

Life Sciences and Medicine

Special Topic: Gene Editing towards Translation

Anthocyanin-assisted *Agrobacterium* infiltration for the rapid evaluation of genome editing efficiencies across multiple plant speciesSinian Xing¹, Yu Sun^{1,2}, Boshu Li^{1,3}, Hongchao Li¹, Kevin T. Zhao¹, Kunling Chen^{1,*} & Caixia Gao^{1,3,*}¹State Key Laboratory of Plant Cell and Chromosome Engineering, Center for Genome Editing, Institute of Genetics and Developmental Biology, Innovation Academy for Seed Design, Chinese Academy of Sciences, Beijing 100101, China;²College of Life Sciences, University of Chinese Academy of Sciences, Beijing 100101, China;³College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing 100049, China*Corresponding authors (emails: cxgao@genetics.ac.cn (Caixia Gao); klchen@genetics.ac.cn (Kunling Chen))

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Abstract: CRISPR-based genome editing technologies continue to drive major advances in life sciences. A major challenge for realizing widespread use of genome editing in plants and agriculture is establishing methods that enable the rapid, comprehensive, and precise evaluation of editing technologies using transient methods. Here we report a new and rapid genome editing evaluation method using *Agrobacterium* infiltration techniques to enable broad-spectrum, simplistic, and precise assessments of genome editing efficiencies. We employed an anthocyanin marker to facilitate visual screenings of genome-edited cells for use in adult strawberry fruits as well as tomato fruits, cotton leaves, and sugar beet leaves. Using this method, we demonstrate the ability to quickly measure genome editing efficiencies mediated by SpCas9, LbCas12a, A3A-PBE, ABE8e, and PPE. This new method will allow researchers to rapidly and easily evaluate genome editing tools across a broad spectrum of plant species, further expediting the development of genome-edited agricultural crops.

Keywords: genome editing, *Agrobacterium* infiltration, anthocyanin, transient transformation, geminivirus replicon, strawberry, tomato

INTRODUCTION

Genome editing technology can generate precise modifications at target genomic loci [1]. CRISPR-Cas systems have made genome editing a widely adopted, low-cost, easy-to-use targeted genetic manipulation tool that has been applied to many crops, such as rice, wheat, and corn, as well as horticultural crops such as tomato, sugar beet, and strawberry [2–4]. New precision genome editing technologies based on CRISPR-Cas proteins, such as base editing and prime editing, have further expanded the scope of genome editing, enabling the production of precise base substitutions, small deletions and insertions [5–9]. These technologies have been used to improve crucial agronomic traits such as crop yield, quality, stress resistance and more [10–13], and, combined with modern breeding approaches, will greatly accelerate crop improvement.

Transient gene expression techniques generally require short periods of time, so genome editing can be

achieved rapidly using transient gene expression methods [14–16]. Transient expression approaches are used to rapidly verify editing efficiency, edited product purity and editor or target specificity. Common transient expression techniques typically involve the use of protoplasts and *Agrobacterium* infiltration. At present, protoplast transient expression technology is the most commonly used approach in wheat, rice and other plants, but this technology requires the production of protoplasts, which is extremely time-consuming and challenging. By comparison, the use of *Agrobacterium* infiltration to infect fruits of strawberry and tomato, and the leaves of cotton, sugar beet, and tobacco, is much simpler [17–19].

In this study, we present a new approach leveraging anthocyanin production to mark cells that have been successfully infected by CRISPR-Cas-containing *Agrobacterium* to increase the efficiency, robustness, and speed of screening genome editing technologies in plants. Anthocyanin, a natural water-soluble pigment is widely distributed in plants and is one of the main pigments in flowers, fruits and leaves [20]. The synthesis of anthocyanins is mainly determined by structural genes and regulatory genes. MYB proteins are an important class of transcription factors regulating secondary metabolism in plants and are the key transcription factor elements regulating anthocyanin production. MYB homologs that regulate anthocyanin metabolism have been identified in many plant varieties [21–23], and overexpression of the corresponding *MYB* gene results in a strong accumulation of anthocyanins. For example, overexpression of *FvMYB10* in strawberries turns the whole plant red [24] and overexpression of *SLAN2-like* (a MYB homolog) in tomato turns tomato plants purple [25]. Furthermore, it was demonstrated that transient expression of *MYB* mediated by *Agrobacterium* infiltration can promote anthocyanin accumulation [18].

In this study, we established a new, efficient, and rapid approach to evaluate genome editing technologies across a variety of plant species using anthocyanin-assisted screening combined with *Agrobacterium* infiltration transient expression delivery methods. This approach does not require any special equipment, expensive chemicals, or invasive treatments. Due to its visible color signal, tissues and cells successfully delivered with anthocyanin-containing genome editing vectors can be accurately identified by the red color, thus facilitating isolation and subsequent sequencing. With this method, we achieved efficient genome editing in strawberry fruits, tomato fruits, cotton leaves and sugar beet leaves.

Plant viruses have been used as vectors to deliver and express proteins in plants [26–29]. Several characteristics of geminiviruses make them ideal vectors for expressing genome editing tools in plant cells. A crucial property of geminiviruses is that they have a relatively high replication efficiency in plants, thus achieving a high viral copy number [30]. In tomato fruits, we demonstrate that installing geminiviral replicon signals to *Agrobacterium* vectors containing genome editing components can significantly improve editing efficiencies across multiple endogenous genomic target sites. This editing enhancement combined with the anthocyanin-mediated transient editing efficiency evaluation method enables rapid comparisons of editing approaches across a variety of plant species and target sites.

