

# New Breeding Techniques in Citrus

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Contributor: Lara Poles , Concetta Licciardello , Gaetano Distefano , Elisabetta Nicolosi , Alessandra Gentile , Stefano La Malfa

The development of novel citrus varieties with improved quality and resistance to biotic and abiotic stresses is one of the main purposes of breeding programs. Thus far, the use of conventional breeding techniques in citrus has been shown to be time consuming and difficult due to the many limitations of typical of tree crops, such as the long juvenility and high heterozygosity. The application of NPBTs could overcome these problems, offering new tools that combine site-specific and targeted editing with a reduction in the time for plant breeding, thus leading to lower production costs. Many aspects need to be considered to apply transgenesis to citrus, among them transformation efficiency, and regeneration potential of citrus commercial varieties

genome editing

citrus

crispr/cas

transformation

## 1. Introduction

Citrus is one of the most important fruit crops in the world. The development of novel varieties with improved resistance to various pests and pathogens is one of the main aims of citrus breeding programs; conventional breeding strategy in citrus has demonstrated numerous limitations due to biological characteristics common to woody plants, such as long juvenile period, large size, long generation time, and also the lack of knowledge on how the most important horticultural traits are inherited. In addition, citrus display other limitations, such as nucellar polyembryony, self-incompatibility, and high heterozygosity, that genetic engineering and New Plant Breeding Techniques (NPBTs) <sup>[1][2]</sup> can overcome, leading to the development of novel varieties with the incorporation of selected traits, while retaining the unique characteristics of the original cultivar. NPBTs include different biotechnological tools that are used to induce DNA modification, such as insertion, deletion, gene replacement, or stable gene silencing.

Genetic engineering has been strongly considered for the development of novel citrus varieties, offering a wide range of tools and strategies that enable the insertion or the editing of desirable traits into elite commercial cultivars. The applications of transgenesis are wide and include resistance to biotic and abiotic stresses and the control of fruit quality traits. Several traits have been considered for genetic transformation, including early flowering <sup>[3][4][5][6][7]</sup>, tree architecture and growth habitus <sup>[8][9][10]</sup>, tolerance to abiotic stresses <sup>[11][12][13][14]</sup>, improvement of fruit quality <sup>[15][16][17]</sup>, in particular carotenoid content <sup>[7][18]</sup>, and seedlessness <sup>[19][20][21]</sup>. Thus far, the main aspects rely on biotic stresses, as these are the most limiting factors for citriculture worldwide. In the last years, great interest has been devoted to the development of novel varieties showing resistance to citrus greening (Huanglongbing, HLB).

## 2. Development

One of the most important NPBTs is genome editing, that involves the production of a permanent and inheritable mutation in a specific DNA sequence that can be inaccurately repaired by the plants' own repair mechanism (leading to gene knock-out), or that can be accurately repaired using a DNA-repair template (leading to target mutation or gene replacement) [22][23][24]. In the CRISPR-Cas system, an adaptive immune system of prokaryotes [25], Cas nuclease is directed by a single guide RNA (sgRNA) that recognizes a target DNA sequence flanked by a protospacer adjacent motif (PAM) and generates specific DNA double-strand breaks (DSBs). Nuclease-induced DSBs can be repaired by the non-homologous end-joining (NHEJ) pathway, which leads to the introduction of insertion/deletion mutations (indels) of various lengths, or by homology-directed repair (HDR), which is useful to introduce specific point mutations or to insert desired sequences through recombination of the target locus using DNA 'donor templates' present at the moment of DSB formation [26].

In citrus, Jia and Wang [27] reported the first genome editing using the Cas9/sgRNA system and Xcc-facilitated agroinfiltration on Valencia orange. The delivery of Cas9 and sgRNA were accomplished with a particular agroinfiltration that consists of an initial inoculation of Xcc followed by an *Agrobacterium* infiltration on Valencia leaves; the target gene was the endogenous Citrus phytoene desaturase (CSPDS) gene, an enzyme required for the biosynthesis of carotenoid pigments that results in a white-colored (albino) phenotype when it is silenced or mutated [28]. Jia and Wang [29] applied the same strategy on Duncan grapefruit and, being a grapefruit hybrid between pummelo and sweet orange [30], they were able to apply the Cas9/sgRNA system to specifically modify one of the two CSPDS alleles of the variety.

Subsequent application of genome editing has focused on editing genes involved in citrus disease resistance, especially in citrus canker. Most of the studies were performed to target the CsLOB1 gene (*C. sinensis* Lateral Organ Boundaries 1), a disease-susceptibility gene upregulated by PthA4, a transcription activator-like effector of Xcc [31][32], in particular to target the effector binding elements (EBEs) of PthA4, which are located in the promoter of the CsLOB1 gene (EBEPthA4-CsLOBP), and should confer resistance to the disease without losing CsLOB1 function. Peng et al. [33] edited Wanjincheng orange using 5 different constructs to modify different regions along EBEPthA4-CsLOBP; through the transformation of epicotyl segments, they obtained 16 lines (42% TE) with EBEPthA4 modifications and 4 mutation lines that showed enhanced resistance to citrus canker.

