CRISPR CAS SYSTEM IN PLANT GENOME EDITING A NEW OPPORTUNITY IN AGRICULTURE TO BOOST CROP YIELD

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Abstract

Clustered regularly interspaced short palindromic repeats CRISPR/Cas9 technology evolved from a type II bacterial immune system develop in 2013 This system employs RNA-guided nuclease, CRISPR associated (Cas9) to induce double-strand breaks. The Cas9-mediated breaks are repaired by cellular DNA repair mechanisms and mediate gene/genome modifications. 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 have already breach the boundaries and made genetic engineering much more versatile, efficient and easy also it has been reported to have increased rice grain yield up to 25-30 %, and increased tomato fruits size, branching architecture, and overall plant shape. CRISPR/ Cas also mediated virus resistance in many agricultural crops. In this article, we reviewed the history of the CRISPR/Cas9 system invention and its genome-editing mechanism. We also described the most recent innovation of the CRISPR/Cas9 technology, particularly the broad applications of modified Cas9 variants, and discuss the potential of this system for targeted genome editing and modification for crop improvement.

Key words: Crispr-Cas9; Precise genome engineering; Crop improvement, Crop Yield, Gene editing technology.

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR associated; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA; PAM, protospacer adjacent motif; sgRNA, single guide RNA; gRNA, guide RNA; ssODN, single-stranded DNA oligonucleotide; DSB, double-strand break; NHEJ, non-homologous end joining; HDR, homology directed repair, CRISPRi ,CRISPR interference

INTRODUCTION

Since agriculture became practice, new innovations are consistently introduced to increase the yield and quality of crops. In present scenario where increasing world's population, decreasing arable land and adversely changing climate are posing serious threats for sustainable agriculture, researchers are actively exploring the vast arena of biology for finding new tools for increasing crop yield. Although crops have been improved through conventional plant breeding methods these methods are now constrained by limited genetic variations. In the 1970s, genetic engineering surfaced as a swift and robust way of modifying plant genome to understand gene functions and improve crop productivity under adverse environmental conditions. Where useful genes from same or different species were introduced in plants for crop improvement. These genetically modified (GM) crops are still not readily acceptable to the public for a variety of reasons. (Giovannetti, M., Sbrana, C., Turrini, A., 2005). In last few decades, several gene editing technologies have been developed which are capable of generating targeted double-strand break (DSBs). Most of these methods use specific DNA recognition and binding properties of specialized proteins such as customized homing nuclease (meganuclease), zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Puchta H, Fauser F, 2014) (Voytas DF, Gao C, 2014). Each of them had their own importance but also has a set of associated advantages and disadvantages (Voytas DF, Gao C, 2014). The latest ground-breaking technology for genome editing is based on RNA-guided engineered nucleases, which already hold great promise due to their simplicity, efficiency and versatility. The most widely used system is the type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated) system from *Streptococcus pyogenes* (Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E., 2012).CRISPR/Cas systems are part of the adaptive immune system of bacteria and archaea, protecting them against invading nucleic acids such as viruses by cleaving the foreign DNA in a sequence-dependent manner.

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The immunity is acquired by the integration of short fragments of the invading DNA known as spacers between two adjacent repeats at the proximal end of a CRISPR locus. (Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. , 2007). In this review, we described how this RNA-guided CRISPR-Cas9system works and its significance in plant genome editing. We also discuss the limitations of this system and the current advancements in this technology.

The CRISPR/Cas9 system: a journey from bacterial immunity to genome editing.

Discovery of CRISPR and its function

In 1993 - 2005 Francisco Mojica was the first researcher to portray what is now called a CRISPR locus, He worked on them throughout the 1990s, and in 2000, he recognized that what had been reported as disparate repeat sequences actually shared a common set of features, now known to be hallmarks of CRISPR sequences (he coined the term CRISPR through correspondence with Ruud Jansen, who first used the term in print in 2002). In 2005 he reported that these sequences matched snippets from the genomes of bacteriophage (Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. , 2005). This finding led him to hypothesize, correctly, that CRISPR is an adaptive immune system. Another group, working independently, published similar findings around this same time (Pourcel C, Salvignol G, Vergnaud G, 2005).

