CRISPR/CAS9 MEDIATED GENOME EDITING AND ITS POTENTIAL APPLICATIONS IN GENETIC IMPROVEMENT OF *HEVEA BRASILIENSIS*

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Biotechnology is now entering a new era, equipped with tools, which are quick and more precise than the conventional methods of genetic engineering. Genome editing with the help of engineered nucleases is the latest technology, where precise manipulation of specific genomic sequences is possible by knocking out undesirable genes or modifying genes to gain new functions. This technique was developed based on a naturally occurring bacterial immune mechanism and relies up on sequence specific endonucleases which are capable of generating DNA double strand breaks at specific locations within the genome. With the help of error-prone natural endogenous DNA repairing mechanism in the cell, site-specific mutations can be introduced. The nucleases can be programmed theoretically in such a way that precise editing of any gene in an organism would be possible to gain desirable phenotypes. Among the various genome editing platforms, CRISPR/Cas9 mediated genome editing is the most popular technology owing to its simplicity and versatility. Since variations generated through these techniques are precise and more similar to natural variations and are more acceptable than conventional GMOs where the possibility of retaining undesirable gene sequences and unforeseen ill effects are relatively high.This paper aims to givean overview to this new technology which holdsmajor implications in different areas of life science. The concept, history, mechanism, applications and thelimitations of this technology are discussed in detail. The potential application of the technology in genetic improvement of *Hevea brasiliensis* and its implications are also discussed.

Key words: CRISPR/Cas9, Gene editing, Guide RNA, Gene knockout, DNA free editing, *Hevea*

INTRODUCTION

Creation, identification and utilization of genetic variation is the base of any crop improvement programme. Twentieth century witnessed the green revolution mainly due to development of high yielding varieties by conventional breeding techniques producing hybrids that responded to application of inorganic fertilizers. Conventional breeding strategies are not sufficient enough to meet the demand of increasing global population in the 21st century even as the extent of arable land is declining and several external factors such as climate change, land degradation *etc*. adversely affect productivity. In conventional breeding, breeder has absolutely no control over the process of recombination of genes during sexual reproduction except for the selection of

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parents. Another method of creating variability in plants is by inducing mutations, either by the use of chemicals or by irradiation (Harden, 1998). Mutagenesis is also random and unpredictable. Genetic modification of a plant by transgenic approach is another method and it can ensure the integration and expression of desired genes or genomic region in the genome of any plant. But the random integration, presence of genes unrelated to the final-target trait such as marker genes, promoter sequences are the drawbacks. With the advancements in molecular biology and biotechnology, gene editing technologies were emerged which are quick and more precise than the already established genetic engineering methods. Faster development of smart varieties with better yield, disease tolerance, and climate resilience are expected through this technology. Among the various genome editing platforms, CRISPR/ Cas9 mediated genome editing is the latest and widely accepted technology. This paper aims to give an introduction to this new technology, which may have major applications in genetic improvement of *Hevea* in future. The concept, history, mechanism, applications and the limitations and its implications on *Hevea* breeding are discussed.

Genome editing

The technology of genome editing relies upon sequence specific nuclease (SSNs), which are capable of generating DNA double strand breaks at specific locations within the genome. As a result, the errorprone natural endogenous DNA repairing mechanism will be triggered to repair the DNA damage which aids in targeted mutagenesis. SSNs are programmable and can theoretically aid in precise editing of any genes in any plant species to achieve the desirable phenotype. This is achieved either through knocking out of undesirable genes or modifying the genes to gain new function.There are mainly three site specific engineered nucleases namely Zinc Finger Nucleases (ZFNs), Transcription Activator Like Effector Nuclease (TALENS), and CRISPR/Cas9 which have been used for genome editing in plants. Both ZFNs and TALENS are complex techniques because of the difficulties in the development of constructs (Christian *et al.*, 2010).

CRISPR/Cas9

The clustered, regularly interspaced, short palindromic repeat (CRISPR) and CRISPR associated protein 9 (Cas9) system was discovered as an adaptive immunity mechanism in prokaryotes against bacteriophages and other mobile genetic elements. This has emerged as the most popular tool for precise genome modifications recently. They are segments of DNA containing short repeats followed by short segments of spacer DNA. CRISPR locus was first observed in *Escherichia coli*, and according to the most recent reports, it is present in about 84 per cent of *archaea* and 45 per cent of bacteria (Bhaya *et al.*, 2011). Cas9 is an endo nuclease enzyme associated with the CRISPR adaptive immunity system in *Streptococcus pyogenes*.This bacterial immune system has been utilized as a powerful tool for efficient RNA guided genome editing practically in any organism.

History

CRISPR arrays were first discovered from *Escherichia coli* genome in 1987, by Ishino and

colleagues as an unusual structure with five identical segments of DNA (repeats) having 29 nucleotides found in the 3'-end flanking region of isozyme alkaline phosphatase. In between the repeats there were 32 nucleotides as spacers. In 2002, Ruud Jansen and colleagues of Utrecht University in the Netherlands named these sandwiches "clustered regularly interspaced short palindromic repeats" (CRISPR). Jansen's team could also identify that CRISPR arrays were always accompanied by a group of genes which encoded enzymes that could cut DNA. They called these genes *Cas* genes, *i.e.* CRISPR-associated genes.

