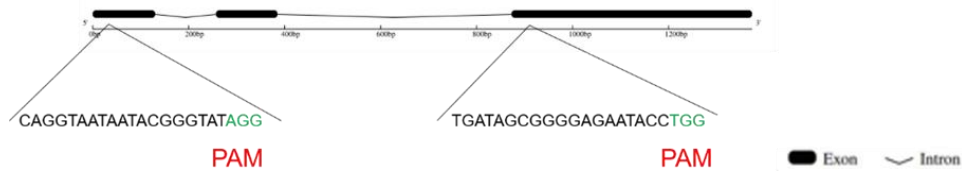


### (a) Design of CRISPR/Cas9 Gene Editing Target Sites



### (b) Primer dilution protocol

The primers for target site T1 (LsT1-F/LsT1-R) and T2 (LsT2-F/LsT2-R) were first diluted to a working concentration of 10  $\mu$ M. The reaction mixture was composed of 1  $\mu$ L of forward primer, 1  $\mu$ L of reverse primer, and 8  $\mu$ L of ddH<sub>2</sub>O. The thermal conditions were set as follows: incubation at 90°C for 30 sec, followed by natural cooling to room temperature.

### (c) Cut-and-link PCR reaction and multi-round PCR

Table 1 Cut-and-link PCR reaction system

Groups	Volumes
The primers diluted in the previous step	1 ul
sgRNA vector plasmid	1 ul
T4 DNA ligase Buffer	0.5 ul
T4 DNA ligase	0.5 ul
BsaI HF Buffer	1 ul
BsaI HF	0.5 ul
ddH <sub>2</sub> O	up to 10 ul

Reaction program: 37°C for 5 min and 20°C for 5 min, repeated for 5 cycles. During this process, the reactions for the two primer pairs were carried out separately.

Table 2 First round of PCR

Groups	Volumes
2×Phanta Flash Master Mix	10 ul
U-F (10 $\mu$ M)	2 ul
T1-R (10 $\mu$ M)	2 ul
The ligation product	1ul
ddH <sub>2</sub> O	up to 25 ul

The PCR product from this step was designated as u1.

Table 3 First round of PCR

Groups	Volumes
2×Phanta Flash Master Mix	10 ul
gRNA-R (10 μM)	2 ul
T1-F (10 μM)	2 ul
The ligation product	1 ul
ddH <sub>2</sub> O	up to 25 ul

The PCR product from this step was designated as **g1**.

Table 4 PCR reaction program

Temperature	Time	Cycle times
95°C	3min	} 32 cycles
95°C	15s	
56°C	15s	
72°C	1min	
72°C	5min	

For the **T2** target site, primers **U-F/T2-R** and **T2-F/gRNA-R** were used with the same PCR reaction system and program to generate the products **u2** and **g2**, respectively. The PCR products **u1**, **g1**, **u2**, and **g2** were examined by 1% agarose gel electrophoresis for quality control. Products that passed quality control were used for the **second-round PCR**.

Table 5 Second-round PCR

Groups	Volumes
2×Phanta Flash Master Mix	10 ul
B ' (10 μM)	2 ul
B2 (10 μM)	0.5 ul
u1+g1	1 ul
ddH <sub>2</sub> O	up to 25 ul

The PCR reaction program was the same as described in Table4. The PCR product from this step was designated as **6-T1**.

For the **T2** target site, primers **B2'** and **BL** were used with the same PCR reaction system and program to generate the products **6-T2**. The PCR products **6-T1** and **6-T2** were examined by 1% agarose gel electrophoresis for quality control.

#### (d) Purification of the overlapping extension products

An aliquot of 20 μL of the overlapping extension products was mixed thoroughly with 70 μL of ddH<sub>2</sub>O and 10 μL of 3 M sodium acetate (pH 5.2). Subsequently, 200 μL of anhydrous

ethanol pre-chilled to -20°C was added. After centrifugation, the supernatant was removed, and the pellet was washed once with 75% ethanol. Following another centrifugation to remove the supernatant, the pellet was air-dried and then dissolved in 15 µL of ddH<sub>2</sub>O.

**(e) The final digestion-ligation product**

Table 6 Cut-and-link PCR reaction system

Groups	Volumes
6-T1(10-20ng)	1 ul
6-T2(10-20ng)	1 ul
BsaI Buffer	1.5 ul
BsaI HF	0.5 ul
pYLCRISPR /Cas9P <sub>35S</sub> -H plasmid	1.5 ul
ddH <sub>2</sub> O	up to 10 ul
37°C, after 10 min of digestion, the following solutions were added	
T4 DNA ligase Buffer	0.5 ul
T4 DNA ligase	0.5 ul

Table 7 PCR reaction program

Temperature	Time	Cycle times
37°C	2min	15 cycles
10°C	3min	
20°C	5min	

**(f) PCR Detection of Positive Strains Harboring the Gene-Editing Vector**

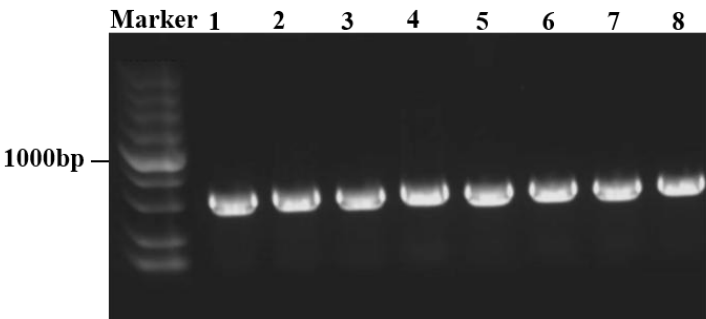


Figure Legend: PCR Detection of Positive Strains Harboring the Final Vector.

**(g) Identification of Positive Gene-Edited Strains**

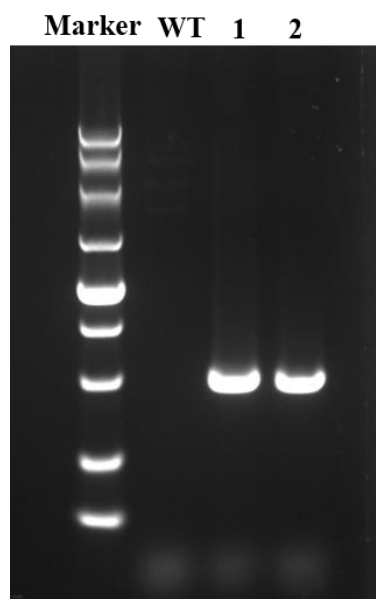


Figure Legend: DL5000 DNA marker; WT: wild-type; 1, 2: transgenic lines.

**Figure S1. Schematic diagram of the CRISPR/Cas9 vector construction workflow and detailed PCR conditions.**