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Construction and Verification of CRISPR/Cas9 Gene Editing Vector for Cassava *MeSSIII* Gene

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Abstract Starch glucan chain structure of cassava root is the key factor to determine starch quality. Soluble starch synthase III (SSIII) is the key enzyme to regulate the synthesis of long chain in plant amylopectin glucan. Cassava has two *MeSSIII* homologous genes *MeSSIII-1* and *MeSSIII-2*. To study the effect of cassava *MeSSIII* on the quality formation of cassava root starch, a double gene editing vector for *MeSSIII-1* and *MeSSIII-2* was constructed. The sgRNA target for *MeSSIII-1* and *MeSSIII-2* was designed simultaneously by online software CRISPR-Pv2.0 based on the conserved segments, and the recombinant pCAMBIAP1301-Cas9-MeSSIII-gRNA plasmid was constructed by digestion and ligation. The gene editing vector was transformed into LBA4404 Agrobacterium competent cells and used to infect the friable embryogenic callus of cassava, and the their DNA was extracted. The target segments of *MeSSIII-1* and *MeSSIII-2* were amplified by PCR for Sanger sequencing, and analyzed the editing of target position. The results showed that the target sites of *MeSSIII-1* and *MeSSIII-2* were successfully edited. This study helps to further obtain mutants of the *MeSSIII* gene to analyze the role of this gene in the cassava starch synthesis pathway.

Keywords Cassava (*Manihot esculenta Crantz*); Starch synthase; *MeSSIII*; CRISPR

Cassava (*Manihot esculenta* Crantz) is a tropical and sub-tropical tuberous cash crop with starch as 85% of the dry matter in the tuber and is the sixth most important food crop in the world after wheat, rice, corn, potatoes and barley (da Silva et al., 2017). Cassava starch has many special physicochemical properties, such as high viscosity, transparency, and strong stability of frozen and melt. Therefore, cassava starch is a good industrial raw material, which has been widely used in food industry, light industry, renewable resources, and other industries (Kiatkamjornwong et al., 2000; Ihemere et al., 2006). The structure of cassava starch, like other plant starch, is composed of two dextran polymers, which can be divided into two categories: amylose and amylopectin. The ratio of amylose to amylopectin is an important factor to determine the physicochemical properties of starch. Amylopectin has higher temperature gelatinization and viscosity. Amylose has better resistance to swelling and flexibility. Starch biosynthesis occurs in amyloid after a series of synergetic effects of starch synthesis-related enzymes, including adenine glucosamine pyrophosphorylase (AGPase), granule-bound amylase (GBSS), soluble amylase (SS), amylase (SBE), desorylase (DBE), and amylase phosphorylase (Pho1) (Jeon et al., 2010). SS catalyzes the transfer of glycosyl ADP- glucose to the non-reducing end of the dextran chain and participates in the extension of the amylopectin dextran chain (Hirose and Terao, 2004). Cassava has 5 SS isozymes, contains MeSSI, MeSSII, MeSSIII, MeSSIV, and MeSSV. MeSSIII is encoded by two homologous genes *MeSSIII-1* and *MeSSIII-2*.

At present, the mechanism of complex formed by starch synthesis-related enzymes regulating tapioca root starch synthesis is unclear. Analysis of the mechanism of cassava starch synthesis by using CRISPR/Cas9 technology to create mutants related to starch synthesis pathway is an effective means to improve starch quality. This study used

CRISPR/Cas9 technology to construct two encoded gene *MeSSIII-1* and *MeSSIII-2* dual mutant vectors of cassava soluble starch synthase SSIII, and verified the editing effect of the vectors. It is helpful to further explore the function of SSIII regulating cassava starch synthesis.

1 Results and Analysis

1.1 Design of gene editing targets

Target sequence CTCAAATCTGAGAGAAAGGA (CG40%), that can be edited simultaneously *MeSSIII-1* and *MeSSIII-2* was designed by online software CRISPR-P 2.0 based on the cassava *MeSSIII-1* and *MeSSIII-2* sequences. *MeSSIII-1* target location is 1 541~1 520 bp (20 bp), PAM sequence is CGG. Meanwhile, *MeSSIII-2* target location is 1 511~1 520 bp (19 bp), PAM sequence is GGG (Figure 1). Analysis of the sgRNA secondary structure, the prediction results showed that the target sequence secondary structure is relatively loose, which is conducive to binding the target position (Figure 2).

