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Original article

Efficient floral dip transformation method using *Agrobacterium tumefaciens* on *Cosmos sulphureus* Cav.

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ABSTRACT

Yellow cosmos (*Cosmos sulphureus* Cav.) is a specific flowering plant and considered a suitable genetic engineering model. *Agrobacterium*-mediated plant transformation is commonly used for plant genetic engineering. Floral dip transformation is one of the plant genetic transformation methods, and it involves dipping flower buds into an *Agrobacterium* suspension. Studies on floral dip transformation of yellow cosmos have never been reported. Therefore, an efficient method in plant genetic engineering must be established. This study developed an effective and efficient floral dip transformation method for yellow cosmos.

In this study, flower buds with sizes of 5–7 mm were used. Several parameters have been observed to optimize the floral dip method. These parameters included the optical density (OD_{600}) of *Agrobacterium* culture, concentration of surfactant, and duration of flower bud dipping into the *Agrobacterium* suspension.

The results showed that the floral dip method was most efficient when the flower buds were dipped into *Agrobacterium* suspension with OD_{600} = 0.8 and containing 5% sucrose and 0.1% Silwet L-77 for 30 s. This method enhanced the transformation efficiency at a rate of 12.78 ± 1.53%. The *neomycin phosphotransferase* II and green fluorescent protein genes with sizes of 550 and 736 bp, respectively, were confirmed by polymerase chain reaction. In addition, the transgenic plants were kanamycin resistant and fluorescent under ultraviolet light observation. This finding suggests that the proposed floral dip transformation provides new insights into efficient plant genetic engineering methods for yellow cosmos.

1. Introduction

Yellow cosmos (*Cosmos sulphureus* Cav.) is an annual tropical plant that belongs to the Asteraceae family and has a golden-yellow flower. In general, each flower has approximately 15–30 flower tubes with a stalk length of 15 cm (Hilmi et al., 2020; Win, 2016). Recently, yellow cosmos has been reported to contain high levels of secondary metabolites, such as flavonoids, phenolics, tannins, and gallic acid, which can be used as natural pesticides and herbicides (Aftab et al., 2021; Respatie et al., 2019; Saleem et al., 2017). Yellow cosmos produces daisy-like flowers with seeds atop long and slender stems. Moreover, the cosmos plant has many flowers, pollination can be controlled, and seeds can be produced quickly. This plant is a suitable model for genetic engineering using the floral dip method.

Several methods, including *Agrobacterium*-mediated plant transformation, are used for plant genetic engineering (Keshavareddy et al., 2018; Mayavan et al., 2015). *Agrobacterium*-mediated transformation is the most widely used plant genetic engineering method because this technique is low cost, simple, and efficient for plant genetic transformation methods. *Agrobacterium tumefaciens* from soil bacteria causes crown gall disease through open tissues to colonize plant cells. The advantage of

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Agrobacterium is that it contains a tumor-inducing plasmid (Ti-plasmid), virulence genes (*vir*), and chromosomal virulence (*chv*) (Silalahi et al., 2021; Subramanyam et al., 2015). Therefore, plant genetic transformation heavily relies on A. tumefaciens as a powerful tool for the transfer of genes into the plant genome.

To date, genetic transformation methods are often carried out through plant tissue culture. The disadvantages of plant genetic transformation using tissue culture-based methods are the high cost, laboriousness, and easy contamination by bacteria and fungi Morphogenesis and organogenesis in tissue culture processes require a considerable long time from culture until acclimatization (Handayani et al., 2022; Jakhar et al., 2019). Therefore, an efficient genetic transformation method must be developed. Tissue culture-independent–based transformation using the *in planta* method may be an option to overcome this problem without conducting tissue culture (Chumakov and Moiseeva, 2012; Yaroshko et al., 2020).

Floral dip method is one of the *in planta* genetic transformation procedures, and it is performed by dipping flowers into an *Agrobacterium* suspension in a time-dependent manner (Bastaki and Cullis, 2014; Rod-In et al., 2014). The floral dip method does not require a particular tissue culture skill, eliminates soma-clonal variations, and is affordable (Chumakov et al., 2006; Eck, 2018; Zhang et al., 2017). The floral dip method is influenced by several factors, such as the density of bacteria, the presence of sucrose, concentration of surfactants, and dipping duration. Bacterial density indicates the amount of bacterial growth in a medium suspension (Clough and Bent, 1998). Sucrose serves as an energy source for bacterial growth (Subramanyam et al., 2015). The duration of target organ dipping into the *Agrobacterium* suspension provides the bacteria with the opportunity to process gene insertion (Zhang et al., 2006). Meanwhile, surfactants can reduce the surface tension of target organs and act as attractants to increase the penetration of bacteria into plant tissues (Curtis and Nam, 2001). Studies of floral dip transformation has been reported for *Arabidopsis thaliana* (Bent, 2006; Clough and Bent, 1998; Narusaka et al., 2010; Zhang et al., 2006), *Raphanus sativus* (Curtis and Nam, 2001), *Scherenkiella parvula* (Wang et al., 2019), *Amaranthus caudatus* L. (Yaroshko et al., 2018) and *Zea mays* (Guiqin et al., 2012). However, the transformation efficiency was<5%. A higher transformation efficiency was obtained in *Solanum lycopersicum* L.(Yasmeen et al., 2009), *Oryza sativa* (Rod-In et al., 2014), *A. thaliana* (Ali et al., 2022) and *Linum usitatissimum* (Bastaki and Cullis, 2014).

