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Genome Editing Using Crispr/Cas System: New Era Genetic Technology in Agriculture to Boost Crop Output

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Abstract

Genome engineering with the RNA-guided CRISPR-Cas9 system in animals and plants is revolutionizing biology. First techniques of genome editing like zinc finger nucleases and synthetic nucleases called TALENs were a starting point but turned out to be expensive, difficult to handle and time-consuming to engineer, limiting their widespread use, particularly for large scale, high-throughput studies. Moreover, these existing technologies depending on proteins as address labels and customizing new proteins for any new change to introduce in the DNA is a cumbersome process. Of the current generation of genome editing technologies, CRISPR-Cas9 is easier to use and more efficient and can be easily targeted to almost any genomic location of choice by a short RNA guide and has been successfully applied in many organisms, including model and crop plants. Together the system has the ability to detect specific sequences of letters within the genetic code and to cut DNA at a specific point. Simultaneously with other sequence-specific nucleases, CRISPR/ Cas9 has already breach the boundaries and made genetic engineering much more versatile, efficient and easy. There really doesn't seem to be a limit in applications of CRISPR system extendable from bacteria to complex eukaryotic organisms including plants changing the pace and course of agricultural, Biomedicine and Biotechnological research in the future. This review provides an overview of recent advances in genome editing technologies in plants, and discusses how these can provide insights into current plant molecular biology research and molecular breeding technology.

Keywords: Crispr-Cas9; Precise genome engineering; Crop improvement

CRISPR-Cas9: Big Bang in Molecular Biology

Eukaryotic genomes contain billions of DNA bases and are difficult to manipulate through ordinary tools and techniques of molecular biology. To triumph over these challenges, a chain of nuclease-based genome editing technologies have been evolved in recent years, enabling targeted and efficient modification of a variety of eukaryotes particularly mammalian species [1]. To achieve effective genome editing via introduction of site specific DNA double stranded breaks, there are presently four major classes of DNA binding proteins: engineered homing endonucleases or meganucleases, zinc finger (ZF) nucleases, transcription activator-like effectors (TALENs), and most recently the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR. Meganuclease, zinc finger nucleases, and TALE proteins all recognize specific DNA sequences through protein-DNA interactions. Each of these classes of DNA binding proteins however has distinctive limitations. Meganucleases can be engineered to recognize new sites; however, it is not easy to achieve the changes in target site specificity and more often result in a reduction of catalytic activity; which has restricted their widespread use. ZFNs, in contrast, bind DNA through an array of engineered zinc finger proteins, which are fused to the catalytic domain of the FokI endonuclease [2,3]. Likewise, TALE proteins can still suffer from context-dependent specificity, no doubt they are for the most part modular. Moreover, their repetitive sequences make construction of novel TALE arrays labor intensive and costly.

It's the first time in the history of biological research, scientists are able to directly edit or modulate the function of DNA sequences using so called CRISPR/cas9 technology by making precise and targeted changes to the genome of living organisms, its contexts (e.g., epigenetic marks), or its outputs (e.g., transcripts). Focusing on proficiency, or the rate of wanted transformation accomplished, is a standout amongst the most essential parameters by which to survey a genome-altering apparatus [4]. The focusing on productivity of Cas9 contrasts positively and more settled strategies, for example, TALENs or ZFNs. The outline of the CRISPR/Cas framework with the steady Cas9 protein and the sgRNA-determined target specificity gives the opportunity to focus on various locales without a moment's delay, similar to the case in the regular bacterial framework. However, researchers have begun to adventure this open door and thriving impersonation of the bacterial framework with

