

Research Article

A New Reference Plasmid "pGMT27" Provides an Efficient Transgenic Detection Method for Flue-Cured Tobacco

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Owing to the economic value of its foliage, tobacco (*Nicotiana tabacum*) is cultivated all across the world. For the detection of genetically modified (GM) tobacco, there is a lack of universal standard material which ultimately limits the detection methods because the accuracy and comparability of the results cannot be ensured. Here, we prepared a reference plasmid "pGMT27" for the detection of GM tobacco, which was 18,296 bp in length harboring two of the tobacco endogenous and seven exogenous genes. By using qualitative PCR test for the nine genes, 10 copies were used for plasmid sensitivity. In the quantitative real-time PCR (qPCR) assays with pGMT27 as a calibrator, the reaction efficiencies for *P-35S* and *NR* were 101.427% and 98.036%, respectively, whereas the limit of detection (LOD) and limit of quantification (LOQ) were 5 copies and 10 copies per reaction. For standard deviation (SD) and relative standard deviation (RSD) of the Ct values, the repeatability values were from 0.04 to 0.42 and from 0.18% to 1.29%, respectively; and the reproducibility values were from 0.04 to 0.39 and from 0.18% to 1.14%, respectively. For the unknown sample test, the average conversion factor (Cf) was 0.39, and the accuracy bias was from -15.55% to 1.93%; for precision, the SD values ranged from 0.02 to 0.62, while RSD values were from 1.34% to 10.6%. We concluded that using the pGMT27 plasmid as a calibrator provided a highly efficient transgenic detection method for flue-cured tobacco.

1. Introduction

Tobacco is one of the most widely cultivated nonfood crops and has emerged as an extensively investigated model plant with the accelerated development of molecular biology research. Attributable to its agronomic importance, tobacco is grown in over 125 countries for its foliage, mainly consumed as cigarettes, cigars, and snuff. According to the FAO (Food and Agriculture Organization) data, approximately 4 million hectares of lands are used for cultivating tobacco, one-third of which is in China alone [1]. As smoking is health related [2–4], consumers appear to have more cautious attitudes to tobacco than other crops and GM tobacco, as well as other GM crops. Though applications from biotechnology to tobacco products have been limited, unintentional release of GM tobacco seeds and leaves of nonpermissible sort to the market is possible [5]. Now, GM composition detection in tobacco is becoming an international trade barrier for monitoring the presence of adventitious GM tobacco. So, when entering into the market, it is indispensable for the tobacco companies to enhance controls on import and export of the tobacco product. In many countries, respecting public's right to information and education, the GMO threshold levels of food and feed products have already been established [6–8], Consequently, to ensure the accurate and comparable measurements, selection of standard material for GMO detection becomes critical [9]. The standard reference material includes matrix and plasmid. Plasmid has

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many advantages, such as ease of availability, straightforward manipulation, repeatable production, and low cost [10]. Some plasmids as calibrators have been used for transgenic soybean, maize, cotton, and other crops, including ERM-AD413, ERM-AD415, ERM-AD427, and ERM-AD425. They are also used to calibrate the transgenic maize MON810, NK603, 98140, and transgenic soybean 356043 [11].

However, the lack of standard material has limited the GM tobacco detection methods. Up until now, no standard material as the calibrator for detecting GM tobacco has been found, and no corresponding products have been reported. In the tobacco trade market, tobacco is usually sold in the form of tobacco product, including flue-cured tobacco, redried leaf tobacco, and cigarettes. Here, we reported a plasmid for the GM tobacco test, which was designated as pGMT27. It was constructed based on plant expression vector pBI121. pGMT27 contained two tobacco endogenous genes (actin and NR), genetic elements of the P-35S promoter, T-NOS terminators, selective marker genes of NPT II, HPT, Bar, and aadA, and GUS reporter gene. These seven exogenous genes could make the screening rate of transgenic tobacco reach 100% theoretically in our previous screening strategy [12]. Accordingly, a qualitative PCR detection method for detecting the nine targets was developed. Furthermore, a qPCR assay for P-35S and NR was also developed by using plasmid DNA as the calibrator. Thereby, we concluded that using the pGMT27 plasmid as a calibrator provided a highly efficient transgenic detection method for flue-cured tobacco.