RESULTS

Efficient genome editing in strawberry fruits by SpCas9 and LbCas12a

Strawberry is an important horticultural crop, with a pleasant aroma, and rich in vitamins, anthocyanins and other ingredients, which are beneficial to human health [31]. The establishment of a simple and efficient

transient expression technique for evaluating genome editing efficiencies could accelerate the investigation and breeding of strawberry. Fruits of the diploid strawberry *F. vesca* ssp. *vesca* accession Hawaii 4 are always white due to a mutation in the *FvMYB10* gene, but transient expression of wild type *FvMYB10* in white fruits can rescue the red color phenotype [18]. In this study, genome editing tools were transiently expressed at the same time as *FvMYB10* in Hawaii 4 fruits by *Agrobacterium* infiltration (Figure 1A). Transient expression of *FvMYB10* in Hawaii 4 fruits turns the fruits red in three to five days and can be monitored in real time. Tissues successfully delivered using this transient expression method can be easily identified and harvested to evaluate editing events, which mitigates the impact of low transient delivery and expression on genome editing efficiencies (Figure 1A).

SpCas9 from a type-II CRISPR system is one of the most widely used genome editing tools, so we used it in constructing plasmid pCas9-FvMYB10 (Figure 1B), which consists mainly of an sgRNA expression cassette, an SpCas9 expression cassette and a *FvMYB10* expression cassette. Expression of the sgRNA is driven by an *Arabidopsis* RNA polymerase III promoter, AtU6, expression of SpCas9 is driven by a 2x CaMV35S promoter, and expression of *FvMYB10* is driven by a CaMV35S promoter. When *Agrobacterium* containing pCas9-FvMYB10 was injected into Hawaii 4 fruits by syringe, areas of red anthocyanin accumulation appeared after three to five days (Figure 1C). We evaluated genome editing efficiencies mediated by SpCas9 at 12 endogenous targets in strawberry fruits by amplicon deep sequencing and successfully detected editing efficiencies ranging from 2.4% to 29.1% (Figure 1D). Of the 12 target sites, the editing efficiency at the *FvbZIP* target was the highest. At *FvARF* and *FvPG* it also reached above 20%, 26.1% for *FvARF* and 22.6% for *FvPG*. The editing efficiency of the remaining nine targets remained around 10%.

Fruit softening is a major determinant of shelf life and commercial value. Strawberry is a soft fruit with a short postharvest life. Pectate lyase (PL) genes have been documented as excellent candidates for improvement of fruit firmness [32]. To control the strawberry fruit softening, we created pectate lyase gene mutant plants in diploid wild strawberry and octoploid culture strawberry, using the *FvPL* target site assessed above (editing efficiency at the *FvPL* target was 14.2%). After *Agrobacterium*-mediated transformation, we obtained 24 transgenic diploid strawberry plants and 12 transgenic octoploid strawberry plants. Sequencing revealed that nine diploid plants and two octoploid plants harbored mutations in the target region, editing efficiency was 37.5% and 16.7% (mutant plants/transgenic plants), respectively (see Supplementary Table S1). The above results show that the assessed target sites can be used to create mutant plants in diploid and octoploid strawberry.

LbCas12a is a class 2/type-V CRISPR system and has also been widely used for genome editing [33]. It has some features that differ from SpCas9. It requires a T-rich PAM (TTTN), which is orthogonal to the PAM (NGG) of SpCas9, and generates staggered cuts distal to the PAM sequence. LbCas12a is an attractive tool for plant editing. Editing with LbCas12a has not been previously reported in strawberry. We therefore constructed a pCas12a-FvMYB10 plasmid (Figure 1E) with LbCas12a expressed from a 2x CaMV35S promoter and a crRNA ribozyme cassette expressed from the CmYLCV promoter and tested for editing at eight targets in strawberry (Figure 1F). Efficient mutagenesis was obtained at seven of eight targets. Genome editing of the target sites assessed by amplicon deep sequencing revealed editing changes at frequencies ranging from 0.8% to 7.0% across the seven edited targets. Editing efficiencies at *FvYUCg7* and *FvuORFg11* were 5.8% and 7.0%, respectively and substantial changes were also detected at the other five targets (average over the seven edited targets, 3.0%) (Figure 1G). The deletions detected mostly occurred 13–23

