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Establishing a CRISPR–Cas-like immune system conferring DNA virus resistance in plants

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CRISPR–Cas (clustered, regularly interspaced short palindromic repeats–CRISPR-associated proteins) is an adaptive immune system in many archaea and bacteria that cleaves foreign DNA on the basis of sequence complementarity. Here, using the geminivirus, beet severe curly top virus (BSCTV), transient assays performed in *Nicotiana benthamiana* demonstrate that the sgRNA–Cas9 constructs inhibit virus accumulation and introduce mutations at the target sequences. Further, transgenic *Arabidopsis* and *N. benthamiana* plants overexpressing sgRNA–Cas9 are highly resistant to virus infection.

Many archaea and bacteria have adaptive immune systems in which CRISPR RNAs direct Cas endonucleases to cleave foreign nucleic acids on the basis of sequence complementarity[1]. In recent years, CRISPR–Cas-based systems have been developed as a powerful tool for genome editing. In the widely used CRISPR–Cas9 system, a single guide RNA (sgRNA) directs a Cas9 nuclease to make a sequence-specific double-stranded break (DSB) and modify the targeted DNA sequences[2]. Geminiviruses are circular single-stranded DNA (ssDNA) viruses that replicate within the nuclei of plant cells, causing serious damage to many dicotyledonous crop plants, including tomato, cassava, cotton, sugar beet and pepper[3,4]. During geminivirus replication, the ssDNA is converted to a double-stranded DNA (dsDNA) intermediate, from which new ssDNA is generated by rolling-circle replication[5]. Here, we sought to use CRISPR–Cas9 to specifically target the dsDNA of a geminivirus as a way of inhibiting virus replication and conferring virus resistance to host plants (Fig. 1a).

To test our approach, we employed the monopartite-genome geminivirus BSCTV and two of its hosts, *Arabidopsis thaliana* (Col-0) and *N. benthamiana*[6,7]. As BSCTV can be inoculated into both of these hosts by agroinoculation, a pCambia T-DNA carrying 1.8 copies of the BSCTV genome was used[8]. After *Agrobacterium*-mediated transformation of this pCambia–BSCTV construct into plants, the complete circular virus genome is generated by recombination. The BSCTV genome is small (2.9 kb) and encodes only seven proteins (Supplementary Fig. 1a). To identify highly efficient sgRNA–Cas9 target sites, we chose 43 candidate sites (Supplementary Fig. 1 and Table 1), designed sgRNA to target these sites and constructed 43 pHSN401 vectors[9], each containing Cas9 driven by a 2 × 35S promoter and one of the sgRNAs driven by an AtU6 promoter (Fig. 1a). All of the candidate sites are within one of three 300-nt regions in coding or non-coding sequences of the BSCTV genome (Supplementary Fig. 1 and Table 1).

We first transiently expressed sgRNA–Cas9 in the vectors of pHSN401–sgRNA together with pHSN401 vector (Cas9 alone) as control in *N. benthamiana* by agroinoculation. Two days later, we infected the same leaves with the pCambia–BSCTV construct also by agroinoculation (Supplementary Fig. 2). Ten days after that, typical BSCTV symptoms, such as leaf curling, were observed on infected control plants (Fig. 1b). Quantification of virus DNA by qPCR showed that all the sgRNA–Cas9 constructs inhibited virus accumulation in the injected (local) leaves to varying levels (Fig. 1b and Supplementary Table 2). Compared with the control vector, 38 of the 43 constructs reduced viral DNA by over 60%, and 20 constructs reduced it by over 80%. Because geminiviruses can be systemically transmitted within plants, symptoms were also observed in non-inoculated (systemic) leaves of the control plants (Fig. 1b). However, in plants containing the highly efficient sgRNA candidates, such as those targeting the A7, B7 and C3 sites, no severe leaf-curling symptoms were observed in systemic leaves, and virus accumulation in local leaves was reduced by 93, 90 and 97%, respectively (Fig. 1b and Supplementary Table 2). These results suggest that we have established an efficient system for screening suitable sgRNA target sites on the viral genome.

