

Establishment of Agrobacterium-mediated genetic transformation and application of CRISPR/Cas9 gene-editing system to Chinese cabbage (*Brassica rapa* L. *ssp. pekinensis*)

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Research

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Abstract

Background: Chinese cabbage, belonging to *Brassica rapa* species, is an important vegetable in Eastern Asia. It is well known that Chinese cabbage is quite recalcitrant to genetic transformation and the transgenic frequency is generally low. The lack of an efficient and stable genetic transformation system for Chinese cabbage has largely limited related gene functional studies.

Results: In this study, we firstly developed a regeneration system for Chinese cabbage by optimizing numerous factors, with 93.50% regeneration rate. Based on this, a simple and efficient *Agrobacterium*-mediated genetic transformation method was established, without a pre-culture procedure and concentration adjustment of hormone and AgNO₃ in co-cultivation and selection media. Using this system, transformants could be obtained within 3.5 to 4.0 months. Average transformation frequency is up to 10.83%. Furthermore, using this transformation system, the CRISPR/Cas9 technology was successfully applied in Chinese cabbage by knocking out a self-incompatibility-related gene *SRK*. Gene sequencing analysis in the positive transgenic lines revealed various mutations, including deletions, insertions, and substitutions.

Conclusion: A simple, stable and efficient genetic transformation method was established for Chinese cabbage and successfully applied to the CRISPR/Cas9 system. The results of this study pave the way for further gene functional studies and genome editing in Chinese cabbage.

Background

Advances in genetics and genomics of *Brassica* crops have resulted in the need to have a better knowledge of its gene functions and genetic engineering. Reverse genetic approaches involving insertional mutagenesis, gene knockdown, or RNAi have relied on transformation technologies to elucidate gene functions. Trait improvement by genetic engineering and recent genome editing technology, which could shorten the duration of conventional breeding, is also based on gene transformation techniques. *Agrobacterium*-mediated transformation is the most widely used method for gene delivery to all six major economically *Brassica* species [1–4].

B. rapa species embrace various cultivars and are consumed throughout the world and grown for the production of vegetables, seed oil, fodder crops, and condiments. Chinese cabbage, the most important leafy vegetables in China, Korea, and other East Asian countries, provides health-promoting nutrients such as vitamins, glucosinolate, and carotenoids [5]. Genomic sequencing of Chinese cabbage as a model for the *B. rapa* A genome [6] has facilitated the genetic identification and cloning of candidate genes governing traits that are involved in plant architecture [7, 8], metabolites [9, 10], and biotic and abiotic stress tolerance [11, 12]. However, verification of gene functions revealing the genetic mechanisms of trait formation has remained stagnant due to the lack of an efficient genetic transformation system. *B. rapa* is widely known as the most recalcitrant species for genetic transformation among *Brassica* species, and this is due to its shoot regeneration capacity and sensitivity

of explants to *Agrobacterium* [13]. To a large extent, transformation efficiency is dependent on the genetic background of the *B. rapa* species [13, 14]. Various factors influencing *B. rapa* regeneration and *Agrobacterium*-mediated gene transformation have been optimized and discussed, including *Agrobacterium* strain, genotype, explant type, hormone concentration, and infection time [3, 15].

Recently, a limited number of studies on gene manipulation and trait improvement through transgenic in Chinese cabbage have been reported [16, 17]. A soft rot disease tolerance gene has been successfully transformed into Kenshin [18], a Chinese cabbage inbred line, as one of the parents for *B. rapa* reference genetic map construction [19]. A Lepidopteran insect pest resistance gene *cry1C Bt* with a transformation efficiency of 0.4–8.9% was introduced to a Chinese cabbage hybrid line [20]. Zang et al. generated transgenic Chinese cabbage lines with high levels of aliphatic glucosinolate compound by overexpressing three genes from *Arabidopsis*, namely, *MAM1*, *CYP79F1*, and *CYP83A1* [21]. Despite reports of successful transformations in a few of *B. rapa* subspecies, the transformation efficiency remains low, thereby preventing its application to gene editing.

CRISPR/Cas9 system can precisely and effectively edit target gene/sites in the genome and is easier to operate; therefore, it has been rapidly applied for genome editing and gene functional studies [22, 23]. Most recently, it also has been successfully utilized for targeted mutagenesis in various *Brassica* crops, including *B. napus* [24, 25, 26], *B. carinata* [27], and *B. oleracea* [28, 29]. In *B. rapa*, Xiong et al. (2019) reported the application of the CRISPR/Cas9 system to caixin (*B. rapa* ssp. *parachinensis*) as explants [30]. Jeong et al. (2019) generated early-flowering Chinese cabbage by introducing the targeted mutation into multiple *BraFLC* genes using the CRISPR system [31]. However, due to the lack of a stable genetic transformation system, the CRISPR/Cas9-mediated gene editing method has rarely been applied to Chinese cabbage to date yet. Additionally, most of the Chinese cabbage exhibits self-incompatibility (SI), which leads to difficulty in transgenic plant propagation. Thus, attempting to knock out SI-related genes using the CRISPR/Cas9 method allows the creation of Chinese cabbage self-compatibility lines.

In this study, we first optimized several factors affecting *in vitro* regeneration of Chinese cabbage, including explant type, sucrose concentration, hormone composition, and AgNO₃ concentration. The average regeneration rate was 93.5%, which guarantees the generation of putative transgenic plants. Subsequently, factors on *Agrobacterium*-mediated transformation efficiency have been investigated, including antibiotics concentration, pre-culture time, AS concentration, *Agrobacterium* strains, and plant hormone combination for rooting. Each appropriate factor was combined to verify the stability and reproducibility of the transgenic system, resulting in a 10.83% average transformation efficiency. Moreover, the high transformation efficiency and stability of this system we developed facilitate the application of the CRISPR/Cas9 technology to Chinese cabbage by successfully knocking out a *B. rapa* SI-related gene, *BrSRK*. This study may be utilized in functional genomic studies and advance the molecular improvement of traits in Chinese cabbage.

Materials And Methods

Overexpression vector construction and *Agrobacterium* strain

A Chinese cabbage orphan gene *BraA1000785* with unknown function identified from “Chiifu,” a material for *B. rapa* reference genomic sequencing, was used as target gene [32] for vector construction. The sequence of *BraA1000785* was isolated from a Chinese cabbage inbred line “C-24” and used as material for genetic transformation using gene-specific primers (Additional file 1 Table S1). The sequence of the 35S Promoter-MS (multi-cloning sites)-OCS terminator from a medium vector 35S-ASC was cloned and inserted into the binary vector pCAMBIA1305.1 that contained hygromycin phosphotransferase (HPT), neomycin phosphotransferase II (nptII) gene conferring kanamycin (Km) resistance, and GUS gene (referred to as pCAMBIA1305.1-35S). The *BraA1000785* gene was then infused into pCAMBIA1305.1-35S using an In-fusion HD cloning kit (Takara) (Additional file 2). The reconstructed *BraA1000785* overexpression vector was introduced into *A. tumefaciens* strain GV3101, LB4404, EHA105, and GA101 by the freeze-thaw method.

Media preparation

LB and MS media [33] were used in this study. The MS media consisted of 0.7% agarose, and its pH was adjusted to 5.8; it was autoclaved at 121 °C for 20 min. Acetosyringone (AS), silver nitrate (AgNO₃), and antibiotics (kanamycin, hygromycin, and timentin) were filtered and sterilized and added to the media that was cooled to 50–60 °C after autoclaving. Culture medium components for the transformation procedure are listed in Table 1.

