filamentous fungi industrially relevant for enormous biotechnological applications are non-domesticated difficult to engineer, have intricate genomes and have little molecular tools, making their genome engineering a complex task. But precise genome editing which mimics the naturally occurring mutations has been used to overcome the biological engineering challenges posed by these organisms. Application areas of precise genome editing are diverse and potentially limitless as it is capable of altering any component of any genome. The technique enables to open the genome like a book and proceed to words; in this case the DNA sequences then engineer the sequences to end up with the desired product. Focus areas of precise genome editing includes but not limited to; genome engineering, knockout, activation, RNA editing, in disease models, gene drive, biomedicine, gene function and in *vitro* gene depletion. With precise genome editing approach particularly Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR), there are visions of another green revolution - where plant yields could be improved significantly and worries might fade about how to feed the world in 2050 with projected population of nine billion people. For various applications precise genome editing has been successfully employed in several plants including; *Arabidopsis thaliana, Nicotiana tobacum,* sweet orange, rice, wheat, tomato, soybean, maize, sorghum and popular. Although CRIS-

PR/Cas9 is a rising star in genome editing taking the technology to its full potential requires tackling off-target mutations among others.

Keywords: CRISPR/Cas9, Double strand breaks, Genome engineering, Targeted mutagenesis

INTRODUCTION

Over the next 30 years, the world is expected to look for ways to feed additional two billion people. This implies we are responsible to produce a lot more food. Among the different options, one way we might be able to accomplish this is by engineering plants to make them more nutritious, grow faster and more resilient to various stresses [1].

As a result of the rapid growth of genome editing techniques, commercially engineered targeted nucleases became available. But, such methods utilized for genome engineering are with the problem of low efficiency and target organisms and cell types are limited [2]. In the past, strategies for introducing precise and efficiently targeted genomic alterations were limited to model organisms that had well established collection of molecular biology tools such as markers, plasmids and strong means of inducing homologous recombination. But, all such limitations changed over time with the advent of customized DNA endonucleases such as transcription activator -like effector nucleases (TALENs) and zinc finger nucleases (ZFNs). TALENs were discovered at the end of 2009, immediately after the advent of TALE-DNA [3]. Recently the most advanced technique of genome editing, CRISPR/Cas9 enter to applicability although its discovery was in 1987 [4].

Genome editing

Genome engineering, the ability to manipulate and alter DNA sequences in living cells is entering to its golden age [5]. Fast advancing techniques that enable to engineer the genome with significant impact is emerging. The technology is enabling to have easy and precise removal, insertion or edition of DNA sequences that attracts the attention of scientific community in wide areas, such as agriculture, energy, biomedicine and environment. Many of the plants, yeast strains and filamentous fungi industrially relevant for enormous biotechnological applications are non-domesticated difficult to engineer, have intricate genomes and little molecular tools, making their genome editing a complex task. Genome editing especially with CRISPR/Cas9 has been used to overcome the genetic engineering challenges posed by these organisms [6]. It is believed that the ideal genome editing tool should fulfill the following three criteria. (a) With-out off target mutation, (b) high frequency of the desired sequences in the target cell population, and (c) assembling of the nucleases should be rapid and efficient [7].

When we look details of genome editing techniques emerged before the advent of CISPR/Cas9, ZFNs use about 30 amino acid fingers that fold around a zinc ion to form a compact structure that recognizes a 3-base pair of DNA. Consecutive finger repeats are able to recognize and target a wide area of the target DNA [5]. When compared with ZFNs, TALENs have better ability in targeting their chromosomal site, have a wide spectrum of sequences that can be targeted and has less chance for off-target since their design is intended to recognize 30 -36 base pairs from the target site while ZFNs is designed to recognize 18-24 base pairs [7].

Although ZFNs and TALENs enable genome editing in many species, their wider applications is hindered by their costs in time and labor, have access to the chromatin site but not other part of the genome, unable to have multiplex genome editing and both require engineering of new proteins for each DNA sequences to be targeted [5, 8, 9].

