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REVIEW

Use of genome editing technologies for genetic improvement of crops of tropical origin

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Abstract

Population growth and climate change demand the constant development of new crop varieties that can produce higher yields, and better organoleptic and nutritional value under adverse biotic, and abiotic conditions. In this sense, traditional breeding and genetic transformation have been used for decades. Nevertheless, the first approach is time consuming endeavor, and is unable to keep up with increasing food demands. On the other hand, genetic transformation is often limited by consumer acceptance. Recent genome editing technologies, such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (CRISPR-Cas9) system allows precise, specific, and low cost edition in a targeted genome region. The wide variety of applications for this technology includes increased yields and nutritional value, stress tolerance, and herbicide resistance. Crops of tropical origin have nutritional and economic importance; therefore, this review will analyze the advances and applications of CRISPR in crops of tropical origin to obtain varieties better adapted to current environmental conditions and market requirements.

Key message

Genome editing technologies, such as CRISPR, allows precise and specific modification of genetic information for the improvement of crops of tropical origin, including rice, maize, tomato, coffee, cacao, and citrus, to produce varieties with resistance or tolerance to biotic and abiotic factors.

Keywords Gene editing · sgRNA · Mutations · *Agrobacterium tumefaciens* · Particle bombardment

Abbreviations

bp	Base-pair
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated protein 9
DBS	DNA double-strand break
DNA	Deoxyribonucleic acid
HDR	Homology directed repair
Indel	Insertion or deletion of base(s)
NHEJ	Non-homologous end joining
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
sgRNA	Single guide RNA

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Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), the impact of climate change on crop yield will depend on factors such as temperature, CO_2 increases, and precipitation changes (FAO 2016). In this sense, food production systems rely on highly selected cultivars adapted to specific environments and growth conditions. Because of climate change, these cultivars may be grown under conditions for which they are not adapted, making them more vulnerable to different biotic (bacteria, fungi, insects, and viruses), and abiotic stresses (salinity, drought, temperature) (Govindaraj et al. 2015).

The progress achieved in the last decades in biotechnology and molecular biology has increased the available tools for improvement of agricultural crops. Some of these tools include techniques for the introduction of transgenes, directed mutagenesis, and accelerated reproduction techniques, among others (Lusser et al. 2012). Genetic improvement using *Agrobacterium tumefaciens* or particle gun bombardment has some limitations, such the inability to control the random integration of the transgene in the genome affecting the level, and stability of gene expression (Kohli et al. 2006). More specific, in the case of particle gun bombardment, the transgene copy number can be very high which depends on the amount of DNA delivered into cells. Also, some studies showed that it can cause extreme genome damage in the form of chromosome truncations, large deletions, and partial trisomy (Liu et al. 2019a, b). On the other hand, *Agrobacterium*-mediated transformation system has host-range limitations and it cannot be used to directly deliver molecules like proteins and RNA (RNP) unlike particle gun bombardment (Mello-Farias and Chaves 2008; Mohammed et al. 2019; Liu et al. 2019a, b).

In recent years, new precision plant biotechnologies (NPBT) such as zinc finger domains (ZFN), effector nucleases similar to transcriptional activators (TALEN), and short palindromic repeats grouped regularly interspaced associated with the Cas9 endonuclease (CRISPR/Cas9) have been developed and allow precise, and specific edition in a targeted genome region. The editing of a genome consists of producing directed, permanent, and inheritable mutations in a specific place of the genome, mediated by DNA repair systems in the cell, with the lowest probability of committing unwanted errors (off-targets) and leaving no foreign DNA sequences.

The newest and most widely used genome editing technology for the study of the function of genes and for the development of mutant lines with enhanced tolerance to biotic and abiotic stresses, herbicide resistance or improved yield is CRISPR/Cas (Petolino 2015; Cao et al. 2016; Khan et al. 2016; Lassoued et al. 2018a, b). This technology differs from ZFN and TALEN in terms of the DNA-binding system. Cas9 can be targeted to a specific genomic sequence by an easily engineered 20 base pair (bp) RNA guide sequence that binds to its DNA target by base-pairing. The wide variety of applications for this technology includes simple non-homologous end joining, homologous recombination, gene replacement, and base editing, among others. The applications and challenges of CRISPR/Cas9 in tropical crops has been recently reviewed (Haque et al. 2018). However, in this review, we will present an in-depth study of genome editing technologies research that has been reported in crops of tropical origin emphasizing in the pre-requisites, limitations, and advantages. Literature from 2013 to mid-2019 was searched using the PubMed database with the keywords "NAME OF THE CROP" and "CRISPR".

Importance for breeding crops of tropical origin

One of the challenges that crops breeders face is the generation of varieties resistant to the adverse environmental conditions produced by climate change (Pereira 2016). Crops are susceptible to a large set of pathogens and pest, including fungi, bacteria, nematodes, insects, and viruses that cause important economic losses, especially in tropical climates. It is estimated that biotic stress cause losses worldwide up to 35% of the total food production (Bainsla and Meena 2016). As an example, losses in rice due to insects have been estimated in 40% (Oerke and Dehne 2004); and in sorghum the losses been valued over $1079 billion. On the other hand, abiotic stress (drought, salinity, cold, and heat) represent the primarily cause of crop losses worldwide, and yield losses are estimated around 50% of crop production (Ashraf et al. 2008).

This adverse biotic and abiotic factors influence negatively the survival, production and yields of staple food crops threatening food security (Meena et al. 2016). To counteract these losses, plant breeding programs focus the efforts on developing crops with improved yield, field performance and nutritional quality, as well as resistance to biotic and abiotic stresses (Ferrão et al. 2017). For decades, breeding strategies include selection, hybridization, mutation induction using chemical and physical agents, and somaclonal variation. However, advances for breeding crops against biotic and abiotic stresses using conventional strategies has been slow, principally due to the long time necessary to achieve the desired phenotype, and it is limited by the lack of resistance genes in the germoplasms (Bainsla and Meena 2016; Parmar et al. 2017).

Moreover, several crops of tropical origin are an important source of nutrients and carbohydrates for humans. Therefore, efforts have been focused to the enrichment of nutrients and the elimination of toxic elements. Several strategies including conventional and transgenic approaches have been used for the bio fortification of staple crops (Ricachenevsky et al. 2019). As an example, efforts have been made to increase the content of pro-vitamin A in banana (Paul and Qi 2016).

In this sense, modern biotechnology tools, such as tissue culture and genetic engineering, offer an alternative to conventional breeding in order to generate new cultivars with enhanced agronomic and nutritional characteristics (Parmar et al. 2017). In the last decades, transgenic crops, including those of tropical origin, have been developed and genetic modification has been performed to confer resistance against insects, bacteria and fungi diseases, and viruses; as well as tolerance against drought, high temperatures, salinity, and herbicides (Parmar et al. 2017). More

recently, the availability of genome editing technologies, genome sequences, efficient tissue culture, and transformation methodologies could remarkably facilitate the breeding of crops of tropical origin.

Overview of CRISPR/Cas9 technology

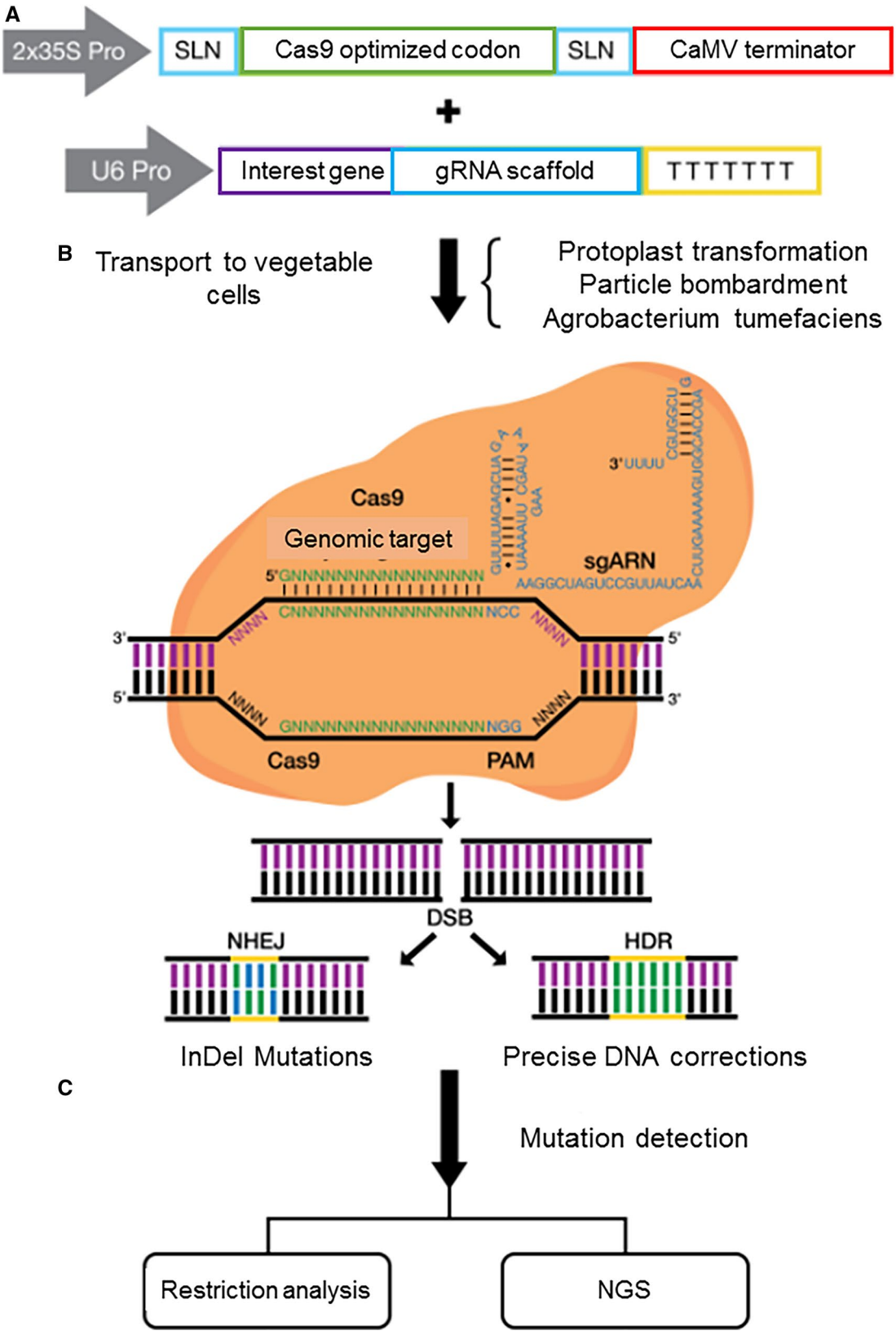
CRISPR/Cas9 originates from the immune response of bacteria and archaea against foreign DNA from viruses and plasmids. When bacteria survive initial infection by a bacteriophage, segments of the phage genome are inserted into the bacterial genome. Then, when the bacteria is infected a second time, CRISPR RNA is synthesized and guides the Cas endonuclease to the phage DNA for its degradation (Johnson et al. 2015). Several CRISPR systems which differ in the diversity of their protein components have been identified in bacteria. In general, the three main CRISPR systems are type I, II, and III, which use the endonucleases Cas3, Cas9, and Cas10, respectively (Chylinski et al. 2014). More information on the systems can be found in the work by Ishino et al. (2018). The CRISPR/Cas technology combined the two RNAs naturally involved in the CRISPR system (tracrRNA and crRNA) in a single artificial chimeric molecule called guide RNA or single guide RNA (sgRNA) (Fig. 1a). The sgRNA is designed in silico to guide the Cas9 to specific sites in the genome of the organism that need to be modified or improved. This results in a double strand break in the DNA (deoxyribonucleic acid). The DNA breakage triggers the Non-Homologous End Joining (NHEJ) repair system (Fig. 1b) which leads to insertions or deletions of small sequences (InDels) adjacent to the excision site (Porteus 2016; Scheben et al. 2016). NHEJ mediated gene inactivation is the simplest way of directed modification, and it is generally used to knockout genes that have a negative influence on a trait of interest in the plant (Bortesi and Fischer 2015). Homology directed repair (HDR) can also be generated when a DNA template with high sequence homology is introduced into the cleaved zones (Sander and Joung 2014). HDR is the repair mechanism used to insert a sequence or repair a mutation in a specific part of a gene (Kamburova et al. 2017; Malzahn et al. 2017).