Table 1
Media composition and procedure for Chinese cabbage genetic transformation

Procedure	Media composition	Period
Seed sterilization and seedling	M0 : 1/2MS	4days
<i>Agrobacterium</i> infection	DM : MS + 100 µM AS + 30 g/L sucrose (pH = 5.8)	15 min
Pre-cultivation	Mp : -	-
Co-cultivation	Mc : MS + 0.5 mg/L NAA + 4 mg/L 6-BA + 4 mg/L AgNO ₃ + 7 g/L agarose + 30 g/L sucrose + 100 µM AS (pH = 5.8)	2days
Selection	Ms : MS + 0.5 mg/L NAA + 4 mg/L 6-BA + 4 mg/L AgNO ₃ + 7 g/L agarose + 30 g/L sucrose + 200 mg/L TMT + 25 mg/L Hyg (pH = 5.8)	14days
Rooting cultivation	Mr : MS + 1 mg/L NAA /IBA + 200 mg/L TMT + 7 g/L agarose + 30 g/L sucrose (pH = 5.8)	30days
Planting	-	20days

Surface seed sterilization

The seeds of "C-24" with roughly the same size were surface-sterilized by soaking and shaking the seeds with 75% ethanol for 30 s and then immersed in 10% sodium hypochlorite for 1 min. Then, the seeds were submerged in 2% sodium hypochlorite for 15 min, followed by rinsing 4–5 times with sterilized ddH₂O. Approximately 20 seeds were placed in a 5.5 × 9 cm glass culture bottle, each containing 30 mL ½ MS basal medium with 20 g/L sucrose and 0.7 g/L agarose (pH 5.8) and maintained at 25 ± 2°C in 16 h/8 h (light/dark) photoperiods for germination.

Optimization of plant regeneration efficiency conditions

To increase plant regeneration efficiency, the following single factors were optimized based on a preset general regeneration media (MS + 30 g/L sucrose + 7 g/L agarose + 0.5 mg/L NAA + 4 mg/L 6-BA + 4 mg/L AgNO₃, pH 5.8), explant type (hypocotyl, cotyledon-petiole, and root), sucrose concentration (0, 10, 20, 30, 40, and 50 g/L), 6-BA concentration (0, 2, 4, 6, 8, and 10 mg/L), and AgNO₃ concentration (0, 2, 4, 6, and 8 mg/L). Thirty-six explants were used for each treatment with three replicates. The rate of adventitious shoots was evaluated after cultivation for 20 days. The optimal conditions with the appropriated factor combination were selected for subsequent transformation experiments.

Optimization of Agrobacterium-mediated transformation factors

To improve genetic transformation efficiency, single-factor experiments were designed to screen optimal conditions. The effect of Hyg was first evaluated because it is critical for resistant shoot screening. Based on the previous studies and the plant regeneration conditions we established, the preset transformation conditions were as follows: pre-cultivation for one day, OD₆₀₀ value = 0.5, infection 15 min, and co-cultivation for two days. The selection medium was preset as MS + 30 g/L sucrose + 7 g/L agarose + 0.5 mg/L NAA + 4 mg/L 6-BA + 4 mg/L AgNO₃ + 200 mg/L TMT + 20 mg/L Hyg (pH 5.8, 25 ± 2°C, 16 h/d). The root induction medium was preset as MS + 1.5 mg/L IBA + 1.5 mg/L NAA + 200 mg/L TMT.

Different concentrations of Hyg (0, 5, 10, and 15 mg/L) were first screened to determine the threshold concentration for untransformed explant elimination. Two replicates with 100 untransformed explants each were used. After that, Hyg concentrations (10, 15, 20, and 25 mg/L) were evaluated for resistant shoot differentiation using 50 explants with two replicates. Other factors, including TMT concentration (0, 100, 150, and 200 mg/L), AS concentration (50, 100, 150, and 200 µM), pre-culture duration (0, 1, 2, and 3 days), *Agrobacterium* strains (GV3101, LB4404, EHA105, and GA101), and suitable hormone proportion for root induction were optimized.

Agrobacterium-mediated plant transformation

The plasmid of pCAMBIA1305.1-35S-BraA1000785 was transformed into the *Agrobacterium* strains and cultured in 1 mL of LB medium containing 50 mg/L kanamycin and 50 mg/L gentamicin at 28°C overnight. Positive transformation was confirmed by PCR using BraA1000785-F/R primers (Additional file 1 Table S1). A transformant was picked and inoculated in LB liquid medium with an agitation of 220 rpm for 12 h until OD₆₀₀ was 0.5. Then, a 2-mL aliquot was collected and centrifuged at 6,000 rpm for 10 min.

The bacterial precipitate was suspended in an equal volume of DM medium (MS medium with 100 µm/L AS, Table 1) and repeated twice. Finally, the bacterial solution was diluted 10-fold with DM medium for explant infection.

The explants were immersed in an infection solution of *Agrobacterium* for 15 min (gently shaking at 5-min interval). After infection, the bacterial suspensions were removed, and the explants were blotted dry on sterilized paper towels and subsequently cultured on co-cultivation medium (Table 1) in a controlled growth room at dark conditions. After two days of co-cultivation, the explants were transferred onto a selection medium (Table 1) for shoot induction at 14-day intervals until resistant shoots developed. Hygromycin-resistant shoots 2–3 cm in length were cut and transferred to a root-induction medium (Table 1). Regenerated plantlets with well-developed roots were thoroughly washed in tap water to remove Phytagar™ and then transferred to pots and grown for 25 days in the same environmentally-controlled growth chamber as described above. The acclimatized plantlets were then transplanted to the greenhouse.

The optimal conditions and the entire procedure for transformation are presented in Table 1 and Fig. 1. To test the stability of the system, three replicate experiments were conducted using the established optimal conditions.

Confirmation of transgenic plants

Polymerase chain reaction (PCR) analysis

The primer pairs of the Hgy gene were used for DNA amplification to confirm the presence of the transgene in putative transgenic plants (Additional file 1 Table S2). Total genomic DNA was isolated from young leaves of greenhouse-grown plants using a modified CTAB method [34]. Untransformed wild-type plants “C-24” and plasmid vector were used as negative and positive controls, respectively.

The total volume of the PCR reaction mixture was 20 mL and comprised 10 mL of the PCR mix (Takara, ExTaq), 6 mL of distilled sterile water, 2 µL of genomic DNA, and 1 mL of each primer. The PCR conditions were as follows: an initial denaturation step of 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 55 °C for 30 s, and 72°C for 30 s; and a final extension of 72 °C for 5 min. The PCR products were resolved by 1% agarose gel electrophoresis and viewed under a UV transilluminator.

Histochemical GUS assay

Histochemical GUS assay was used to further identify putative transgenic plants. Wild-type plants were used as control. All the leaves were rinsed thrice in double-distilled water and stained in a 2-mM X-Gluc (5-bromo-4-chloro-3-indolyl-D-glucuronide and cyclohexylammonium salt) solution overnight at 37 °C. After staining, the leaves were washed in 70% ethanol until no visible chlorophyll remained. Transient GUS expression was documented by recording the number of explants with at least one blue focus.