polycistronic crRNAs and tracrRNAs has not yet been accounted for plants [5,6]. In this way, the most widely recognized approach by a few gatherings to accomplish multiplex sgRNA expression is to just amass various sgRNA expression frameworks, each with its own promoter. Multiplexed altering could take care of the issue by permitting the cross examination of quality or protein systems at a bigger scale. In any case, this method has a limitation since builds turn out to be substantial with a Pol III promoter required for each sgRNA. Researchers built up a methodology to beat this difficulty by embeddings tRNA groupings in the middle of the sgRNA successions on the develop so that a solitary polycistronic quality is made. Communicated RNA is then severed at the fringes amongst tRNA and sgRNA by two host endogenous nucleases making individual sgRNAs. Concurrent multiplex mutagenesis was shown in rice protoplasts and transgenic plants for various quantities of sgRNAs (up to eight) in a tRNA sgRNA exhibit. Researchers from KAUST in Saudi Arabia showed the capability of plant infections for multiplex genome building [7-9]. By utilizing the Tobacco shake infection (TRV) to convey sgRNAs to transgenic N. benthamiana that steadily over-communicated Cas9. Using leaf agroinfiltration system, two TRV RNAs were carried into the plants with a mix of two bacterium social orders, one with a plasmid for RNA1, one for RNA2. The last contained the sgRNA expression framework. A systemic disease all through the plant prompts sgRNA expression in all tissues and thusly productive mutagenesis after the reconstitution of TRV in the invaded tissue. By blending bacterial societies with various RNA2 vectors, Moreover, synchronized mutagenesis of two loci was accomplished by blending the bacterial societies with various RNA2 vectors, speaking to the potential for multiplex genome building by TRV-interceded sgRNA conveyance [10].

Off-Target Effects: A Major Concern

In any case, the occurrence of off-target transformations are probably going to show up in locales that have contrasts of just a couple of nucleotides contrasted with the first grouping, the length of they are neighboring a PAM arrangement, since Cas9 can endure up to 5 base bungles inside the protospacer district or a solitary base distinction in the PAM grouping. Off-target transformations are for the most part more hard to identify, requiring entire genome sequencing to preclude them totally [11]. If there should be an occurrence of plants, there is little data directly accessible that locations off-site movement. Sequencing of bioinformatically recognized putative off-target locales demonstrated no perceptible occasions in A. thaliana, N. benthamiana, wheat, rice and sweet orange. Besides, changed A. thaliana plants additionally demonstrated no off-target occasions when subjected to entire genome sequencing. On the in spite of these outcomes, a researchers utilizing Cas9 in rice found a putative off-target site to be changed in 1.6 % of the aggregate plants, despite the fact that this was as yet five times less continuous than the on-target site. For decreasing those off-target transformations most recent enhancements to the CRISPR framework have been made using truncated gRNA (truncated inside the crRNA-inferred grouping) or by including two additional guanine (G) nucleotides to the 5' end [12,13]. Analysts have likewise endeavored to limit off-target impacts in another route by using "matched nickases". This approach utilizes D10A Cas9 and two sgRNAs integral to the nearby zone on inverse strands of the objective site (Crispr Cas9 and focused on genome altering another period in mol. science word record). While this instigates DSBs in the objective DNA, it is relied upon to make just single scratches in off-target areas and, in this manner, result in insignificant off-target transformations [14,15].

Precise Genome Modification via Targeted dsbs

The repair process of double stranded breaks is vital for CRISPR efficiency after the cleavage by cas9 is done, which is necessary to be alarmed. The repairing pathways incorporate homology coordinated repair (HDR) and non-homologous end joining (NHEJ). Dominance of NHEJ has been shown during G1,S and G2 phases while homology directed repair (HDR) dominates in late S and G2 phase. With the passage of time, these two pathways have been manipulated by researchers for genome editing using CRISPR in mammalian cell [16-19]. One of the mechanisms for break repair being non-homologous end-joining after the breaks are introduced into the chromosomes. At the junction of the newly rejoined chromosome, small deletions or more rarely insertions can be introduced as the non-homologous end-joining being precise in nature. A knock-out mutation (loss of function) results if the sequence alteration causes the frameshift mutation or changes key amino acid residues in the target gene product [20]. With the SSN, the broken ends can be ligated to the other DNA molecules simultaneously that are introduced in the cell. A targeted gene knock-in can be achieved by capturing of heterologous DNA sequence. Ultimately, if the two breaks are introduced into the chromosome simultaneously, targeted gene deletions or other rearrangements can result [21-24]. Thus the powerful means to achieve the targeted DNA modification is the DNA repair through NHEJ.