The application of floral dip transformation method in yellow cosmos has not been reported. Therefore, this study is important for improving the efficiency of plant breeding through a biotechnological approach. This research aimed to develop an effective and efficient floral dip transformation method for yellow cosmos.

2. Materials and methods

2.1. Plant material

C. sulphureus Cav. Plants were planted in a mixture of soil medium and organic manure (1:1) in a polybag at the Green House, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. The plants were maintained until the generative phase after 10 weeks for floral dip transformation.

2.2. Agrobacterium tumefaciens preparation

An *Agrobacterium tumefaciens* strain GV3101 containing the recombinant vector pRI101AN- green fluorescent protein (GFP) was provided by Prof. Bambang Sugiharto of The Center for Development of Advanced Science and Technology, University of Jember, Indonesia. *Agrobacterium* containing the GFP-encoding gene was confirmed by polymerase chain reaction (PCR) colony using two pairs of primers: *neomycin phosphotransferase* II (*NPT*II) and GFP. Detection vector using two genes was utilized to confirm the presence of T-DNA (Fig. 1). The composition of the PCR mix will be described Section 2.7. The *NPT*II gene had a band size of 550 bp, and the full-length GFP had band size of 736 bp.

2.3. Floral dip transformation

The *Agrobacterium* suspension was prepared by the addition of 5% sucrose and Silwet L-77. To establish the floral dip transformation method in yellow cosmos, we applied two different optical densities (OD_{600}) of *Agrobacterium* culture, i.e., 1.5 and 0.8, in the first experiment. The different durations of bud dipping were 30, 60, and 90 s. In the second experiment, the suspension was added with varied concentrations of Silwet L-77, i.e., 0%, 0.05% and 0.1%. Ten buds were used in each treatment.

The floral dip transformation method was started by preparing an *Agrobacterium* suspension and then dipping the flower buds until they were covered by the suspension. Fig. 2 presents the procedure of the floral dip transformation method. A colony of *A. tumefaciens* strain GV3101 carrying the recombinant vector pRI101AN-GFP was cultured in a starter medium. It was cultured in 5 mL Luria Bertani (LB) medium containing 50 ppm kanamycin, 100 ppm rifampicin, and 12.5 ppm gentamicin in the dark for overnight and with shaking at 200 rpm at 27 °C. The starter was transferred to 50 mL LB medium containing the same antibiotics and then shaken at 250 rpm at 27 °C to obtain OD_{600} = 1.5, 0.8. Afterward, the suspension at OD_{600} = 0.8 was transferred to a new conical tube, and 5% sucrose and Silwet L-77 were added to each treatment. Flower buds with sizes of 0.5–0.7 mm were used (Fig. 3**a**). The infection was carried out by dipping the flower buds into the *Agrobacterium* suspension for each treatment period (Fig. 3**b**) and covering them with a paper for 48 h to maintain high humidity (Fig. 3**c**). Finally, the flowers were maintained for 3 weeks until the seeds were physiologically ripe. The seeds were harvested and dried at room temperature.

2.4. Screening of seedling on the medium containing kanamycin

The harvested seeds were soaked in 2 gL^{-1} fungicide (Dhitane™ M−45) containing mancozeb 80% and bactericide (Agrept® 20WP) containing 20% streptomycin sulfate for 30 min. Then, the seeds were rinsed with distilled water and germinated on a medium using a filter paper with 50 ppm kanamycin for 7 days. Kanamycin-resistant seedlings were transplanted to the growth medium composed of mixtures of soil and manure in a screen house until the generative period.

2.5. Detection of GFP in the assay of T1 cosmos resistant to kanamycin

Both leaves and steam collected from one month after planting screened and wild-type plants were used for the detection of fluorescence GFP gene. The samples were visualized using an ultraviolet (UV) transilluminator (Scope WD, Japan). Then, they were documented in light and dark conditions using a digital camera.

