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Research Article

Genome- and transcriptome-wide off-target analyses of a high-efficiency adenine base editor in tomato

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Abstract

Adenine base editors (ABEs) are valuable, precise genome editing tools in plants. In recent years, the highly promising ADENINE BASE EDITOR8e (ABE8e) was reported for efficient A-to-G editing. However, compared to monocots, comprehensive off-target analyses for ABE8e are lacking in dicots. To determine the occurrence of off-target effects in tomato (*Solanum lycopersicum*), we assessed ABE8e and a high-fidelity version, ABE8e-HF, at 2 independent target sites in protoplasts, as well as stable T0 lines. Since ABE8e demonstrated higher on-target efficiency than ABE8e-HF in tomato protoplasts, we focused on ABE8e for off-target analyses in T0 lines. We conducted whole-genome sequencing (WGS) of wild-type (WT) tomato plants, green fluorescent protein (GFP)–expressing T0 lines, ABE8e-no-gRNA control T0 lines, and edited T0 lines. No guide RNA (gRNA)–dependent off-target edits were detected. Our data showed an average of approximately 1,200 to 1,500 single-nucleotide variations (SNVs) in either GFP control plants or base-edited plants. Also, no specific enrichment of A-to-G mutations were found in base-edited plants. We also conducted RNA sequencing (RNA-seq) of the same 6 base-edited and 3 GFP control T0 plants. On average, approximately 150 RNA–level SNVs were discovered per plant for either base-edited or GFP controls. Furthermore, we did not find enrichment of a TA motif on mutated adenine in the genomes and transcriptomes in base-edited tomato plants, as opposed to the recent discovery in rice (*Oryza sativa*). Hence, we could not find evidence for genome- and transcriptome-wide off-target effects by ABE8e in tomato.

Introduction

CRISPR-mediated base editing enables direct, irreversible conversion of 1 target nucleotide into another in a programmable manner, which presents a precise genome editing technology with applications in genetics, medicine, and agriculture. Base editing was demonstrated in mammalian cell lines with cytosine base editors (CBEs) (Komor et al. 2016), followed by adenine base editors (ABEs) (Gaudelli et al. 2017), and cytosine to guanine base editors (CGBEs) (Chen et al. 2021; Kurt et al. 2021; Zhao et al. 2021), and an adenine transversion base editor (AYBE, Y = C, T) (Tong et al. 2023). During adenine base editing, ABE is recruited to the target site, and deamination of adenine produces inosine (I), which is converted to guanine (G) during either DNA replication or cellular mismatch repair (Gaudelli et al. 2017). Since none of

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the reported naturally occurring adenosine deaminases work on DNA but only on RNA, *Escherichia coli* transfer RNA adenosine deaminase (TadA) was engineered to work on single-stranded DNA (ssDNA) and coupled to Cas9-D10A nickase in the ABEs (Gaudelli et al. 2017). Several versions of engineered TadA were tested (Gaudelli et al. 2020; Chen et al. 2023a, b) with a TadA-WT-TadA-7.10 heterodimer showing potent A-to-G base editing activity (Gaudelli et al. 2017) and a TadA8e monomer in ABE8e substantially increased deamination kinetics over the previous versions (Richter et al. 2020). Recently, several research groups have further engineered TadA deaminase to work as a cytidine deaminase in CBEs (Chen et al. 2023b; Lam et al. 2023; Neugebauer et al. 2023) or have both cytosine and adenine editing potential in the TadDE dual base editor (Neugebauer et al. 2023). ABEs have also been demonstrated in various plants, including Arabidopsis (*Arabidopsis thaliana*) (Kang et al. 2018; Niu et al. 2023), benth (*Nicotiana benthamiana*) (Wang et al. 2021), poplar (*Populus tremula* × *Populus alba* hybrid) (Li et al. 2021a), moss (*Physcomitrium patens*) (Guyon-Debast et al. 2021), and cotton (*Gossypium hirsutum*) (Wang et al. 2022). They have also been demonstrated to be effective in food crops such as rice (*Oryza sativa*) (Hua et al. 2018; Li et al. 2018; Yan et al. 2018; Hao et al. 2019; Li et al. 2020; Molla et al. 2020; Wei et al. 2021; Li et al. 2022; Tan et al. 2022; Wu et al. 2022), wheat (*Triticum aestivum*) (Li et al. 2018), rapeseed (*Brassica napus*) (Kang et al. 2018), tomato (*Solanum lycopersicum*) (Niu et al. 2023), soybean (*Glycine max*) (Niu et al. 2023), and grapefruit (*Citrus paradise*) as well as sweet orange (*Citrus sinensis*) (Huang et al. 2022).

As with other genome editing technologies, ABEs' editing specificity has been a focus of intensive investigation. Several studies have found that earlier versions of ABEs in rice, wheat, and cotton did not induce genome-wide off-target mutations (Hua et al. 2018; Kang et al. 2018; Li et al. 2018; Jin et al. 2019; Wang et al. 2022), albeit unintended proximal base editing to the target sites in rice (Molla et al. 2020). However, our recent study in rice reported substantial genome-wide off-target A-to-G mutations by ABE8e, a highly efficient ABE (Wu et al. 2022). Editing with earlier versions of ABEs, when both wild-type (WT) and engineered TadA adenosine deaminases were used, induced transcriptome-wide off-target A-to-I RNA editing in mammalian cell lines (Grünewald et al. 2019; Zhou et al. 2019). RNA–level off-targeting was also observed in rice (Li et al. 2022) and cotton (Wang et al. 2022) when ABEs were overexpressed. To reduce such DNA– and RNA–level off-target effects, several high-fidelity versions of TadA adenosine deaminase were engineered (Li et al. 2021b; Cao et al. 2022), including TadA8e-HF with a V106W mutation that has been used in the ABE8e-HF (Richter et al. 2020).

While it is crucial to improve the precision of ABEs for base editing in plants, it is also critical to assess the off-target potential for such ABEs at both genome and transcriptome levels. As one of the most promising ABEs, ABE8e was found to generate substantial off-target A-to-G mutations in the genome and A-to-I mutations in the transcriptome in rice (Li et al. 2022; Wu et al. 2022). However, it is unclear for ABE8e's potential off-target effects in a dicot plant. Tomato is a dicot model crop and an important vegetable crop very suitable for demonstrating genome editing technologies and assessing their potential off-target effects. In this study, we assessed genome- and transcriptome-wide off-target effects of ABE8e using whole-genome sequencing (WGS) and RNA sequencing (RNA-seq) in tomato.

Results

Assessment of ABE8e and ABE8e-HF base editors in tomato protoplasts

ABE8e is a widely used base editor due to its compactness and high on-target editing activity, as demonstrated in human cell lines (Richter et al. 2020) and several plant species (Wang et al. 2021; Huang et al. 2022; Tan et al. 2022; Niu et al. 2023), albeit with increased RNA and DNA off-target editing as revealed in human cells (Richter et al. 2020). Since the V106W mutation in adenosine deaminase TadA8e (in ABE8e-HF) was shown to decrease off-target editing in human cell lines (Richter et al. 2020), we tested both ABE8e and ABE8e-HF in tomato protoplasts. Both base editors consisted of a maize codon–optimized Cas9(D10A) nickase fused to a rice codon–optimized TadA8e with or without the V106W mutation. The base editor(s) and guide RNA (gRNA) were expressed using a 2×35S promoter and an AtU3 promoter, respectively (Fig. 1A and Supplemental Fig. S1). These 2 base editors were tested at 2 independent target sites in *S. lycopersicum acetolactate synthase* (*SlALS*), a gene involved in the synthesis of the branched-chain amino acids and a popular target for generating herbicide-resistant plants (Yu and Powles 2014). A-to-G base editing activity was first assessed in tomato protoplasts and quantified by next-generation sequencing (NGS) of PCR amplicons. ABE8e demonstrated higher A-to-G base editing activity of approximately 5% to 8% at both tested target sites compared to ABE8e-HF at approximately 1% to 2% (Fig. 1, B and C). A-to-Y editing activity (Y = C, T) remained at WT/background levels (Fig. 1, B and C), suggesting high editing purity. ABE8e, as well as ABE8e-HF, showed a rather wide base editing window spanning from the 3rd to 9th nucleotide position within the target site (Fig. 1, D and E).