Southern blot analysis

Southern blot hybridization was conducted to determine the copy number of the transgene in the transgenic plants. The probe was prepared by PCR amplification with the primers designed for *BraA1000785* (Additional file 1 Table S3) using a PCR DIG probe synthesis kit (Roche, 11636090910). The genomic DNA of wild-type “C-24” and PCR products served as negative and positive controls, respectively. Genomic DNA was digested with *Hind*III overnight at 37 °C. The digested DNA was extracted with an equal volume phenol/chloroform and separated by electrophoresis on a 0.7% agarose gel. Then, the DNA was blotted onto nylon membrane. The subsequent hybridization was performed according to DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Cat. No. 11585614910).

Statistical analysis

In this study, the SPSS statistical analysis software was used for ANOVA analysis, and significant differences were set at $P < 0.05$.

The following parameters were calculated as follows: Adventitious shoot induction rate = Number of explants forming adventitious shoot/Total explants \times 100%; Resistant calli induction rate = Number of calli-induced explants/Total number of explants \times 100%; Resistant shoot induction rate = Number of resistant shoot-induced explants/Total number of explants \times 100%; Transformation efficiency = Total number of positive transformants/Total number of infectious explants \times 100%; Contamination rate = Number of contaminated explants/Total number of explants \times 100%.

Construction of CRISPR/Cas9 targeting vector and transformation in Chinese cabbage

The SI-related gene *BrSRK* was selected as target gene for CRISPR/Cas9-induced gene editing. The full-length gene sequence of *BrSRK* was obtained from Chinese cabbage “C-24”. The *BrSRK* gene sequence was scanned for PAM sequences on both genomic DNA strands. Potential targeted knockout sites were searched using the online tools Cas-OFFinder (<http://www.rgenome.net/cas-offinder>) and TargetFinder (<http://www.targetfinder.flycrispr.neuro.brown.edu/>). The sgRNA was designed using a CRISPR online tool (<http://crispr.mit.edu/>) using the following standards: GC content: 40–70%, to be located within an exon and sequence length of around 20 nt, avoid ending with the TTTT sequence. The sgRNA sequences were cloned into spCas9gRNA vector construct kit (VK005-101) and then transformed into GV3101 for “C-24” transformation.

Results

Plant regeneration conditions

Explant type

In this study, to evaluate the regeneration efficiency of different explant types, adventitious bud differentiation of hypocotyls, cotyledon-petioles, and roots cultured for 20 days was assessed. The

results showed that the differentiation rate of adventitious buds of three explants was significantly different ($P < 0.05$) (Fig. 2a). The average differentiation rate of cotyledon-petioles was 81.15%, whereas the cultured hypocotyls and roots could not form regenerative shoots. In addition, the wound of cotyledon-petioles explants began to swell after two days and gradually formed calli. The regenerated shoots appeared on the 7th day, and more than 80% of the explants formed regenerated buds at about 20 days (Fig. 3a). Hypocotyl explants expanded to form calli only (Fig. 3b). During culture, root explants differentiated into numerous robust lateral roots in the medium (Fig. 3c).

The effect of sucrose concentrations

Six levels of sucrose concentrations were measured (0, 10, 20, 30, 40, and 50 g/L). The results showed that the rate of adventitious bud differentiation was highest using a sucrose concentration of 30 g/L (Fig. 2b). Neither higher (40 and 50 g/L) nor lower concentration (10 g/L) led to chlorosis and wilting of explants.

Optimal concentration of AgNO_3

The regeneration of explants was observed after 20 days of culture under five levels of AgNO_3 concentration (Fig. 2c). The differentiation rate of adventitious buds under five concentration treatments was significantly different. The explants showed the highest regeneration rate (87.5%) at an AgNO_3 concentration of 4 mg/L. The regeneration rate was also relatively higher at 6 and 8 mg/L AgNO_3 ; however, numerous abnormal shoots formed.

Determination of 6-BA concentration

The effects of hormones on shoot differentiation were measured using different concentrations of 6-BA (0, 2, 4, 6, 8, and 10 mg/L) combined with 0.5 mg/L NAA. The adventitious bud differentiation rate was 0 when 6-BA was not added, and numerous roots were induced in the presence of NAA. When 6-BA concentration was increased to 4 mg/L, the highest adventitious bud differentiation rate was observed (79.2%) (Fig. 2d). Therefore, the optimal concentration of 6-BA, i.e., 4 mg/L, was selected for further genetic transformation.

Optimization of system stability

Three replicate experiments were conducted with the established optimal conditions (MS + 30 g/L sucrose + 7 g/L agarose + 0.5 mg/L NAA + 4 mg/L AgNO_3 + 4 mg/L 6-BA, pH 5.8). The number of explants forming regenerating buds was counted within 20 days to calculate the adventitious bud differentiation rate. Cotyledon explants showed a higher rate of adventitious bud differentiation in three replicates, which were 91.5%, 95%, and 94% (Table 2), with an average of 93.5%. These results demonstrate the stability and efficiency of the optimized *in vitro* regeneration system.

Table 2
Adventitious shoot induction rates under optimized system in repeatability testing

Replication	Total explant number	Percentage of in vitro shoot regeneraton (%)
1	200	91.50
2	200	95.00
3	200	94.00
Average		93.50

Factors influencing on *Agrobacterium*-mediated transformation

Optimization of Hyg concentration

Appropriate Hyg concentrations may promote the growth of positively transformed plants and inhibit untransformed plants. Untransformed explants were cultured in the optimized shoot induction medium containing different Hyg concentrations (0, 5, 10, and 15 mg/L) for threshold value determination. The results showed that 5 mg/L Hyg could inhibit the adventitious bud differentiation, and Hyg at 10 and 15 mg/L induced all explants to die (Table 3). Thus, 10 mg/L Hyg was selected as the threshold value for genetic transformation screening.

Hyg concentrations were set to 10, 15, 20, and 25 mg/L to measure its effects on transformation efficiency. The results showed that with increasing Hyg concentration, the differentiation rate and transformation efficiency of resistant buds decreased, but there was no significant difference among Hyg concentrations of 15, 20, and 25 mg/L (Table 3). During the detection of resistant buds, false-positive plants were detected using 20 mg/L Hyg. Therefore, 25 mg/L Hyg was determined as the optimal concentration for genetic transformation that eliminates false-positive plantlets.

Optimization of termitomycin (TMT) concentration

In this study, TMT was selected as the antibiotic for inhibiting the growth of *Agrobacterium*. The co-cultured explants were cultured on selection media supplemented with different concentrations of TMT (0, 100, 150, and 200 mg/L), and the contamination rate was calculated. All explants were contaminated with *Agrobacterium* when TMT was not added. When the concentration of TMT was increased to 100 mg/L and 150 mg/L, some of the explants were contaminated. However, the contamination rate was 0% when the concentration of TMT was increased to 200 mg/L (Table 3). In addition, the differentiation of resistant buds did not significantly differ with increasing TMT concentrations.

AS concentration

The effects of different AS concentrations that were added to the DM and Mc media were measured on *Agrobacterium*-mediated transformation. As shown in Table 3, the highest mean percentage of resistant calli and transformation efficiency was 22.33% and 8.57% with 100 μ M AS. The transformation efficiency decreased to 0% with 150 and 200 μ M AS.

