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GENETIC CHARACTERIZATION OF *CPTI* AND *NPTII* GENES IN THE HYBRIDS OF TRANSGENIC AND NON-TRANSGENIC APPLE

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Abstract

To investigate the inheritance and expression of exogenous cowpea trypsin inhibitor (*CpTI*) and neomycin phosphotransferase II (*nptII*) genes in the progenies of transgenic apples (*Malus domestica* Borkh.), hybrid (F1) fruits and seeds were obtained using transgenic Gala and non-transgenic Fuji for cross and reciprocal cross experiments. Transgenic apple hybrid F1 plants were obtained using the established apple embryo culture system. The law of segregation of *nptII* in the hybrid progeny was studied through successive selections under high-level kanamycin pressure. The genetic linkage between *CpTI* and *nptII* was tested by PCR analysis. The effects of exogenous genes on transgenic apple fruits were measured by nptII enzyme activity, fruit weight, fruit shape index, fruit hardness, soluble solids content, acid content, and other related indicators in the transgenic Gala fruit. The kanamycin resistance test in F1 plantlets 1:1 Mendelian ratio of kanamycin resistance to non-resistance. Thus, the *nptII* marker gene, a dominant heterozygous gene, passed onto the progenies through sexual hybridization. PCR analysis also showed that *CpTI* was closely linked to *nptII*. The activity of nptII enzyme was detected in most transgenic apples, but the intensity of nptII enzyme activity in different fruits was different.. Transgenic Gala fruit and the control fruit were oblate or nearly round, with the fruit shape index ranging between 0.83 and 0.90. Acid content of a transgenic fruit was not significantly different compared to the control. Fruit hardness and soluble solids content were significantly lower compared to the control. The results provide a reference for further studies on the genetic characteristics of transgenic apples and the utilization of transgenic products.

Introduction

Transgenic technology is a comprehensive biotechnology developed in the 1970s, which transfers ideal genes to target organisms through modern cellular and genetic engineering. It is also an important means to study gene function and regulation mechanisms. With the development of transgenic technology, new germplasms with high quality and resistance, which are typically difficult to obtain by traditional breeding methods, were developed. The production of transgenic crops, including cotton, soybean, rice, tomato, and tobacco, conferred social and economic benefits. Transgenic technology is also used to transform fruit tree genes and overcome the limitations of traditional fruit tree breeding. Apple is a perennial woody plant with highly heterozygous genes, a long juvenile period of seedling offspring, and a low selection rate of hybrid offspring. The first transgenic apple plant was cultivated in 1989 (James *et al*. 1989), with many transgenic apple plants being obtained thereafter (Espley *et al.* 2007, Chen *et al.* 2012, Yao *et al.* 2013, Weig *et al.* 2015, Dai *et al.* 2014). For example, the introduction of the Malus x domestica *TERMINAL FLOWER 1* (*MdTFL1*) gene into the 'Orin' apple promoted early flowering of the apple plants (Kotiba *et al.* 2006), and the introduction of the *root loci B* (*rolB*) gene into the 'Jork 9' apple produced dwarf apple trees (Sedira *et al.* 2001).

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Stable inheritance and expression of an exogenous target gene in transgenic plants after being integrated into a plant genome is directly related to its commercial and practical value. Foreign genes integrated into a plant genome can be stably transmitted to their offspring through sexual reproduction and stably expressed through conventional cross-breeding techniques (Fearing *et al.* 1977, Webb *et al.* 1999). This was confirmed in transgenic rice (Chikkappa *et al.* 2011, Ma *et al.* 2001), soybean (Dufourmantel *et al.* 2006, Cai *et al.* 2011, Liu *et al.* 2012), corn (Wang *et al.* 2019), cotton (Guo *et al.* 2010, Wang *et al.* 2016), apple (Briviba *et al.* 2004), etc. The exogenous *nitric oxide synthase* (*nos*) and *nptII* genes were stably expressed in transgenic "green sleeve" apples. Additionally, in the F1 progeny, *nos*, inserted at a unit point, was segregated at a 1:1 ratio, and *nptII*, inserted at double sites, was segregated at a 3:1 ratio, which was in line with Mendel's law of segregation (James *et al.* 1996). In transgenic rice with three insecticidal genes (the *Bt* genes *cry1Ac* and *cry2A*, and the snowdrop lectin gene *gna*), the three insect-resistant genes were expressed as a single dominant gene, passed onto the next generation, and stably expressed (Maqbool *et al.* 2001). Other studies also showed that most exogenous genes could be stably integrated and expressed in the genome of recipient plants, conforming to the Mendelian genetic model (Liu *et al.* 2012, Fromm *et al.* 1990, Guo *et al.* 2007, Wu *et al.* 2011).