Assessment of ABE8e and ABE8e-HF base editors in T0 tomato lines

While ABEs showed only a single-digit percentage of editing efficiency in protoplasts, both ABE8e and ABE8e-HF generated a higher percentage of edited T0 tomato lines through the stable transformation of tomato utilizing *Agrobacterium*-mediated T-DNA delivery. This phenomenon has been

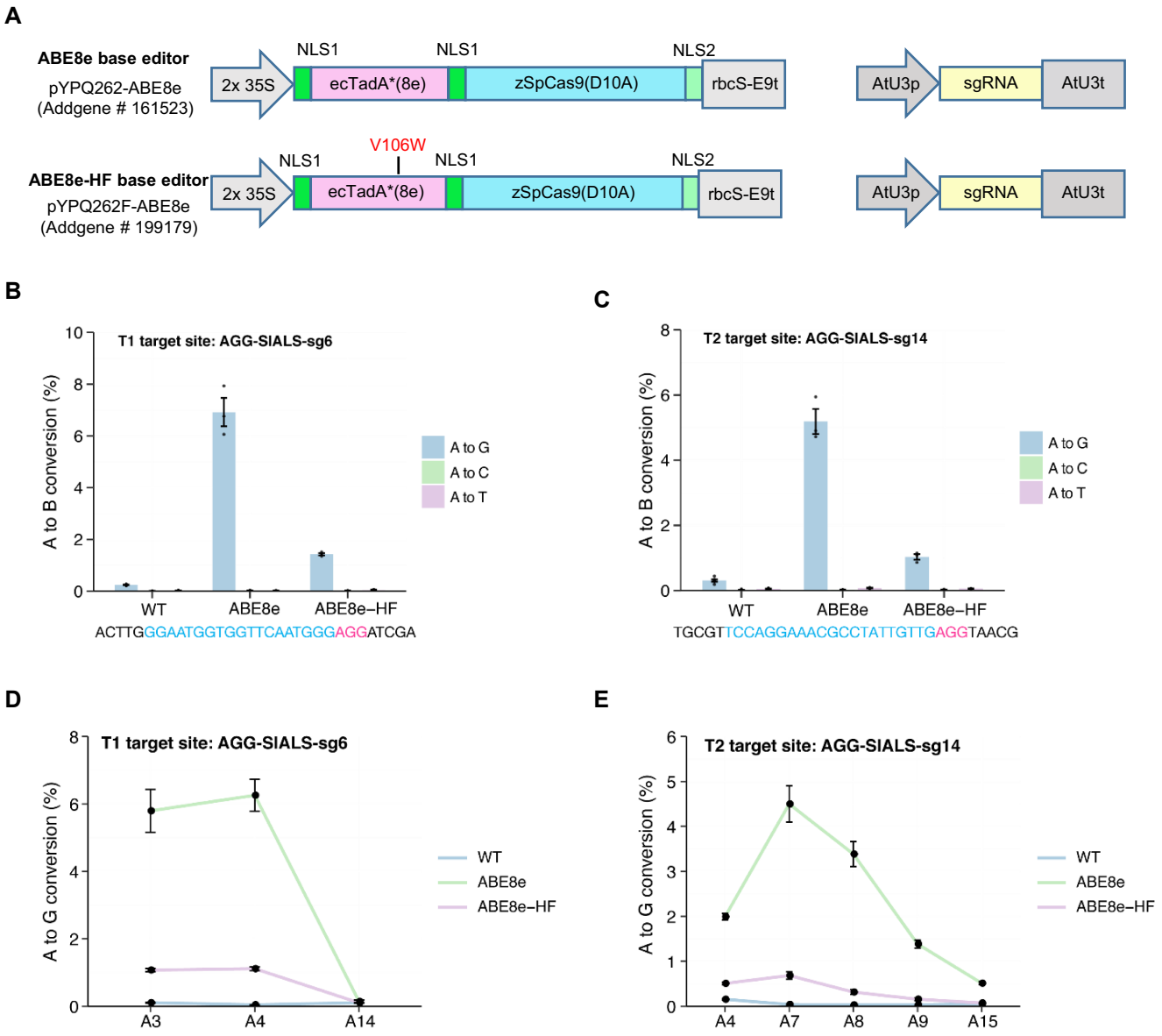


Figure 1. Testing ABE8e base editors in tomato protoplasts. **A)** Schematics of ABE8e and ABE8e-HF base editors consisting of maize codon–optimized canonical SpCas9(D10A) nickase and rice codon–optimized engineered *E. coli* TadA8e adenosine deaminase. High-fidelity ABE8e base editor carries V106W mutation in engineered TadA8e adenine deaminase. Base editor was transcribed using 2× cauliflower mosaic virus (CaMV) 35S promoter and Rubisco small subunit rbcS-E9 terminator. gRNA was transcribed using the *A. thaliana* snRNA U3 (AtU3) promoter and terminator. NLS stands for nuclear localization signal. **B, C)** A to B (B = C, G, T) conversion rates at T1: AGG-SlALS-sg6 (B) and T2: AGG-SlALS-sg14 (**C**) target sites by ABE8e and ABE8e-HF base editors. Both target sites are located within *SlALS* gene (Solyc03g044330.1); T1 at position 8143100-8143131 and T2 at 8144219-8144186. **D, E)** Base editing windows of ABE8e and ABE8e-HF editors at T1 and T2 target sites. **B to E)** Data are presented as averages and standard deviation of 3 biological replicates.

demonstrated previously by us (Sretenovic et al. 2021; Ren et al. 2021b) as well as other researchers (Kang et al. 2018; Li et al. 2018) and was probably due to no protoplast division as compared to the generation of stable T0 lines that requires cell division for plant regeneration to occur. At the T1 target site, ABE8e-HF showed comparable A-to-G editing efficiency to ABE8e in transgenic tomato lines: 52.6% vs 45.9% (Fig. 2A). At the T2 target site, ABE8e showed higher A-to-G editing efficiency than ABE8e-HF: 23.1% vs 9.8% (Fig. 2A). All edited lines were either monoallelically or mosaically/chimerically A-to-G edited, without A-to-Y (Y = C, T) undesired by-product editing and indel introduction at the 2 tested target sites (Fig. 2A). Sanger sequencing chromatograms of selected edited lines show that A3, A4, or both within the T1 target site and A7 or A7 and A8 within the T2 target site were A-to-G edited by both ABEs (Fig. 2, B to E), which is consistent with the base editing window identified from the data obtained in tomato protoplasts (Fig. 1, D and E).