A requirement for the CRISPR endonucleases to adhere to the target site of the DNA, aside from the pairing of the sgRNA to the target sequence, is the presence of a PAM (Protospacer adjacent motif). The PAM is a sequence of 2 to 6 nucleotides, generally found after the joining site of the leader sequence in the DNA. This sequence may vary depending on the type of Cas protein being used. The most commonly used is Cas9 from *S. pyogenes*, which recognizes the PAM sequence 5′NGG3′ (Endo et al. 2018). More information on the CRISPR/Cas mechanism and structures can be found in the work by Jiang and Doudna (2017).

In plants, the first step involved for using CRISPR, consist in the design and construction of the sgRNA for the specific gene or site where the modification will take place (Fig. 1a). The next step is the introduction of the CRISPR/Cas9 components into the plant cells using particle bombardment or *Agrobacterium*-mediated transformation (Fig. 1b). Finally, plants are regenerate from the genetically edited cells, tissues, or organs, and the mutations are verified through restriction analysis, PCR (polymerase chain reaction) and sequencing (Fig. 1c) (Kangquan et al. 2017).

The CRISPR/Cas9 system offers some advantages, such as simplicity, accessibility, versatility, and low cost, in comparison with other editing technologies, such as ZNFs (zinc/finger nucleases), and TALENs (transcription activator-like effector nuclease). For example, ZNFs and TALENs technologies recognize their target site by proteins so designing these proteins are far more complicated and expensive that designed a single guide RNA for CRISPR (Farooq et al. 2018). Also CRISPR/Cas9 make possible to target several different genes by multiples sgRNA in a single CRISPR array (Li et al. 2017). Another advantage of the CRISPR system is the ability to excise methylated DNA, which is particularly appropriate for monocotyledons with a high genomic GC content, such as rice (Bortesi and Fischer 2015).

The main drawback of CRISPR/Cas9 is the possibility to generate off-target mutations, which are the product of erroneous pairings of the sgRNA on unwanted sites in the DNA. Studies in *Arabidopsis* (Zhang et al. 2018a, b), citrus (Jia et al. 2017), rice (Baysal et al. 2016) and tomato (Li et al. 2017) genomes mutated by CRISPR/Cas9 have demonstrated that this system is very specific in plants, and off-target mutations do not happen often. Nevertheless, there are some cases in rice where off-target mutations were detected (Xie and Yang 2013). Therefore, the sequencing of the complete mutant genome is the most reliable way to detect off-target mutations. A less expensive method would be sequencing only the potential off-target regions predicted in silico (Wolt et al. 2016; Puchta 2017). Although problems caused by off-target mutations are not as severe in plants as in humans and animals (Kangquan et al. 2017), there are strategies to minimize their occurrence, like the use of alternatives to SpCas9. For example, SaCas9 is usually more precise due to a longer PAM sequence, and Cas12a uses a different molecular mechanism for DNA targeting, recognition and cutting (Schindele et al. 2018). There are also reports that insertion of the CRISPR system as an RNP (Ribonucleoprotein) reduces off-target mutations (Hahn and Nekrasov 2018). Another limitation is that for the proper recognition of the target site by the sgRNA a PAM sequence is required, this decreases the flexibility of possible targets sites, and also affects the design of sgRNA (Zhou et al. 2018b).


A
2x35S Pro
SLN
Cas9 optimized codon
SLN
CaMV terminator
U6 Pro
Interest gene
gRNA scaffold
TTTTTTT
B
Transport to vegetable cells
Protoplast transformation
Particle bombardment
Agrobacterium tumefaciens
Cas9
Genomic target
sgARN
AAGGCUAGUCCGUUAUCAA
5'GNNNNNNNNNNNNNNNNNN
CNNNNNNNNNNNNNNNNNN NCC
GNNNNNNNNNNNNNNNNNN NGG
NNNN
Cas9
PAM
DSB
NHEJ
HDR
InDel Mutations
Precise DNA corrections
C
Mutation detection
Restriction analysis
NGS

◂**Fig. 1** Mechanism of genome editing through CRISPR/Cas9 in plants. (**A**) The cassette expressing Cas9 is driven by the 35S promoter and the guiding RNA is usually driven by the U6 promoter. (**B**) The CRISPR/Cas9 system is introduced into plant cells by protoplast transformation, particle bombardment or *Agrobacterium* transformation. Once in the cells, the sgRNA directs the Cas9 to the target site of the genome. Cas9 recognizes the PAM sequence and performs the double DNA chain break. Through the NHEJ repair system, deletions or insertions of bases (InDel) are generated in the target site, on the other hand, by means of the HDR repair system, precise corrections can be made in the DNA or directed sequences can be inserted. (**C**) Finally, gene editing could be detected by restriction enzymes or by sequencing. *Source* Own elaboration based on Tang and Tang (2017)

Applications of CRISPR/Cas9 in crops of tropical origin

CRISPR/Cas9 was used for the first time in the model plants *Arabidopsis thaliana* (Li et al. 2013), *Nicotiana benthamiana* (Li et al. 2013), and *Oryza sativa* (Shan et al. 2013) in 2013. Since then, this technology has been widely used as a genetic editing method for several plants (Zhang et al. 2017a, b; Belhaj et al. 2015). A review of 52 studies between 2014 and 2017 revealed that this technology has been mainly focused on rice, tomato, citrus, tobacco, maize, and wheat for tolerance to abiotic (drought, salinity), and biotic (bacteria, fungus, and viruses) factors, yield improvement, and bio-fortification (Ricroch et al. 2017) (Fig. 2). Nevertheless, in the last few years, applications of this technology have been extended to other crops such as cacao, maize, sorghum, and sweet orange (Table 1). Since 2013, the number of publications where CRISPR/Cas9 has been used in crops of tropical origin has increased especially in rice, tomatoes, and maize according to the studies present in the PubMed database (Fig. 3).

The first approaches using CRISPR/Cas9 in crops of tropical origin aimed to evaluate the efficiency and to experiment with this technology. Therefore, many studies have used marker genes such as phytoene desaturase (*PDS*) as proof-of-concept for genome editing. Phytoene desaturase gene encodes an enzyme that limits carotenoid synthesis and converts phytoene into colored ζ-carotene in a two-step desaturation reaction. Successful knock out of this gene leads to albino phenotypes and allow the screening of mutants (Wang et al. 2009). This has been applied in banana (Hu et al. 2017a, b; Kaur et al. 2018), cassava (Odipio et al. 2017), citrus (Jia and Wang 2014; Zhang et al. 2017b; LeBlanc et al. 2018), coffee (Breitler et al. 2018), maize (Svitashev et al. 2016), rice (Miao et al. 2013; Liang et al. 2018), sorghum (Jiang et al. 2013), tomato (Pan et al. 2016), and

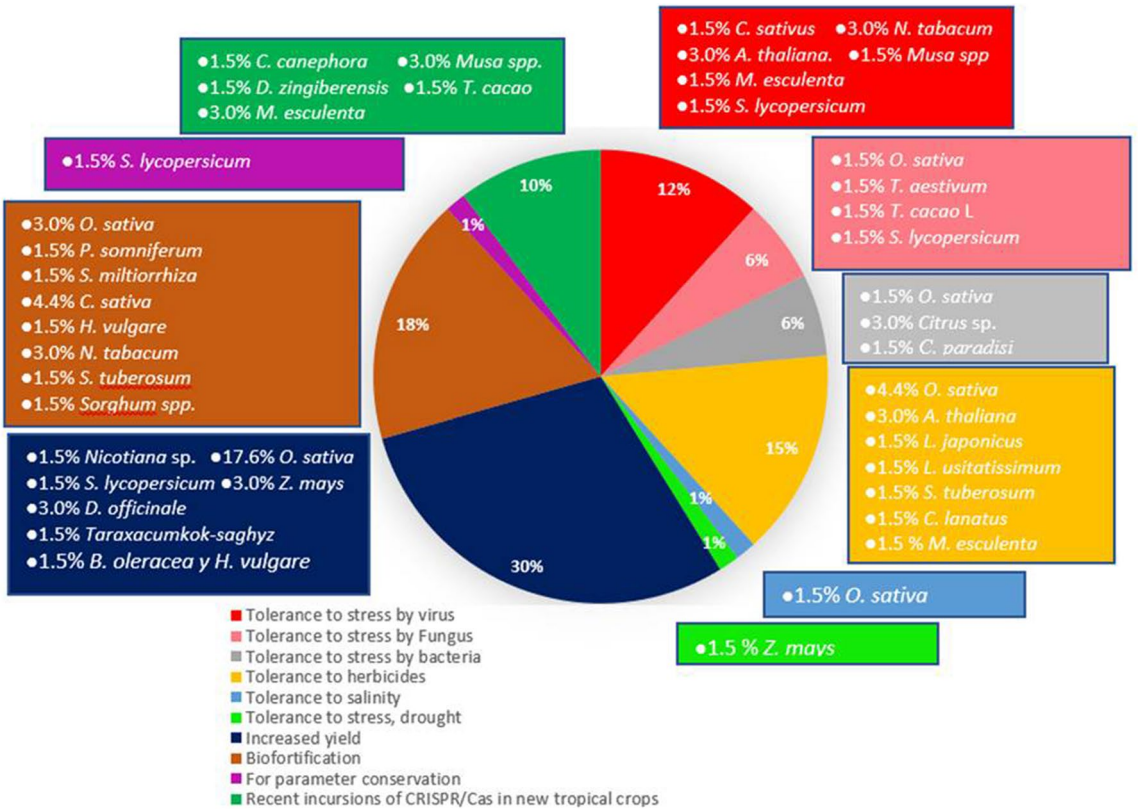


Fig. 2 Applications of the CRISPR/Cas9 system for crop improvement. Based on the 52 studies reported between 2014 and 2017 by Ricroch et al. (2017) and 15 studies published in 2018–2019 which are mentioned in this review

Table 1 Applications of CRISPR/Cas9 in species of tropical origin

Common name	Specie	Application target	Target gene	sgRNA design tool	Molecular function of the target gene	Tissue culture system	DNA delivery	Screening of mutants	References
Banana	*Musa* spp.	Proof-of concept	*PDS*	ZiFiT SSFinder n.i	Encodes the phytoene desaturase enzyme	Embryogenic cell suspensions	*A. tumefaciens*	PCR and sequencing	Hu et al. (2017a, b) Kaur et al. (2018) Naim et al. (2018)
		Plants resistant to banana streak virus (eBSV)	BSOLV	n.i	Conserved parts of The BSV strain Obino l'Ewai	Embryogenic cells	*A. tumefaciens*	PCR band shift analysis, T7E1 assay, and sequencing	Tripathi et al. (2019)
		Semi dwarf plants	*MaGA20ox2*	n.i	Encodes an important enzyme for the biosynthesis of gibberellin	Embryogenic cell suspension	*A. tumefaciens*	PCR and sequencing	Shao et al. (2019)
Cacao	*Theobroma cacao* L.	Generate plants resistant to *Phytophthora tropicalis*	*TcNPR3*	*Geneious and* CRISPR site tool	Cacao Non-Expressor of Pathogenesis-Related 3	Secondary somatic embryo cotyledons	*A. tumefaciens*	PCR and sequencing	Fister et al. (2018)
Cassava	*Manihot esculenta*	Proof-of concept test	*MePDS*	n.i	Encodes the phytoene desaturase enzyme	Embryogenic calli	*A. tumefaciens*	PCR and Sequencing	Odipio et al. (2017)
		Plants resistant to the Cassava Brown Streak Disease (CBSD)	*IF4E nCBP1* and *nCBP-2*	CRISPR-P	Genes involved in susceptibility to the *Potyviridae* family	Embryogenic calli	*A. tumefaciens*	Restriction enzymes, PCR and sequencing	Gomez et al. (2018)
		Resistance to glyphosate	*EPSPS*	n.i.	Encodes the 5-enolpyruvylshikimate-3-phosphate synthase	Embryogenic calli	*A. tumefaciens*	PCR and sequencing	Hummel et al. (2018)
		Resistance to geminivirus	*AC2* and *AC3*	Software from the Broad Institute	Viral genes	n.i	PCR and sequencing		Mehta et al. (2019)
	Citrus sinensis	Proof-of concept and optimization of CRISPR/Cas9	*CsPDS*	n.i n.i n.i	Encodes the phytoene desaturase enzyme	Epicotyl explants Leaves	*A. tumefaciens*/Agroinfiltration	Restriction enzymes and sequencing	Jia and Wang (2014) Zhang et al. (2017b) LeBlanc et al. (2018)

Table 1 (continued)