However, as foreign invaders, the genetic behavior of foreign genes is more complex compared to endogenous genes. Due to different integration modes and copy numbers of some foreign genes, as well as silencing, loss, rearrangement, and abnormal separation of foreign genes, irregular inheritance creates difficulties in the research and utilization of transgenes (Brandle *et al.* 1995, Zhou *et al.* 2006, Ren *et al.* 2006). Therefore, in the breeding and practical application of transgenic crops, it is important to study the genetic characteristics of foreign genes in the sexual reproduction of transgenic plants. In 1998, cowpea trypsin inhibitor (*CpTI*) and *nptII* genes were successfully introduced into apple cultivars (Shi *et al.* 2000). Further studies showed that exogenous *CpTI* and *nptII* were stable in apple tissue-cultured seedlings after 6-8 years of conventional subculture and that they were expressed in most lines (Zhou *et al.* 2011). To further understand the genetic characteristics of exogenous genes in transgenic apple hybrids, the genetics and expression of foreign genes in the progenies of transgenic Gala apple plants transformed with *CpTI* and *nptII* was studied in 2005, 2006, 2011, and 2021, respectively. The fruit characteristics of transgenic hybrids were primarily analyzed and, the results provided a basis for further research on the genetic stability of sexual reproduction of exogenous genes in woody plants.

Materials and Methods

The *Agrobacterium* tumefaciens strain is LBA4404, and the high-efficiency expression vector pARTCP (carrying the *NPTII* screening gene) is linked with the target gene *CpTI* in the expression vector pARTC940 containing a high-efficiency promoter. Transgenic apple lines (*Malus* × *domestica* 'Gala') carrying the exogenous *CpTI* gene were obtained via *Agrobacterium tumefaciens*-mediated transformation (Shi *et al.* 2000). Transgenic lines and non-transgenic *in vitro* plantlets of Gala and Fuji were rooted, acclimated, and planted in the specimen orchard at the Agricultural University of Hebei, Baoding, Hebei, China, in 2004.

Flowers of transgenic Gala, non-transgenic Gala, and non-transgenic Fuji were isolated with bags at the balloon stage (apple is a cross-pollinating plant and hence, was not emasculated). Simultaneously, 50 healthy flowers were picked from transgenic Gala, non-transgenic Gala, and non-transgenic Fuji plants. Pollen was collected after drying and removing impurities. Transgenic Gala as a maternal plant was crossed with non-transgenic Fuji as a paternal plant, and non-transgenic Fuji as a maternal plant was crossed with transgenic Gala as a paternal plant. Non-transgenic Gala (♀) × non-transgenic Fuji (♂) and non-transgenic Fuji (♀) × non-transgenic Gala (♂) were used as controls.

Both transgenic and non-transgenic Gala fruits were collected on July 25th, 2011, and the quality indexes of the fruits were measured. Twenty fruits were investigated for each plant line, and five fruits were divided into a plot and repeated four times. Fruit weight, diameter (D), and height (H) were determined, and the fruit shape index (D/H) was calculated. Fruits were peeled to measure fruit hardness using a GY-1 (TOP Instrument, China) fruit hardness tester. The soluble solids content and titratable acid content were determined using a PAL-1 (Atago, Japan) digital display saccharometer and GMK-835F (G-WON, Korea) fruit acidity meter, respectively.

The activity of the nptII enzyme in transgenic fruits was detected by a ^{32}P marker (Wang *et al*. 2002). The nptII enzyme was extracted from transgenic and non-transgenic Gala fruits (negative control) and an *Agrobacterium* solution containing the *nptII* gene (positive control). Phosphate cellulose paper Whatman P81 was immersed in a blocking solution, saturated, and dried. The extracted enzyme solution was mixed with a reaction solution at 37°C for 30 min, centrifuged at 10,000 r/min for 5 min at room temperature, and the supernatant was retained. Then, 20 μL of the supernatant was taken on the phosphate cellulose paper and dried. The phosphate cellulose paper was rinsed in an 80°C rinse solution for 2 min and transferred to a room temperature rinse solution for 10 min. This was repeated five times and the cellulose paper was removed and dried. The phosphate cellulose paper was wrapped with a cling film, pressed on the 'storage phosphor screen' for 24-48 h, and the black exposure point was then scanned.