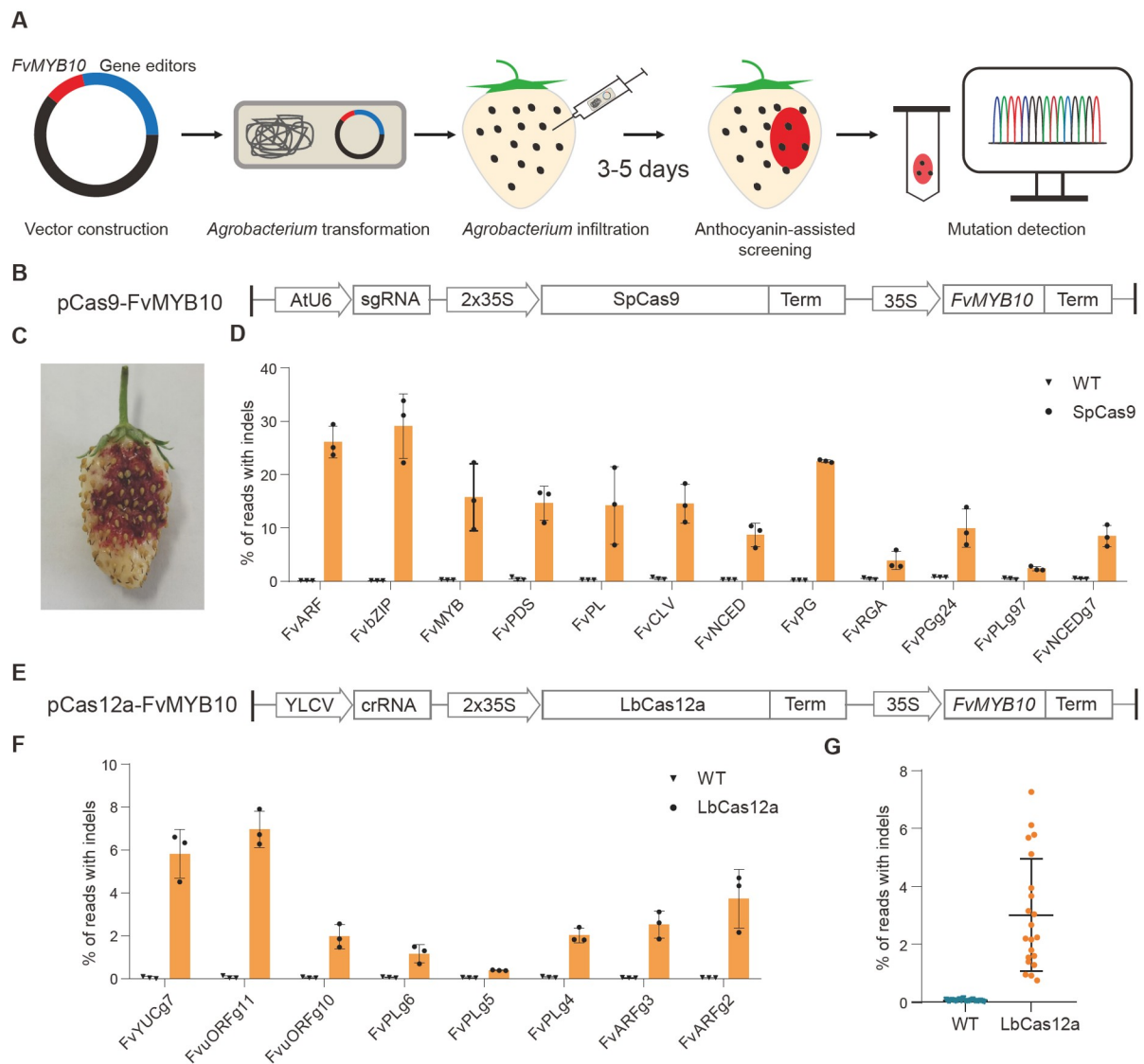


Figure 1 Genome editing in strawberry fruits by SpCas9 and LbCas12a. (A) Diagram of the anthocyanin-assisted screening and *Agrobacterium* infiltration transient expression techniques; (B) schematic representation of the pCas9-FvMYB10 vector; (C) phenotype of *Agrobacterium* infiltration with *FvMYB10* in strawberry fruits; (D) frequencies of SpCas9 at 12 endogenous genomic targets in strawberry fruits; (E) schematic representation of the pCas12a-FvMYB10 vector; (F) frequencies of LbCas12a at eight endogenous genomic targets in strawberry fruits; (G) average efficiency of LbCas12a-mediated genome editing in strawberry fruits. An untreated fruit sample served as control in (D) and (F). Frequencies (mean ± S.D.) were calculated from three biological replicates ($n = 3$).

nucleotides distal to the PAM site, and were generally of 3–20 bp, somewhat larger than those generated by SpCas9 (see Supplementary Figures S1 and S2).

Base editing in strawberry fruits by A3A-PBE and ABE8e

Genome-wide association studies have shown that many elite traits of crops are dependent on single base changes. Since base editing creates base substitutions at target loci without the need for double-stranded DNA breaks (DSBs), homology-directed repair (HDR) processes, or donor DNA templates, it has greatly

advanced agricultural research in recent years. Two main classes of base editors have been developed, cytosine base editors (CBEs) that convert C-to-T and adenine base editors (ABEs) that convert A-to-G. A3A-PBE is an efficient plant CBE with a 17-nucleotide editing window [34]. To see whether efficient C-to-T base editing can be achieved in strawberry using the present transient expression method, we constructed a new vector, pA3A-FvMYB10 (Figure 2A), and tested it across 12 endogenous genomic targets in strawberry fruits using our previously discussed anthocyanin-associated *Agrobacterium* transient delivery method (Figure 2B). Amplicon deep sequencing showed that A3A-PBE induced efficient C-to-T base editing at all 12 targets, with the frequency of single C-to-T substitutions reaching 20.1% at C9 in the *FvYUC2* target. Frequencies >10% at the most frequently edited position were obtained at targets in *FvARF2*, *FvPL2* and *FvPDS*, and maximal frequencies ranging from 1%–10% at the remaining eight targets. Few undesired editing and indels were detected in the amplicon deep sequencing data at the four highly efficient targets (C-to-T efficiency >10%) (see Supplementary Tables S2 and S3).

ABE8e, which was generated by phage-assisted evolution of the TadA deaminase component of ABE7.10, has been reported to generate very high A-to-G editing frequencies in human cells and plants [35]. ABE8e contains eight additional mutations that increase activity 590-fold compared with ABE7.10 [35]. No study has yet reported genome editing with ABEs in strawberry. To see whether efficient A-to-G conversion can be achieved in strawberry using this transient expression method, we constructed a new vector, pABE8e-FvMYB10 (Figure 2C), in which ABE8e is expressed from 2x CaMV35S promoter, and tested it across 12 endogenous genomic target sites (Figure 2D). Efficient A-to-G conversion was induced at all 12 targets. The frequency of single A-to-G substitutions was 15.4% at the *FvYUC* target; it exceeded 10% at the highest edited positions in the *FvPG* targets and ranged from 1%–3% at the other ten targets. ABE8e converted A-to-G very “cleanly” in strawberry at two highly sensitive targets (A-to-G efficiency >10%) (see Supplementary Tables S4 and S5).