Discovery of Cas9 and PAM

In 2005 — Alexander Bolotin, was studying the bacteria *Streptococcus thermophilus*, which had just been sequenced, revealing an unusual CRISPR locus (Bolotin A, Quinquis B, Sorokin A, Ehrlich SD, 2005;). Although the CRISPR array was similar to previously reported systems, it lacks some of the known Cas genes and instead contained novel Cas genes, including one encoding a large protein they predicted to have nuclease activity, which is now known as Cas9. Furthermore, they noted that the spacers, who have homology to viral genes, all share a common sequence at one end. This sequence, the protospacer adjacent motif (PAM), is required for target recognition. (Bolotin A, Quinquis B, Sorokin A, Ehrlich SD, 2005;)

Hypothetical scheme of adaptive immunity

In 2006, Eugene Koonin was studying clusters of orthologous groups of proteins by computational analysis and proposed a hypothetical scheme for CRISPR cascades as the bacterial immune system based on inserts homologous to phage DNA in the natural spacer array, abandoning the previous hypothesis that the Cas proteins might comprise a novel DNA repair system. (Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I., Koonin, E.V. , 2006)

Experimental demonstration of adaptive immunity

In 2007 — Philippe Horvath, and Danisco did a study with *S. thermophilus* that is widely used in the dairy industry to make yogurt and cheese, and scientists at Danisco wanted to explore how it responds to phage attack, a common problem in industrial yogurt making. Horvath and colleagues showed experimentally that CRISPR systems are indeed an adaptive immune system: they integrate new phage DNA into the CRISPR array, which allows them to fight off the next wave of attacking phage (Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. , 2007). Furthermore, they showed that Cas9 is likely the only protein required for interference, the process by which the CRISPR system inactivates invading phage, details of which were not yet known.

Spacer sequences are transcribed into guide RNAs

By the year 2008 Scientists began to fill in some of the details on exactly how CRISPR-Cas systems "interfere" with invading phage. The first piece of critical information came from John vander Oost and colleagues who showed that in *Escherichia coli*, spacer sequences, which are derived from phage, are transcribed into small RNAs, termed CRISPR RNAs (crRNAs), that guide Cas proteins to the target DNA (Brouns, S.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J., Snijders, A.P., Dickman, M.J., Makarova, K.S., Koonin, E.V., van der Oost, J. , 2008)

CRISPR acts on DNA targets 2008

The next key piece in understanding the mechanism of interference came from Marraffini and Sontheimer, who elegantly demonstrated that the target molecule is DNA, not RNA (Marraffini, 2008). This was somewhat surprising, as many people had considered CRISPR to be a parallel to eukaryotic RNAi silencing mechanisms, which target RNA. (Marraffini, 2008).Explicitly noted in their paper that this system could be a powerful tool if it could be transferred to non-bacterial systems. (It should be noted, however, that a different type of CRISPR system can target RNA (Hale, 2009).

Cas9 cleaves target DNA

In 2010 Moineau and colleagues demonstrated that CRISPR-Cas9 creates double-stranded breaks in target DNA at precise positions, 3 nucleotides upstream of the PAM (Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P., 2010).

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They also confirmed that Cas9 is the only protein required for cleavage in the CRISPR-Cas9 system. This is a distinguishing feature of Type II CRISPR systems, in which interference is mediated by a single large protein (here Cas9) in conjunction with crRNAs.

Discovery of, trans-activating RNA (tracrRNA) for Cas9 system

In 2011 the final piece to the puzzle in the mechanism of natural CRISPR-Cas9-guided interference came from the group of Emmanuelle Charpentier. They performed small RNA sequencing on *Streptococcus pyogenes*, which has a Cas9-containing CRISPR-Cas system. They discovered that in addition to the crRNA, a second small RNA exists, which they called trans-activating CRISPR RNA (tracrRNA) ((Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, 2011). They showed that tracrRNA forms a duplex with crRNA, and that it is this duplex that guides Cas9 to its targets.

CRISPR systems can function heterologously in other species

In *2011* Siksnys and colleagues cloned the entire CRISPR-Cas locus from *S. thermophilus* (a Type II system) and expressed it in *E. coli* (which does not contain a Type II system), where they demonstrated that it was capable of providing plasmid resistance (Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., and Siksnys, V. Sapranauskas, R., Gasiunas, G., Fremaux, C., Barrangou, R., Horvath, P., and Siksnys, V. , 2011). This suggested that CRISPR systems are self-contained units and verified that all of the required components of the Type II system were known.

