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Effective transformation of wild tomato species Solanum pimpinellifolium mediated by Agrobacterium tumefaciens

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Research Article

Keywords: Solanum pimpinellifolium, Agrobacterium tumefaciens, Genetic transformation

Posted Date: August 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2805472/v1

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Abstract

Genetic transformation has become a routine method for understanding the growth and development of crops and promoting sustainable agricultural production. The standard transformation methodology for tomato crops, as a model plant, has been extensively established. However, most studies have focused on tomato cultivars with typical genotypes, while less information is known about wild species that have the potential for crop improvement. This study aimed to construct an effective transformation system for the wild species *Solanum pimpinellifolium*based on a routine method.The results indicate that the addition of acetosyringone promotes shoot regeneration in the wild species *S. pimpinellifolium*. A reduced IAA content along with an appropriate Zeatin level plays a key role in improving regeneration efficiency. Using a lower bacterial solution (OD600 = 0.25) and a shorter infestation time (15 minutes) may also benefit the regeneration efficiency. Although the transformation of *S. pimpinellifolium* has shown genotype dependence, a medium named B2 in this study shows potential for broad applicability in the wild species *S. pimpinellifolium*. The findings from this study will contribute to the understanding of wild species and their potential role in tomato improvement.

Key message

the reduced IAA content with an appropriate Zeatin level plays a key role to increase the regeneration efficiency of wild tomato species *S. pimpinellifolium*.

Introduction

Tomato (*Solanum lycopersicum*) is a globally cultivated vegetable crop. As a model plant, it has been extensively studied to uncover the genetics and regulatory mechanisms behind crop production, in order to meet the needs of continually growing populations. However, throughout its long history, cultivars have been subject to heavy human selection pressure, resulting in a limited genetic background. This loss of genetic diversity has made it more difficult for cultivars to adapt to the frequently changing environments, particularly in recent years. In contrast, wild tomato species, such as *S. pimpinellifolium*, exist in suboptimal environments (Woodward and Barne, 1988) and have evolved various adaptations to extreme conditions. These wild species are considered a potential resource for improving cultivars, especially in challenging harsh conditions such as extreme temperature, drought, and various biotic stresses (Foolad, 2003; Salinas et al., 2013).

Traditional breeding methods for varietal improvement, relying on these wild species, are slow due to the tedious process of accumulating a large number of elite alleles and the difficulty of predicting the effects of multiple allele interactions (Østerberg et al., 2017). An attractive strategy called *De novo* domestication of wild species has been proposed as an alternative approach to accelerate the exploration of these wild species (Zsögön et al., 2017; Østerberg et al., 2017). In a study, by employing multiple CRISPR-Cas9 gene editing techniques on the wild species *S. pimpinellifolium*, researchers successfully created domesticated tomato phenotypes that retained the parental disease resistance and salt tolerance (Li et al., 2018). This

strategy holds promising prospects for crop improvement by utilizing the genetic resources of wild relatives.

Efficient transformation methods are crucial for the rapid advancement of genome editing technologies (Gerszberg et al., 2015). However, most of transformation methodologies for tomatoes presently focused on the cultivars with several genotypes (Frary et al., 1996; Raj et al., 2005; Chaudhry et al., 2007; Yasmeen et al., 2009; Somayaji et al., 2014; Lee et al., 2020). There is currently limited clear information available regarding the transformation of wild tomato species. In our study, we attempted to apply a routine transformation method used in our lab for the wild species *S. pimpinellifolium*, which is a close relative of cultivated tomatoes (Lu et al., 2021). We found that it is unexpectedly infeasible. This highlights the necessity of establishing an effective protocol specifically tailored for wild tomatoes.To address this need, we optimized several key factors based on previously accumulated knowledge from relevant studies (Yasmeen et al., 2009; Gerszberg et al., 2015; Gupta and Van Eck, 2016). These factors included acetylsyringone, medium composition, exogenous hormones, *Agrobacterium* strain and concentration, infestation time, and others. One modified method established in this study might provide the broad applicability for the transformation of wild species *S. pimpinellifolium*. It would facilitate the exploration of wild tomato species.