Later, three groups of scientists independently noticed that the spacer sequences have similarity with viral genomes, which eventually lead to the discovery of its role in adaptive immunity (Bolotin *et al.,* 2005; Mojica *et al.,* 2005; Pourcel *et al.,* 2005). At the same time, Koonin(2005), an evolutionary biologist at the National Centre for Biotechnology Information (NCBI) in Bethesda, proposed that bacteria use *Cas* enzymes to grab fragments of viral DNA. To verify this hypothesis, Barrangou and his team infected the milk-fermenting microbe *Streptococcus thermophilus* with two different strains of viruses. Eventhough most of the bacteria were killed by the virus, a few of them survived. Also this property was passed on to their descendants. However, when the new spacer regions were chopped out, the bacteria lost their resistance. Thus, it was confirmed that CRISPR arrays provides protection against invading viruses with the combined action of Cas genes (Barrangou *et al.,* 2007). Further, the mechanism of the system was elucidated and demonstrated by different groups (Brouns *et al.,* 2008; Deltcheva *et al.,* 2011; Garneau *et al.,* 2010; Marraffini and Sontheimer, 2010).

The development of genome engineering tool utilizing this biological phenomenon came out when it was shown that the target DNA sequence could be reprogrammed (Jinek*et al.,* 2012).The functional validation of this system was demonstrated later by different groups in eukaryotes like humans, mouse and zebra fish (Cho *et al*., 2013; Cong *et al*., 2013; Hwang *et al*., 2013; Jinek *et al.,* 2012; Mali *et al.,* 2013). The possibility of multiplex genome engineering in a single experiment was also validated (Cong *et al.,* 2013; Mali *et al.,* 2013).

Thus the CRISPR/Cas9 system was established as a simple, inexpensive and versatile tool for genome editing having applications in different fields of science including medicine. Recently Chinese scientist He Jiankui announced his experiment on CRISPR, editing the genes of twin babies 'Lulu and Nana' (Cohen, 2019). The target gene was CCR5 that codes for a protein that HIV virus requires for entering the host cell. *In vitro* fertilisation was conducted with sperm and egg collected from a childless HIV infected couple and the genome of the embryos was edited using CRISPR/Cas9. It was claimed that one among the two babies is resistant to HIV. This raised a lot of ethical issues and He Jiankui, was found guilty of conducting "illegal medical practices" by Chinese court and sentenced to three years in prison.

CRISPR mechanism

As mentioned earlier, CRISPR/Cas9 system was developed from naturally occurring bacterial adaptive immune system.

a. Mechanism of adaptive immunity in prokaryotes

CRISPRs are segments of DNA containing palindromic repeats of bases which can target DNA or RNA of viruses and other mobile elements as a way of protection against invaders. During the attack of viral/ bacteriophage DNA, the bacterial immune system will integrate short fragments of foreign DNA into the CRISPR repeat-spacer array within the host chromosome as a new spacer. This forms a genetic record of infection and act as a genetic vaccination card and enables the host to prevent future invasion of the same invader. The spacers act as recognition elements to find matching virus genomes and destroy them. The integrated spacer sequences are transmitted to the next generationand thusthe offspring's also inherit the protection. It is interesting to see that the addition of new spacers usually occurs at one side of the CRISPR, so that a chronological record of the invaders in the cell and ancestors are maintained. Usually CRISPR sequences are found on both chromosomal and plasmid DNA. Adjacent to the CRISPR, a set of CRISPR associated (Cas) genes are usually found which code for an endonuclease protein,