A

pLR#	Base Editor	Target Site	Screened T0 Lines (number)	Monoallelic or Mosaic A-G Base Edited T0 Lines (number; percentage)	Biallelic A-G Base Edited T0 Lines (number; percentage)	A-Y Base-Edited T0 Lines (number; percentage)	Indels in T0 Lines (number; percentage)
3318	ABE8e	T1: AGG-SlALS-sG6	37	17; 45.9%	0; 0%	0; 0%	0; 0%
3433	ABE8e	T2: AGG-SlALS-sG14	26	6; 23.1%	0; 0%	0; 0%	0; 0%
4858	ABE8e-HF	T1: AGG-SlALS-sG6	19	10; 52.6%	0; 0%	0; 0%	0; 0%
4859	ABE8e-HF	T2: AGG-SlALS-sG14	41	4; 9.8%	0; 0%	0; 0%	0; 0%

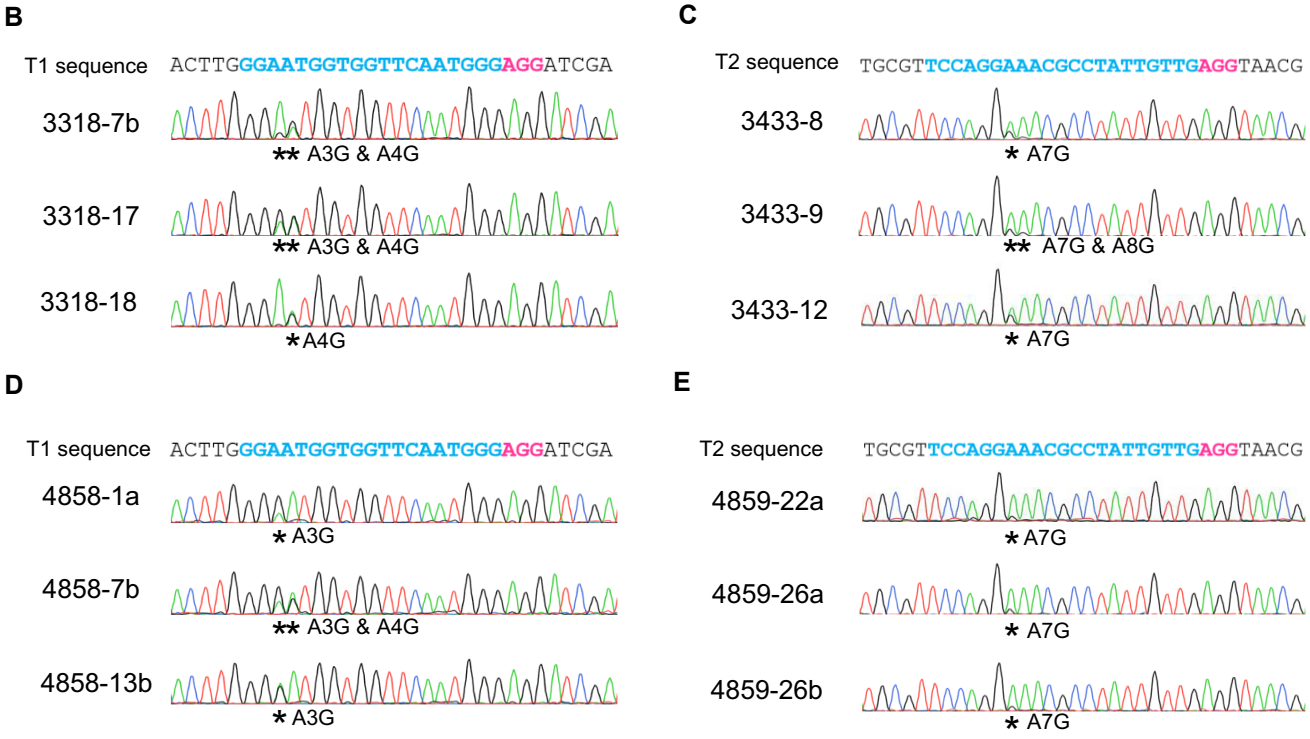
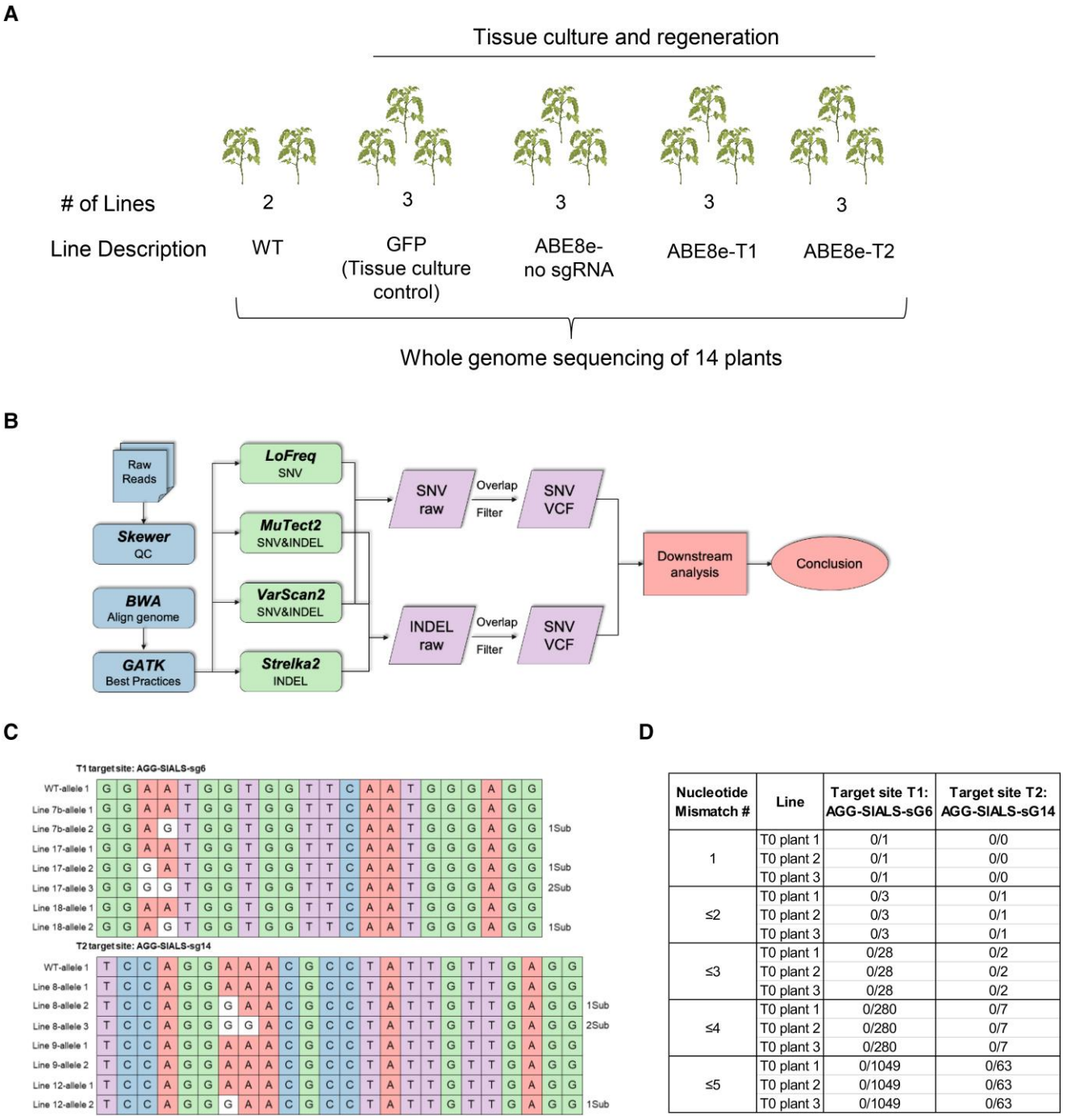


Figure 2. Testing ABE8e base editors in T0 tomato plants. **A)** Adenine base editing in stable T0 tomato lines with ABE8e and ABE8e-HF base editors at T1: AGG-SlALS-sg6 and T2: AGG-SlALS-sg14 target sites. **B)** Sanger sequencing chromatograms of base-edited T0 lines at T1 target site by ABE8e. **C)** Sanger sequencing chromatograms of base-edited T0 lines at the T2 target site by ABE8e. **D)** Sanger sequencing chromatograms of base-edited T0 lines at the T1 target site by ABE8e-HF. **E)** Sanger sequencing chromatograms of base-edited T0 lines at the T2 target site by ABE8e-HF. **B to E)** Asterisks represent nucleotide position within the target site where base editing had occurred.

