**Supplemental Materials**

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

**Xiaomei Tang1 · Shulin Chen1 · Huiwen Yu2 · Xiongjie Zheng1, 3 · Fei Zhang1 · Xiuxin Deng1 · Qiang Xu1\***

1 Key Laboratory of Horticultural Plant Biology Ministry of Education, Huazhong Agricultural University, Wuhan, the People’s Republic of China

2 Key Laboratory of Landscape Plants with Fujian and Taiwan Characteristics of Fujian Colleges and Universities, Minnan Normal University, Zhangzhou 363000, China

3 Present address: Division of Biological and Environmental Science and Engineering, Center for Desert Agriculture, the BioActives Lab, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia

\* xuqiang@mail.hzau.edu.cn

Xiaomei Tang and Shulin Chen contributed equally to this work.

**Supplementary Tab. S1** Primers used in this study for the PCR amplification of PTG/Cas9 vectors and *CsPDS* gene, sequencing of AtU6-26-sgRNA-SK and PTG/Cas9 and amplification of off target gene.

|  |  |  |
| --- | --- | --- |
| Primer name | Sequence of primers (5’-3’) | Usage |
| tRNA-F | AACAAAGCACCAGTGGTCTAGTGGTAGAATAGTACCCTGCCACGGTACAG | tRNA fragment amplifying |
| tRNA-R | TGCACCAGCCGGGAATCGAACCCGGGTCTGTACCGTGGCAGGGTACTATTC |
| BsaI-tRNA-F | GCTAGAGTCGAAGTAGTGATTGAACAAAGCACCAGTGGTCTAG | PTG/Cas9 vectors construction |
| BsaI-tRNA-gRNA1-R | GCTATTTCTAGCTCTAAAACGCACCAGCAATTACAACTT |
| BsaI-tRNA-gRNA2-R | GCTATTTCTAGCTCTAAAACGATGAACTGTCAATGCAATG |
| BsaI-tRNA-gRNA3-R | GCTATTTCTAGCTCTAAAACGTACAATTGCTTGTGCACAC |
| sgRNA-tRNA-F | GCACCGAGTCGGTGCTTTTTTTAACAAAGCACCAGTGGTCTAGTGG |
| sgRNA-tRNA-R | CCACTAGACCACTGGTGCTTTGTTAAAAAAAGCACCGACTCGGTGC |
| SK-gRNA-F | CTCACTATAGGGCGAATTGG | Primers for gene editing vectors verification |
| 1300-gRNA-F | CCAGTCACGACGTTGTAAAAC |
| 1300-gRNA-R | CAATGAATTTCCCATCGTCGAG |
| gRNA1-F | TTTCCGAGATAGTGAACCGATGG | Amplification primers for target gRNA1 |
| gRNA1-R  gRNA2-F | ACACACCCATAGGCTCCATTTCA |
| CAACCTTCAGATTTGGTGAGAGA | Amplification primers for target gRNA2 |
| gRNA2-R | ATACAGGTTTCACAAGGCAGAGA |
| gRNA3-F | TCCGAGCCATACAAATACTACGA | Amplification primers for target gRNA3 |
| gRNA3-R | CGAGTCGGAGATTAGCAACAAGT |
| gRNA1-DQ-F | ggagtgagtacggtgtgcAGGTTTGGTTGTTATTATTCTT | Amplification primers of target gRNAs for Hi-Tom sequencing |
| gRNA1-DQ-R  gRNA2-DQ-F | gagttggatgctggatggTAGAGGAGAAGAAAATGAAGTC  ggagtgagtacggtgtgcTTCTCCATCTTCAAAAGTTCAA |
| gRNA2-DQ-R | gagttggatgctggatggATACAGGTTTCACAAGGCAGA |
| gRNA3-DQ-F | ggagtgagtacggtgtgcTGTGAACCTTGCCGTCCCTTA |
| gRNA3-DQ-R | gagttggatgctggatggCAGCAATGCCAAGCCACAAAG |
| Off target1-F | ATTTTTAAGTTCGAAGATATG | Off target primers for gRNA1 |
| Off target1-R | ATCTTACTCCAGTCATTTAATC |
| Off target2-F | CCATCTTCTAATCTTTCTCTG | Off target primers for gRNA2 |
| Off target2-R | ATTTTGTCAAAGTAGTGTTAC |
| Off target3-F | CCACTGGAACTGGTGGAATC | Off target primers for gRNA3 |
| Off target3-R | TGATCATGGATATAAGAAGAC |