Random mutagenesis is complex task due to low homologous recombination in plants. But as a result of growing demand in boosting plant productivity, robust and versatile genome editing tools became highly demanding. Due to availability of more plant genomes, exploiting the powerful approach of genome editing, CRIPSR/Cas9 in various plant species is becoming realistic [10]. CRISPR/Cas9 genome editing tool is successfully employed in several plants including model plants and food crops; *Arabidopsis thaliana, Nicotiana tobacum,* sweet orange, rice, wheat and tomato and few energy crops; popular, switch grass, soybean, maize and sorghum [11]. The genomes of both dicots and monocots have been edited using the same vector system for CRISPR-Cas9, even though codon optimized versions of Cas9 are available for each plant type [10, 11, 12, 13, 14]. Success of genome editing techniques varies from species to species for instance, CRISPR/Cas9 works well in rice and barley, but TALENs works well in wheat than CRISPR/Cas9 [15].

CRISPR/Cas9 Technology

The search for an evolutionary genome editing approach results in to the advent of a system called Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR/Cas9). When compared with previous genome editing systems (TALENs and ZFNs), CRISPR/Cas9's simplicity, efficiency, specificity, minimal off-target effects, and amenability to multiplexing has brought hasty genetic manipulation in almost all tested eukaryotes

[16,17, 18].
In 1987, CRISPR/Cas9 system was first discovered by Japanese scientist in bacteria as an adaptive immune system by which enables the bacteria to defend against invading foreign DNA, like bacteriophage. Later on they were found in 40 % of sequenced bacterial genomes and 90% of the archaea [19, 20]. The CRISPR system is composed of CRISPR loci in the genome and a Cas9 protein. CRISPRs is a genomic locus of tandem direct repeat sequences and protospacers , the space in between repeat sequences, both of which are derived from the invading elements. The CRSPR loci contains a combination of Cas9 genes; sequences for non-coding RNA elements called CRISPR RNA(crRNA) and sequences for small trans-encoded CRIPSR RNA, i.e., trans-activating crRNA(tracrRNA). The two RNA sequences (crRNA and tracrRNA) are responsible in forming a complex known as guide RNA, which again determines the specificity of the cleavage of the target sequences in the nucleic acid along with the Protospacer Adjacent Motif (PAM), a 5'-NGG sequences [21, 22].
CRISPR/cas9 combines Cas9 endonuclease of Streptococcus pyogenes and a synthetic single guide RNA (sgRNA), responsible for directing Cas9 endonuclease to a target sequences complementary to the 20 nucleotides preceding the post spacer-associated motif(PAM) NGG, which is required for Cas9 activity [23, 24]. Then it is possible to engineer the system to the required target by the addition of only 20 nucleotides to the sgRNA molecule allowing easily targeted genome editing and regulation. Again by using multiple sgRNAs it is possible to have simultaneous targeting of several genomic loci (multiplexing) [25]. Cas9 is directed to its DNA target by base pairing between the gRNA and DNA. A Protospacer Adjacent Motif (PAM) downstream of the gRNA-binding region is required for Cas9 recognition and cleavage as illustrated in Fig1.

