Base editors for citrus gene editing

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Abstract

Base editors, such as adenine base editors (ABE) and cytosine base editors (CBE), provide alternatives for precise genome editing without generating double-strand breaks (DSBs), thus avoiding the risk of genome instability and unpredictable outcomes caused by DNA repair. Precise gene editing mediated by base editors in citrus has not been reported. Here, we have successfully adapted the ABE to modify the TATA box in the promoter region of the canker susceptibility gene LOB1 from TATA to CACA in grapefruit and Hamlin sweet orange. Inoculation of the TATA-edited plants with the canker pathogen Xanthomonas citri subsp. citri (*Xcc*) demonstrated that the TATA-edited plants were resistant to *Xcc*. In addition, CBE was successfully used to edit the *acetolactate synthase (ALS)* gene of Carrizo citrange, a hybrid of *Citrus sinensis* 'Washington' sweet orange X *Poncirus trifoliata*. Editing the ALS genes conferred resistance of Carrizo to the herbicide chlorsulfuron. Two *ALS*-edited Carrizo plants did not show green florescence although the starting construct for transformation contains a GFP expression cassette. We performed PCR amplification for *Cas9* gene in the mutant plants and found that *Cas9* gene was undetectable in the herbicide resistant citrus plants. This indicates that

24 the *ALS* edited plants are transgene-free, representing the first transgene-free gene-edited citrus
25 using the CRISPR technology. In summary, we have successfully adapted the base editors for
26 precise citrus gene editing. The CBE base editor has been used to generate transgene-free citrus
27 via transient expression.
28
29
30 Nickase Cas9 (nCas9) derived base editors, such as adenine base editors (ABE) and cytosine
31 base editors (CBE), are a useful tool for precise genome editing (Kluesner et al., 2021). Precise
32 gene editing mediated by base editors in citrus has not been reported. Here, we first tested if
33 ABE, such as ABE8e which can mediate A•T-to-G•C base changes (Richter et al., 2020), can
34 edit citrus genes. A citrus optimized ABE8e vector was constructed (Figure 1a). TadA8e
35 (Richter et al., 2020) was fused with $Cas9^{D10A}$ nickase at the N-terminal. There is a nucleus
36 localization signal (NLS) at each end of TadA8e- $Cas9^{D10A}$ for nucleus transportation. NLS-
37 TadA8e-$Cas9^{D10A}$-NLS is driven by the CmYLCV promoter, which confers high gene editing
38 efficiency in citrus. We aimed to edit the TATA box (Figure 1b) located upstream of the effector
39 binding elements (EBE) of the promoter of the citrus canker susceptible (S) gene *LOB1* with
40 ABE8e. The EBE in the promoter of susceptibility genes usually overlaps with or locates
41 immediately downstream of the TATA box of the S gene promoter (Huang et al., 2017). PthA4,
42 a TAL effector from citrus canker pathogen *Xanthomonas citri* subsp. *citri* (Xcc), binds to the
43 EBE region of *LOB1* to activate its expression. The TATA box of the *LOB1* promoter overlaps
44 with the EBE region (Figure 1d). We hypothesized that editing of the EBE-associated TATA box
45 may abolish or reduce the induction of *LOB1* by *Xcc*, thus generating canker resistant citrus
46 plants. The transient assay (Figure 1c) through *Xcc*-facilitated agroinfiltration of citrus leaves

47 showed that the ABE construct edited the TATA box as anticipated (Figure 1d). The ABE
48 construct mutated TATA to CACA (from TATA to TGTG for the complementary strand). Test
49 on another gene *CsTub* (Cs1g21050) through transient assay also demonstrated precise editing
50 (Figure 1e). Next, *Agrobacterium*-mediated stable transformation of grapefruit and Hamlin sweet
51 orange epicotyl tissues was conducted to edit the TATA box in the *LOB1* promoter of both
52 varieties. Two transgenic grapefruit and one transgenic sweet orange plants were obtained.
53 Genotyping showed the TATA box was successfully edited in the three transformed plants
54 (Figure 1f). The TATA in the *LOB1* promoter was edited into CACA. By using the ABE system,
55 we achieved 100% biallelic/homozygous editing of the TATA box in the *LOB1* promoter. After
56 micro-grafting, one transgenic grapefruit plant and one transgenic sweet orange plant survived.
57 Inoculation of the TATA-edited plants with *Xcc* demonstrated that the TATA-edited plants were
58 resistant to *Xcc* (Figure 1g). On the contrary, all plants were susceptible to *Xcc pthA4*:Tn5
59 dLOB2, which carries a designer TAL dLOB2 for *LOB2* induction to cause canker disease in
60 citrus plants. These results showed that base editor ABE8e can efficiently and precisely edit
61 citrus genome.