Materials and methods

Plant materials and explant preparation

All tomato materials, including the wild species *S. pimpinellifoliu*m LA2179, LA2345, LA2423, as well as the tomato cultivars *S. lycopersicum* M82, Moneymaker, and Ailsa Craig, were generously provided by the Tomato Genetic Resource Center (TGRC). The seeds were soaked in a 50mL centrifuge tube and then placed in a shaking table under 37°C dark conditions for 30min (200rpm /min). After pouring out the water into the centrifuge tube, the seeds were washed repeatedly 3 times. The treated tomato seeds are subject to the following operations on the ultra-clean workbench: (1) Sterilized in 75% ethanol for 30s and then washed with sterile water 3 times (the 30s each time). (2) Disinfect the seeds with 5% sodium hypochlorite for 8 minutes, add the same volume of sterile water after the seed coat turns pale yellow, and clean the seeds with sterile water 6 times after sedimentation. After pouring out the water from the centrifuge tube, the tomato seeds were subjected to the following procedures on an ultra-clean workbench:(3) The sterilized seeds are placed in a 100mL conical flask and placed on a shaking table (dark, 28°C, 80 rpm /min) for germination. The germinated seeds are placed on the seeding medium (A0 medium: MS 2.22g /L + Sucrose 15g /L + Agar 6.8 g /L, pH 5.8–5.85) and cultured at 25°C, 16h light/8h dark and light intensity 1800 Lux.

Bacterial strain and plasmid

The CRISPR/Cas9 vector containing *NPTII*, *GFP* and *Cas9* as the selectable markers was used. The recombinant vector was transformed into *Agrobacterium tumefaciens* EHA105 and GV3101 by the

heating shock, and then cultured in a YEB-selective medium containing 50 mg/L kanamycin sulfate, 50 mg/L rifampicin at 28°C and 200 rpm under dark conditions until OD_{600} = 0.5.

Plant transformation

The fully expanded cotyledons were cut and the middle part approximately 5-10mm in length was pre-cultured in dark conditions for 36–48 hours. Then the pre-cultured cotyledons were exposed to the infection solution for 15 or 30 minutes, and placed in A1 medium (MS 4.44 g/l, sucrose 30 g/l, agar 6.8 g/l, IAA 1 mg/l, Zeatin 1.75 mg/l, pH 5.8–5.85) for 48 hours in dark. Four selected media were used. A2 is a routine medium for the cultivars (MS4.44 g/L + Sucrose 30 g/L + Agar 6.8 g/L + Zeatin 1.75 mg/L + IAA 1 mg/L + Kan 100 mg/L + Tim 200 mg/L) (Lu et al., 2021). Three additional medium special for wild species *S. pimpinellifolium* are B2 (MS4.44 g/L + Sucrose 30 g/L + Agar 6.8 g/L + Zeatin 2 mg/L + IAA 0.5 mg/L + Kan 100 mg/L + Tim 200 mg/L), C2 (MS4.44 g/L + Sucrose 30 g/L + Agar 6.8 g/L + Zeatin 2 mg/L + IAA 0.2 mg/L + Kan 100 mg/L + Tim 200 mg/L) (Sandhya et al. 2022), and D2 (MS4.44 g/L + Sucrose 30 g/L + Agar 6.8 g/L + Zeatin 1 mg/L + IAA 0.1 mg/L + Kan 100 mg/L + Tim 200 mg/L; pH 5.8–5.85). The components of rooting medium are MS4.44 g/L + Sucrose 30 g/L + Agar 6.8 g/L + IBA 2 mg/L + Kan 25 mg/L + Tim 200 mg/L, pH 5.8–5.85.

Polymerase chain reaction (PCR) detection

Genomic DNA was extracted from plant tissue using the CTAB method. PCR were performed to detect *NPTII*, *GFP*, and *Cas9* genes and confirm the insertion of T-DNA. The primers for *NPTII*, *GFP*, and *Cas9* genes were 5'-GGCTATCGTGGCTGGCCA-3' and 5'-ACGCTATGTCCTGATAGCGGT-3', 5'-GTGTTCTGCTGGTAGTGGTC-3' and 5'-CCGACCACATGAAGCAGCAC-3', 5'-GGGACCTACCACGATCTCCT-3' and 5'-ATATCGCGGCCATTCTGGAG-3', which produced respectively a DNA fragment of 471 bp, 334 bp and 692 bp. The PCR procedure was 95℃ for 3 min, followed by 35 cycles of heating at 95°C for 15 s, 60°C for 15 s, 72°C for 30 s and final extension at 72℃ for 7 min. To detect *Solyc07g062270* and *Solyc10g079830* target sequence, the primers were 5'-TAGAGCAGTTTCACAATCTATT-3' and 5'-ATAATCATACTTCCAGCATAATTTG-3', 5'-CTCCTTAGCAAGAAGGTCAG-3' and 5'-ATTCTGAGGTCTCGTCTGTA-3', which respectively produced a DNA fragment of 731 bp and 723 bp. The PCR procedure was 95℃ for 3 min, followed by 35 cycles of heating at 95°C for 15 s, 52°C for 15 s, 72°C for 30 s and final extension at 72℃for 7 min.