Common name	Specie	Application target	Target gene	sgRNA design tool	Molecular function of the target gene	Tissue culture system	DNA delivery	Screening of mutants	References
Citrus	*Citrus sinensis*	Citrus canker disease resistance (*Xanthomonas axonopodis* pv *citri.*)	*CsLOB1*	n.i	Susceptibility gene for citrus canker disease	Epicotyl explants	*A. tumefaciens*	PCR and Sequencing	Peng et al. (2017)
	Citrus paradisi	Citrus canker disease resistance (*Xanthomonas axonopodis* pv *citri.*)	*CsLOB1*	n.i	Susceptibility gene for citrus canker disease	Epicotyl explants Leaves	*A. tumefaciens*/Agroinfiltrations	PCR and Sequencing	Jia et al. (2015, 2017, 2019)
Coffee	*Coffea canephora*	Proof-of concept	*CcPDS*	CRIP	Encodes the phytoene desaturase enzyme	Embryogenic calli	*A. tumefaciens*	PCR and Sanger sequencing	Breitler et al. (2018)
Cucumber	*Cucumis sativus* L.	*Potyviridae* virus resistance	*eIF4E*	n.i	Essential translation factor for life cycle of *Potyviridae*	Cotyledons	*A. tumefaciens*	Restriction enzymes, PCR, and Sequencing	Chandrasekaran et al. (2016)
		Increase CRISPR/Cas9 efficiency	*CsWIP1, CsVFB1, CsMLO8 CsGAD1*	n.i	Acts as an inhibitor of carpel development	Cotyledonary nodes	*Agrobacterium* sp.	T7EI and Sanger sequencing	Hu et al. (2017b)
Maize	*Zea mays*	Reduce phytic acid in seeds	ZmIPK	n.i	Encodes the phosphoinositol 3-kinase gen	Immature embryos/ mesophyll protoplasts	*Agrobacterium* sp./protoplast transformation	Restriction enzymes, PCR and sequencing	Liang et al. (2014)
		Increase the yield of grains in drought conditions	Overexpression of the ARGOS8 gene	n.i	Overexpression of this gene reduces ethylene sensitivity	Immature embryos	Particle bombardment	PCR and sequencing	Shi et al. (2017)
		Proof-of concept	*ZmAgo18a* and *ZmAgo18b*	n.i	Implicated in the biogenesis of interfering RNA in the anthers	Immature embryos	*A. tumefaciens*	PCR, T7EI mutation detection assay and sequencing	Char et al. (2016)
		Proof-of concept	*LIG ALS2 MS26* and *MS45*	n.i.	Liguleless1 Acetolactate synthase Male fertility genes	Immature embryos	Particle bombardment	PCR and sequencing	Svitashev et al. (2016)
		A maize haploid inducer line carrying a CRISPR/Cas9 cassette for agronomic traits	*ZmLG1 UB2*	n.i	Liguleless1 SBP transcription factor	n.i	*A. tumefaciens*	PCR and sanger sequencing	Wang et al. (2019a, b, c, d)

Table 1 (continued)

Common name	Specie	Application target	Target gene	sgRNA design tool	Molecular function of the target gene	Tissue culture system	DNA delivery	Screening of mutants	References
Rice	*Oryza sativa* L.	Proof-of concept	*OsMPK5* (mitogen-activated protein kinase)	n.i	Stress response activated protein kinase	Rice protoplast	Protoplast transformation	Restriction enzymes, PCR and sequencing	Xie and Yang (2013)
		Proof-of concept	*CAO*1	n.i	Encodes the chlorophyll oxygenase genes	Embryogenic calli	*Agrobacterium* sp. and particle bombardment	PCR and sequencing	Miao et al. (2013)
		Confer resistance to bentazon and sulfonylurea herbicides	BEL	n.i	Bentazon Sensitive Lethal	Embryogenic calli	*A. tumefaciens*	PCR and Sanger sequencing	Xu et al. (2014)
		Proof-of concept	*OsADH2*	n.i	Alcohol dehydrogenease 2	Embryogenic calli	*A. tumefaciens*	Restriction enzymes, PCR and sequencing	Mikami et al. (2015)
		Rice Blast-resistant plants (*Magnaporthe oryzae*)	*OsERF922*	n.i	Acts as a negative regulator of resistance to *Magnaporthe oryzae*	Embryogenic calli and protoplast	*A. tumefaciens* and protoplast transformation	PCR, sequencing and Degenerate Sequence Decoding method (DSD)	Wang et al. (2016)
		Herbicide-resistant plants (bispyribac-sodium and chlorsulfuron)	*ALS*	n.i	Encodes the acetohydroxyacid synthase enzyme	Embryogenic calli	*Agrobacterium* sp. and particle bombardment	Restriction enzymes, PCR and sequencing	Sun et al. (2016)
		Increase the content of resistant starch	*SBEI* and *SBEIIb*	n.i	Encodes the starch-branching enzyme	Embryogenic calli	*A. tumefaciens*	PCR-based restriction enzyme and sequencing	Sun et al. (2017)
		Positive regulator of stomatal development	*OsEPFL9*	n.i	Encodes the epidermal Patterning Factor like-9	Immature embryos/ Embryogenic calli	*Agrobacterium* sp.	Restriction enzymes, PCR and sequencing	Yin et al. (2017)
		Low cesium accumulation	*OsHAK-1*	CRISPR-P	Encodes a Cs + -permeable K + transporter	Embryogenic calli	*A. tumefaciens*	PCR and sequencing	Cordones et al. (2017)
		Ideal grain architecture and enhanced grain yield	*OsSPL14*	n.i	Encodes for the squamosa promoter binding protein-like 14. (transcriptional regulator)	n.i	*Agrobacterium* sp.	PCR and Sanger sequencing	Hua et al. (2018)

Table 1 (continued)

Common name	Specie	Application target	Target gene	sgRNA design tool	Molecular function of the target gene	Tissue culture system	DNA delivery	Screening of mutants	References
		High oleic/low linoleic content in seeds	*FAD2-1*	CRISPR-P program	Fatty acid desaturase 2	Embryogenic calli	*A. tumefaciens*	Restriction enzymes, PCR and sequencing	Abe et al. (2018)
		Study the effect of *GBSSI* knockdown in starch biosynthetic machinery	*GBSSI*	E-CRISP	Granule-bound starch synthase I	Embryogenic calli	Particle bombardment	PCR and sequencing	Pérez et al. (2019)
Sorghum	*Sorghum* spp.	Proof-of concept	*DsRED2*	n.i	Red fluorescent protein gen	Immature embryo	*A. tumefaciens*	Restriction enzymes, PCR and sequencing	Jiang et al. (2013)
		Proof-of concept	*CAD* *PDS*	CRISPR-P 2.0 CRISPOR 4.4	Cinnamyl alcohol dehydrogenase Phytoene desaturase	Immature embryo	Biolistics	PCR and sequencing	Liu et al. (2019a, b)
		Induce haploid plants	*b-CENH3*	n.i	Participation in the regulation of chromosome segregation	Immature embryo	*Agrobacterium* sp.	PCR and sequencing	Che et al. (2018)
		Increase digestibility and protein quality	*k1C* gene family	n.i	Encode most of kafirins	Immature embryo	*A. tumefaciens*	PCR, T7EI mutation detection assay and sequencing	Li et al. (2018a, b, c)
Tomato	*Solanum lycopersicum*	Proof-of concept	*SlPDS* *SlPIF4*	CRISPR-P	Phytoene desaturase PIF4 factor that interacts with phytochrome	Leaf discs and calluses	*A. tumefaciens*	PCR, T7 Endonuclease I (T7EI) assay and sequencing	Pan et al. (2016)
		Reduce the concentration of γ-aminobutyric acid, which could cause health problems for humans	*GABA-TP1, GABA-TP2, GABA-TP3, CAT9 and SSADH*	CRISPR-P	Essential genes for the γ-aminobutyric acid (GABA) path	n.i	*Agrobacterium* sp.	PCR, sequencing and Degenerate Sequence Decoding method (DSD)	Li et al. (2017)
		Parthenocarpic plants	*SlIAA9*	Web-tool "focas"	Key gene for parthenocarpy	Leaf disc and calli	*A. tumefaciens*	PCR, HMA, PCR–RFLP and Sequencing	Ueta et al. (2017)
		Proof-of concept	*CRTISO* *PSY1*	n.i	Carotenoid isomerase Phytoene synthetase 1	Cotyledon	*A. tumefaciens*	PCR and Sanger sequencing	Dahan-Meir et al. (2018)

Table 1 (continued)

Common name	Specie	Application target	Target gene	sgRNA design tool	Molecular function of the target gene	Tissue culture system	DNA delivery	Screening of mutants	References
		Tomato Yellow Leaf Curl Virus (TYLCV) resistance	CP or RP sequences	n.i	Encodes Viral Coat Protein or Replicase, respectively	Cotyledon	*A.tumefaciens*	T7EI mutation detection assay	Tashkandi et al. (2018)
		Proof-of concept	*Psy1* *CrtR-b2*	CRISPR-P	Phytoene synthase 1 Beta-carotene hydroxylase 2	n.i	*A. tumefaciens*	PCR, sequencing and Degenerate Sequence Decoding method (DSD)	D'Ambrosio et al. (2018)
		Tolerance to heat stress Susceptible to drought stress	*SlMAPK3*	CRISPR-P	Mitogen-activated protein kinase 3	Cotyledons	*A. tumefaciens*	Sequencing	Wang et al. (2017) Yu et al. (2019)
		Resistance to *Phelipanche aegyptiaca*	*CD7* and *CCD8*	CRISPR-Scan web tool	Carotenoid cleavage dioxygenases 7 and 8	n.i	*A. tumefaciens*	Restriction enzymes, PCR and sequencing	Kumar et al. (2019)
Currant tomato	*Solanum pimpinellifolium*	Genes for improving yield and productivity	*SELF-PRUNING (SP)–OVATE (O)* *FRUIT WEIGHT 2.2 (FW2.2)* *LYCOPENE BETA CYCLASE (CycB)*	n.i	Involved respectively in: plant growth habit fruit shape fruit size nutritional quality	Calli	*A. tumefaciens*	PCR, sequencing	Zsögön et al. (2018)
Watermelon	*Citrullus lanatus*	Proof-of concept	*ClPDS*	n.i	Encodes the phytoene desaturase enzyme	Embryogenic calli	*A. tumefaciens*/protoplast transformation.	Restriction enzymes and sequencing	Tian et al. (2017)
		Herbicide resistant	*ALS*	n.i	encodes acetolactate synthase enzyme	Cotyledon	*A. tumefaciens*	PCR and Sanger sequencing	Tian et al. (2018)
Yam	*Dioscorea zingiberensis*	Proof-of concept	*Dzfps*	CRISPR-P	Farnesylpyrophosphate synthase gene involved in diosgenin biosynthesis	Embryogenic calli	*A.tumefaciens*	PCR and Sequencing	Feng et al. (2018)

n.i. not indicated

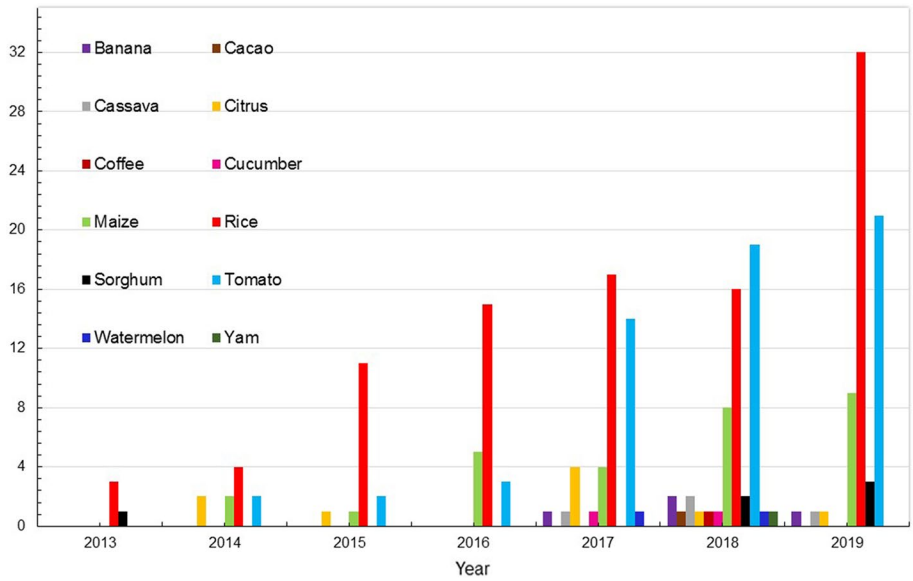


Fig. 3 Number of publications per year where CRISPR/Cas9 tool has been employed in crops of tropical origin according with data base PubMed from 2013 to August 2019

watermelon (Tian et al. 2017) (Table 1). Recently, the green fluorescent protein (*EGFP*) has been used as an indicator for the screening of tobacco and grapes mutants generated by CRISPR/Cas9 as a result of the activation of the non-functional *EGFP* protein (Ren et al. 2019). This method could be applied for the identification of large populations of mutants of crops of tropical origin derived by new breeding technologies without the necessity of isolating DNA or generating mutants with an albino phenotype.