Mature fruits were picked on August 18th, 2006. The seeds were removed, washed, and placed at low temperature (4-8°C) for 60 days. Then, they were placed on the gauze of a culture dish, moistened, and left to sprout at approximately 25°C. After seven days, the sprouted seeds were sterilized with 0.1% mercuric chloride for 10 min, washed with sterile water 3-4 times, and cultured in Murashige and Skoog (MS) medium with 6-benzyl adenine (BA) (0.2 mg/c) + gibberellic acid (GA) (2.0 mg/c) + sucrose (50 g/c) + agar (7.0 g/c) (see Table 1 for specific inoculation methods) at pH 5.8-6.0. The culture temperature was 25 ± 2˚C, light intensity was 1500-2000 lux, and the light cycle was 14/10 h light/dark. After 30 days, contamination and seedling rates were calculated. The sign of a seedling is the embryo developing into stems and leaves.

The calculation of contamination rate and seedling rate was as follows:

Contamination rate = number of contaminated seeds/number of inoculated seeds × 100%

Seedling rate = number of seedlings/number of inoculated seeds × 100%

Progenies of F1 apples were transferred to an MS + BA (0.5 mg/L) + α-naphthalene acetic acid (NAA) (0.04 mg/l) + white sugar (30 g/l) + agar (6.0 g/l) + kanamycin (50 mg/L) subculture medium for three generations. After cultivation, albinism in plants was investigated. The leaves that remained green and did not change to yellow were resistant plants, while those that turned yellow were non-resistant plants. The proportion of kanamycin resistance characters in the progeny of three generations were counted, and a chi-square (x^2) test was carried out to determine whether the proportion of kanamycin resistance characters in the progeny followed the Mendel's law of segregation.

$$\chi_c^2 = \sum \left[\frac{(|O - E| - 0.5)^2}{E} \right]$$

Where O represents the actual survey value and E the theoretical value.

DNA was extracted from the leaves of F1 progenies in the embryo culture. CpTI plasmid DNA was used as a positive control and the non-transformed apple tissue culture DNA as a negative control. The leaf DNA of apple tissue-cultured seedlings was extracted according to the

method described by Du *et al*. (1999). *Agrobacterium* plasmid DNA was extracted by the sodium dodecyl sulfate (SDS) method (Sambrook *et al.* 2002). Polymerase chain reaction (PCR) detection primer sequence of the *CpTI* gene, forward 5'-GATGATGGTGCTAAAGGTGT-3', reverse 5'-CTTACTCATCATCTTCATCC-3', were synthesized by TaKaRa (Dalian, China). MyCycler^{TM} PCR system (BIO-RAD, USA) was used for amplification. PCR reaction system: 20 µl containing *Taq* DNA polymerase 0.1 µl, dNTPs1.0 µl, upstream primer 1.0 µl, downstream primer 1.0 µl, Mg^{2+} 2.0 µl, template DNA 2.0 µl, 1 × PCR Buffer 2.0 µl and ddH_2O 10.9 µl. The reaction procedure consisted of pre-denaturation at 94°C for 6 min and amplification for 30 cycles. Each cycle included denaturation at 94°C for 60 s, annealing at 56°C for 90 s, and extension at 72°C for 90 s. Finally, the target fragment of 326 bp was amplified by extension at 72°C for 10 min. The PCR amplified product was detected by 5 µl agarose electrophoresis and the results observed and ph. otographed by the Universal Hood II gel imaging system (Bio-Rad, USA).

SPSS v.19.0 (IBM) and Microsoft Excel 2010 (Microsoft) were used to analyze the data.

Results and Discussion

The seeds were germinated before inoculation, which could effectively reduce the contamination rate of embryo. The physiological metabolism of seed embryos was active after germination. Also, as the seed embryos were in close contact with the culture medium, they absorbed nutrition better, and hence, were more conducive to differentiation. The seedling rate was the highest (96.03%) with this treatment, whereas the seeds that did not germinate after germination treatment and were directly inoculated showed the lowest seedling rate (14.46%) due to the thick seed coat, which affected seed germination. For seeds that did not undergo germination treatment or did not germinate in time after treatment, the seed coat was peeled off and inoculated (Fig. 1, Table 1) to obtain a higher seedling rate.