2. Materials and Methods

2.1. Flue-Cured Tobacco and Genomic DNA Isolation. Transgenic flue-cured tobacco tested by the FAPAS (Food Analysis Performance Assessment Scheme) and CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) proficiency test in 2014 was defined as 100% positive GM with *P-35S* and *T-NOS*. Nontransgenic fluecured tobacco was collected in our laboratory. All materials were leaves' flour. Plant genome extraction kit (TIANGEN Biotech, China) was employed to extract DNA according to the manufacturer's protocol. Total DNA quantification was performed using a NanoDrop ND-2000 Spectrophotometer (NanoDrop Technologies, USA), OD260/OD280 \approx 1.80–1.90, and it was diluted to a 100 ng/µL working stock with sterile distilled water, which was stored at -80° C.

2.2. Construction of the pGMT27 Plasmid. According to the screening strategy previously reported [12], we synthesized a 3,818 bp fragment of HPT + Bar + aadA + actin + NR junction and subcloned it into pMD18-T simple vector by restriction enzyme sites, EcoR I and Sac I. Furthermore, the full-length fragment of five targets was digested with EcoR I/ Sac I and cloned into the plant expression vector pBI121 instead of "T-NOS" with the same enzyme sites, so as to create the recombinant pGMT27 plasmid, which includes nine target DNA fragments consisting of seven exogenous genes and two endogenous. Then, pGMT27 plasmid was isolated and purified by using TIANGEN Plasmid Miniprep Kit (TIANGEN Biotech, China) according to the manufacturer's guidelines. Afterwards, it was identified by Sac II and EcoR I restriction digests and DNA sequencing (Shanghai Generay, China). Total DNA quantification was performed on a Qubit[®] 2.0 Fluorometer (Invitrogen Life Technologies, USA) by using the Qubit[®] dsDNA BR Assay Kit (Invitrogen Life Technologies, USA) following the manufacturer's directions. The copy numbers were calculated on the basis of pGMT27 plasmid size (18,296 bp) by using Avogadro's constant (6.023×10^{23}). The plasmid DNA molecular weight (660 g/mol/dp) was calculated with formula (A) proposed by Lee et al. [13].

Formula (A): DNA copies =
$$\frac{6.02 \times 10^{23} (\text{copy/mol}) \times \text{DNA amount (g)}}{\text{DNA length (dp)} \times 660 (g/\text{mol/dp})}.$$
(1)

2.3. Qualitative PCR Assay for the pGMT27 Plasmid. For the LOD test of the qualitative PCR, the plasmid copy number was prepared by $0.1 \times \text{TE}$ buffer (10 mM Tris, 0.1 mM EDTA, and pH 8.0). All samples were prepared by serially diluting 10^6 copies/ μ L to 1 copy/ μ L, and all the reactions were performed in $20 \,\mu$ L volume containing 1.0 U of rTaq DNA polymerase (TAKARA, Japan) and $2 \,\mu$ L of $10 \times \text{PCR}$ buffer along with $125 \,\mu$ M dNTP, $0.5 \,\mu$ M of primers, and $1 \,\mu$ L of plasmid DNA. PCR amplifications were performed in C1000 Touch PCR (Bio-Rad, USA) with the program as follows: one step of 5 min at 95°C; 40 cycles of 30 s at 94°C, 45 s at 63–65°C (*T-NOS* annealing temperature was 63°C, and the others were 65°C), and 45 s at 72°C; and 5 min at 72°C. Amplification products were electrophoresed in 2% agarose gels for approximately 40 min at 100 V. Primers were synthesized

and purified by Shanghai Generay Biotech Co., Ltd. (Table S1).

2.4. Quantitative PCR Assay for the pGMT27 Plasmid

2.4.1. Reaction System and Conditions. All qPCR amplification reactions were performed in a 20 μ L final volume containing 10 μ L of Premix Ex Taq Mix (TAKARA, Japan), 0.5 μ M of each primer, 0.2 μ M of probe, 0.4 μ L of ROX Dye II (50×), and 1 μ L of plasmid DNA. PCR was in accordance with the following cycling conditions: 95°C 30 s, 40 cycles of 5 s at 95°C, and 34 s at 60°C. Reactions were run in a ViiATM 7 Real-Time PCR System (Invitrogen Life Technologies, USA), and the data were analyzed by ViiA 7 software v1.2. PCR primer and probes for *P-35S* and *NR* were synthesized by Shanghai Generay Biotech Co., Ltd. (Table S2).