Data analysis and statistics

Shoot regeneration efficiency=(number of buds differentiated/total number of cotyledon explants infected with *Agrobacterium*)×100%. SPSS 23 software was used to analyze the data. One-way ANOVA was used to determine the significance.

Results

Shoot regeneration is unavailable for S. pimpinellifolium by A2 medium

To test the transformation efficiency of a routine method (with an OD600 of 0.5, infection time of 15 minutes, and *Agrobacterium* strain of EHA105) generally used for the tomato cultivars, three genotypes *S. lycopersicum* M82, Moneymaker, and Ailsa Craig) and one *S. pimpinellifolium* LA2345 were employed for the transformation. The results presented in Table 1 clearly demonstrated that the transformation efficiency of the three cultivars was significantly higher than that of LA2345. Although the transformation efficiency was greatly depend on the different genotypes of the cultivars, the shoot differentiation of *S. pimpinellifolium* LA2423 did not occurred at all. This indicated that the routine medium is not effective for wild species *S. pimpinellifolium*..

Table 1
Effect of genotype difference on shoot regeneration regeneration

Material	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency (±) SE (%)
	Replication 1	Replication 2	Replication 3	
M82	11.32	16.36	13.64	13.77 ± 2.52ab
MM	10.00	0.00	8.89	6.30 ± 5.48bc
AC	16.67	22.73	18.18	19.19 ± 3.15a
LA2345	0.00	0.00	0.00	0.00 ± 0.00c

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.01$)

Acetylsyringone promotes shoot regeneration

The previous study has shown that the acetylsyringone can improve transformation efficiency by promoting the integration of foreign genes (Shahla et al., 1987). In particular, a concentration of 100 µmol/L of acetylsyringone has been found to be more effective for Arka Vikas (Somayaji et al., 2014). Along with the treatment of cotyledons by 100 µmol/L acetylsyringone, the shoot regeneration efficiency of LA2345 was increased up to 3.2% (Table 2). It proved that AS can effectively assist the shoot differentiation of *S. pimpinellifolium*.

Table 2
Effect of acetylsyringone on shoot differentiation

Material	AS(µmol/L)	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency (±) SE (%)
		Replication 1	Replication 2	Replication 3	
LA2345	0	0	0	0	0 ± 0a
	100	4.44	5.17	0	3.20 ± 2.80a

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.05$)

The appropriately decreased IAA is essential for shoot regeneration

Based on A2 medium, three mediums named B2, C2, and D2 were designed with different concentrations of IAA and Zeatin in order to test the efficiency of these components. The results indicated that the shoot regeneration efficiency of B2, C2, and D2 mediums was significantly enhanced compared to A2 (Table 3).Moreover, C2 medium showed the highest average shoot regeneration efficiency but the difference was not significant as compared to B2 and C2. Considering the impact of different combinations of IAA and Zeatin on transformation efficiency, based on previous research results(Yasmeen, 2009; Gupta and Van Eck, 2016), it has been suggested that the combination of 0.5 mg/L IAA and Zeatin may exhibit a higher transgenic positive rate. Therefore, the B2 medium containing 2.0 mg/L Zeatin and 0.5 mg/L IAA was ultimately selected to optimize other factors, including OD_{600}, infection time, and different strains of *Agrobacterium*.

Table 3
Effect of medium difference on shoot differentiation

Material	Culture medium	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency (±) SE (%)
		Replication 1	Replication 2	Replication 3	
LA2345	A2	8.57	0.00	2.86	3.81 ± 4.36b
	B2	17.78	14.29	2.50	11.52 ± 8.01ab
	C2	11.86	20.83	20.00	17.56 ± 4.96a
	D2	7.69	16.33	22.00	15.34 ± 7.21ab

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.05$)

Low OD_{600} value is helpful for improving the shoot regeneration efficiency

A higher concentration of *Agrobacterium* can lead to a hypersensitive response in the explants (Saini et al. 2007). To find an appropriate OD_{600} value, B2 medium with OD_{600} values of 0.25 and 0.5 were tested (Table 4). The results in Table 4 indicate that compared to an OD_{600} value of 0.5, the average conversion efficiency of 0.25 has increased by approximately 6.41%. Meanwhile, we found that OD_{600} of 0.5 usually lead to bacterial overflow in the cotyledons, while OD_{600} of 0.25 showed much less. Thus, a bacterial solution with OD_{600} of 0.25 might be more suitable for the infestation and transformation of *S. pimpinellifolium*.