Banana

Musa spp. is one of the most important commercial commodities in tropical and subtropical developing countries. It is cultivated over 130 countries, and is a source of carbohydrates for millions of people worldwide (FAOSTAT 2017). Conventional banana breeding is limited by factors, such as sterility, poliploidy, and asexual propagation (Rout et al. 2008). Therefore, number of biotechnological tools, including tissue culture, and genetic engineering protocols have been developed to complement traditional breeding (Rout et al. 2008). Moreover, the sequencing of the banana genome (D'Hont et al. 2012) provides access and information about each gene making it possible to apply genome editing technologies for improving it (Table 2).

Among the first studies of CRISPR/Cas9, Hu et al. (2017a, b) modified the gene *MaPDS* in embryogenic cell suspensions of Boxi cultivar via *A. tumefaciens*. As a result, approximately 55% of the genetically modified lines displayed an albino and/or chimeric phenotype. Similarly, Kaur et al. (2018) edited the *MaPDS* gene in the Rasthali cultivar transforming cells suspensions via *A. tumefaciens*. In this case, most of the resulting mutants displayed a complete albino phenotype and did not survive the rooting phase. Also, some mutants displayed chimeric phenotypes (green and albino). Moreover, Naim et al. (2018) edited the *PDS* gene in Cavendish *banana* cv. Williams and genotyping of 19 independent events showed a 100% PDS mutation rate like insertions or deletions. These three studies demonstrated the efficiency of CRISPR/Cas9 for genome editing of endogenous genes in banana and opens the possibility to apply this system for the targeting of genes for the improvement of important agronomic characteristics. In this sense, CRISPR/Cas9 was used to confer resistant to Banana Streak Virus (eBSV) by editing the virus sequences in the B genome in a plantain with AAB genome called "Gonja Manjaya". For this, three sgRNAs were designed to target the most conserved parts of the BSV strain Obino l'Ewai (BSOLV). The mutation efficiency was 85% at the target sites. After 1 month, six of eight edited lines remained asymptomatic and two lines showed moderate symptoms compared to wild type control plants (Tripathi et al. 2019). This study shows that targeted mutagenesis by CRISPR/Cas could be used for the rapid generation of disease-resistant plants. A similar approach is expected in the near future in banana against *Fusarium oxysporum* Tropical race 4 or TR4 ("Panama disease"). This is an aggressive and fast-spreading disease that has already affected Cavendish banana in Africa, Asia, and Central America, endangering this variety which is the most important worldwide (Pérez et al. 2014). For example, genes like *MaAPS1* and *MaAPL* could be edited to generate resistance not only to abiotic stress (cold, and salinity), but also to TR4 (Haque et al. 2018).

Moreover, semi dwarf plants were obtained using CRISPR/Cas9 by editing the *MaGA20ox2* gene in *Musa acuminate* (AAA group) (Shao et al. 2019). The Ga20ox2 (GA_{20} oxidase) is an important enzyme in the biosynthesis

Table 2 Availability of in vitro regeneration, genetic transformation protocols and of genomic resources for some crops of tropical origin

	Species	Regeneration[a]	Transformation[b]	Genome[c]	References
Banana	*Musa* spp.	+/++	+/++/+++	+	Rout et al. (2008), D'Hont et al. (2012) and Liu et al. (2017)
Cacao	*Theobroma cacao* L.	+/++	+/++/–	+	Gotsch (1997). Argout et al. (2011), Motamayor et al. (2002) and Wickramasuriya and Dunwell (2018)
Cassava	*Manihot esculenta*	+/++	+/++	+	Prochnik et al. (2012), Chavarriaga-Aguirre et al. (2016), Fondong and Rey (2018)
Citrus	*Citrus* sp.	+/++	+/++/+++	+	Singh and Rajam (2009) and Xu et al. (2012)
Coffee	*Coffea canephora*	+/++	+/++/+++	+	Ribas et al. (2006) and Denoeud et al. (2014)
Cucumber	*Cucumis* sp.	+/++	+/++/+++	+	Huang et al. (2009), Navrátilová et al. (2011) and Wang et al. (2015)
Maize	*Zea mays*	+/++	+/++	+	Lyznik et al. (1989), Emons and Kieft (1995), Wright et al. (2001), Lee and Zhang (2013), Nannas and Dawe (2015) and Mushke et al. (2016)
Rice	*Oryza sativa* L.	+/++	+/++/+++	+	Hayashimoto et al. (1990), Li et al. (1993), Hiei et al. (1994), Yoshida et al. (1994), Nhut et al. (2000) and Yu et al. (2005)
Sorghum	*Sorghum* spp.	+/++	+/++/+++	+	Battraw and Hall (1991), Tadesse et al. (2003), Nirwan and Kothari (2004), Girijashankar et al. (2007), Paterson et al. (2009) and Do et al. (2016)
Tomato	*Solanum lycopersicum* and *Solanum pimpinellifolium*	+/++	+/++/	+	Newman et al. (1996), Ma et al. (2015), Ruma et al. (2009), The 100 tomato Genome Sequencing Consortium (2014), Ray et al. (2015), Razali et al. (2018) and Zsögön et al. (2018)
Watermelon	*Citrullus lanatus*	+/++	+/++/+++	+	Compton and Gray (1993), Suratman et al. (2010), Hema et al. (2004), Krug et al. (2005), Cho et al. (2008) and Guo et al. (2013)
Yam	*Dioscorea sp.*	+/++	+/++/+++	+	Tör et al. (1993, 1998), Shu et al. (2005), Shi et al. (2012), Anike et al. (2012) and Zhou et al. (2018a, b)

[a]"+" Protocols available for organogenesis; "++" Protocols available for somatic embryogenesis; "–" not available

[b]"+" Protocols available for *Agrobacterium*-mediated transformation; "++" Protocols available for particle bombardment transformation; "+++" Protocols available for PEG or electroporation; "–" not available

[c]"+" available; "–"not available

of gibberellin. It has been proven that mutation of this gene (383-base-pair deletion) has caused a semi-dwarf phenotype in rice (Sasaki et al. 2002). In the banana study, five GA20ox2 homologous genes were identified in the cultivar "Gros Michel", and two sgRNAs were designed targeting exon 2 of each gene. At the end, seven mutant lines with semi-dwarf phenotype were obtained showing short insertions in the target regions (Shao et al. 2019). This study is important because generating dwarf or semi-dwarf fruit plants could have agronomical advantages like an easier maintenance, and harvesting. However, it has to be considered that inducing this phenotype does not affect yield or generate adverse effects in plants.

Cacao

Theobroma cacao L. is a diploid tree endemic to tropical jungles in South America (Argout et al. 2011). The seeds are used as a raw material for the chocolate industry, which is estimated at 90 billion dollars per year and it is grown by approximately 6 million small holders around the world (Anga 2014; Wickramasuriya and Dunwell 2018). Recent advances in tissue culture, and genetic transformation applications have allowed cacao improvement using modern biotechnological tools (Wickramasuriya and Dunwell 2018) (Table 2). A previous study using microRNA targeting the *TcNPR3* (Non-Expressor of Pathogenesis-Related 3) showed that knockout of this gene induce resistance to infection with *Phytophthora capsici* (Shi et al. 2013). In this sense, CRISPR/Cas9 was used to knockout the *TcNPR3* gene in this crop. The system was first tested in leaves and confirmed the resistance to *P. tropicalis* and an elevated expression of downstream defense genes. Afterward, stable transformation and edition of somatic embryos was performed to analyze the resistance in the whole plant (Fister et al. 2018). The availability of the draft genome of *Theobroma cacao* (Argout et al. 2011; Motamayor et al. 2002) could make possible in a near the application of genome editing

technologies for the modification of genes associated with biotic, and abiotic stress, and improvement of bean quality.

Cassava

Manihot esculenta is a tropical crop of high economic importance and nutritional value as a source of carbohydrates for human consumption. Moreover, it is a staple food for 800 million people around the world and it is cultivated mainly by small farmers (Fondong and Rey 2018). Therefore, genetic improvement of this crop is very important. Nevertheless, conventional breeding and genetic engineering are very challenging (Fondong and Rey 2018). In this sense, genome editing technologies offer an opportunity to complement and accelerate conventional and modern (tissue culture and genetic transformation) breeding (Table 2) (Chavarriaga-Aguirre et al. 2016; Fondong and Rey 2018).

As a proof-of-concept, *MePDS-1* and *MePDS-2* genes were edited by delivering the CRISPR/Cas9 components via *A. tumefaciens* in friable embryogenic callus of cultivar 60444 and TME 204. The altered phenotype (albino or partially albino) was observed in 97.1% and 98.9% of the lines obtained for *MePDS-1* and *MePDS-2,* respectively. Sequence analysis showed that 100% of the examined lines carried mutations on the target site, and found out that plants with complete albinism had homozygous mutations while the chimeric plants had heterozygous mutations (Odipio et al. 2017). This study and the availability of the cassava draft genome (Prochnik et al. 2012) open the possibility to use the CRISPR/Cas9 technology to confer virus resistance, and drought tolerance, increase the protein and amylose content, and reduce the content of hydrogen cyanide (Fondong and Rey 2018). For example, CRISPR/Cas9 has already been used to generate cassava plants resistant to *Potyviridae* family viruses that cause Cassava Brown Streak Disease (CBSD) (Gomez et al. 2018). For this, the target genes were two isoforms of the *IF4E* gene (*nCBP1* and *nCBP-2*). Plants with both isoforms mutated showed a lower incidence of symptoms or delayed symptom onset, and a lower incidence of root necrosis (Gomez et al. 2018).

Additionally, glyphosate resistance was achieved by editing the endogenous promoter and first two exons of the gene *EPSPS* in embryogenic calli of TME 419 cassava cultivars using *Agrobacterium tumefaciens*. Homologous recombination (HR) or non-homologous end-joining repair mechanisms were employed for correcting the 3.2-kb deletion cause in the locus. As a result, six edited lines showed glyphosate resistance. This study demonstrated that the highest recovery of edited events was obtained using the HR mechanisms and opens the possibility for further development of glyphosate traits in other crops (Hummel et al. 2018).

On the other hand, AC2 and AC3 viral genes were also targeted using CRISPR/Cas9 in order to confer resistance to geminivirus (Mehta et al. 2019). In the past, resistance to geminivirus was achieved targeting AC1 viral gene through RNAi (Vanderschuren et al. 2009). Nevertheless, the results in the CRISPR/Cas9 study showed that during the inoculation in the greenhouse an effective resistance was not achieved. Moreover, it was shown that some virus genomes develop a conserved mutation conferring resistance to the excision of CRISPR-Cas9 (Mehta et al. 2019). This research is important because it shows that viruses could evolve to escape from antiviral CRISPR transgenics plants.

Citrus

Citrus fruits are economically important, especially in tropical zones (Kumar et al. 2018). Between 2012 and 2013 an average of 119.164 thousand tons of citrus were produced worldwide (FAO 2015). Various protocols for organogenesis and somatic embryogenesis, as well as, transformation techniques, including *Agrobacterium*-mediated transformation, particle gun bombardment and PEG-mediated transformation has been successfully developed and applied for the breeding of some species of this genus (Singh and Rajam 2009). Also, advances in sequencing of sweet orange (*Citrus sinensis*) (Xu et al. 2012) allow the application of genome editing technologies for the improvement of this crop.

The first report of CRISPR/Cas9 in citrus was made in sweet orange (*Citrus sinensis*, Valencia cultivar) where the *CsPDS* gene was edited by infiltrating leaves with *A. tumefaciens*. Mutation rates of 3.2% to 3.9% were obtained, with no evidence of off-target sites. In this study, 11 types of InDels were obtained, among which deletions of 1 bp to 12 bp were predominant (Jia and Wang 2014).