2.5. Dilution for Standard Curves, Reproducibility, Repeatability, and Sensitivity. To test the availability of pGMT27, the plasmid was prepared as a calibrator and was serially diluted to 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 copies/ μ L with $0.1 \times TE$ buffer to construct the standard curves. For the repeatability and reproducibility test, the same five dilution points were also used. Each dilution point was amplified in three replicates. For the reproducibility test, the amplification was finished in three different days. For sensitivity, it included LOD and LOQ, and the pGMT27 plasmid was diluted to 50, 25, 10, and 5 copies/ μ L with 0.1 × TE buffer.

2.6. Conversion Factor (Cf) Calculation and Unknown Samples' Test. In order to minimize the differences between plasmid DNA and plant genomic DNA in PCR efficiencies, calculating the Cf value is a prerequisite [14]. For the unknown samples' test, 100% transgenic flue-cured tobacco DNA was diluted into a gradient of 100%, 50%, 25%, 12.5%, and 6.25% (V/V) with the content of 0% transgenic fluecured tobacco DNA, reactions were run in ViiATM 7 Real-Time PCR System, and then target gene and endogenous gene copy number were calculated according to the pGMT27 standard curve. The calculations were performed according to formula (B) suggested by Pi et al. [15], and the final Cf value represented the mean of five calculated Cfs. The unknown sample GM content (%) was calculated by using formula (C) according to Pi et al. [15]. Each reaction was repeated thrice.

Formula(B): Cf =	copy number of the exogenous gene copy number of the endogenous gene	(2)
Formula(C): GM content(%) =	copy number of the exogenous gene $\frac{1}{2}$	(2)

3. Results

3.1. Construction of the pGMT27 Plasmid and LOD Test by Qualitative PCR. Figure 1 represents a plasmid pGMT27 (18,296 bp), constructed in this study for the qualitative detection of GM tobacco. It harbored nine targets including seven exogenous and two endogenous targets shown in Table 1. Sac II and EcoR I restriction digests (Figure S1) and DNA sequencing (data not given) validated the acquisition of expected plasmids. For testing sensitivity in the qualitative PCR, nine targets by using 10⁶, 10⁵, 10⁴, 10³, 10², 50, 10, 5, and 1 copies of the pGMT27 plasmid as templates were measured by the LOD method. Nine targets were detected as low as 5 copies, which meant that the lowest testing level was 5 copies (Figure 2). However, we recommend that the template should be more than 10 copies if pGMT27 is a positive control plasmid in the practice test work because some groups have no amplification signals in the three repeats when only 5 copies of the templates were present (data not shown); therefore, the uncertainty is obviously greater because of the low template amount.

3.2. Preparation of the pGMT27 Plasmid as the Calibrator. The pGMT27 plasmid was diluted to 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 copies/µL, so as to establish standard curves for *P*-35S and *NR* targets for qPCR. Standard curves of the two reactions are displayed in Figure 3. The correlation coefficient (R^2) values of the standard curves were 0.997 and 0.998 for both *P*-35S and *NR* which indicated excellent linearity. PCR efficiencies were 101.427% and 98.036%, respectively, and data were generated by equation $E = 10^{-1/\text{slope}} - 1$ [16] and analyzed by ViiA 7 software v1.2, and standard curves of the two targets showed excellent linearity.

3.3. Repeatability, Reproducibility, LOD, and LOQ for qPCR Assays. For repeatability and reproducibility of Ct from P-35S and NR, targets of the pGMT27 plasmid were diluted and amplified for one day and three different days, respectively, and the data are shown and analyzed in Table 2. For the repeatability test, the SD of the Ct values ranged from 0.04 to 0.42, and the RSD was less than 1.29%. For the reproducibility test, the SD and RSD values were in the range of 0.04 to 0.39 and 0.18% to 1.14%, respectively. For qPCR assays, usually, repeatability of RSD less than 25% and reproducibility below 35% were accepted according to the European Network of GMO Laboratories (ENGL) guideline in 2015 [17], and our results indicated good repeatability and reproducibility, which were all in the acceptable range. To test the LOD and LOQ of plasmid pGMT27 in the qPCR, four kinds of concentrations (50, 25, 10, and 5 copies/ μ L) were prepared. According to ENGL [17], the acceptance criterion of LOD and LOQ should be less than 25 copies and 50 copies, respectively. In the assays, the results determined that the LOD and LOQ were 5 copies and 10 copies per reaction for *P-35S* and *NR*, respectively (Table 3).