In the above experiment, viral infection was achieved by agroinoculation. Because plasmids are double-stranded DNA, it seemed possible that Cas9 might cleave the pCambia–BSCTV plasmid before it generated a single-stranded circular viral genome. To exclude this possibility, we developed a method to directly determine whether sgRNA–Cas9 could inhibit actively replicating virus (Fig. 1c). In this method, we selected the sgRNAs targeting the A7, B7 and C3 sites, which substantially inhibited virus accumulation in the targeting site screening assays (Fig. 1b). Five groups of constructs (experimental vectors) were tested: pHSN401–sgRNA (A7, B7 and C3), pHSN401 (Cas9 alone), pHSN401–A7/B7/C3%Cas9 (sgRNA alone), pHSN401–mA7/mB7/mC3 (mutated sgRNA) and pHSN401–A7/B7/C3–dCas9 (dead Cas9). The mock vector pHSN401%Cas9%sgRNA (without Cas9 and sgRNA) was used as the control (Supplementary Fig 3a), and we simultaneously injected *Agrobacteria* containing a given experimental vector and the control vector together with pCambia–BSCTV into individual 30-day-old *N. benthamiana* leaves (Fig. 1c). The pCambia–BSCTV construct was injected into the top part of the leaf and one of the experimental vectors and the control vector were injected into separate areas of the bottom part of the leaf (Fig. 1c). Because of virus movement, the BSCTV generated by the pCambia–BSCTV constructs in the top part of the leaf should be able to move to the bottom part and replicate. Therefore, if the BSCTV reaching that part of the leaf could be inactivated, this would show that the cutting acts on actively replicating virus rather than on incoming plasmids. Six days after injection, we harvested leaf punches from areas in the bottom of the leaves injected with *Agrobacteria* containing a given experimental vector or the control vector and quantified the virus in these areas. The qPCR results showed that virus accumulation was reduced by 65, 66 and 70% in the regions containing sgRNA–Cas9 targeting the A7, B7 and C3 sites (Fig. 1c), respectively, while virus accumulation in the regions containing the other experimental vectors remained at a level