The region of the R-loop that supports efficient base editing defines the base editing “activity window”. We examined the base editing window of A3A-PBE and ABE8e in strawberry. By analyzing editing efficiencies at every protospacer position across all 12 target sites of A3A-PBE and ABE8e, we found that the base editing activity window of A3A-PBE in strawberry was very wide, with efficient C-to-T base editing being detected from 2 to 16 (Figure 2E), taking the PAM as positions 21–23, while that of ABE8e was approximately protospacer positions 3 to 9 (Figure 2F).

Extending this method to cotton, sugar beet and tomato

Using this simple and rapid transient delivery method, we successfully achieved efficient targeted genome editing mediated by SpCas9 and LbCas12a, and efficient base editing with A3A-PBE and ABE8e in strawberry fruits. To see if this approach could be applied to other plants, we replaced the *FvMYB10* gene in the pCas9-FvMYB10 vector with the homologous genes of other plants (Figure 3A, 3E and 3I), and observed anthocyanin accumulation and efficient genome editing in cotton leaves, sugar beet leaves, and tomato fruits (Figure 3B, 3F and 3J). In cotton leaves, numerous genetic changes were obtained at seven of ten targets, with a maximum frequency of 11.4% across the seven edited targets and an average of 6.0% (Figure 3C and 3D). In sugar beet leaves, mutagenesis was observed at five of eight targets (Figure 3G). Frequencies exceeding 10% were obtained at two targets, 11.8% for *BvWRKYg1* and 15.6% for *BvWRKYg2*. The average efficiency

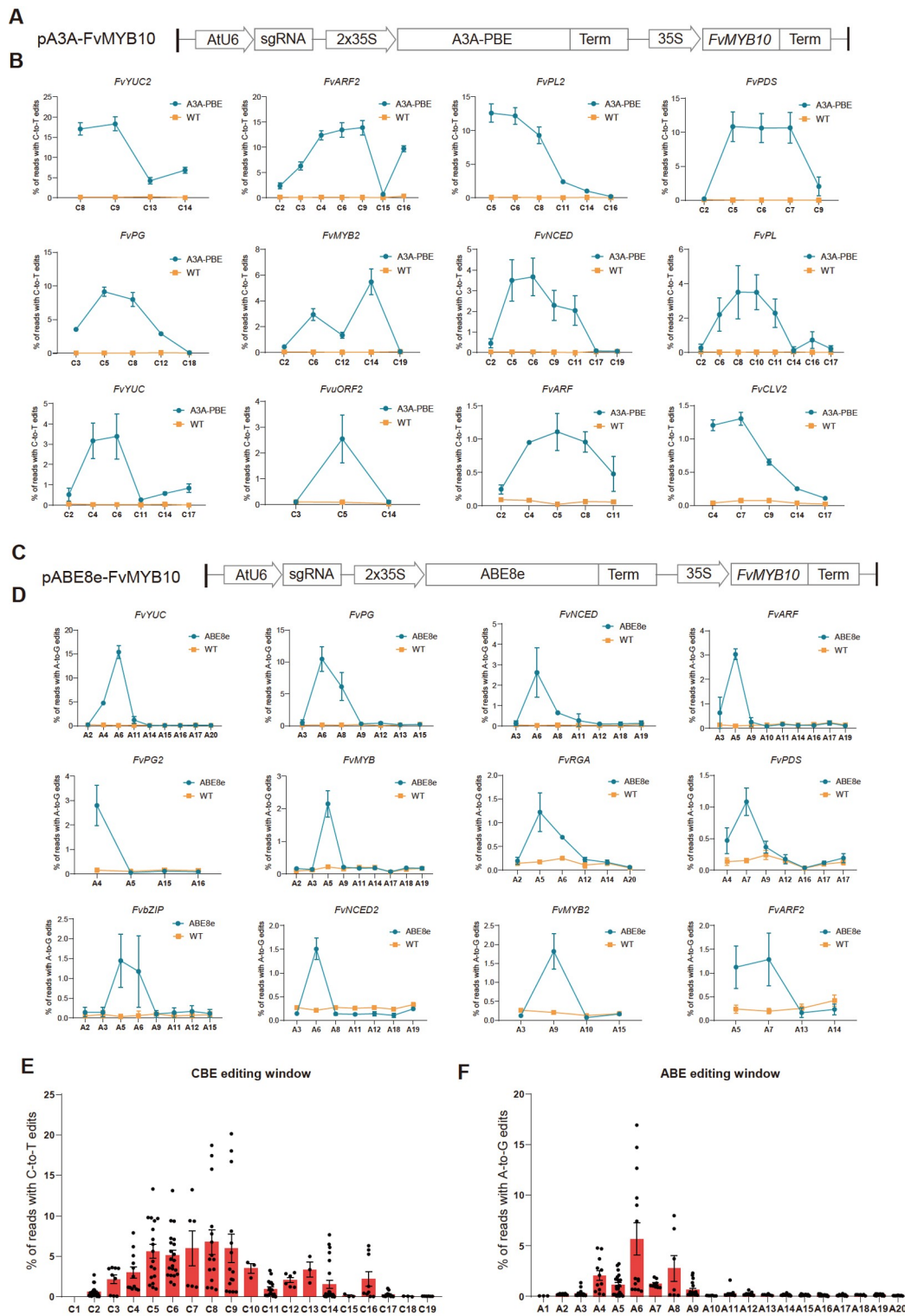


Figure 2 Base editing in strawberry fruits by A3A-PBE and ABE8e across 12 endogenous genomic sites. (A) Schematic representation of the pA3A-FvMYB10 vector; (B) frequencies of A3A-PBE in strawberry fruits; (C) schematic representation of the pABE8e-FvMYB10 vector; (D) frequencies of ABE8e in strawberry fruits; (E) editing window of A3A-PBE in strawberry; (F) editing window of ABE8e in strawberry. An untreated fruit sample served as control in (B) and (D). Frequencies (mean \pm S.D.) were calculated from three biological replicates ($n = 3$).