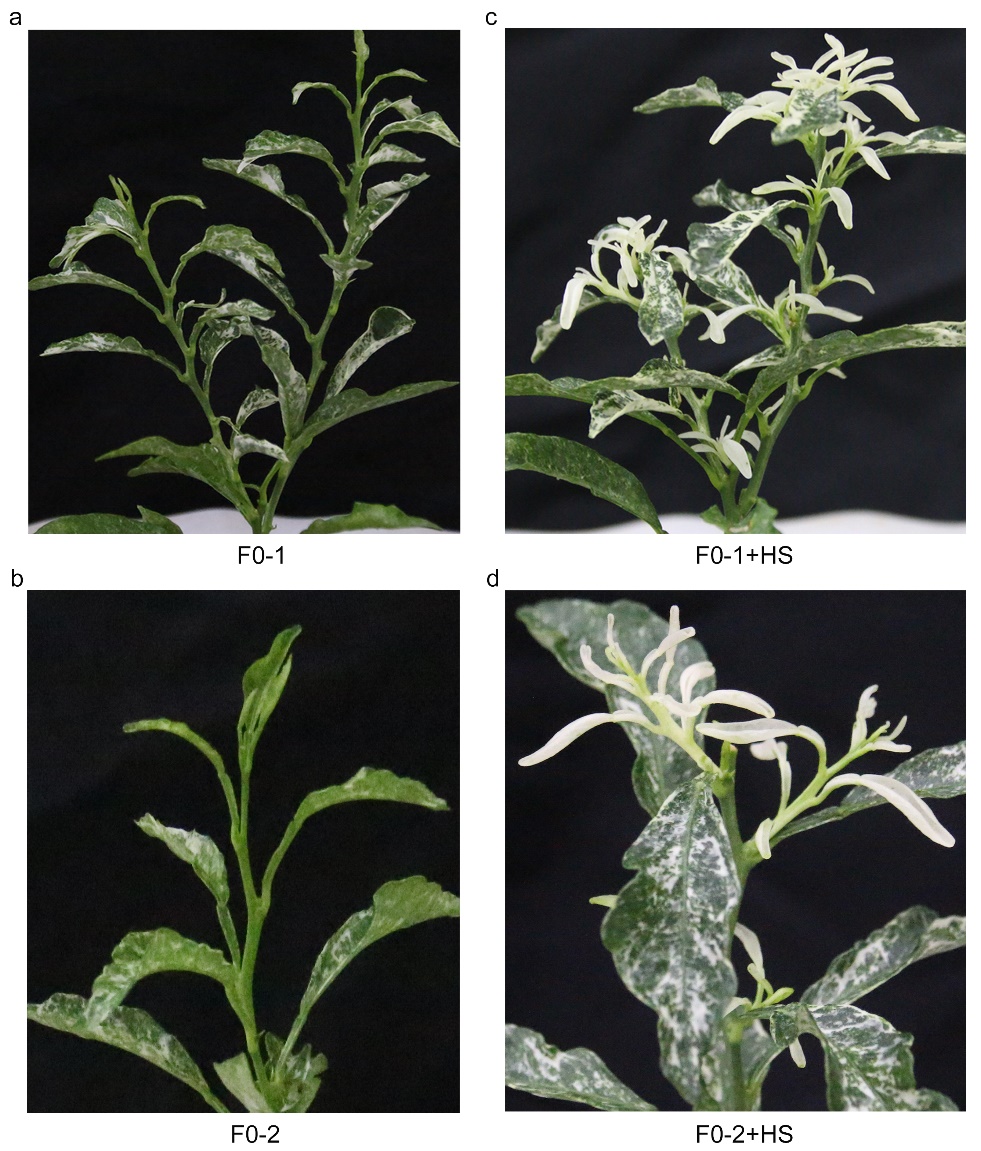
Note: the nucleotides in red color represent Hi-Tom sequencing adapter primers.



**Supplementary Fig. 1** Mutation detection of three target sites induced by PTG/Cas9. T0 lines are A1#12, B1#2, and C1#31. The sequences in green indicates three gRNAs, PAM are marked in blue. Deleted nucleotides are listed as dots, the mutated nucleotides are shown in red. The lengths of the deletions (D), insertions (I) and mutations are presented on the right of the sequences.



**Supplementary Fig. 2** *Agrobacterium*-mediated highly efficient genetic transformation of Anliu sweet orange. (**a**) Schematic represents the complete procedure for the generation of gene-edited sweet orange. (**b**) The transformation efficiency of Anliu sweet orange transformed with PTG/Cas9 vectors A1, B1 and C1.



**Supplementary Fig. 3** (**a-b**)Phenotypes of grafted *CsPDS* gene-edited chimeric seedlings (F0-1 and F0-2) cultivated in green house at 25°C. (**c-d**) New albino leaf-shoots formed on F0-1 and F0-2 chimeric seedlings 15 days after heat stress treatment of 37°C (The shoots of F0-1 and F0-2 chimeric seedlings were removed before heat stress treatment).