ABE8e's on-target and gRNA–dependent off-target editing by WGS

Having identified ABE8e as an efficient and pure base editor in tomato, we focused on investigating ABE8e's potential genome-wide off-target effects. WGS was carried out on 2 WT tomato plants, 3 green fluorescent protein (GFP)–expressing control T0 lines, 3 ABE8e-no-gRNA control T0 lines, and 6 edited T0 lines (3 independent T0 lines for each of the T1 and T2 target sites) (Fig. 3A). For all 14 samples, the genome sequencing depth spanned from 25× to 51×, and sequencing reads were mapped to the genome at 97.07% or higher with genome coverage of 96.84% or higher (Supplemental Table S1). To analyze the WGS data, a similar analysis workflow was adopted as we previously used for assessing genome-wide off-target effects by Cas9 and Cas12a in rice (Tang et al. 2018) and by CBE editing in tomato (Randall et al. 2021). This rigorous pipeline utilized 3 independent calling programs to identify single-nucleotide variations (SNVs) as well as insertions and deletions (indels) (Fig. 3B). Based on WGS, we reconfirmed A-to-G base editing events at the 2 target sites in the 6 selected lines that were previously identified by Sanger sequencing as either monoallelic or mosaic/chimeric (Fig. 3C).

We next sought to investigate gRNA–dependent off-targeting in ABE8e-edited lines. We used Cas-OFFinder (Bae et al. 2014) to identify similar target sites with up to 5 nucleotide mismatches compared to the T1 and T2 target



D

Nucleotide Mismatch #	Line	Target site T1: AGG-SIALS-sG6	Target site T2: AGG-SIALS-sG14
1	T0 plant 1	0/1	0/0
	T0 plant 2	0/1	0/0
	T0 plant 3	0/1	0/0
≤2	T0 plant 1	0/3	0/1
	T0 plant 2	0/3	0/1
	T0 plant 3	0/3	0/1
≤3	T0 plant 1	0/28	0/2
	T0 plant 2	0/28	0/2
	T0 plant 3	0/28	0/2
≤4	T0 plant 1	0/280	0/7
	T0 plant 2	0/280	0/7
	T0 plant 3	0/280	0/7
≤5	T0 plant 1	0/1049	0/63
	T0 plant 2	0/1049	0/63
	T0 plant 3	0/1049	0/63

Figure 3. WGS for gRNA–dependent on- and off-target editing by ABE8e. **A)** Line description and number of lines that were whole-genome sequenced. **B)** A workflow for whole-genome detection of SNV and indel mutations. SNV analysis includes using 3 computer programs: LoFreq, VarScan2, and MuTect2. Indel analysis also involves using 3 programs: VarScan2, MuTect2, and Strelka2. **C)** Targeted adenine base editing as determined by WGS in lines 7b, 17, and 18 at the T1 target site as well as in lines 8, 9, and 12 at the T2 target site, respectively. **D)** Number of off-target sites identified in edited T0 lines versus the number of all potential off-target sites in the tomato genome, predicted by Cas-OFFinder, allowing up to 5-nucleotide mismatches in both T1 and T2 target sites.

sites. Cas-OFFinder predicted 1,049 and 63 putative off-target sites for the T1 and T2 target sites, respectively, indicating that the T2 target site is more unique in the tomato genome compared to the T1 target site (Fig. 3D). However, WGS analysis did not reveal any gRNA–dependent mutations at these putative target sites (Fig. 3D), suggesting the different levels of mismatch mutations all prevented ABE8e editing on the putative off-target sites in these lines.

ABE8e's gRNA–independent off-target editing by WGS

However, the major concern of ABE8e's off-target effects is TadA8e's promiscuous binding to DNA or RNA, resulting in gRNA–independent off-target editing of DNA and RNA as reported in rice lately (Li et al. 2022; Wu et al. 2022). We first investigated such gRNA–independent off-target effects on DNA. Our WGS analysis revealed T-DNA insertion sites of 3 ABE8e-no-gRNA control T0 and 6 edited T0 lines, and we observed a random pattern of T-DNA insertions spread among the 12 chromosomes of the tomato genome (Fig. 4A). The ABE8e-T2-line 8 (T2-1), ABE8e-no-gRNA line 9 (BB-1), and ABE8e-no-gRNA line 13 (BB-2) T-DNAs contained 2 T-DNA insertion events. Interestingly, the ABE8e-T1 7b (T1-1) and 17 (T1-2) lines shared the same T-DNA integration site, indicating that the 2 lines shared the same transgenic event (Wu et al. 2022) (Fig. 4A). To identify gRNA–independent and deaminase-dependent off-target mutations, we first compared the total SNVs per line for each sample group. Approximately 200 SNVs were identified in each WT plant (Fig. 4B), indicating a spontaneous SNV mutation rate. On average, approximately 1,200 SNVs were identified in each GFP–expressing control T0 line, defining a level of somaclonal variation for SNVs attributed to the tissue culture process and *Agrobacterium*-mediated transformation in our experimental conditions (Fig. 4B). Approximately the same number of SNVs were identified in each ABE8e-no-gRNA control T0 line indicating ABE8e without gRNA did not introduce additional SNVs beyond the level of somaclonal variation (Fig. 4B). For the base-edited lines, an average of approximately 1,500 SNVs were found per line; however, the numbers of SNVs in base-edited, ABE8e-no-gRNA control, and GFP–expressing control lines are not statistically significantly different (Fig. 4B). The SNVs were evenly distributed across the 12 tomato chromosomes (Supplemental Fig. S2). A further breakdown of the SNVs showed edited lines had similar levels of A:T>G:C mutations compared to ABE8e-no-gRNA and GFP-expressing control plants, as well as WT (Fig. 4C). For all sample types, more SNVs were found in the transposable elements (TEs) and repeats than in exons, intergenic regions, or introns (Fig. 4E). Therefore, we concluded that the tomato tissue culture process introduced approximately 1,200 SNVs per regenerated plant as a result of tissue culture and *Agrobacterium*-mediated transformation. No off-target SNVs were identified in the ABE8e-edited lines.

Since no indels were detected at the T1 or T2 target sites in ABE8e base-edited lines (Fig. 2A), we investigated the potential occurrence of indels genome wide by comparison of the indel counts among different sample groups. Approximately 100 indels were identified in each WT plant (Fig. 4D), indicating a spontaneous indel mutation rate. On average, approximately 250 indels were identified in each GFP–expressing control T0 line and 350 indels in each ABE8e-no-gRNA T0 line (Fig. 4D). The base-edited plants at the T1 and T2 target sites carried approximately 500 and 350 indels, albeit with large variations among the 6 plants. Consequently, the differences in indel counts between WT, GFP–expressing control T0, ABE8e-no-gRNA T0, and base-edited plants are not statistically significant (Fig. 4D). The indels were enriched in intergenic regions, intron regions, and TE & repeats (Fig. 4F). Since the GFP–expressing control T0, ABE8e-no-gRNA T0, and edited plants showed very similar indel profiles, we concluded that very few indels, if any, resulted from the off-target effects of ABE8e. This finding is consistent with the fact that ABEs tend not to generate indel mutations.

