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Ushering in CRISPR/Cas Mediated Genome Engineering for Crops

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Abstract **Original Research Article**

The efficacy, diversity and modularity of CRISPR/Cas system are a driving force in the biotechnological revolution. Cas enzymes have been adopted as tools for manipulation of genomes in planta as a means to accelerate fundamental research and enable agricultural breakthroughs. Here, we review the working principles and components of CRISPR/Cas system for efficient gene editing in plants. We have also tabulated the recent work that has utilised CRISPR/Cas to improve economically important traits in plants. Although the apparent use of CRISPR/Cas mediated editing may make it appear as though researchers are toying with plant genomes, the cumulative power of this tool has made optimized and adaptable plant species towards permitting crucial advances in crop improvement.

Keywords: CRISPR/Cas, nutrition, crop improvement, genome editing, plant breeding.

INTRODUCTION

Among crops phenotypic variations have been related directly to the erosion or loss of genetic diversity with the observations at both the species level during domestication and the gene level through practices in breeding. Such genetic consequences have made the modern-day crop plants more sensitive to stress in comparison with their wild type. In order to overcome this issue, breeders and researchers alike have involved themselves in various strategies to elevate diversity for better yield and/or adaptability by inducing mutations.

Various tools and methodologies that have been used for inducing mutagenesis include T-DNA transformation [1], TILLING [2], EcoTILLING [3], antisense RNA and RNAi [4, 5]. The former two methods provide a better understanding of the function and regulation of genes whereas the latter two are used for disrupting the function of specific genes to indirectly or partially decrease gene function. Although the contributions by these methodologies have greatly helped understand gene functions, unintended random and deleterious mutations have raised alarms for the consumption of resultant crops. Following this, the discovery of sequence-specific nucleases has enabled customisable gene editing thus achieving precise mutagenesis and genome editing.

The sequence specific nucleases are engineered for the induction of double-stranded (ds) breaks at specific sites within the genome followed by repair by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). Currently the sequence-specific nuclease system, clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein (Cas) has been widely utilized for many plant species. CRISPR/Cas is practiced by engineering single-guide RNAs to target specific DNA sequences as a simpler and more efficient tool than its previous technologies. This RNA-guided DNA recognition toolbox provides diverse yet important scientific opportunities to engineer desirable genetic traits with integration of high-throughput functional genomic screens [6]. Such amalgamation of various techniques with CRISPR toolbox transcends genetic programmable applications lending itself to impact a revolution in molecular biology and can be adapted for DNA, RNA and transcriptome (Figure-1).

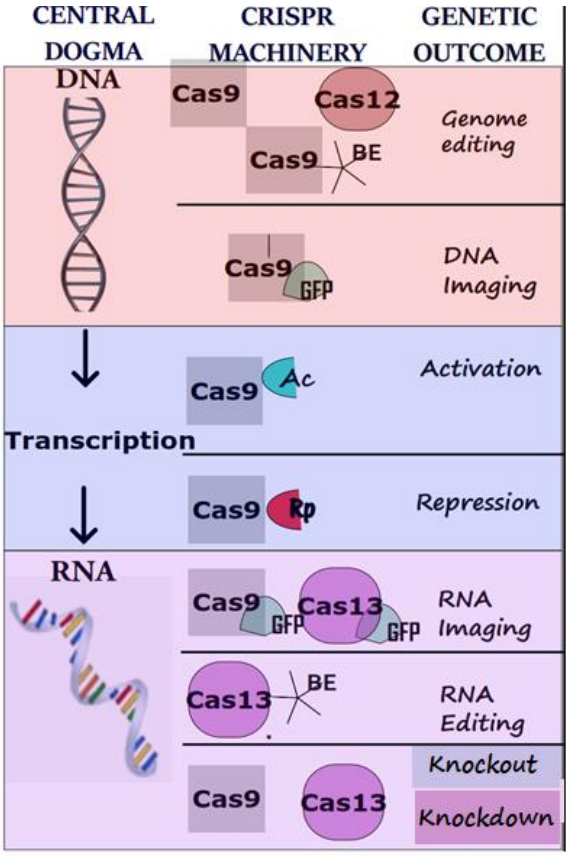

Fig-1: Genetic manipulation of CRISPR/Cas systems *in planta* at every stage of the central dogma. Cas9 and Cas12a are used to induce double stranded breaks (DSBs) for genome editing such as knock-in or knock-out. nCas9 can be fused with Base Editors (BE) to modify the nucleotides without inducing a DSB. To regulate transcription dCas9 can be fused with epigenetic modifiers, activators or repressors to regulate transcription. Cas9 can bring about knockout of RNA whereas Cas13a can be used for knockdown [7]. Cas13a can be fused with BE to render a modification in RNA nucleotides. Visualization of DNA or RNA can be achieved by the fusion of dCas9 or dCas13a to green fluorescent protein (GFP)