1.2 Construction of pCAMBIA1301-Cas9-MeSSIII-gRNA gene editing vector

Nuclear localization signals were added on both sides of the Cas9 protein of the pCAMBIA1301-Cas9-gRNA gene editing vector and expressed by 35S promoters. sgRNA expression driven by Arabidopsis AtU6-26 promoter. The vector was linearized by Bsa I enzyme digestion, and the linearized vector was connected with the annealed target primer to clone the target site sequence to the upstream position of the sgRNA. The ligation products were transferred to escherichia coli receptive cells to screen positive clones. The results showed that the PCR amplified the bands about 350 bp, which was consistent with the expected target fragment size (Figure 3; Figure 4). The gene editing target sequence has been successfully constructed onto the gene editing vector by Sanger sequencing. The new vector is named pCAMBIA1301-Cas9-MeSSIII-gRNA, saved to-20℃ standby.

1.3 Gene editing vector transformation LBA4404 *Agrobacterium* receptive cells

The gene editing vector pCAMBIA1301-Cas9-MeSSIII-gRNA plasmid was transformed LBA4404 *Agrobacterium* receptive cells by freeze-thaw method .5 single colonies were selected for bacterial liquid PCR detection and positive clones were screened. The results showed that the amplified obtained fragments were about 350 bp, consistent with the expected fragment size, which is indicating that the plasmid pCAMBIA1301-Cas9-MeSSIII-gRNA gene editing vector was successfully transformed into LBA4404 *Agrobacterium* (Figure 5).

