In Silico Tools and Approach of CRISPR Application in Agriculture

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Abstract

The emergence of new gene editing technologies poses the ability to transform human healthcare, lifestyle, and agriculture. The groundbreaking implications of genome editing have already been showcased in crops and agricultural system. Its successful applications span from breeding of animals and plants to exhibiting resistance to pests and diseases. CRISPR being the pioneered product of research-academia has gained tremendous importance and dominance in the field of genetic engineering. Advances in clustered regularly interspaced short palindromic repeats (CRISPR) have provided platform for large-scale production of engineered products. However, successful application of CRISPR requires precise design and target strategy for Cas protein and guide RNA. The application of CRISPR/Cas systems is limited by the inconsistent efficiency of endonucleases and cleavage at off-targets. Computational tools, platforms, and programs have reduced the hindrance in achieving cleavage efficiency and specificity. This review provides information on tools and platforms that are available in designing of guide RNA, selecting target sites, analyzing output results and efficiency, etc. The updated information on online and off-line tools will prevent CRISPR/Cas off-targeting during in vivo application.

Keywords

CRISPR · Gene editing · Crop improvement · Protospacers · Guide RNA

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10.1 Introduction

The burden of achieving global food security over the years is increasing and becoming a major challenge for many countries, which needs immediate attention. Limited arable land, crop loss by disease and pests, and maintaining of nutritional quality intensify the problem of food scarcity. Without hampering the natural resources and balance, the focus shifted to enhancing the inherent quality of the plants for increased food production. Traditionally, farmers have to rely on seeds from fewer varieties of crops with higher quality attributes and improved vigor. However, the solution to issues like marginal difference in the selected traits and longer duration of plant breeding programs may take 10–20 years and unpredictable weather change makes it more difficult to increase food production. On the other hand, introduction of new crop species, maximized use of degraded land for agricultural production, plants resisting stressful climate, or geographical areas with drastic climate change would meet the challenge of global food security (Zhang et al. 2018a). Currently, the genetic and species diversification in agricultural systems will solve diverse range of food production challenges (Fernie and Yan 2019).

In the field of biological research, genome editing or genome engineering had created a revolution by manipulating the genome of an organism either directly or indirectly through gene silencing. These genome alterations have proved to be effective in expressing the desirable trait within the organism to fulfil the desired purpose. Since 1960s, the discovery of restriction endonuclease has led the gene alteration and manipulation process. Moreover, metagenomics is also a key player in exploring hidden genetic features and advancing the application of biotechnology in finding novel bioactive compounds, improved biochemical functions, and gene of interest (Baliyarsingh 2020). Subsequently, advances in the recombinant DNA technology as well as traditional homologous recombination methods have quickened the genomics and genomic manipulation. But due to their minimized efficiency, at present new types of restriction endonuclease are being developed and designed like clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein, zinc-finger nucleases (ZNF), and transcription activator-like effector nucleases (TALEN).

Out of these new methods available, the focus here would be widely on clustered regularly interspaced short palindromic repeats/Cas method that is believed to show the best efficiency so far. This method is an easy, comfortable, user-friendly, and well-adopted genome editing tool using RNA-guided endonuclease for producing double-stranded break (DSB) (Khatodia et al. 2016). The CRISPR/Cas (CRISPR-associated protein) system has turned out to be an efficient technology that has the ability to bring transformation in the field of genome engineering. In the present scenario, the Cas9 nuclease is predominantly used when there is a need of target-specific DNA cleavage. In its natural settings, the CRISPR/Cas9 system provides adaptive immunity of bacteria and archaea against the introduced mobile genetic elements. The prokaryote keeps records of viral infection occurred in genome as CRISPR arrays. These CRISPR arrays consist of acquired viral DNA fragments

interspersed by palindromic repeats (called spacers). Majorly studied Cas 9 system is guided by single-guide RNA (sgRNA) or a hybrid of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) to target a specific DNA with sgRNA complementarity (Jinek et al. 2012). After the double-stranded break has been created the genome can be modified as per required, like gene addition, disruption, or correction (site directed) by activating recombinase repair activity (Kim et al. 2011). The whole principle of CRISPR/Cas technology is now adapted in computational tools (online/off-line) to help in designing experiment, finding target sites, constructing target-specific guide RNA, predicting off-target sites, etc. (Sangar et al. 2016). These software have eased the method of performing the experiment with predetermination of the approximate efficiency of result. There is a great need of editing plant genome (especially crops) to develop or to have improved varieties of them that were previously achieved by plant breeding process. Targeted genome editing by CRISPR/Cas is believed to have potential in crop improvement by modifying the plant genome to produce a more valuable product of interest and to meet the surge of food demand globally (Liu et al. 2013). This topic would summarize on the mechanism of CRISPR/Cas in genome editing of plant species and the online tools available for designing the experiment as well as on finding the targets.

