New Breeding Techniques in Citrus

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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 diffcult 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

Keywords: 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) [1][2] 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 [3][4][5][6][7], tree architecture and growth habitus [8][9][10], tolerance to abiotic stresses [11][12][13][14], improvement of fruit quality [15][16][17], in particular carotenoid content [7][18], and seedlessness [19][20][21]. 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 e_ector 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 e_ect 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].

References

1. Eriksson, D.; Harwood, W.; Hofvander, P.; Jones, H.; Rogowsky, P.; Stöger, E.; Visser, R.G.F. A Welcome Proposal to Amend the GMO Legislation of the EU. Trends Biotechnol. 2018, 36, 1100–1103.
2. Limera, C.; Sabbadini, S.; Sweet, J.B.; Mezzetti, B. New Biotechnological Tools for the Genetic Improvement of Major Woody Fruit Species. Front. Plant Sci. 2017, 8, 1418.
3. Peña, L.; Martín-Trillo, M.; Juárez, J.; Pina, J.A.; Navarro, L.; Martínez-Zapater, J.M. Constitutive expression of Arabidopsis LEAFY or APETALA1 genes in citrus reduces their generation time. Nat. Biotechnol. 2001, 19, 263–267.
4. Cervera, M.; Navarro, L.; Peña, L. Gene stacking in 1-year-cycling APETALA1 citrus plants for a rapid evaluation of transgenic traits in reproductive tissues. J. Biotechnol. 2009, 140, 278–282.
5. Endo, T.; Shimada, T.; Fujii, H.; Kobayashi, Y.; Araki, T.; Omura, M. Ectopic expression of an FT homolog from Citrus confers an early flowering phenotype on trifoliate orange (Poncirus trifoliata L. Raf.). Transgenic Res. 2005, 14, 703–712.
6. Nishikawa, F.; Endo, T.; Shimada, T.; Fujii, H.; Shimizu, T.; Kobayashi, Y.; Araki, T.; Omura, M. Transcriptional changes in CiFT-introduced transgenic trifoliate orange (Poncirus trifoliata L. Raf.). Tree Physiol. 2010, 30, 431–439.
7. Pons, E.; Alquézar, B.; Rodríguez, A.; Martorell, P.; Genovés, S.; Ramón, D.; Rodrigo, M.J.; Zacarías, L.; Peña, L. Metabolic engineering of β-carotene in orange fruit increases its in vivo antioxidant properties. Plant Biotechnol. J. 2014, 12, 17–27.
8. Gentile, A.; Deng, Z.N.; la Malfa, S.; Domina, F.; Germanà, C.; Tribulato, E. Morphological and physiological effects of rolABC genes into citrus genome. Acta Hortic. 2004, 632, 235–242.
9. Distefano, G.; Cirilli, M.; las Casas, G.; la Malfa, S.; Continella, A.; Rugini, E.; Thomas, B.; Long, G.; Gentile, A.; Muleo, R. Ectopic expression of Arabidopsis phytochrome B in Troyer citrange affects photosynthesis and plant morphology. Sci. Hortic. 2013, 159, 1–7.
10. Fagoaga, C.; Tadeo, F.R.; Iglesias, D.J.; Huerta, L.; Lliso, I.; Vidal, A.M.; Talon, M.; Navarro, L.; García-Martínez, J.L.; Peña, L. Engineering of gibberellin levels in citrus by sense and antisense overexpression of a GA 20-oxidase gene modifies plant architecture. J. Exp. Bot. 2007, 58, 1407–1420.
11. Cervera, M.; Ortega, C.; Navarro, A.; Navarro, L.; Peña, L. Generation of transgenic citrus plants with the tolerance-to-salinity gene HAL2 from yeast. J. Hortic. Sci. Biotechnol. 2000, 75, 26–30.
12. Molinari, H.B.C.; Marur, C.J.; Filho, J.C.B.; Kobayashi, A.K.; Pileggi, M.; Júnior, R.P.L.; Pereira, L.F.P.; Vieira, L.G.E. Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (Citrus sinensis Osb. × Poncirus trifoliata L. Raf.) overproducing proline. Plant Sci. 2004, 167, 1375–1381.
13. Orbović, V.; Ćalović, M.; Dutt, M.; Grosser, J.W.; Barthe, G. Production and characterization of transgenic Citrus plants carrying p35 anti-apoptotic gene. Sci. Hortic. 2015, 197, 203–211.
14. Orbović, V.; Fields, J.S.; Syvertsen, J.P. Transgenic citrus plants expressing the p35 anti-apoptotic gene have altered response to abiotic stress. Hortic. Environ. Biotechnol. 2017, 58, 303–309.
15. Guo, W.; Duan, Y.; Olivares-Fuster, O.; Wu, Z.; Arias, C.R.; Burns, J.K.; Grosser, J.W. Protoplast transformation and regeneration of transgenic Valencia sweet orange plants containing a juice quality-related pectin methylesterase gene. Plant Cell Rep. 2005, 24, 482–486.
16. Dutt, M.; Stanton, D.; Grosser, J.W. Ornacitrus: Development of genetically modified anthocyanin-expressing citrus with both ornamental and fresh fruit potential. J. Am. Soc. Hortic. Sci. 2016, 141, 54–61.
17. Hijaz, F.; Nehela, Y.; Jones, S.E.; Dutt, M.; Grosser, J.W.; Manthey, J.A.; Killiny, N. Metabolically engineered anthocyanin-producing lime provides additional nutritional value and antioxidant potential to juice. Plant Biotechnol. Rep. 2018, 12, 329–346.
18. Alquézar, B.; Rodrigo, M.J.; Zacarías, L. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochemistry 2008, 69, 1997–2007.
19. Li, D.D.; Shi, W.; Deng, X.X. Agrobacterium-mediated transformation of embryogenic calluses of Ponkan mandarin and the regeneration of plants containing the chimeric ribonuclease gene. Plant Cell Rep. 2002, 21, 153–156.

