Biotechnological Approaches for the Resistance to Citrus Diseases

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Abstract

Citrus is one of the top fruit crops worldwide. Citrus production faces many challenges such as diseases, insects, and abiotic stresses. Genetic improvement of citrus using conventional breeding is a lengthy, costly, and time-consuming process. Biotechnological approaches such as *Agrobacterium*-mediated transgenic expression, Citrus tristeza virus (CTV)-mediated transient expression and CRISPR-based genome editing have shown tremendous potential to improve citrus against different diseases. Here, we summarize the progress in generating disease-resistant/tolerant citrus plants via biotechnological approaches.

Citrus is an economically important fruit crop grown in tropical and subtropical regions of the world. In recent years, citrus industry has been under immense pressure to develop new germplasm to overcome barriers to production from diseases, insects, and abiotic stresses. Especially, citrus Huanglongbing caused by *Candidatus* Liberibacter presents an unprecedented challenge to citrus production worldwide (Wang et al. 2017). Genetic improvement of citrus using conventional breeding is a lengthy and challenging process due to the complex reproductive biology of citrus including sexual incompatibility, highly heterozygous nature, nucellar seedlings, male or female sterility, and the long juvenile phase (Omura and Shimada 2016). Biotechnological approaches provide a promising alternative to engineer citrus plants that can resist the many abiotic and biotic stresses. The biotechnological approaches include the commonly used *Agrobacterium*-mediated transgenic expression, citrus tristeza virus (CTV)-mediated transient expression, and the forthcoming CRISPR-based genome editing. Here, we summarize the progress in generating disease-resistant/tolerant citrus plants via biotechnological approaches.

14.1 *Agrobacterium*-Mediated Transgenic Expression

The advent of genetic transformation technologies has facilitated the rapid germplasm improvement of citrus. *Agrobacterium*-mediated transformation, protoplast transformation, and particle bombardment methods have been successfully applied in various crop plants (Hansen

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and Wright 1999). These techniques allow us to introduce the gene(s) of interest into the genome of cultivars, modify, or silence selected genes of a cultivar.

Citrus tissues can be transformed by several methods. The *Agrobacterium*-mediated transformation process is the most commonly used technique. This process utilizes different explants as source tissues for transformation by the *Agrobacterium*. Among them, juvenile in vitro epicotyl segments (Dutt and Grosser 2009; Moore et al. 1992; Luth and Moore 1999) and many others, mature internode segments obtained from greenhouse-grown plants (Cervera et al. 1998; Almeida et al. 2003), or embryogenic callus obtained from unfertilized ovules (Dutt and Grosser 2010; Li et al. 2002) are the commonly used source tissues. Direct incorporation of DNA into protoplasts using electroporation (Niedz et al. 2003), biolistics (Wu et al. 2016), or PEG mediated (Olivares-Fuster et al. 2002; Guo et al. 2005; Omar et al. 2007; Fleming et al. 2000) have also been utilized.

Enhanced Biotic and Abiotic Stress Management Using a Transgenic Approach

Huanglongbing (HLB) caused by the phloem limited *Candidatus* Liberibacter asiaticus (*C*Las) (Jagoueix et al. 1996) has become a major issue globally, especially in citrus growing regions of the United States, China, and Brazil (da Graça et al. 2016). Several transgenic solutions have been devised to combat this disease. Mirkov and Gonzalez-Ramos (2013) claimed that constitutive overexpression of a spinach defensin gene resulted in enhanced HLB tolerance. Both constitutive overexpression and phloem targeted expression of the *Arabidopsis* NPR1 in the sweet orange cultivars Hamlin and Valencia (Dutt et al. 2015) resulted in the production of HLB tolerant transgenic sweet oranges. This was the first scientific report on transgene mediated resistance to HLB and its overexpression resulted in significantly lower HLB incidence when compared to non-transformed plants. Antimicrobial peptides have demonstrated promise against combating HLB. Dutt et al. (2008) overexpressed several antimicrobial peptides in the sweet oranges Hamlin and Valencia. Stover et al. (2013) screened several antimicrobial peptides in vitro for use in developing transgenic citrus resistant to HLB. A modified thionin peptide gene was observed to reduce the Liberibacter asiaticus (Las) titer in roots and scion of transgenic Carrizo rootstock, 12 months after graft inoculation (Hao et al. 2016). Similarly, phloem targeted expression of the *cecropin* B gene resulted in decreased susceptibility to HLB in sweet orange (Zou et al. 2017).