10.2 Mechanism of Action

The CRISPR/Cas is now the most powerful technique of gene editing which was discovered from bacterial species back in 1987 as their defense mechanism. This is an important machinery of prokaryotic representation of adaptive immunity, containing Cas protein along with a pair of RNA fragments needed to guide for the specific cleavage of viral genome, ultimately providing the protection against infection. Different forms of Cas proteins are expressed in bacterial cells which perform with minor difference of functions from each other. Short palindromic repeat sequences generated from the foreign DNA get incorporated as spacers (approximately of 20 nucleotides) in the CRISPR array after fragmentation by nuclease activity of Cas (Mei et al. 2016). The spacers are known to help Cas9 protein in recognizing the same viral DNA (as template strand) if encountered again in future (immunity stage or expression) to perform specific fragmentation. During the condition when the cell confronts foreign genome for the first time, regarded as prokaryotic immunization stage or adaptation stage, a different form of Cas is activated and it results in integrating a short fragment of it into the host genome (CRISPR array). There are three different types of CRISPR/Cas editing, type I, II, and III, and these are distinguished mainly based on the type of Cas protein involved. The Cas 3/Cas 6, Cas 9, and Cas 10 are involved in type I, type II, and type III, respectively, having their own cascade protein system required for the activity (Barrangou and Horvath 2017).

Cas protein is a bilobed structural protein with nuclei acid sites, involved in RNA binding (DNA-binding motifs) and restricted core domain. These sites are responsible for forming recognition lobe (REC) followed by a nuclease lobe (NUC)

connected by a helix bridge (Song et al. 2016). Type II system (RNA-guided endonuclease) with Cas 9 cleaves double-stranded DNA with HNH (His-Asn-His) nuclease domain and RuvC-like domain cutting each strand (Wang et al. 2013). The Cas protein is composed mainly of six functional domains and these are REC-I, REC-II, HNH domain, RuvC, bridge helix, and protospacer adjacent motif (PAM) sequence. The main role of PAM sequence is to differentiate self-DNA from non-self-DNA, hence protecting CRISPR arrays from Cas protein's activity against its own genome (Sternberg et al. 2014).

Agricultural methods need a desired change in plant genome to introduce the desired trait into them. CRISPR/Cas and Cpf1 (centromere and promoter factor-1) system has been proved to be a revolutionary method in producing the variant types in plants (Zetsche et al. 2015). CRISPR is provided into plant cells as DNA, RNA, or protein that induces double-stranded break which is then repaired by the cells through annealing of DNA ends (gene KO). The strand joining can also involve inserting different gene sequences at DSB or by sequence replacement (Gao 2018).

10.3 CRISPR Role in Agriculture Advancement

10.3.1 Overview of CRISPR Application in Agriculture

CRISPR/Cas system is able to produce required plant germplasm by specific alteration of gene and developing mutated genome that showed gain of trait by insertion and/or loss of function of undesired gene of interest by deletion. Initial studies of crop improvement were focused around increasing the yield by manipulating factors affecting it (Zhu et al. 2020). Practically, expression of cytokinin is the likely target for improving the yield of cereals and the enzymes involved are cytokinin activation enzyme and cytokinin dehydrogenase (CKX). Thus, modifying the ends of cytokinin activation enzyme and knocking out CKX from the cell increase the yield in different environmental conditions (Zhang et al. 2019; Wang et al. 2020). Along with increasing yield, quality improvement is equally essential for a crop to be considered as healthy for consumption. For example, amylose content in crops is desired at different levels, where low amylose is better suited in grains and high amylose content in cereals is valuable to human health. The enzyme granule-bound starch synthase 1 (GBSS1) is important for amylose biosynthesis and CRISPR technique proven to be successful in targeting its pathway of biosynthesis (Sun et al. 2017).