Optimization of pre-culture time

Explants treated with different pre-culture times showed different differentiation rates in the resistant shoot selection medium. Without pre-incubation, the mean resistant calli rate, mean resistant shoot induction rate, and transformation efficiency were 11%, 3.25%, and 3%, respectively. However, the rates decreased to 0% after pre-culture. Therefore, the Chinese cabbage “C-24” did not need to be pre-cultured for transformation using cotyledon-petiole as explants (Table 3).

Screening of *Agrobacterium* strains

The ability of four *A. tumefaciens* strains AGL1, EHA105, GV3101, and LB4404 harboring the expression plasmid pCAMBIA1305.1-35S-BraA1000785 for transformation was evaluated. The mean transformation efficiency of GV3101 was highest at 9.33% (Table 3). However, the resistant calli and shoot induction rate and transformation efficiency of AGL1, EHA105, and LB4404 were significantly lower than GV3101. These results indicate that *Agrobacterium* GV3101 is the most suitable strain for the genetic transformation of explants.

Root induction of resistant buds

When the resistant buds had extended to 2–3 cm in length, the shoots were excised from the base and inoculated into the optimal rooting medium. To select the optimal condition for root induction, different ratios of plant growth regulators were adjusted. Figure 3a shows that resistant shoots did not differentiate into robust adventitious roots but instead formed chunks of calli in culture medium supplemented with 1.5 mg/L IBA + 1.5 mg/L NAA. When only 1.5 mg/L NAA was added to the culture medium, some adventitious roots were found, but some callus still formed (Fig. 3b). To avoid the formation of calli, plant hormone concentrations were again reduced. Two kinds of rooting media with 1 mg/L NAA or 1 mg/L IBA were used. Adventitious roots were more strongly induced by 1 mg/L NAA (Fig. 3c) rooting medium than 1 mg/L IBA (Fig. 3d), whereas all plants survived after transplanting into soil.

Stability testing of the transformation system

To test the stability of the transformation system of “C-24,” three experimental replicates were conducted using the optimal combination of factors for genetic transformation. The final media composition and procedure scheme are shown in Table 1 and Fig. 1. The results showed that the system was stable, and the mean transformation efficiency was 10.83% (Table 4). In addition, it took approximately 3.5–4.0 months for transformants to form using this system.

Table 4
Repeatability test of transformation system

Replication	Number of explants	Transformation efficiency (%)
1	112	11.61
2	84	10.71
3	59	10.16
Average		10.83

Confirmation of transgenic plants

PCR amplification was performed using specific primers for the HPT gene. The PCR product of the HPT gene (1088 bp) was amplified from 13, 10, and 7 lines out of 15, 11, and 7 resistant plants in three replicated experiments of the stability test (Fig. 5).

GUS histochemical staining was further used to verify the positive transformants. The leaves of candidate positive transgenic plants confirmed by PCR reaction were collected for GUS staining. As shown in Fig. 6, wild-type “C-24” and false-positive plants without the HPT gene did not exhibit any blue GUS staining, whereas GUS expression was observed in HPT gene-induced plants.

To further confirm the integration of the target gene into the Chinese cabbage genome and determine copy number, southern blot analysis was performed using both PCR and GUS-positive transgenic plants. The results showed that two copies of targeted gene existed in the wild-type (Fig. 7). Five transgenic lines have hybridization signals and occurred as multiple copies (Lane 2, 3, 5, 6, 7 in Fig. 7), indicating the insertion of T-DNA into the “C-24” genome.

Mutation generation in the Chinese cabbage transformation system using the CRISPR/Cas9

To establish the CRISPR/Cas9 gene-editing system using the Chinese cabbage genetic transformation method, we selected an SI gene *BrSRK* as target gene. We chose the spCas9gRNA vector to generate the Cas9-*BrSRK* plasmid construct, which used the *AtU6* promoter to drive sgRNA expression. The two target sequences are shown in Fig. 8.

A total of 250 explants of “C-24” were used for Cas9-*BrSRK* transformation following the procedure we developed. Twenty-one Hyg-resistant transgenic shoots were obtained. To analyze the mutation type in target sites in resistant plants, the specific primers across the target sequence were designed to amplify target fragment for Sanger sequencing. Three out of five resistant transgenic lines exhibited mutations. Out of nine clones from BrL1, one clone exhibited a 7-bp deletion in target 1 and one clone presented a 1-bp deletion in target 2 (Fig. 8). Among 10 clones from BrL2, 6 clones showed a 10-bp deletion in target 1, and 1 clone has a 1-bp insertion within target 2 (+ 1G) 3-bp upstream of PAM (Fig. 8). However, in BrL3,

target 2 was not edited, and four clones showed a 10-bp deletion in target 1. The successful editing of the *BrSRK* gene would be helpful in generating self-compatibility lines in Chinese cabbage.

Discussion

Stable and high-efficiency transformations have been conducted in a variety of *Brassica* crops, particularly *B. napus* and *B. oleracea* [2, 35, 36]. This study identified various factors that influence *Agrobacterium*-mediated plant transformation in Chinese cabbage. An efficient system for plant *in vitro* regeneration is the requirement for genetic transformation of Chinese cabbage. Previous studies have shown that regeneration frequencies vary with genotype. Among 123 genotypes of Chinese cabbage, a large variation in regeneration frequency, ranging from 0–95%, was observed [14]. Hypocotyls are usually selected as explants for regeneration and transformation in *Brassica* crops [3, 35, 37]. However, in this study, hypocotyl explants expanded to form calli only. The use of AgNO₃ as a potential ethylene inhibitor could promote adventitious bud formation in tissue culture. It has been commonly used in *Brassica* spp. transformation [38, 39, 40]. Here, the addition of 4 mg/L AgNO₃ resulted in a 20% increase in shoot regeneration frequency. As the concentration was increased to > 6 mg/L, shoot regeneration frequency dramatically decreased and readily caused vitrification and tissue necrosis, which was also described by Khan et al. (2003) in *B. napus* [41]. Explants with high levels of auxin followed by treatment with cytokinin are essential for shoot regeneration [42]. The highest frequency of shoot regeneration from cotyledon explants (80%) was observed using 4 mg/L 6BA along with 0.5 mg/L NAA. A much higher concentration of 6 BA (8 mg/L) has been used in *B. napus*, resulting in 100% shoot regeneration frequency [43].

The transformation of Chinese cabbage is generally difficult (transformation rate: <1%) [15]. Based on the high regeneration system of “C-24” explants, we investigated numerous factors influencing *A. tumefaciens*-mediated transformation. Genotype “C-24” is sensitive to Hgy, and 5 mg/L Hyg could eliminate most of the nontransformants. This study also determined that 10 mg/L Hyg was the threshold value for screening transgenic plants, which is concordant to the findings of previous studies [3, 18]. In our experiments, Hyg concentration was increased to 25 as a selection value to avoid false positive plants. Different *A. tumefaciens* strains have been used in *B. rapa* transformation, including GV3101 [21], AGL1 [40], and LBA4404 [15, 44]. LBA4404 has been shown to be the optimal one with high transformation efficiency [38]. In our study, only strain GV3101 was determined to be suitable for genetic transformation of explants. However, this comparison is probably genotype-dependent. AS is an effective phenolic compounds for *Agrobacterium*-mediated transformation that induces the virulence of the bacterium [45]. It has been commonly used in bacteria cultivation process at a concentration of ~ 200 µM [3, 15]. High transformation efficiency was observed with 100 µM AS added in DM and Mc Medium. In this study, a large number of calli was formed from the resistant buds, which may be caused by the endogenous hormones change in the transformed plants. Thus, a lower hormone concentration (1 mg/L NAA or 1 mg/L IBA) is effective for root formation.