Table 4
Effect of OD_{600} value on shoot differentiation

Material	OD600	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency(±) SE (%)
		Replication 1	Replication 2	Replication 3	
LA2345	0.5	17.78	14.29	2.5	11.52 ± 8.01a
	0.25	13.04	17.5	23.26	17.93 ± 5.25a

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.05$)

Less infestation time is beneficial for shoot regeneration

The infestation time greatly affects the degree of hypoxia-induced explant death. Herein, we tested two different duration time, 15 minutes and 30 minutes respectively. The results proved that the average shoot regeneration efficiency was significantly higher with the 15-minute infection treatment as compared to the 30-minute treatment (Table 5). It was observed that most of the cotyledon explants of LA2345 turned brown after 2 days during co-culture with the 30-minute infection treatment and were unable to go the callus differentiation. In contrast, the 15-minute infection-treated explants differentiated the callus after 7–10 days and produced the bud within 20 days.

Table 5
Effect of infection time on shoot differentiation

Material	Infection time/min	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency(±) SE (%)
		Replication 1	Replication 2	Replication 3	
LA2345	15	13.04	17.5	23.26	17.93 ± 5.12b
	30	6.38	2.5	0.0	2.96 ± 3.21a

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.05$)

No difference between two Agrobacterium strains EHA105 and GV3101

To date, several strains of Agrobacterium have been used for tomato transformation (Chettyet al. 2013;Van Eck, 2019). Herein, the effect of two *Agrobacterium* strains EHA105 and GV3101 were investigated on the shoot regeneration of *S. pimpinellifolium*. The results indicated no significant difference between the effects of EHA105 and GV3101 (Table 6).

Table 6
Effect of *Agrobacterium* strains on shoot differentiation

Material	*Agrobacterium* strains	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency (±) SE (%)
		Replication 1	Replication 2	Replication 3	
LA2345	EHA105	13.04	17.5	23.26	17.93 ± 5.12a
	GV3101	10.63	20.93	20	17.19 ± 5.70a

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.05$)

The detection of transgenic plants

To further confirm the presence of transgenic plants, genomic DNA was extracted from the seedlings, and PCR was performed using specific primers for the selectable markers *Cas9*, *NPTII*, and *GFP* to detect T-DNA insertions (Fig. 1). The results revealed that 18 transgenic lines of *S. pimpinellifolium* LA2345 tomato were successfully found to have integrated T-DNA. This indicates the suitability of this system for genetic transformation of *S. pimpinellifolium* LA2345.

One important factor to affect the plant transformation is the genotype differences (Jabeen et al., 2009). In order to evaluate the effectiveness of B2 medium in inducing adventitious shoot formation, we conducted tests on two genotypes *S. pimpinellifolium* LA2179 and LA2423. As indicated in Table 7, all of B2, C2 and D2 treatments showed the higher shoot regeneration efficiency with two genotypes *S. pimpinellifolium* LA2179 and LA2423 when compared to the original A2. The highest average shoot regeneration efficiency for LA2179 was 21.83% with C2 medium, and LA2423 was 16.08% with B2 medium. However, C2 and D2 treatments showed the genotype dependent because both of them showed the almost same efficiency with B2 for LA2179 but the great difference for LA2423. Hence, we considered that B2 might be more applicable for the different genotypes of *S. pimpinellifolium*.

Table 7
Effect of medium difference on shoot differentiation

Material	Culture medium	Shoot regeneration efficiency (%)			Average shoot regeneration efficiency (±) SE (%)
		Replication 1	Replication 2	Replication 3	
LA2179	A2	3.45	4.44	7.27	5.05 ± 1.98b
	B2	19.35	15.22	18.87	17.81 ± 2.26a
	C2	18.87	23.4	23.21	21.83 ± 2.56a
	D2	14.00	20.41	18.00	17.47 ± 3.24a
LA2423	A2	1.92	0.00	0.00	0.64 ± 1.11b
	B2	16.67	10.00	21.57	16.08 ± 5.81a
	C2	5.00	5.00	0.00	3.33 ± 2.89b
	D2	5.45	11.43	2.63	6.50 ± 4.49b

The lowercase letters after the average shoot regeneration efficiency in the table indicate significant differences among the treatments ($P \leq 0.05$)

To evaluate the effectiveness of this protocol, the transformation experiments with genes *Solyc07g062270* and *Solyc10g079830* were conducted on the genotypes LA2179 and LA2423 respectively. The results showed in total respectively 9 positive transgenic plants for LA2179 and 18 for LA2423 were obtained (Fig. 2), of which LA2179-4 and LA2423-7 were mutant plants (Fig. 3, 4).