Antimicrobial peptides have been more extensively evaluated for citrus canker tolerance. Citrus canker caused by *Xanthomonas citri* ssp. *citri* is also a global problem resulting in leaf-spotting and fruit rind blemishing and can result in fruit drop and unmarketable fresh fruit (Brunings and Gabriel 2003). Expression of a dermaseptin gene in sweet orange plants reduced citrus canker symptoms (Furman et al. 2013) while the sarcotoxin IA gene reduced canker symptoms in transgenic sweet orange (Kobayashi et al. 2017). Introduction of the Attacin A (*attA*) gene into sweet orange cultivars Hamlin and Valencia reduced the canker disease symptoms (Cardoso et al. 2010; Boscariol et al. 2006). Transgenic plants regenerated via *Agrobacterium* transformation of mature axillary buds with antibacterial peptide genes Shiva A and *Cecropin* B showed enhanced resistance to canker (He et al. 2011). More recently, transgenic Carrizo plants expressing D2A21 peptide were developed which showed significant resistance to canker but not HLB (Hao et al. 2017). In addition to antimicrobial peptides, pathogen-related genes responsible for systemic acquired resistance such as *hrpN* or the *AtNPR1* were used with *Agrobacterium* transformation experiments to develop canker-resistant plants (Barbosa-Mendes et al. 2009; Zhang et al. 2010). Also, the rice derived *Xa21* gene was introduced into citrus via *Agrobacterium* (Mendes et al. 2010) and protoplast transformation systems (Omar et al. 2007) for citrus canker resistance. Recently, canker

tolerance was also demonstrated in transgenic W. Murcott plants overexpressing Xa21 under greenhouse conditions (Omar et al. 2018). Reduced susceptibility to citrus canker was also observed in transgenic sweet orange plants overexpressing the *MdSPDS1* gene responsible for polyamine biosynthesis (Fu et al. 2011a).

In addition to HLB and citrus canker resistance, transgenic strategies have also been utilized for resistance against CTV (Febres et al. 2003; Dominguez et al. 2000; Ghorbel et al. 2000; Gutierrez et al. 1997), C*itrus psorosis virus* (Zanek et al. 2008), *Phytophthora* spp (Fagoaga et al. 2001; Azevedo et al. 2006) and other biotic stresses. A commercially important Rio Red grapefruit was transformed with CTV untranslatable coat protein gene (*uncp*) and plant-derived insecticidal *Galanthus nivalis* agglutinin (*gna*) gene with an aim to protect the plants from CTV and aphids that transmit this virus (Yang et al. 2000). In addition to coat protein, transgenic plants developed by introduction of antisense constructs of CTV *RdRp* gene into epicotyl segments of grapefruit also exhibited enhanced resistance to CTV infection (Cevik et al. 2006). Fruit aroma chemistry has also been modified to improve resistance to pathogens and insect pests by introducing antisense constructs of genes responsible for terpene biosynthesis that down-regulate the production of terpenes in fruit peels (Rodriguez et al. 2011).

Apart from biotic stresses, transgenic approaches have been utilized for reducing chilling injury in Carrizo citrange and *Poncirus trifoliata* by suppressing the ethylene production with the introduction of ACC synthase antisense transgene *CS-ACS1* (Wong et al. 2001). Drought tolerance and osmotic adjustment were enhanced in rootstock Carrizo citrange by incorporating proline synthesis *p5cs* gene (Molinari et al. 2004). Being a commercially important rootstock, halotolerance gene *HAL2* extracted from *Saccharomyces* yeast was introduced into Carrizo citrange via *Agrobacterium* method for improving the performance of this rootstock in saline conditions (Cervera et al. 2000). Salt tolerance was also enhanced in trifoliate orange rootstock with the incorporation of betaine aldehyde dehydrogenase (*BADH*) gene which leads to synthesis of the osmoprotectant glycine betaine (GB) (Fu et al. 2011b).

