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A Comprehensive Method to Generating and Identifying Transgenic Tobacco Lines with a Single Transgene Integration Locus for Functional Analysis

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ABSTRACT

Tobacco is a popular model plant used for studying gene function. The generation of transgenic tobacco is tremendously essential in functional genomics. The generated transgenic plants must undergo careful selection and analysis before being used. However, most published protocols for generating transgenic tobacco for functional genomics are not comprehensive and involve sophisticated equipment. This study demonstrates an efficient and comprehensive method for developing and selecting transgenic tobacco lines without involving sophisticated equipment. Transgene was delivered into the genome of a tobacco plant via *Agrobacterium tumefaciens*. Polymerase Chain Reaction (PCR) was performed to verify the integration of transgenes in the putative primary transformants. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) examined transgene expressions. The number of transgene integration loci (TIL) was determined by transgene segregation analysis. PCR results revealed that ≈97% of the primary transformants were positive. The transgene was highly expressed in the transgenic plants. Segregation analysis showed that 47.6%–66.7% of the transgenic plants contained a single TIL, and the T1 and T2 progenies inherited the transgene. Homozygous transgenic lines with

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a single TIL were successfully developed by using our method. This manuscript encompasses detailed guidance on genetic transformation, molecular analysis, seed production, and transgene segregation analysis. It serves as a guideline for the researchers to produce transgenic tobacco lines that can be used for functional analysis. The procedures described here can be conducted in standard laboratories as they require no high-end equipment. This comprehensive and efficient method for generating transgenic tobacco will foster functional genomics.

Keywords: *Agrobacterium*, functional analysis, transgene integration locus, transgenic tobacco

INTRODUCTION

Plant genome databases provide tremendous sequence information for genetic study and plant breeding programs. Many plants of economic importance have their genomes completely sequenced (Guo et al., 2021; Robbins et al., 2023; Wang et al., 2023). A genome consists of genes encoding specific proteins and the intergenic regions (promoter and terminator) regulating gene expression. Without functionally characterizing individual genes in the genome, the genome sequence alone has little impact on any breeding program. Functional characterization aims to disclose the functions of particular genes by studying the coding sequences and their regulatory regions. Such a study often involves transgenic analysis in model plants. Having a plant genome database in which the individual genes are well-characterized would facilitate the breeding program for that plant.

Although *in silico* analyses, including phylogenetic analysis and protein modeling, have been used to predict gene function, further functional analysis is still necessary to elucidate the gene function accurately (Hu et al., 2023; Kiyak & Mutlu, 2023; Liu et al., 2020). Generally, a functional analysis could be performed through a gain- or loss-of-function approach. In the gain-of-function approach, a gene-of-interest (GOI) is overexpressed, whereas in the loss-of-function approach, a GOI is repressed. The consequences of this overexpression or repression are evaluated in the host plant. Heterologous expression of a GOI in model plants such as *Arabidopsis* or tobacco plants has been the most popular approach to functional gene analysis (Kim & Huh, 2019; Wang et al., 2021; Yao et al., 2020). This approach is frequently employed on genes from plant species that show poor *in vitro* regeneration capacity or when an efficient transformation method has not been established. Generating stable transformants allows detailed analysis and characterization of the transgenic plants, providing a better understanding of the transgene's role.

When generating a transgenic plant, the expression level of transgene is one of the major concerns. Transgene silencing associated with integrating transgene at multiple loci has been reported by Rajeevkumar et al. (2015). Previous studies showed that the particle bombardment method always results in transgenic plants carrying a high copy number of transgenes compared to the *Agrobacterium*-mediated method (Hwang et al., 2017; Jackson et al., 2013). Apart from that, a varying extent of genome damage was reported in the

transgenic rice and maize plants transformed by the particle bombardment method (Liu et al., 2019). Even though recent studies showed that transgenic lines with a low copy number of transgenes could be generated via the particle bombardment method by reducing the quantity of Deoxyribonucleic Acid (DNA) used during DNA/gold coating (Ismagul et al., 2018; Jackson et al., 2013), the *Agrobacterium*-mediated transformation method remains preferable due to its simplicity and affordability. As a result, the *Agrobacterium*-mediated transformation method has become the most popular method of plant genetic transformation since it was introduced in the 1980s (Fraley et al., 1983).

Tobacco (*Nicotiana tabacum*) is one of the popular model plants used in functional genomics due to its desirable transformation and regeneration efficiency (Niedbala et al., 2021). Compared with *Arabidopsis thaliana*, tobacco has a real stem and produces a more significant amount of plant biomass for experimental purposes. Owing to its showy and conspicuous flowers, the introduction of a transgene(s) from one transgenic line to another through cross-pollination can be efficiently conducted in tobacco (Zhang et al., 2020). It is also worth noting that the tobacco plant is self-fertile and generates many seeds after flowering, enabling the development of a homozygote line for comprehensive genetic analyses (Lewis et al., 2020; Schmidt et al., 2020). Apart from producing stable transformants, tobacco is also a popular choice of host plant for transient expression analysis through agroinfiltration (Duxbury et al., 2020; Shokouhifar et al., 2019). Due to its fascinating characteristics, tobacco has been used to study the genes from dicot and monocot species (Alexander et al., 2021; Boyidi et al., 2021; Fan et al., 2021; Wang et al., 2021).

Generating transgenic tobacco plants for functional analysis is a lengthy and complicated procedure. It encompasses the *in vitro* regeneration of transformants from the transformed cells and molecular analyses to verify the integration and functionality of transgene in the transgenic lines generated. In most cases, individual transgenic lines with a single transgene integration locus (TIL) are preferable for functional analysis to avoid a transgene silencing phenomenon caused by the insertion of transgenes at multiple loci (Rajeevkumar et al., 2015; Tang et al., 2007). The selection of transgenic lines that fulfill the criteria for functional analysis is time-consuming as it always involves the study of transgene inheritance in several generations. In certain studies, homozygous transgenic lines were generated for experimental purposes to offset the effects caused by the genetic variability within a heterogeneous population (Aleem et al., 2022; Dutta et al., 2023; Xie et al., 2024). Therefore, the researchers engaging in transgenic study need a detailed and comprehensive method for generating and identifying transgenic tobacco lines that comply with the requirements of functional analysis. Our method describes the complete procedures for the *Agrobacterium*-mediated transformation of tobacco plants and the steps involved in generating and identifying transgenic plants, fulfilling the criteria for functional analysis.

METHODS

To demonstrate the practicality of this method in generating transgenic tobacco plants with a single TIL, two GOI, namely *EgCAD1* reported by Yusuf et al. (2022) and β-glucuronidase (GUS), were used. The steps involved in the method are outlined in Figure 1.

Section 1: Construction of Transformation Vector

A PCR mixture to amplify the GOI using high-fidelity DNA polymerase was prepared on ice. A total of 10 µL of 5X Q5 reaction buffer, 1 µL of 10 mM dNTPs, 2.5 µL of 10 µM forward primer, 2.5 µL of 10 µM reverse primer, 1 µL of template DNA (10 ng/µL plasmid containing the GOI), 0.5 µL of Q5 High-Fidelity DNA Polymerase (NEB, USA) and 32.5 µL of water were added into a 0.2 ml thin-wall PCR tube. In this study, the *EgCAD1* gene was amplified using CAD1-F (5'-CAC CAT GGC TGG TGC CGG ATC-3') and CAD1-R (5'-TCA GAG TTT GCT GCG AGC CAC A-3') primers. While the GUS gene was amplified using GUS-F (5'- CAC CAT GTT ACG TCC TGT AGA AAC CCC AA-3') and GUS-R (5'-TCA TTG TTT GCC TCC CTG CTG CGG T-3') primers. The PCR was performed using the following thermocycling profile: to 98°C (30 s), 98°C (10 s), and 72°C (1 min) for 30 cycles; 72°C (2 min). Per the manufacturer's instructions, the PCR product was purified using the Monarch® DNA Gel Extraction Kit (NEB, USA). The transformation vectors were developed using the Gateway cloning method described by Yusuf, Abdullah, et al. (2018).

