BIODIVERSITAS
Volume 23, Number 12, December 2022
Pages: 6097-6105

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231202

Molecular and phenotypic characteristics of T1 transgenic yellow cosmos (*Cosmos sulphureus*) carrying neomycin phosphotransferase II gene

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Manuscript received: 3 October 2022. Revision accepted: 26 December 2022.

Abstract. *Irsyadi MB, Sawitri WD, Purwantoro A. 2022. Molecular and phenotypic characteristics of T1 transgenic yellow cosmos (*Cosmos sulphureus*) carrying neomycin phosphotransferase II gene. Biodiversitas 23: 6097-6105.* Yellow cosmos (*Cosmos sulphureus*) is a tropical ornamental flower that contains secondary metabolites useful for natural pesticide application. Plant genetic transformation is one genetic engineering method used to increase secondary metabolite accumulation. However, published reports on this issue have yet to be made available. This is the first report of genetic transformation in transgenic yellow cosmos using neomycin phosphotransferase II (*npt*II) encoding gene. This study aimed to determine the efficiency transformation and phenotypic character of the T1 yellow cosmos transgenic *npt*II. This genetic transformation was carried out by the floral dip method mediated with *Agrobacterium tumefaciens* and characterized based on quantitative and qualitative observations, as well as a cluster analysis of transgenic plants. The T1 transgenic yellow cosmos was confirmed using PCR with a transformation efficiency of 73.33% based on the total plants resistant to Kanamycin and 10.57% based on the total seeds transformed. The presence of the *npt*II encoding gene was shown in transgenic plant samples with a size of 550 bp. In general, the introduction of the *npt*II gene generated no novel traits except the resistance to the kanamycin antibiotic. However, there was a decrease and increase in the number of stomata and the size of stomata in transgenic plants, respectively. In addition, there was a change in the type of ray floret to a mixture of ligulate and tubular, indicating that the *npt*II gene affected the phenotype of yellow cosmos. The result revealed that the *npt*II gene was inserted fairly randomly into the plant genome.

Keywords: Cosmos, *npt*II, phenotypic, transgenic

INTRODUCTION

Yellow cosmos (*Cosmos sulphureus*) is a tropical ornamental flower that originated in South America. The habitat cosmos is spread out in the tropics, but the yellow cosmos is a not-consumed by humans and animals (Zhou et al. 2018). The yellow cosmos contains high secondary metabolites such as phenolics, flavonoids, saponin, and tannins that can be used for biopharmaceutical and biopesticide applications (Saleem et al. 2017; Aftab et al. 2021). The previous study reported that yellow cosmos also contains gallic acid, which has the potential as a natural herbicide (Respatie et al. 2019). The secondary metabolites of yellow cosmos can be increased through plant genetic transformation using the Agrobacterium-mediated technique. *Agrobacterium tumefaciens* is a soil bacteria that contains a tumor-inducing plasmid (Ti-plasmid), virulence genes (vir), and chromosomal virulence (chv) (Subramanyam et al. 2015).

Plant genetic transformation is a genetic transfer method into the plant's genome for generating genetically modified crops (Keshavareddy et al. 2018). This method aims to produce new plant varieties with the desired characteristics (Mayavan et al. 2015). However, there are some disadvantages in plant genetic transformation through in vitro cultures, such as low-efficiency transformation, expensive cost, and is easily contaminated with bacteria and fungi. In addition, the morphogenesis and organogenesis of explants need a long time of culture period (Jakhar et al. 2019; Handayani et al. 2022). Therefore, plant genetic transformation through in planta method could be an option for generating genetically modified crops without in vitro culture, such as the floral dip method. This method is performed by dipping flowers into *Agrobacterium* suspension for a few seconds. Furthermore, this method does not require a particular skill for generating transgenic plants (Bastaki and Cullis 2014; Rod-In et al. 2014).

Neomycin phosphotransferase II (*npt*II) is a selectable marker gene in the plant genetic transformation as a gene conferring resistance to the kanamycin antibiotic (Rashid 2017). This antibiotic inhibits protein synthesis in plants by binding to ribosomes in non-transgenic plants. In addition, the insertion of *npt*II can catalyze the phosphorylation of Kanamycin. Therefore, Kanamycin could not inhibit protein synthesis in kanamycin-treated plants (Davey et al. 2010; Marenkova et al. 2020). Several binary vectors that contain antibiotic resistance gene *npt*II are pCAMBIA 2300 (Rai et al. 2012), pBI121 (Kadasa et al. 2021), and pRI101AN (Anur et al. 2020).

