A long shelf-life melon created via CRISPR/Cas9 RNP-based *in planta* genome editing

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Keywords: CRISPR/Cas9, ethylene, genome editing, melon, shoot apical meristem, shelf-life

Abstract

We developed a non-culture and DNA-free genome editing technique for melon using in plant Particle Bombardment (iPB-RNP). The method was employed to create CmACO1 mutants. One of the mutant lines exhibited an extended shelf life due to ethylene deficiency during fruit ripening. As no cell culture step is involved, the iPB-RNP method is expected to overcome limitations associated with conventional genome editing, such as genotype dependency and somatic variations. Thus, this methodology holds significant potential for application in commercial melon breeding and across a wide range of Cucurbitaceae species.

Melon (*Cucumis melo* L.) is a widely consumed fruit globally. Although conventional breeding has enhanced the quality and productivity of melon varieties, there is a pressing need for rapid and diverse genetic improvements. Genome editing is anticipated to fulfill these demands. Most current genome editing protocols depend on genetic transformation and tissue culture, yet melon exhibits low transformation efficiency due to its limited regeneration capabilities and the occurrence of escapes (Shirazi Parsa *et al.*, 2023). Additionally, melon is prone to ploidy changes during tissue culture, altering morphogenesis in regenerated plants (Nonaka *et al.*, 2023; Nuñez-Palenius *et al.*, 2008). Therefore, an efficient and robust system for melon genetic enhancement through genome editing is required.

In a prior study, we developed a transgene-free genome editing approach, *in planta* particle bombardment-ribonucleoprotein (iPB-RNP), applied in wheat and barley (Kumagai *et al.*, 2022; Tezuka *et al.*, 2024). This technique employs the CRISPR/Cas9 ribonucleoprotein, introduced directly into the shoot apical meristem (SAM) to specifically target subepidermal L2 cells, potential germline cells (Goldberg *et al.*, 1993). Since it bypasses cell culture and regeneration processes, iPB-RNP could be applicable to recalcitrant crop species, including melon.

To assess the feasibility of genome editing in melon via iPB-RNP, we first evaluated the efficiency of gold particle delivery into the meristematic cells . Following 20 hours of imbibition, we removed half of the cotyledon from germinated seeds (cv. "Harukei-3") to expose the SAM, and positioned the remaining seeds on an agar plate with the exposed SAM facing upwards (Figure 1a). Gold particles coated with a GFP-expressing vector were bombarded towards the SAMs on the plate (Figure 1a). After 16 hours in darkness, GFP signals were observed on the SAM surface in almost all bombarded plants, unlike the controls (Figure 1a and S1). These results confirmed the proper preparation of SAMs and the suitability of the bombardment conditions for melon iPB.

To implement the iPB-RNP method in melon, we targeted *CmGAD1*, a glutamate decarboxylase gene (You *et al.*, 2024). Pre-assembled SpCas9 RNP targeting *CmGAD1* (Figure S2) was coated onto gold particles and delivered to the SAM of germinated seeds. The plants grown from the bombarded seeds were analyzed after 4-5 weeks, and their 6th leaf was subjected to cleaved amplified polymorphic sequences (CAPS) analysis for mutation detection (Figure 1b). Seven out of 153 initially bombarded plants showed undigested bands in the CAPS assay (Figure 1c and Table S1), with subsequent sequencing confirming mutations at the target site (Figure 1d). Since the E_0 plants exhibit chimeric mutations, E_1 plants were analyzed to confirm fixed mutations. The CAPS assay detected a mutant allele of *CmGAD1* in two out of the seven E_0 progenitors (Figure 1e and Table S1). Among these, #2-13_2, #2-13_3, #2-16_5, and #2-16_6 were homozygous mutants for *CmGAD1* (Figure 1e), and sequence analysis showed that mutations in the E_1 plants were consistent with those detected in the E_0 generation (Figure 1d and S3). The efficiency of iPB-RNP editing for *CmGAD1* was

1.31% (two out of 153 bombarded plants), comparable to efficiencies observed in wheat and barley (Kumagai *et al.*, 2022; Tezuka *et al.*, 2024).