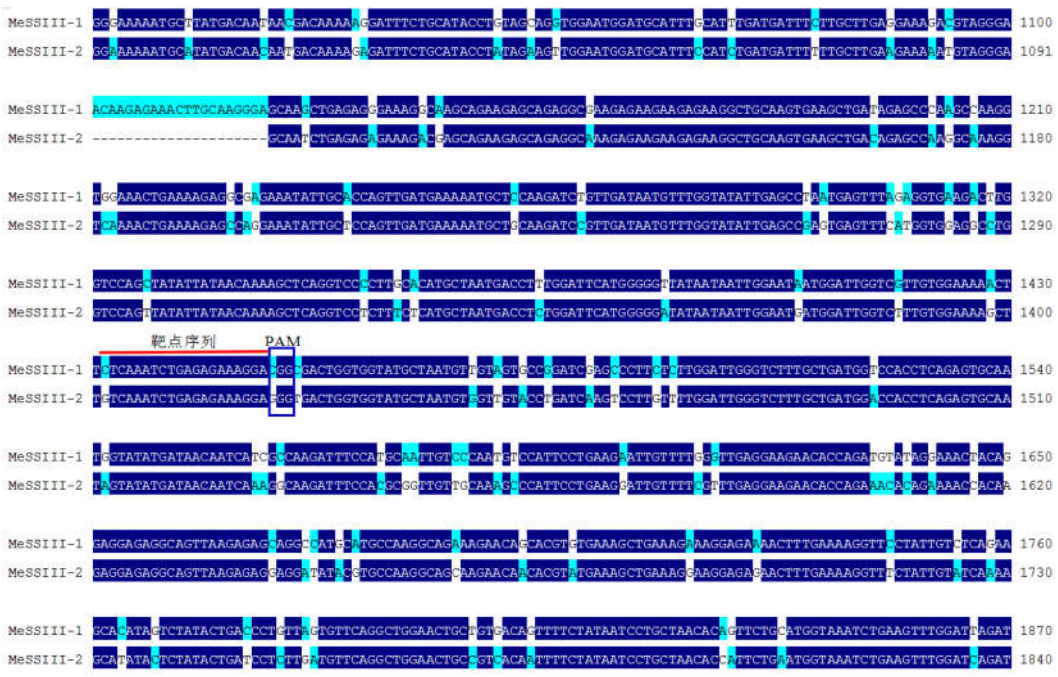

Figure 1 Location of gene editing targets

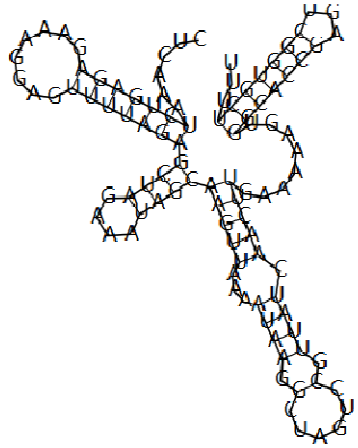

Figure 2 Predicted secondary structure of sgRNA

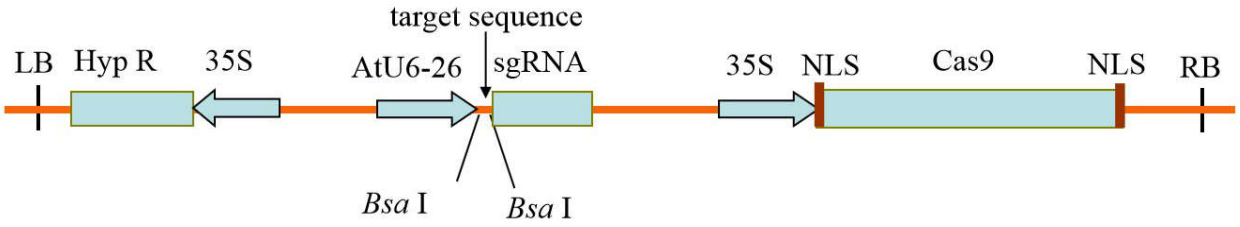

Figure 3 T-DNA structure diagram of vector

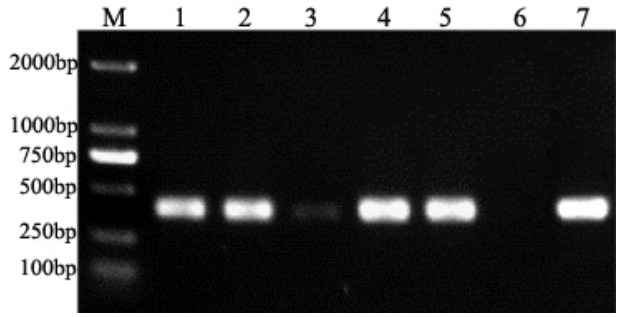

Figure 4 PCR test results of recombinant plasmid

Note: M: DL2000 DNA Marker; 1~5: 5 monoclonals; 6: Negative control; 7: Positive control

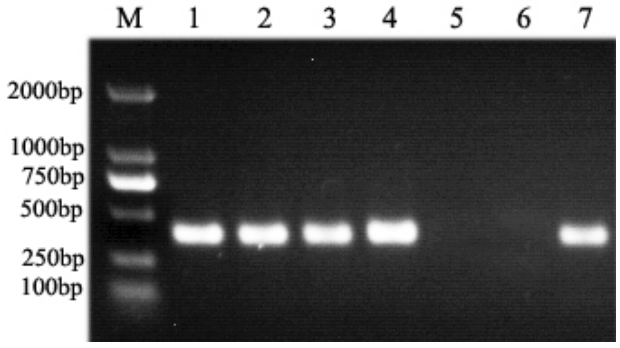

Figure 5 Screening for positive clones by PCR

Note: M: DL2000 DNA Marker; 1~5: 5 monoclonals; 6: Negative control; 7: Positive control

1.4 Validations of transformed cassava friable embryogenic calli and editing effect

The receptor material of cassava genetic transformation was obtained by inducing axillary bud, somatic embryo, and friable embryogenic calli with the tissue culture seedling of Manihot esculenta Crantz.cv.M.SC8. *Agrobacterium* infection was used to transform pCAMBIA1301-Cas9-MeSSIII-gRNA vector into cassava friable embryogenic calli (Figure 6).

Using DNA of Agrobacterium tumefaciens-infected and uninfected cassava friable embryogenic calli as templates, fragments of *MeSSIII-1* and *MeSSIII-2* gene editing target regions were amplified with PCR. PCR fragments were sequenced by Sanger sequencing. Analysis of sequencing peak degree changes to identify whether the target gene was edited successfully. The results showed that many peaks appeared near the PAM of the sequencing peaks of *MeSSIII-1* and *MeSSIII-2* gene fragments in the transformed cassava friable embryogenic calli, and were no peaks in the untransformed samples (Figure 7). It indicted that the gene editing vector pCAMBIA1301-Cas9-MeSSIII-gRNA we constructed can edit both *MeSSIII-1* and *MeSSIII-2*.