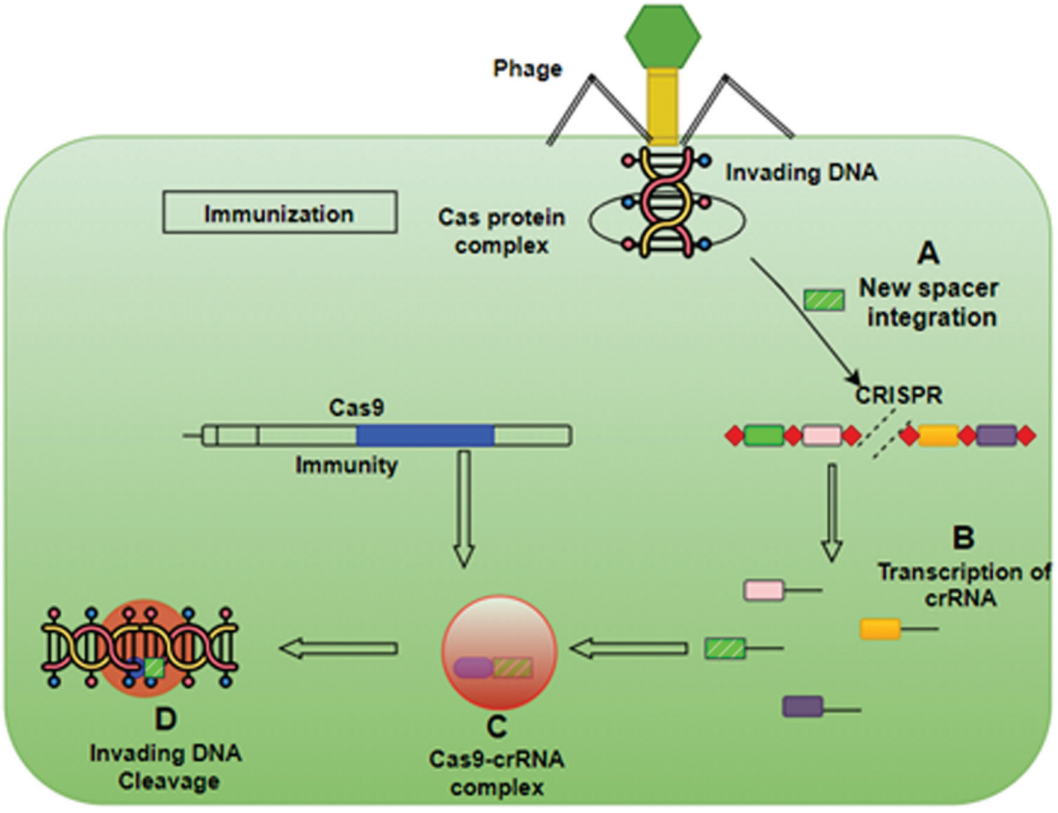

Fig.1. CRISPR activity in bacterial immune system
A.Integration of new spacer, B. Transcription of crRNA during invasion, C. Cas9 –cr RNA complex formation, D. Cleavage of invading DNA.

capable of cleavingthe double stranded DNA (Jansen *et al*., 2002). Upon infection of foreign DNA,the CRISPR loci will be transcribed, and transcripts are then processed to generate small RNAs (crRNA – CRISPR RNA), which will guide the Cas9 endonucleases to the target invading DNA based on sequence complementarity (Fig.1). Cas9 endonuclease in turn depends on PAM sequences (Protospacer Adjacent Motif) for targeting and effecting cleavage. PAM is a short specific sequence of 2-6 base pair DNA immediately following the target DNA which distinguishes bacterial DNA fromforeign DNA and thereby preventing the CRISPR locus from being targeted and destroyed by nuclease.

b. Mechanism for CRISPR mediated gene editing

Scientists could successfully repurpose the bacterial immune system for genome editing as follows. Engineered CRISPR systems contain two components:

A guide RNA (gRNA or sgRNA) and a CRISPR-associated endonuclease (Cas9 protein).

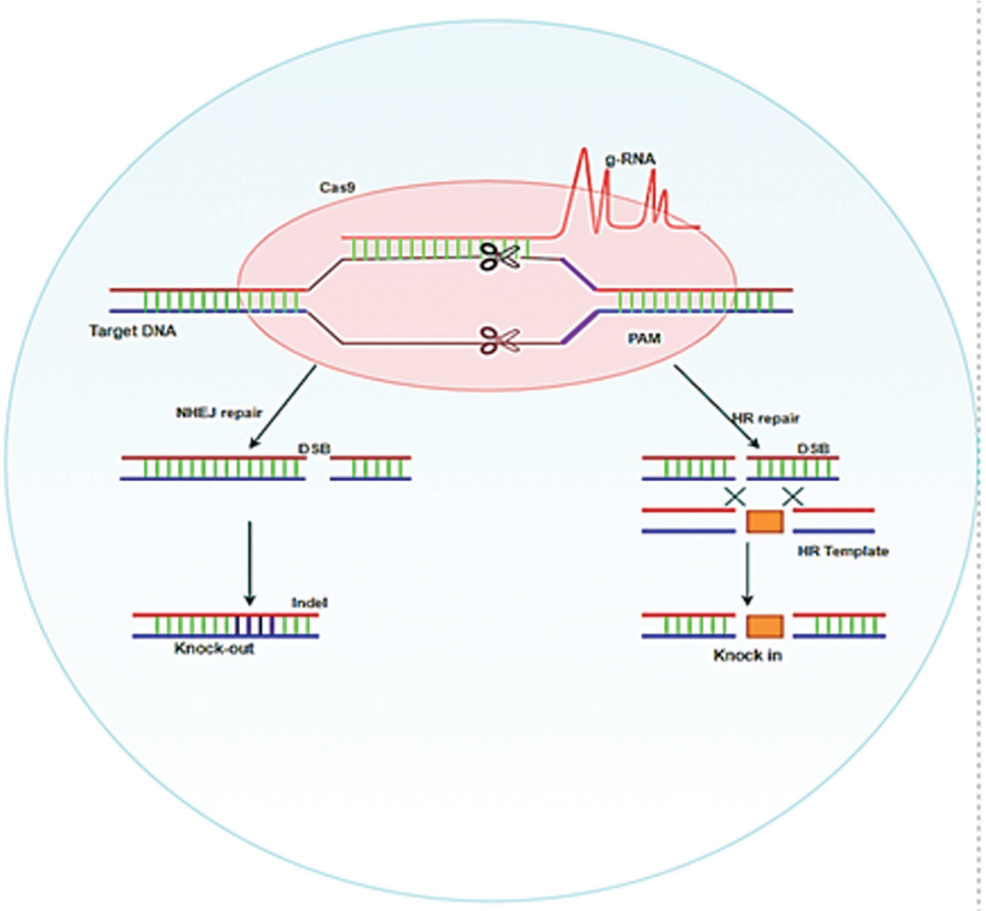

Fig. 2. Schematic representation of CRISPR /Cas9 mediated editing

1. Guide RNA (gRNA): It is a synthetic 100 nucleotides (nt) RNA molecule of which the first 20 nt are the targeting site and the 3' end forms a hairpin structure which interacts with the Cas9 protein. GRNA is composed of a scaffold sequence necessary for Cas-binding and a 20 nucleotide spacer which defines the genomic target to be modified. It is possible to change the genomic target of the Cas protein by changing the sequence present in the gRNA.