Having established the iPB-RNP in melon, we then applied this method to extend the shelf-life of melon, a desirable trait. Since the gaseous phytohormone ethylene controls fruit maturation, our target was *CmACO1*, an ethylene biosynthesis gene (Nonaka *et al.*, 2023). We designed a guide RNA for *CmACO1* (Figure 1f) and synthesized it either enzymatically or chemically (Figure 1g). Cas9 RNPs targeting *CmACO1* were delivered into SAMs by iPB-RNP. Ultimately, we obtained one and three genome-edited lines, corresponding to 1.27% and 1.32% of the total, with enzymatically and chemically synthesized gRNAs, respectively (Figure 1g and S4).

Given the role of *CmACO1* in fruit ripening, we compared the fruit phenotype between the wild-type and the #3-2 homozygous mutant (Figure 1h). The #3-2 line harbors a one-base insertion within the *CmACO1* gene, suggesting a loss of *CmACO1* function (Figure 1i and S4f). The fruit size and shape of the *cmaco1* mutant were comparable to those of the wild-type. Ten days after harvest, the pericarp color changed from green to yellow in the wild-type fruit but remained green in the *cmaco1* mutant (Figure 1j). Cross-sectional analysis of the fruit after harvest revealed a mushy texture in the wild-type, which was absent in the *cmaco1* mutant (Figure 1k). To confirm that the delay in fruit ripening was caused by reduced ethylene production in the *cmaco1* mutant, ethylene levels were measured post-harvest. Wild-type fruit exhibited a significant ethylene burst, peaking 6 days after harvest, whereas the *cmaco1* mutant consistently produced significantly lower ethylene levels up to 14 days after harvest (Figure 1l). In a previous study, a *cmaco1* mutant generated by conventional *Agrobacterium*-mediated transformation displayed smaller and flattened fruit due to somatic mutations that induced tetraploidy (Nonaka *et al.*, 2023). Our findings demonstrate that the iPB-RNP method effectively circumvents complications associated with cell cultures, ultimately enabling the creation of melons with an extended shelf life without inducing morphological or growth defects.

In summary, we developed a non-culture and DNA-free editing technique for melon using iPB-RNP. The efficiency for obtaining positive plants in the E_1 generation reached 1.27% and 1.32% for *CmACO1*, and 1.31% for *CmGAD1* (Figure 1g and Table S1). This editing efficiency in melon is comparable with those in wheat and barley (Kumagai *et al.*, 2022; Tezuka *et al.*, 2024). As no cell culture step is involved, the iPB-RNP method is expected to overcome limitations associated with conventional genome editing, such as genotype dependency and somatic variations (Nuñez-Palenius *et al.*, 2008). Thus, this methodology holds significant potential for application in commercial melon breeding and across a wide range of Cucurbitaceae species.

Acknowledgements

This work was supported by the Cross-ministerial Strategic Innovation Promotion Program (SIP), "Technologies for Smart Bio-industry and Agriculture", with funding provided by the Bio-oriented

Technology Research Advancement Institution to H.E. and R.I.

Conflict of interests

The authors declare no conflict of interest.

Author contributions

H.E., and R.I. designed research; K.S., N.M., and S.N. performed research; K.S., N.M., H.E., and R.I. analyzed data; and K.S. and R.I. wrote the paper.

References

Goldberg, R.B., Beals, T.P., and Sanders, P.M. (1993) *Anther development: Basic principles and practical applications*. *Plant Cell*, **5**, 1217–1229.

Kumagai, Y., Liu, Y., Hamada, H., Luo, W., Zhu, J., Kuroki, M., et al. (2022) *Introduction of a second "Green Revolution" mutation into wheat via in planta CRISPR/Cas9 delivery*. *Plant Physiol.*, **188**, 1838–1842.