2.6. Genomic DNA extraction

Yellow cosmos leaves were extracted using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) with modifications. Then, 100 mg leaves were grinded using pastel stick added with liquid N_2 in a 2 mL tube. The samples were added with 1.400 µL buffer (2% CTAB, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 M ethylenediaminetetraacetic acid, 2% polyvinylpyrrolidone, 2%

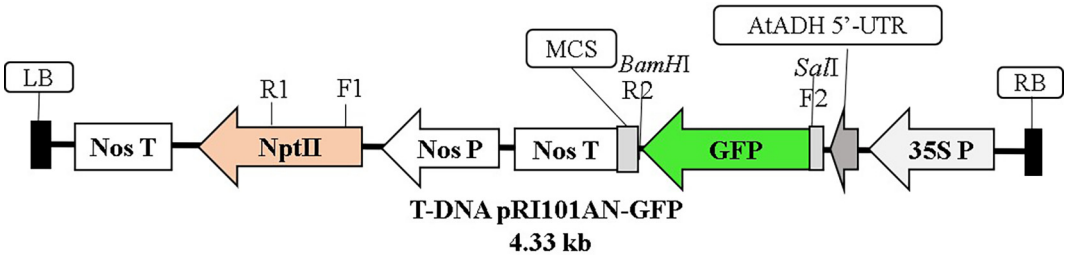

Fig. 1. T-DNA physical map of the recombinant vector pRI101AN-GFP.

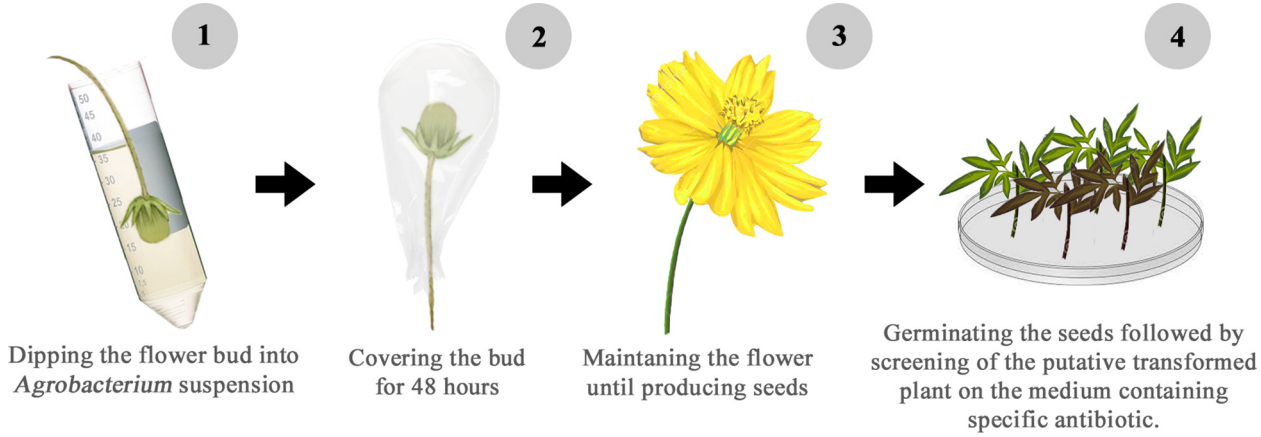

Fig. 2. Floral dip transformation of yellow cosmos *C. sulphureus* Cav.

Fig. 3. Materials used in floral dip transformation of yellow cosmos *C. sulphureus* Cav. **a)** Cosmos flower buds with sizes of 5–7 mm, **b)** floral dip transformation process, and **c)** covering of flower buds for 48 h.

β-mercapto-ethanol, and ddH_2O) and then incubated in a water bath at 65 °C for 60 min. Afterward, the samples were centrifuged at 12,000 rpm for 10 min using Minispin® (Eppendorf, Germany). The supernatants were transferred to a new 1.5 mL tube and added with 500 µL chloroform isoamyl alcohol (CIA). The samples were vortexed and centrifuged at 12,000 rpm for 15 min. The supernatants were transferred to a new tube and added with 500 µL CIA followed by vortexing and centrifugation in a similar manner. The supernatants were transferred to a 1.5 mL new tube and added with 1/10 from a solution of sodium acetate and 2/3 from a total solution of cold isopropanol. The solutions were stored overnight at – 20 °C. Afterward, the solutions were centrifuged at 12,000 rpm for 15 min, and the flowthrough was discarded. The tubes were added with 500 µL 70% ethanol and centrifuged at 12,000 rpm for 5 min. The flowthrough was discarded, and 500 µL ethanol absolute was added. The solutions were centrifuged at 12,000 rpm for 5 min, the flowthrough was discarded, and the pellets were dried at room temperature. Finally, the pellets were added with 50 µL ddH_2O, and the DNA samples were stored at – 20 °C.