Single fruit weight, fruit shape index, fruit hardness, soluble solids content, and acid content of transgenic Gala and non-transgenic Gala control fruits of nine strains were measured. The single fruit weight of the No. 6 plant line was significantly lower. The single fruit weight of the remaining 8 lines was not much different from that of the control. Transgenic Gala fruit and the control fruit were oblate or nearly round, with the fruit shape index ranging between 0.83 and 0.90 (Fig. 2). Flesh hardness, soluble solids, and acid content are important indicators of fruit quality. They affect not only the taste and nutrition of fresh fruits but also relate to the fruits' storage and processing properties. There was no significant difference in the acid content between the tested transgenic lines and the control. However, fruit hardness and the soluble solids content were lower compared to the control (Table 2).

Table 1. Effect of seed treatment and inoculation on seed embryo culture of apple.

Seed treatment and inoculation method	Contamination rate/%	Seedling rate/%
Non-germination and seed coat cut off for inoculation	21.22	42.19
Germination and inoculation after germination	5.73	96.03
Germination and non-germination with seed coat inoculation	63.57	14.46
Germination and seed coat cut off for inoculation	25.54	59.92

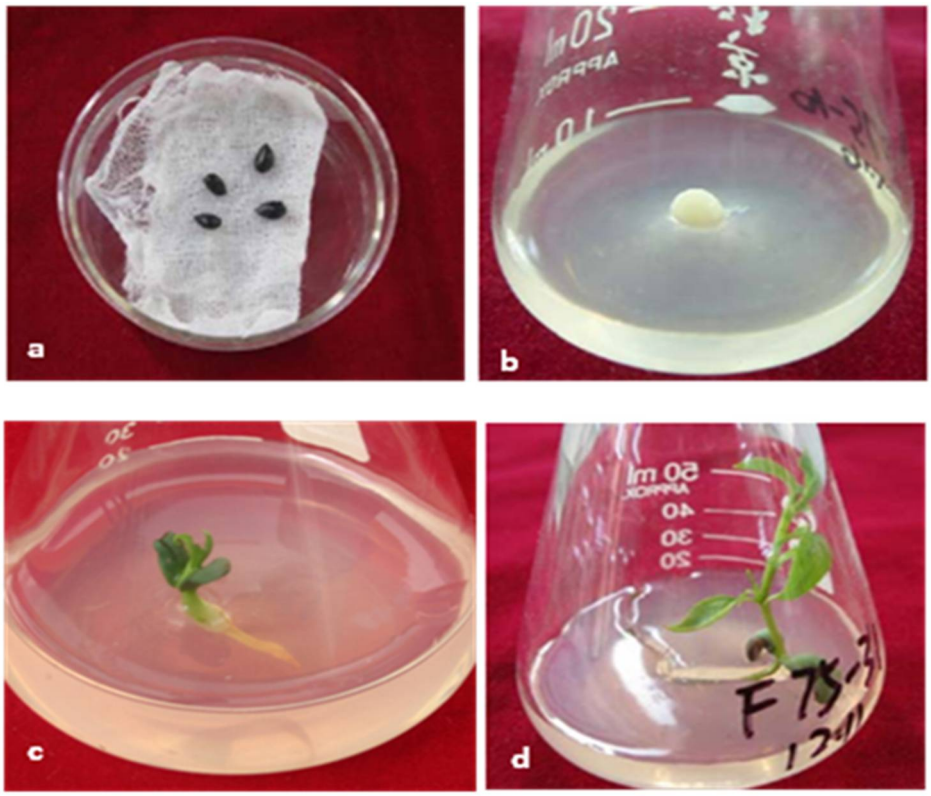

Fig. 1. Embryo culture of apple hybrids. (a) Seed germination, (b) Embryo inoculated on medium, (c) Plumule 40 days after inoculation and (d) Plantlets regenerated from an embryo.

Fig. 2. Comparison of fruit. (A1-3) Transgenic Gala. (B1-3) Non-transgenic Gala.