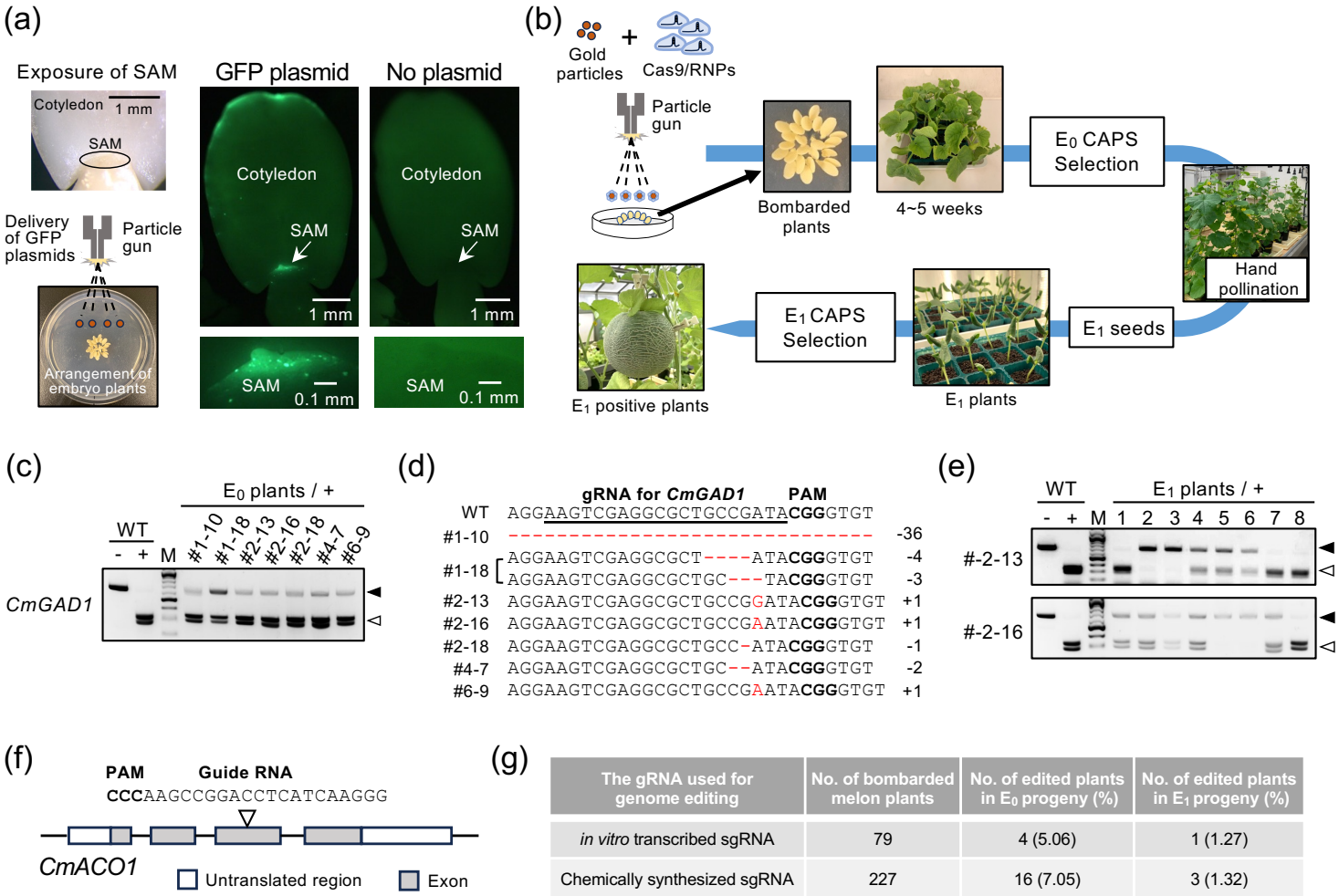
Nonaka, S., Ito, M., and Ezura, H. (2023) *Targeted modification of CmACO1 by CRISPR/Cas9 extends the shelf-life of Cucumis melo var. reticulatus melon*. *Front. Genome Ed.*, **5**, 1–17.