**Materials and Methods**

**Plant materials and growth conditions**

Seeds of Anliu sweet orange (*Citrus sinensis* Osbeck) were collected and sterilized with 30% (v/v) sodium hypochlorite for 15-20 mins, following with sterile water for three to five times, the seeds were sowed in MT basal medium and cultured in the dark room at 25oC for 20-30 days. The etiolated seedlings were transferred into light room for 7-10 days with 16 h of light and 8 h of darkness.

**Expression vector construction**

Phytoene desaturase (*PDS*) gene was selected as targeted gene. Three gRNAs were selected in the coding region, namely gRNA1, gRNA2 and gRNA3 as previously reported (Jia and Wang 2014; Zhang et al. 2017), and the Cas9 binary construct was pCAMBIA1300-pYAO:Cas9-eGFP (Yan et al. 2015; Zhang et al. 2017). Fragments containing tRNA and gRNA were amplified from the vector pGTR (Xie et al. 2015) using the forward primer BsaI-tRNA-F and the reverse primers BsaI-tRNA-gRNA1-R/BsaI-tRNA-gRNA2-R/BsaI-tRNA-gRNA3-R, and inserted into the vector AtU6-26-sgRNA-SK (Yan et al. 2015) linearized with *Bsa* I. Fragments g1, g2 and g3 were amplified from vector AtU6-26-sgRNA-SK containing tRNA and gRNAs using primers BsaI-tRNA-F/sgRNA-tRNA-R, sgRNA-tRNA-F/BsaI-tRNA-gRNA2-R or BsaI-tRNA-gRNA3-R. Fragments g12 and g13 were amplified from fragments g1 and g2 or g1 and g3 using primers BsaI-tRNA-F, BsaI-tRNA-gRNA2-R/BsaI-tRNA-gRNA3-R, and inserted into vector AtU6-26-sgRNA-SK. Fragment g12-1 was amplified from vector AtU6-26-sgRNA-SK containing g12 using primers BsaI-tRNA-F/sgRNA-tRNA-R, Fragment g123 was amplified from g12-1 and g3 using primers BsaI-tRNA-F/BsaI-tRNA-gRNA3-R and inserted into vector AtU6-26-sgRNA-SK. The constructs of AtU6-26-sgRNA-SK containing corresponding gRNAs were sequenced by primer SK-gRNA-F, the positive plasmids were digested with *Spe* I and *Nhe* I, and transferred into the binary vector of pCAMBIA1300-pYAO:Cas9-eGFP as previous reported (Yan et al. 2015; Zhang et al. 2017).

**Genetic transformation of sweet orange**

The three constructs were transferred into *Agrobacterium tumefaciens* strain EHA105. The stems (1-2 cm) were dipped into *Agrobacterium tumefaciens* suspension medium containing different vectors and shaken for 15 mins at 200 rpm, following with vacuum negative pressure of 0.6 Mpa for 5 mins. The stems were transferred into MS medium containing 20 mg/L Acetosyringone (AS), 0.5 g/L Malt extract and 1.5 g/L L-glutamine at 21-23oC for 3 days. The stems were washed three times with sterilized water and transferred to the regeneration medium (MT + 40 g/L sucrose + 0.5 mg/L 6-BA + 0.5 mg/L KT + 0.1 mg/L NAA + 8.0 g/L Agar+ 25 mg/L Km + 400 mg/L Cef, pH 5.85) under a 7 days dark culture at 25oC, following with 16 h of light / 8 h of darkness at 25oC for 30-60 days.

**Mutation detection and off-targets analysis**

Genomic DNA was extracted from Anliu sweet orange leaves according to previous report (Cheng et al. 2003). In order to validate T-DNA insertion, target specific primers (S1 Table) were designed and used for PCR amplification containing the gRNAs, the PCR products were sequenced using Hi-Tom sequencing technology (Liu et al. 2019) at the experimental base of National Rice Research Institute in Fuyang city of China. We further verified the mutation types by introducing the PCR products to the TA/Blunt-Zero vector (Vazyme, Nanjing, China), eight positive clones were selected for sanger sequencing. The primers to detect the potential off-target sites of three gRNAs were listed in S1 Table, the amplified products were purified for sanger sequencing. The sequence alignment analysis was performed using the BioEdit and DNAMAN software.

**Grafting and heat stress treatment of transgenic seedlings**

The transgenic seedlings with 3-4 true leaves were used for grafting and 30-60 days old Anliu sweet orange seedlings were used as rootstocks. The base of the transgenic scions were cut to a matching V-shape and inserted into the rootstocks, the grafting point was wrapped with parafilm. The grafted seedlings were cultured in the tube with running water and transferred into light room with 16/8 h (day/night) photoperiod about 2 weeks until new leaves came out. Decapitated 10-month old grafting plants were subjected to heat stress treatments (37°C for 24 h, 25°C for 24 h) for 15 days.

**Supplementary references**

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