of editing on the five edited targets in sugar beet leaves was 7.6% (Figure 3H). We also obtained a phenotype of anthocyanin accumulation in tomato, mainly in the interior of the fruit: the pith (Figure 3J). In tomato, efficient mutagenesis was obtained at seven of nine targets (Figure 3K). Mutation frequencies were 20.8% at *SIER2* and 14.2% for *SIWUS2*. The average efficiency over the seven edited targets was 9.0% (Figure 3L). The above results show that the method presented is widely applicable for rapidly and easily evaluating genome editing tools across a variety of plant species and genomic targets.

Genome editing efficiency is improved by the BeYDV replicon in tomato fruits

Geminiviruses require only one protein, Rep (replication associated protein), to initiate viral replication in host cells. In the present study, we placed the sgRNA expression cassette, SpCas9 expression cassette and *SIAN2-Like*-containing expression cassette between geminivirus replicon elements LIR and SIR, thus generating pCas9-SIAN2L-YDV (Figure 4A). This geminivirus replicon enables replication in cells, thus achieving relatively high copy numbers and enhancing genome editing efficiencies (Figure 4B). After transient expression by *Agrobacterium* infiltration, the distinctive red color phenotype of anthocyanin accumulation was seen in the tomato fruits, with the area of anthocyanin accumulation significantly increased compared with the control treatments (Figure 4C).

We compared the genome editing efficiency of SpCas9 and SpCas9 + BeYDV across nine endogenous genomic targets, and found that SpCas9 + BeYDV significantly improved genome editing efficiencies (Figure 4D). For SpCas9 + BeYDV, editing efficiencies ranged from 10.6%–32.9% at the nine targets tested compared with 0.1%–20.7% for SpCas9 (averages 6.0% for SpCas9 and 19.2% for SpCas9 + BeYDV) (Figure 4E). In particular, mutation by the SpCas9 + BeYDV strategy was greatly improved at *SIWUS10*, *SISP2* and *SISP5G14*, at which the SpCas9 produced virtually no mutations (Figure 4D).

Prime editing in tomato fruits

Prime editing is a recent genome editing technology that can introduce all 12 possible types of point mutation, as well as small insertions and small deletions in a precise and targeted manner [36–39]. Prime editors are fusion proteins using a catalytically impaired Cas9 endonuclease fused to an engineered reverse transcriptase programmed with a prime editing guide RNA (pegRNA) that both specifies the target site and encodes the desired edit [36]. Previous studies showed that the plant prime editor PPE2 has similar editing efficiency in plants to PPE3 [37]. Therefore, in this study, we used PPE2 to evaluate editing efficiency. The pegRNA expression cassette, prime editing expression cassette and *SIAN2-Like* expression cassette were placed between the geminivirus replicon elements LIR and SIR, yielding pPE-SIAN2L-YDV (Figure 4F). We tested prime editing on 20 endogenous genomic targets in tomato fruits. PBS length and the RT template sequence were designed using PlantPegDesigner, a user-friendly web application (<http://www.plantgenomeediting.net/>) [37] and prime editing efficiencies were measured by amplicon deep sequencing. Prime editing was obtained at 14 of the 20 targets (Figure 4G). The PPE system was found to induce nucleotide substitutions at frequencies up to 0.9%, with the edited bases at positions +1 to +3 (Figure 4G). Single nucleotide substitution frequencies exceeding 0.5% (sequencing depth: 70,000–310,000 reads per locus) were found at two targets: A-to-T substitutions at a frequency of 0.7% at *SLACOG5*, and C-to-A substitutions