Firstly, to generate the entry clones, the gel-purified PCR products were cloned into the pENTR/D-TOPO vector using the pENTR™ Directional TOPO® Cloning Kits (Life Technologies, USA) according to the manufacturer's instructions. To transfer the GOI from the entry clone to a destination vector (pMDC32), LR recombination was performed using LR Clonase™ II enzyme mix (Life Technologies, USA). The

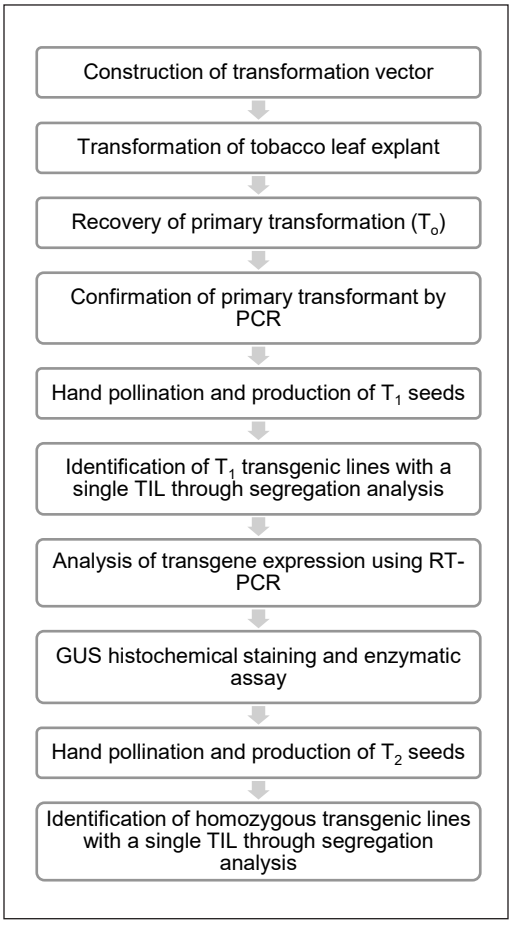

Figure 1. Flow chart outlining the procedures involved in generating and identifying transgenic tobacco lines with a single transgene integration locus

expression cassettes developed in this study are illustrated in Supplementary Figure 1. The sequence of the insert DNA was verified by sequencing.

Important Note. To ensure error-free amplification of GOI, high-fidelity DNA polymerase is highly recommended.

Section 2: Transformation of *Agrobacterium* Through the Electroporation Method

The electrocompetent cells of *A. tumefaciens* Strain LBA4404 were thawed on ice for about 5 min. Approximately 50 ng of plasmid DNA was added into 100 µL of the competent cells and mixed by gently tapping the tube. The mixture (competent cells and DNA) was transferred to a pre-cooled electroporation cuvette (gap width: 1 mm), and the electroporation was performed at 2400 V using Electroporator 2510 (Eppendorf, Germany). One milliliter of Luria-Bertani (LB) broth (Miller) was immediately added to the cuvette after the pulse, and the *Agrobacterium* cell suspension was transferred to a 15 ml Falcon tube. The *Agrobacterium* cell suspension was agitated at 250 rpm in an incubator shaker at 28°C under dark conditions for 2 h. Then, 200 µL of the *Agrobacterium* cell suspension was spread on an LB agar (1.5% w/v) plate (90 mm × 15 mm disposable Petri dish) supplemented with 50 µg/ml kanamycin, 25 µg/ml rifampicin, and 100 µg/ml streptomycin. The plate was incubated at 28°C under dark conditions for three days. A single colony was picked using a toothpick and transferred to a 50 ml Falcon tube containing 5 ml of LB broth with the above antibiotics. The bacterial culture was incubated at 28°C overnight under dark conditions using an incubator shaker run at 250 rpm. The next day, 500 µL of the bacterial culture was transferred to a 1.5 ml microcentrifuge tube, and the same volume of glycerol was added to prepare a glycerol stock. The glycerol stock was stored at -80 °C.

Important Note. *Agrobacterium* strains, such as AGL1 and GV3101, may be used for tobacco transformation. However, our studies have shown that LBA4404 showed satisfactory results. Furthermore, Niedbala et al. (2021) reported that LBA4404 produced the highest transformation efficiency in tobacco plants. Apart from the electroporation method, plasmid DNA also can be introduced into *Agrobacterium* cells through the freeze-thaw method. However, a reduced transformation efficiency will be obtained, and the method requires liquid nitrogen.

Section 3: Preparation of Plant Materials

About 20–30 tobacco (*Nicotiana tabacum* cv. SR1) seeds were transferred into a 1.5 ml microcentrifuge tube and soaked with distilled water for 2 h. The seeds floating on the water surface were removed, and the water was carefully decanted. Seed surface sterilization was performed in a laminar airflow; 1 ml of 0.5% (v/v) sodium hypochlorite solution was added to the seeds and mixed well by inverting the tube for 10 min. The sodium hypochlorite

solution was decanted carefully. The seeds were rinsed with 1 ml of sterile distilled water for 1 min. The sterile distilled water was decanted, and the washing step was repeated twice. Using a 1 ml pipette tip, about 20–25 sterilized seeds were transferred and distributed evenly on a germination medium. The Petri dish was sealed with a parafilm strip and kept at 4°C under dark conditions for three days. Then, the Petri dish containing the tobacco seeds was moved to a growth chamber, and the seedlings were allowed to grow at 25°C under 16 h light/8 h dark conditions for four weeks.

Important Note. All solid tissue culture media were prepared using a 90 mm × 15 mm disposable Petri dish unless specified otherwise. The composition of the culture media used in this protocol is listed in Supplementary Table 1. Incubating the surface-sterilized tobacco seeds at 4°C for three days is optional. However, performing this step would allow uniform germination of the tobacco seeds.

Section 4: Preparation of Bacterial Cultures

An inoculating loop was used to streak the glycerol stock of *Agrobacterium* cells carrying the desired construct on an LB agar supplemented with 50 µg/ml kanamycin, 25 µg/ml rifampicin, and 100 µg/ml streptomycin four days before plant transformation. A single colony was picked using a toothpick and transferred into a 100 ml conical flask containing 10 ml of LB broth with antibiotics specified above. The bacterial culture grew at 28°C overnight under dark conditions in an incubator shaker at an agitation speed of 250 rpm. The next day, 5 ml of the overnight bacterial culture was transferred to a 500 ml conical flask containing 50 ml LB broth supplemented with the same antibiotics.

Important Note. The volume of bacterial culture can be upscaled or downscaled, depending on the number of leaf discs to be transformed. However, the value of OD_{600} should be maintained between 0.8 and 1.0, as this is the optimum concentration of *Agrobacterium* to achieve a high transformation efficiency.

Section 5: Plant Transformation and Establishment of Transgenic Plant

In a laminar airflow, the leaves of four-week-old tobacco plants prepared in Section 3 were punched with a sterile paper puncher or cork borer to produce leaf discs of 0.6 cm in diameter. About 50 leaf discs were collected in a 90 mm × 15 mm disposable Petri dish containing a piece of sterile filter paper moistened with 1 ml of sterile distilled water. Subsequently, the inoculation medium prepared earlier was poured into the Petri dish, and the leaf discs were soaked for 30 min with gentle shaking every 3 min. The inoculation medium was drained off, and the leaf discs were blot-dried on sterile tissue paper before being placed on a co-cultivation medium with the abaxial side facing upward. The co-cultivation was performed at 28°C for one to two days under dark conditions. The growth of *Agrobacterium* was observed during this period. The co-cultivation time may vary with

the *Agrobacterium* strain used. The leaf discs were transferred to a regeneration medium 1 when the growth of *Agrobacterium* at the edge of the leaf discs was visible. The leaf discs were subcultured to a fresh regeneration medium once every two weeks or once the *Agrobacterium*'s growth was visible. After one month of culturing on regeneration medium 1 (when the adventitious shoots were about 0.3–0.5 cm in height and forming a multiple shoot cluster), multiple shoot clusters were transferred to regeneration medium 2 for shoot elongation. When the regenerated shoots were about 1–1.5 cm in height, only one shoot from each multiple-shoot cluster was excised and transferred to a rooting medium. The plantlets were acclimatized when the roots were about 1–2 cm.