2.7. PCR analysis

PCR was used to detect *NPT*II and GFP genes in transgenic plants. The PCR reaction mixture consisted of 5 µL Powerpol 2X PCR Mix with Dye® (ABclonal, USA), 2 µL nuclease-free water, 1 µL DNA sample, and 1 µL of each forward and reverse primer (10 mM). Amplification of the *NPT*II gene was performed using a forward primer and a reverse primer with a size product of 550 bp. The GFP gene was amplified using a forward primer and

a reverse primer with a product size of 736 bp (Table 1). DNA amplification was conducted using a PCR T100™ Thermal Cycler (Bio-Rad, USA). Amplification was carried out with thermocycling settings consisting of predenaturation at 95 °C for 3 min, followed by 39 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for the *NPT*II gene and 58 °C for the GFP gene, elongation at 72 °C for 1 min, and final elongation 72 °C for 5 min. The PCR products were separated by electrophoresis Powerpac-Basic™ (Bio-Rad, USA) in 1% agarose gel added with 5 μL DNA stain for a 30 mL solution gel. The plate was added with 5 μL ladder AccuBand™ 100 bp + 3 K DNA Ladder II (Smobio, Taiwan) as the marker band, 10 μL PCR product of the plasmid as the positive marker, wild-type plant, and each putative transgenic plant. Lastly, the agarose gel was visualized in a UV transilluminator (Scope WD, Japan).

2.8. Data analysis

Data from different levels of OD, durations, and Silwet L-77 were analyzed using the analysis of variance. The significant data were analyzed using DMRT $\alpha < 0.05$. Then, the comparison of level bacterial density was analyzed by *t* test with $\alpha < 0.05$ using R studio.

3. Results

3.1. Confirmation of the presence of binary vector in Agrobacterium tumefaciens

A. tumefaciens strain GV3101 containing the binary vector pRI101AN-GFP was cultured in LB medium with selective antibiotics. The results of the PCR colony *A. tumefaciens* showed that the single amplicon size was 550 bp for *NPT*II and 736 bp for GFP (Fig. 4). The target sequences were amplified using *NPT*II and GFP genes as selectable markers. This *A. tumefaciens* strain can be used for genetic transformation using the floral dip method.

3.2. Effect of floral dipping on the number of seeds produced

The results showed that the difference in OD and dipping duration had an effect on the average number of seeds produced per flower. A high OD resulted in a small number of seeds. Likewise, the increase in dipping duration produced a few seeds (Table 2). This result showed that the high density of *Agrobacterium* suspension and long dipping period caused a decrease in the amount of seeds produced.

3.3. Screening of seedlings resistant to kanamycin

Based on experiment 1 (Table 2), a lower percentage of kanamycin-resistant plants were obtained using *Agrobacterium* suspension at OD_{600} = 0.8 for 30 s obtained compared with those at OD_{600} = 1.5, whose percentage reached 45.32%. The plants survived on the selection media were suggested as putative transformants. These results indicate that the plants contained the *NPT*II gene as a kanamycin resistance antibiotic. *In vivo* plant screening

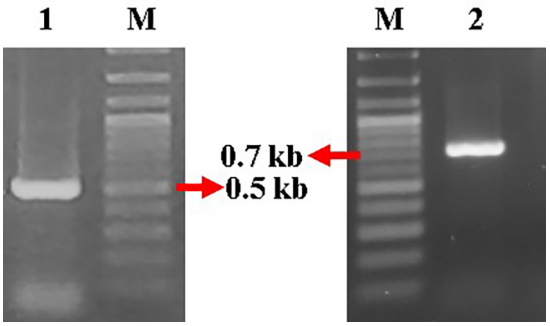

Fig. 4. Confirmation of *A. tumefaciens* colonies containing the recombinant vector pRI101AN-GFP in PCR using the primers of *NPT*II and GFP. M: 100 bp ladder, 1: amplicon *NPT*II gene with a band size 550 bp; 2: amplicon GFP gene with a band size 736 bp.

using filter paper media with kanamycin was more efficient than in vitro screening (Fig. 5**a**). The grown plants were then transferred to the soil media in polybags maintained in the greenhouse until T2 seeds were obtained (Fig. 5**b**).