Crops are threatened by many types of disease-causing organisms during development, mainly by bacteria and viruses. On the other hand, microbes of soil are key players in maintaining soil structural integrity, promoting plant growth, and thereby increasing food productivity (Baliyarsingh et al. 2017). CRISPR/Cas technique can help in reducing the biotic stress over crops. In case of viruses, Cas9 protein can be programmed to cleave the DNA of infecting virus and can also trigger transcription of certain genes whose products are required for inhibiting bacterial infection to confer virus and bacterial infection, respectively (Ji et al. 2018). Similarly inducing herbicide resistance in plant maintains and improves high crop productivity. The key

enzyme, acetolactate synthase (ALS) which is involved in amino acid synthesis, is the primary target of many herbicides (like sulfonylurea and imidazolinone). Thus studies on introduction of specific substitution of amino acid by CRISPR/Cas showed herbicide tolerance (Powles and Yu 2010).

Breeding technologies can be approached using CRISPR/Cas for add-on benefits to agricultural production. To fix the genetic background of hybrid plants haploid induction can be achieved in fewer generations with CRISPR than traditional methods. Targeting certain genes for mutation like MTL (coding phospholipase A1), CENH3, and DMP by CRISPR can cause haploid formation (Liu et al. 2017a; Zhong et al. 2020). Hybrid seeds are effectively produced by eliminating self-pollination of female organ, i.e., by inducing male sterility in maternal plants (Okada et al. 2019). Hybrid vigor can be fixed by eliminating meiosis recombination (by passing second meiosis) and keeping mitosis to develop clonal multiploidy gametes (Wang et al. 2019).

10.3.2 In Silico-Assisted Gene Editing Using CRISPR/Cas

The dependency of engineered nucleases and guide RNAs on gene editing processes is well established. However, its application is limited by their off-target DNA cleavage leading to cellular toxicity. Different organisms possess a variety of Cas9 proteins that utilize different PAM sequences. Moreover, evidence of RNA-guided endonucleases (RGENs) cleaving DNA at off-targets with several mismatches (Fu et al. 2013; Cho et al. 2014) or causing addition/deletion of nucleotides (Lin et al. 2014) has hindered the application in the healthcare to agriculture. In silico approach and tools have benefited in overcoming these issues. Although the CRISPRs' in silico analyses began in mid-1990s (Mojica et al. 1995), the progress in the development of CRISPR software tool has been slow. Initial software tools used to identify particular repeats had to screen and discard the background manually and sometimes short CRISPR clusters were missed or neglected. Since then many researchers have been developing and presenting computational tools that help in selecting appropriate targets, designing guide RNAs, PAMs, and output analysis (Sander et al. 2010; Bae et al. 2014; Heigwer et al. 2014; Naito et al. 2015) (Table 10.1).

10.4 Applications of CRISPR in Agriculture

Gene editing by CRISPR is being widely accepted for creating noble plant varieties with desired phenotype that further helps in yield improvement, quality improvement, and stress resistance to abiotic and biotic factors. The gene of interest is altered to generate In-Dels or to produce a desired type of crop variety by changing the level of expression. Online tools, software, and databases are providing the medium to access the gene and its target sequences for gene editing. With crop improvement being the major objective of genome editing process, the knocking-out and

Table 10.1 In silico tools that are helpful in designing and guiding CRISPR/Cas gene editing

Sl. no.	Software	Molecule involved	Target	Application	Reference
1.	CRISPR-GE	Cas9 or Cpf1	sgRNAs	Off-target site prediction and primer designing	Xie et al. (2017)
2.	PhytoCRISP-Ex	Cas9	PAM sequence	Searching target sites of Cas9	Rastogi et al. (2016)
3.	CRISPR-P	Cas9	Guide sequence	Searches for highly specific Cas9 targets in interested DNA sequence	Lei et al. (2014)
4.	GuideScan	PAM and gRNA	Guide sequence	Designing comprehensive guide RNA database	Perez et al. (2017)
5.	sgRNAcas9	Cas9	sgRNA	Quick designing of sgRNA with low off-target effects	Xie et al. (2014)
6.	PrimeDesign	pegRNA and ngRNA	Design of PE experiment	Automatic designing of pegRNA and ngRNA	Hsu et al. (2021)
7.	CRISPRseek	PAM and gRNA	gRNA	Constructing target-specific guide RNA with known PAM sequence	Zhu et al. (2014)
8.	CRISPRdirect	PAM	gRNA selection	Finding target sites with minimum off-target candidates and it is a repository of off-target sites from few organisms	Naito et al. (2015)
9.	CHOPCHOP	Genome sequence	Off-target sites	Prediction of binding off-target with TALENs	Montague et al. (2014)
10.	CRISPRTarget		Protospacers	Identification of protospacer targets	Biswas et al. (2013)
11.	CRISPRer	PAM and seed sequence	Protospacers	Selection of CRISPR/Cas protospacer by comparing with seed sequence	Sangar et al. (2016)
12.	E-CRISP	Cas9 nuclease	gRNA	Used to design gRNA and it is a fast approach to find the binding sites (complementary to gRNA)	Heigwer et al. (2014)
13.	CRISPR-ERA		sgRNA	Designing of sgRNA for editing	Liu et al. (2015)
14.	CRISPRfinder		CRISPR loci	A tool for detecting CRISPR and PAM sequence	Grissa et al. (2007)
15.	Cas-OFFinder		Off-target sites	Finds potential off-target sites in user-defined sequence	Bae et al. (2014)
16.	Cas-Designer	PAM sequence	gRNA	Used for gRNA selection and finding potential off-targets	Park et al. (2015)