It has always been a major challenge in plant genetic engineering to modify or alter the plant genomes precisely (gene targeting) or site-specific integration of trangenes, as NHEJ being, beyond a doubt, the preferred mechanism to repair DNA breaks in somatic plant tissues [25].

However, large number of flourishing genome targeting experiments has been performed utilizing different classes of engineered nucleases. Development of the I-SceI-based in planta GT system, that permitted for genome targeting rates of more than 1% in *Arabdopsis thaliana* without depending on high transformation rates was one of the major steps to be taken [26,27]. This strategy was enhanced further by utilizing cas9 in which the three T-DNA successions (giver grouping, fake I-SceI target arrangement and I-SceI expression framework) required for the framework to work legitimately was lessened to just a single, harboring both the benefactor arrangement and the Cas9/sgRNA expression tapes. Besides, an endogenous locus in the *Arabidopsis* genome was focused on which ended up noticeably conceivable due to target adaptability of this system upsetting plant science [28].

In an another inventive stragety, quality focusing on and Cas9-intervened mutagenesis were joined by the gathering of analysts, where in *Arabidopsis* plants were steadily changed with a particular T-DNA flanked by the viral extensive intergenic locales (LIRs) [29]. Endless supply of the viral replication-start protein, replicational discharge, circularization and moving circle replication of the replicon started at the LIRs. The circularization prompt two 35S promoters winding up noticeably accurately situated in front of the coveted quality, i.e., the nuclease ORF [30,31].

Subsequently, NHEJ-interceded mutagenesis was exhibited with ZFNs, TALENs and Cas9 utilizing this approach. Besides, it was demonstrated that when a GT benefactor grouping was added to the replicon, ZFN-intervened quality focused on could be accomplished. This methodology was likewise appeared to be material utilizing Cas9 in tomato [32]. CRISPR/Cas-mediated genome targeting has also been reported recently in soyabean by Du Pont Pioneer researchers.

Another alternative means to repair a broken chromosome is homologous recombination (HR). In HR, a repair template is used as a source of DNA sequence information that is copied to the broken chromosome to restore its integrity [33,34]. By introducing into the cell both a SSN and a DNA repair template with sequence similarity to the break site (gene targeting) into the cell is a way to harness the HR so that targeted DNA sequence modifications can be achieved. Also, sequence variation that is carried by the repair template is copied by HR into the chromosome. Moreover, HR offers various possibilities for manipulating plant genomes as the type of sequence variation in the repair templates can be specified [35,36]. In addition, DNA sequence modifications can also be attained, or by bringing changes in promoter elements or other Cis-acting motifs that control gene expression or inducing alterations to key amino acid residues within a gene's coding sequence. Therefore, DNA repair by HR provides an exceptional ability to manipulate a plant's genotype and so its phenotype [37].