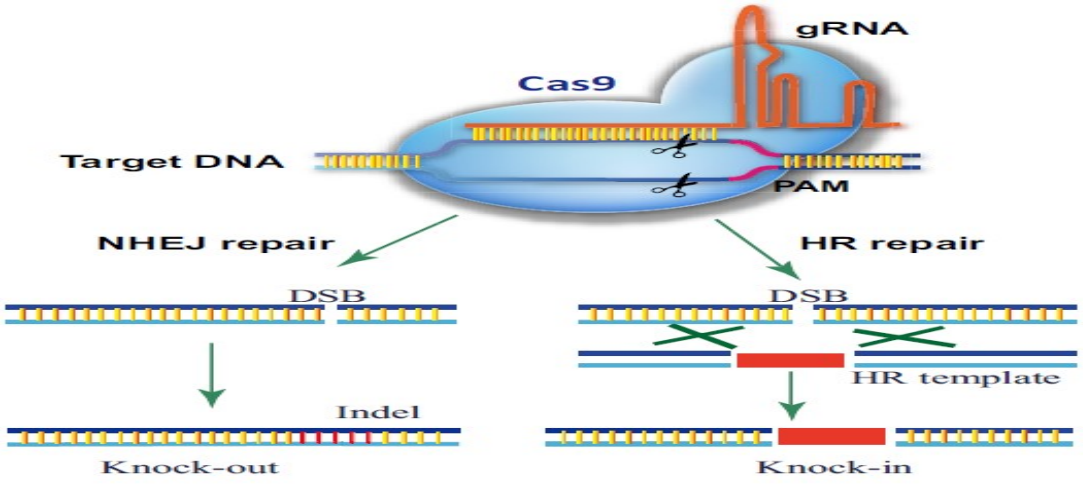

Figure 1: Schematic of double strand break repair, Adopted from Ding et al., 2016, Ref.26

Cas9/gRNA cuts both strands of the target DNA, triggering endogenous Double Strand Break (DSB) repair. For a knockout experiment, the DSB is repaired via the efficient but error-prone Non Homologus End Joining (NHEJ) pathway, which introduces an indel at the DSB site that knocks out gene function. In a knock-in experiment, the DSB is repaired by Homology Repair (HR) using the donor template present, resulting in the donor DNA sequence integrating into the DSB site.

Application of CRISPR/Cas9 genome editing in plant improvement
Application areas of genome editing are diverse and potentially limitless. Precise genome editing is capable of altering and/or replacing any component of any genome. Application areas of CRISPR/Cas9 includes but not limited to; genome engineering, knockdown/knockout, activation, RNA editing, in disease models, gene drive, biomedicine, gene function and *in vitro* gene depletion [27, 28, 29, 30, 31, 32, 33, 34, 35, 36].
CRISPR/Cas9 and its modified versions have been widely employed for genome editing in various organisms. In a model plant, *Arabidopsis*, many genes including; *AtSPL4, AtBRI1, AtPDS3, AtFLS2, AtADH* and *AtFT* have been targeted with *varying* mutational efficiencies, from 1.1% up to 84.8%, in the first generation. Up to 79.4% of mutations have been stably inherited across multiple generations of plants. In rice several genome editing attempts have been successful [37, 38, 39, 40].
For instance, in rice mutation rates of 9.4% and 7.1% were achieved in the knockouts of *OsPDS* and *OsBADH2* genes respectively. But, later on with improved CRISPR/Cas9 components much higher mutation rates (average 85.4 %) with more of homozygous and biallelic mutations were obtained. Again in rice with the right combina-

tions of sgRNA, the system has successfully deleted 115-245 kb of chromosomal fragments [41, 42, 43]. Rice bacterial blight susceptibility genes *OsSWEET14* and *OsSWEET11* have been targeted by Protoplast transfection with transient expression of sgRNA/Cas9 (variant of the CRISPR/Cas9 system) [12]. CRISPR/Cas9 mediated multiplex genome editing has employed for rapid improvement of grain weight in rice [44].

In a study where Cas9 targeting of a GFP gene in soybean hairy roots has resulted in average indel frequencies greater than 70% for targeted seven genes(*GFP 5', 01gDDM1, 11gDDM1, Glyma04g36150, Glyma06g18790, miR1509,* and *miR1514)* (14). But prior gene editing experiment by transcription-activator like effector nucleases (TALENs) resulted in 3-7 % mutation in soybean hairy roots [45].