Important Note. All the tissue culture steps described in this section should be performed under sterile conditions in a laminar airflow. The composition of the culture media used in this method is listed in Supplementary Table 1. A co-cultivation period longer than two days should be avoided, as this will result in the overgrowth of *Agrobacterium* and pose a problem in eliminating it during shoot regeneration.

Section 6: Acclimatization of Transgenic Plantlet

The plantlets from the culture jar were carefully removed, and the roots were washed with tap water to remove the culture medium. The plantlets were transferred to a 16 oz disposable plastic cup filled with a potting mix for gardening purposes, and each plantlet was immediately covered with another plastic cup to create and maintain high air humidity. The plantlets were cultivated in a growth room at 24°C under a 16 h photoperiod. After two weeks (when the leaf and root development was noticeable), the cover (plastic cup) was slightly opened to reduce the air humidity. Then, gradually increase the opening day by day to allow a gradual reduction of air humidity. Finally, the cover was removed after one week when no wilting was observed.

Important Note. Ensure the culture medium is completely removed from the roots. Nutrients and sugar in the tissue culture medium will encourage the growth of bacteria and fungi in the soil, killing the plantlets. The young roots are very vulnerable; avoid breaking them when washing them. A sudden or too early cover removal will cause the plantlets to wilt and die. Plantlet acclimatization is recommended in a disposable plastic cup, as the transparent plastic material will allow root growth to be observed easily.

Section 7: Verification of Transgenic Plant

About 100 mg of leaf tissues from a putative transgenic plant were collected in a 1.5 ml microcentrifuge tube. The tube and a disposable pestle were dipped in liquid nitrogen for 10 s, and the sample was immediately ground into a fine powder using the pre-cooled disposable pestle. According to the manufacturer's protocol, the sample's genomic DNA (gDNA) was extracted using the GF-1 Plant DNA Extraction Kit (Vivantis, Malaysia).

The gDNA sample was quantified using an ultraviolet (UV) spectrophotometer (Thermo Scientific, USA) to obtain the A260, A280, and A230 values. A DNA sample with A_{260}/A_{280} and A_{260}/A_{230} ratios ≥ 1.8 is considered pure. A PCR mixture (20 µL final volume) was set up for each transgenic line tube to detect the transgene (*EgCAD1* or GUS in this case) by adding 2 µL of 10X DreamTaq Green Buffer, 2 µL of 2 mM dNTPs, 1 µL of 10 µM forward primer and 1 µL of 10 µM reverse primer, 1 µL of gDNA (50 ng/µL), 0.1 µL of DreamTaq Green DNA Polymerase (Thermo Scientific, USA) and 12.9 µL H_2O into a 0.2 ml thin-wall PCR. The primer pairs used to develop the transformation constructs (refer to Section 1) were utilized to verify the transgenic plants. The PCR mixture was set up on ice, and a positive control reaction was set up in parallel. Plasmid DNA (the transformation vectors developed in Section 1) was used as a template for the positive control reaction. The PCR was performed using the following thermocycling profile: 3 min at 95°C (1 cycle); 30 s at 95°C, 30 s at 63°C (for *EgCAD1*) or 66°C (for GUS), 30 s at 72°C (35 cycles); 5 min at 72°C (1 cycle). The PCR products (5 µL) were analyzed on a 1.2% Tris-acetate-EDTA (TAE) agarose gel. DNA ladder was loaded next to the samples. The agarose gel was viewed using a gel documentation system or UV transilluminator when the gel electrophoresis was completed.

Important Note. To ensure that the transformants are not carrying a truncated GOI, PCR primers that amplify the entire sequence of GOI (instead of a partial sequence) were used to verify the transgenic plants. If a detailed gel electrophoresis protocol is required, please refer to Lee et al. (2012).

Section 8: Seed Production

The PCR-positive transgenic plants (T_0 generation) were cultivated in a growth room at 24°C under a 16 h photoperiod until the plants bloom. The plants were watered daily, and one teaspoon of fertilizer rich in phosphorus and potassium was applied to each plant every three weeks (fertilizer with an N:P:K ratio of 8:16:24 or similar will do). Flower buds were bagged with a pollination bag when they were about to bloom. The pollination bag was removed on the second day after blooming, and the flowers were self-pollinated by transferring pollen to the stigma using a cotton bud to maximize the number of seeds produced. After hand-pollination, the flowers were bagged again to prevent cross-pollination. The pollination bag was removed when seed pod development was noticeable. The seeds were harvested when the seed pods turned brown. The seeds were kept in a 1.5 ml microcentrifuge tube and stored in a chiller until used for transgene segregation analysis.

Important Note. Seeds can be produced in tobacco plants without hand-pollination. However, fewer seeds will be obtained due to the absence of pollinating agents. The best time to perform hand pollination is the second day after blooming, as all anthers have dehisced at this time.

Section 9: Analysis of the Number of Transgene Integration Loci

The number of TIL of a transgenic line was determined by analyzing the transgene inheritance pattern. According to Mendel's law of inheritance, transformants harboring one, two, and three TIL are expected to have their transgenes segregated in their progenies with a segregation ratio (transgenic:non-transgenic) of 3:1, 15:1, and 63:1, respectively (Tizaoui & Kchouk, 2012). Segregation of hygromycin resistance genes among individuals was analyzed in the T_1 generation. Approximately 120 seeds (T_1 generation) collected from a transgenic line were prepared in a 1.5 ml microcentrifuge tube. Surface sterilization was performed as described in Section 3, and the seeds were evenly distributed on a screening medium containing 50 mg/L hygromycin. The Petri dish was stored in a 4°C chiller under dark conditions for three days and then moved to a growth chamber at 25°C under a 16 h light photoperiod for the seeds to germinate. The Petri dish was kept under dark conditions for two days after seed germination and then returned to normal growth conditions. The numbers of hygromycin-resistant (hyg^r) and hygromycin-sensitive (hyg^s) seedlings were determined based on hypocotyl length. A seedling with a longer hypocotyl was regarded as hygromycin-resistant (hyg^r), while a seedling with a shorter hypocotyl was regarded as hygromycin-sensitive (hyg^s). The data obtained was analyzed with the Chi-square (χ^2) test using the formula as follows:

$$\chi^2 = \frac{(\text{observed hyg}^r - \text{expected hyg}^r)^2}{\text{expected hyg}^r} + \frac{(\text{observed hyg}^s - \text{expected hyg}^s)^2}{\text{expected hyg}^s}$$

The transgenic lines were hypothesized to have a Mendelian segregation ratio of 3:1, 15:1, or 63:1 (hyg^r: hyg^s), and the hypothesis was accepted when the value of $\chi^2 \leq 3.841$.

Important Note. The selective agent may vary with the transformation construct used. Storing the germinating seeds in the dark for two days reduced the time needed to distinguish hyg^r seedlings from hyg^s ones based on their phenotypes. The Chi-square analysis can be easily performed using our prepared spreadsheets (http://surl.li/uhzmm). The southern blot method can also determine the number of transgene integration loci in a transgenic line. However, special equipment is required and involves lengthy procedures, limiting usage.