Radiolabeled [γ32P] ATP was used to generate radioactive kanamycin phosphate by γ-phosphate group transfer and nptII enzyme activity in transgenic Gala fruit detected. From the results of the nptII analysis (Fig. 3), some of the samples tested were similar to the *Agrobacterium* positive control, showing a strong nptII enzyme activity reaction, while the untransformed control only had a weak background reaction, with the water sample control being blank. Transgenic fruit 7 was similar to the negative control and the spotted background was weak. This showed that most of the fruits obtained from the hybridization of transgenic Gala as a female parent and common Fuji as a male parent contained *nptII*, with the nptII enzyme activity being strong.

Table 2. Parameters for apple fruit quality of transgenic Gala lines.

Clone	Fruit weight/g	Fruit shape index	Fruit firmness/kg.cm^{-2}	SSC /%	Acid content/%
1	97.55	0.90	11.08	10.05	0.17
2	113.90	0.85	11.17	9.77	0.14
3	100.56	0.89	12.43	10.46	0.17
4	101.50	0.86	11.08	9.45	0.18
5	101.02	0.85	11.24	9.82	0.17
6	95.66	0.85	11.74	10.31	0.17
7	99.92	0.86	12.13	10.81	0.15
8	97.81	0.84	12.32	9.70	0.18
9	100.61	0.85	12.99	12.22	0.17
ck	113.90	0.83	13.99	13.04	0.14

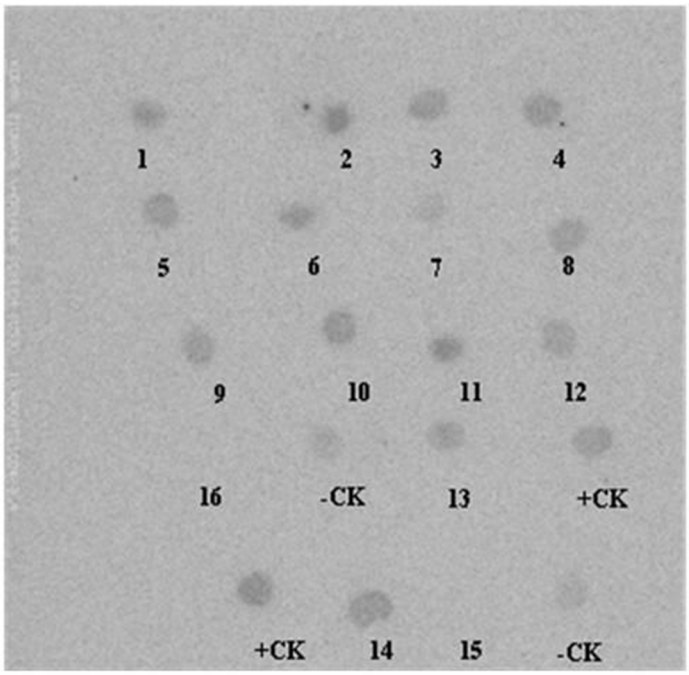

Fig. 3. Enzyme activity dot blot assay of *nptII* in transgenic Gala fruits. Positive control (+CK): *CpTI* plasmid. Negative control (-CK): Fruit of non-transgenic control Gala. (1~14) and Fruit of transgenic Gala. (15~16) H_2O.

After the F1 generation plants of fruit embryo culture were cultured in a subculture medium with 50 mg/L kanamycin for three generations, a few plants showed normal differentiation and growth, i.e., were kanamycin-resistant, whereas the others gradually whitened and even died, i.e., kanamycin-sensitive. The *nptII* marker gene was separated in the transgenic progeny. In the analysis of different single-fruit progeny, no typical separation rule was found due to the small number of individuals (Table 3).

Table 3. Kanamycin sensitivity analysis of transgenic Gala × non-transgenic Fuji progeny in a single fruit.

Transgenic Gala (♀) × Non-transgenic Fuji (♂)	The total number of fruit seeds	Resistant seeds	Sensitive seeds	Transgenic Gala (♀) × Non-transgenic Fuji (♂)	Total number of fruit seeds	Resistant seeds	Sensitive seeds
Fruit 1	5	3	2	Fruit 1	5	2	3
Fruit 2	4	4	0	Fruit 2	4	1	3
Fruit 3	5	3	2	Fruit 3	5	2	3
Fruit 4	5	4	1	Fruit 4	4	3	1
Fruit 5	4	1	3	Fruit 5	4	2	2
Fruit 6	4	1	3	Fruit 6	4	1	3
Fruit 7	4	2	2	Fruit 7	4	0	4

The segregation ratio of kanamycin sensitivity and kanamycin resistance in F1 hybrid lines was investigated in 2005, 2006, 2011 and 2021. In the chi-square analysis, the segregation ratio of 1:1 in F1 seeds by reciprocal crosses was in accordance with the Mendel's law of segregation (Table 4), indicating that *nptII* was inherited in line with the Mendelian's law. Therefore, *nptII* was a dominant heterozygous gene stably inherited in transgenic plants through sexual reproduction.