Genetically, it has been accepted that the pre-culture of explants promotes cell division and improves transformation rates [46, 47]. Numerous studies have shown that pre-culturing is essential for genetic transformation in *Brassica* species [20, 36, 48]. A previous study demonstrated that pre-cultured hypocotyls prior to exposure to *Agrobacterium* show increased shoot regeneration rates [3]. However, our system did not require a pre-culture step, thereby would simplify and shorten the transformation procedure. In addition, the NAA concentration used in the pre-culture stage widely varies from $0.1 \text{ mg}\cdot\text{L}^{-1}$ [18, 49] to $3 \text{ mg}\cdot\text{L}^{-1}$ [3, 44]. Omitting the pre-culture step thus indicates that there is no need to adjust NAA concentrations. Additionally, the concentration of MC and Mp composition is identical in this transformation system (MS + 0.5 mg/L NAA + 5 mg/L 6-BA + 4 mg/L AgNO_3 + 7 g/L agarose + 30 g/L sucrose), which guarantees the simplicity and rapidity of our experiment procedure.

An increase in information relating to the function of genes and the development of plant transformation methods may facilitate genetic improvement of crops, either as a genetically modified (GM) product or by conventional breeding. To date, *B. napus* is the only *Brassica* crop that has received GM commercial regulatory approval [50]. The birth of the CRISPR/Cas 9 editing system is a simple and inexpensive technology that has been widely applied to basic gene functional studies, particularly crop improvement. The offspring of crops edited by CRISPR/Cas 9 could be considered as non-genetic-modified (non-GM) products and may be helpful for improving agricultural productivity and environmental friendliness. Due to a lack of genetic transformation, no GM or non-GM using the CRISPR/Cas 9 system Chinese cabbage has been obtained. In this study, we established a simple and stable genetic transformation system and successfully applied the CRISPR/Cas 9 technique in Chinese cabbage by editing an SI gene, possibly accelerating the studies on gene function and trait improvement.

Conclusions

A simple, stable and high efficient *Agrobacterium*-mediated genetic transformation method for Chinese cabbage was developed, by optimizing numerous factors influencing on regeneration capacity and transformation. The advantage of the method is no need for pre-culture and the concentration of hormone, AgNO_3 , sucrose and agarose in Mc and Ms is identical. Positive transgenic plants could be achieved within 3.5 to 4.0 month. A high regeneration and transformation efficiency of 93.5% and 10.83% was obtained, respectively. Additionally, based on this method, CRIPSPR/Cas9 technology was successfully applied on Chinese cabbage by editing a SI related gene.

Table 3
Factors influencing on Agrobacterium-mediated transformation in Chinese cabbage

Factors	Total explant number	Percentage of resistant calus (%)	Percentage of resistant shoot(%)	Percentage of transformation efficiency(%) [□]	Percentage of pollution explant(%)
Concentration of Hyg (mg/L)					
10	50		9 ± 1 ^a	5 ± 1 ^a	
15	50		7 ± 1 ^{fe}	3 ± 1 ^b	
20	50		5 ± 1 ^c	2 ± 1 ^b	
25	50		2 ^d	2 ^b	
Concentration of TMT (mg/L)					
0	100		0 ^c		100 ^{a□□}
100	100		3.5 ± 0.5 ^a		8.5 ± 1.5 ^{b□□}
150	100		2.5 ± 0.5 ^b		3 ± 1 ^c
200	100		2 ± 0 ^b		0 ^{d□□}
The concentration of AS(uM)					
50	66	8 ± 0.97 ^b		0.46 ± 0.46 ^b	
100	47	22.33 ± 2.39 ^a		8.57 ± 1.10 ^a	
150	54	12.42 ± 3.71 ^b		0 ^b	
200	58	5.95 ± 1.19 ^b		0 ^b	
Time of per-culture					
0	200	11 ± 3 ^a	3.25 ± 0.75 ^a	3 ± 0.5 ^{a□□}	
1	200	4.5 ± 0.5 ^b	0.75 ± 0.25 ^b	1.25 ± 0.25 ^{b□□}	

Factors	Total explant number	Percentage of resistant calus (%)	Percentage of resistant shoot(%)	Percentage of transformation efficiency(%) [‡]	Percentage of pollution explant(%)
2	200	1.75 ± 0.25 ^{bc}	0 ^c	0 ^c	
3	200	0 ^c	0 ^c	0 ^c	
Agrobacterium strain					
GV3101	200	20.00 ± 2.18 ^a		9.33 ± 0.44 ^a	
EHA105	200	7.17 ± 0.44 ^b		0.83 ± 0.33 ^b	
LB4404	200	2.50 ± 1.04 ^c		0.17 ± 0.17 ^b	
GA101	200	0.17 ± 0.17 ^d		0 ^b	

Abbreviations

MS Murashige and Skoog

AS Acetosyringone

6-BA N6-benzyladenine

NAA Naphthalene acetic acid

Hyg Hygromycin phosphotransferase

TMT Termitomycin

GUS beta-glucuronidase

Declarations

Author contribution statement

X Li and H Li participated in the experiment and wrote the manuscript. Y Zhao carried out the genetic transformation. P Zong participated in the experiments for optimizing factors on tissue regeneration. Z Zhan designed the CRISPR/Cas 9 experiment. Z Piao conceived the project and designed the research.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figures