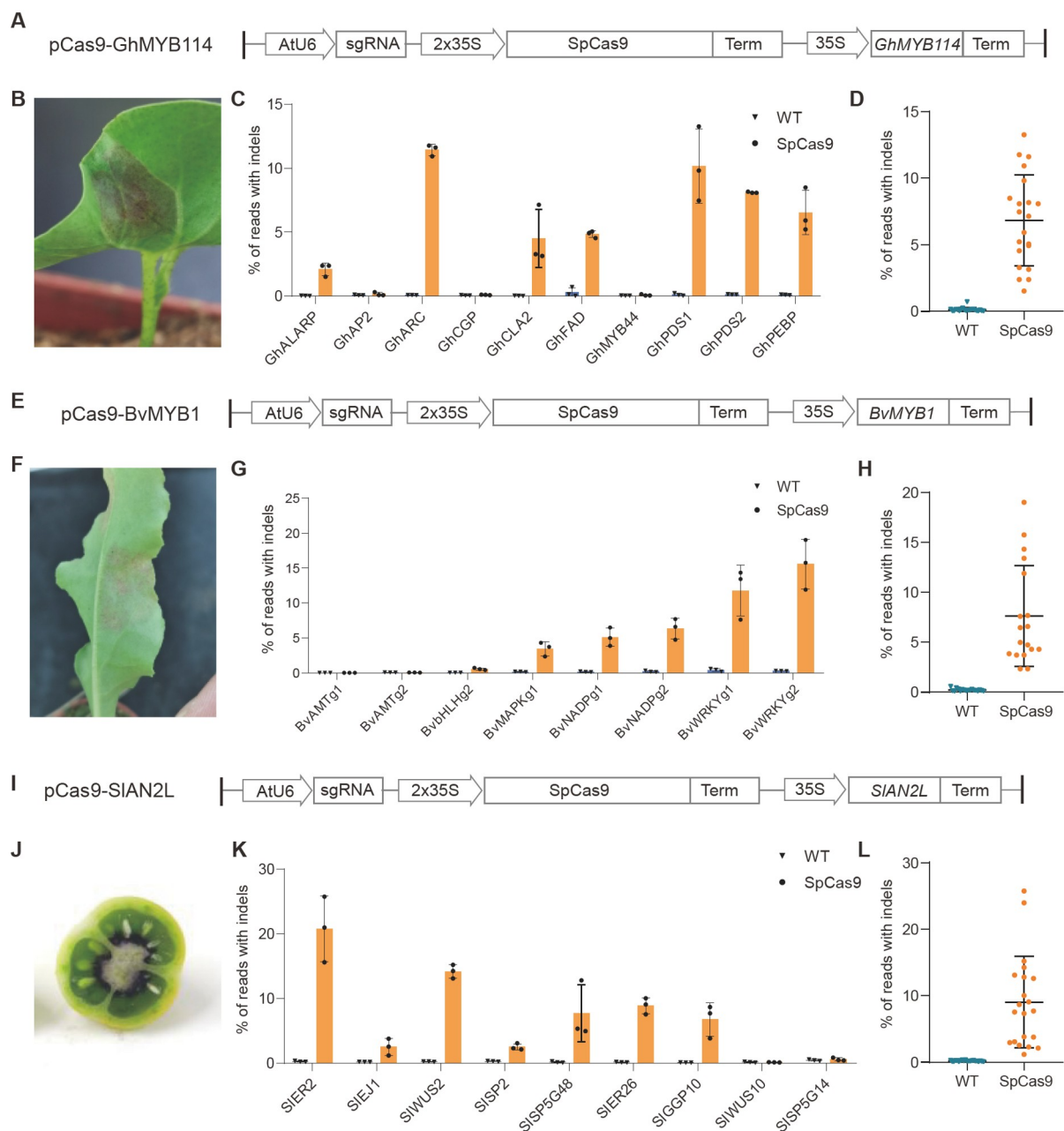


Figure 3 Genome editing in cotton leaves, sugar beet leaves and tomato fruits by SpCas9. (A) Schematic representation of the pCas9-GhMYB114 vector; (B) phenotype resulting from *Agrobacterium* infiltration with *GhMYB114* in cotton leaves; (C) frequencies of SpCas9 at ten endogenous genomic targets in cotton leaves; (D) average efficiency of SpCas9-mediated genome editing in cotton leaves; (E) schematic representation of the pCas9-BvMYB1 vector; (F) phenotype of *Agrobacterium* infiltration with *BvMYB1* in sugar beet leaves; (G) frequencies of SpCas9-mediated genome editing at eight endogenous genomic targets in sugar beet leaves; (H) average efficiency of SpCas9-mediated genome editing in sugar beet leaves; (I) schematic representation of the pCas9-SIAN2L vector; (J) phenotype resulting from *Agrobacterium* infiltration with *SIAN2-like* in tomato fruits; (K) frequencies of SpCas9-mediated editing at nine endogenous genomic targets in tomato fruits; (L) average efficiency of SpCas9-mediated genome editing in tomato fruits. An untreated fruit or leaf sample served as control in (C), (G) and (K). Frequencies (mean \pm S.D.) were calculated from three biological replicates ($n = 3$).

at a frequency of 0.9% at *SIARF7g6*. Single nucleotide substitutions frequencies at six targets ranged from 0.1% to 0.5%; at the other six target sites, prime editing efficiencies were below 0.1%.