In the plant genetic transformation system, it has been reported that the application of this method through the in-planta approach is more efficient compared to in vitro culture approach (Zhang et al. 2017). The transformed transgenic plants are confirmed by molecular analysis by amplifying target sequences with a selective molecular marker (Fatmawati et al. 2021). In addition, the plant phenotype based on plant morphological observations was also necessary to be characterized. These are determining morphological characteristics of wild-type cosmos and transgenic plants (Kuluev et al. 2012; Hilmi et al. 2020). Furthermore, these observation is important for showing qualitative and quantitative characteristics (Pons et al. 2012; Lukmanasari et al. 2020).

Phenotypic characterization of transgenic plants carrying the *npt*II gene has been reported in rice plants (*Oryza sativa*) (Lynch et al. 1995), *Arabidopsis thaliana* (Rashid 2017), Tobacco (Kuluev et al. 2012), and *Bidens pilosa* (Wang et al. 2012), *Bacopa monnieri* (Largia et al. 2016). Based on these reports, the phenotypes of *npt*II transgenic plants are mostly similar, except for the petal size of *B. pilosa* (Wang et al. 2012). In addition, transgenic rice plant has a smaller plant size than wild-type ones (Lynch et al. 1995). To date, there is no report on the phenotypic characteristics of yellow cosmos transgenic plants. Therefore, this study is important to provide new information for developing genetic engineering and plant breeding by transgenic plants carrying the *npt*II gene. This study aimed to obtain the plant genetic transformation efficiency through molecular analysis and phenotypic characteristics of the T1 transgenic yellow cosmos plant.

MATERIALS AND METHODS

Plant material

The binary vector of pRI101AN and *Agrobacterium* were obtained from the Center Development of Advanced Science and Technology (CDAST), University of Jember, Indonesia. First, the *Agrobacterium* strain LBA4404 with 50 ppm Rifampicin and 30 ppm Streptomycin antibiotics were used to infect genetically transformed yellow cosmos. Then, the yellow cosmos seeds were planted in soil media and manure 1:1 in polybags for plant material preparation. After that, the plants were maintained until the generative stage for a floral dip transformation.

Transformation vector into *Agrobacterium*

The binary vector pRI101AN (Takara, Japan) is transformed into *Agrobacterium* using the cold shock method. *A. tumefaciens* strain LBA4404 was cultured in 2 ml YEP media containing 50 ppm Rifampicin, 30 ppm Streptomycin, and 50 ppm Kanamycin during overnight shaking at 25°C. The suspension was transferred into 50 mL of YEP media and then shaken at 250 rpm for 7 hours at 25°C to obtain OD_{600}: 0.6. The suspension was incubated on ice for 10 minutes. After that, it was centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was added with 1 mL of 20 mM cold $CaCl_2$ and then resuspended. One hundred µl aliquots plus 1 ng of pRI101AN vector DNA was transferred into a new tube. Then it was incubated on ice for 90 minutes. After that, the cells were frozen using liquid N_2, and then the cells were thawed at 37°C for 5 minutes; then, 1 mL of YEP media was added and incubated at 28°C, shaken at 150 rpm. After that, the suspension was centrifuged at 10,000 rpm for 1 minute at 4°C, the supernatant was discarded, and the cells were resuspended in 100 mL YEP media. Then, it was spread on YEP media containing specific antibiotics strain, then incubated at 28°C for 2 days. The colonies were confirmed by PCR to detect the presence of the *npt*II gene.

Agrobacterium-mediated transformation in planta

Genetic transformation in planta was carried out using the floral dip method (data not shown) (Irsyadi 2022). *Agrobacterium* strain LBA4404 was used to transform the gene into yellow cosmos. The yellow cosmos seeds from floral dip were harvested 3 weeks after transformation and dried as T1 seeds.