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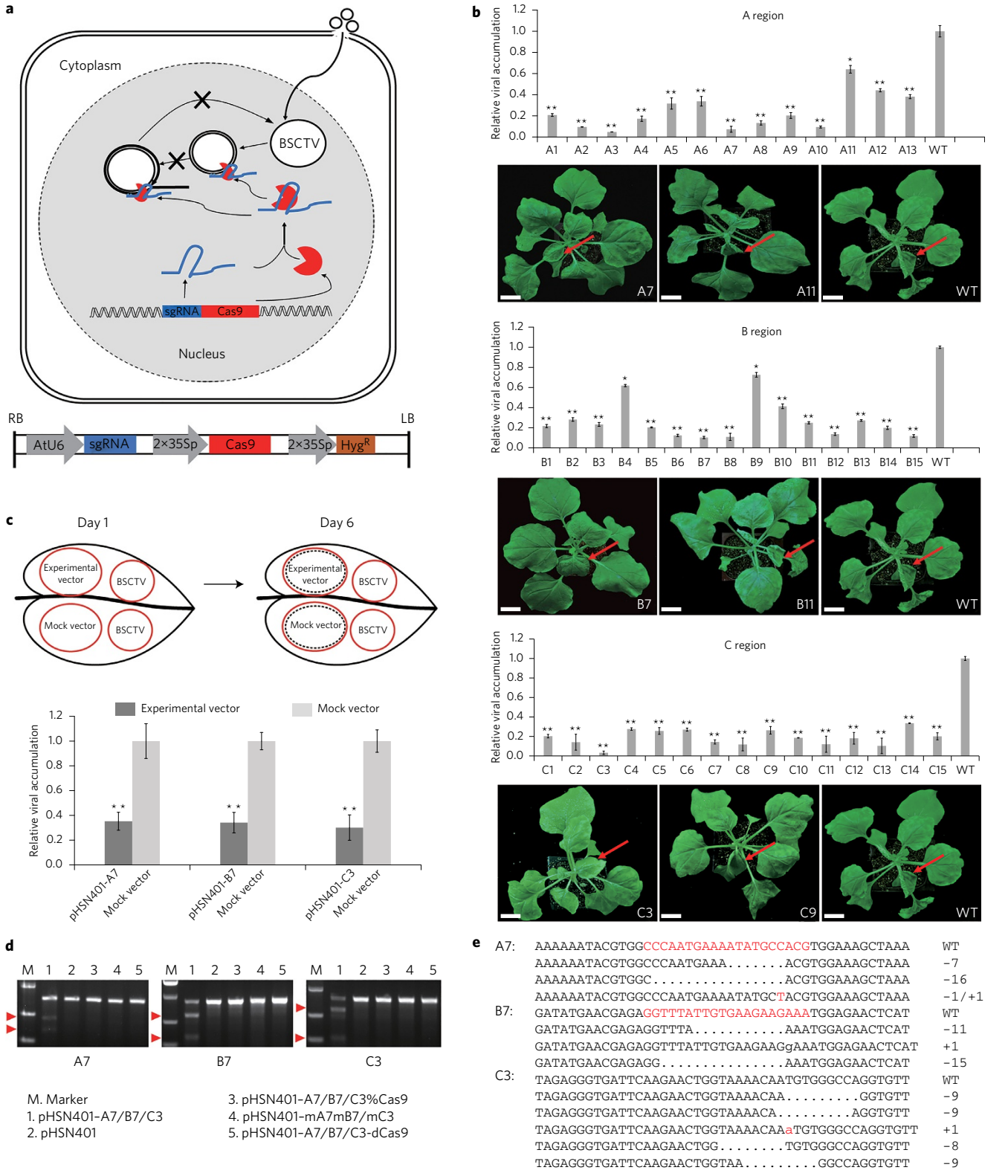

Figure 1 | Screening viral target sites and direct cutting of virus in *N. benthamiana*. **a**, The overview of sgRNA–Cas9-based sequence-specific system for conferring geminivirus resistance in plants. **b**, The activities of 43 sgRNAs targeting the BSCTV genome. Red arrows indicate the systemic leaves with different phenotypes after BSCTV infection. Each region has sgRNAs conferring strong resistance (left photograph) and others conferring mild resistance (middle photograph) compared with WT (right photograph). WT represents the pHSN401 vector. Scale bar, 2 cm. **c**, Confirmation that the sequence-specific sgRNA–Cas9 system cuts replicating virus. A leaf from a 30-day-old *N. benthamiana* plant was marked with four circular regions. The pCambia-BSCTV construct was injected into the top part of the leaf and one of the experimental vectors and the control vector were injected into separate areas of the bottom part of the leaf. Six days after injection, the DNA in the bottom regions (marked by dashed lines) was extracted and viral accumulation was measured by qPCR (as shown by the bar charts). Error bars represent s.d., asterisks indicate significance $^{*}P < 0.05$, $^{**}P < 0.01$. **d**, T7E1 assay detecting sgRNA–Cas9-induced mutations in the BSCTV genome. Red arrowheads indicate fragments generated by T7E1. **e**, sgRNA–Cas9-induced mutations at the targeting sites. –/+ indicates deletion/insertion of nucleotides.