Section 10: Analysis of Transgene Expression

A semi-quantitative RT-PCR was performed to analyze the transgene expression, which was the most economical and easiest method available. In our study, the transgene expression was analyzed in four-week-old CAD1-OE1, CAD1-OE6, CAD1-OE24, GUS-OE1, and GUS-OE3 plants by using the method described by Yusuf, Abu Seman, et al. (2018) with minor modifications. Approximately 100 mg of young leaf sample was collected and homogenized as described in Section 7. According to the manufacturer's instructions, the total RNA from the leaf tissues was extracted using the GF-1 Total RNA Extraction Kit

(Vivantis, Malaysia). The total RNA sample was quantified using a UV spectrophotometer (Thermo Scientific, USA), and the A_{260}, $A_{280,}$ and A_{230} values were obtained. An RNA sample with A_{260}/A_{280} and A_{260}/A_{230} ratios ≥ 2.0 is considered a pure RNA sample. Based on the manufacturer's protocol, cDNA was synthesized from 1 µg of total RNA using the Viva cDNA Synthesis Kit (Vivantis, Malaysia). The cDNA was diluted with 40 µL (two volumes) of nuclease-free water, and the diluted cDNA sample was stored at -20°C. The PCR mixture was set up as described in Section 7 to amplify the transgene from 1 µL of cDNA sample instead of gDNA. NtEF-F (5'- TCC CCA TCT CTG GTT TTG AAG-3') and NtEF-R (5'-CAG GCT TGA GGA CAC CAG TT-3') primers were used to amplify the *Elongation factor 1-α* (*NtEF-1α*) gene (accession number: AF120093) which acts as the internal control of gene expression analysis. PCR was performed using the following thermocycling profile: 3 min at 95°C (1 cycle); 20 s at 95°C, 25 s at 63°C (for *EgCAD1*) or 66°C (for GUS) or 55°C (for *NtEF-1α*), 60 s at 72°C (32 cycles); 5 min at 72°C (1 cycle). The PCR products were analyzed through gel electrophoresis as described in Section 7.

Important Note. DNA-free total RNA samples can be obtained using the GF-1 Total RNA Extraction Kit (Vivantis, Malaysia) as a DNase treatment step included in the protocol. If a conventional method or other RNA extraction kits are used for RNA extraction, an additional DNase treatment step should be conducted to remove contaminating gDNA before cDNA synthesis. If an accurate quantification of transgene expression levels is required, quantitative PCR (real-time PCR) might be used.

Section 11: GUS Histochemical Staining

The protocol developed by Jefferson et al. (1987) with minor modifications was applied to examine the GUS expression in this study. The tobacco seedlings were collected in a 1.5 ml microcentrifuge tube. One milliliter of 90% (v/v) acetone (Sigma-Aldrich, USA) was added, and the sample was incubated at room temperature for 20 min. The 90% acetone was discarded, and the sample was washed with 1 ml prechilled staining buffer [50 mM sodium phosphate buffer (pH 7.2, Sigma-Aldrich, USA)], 0.2% (v/v) Triton X-100 (Sigma-Aldrich, USA), 2 mM potassium ferrocyanide (Sigma-Aldrich, USA), and 2 mM potassium ferricyanide (Sigma-Aldrich, USA)]. The staining buffer was discarded, and 1 ml prechilled staining solution [50 mM sodium phosphate buffer (pH 7.2), 0.2% (v/v) Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide and 2 mM X-Gluc] was added to the sample. Then, the sample was placed on ice in a vacuum desiccator connected to a vacuum pump, and the vacuum infiltration was performed at 700 mmHg for 15 min. The vacuum was slowly released, and the sample was incubated overnight at 37°C. The next day, the sample was successively treated with 1 ml of 20%, 35%, and 50% ethanol (Nacalai Tesque, Japan) for 30 min each at room temperature. The tube was gently inverted several times every 5 min. After that, the 50% ethanol was decanted, and the sample was treated with 1 ml of FAA

fixative (50% ethanol, 10% glacial acetic acid, and 5% formaldehyde) at room temperature for one hour. Lastly, the FAA fixative was replaced with 1 ml of 70% ethanol (Nacalai Tesque, Japan). A photograph of the stained sample was taken using a digital camera.

Important Note. Potassium ferrocyanide, potassium ferricyanide, and X-Gluc must be stored in dark conditions as they are light-sensitive. If a transgenic seedling is required for other purposes, use a detached organ from the seedling instead of the whole seedling for histochemical staining of GUS activity, as this assay is destructive to plant tissue. The sample can be stored at 4°C if not photographed immediately.

Section 12: GUS Enzymatic Assay

The leaf tissues of the transgenic tobacco plant overexpressing the GUS gene were ground using a mortar and pestle to form a fine powder in the presence of liquid nitrogen. Approximately 0.5 g of fine powder was transferred to a 15 ml Falcon tube containing 4 ml pre-cooled GUS extraction buffer [50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol (Sigma-Aldrich, USA), 10 mM Na_2EDTA (Sigma-Aldrich, USA), 0.1 % sarkosyl (Sigma-Aldrich, USA) and 0.1% (v/v) Triton X-100 (Sigma-Aldrich, USA)] and mixed by vortexing for 1 min. The sample was centrifuged at 4ºC, 10,000 x *g* for 45 min. Then, 2 ml of the supernatant was transferred to a 2 ml microcentrifuge tube, and the centrifugation step was repeated. Subsequently, 1.5 ml of the supernatant (crude protein sample) was transferred to a new 1.5 ml microcentrifuge tube. The concentration of the crude protein sample was determined using the Easy Protein Quantitative Kit (TransGen Biotech, China) according to the manufacturer's protocol. A 1.5 ml microcentrifuge tube containing 800 uL of GUS assay buffer [50 mM sodium phosphate (pH 7.0, Sigma-Aldrich, USA), 1 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, USA), 5 mM Dithiothreitol (DTT, Thermo Scientific, USA), and 1.25 mM *p*-Nitrophenyl-β-D-glucuronide (PNPG, Sigma-Aldrich, USA)] was prewarm at 37°C for 5 min. A total of 200 µL of crude protein sample was added to the pre-warmed GUS assay buffer. The reaction buffer was vortexed shortly and incubated at 37°C for 30 min (or up to several hours). The development of a yellow color was observed during the incubation period, indicating the formation of *p*-nitrophenol (product). When the yellow color was visible, 100 µL of the reaction mixture was transferred to a 1.5 ml microcentrifuge tube containing 800 µL of 0.4 M Na_2CO_3 (stop solution), and the reaction time was recorded. The absorbance of the sample was measured at 405 nm using a UV spectrophotometer (Thermo Scientific, USA) against a stopped blank reaction. The rate of reaction (nanomoles product/ min/ mg protein) was calculated using the formula as follows:

$$\text{Rate of reaction} = \frac{OD_{405}}{\text{Time (min)} \times \text{Protein concentration } (\frac{\text{mg}}{\text{ml}}) \times 0.02 \text{ ml} \times 0.02}$$

Where 0.02 is a value derived from the molar extinction coefficient of *p*-nitrophenol. An absorbance of 0.02 is equivalent to 1 nmol of *p*-nitrophenol formed under the conditions used in this method.

Important Note. The GUS enzymatic assay was performed in triplicates. The assay allows quantitative determination of GUS activity in the transgenic lines obtained. The incubation period varies according to the promoter used. A strong constitutive promoter, such as the CaMV 35S promoter, may require a shorter time to produce a detectable product. When an uncharacterized promoter is used, it is recommended to perform the enzymatic assay with several different reaction times to ensure the reaction rate is measured in the linear phase of the enzymatic reaction.