3.4. GFP assay of T1 putative transformant plants

Based on the results and detection of GFP signals, some plants have been selected from the medium containing kanamycin. Their leaf and stem samples were fluorescent under UV light. In addition, other plant and wild-type samples showed no fluorescence (Fig. 6). This finding indicates that the GFP gene has been inserted into the genome of the cosmos plant. GFP is a reporter gene whose expression can be detected visually without molecular analysis. It can assist in the selection process of transgenic plants. To ensure that transgenic plants were obtained, we performed PCR to detect the *NPT*II and GFP genes in the plant DNA.

3.5. PCR analysis of T1 transgenic yellow cosmos

Molecular analysis of DNA genome from putative transformed plants in the selection medium and wild-type plants was carried out by amplification of specific primers for *NPT*II and GFP genes. Based on the first experiment, bacterial density OD600 = 0.8 for 30 s resulted in a high average transformation efficiency (8.26 ± 6.5%). The results showed that transformed plants were indicated by the presence of approximately 550 bp amplicon region of *NPT*II gene (Fig. 7**a**) and 736 bp amplicon GFP gene in ten samples (Fig. 7**b**) of 44 T1 transgenic plants in the second experiment. This result indicated that the genomic DNA-presenting gene amplicon was detected in the transgenic plants.

3.6. Factors affecting the transformation efficiency of floral dip on C. sulphureus Cav.

3.6.1. Effect of bacterial density

Different bacterial densities were used in floral dip transformation in *Agrobacterium* suspension (OD_{600} = 0.8, 1.5). The flower

Table 1
Primers used for PCR analysis of *NPT*II and GFP genes in putative transformed plants.

Primer	Sequence	GC contents	Tm (°C)	Size product
*NPT*II-F1	5′- GTCATCTCACCTTGCTCCTGCC −3′	59%	60	550 bp
*NPT*II-R1	5′- GTCCGTTGGTCGGTCATTTCG −3′	57.14%		
GFP-F2	5′- GCTGTCGACATGCGTAAAGG −3′	52.38%	58	736 bp
GFP-R2	5′- GCGCGGATCCTTATTTGTATAG −3′	45.45%		

Table 2
Effect of OD, dipping duration, and Silwet L-77 on the average number of seeds and plants resistant to kanamycin and transformation efficiency of T1 *C. sulphureus* Cav.

Treatments				Results			
Experiment	***Agrobacterium* suspension**	**Silwet L-77 (%)**	**Duration (s)**	**Number of seeds**	**Number of seedlings**	**Resistant to Kanamycin (%)**	**Transformation efficiency (%)**
	Control	0	0	14.5 ± 2.7^{a}	0 ± 0^{d}	0 ± 0^{c}	0 ± 0^{b}
I		0	30	3.40 ± 1.5^{d}	0.8 ± 1.0^{c}	23.06 ± 23.6^{b}	0 ± 0^{b}
	OD_{600} = 1.5	0	60	10.2 ± 3.2^{bc}	4.6 ± 1.6^{a}	45.32 ± 11.7^{a}	0 ± 0^{b}
		0	90	2.00 ± 1.7^{d}	0.9 ± 1.1^{c}	26.00 ± 28.6^{b}	0 ± 0^{b}
		0	30	12.3 ± 2.0^{a}	1.8 ± 1.3^{bc}	14.63 ± 11.9^{b}	8.26 ± 6.5^{a}
	OD_{600} = 0.8	0	60	9.00 ± 2.7^{c}	2.2 ± 1.4^{b}	24.97 ± 15.8^{b}	2.26 ± 4.9^{b}
		0	90	11.1 ± 2.4^{ab}	1.3 ± 1.4^{bc}	11.71 ± 10.5^{b}	0 ± 0^{b}
II	OD_{600} = 0.8 + 5% sucrose	0	30	15.4 ± 4.0^{a}	4.2 ± 3.2^{a}	25.35 ± 19.2^{a}	9.74 ± 2.0^{b}
		0.05	30	12.9 ± 2.5^{a}	4.2 ± 3.6^{a}	32.52 ± 29.2^{a}	9.38 ± 2.3^{b}
		0.1	30	13.3 ± 4.8^{a}	4.5 ± 3.0^{a}	35.11 ± 19.4^{a}	12.78 ± 1.5^{a}

Note that the number followed by the same letters is not significantly different based on the DMRT test; $\alpha < 0.05$.