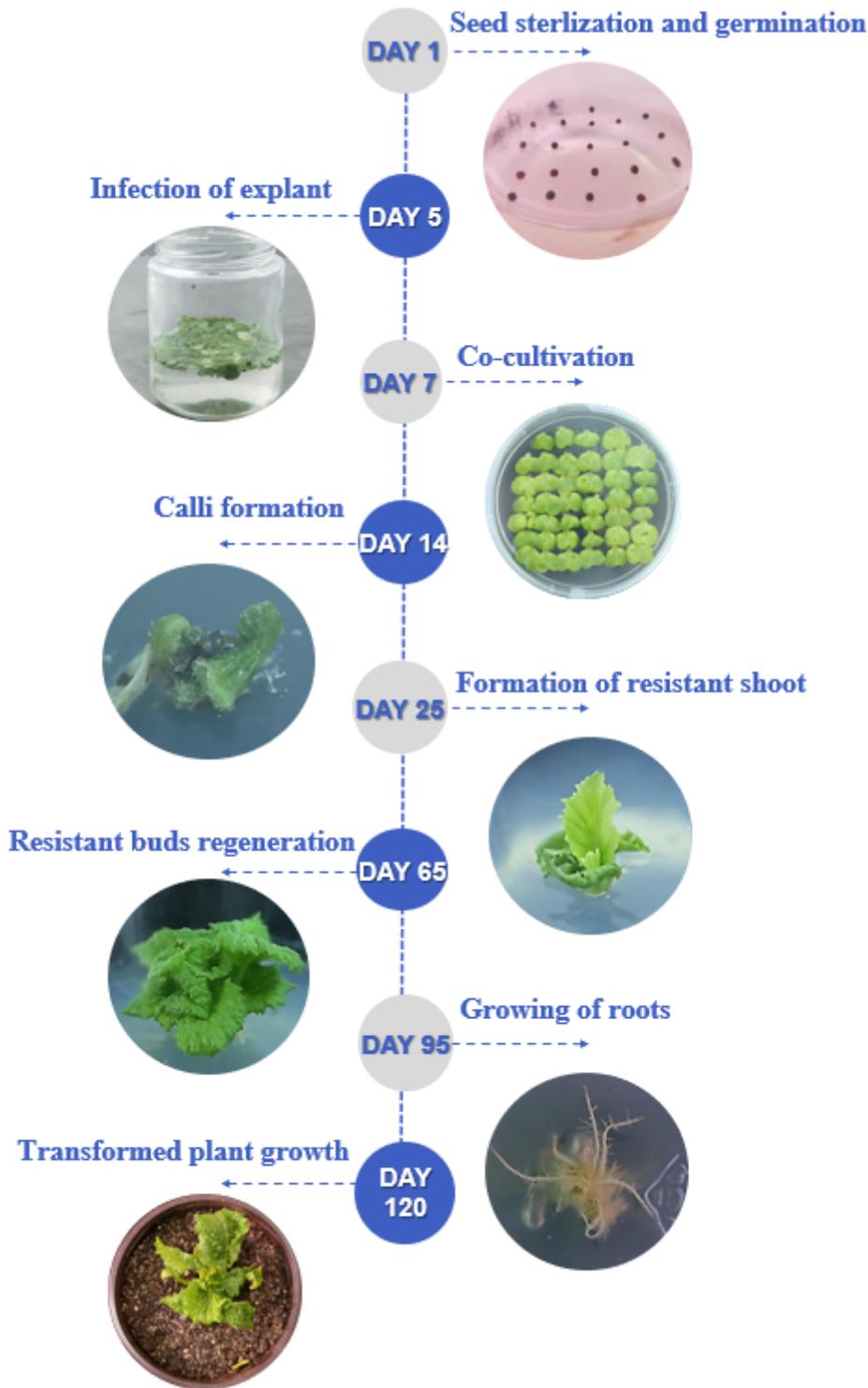
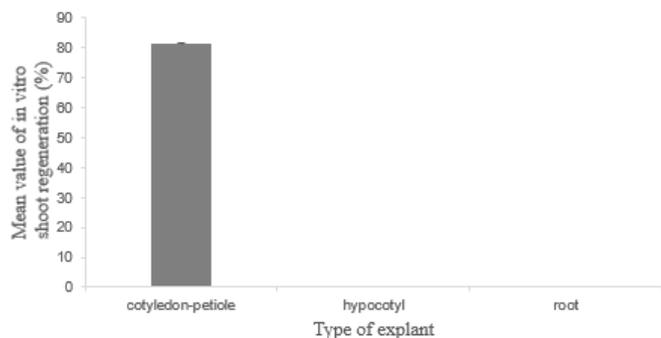


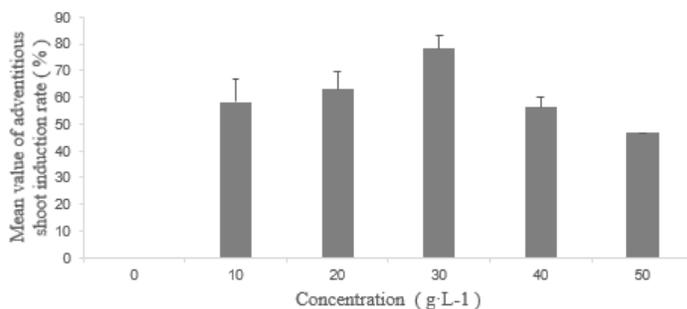
Figure 1

Overview of the process for Agrobacterium-mediated genetic transformation in Chinese cabbage, including seed sterilization and germination in M0, infection of explants using DM, co-cultivation in Mc, calli formation, formation of resistant shoot in Ms,, resistant buds regeneration, growing of roots and transformed plant growth in soil. The whole procedure spend 3.5-4.0 month.

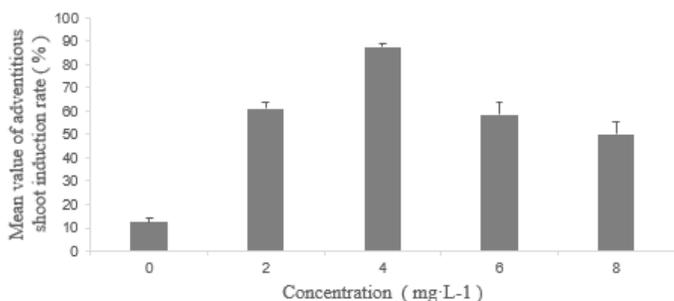
A Effect of the explant type on shoot regeneration in vitro



B Effect of different concentrations of sucrose on adventitious shoot induction rates



C Effect of different concentrations of AgNO₃ on adventitious shoot induction rates



D Effect of different concentrations of 6-BA on adventitious shoot induction rates

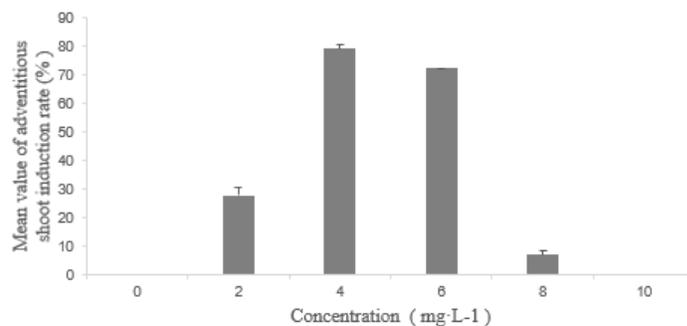


Figure 2

Factors affecting shoot regeneration efficiency of Chinese cabbage. A. explants type; B. sucrose concentration; C. different concentration of AgNO₃; D. different concentration of 6-BA

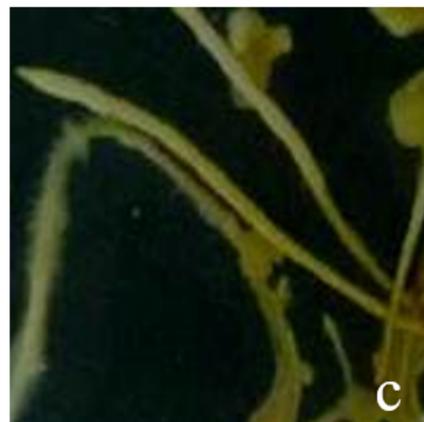
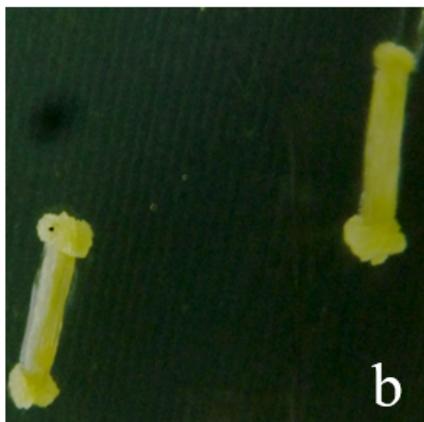