Moreover, the mutation efficacy of CRISPR/Cas9 has been improved by editing the *CsPDS* gene using the YAO promoter (promoter of the active meristem of the apical apex) instead of CaMV35S for Cas9 expression. As a result, 65% of plants displayed an altered phenotype, of which 85% were albino (Zhang et al. 2017b). Another study showed that thermal stress (37 °C) promoted higher levels of CRISPR/Cas9 mutations in the *CsPDS* gene and most of the transformed plants with CRISPR plasmid and subjected to thermal stress showed the expected albino phenotype. This study concludes that temperatures closer to the optimal growth temperature of *S. pyogenes* (37 °C) favored higher Cas9 activity (LeBlanc et al. 2018). Despite the promising results of this study, thermal stress could only work in species or varieties with high tolerance to heat stress otherwise it can generate physiological problems, such as foliar senescence and abscission, inhibition of shoot and root growth, reduce of the photosynthesis rate (Nievola et al. 2017).

On the other hand, several studies demonstrated the feasibility to use CRISPR/Cas9 for inducing biotic resistance plants in the Citrus genus. In this sense, this system was used to modify the *CsLOB1* gene, which is responsible for susceptibility to Citrus Canker disease produced by *Xanthomonas axonopodis* pv *citri* bacteria, in Duncan grapefruit (*Citrus x paradise*). As a result, several genetically modified lines were obtained with a mutation efficiency of 23.80% to 89.36%. In some of these lines, plants inoculated with the pathogen displayed delayed and milder symptoms of the disease than the wild type plants (Jia et al. 2017, 2019). In *Citrus sinensis* the promoter of *CsLOB1* gene was also edited by using five sgRNAs to modify $EBE_{PthA4,}$ which is an effector binding element to the promoter of the *CsLOB1* gene in both alleles and activates its expression. Mutation rates of 11.5% to 64.7% were obtained. In four mutant lines, resistance to citrus canker disease was higher than in wild type plants and two of these lines showed no symptoms of the disease (Peng et al. 2017). The PthA4 effector binding elements (EBEs) in the *CsLOB1* promoter was also edited in Duncan grapefruit but edited transgenic plants developed canker symptoms similarly as wild type and the authors concluded that mutation in the promoters of both alleles of *CsLOB1* is probably needed to generate citrus canker-resistant plants (Jia et al. 2015).

Coffee

Coffee (*Coffea* spp.) is a crop of great relevance worldwide for its high value as a beverage (Alemayehu 2017) and for being one of the most important agricultural products, occupying the second place in international trade after oil (Labouisse et al. 2008). This crop is mainly cultivated in tropical and subtropical regions of the world deriving directly or indirectly the income of more than 125 million people (Tran et al. 2016). Previously, modern biotechnology tools (such as organogenesis, somatic embryogenesis, and genetic engineering) have been used to confer insect and herbicide resistance, and reduce the caffeine content (Ribas et al. 2006).

In a first attempt, Breitler et al. (2018) designed an algorithm, called CRIP (Coffee gRNA Identification Program), to detect target sites in the diploid *C. canephora* genome for CRISPR/Cas9 editing. As a result, 8.145.748 possible sgRNAs were found for *C. canephora* genome. These authors edited the *CcPDS* gene in embryogenic calluses via *A. tumefaciens* mediated transformation. Sequencing revealed that 30% of the modified plants displayed a mutation; however, none of the plants presented a completely albino phenotype and some anomalies like leaf size (very small) and pigmentation (yellowish, chlorosis or anthocyanins) were also observed in mutated plants.

Previously, the genetic transformation of coffee has been employed as a tool for the validation of gene function, and for the production of transgenic crops with agronomical important characteristics (Mishra and Slater 2012). Nevertheless, the application of the genome editing technologies in this crop requires knowledge of the specific target genes that will be modified. In this sense, the recent release of the Arabica (*C. arabica* L.) (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Carabica_er), and robusta (*Coffea canephora*) (Denoeud et al. 2014) genomes opens the possibility to modify genes related with cup quality, resistance to biotic and abiotic factors, or production.

Cucumber

Cucumis sativus L. is one popular member of the Cucurbitaceae family with high economic value (Huang et al. 2009; Maurya et al. 2015). Biotechnological methods utilized in the genus *Cucumis* for genetic improvement include embryo rescue, in vitro pollination, protoplast isolation, culture and fusion, organogenesis, somatic embryogenesis, *Agrobacterium* and particle gun-mediated transformation, and electroporation (Navrátilová et al. 2011; Wang et al. 2015). Moreover, the genome of *Cucumis sativus* var. *sativus* L. has been sequenced (Huang et al. 2009), providing useful information for improving cultivars with biotic and abiotic resistance and horticultural traits (Wang et al. 2015). Although significant progress has been made for genetic transformation of cucumber, it is still necessary to increase regeneration and transformation efficiency and to explore other methods that allow genetic improvement without modifying desirable characteristics of the cucumber plants (Wang et al. 2015). In this sense, genome editing technologies could be considered as an alternative for genetic improvement of this crop. In a first attempt, CRISPR/Cas9 technology was used to generate virus resistant cucumber plants by editing the *eIF4E* gene (Chandrasekaran et al. 2016). This gene encodes an essential translation factor essential for the life cycle of the *Potyviridae* (Piron et al. 2010). As a result, the homozygous edited lines showed resistance to different viruses, including cucumber vein-yellowing virus (CVYV); whereas, the heterozygous mutants were susceptible just as the unedited control plants (Chandrasekaran et al. 2016). An attempt has been made to improve the efficiency of CRISPR/Cas9 in this crop by using endogenous U6 protomers for sgRNA expression. For this, the *CsWIP1*, *CsVFB1*, *CsMLO8,* and *CsGAD1* genes were targeted for mutation (Hu et al. 2017b). As a result the transformation efficiency was 1% which was better than the previous study made by Chandrasekaran et al. (2016).

Maize

Zea mays is an economical important crop used as food and feed as well as for bioenergy production, and industrial materials (Guo et al. 2018). Advances in in vitro regeneration and genetic transformation protocols, as well as the availability of the genome sequence have allowed the application of modern biotechnology for the genetic improvement of this crop (Table 2).

In order to create plants with better nutritional values, the gene *ZmIPK* (phosphoinositide 3-kinase) was edited by CRISPR/Cas9 with the purpose of reducing the phytic acid concentration in seeds (Liang et al. 2014). Phytic acid is an anti-nutrient as it reduces the bioavailability of important micronutrients; also it is a compound that cannot be digested and can also cause environmental pollution (Perera et al. 2018). Two sgRNAs were designed to edit the *ZmIPK* gene and mutation efficacy of 16.4% and 19.1% was achieved, respectively. Unfortunately, this study does not report whether the edited plants had lower levels of PA in the seeds (Liang et al. 2014).

Svitashev et al. (2016) successfully introduced the CRISPR/Cas9 system as ribonucleoproteins (RNP) into maize tissue via particle bombardment for editing the genes liguleless1 (*LIG*), acetolactate synthase (*ALS2*), and two male fertility genes (*MS26* and *MS45*). These genes were chosen in order to compare the results with a previous study made by the same authors where they used CRISPR plasmids instead of RNPs (Svitashev et al. 2015). As a result, these authors found fewer off-target mutations when RNPs were used instead of plasmids.

Also in maize, Char et al. (2016) edited two polymorphic Argonaute (Ago) genes, *ZmAgo18a* and *ZmAgo18b,* implicated in interference-RNA biogenesis in anthers. The gene for dihydroflavonol 4-reductase (*a1*) enzyme and its homolog (*a4*) were also modified at two different sites, following the same procedure used for the AGO genes. In order to do that, they designed two sgRNA for each gene. Mutations were observed in one or two alleles of the Ago genes in the edited lines at percentages higher than 70%. The mutation frequency of the *a1* and *a4* genes was higher than 15% (Char et al. 2016).

For abiotic stress tolerance, the CRISPR/Cas9 system was used to insert the constitutive GOS2 promoter in the 5′ UTR end of the *ARGOS8* gene through HDR. For this, the CRISPR/Cas9 system and DNA repair template (GOS2 promoter) were introduced into immature maize embryos by particle gun bombardment. The overexpression of this gene reduced ethylene sensitivity and increased grain yield in drought conditions in comparison with wild type plants (Shi et al. 2017). This study is a clear example of how CRISPR/Cas9 can be used in other ways besides inducing gene knockout and mutations.

Regarding herbicide resistance, specific amino acid in the *ALS2* gene (proline to serine at position 165) was changed using CRISPR/Cas9 to generate resistance to the herbicide chlorsulfuron. The difference with a similar study in rice (Sun et al. 2016) was that these authors introduced the CRISPR/Cas9 system as a ribonucleoprotein (RNP) together with a DNA template of 127 nt for HDR repair system. As a result, all edited plants with this amino acid change were chlorsulfuron-resistant (Svitashev et al. 2016).

A more recent application is the generation of a maize haploid inducer line carrying a CRISPR/Cas9 cassette for agronomic traits. In this study, the authors introduced a cassette for editing the *ZmLG1* gene (liguleless1), which loss-of function mutants dramatically reduces leaf angle. Also, they introduced a cassette for the *UB2* gene, which is involved in regulating tassel branch number. After getting the haploid modified lines with CRISPR/Cas9 cassettes, they were crossed with the B73 line. As a result, the next generation manifested mutant phenotypes and presented the expected editions (Wang et al. 2019a, b, c, d).

The CRISPR/Cas9 system has also been used for studying the fertility restoration of maize CMS-C (Type C Cytoplasmic male sterility) (Jaqueth et al. 2019), the role of *ZmACD6* for *Ustilago maydis* resistance (Zhang et al. 2019), for the characterization of Phytochrome-Interacting Factor (PIF) genes *Zmpif3, Zmpif4,* and *Zmpif5* (Wu et al. 2019), for the induction of haploid plants thought the mutation of the *ZmDMP* gene (Zhong et al. 2019), for the study of *ZmbZIP22* transcription factor (Li et al. 2018b) and *ZmCCT9* gene (Huang et al. 2018), among others.

Rice

Oryza sativa L. is one of the most important crops in tropical and subtropical humid regions around the world. This crop feeds approximately 50% of the world population (Wang et al. 2016; Kumar and Tuteja 2012). In the last decades, in vitro regeneration and genetic transformation protocols, as well as the development of genomic resources have allowed the application of modern biotechnology for the genetic improvement of this crop (Table 2).

Since first applications of the CRISPR/Cas9 technology in rice (Miao et al. 2013; Shan et al. 2013; Xie and Yang 2013), the number of studies in this crop keep growing at a fascinating rate (Fig. 3). Here we present only some applications of this genome editing technology and more detailed review can be consulted in Romero and Gatica-Arias (2019).

A concept proof research on the use of CRISPR/Cas9 in rice was reported by Miao et al. (2013). These authors used the modified *GUS* (β-Glucuronidase) reporter gene in a transgenic line to evaluate whether the CRISPR/Cas9 system was capable of generating a DNA double-strand break (DBS) in plant cells. As a result, expression of the *GUS* gene

was achieved by excising repeated regions of the *GUS* gene either using the chimeric sgRNA or the two RNA molecules crRNA-tracrARN. Moreover, in the same study, the oxygenase gene in chlorophyll a (*CAO*1), whose mutants have a lighter green color, and the *LAZY* gene, which regulates shoot gravitropism were edited. Mutations in T1 generation were observed in 83.3% of the edited lines for *CAO1* gene and 91.6% for LAZY gene (Miao et al. 2013).

In one of the first studies in rice, the *OsMPK5* (mitogen-activated protein kinase) gene has been edited in protoplasts (Xie and Yang 2013). This gene codes for a kinase protein activated by the stress response (Jagodzik et al. 2018). Using three sgRNAs for different sites in the gene, the mutations average was 3%-8% on the target sites. Off-target mutations were detected on a site in chromosome 12 with an incidence of 1.6% (Xie and Yang 2013).

The CRISPR/Cas9 system has been used to generate new rice varieties with tolerance to abiotic stress (Cordones et al. 2017). For example, CRISPR/Cas9 system has been used as a strategy to develop new varieties with reduced cesium (Cs^+) accumulation. Cesium is a toxic metal present in polluted soils that affects rice production and that has become a problem, especially in places like Fukushima in Japan, where soils are contaminated with radioactive cesium (Nihei et al. 2015). In this sense, Cordones et al. (2017) inactivated the Cs+-permeable K+ transporter (OsHAK1) using two sgRNAs targeting exons 1 and 2 of this gene and obtained a mutation rate of 83%. As a result, the plants carried homo- or bi-allellic frameshift mutations causing the inactivation of the *OsHAK1* gene. Also, Cs^+ uptake in roots of the edited lines was 35 times lower than in wild type plants (Cordones et al. 2017).