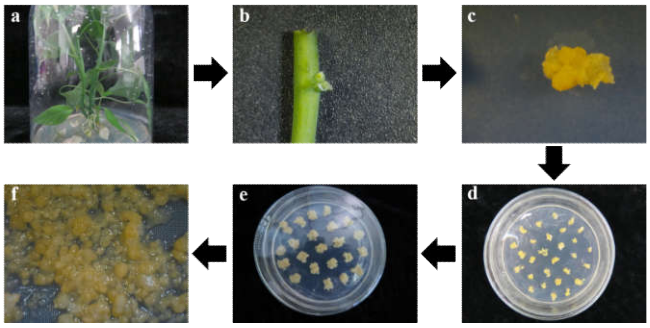

Figure 6 Induction and infection process of cassava friable embryogenic calli

Note: A: Sterile seedlings; B: Swelling of lateral buds on the stem segment; C: Somatic embryos induced by lateral buds; D: Somatic embryos of expanded culture; E: Friable embryogenic calli; F: Transformed friable embryogenic calli

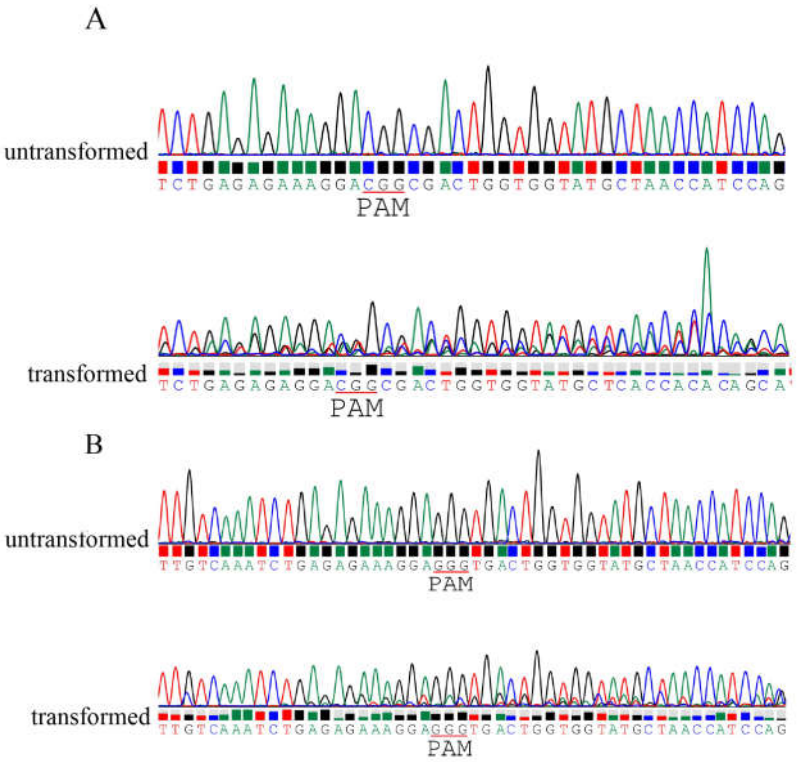

Figure 7 Analysis of gene editing effects

Note: A: Peak map of MeSSIII-1 sequencing; B: A: Peak map of MeSSIII-2 sequencing

2 Discussion

Plant mutants are important materials for basic research of functional genomics and molecular design breeding. Conventional plant mutants are derived from spontaneous mutations or chemical, physical, and biological mutagenesis, with great randomness and limitations, which cannot meet the requirements for large-scale functional genomics research and molecular design breeding. In recent years, CRISPR/Cas9 gene editing technology has been developed rapidly, which can conveniently, quickly and efficiently edit and knock out plant target genes and create plant mutants (Liao et al., 2016; Chen et al., 2019). The mutants obtained by RISPR/Cas9 gene editing can be self-cross-bred or cross-bred, and non-transgenic plants with homozygous mutations can be screened from the offspring (Wang et al., 2019). CRISPR/Cas9 technology was used to edit genes related to starch synthesis pathway. Studies on improving starch quality of crops mainly focused on GBSS and SBE genes, and there were no editing reports on *SS* genes. Prez et al. (2019) successfully mutated the *Waxy* (*Wx*) gene of starch synthase I (GBSSI) by using CRISPR/Cas9 technology, the amylose content in heterozygous seeds decreased to 8%~12%, the amylose content in pure seeds decreased to 5%, and the activity of AGPase and sucrose synthase in endosperm was also affected. Yang et al. (2020) edited *Wx* gene of early rice variety "Zhongzao 35" (24.6% amylose content), and obtained a mutant with greatly reduced amylose content (4.9%~12.2% amylose content). Wang et al. (2019) edited the granule bound starch synthase I (*IbGBSSI*) gene and starch branching enzyme II (*IbSBEII*) gene of sweet potato starch biosynthesis pathway. The results showed that the amylose content in the tuber of *IbGBSSI* mutant was decreased, while that of *IbSBEII* mutant was increased. Finally, the physical and chemical properties of sweet potato starch were changed. It was proved that the key gene of starch synthesis pathway can change the quality of sweet potato starch. Bull et al. (2018) edited cassava amylose biosynthesis gene *ptst1* and granule bound starch synthase gene *GBSS* by CRISPR/Cas9 technology. The results showed that amylose content decreased or eliminated, and changed the physical and chemical properties of cassava starch and expanded its industrial utilization scope.