Selection seeds T1 transgenic yellow cosmos carrying *npt*II

The T1 yellow cosmos seeds were soaked in 2 gL^{-1} bactericide and fungicide for 30 minutes. Afterward, the seeds were rinsed with distilled water and then germinated on a selected media on filter paper provided with 50 ppm Kanamycin antibiotic for a week. Seeds germinating indicated their resistance to Kanamycin antibiotics; thus, they were transplanted into polybags containing mixtures of soil and manure as the growth medium. The transplanted seeds were raised in the screen house. Plants were grown until the generative period for PCR analysis and phenotypic characterization of transgenic plants.

PCR analysis

DNA of transgenic cosmos leaves was obtained from randomly selected 60 plants. The 100 mg leaves were extracted using a 2% CTAB buffer described in the previous report (Setiawan et al. 2020). The concentration of DNA was measured using a Spectrophotometer GeneQuant1300® (Fisher Scientific, Austria). Furthermore, the DNA samples were used for PCR analysis. PCR analysis was used to detect the presence of the *npt*II gene as a marker gene in T-DNA. The amplification of the *npt*II gene was done using a forward primer (5'-GTCATCTCACCTTGCTCCTGCC-3') and a reverse primer (5'-GTCCGTTGGTCGGTCATTTCG-3'), which resulted in band size product of 550 bp length. The PCR reaction mixture consisted of 5 µL Powerpol® Master Mix, 2 µL nuclease-free water, 1 µL for each forward and reverse primer (10 mM), and 1 µL DNA sample. Amplification DNA was done using PCR T100™ thermal cycler (Bio-Rad, USA). Amplification was carried out with thermocycling settings consisting of pre-denaturation at 95°C for 3 min, followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR products were separated using electrophoresis (Bio-Rad, USA) in 1% agarose gel plus DNA staining and 5 µL ladder Smobio®. Then the gel was visualized under UV Transilluminator.

Phenotypic characterization

Phenotyping was carried out on yellow cosmos transgenic and wild-type plants aged 3 months that had entered the generative phase. Plant phenotyping was done using morphological markers based on quantitative and qualitative characters (UPOV 2015) using a completely randomized design (CRD) method. Characters observed included plant height, leaf length, leaf width, lob width, petiole length, leaf type, leaf tip shape, leaf color, stomata, flower type, collar segments, ray floret type, color, length, width, and numbers, flower diameter, disk diameter.

Statistical analysis

Quantitative phenotypic data were analyzed using T-test α: 0.05 and standard deviation using R studio to compare transgenic cosmos plants with wild types. Qualitative data were analyzed descriptively and cluster analysis using GenAlEx 6.5.1b2. Plant stomata were analyzed using Obtilab Viewer Ver.2.1. observed using ImageRaster.

RESULTS AND DISCUSSION

Transformation vector into *Agrobacterium*

The results showed that *the npt*II gene was successfully transferred into *Agrobacterium tumefaciens* strains LBA4404 and GV3101. *Agrobacterium* strain LBA4404 was selected for genetic transformation into a yellow cosmos plant. The binary vector pRI101AN carried T-DNA containing *npt*II as a marker gene that confers resistance to Kanamycin and the 35S CaMV and NOS promoters (Figure 1). The successfully transformed vector was indicated by the presence of a DNA amplicon in the PCR analysis of the *Agrobacterium* colonies of each strain with a size of 550 bp (Figure 2a).

Selection and molecular analysis of T1 transgenic yellow cosmos carrying *npt*II

The selection of transgenic plants was carried out at the germination stage. The germination rate of seeds in the selection media was evaluated using filter papers provided with 50 ppm kanamycin antibiotic. Table 1 showed that out of 416 seeds germinating in the selection media, only 60 had good growth and were resistant to Kanamycin. By using an antibiotic screening medium, the percentage of resistance to Kanamycin was about 14.42% for putative transgenic yellow cosmos. Therefore, it is suspected that these plants have carried the *npt*II gene. The remaining seeds were unable to grow since they were susceptible to Kanamycin. Putative transformant plants were confirmed by PCR to detect the *npt*II gene in the plant genome. Based on molecular analysis, 44 out of 60 DNA samples were confirmed with a band size of 550 bp (Figure 2b). Indicating that these 44 yellow cosmos plants contain the *npt*II gene with a transformation efficiency was 73.33% based on total plants resistant to Kanamycin and 10.57% based on the total seeds transformed. The results also indicated that the T-DNA vector pRI101AN had been successfully inserted into the yellow cosmos genome.

