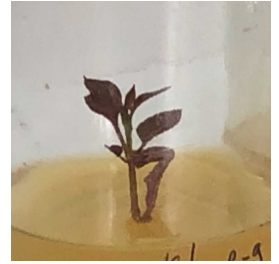


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Breaking the Glass Ceiling of Stable Genetic Transformation and Gene Editing in the Popular Pepper cv Cayenne

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Manoj Kumar¹, Dana Ayzenshtat¹, Gulzar A. Rather¹, Emmanuella Aisemberg¹, Alexander Goldshmidt¹, Samuel Bocobza¹

¹Institute of Plant Sciences, Agricultural Research Organization – Volcani Institute, Rishon LeZion, Israel



Samuel Bocobza

Agriculture Research Organization - Volcani Institute

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Abstract

A commercial cultivar of pepper *C. annum* named Cayenne was used in this study. Seeds were surface sterilized with 1.2% sodium hypochlorite and 0.01% tween-20 for 13 minutes, rinsed three times with distilled water, and incubated on half-strength MS medium including Gamborg vitamin mixture supplemented with 2% sugar and 0.8% plant agar. Seeds were kept for two days in dark conditions. The pH was adjusted to 5.8, and all media were autoclaved for 20 min at 121 °C. Germinated seeds and plantlets were grown at 25 °C ± 2 °C, 16 h photoperiod of 100 µmol m⁻² s⁻¹ irradiance, and relative humidity of ~50%. After 14 days following germination, cotyledons were excised and used for *Agrobacterium*-mediated genetic transformation.

Guidelines

See the corresponding file for a full description of the method.

Materials

All tissue culture materials were purchased from Duchefa Biochemie (Netherlands).

Safety warnings

 Avoid contaminations.

Determination of the Minimum Inhibitory Concentration of Kanamycin for Pepper Cotyledon Explants

- 1 The minimum inhibitory concentration of kanamycin was determined by placing 14-day-old pepper cotyledon explants on regeneration media supplemented with increasing concentrations of kanamycin (0, 25, 50, 75, and 100 mg/l).
- 2 After five weeks, events of regeneration and tissue senescence were monitored, and the percentage of vigorous explants was calculated.
- 3 100 mg/l kanamycin was needed to completely inhibit de novo organ formation in this pepper cultivar.

Constructs Assembly and Agrobacterium Preparation

- 4 All plasmids were assembled using the GoldenBraid cloning system.
- 5 Promoter and terminator regions were cloned into entry vectors and used to generate expression cassettes.
- 6 Inserts corresponding to genes, promoters, terminators, tRNA, and gRNA were cloned into the pUPD entry vector and assembled into expression cassettes in alpha vectors.
- 7 gRNA expression cassettes were generated using the tomato glycine tRNA.
- 8 The RED FLUORESCENT PROTEIN (RFP), CaAN2, and NPT2 genes were assembled with various promoters and fused to the terminator region of the tomato TH14.
- 9 The pepper ortholog of the tomato SIAGO7 was identified, and two gRNAs were designed to target the second exon of CaAGO7.
- 10 The gRNA cassettes were fused and combined with the NPT2, RFP, and SpCAS9 genes.
- 11 The pepper ortholog of the tomato SIPPO was identified, and one gRNA was designed.



- 12 The ortholog of the arabidopsis SGS3 was identified, and a silencing cassette was built using RNAi.
- 13 Binary vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw method.

Agrobacterium-mediated Transformation of Pepper Plants

- 14 Thirty pepper cotyledons were excised from 2-week-old in vitro-grown pepper plantlets.
- 15 Cotyledons were cut at both ends and incubated for 30 min with the *Agrobacterium* suspension cultures.
- 16 Cotyledons were dried on sterile filter papers and placed onto the regeneration medium for 48 hours in the dark at 25 ± 2 °C.
- 17 Explants were transferred to the selection medium supplemented with 100 mg/l kanamycin and 250 mg/l ticarcillin.
- 18 Kanamycin-resistant explants were transferred to the elongation medium for two rounds of selection of 14 days each.
- 19 Green, healthy kanamycin-resistant shoots were transferred to the rooting medium.
- 20 Rooted plants were transferred to sterile peat pellets and kept under transparent polybags.
- 21 When plants reached about 20 cm in height, they were transferred to 3-liter pots and then to the greenhouse.

Fluorescent Microscopy

- 22 RFP fluorescence was observed in the transformants using fluorescent microscopy at different stages of plant development.



- 23 A fluorescent binocular was used, with settings adjusted to 3x magnification for calli and 1x magnification for shoots.
- 24 Non-transformed plant cotyledons were used as a control for imaging under the same settings.

DNA Extraction, PCR Genotyping, and Sanger Sequencing

- 25 Genomic DNAs were extracted from approximately 100 mg of leaves using the C-TAB method.
- 26 Quantification of gDNA was performed using a nanodrop spectrophotometer.
- 27 For genotyping, a PCR reaction mixture was prepared and run on a PCR machine.
- 28 The PCR program consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 kb/min.
- 29 The final extension was performed at 72 °C for 5 min, and the amplicons were observed in 1% agarose gel.
- 30 Primers corresponding to specific genes were used for each reaction.

Mutation Analysis

- 31 To determine the presence of mutations at the CaAGO7 target site, genomic DNAs were used for PCR amplification.
- 32 The resulting amplicons were subjected to Sanger sequencing, and chromatographs were analyzed using the Synthego CRISPR analysis tool.
- 33 Amplicons from four independent lines were cloned into the pUPD2 entry vector and subjected to Sanger sequencing.



- 34 CRISPR/Cas9-induced mutations were detected using DNA sequence alignments.

Generation Advancement and Segregation Analysis of Transgenic Events

- 35 Transgenic plants were grown in greenhouse conditions until flowers were obtained and were self-fertilized or crossed with wt flowers.
- 36 Thirty T1 seeds from each line were germinated in soil under controlled conditions.
- 37 The number of green and purple T1 seedlings was recorded, and PCR analyses were conducted to confirm the presence of the transgene.

RNA Extraction, cDNA Synthesis, and Real-time PCR Analysis

- 38 Leaf samples from T1 peppers were harvested for RNA extraction.
- 39 Total RNA was isolated using TRI-ZOL reagent.
- 40 The quality and quantity of RNA samples were analyzed by agarose gel electrophoresis and Nanodrop spectrophotometer.
- 41 High-quality total RNA samples were used for cDNA synthesis.
- 42 The synthesized cDNAs were diluted and used for qRT-PCR.
- 43 The reaction was carried out in a Rotor-Gene 6000 Real-time PCR machine using specific thermal cycling conditions.
- 44 The pepper BETA TUBULIN gene was used as an internal control for normalization.



- 45 The transcript levels were calculated using the standard $2^{-\Delta\Delta CT}$ method.
- 46 Significant differences among means were calculated using Tukey's HSD test.

Enzyme Extraction and Measurements of PPO Activity

- 47 Leaf samples from T1 pepper plants were homogenized in liquid nitrogen and resuspended in sodium phosphate buffer.
- 48 Following centrifugation, the supernatant was transferred and kept on ice.
- 49 Protein concentration was determined using the Bradford method.
- 50 The PPO enzymatic activity was determined by measuring the absorbance at 420 nm.