Biochemical characterization of Cas9-mediated cleavage

In 2012 Taking advantage of their heterologous system, Siksnys and his team purified Cas9 in complex with crRNA from the E. coli strain engineered to carry the *S. thermophilus* CRISPR locus and undertook a series of biochemical experiments to mechanistically characterize Cas9's mode of action (Gasiunas, G., Barrangou, R., Horvath, P., and Siksnys, V. , 2012).They verified the cleavage site and the requirement for the PAM, and using point mutations, they showed that the RuvC domain cleaves the non-complementary strand while the HNH domain cleaves the complementary site. They also noted that the crRNA could be trimmed down to a 20-nt stretch sufficient for efficient cleavage. Most impressively, they showed that they could reprogram Cas9 to a target a site of their choosing by changing the sequence of the crRNA.

Similar findings as those in Gasiunas et al 2012). were reported at almost the same time by Emmanuelle Charpentier in collaboration with Jennifer Doudna at the University of California, Berkeley (Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E., 2012). Charpentier and Doudna also reported that the crRNA and the tracrRNA could be fused together to create a single, synthetic guide, further simplifying the system. (Although published in June 2012, this paper was submitted after (Gasiunas, G., Barrangou, R., Horvath, P., and Siksnys, V. , 2012)

CRISPR-Cas9 harnessed for genome editing

2013 — Zhang, who had previously worked on other genome editing systems such as TALENs, was first to successfully adapt CRISPR-Cas9 for genome editing in eukaryotic cells (Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., , 2013) (Cong et al., 2013). Zhang and his team engineered two different Cas9 orthologs (from S. *thermophilus and S. pyogenes*) and demonstrated targeted genome cleavage in human and mouse cells. They also showed that the system (i) could be programmed to target multiple genomic loci, and (ii) could drive homology-directed repair. Researchers from George Church's lab at Harvard University reported similar findings in the same issue of Science (Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE Church GM., 2013).

Applications of CRISPR/Cas9 in plant genome editing

One of the foremost requirements for crop improvement is the precise editing of candidate genes for their functional characterization. The recent discovery of a bacterial CRISPR-Cas9 system has offered tremendous potential to meet this demand. The use of CRISPR-Cas9 the system has also been extended to understand transcriptional regulation (Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW., 2013), It is being further modified for achieving different goals, such as gene replacement, knockout, chromosomal deletions and raising marker-free GM plants. In the following section, we describe a few variants of the CRISPR system being used in understanding plant functions and their response to the environment.

Targeted mutagenesis or gene knock-out

Target sequence-specific sgRNA fused with Cas9 induces double-strand breaks at a precise position in the genome and facilitates random insertions or deletions by NHEJ ((Voytas DF, Gao C, 2014). Targeting specific genes for down-regulation or disruption has been well established in many organisms (Gaj, T., Gersbach, C.A., 2013)

CRISPR-Cas9 induced targeted mutation in rice transcription factor encoding gene, OsERF922, enhanced its resistance to a rice blast fungal pathogen (Wang,F.,Wang,C.,Liu,P.,Lei,C.,Hao,W.,Gao,Y., Liu,Y.-G.,andZhao, K., 2016) Thermo-sensitive genic male sterile rice has also been raised for hybrid rice production by knocking out OsTMS5 gene using CRISPRCas9 system (Zhao, Y., Zhang, C., Liu, W., Gao, W., Liu, C., Song, G., Li, W.X., Mao.L., Chen, B., Xu, Y., 2016). Whole genome analysis of rice suggested that 90% of rice transcripts could be precisely altered by specifically designed gRNAs (Xie K, Yang Y., 2013)