There are other rice genes which are expected to be modified in the future using CRISPR/Cas to generate new varieties resistant to abiotic stress. For example, overexpression of the gene *OsPRX2,* which encodes 2-Cys peroxiredoxins, contributes to stomatal closure and increases tolerance to potassium deficiency (Mao et al. 2018). Thus, this gene could be overexpressed by CRISPR/Cas9 system using a similar approach to the study in maize made by Shi et al. (2017).

On the other hand, in the case of biotic stress, CRISPR/Cas9 technology has been used to obtain resistance to rice blast disease (*Magnaporthe oryzae*) by editing the *OsERF922* gene, which acts as a negative regulator of resistance to this disease. The frequency of mutations on the target site in edited T_0 was 42%. Afterwards, the inserted transgenes (genes from the CRISPR/Cas9 plasmid) were eliminated through segregation, but the induced mutations remained in the T1 and T_2 generations. At the end, lesions caused by the pathogen significantly decreased in the edited lines in comparison to the control plants at the seedling and bunch stages (Wang et al. 2016).

In the case of herbicide resistance, the *ALS* (*acetolactate synthase*) gene was edited to achieve plants resistant to chlorsulfuron and bispyribac sodium (Sun et al. 2016). In order to do this, CRISPR/Cas9 plasmid was designed with two sgRNAs under the control of the U3 promoter and a donor template for HDR. The donor sequence consisted of a 476 bp fragment which, after insertion, would substitute two amino acids (W548 to L, and S627 to I) in the coding protein. In all of the analyzed lines, the W548 amino acid had been substituted with a L. The plants were sprayed with bispyribac sodium for 36 days and, as result, control non-edited plants died while the edited plants grew normally (Sun et al. 2016).

To enhance nutritional value, the CRISPR/Cas9 system was used to mutate the *SBEI* gene and its homolog *SBEIIb*, which encode the starch-branching enzyme (Sun et al. 2017). The first and third exons were edited for *SBEI* gene and *SBEIIb* gene, respectively. As a result, 32 and 21 mutants were obtained from 40 and 30 transgenic plants for the SBEI gene and *SBEIIb* gene, respectively. As a result, the *SBEII* mutants had a higher proportion of long unbranched amylopectin chains and an increased content of amylose (25%) and resistant starch (9.8%) (Sun et al. 2017). This is important because a cereal with higher amylose content is a good source of resistant starch, which is beneficial to human health (Higgins 2004). Also, the CRISPR/Cas9 technology has been used to disrupt one of the three fatty acid desaturase 2 genes, *FAD2-1*, in order to generate plants with high oleic and low linoleic content in seeds. The content of oleic acid increased to more than twice in homozygous mutants and increase 10% in heterozygous mutants compared to non-edited control plants (Abe et al. 2018). This study showed that targeting only the genes with dominant or higher expression could be enough to change agronomic traits.

In several studies, the CRISPR/Cas9 technology has been used to generate knockout mutants in order to study genes and their molecular roles. For example, each rice SR (Serine/arginine-rich proteins) locus has been target to produce single knockouts (Butt et al. 2019); *OsPIN1* mutants have also been created to study the role of this gene (Li et al. 2019a, b). Some other mutants generated by CRISPR/Cas9 in rice include: *OsDGD2β* mutants (Basnet et al. 2019), *OsPPa6* mutants (Wang et al. 2019b), *PE-1* mutants (YuChun et al. 2019), *OsLHT1* mutants (Wang et al. 2019c), *OsPRP1* mutants (Nawaz et al. 2019), *OsPKS2* mutants (Zou et al. 2018), *OsPT4* mutants (Ye et al. 2017) (Fig. 3). A recent study demonstrated that using a soybean heat-shock protein gene promoter for Cas9, it was possible to successfully develop a heat-shock -inducible CRISPR/Cas9 system in rice. This system offers the advantage of increasing the mutation rate and off-target effects were low or not found (Nandy et al. 2019). In this sense, this system culd be applied

for the improvement of the CRISPR/Cas9 platforms for targeting genes in other crop of topical origin.

Sorghum

Sorghum bicolor L. is the fifth cereal more important in the world providing food for millions of people in subtropical and semi-arid regions in Africa and Asia (Hariprasanna and Rakshit 2016; Che et al. 2018). Regarding the application of modern biotechnology for genetic improvement of this crop, several protocols for tissue culture, and genetic transformation have been developed (review by Girijashankar Swathisree 2009) (Table 1). Additionally, the genome of *Sorghum bicolor* has been sequenced (Paterson et al. 2009), and it represents a valuable resource for genetic improvement of this crop using modern biotechnological tools, such genome editing technologies.

In a first incursion, Jiang et al. (2013) verified the activity of the CRISPR system within the cells of immature sorghum embryos with a plasmid that encoded the Cas9 gene, one green fluorescent protein (GFP) gene, one sgRNA, and one out of frame red fluorescent protein gene (*DsRED2*). The sgRNA/Cas9 complex was designed to excise the *DsRED2* gene and achieved an active gene via NHEJ repair. In their results, five of eighteen groups of transformed cells with positive GFP expression included sectors with *DsRED2* expression (Jiang et al. 2013).

Five years later, the CRISPR/Cas9 system was used to knock out the endogenous histone H3 gene (*b-CENH3*) in immature sorghum embryos. It is known that this gene is involved in the regulation of chromosome segregation; therefore, its knockout can induce haploids. These researchers obtained lower regeneration percentages (8 to 16%) and the result was attributed to the possible lethality of the biallelic knockout of the *Sb-CENH3* gene. In general, the inactivation efficacy in edited immature embryos was between 1% and 5%, and biallelic knockout was not observed (Che et al. 2018).

On the other hand, the CRISPR/Cas9 system has been used to increase the nutritional value of this crop by increasing the digestibility and protein quality (Li et al. 2018c). Kafirins (sorghum prolamins), especially a-Kafirin, are the most abundant storage proteins in the endosperm. Nevertheless, these proteins form bodies with poor digestibility (Oria et al. 2000). In order to increase the protein quality, the *k1C* gene families, which encode most of kafirins, were targeted. As a result, the T1 and T2 generations showed lower α-kafirin accumulation compared to non-edited control plants, also protein digestibility in the edited lines increased 1.3- to 1.5-fold (Li et al. 2018c).

Recently, Char et al. (2019) used the CRISPR/Cas9 system for targeting the genes *SbFT* and *SbGA2ox5*. As a result, the *SbFT* mutant plants exhibit significant difference in flowering time. Also, Liu et al. (2019b) edited the genes cinnamyl alcohol dehydrogenase (*CAD*) and phytoene desaturase (*PDS*) though the particle bombardment of the CRISPR/Cas9 components. In order to establish an efficient genome editing system in sorghum, these authors mentioned that it is important to have an efficient transformation and delivery system for the effective expression of the sgRNA and Cas proteins.

Tomato

Solanum lycopersicum is a vegetable crop of high economic importance and widely cultivated around the world, including tropical and subtropical regions (Hanson and Yang 2016). Conventional breeding of tomato is challenging due to the narrow genetic diversity existing in the germoplasm of cultivated tomatoes, the crossing incompatibility among cultivated and wild species, and the long time required for the introgression (Chaudhary et al. 2019). In the last years, efforts have been made to develop efficient tools for plant tissue culture, plant transformation, and sequence the genome as a complement for the conventional breeding programs (Table 2). The CRISPR/Cas9 system has been employed in *S. lycopersicum* and its wild relative *S. pimpinellifolium*. In this sense, Pan et al. (2016) edited the *SPIDS* (phytoene desaturase) and *SIPIF4* (the PIF4 factor that interacts with phytochrome) genes with two independent sgRNAs for each gene. The mutation efficiency was 54.54% and 57.14% for the two sgRNAs in the *SIPDS* gene, and the mutants showed different types of albinism. In the case of the *SIPIF4* gene, the mutation efficacy for the two sgRNAs was 84.00% and 89.47%, respectively. This study demonstrated the ability to mutate genes with high efficacy in tomato. The researchers concluded that high percentages of guanine and cytosine in the sgRNAs led to higher efficacy of editing.

Dahan-Meir et al. (2018) used CRISPR/Cas9 system in the Micro-Tom variety to edit the carotenoid isomerase (*CRTISO*) and phytoene synthase 1 (*PSY1*) genes of the carotenoid biosynthetic pathway. These genes were chosen as targets because of the easily detectable phenotype in the mutants. The *crtiso* mutant had orange fruits, yellowish young leaves, and pale petals, while the *psy1* mutant displayed only yellow fruits. Mutation efficacies of 90.4% and 56.4% were obtained in the *CRTISO* and *PSY1* loci, respectively. These authors also used a tomato variety with defective *CRTISO* genes (with one deletion) and inserted a template of the gene without the mutation by HDR. The deletion was repaired in 25% of the genetically edited plants.

In another study, D'Ambrosio et al. (2018) confirmed the efficacy of CRISPR/Cas9 as a rapid method for generating allelic variants in tomato. For this, the authors edited the marker genes *Psy1* (phytoene synthase 1) and *CrtR-b2* (beta-carotene hydroxylase 2), which are key genes in the

biosynthesis of carotenoids and their knockout generates visible phenotypes in fruit (silencing of *Psy1* leads to yellow coloring), and flowers (silencing of *CrtR-b2* results in white petals). Two sgRNAs were designed for exon 1 of each gene and the plasmids were introduced in tomato cotyledons by *A. tumefaciens*. The mutated phenotype was observed in 69% of the modified plants.

Generating tomato plants tolerant to abiotic stress, such as heat stress, is an important objective in the breeding programs since higher temperatures cause physiological and biochemical damages. For example, heat stress in tomato decrease shoot weight, accumulate soluble phenols, and generate lowest peroxidase and polyphenol oxidase activity (Rivero et al. 2001). In this case, the CRISPR/Cas9 system was used to edit the *SlMAPK3* gene in tomato (Wang et al. 2017). It has been previously demonstrated that mitogen-activated protein kinases (MAPKs) are genes involved in plant response to environmental stresses (Jalmi and Sinha 2015). In this study, sgRNA were designed to target two different sites of third exon with a mutation efficiency of 41.82%. As a result, editing types were almost small deletions, insertions, or substitutions. The authors of this study concluded that *slmapk3* mutants had a lower tolerance to drought stress compared to non-edited plants (Wang et al. 2017). However, a more recent study of the same mutants lines also showed that knocking out *SlMAPK3* gene enhances tolerance to heat stress in tomato plants because mutant lines showed less severe wilting and less membrane damage, lower reactive oxygen species (ROS) contents, and higher levels of antioxidant enzymes compared to non-edited plants (Yu et al. 2019). As a conclusion, both studies showed that these mutant lines were more susceptible to drought stress but more tolerant to heat stress than non-edited plants.

Regarding biotic stress, Tashkandi et al. (2018) used CRISPR/Cas9 to target the Tomato Yellow Leaf Curl Virus (TYLCV) genome to produce virus resistant plants. The authors generated tomato plants expressing sgRNA targeting the CP (viral coat protein) or RP (replicase) sequences of TYLCV. Six lines were obtained for each target (CP or RP) expressing the Cas9 gene. This study is important because target genes for editing were those of the virus and not those of the plant. Nevertheless, the problem of this approach is that next generations must keep the transgenes in order to maintain the resistance.

Tomato is also highly vulnerable to *Phelipanche aegyptiaca*, a weedy root parasitic, which causes many economic problems. There are no effective methods to control it (Hershenhorn et al. 2009). It has been proven that strigolactones in host roots stimulate seed germination of parasitic plants, such as *Phelipanche aegyptiaca*. This phytohormones are derived from carotenoids via a pathway involving the *carotenoid cleavage dioxygenases 7 (CCD7),* and *CCD8* (Aly et al. 2014). The CRISPR/Cas9 system was used to edit the *CCD8* gene in order to generate mutant lines resistant to this parasite. For that, a sgRNAs were designed for targeting exon 2 of this gene, and several mutated lines were obtained with no off-target mutation. The mutated lines in *CDD8* gene presented insertions and deletions in the target site and also many morphological changes like dwarfing, excessive shoot branching, and adventitious root formation compared to non-edited plants. Also mutants showed a reduction in parasite infection compared to non-edited plants (Kumar et al. 2019).