Duncan grapefruit epicotyl transformation was achieved by Jia et al. [34] and resulted in 4 lines with targeted modification of only EBEPthA4 CsLOBP Type I with a mutation rate of 15.63–81.25%; the transgenic plants were susceptible to Xcc infection. In 2017, Jia et al. [35] succeeded in disrupting the coding regions of both alleles of CsLOB1, and no canker symptoms were observed in the lines DLOB9 (mutation rate of 89.36%), DLOB10 (88.79%), DLOB11 (46.91%), and DLOB12 (51.12%) after Xcc inoculation. In both studies, no off-target mutation was detected, but only a few among the possible off-targets were subjected to analysis; an alternative strategy to reduce off-target mutations is the use of a different type of nuclease, such as CRISPR derived from *Prevotella* and *Francisella* (CRISPR-Cpf1), a new class II CRISPR-Cas system [36][37] that has been used to edit tobacco, rice and soybean [38][39][40][41].

Another approach was used by Wang et al. [42], editing the transcription factor CsWRKY22 that was negatively correlated with citrus canker resistance. Epicotyls of Wanjincheng orange were transformed, and the transgenic plants W1-1, W2-2, and W2-3 showed 85.7%, 79.2%, and 68.2% mutation rates, respectively, with off-target frequencies of 3.0-16.0%; resistance evaluation indicated that transgenic plants delayed the development of canker symptoms.

Although all these studies demonstrate how CRISPR/Cas9 technology can be exploited for citrus genome editing, accelerating the breeding process and combining multiple favorable traits, there is a need for more precise biotechnology tools than those that are currently available. One of the problems is the efficiency of the editing obtained; despite the fact that several computational tools are now available for designing guide RNAs targeting a specific gene, the editing efficiencies might be different due to the existence of variant alleles not included in online citrus genome databases [30]; for this reason, the investigation of the sequence of the gene of interest [34][35][33],

the functionality evaluation of many sgRNAs using Xcc-facilitated agroinfiltration [27][34][35][43], and the in vitro cleavage analysis of the construct before citrus transformation [42] represent fundamental steps to increase editing efficiency.

The low frequencies of mutations induced by the CRISPR/Cas9 system used in citrus were improved by Zhang et al. [44], who used a different promoter to drive Cas9 expression, replacing the CaMV35S promoter with the *A. thaliana* YAO sequence [45] and increasing the frequency of mutational events from 3.2–3.9% [44] to 75% using the same sgRNA. Le Blanc et al. [46] also demonstrated that temperature has an effect on mutation rate achieved by the CRISPR/Cas9 system; Carrizo citrange transgenic plants containing pYAO:SpCas9 and sgRNA targeting CsPDS genes that were exposed to several heat stress treatments (24 h at 37 °C and 24 h at 24 °C repeated seven times) showed an increase in targeted mutagenesis (100% CsPDS alleles mutated) with respect to those continuously grown at 24 °C (approximately half of the CsPDS alleles mutated). This result suggests that all CRISPR/Cas9 systems require higher temperatures to achieve optimal editing efficiency, regardless of the promoter used to regulate Cas9 expression [46], and that many aspects of the functioning of this technology are still to be explored.

Jia and Wang [47] generated homozygous and biallelic canker-resistant pummelo in the T0 generation via the CRISPR-Cas9 system with a 100% mutation rate in the EBE region of the LOB1 promoter. Zhang et al. [44] also developed a bifunctional selectable and visible marker for citrus (eGFP-NPTII) that improved the recovery of transgenic events expressing high levels of Cas9, reducing the number of promoters present in the vector. In citrus, special efforts to control CRISPR/Cas9-mediated chimeric mutation are required, and the optimization of regeneration protocols will offer a great opportunity to select transgenic events and reduce the formation of chimeric mutations [42].

Other options include the use of embryogenic calli transformation that rarely produce transgenic chimeras [48][19] and a transient approach using purified CRISPR/Cas9 ribonucleoproteins to edit plant protoplasts, which has been tested in wheat [49] and applied to grape and apple [50]. Other concerns are related to the findings of new

target genes for editing and to genetically modified organisms legislation; knowledge of plant pathogen interactions and mechanisms is critical to the development of new varieties with improved quality or resistance to disease via the CRISPR/Cas system [51]. The legislation of genome-edited plants is still a debated issue at international scientific and political forums, and many countries are in the process of drafting the regulatory frameworks for their use [52].

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