14.2 CTV-Mediated Expression

The effect of HLB on the citrus industry is devastating and a quick solution is necessary to maintain it in Florida. As the causal bacterium is not cultured yet, direct in vitro screening is not possible. CTV and Las co-localize to the phloem tissue of their citrus host where the Asian citrus psyllid (ACP) feeds. Thus, CTV-based expression and/or RNAi vectors are being used as a screening tool to identify potential therapeutic products. Furthermore, due to its unusual stability and because it can be deployed rapidly, it is being considered as an interim control measure until transgenic or CRISPR/Cas9 plants can become available.

CTV was first reported in Argentina causing quick decline on the sour orange rootstock (Bar-Joseph et al. 2010). CTV is endemic to Florida. Some isolates of CTV in other parts of the world are extremely virulent and prevent profitable production of citrus. Most Florida isolates cause mild symptoms in most citrus genotypes. One exception is T36 that causes decline and death of trees on the sour orange rootstock. The decline has been avoided in Florida simply by using other rootstocks. On other rootstocks T36 isolates cause even milder symptoms than other isolates. Currently, a T36-based CTV vector is being used to overexpress genes or induce RNA interference (RNAi).

CTV, a positive-sense 19.3 kb single-stranded RNA virus, is a member of the closteroviridae. The RNA genome is organized into 12 open reading frames (ORFs), which are expressed via three different strategies (Karasev et al. 1995). The first strategy is a poly-protein strategy with post-translational processing. The second strategy is a +1 ribosomal frameshift that allows continued translation beyond a stop codon. Both strategies are used to express ORF1a and 1b,

which are involved in RNA replication. The 3' half of the genome is organized into 10 ORFs. Hsp70 homologue, p61, CPm, CP, and p20 ORFs are involved in virion formation (Peremyslov et al. 2004; Satyanarayana et al. 2000). CP, p20, and p23 are silencing suppressors with different modes of action (Lu et al. 2004). P6 is a potential movement protein. All these genes are essential for the systemic infection of all citrus plants. P33, p18, and p13 are host range determinants not necessary for the infection of some citrus hosts (Tatineni et al. 2008). The 10 ORFs are expressed via a nested set of 3'coterminal sub-genomic RNAs (Hilf et al. 1995). The transcription of each ORF is driven by a subgenomic controller element upstream of the coding sequence (Gowda et al. 2001). The 3' half of the genome was explored for expression of foreign sequences via different strategies to develop CTV-based expression/RNAi vectors (Folimonov et al. 2007; El Mohtar 2011; EL Mohtar and Dawson 2014; Hajeri et al. 2014).

CTV as an Expression/RNAi Vector

The first CTV vector had an extra gene inserted between the minor and major coat protein ORFs (Folimonov et al. 2007). Further studies identified three new locations within CTV that can differentially express genes of different lengths (El Mohtar 2011; EL Mohtar and Dawson 2014). The first two positions had the extra gene added between p13 and p20 or between p23 and 3' nontranslated region (NTR). In addition, the substitution of the p13 ORF and its controller element was successful. The different positions could be combined to express multiple foreign genes from the same vector. Using green fluorescent protein (GFP) as a reporter gene, CTV vectors have been shown to be exceptionally stable. There are trees infected with CTV vectors that are still expressing GFP almost 15 years after inoculation. In 2012, the vector was used to efficiently induce silencing of the phytoene desaturase (PDS) citrus gene via RNAi (Hajeri et al. 2014). The plants are still showing the bleaching phenotype characteristic of PDS silencing 6 years after inoculation.