Transcriptome-wide off-target analysis of ABE8e by RNA-seq

Since *E. coli* TadA deaminase, used in ABE8e, evolved to recognize DNA from naturally recognizing RNA, we were curious about the potential RNA–level deaminase-dependent off-target effects. This concern was raised given the recent report of A-to-I off-targeting by ABE8e in rice (Li et al. 2022). Hence, we conducted RNA-seq on the same 3 GFP–expressing control T0, 3 ABE8e-no-gRNA control T0, and 6 edited T0 lines that were used for WGS. For analyzing the RNA-seq data, we used a previously established pipeline (Fig. 5A) (Randall et al. 2021). The total mapped reads for each sample spanned from 58 to 94 M (Supplemental Table S2). An average of approximately 150 RNA–level SNVs were identified in GFP–expressing control T0, ABE8e-no-gRNA control T0, and edited lines with no statistically significant differences in SNVs among the samples (Fig. 5B). For all 12 samples, only a minority of the SNVs found at the transcriptome level originated from genomic SNVs, with a Pearson correlation coefficient ≤ 0.23 among these 2 groups of SNVs in each plant (Fig. 5C). The RNA–specific SNVs constituted 25.8% to 39.9% SNVs detected in the GFP–expressing control T0 plants, 31.2% to 49.5% SNVs detected in the ABE8e-no-gRNA control T0 plants, and 31.6% to 37.7% in edited plants (Fig. 5D). Among the SNVs, A-to-I changes represented between 22.0% and 31.5% for all twelve samples (Supplemental Fig. S3). Considering unidirectional gene transcription, we further compared the GFP–expressing control T0 plants, ABE8e-no-gRNA control T0 plants, and edited plants for all 12 possible nucleotide combinations, and again, no differences were found among these plants (Supplemental Fig. S4). In addition, no specific motif preference around mutated adenines (As) was observed to show any preferred activity by ABE8e with or without gRNAs (Fig. 5E), indicating no evidence of RNA editing. A low coefficient of determination between ABE8e mRNA expression and DNA– or RNA–level mutations further illustrates a lack of evidence for RNA editing (Supplemental Fig. S5). Together, our findings suggest ABE8e did not elicit any detectable off-target A-to-I mutations at the transcriptome level in base-edited tomato plants.

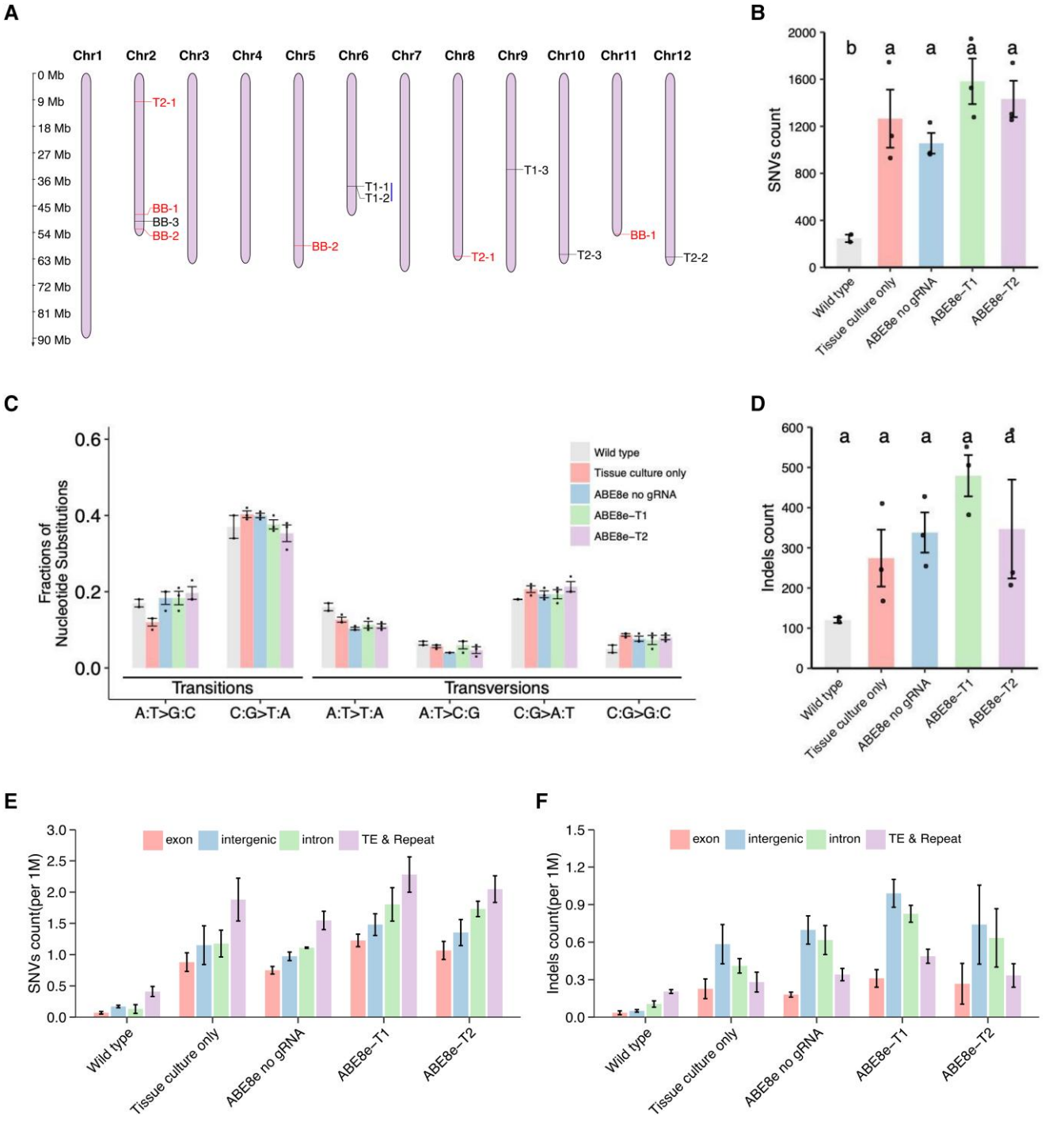


Figure 4. Genome-wide distribution of mutations in ABE8e-edited and control plants. **A)** Genome-wide mapping of T-DNA integration sites in the tomato genome for all 9 T0 lines (3× ABE8e-no gRNA, 3× ABE8e-T1, and 3× ABE8e-T2). T-DNAs that were integrated more than once into the tomato genome during an A*grobacterium*-mediated transformation event are depicted in red. The 2 T0 lines that share the same T-DNA integration site are marked with a solid line indicating the 2 lines share the same transgenic event. **B)** The average numbers of DNA SNVs identified in 2 independent lines of the WT and 3 independent lines of tissue culture–only control plants, ABE8e-no gRNA, ABE8e-T1, and ABE8e-T2 plants. **C)** Fractions of nucleotide substitutions in the WT, tissue culture–only control plants, ABE8e-no gRNA, ABE8e-T1, and ABE8e-T2 plants. Error bars represent standard error of mean (SEM), and points represent the fraction of nucleotide substitutions from an individual line. **D)** The average number of DNA indels identified in the WT, tissue culture–only control plants, ABE8e-no gRNA, ABE8e-T1, and ABE8e-T2 plants. **E)** Annotation of genome-wide distribution of SNVs in the WT, tissue culture–only control plants, ABE8e-no gRNA, ABE8e-T1, and ABE8e-T2 plants. **F)** Annotation of genome-wide distribution of indels in the WT, tissue culture–only control plants, ABE8e-no gRNA, ABE8e-T1, and ABE8e-T2 plants. **B, D)** Error bars represent SEM, and points represent the number of SNVs (B) or indels (D) from an individual line. Letters denote statistical differences assessed with the Student–Newman–Keuls (SNK) test ($P \leq 0.05$). **B to F)** Error bars represent SEM.