Table 4. Segregation of transgenic *nptII* in F_1 generation.

Treatment	Year	No. of plants	No. of actual plants (O)		No. of theoretical plants (E)		$\chi_c^2 = \sum\left[\frac{(\lvert O-E\rvert-0.5)^2}{E}\right]$
			Resistance	Sensitivity	Resistance	Sensitivity	
Transgenic Gala (♀) × non-transgenic Fuji (♂)	2005	41	26	15	20.5	20.5	2.4390
	2006	41	27	14	20.5	20.5	3.5122
	2011	68	40	28	34	34	1.7794
	2021	287	136	151	143.5	143.5	0.6829
Non-transgenic Fuji (♀) × transgenic Gala (♂)	2005	26	8	18	13	13	3.1154
	2006	322	163	159	161	161	0.0280
	2011	31	10	21	15.5	15.5	3.2258
	2021	306	147	159	153	153	0.3954

Note: $X^2_{0.05} = 3.84$.

PCR results showed that specific bands of *CpTI* were amplified in kanamycin-resistant but not kanamycin-sensitive seedlings, indicating that *CpTI* and *nptII* in kanamycin-resistant seedlings were closely linked and not isolated in the hybrid progenies of transgenic plants (Fig. 4). Wang et al. (2000) revealed that the synergistic expression rate of *β-glucuronidase* (*GUS*) and *cry1Ab* genes was 99.49% in the cross and backcross progenies of *Bacillus thuringiensis* (*Bt*) rice. In this study, *nptII* and *CpTI* genes were also tightly linked in transformed apple plants and their F1 generation. Therefore, it was reliable to analyze the genetic law of transgenic plants through the detection of *nptII* activity.

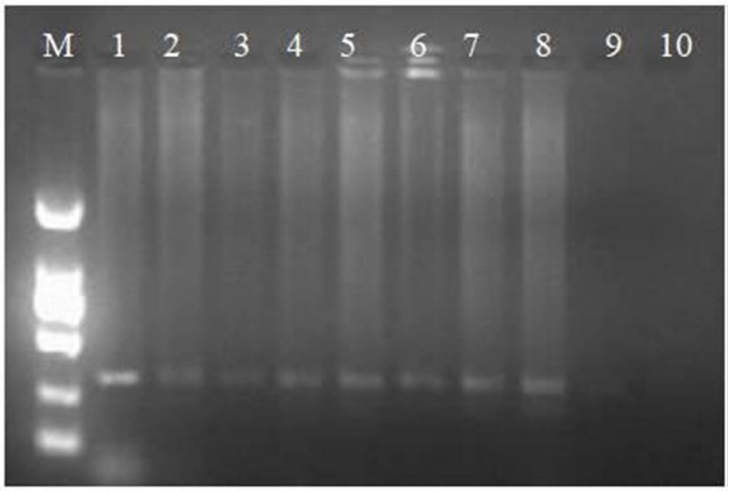

Fig. 4. Electrophoresis of PCR products of F1 plant DNA to observe amplifications in kanamycin-resistant seedlings. (M) 100 bp Marker. (1) *CpTI* plasmid. (2-8) Kanamycin-resistant plants. (9) Kanamycin-sensitive plant. (10) Non-transgenic control.