At present, reports on the functional analysis of soluble starch synthase SSIIII mainly focus on *Arabidopsis thaliana* and starch crops such as *Oryza sativa* L., *Zea mays* L. and *Solanum tuberosum*. Rice *OsSSIIIa* gene is mainly expressed in endosperm. The amylopectin long chain (DP 35-50) decreased, the short chain (DP 10-20) increased, the content of amylose and resistant starch increased, the peak viscosity decreased, the gelatinization temperature increased, and the total lipid content of starch increased 2~3 times. *OsSSIIIb* gene is mainly expressed in rice leaves, but its function has not been reported (Zhou et al., 2016). With the loss of SSI3 activity in Maize, the amylose content increased, the amylose content decreased, the amylopectin long chain (DP 36-56) decreased, the starch granule became smaller, the total starch content decreased slightly, and the enthalpy change ΔH of starch gelatinization process decreased (Zhu et al., 2016; Lin et al., 2012). The decrease of SSSIII activity resulted in the decrease of amylopectin content and long chain (DP 25-35), the formation of cracks in starch grains and the increase of peak viscosity (Nazarian-Firouzabadi and Visser, 2017). Arabidopsis SSIII negatively regulated the total activity of starch synthase in leaves. In AtSSIII mutants, the content of starch temporarily increased, the content of starch phosphate increased, and the chain length distribution of amylopectin changed: DP 5-10 chain increased, DP14-20 chain decreased, DP26-37 chain increased, and longer DP43-46 chain decreased (Zhang et al., 2005). Therefore, SSIII is involved in the synthesis of amylopectin long chains, affecting starch grain morphology, amylopectin/amylopectin ratio, phosphate content, and finally affecting the physical and chemical properties of starch. However, when SSIIII activity decreased in different plants, the change direction of physicochemical properties of starch was different. For example, the peak viscosity of rice starch decreased, while that of potato starch increased. The results showed that SSIIII had different functions in regulating starch quality formation among different starch crops. In this study, CRISPR/Cas9 technology was used to construct the double editing vector of two homologous genes of cassava soluble starch synthase *MeSSIII*. It was proved that the vector could edit *MeSSIII-1* and *MeSSIII-2* at the same time by transforming friable embryogenic callus. This study will help to obtain the *MeSSIII* gene mutant in order to deeply analyze the role of this gene in cassava starch synthesis pathway.

3 Materials and Methods

3.1 Experimental materials

The competent cells of *E.coli* DH5 α and LBA4404 *Agrobacterium* were purchased from Haikou Lvhengyuan Biotechnology Co., Ltd. pCAMBIA1301-Cas9-gRNA plasmid was preserved in our laboratory. T4 DNA ligase and *Bsa* I-HFR v2 restriction enzymes were purchased from New England Biolabs. The plasmid extraction kit was purchased from Shanghai Sangon Biological Engineering Co., Ltd. The plant DNA extraction kit was purchased from Chengdu Fuji Biology Co., Ltd. The DNA polymerase Ex *Taq* kit was purchased from TaKaRa.