Multiplexing: editing of multiple loci simultaneously

Gene's functional redundancy is a common phenomenon of complex plant genome due to the presence of gene families. Functional characterization of genes in such complex systems requires simultaneous targeting of multiple genes. CRISPR-Cas9 system enables editing of multiple genes in plants concurrently (Xie, K., Minkenberg, B., Yang, Y., 2015). Multiplexing is achieved by fusing Cas9 with multiple sgRNAs specific for different target genes. Expression of multiple sgRNAs can be achieved by assembling multiple sgRNAs expression systems with each having own promoter ((Schiml S, Fauser F, Puchta H. , 2014); (Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., 2015). Moreover, multiple sgRNAs can also be generated inside the cell by a single polycistronic tRNA-gRNA gene (PTG) that contains sgRNA sequences between tRNA sequences. This is processed by the endogenous tRNA system to generate multiple sgRNAs by recognizing and cleaving tRNA end sequences (Xie, K., Minkenberg, B., Yang, Y., 2015). This strategy has been successfully demonstrated to produce up to eight sgRNA that cleave eight target sites in DNA (Xie, K., Minkenberg, B., Yang, Y., 2015) . CRISPR-Cas9 system has been efficiently utilized in conferring powdery mildew resistance to hexaploid bread wheat by simultaneous knock-out of three mildew-resistance loci (MLO) proteins ((Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu J-L:, 2014). In a recent report, a chimeric guide RNA (cgRNA) was used for targeting two different genes inositol oxygenase (inox) and phytoene desaturase (pds) in wheat (Upadhyay SK, Kumar J, Alok A, Tuli R., 2013).

Gene replacement and trait stacking

Targeted gene replacement or targeted gene knock-in involves DNA integration at the desired target site in the genome. This can be efficiently achieved through the CRISPR-Cas9

system in plants (Schiml S, Fauser F, Puchta H. , 2014). One of the best examples of such targeted gene insertion was demonstrated in soybean where hygromycin phosphotransferase (HPT) gene was integrated on chromosome 4 by homology-directed recombination (HDR) (Li Y, Lin Z, Huang C, Zhang Y, Wang Z., 2015). Candidate gene (e.g HPT) can be placed on a donor fragment, flanked by appropriate sequences homologous to the target site. Gene replacement can be achieved by either co-transformation of Cas9-sgRNA constructs or a donor template or by synthesizing single construct harboring both donor sequence and Cas9/sgRNA expression cassette. SSNs allowed molecular stacking of multiple traits at single loci using HDR method (Ainley WM, Sastry-Dent L, Welter ME, Murray MG, Zeitler B, Amora R, 2013)

Gene regulation: RNA interference and activation

Apart from knock-out and targeted mutation of desired genes, the CRISPR-Cas9 system has the potential to alter the regulation of gene expression. Therefore, the CRISPR system with modified Cas9 i.e. dCas9 allowed researchers to manipulate not only gene structure but also its regulation. (Piatek A, Ali Z, Baazim H, Li L, Abulfaraj A, 2015) Successful used the modified CRISPR-Cas9 system for modulating gene expression in plants. For down-regulation of the target gene, dCas9 was fused with the transcriptional repressor domain and for up-regulation of the target gene; dCas9 was fused with a transcriptional activator domain. This modified dCas9 with either transcription repression or transcription activation domain is carried to the promoter element specified by the sgRNA for the target gene and influences the expression of target gene depending upon the type of domain fused to dCas9. Expression of a transcriptional activator or transcriptional repressor fused Cas9 with multiple gRNAs targeting promoter regions of desired genes can help in achieving co-regulation of multiple genes. Similarly, CRISPR-dCas9 also allows epigenetic modifications like acetylation and methylation of the genome (Thakore, P.I., D'ippolito, A.M., Song, L., Safi, A., Shivakumar, N.K.., 2015). These modifications of Cas9 expanded the applications of CRISPR-Cas9 from a gene targeting tool. to a more versatile transcription regulatory tool.

Visualization of loci in large genomes

GFP-tagged CRISPR-dCas9 is one of the most useful tools for fluorescent labeling of native repetitive DNA sequences and visualization of various gene interactions in different chromatin states (Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW., 2013); (Hu, J.,

Lei, Y., Wong, W.K., Liu, S., Lee, K.C. , 2014). While, traditional DNA labeling techniques require a lengthy procedure of sample fixation and are not appropriate for live imaging, fluorescently tagged Cas9 is a potent tool for live-cell-imaging of DNA. For tracking multiple genome sites simultaneously multicolor imaging method has also been devised (Chen, B., Hu, J., Almeida, R., Liu, H., Balakrishnan, S., 2016). Additionally, GFP tagged CRISPR system can also track chromosome translocation and transposition (Roukos, V., Voss, T.C., Schmidt, C.K., Lee, S., Wangsa, D., Misteli, T., 2013)