(continued)

Table 10.1 (continued)

Sl. no.	Software	Molecule involved	Target	Application	Reference
17.	DESKGEN		Experiment design	Designing CRISPR experiment: setting up and analyzing the experiment	Hough et al. (2016)
18.	caRpools		Result screen analysis	Experimental data analysis and workflow analysis	Winter et al. (2016)

Cas9 CRISPR-associated protein 9; *Cpf1* CRISPR from *Prevotella* and *Francisella* 1; *sgRNA (gRNA)* single-guide RNA (guide RNA); *PAM* protospacer adjacent motif; *PE experiment* prime editing experiment; *pegRNA* prime editing guide RNA; *ngRNA* nicking single-guide RNA; *TALEN* transcription activator-like effector nuclease

knocking-in techniques are major players in achieving the quality improvement in crops over the wild variety. A notable example is targeting of GW5 protein (inhibiting the kinase activity of GSK2), a positive regulator of signaling pathway that controls grain width and weight of rice. The expression of GW5 gene can be altered by knockout-based method according to the yield requirement in crops (Liu et al. 2017b). In a similar study, by pedigree analysis, whole-genome sequencing (WGS), and CRISPR/Cas-based knockout a large gene in rice involved in high yield was identified. The genes essential for the production of rice also showed associated phenotype alteration at different loci such as plant height and flowering time (Huang et al. 2018).

Quantity improvement by gene editing is focused on altering nutritional value, storage capacity, and major content of the crop. With rice being the major dietary food of many countries, the starch content is targeted for reduction for improved cooking and rice eating. Waxy gene knockout led to the production of low amylose content in grains by CRISPR/Cas9 editing which did not affect any other trait in the crop (Zhang et al. 2018b). And same waxy gene is also deleted in case of corn line (six genes coding for polyphenol oxidase) for high yield and quality crop production (Waltz 2016). The alpha-gliadin genes in cereal are reduced to downregulate gluten protein (that causes celiac disease in humans). CRISPR is used to knock down alpha-gliadin in wheat with no off-target mutation other than the potential target observed (Sánchez-León et al. 2018). Seeds are edited with CRISPR/Cas9 for high oleic acid content to reduce steroid toxicity level and to increase the shelf life of camelina (Morineau et al. 2017) and tomatoes (mutation in lncRNA 14,559 gene) (Li et al. 2018a).

CRISPR is not limited to increasing the yield but to alleviate various other challenges of crop production and protection (Table 10.2). This site-specific gene editing technique is helpful in facing the biotic stress by inducing resistance to bacteria, viruses, fungus, and insects. Rice genes are targeted to produce resistance to fungal disease by knockout of OsERF922 transcriptional factor gene (Wang et al.