Mechanism behind Efficient and Site-Specific Genome Editing Tool: CRISPR/Cas

Previous studies led to the apprehension that targeted DNA double-strand breaks (DSBs) could significantly stimulate genome editing through HR-mediated recombination events. CRISPR/CAS depends on the insusceptible arrangement of microscopic organisms and archaea but at the same time is of incentive in the research facility [38,39]. CRISPR is short frame for Clustered Regularly Interspaced Palindromic Repeats, though Cas basically remains for the CRISPR-related protein. These rehashes were at first found in the 1980s in *E. coli*, however their capacity wasn't affirmed until 2007 by Barrangou and partners, who exhibited that *S. thermophilus* can procure resistance against a bacteriophage by incorporating a genome piece of an irresistible infection into its CRISPR locus [40-42]. At first a novel RNA, in particular tracrRNA, related to the CRISPR-Cas9 framework was recognized, which was distributed in 2011 in Nature. Thusly a long haul thinking come about into Cas9, a chemical that capacities with two RNAs was affirmed. Together the framework can recognize particular groupings of letters inside the genome and to cut DNA at a particular point. In this game-plan the Cas9 protein works as the scissors and a RNA scrap as the address mark guaranteeing that the cut occurs in the ideal place [43,44]. This framework is straightforward and simple to use as an all-inclusive innovation since it simply include the substitution of succession of this RNA to target for all intents and purposes any arrangement in the genome. Outside plasmid or viral DNA entering the bacterial cells are debased by a solitary protein, the nuclease Cas9. CRISPR-RNA (crRNA), which is encoded in the CRISPR locus administers the objective specificity and being integral to the attacking DNA, it can tie specifically to the remote DNA utilizing an extend of 20 nts [45]. The right acknowledgment of the objective site requires an extra short succession theme besides target grouping known as protospacer which lies contiguous PAM. A moment short RNA, the trans-actuating CRISPR-RNA (tracrRNA), ties to the crRNA, and an enduring complex is framed with Cas9. Two nuclease spaces of Cas9 then cut any remote DNA grouping with exact specificity [46]. Later on, it was uncovered that the two RNAs can likewise be melded to shape a supposed single-guide RNA. In perspective of the reality, the CRISPR/Cas framework wound up plainly outstanding device for genome building because of its straightforwardness in cloning new succession particular nucleases and in actuality working in any living being. Since these initial studies, Cas9 has been used by thousands of laboratories for genome editing applications in a variety of experimental model systems. The rapid adoption of the Cas9 technology was also greatly accelerated through a combination of open-source distributors such as Add gene, as well as a number of online user forums. CRIPSR/Cas9 reagents are rapidly emerging as the SSN of choice [47,48]. Engineering SSNs with the requisite DNA specificity was for a long time a bottleneck for genome engineering, DNA targeting can now be achieved with much greater efficiency. Indeed many challenges continued in terms of the delivery of genome engineering reagents to plant cells, but progress on this front is also advancing at a rapid pace [49,50].

CRISPR/Cas9: A Remarkable Genome Modification Tool in Plants

In past few years, there were many independent studies reporting the applications of the CRISPR/Cas9 system repair in genomic DNA, an innate error-prone DNA repair mechanism that is ideal for mutagenesis. Knowledge gained from the remarkable nucleic acid based adaptive immune system from bacteria or archaea, researchers have reprogrammed the CRIS-PR/Cas9 system into a molecular technology for creating DSB at specific genomic sites to facilitate site specific genome editing [51,52].

The creation of a single-guide RNA (sgRNA) that is capable of accurately guiding Cas9 to a predetermined site in the host genome was the most innovative modification. Hence, CRISPR/Cas system was not only brought down from three to two component number by this modification, but also enabled to design the DNA expression modular or vector for potentially simple and high throughput targeting of DNA sites throughout the genome in all organisms, including human, animal and plant.

By creating such a DNA expression modular, synthesizing nucleotide oligos from the targeting sites was required for a new targeting and then assembling them into the modular in place originally for a spacer in crRNA. This technical simplicity represents a significant reduction in required resource from what was needed to create previous genome editing tools such as TALENs to target a new site as TALENs work in pairs and each creation of a TALEN would need to synthesize or assemble a DNA fragment of 2000 bp or more. This system has been tested in many plant species which include *Arabidopsis*, rice, sorghum, tobacco and wheat. This is unique in terms of the rapidity and degree in adapting the CRISPR/Cas9 system in plants as the system was demonstrated in bacteria in August 2012 [53,54].