Used of CRISPR /Cas to increase Crop Yield
Development of superior crop varieties has relied on securing genetic gains through sexual recombination, induced random mutagenesis, and transgenic approaches. The recent emergence of targeted genome-editing technologies offers a new avenue for incorporating beneficial genetic changes in the world's most important crop species (Zhang, K., Raboanatahiry, N., Zhu, B., and Li, M. , 2017) Scientists have finally harnessed the untapped power of genome editing to improve agricultural crops. In the tomato plant, they have mobilized CRISPR to rapidly generate variants of the plants displaying a continuum of three agriculturally important traits: fruit size, branching architecture, and overall plant shape. All are major components in determining yield. The method is designed to work in all food, feed, and fuel crops, including staples rice, maize, sorghum, and wheat. (Daniel Rodríguez-Leal, Zachary H. Lemmon, Jarrett Man, Madelaine E. Bartlett, Zachary B. Lippman. , 2017) A team of scientists from Purdue University and the Chinese Academy of Sciences has used CRISPR/Cas9 gene-editing technology to develop a variety of rice that produces 25-31 percent more grain and would have been virtually impossible to create through traditional breeding methods. (Jian-Kang Zhu, 2018)

CRISPR/Cas9 mediated virus resistance in agriculturally important crops.
Plant viruses infect many agriculturally important crops, from cereals to vegetables limiting the crop yield and posing a serious threat to the food security for feeding the increasing world population ((Zaidi SS, Tashkandi M, Mansoor S, Mahfouz MM., 2016) (Andolfo G, Iovieno P, Frusciante L, Ercolano MR., 2016) The CRISPR/Cas9 technology has recently become a novel antiviral tool for plants (Zhang D, Li Z, Li J-F. , 2015) to combat viral infection in plants to destruct invading viral DNA for virus resistance development in plants was first reported in 2015 by 3 independent

communications; 2 in Nature Plants by (Unniyampurath U, Pilankatta R, Krishnan MN. , 2016) (Ji X, Zhang H, Zhang Y, Wang Y, Gao C., 2015) (Baltes NJ, Hummel AW, Konecna E, Cegan R, Bruns AN, Bisaro DM, Voytas DF., 2015) followed by one in Genome Biology by (Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz MM. , 2015) against geminiviruses. Then after many research groups started testing this and have reported the feasibility of viable virus resistance development in different plants using CRISPR/Cas9 technology the reports of CRISPR/Cas9 mediated virus resistance development in plants can be basically divided considering 2 broad strategies used. The first approach targets the viral factors for viral genome editing in viruses and the second approach targets the host plant factors responsible for the viral cycle for plant genome editing. The applications of CRISPR/Cas9 mediated virus resistance in plants have been so far limited mainly to model species demonstration like Tobacco and Arabidopsis targeting the viral genes responsible for replication (Baltes NJ, Hummel AW, Konecna E, Cegan R, Bruns AN, Bisaro DM, Voytas DF., 2015)

Other applications of the CRISPR-Cas9 system beyond genome editing
In addition to genome editing, the CRISPR–Cas system has been employed to regulate gene expression. Recently, a CRISPR interference (CRISPRi) platform has been developed for gene silencing, which provides a complementary approach to RNA interference (Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP,Lim WA, 2013); (Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP,Lim WA, 2013). The co-expression of dCas9 with a specific sgRNA of the gene of interest facilitates the binding of dCas9-sgRNA to the non-template DNA strand of the coding region and blocks the transcription elongation process. This approach has also been adopted for preventing the interaction between key cis-acting DNA motifs and their corresponding transacting transcription factors in the promoter region to block transcription initiation (Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP,Lim WA, 2013). The gene knockdown by CRISPRi is highly specific and reversible in action. One more advantage of CRISPRi over RNA interference is that it can be used simultaneously for multiple target genes (Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP,Lim WA, 2013). Thus, the CRISPRi approach shows enormous potential for gene silencing applications. In addition, inactive Cas9 has been fused with different

effector domains (repressor/activator) for recruiting fusion proteins to specific genomic loci (Bikard D, 2013); (Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW., 2013); (Rusk N., 2014).