3.4. Estimation of Cf Values and Quantitative Analysis of Unknown Samples. In order to analyze the GM tobacco content of unknown samples by using the pGMT27 plasmid as a calibrator in qPCR, the Cf values were calculated by the copy ratios of *P*-35S to *NR*. The endogenous gene *NR* was a single-copy gene in the tobacco genome [5]. In Table 4, Cf values for each time, including three parallel reactions, were 0.34, 0.39, 0.40, 0.40, and 0.41, and the mean value of the Cfs was 0.39. The SD and RSD values of the Cf were 0.03 and 6.77%, respectively, which were all in the acceptable range. With the aim of testing the GM content of the unknown



FIGURE 1: Schematic diagram of pGMT27 for the seven GM tobacco events. *P-35S, T-NOS, NPT II, GUS, HPT, Bar*, and *aadA*: fragment of the tobacco exogenous gene in red font; *actin* and *NR*: fragment of the tobacco endogenous gene in blue font.

Targets		Description of genetic elements	NCBI (accession no. and position of corresponding bp on the sequence of the accession)			
	P-35S ^A	Cauliflower mosaic virus 35S promoter	AF485783 (49745808)	835		
Exogenous	NPT II ^A	Neomycin phosphotransferase II	AF485783 (28383632)	795		
	T- NOS ^A	Agrobacterium tumefaciens nopaline synthase terminator	AF485783 (40224277)			
genes	GUS^A	Beta-glucuronidase (uidA) gene	AF485783 (58457656)	1,812		
	$HPT^{\mathbb{B}}$	Hygromycin phosphotransferase gene	AF354045 (89489973)	1,026		
	Bar^{B}	Herbicide-resistant gene	KF840400 (23812932)	552		
	$aadA^{B}$	Spectinomycin-resistant gene	EU497669 (35444335)	803		
Endogenous genes	Actin ^B	N. tabacum gene for actin	X63603 (42694640)	372		
	NR ^B	N. tabacum gene for nitrate reductase (nia-1)	JN384019 (4451455)	1,011		

TABLE 1: Description of the fragments present in the pGMT27 plasmid (18,296 bp).

A: from plant expression vector pBI121; B: synthesis.



FIGURE 2: LOD of pGMT27 using qualitative PCR for nine targets. Lane M: TAKARA 100 bp DNA ladder; lanes 1–9: 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 50, 10, 5, and 1 copies/ μ L; lane 10: NTC.



FIGURE 3: Calibration curves for *P*-35S and *NR* in qPCR. The dilutions contained approximately 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 copies per PCR. (a) Amplification plot of *P*-35S. (b) Amplification plot of *NR*. (c) Standard curve of *P*-35S (y = -3.288x + 40.022, $R^2 = 0.997$, Eff% = 101.427). (d) Standard curve of *NR* (y = -3.37x + 39.38, $R^2 = 0.998$, Eff% = 98.036).

Targets	True copy number	Ct repeatability (one day)		SD RSD (%)		True copy number	Ct reproducibility (different days)			SD	RSD (%)			
		1	2	3	Mean				1	2	3	Mean		
	100	32.96	33.68	32.94	33.19	0.42	1.28	100	33.24	32.90	33.26	33.14	0.20	0.62
	1,000	30.21	30.37	30.33	30.31	0.08	0.27	1,000	30.66	30.47	30.41	30.51	0.13	0.43
P-35S	10,000	27.10	27.22	27.07	27.13	0.08	0.29	10,000	27.38	27.33	27.11	27.27	0.14	0.52
	100,000	23.60	23.41	23.86	23.62	0.23	0.97	100,000	23.71	23.77	23.54	23.67	0.12	0.50
	1,000,000	20.07	20.14	20.07	20.09	0.04	0.18	1,000,000	20.20	20.14	20.13	20.16	0.04	0.18
	100	32.16	32.99	32.65	32.60	0.42	1.29	100	33.66	33.72	34.35	33.91	0.39	1.14
	1,000	29.42	29.25	28.99	29.22	0.22	0.74	1,000	29.99	29.88	30.04	29.97	0.08	0.27
NR	10,000	26.20	26.00	25.80	26.00	0.20	0.76	10,000	26.77	26.59	26.82	26.73	0.12	0.46
	100,000	22.61	22.54	22.72	22.63	0.09	0.41	100,000	23.05	22.93	23.12	23.03	0.09	0.40
	1,000,000	19.21	19.18	18.77	19.05	0.25	1.29	1,000,000	19.70	19.57	19.59	19.62	0.07	0.35

