Development of a gRNA–tRNA array of CRISPR/Cas9 in combination with grafting technique to improve gene-editing efficiency of sweet orange

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Abstract

Key message Here, we developed a reliable protocol for the fast and efficient gene-edited Anliu sweet orange plants production. The application of in vitro shoot grafting technology significantly reduced the growth cycle of transgenic seedlings, and the survival rate of cleft grafting was more than 90%. In addition, the mutation efficiency of the grafted gene-edited sweet orange was significantly improved by short-term heat stress treatments. Thus, the combination strategy of grafting and heat stress treatments provided a reference for the fast and efficient multiplex gene editing of sweet orange.

Keywords PTG/Cas9 · Sweet orange · Grafting · Heat stress · Efficient gene editing

Sweet orange is one of the most popular fruit crops worldwide. Traditional breeding approaches in sweet orange are impractical due to the apomixis and long juvenility, making it difficult to obtain hybrids and selection of ideal genotypes. Anliu sweet orange is a local variety in Guangdong, Fujian and Guangxi provinces in Southern China. Seeds from Anliu sweet orange were used to verify multiple gene functions of citrus using Agrobacterium-mediated genetic transformation of Anliu sweet orange epicotyl. The development of targeted genome engineering technologies made it possible for the precise modification of target genes. Recently, a more efficient gene-editing tool has emerged based on the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system (Jinek et al. 2012). The development of CRISPR/Cas9 technology is promising to accelerate the process of genetic improvement in perennial crops. Here, we developed a reliable protocol for the fast and efficient gene-edited Anliu sweet orange plants production. The application of in vitro shoot grafting technology significantly reduced the growth cycle of transgenic seedlings, and the survival rate of cleft grafting was more than 90%. In addition, the mutation efficiency of the grafted gene-edited sweet orange was significantly improved by short-term heat stress treatments. Thus, the combination strategy of grafting and heat stress treatments provided a reference for the fast and efficient multiplex gene editing of sweet orange.

Gene editing in sweet orange is usually faced with difficulties including low editing efficiency and low root formation rate (de Oliveira et al. 2009), which severely limit the advancement of gene function studies and application in molecular breeding in sweet orange. Recently, the application of Arabidopsis YAO promoter-driven CRISPR/Cas9 system in citrus rootstock cultivar Carrizo Citrange significantly improved gene-editing efficiency over the 35S promoter-driven CRISPR/Cas9 system (Jia and Wang...
2014; Zhang et al. 2017). Meanwhile, studies have shown that polycistronic tRNA–gRNA (PTG) /Cas9 is more efficient for multiplex gene editing than the CRISPR/Cas9 system with a single gRNA under a Pol III promoter such as AtU6 (Xie et al. 2015; Wang et al. 2018), and for Carriço Citrange (Huang et al. 2020). Thus, it is possible to further improve the editing efficiency by adding the PTG to the YAO-driven Cas9 system in sweet orange. In
Fast and efficient gene-editing technology in sweet orange by using YAO promoter-driven PTG/Cas9 system in combination with grafting. A. Schematic map of the PTG/Cas9 system. hSpCas9 gene was driven by the Arabidopsis YAO promoter. Purple rounded rectangles represent tRNA. Red, yellow and orange rounded rectangles represent gRNA spacers, and the white rounded rectangles show gRNA scaffold. The black rectangle represents Pol III terminator. b. Mutation efficiency induced by three PTG/Cas9 vectors. The frequencies of induced mutations were the ratio of mutated lines to the transgenic lines. c. Phenotypes induced by PTG/Cas9 system in transgenic Anliu sweet orange. d. e. Sanger sequencing of site-target mutations in Anliu sweet orange. The nucleotide mutations compared with WT (wild type) are displayed in DNA sequence of CsPDS. The sequences in green represent gRNAs, and the PAM sites are in blue. The deleted nucleotides are shown in black dots. The inserted nucleotides are shown in red. f. Transgenic lines used as scions for grafting. g. Rootstocks preparation for grafting. The rootstocks were 1–2 months seedlings of the Anliu sweet orange grown on the MT basal medium. h. i. V-shaped scions were grafted onto prepared sweet orange rootstocks. Graft junctions were wrapped with parafilm. j. Grafted seedlings were cultured in vitro with tap water at 25 °C in the cycle of 16 h of light and 8 h of darkness. k. Statistical analysis of graft survival rate of Anliu sweet orange. l. Phenotypes of 10 months old grafted CsPDS gene-edited chimeric seedlings cultivated at 25 °C. m. New albino tissues formed 15 days after heat stress treatments (37 °C for 24 h, 25 °C for 24 h). n. Analyzing of the mutation efficiency in CsPDS gene-edited citrus plants exposed to heat stress. Old and young leaves formed before heat stress (HS) treatments (continuously grown at 25 °C) and after heat stress treatments were used (color figure online).

In addition, improving the survival rate of transgenic sweet orange seedlings by avoiding the high risk of root induction process still requires further investigation (Belide et al. 2011). To further explore the application of the PTG/Cas9 system in sweet orange, three gRNAs targeting phytoene desaturase (PDS) in Anliu sweet orange were used (Jia and Wang 2014; Zhang et al. 2017). The three targets were assembled into pCAMBIA1300-pYAO:hSpCas9-eGFP vector (Fig. 1a). We obtained 145 transgenic lines using eGFP selection marker, and the mutation frequencies of the transgenic lines transformed with three PTG/Cas9 vectors were analyzed by Hi-Tom sequencing. Statistical analysis revealed that 36.7% (18 of 49), 37.1% (13 of 35) and 49.2% (30 of 61) of the sweet orange lines transformed with A1, B1 and C1 were mutated, respectively (Fig. 1b). The albino phenotypes can be precisely induced using the PTG/Cas9 system after 1–2 months (Fig. 1c). Sanger sequencing revealed that most of the mutations induced by PTG/Cas9 in sweet orange were small InDels (Fig. 1d, e, Fig. S1).