62 Next, we tested if CBE, which mediates C•G-to-T•A base changes, can edit citrus genes.
63 A citrus optimized CBE vector was constructed (Figure 1h). We chose APOBEC3A (A3A)
64 deaminase for citrus CBE considering its wide deamination window and high editing efficiency
65 (Zhang et al., 2020; Zong et al., 2018). This CBE system contains CsU6-tRNA-gRNA-scaffold
66 unit for multiplex editing. The final CBE construct also contains a GFP expression cassette for
67 direct visualization of transgene integration. We first determined if citrus *acetolactate synthase*
68 *(ALS)* gene can be edited with this CBE. Acetolactate synthase (ALS) is an enzyme required for
69 biosynthesis of some branched-chain amino acids, such as valine, leucine, and isoleucine. A

single amino acid mutation in *ALS* genes in diverse plants confers resistance to herbicides such as chlorsulfuron (Kuang et al., 2020; Malabarba et al., 2021). The growth of Carrizo citrange, a hybrid of *Citrus sinensis* 'Washington' sweet orange X *Poncirus trifoliata*, was completely inhibited in the presence of chlorsulfuron (Figure 1i). We designed two gRNAs targeting the two *ALS* alleles of Carrizo (Figure 1j). The transient expression result showed that the construct edited both alleles of the *ALS* gene of Carrizo (Figure 1j). *Agrobacterium*-mediated stable transformation of Carrizo epicotyl tissues was performed with herbicide chlorsulfuron selection in culture media. Two chlorsulfuron-resistant plants grew on the chlorsulfuron containing medium while most of the transformed epicotyl tissues did not grow (Figure 1k). Genotyping of the chlorsulfuron-resistant plants showed that both alleles of the *ALS* gene were edited in the two plants (Figure 1l and m). Intriguingly, these two plants did not show green florescence although the starting construct for transformation contains a GFP expression cassette. We performed PCR amplification for *Cas9* gene in the mutant plants and found that *Cas9* gene was undetectable in the herbicide resistant citrus plants. This indicates that the *ALS* edited plants are transgene-free, which were obtained from transient expression of the CBE construct, representing the first transgene-free gene-edited citrus using the CRISPR technology.

In summary, we have successfully adapted the base editors for precise citrus gene editing. The CBE base editor has been used to generate transgene-free citrus via transient expression.

Conflicts of interest

The authors declare no competing financial interests.

Author contributions

XH designed and performed the experiments. YW performed micro-grafting. XH and NW wrote the paper. NW supervised the study.

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Figure legend

Figure 1 Highly efficient base editors for citrus gene editing. (a) Illustration of ABE8e adenine base editing (ABE) system for citrus. CmYLCV, *Cestrum yellow leaf curling virus* promoter;

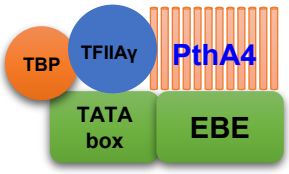
131 TadA8e, evolved *Escherichia coli* tRNA adenosine deaminase; Cas9^{D10A}, Cas9 nickase. (b) The
132 TATA box located upstream of the *LOB1* EBE region is associated with general transcription
133 factors and TAL effector PthA4. (c) *Xcc*-facilitated transient expression of ABE8e construct in
134 citrus leaf. The construct carries a GFP expression cassette. (d) Editing of TATA in the TATA
135 box of the *LOB1* promoter into CACA in transient assay from (c). Underlined nucleotides were
136 the target region for gRNA design. (e) Editing of the *CsTub* gene using transient assay. (f)
137 Chromatograms for the TATA-edited grapefruit mutant line 1 (upper), line 2 (middle) and
138 Hamlin sweet orange mutant line (lower). (g) Inoculation of WT and TATA-edited mutants with
139 *Xcc* or *Xcc pthA4*:Tn5 dLOB2. *Xcc pthA4*:Tn5 is a Tn5 insertion mutant of *pthA4* gene of *Xcc*.
140 Leaf area 1 inoculated with *Xcc*; leaf area 2 inoculated with *Xcc pthA4*:Tn5 dLOB2. (h)
141 Illustration of cytosine base editing system (CBE). (i) Growth of citrus seedlings (Carrizo
142 citrange) was inhibited by herbicide chlorsulfuron. (j) CBE base editing of the *ALS* gene via
143 transient assay. The restriction enzyme *Dra*II-resistant PCR amplicon was subject to cloning and
144 sequencing. (k) Selection of herbicide resistant Carrizo citrange on chlorsulfuron-containing
145 media (150 nM). (l) Sequencing results from chlorsulfuron-resistant mutant plants. (m)
146 Chromatograms for (l). (n) PCR of *Cas9* and *ALS* for control plant (GFP positive) and herbicide
147 chlorsulfuron-resistant citrus plants.