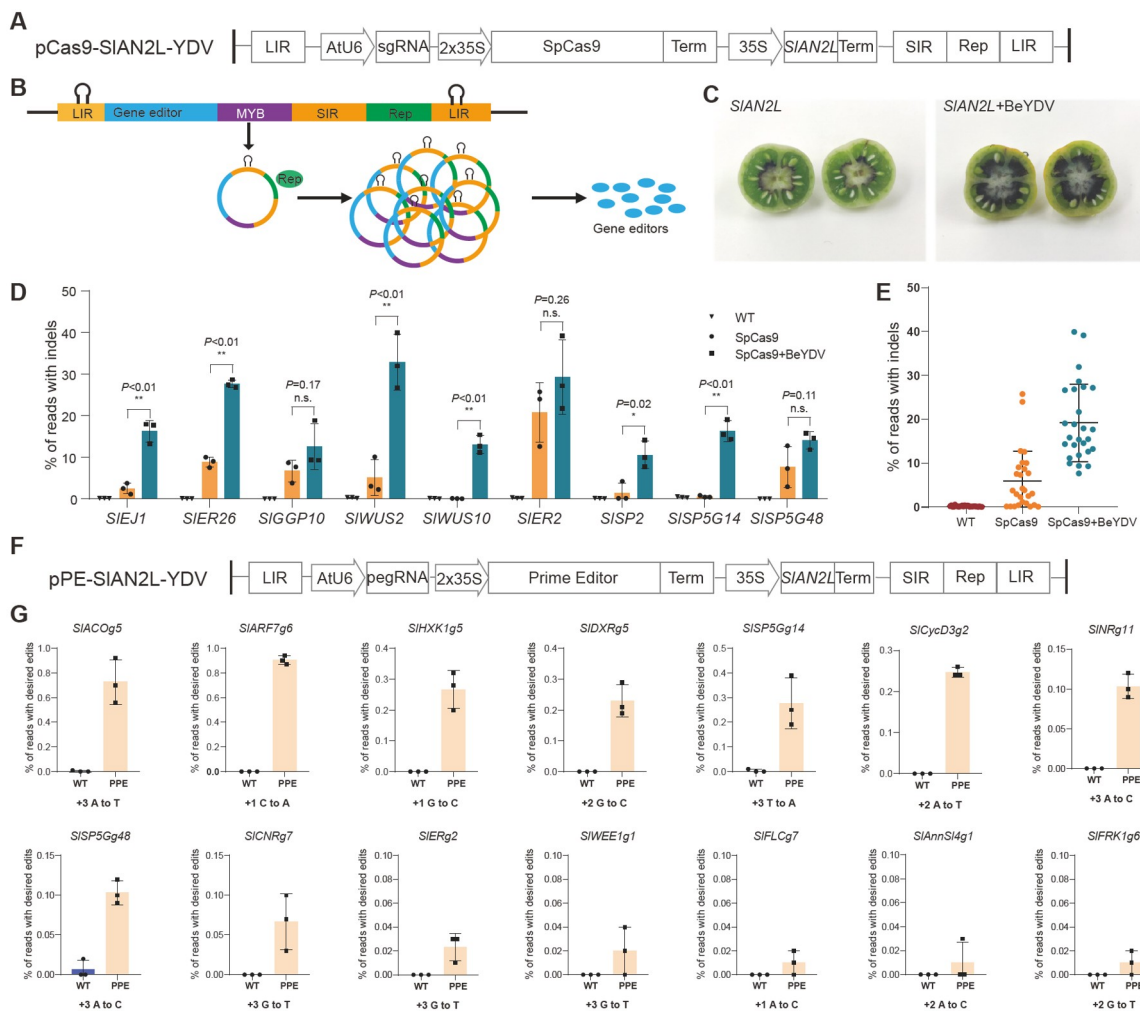


Figure 4 Genome editing in tomato fruits with addition of BeYDV. (A) Schematic representation of the pCas9-SIAN2LYDV vector; (B) diagram of geminivirus-based replicons used to enhance genome editing efficiency; (C) phenotypes resulting from *Agrobacterium* infiltration with SIAN2L and SIAN2L+BeYDV in tomato fruits; (D) frequencies of SpCas9 and SpCas9+BeYDV at nine endogenous genomic targets in tomato fruits. *P* values were obtained using two-tailed Student's *t*-tests. **P* < 0.05, ***P* < 0.01, n.s., *P* > 0.05. (E) Average efficiency of SpCas9 and SpCas9+BeYDV mediated genome editing in tomato fruits; (F) schematic representation of the pPE-SIAN2L-YDV vector; (G) frequencies of prime editing at 14 endogenous genomic targets in tomato fruits. An untreated fruit sample served as control in (D) and (G). Frequencies (mean ± S.D.) were calculated from three biological replicates (*n* = 3).

DISCUSSION

In this study, we demonstrate the use of anthocyanin-assisted *Agrobacterium* infiltration-induced transient expression of genome editing technologies as a rapid and facile approach to evaluate editing efficiencies across a variety of plant species and genomic sites. We achieved efficient genome editing in strawberry, tomato, cotton, and sugar beet, but this approach could be generalized to more plants in the future. The *Agrobacterium* infiltration transient expression technique has been proven to be applicable to many plants, such as kiwi fruit, apple fruit, *Medicago truncatula* leaves, and rose petals [40–42]. It was shown earlier that the transient expression of MYB in kiwi fruit and *Medicago truncatula* leaves by *Agrobacterium* infiltration

caused fruits and leaves to accumulate anthocyanins [43,44]. The expression patterns of MYB activators in vegetative and reproductive plant tissues are variable. For example, in nectarine (*Prunus persica*), *PpMYB10.1*, *PpMYB10.4*, and *PpMYB10.2* are positive regulators of anthocyanin accumulation in fruits, leaves and flowers, respectively [45]. Therefore, when performing *Agrobacterium*-dependent transient expression in different tissues, different MYB factors may be necessary.

As an efficient transient expression system to evaluate plant genome editing efficiency, anthocyanin-assisted *Agrobacterium* infiltration method provides a convenient alternative to protoplast transient expression system. Although MYB genes are the key transcription factor elements regulating anthocyanin production, anthocyanins synthesis involves multiple genes. Thus, its use is limited to cases where the anthocyanin biosynthetic pathway is complete and can be activated by one MYB factor. In addition, *Agrobacterium* infiltration is commonly used in dicotyledonous plants, with few reports in monocotyledonous plants. Thus, for the study of monocotyledonous plants, use of anthocyanin-assisted *Agrobacterium* infiltration method may be limiting.

Geminiviruses are widespread and have the ability to infect a wide variety of plant species such as wheat, maize, cotton, tomato, cucurbits, beans, legumes, fruits, ornamental plants, and common weeds. We leverage geminiviral replicons as a new method to enhance gene copy number and ultimately editor expression in target plant cells to increase editing efficiencies. Therefore, the combination of geminiviral replicons with the above mentioned anthocyanin-mediated *Agrobacterium*-infiltration enables the rapid, cheap, and efficient evaluation of genome editing technologies across a variety of plant species.