Section 13: Production and Identification of Homozygous Line

Surface sterilization on T_1 seeds derived from the selected transgenic lines containing a single TIL was performed, as mentioned in Section 3. The seeds were cultivated on a screening medium containing 50 mg/L hygromycin under conditions specified in Section 9. Six hyg^r seedlings (T_1) from each transgenic line were randomly selected and transferred to the soil. The plants were grown until they set seeds (T_2) for transgene segregation analysis following the steps in Section 8. About 50 seeds (T_2) derived from a specific transgenic line (T_1) were surface sterilized and cultivated on a screening medium. The T_2 seedlings' phenotype was observed and recorded one week after seed germination. The T_1 individuals producing hyg^r and hyg^s progenies (T_2 generation) are regarded as hemizygotes, while those producing only hyg^r seedlings are considered homozygotes. The concept of transgene inheritance in transgenic lines with a single TIL was illustrated in Figure 2.

Important Note. Precautions should be taken to avoid cross-pollination between the T_1 plants, even though they are derived from the same origin (T_0), because the T_1 population consists of homozygous and hemizygous individuals. The probability of obtaining a homozygous individual among the hyg^r individuals in the T_1 generation is 1/3 (0.33). One could cultivate more than six hyg^r plants derived from a selected transgenic line for zygosity determination to increase the possibility of obtaining a homozygous individual. However, it is not necessary to determine the zygosity of all T_1 individuals at once. The transgene segregation analysis can be stopped once a homozygous individual is identified.

RESULTS

Transformation and Regeneration of Transgenic Plants

The *Agrobacterium*-infected leaf discs expanded and curled after being cultured on a regeneration medium 1 for one week (Figure 3a). The emergence of adventitious shoots at the edges of the leaf discs was visible in the second week (about 11 days after co-cultivation) (Figure 3b). Next, the regenerated shoots developed into a multiple-shoot cluster in 10

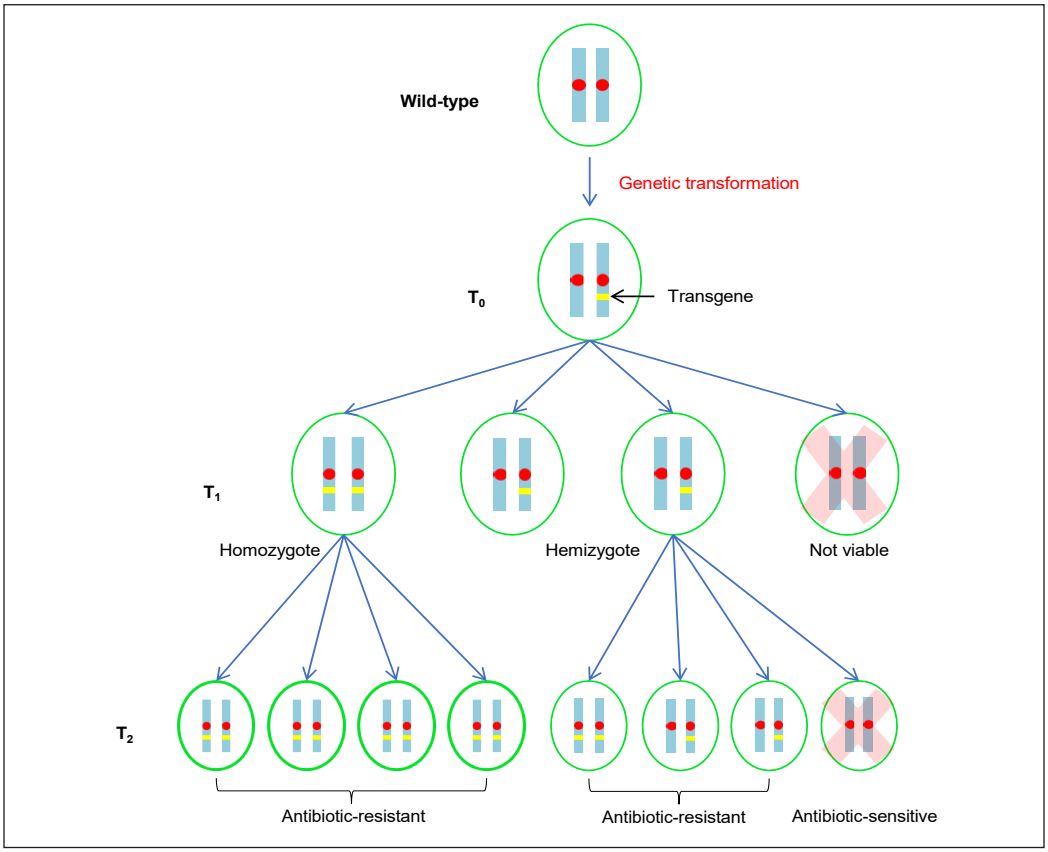

Figure 2. Diagrammatic representation of the inheritance of a transgene in the transgenic plant carrying the transgene at a single locus. The green circle represents the cell nucleus, the blue bars represent the homologous chromosomes, the red dot indicates the centromere, and the yellow line denotes the transgene. Only a single pair of homologous chromosomes is shown

days (Figure 3c), where each cluster consisted of 2–5 shoots. The shoots started to produce roots 7–10 days after being transferred to a rooting medium (Figure 3d). Figure 3e shows a transgenic plantlet ready for acclimatization. Putative transformants carrying the *EgCAD1* gene were designated CAD1-OE lines, while the GUS gene-containing lines were designated GUS-OE lines. A total of 22 putative CAD1-OE plants and nine putative GUS-OE plants were successfully obtained after acclimatization (Figure 3f).

Verification of Transgenic Plants by PCR

PCR analysis was performed on the putative CAD1-OE and GUS-OE plants to verify transgene incorporation. PCR results showed that a band (approximately 1 kb) corresponding to the *EgCAD1* gene was amplified from the genome of all the CAD1-OE lines except for line 25 (Supplementary Figure 2a). The result indicated that all the transgenic plants

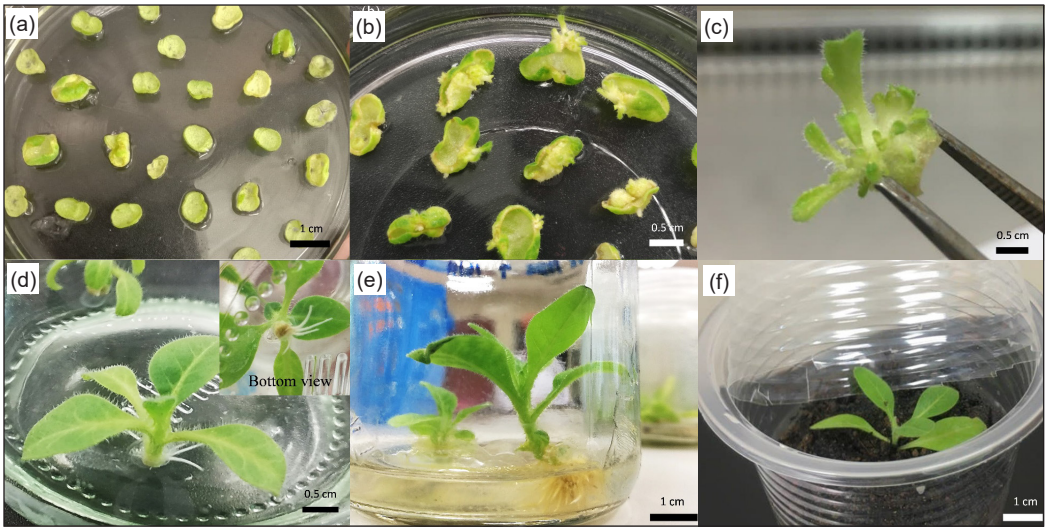

Figure 3. Different stages in the generation of putative primary transformants: (a) *Agrobacterium*-infected leaf discs cultured on regeneration medium 1; (b) Adventitious shoots regenerated from leaf discs; (c) Multiple-shoots cluster developed from transformed cells; (d) Roots produced from regenerated shoots; (e) Putative primary transformants; and (f) Acclimatized T_0 transgenic plants

carried the *EgCAD1* gene except line 25. On the other hand, all the GUS-OE plants analyzed contained the GUS gene (Supplementary Figure 2b). Altogether, 21 and nine primary transformants (T_0 generation) carrying the *EgCAD1* and GUS genes, respectively, were successfully generated. By using our established protocol, a transformation efficiency of 30% was obtained in this study.