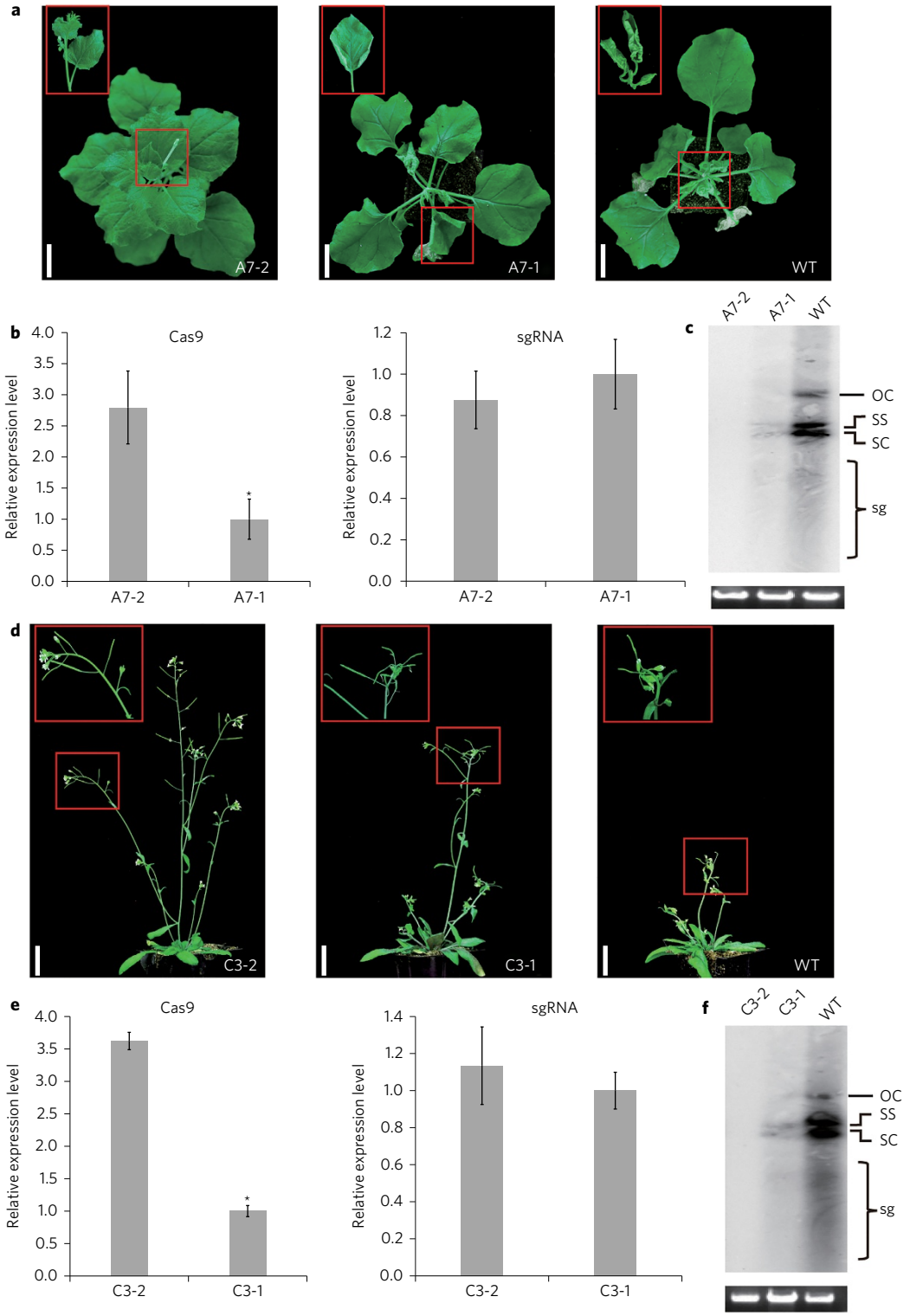

Figure 2 | Transgenic *N. benthamiana* and *Arabidopsis* plants develop resistance to BSCTV. a,**d**, The symptoms of transgenic and WT *N. benthamiana* (**a**), transgenic and WT *Arabidopsis* (**d**) after virus inoculation. Scale bar, 2 cm (**a**) and 3 cm (**d**). **b**,**e**, Expression levels of Cas9 and sgRNA in transgenic *N. benthamiana* (**b**) and transgenic *Arabidopsis* (**e**). Error bars represent s.d. $^{*}P < 0.05$. **c**,**f**, Virus accumulation in *N. benthamiana* (**c**) and *Arabidopsis* (**f**) assessed by Southern blots. OC, open circle double-stranded DNA; SS, single-stranded DNA; SC, supercoiled double-stranded DNA; sg, subgenomic forms of virus DNA.