RNA-programmable CRISPR-Cas enzymes

The CRISPR toolbox is adapted from a prokaryotic RNA-mediated type II system that is involved in providing defence against invading viruses. This RNA-guided adaptive immunity to foreign genetic elements by directing nucleases to cut and bind specific nucleic acid sequences. Microbes capture small reads of foreign genetic elements and incorporate them into CRISPR array. Transcription of CRISPR arrays creates CRISPR RNAs (crRNAs) that bind to Cas nucleases and provide specific pairing with target nucleic acids. Class II CRISPR-Cas systems constitute a single large RNA-guided Cas nuclease that mediate cleavage or interference in the target sequence / gene. Several properties of class 2 type II DNA – targeting endonuclease Cas9 such as assembly with intended gRNA alone, specific recognition of crRNA, interaction of the crRNA with a trans-activating crRNA (tracrRNA), stable binding to target DNA adjacent to specific protospacer adjacent motif (PAM), switch-based binding to correct nucleotide sequence and introduction of a dsDNA break [8].

Among the many Cas effectors, *Streptococcus pyogenes* Cas9 (SpCas9) is the most commonly used effector [9-11]. In addition to this numerous functionally distinct CRISPR-Cas system that maintain programmable characteristic keys similar to SpCas9. Expansions in the Class II systems was observed by the inclusion of two other candidate systems designated as type V CRISPR/Cas12a (formerly called Cpf1) [12] and type VI CRISPR-Cas13 (previously designated C2c2) [13]. The present-day scientific scenario of programmable RNA-guided nucleases reflects an equally shared spotlight between Cas9 homologs, RNA-targeting Cas13 and DNA-targeting Cas12.

Precision editing of CRISPR remains challenging particularly due to the competing repair outcomes that retrain the efficiency of a more desirable HDR repair outcomes. Apart from the distinct DNA cleavage and repair, nickase Cas9 (nCas9)-mediated base editing carries a single base editor to the target that facilitates conversion of base without dsDNA cleavage [14]. The toolbox of nCas9 has further expanded recently by an invitro evolved deaminase that now enables nCas9-base editors to catalyse A-T to G-C transitions. Although such single-base editors provide a great potential to induce mutations without a dsDNA break, limiting the off-targets requires further technological advancement.

Regulation with dCas9

The functionally distinct DNA binding and nuclease activities has made Cas9 as a modular platform. An explosion of information can now be gained by the use of deficient Cas9 (dCas9) that is created by segregating the DNA binding from the enzymatic activity of Cas9 by mutating the nuclease domains. dCas9, thus results in a scaffold that is functional to recruit proteins or components of RNA that is specific, perturbs transcription without any alteration of the DNA [15, 16]. Recently, dCas9 proteins were fused with transcriptional activator or repressor domains to modulate the expression of transcript levels in planta. CRISPR-dCas9 has been made as chimeric effector regulators that act as artificial transcriptional factors has been studied for the gene function and interaction within various regulatory networks in *Arabidopsis thaliana*. Plant specific VP64, HSF1 activation domain and NF-kappa B trans activating subunit were fused to dCas9 and resulted in the gRNA guided transcription activation of AVP1 and PAP1. The enhancement in the expression of AVP1 lead to increased drought tolerance due to increase in the leaf size and number whereas an enhancement in the production of anthocyanin was achieved by PAP1 overexpression [17]. Challenges in

the dCas9-effector fusions remain with the complex off target effects generated by the fused catalytic domains targeting neighbouring or unrelated loci [18]. Such locus-specific effects, if unpredicted, can confound analysis if the chromatin or transcription processes are involved or affected.

Engineering Posttranscriptional Effects with RNA-Targeting Cas

Alternatives to permanent genetic alteration can be achieved by transient disturbance of the transcriptome by using Cas effectors by targeting the RNA directly. By utilising PAM-presenting oligonucleotide [19] a programmable RNA-targeting system was created by engineering SpCas9 that furthered applications for RNA-targeting with Cas9 (RCas9) [20]. The potential applications of using RCas9 helps to eliminate pathogenic RNA, alleviate mRNA splicing defects and/or lessen the protein production from RNAs with CAG repeats. Until recently, the arsenal of RNA targeting Cas9s also included its homologs with programmable RNA-targeting activity that is PAMmer-independent [21-23].

Cas13 has also contributed with its high versatility for RNA targeting. Cas13a has been employed as a tool for specific knockdowns in plant [7] and mammalian cells alike and has established type VI systems as RNA-guided ribonuclease (RNase). In addition to Cas13a, enzymatic Cas13b have been confirmed for their functional and efficient RNA interference and RNA editing mechanisms [24]. More recently Cas13d was identified for modulating splicing *in vivo* [25]. Future studies are required to determine how RNA-targeting Cas-effectors interact with structured proteins and how trans-RNA cleavage can be achieved by Cas13 *in vivo*.