Nuñez-Palenius, H.G., Gomez-Lim, M., Ochoa-Alejo, N., Grumet, R., Lester, G., and Cantliffe, D.J. (2008) *Melon fruits: Genetic diversity, physiology, and biotechnology features*. *Crit. Rev. Biotechnol.*, **28**, 13–55.

Shirazi Parsa, H., Sabet, M.S., Moieni, A., Shojaeiyan, A., Dogimont, C., Boualem, A., and Bendahmane, A. (2023) *CRISPR/Cas9-Mediated Cytosine Base Editing Using an Improved Transformation Procedure in Melon (Cucumis melo L.)*. *Int. J. Mol. Sci.*, **24**, 11189.

Tezuka, D., Cho, H., Onodera, H., Linghu, Q., Chijimatsu, T., Hata, M., and Imai, R. (2024) *Redirecting barley breeding for grass production through genome editing of Photoperiod-H1*. *Plant Physiol.*, **195**, 287–290.

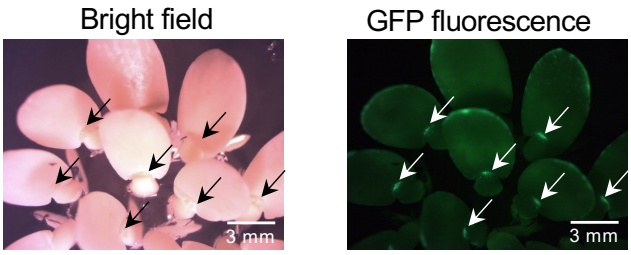
You, W., Zhang, J., Ru, X., Xu, F., Wu, Z., Jin, P., et al. (2024) *CmCML11 interacts with CmCAMTA5 to enhance γ-aminobutyric acid (GABA) accumulation by regulating GABA shunt in fresh-cut cantaloupe*. *Plant Physiol. Biochem.*, **206**, 108217.



The gRNA used for genome editing	No. of bombarded melon plants	No. of edited plants in E_0 progeny (%)	No. of edited plants in E_1 progeny (%)
in vitro transcribed sgRNA	79	4 (5.06)	1 (1.27)
Chemically synthesized sgRNA	227	16 (7.05)	3 (1.32)

Figure 1 *In planta* RNP-mediated genome editing in melon. (a) Microprojectile-mediated GFP gene transfer to melon SAMs. (b) Overview of the iPB-RNP method for genome editing in melon. (c) CAPS analysis of positive E_0 plants carrying mutations at the targeted *CmGAD1* locus. Symbols "–" and "+" represent digestion without and with Cas9 RNPs, respectively. Black and white triangles indicate undigested and digested bands following Cas9 RNP treatment, respectively. (d) Alignment of CRISPR/Cas9 target sequences from positive E_0 plants with that of the wild-type, with insertions and deletions highlighted in red letters. (e) Gene-specific CAPS analysis of E_1 plants derived from two E_0 plants (#2-13 and #2-16), using the same symbols as in (c). (f) Schematic of the *CmACO1* gene with gRNA design. (g) Summary of genome editing experiments targeting *CmACO1*. (h) Gene-specific CAPS analysis of the *cmaco1* homozygous mutant line (#3-2, E_2 generation), using symbols consistent with (c). (i) Amino acid sequence comparison of CmACO1 in wild-type and the *cmaco1*, with target sites for CRISPR/Cas9 underlined and sequence changes in red letters. An asterisk denotes the stop codon. (j and k) Appearance (j) and longitudinal section (k) of fruit from wild-type and the *cmaco1* post-harvest. (l) Ethylene production in fruits from wild-type and *cmaco1* (individual plants E2-1 and E2-2 in the E_2 generation) measured 40 days after pollination. Data are presented as means ± SE (n = 3).