Regarding the nutritional value of tomato, the γ-aminobutyric acid (GABA) is a health-promoting functional compound that helps people with nervousness, depression, and insomnia (Liu et al. 2015). In this crop, GABA has been manipulated through gene editing to modify six targets in five key genes for GABA metabolism (*GABA-TP1, GABA-TP2, GABA-TP3, CAT9 and SSADH*) using the pYLCRISPR/Cas9 multiplex system (Li et al. 2017). The edited efficiencies of the six target sites were 50.0%, 56.8%, 0.0%, 46.6%, 6.8%, and 9.1%, respectively. As a result, heterozygous mutations were the most predominant and homozygous mutations were least abundant. Plants with mutations in four genes at the same time were obtained and not off-target changes were found. Almost all edited plants had higher GABA accumulation in leaves compared to wild type control plants. Specifically, the edited lines with four knocked-out genes at the same time had the highest GABA levels. This study demonstrated the possibility to efficiently edit multiple genes at the same time in a metabolic pathway by creating multi-site knockout mutations (Li et al. 2017).

The CRISPR/Cas9 gene editing technology has also been implemented to enhance agronomic traits, such as parthenocarpy (seedless fruit without prior fertilization) in tomato. In order to achieve this, a knock down of *SlIAA9* gene, which is important for control of parthenocarpy, was performed targeting exon 2 in the commercial cultivars Micro-Tom and Ailsa Craig using three different sgRNAs. The mutation rates in the Micro-Tom were 11.1% for sgRNA1, and 40.0–46.2% for sgRNA2, and sgRNA3, respectively. Parthenocarpic fruits were observed in the mutants, but not in wild type plants. Furthermore, fruit morphology of mutant plants was very similar to wild type (Ueta et al. 2017). Induced parthenocarpy is important because it confers many agricultural and industrial benefits. For example, pollination and fertilization are affected by the environment, so parthenocarpy could help to stabilize crop production in unstable environments. Also, seedless fruits facilitate many industrial processes of the fruits (Pandolfini 2009).

Additionally, the CRISPR/Cas9 system has been used in many studies to determine the functions and molecular roles of genes and regulatory elements in tomato. Some recent examples are: *GID1* mutants (Illouz-Eliaz et al. 2019), *SlMYB21* mutants (Schubert et al. 2019), *LOL1* mutants (Borovsky et al. 2019), *AP2a* mutants (Wang et al.

2019d), *SlNPR1* mutants (Li et al. 2019b), and SlMET1 mutants (Yang et al. 2019).

Genome editing has also been used for de novo domestication of plants, such as wild *Solanum pimpinellifolium* (currant tomato). For this, Zsögön et al. (2018) designed six sgRNAs to target six important loci that control important traits for yield and productivity, like plant growth habit (*SELFPRUNING*), fruit shape (*OVATE*) and size (*FASCIATED FRUIT WEIGHT* 2.2), fruit number (*MULTIFLORA*), and nutritional quality (*LYCOPENE BETA CYCLASE*). Four of the six targeted loci were edited successfully in 50 lines from T1. The edited loci were SELF-PRUNING (SP), OVATE (O), FRUIT WEIGHT 2.2 (FW2.2), and LYCOPENE BETA CYCLASE (CycB). These authors demonstrated the feasibility of using CRISPR/Cas9 system for rapid domestication of wild plants that could be extended to other crops of tropical origin.

Watermelon

Citrullus lanatus is an economic important tropical fruit with an estimated worldwide production of 29.6 million tonnes. Moreover, watermelon contains several compounds (vitamins, minerals, and other antioxidants) which play an important role in human health (Reetu and Maharishi 2017). Regarding biotechnological tools, protocols have been developed for somatic embryogenesis (Compton and Gray 1993), biolistic-mediated transformation (Suratman et al. 2010), electroporation of zygotic embryos and nodular buds (Hema et al. 2004); organogenesis (Krug et al. 2005), and *Agrobacterium*-mediated transformation (Cho et al. 2008). Moreover, the draft genome of watermelon has been released (Guo et al. 2013). These advances open the possibility of applying new genome editing technologies might be impact positively in the watermelon genetic improvement. In a first approach, phytoene desaturase gene (*CIPDS*) in protoplasts and calluses of this crop were knocked out using the CRISPR/Cas9 system (Tian et al. 2017). In transformed protoplasts with two CRISPR plasmids editing efficacy was 52.0% and 40.7%, respectively. From the calluses transformed with *A. tumefaciens* with a third plasmid, 16 transformed lines showing albino or quimeric phenotypes and not off-target mutations were found (Tian et al. 2017). Later in this crop, CRISPR/Cas9 was used to generate resistant plants to herbicides by editing *ALS* gene with a base editing efficiency of 23% at T0 generation and not off-target mutations were found. At the end, some edited plants were not affected after treated with tribenuron while non-edited control plants were severely damaged at 14 days after treatment (Tian et al. 2018).

Yam

The Dioscorea genus include about 600 species but only *D. rotundata* and *D. alata* are an economic important crops proving around 90% of the world consumption. It has been used in traditional medicine, and is an important source of carbohydrates, vitamins, and minerals (Aighewi et al. 2015). Breeding programs of yam face some challenging due to the long breeding cycle, polyploidy, heterozygous genetic background, and clonal propagation. In the genus *Dioscorea* protocols for organogenesis (Anike et al. 2012), somatic embryogenesis (Shu et al. 2005), protoplast culture (Tör et al. 1998), *Agrobacterium tumefaciens*-mediated transformation (Shi et al. 2012); particle gun mediated transformation (Tör et al. 1993) have been reported. Moreover, the genome of *Dioscorea zingiberensis* has been recently analyzed (Zhou et al. 2018a, b). Therefore, genetic engineering and genome editing technologies are valuable tools for improvement of this crop (Tripathi 2018). Diosgenin, a compound isolated from the rhizomes, is a precursor of many steroid hormones used as anti-inflammatory, anti-allergic, and antioxidant drugs (Jesus et al. 2016; Zhang et al. 2018b). In a first incursion of the CRISPR/Cas9 technology in *Dioscorea zingiberensis*, the farnesylpyrophosphate synthase (*Dzfps*) gene, which encodes an essential enzyme involved in the biosynthesis of secondary metabolites like diosgenin, has been edited. As a result, 9 out of 15 genetically edited plants showed mutations on the target site, and the expression of the *Dzfps* gene and the content of squalene were significantly less than in the control plants (Feng et al. 2018). This study is important because editing genes involved in diosgenin biosynthesis could be an alternative to increase the production of this metabolite, especially when germoplasm resources with high diosgenin content are needed. The study revealed that CRISPR-Cas9 can be an effective approach for genome modification in yam allowing the development of cultivars with increased pharmacological compounds (such as sapogenin, alkaloids, diterpenes, and steroidals), resistance to diseases, pests, and herbicides (Tripathi 2018).

Design of sgRNA for genome editing of plant cells of crops of tropical origin

At the time of writing, multiple software's have been developed for predicting and designing CRISPR sgRNA constructs to target specific plant genomic loci. Nevertheless, all these tools are different and have some limitations. For example, some of them are user friendly and are available via web servers while others are not. Moreover, the genomes accessible in some tools are limited, and very few tools have been subjected to peer-review (Brazelton et al. 2015). Off-target effect determination is a challenging task and could be

minimized at the time of choosing the appropriate designed sgRNA. Therefore, tools that search the rest of the genome for off-target prediction and allow using alternate PAM sequences should be selected. In the case of the studies analyzed in this review several different computational tools were used for the design of the sgRNA (Table 1).

Various systems that may be used for delivery of CRISPR into plant cells of crops of tropical origin

Several methods are available for delivering CRISPR reagents to plant cells (Fig. 4). CRISPR components can be expressed in the plant cell genome using heterologous DNA or RNA transgenes, or integrated directly into the nuclei as ribonucleotide protein complexes. The delivery systems of the CRISPR components depend on several factors, such as plant species, research purposes, experience, and available equipment. Moreover, the commercialization of non-GM genome edited crops can be favored by using specific delivery systems that circumvent restrictive regulatory burdens (Lowder et al. 2016).

Agrobacterium mediated T-DNA delivery

Specific *Agrobacterium* transformation methods have been developed and optimized for different plant species, including crops of tropical origin (Wang 2015). The *Agrobacterium* transformation method uses pathogenic bacteria that have the ability to infect plant cells and transfer exogenous DNA horizontally or directly to the genome of the host plant (Mohammed et al. 2019).

Not surprisingly, *Agrobacterium* mediated T-DNA transformation method, followed by plant tissue culture dependent regeneration of stable mutants, and has been used for generating genome edited crops of tropical origin (Table 1). Nevertheless, the CRISPR systems have the advantage that desirable results can often be accomplished using transient expression in plant cells. Hence, it is not necessary the integration of the CRISPR directly into plant genomes.

In this sense, the delivery of CRISPR expression DNA cassettes into plant cells has been carried out using *in planta* transformation methods, such as floral dip in the model plant *Arabidopsis* (Feng et al. 2013; Jiang et al. 2013; Li et al. 2013) (Fig. 4a), and infiltration of tobacco somatic cells (Gao et al. 2015) (Fig. 4b). *In planta* methodologies allows that the transformation strategies could be used directly and quickly by being independent of in vitro tissue culture techniques. This strategy could be applied to recalcitrant crops of tropical origin.

Viral delivery

Plant RNA and DNA viruses offer a great potential for efficient delivery of CRISPR components into plant cells (Fig. 4c). *Beet necrotic yellow vein virus*-based vectors were employed to deliver *NbPDS* (*N. benthamiana* phytoene desaturase gene) guide RNAs for genome editing in tobacco transgenic plants expressing Cas9, leading to the generation of photo bleached phenotype in infected leaves (Jiang et al. 2019). Viral systems provide great opportunity for genome editing of crops of tropical origin as plants can be transiently infected moderately rapidly and viral replication of CRISPR components can spread to systemic infection of whole plants. Moreover, such systems offer a more facile and efficient delivery option compared to the laborious and highly technical process of *Agrobacterium*-mediated transformation and biolistic transformation. Nevertheless, the application of such systems is limited by the low editing efficiency (Lowder et al. 2016).

Plasmid delivery

Instead of being delivered by *Agrobacterium*, CRISPR components can be transferred into plant cells by expression plasmids using polyethylene glycol–calcium (PEG–Ca^{2+}) transfection of protoplasts or biolistic particle delivery using a gene particle gun (Fig. 4d, e). The first approach has been mainly used for rapid testing of CRISPR activity in plant cells and such assays have been performed in watermelon, rice, and maize (Table 1). Whereas the biolistics transformation system consists of firing micro particles at high speeds, usually of gold or tungsten, coated with the DNA fragments to the plant cells. This technique has been successfully applied to rice, and maize (Table 1). Both approaches are robust enough and generally can be applied to deliver CRISPR-Cas9 reagents to almost any crops of tropical origin.

Ribonucleotide protein complex delivery

Previously, ZFNs (zinc-finger nucleases) and TALENs (transcription activator-like effector nucleases) were successfully delivered as proteins into cells to mediate genome editing (Lowder et al. 2016). This approach consists in the delivery of preassembled Cas protein-gRNA ribonucleoproteins (RNPs) into protoplasts derived from somatic tissues by PEG–Ca^{2+}-mediated transfection or biolistic particle delivery (Fig. 4f). This technique has been successfully applied to plant protoplasts of rice (Woo et al. 2015) or into embryo cells by biolistic bombardment in maize (Svitashev et al. 2016). Nevertheless, the isolation and culture of protoplasts is a challenging task in most plant species and the frequency of obtaining genome-edited plants through biolistic