CTV-Mediated Expression of Antimicrobial Peptides and Proteins

CTV is being used to transiently express small antimicrobial peptides (AMPs, 10-70 amino acids) to either directly target Las or to help the plant tolerate the infection. Targeting the bacteria directly is based on expressing small antimicrobial peptides that kill or reduce Las titer in the plant. More than 100 AMPs with different modes of action have been or are being screened for activity against Las. Furthermore, potential PAMPs peptides of Las are being expressed through CTV-based expression vectors to trigger the citrus plant defenses. CTV vectors are also being used to express lytic phage proteins that are directed against the bacterial cell wall causing its disintegration and killing the bacteria. Two proteins from the Las prophage have been selected for CTV-mediated expression.

Use of CTV RNAi Vectors Against Psyllids

A major goal is the deployment of CTV RNAi vectors against the psyllid. The idea is to introduce truncated sequence of psyllid genes into the CTV vector. The plant will load these genes into its silencing machinery producing abundant amounts of siRNA in phloem cells. Upon feeding on the citrus phloem, the ACP insect acquires the siRNA, which silences the psyllid gene and prevents reproduction of the next generation of psyllids. More than 20 ACP genes are being targeted for silencing. For example, it has been used to transiently express truncated abnormal wing disc (*Awd*) gene of *Diaphorina citri*, the insect vector of Las. Consequently, feeding *D. citri* nymphs led to altered *Awd* expression and malformed-wing phenotype in adults and increased adult mortality (Hajeri et al. 2014).

Prescreening for CRISPR-Cas9 Genome Editing Genes

Many researchers believe that CRISPR-Cas9 could be used to successfully engineer resistance/tolerance to HLB in citrus by editing either susceptibility genes or negative regulators of plant defense. However, CRISPR-Cas9 is a difficult technique to employ in citrus on a list of potential target genes. Thus, CTV is being used to

induce RNAi against citrus genes to prescreen potential genes for targetting using the CRISPR/Cas9 technique. Around 40 plant genes are being targeted for silencing by CTV RNAi vectors. The major advantage of using the CTV vector to silence genes is speed. Although nothing is fast in citrus, silencing a potential gene using the CTV vector is much quicker and easier than directly using CRISPR/Cas9 modification. Using CTV also has additional advantages. One is that several different vectors with potential targets can be examined in parallel. Most of the potential targets will be examined in the first screening run. Also, more than one target gene sequences can be inserted into the same vector to silence more than one plant gene. Thus, if more than one gene was predicted to be modified by CRISPR to provide tolerance, these genes could be silenced simultaneously by the CTV vector. Perhaps more importantly, the CTV vector can be graft transmitted to mature plants, allowing the determination of the effect of the silenced gene on mature characteristics such as fruit development and juice flavor. Finally, if silencing a target gene using the CTV vector results in HLB resistance or tolerance, this in itself could be used as a short-term management possibility for HLB in the field.

14.3 CRISPR Technology in Citrus Disease Management

The CRISPR/Cas modules are adaptive immune systems of prokaryotes against invading phages and plasmids by cleaving the foreign DNA, or, in some cases, RNA, in a sequence-dependent manner (Jinek et al. 2012; Barrangou et al. 2007). Approximately 84% of archaea and 48% of bacteria genomes contain CRISPR-Cas systems (Marraffini 2013). A CRISPR locus consists of a CRISPR array and diverse *cas* genes. The CRISPR array comprises short direct repeats interspaced by variable DNA spacer sequences which are acquired from virus and plasmid genes. The spacers enable the recognition and cleavage of the invasive viruses and plasmids (Barrangou et al. 2007). CRISPR/Cas-mediated adaptive immunity consists of three stages: adaptation, expression, and interference (van der Oost et al. 2009; Wiedenheft et al. 2012; Barrangou and Marraffini 2014; Marraffini 2015). During the adaptation stage, short pieces of foreign DNA (called protospacers) from invading viruses or plasmids are processed and incorporated into the CRISPR loci (Barrangou et al. 2007; Garneau et al. 2010). In the expression stage, the CRISPR array is transcribed, which is further processed into mature CRISPR RNAs (crRNAs). The pre-crRNA binds to either Cas9 or to a multisubunit complex, forming the crRNA-effector complex after further processing involving bacterial RNase III and transactivating CRISPR RNA (tracrRNA) (Deltcheva et al. 2011) or by an endonuclease subunit of the multisubunit effector complex. The interference stage involves crRNA-directed cleavage of invading cognate virus or plasmid nucleic acids by Cas nucleases.