The Krüppel-associated box, a transcriptional repressor, has been grafted with inactive Cas9, which showed relatively higher repression efficiency than inactive Cas9 alone in HEK293T cells (Bikard D, 2013) Conversely, the ω subunit of RNA polymerase has been used with inactive Cas9 to activate gene expression in Escherichia coli (Bikard D, 2013). Recently, an inactive Cas9 fused with an enhanced green fluorescent protein was co-expressed with specific sgRNA to enrich the fluorescent signal at the targeted genomic loci, which demonstrated the use of the CRISPR–Cas9 system as a robust and flexible platform for visualization of the genetic element dynamics ((Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW., 2013) (Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE Church GM., 2013) has extended the utility of the CRISPR-Cas9 system by demonstrating it's used in targeted gene disruption positive-selection screening in vitro and in vivo. The idea of a fusion of inactive Cas9 with histone-modifying enzymes to introduce custom changes in the complex epigenome has also been documented (Rusk N., 2014). Recently, the CRISPR–Cas system has been

Exploited to purify a specific portion of the chromatin and identify the associated proteins, thus elucidating their regulatory roles in transcription (Waldrip ZJ, Byrum SD, Storey AJ., 2014). These reports demonstrate the utility of the CRISPR–Cas9 system as a potential molecular tool with broader applications

Technical pitfalls of using CRISPR/Cas9 system.

Similarly to ZFNs and TALENs, the CRISPR/Cas9 system has the problem of off-target effects that may introduce unexpected mutations. First, an improper concentration ratio between Cas9 and sgRNA may lead to off-target cleavage, and the higher the Cas9: sgRNA ratio, the more severe the effect (Hsu PD, Lander ES, Zhang F, 2014), (Pattanayak V, Lin S, Guilinger JP., 2013) Optimal mutagenesis was reported in Arabidopsis with a Cas9: sgRNA ratio of 1:1 when two genes (AtPDS3 and AtFLS2) were tested with different Cas9:sgRNA ratios((Li J-F, Norville JE, Aach J, McCormack M, Zhang D, Bush J,, 2013) Second, promiscuous PAM sites may lead to undesired cleavage of DNA regions

(Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA., 2014). To avoid this event, bioinformatics tools such as E-CRISPR and Cas OT (Xie, K., Minkenberg, B., Yang, Y., 2015), (Xiao A, Cheng Z, Kong L, Zhu Z, Lin S, Gao G, Zhang B, 2014) third, insufficient Cas9 codon optimization may lead to inefficient translation of Cas9 proteins in target species. Several codon-optimized versions of Cas9 genes are available, such as for Arabidopsis (Schiml S, Fauser F, Puchta H. , 2014), rice (Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP, 2013) (hang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Qiu, J.L. , 2016) and tobacco (*Nicotiana benthamiana*) ((Gao, Y., and Zhao, Y, 2014). Codon efficiency should be considered when these vectors are used for other crop plants. Fourth, given that most CRISPR/Cas9 systems use exogenous promoters for Cas9 and sgRNA expression, vectors with optimal promoters should be selected. Fifth, homolog's or gene family member (Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, Wu Y, Zhao P, Xia Q., 2015)s may complicate target sequences to be edited. (Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, Wu Y, Zhao P, Xia Q., 2015) to ensure the function of the CRISPR/Cas9 system for knockout mutation, the position of sgRNAs is best located in the 5′ region of the targeted gene. Finally, epigenetic factors such as DNA methylation or histone modification, which are known to limit protein binding or RNA pairing, should also be considered in regions with complex DNA compositions, such as those with repetitive sequences.

Perspectives

Although CRISPR-mediated genome-editing can be considered a relatively new technology, it has already garnered widespread adoption much progress has been made in CRISPR/Cas9-based genome editing technology in the last few years, some problems need to be solved: off-target effects, the influence of chromatin structure, side effects on nearby genes, mechanisms underlying the different effects of different single guide RNA (sgRNAs) on mutation efficiency, and methods for efficient delivery in polyploidy plants. Despite these challenges, with the tremendous enthusiasm of the research community, gene editing technologies as represented by the CRISPR/Cas9 system will improve rapidly. Been simple, affordable, and the elegant genetic scalpel is expected to be widely applied to enhance the agricultural performance of most crops in the near future.