Identification of Transgenic Lines with a Single Transgene Integration Locus

To avoid the phenomenon of transgene silencing caused by multiple TIL, transgenic lines with a single TIL were identified. The number of TIL in the primary transformants generated was determined by studying the inheritance of selectable marker genes in T_1 progeny. The hygromycin-resistant (hyg^r) seedlings can be distinguished from the hygromycin-sensitive (hyg^s) seedlings based on their distinctive phenotypes as early as five days after seed germination. The hyg^r seedlings possessed a longer hypocotyl compared to the hyg^s seedlings after being cultured on a screening medium for two days under dark conditions (Figure 4a and 4b). After three weeks, the hyg^r seedlings thrived on the screening medium and displayed visible root growth, expanded cotyledon, and the development of true leaves. In contrast, the growth of hyg^s seedlings was retarded (Figures 4c and 4d).

Among the CAD1-OE lines produced, 10 lines (47.6%) yielded a hyg^r to hyg^s ratio that fits the Mendelian ratio of 3:1 (Table 1), indicating that these transgenic lines contain

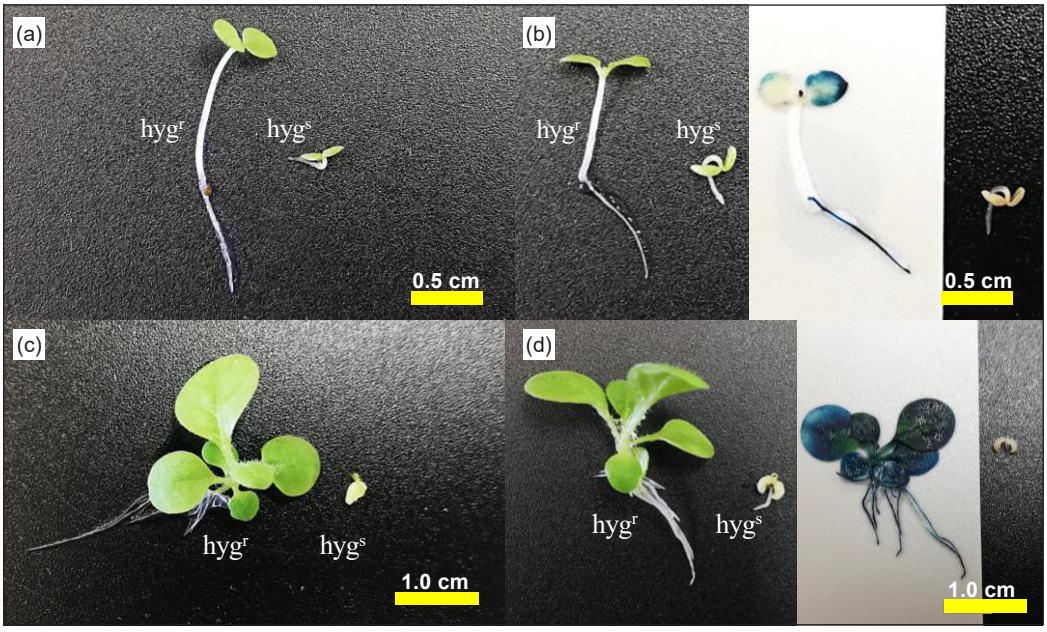

Figure 4. Phenotypes of hygromycin-resistant (hyg^r) and hygromycin-sensitive (hyg^s) seedlings. The seedlings were observed for one week (a & b) and three weeks (c & d) after germination. The CAD1-OE seedlings (a & c) displayed similar phenotypes to the GUS-OE seedlings (b & d). The GUS-OE seedlings (b & d, right panel) turned blue after being subjected to GUS staining

a single TIL. Besides, the hygromycin resistance gene segregated in the T_1 progenies of seven transgenic lines (33.3%) in a segregation ratio (hyg^r:hyg^s) that fits the Mendelian ratio of 15:1, reflecting that there are two TIL present in these transgenic lines. In addition, three CAD1-OE lines (14.3%) carried three TIL, judging by their segregation ratios that fit the Mendelian ratio of 63:1. Another CAD1-OE line (4.8%) exhibited an aberrant transgene inheritance pattern by producing only hyg^r seedlings in the T_1 generation.

Contrary to CAD1-OE lines, no GUS-OE plants with distorted segregation ratios were generated in this study. Indeed, the GUS-OE lines only displayed a hyg^r to hyg^s ratio that fits the Mendelian ratios of 3:1 or 15:1 (refer to Table 1), showing that 66.7% of the population contain a single TIL, and the rest possess two. Details of segregation analysis performed on the transgenic lines generated in this study are shown in Table 1. The results indicate that the *EgCAD1* and GUS genes (individually) were stably integrated into the tobacco genome and transmitted to the progeny.

Analysis of Transgene Expression

The *EgCAD1* or GUS gene transcript was detected using semi-quantitative RT-PCR in selected transgenic lines containing a single TIL to validate the transgene expression in the transgenic plants. Figure 5 shows an intense band corresponding to the *EgCAD1* or GUS

Table 1
Analysis of the segregation of hygromycin resistance gene in the T_1 progeny of primary transformants

Transgenic plant	Line	Germinated seedlings	Hyg^r	Hyg^s	Observed ratio (hyg^r:hyg^s)	Expected ratio (hyg^r:hyg^s)	χ^2	No. of transgenic loci
CAD1-OE	1	192	143	49	2.9:1	3:1	0.028	1
	2	124	91	33	2.8:1		0.172	
	3	122	95	27	3.5:1		0.536	
	6	116	88	28	3.1:1		0.046	
	8	202	157	45	3.5:1		0.799	
	14	230	169	61	2.8:1		0.284	
	21	147	103	44	2.3:1		1.907	
	24	145	110	35	3.1:1		0.057	
	31	165	129	36	3.6:1		0.891	
	32	131	91	40	2.3:1		2.140	
	7	119	112	7	16.0:1	15:1	0.027	2
	10	189	179	10	17.9:1		0.297	
	20	180	167	13	12.8:1		0.290	
	26	116	109	7	15.6:1		0.009	
	27	97	91	6	15.2:1		0.001	
	28	107	100	7	14.3:1		0.016	
	30	120	114	6	19.0:1		0.320	
	5	131	129	2	64.5:1	63:1	0.001	3
	18	80	78	2	39.0:1		0.457	
	29	132	128	4	32.0:1		1.849	
	17	73	73	0	73.0:0	-	ND	Unknown
GUS-OE	1	234	176	58	3.0:1	3:1	0.006	1
	3	181	134	47	2.9:1		0.090	
	4	165	120	45	2.7:1		0.455	
	5	109	85	24	3.5:1		0.517	
	7	102	72	30	2.4:1		1.059	
	8	113	83	30	2.8:1		0.145	
	2	142	136	6	22.7:1	15:1	0.993	2
	6	106	96	10	9.6:1		1.834	
	9	78	72	6	12.0:1		0.277	

Note. ND = Not determined; Hyg^r = Hygromycin-resistant; Hyg^s = Hygromycin-sensitive

gene produced by RT-PCR from the cDNA samples of transgenic plants but not from that of wild-type plants. The RT-PCR result reflects that the T_1 progenies inherited the *EgCAD1* or GUS gene, and the transgene was actively transcribed in the transgenic plants analyzed. Apart from the gene expression analysis, the expression of GUS was also confirmed by histochemical staining (Figure 4b & 4d) and enzymatic assay (Data not shown).