Specific genomic sites in some plant genes were targeted with this system, and the desired site specific mutation rates were considerably high as shown in **Table 1**. Transient expression of the CRISPR/Cas9 systems in protoplasts (plant cells without cell wall) or tissues, recorded mutagenesis rates ranged from 1.1% (*Arabidopsis* protoplast) to 90.1% (rice immature embryo). In the cases of stable expression of the systems in regenerated plants, the mutation rates were even higher, which varied from 4.0% to 91.6%. The highest mutation rate of 91.6% was observed from regenerated rice plants in which the Lazy1 gene was targeted. It has been demonstrated that the rice Lazy1 gene plays an important role in determining rice tiller angle and disrupting Lazy1 would result into plants having wide-spreading tillers. Interestingly, a recent report has demonstrated that the TAC1 gene that controls the pillar growth habit in peach is closely related to Lazy1. It may be possible that targeting the apple counterpart of the TAC1 or Lazy1 gene might lead to large angle of branches in apple, an ideal and preferred tree form in the current orchard system [55]. In case of *Arabidopsis*, rice and tomato CRISPR/Cas9-induced homozygous and biallelic mutations have been reported in first-generation transgenics allowing early gene-function studies. If homozygous or biallelic mutants are not generated as primary transformants, they must be progressed to the next generation for loss-of-function phenotype analysis. Single spacer sequence was mostly used to accomplish the targeting of the plant genes in the DNA expression modular or vector, which targeted one specific site at a time.

To attain the effect of 'one arrow multiple birds', two or more spacer sequences (in sgRNAs) were engineered together and analyzed in both *Arabidopsis* and tobacco. RNA-guided Cas9 was connected by Chen et al. from Beijing to *A. thaliana* and maize by utilizing an arrangement of various develops (maize codon-improved zCas9 or hCas9, Ubiquitin or 2 × 35S promoter, AtU6-26, OsU3 or TaU3 promoter for the sgRNA). Directed mutagenesis was shown in both maize protoplasts and transgenic plants, with the blend of zCas9 and TaU3p displaying the most astounding effectiveness. By utilizing distinctive Pol III promoters, it was likewise conceivable to collect up to four diverse sgRNA expression tapes on one vector for multiplex genome building.

By checking for transgene free T2 *A. thaliana* plants by means of PCR and ensuing sequencing of the Cas9 target destinations, heritability of changes was affirmed [56]. The results revealed that this approach not only successfully caused mutations in multiple sites as expected, but also performed as efficient as the common approach that uses single spacer sequence. This implies that multiple genes could be efficiently targeted by a single DNA expression modular or vector, which is difficult to do with the TALENs based biotechnology.

Table 1: Specific genomic sites in some plant genes.

Crop	Gene targeted
Solanum lycopersicum	DMR6
Arabidopsis thaliana	AtADH1, AtTT4
Arabidopsis thaliana	AtBrI1,AtJAZI, AtGAI
Arabidopsis thaliana	AtchI1, AtTT4, AtGAI
Arabidopsis thaliana	AtCHL1, AtTT4, AtAP1, AtGUUS
Hordeum vulgare	HvPMI9
Triticum aestivum	TaLOX2
Triticum aestivum	TaMLO-A1
Oryza sativa	OsELB
Oryza sativa	OsERF922
Oryza sativa	OsSWEET11, OsSWEET14
Oryza sativa	46 genomic target
Orya sativa	OsMpKs
Oryza sativa	OsROC5, OsSPP, OsYSA
Oryza sativa	OsCAO1, Oslazy
Citrus sinesis	CsPDs
Glycine max	G Lymap7g1450, GmDDM1S, GmMIRs
Sorghum bicolor	DsRED2
Zea mays	ZmLIG1, ZmM26, Zm45, ZmALS1
Zea mays	ZmIPK
N. benthamania	NbPDS, NbPCNA,
N. benthamania	NbPDS, NbPDR6
Nicotiana tabacum	NtPDS, NtPDR6, ALS
Marchantia polymorpha	MpARF1
Populus tomentosa	PtPDS1 & PtPDS2
Malus domestica	PDS

Cpf 1: Substitute to Cas9

Cpf1 is a RNA-guided endonuclease of a class II CRISPR/Cas framework. Cpf1 qualities are related with the CRISPR locus, coding for an endonuclease that utilization a guide RNA to discover and divide viral DNA. Cpf1 is a littler and less complex endonuclease than Cas9, conquering a portion of the CRISPR/Cas9 framework impediments.