2. Cas9 protein: It is a bacterial RNA guided endonuclease that recognize and cleave target DNA having sequence complementarity to the RNA.Cas9 proteins can be derived from different bacteria, including *Brevibacillus laterosporus*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus thermophilus.* The commonly used Cas9 is derived from *Streptococcus pyogenes.*

As in the case of bacterial immune system, in CRISPR/Cas9-mediated genome editing also, gRNA binds to Cas9 protein and induces a conformational change to form a riboprotein complex and directs the protein to the target sequence. The gRNA recognizes its target sequence in the genome through complementary base pairing. Cas9 nuclease then makes a double strand break (DSB) at a site three base pairs away from the Protospacer Adjacent Motif (PAM) sequence. For different Cas endonucleases isolated from different bacterial species, there are distinct PAM sequences. Double-strand breaks induced by Cas9 nuclease at a specific site will be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR) repair mechanism in the cell. Repair by NHEJ usually results in the insertion or deletion of random base pairs, causing gene knockout by disruption.HR with a donor DNA template can be exploited to modify a gene by introducing specific nucleotides designed for our purpose. Thus CRISPR/Cas9 can efficiently edit the genome of diverse organisms, including humans, animals and plants. A schematic diagram of genome editing mechanism is given in Figure 2.

Methodology

a. Design of Sg RNA +Cas 9 complex

As mentioned earlier, engineered CRISPR/Cas9 system contains two components: guide RNA and a CRISPR-associated endonuclease (Cas9 protein). The gRNA is a short synthetic RNA composed of a scaffold sequence necessary for Cas binding and a user defined 20 nucleotide spacer that defines the genomic target to be modified. The gRNAs should have three important features such as low potential off targets, highest predicted cleavage efficiency and should target the earliest possible exons.gRNA target sequences are designed to be immediately upstream of a Protospacer Adjacent Motif (PAM) which has a sequence of 5′-NGG-3′for the most popular spCas9 from *Streptococcus pyogenes* and 5-NNGRR(N)-3′sequence for saCas9 from *Staphylococcus aureus*. The PAM, NGG, is located at 3[1] downstream of the target sequence and is necessary for the CRISPR/Cas9 system to recognize and cleave the target site to generate double strand breaks.In CRISPR/Cas9 editing, three different design strategies are followed for the development of components for the delivery. (a) DNA plasmid encoding both the Cas9 protein and the guide RNA, (b) mRNA for Cas9 translation alongside a separate guide RNA, and (c) Cas9 protein with guide RNA (ribonucleoprotein complex).

b. Delivery of the CRISPR components into the genome

The cassettes are delivered in to plant cells using already established DNA delivery methods used for gene transfer including *Agrobacterium*, particle bombardment, electroporation and microinjection.

c. Screening and confirmation

Screening for edited events is carried out by different ways.Use of reporter system is the simplest one whenever gene integration strategies are adopted. Use of endonuclease, PAGE, use of high resolution melting of the PCR products and sequencing are the other methods to detect the mutants by locating sequence variation at the edited site.

Applications of CRISPR/Cas9 in Plant Science

Research on CRISPR/Cas9 in model systems and other plants provided strong evidence for the use of this technology for developing improved varieties with genome modification and stable inheritance. Lack of inheritance stability is one of the problems associated with conventional GM developed through genetic transformation, where the introduced genes were lost /silenced within a few generations.Generation of stable and heritable mutants without disturbing the existing genetic composition and the ability to develop homozygous edited plants in one generation make the CRISPR technology more attractive than conventional GM approach and has been used successfully in many plant species for precise genome editing. First report on the genome editing *via* CRISPR/Cas9 in plants came during 2013 using model systems like *Arabidopsis* protoplasts and tobacco cells (Li*et al.,*2013). Researchers could successfully induce mutations in *AtPDS3* (*Arabidopsis phytoene desaturase*) and *AtFLS2* (*flavonol Synthase*) genes and the tobacco *NbPDS* (*Phytoenedesaturase*) gene through CRISPR technology.

1. Methods of gene editing in plants

Gene editing is carried out in three different ways:

a. *Gene disruption (knockout*). This is the most applied technique as it can turn one of the genes functionless or inoperative by simply introducing small deletion or insertion of DNA sequences through NHEJ-mediated CRISPR/Cas9. This can be utilized for elucidating gene function (Table1)

b. *Gene insertion/Replacement* Gene insertion or addition is a technique where nucleotides encoding a protein for a positive trait are added. This is not an easy task as it has a risk of unpredictable events. With the CRISPR technology it is possible to incorporate complex traits thereby minimising the disadvantages of gene stalking (Leong *et al.,* 2019). CRISPR has been used for targeted mutagenesis in rice by gene insertion for achieving resistance against rice blast (Xu *et al*., 2019).