20. Li, D.D.; Shi, W.; Deng, X.X. Factors influencing Agrobacterium-mediated embryogenic callus transformation of Valencia sweet orange (Citrus sinensis) containing the pTA29-barnase gene. Tree Physiol. 2003, 23, 1209–1215.
21. Koltunow, A.M.; Brennan, P.; Protopsaltis, S. Regeneration of West Indian Limes (Citrus aurantifolia) containing genes for decreased seed set. Acta Hortic. 2000, 535, 81–91.
22. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 2012, 337, 816–821.
23. Liu, X.; Xie, C.; Si, H.; Yang, J. CRISPR/Cas9-mediated genome editing in plants. Methods 2017, 121, 94–102.
24. Bortesi, L.; Fischer, R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol. Adv. 2015, 33, 41–52.
25. Barrangou, R.; Fremaux, C.; Deveau, H.; Richards, M.; Boyaval, P.; Moineau, S.; Romero, D.; Horvath, P. CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. Science 2007, 315, 1709–1712.
26. Sander, J.D.; Joung, J.K. CRISPR-Cas systems for genome editing, regulation and targeting. Nat. Biotechnol. 2014, 32, 347–355.
27. Jia, H.; Wang, N. Targeted genome editing of sweet orange using Cas9/sgRNA. PLoS ONE 2014, 9, e93806.
28. Agüero, J.; del Carmen Vives, M.; Velázquez, K.; Pina, J.A.; Navarro, L.; Moreno, P.; Guerri, J. Effectiveness of gene silencing induced by viral vectors based on Citrus leaf blotch virus is different in Nicotiana benthamiana and citrus plants. Virology 2014, 460–461, 154–164.
29. Jia, H.; Wang, N. Xcc-facilitated agroinfiltration of citrus leaves: A tool for rapid functional analysis of transgenes in citrus leaves. Plant Cell Rep. 2014, 33, 1993–2001.
30. Xu, Q.; Chen, L.L.; Ruan, X.; Chen, D.; Zhu, A.; Chen, C.; Bertrand, D.; Jiao, W.B.; Hao, B.H.; Lyon, M.P.; et al. The draft genome of sweet orange (Citrus sinensis). Nat. Genet. 2013, 45, 59–66.
31. Hu, Y.; Zhang, J.; Jia, H.; Sosso, D.; Li, T.; Frommer, W.B.; Yang, B.; White, F.F.; Wang, N.; Jones, J.B. Lateral organ boundaries 1 is a disease susceptibility gene for citrus bacterial canker disease. Proc. Natl. Acad. Sci. USA 2014, 111.
32. Yang, L.; Hu, C.; Li, N.; Zhang, J.; Yan, J.; Deng, Z. Transformation of sweet orange [Citrus sinensis (L.) Osbeck] with pthA-nls for acquiring resistance to citrus canker disease. Plant Mol. Biol. 2011, 75, 11–23.
33. Peng, A.; Chen, S.; Lei, T.; Xu, L.; He, Y.; Wu, L.; Yao, L.; Zou, X. Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus. Plant Biotechnol. J. 2017, 15, 1509–1519.
34. Jia, H.; Orbović, V.; Jones, J.B.; Wang, N. Modification of the PthA4 effector binding elements in Type I CsLOB1 promoter using Cas9/sgRNA to produce transgenic Duncan grapefruit alleviating XccΔpthA4: DCsLOB1.3 infection. Plant Biotechnol. J. 2016, 14, 1291–1301.
35. Jia, H.; Zhang, Y.; Orbović, V.; Xu, J.; White, F.F.; Jones, J.B.; Wang, N. Genome editing of the disease susceptibility gene CsLOB1 in citrus confers resistance to citrus canker. Plant Biotechnol. J. 2017, 15, 817–823.
36. Zetsche, B.; Gootenberg, J.S.; Abudayyeh, O.O.; Slaymaker, I.M.; Makarova, K.S.; Essletzbichler, P.; Volz, S.E.; Joung, J.; van der Oost, J.; Regev, A.; et al. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. Cell 2015, 163, 759–771.
37. Zaidi, S.S.E.A.; Mahfouz, M.M.; Mansoor, S. CRISPR-Cpf1: A New Tool for Plant Genome Editing. Trends Plant Sci. 2017, 22, 550–553.
38. Endo, A.; Masafumi, M.; Kaya, H.; Toki, S. Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from Francisella novicida. Sci. Rep. 2016, 6, 1–9.
39. Hu, X.; Wang, C.; Liu, Q.; Fu, Y.; Wang, K. Targeted mutagenesis in rice using CRISPR-Cpf1 system. J. Genet. Genomics 2017, 44, 71–73.
40. Xu, R.; Qin, R.; Li, H.; Li, D.; Li, L.; Wei, P.; Yang, J. Generation of targeted mutant rice using a CRISPR-Cpf1 system. Plant Biotechnol. J. 2017, 15, 713–717.
41. Yin, X.; Biswal, A.K.; Dionora, J.; Perdigon, K.M.; Balahadia, C.P.; Mazumdar, S.; Chater, C.; Lin, H.C.; Coe, R.A.; Kretzschmar, T.; et al. CRISPR-Cas9 and CRISPR-Cpf1 mediated targeting of a stomatal developmental gene EPFL9 in rice. Plant Cell Rep. 2017, 36, 745–757.
42. Wang, L.; Chen, S.; Peng, A.; Xie, Z.; He, Y.; Zou, X. CRISPR/Cas9-mediated editing of CsWRKY22 reduces susceptibility to Xanthomonas citri subsp. citri in Wanjincheng orange (Citrus sinensis (L.) Osbeck). Plant Biotechnol. Rep. 2019, 13, 501–510.