Discussion

In the past decades, significant progress has been made in establishing a relatively effective genetic transformation method for the model crop tomato. This method has played a crucial role in elucidating various aspects of crop growth, development, and genetic enhancement (Soyk et al., 2017b; Ueta et al., 2017). With the rapid advancements in biotechnology, gene editing techniques have emerged as a powerful tool for improving wild plant resources. They have expedited the exploration and utilization of wild species (Alba et al., 2009; Ashrafi et al., 2012).However, it has been observed that the conventional genetic transformation methods used for cultivated tomato varieties are not as efficient when applied to the wild species *S. pimpinellifolium*,which greatly hinders the use of wild species in tomato crop improvement. This study established a genetic transformation system for wild species *S. pimpinellifolium* by manipulating factors that affect genetic transformation (Gerszberg et al., 2015), including acetosyringone, culture medium, *Agrobacterium* strain and concentration, and infection time. The development of this effective transformation system will provide certain assistance in harnessing the potential of wild species in tomato crop improvement.

We observed that the cultivated tomatoes were capable of shoot regeneration without acetosyringone, but *S. pimpinellifolium* did not. It is possible that the cultivated tomatoes can independently generate sufficient acetylsyringone to facilitate T-DNA insertion for transformation, whereas exogenous acetylsyringone is required for *S. pimpinellifolium*. Park (2003) discovered that the combination of Zeatin and IAA had a more positive impact on the genetic transformation of tomatoes. This study also found that under the condition of a certain level of Zeatin, reducing the concentration of IAA could improve the regeneration efficiency of *S. pimpinellifolium*. This is consistent with the cultivars responding to exogenous hormones(Gupta and Van Eck, 2016).

Li et al. (2011) demonstrated that the *Agrobacterium* OD_{600} level was a crucial factor influencing tomato genetic transformation. In this study, we discovered that a lower OD_{600} value (0.25) was more advantageous for *S. pimpinellifolium*. In the process of genetic transformation, both too short and too long infection times can have adverse effects on transformation efficiency (Li et al., 2011). This study observed that an extended infection duration can lead to excessive *Agrobacterium* growth on the explants, resulting in a reduction in shoot regeneration efficiency. Moreover, significant variations were observed among tomato cultivars due to different *Agrobacterium* strains (Van Eck, 2019). For example, the efficiency of the strain GV3101 is significantly higher than that of EHA105 for the cultivar Micro-Tom (Chetty et al., 2013) In contrast, we found that GV3101 and EHA105 had no shoot regeneration efficiency for wild species *S. pimpinellifolium*. Although the previous studies found that the transformation of tomato cultivars is generally genotype-dependent, herein the established B2 medium seemly showed the broad applicability to *S. pimpinellifolium* as proved by three accessions of *S. pimpinellifolium* with quite different genetic background (unpublished data).

Moreover, the reproducibility of our three replicated data was poor, which could be attributed to the limited sample size and uneven infestation levels between the cotyledon and the bacterium. To further validate the results, two additional accessions of *S. pimpinellifolium* tomatoes were subjected to genetic transformation using the B2 medium. Following editing of the target genes (LA2179 and LA2423, *Solyc07g06227*0, and *Solyc10g079830*), mutant plants were successfully obtained, providing clear evidence of the B2 medium's suitability for genetic transformation in *S. pimpinellifolium*. Furthermore, 13 wild relatives of tomato are existed, which are more distant genetic relationship with cultivated tomatoes than *S. pimpinellifolium*. Further investigation is required to determine whether the transformation method established in this study would be effective for these wild resources. However, regardless of its applicability to other wild resources, we have successfully demonstrated the effectiveness of the method based on the B2 medium for the genetic transformation of *S. pimpinellifolium* tomatoes.

Declarations

Acknowledgments This work was financially supported by the Finance Science and Technology Project of Hainan Province (SQ2022JSKFZD0060), the Fundamental Research Funds for Central Non-profit Scientific Institution (IVF-BRF2018006), the Key Laboratory of Biology and Genetic Improvement of

Horticultural Crops, Ministry of Agriculture, China, and the Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (Grant No. CAAS ASTIP-IVFCAAS).