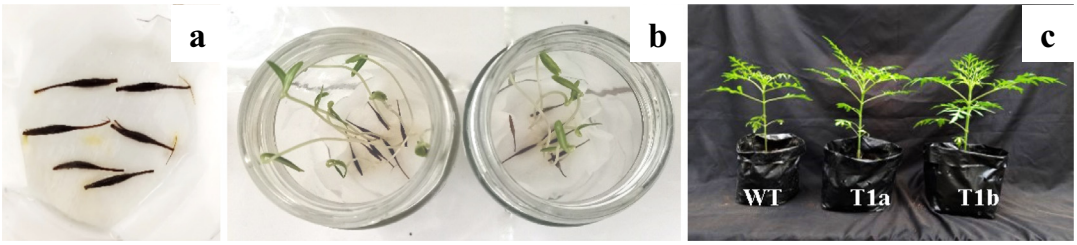

Fig. 5. Screening of *C. sulphureus* Cav. putative transformants in the selection medium containing 50 ppm kanamycin. **a)** Wild-type seeds did not germinate on the selection medium after 7 days. **b)** Seedlings selected from the selection medium containing 50 ppm kanamycin after 7 days. **c)** Transformed cosmos plants at age of 6 weeks. WT: Wild type; T1a and T1b: first-generation transgenic cosmos plants.

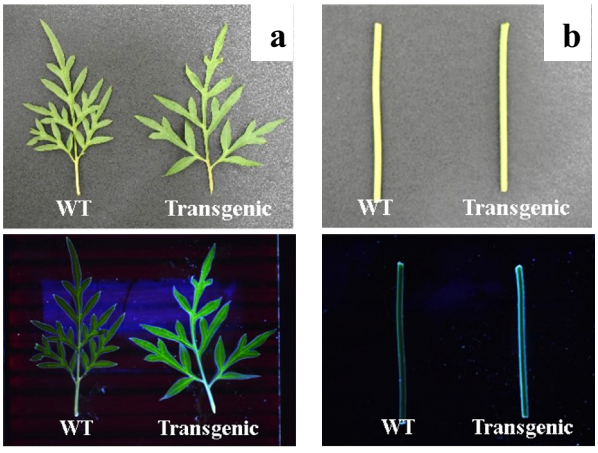

Fig. 6. GFP assay of T1 yellow cosmos organs using UV light: **a)** leaves from wild-type and transgenic plants in light condition (upper) and UV transilluminator (lower); **b)** stems from wild-type and transgenic plants in light condition (upper) and UV transilluminator (lower).

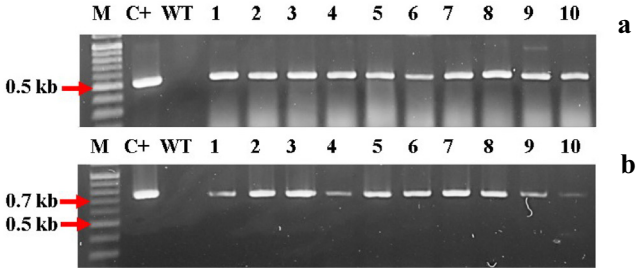

Fig. 7. Amplicon genes from PCR product in 1% agarose gel, **a)** *NPT*II gene with a size of 550 bp, **b)** GFP gene with a size of 736 bp. M: 100 bp ladder, C+: control (recombinant plasmid), WT: wild-type; No. 1–10: DNA transgenic plant samples.

buds infected in *Agrobacterium* suspension at OD_{600} = 0.8 obtained the highest transformation efficiency of (3.74 ± 3.24%) than those at OD_{600} = 1.5, which caused no plant transformation according to the *t* test (Fig. 8**a**). In addition, the high bacterial density caused browning of the buds after dipping into the *Agrobacterium* suspension. This step disturbed flower blooming, which decreased the number of seeds produced.

3.6.2. Effect of dipping duration

The flower buds were infected by *Agrobacterium* suspensions for various intervals from 30 to 90 s. Dipping duration for 30 s obtained the highest transformation efficiency of (4.13 ± 6.16%) (Fig. 8**b**). The low duration effectively increased the transformation efficiency of floral dipping of yellow cosmos.

3.6.3. Effect of silwet L-77 concentration

Bacterial density of OD_{600} = 0.8 with 5% sucrose and duration dipping of 30 s were used in various concentration levels of Silwet L-77 (0%, 0.05%, and 0.1%). The different concentration levels of Silwet-77 affected the increase in the transformation efficiency. The best treatment was observed with 0.1% concentration of Silwet L-77, with a transformation efficiency of (12.78 ± 1.53%). The 0% and 0.05% concentrations of Silwet L-77 attained transformation efficiencies of (9.74 ± 2.01%) and (9.38 ± 2.38%), respectively (Fig. 8**c**). Silwet L-77 as surfactant assisted in the increase in insertion level.