Genetic transformation in sweet orange has been vastly studied, and the transformation efficiency varies among sweet orange varieties. In this study, we optimized the transformation conditions by using vacuum negative pressure with the Agrobacterium-mediated genetic transformation of epicotyls for 5 min by using Anliu genotype (Fig. S2a). The transformation efficiency was 18.22%–21.15% (Fig. S2b). To shorten the time and improve the survival rate of transgenic sweet orange, transgenic seedlings with three to four true leaves were grafted on the sweet orange rootstocks in vitro (Fig. 1f), the rootstocks were 1–2 months seedlings of the Anliu sweet orange grown on the MT basal medium (Fig. 1g), and all the grafting operations did not require aseptic treatment. The grafted seedlings were cultured in the tube with tap water, and the tubes were covered with parafilm (Fig. 1h–j), grown in a light room with 16/8 h (day/night) photoperiod for 2 weeks until new leaves came out. Our data showed that the survival rate of cleft grafting was more than 90% (Fig. 1k). As previous studies illustrated, heat stress treatments could increase targeted mutagenesis induced by CRISPR/Cas9 (LeBlanc et al. 2018). To improve the mutation efficiency of grafted mosaic sweet orange (Fig. 1l), 10-month-old grafted mosaic plants were exposed to heat stress treatments (37 °C for 24 h, 25 °C for 24 h) for 15 days. We observed new albino tissues grown from mosaic seedlings after heat stress treatments (Fig. 1m, S3c–d), as well as the increased mutation efficiency (Fig. 1n). The endogenous tRNA processing system exists in almost all species, the endogenous tRNA-processing RNases can process and cleave multiple gRNAs in one gRNA expression cassette, and the tRNA gene contains internal promoter elements, suggesting that it may act as transcription enhancers to enhance the expression of gRNAs (Xie et al. 2015). However in this study, the mutation frequencies of sweet orange (36.7–49.2%) induced by the three PTG/Cas9 vectors were not as efficient as the mutation frequencies (45.5–75%) induced by CRISPR/Cas9 in Carrizo Citrange (Zhang et al. 2017). We inferred that this may be related with that the gene-edited seedlings in this study were directly induced from stem sections from 1–2 months seedlings (Fig. S2a), while the transgenic plants of Carrizo Citrange were regenerated from callus, in which the prolonged YAO promoter activity may modulate the expression of SpCas9 (Zhang et al. 2017; Li et al. 2010), and a single AtU6 promoter was used to drive the expression of several gRNAs, which required further processing. In addition to promoters, temperature could also regulate the activity of SpCas9 and the mutation efficiency (LeBlanc et al. 2018). We found that the mutation efficiency of chimera sweet orange plants was also significantly increased after heat stress treatments (Fig. 1n), and our result was consistent with a previous report on Arabidopsis and Carrizo Citrange (LeBlanc et al. 2018).

We further evaluated the site-target mutations induced by the three PTG/Cas9 constructs using Agrobacterium-mediated genetic transformation of sweet orange epicotyls. For the target sites, the majority of the mutated alleles identified consisted of some deletions and therefore caused
a shift of reading frame, while the majority of mutations mediated by YAO promoter-driven CRISPR/Cas9 system were insertions (Zhang et al. 2017). This difference may be because the PTG/Cas9 system enables the generation of multiple double-strand breaks (DSB) in genomic DNA (Xie et al. 2015). Meanwhile, we examined the potential off-target effects of the three target sites, and no off-target mutations were detected in the five potential off-target sites (Table S2).

Cleft grafting in vitro significantly improving the survival rate of transgenic seedlings, and this is a suitable method for those plants which are hardly to root or susceptible to various soil pathogenic bacteria (Belide et al. 2011). The combination of grafting and heat stress treatments may be a suitable strategy for the knockout of plant reproduction- and development-related genes, which may affect plant regeneration or rooting if they are mutated at early growth stages. Here, we developed a fast and efficient gene-editing method for sweet orange; our approach offers a new way to facilitate the efficient multiplex gene editing of sweet orange, and this approach is especially suitable for the genetic improvement of citrus varieties.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00299-021-02781-7.

Acknowledgements We are grateful to Dr. Qi Xie from Institute of Genetics and Development, Chinese Academy of Sciences for providing the YAO promoter-driven CRISPR/Cas9 vector and Kabin Xie from College of Plant Sciences and Technology, Huazhong Agricultural University for the guidance of the vectors construction. This work was supported by the National Key Research and Development Program of China (NO. 2018YFD1000101), National Natural Science Foundation of China (NOS. 31925034 and 31872052), and the Fundamental Research Funds for the Central Universities.

Author contribution statement X.T., Q.X. and X.D. conceived and designed the experiments, X.T., S.C., H.Y. and X.Z. conducted the experiments. X.T. and S.C. analyzed data. X.T. wrote the manuscript. Q.X. and F.Z. directed the study and revised the manuscript. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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