c. Gene *regulation:* Gene regulation refers to changes in transcription rate that result in changed gene expression levels. The activation and repression of genes are possible using inactivated Cas9 protein combining with activators /repressors respectively. Activation and repression of PDS gene and activation of AtPAP1 (production of anthocyanin pigment1) was successfully demonstrated in *Arabidopsis* using dCas9 along with activators and repressors *(Lowder et al*., 2017). Reversion of methylation-induced gene silencing of AtFIS2 (fertilization- independent seed 2) in *Arabidopsis* is another example. Thus

Table 1. **Overviewof gene editing experiments carried out in different plant species and keyresults**

Crop Category	Genes modified	Gene function	Results	References	Country
Model plants					
Arabidopsis thaliana	*BR11, JAZ1, GAI*	Growth regulator synthesis	Gene knock out resulted in retarded growth	Feng *et al.* (2013)	China
	Chl11,CHL2	Magnesium chelatase subunit1 genes	Knock out resulted in albino plants	Mao *et al* . (2013)	China
	ABP1	Perception of auxin and developmental process	Knockout, no identifiable phenotype	Gao *et al.* (2014)	China
	ADH1	Turns allyl alcohol to highly toxic acrylaldehyde	Knockout, survival after allyl alcohol treatment	Fauser *et al*. (2014)	Germany
Nicotian abentha miana	β-1,2-xylose and α-1,3-fucose	Genes involved in N glycosylation machinery	lines deficient in plant-specific α-1, 3-fucosyltransferase and β-1,2-ylosyltransferase activity	Jansing *et al.* (2019)	Germany
Nicotiana tabaccum	*NtPDS NtPDR6* genes,	Phytone desaturase	Etiolated leaves for PDS mutant more number of branches for PDR6 mutants	Gao *et al.* (2015)	China
Cereals					
Hordeumvulgare	HvPM19-gene	Genes for regulating grain dormancy	Gene disruption resulted in plants showing signs of dormancy	Lawrenson *et al*. (2015)	U.K
Oryza sativa	*OsPDS,OsMPK2, OsBADH2*	Phyteone desaturase	Plants shows dwarfism and albinism after editing	Shan *et al*. (2013)	China
	CAO1	Synthesis of chlorophyll b	Knockout, plants with pale green leaves	Miao *et al*. (2013)	China
	LAZY1	Regulate shoot gravitropism and control tiller angle	Knock out pronounced tiller spreading	Miao *et al*. (2013)	China
	OsPDS	Coding for Phytone desaturase	Knock out resulted in albino and dwaf plant	Shan *et al.* (2013)	China
	OsWaxy	Amylose synthesis	Knockout, glutinose rice	Ma *et al.* (2015)	China

	ALS	Coding for acetolactate synthase involved in branched amino acid biosynthesis	Knockin, developed resistance to sulfonyl urea herbicides	Sun *et al.* (2016)	China
Triticum aestivum	*TaMLO* homologs	Repress the resistance pathway to powdery mildew	Knockout, resistance to powdery mildew	Wang *et al.* (2014)	China
Sorghum bicolor	Ds red reporter	Remove the reporter effect	Gene insertion	Jiang *et al.* (2013)	China
Zea mays	*L1G1* gene (liguleless)	Genes for ligule formation	Gene disruption, mutants identified	Svistashev *et al.*, 2016	USA
	Ms26&*Ms 45*	Male sterility genes	Male sterile plants identified	Chen *et al.* (2018)	China
Vegetables					
Solanum ly copersicum	*SIAGO7*-	Biogenesis of trans-acting short interfering RNAs	Narrow, needle like leaves	Brooks *et al.* (2014)	USA
Solanum tuberosum	Starch synthase gene	Gene insertion	Increased herbicide resistance	Anderson *et al.*(2017)	Sweden
Cucumis sativus	*ElF4E*-gene	translation iniciation factor gene	Plants showed resistance to wide range of viruses	Chandrase-kharan *et al.* (2016)	Israel
Brassica oleraceae	*BolC.GA4.A*-gibberellin synthesis gene	Gene for gibberellin synthesis	Dwarf phenotype was observed	Lawrenson *et al.* (2015)	U. K
Tree crops					
Citrus sinensis	*CsPDS*-Carotenoid biosynthesis	Carotinoid biosynthesis	Gene regulation expressed albinism	Hongge *et al.* (2017)	USA
Populus tomentosa	*PDS*-	Phytone desaturase	Gene disruption expression of albinism	Fan *et al.* (2015)	China
Actinidia chinensis (kiwifruit)	*PDS*	Phytone desaturase	Gene disruption resulted in 62% mutations	Wang *et al.* (2018)	China
Coffea canephora	*PDS*	Phytonedesaturase	3% Mutagenesis	Breitler *et al.* (2018)	France
Theobroma cacao (cacao)	*NPR3*	Defence related gene	27% Mutagenesis	Fister *et al.* (2018)	USA

Others					
Dandeleon rubber (*Taraxacum kok-saghyz)*	1-*FFT*	gene for inulin biosynthesis	Knockout alleles were present in the regenerants	Laffaldano *et al.* (2016)	USA
Vitis vinifera	*IdnDH*	Tartaric acid biosynthesis	Gene disruption, no sign of tartaric acid in the edited plants	Ren *et al*. (2016)	China

CRISPR/Cas9 is a powerful tool for gene regulation by transcriptional activation/ repression of genes and by reversing gene silencing caused by methylation.