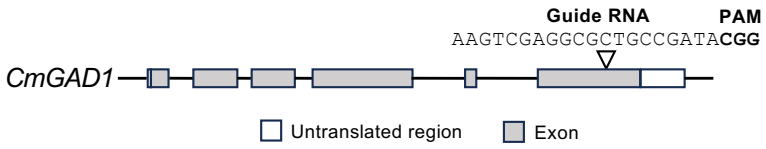
(a)



(b)

Expt.	No. of bombarded plants	No. of plants carrying GFP within SAM (%)
1	14	13 (92.9)
2	14	14 (100)

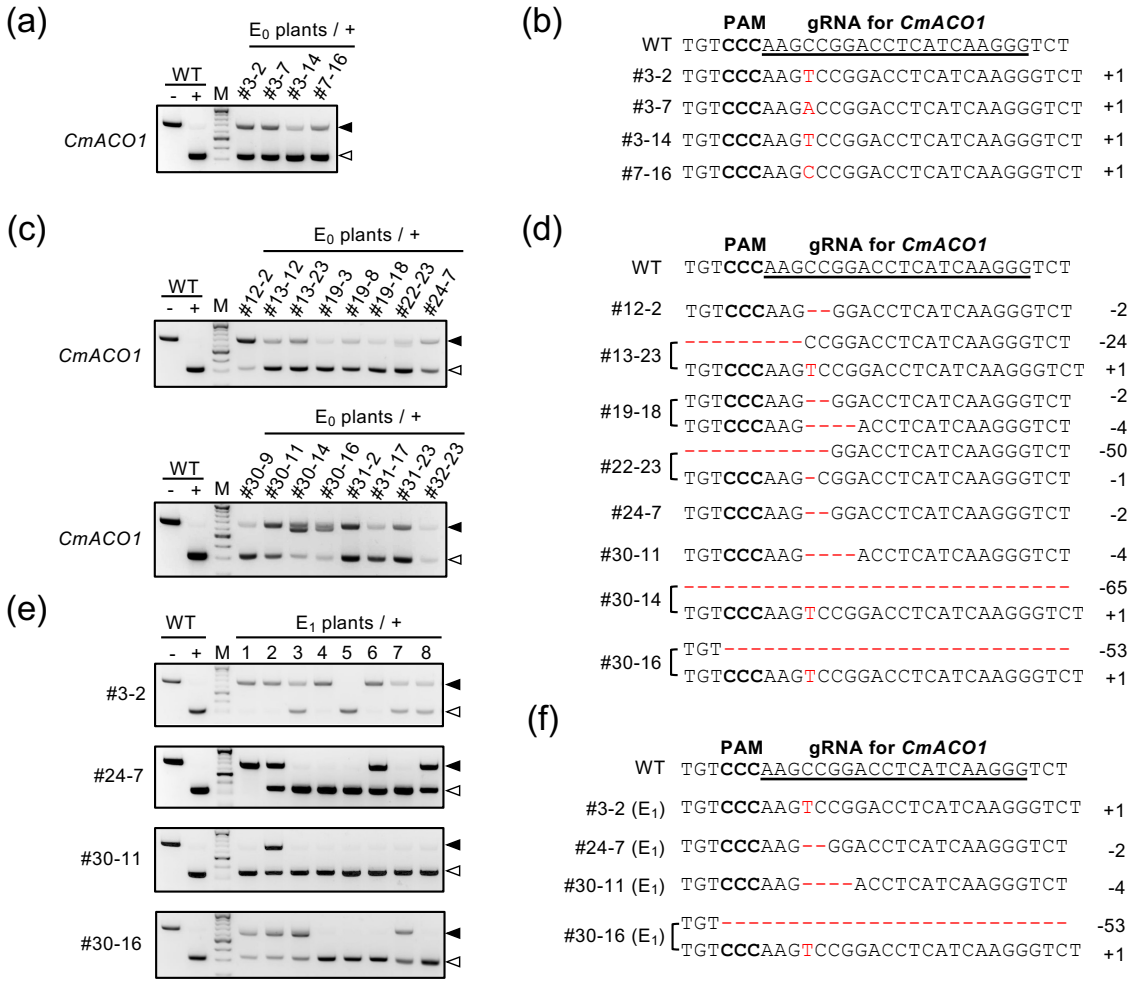
Supporting Figure S1 Microprojectile-mediated GFP gene transfer to melon SAMs. (a) Bright field and fluorescence images of plants 16 hours post-bombardment, with SAMs highlighted by arrows. (b) Microprojectile-mediated delivery efficiency of the GFP plasmid in melon.