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FIGURES

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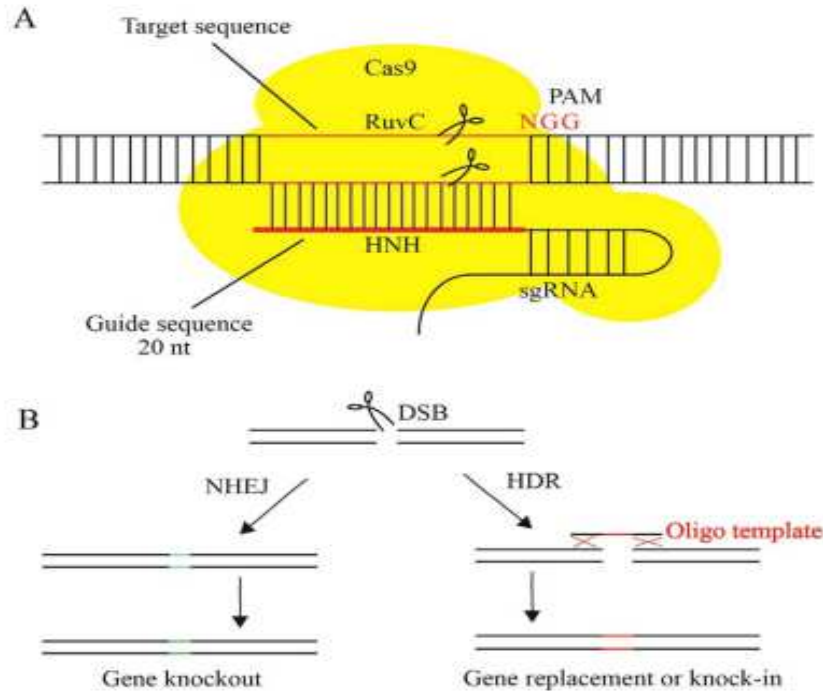
Schematic diagram of CRISPR/Cas9 editing of target genes. (A) A sketch of CRISPR/Cas9 system. The sgRNA (black and red) can identify the target gene, and then the two domains of Cas9 (yellow) cleave the target sequence. (B) Two ways DSB can be repaired. NHEJ is imprecise and always results in a gene knockout mutation. When a template is present, HDR can be activated and results in gene replacement or knock-in. PAM, protospacer adjacent motif; sgRNA, single guide RNA; DSB, double-strand break; NHEJ, nonhomologous end-joining; HDR, homology-directed repair. (Source Xuan Liu 2017)
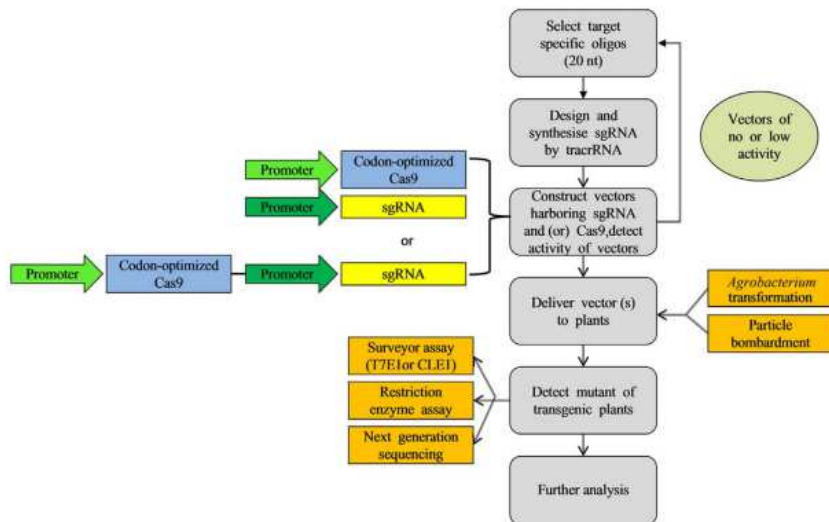

Figure 2 The basic flow of CRISPR/Cas9 editing of target genes. (Source Xuan Liu 2017)