Author contributions Study

Conception and design—Xu MM, Liu JC; performed experiments—Xu MM, Liu JC; analysis of data—Xu MM, Liu JC, Li X; drafting of the manuscript—Xu MM, Liu JC, Li X, Li JM; improvement of the manuscript —Xu MM, Liu JC, Li X, Li JM; final approval of the version to be published—Xu MM, Liu JC, Gao J, Li JM, Li X, Liu L, Huang ZJ, Wang XX, Du YC, Zhang H, Su WY, Zhang M, Chen YS

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Figures

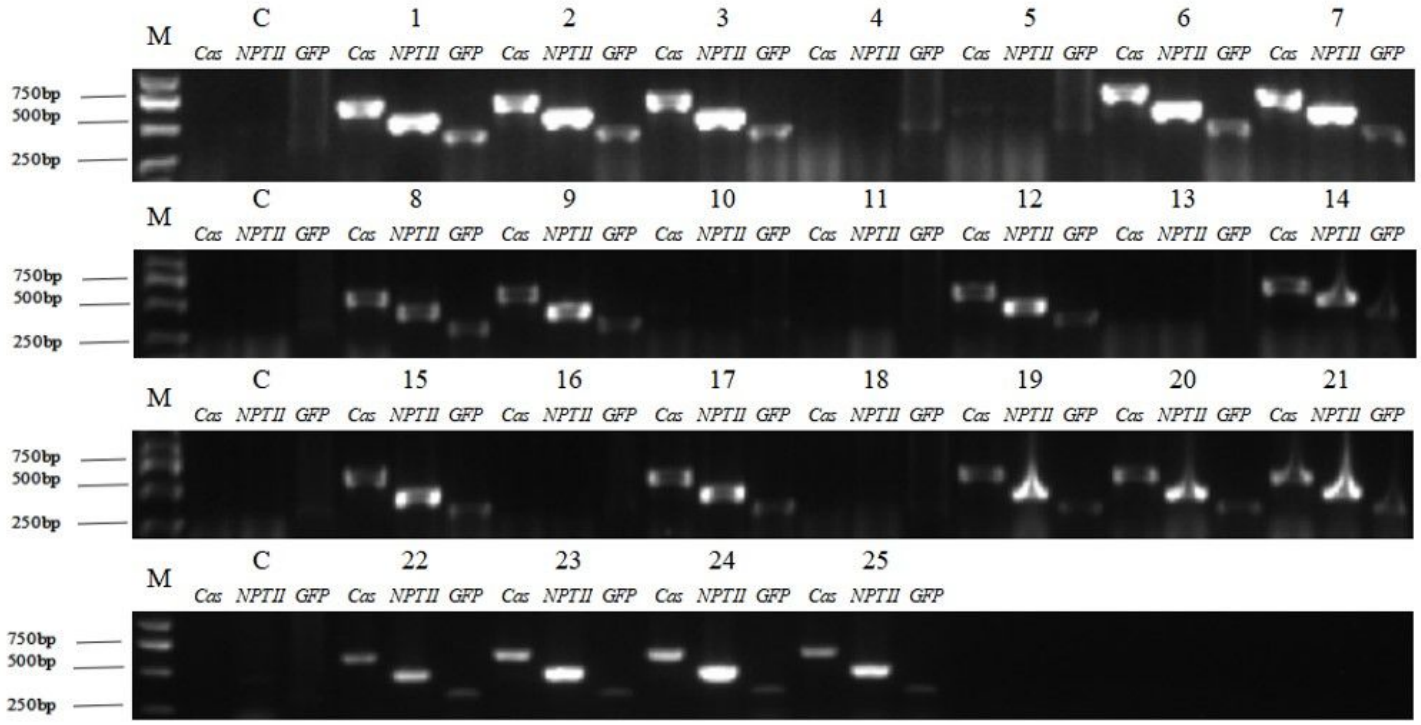


Figure 1

Agarose gel PCR products showed that *Cas9*, *NPTII* and *GFP* selected marker genes were amplified from independent transgenic lines, and the products were 692bp, 471bp and 334 bp. M= DL2000bp plus DNA marker, C= Non-transgenic control plant, Lane (1-25) = Transgenic plants