Delivery of sgRNAs specific for coding and non-coding sequences of tomato yellow leaf curl virus (TYLCV) into *Nicotiana benthamiana* plants stably over expressing the Cas9 endonuclease and subsequently challenge these plants with TYLCV. This demonstrates that the CRISPR/Cas9 system targeted TYLCV for degradation and introduction of mutations at the target sequences. This confirms the efficacy of the CRISPR/Cas9 system for virus interference by targeting TYLCV, providing new possibilities for engineering plants resistant to DNA viruses ([5].

Targeted gene mutagenesis was detected for 90 loci by maize protoplast assay, with an average cleavage efficiency of 10.67%. Stable knockout transformants for maize phytoene synthase gene (*PSY1*) were obtained. Mutations occurred in germ cells can be stably inherited to the next generation. Moreover, no off-target effect was detected at the computationally predicted putative off target loci [46]. Targeting of *Zmzb7 gene* that encodes the IspH protein resulted in the mutation efficiency ranged from 19%–31% in maize [47].

As stated in Table1, several successful experiments have been reported in diverse plants with more complex genomes including, sorghum (*Sorghum bicolor*), maize (*Zea mays*), poplar (*Populus tricocarpa*), tomato (*Solanum esculentum*) citrus (*Citrus sinensis*) and wheat (*Triticum aestivum*). All this fruitful trails indicates the potential of CRISPR/Cas9 system as feasible and pragmatic technology in plant genome editing for various novel applications [10, 12, 48, 49].

Plants species	Target genes	Ref.
Gene knockout or editing with Cas9 paired nickase/sgRNA		
Arabidopsis thaliana	*RTEL1, ADH1, TT4*	50, 39
Gene knockout or editing with Cas9/sgRNA		
Arabidopsis thaliana	*AtPDS3, AtFLS2, TT4,BRI1, JAZ1, GAI,ADH1, CHLI, AP1, FT, SPL4, AtCRU3, At1g56650*	40,43, 51, 52
Nicotiana benthamiana	*NbPDS, PDS, NbPDS, NbPDS3, NbIspH*	53, 54
Oryza sativa	*CAO1, LAZY1, OsMPK5, OsMYB1, ROC5,SPP, YSA.BEL,SWEET13/1a/1b, PMS3, EPSPS, DERF1, MSH1, MYB5, CDKB2, OsGSTU, OsMRP15, OsAnP, OsAOX1a, OsAOX1b, OsAOX1c, OsBEL and many more*	12, 37,41,55, 56, 57
Nicotiana tabacum	*NtPDS, NtPDR6, ALS*	58, 59
Solanum tubersum	*StIAA2, StALS1*	60, 61
Medicago trancatula	*GUS*	62
Populus tomentosa	*PtoPDS, 4CL, PtPDS*	11, 63
Glycine max	*Glyma07g14530,Glyma06g14180, Glyma08g02290, Glyma12g37050, Glyma18g04660, Glyma20g38560; GmFEI2, GmSHR*	14 , 62, 64
Zea mays	*ZmIPK, LIG1, Ms26, Ms45, ALS2*	10 ,49
Solanum lycopersicum	*SlAGO7,mGFP5, eGFP, RIN, ANT1*	65, 66, 67
Citrus sinensis	*CsPDS*	48
Marchantia polymorpha	*MpARF1*	68
Sorghum bicolor DsRED2	*DsRED2*	12
Triticum Aestivum	*TaMLO,INOX, PDS,TaMLO-A1*	41,53, 69