148

149

(a)

(b)



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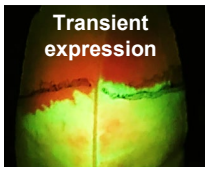


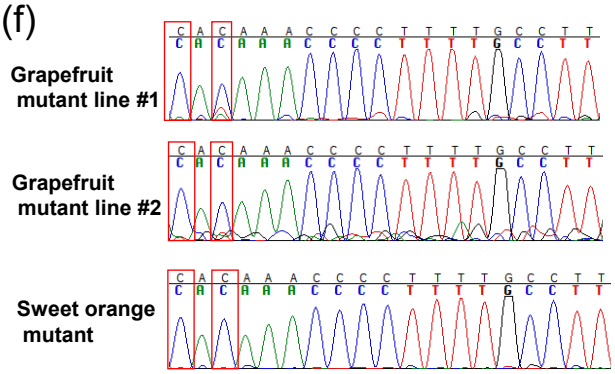
(d)

	PAM	Target region	TATA box	EBE
LOB1 WT	CCT	TTTCCTTTCTCTA	TATAAAC	CCCTTTTGCCTT
mutant	CCT	TTTCCTTTCTCTA	CACAAAC	CCCTTTTGCCTT

(e)

	PAM	Target region	
CsTub WT	CCT	AGGATCCACTTCATGCTTTCA	
	P R I H F M L S		Amino acid
mutant	CCT	AGGATCCACTTCACGCTTTCA	
	P R I H F T L S		Amino acid

(f)

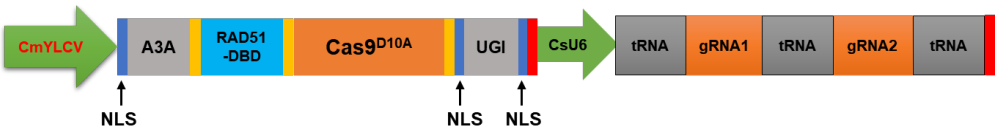


(g)

(i)

+ Chlorsulfuron

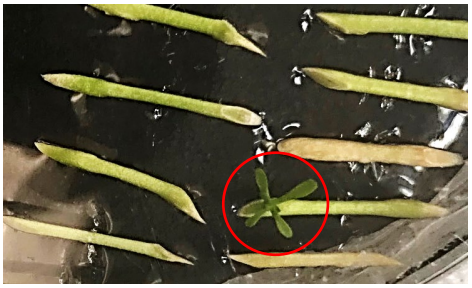
(h)



(j)

	DraII / Target region	PAM	
	CAGGTCCCGCGGAGGATGATCGG		WT allele 1
	CAGGTCCCTCGGAGGATGATCGG		WT allele 2
	Q V P R R M I		amino acid
	CAGGTTTTGTGGAGGATGATCGG		mutant 1
	Q V L W R M I		
	CAGGTTTTTTGGAGGATGATCGG		mutant 2
	Q V F W R M I		

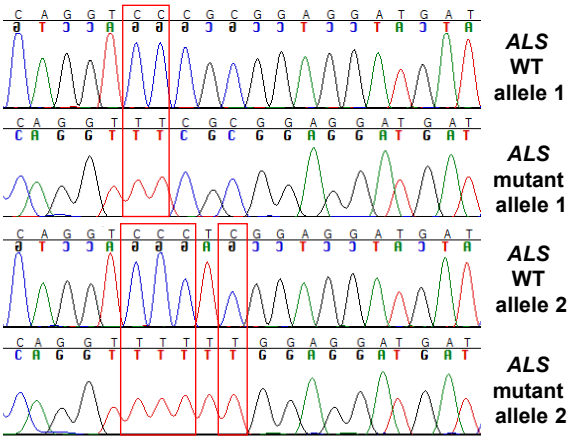
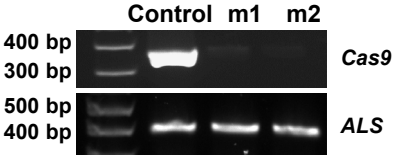
(k)



(l)

	Target region	PAM	
	CAGGTCCCGCGGAGGATGATCGG		WT allele 1
	CAGGTCCCTCGGAGGATGATCGG		WT allele 2
	Q V P R R M I		amino acid
	CAGGTTTCGCGGAGGATGATCGG		mutant 1
	Q V S R R M I		
	CAGGTTTTTTGGAGGATGATCGG		mutant 2
	Q V F W R M I		

(m)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