Specificity and Delivery of CRISPR/Cas

The major challenge posed for technologies in genetic manipulation is inadvertent modification, binding and/or cleavage of nucleic acids. In addition to the off-target interactions, CRISPR tool also involves the permanence of genome editing by deleterious off-target Cas nuclease activity. In order to negate this, evolving and engineering of Cas enzymes, gRNAs have been established by computational resources to improve nuclease specificity (Table-1). Beyond this approach, researchers are now focusing on a deeper understanding of repair mechanisms involved in cellular DNA to achieve a desired editing. Alternatively, optimizing vehicles for specific and efficient delivery of Cas *in planta* is also important. The major routes for the delivery of Cas include transfection, electroporation, direct injection and viral vectors. Alternatively, functionalised nanomaterials are being used to enable the specific delivery to a cell of interest [26].

Table-1: Online resources for CRISPR/Cas

Software	CRISPR component	Link	Created by	Features	Reference
CGAT	gRNA	http://cbc.gdcb.iastate.edu/cgat/	Iowa State University	Identify potential target and off-target sites	Brazelton *et al.*, 2015 [27]
ge-CRISPR	gRNA	http://skl.scau.edu.cn/	The Genetic Engineering Laboratory of South China Agricultural University	sgRNA sequences, constructs and germplasm resources	Kaur *et al.*, 2015 [28]
CRISPR-PLANT	gRNA	https://www.genome.arizona.edu/crispr/	Arizona Genomics Institute	sgRNA design, no off-targets, target restriction enzyme sequence analysis, eight species	Xie *et al.*, 2014 [29]
CRISPR-P	gRNA	http://crispr.hzau.edu.cn/crispr/	Huazhong Agricultural University	robust sgRNA design, visual interface for sgRNA off-targets, restriction enzyme analysis	Lei *et al.*, 2014 [30]
CRISPR-P v 2.0	gRNA	http://crispr.hzau.edu.cn/CRISPR2/	Huazhong Agricultural University	scores sgRNA target efficiency and specificity, analyses secondary structure, GC content and flanking regions of targets	Liu *et al.*, 2017 [31]

CRISPy-web	gRNA	http://crispy.secondarymetabolites.org/	The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark	design gRNAs for any user-provided microbial genome	Blin *et al.*, 2016 [32]
CRISPR DESIGN	Off-target	http://crispr.mit.edu/	Massachusetts Institute of Technology	Off target minimization	Beisel *et al.*, 2014 [33]
E-CRISP	Constructs	http://www.e-crisp.org/E-CRISP/	German Cancer Research Center	Design of CRISPR constructs	Heigwer *et al.*, 2014 [34]
CCTOP	Constructs	https://crispr.cos.uni-heidelberg.de/	Center for Organismal Studies Heidelberg	CRISPR/Cas9 online prediction tool	Stemmer *et al.*, 2015 [35]

Detection of Targeted Mutations

The detection of the desired alteration to the genome is the first step to confirm the genotypes of CRISPR/Cas mediated mutants and validate the results before further analysis. The means of detection is quite varied and largely depends on the editing introduced. If the melting temperatures of the mutated and native PCR amplicons are different, mutations can be detected by high-resolution melting technology (HRMT) [36]. Although this tool limits its efficiency by its inability to sequence the altered DNA, it can be adapted as a pre-screening tool that reduces the cost of the number of sequencing samples needed. If the target DNA sequence has a restriction enzyme site detected by a specific endonuclease, the mutation in the target site could modify the restriction site. Reporter genes such as genes encoding fluorescent proteins such as beta-glucuronidase, GFP, YFP or RFP can be used to identify successful editing events [37]. Although Sanger sequencing is successfully used to identify chimeric or simple mutations, next-generation sequencing (NGS) methods are consistent, efficient and powerful to identify rare, complex, multiple off target mutations. In spite of high-costs incurred when many samples need evaluation, the effectivity and reliability of NGS methods makes them a dependable evaluation.

Target Precision Achieved in CRISPR/Cas System Using Databases

Off-target or unintended mutations can occur if the spacer sequences are imperfectly matched due to the guide RNAs being unspecific. Extensive use of bioinformatics tools such as RISPRGE, CGAT, CRISPR-PLANT, CRISPR-P and CRISPR-P 2.0 have been discussed for the selection of specific guide RNAs with zero or minimal off-target sites (Table-1). Off-target mutations resulting from insufficient optimization of Cas9 codon can be avoided by using plant-optimized Cas9 codon. The use of dCas9 and Cas9 nickase can also be used to eliminate off-target mutations. It is well recognised that the inclusion of all these above-mentioned steps in the working of a researcher will minimize or eliminate off-target mutations as reflected by the explosion of studies done in many crop and plant species over the past two years (Table-2). Our laboratory has also utilised CRISPR/Cas9 system to generate herbicide tolerant maize, glyphosate tolerant rice and is presently utilizing this tool for tomato and pigeon pea [38].