Gene editing using CRISPR is now being used for the yield and nutritional aspects, combating abiotic and biotic stress tolerance, and metabolic engineering in different crops (Table 1)

Limitations of CRISPR and further improvement strategies

Inspite of the simplicity and potential applications in different fields of science including agriculture, medicine and industry, CRISPR technology has some limitations also. Low efficiency of HR, offtarget effects, restrictive PAM sequences etc., are the major limitations. In order to overcome these limitations scientists are trying to bring improvements to the existing technology.

1. Base editing

Genome wide association studies have shown that single base changes can create variations in the elite traits in crop plants. Hence strategies have been developed for inducing single nucleotide changes.Base editing is a technology that includes the conversion of one DNA base into another without the use of a DNArepair template. Here a different Cas9 is employed for the purpose. Cas9 nickase (nCas9) or dead Cas9 (dCas9) is fused to an enzyme with base conversion activity.For example,cytidine deaminase convert cytosine (C) to uracil (U), and during DNA repair or replication, uracil will be treated as thymine (T) leading to CG to TA substitution.

2.CRISPR/Cpf1 system

The major limitation of the popular type II CRISPR /Cas9 system is that it can recognize only DNA sequence upstream of the appropriate PAMs,thus restricting potential target sites. CRISPR/Cpf1 system is a variant and has potential to overcome this limitation. It can recognise T-rich PAMs and generates cohesive ends with four or five nucleotide overhangs instead of blunt-end breaks, which complements the characteristics of Cas9 to a larger extent. Higher rate of mutagenesis with better efficiency was reported with Cpf1 and other orthologs in rice, soybean and human cells (Zhong *et al*., 2018).

3. Prime editing

Prime editing is a "search-and-replace" genome editing technique which can be used

for targeted insertions, deletions, and base-to-base conversions. Unlike CRISPR/ Cas9 system editing is done without double strand breaks (DSBs) or donor DNA templates. An engineered prime editing guide RNA called pegRNA having both target site and the desired edit(s) is the main construct which engages a prime editor protein. This consists of a Cas9 nickase fused to a reverse transcriptase. The Cas9 nickase part of the protein is guided to the DNA target site by the pegRNA and creates only a single stand break. Once the Cas9 creates a nick at the target site, the reverse transcriptase domain uses the pegRNA to generate a DNA template of the desired edit by directly polymerising DNA onto the nicked target DNA strand. The original DNA strand is replaced by the edited DNA strand and as a result, a heteroduplex containing one edited strand and one unedited strand will be created. Lastly, the non-edited strand is nicked by an additional gRNA which prompt the cell to remake that strand using the edited strand as the template (Ledford, 2019.

Rationale for genome editing in *Hevea*

Being a perennial tree crop with long breeding cycle and high gestation period, crop improvement through conventional breeding methods are difficult and takes long years of field experimentation (Varghese and Mydin, 2000). Low fruit set, seasonal flowering, asynchrony of flowering among clones and premature withering of immature fruits at different stages of maturity etc., further delays breeding efforts in rubber (Mydin, 1998).

Genetic engineering is a viable alternate strategy for *Hevea* breeding to overcome some of the above problem as it may alter the character of an already accepted cultivated variety without affecting the existing genetic constitution. Thus, attempts to genetically transform *Hevea* using different techniques were initiated during the early 1990s by different rubber growing countries. From India the first output of this extensive research came during 2003 (Jayashree *et al.*, 2003) as the transgenic plant incorporated with a functional gene HbMnSOD which enhanced the stress tolerance of the elite Indian clone RRII 105. Later on transgenic plants with the integration of other new genes were reported in *Hevea (*Sobha *et al.*, 2019). Unfortunately in contrary to the huge expectations about this technology, the results were not so promising from the crop improvement point of view due to several factors like difficulties in plant regeneration, hardening etc. Moreover screening of a large number of events is necessary since we have no control over the site of integration in the *Agrobacterium* mediated transformation. Even though high transformation frequency is reported, only a few events could be developed as plantlets due to constraints in plant regeneration and hardening.

In spite of the above constraints, transgenics were developed using different introduced traits of agronomic importance in India. However the release of a transgenic rubber variety is yet to be realised. A confined field trial (CFT) for assessing the field performance of transgenics developed could not be materialised so far, due to the stringent biosafety regulations and public protest against GM crops in India. The only reported field trial with transgenic rubber was in Malaysia where they have

incorporated genes encoding proteins of therapeutic value in rubber (Sunderesan, 2016).

In this context, genome editing through CRISPR/ Cas 9 gains importance since it eliminates many of the associated hurdles in the development and field planting of transgenic rubber varieties developed using conventional plant transformation technologies. As in any other crops, genome editing can accelerate *Hevea* breeding by allowing the introduction of precise and predictable modifications in elite clones. The CRISPR/Cas9 system is particularly beneficial in a perennial recalcitrant tree crop like *Hevea*, because of its ability to alter multiple traits simultaneously with defined site targeting which is difficult to achieve by classical breeding or conventional genetic engineering. Editing of defined loci eliminates the possibility of interfering with the activity of other genes. Moreover, once the rubber plant is genetically transformed through gene editing, the trait could be fixed in the T1 generation itself with the help of large-scale clonal propagation.

Availability of *Hevea* whole genome sequence is an added advantage for selecting specific target regions for editing which will significantly minimise the problems associated with random integration of the genes and related unintended effects such as disrupting host metabolism and/or producing toxic or allergenic compounds often observed, in edited organisms. Screening of large number of events can also be avoided as the editing is precise and site specific and regeneration of one edited event is enough to serve the purpose.