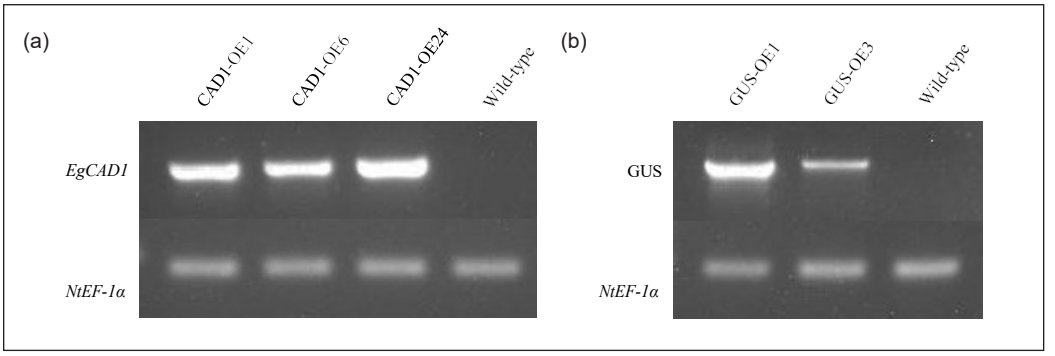

Figure 5. Analysis of RT-PCR products by agarose gel electrophoresis. The expressions of *EgCAD1* (a) and GUS (b) genes were examined in the leaf tissue of four-week-old transgenic plants using semi-quantitative RT-PCR. Wild-type tobacco plants were used as the negative control. The tobacco Elongation factor 1-α (NtEF-1α) gene serves as the internal control for the analysis

Identification of Homozygous Line

The T_1 generation consists of homozygous and hemizygous individuals (Figure 2), but they are visually indistinguishable. Therefore, homozygous individuals were identified by studying the inheritance of the selectable marker gene in T_2 progenies. As anticipated, the hemizygous individual produced both hyg^r and hyg^s progenies in a 3:1 ratio, while the homozygous individual produced only hyg^r progenies. The hyg^r and hyg^s seedlings were easily distinguishable by the length of their hypocotyls, with the former displaying a longer hypocotyl compared to the latter after being cultured under dark conditions. The present study successfully developed homozygous plants for the CAD1-OE1, CAD1-OE6, CAD1-OE24, GUS-OE1, and GUS-OE3 transgenic lines. These homozygous plants carry the transgene of interest at a single TIL.

DISCUSSION

Genetic engineering has become a powerful tool in functional genomics as it allows the direct introduction of foreign DNA into the genome of a species. Plant genetic transformation is commonly performed through the *Agrobacterium*-mediated transformation method owing to the ability of *Agrobacterium* to transfer a portion of its DNA to the host plant genome. Nevertheless, some plant species are recalcitrant to *Agrobacterium*-mediated transformation as the transformation efficiencies are relatively low (Kumar et al., 2021; Masters et al., 2020; Sharma et al., 2020). This problem greatly impedes the progress of functional genomics in plant species that are not amenable to *Agrobacterium*-mediated transformation. Hence, performing a functional study using a model plant can be a strategy to circumvent the bottleneck. This article provides a complete and comprehensive guide to generating and identifying transgenic tobacco lines with a single TIL. This article also provides a guideline for developing a homozygous transgenic plant from selected transgenic lines. Using the

method presented in this manuscript, transgenic tobacco plants carrying the intended GOI were successfully produced. It is worth mentioning that more than 50% of the transgenic tobacco plants generated contained a single TIL (Table 1). Furthermore, homozygous lines were developed from selected transgenic lines. The procedures outlined in our method do not require high-end and sophisticated equipment. Hence, they can be performed in most laboratories at minimal cost. The transgenic tobacco plants produced are appropriate for functional analysis to dissect the roles of a genetic element in plants. Undoubtedly, this method will foster functional genomics.

Tobacco as a Model Plant

The choice of a model plant is of great importance in dissecting the role of a plant gene. Several model plants, such as *Arabidopsis*, petunia, and tobacco, have been used in functional studies (Naing et al., 2022; Shingote et al., 2020; Sun et al., 2021). Being a dicotyledonous plant does not limit the tobacco plant from being used in the functional study of a gene from monocots. The use of dicotyledonous model plants to study a gene from monocots is not uncommon. Previously, transgenic tobacco plants carrying a gene from wheat, maize, rice, and sugarcane had been reported (Li et al., 2018; Miftahudin et al., 2021; Su et al., 2020). Tobacco is a popular model plant due to its efficient and straightforward transformation protocol. Transgenic tobacco plants can be produced by the leaf disc transformation method, which involves co-culturing tobacco leaf disc explants with *Agrobacterium,* followed by a shoot regeneration on a cytokinin-rich medium (Gallois & Marinho, 1995). Alternatively, stable transgenic lines can be regenerated from tobacco leaf tissues infiltrated with *Agrobacterium* (Sparkes et al., 2006). Apart from the direct organogenesis pathway commonly used to obtain transgenic plants, Pathi et al. (2013) also reported the recovery of transgenic tobacco plants through direct somatic embryogenesis.

Regeneration of Escape

The emergence of escapes in genetic transformation works is a common phenomenon, as it has also been reported in other studies (Hayta et al., 2019; Maggini et al., 2021; Miguel et al., 2020). The escaped plant (CAD1-OE25) recovered in this study could be an untransformed plant that escaped the selection procedure, or it has incorporated a truncated T-DNA that only contains the selectable marker gene that renders it resistant to hygromycin. It is also possible that this escape is a chimera that contains both transformed and untransformed cells. Factors affecting the regeneration of escapes in genetic transformation works include the transformation construct used (Hayta et al., 2019), the dose of selective agent (Hu et al., 2016; Li et al., 2013), and the number of subcultures during shoot regeneration (Li et al., 2009). A double selection system that combines selectable makers and fluorescence protein genes could improve selection efficiency.

Inheritance of Transgene

The transgenic plants produced must undergo a series of molecular analyses before being used in the functional study. Transgene silencing tends to occur in transgenic plants with multiple TIL (Rajeevkumar et al., 2015; Tang et al., 2007). Hence, obtaining a transgenic plant with a single TIL is essential before characterization. The number of transgenic loci in a transgenic line is determined by studying transgene segregation in the offspring generation. Segregation analysis is a simple, reliable, and cost-effective method to determine the number of transgenic loci in transgenic plants. Besides, segregation analysis also helps to illustrate the inheritance of the transgene by the T_1, T_2, and subsequent generations, confirming stable integration of a transgene in the primary transformant (T_0 generation). Unfortunately, the use of this method is limited to plant species that produce a large number of seeds, as many seeds (suggested around 120 seeds) are required in a segregation analysis to obtain a reliable result. For model plant species that produce a lot of seeds (like tobacco, tomato, *Arabidopsis,* and *Brachypodium*), there is no problem with conducting a segregation analysis to determine the number of transgenic loci. Unfortunately, segregation analysis is not feasible for plant species that produce fewer seeds or do not produce seeds (such as orchids, bananas, sugarcane, and cassava). In this regard, Southern blot analysis can determine the number of transgenic loci in transgenic plants. However, it is more tedious and technically challenging compared to segregation analysis. Furthermore, it requires special equipment (Gebbie, 2014). This is why segregation analysis determines the number of transgenic loci in this manuscript. Apart from Mendelian inheritance, non-Mendelian transgene inheritance has also been reported in transgenic rapeseed and wheat plants (Miroshnichenko et al., 2018; Raldugina et al., 2021). Factors leading to the non-Mendelian inheritance of transgene were reviewed by Yin et al. (2004).