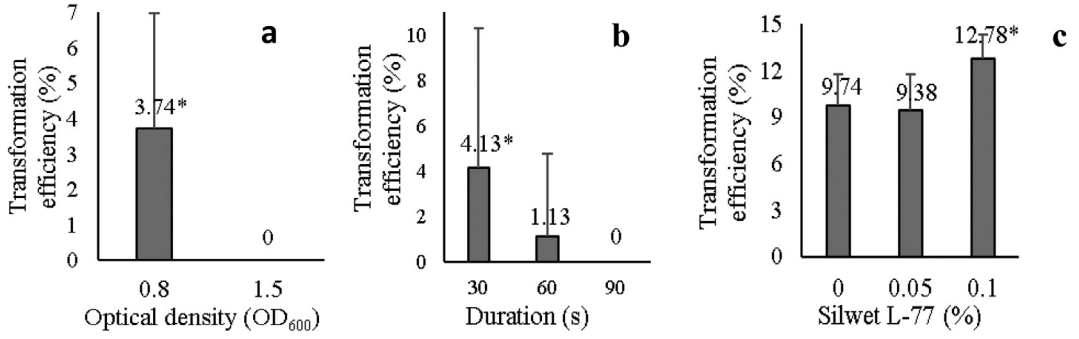

Fig. 8. Effect of different treatments on transformation efficiency of floral dipping of *C. sulphureus* Cav. in the presence of *NPT*II and GFP genes. **a)** Effect of *Agrobacterium* OD_{600}, **b)** dipping duration (s) in the first experiment; **c)** effect of Silwet L-77 (%) in the second experiment. (*): significant based on the T-test and DMRT test $\alpha < 0.05$.

4. Discussion

The *Agrobacterium* strain GV3101 containing the recombinant vector pRI101AN-GFP was successfully used for floral dip transformation of yellow cosmos (*C. sulphureus* Cav.). Floral dip transformation is a simple and efficient genetic transformation method. The floral dip transformation method does not require a particular skill and rapidly obtains transgenic plants. This method is faster than tissue culture in obtaining genetically modified organisms (Chumakov and Moiseeva, 2012; Eck, 2018). In addition, it can be performed outside the laboratory without using laminar air flow and unsterile conditions (Ali et al., 2022b; Yaroshko et al., 2020). Several factors, such as flower bud size, bacterial density, sucrose content, dipping duration, and concentration of surfactant agent (Silwet L-77), affect the increase in transformation efficiency.

A. tumefaciens strain GV3101 has a c5b chromosomal background with the Ti-plasmid pTiC5BD, opine of nopaline, and genes resistant to rifampicin and gentamicin (Yadav et al., 2014). A high bacterial density causes necrosis in the target organ. Necrosis can inhibit the development of flower tubes until they dry out. This condition provides a hypersensitive response that reduces the potential for postinfection cell regeneration and recovery (Jaberi et al., 2018; Yong et al., 2006). Moreover, the dipping duration causes flower bud damage and inhibits seed development (Smagur et al., 2009). A significant reduction in the number of seeds has been reported in the floral dipping of *Triticum sp*., which was caused by the long duration of infection (Zale et al., 2009).

The recombinant vector pRI101AN-GFP has two inserted genes, namely, *NPT*II and GFP, as selectable marker and reporter gene, respectively. *NPT*II is used as a selectable marker for resistance to kanamycin antibiotics. In this study, the plants were germinated as putative transformants in a selection medium. Thus, the seedlings germinated had kanamycin resistance. The *NPT*II gene cannot inhibit protein synthesis and ribosome binding in transformant plants (Irsyadi et al., 2022). However, the selected plants can be affected by their resistance properties. High concentrations caused seedling etiolation and plant death (Chen et al., 2020). A concentration of 50 ppm kanamycin was effectively used in the selection of transformants of *Glycine* max L. (Isda, 2012) and *Saccharum officinarum* (Fibriani et al., 2019).

Plants selected from the selection medium containing kanamycin lacked *NPT*II and GFP gene amplicons according to the PCR. Thus, the seedlings escaped at 50 ppm kanamycin concentration. Irsyadi et al. (2022) reported that in 50 ppm kanamycin concentration, selected putative transformants of *C. sulphureus* that escaped kanamycin reached 3.84%. Dalton et al., (1995) reported the extremely low concentration of selective agents, which allowed the undesirably high numbers of escape and loss of resistant transformants. Moreover, each plant species has a different tolerance level for various concentrations of kanamycin antibiotics. Seedlings of wild-type *A. thaliana* tolerated up to 1,400 ppm kanamycin (Chen et al., 2020), *Gossypium hirsutum* L. tolerated up to < 50 ppm kanamycin (Unbeck et al., 1989), *Lycopersicon esculentum* Mill was putatively transformed under 100 ppm kanamycin (Subaila and Saleh, 2010), and *Artemisia annua* L. tolerated up to < 20 ppm kanamycin (Chen et al., 2000).