43. Jia, H.; Orbovi, V.; Wang, N. CRISPR-LbCas12a-mediated modification of citrus. Plant Biotechnol. J. 2019, 17, 1928–1937.
44. Zhang, F.; LeBlanc, C.; Irish, V.F.; Jacob, Y. Rapid and efficient CRISPR/Cas9 gene editing in Citrus using the YAO promoter. Plant Cell Rep. 2017, 36, 1883–1887.
45. Yan, L.; Wei, S.; Wu, Y.; Hu, R.; Li, H.; Yang, W.; Xie, Q. High-Efficiency Genome Editing in Arabidopsis Using YAO Promoter-Driven CRISPR/Cas9 System. Mol. Plant 2015, 8, 1820–1823.
46. LeBlanc, C.; Zhang, F.; Mendez, J.; Lozano, Y.; Chatpar, K.; Irish, V.F.; Jacob, Y. Increased efficiency of targeted mutagenesis by CRISPR/Cas9 in plants using heat stress. Plant J. 2018, 93, 377–386.
47. Jia, H.; Wang, N. Generation of homozygous canker-resistant citrus in the T0 generation using CRISPR-SpCas9p. Plant Biotechnol. J. 2020, 1–3.
48. Dutt, M.; Erpen, L.; Grosser, J.W. Genetic transformation of the ‘W Murcott’ tangor: Comparison between different techniques. Sci. Hortic. 2018, 242, 90–94.
49. Liang, Z.; Chen, K.; Li, T.; Zhang, Y.; Wang, Y.; Zhao, Q.; Liu, J.; Zhang, H.; Liu, C.; Ran, Y.; et al. Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat. Commun. 2017, 8, 6–10.
50. Malnoy, M.; Viola, R.; Jung, M.H.; Koo, O.J.; Kim, S.; Kim, J.S.; Velasco, R.; Kanchiswamy, C.N. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. 2016, 7, 1–9.
51. Caserta, R.; Teixeira-Silva, N.S.; Granato, L.M.; Dorta, S.O.; Rodrigues, C.M.; Mitre, L.K.; Yochikawa, J.T.H.; Fischer, E.R.; Nascimento, C.A.; Souza-Neto, R.R.; et al. Citrus biotechnology: What has been done to improve disease resistance in such an important crop? Biotechnol. Res. Innov. 2020.
52. Kim, J.I.; Kim, J.Y. New era of precision plant breeding using genome editing. Plant Biotechnol. Rep. 2019, 13, 419–421.