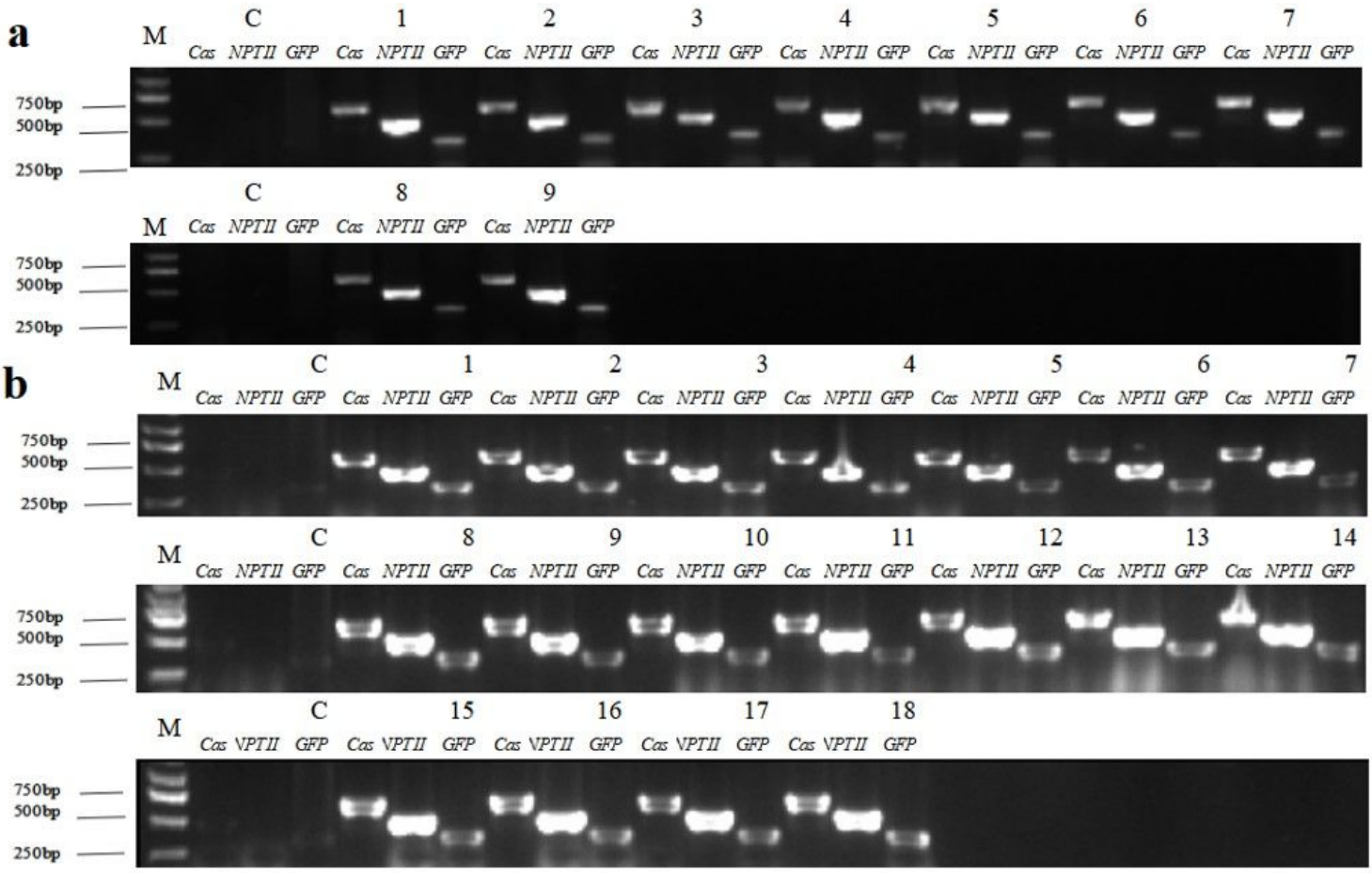


Figure 2

Agarose gel PCR products showed that *Cas9*, *NPTII* and *GFP* selected marker genes were amplified from independent transgenic lines, and the products were 692bp, 471bp and 334 bp. M= DL2000bp plus DNA marker, C= Non-transgenic control plant, Lane (1-25) = Transgenic plants