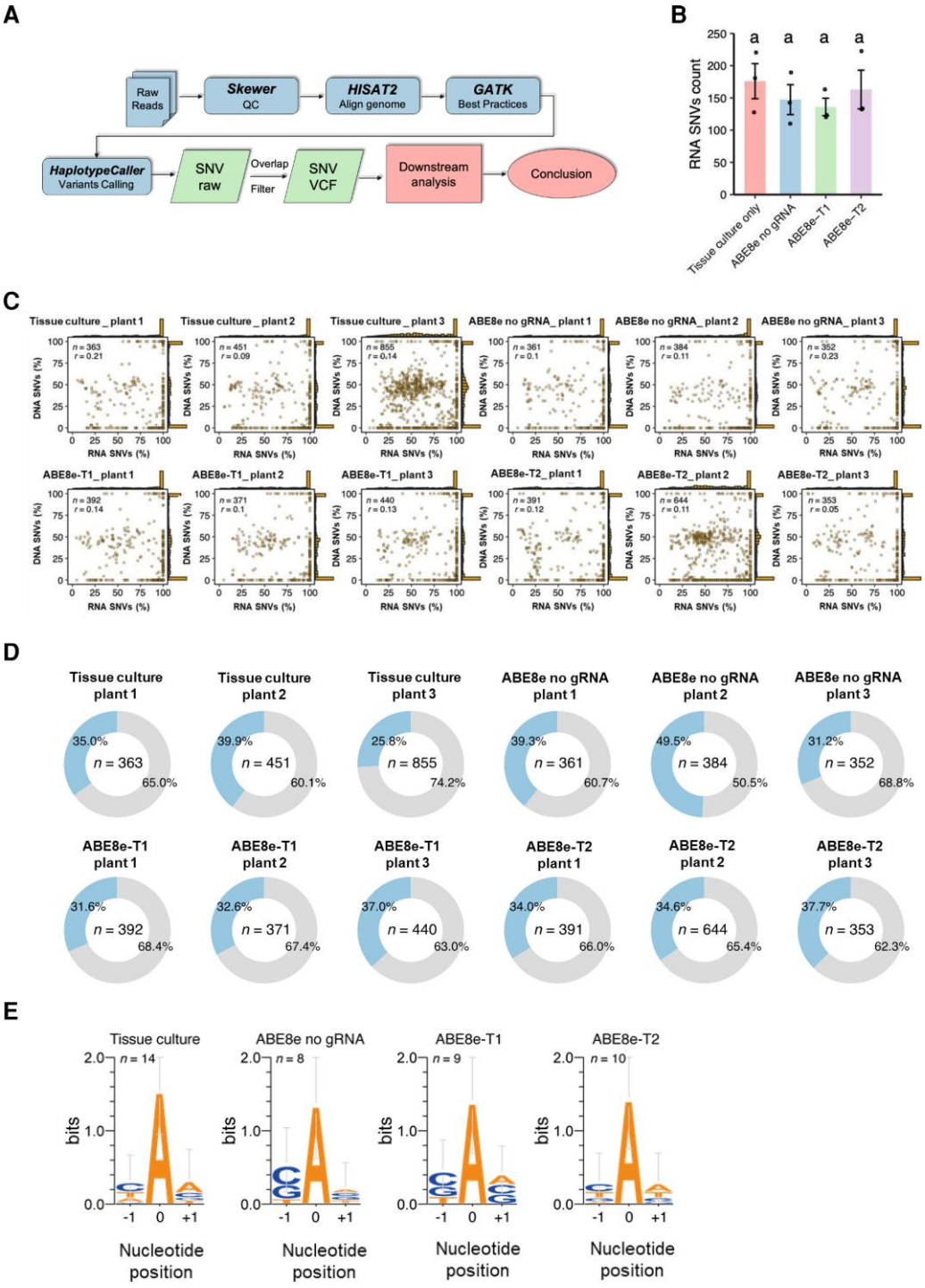


Figure 5. Transcriptome-wide off-target assessment of adenine base-edited plants. **A)** Workflow of detection of RNA–level SNVs. RNA SNVs were identified by HaplotypeCaller. **B)** Total number of RNA–level SNV counts averaged over 3 independent lines of the tissue culture–only control plants, ABE8e-no gRNA, ABE8e-T1, and ABE8e-T2 plants. Error bars represent standard error of mean (SEM), and points represent the number of RNA–level SNVs from an individual line (3 in total per sample). Letters denote statistical differences assessed with the Student–Newman–Keuls (SNK) test ($P \leq 0.05$). **C)** Scatter plot correlating RNA mutation rates of RNA SNVs as identified by HaplotypeCaller with DNA mutation rates as determined by WGS. The *x*-axis depicts fractions of RNA SNVs due to RNA–level mutations. The *y*-axis depicts fractions of RNA SNVs due to DNA–level mutations. Each dot represents an RNA SNV mutation. Person's correlation (*r*) was calculated to measure the correlation between DNA and RNA mutation rates. **D)** Comparison of RNA–level SNVs in each plant. All identified RNA SNVs were divided into DNA–level SNVs (>5%, gray) and RNA–level SNVs (≤5%, blue) according to their DNA mutation rates. The "*n*" means total SNVs including DNA level and RNA level. **E)** Hidden Markov model (HMM) sequence logos derived from specific strand RNA–level SNVs for tissue culture–only control, ABE8e-no-gRNA, ABE8e-T1, and ABE8e-T2 edited plants. 0 position on *x*-axis indicates mutated A position.

Discussion

It was a remarkable accomplishment when ABEs were developed considering that adenosine deaminase needed to be engineered to work on DNA instead of RNA as its natural substrate (Gaudelli et al. 2017). After this report, several research groups tried to further engineer adenosine deaminase to ameliorate A-to-G base editing efficiency (for example, with ABE8e (Richter et al. 2020) or ABE9 (Chen et al. 2023a, b) or abate the off-target effects (for example, with ABE8e(V106W)-HF) (Richter et al. 2020). Comparing both ABE8e and ABE8e-HF efficacy in tomato protoplasts, we observed low on-target base editing activity as determined by NGS of PCR amplicons (Fig. 1, B and C). The activities were comparable to base editing activities observed by others in *Arabidopsis* and rapeseed protoplasts by ABE7.10 (Li et al. 2021b). Interestingly, observed editing efficiencies of ABE7.10, ABE8e, and ABE8e-HF in mammalian cell lines were approximately an order of magnitude higher than in plant protoplasts, with ABE8e-HF not having a drastically reduced on-target editing activity compared to ABE8e (Richter et al. 2020). Low editing activities in tomato protoplasts can partly be explained by no cell division or unfavorable DNA repair pathways for A-to-G base conversion in protoplasts. However, we observed a higher percentage of editing at 2 independent target sites by ABE8e in tomato lines, consistent with a recent report (Niu et al. 2023).

WGS is an established method to assess potential off-target effects of genome editing technologies. Previously, we utilized WGS for genome-wide analysis of off-target effects of Cas9 and Cas12a in rice (Tang et al. 2018), CBEs in rice (Ren et al. 2021a) and tomato (Randall et al. 2021), and ABEs with PAM–relaxed properties in rice (Wu et al. 2022). While several studies in various plants found no potential genome-wide off-target effects by earlier versions of ABEs that are less efficient (Hua et al. 2018; Kang et al. 2018; Li et al. 2018; Jin et al. 2019; Wang et al. 2022), we and others documented substantial genome-wide off-target editing in rice by the highly efficient ABE8e (Li et al. 2022; Wu et al. 2022). In A-to-G base editing applications, gRNA–independent off-target editing is typically attributed to nonspecific deoxyribonucleotide conversions caused by the adenosine deaminase. In this work, we found no evidence for gRNA–dependent off-target effects by the 2 gRNAs examined (Figs. 3D). Importantly, we did not find evidence for gRNA–independent off-target editing by ABE8e as the number of SNVs in ABE8e-expressing plants either with or without a gRNA was similar to that of GFP–expressing controls (Figs. 4B). Further supporting this, detailed analysis of the SNVs discovered in edited and control plants showed similar transition and transversion types (Fig. 4C). Hence, the ~1,000 to 1,500 SNVs identified in the controls and ABE8e-edited tomato plants can be attributed to somaclonal variation resulting from the transformation and tissue culture process. Among all the SNVs discovered, only a fraction were in exons (Fig. 4E), suggesting that such somaclonal variation mutations are less likely to affect gene function.