Genome editing is giving rise to precision plant breeding techniques and is set to revolutionize plant breeding [11]. As we all know, plant transformation and regeneration are labor-intensive and time-consuming process. Evaluation of genome editing efficiencies of the target sites before plant transformation helps to reduce workload and successfully obtain genome-edited plants. Especially for prime editing, the efficiency of prime editing is strongly affected by the design of the pegRNA [37]. The primer binding site and reverse transcriptase template of pegRNA need to be optimized for highly efficient prime editing. This new method will allow researchers to rapidly and easily evaluate target sites and expediting the development of genome-edited agricultural crops.

METHODS

Plasmid construction

The pCas9-FvMYB10 vector was modified from pHSE401 [46] by replacing HygR (hygromycin phosphotransferase) with *FvMYB10*. The pCas12a-FvMYB10, pA3A-FvMYB10 and pABE8e-FvMYB10 vectors were derived from pCas9-FvMYB10 by replacing SpCas9 with LbCas12a, SpCas9 (D10A)-APOBEC3A and SpCas9 (D10A)-TadA8e fusion protein sequences, respectively. The pCas9-GhMYB114, pCas9-BvMYB1 and pCas9-SIAN2L vectors were developed from pCas9-FvMYB10 by replacing *FvMYB10* with *GhMYB114*, *BvMYB1* and *SIAN2-Like*, respectively. pCas9-FvMYB10-YDV was generated from pCas9-FvMYB10 by inserting a geminivirus replicon into the T-DNA while pPE-FvMYB10-YDV was modified from pCas9-FvMYB10-YDV by replacing SpCas9 with SpCas9 (H840A)-M-MLV fusion protein sequences. A ClonExpress II One Step Cloning Kit (Vazyme) was used for fragment replacement. For PCR,

we used TransStart FastPfu DNA Polymerase (TransGen Biotech). All primers were synthesized at the Beijing Genomics Institute (see Supplementary Table S6). sgRNA target sites, sequences and primers for constructing binary vectors are listed in Tables S7 and S8 in Supplementary information.

***Agrobacterium* infiltration**

For *Agrobacterium* infiltration, we used strawberry and tomato fruits, cotton and sugar beet leaves. The criteria of selecting fruits or leaves are whether it is suitable for *Agrobacterium* infiltration and whether it can accumulate anthocyanins. Strawberry leaves are too thin for *Agrobacterium* infiltration, and tomato leaves do not accumulate anthocyanins after *Agrobacterium* infiltration. Thus, we used strawberry and tomato fruits for *Agrobacterium* infiltration.

Agrobacterium tumefaciens strain EHA105 containing the appropriate construct was grown at 28°C in Luria-Bertani liquid medium containing appropriate antibiotics. When the culture reached an optical density at 600 nm of approximately 1.0, the cells were harvested and resuspended at a final optical density at 600 nm of 0.6 in infection buffer (liquid MS medium containing 2.0% sucrose and 5.0 mg/L Acetosyringone) and shaken for two hours at room temperature, followed by use for infiltration into fruits or leaves by syringe.

Strawberry fruits ranging from “white” to “turning” stages were injected once and were left on the plants to continue growth at room temperature. The injections need to be very careful to minimize the wound damages to the strawberry fruits. Patches of red color appeared three to five days later. The fraction with the highest concentration of anthocyanin accumulation was selected. About 0.1 g of red tissue was sampled for DNA extraction five days after infiltration.

To infiltrate the tomato fruits, the *Agrobacterium* suspension was evenly injected throughout the entire green tomato fruits. These completely agroinfiltrated fruits were left on the plants to continue growth at room temperature. Anthocyanin accumulates mainly in the interior of the fruit three to five days later. The fraction with the highest concentration of anthocyanin accumulation was selected. About 0.1 g of red tissue was sampled for DNA extraction five days after infiltration.

Cotton cotyledons and first true leaves of sugar beet were best used for *Agrobacterium* infiltration. About 0.2 mL suspension was slowly infiltrated into cotton or sugar beet leaves. These agroinfiltrated leaves were maintained on the plants to continue growth at room temperature. Patches of red color appeared three to five days later. The fraction with the highest concentration of anthocyanin accumulation was selected. About 0.25 cm² of red tissue was sampled for DNA extraction five days after infiltration.

Amplicon deep sequencing and data analysis

Genomic DNA was extracted from red tissues and used as template. In the first round of PCR, the target region was amplified from the DNA with site-specific primers, and in the second round, forward and reverse barcodes were added to the ends of the PCR products for library construction (see Supplementary Tables S9 and S10). Equal amounts of PCR products were pooled and sequenced commercially (Novogene) using the NovaSeq platform, and the sgRNA target sites in the sequenced reads were examined for substitutions and indels. Amplicon sequencing was repeated three times for each target site using DNA extracted from three independent pieces of tissue.

Statistical analysis

GraphPad Prism 8 software was used to analyze the data. All numerical values are presented as mean \pm S.D.

Data availability

All data supporting the findings of the present study are available in the article and supplementary tables, or are available from the corresponding author on request. The amplicon deep sequencing data have been deposited in an NCBI BioProject database (accession No. PRJNA824659). All plasmids in this study will be available through Addgene. Other materials are available upon reasonable request.

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Author contributions

S.X., K.C., K.Z. and C.G. designed the project. S.X., Y.S., B.L. and H.L. performed the experiments. S.X., K.Z. and C.G. wrote the manuscript. C.G. supervised the project.

Conflict of interest

The authors declare no conflict of interest.

Supplementary information

The supporting information is available online at <https://doi.org/10.1360/nso/20220052>. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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