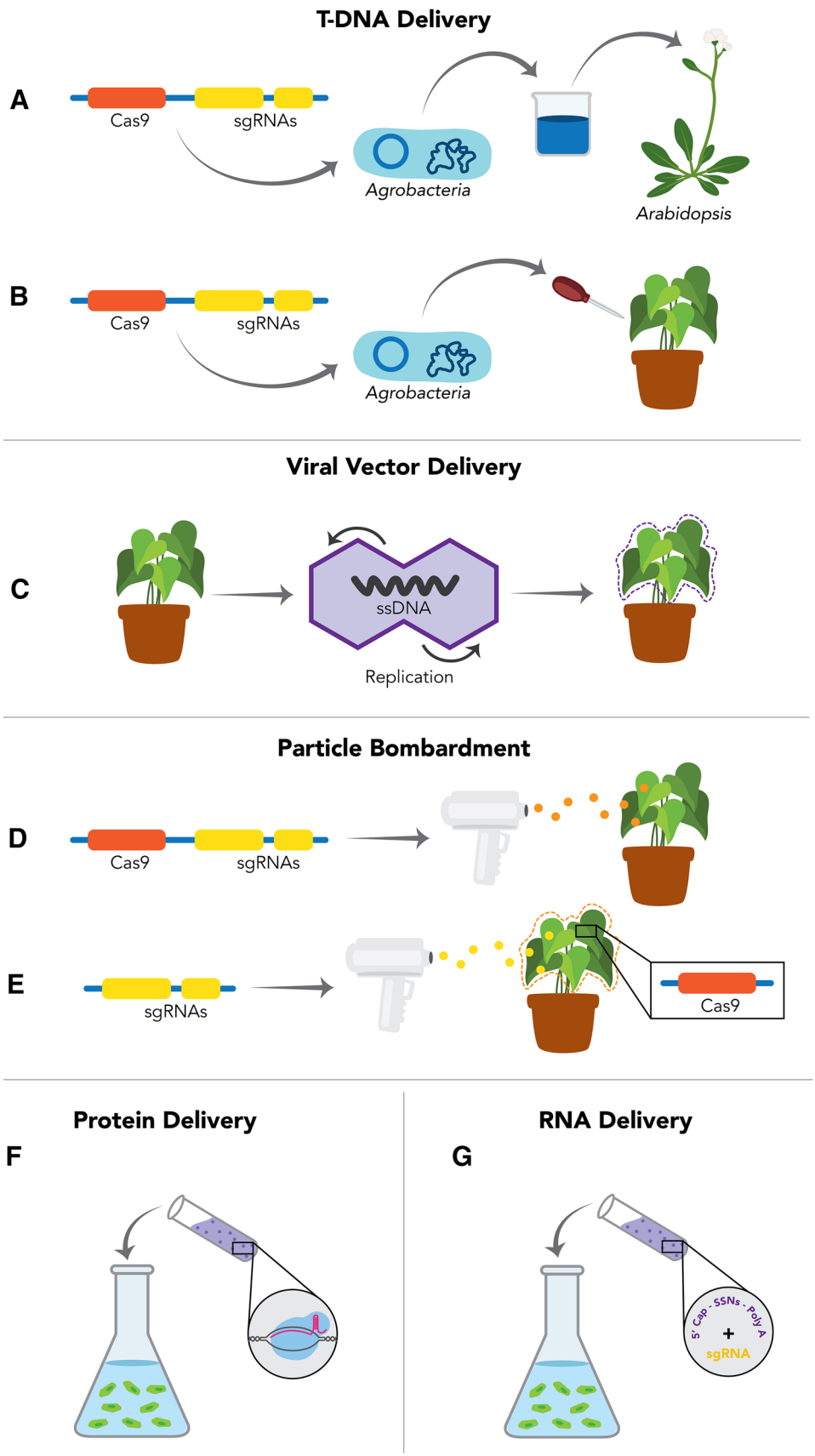


Fig. 4 Methods for the delivery of CRISPR components into plant cells and tissues. **a** Floral dip transformation with transgenic T-DNA carrying *Agrobacteria*. **b** Transient inoculation of plant leaf tissue or calli with *Agrobacteria* harboring Cas9 and gRNA T-DNA. **c** Viral vector delivery causes a transiently transformed plant (at left) to develop systemic infection upon viral capsid replication. **d** Transient gun bombardment of plant leaf tissue using a cassette containing Cas9 and gRNA or **e** delivery of gRNAs only to plant leaf tissues. **f** Delivery of ribonucleoprotein (RNP) complex to protoplasts using PEG transformation or **g** RNA delivery directly to protoplasts. *Source* Own elaboration based on Lowder et al. (2016)

bombardment is relatively low. Therefore, Toda et al. (2019) developed a system for the direct delivery of Cas9–gRNA RNPs into plant zygotes of rice produced by in vitro fertilization of isolated gametes (Toda et al. 2019). The RNPs delivery may offer advantages for certain applications such as avoiding potential GMOs regulatory laws or genome editing of asexually propagated crops, such as bananas (Lowder et al. 2016). Therefore, this plant-genome-editing system has enormous potential for the improvement of other important crop of tropical origin.

RNA delivery

This strategy consists in the transfer of RNA encoding genome editing components directly into the plant cells (Fig. 4g). This system has been used for the successfully delivery of mRNA transcripts of CRISPR-Cas9 and gRNA into wheat calli, although mutation frequencies were very low-1.1% (Zhang et al. 2016). Nevertheless, the RNA delivery could induce genome editing without transgene insertion into host genomes (Lowder et al. 2016). An additional benefit of this system could be the reduction of the probability of off-targeting that could negatively impact plant function or growth, since RNPs and mRNA degrade quickly after mutagenesis (Luo et al. 2015).

Screening of mutants

Various tools, including PCR (Polymerase chain reaction), sequencing PCR amplicons, next generation sequencing (NGS), whole genome sequencing (WGS), Southern blotting, DNA microarray, ELISA, genotypic and phenotypic screening, have been established and could be used to corroborate mutagenesis in genome edited plants (Grohmann et al. 2019). One of the most common, simply, cheaper, sensitive, and specific method for detection of mutations is PCR. This method requires information about the target DNA sequence and the design of complementary primers for the amplification of changes induced by genome editing. PCR-based methods have been used for screening of mutants generated by CRISPR in crops of tropical origin (Table 1). Short sequence changes, such as substitutions or InDels of few nucleotides, could be detected by real-time PCR or digital PCR (Grohmann et al. 2019).

Sanger sequencing of PCR amplicons and NGS are suitable methods for the detection of changes in the targeted gene, even if the modifications are small (Grohmann et al. 2019). Nevertheless, sequencing errors and bioinformatic analysis problems should be taken into account in order to avoid false positives results. These two methods have been used for the detection and corroboration of mutations in generated by CRISPR in crops of tropical origin (Table 1).

Moreover, WGS has been used for GMOs (Genetically Modified Organisms) detection, and might be adapted for genome-edited plants. This tool does not require prior information on a specific genetic modification and can be used as a detection approach for detection of off-target alterations. Nevertheless, efforts and costs are significantly increased compared to NGS. Also, it requires a high quality reference genome, derived from the parental plant, and bioinformatics capacity for the processing of millions of small DNA sequence reads. Furthermore, another limitation for the application of WGS is the size of the genome in question and the presence of repetitive sequences in the genome (Grohmann et al. 2019). This applies for a variety of crop plants of tropical origin, e.g., the genome of the arabica coffee (*Coffea arabica* L.) and maize.

DNA hybridization based methods, such as Southern blotting and DNA microarray, have been used for the detection of GMOs; nevertheless, the application for the detection of genome-edited plants is inappropriate since it requires a large amount of genetic material and the sensitivity is low compared to DNA-amplification and sequencing methods. Moreover, these methods are commonly used for the detection of longer altered nucleotide sequences and/or integrated foreign DNA, therefore, the detection of small or single nucleotide modifications are challenging (Grohmann et al. 2019).

Although, protein based-methods, such as ELISA, are available and have been used the characterization of GMO, the corroboration of changes induced by genome editing should be confirmed by subsequent DNA analyses (Grohmann et al. 2019). Similarly, metabolite-based methods allow the detection and identification of a wide variety of substances in a plant metabolite profile. Nevertheless, identified difference in the metabolite profile is no proof of a genetic modification since it is highly dynamic and fluctuating in response to developmental and environmental conditions (Grohmann et al. 2019).

Novel CRISPR technologies that may be used in breeding of crops of tropical origin

CRISPR/Cas12a system

This system, previously known as CRISPR/Cpf1, allows double strand breaks with efficiencies similar to those of CRISPR/Cas9. The nuclease Cas12a requires a small crRNA for its activity and the maximum efficiency and specificity is determined by 22 nt spacer (Schindele et al. 2018). Moreover, this nuclease identifies a T-rich PAMs located upstream of the guide and generated staggered ends (Schindele et al. 2018). In plants, LbCas12a from *Lachnospiraceae bacterium* and FnCas12a from

Francisella novicida, promoted mutagenesis by genome editing in rice and tobacco (Endo et al. 2016) and citrus (Jia et al. 2019). Also, Cas12a could be used as a specific DNA binding protein and downregulate gene expression as demonstrated in *Arabidopsis* (Tang et al. 2017). Therefore, Cas12a nucleases could be a potential option for targeting genes in crops of tropical origin, specifically those genes with low GC content.

CRISPR/Cas13 system

This system, previously called C2c2, recognizes and cleaves specific single strand RNA in eukaryotic cells, offering a wide range of applications in bacteria, mammals, and plants (Ali et al. 2018; Schindele et al. 2018; Wolter and Puchta 2018). It offers great potential for agriculture and could be used for post-transcriptional repression (Abudayyeh et al. 2017), for combating RNA viruses as demonstrated in *Nicotiana benthamiana* (Aman et al. 2018), and as RNA binding protein for the discrimination of specific nucleic acids as reported for Zika virus (ZIKV) and the related flavivirus Dengue (DENV) (Gootenberg et al. 2017).

Base editing

Base editing is a recently new tool that allows the conversion of nucleotides without inducing double-stranded DNA breaks or using donor templates. In this sense, it is rapidly adopted for changing a C-G base pair into T-A, or A-T into G-C (Marx 2018). As proof-of-concept, the system CRISPR-Cas9 nickase-cytidine deaminase was successfully employed in protoplasts and regenerated rice and maize plants for targeting the conversion of cytosine to thymine within the protospacer with efficiency up to 40% (Zong et al. 2017). In tomato (*Solanum lycopersicum*), the CRISPR-Cas9 and activation-induced cytidine deaminase was used to develop marker free plants (Zenpei et al. 2017). Moreover, herbicide-tolerant rice plants have been developed through a substitution of T to C in a single amino acid (C2186R) of the gene Acetyl-coenzyme A carboxylase (*ACC*) (Li et al. 2018a, b, c).

Modification of gene expression and epigenome

The CRISPR/Cas9 system could be used in synthetic biology for engineering gene circuits and regulate ectopic gene expression by combining dCAS9 with different activator/inhibitor domains of transcription factors. In this sense, dCAS9 could be employed to edit epigenetic marks in the genome (Bortesi and Fischer 2015).

Challenges for genome editing in crops of tropical origin

There is still much work to do in this area of plant biotechnology and plant genome editing still faces some challenges. For example, for effective genome editing in crops of tropical origin, the genome of the target plant must first be sequenced. Moreover, plant transformation methods (via *Agrobacterium*, biolistic bombardment or protoplast transformation) is a major limiting factor for adopting CRISPR-Cas9 technology to many crops of tropical origin, especially those recalcitrant plants. Additionally, an efficient plant tissue culture regeneration system must be established. For example, regeneration of plants from protoplasts and intact somatic cells can be challenging and time consuming. Improving plant regeneration protocols and delivery approaches will become an important research priority to open up CRISPR delivery to these plants.

Regulatory aspects

Genome editing technologies can introduce advantageous traits for the improvement of crops of tropical origin that could be commercially available very soon. Some gene-edited crops such as white button mushrooms, wheat, soybeans and waxy corn have overcome USDA regulation. However, there is still a need to clarify their regulatory status, particularly with regard to global regulations on Genetically Modified Organisms (GMOs) (Kinderlerer 2008; McHughen and Smyth 2008). In many countries, the use of GMOs is regulated (Rosado and Craig 2017). Nevertheless, with respect to genome editing technologies, governments should consider their regulatory status and establish appropriate regulations, if necessary, without representing an obstacle to the commercialization of products derived from NPBTs (Seyran and Craig 2018). However, countries differ in how they regulate these technologies, which fall between genetic engineering and traditional techniques (Sprink et al. 2016; Eckerstorfer et al. 2019; Eriksson et al. 2019; Smyth 2019). In some jurisdictions, some genome editing technologies have been considered as simply as a variation of existing conventional plant breeding, while other countries have not determined what to do or how to proceed to regulate them (Lassoued et al. 2018b). So far, countries such as Argentina, Australia, Brazil, Chile, Colombia, the United States, and Israel have regulatory approaches that allow the cultivation and use of the products obtained by gene editing. The premise is that the final products do not contain genes

from other organisms, unlike the transgenic ones, and the genetic changes produced by the human being, precisely resemble those genetic changes that occur spontaneously in nature (Smyth 2019).

Conclusions

Many studies have demonstrated the efficacy of the genome editing technologies for gene editing in highly important tropical crops, such as rice, maize, tomato, and citrus. On the other hand, the use of this technology in other tropical crops with similar commercial value is just beginning. This is the case for coffee, yams, bananas, and many other species in which the technology has not been applied. Without a doubt, due to the low cost, precision, and rapidity, the CRISPR system could accelerate the genetic improvement of tropical crops of economic importance and as a consequence contribute to global food security. In this sense, this new breeding technology could improve a variety of crop traits, including yield, nutritional value, and stress tolerance, and pest and herbicide resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest. All authors read and approved the final review.

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