similar to that obtained with the control vector (Supplementary Fig. 3b–e), indicating that cutting acts on actively replicating virus and that an active and complete CRISPR–Cas9 system is required.

We also speculated that DSBs in the BSCTV genome created by sgRNA–Cas9 would be repaired by non-homologous end joining, which can introduce small deletions or insertions (indels) at the break site. We therefore performed a T7E1 assay[10] using the same DNA samples from leaf patches infiltrated with the experimental vectors as described above. All three pHSN401-sgRNA vectors (A7, B7 and C3) generated indel mutations in the BSCTV genome, while the other experimental vectors did not (Fig. 1d). Most of the mutations within the target regions were small deletions of 1–10 bp (Fig. 1e).

Next, we examined the ability of sgRNA–Cas9 to inhibit BSCTV replication in stable transgenic plants. Using *Agrobacterium*-mediated transformation, we introduced pHSN401-A7 into *N. benthamiana*, and pHSN401-C3 into *Arabidopsis*.

First, two T1 transgenic *N. benthamiana* lines, A7-1 and A7-2, expressing different levels of Cas9 along with control non-transgenic plants, were selected and infected with the pCambia–BSCTV construct by agroinoculation as in the transient system (Supplementary Fig. 4). Twenty days later, we observed severe symptoms in the control plants. In particular, their shoot-tip leaves were severely curled and stunted (Fig. 2a). In the transgenic line (A7-1) with the lower level of Cas9 expression, one could see curled shoot-tip leaves, but the symptoms were mild (Fig. 2a,b), while in the transgenic line (A7-2) with the higher level of Cas9 expression, no obvious symptoms were evident (Fig. 2a,b). The sgRNA expression levels of these transgenic plants were similar (Fig. 2b). Southern blots with DNA extracted from shoot-tip leaves to measure BSCTV accumulation in these plants (Fig. 2c) showed that sgRNA–Cas9 indeed inhibited BSCTV replication in these plants, and the efficiency was correlated with the Cas9 expression level (Fig. 2b,c).

We also examined inhibition of BSCTV in two T2 transgenic *Arabidopsis* lines, C3-1 and C3-2. Twenty days after injection (Supplementary Fig. 4), severe symptoms, such as curling and stunted inflorescences, deformed floral structures and leaf curling were observed in the infected non-transgenic *Arabidopsis* plants (Fig. 2d). Southern blot analysis of DNA from the whole stem part showed that the extent of virus inhibition depended again on the Cas9 expression level (Fig. 2e,f). Our results demonstrate that over-expressing sgRNA–Cas9 specifically targeting sequences in the viral genome is an effective way to generate virus-resistant plants.

In conclusion, we have demonstrated that a CRISPR–Cas-like immune system directed against a geminivirus can be established in plants. By introducing sgRNA–Cas9, we generated geminivirus-resistant transgenic plants of both *Arabidopsis* and *N. benthamiana*. This sgRNA–Cas9 immune system could be used in other host plants to confer resistance to this and other DNA viruses. Moreover, by screening target sites, we showed that it is possible to identify efficient anti-virus sgRNAs that recognize sites in any region of the viral genome.

Methods

Plants materials. We used *Arabidopsis* ecotype Col-0 and *N. benthamiana* in this study. Plant growth conditions and the production of transgenic plants were performed following previous protocols[11,12].