Using Specific Promoters for Inducing Genomic Changes by Cas9

It has been observed that the frequency of mutagenesis for Cas9 is more by using constitutive promoters, although other promoters can also be utilized. It can be a means for accomplishing stable mutations more rapidly particularly in case of plant species. The utilization of individual promoters can make probability of restrictive knockouts or the mutagenesis of particular qualities in one of kind organs. Qi-Jun Chen's Group of specialists from Beijing exhibited in a review that when the zCas9 was put under control of the promoter from the egg cell-particular EC1.2 of *A. thaliana* to build heritability by instigating transformations in egg cells [57,58]. At the same time with individual sgRNAs, T1 twofold and triple mutant *Arabidopsis* plants could be gotten that additionally isolated in the T2 era. Additionally, not at all like blends out of eight promoters and two eliminators were broke down for their mutagenesis effectiveness. Together with the rbcS E9 eliminator, blends of EC1.1 promoter and EC1.2 enhancer were utilized which brought about the most elevated number of T1 triple mutants (17%). Yet, the physical expression framework is by all accounts a great deal more proficient in controlling mutagenesis through various formative stages, in this manner, can be most critical in concentrate the elements of specific qualities as approach utilized as a part of this examination does not appear to fundamentally build heritability of transformations [59-61].

Transcriptional Control of Cas9: Unbelievable Success Story of CRISPR

Controlling the process of transcription by cas9 besides targeted genome modifications with RNA-guided Cas9 is an incredible achievement for the researchers since CRISPR-Cas9 technology came into existence. Interpretation can be viably hindered by directing chemically latent dead Cas9 to a promoter or a coding district. It has been exhibited that the combination of repressor protein with the Cas9 (dead) improves the blockage of interpretation [62,63]. Correspondingly, an activator, for example, VP64 can likewise be combined to focus Cas9 to actuate interpretation of a particular quality. This framework has been effectively exchanged to plants by a gathering of specialists of KAUST. Counterfeit activators were made by combination of C-end of the human codon upgraded Cas9 with the EDLL space or the TAL initiation area. A repressor was made by combining the SRDX EAR theme. Invaded *N. benthamiana* leaves indicated effective transcriptional enactment or constraint when expression levels of a transient GUS quality or the endogenous PDS were watched [64,65]. At the point when the activator was guided to the sense strand of the promoter close to the transcriptional begin site abnormal state of initiation was watched and both activator builds performed at an equivalent level. Suppression of PDS was exhibited for both dCas9 alone and for the dCas9: SRDX combination develops and could be expanded by managing the complex to a few target locales inside the promoter and the main exon of the quality at the same time [66].

Concluding Remarks

CRISPR/Cas9 system has been recently developed by reprogramming the bacterial type II nucleic acids based immune system, a novel site specific genome editing tool. Given its notable technological simplicity, the CRISPR/Cas9 system is becoming the principal technique of choice replacing the central role of the TALENs based biotechnology in site specific genome editing. Crops produced through genome editing/Cas9-RNP technology will certainly enhance the precision breeding approach for useful traits and minimize the hurdle of deregulation for a sustainable agriculture. The CRISPR/Cas9 system has been rapidly adapted in both model and crop plants and established with a desirable efficacy in site specific gene targeting. This system will likely become more efficient with time, allowing high throughput applications that will target the entire genome in plants. Ultimately the shockwave sent to the genome engineering community today by the discoverers of this brain storming genome editing technique will be felt by the agriculture in a positive way tomorrow.

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