Gene modification methods such as gene expression regulation and modulation in epigenetics can be adapted for agricultural purposes. Further, CRISPR/Cas provides alternative approaches for delivering genes of interest into crops by transformation using pre-assembled Cas protein-guide RNA ribonucleoproteins, viral infection or agroinfiltration with no transgenic footprint. Gene editing technology represented by CRISPR/Cas system is an affordable, elegant and simple genetic scalpel that can be widely applied to enhance the agricultural performance in crops.

Table-2: CRISPR/Cas9 mediated improvement in quality, yield, herbicide pathogen and stress traits in select plant species

Species	Gene of target	Trait / quality improved	References
Rice	OsIPA1	Number of tillers	Li *et al.*, 2016 [39]
	OsGS3	Grain size and higher yield	
	OsDEP1	Dense and erect panicles	
	OsGn1a	Grain number	
	OsGW2	Grain weight and higher yield	Xu *et al.*, 2018 [40]
	OsHD2	Early heading and maturity	Li *et al.*, 2017 [41]
	OsSWEET11	Grain filling and sugar transport	Ma *et al.*, 2017 [42]
	OsSBEI, OsSBEIIb	Amylose resistant starch	Sun *et al.*, 2017 [43]
	OsERF922	Resistance to *Magnaporthe oryzae*	Wang *et al.*, 2016 [44]

	OsEPSPS	Glyphosate tolerance	Jung *et al.*, 2018 [45]
	OsSAPK2	Drought, osmotic, salinity tolerance; stomata and ABA signalling	Lou *et al.*, 2017 [46]
	OsALS	Bispyribac sodium resistance	Butt *et al.*, 2017 [47]
	OsMPK1, 2	Biotic/abiotic signalling	Khatodia *et al.*, 2016 [48]
Tomato	SlSP5g	Early flowering and yield	Karkute *et al.*, 2017 [49]
	SlJ2	Less fruit dropping and jointless fruit stem	Soyk *et al.*, 2017 [50]
	SIEJ2	Higher yield with larger fruit	
	SlSP5G	Improves inflorescence architecture and fruit yield	
	SlMlo	Resistance to *Podosphaera xanthii*	Nekrasov *et al.*, 2017 [51]
	SIAA9	Parthenocarpic fruits	Ueta *et al.*, 2017 [52]
Maize	ZmRPL and ZmPPR	Reduced protein level in kernels	Qi *et al.*, 2016 [53]
	ZmARGOS8	High yield under drought stress	Shi *et al.*, 2017 [54]
	ZmALS2	Chlorsulfuron resistance	Svitashev *et al.*, 2016 [55]
	Phytoene synthase	White kernels and albino seedlings	Zhu *et al.*, 2016 [56]
Soybean	FAD2- 1A and 1B	Reduced linolenic acid levels	Kim *et al.*, 2017 [12]
	GmFT2	Late flowering under long and short-day conditions	Cai *et al.*, 2018 [57]
Watermelon	Phytoene desaturase (PDS)	Albino phenotype	Parmar *et al.*, 2017 [58]
Sweet orange	CsPDS	Increase in fruit size with albino phenotype	Jia and Wang, 2014 [59]
Citrus	PDS	Early stages of shoot generation; albino phenotype	Jia *et al.*, 2017 [60]
Grape	PDS	Albino Leaves	Nakajima *et al.*, 2017 [61]
	MLO7	Resistance to Powdery mildew	Malnoy *et al.*, 2016 [62]
	VvWRKY52	Increase disease resistance to *Bitrytis cinerea*	Wang *et al.*, 2018 [63]
Wheat	TaGW2	Increase in grain size and weight	Wang *et al.*, 2018 [64]
	Ms45	Rapid generation of male sterile bread wheat	Singh *et al.* 2018 [65]
Flax	FAD2- 1A and 1B	Reduced linolenic acid levels	Jiang *et al.*, 2017 [66]

CONCLUSION

An accessible and adaptable platform is created by means of CRISPR/Cas toolkit that empowers applications involving alteration and regulation of genomes. The impact of CRISPR/Cas has bloomed in the agricultural sector in a rapid manner creating products for various markets and expanding the repertoire of applications seeking regulatory rulings in India, EU and USA. In light of its early successes and the multiplexing of CRISPR/Cas, the utility of this toolkit can be limited only by a human understanding of the function of the target gene, understanding the mechanisms, and its associated technological advancements.

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