Supporting Figure S2 *CmGAD1* gene with gRNA design. This schematic provides an overview of the gene structure and the regions targeted by the guide RNA (gRNA).

	gRNA for *CmGAD1*	PAM	
WT	AGGAAGTCGAGGCGCTGCCGATA	**CGG**GTGT	
#2-13 (E_1)	AGGAAGTCGAGGCGCTGCCGGATA	**CGG**GTGT	+1
#2-16 (E_1)	AGGAAGTCGAGGCGCTGCCGAATA	**CGG**GTGT	+1

Supporting Figure S3 Sequence evaluation of *cmgad1* mutant lines in the E_1 generation. The CRISPR/Cas9 target sequence of the positive E_1 plants (#2-13 and #2-16) is aligned with that of the wild-type, with insertions highlighted in red letters.



Supporting Figure S4 Introduction of a mutation in *CmACO1*. (a) CAPS analysis of positive E_0 plants created using *in vitro*-transcribed sgRNA. Symbols "–" and "+" denote digestion without and with Cas9 RNPs, respectively. Black and white triangles represent undigested and digested bands after Cas9 RNP treatment, respectively. (b) The CRISPR/Cas9 target sequences of positive E_0 plants in (a) are aligned with the wild-type sequence. Insertions are highlighted in red letters. (c) CAPS analysis of positive E_0 plants generated using chemically synthesized sgRNA. The symbols have the same meaning as in (a). (d) The CRISPR/Cas9 target sequences of some positive E_0 plants in (c) are aligned with the wild-type sequence. Insertions and deletions are indicated in red letters. (e) Gene-specific CAPS analysis of E_1 plants derived from four positive E_0 plants (#3-2, #24-7, #30-11, and #30-16). The symbols are the same as in (a). (f) The CRISPR/Cas9 target sequence of positive E_1 plants (#3-2, #24-7, #30-11, and #30-16) is aligned with that of the wild-type. The symbols are the same as in (d).

Supporting Table S1 Summary of the genome editing experiment targeting *CmGAD1*. This table presents the editing efficiency calculated based on the number of bombarded plants.

Target gene	No. of bombarded melon plants	No. of edited plants in E_0 progeny (%)	No. of edited plants in E_1 progeny (%)
CmGAD1	153	7 (4.58)	2 (1.31)

Supporting Table S2 Primers used in this study.

Primer name	Primer sequence	Note	Purpose
CmGAD1-2Fw	GGGTACAAAAACGTGATGGAAAACTG	For E_0 and E_1 screening	To amplify the *CmGAD1* target region
CmGAD1-2Rv	TGGTGGAAAAAGGGTGTGTATTGTG		
CmACO1-1Fw	TGGGAAAGCACCTTTTTCTTACGCCA	For E_0 and E_1 screening	To amplify the *CmACO1* target region
CmACO1-1Rv	ATCGACATTCGCCCAGTTCC		
CmGAD1_gRNA_Fw	TAATACGACTCACTATAGAAGTCGAGGCGCTGCCG	CmGAD1	Template DNA amplification to synthesize gRNA for CAPS analysis
CmGAD1_gRNA_Rv	TTCTAGCTCTAAAACTATCGGCAGCGCCTCGACTT		
CmACO1_gRNA_Fw	TAATACGACTCACTATAGCCCTTGATGAGGTCCGG	CmACO1	Template DNA amplification to synthesize gRNA for genome editing and CAPS analysis
CmACO1_gRNA_Rv	TTCTAGCTCTAAAACAAGCCGGACCTCATCAAGGG		

Supporting Table S3 Guide RNA target sites.