Table 10.2 Application of CRISPR tools in agriculture

Sl. no.	Online tool	Targeted crop	Desired effect	Reference
1.	CRISPR-GE	Maize	Comparison of gene editing efficiency with different Cas proteins to detect mutation at the desired site in *O2* gene	Gong et al. (2021)
2.	CRISPR-GE	Rice	Controlling amylose synthesis by editing *Waxy (Wx)* gene for quality improvement	Zeng et al. (2020)
3.	CRISPR RGEN and CRISPR-P	Grape	Deleting *VvWRKY52* gene (transcriptional factor) increases biotic stress resistance (resistance against pathogen)	Wang et al. (2018)
4.	Cas-OFFinder	Rice	Analysis of any off-target mutation in rice due to CRISPR editing	Liu et al. (2021)
5.	CRISPR-P	Chardonnay	Site-specific mutation in L-idonate dehydrogenase gene (*IdnDH*) to reduce synthesis and accumulation of tartaric acid	Ren et al. (2016)
6.	Guide design resources	Potato	Mutation in granule-bound starch synthase (*GBSSI*) gene to produce waxy potatoes having amylopectin	Andersson et al. (2017)
7.	CRISPR-P	Soybean	sgRNA to edit soybean hairy root and its gene function analysis (*GmFEI2* and *GmSHR* endogenous gene) by CRISPR	Cai et al. (2015)
8.	CRISPR-P	Tomato	Targeting insertion and deletion in *RIN* (MADS-box transcription factor) gene of tomato genome to state its role in fruit ripening	Ito et al. (2015)
9.	CRISPR-GE	Rice	Developing herbicide tolerance allele by generating In-Dels in acetolactate synthetase (*OsALS*) gene by CRISPR/Cas9 editing	Wang et al. (2021a)
10.	CRISPR-P	Tomato	*SBP-CNR* and *NAC-NOR* transcriptional factor gene editing which is involved in fruit ripening	Gao et al. (2019)
11.	CRISPR-P	Tomato	Knocking out *SGR1*, *Blc*, and *LCY* gene to reduce conversion of lycopene to carotene	Li et al. (2018b)
12.	SSFinder	Banana	Termination of *RAS-PDS1* and *RAS-PDS2* gene by inserting a stop codon in between resulting in carotenoid content	Kaur et al. (2018)
13.	CRISPR-P and CHOPCHOP	Maize	Targeting functional genes of maize to find its effectivity in editing	Hunter (2021)
14.	CRISPR RGEN and CRISPR-P	Grapevine	Targeting *VvbZIP36* gene (transcriptional factor) to find any off-target regions edited through WGS	Wang et al. (2021b)
15.	CRISPR-P	Cotton	Knocking out GhFAD2 gene to increase oleic acid content reducing linoleic acid to have better oxidative stability in cottonseed oil	Chen et al. (2021)

2016), bacterial blight by deleting OsSWEET13 gene (Zhou et al. 2015), and viral disease resistance rice varieties (Macovei et al. 2018). The CRISPR system has been used to make improved crops, for example cassava resistance to brown streak disease and mosaic virus, resistance in spinach to downy mildew, and resistance to fire blight diseases (Ricroch et al. 2016).

10.5 Future Prospects

In plant breeding process the commonly accepted practice is to use improved variations that have aroused from natural or induced mutagenesis. Both the traditional breeding strategies and gene modification technologies have facilitated the finding and generation of newer traits. Genome editing in crops not only holds the promise of speeding up plant breeding programs but also helps in achieving novel agro-traits like resistance against stress, pests, and diseases; improvement of food quality; increase of yield; and limited use of natural resources. Genome editing techniques like CRISPR that uses site-directed nucleases have proven to advance the crop improvement process by precisely editing the required gene of interest. This target-specific gene editing technique is well controlled than other gene alteration techniques as the risk of off-target/site mutations is minimal. The Cas protein has been very precise in cleaving gene at a particular site and has been in use in designing sgRNA for target-specific gene editing. Having such incomparable ability, they have been used to modify large crop and plant varieties as well as wild crop varieties are being targeted for manipulation in order to incorporate change to meet the current food demand of the society.

Moreover, a number of online tools or software have been developed to ease the process of guide sequence generation and identification of CRISPR targets. Apart from sgRNA, PAM sequence and protospacer recognition are also feasible using the online tools. Online tools are serving as a fast and accurate platform for CRISPR experiment designing with additional advantages of off-target prediction which may have partial dysfunctional effect over other exons, not intended for editing. All the potential parameters can be considered at once with online tools and it reduces the hit-and-trial success. Most of the CRISPR gene editing done with the help of online tools is showing effective results than traditional methods or manual designing of targets. CRISPR/Cas is evolving slowly and will tend to improve crops and plant biotechnology over the ages. Addressing food security and sustainability of world, genome editing holds promise in developing new plant and animal varieties that would meet the global challenge while preserving the environment and natural resources.

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