The *nptII* gene, derived from ahpA2 on the bacterial transposon Tn5 (Beck *et al.* 1982), encodes aminoglycoside-3'-phosphate transferase, which inactivates aminoglycoside antibiotics (neomycin, kanamycin, gentamicin, paromomycin, and G418) by phosphorylation (Anfossi *et al.* 2004). The mechanism of the toxicity of these antibiotics to plant cells is through their binding to the ribosomal 30S subunit in chloroplasts and mitochondria of plant cells. This affects the formation of the 70S initiation complex, interferes with the protein biosynthesis of chloroplasts and mitochondria, and ultimately leads to plant cell death. NptII transfers the γ-phosphate group on the adenosine-triphosphate (ATP) molecule to the antibiotic molecule, affecting the binding of antibiotics to ribosomal subunits, thereby inactivating antibiotics. Therefore, the principle of nptII enzyme detection was to use radioactively labeled [γ32P] ATP to generate radioactive kanamycin phosphate through the γ-phosphate group transfer (Reiss *et al.* 1984). Based on this principle, this study used transgenic fruits as test materials to detect the activity of antibiotic-resistance genes in fruits. The results showed that most of the transgenic fruits contained nptII enzyme activity, with intensity varied in different fruits. The response signal of one transgenic fruit was extremely weak, while it was strong in other fruits. However, it is of concern whether the transgenic fruits containing nptII are safe for consumption. Kanamycin-resistant organisms in human and animal guts and in soil were determined to be so common, and allergic reactions to the kanamycin-resistance protein were also determined to be highly unlikely (Bruening *et al.* 2000). Therefore, the *nptII* gene was a relatively safe marker gene. In transgenic FLAVR SAVR tomatoes, the copy number of marker genes in each cell does not exceed 10. Also, the number of kanamycin resistance genes in humans ingested daily by eating transgenic tomatoes does not exceed 0.33-1.00 pg. Under normal circumstances, the amount of DNA entering the human digestive tract through food daily is 200-500 mg in the small intestine and 20-50 mg in the colon. It can be seen that the amount of exogenous marker genes ingested by eating genetically modified foods is negligible compared to the amount of DNA persisting in the digestive tract from other foods (Redenbaugh *et al.* 1992). Our study showed that though the transgenic fruits had hybridization signals, they were weaker compared to the positive control, and their specific content needed to be quantitatively analyzed.

Foreign genes are randomly integrated into recipient chromosome DNA by homologous recombination or non-homologous recombination using a carrier plasmid. The foreign DNA, integrated into the recipient plant genome, can be maintained by meiosis, and stably transmitted to the next generation through sexual processes, ensuring high stability (Botella *et al*. 2006, Zhang *et al*. 2020). Exogenous genes are generally inherited by offspring as dominant genes, following the Mendelian law of genetic segregation. Self-pollinated progenies exhibit a 3:1 segregation ratio, whereas progenies with non-transformed parents exhibit a 1:1 segregation ratio, showing single gene inheritance inserted at a unit point (Wang *et al*. 2004). However, the non-Mendelian genetic phenomenon caused by the abnormal isolation of foreign genes is not uncommon in transgenic plants and its causes are relatively complex. The characteristics of a recipient genome and the transformation gene, the interaction between them, and the methods/ strategies used in transformation affect the inheritance and expression of foreign genes, which may result in the abnormal isolation of foreign genes (Zhou *et al*. 2006). Wu et al. showed that the individual transformants in the T1 generation of transgenic cotton deviated from the principle of segregation, which could be related to the loss of insect-resistant genes and that the pollen activity of the plant with insect-resistant genes was lower compared to the plant with no insect-resistant genes (Wu *et al*. 2003). Further, Wang et al. reported that the chromosome structure and the number of pollen mother cells in the early generation of transgenic cotton plants varied, resulting in a variation in pollen mother cell abortions. This partly explained the deviation of insect resistance characteristics of transgenic cotton in the early generation (Wang *et al.* 2001).

In this study, F1 generation plants were obtained using transgenic and non-transgenic apple plants as parents, where kanamycin resistance and kanamycin sensitivity showed a 1:1 separation ratio. Based on the genetic theory, kanamycin-resistant offspring plants not only contained heterozygous plants but also homozygous plants, as genetic characteristics are controlled by a pair of dominant heterozygous genes. The results of segregation showed that the exogenous gene *nptII* was inherited by single dominant inheritance. These results provide important information to further study and utilize transgenic apple plants.

Exogenous *nptII* genes can be stably inherited in apple hybrid offspring plants through sexual reproduction, at a 1:1 separation ratio following Mendel's law. Both *CpTI* and *nptII* genes were closely linked in the hybrid offspring of transgenic apple plants, with no separation. The nptII enzyme was present in the fruits of transgenic apple hybrid offspring, with the detection rate of nptII enzyme activity being 92.86%. There was no significant difference between the fruit shape index and acid content of the transgenic fruit and control, whereas the weight of single fruit, fruit hardness, and soluble solids content were lower compared to the control.

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