The continuous arms race between prokaryotes and invading viruses and plasmids have driven rapid evolution of highly diverse CRISPR-Cas systems (Takeuchi et al. 2012; Koonin and Wolf 2015). Based on the repertoire of *cas* genes, the sequence similarity between Cas proteins and the locus architecture, the CRISPR-Cas systems have been classified into two classes that are subdivided into six types (Makarova and Koonin 2015; Shmakov et al. 2015). The Class 1 systems are present in bacteria and archaea and include the most common and diversified type I, type III that is mainly presented in archaea, as well as the rare type IV (Koonin et al. 2017). Class 1 systems encompass effector complexes composed of four to seven Cas protein subunits. The Class 2 systems (types II, V, and VI) are less common and are mostly restricted to bacteria. Class 2 effector complex consists of a single multidomain protein represented by Cas9 and Cpf1 (Makarova et al. 2015). The ability to easily program sequence-specific DNA targeting and cleavage by CRISPR-Cas components render them a very useful tool for genetic engineering in a wide range of eukaryotes including various plant species and prokaryotes (Mohanraju et al. 2016).

CRISPR-Cas9 mediated genome editing of citrus has been successfully conducted previously (Jia and Wang 2014a; Jia et al. 2016b; LeBlanc et al. 2017; Peng et al. 2017; Zhang et al. 2017; Jia et al. 2017b).

Major Tasks in the Application of CRISPR Technology for Genome Editing of Citrus

There are multiple tasks or challenges facing the application of CRISPR technology for gene editing of crops especially citrus: identification of critical traits for targeting, foreign DNA free in modified plants, off-target issue, expanding the toolbox of genome editing, and optimizing the procedure and improving the efficacy.

Critical traits for targeting. Genome editing can be used to improve many different aspects of citrus such as color, nutrition, metabolic engineering, quality, yield, seedlessness, and stress resistance (both biotic and abiotic stress). CRISPR-mediated genome editing has been successfully used to generate disease-resistant citrus varieties against bacterial canker disease caused by *Xanthomonas citri* (Jia and Wang 2014a; Jia et al. 2017b; Peng et al. 2017). Specifically, Cas9/sgRNA has been utilized to modify the PthA4 effector binding elements (EBEs) in the promoter region as well as the coding region of the *CsLOB1* (*Citrus sinensis* Lateral Organ Boundaries) gene (Jia et al. 2016a, b). *CsLOB1* is a susceptibility gene for citrus canker, which is induced by the pathogenicity factor PthA4 via its binding of the EBE_{PthA4}-CsLOBP (Hu et al. 2014). Genome editing of the coding region of the disease susceptibility gene *CsLOB1* in citrus leads to the development of canker resistant plants (Jia et al. 2016a, b). Deletion of the entire EBE_{PthA4} sequence from both *CsLOB1* alleles confers a high degree of resistance to citrus canker (Peng et al. 2017).