3.2 Design and anneal of target primers

According to the CDS sequences of *MeSSIII-1* (Manes.02G082800) and *MeSSIII-2* (Manes.S044400) of cassava soluble starch synthase gene in cassava genome database (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Mesculenta), the potential targets of MeSSIII-1 and *MeSSIII-2* was analyzed using the online gene editing target design software CRISPR-P 2.0 (crispr.hzau.edu.cn/CRISPR2.0/). And the target that can edit two genes at the same time is selected for gene editing. Target primers MeSSIII sgRNA-F: GATTGCTTATTGCGCGG GCATACCG and MeSSIII-1-sgRNA-F: AAACCGGTATGCCCGCGCAATAAGC were synthesized in Shanghai Sangon Biological Engineering Co., Ltd. The upstream and downstream primers of each target were diluted with ddH2O to 10 μmol/L. The diluted upstream and downstream primers were placed in a water bath at 98°C for 3 min, cooled naturally to room temperature, transferred to 16°C for 10 min, which was used for gene editing vector construction.

3.3 Construction of gene editing vector

The reaction system of *Bsa* I-HFv2 endonucase and pCAMBIA1301-Cas9-gRNA vector was placed at 37℃ and digested for 4 h. Electrophoresis was used to detect the enzymatic digestion effect, then the enzyme fragment was purified and its concentration was determined, and stored at -20℃ for later use. T4 DNA ligase produced by NEB company was used to connect the annealed target sequence with the recovered vector at 16℃ overnight, and transformed into *E. coli*, and then cultured in LB solid medium containing kanamycin. Five single colonies were selected and cultured in liquid LB medium for 8 h. Cas9 vector primer F: GCAAGCTGCTCTAGCCAATACGC and downstream primer Messiii-Sgrna-R were used for identification of bacterial liquid PCR, and the PCR products with a band size of about 350 bp were sent to Shanghai Sangon Biological Engineering Co., Ltd. for sequencing. The recombinant plasmid correctly sequenced was named pCAMBIAP1301-Cas9-MeSSIII-gRNA. The gene editing vector was transformed into LBA4404 *Agrobacterium* by freeze-thaw method, and colony PCR was used to screen positive clones. The positive clones obtained by screening were stored at -80℃ for later use.

3.4 Induction and infection of friable embryogenic callus in cassava

1 cm stem segments with lateral buds were cut from the tissue culture seedlings of cassava and cultured in CAM dark for 4 d. The expanded axillary buds were cut off and transferred to the somatic embryo induction medium (CIM). The somatic embryos were induced by dark culture at 28℃ for 16~18 d. The somatic embryos were selected and cultured in the new CIM medium for 10~14 days at 28℃ for cyclic culture. Somatic embryos were picked up with a needle, transferred to GD medium, and cultured at 28℃ for 21 days to induce friable embryonal callus. The friable embryogenic calli were transferred to a new GD medium, and then cultured in dark at 28℃ for 21 d. After three cycles of culture, they were used for *Agrobacterium* infection. The friable embryogenic callus of cassava was infected by LBA4404 *Agrobacterium* containing pCAMBIAP1301-Cas9-MeSSIII-gRNA plasmid and co-cultured on GD solid medium for 4 d. They were washed with liquid GD medium containing Carbenicillin for 3 times and sterile water for 2 times, and then transferred to solid GD medium containing Carbenicillin for 12 d to detect the effect of gene editing.

3.5 Identification of gene editing effects of *MeSSIII-1* and *MeSSIII-2*

NCBI online primer design software was used to design amplification primers for editing target regions of *MeSSII-1* and *MeSSIII-2* genes. The amplification primer of *MeSSIII-1* is MeSSIII-1-F: GGTCCCCTTGCAC

ATGCTAAT and MeSSIII-1-R: AGTATGCCAAGTTTATCAGCACC. The amplification primer of *MeSSIII-2* is MeSSIII-2-F: TTCTCATGCTAATGACCTCTGGA and MeSSIII-2-R: GCCAAGCTTAAGGTGCCAATC. Plant DNA extraction kit was used to extract DNA from cassava friable embryogenic calli infected and uninfected by *Agrobacterium*. The target region fragments of *MeSSIII-1* and *MeSSIII-2* genes were amplified by Ex Taq kit PCR. The PCR fragments were sent to Shanghai Sangon Biological Engineering Co., Ltd. for Sanger sequencing, and the sequencing peak map was analyzed.

Authors' contributions

LZ and WYJ designed and carried out the study, completed data analysis, and drafted the manuscript. LXH participated in the carrier construction. LRM and LJ participated in the induction of somatic embryo and friable callus of cassava. FSP and HXW participated in the experimental design and analysis of the experimental results. GJC and YY were the experimental project leaders and designers, who guided the experimental design, data analysis, draft, and revision. All authors read and approved the final manuscript.

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