Substantial transcriptome-wide off-target effects by early ABE versions have been observed in mammalian cell lines (Grünewald et al. 2019; Zhou et al. 2019) and only slightly higher transcriptome-wide off-target effects due to ABE overexpression were found in both rice (Li et al. 2022) and cotton (Wang et al. 2022). This is not surprising since in early ABE versions, both TadA-WT and engineered TadA heterodimer were fused, and it was known that TadA-WT could introduce unwanted ribonucleotide conversions in the transcriptome. In our study, we did not find any off-target effects in tomato plants at the transcriptome level for ABE8e, which only contains an evolved TadA-8e monomer coupled to Cas9 nickase. This is important as it can help avoid ABE self-editing at the transcriptome level and ensure high on-target editing activity and specificity.

Compared to recent investigations of ABE8e off-target effects in the genome and transcriptome of rice (Li et al. 2022; Wu et al. 2022), our investigation with similar approaches in tomato did not reveal detectable off-target effects in both genomes and transcriptomes of the ABE8e-edited lines. This discrepancy could be largely attributed to differential editing efficiencies in rice and tomato. Compared to the results in rice (Wei et al. 2021; Li et al. 2022; Wu et al. 2022), ABE8e editing efficiencies in tomato appeared to be lower (Niu et al. 2023). It is not surprising that lower on-target editing efficiency will translate to lower or undetectable off-target editing. Consistent with this, we did not find off-target editing to the CasOFFinder-nominated off sites, even with 1 to 3 mismatches of the protospacers (Fig. 2D). Unlike the recent reports in rice (Li et al. 2022; Wu et al. 2022), the genome-wide A-to-G and transcriptome-wide A-to-I mutations discovered in edited tomato plants did not enrich a TA motif, suggesting these mutations are indeed spontaneous mutations, rather than off-target edits. Together, our results suggest there would be minimal concerns about off-targeting when using our ABE8e vector system in tomato. A major take-home message from our study, along with earlier reports, is that ABE8e off-target effects must be assessed in a case-by-case scenario in different plant species. It is feasible to mitigate its potential off-target effects by controlling ABE8e expression and using different delivery methods. All these warrant future explorations into more plant species.

The adenine base editing field has recently regained momentum with several research groups reporting on engineered TadA adenosine deaminases that introduce C-to-T editing or simultaneous C-to-T and A-to-G editing (Chen et al. 2023b; Neugebauer et al. 2023; Lam et al. 2023). Recently developed CBEs based on engineered TadA deaminase showed several distinct superiorities, such as low indel rate, reduced bystander mutations partly due to narrower editing windows, and a background level of gRNA–independent DNA and RNA off-target effects in mammalian cell lines (Chen et al. 2023b; Neugebauer et al. 2023; Lam et al. 2023). With so many types of activities in these recently developed

CBEs and ABE/CBE dual editors, it will be interesting to see how these base editors fare in plants and what their off-targeting potential might be.

Conclusions

In this work, we demonstrated an efficient ABE8e base editor in tomato protoplasts, as well as stable T0 lines. Based on evidence obtained by WGS and RNA-seq, we did not discover any genome-wide or transcriptome-wide off-target editing by ABE8e in edited tomato plants. Our observation provides insights on mitigating ABE8e off-target effects previously reported in rice. Our data should also help regulatory agencies develop policies and guidelines on regulating or deregulating base-edited crops.

Materials and methods

Vector construction

Gateway-compatible ABE (attL1-attR5) pYPQ262-ABE8e (Addgene #161523) was prepared as previously described (Ren et al. 2021b). Gateway-compatible high-fidelity ABE (attL1-attR5) pYPQ262F-ABE8e (Addgene #199179) was generated by introducing the V106W mutation into the engineered TadA8e adenosine deaminase using a Q5 site-directed mutagenesis kit (NEB, catalog #E0554S) with primers V106W-F and V106W-R (Supplemental Table S3). Successful Gateway-compatible attL1-attR5 entry clone preparation was confirmed by Sanger sequencing with primers listed in Supplemental Table S3.

T-DNA vectors (Supplemental Table S4) for adenine base editing were prepared using Golden Gate and Gateway LR 3-way assembly reactions based on previously described protocols (Lowder et al. 2015). In brief, forward and reverse primers (Supplemental Table S3) were phosphorylated with T4 polynucleotide kinase (NEB, catalog #M0201*), annealed, and ligated with T4 DNA ligase (NEB, catalog #M0202*) into BsmBI (Thermo Fisher, catalog #ER045*) restriction–digested pYPQ141B (Addgene #69291) gRNA entry clones in 1-step Golden Gate reactions. Individual 3-way Gateway LR reactions were conducted using an attL5-attL2 gRNA entry clone, attL1-attR5 ABE entry clone, and attR1-attR2 destination vector pCGS710 containing the 2×35S promoter for ABE expression (Supplemental Fig. S6). Both gRNA and base editor entry clone recombination regions were confirmed by Sanger sequencing. Final T-DNA vectors were confirmed by restriction digestion with EcoRV-HF (NEB, catalog #R3195*). An example of the final T-DNA vector map can be found in Supplemental Fig. S1.

Tomato protoplast isolation and transformation

Tomato (*S. lycopersicum*) protoplasts were isolated from cotyledons of 10- to 14-d-old M82 indeterminate tomato with a functional SELF-PRUNING gene (M82 SP+). M82 SP+ seedlings were grown in vitro, as described by Van Eck et al. (Van Eck et al. 2019). Excised cotyledons were subjected to enzyme digestion (400 mM mannitol, 10 mM $CaCl_2$, 20 mM KCL, 10 mM MES, 0.3% *w/v* Cellulase Onozuka R-10 [Yakult Pharmaceutical], 0.15% *w/v* Macerozyme R-10 [Yakult Pharmaceutical], pH 5.7) for approximately 16 h at 22 °C in the dark with gentle agitation on an orbital shaker at 60 rpm. The protoplast suspension was filtered through a 75 μm cell strainer and centrifuged for 10 min at 200 × *g*. The resulting protoplast pellet was resuspended in 0.55 M sucrose (pH 5.7) and gently overlayed with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) without mixing. After centrifugation for 30 min at 200 × *g*, protoplasts were harvested from the sucrose/W5 interface using a glass Pasteur pipette, washed with fresh W5 solution, and enumerated by Neubauer-improved counting chamber. The final protoplast pellet was resuspended in MMG (500 mM mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7) to a density of 1×10^6 protoplasts/ml.