If editing of single cell could be achieved using DNA free editing options available with CRISPR technology, undesirable foreign genetic elements like viral promoters, terminators, antibiotic resistant genes *etc*. associated with the conventional transgenic approach can be eliminated thereby relieving much of the regulatory burden currently associated with transgenic plants.

Genome editing: Possible applications in genetic improvement of *Hevea brasiliensis*

In *Hevea*, already established gene delivery methods like *Agrobacterium* mediated and particle bombardment can be utilised for the delivery of CRISPR/Cas9 gene editing cassettes into *Hevea* tissues as done in several other crops. Even though precise editing of the target site can be achieved by adopting any of the above delivery mechanism, it will not solve other problems associated with conventional transgenic crops like the presence of antibiotic marker genes and reporter genes, promoter sequences etc. The available option to eliminate these unwanted DNA sequences is by genetic segregation as practiced in other crops. But being a highly heterozygous perennial tree crop, removal of the unwanted sequences through segregation is practically not viable.

However, adoption of DNA free genome editing making use of mRNA for Cas9 translation alongside a separate guide RNA, or Cas9 protein with guide RNA (ribonucleoprotein complex) is a viable option. Methods like particle bombardment as done in wheat (Zhang 2016) and PEG mediated uptake of the above complexes by protoplasts as practiced in *Arabidopsis*, tobacco, lettuce and rice, (Woo *et al*., 2015) grapes and apple (Malnoy *et al*., 2016) are

the probable methods which can be attempted in them. Though plant regeneration from protoplasts in *Hevea* is already reported, selection of edited protoplasts and subsequent plant regeneration in *Hevea* still remains as an uphill task which needs further deliberation.

Microinjection of CRISPR assembly into the germ cells/single celled zygote as practiced in animal systems is yet another option, which can be attempted as an alternate strategy in *Hevea for DNA*-free gene editing. However the isolation of intact and live gametes as well as single celled zygote that too under sterile conditions will be a major challenge. Being a highly cross pollinated and heterozygous trees species, genetic integrity of clones cannot be maintained while using gametes or zygotes. Still this will have some specific advantages in the multiplication of promising events. Since the method involves a zygotic phase, we can exploit the advantage of the juvenility factor also. Selection can be performed at the plant level with the help of molecular techniques; hence antibiotic marker genes are not required. After the selection, promising individuals can be multiplied through bud grafting.

Another possibility is pollen tube mediated transformation followed by natural pollination and fertilization, provided a system for the pollen mediated DNA transfer is standardised. Two probable routes that can be followed are transformation of the gametes in the pollen tube with the use of PEG or pollination using transformed pollen. The method relies upon direct uptake of DNA with the help of PEG. Both the techniques need standardisation. All these possibilities require extensive research to take them to the next level and if successful will promise a new era in genetic improvement of *Hevea*.

What CRISPR technology can do in *Hevea*

Removal of latex allergens

Latex allergy is a common problem among high risk occupational and disease groups. Persons sensitive to latex allergens are likely to develop IgE-mediated urticaria, rhinitis and asthma. Hospital workers and patients with congenital urogenital disease are at high risk.A number of allergens have been isolated and characterized from *Hevea* latex. Inhibition of the pathways of the allergenic protein by gene disruption or modification of the protein using CRISPR is a promising area in *Hevea.*

Metabolic engineering

Metabolic engineering in *H. brasiliensis* by knocking out the genes from competing pathways to improve yield is a promising area. A similar strategy followed in Dandelion rubber is as follows: In Russian Dandelion rubber *Taraxacum kok-saghyz* (TK, Dandelion rubber), knocking out the gene encoding fructan: fructan1-fructosyl transferase (*1-FFT*), involved in inulin biosynthesis was carried out successfully using CRISPR/Cas9 mediated genome editing. Inulin is supposed to be an antagonist of rubber production. The regenerated plants contained knockout alleles with high mutation rates (80.0%). TK plants with edited genomes were obtained within 10 weeks (Laffaldano *et al.,* 2016). In *Hevea brasiliensis* isopentanyl pyro phosphatase (IPP) is the common intermediate for the synthesis of numerous

isoprenoids such as carotenoids, zeatin, diterpenoids, N-glycans, ubiquinone and other isoprenoid quinones along with rubber. Some or all these non-rubber pathways may be suitable targets for tissue specific down regulation in the laticifers so that the post-IPP metabolic flux could be redirected to rubber (cis-polyisoprene) synthesis to increase the yield of natural rubber. Once the rubber tree genome is fully sequenced and annotated, easy identification of genes for editing is possible and will accelerate the promising applications of genome editing tools in *Hevea.*

Elucidation of pathways

Genomics, transcriptomics, and proteomic analyses of the rubber-producing plants could generate high-throughput data, which lead to the comprehensive understanding of the natural rubber biosynthesis process. Many of these genes are identified and characterised (Cherian *et al*., 2019). However,the exact role of each component, multiple role of the same component, rubber particle biogenesis and rubber molecular weight determination still remains elusive. CRISPRtechnology is capable of elucidating these mechanisms and may give new insights about the pathways and how they are interconnected. Compared to other rubber yielding plants *H. brasiliensis* genome showed a significant increase in the number of gene families involved in rubber biosynthesis and can be attributed as the reason for high levels of latex production (Men *et al*., 2019). The arrangement of some of the biosynthetic genes in clusters in the genome suggests the coordinated evolution and expression for latex production. Manipulation of these gene families or clusters and the probable impacts can be studied by CRISPR technology.