Figure 3

Regeneration of different types of explants. a. adventitious shoot inducing by cotyledon-petiole; b. calli formation by using hypocotyl explants; c. adventitious root formation by using root explants

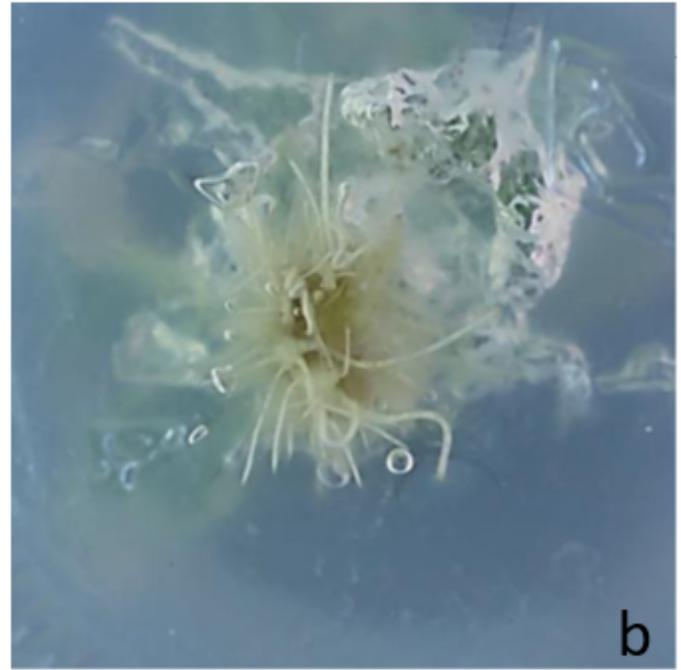


Figure 4

Different hormone concentration effects on root induction from resistant shoots. a. calli formation induced by using both NAA and IBA; b. calli formation induced by 1.5mg/L NAA; c. Adventitious roots induced by using 1mg/L NAA; d. Adventitious roots induced by using 1mg/L IBA

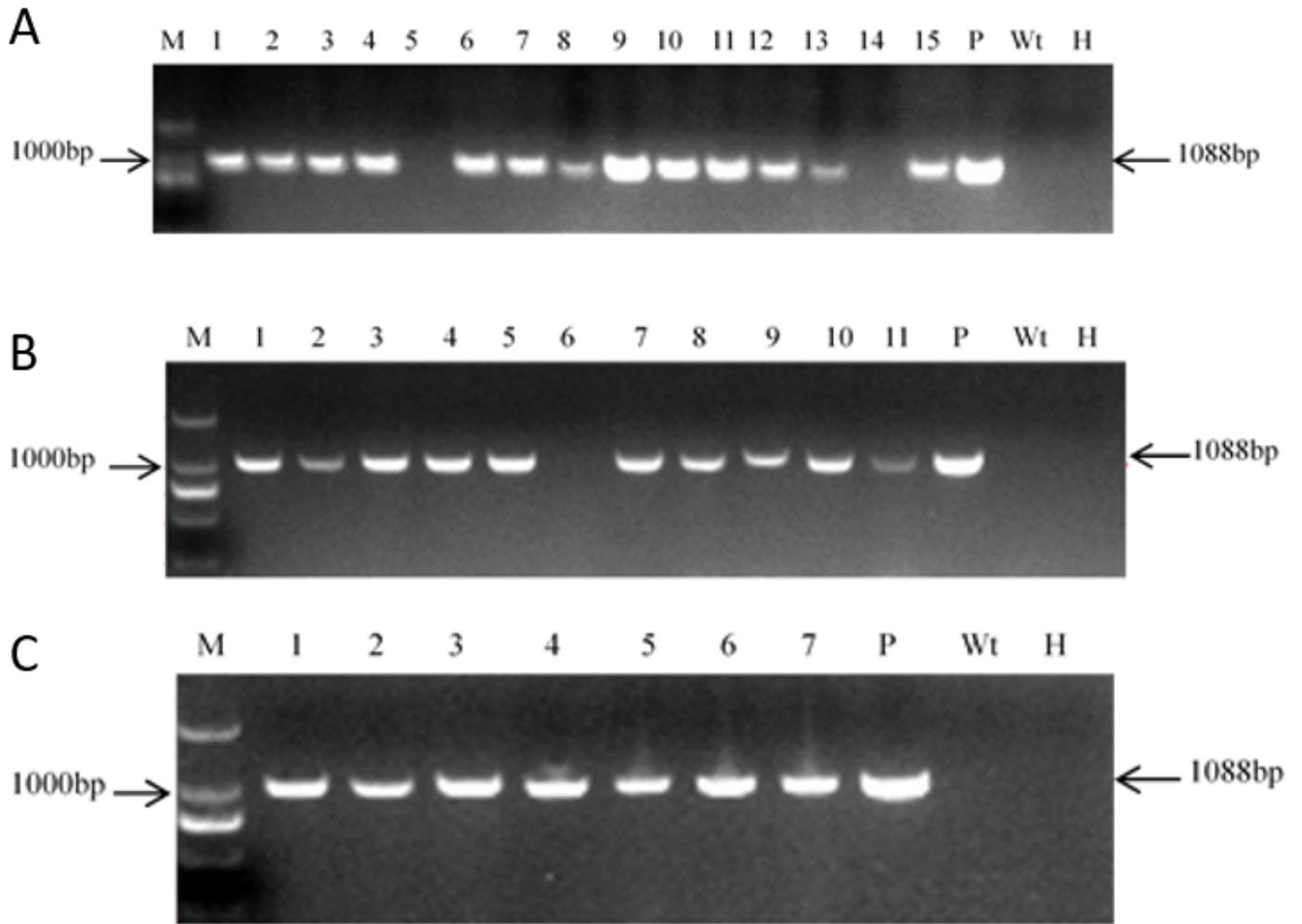


Figure 5

PCR analysis of transgenic plants using Hgy gene. A. 15 transgenic lines are detected in replication 1; B. 11 transgenic lines are shown in replication 2; C. 7 transgenic lines are shown in replication 3; Lane M, DNA marker; Lane W, wild-type "C-24"; Lane P, plasmid control; Lane H, ddH₂O