TABLE 2: Repeatability and reproducibility of the pGMT27 plasmid as a calibrator in qPCR.

samples, six concentrations were used for the analysis, and these samples were 0.5%, 2%, 5%, 6.25%, 12.5%, and 25% (v/v), which were prepared by mixing the samples of several GM and non-GM flue-cured tobacco DNA. GM percentages of unknown samples were calculated by formula (C). The qPCR analysis is shown in Table 5, where the accuracy of bias ranged from -15.55% to 1.93%; however, our bias values were lower than the acceptance criteria (-25% to 25%) according to ENGL [17]. Precision of SD and RSD ranged from 0.02 to 0.62 and from 1.34% to 10.6%. All these results indicated good repeatability, which were all in the acceptable range.

4. Discussion

On a global scale, the number of genetically engineered plants has risen exponentially in recent years, adding up to the complexity of efforts taken by the enforcement laboratories to detect not only the authorized GMOs in food and feed samples but also the unauthorized ones. In order to produce reliable data, research laboratories need to harmonize their techniques to quantify GMOs or to demonstrate that different methods used for the quantification of GMOs are commutable [6, 7]. Quantitative examination of the determination of the determination of the

Target	Template copies	Positive signals/total repeats	Mean Ct	Ct SD
	50	9/9	34	0.33
D 358	25	9/9	35.08	0.54
P-358	10	9/9	36.63	0.89
P-35S	5	8/9	_	NA
	50	9/9	34.04	0.61
	25	9/9	35.22	0.38
NK	10	9/9	36.03	0.76
	5	8/9	_	NA

TABLE 3: The LOD and LOQ of the pGMT27 plasmid as the calibrator in qPCR.

NA: not applicable.

TABLE 4: Conversion factor (Cf) estimation of the reference molecule pGMT27 by qPCR.

Quality of gonomia DNA (ng)	Target	copies	Cf	Marin Of miles	۶D	
Quality of genomic DNA (fig)	P-35S	NR	CI	Mean CI value	5D	KSD (%)
6.25	1675.12	4905.71	0.34			
12.5	3850.91	9848.56	0.39			
25	7782.50	19525.56	0.40	0.39	0.03	6.77
50	15455.08	38570.14	0.40			
100	30893.15	76172.85	0.41			
12.5 25 50 100	3850.91 7782.50 15455.08 30893.15	9848.56 19525.56 38570.14 76172.85	0.34 0.39 0.40 0.40 0.41	0.39	0.03	6.77

TABLE 5: Unknown sample analyses for GMO content (%) using the pGMT27 plasmid as the calibrator (Cf = 0.39).

True value (%)	Experimental value (%)			Accu	iracy	Precision		
	1	2	3	Mean (%)	Bias (%)	SD	RSD (%)	
0.5	0.40	0.39	0.47	0.42	-15.55	0.04	9.84	
2	1.71	1.75	1.75	1.73	-13.29	0.02	1.34	
5	4.26	4.28	3.90	4.15	-17.08	0.21	5.16	
6.25	6.25	5.16	5.32	5.57	-10.80	0.59	10.60	
12.5	12.65	12.54	11.91	12.37	-1.07	0.40	3.26	
25	26.18	25.28	24.99	25.48	1.93	0.62	2.42	

Bias = [(mean experimental value - true value)/true value] * 100.