Foreign DNA free in genome-modified plants. To avoid all the headaches of deregulations related to transgenic and GMO (genome-modified organisms) plants (Hartung and Schiemann 2014), it is critical that the genome-modified plants do not contain foreign DNAs originating from pathogens or other organisms that are not naturally associated with plant chromosomes during evolution, traditional crossing, conventional mutagenesis, or sexually compatible species. Plants modified by the CRISPR technology have potentials to be free of foreign DNAs and to be indistinguishable from plants generated by conventional breeding or mutagenesis. Plants stably transformed with CRISPR/Cas may contain unwanted insertions of plasmid DNA at both on-target and off-target sites (Woo et al. 2015). Even though the foreign DNA may in principle be removed by genetic segregation, this is not feasible in plants that reproduce asexually. Specifically, the crossing approach for citrus is laborious and time-consuming, particularly considering the long juvenile period for citrus. Backcrossing of citrus will lead to loss of traits of the parental cultivars. Additionally, constant expression of Cas9/sgRNA in transgenic plants may lead to accumulation of off-target effects. Transient expression of either Cas9-sgRNA ribonucleoproteins, Cas9/sgRNA DNA or RNA has been used successfully to generate non-transgenic genome-modified plants (Zhang et al. 2016; Liang et al. 2017; Svitashev et al. 2016; Woo et al. 2015). Recently, Cas9/sgRNA DNA and Cas9-sgRNA ribonucleoproteins have been used successfully to edit the genes of protoplast cells of citrus in the Wang lab.

Expanding the toolbox of genome editing. The specificity of CRISPR/Cas9 mediated gene editing is determined by both the sgRNA and PAM, which, on the other hand, also limits the repertoire of sequences that it can target. For Cas9/sgRNA based on *Streptococcus pyogenes*, the PAM sequence of 5'-NGG-3' is required (Cong et al. 2013). Multiple Cas9 orthologs from type II CRISPR-Cas systems, which recognize different PAMs, have been characterized and engineered for genome editing. For example, SaCas9 of *Staphylococcus aureus* recognizes NNGRRT or NNGRR(N) (Kleinstiver et al. 2015a; Ran et al. 2015), the PAM sequence for StCas9 of *Streptococcus thermophiles* is NNAGAAW (Deveau et al. 2008), and the PAM for NmCas9 of *Neisseria meningitides* is NNNNGATT (Hou et al. 2013). Interestingly, Cpf1 is the effector protein for type V CRISPR-

Cas system which recognizes a PAM sequence of 5'-TTN-3' (Zetsche et al. 2015). The different PAM sequences recognized by Cas9 orthologs have significantly increased the repertoire of sequences that are suitable for site-directed mutagenesis. In addition, modification of the PAM-binding domain of Cas9 can change the PAM specificities (Kleinstiver et al. 2015b), which further expanded the use of genome editing. Both SpCas9/sgRNA and SaCas9/sgRNA have been successfully used to conduct genome editing of citrus (Jia and Wang 2014a; Jia et al. 2017a).

Optimization of the CRISPR-Cas9 mediated genome editing of citrus. Optimization of CRISPR-Cas9 mediated genome editing of citrus includes optimization of delivery of genome engineering reagents and improving the design of Cas9-sgRNA. Here, we summarize the current progress in the relevant areas in citrus biotechnology.

Delivery of genome editing reagents into citrus cells is the major barrier for successful genome modification. The delivery methods include plasmid transformation by biological organisms such as Agrobacterium and viruses (e.g., CTV) as well as reagents delivery via protoplast transfection and microprojectile bombardment. Previously, it has been difficult to conduct agroinfiltration-mediated transient expression in citrus leaves. Pretreatment of citrus leaves with *Xanthomonas citri* significantly enhanced transient protein expression in citrus leaves and delivery of Cas9/sgRNA (Jia and Wang 2014a, b). This has been suggested to be due to the excessive cell division caused by *X. citri* infection, which mimics the fast dividing epicotyl segment suitable for *Agrobacterium*-mediated transformation.