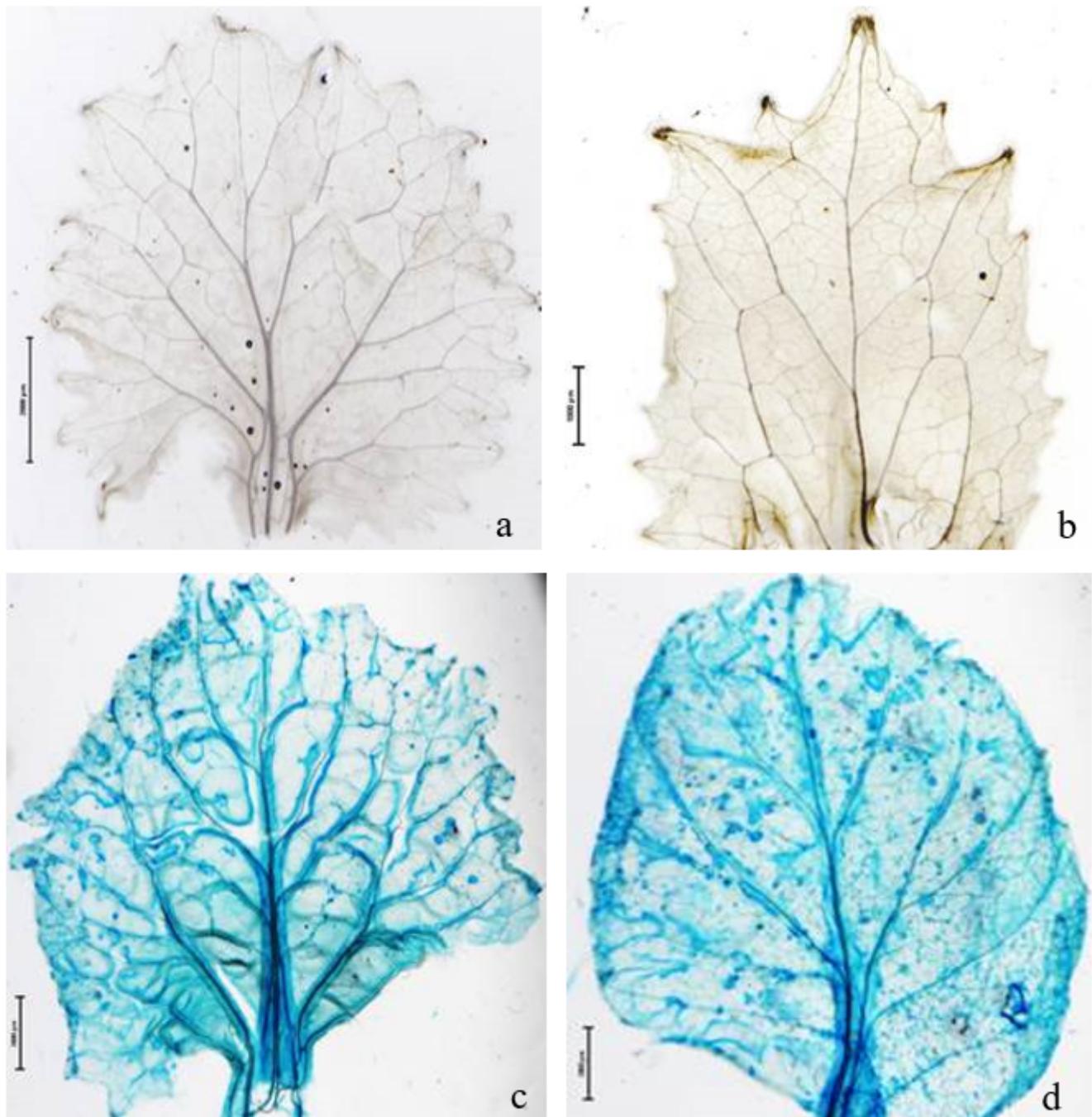


Figure 6

Histochemical GUS staining for transgenic plants. a. wild-type "C-24"; b. false-positive plants; c. and d. positive transgenic plants

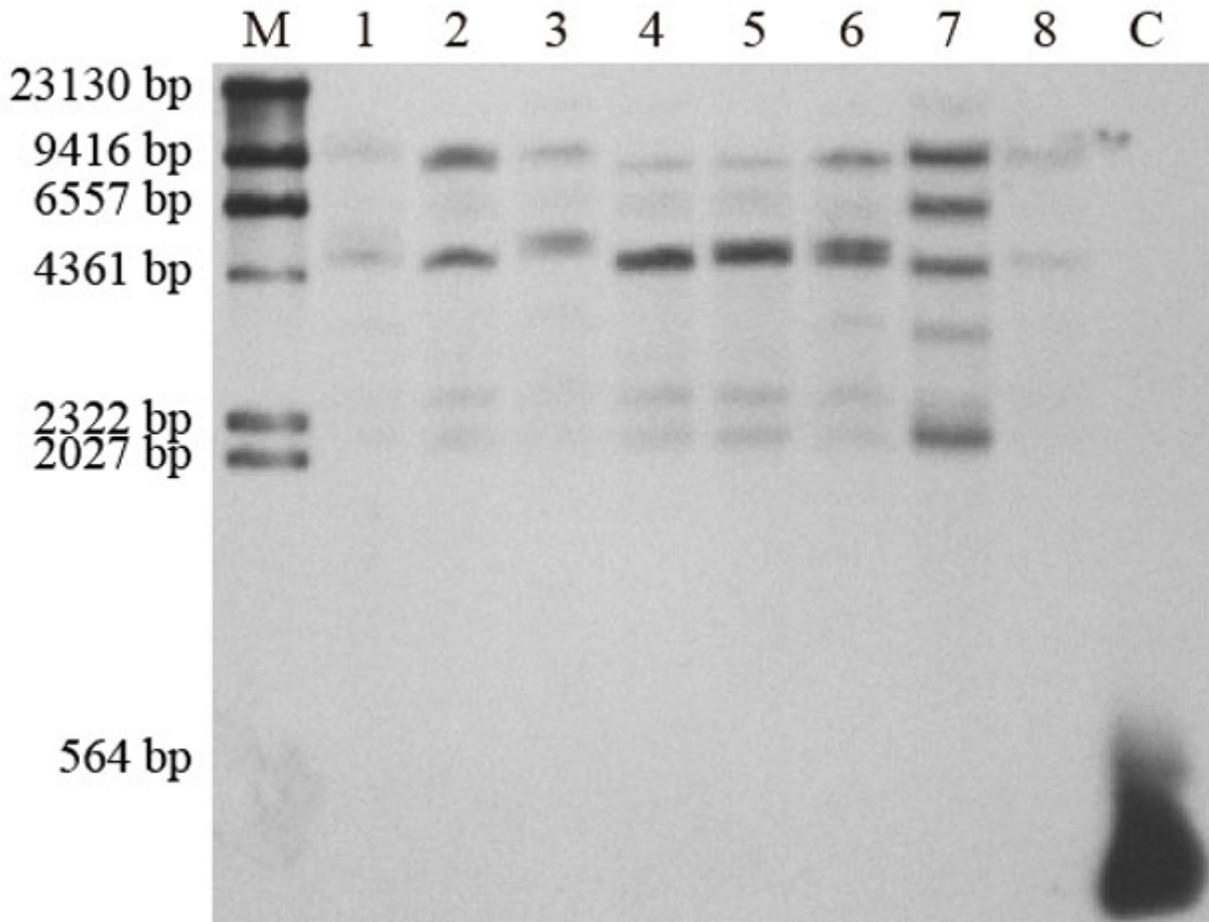


Figure 7

Southern blot analysis of transformants. Lane M, marker; Lane 1-7, PCR and GUS-positive lines; Lane 8, wild-type "C-24"; Lane C, plasmid control.

	Target1	PAM		Target2	PAM
WT	GTCTTGCTTCTTGAAATTATAAG	TGG		ACTTTACTTCCGGAGATGAAACT	AGG
BrL1	GTCTTGCTTCTTGAAATTA-----			ACTTTACTTCCGGAGATGAA--CT	AGG
BrL2	GTCTTGCTTCTTGAAA-----AG	TGG		ACTTTACTTCCGGAGATGAA	GACTAGG
BrL3	GTCTTGCTT-----TAAG	TGG		ACTTTACTTCCGGAGATGAAACT	AGG

Figure 8

CRISPR/Cas9-induced deletion in SRK gene. Color in blue is PAM sequence; letters in red represent insertion; black dashed lines represent deletions.

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