GFP is a reporter gene that can be detected directly without cofactors and certain substrates to exhibit fluorescence in UV or blue light (Chen et al., 2015; Zhao et al., 2016). However, GFP was not detected in all transgenic plants. Such result was due to the autofluorescence of chloroplasts and plant cell walls; solids become a nuisance when detecting GFP fluorescence in plants. In addition, the physiological conditions of the transformant plant and the number of copies of the GFP gene affect the green glow intensity (Cheng et al., 2019). The e*YGFPuv* gene was successfully expressed in fluorescence of *A. thaliana* transformant plants resulting from a floral dip during growth. The fluorescence of the leaf, root, and stem can be observed under UV or blue lights (Yuan et al., 2021).

In floral dip transformation, flower size influences the success of transformation efficiency. As a result, flower buds transform into young organs that have meristem tissues, which can enhance bacterial insertion into plant tissues. Flower buds are still undergoing meiosis to form male and female flowers (Mayavan et al., 2015; Yasmeen et al., 2009). Unpollinated flower buds with sizes of 5–7 mm were used as target organs in this study. In addition, bacterial density affects the increase in the transformation efficiency. *Agrobacterium* growth in the log phase is often used in *Agrobacterium*-mediated transformation. In this phase, the bacteria remain actively growing. This finding usually indicates that the level of bacterial density OD_{600} = 0.4–0.8, which is effective for genetic transformation (Clough and Bent, 1998). Moreover, the use of extremely high bacterial density affects the transformation efficiency level. The bacterial density of OD = 1.5 did not produce transformed plants in yellow cosmos. In addition, the sucrose content can increase the transformation efficiency. Sucrose is a source of energy and additional nutrient for bacterial growth (Rizal et al., 2019; Subramanyam et al., 2015).

The duration of floral bud dipping into the *Agrobacterium* suspension can affect the transformation efficiency. A long dipping duration causes a decrease in the number of seeds and inhibits the development of flower buds (Bastaki and Cullis, 2014; Jaberi et al., 2018). This condition was reported for *Triticum* sp. (Zale et al., 2009). Furthermore, surfactants affected the transformation efficiency. Silwet L-77 is a surfactant with low phytotoxicity and can reduce surface tension and open the pores of plant tissues.

Moreover, Silwet L-77 acts as an attractant that can attract bacteria into the plant genome (Clough and Bent, 1998).

The bud size, sucrose content, and Silwet L-77 content have been reported in the floral dipping of *L. usitatissimum* with bud sizes of 5–10 mm in *Agrobacterium* suspension containing 5% sucrose and 0.1% Silwet L-77, which resulted in the transformation efficiency of 50% (Bastaki and Cullis, 2014), the flower target before pollination with the same suspension content in *S. lycopersicum* was 23% (Yasmeen et al., 2009) and a value of 12.7% was obtained for *O. sativa* (Rod-In et al., 2014). A concentration of 0.1% Silwet L-77 was effective in the floral dipping of *A. thaliana* and obtained a transformation efficiency of 10% (Ali et al., 2022). In addition, flower buds with a diameter of 2 mm were effective in floral dipping of *Brassica rapa* (Hu et al., 2019). A high OD of 1.4 has been reported for the floral dipping of *Capsella grandiflora*, which obtained a transformation efficiency of 0.52% (Dew-Budda et al., 2019). At an OD of 0.8, the floral dipping of *R. sativus* L. (Curtis and Nam, 2001), *Scherenkiella parvula* (Wang et al., 2019), and *A. thaliana* was performed (Zhang et al., 2006). Moreover, 5% sucrose was effectively used in the floral dipping of *S. officinarum* (Mayavan et al., 2015) and *Tricosanthes cucumerina* L. (Subramanyam et al., 2015).

5. Conclusion

The flower bud target organ dipped in *Agrobacterium* suspension at OD_{600} = 0.8 + 5% sucrose + 0.1% Silwet L-77 for 30 s was effective as a floral dipping method for yellow cosmos (*C. sulphureus* Cav.), with a transformation efficiency of (12.78 ± 1.53%). Simple and effective transformation methods enhance the development of plant genetic engineering methods.

CRediT authorship contribution statement

Aziz Purwantoro: Conceptualization, Supervision. **Muhammad Burhanuddin Irsyadi:** Methodology. **Widhi Dyah Sawitri:** Conceptualization. **Nor Chamidah Fatumi:** Methodology. **Shania Nur Fajrina:** Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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