Optimization of expression of Cas9-sgRNA has been used to improve the efficacy of genome editing in plants. Cas9 has been traditionally driven by 35S promoter whereas sgRNA has been driven by U3 or U6 promoter. sgRNA is a small non-coding RNA and requires an accurate 5'-end to keep its target-specific sequence. Transcripts from U3 and U6 promoters start with the nucleotides "A" and "G", respectively, thus restricting the targeting range and potentially the efficiency of Cas9. RNA processing systems have been engineered for sgRNA processing: tRNA processing system (Xie et al. 2015), self-cleaving ribozyme (Gao and Zhao 2014), and the ribonuclease Csy4 (Nissim et al. 2014). Both 35S and U3/U6 have been used to drive expression of sgRNA in citrus (Jia and Wang 2014a; Jia et al. 2017b; Peng et al. 2017; Zhang et al. 2017). 35S promoter is an RNA polymerase II promoter which synthesizes precursors of mRNAs and most snRNA, whereas U6 or U3 are polymerase III promoters, which synthesize tRNAs, rRNA 5S, and other small RNAs. In most studies, sgRNAs are driven by U6 or U3 promoters (Kim et al. 2016; Wei et al. 2017). It seems 35S, U6, and U3 promoters all work in promoting the transcription of sgRNA (Kim et al. 2016; Jia and Wang 2014a). However, U6 or U3 might be more efficient than 35s for driving the transcription of sgRNA since Pol II created RNA will be capped and poly-A-tailed, so the half-life of the RNA in the nucleus will be shorter than that synthesized by RNA polymerase III.

The 35S promoter is the most commonly used to drive the expression of Cas9. However, Cas9/sgRNA gene editing efficacy has been improved by driving the expression of Cas9 using different promoters including the dividing cell-specific INCURVATA2 promoter (Hyun et al. 2015); the cell division-specific YAO promoter (Yan et al. 2015), and the germ-line-specific SPOROCYTELESS promoter (Mao et al. 2016) in Arabidopsis. Besides 35S promoter, the YAO promoter has been successfully used to drive the expression of Cas9 in citrus (Zhang et al. 2017).

Codon-optimization of Cas9 has also been used to maximize Cas9 activity in plants (Bortesi and Fischer 2015). Plant codon-optimized SpCas9 gene has been used in citrus previously (Peng et al. 2017). Codon-optimization of other Cas9 orthologs is also recommended.

In addition, heat stress has also been shown to increase the efficacy of gene editing by CRISPR/Cas9. LeBlanc et al. (2018) demonstrated that Arabidopsis and citrus plants subjected to temperature at 37 °C showed

significantly higher frequencies of CRISPR-mediated mutations compared to plants grown at 22 °C. This seems to have resulted from that SpCas9 is more active in creating double-strand DNA breaks at 37 °C than at 22 °C.

Off-target Issue

Besides their target sites, Cas9 protein and orthologs can also create unwanted cleavages at off-target sites with high sequence similarity to target sequence, thus causing off-targeting mutations. For example, SpCas9 not only recognizes 5'-NGG-3' as the PAM sequence, but also can cleave sites with a 5'-NGA-3' or 5'-NAG-3' PAM sequence at lower efficacy (Hsu et al. 2013). In addition, mismatches in the PAM-distal sequence at the 5' terminus are tolerated, whereas mismatches in the seed region, the 10-12 nucleotides right upstream of PAM are not tolerated. Off-targets can cause negative effect which must be monitored carefully and avoided as much as possible (Koo et al. 2015).

Multiple approaches have been reported to reduce off-target issue associated with Cas9-sgRNA mediated genome editing. First, optimization of sgRNA by selecting unique target sequences which differ from other sequences by at least 2 or 3 nucleotides reduces off-target effects (Cho et al. 2014). Second, application of D10 mutant nickase version of Cas9 pairing with two sgRNAs that each cleaves only one strand decreases off-target effect (Ran et al. 2013). This approach extends the target sequence from 23 bp to 2×23 bp. Third, fusing dead SpCas9 (dCas9, which results from mutations of both cleavage domains of SpCas9, i.e., D10A for RuvC and H840A for HNH) with the FokI nuclease domain at the N-terminus also reduces off-target problem (FokI-dCas9) (Tsai et al. 2014). In addition, nontransgenic genome editing approaches as described above have the potential to reduce off-targets.

Overall, *Agrobacterium*-mediated transgenic expression, CTV-mediated expression, and CRISPR-based genome editing have shown tremendous potential to improve citrus against different diseases. However, their applications remain at the early stage. The scientific community needs to further optimize the tools, rigorously test the end products to avoid negative effects, and appropriately address the public concerns regarding crops containing those elements.

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