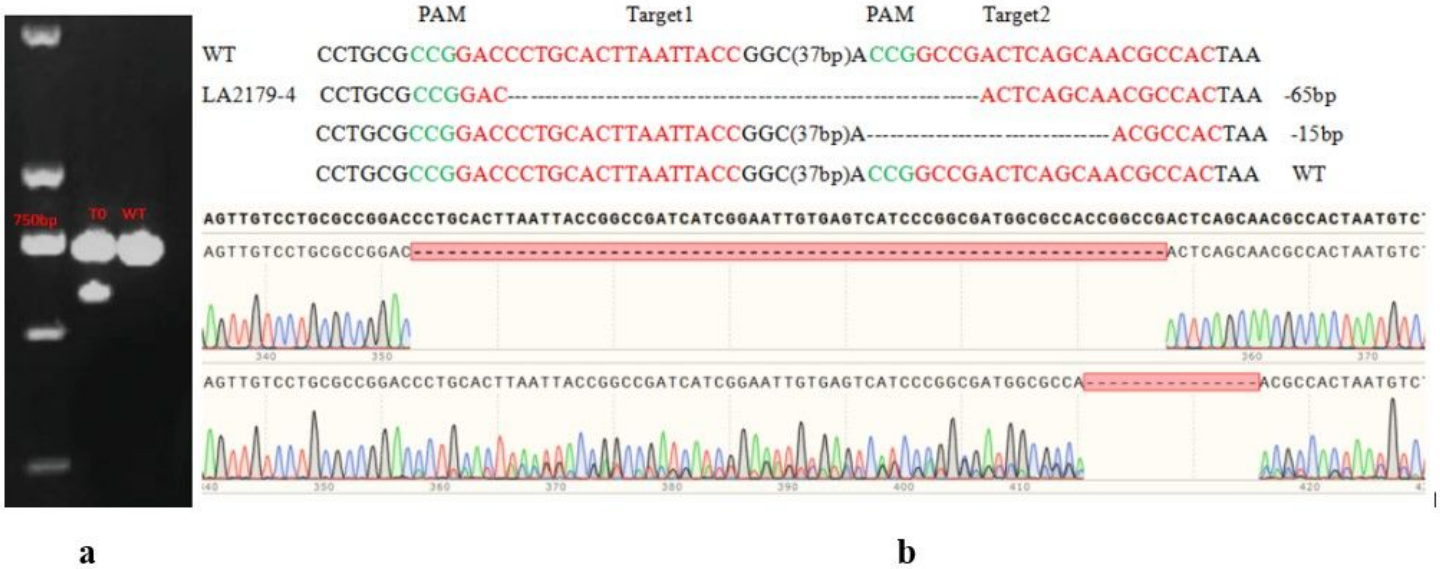


Figure 3

Solyc07g062270 Mutation type detection of transgenic line LA2179-4. a, mutant target agarose gel electrophoresis. b, Mutation type detected by Blunt vector.

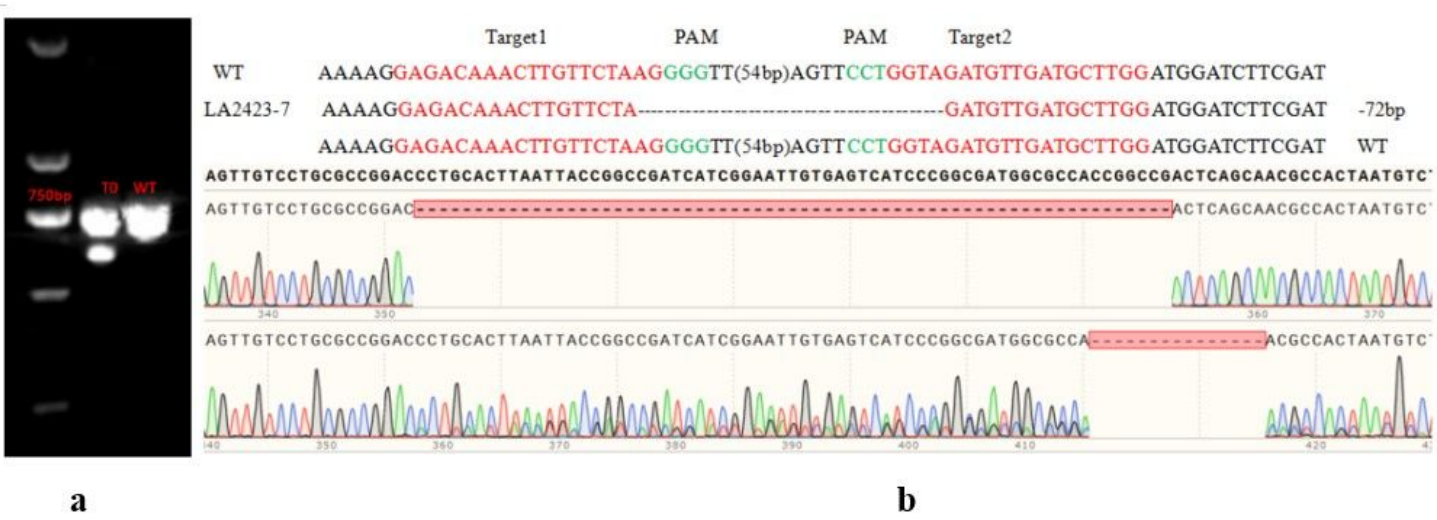


Figure 4

Solyc10g079830 Mutation type detection of transgenic line LA2423-7. a, mutant target agarose gel electrophoresis. b, mutation type detected by Blunt vector.