Multiplex genome editing with Cas9/sgRNA		
Populus tomentosa	*PtPDS1 and PtPDS2*	70
Arabidopsis thaliana	*AtRACK1bCAtRACK1c, HLI1CCHLI2, ETC2,CPC,TRY,andPYL1-6, t5g55580*	37 , 43,71
Nicotiana tabacum	*NtPDSCNtPDR6*	59
Oryza sativa	*CDKB1, CDKA1, MPK1/2/5/6, PDSOsFTL*	43,57, 72
Solanum lycopersicum	*Solyc07g021170CSolyc12g044760*	65
Zea mays	*ZmHKT1*	13
Glycine max	*01gDDM1,11gDDM1,01gC11gDDM1; GmFEI2,andGmSHR*	14,73
Triticum aestivum	*aMLO-A1CTaMLO-B1CTaMLO-D1*	69
Gene insertion/replacement by HDR with Cas9/sgRNA and donor template		
Nicotiana benthamiana	*AvrIIsite ! NbPDS*	71
Zea mays	*UBI:MoPAT*	49
Oryza sativa	*OsPDS,OsBADH2*	41
Arabidopsis thaliana	*YFFP,GU.US,DGU.US ! GUS functional,nptII ! ADH1*	9,37, 50, 55

Table 1: Applications of CRISPR/Cas9 in plants

It is evident that the CRISPR/Cas9 technology continues to evolve with modified versions as well as new technologies coming to the fore-front beyond genome editing. For instance, researchers have been able to reprogram the CRISPR/Cas9 system to recognize and cleave specific RNAs (RCas9) instead of the conventional DNA targets [74, 75]. The advent of this ability opens new avenues in to RNA detection, analysis and manipulation. The system has also been modified to allow targeted control of gene expression. Blocking of transcription of target genes can be possible by utilizing a catalytically inactive dCas9 along with a sequence specific sgRNA to create a DNA recognition complex which can bind to targeted promoter sequences [76, 77].

CRISPR/Cas9 is emerging as a new tool for plant genomics and biotechnology research despite pitfalls to explore the potential of CRISPR/Cas9 for functional genomics and pragmatic plant improvement [78]. Catalytically inactive Cas9 fused with activation or repression domains are important for transcriptional regulation, modification of epigenetic status, or for imaging purposes, thereby, opening new possibilities for genome-wide functional interrogation of genes. When the sciences of CRISPR/Cas9 became matured integration into plant breeding schemes is inevitable. It is also anticipated that synergies between genomics and plant breeding will ease the precise characterization of plant genetics resources and speed up the development of new cultivars [79, 80].

Conclusions

The genome editing technology particularly CRISPR/cas9, a revolutionary bioengineering technique continues to evolve and will have fascinating applications in biological engineering. CRISPR/Cas9 approach of genome editing is a game changer in plant genetics research as it mimics a gene mutation that occurs naturally, but what is wanted today is speed and trait of interest. Though several successful results have been obtained, key advances are still required to maximize the potential of CRISPR/Cas9 for pragmatic plant improvement including:- off-target mutation due to off-target cleavage, side effects on nearby genes, influence on chromatin structure, efficient delivery method in polyploidy plants, the development of Cas9/sgRNA ribonucleoprotein complex (RNP) systems for efficient DNA-free genome engineering, methods for transient delivery of the RNPs to obviate the need for stable transformation and regeneration, efficient gene editing systems that enable gene replacement, fusions and stacking, CRISPR/Cas9-based trait discovery platforms in target plant species and understanding the mechanisms of double strand break induced by Cas9 is also important. It is crucial to expand the availability of plant specific vectors, genome resources and transformation protocols before designing rational strategies that can be used to implement the CRISPR/Cas9 technology to biologically engineer wider economic traits of plants.

Abbreviations
CRISPR/Cas9: Clustered regularly interspaced short palindromic repeat /associated protein 9; TALENs: Transcription activator -like effector nucleases; ZFNs: Zinc finger nucleases; crRNA: CRISPR RNA; tracrRNA : Trans-activating crRNA;PAM : Protospacer adjacent motif ; sgRNA : Single guide RNA ; DSB: Double strand break ;NHEJ : Non homologus end joining ; HR :Homology repair; RNP: Ribonucleoprotein complex; indel: insertion/deletion

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