amount of event-specific target with respect to the reference gene [8]. In the current study, seven exogenous ones were frequent, and the theoretical coverage reached 100% in the tobacco genetic transformation event [12]. Approaches for the detection and quantification of many event-specific targets of commercially available genetically modified organisms and reference genes are available. Additionally, there are many studies that demonstrate the development of methods and their in-house validation [6, 10]. Other studies indicated that the LOD was 5 and 20 copies, respectively, by using the plasmid as a reference molecule for detecting transgenic canola and soybean [18, 19]; correspondingly, in our study, nine targets were detected as low as 5 copies, which meant that the lowest testing level was 5 copies. Nonetheless, it is important to critically evaluate any validation status before implementing a method in the research laboratory for routine sample analyses. For reliable quantification of GMOs, laboratories need appropriate organization and quality management systems, and several critical points should be considered in the analytical procedures, such as sampling, sample preparation, and DNA extraction,

which have been described recently [10]. Differential qPCR for the detection of unauthorized GMOs is based on the occurrence of several common elements in different GMOs (e.g., promoter and genes of interest). A statistical model was developed to study the difference between the number of targets of such a common sequence and the number of event-specific targets that can identify the approved GMO and the donor organism of the common sequence. When this difference statistically deviates from zero, the presence of an unauthorized GMO can be deduced [20]. However, this approach has low sensitivity, and it is reliable only if the presence of an unknown GMO exceeds 30%. Furthermore, a qPCR assay for P-35S and NR was also developed by using plasmid DNA as the calibrator. The R^2 values of the standard curves were 0.997 and 0.998 for both *P*-35S and *NR*, those R^2 values ≥ 0.98 were accepted [9], and other researchers' results ranged from 0.995 to 0.999 [18, 21-23]. Theoretically, the Cf score should be 1.0 for the quantitative PCR test when the exogenous gene and endogenous gene are both one copy [5, 15], but in this study, the Cf value was 0.39, which is far more below 1.0, so we could observe three potential reasons: (1) large differences between plasmid DNA and plant genomic DNA in quantitative PCR efficiencies, and some studies involving crops validate this as Cf values ranging from 0.53 to 0.83 have been reported [14, 21, 23]; (2) these samples' GM content was defined as 100% positive by the FAPAS, but the fact was there was less than 100% GM content; (3) flue-cured tobacco DNA partially degrades after curing [24]. The SD and RSD values of the Cf were 0.03 and 6.77%, respectively, which were all in the acceptable range. The advancement of new approaches that are suitable for the quantitative measurement of specific sequences is a promising solution that can overcome the drawbacks of the currently used ways/methods. In the past, several alternative methods for the detection of GMOs were developed that explored different aspects of amplification, detection, and identification approaches [25, 26]. Recently, additional methods have been developed [27–31]. Consequently, in the current study, a qualitative PCR detection method for detecting the nine targets was developed. Highly linear and efficient reactions indicated that the standard curves of the pGMT27 plasmid were suitable for further quantitative test [32]. Meanwhile, using the pGMT27 plasmid as a calibrator provided a highly efficient method in the transgenic detection of flue-cured tobacco.

5. Conclusion

The newly developed screened plasmid pGMT27 provides better coverage of the GM elements that could be present in a sample and will facilitate advancements in the detection of unauthorized/unknown GM tobacco. It includes two tobacco endogenous genes (*actin* and *NR*), exogenous gene of the *P-35S* promoter and *T-NOS* terminators, selective marker genes of *NPT II*, *HPT*, *Bar*, and *aadA*, and *GUS* reporter gene. Overall, qualitative and quantitative PCR tests infer that the constructed plasmid is a suitable and costeffective strategy for the detection and quantification of GM tobacco.

Data Availability

All the data generated or analyzed during this study are included in the main paper and supplementary information files.

Ethical Approval

Not applicable.

Consent

Not applicable.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jing Yu conceptualized and investigated the study, provided the methodology, performed formal analysis, and wrote the original draft. Xiaolian Zhang visualized the study and wrote the original draft. Muhammad Faheem Adil edited and reviewed the article. Bo Lei investigated the study and provided formal analysis. Mengao Jia provided the methodology and visualized the study. Huina Zhao contributed to data curation and edited and reviewed the article. Shizhou Yu visualized the study and performed formal analysis. Jiemin Liu edited and reviewed the article. Yushuang Guo conceptualized the study, contributed to funding acquisition, provided resources, and reviewed and edited the article. Imran Haider Shamsi conceptualized and supervised the study, contributed to funding acquisition, provided resources, and edited and reviewed the article.

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Supplementary Materials

Figure S1: Sac II and EcoR I restriction digests of pGMT27. Table S1: primers used in qualitative PCR. Table S2: primer and probe sequence of *P-35S* and *NR* in qPCR. (*Supplementary Materials*)

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