Selection of sgRNA targets and construction of sgRNA–Cas9 vectors. Three regions (A–C) of about 300 nt were selected randomly (Supplementary Fig. 1a and Table 1). These sgRNA target sequences were inserted into pHSN401 vector[9] (Supplementary Table 1).

Screening highly active sgRNAs in *N. benthamiana* by transient assays. The *A. tumefaciens* strain EHA105 was used in these assays. The agroinoculation was performed following previous protocols[13]. Firstly, EAH105 containing all PHSN401–sgRNA vectors and pHSN401 vector were grown in LB medium with kanamycin and rifampicin. When A_{600nm} reached approximately 3.0, *Agrobacteria* were harvested by centrifugation and diluted in 10 mM $MgCl_2$ to a final A_{600nm} of 1.5. And two true leaves of 1-month old *N. benthamiana* plants with six to eight true leaves were chosen and injected with 1 ml of solution in each leaf using a 23-gauge needle. Two days later, EHA105 containing the pCambia1300-BSCTV with a final A_{600nm} of 0.5 were injected to the same two leaves with 1 ml of solution in each leaf.

BSCTV inoculation in transgenic *N. benthamiana* and *Arabidopsis*. The *A. tumefaciens* strain GV3101 and EHA105 were used in *Arabidopsis* and *N. benthamiana*, respectively. For transgenic *N. benthamiana* plants, the infiltration method was the same with transient assays. EAH105 containing pCambia1300–BSCTV with a final A_{600nm} of 0.2 were used for infiltration.

For transgenic *Arabidopsis* plants, GV3101 containing pCambia1300-BSCTV were grown at 28 °C in 2 ml LB solution with kanamycin and rifampicin to a final $A_{600\ nm}$ of 1.5. Then, GV3101 were harvested by centrifugation and suspended with 2 ml of LB medium. To infect the *Arabidopsis* plants, we cut the primary inflorescences when they were less than 1 cm in length, and injected the cut stems with the 1 ml of resuspended solution (Supplementary Fig. 4)[14].

RNA extraction, Cas9 and sgRNA expression level analysis. Total RNA was extracted with TRIzol (Invitrogen) then treated with Recombinant DNase I (Takara) and reverse transcribed into cDNA with SuperScript (Invirogen). To measure sgRNA levels, both oligo-dT and an sgRNA-specific primer were used (Supplementary Table 3). Cas9 and sgRNA expression levels were measured by qRT-PCR using Roche Lightcycler @480 SYBR Green I Master. *ACTIN7* in *Arabidopsis* and *PPR* (Pentatricopeptide repeat containing protein) in *N. benthamiana* were used as internal controls. The expression levels of Cas9 and sgRNA in A7-1 (*Arabidopsis*) and C3-1 (*N. benthamian*) were defined to be 1.0.

DNA extraction and quantification of BSCTV accumulation by qPCR and Southern blots. Genomic DNA of *N. benthamiana* and *Arabidopsis* plants was isolated with cetyl trimethyl ammonium bromide (CTAB) buffer. For qPCR assays, *PPR* was used as the internal control. For Southern blots, total DNA (2 μg) was separated by electrophoresis for 14 h in 0.8% agarose gels and transferred to a Hybond N^+ membrane. And a 227 bp fragment in BSCTV genome was used to generate [α-^{32}P]dCTP-labelled probes by a Prime-a-Gene labelling system. DNA accumulation was detected using a PhosphorImager.

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Author contributions

H.W.Z., C.X.G. and X.J. designed the experiments; X.J. performed most of the experiments; H.W.Z. and Y.Z. performed the virus-cutting experiments and mutation analysis; all authors analysed the data; C.X.G., X.J. and H.W.Z. wrote the manuscript. X.J. and Y.P.W. generated the figures. All authors approved the manuscript.

Additional information

Supplementary information is available online. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.G.

Competing interests

X.J., H.W.Z. and C.X.G. filed a patent application in China (priority filing with serial number 201510107492.9).