Tomato protoplasts were transformed according to the method described previously (Zhang et al. 2013). Briefly, 200 μl of MMG protoplast suspension (2×10^5 protoplasts) was mixed with 20 μg of purified plasmid DNA (in 20 μl water) and 220 μl of freshly prepared PEG solution (40% *w/v* PEG-4000, 200 mM mannitol, 100 mM $CaCl_2$). The protoplast suspension was gently mixed and incubated in the dark for 20 min. Afterwards, 900 μl of W5 solution was added, and protoplasts were collected by centrifugation for 5 min at 200 × *g*. The protoplasts were resuspended in 1 ml W5 solution and transferred to a 12-well plate. The plates were incubated in the dark for 60 h at 28 °C. Transformation efficiency for each experiment was estimated by counting the number of GFP–positive protoplasts from a sample transformed with pMDC32-GFP in at least 3 fields of view with a fluorescence microscope. Transformed protoplasts were collected by centrifugation at 10,000 × *g* for 10 min, and pellets were resuspended in 20 μl of Phire Dilution Buffer (Thermo Fisher, catalog # F130WH) and stored at −20 °C.

Mutation analysis of transformed protoplasts

Target regions were PCR amplified from protoplasts with barcoded primers (Supplemental Table S3) using the Phire Plant Direct PCR Kit (Thermo Fisher, catalog #F130WH) per manufacturer's instructions. Amplicons were confirmed by gel electrophoresis, purified with the QIAQuick PCR Purification Kit (QIAGEN, catalog #28104), quantified by Nanodrop One (Thermo Fisher), and combined in equal ratios into pools of 9 amplicons for deep sequencing. Amplicon-EZ sequencing was performed by Genewiz. Mutation analysis was performed on FASTQ sequence files by CRISPRMatch (You et al. 2018). The A-to-B (B = G, C, T) conversion rate for each construct was determined and reported as the average of at least 3 independent biological replicates.

Tomato stable transformation

Agrobacterium-mediated transformation of cotyledons from the M82 SP + tomato genotype was performed with *A.*

tumefaciens AGL1, as previously reported (Van Eck et al. 2019). All in vitro cultures and plants were maintained at 28 °C.

Mutation analysis of stably transformed lines

Sanger sequencing was used to determine genetic modifications at the target sites in T0 plants. DNA was extracted from leaf tissue of well-rooted T0 plants using the Phire Plant Direct PCR Kit (Thermo Fisher, catalog # F130WH) according to the manufacturer's instructions, and genome regions containing target sites were amplified with primers (Supplemental Table S3). Verification of the stable lines was confirmed by PCR amplification for the presence of the Cas9 gene and TadA8e using Phire Plant Direct PCR Kit adhering to the manufacturer's instructions. The specificity of PCR reactions was verified by gel electrophoresis, and amplicons were enzymatically cleaned by ExoSap (NEB). The concentration of purified amplicons was assessed with Nanodrop One (Thermo Fisher), and amplicons were Sanger sequenced at Genewiz (NJ, USA). Sanger sequencing chromatograms were aligned to the tomato reference sequence of the SlALS gene (Solyc03g044330.1) using SnapGene software, and mutations were identified by visual inspection.

Whole-genome sequencing

DNA was extracted from leaf tissue of well-rooted, in vitro-grown T0 plants and 3-wk-old M82 SP+ WT seedlings grown in a soilless mix using DNeasy Plant Mini Kit (QIAGEN, catalog #69204) according to manufacturer's instructions. Genomic DNA was quantified using Nanodrop One (Thermo Fisher). DNA integrity was checked with a ~1% *w*/*v* agarose gel with 50 to 100 ng sample loaded per well. Samples were chosen for library preparation based on the QC results. Library preparation and Illumina sequencing on a HiSeq 4000 platform were performed by Genewiz (NJ, USA).

RNA sequencing

Approximately 30 mg of leaf tissue was excised from each well-rooted, in vitro-grown T0 plant for RNA extraction and sequencing. RNA extraction, quantification, quality check, library preparation, and sequencing were done by Genewiz (NJ, USA).

WGS data analysis

The WGS analysis was performed using a previously established method (Tang et al. 2018) with minor adjustments. SKEWER (v. 0.2.2) (Jiang et al. 2014) was used to remove the adapters from the raw reads. The cleaned reads were aligned to the tomato reference sequence M82 SP+ (https://solgenomics.net/ftp/genomes/tomato100/March_02_2020_sv_landscape/M82_MAS2.0.fasta.gz) using BWA mem (v. 0.7.17) software (Li and Durbin 2010), and Picard and Samtools (v. 1.9) (Li et al. 2009) were employed to filter out reads that mapped to multiple locations. GATK (v. 3.8) (McKenna et al. 2010) was used to realign reads near indels, and whole-genome SNVs and indels were identified by utilizing LoFreq (v. 2.1.2) (Wilm et al. 2012), Mutect2 (Cibulskis et al. 2013), VarScan2 (v. 2.4.3) (Koboldt et al. 2012), and Strelka2 (v2.9.10) (Kim et al. 2018). Bedtools (v. 2.27.1) (Li 2011) were used for overlapping SNVs and INDELs. Additionally, potential off-target sites were predicted using Cas-OFFinder software (v. 2.4) (Bae et al. 2014), with a tolerance of up to 5 nucleotide mismatches. Python and R were utilized to perform data processing and analysis.

Whole-transcriptome sequencing data analysis

The data preparation steps are detailed in the WGS analysis section. Briefly, the cleaned reads were aligned to the tomato reference sequence M82 SP+ (ftp://ftp.solgenomics.net/genomes/tomato100/March_02_2020_sv_landscape/) using the Hisat2 (v. 2.2.0) software (Kim et al. 2019). The BAM files were sorted, and duplicates were marked using Picard tools. Further processing steps included spanned splice junctions, local realignment, and variant calling using SplitNCigarReads, IndelRealigner, and HaplotypeCaller tools from GATK (v. 3.8) (McKenna et al. 2010). The focus was on identifying SNVs on the canonical chromosomes (Chr1 to Chr12), and the VariantFiltration tool was used to filter RNA SNVs with high confidence. All data processing and analysis were conducted using Python and R.

Accession numbers

Addgene numbers for 2 ABE entry clones: pYPQ262-ABE8e (161523) and pYPQ262F-ABE8e (199179). The Amplicon-EZ sequencing data of tomato protoplast, WGS raw data, and RNA-seq data reported in this article have been deposited to the Sequence Read Archive in the National Center for Biotechnology Information (NCBI) under the accession numbers PRJNA954756 and PRJNA953768 and the Beijing Institute of Genomics Data Center (http://bigd.big.ac.cn) under BioProject PRJCA016074.

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Author contributions

Y.Q. and J.V.E. designed the experiments. S.S. generated all base editing vectors. Y.C. conducted tomato protoplast, and Y.G. performed stable transformation. Y.C. analyzed PCR amplicon NGS data from protoplast experiments. Y.G. collected the leaf tissue samples. Y.C. and S.S. analyzed mutagenesis of stable tomato lines. S.S. processed samples for WGS and RNA-seq. Y.W. and T.Z. analyzed the WGS and RNA-seq data. Y.W., Y.C., and S.S. prepared all the figures. S.S., Y.Q., J.V.E., and T.Z. wrote the manuscript. All authors participated in the discussion and revision of the manuscript.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. A representative map of an ABE8e T-DNA vector.

Supplemental Figure S2. The distributions of DNA SNVs and INDELs in tomato chromosomes.

Supplemental Figure S3. Fraction of RNA–level SNV types in each plant.

Supplemental Figure S4. SNV transcripts and motif analysis of altered adenine (A).

Supplemental Figure S5. The coefficient of determination between ABE8e mRNA and the numbers of DNA– or RNA–level mutations in T0 plants.

Supplemental Figure S6. Plasmid map of the Gateway destination vector pCGS710.

Supplemental Table S1. WGS information.

Supplemental Table S2. RNA-seq reads for each sample.

Supplemental Table S3. Oligonucleotides used in this study.

Supplemental Table S4. T-DNA vectors used in this study.

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