Target gene	Guide RNA sequence	PAM
CmGAD1	AAGTCGAGGCGCTGCCGATA	CGG
CmACO1	CCCTTGATGAGGTCCGGCTT	GGG

Supporting Table S4 Chemically synthesized guide RNA used in this study

Name	Sequence	Note
CmGAD1 gRNA	AAGUCGAGGCGCUGCCGAUAGUUUUAGAGCUAUGCUGUUUUG	crRNA
tracrRNA	AAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACOGAGUCGGUGCU	tracrRNA
CmACO1 gRNA	CCCUUGAUGAGGUCCGGCUUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU	single gRNA

Supporting Information for

A long shelf-life melon created via CRISPR/Cas9 RNP-based *in planta* genome editing

Supplemental Materials and methods

Plant materials.

Seeds of the cultivated melon *Cucumis melo* L. var. reticulatus (accession "Earl's favourite Harukei-3") were obtained from the GenBank of the National Agriculture and Food Research Organization (NARO) in Japan.

Preparation of SAMs.

Mature seeds of melon were imbibed at 25ºC for 20 h. One cotyledon, which covers the SAM, was removed from the embryo plant under a stereo microscope using tweezers. The embryos were placed upright in a petri dish containing Murashige and Skoog (MS) basal medium supplemented with sucrose (30 g/L), 2- morpholinoethanesulfonic acid (MES) monohydrate (0.98 g/L, pH 5.8), a plant preservative mixture (3%; Nacalai Tesque, Japan), and phytagel (7.0 g/L; Sigma Aldrich, USA). About twenty embryos were placed on the medium in each petri dish for particle bombardment.

Biolistic delivery of GFP plasmids

Delivery of GFP plasmids (CaMV35S-sGFP(S65T)-NOS3') (Chiu *et al.*, 1996) into melon SAMs by particle bombardment was performed as previously described (Hamada *et al.*, 2017). Briefly, the plasmid (5 μg) was mixed with gold particles (Bio-Rad, USA) with a diameter of 0.6 μm (5 μL, 40 mg/ml), spermidine (10 μL, 0.1 M), and $CaC1_2$ (25 μL, 2.5 M) in a total volume of 44 μL. After incubation at room temperature for 10 min, the DNA-coated gold particles were centrifuged (at 9,100 × g for 1 s) and the supernatant removed. The pellet was washed with ethanol (70 μL, 70%). After that, the final pellet was resuspended in ethanol (30 μL, 99.5%) and sonicated (for 1 s) just before use. Aliquots (6 μL) were spread onto macrocarrier membranes (Bio-Rad, USA) and allowed to evaporate on a clean bench. Bombardment was conducted using a PDS-1000/He™ device (Bio-Rad, USA). The vacuum in the chamber was 27 inches of Hg and the helium pressure was 1,350 psi. Bombardment was repeated four times.

Observation of GFP fluorescence

Fluorescence images of the bombarded embryo plants were observed with an MZFLIII microscope (Leica, Germany) equipped with a GFP filter (excitation wavelength, 470/40 nm; emission wavelength, 525/50 nm).

Preparation of Cas9 protein and guide RNA.

Recombinant *Streptococcus pyogenes* Cas9 protein was purified from *Escherichia coli* as previously described (Svitashev et al., 2016). The guide RNAs were purchased from FASMAC Co.

(Kanagawa, Japan) and Integrated DNA Technologies, Inc. (Coralville, IA) or prepared by *in vitro* transcription using a GeneArt™ Precision gRNA synthesis Kit (Thermo Fisher Scientific, USA). The templates for the *in vitro* transcription were designed and amplified using appropriate primers (Table S2) according to the manufacturer's instructions.

Biolistic delivery of RNPs.