The difference in the transgene expression level observed among different transgenic lines (Figure 5) is likely a result of the transgene position effect. The integration position of a transgene within the genome significantly influences its expression level. Transgenes inserted into regions with active chromatin and high transcriptional activity, such as euchromatin, typically exhibit higher expression levels (Kohli et al., 2006). In contrast, insertion into heterochromatin or regions with dense, inactive chromatin can lead to gene silencing or reduced expression due to positional effects and the influence of surrounding regulatory elements (Nguyen & Bosco, 2015). The local genomic context, including enhancers, repressors, and other regulatory sequences, can either enhance or impede transgene expression (Henikoff, 2000). Therefore, multiple transgenic lines should be produced when generating transgenic plants to obtain transgenic lines with high transgene expression levels.

Challenges in Producing Transgenic Plant

Production of transgenic lines that are usable for functional study is a challenging task. To achieve the goal, in-depth knowledge of genetic engineering and tissue culture is required. Since the *in vitro* culture step is the predominant part of the process, skillful personnel with good aseptic techniques are indispensable to prevent contamination of the cultures. One of the major concerns in *Agrobacterium*-mediated transformation is the elimination of *Agrobacterium* from explants after the inoculation step. It is usually achieved by adding antibiotics to the culture medium coupled with frequent subculture to suppress bacterial growth. Antibiotics such as cefotaxime, carbenicillin, and timentin have been widely used to get rid of *Agrobacterium* contamination in plant transformation (Cano et al., 2021; Haider et al., 2020; Kumar et al., 2019). Previously, several studies have shown that the antibiotics used to eliminate *Agrobacterium* displayed different levels of effectiveness (Teixeira da Silva & Fukai, 2001; Grzebelus & Skop, 2014; Priya et al., 2012). Apart from inhibiting *Agrobacterium* growth, several studies found that some of these selective agents also confer adverse effects on the growth and development of plant tissue when exceeding the optimal amount (Haider et al., 2020; Ma et al., 2015; Song et al., 2020). Hence, the selection of antibiotic(s) and the quantity used in *Agrobacterium*-mediated transformation must be considered carefully.

CONCLUSION

The production of transgenic plants is vital for functional analysis to dissect the roles of a gene. When producing a transgenic plant for functional analysis, the transgene must be stably integrated into the plant genome and expressed in transgenic plants. Hence, the transgenic plants produced must undergo a strict selection process before being used in functional analysis. It ensures that the selected transgenic plants fulfill all the criteria needed for functional analysis. A complete guide to generating and identifying transgenic tobacco lines for functional analysis is presented in this article. This method successfully produced and identified transgenic lines that comply with the requirements of functional analysis. More than 50% of the transgenic tobacco plants generated contained a single TIL. The transgene was stably integrated into the genome of primary transformants (T_0) and passed down to the T_1 and T_2 progenies. In addition, it was also confirmed that the transgenes were abundantly expressed in the selected transgenic lines. It is worth mentioning that no high-end equipment is required in this method, permitting its application in many laboratories. Therefore, this method can be a standard guideline for researchers to produce transgenic tobacco (and other model plants that produce plenty of seeds) plants for functional analysis.

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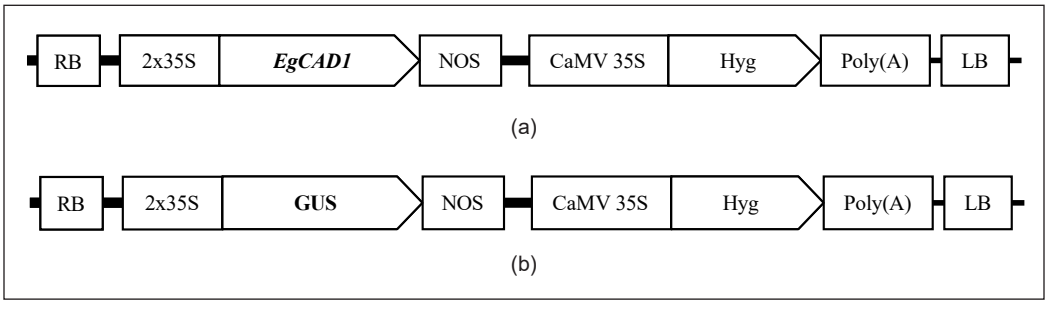
APPENDIX

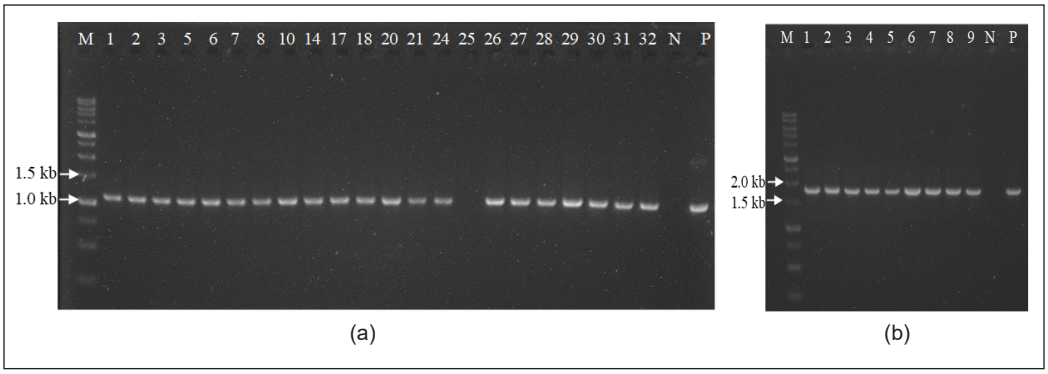
Supplementary Table 1
Composition of tissue culture media used to produce transgenic plants

No.	Medium	Nutrient	Sucrose (g/L)	Gelrite (g/L)	Hormone	Antibiotic
1	Germination medium	Half-strength MS & vitamins	15	2.5	-	-
2	Inoculation medium	MS & vitamins	30	-	-	-
3	Co-cultivation medium	MS & vitamins	30	2.5	2.0 mg/L 6-Benzylaminopurine (BAP) + 0.1 mg/L Naphthaleneacetic acid (NAA)	-
4	Regeneration medium 1	MS & vitamins	30	2.5	2.0 mg/L BAP + 0.1 mg/L NAA	50 mg/L hygromycin + 250 mg/L timentin
5	Regeneration medium 2	MS & vitamins	30	2.5	0.5 mg/L BAP	50 mg/L hygromycin + 250 mg/L timentin
6	Rooting medium	MS & vitamins	30	2.5	0.1 mg/L NAA	50 mg/L hygromycin + 250 mg/L timentin
7	Screening medium	Half-strength MS & vitamins	15	2.5	-	50 mg/L hygromycin

Note. MS = Murashige & Skoog (1962) basal salt mixture including original vitamins (Duchefa Biochemie, Netherlands). The pH of all media was adjusted to 5.7-5.8 before autoclave



Supplementary Figure 1. Schematic representation of the T-DNA region of the constructs used in the present study. (a) pMDC32-*EgCAD1* (b) pMDC32-GUS. RB=right border; 2x35S=dual 35S promoter; *EgCAD1=Elaeis guineensis Cinnamyl Alcohol Dehydrogenase 1* gene; GUS=β-Glucuronidase gene; NOS=nopaline synthase terminator; CaMV 35S=CaMV 35S promoter; Hyg=hygromycin B resistance gene; Poly(A)=CaMV poly(A) signal; LB=left border



Supplementary Figure 2. PCR analysis of putative primary transformants (T_0 generation). (a) PCR products of *EgCAD1* gene (1075 bp) amplified from CAD1-OE transgenic lines. (b) PCR products of GUS gene (1816 bp) amplified from GUS-OE transgenic lines. Numbers above the well represent the individual transgenic lines. M=1kb DNA ladder (1st BASE, Malaysia); N=negative control (wild-type tobacco); P=positive control (pMDC32 plasmid containing *EgCAD1* or GUS gene)