Improving abiotic and biotic stress tolerance

As in any other crop, climate change is likely to seriously affect rubber cultivation and pose a severe risk to productivity and profitability in all rubber producing countries of the world. Genome editing could be an economic and technically feasible solution for developing new *Hevea* clones that are climate-resilient more quickly and precisely with public acceptance (non-GMO).

Abiotic stress response is generally considered as an extremely complex network of interactions among members of many gene families, transcription factors, and cis elements. A major challenge for using CRISPR-Cas genome editing technology for improving abiotic stress tolerance is the identification of potential candidate genes. Recently several reports were published in other crops were the CRISPR-Cas system could discover potential candidate genes as well as to develop edited plants with enhanced abiotic tolerance (Kim *et.al.* 2018; Shi *et al*., 2017). The elucidation of signalling pathways and transcription factors associated with abiotic stress responses in plants lead to the discovery of a significant number of abiotic stress responsive genes and their loci. (Licausi *et al*., 2013). Genes/ regulatory elements with similar functions can be identified in *Hevea* and can be used for inducing abiotic stress tolerance *via* CRISPR. Activating the promoter sequences for increasing the expression of already proven stress tolerance genes like MnSOD, osmotin *etc* is another option.

For inducing disease resistance, the receptor proteins which facilitate pathogen

entry can be targeted. Another strategy is targeting the genes responsible for susceptibility. Development of resistance to citrus canker by targeting the susceptibility gene l*ateral organ boundaries* 1 (CsLOB1) promoter (Peng *et al.*, 2017) is an example. In *Hevea* among the RRII 400 series clones, RRII 429 is a potential high yielder which is susceptible to pink disease. In pink free areas of traditional tract and in Northeast India where pink disease is not prevalent, this clone is equally good as other RRII 400 series clones. Identification of the susceptibility factor/genes to pink disease through molecular mining and targeting is an immediate possibility in *Hevea* which can be achieved by CRISPR.

Biosafety concerns and advantages of CRISPR edited crops

Conventional genome engineering techniques to develop GM crops include the introduction of foreign DNA sequences which are meant to be present in the final plant products and passed onto offspring during sexual reproduction.Because of this foreign gene introduction there is stringent biosafety regulations on planting and cultivation of GM crops all over the world. Potential threats include horizontal gene transfer to other organisms, contamination of natural non GM varieties by gene flow through pollen,possible health hazards to animals and human beings etc.

Inthe case of genome edited crops no alien functional gene is integrated into the genome except for marker genes and promoter sequences.Hence, there is no threat of the escape of integrated gene to the environment. Removal of unwanted sequences like marker gene and promoter sequences is possible through backcrossing and segregation if the edited site is probably away from the site of integration.

In a perennial tree crops like *Hevea*, backcrossing and selection is elaborate and time consuming and nearly impossible. DNA free gene editing approaches like direct delivery of mRNA for Cas9 translation alongside a separate guide RNA as well as delivery of Cas9 protein with guide RNA as a complex (ribonucleoprotein complex) do not necessarily require the stable introduction of recombinant constructs with unwanted sequences. There is no threat of horizontal gene transfer and pollen mediated gene transfer to non GM crops as well. Hence the stringent biosafety regulations may not be warranted for genome edited crops developed through DNA free approach.

Due to these advantages, the new varieties developed through engineered nuclease do not fall under the definition of a GMO under regulatory regimes in many countries. According to United States Department of Agriculture (USDA), CRISPR/Cas9 edited crops can be cultivated and sold free from regulatory monitoring (Waltz, 2016). This can save several million dollars on testing and data collection, reduce time to release a gene edited GMO crop and will remove the uncertainty of consuming traditional GMO crops among the public. Till 2018 there are five crops edited with CRISPR/Cas9 approach in the pipeline that USDA has declared not to regulate including a white button mushroom (*Agaricus bisporus*), Green bristlegrass (*Setaria viridis*) with delayed flowering time,Camelina for increased oil content and drought tolerant soybean (*Glycine max*) (Jaganathan*et al.*, 2018).

CONCLUSION

The CRISPR/Cas9 system is fast emerging as the most popular addition to the genome editing technology mainly because of its simplicity and versatility. Even though it is primarily used to develop single gene knock outs,we can think of replacing genes or genomic regions and knocking out gene families using new vectors and multiple gRNAs. However improvements are needed in its specificity and precision for wider applications in different crop species. Hence continuous upgradations in the system were carried out for achieving better efficiency and specificity which resulted in the introduction of orthogonal CRISPR Cas9 systems. The recently introduced prime editing which allows researchers to edit more types of genetic mutations with more specificity and precision than existing genome-editing approaches is the best example. All these gene editing technologies offer broad promises in genetic improvement of *Hevea* and their application may lead to major break throughs in crop improvement in the years to come. However, we need to gain information on genes, their crucial region for editing, *etc*. through functional genomic approach for the effective use of the technology. Above all,this constantly advancing technologyis evolving with myriad functionalities and capabilities in next generation plant science research.

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