The purified Cas9 protein and the guide RNA for *CmACO1* or *CmGAD1* (Table S2-4) were mixed with 0.6 μm gold particles (Bio-Rad, USA) and then bombarded into SAMs as previously described (Kumagai *et al.*, 2022). CRISPR/Cas9 RNPs were assembled by incubating SpCas9 (250 pmol) and gRNA (chemically synthesized crRNA and tracrRNA targeting *CmGAD1*: 250 pmol each, *in vitro* transcribed sgRNA targeting *CmACO1*: 250 pmol, chemically synthesized sgRNA targeting *CmACO1*: 700 pmol) in a 20 μL CutSmart® buffer (New England Biolabs, USA) and left for 10 min at room temperature. After addition of 5 μL of TransIT®-LT1 transfection reagent (Takara, Japan), the RNP mixture was allowed to incubate at room temperature for 5 min. The mixture was combined with 25 μL of gold particles solution (40 mg/mL) and incubated on ice for 10 min. The resulting mixture was centrifuged at 2,300 × g for 1 s. After centrifugation, the pellet was resuspended in nuclease-free water. The RNP-coated gold particle suspension was spread on a hydrophilic film (3M, USA) and allowed to air-dry at room temperature for 15 min. The biolistic bombardment was performed using a PDS1000/He™ particle bombardment device (Bio-Rad, USA). The vacuum in the chamber was 27 inches of Hg and the helium pressure was 1,350 psi. Bombardment was repeated four times per plate.

Plant growth condition after bombardment.

Three days in the dark (25°C) after bombardment, the embryos were transferred to a growth chamber under long day conditions (16 h light/8 h darkness, 25°C) and cultured for 2–3 weeks. The seedlings were subsequently planted in pots and grown in a phytotron under long day conditions (16 h light/8 h darkness, 22°C). After CRISPR/Cas9-induced mutant screening, positive E_0 plants were grown under the greenhouse condition. For obtaining seeds, female flowers were hand-pollinated using male anthers obtained from the same plant. One fruit was kept on each plant.

Cleaved amplified polymorphic sequences (CAPS) analysis.

Genomic DNA was extracted from the sixth leaf of the E_0 progeny and the first leaf of the E_1 progeny. PCR amplification was conducted using PrimeSTAR® GXL DNA Polymerase (TaKaRa, Japan) with gene specific primers (Table S2) and genomic DNA in a total volume of 20 μl. The mixture was subjected to 30 cycles of amplification (98°C for 10 s, 60°C for 15 s, 68°C for 1 min) in a thermocycler. The amplified PCR products (5 μL) were digested with SpCas9 (600 ng) and *in vitro* transcribed sgRNA (300 ng) in 15 μL of reaction buffer, containing 20 mM HEPES-NaOH, pH 7.5,

100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT and 5% glycerol (Nishimasu *et al.*, 2018). The reaction products were analyzed by agarose gel electrophoresis. Undigested bands from the enzyme digestion were purified and cloned into the pCR-BluntII-TOPO vector (Thermo Fisher Scientific, USA) for sequencing.

Ethylene measurement from fruit

The fruits were harvested 40 days after pollination, and the amount of ethylene production was measured every 2 days for 2 weeks using an SGEA-P3-C1 ethylene detection sensor system (Nissha, Japan). Each fruit was placed in a container, and 5 mL of gas was extracted with a syringe after 4 h and measured three times.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its Supporting Information files). Regarding sequence data, the NCBI GenBank identifiers are: XM_008466382 (CmGAD1), X95551 (CmACO1).

References

Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) *Engineered GFP as a vital reporter in plants*. *Curr. Biol.*, **6**, 325–330.

Hamada, H., Linghu, Q., Nagira, Y., Miki, R., Taoka, N., and Imai, R. (2017) *An in planta biolistic method for stable wheat transformation*. *Sci. Rep.*, **7**, 11443.

Kumagai, Y., Liu, Y., Hamada, H., Luo, W., Zhu, J., Kuroki, M., et al. (2022) *Introduction of a second "Green Revolution" mutation into wheat via in planta CRISPR/Cas9 delivery*. *Plant Physiol.*, **188**, 1838–1842.

Nishimasu, H., Shi, X., Ishiguro, S., Gao, L., Hirano, S., Okazaki, S., et al. (2018) *Engineered CRISPR-Cas9 nuclease with expanded targeting space*. *Science*, **9**, 1259–1262.

Svitashev, S., Schwartz, C., Lenderts, B., Young, J.K., and Mark Cigan, A. (2016) *Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes*. *Nat. Commun.*, **7**, 13274.