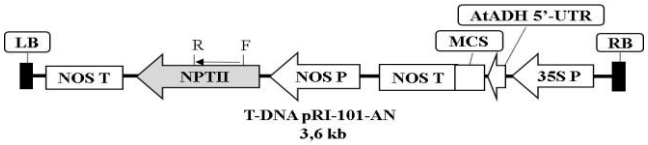

Figure 1. T-DNA mapping of binary vector pRI101AN

Table 1. The efficiency of genetic transformation of yellow cosmos in plants resistant to Kanamycin and transgenic *npt*II

No. of seeds	No. Of plants resistant to kanamycin	Percentage of plants resistant to kanamycin (%)	No. of DNA positive *npt*II	Transformation efficiency (%)	
416	60	14.42	44	73.33 a	10.57 b

Note: the transformation efficiency a: based on the number of samples per number of plants resistant to Kanamycin; b: based on the number of samples per the number of seeds transformed

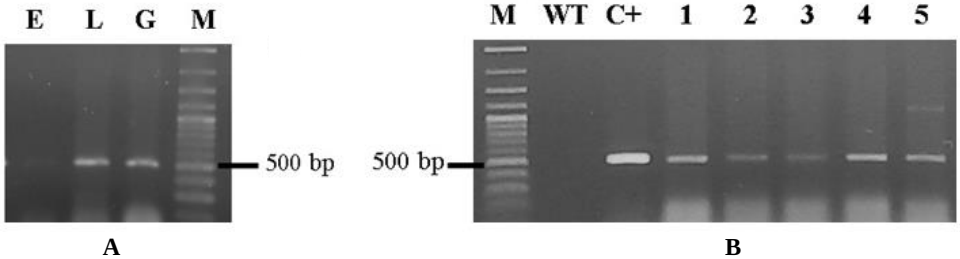

Figure 2. The amplicon of *npt*II gene (A) confirmation of *npt*II in *Agrobacterium* colonies of three strains transformed using vector pRI101AN; (B) confirmation of *npt*II gene from transgenic cosmos plants, M: Ladder 100bp, E: strain EHA105, L: strain LBA4404, G: strain GV3101, WT: wild type DNA, C+: control (pRI101AN), lines 1-5: amplicon *npt*II in transgenic cosmos sample

Phenotypic characteristics of T1 transgenic yellow cosmos carrying *npt*II

Based on the results, nine transgenic yellow cosmos plants named N1, N2, N3, N4, N5, N6, N7, N8, and N9, have been randomly selected and observed for phenotypic characterization. They had similar characters with wild type based on quantitative characteristics. The transgenic yellow cosmos N1 tended to have the highest plant height reaching 135 cm (Figure 2.A). The average height of the transgenic cosmos plants was not significantly different from that of the wild type (Table 2) (Figure 3.A). Transgenic plants N6 tended to have a longer leaf length of 20±1.7 cm and leaf width of 17.3±1.1 cm compared to other plants (Figure 2.B-C), and the average was not significantly different from the wild type (Table 2) (Figure 3.C-D). Plants N1 (10±1 mm) and N4 (9±0.36 mm) had higher lob widths than the others (Figure 3.D) with an average that was not significantly different (Table 2). The leaf stalk length of transgenic plants tended to be longer than that of the wild type (Figure 3.E). The stomata density in the wild type (292.66±8.5 mm^2) was higher than that of the transgenic plant (186.77±39.76 mm^2). In addition, the number of stomata of transgenic plants was significantly lower than the wild type (Figure 3.F) (Table 2). However, the length and width of the stomata of transgenic plants (33.59±2.51 µm) and (23.94±2.99 µm) were significantly longer than those of the wild type (Figure 3.G-H) (Table 2) (Figure 4.K-L).

The transgenic plants N7 tended to have the highest ray florets of 19.4±2.3 pieces. The length of ray floret 3.28±0.07 cm was longer than other plants (Figure 3.I-J) with a significantly higher average than the wild type (Table 2). In addition, the width of the ray florets in transgenic plants was more diverse (Figure 3.J). The average ray floret width in transgenic plants was 1.37±0.31 cm (Table 2). The flower and disc diameter tended to be the same as the wild type (Figure 3.L-M) (Table 2).

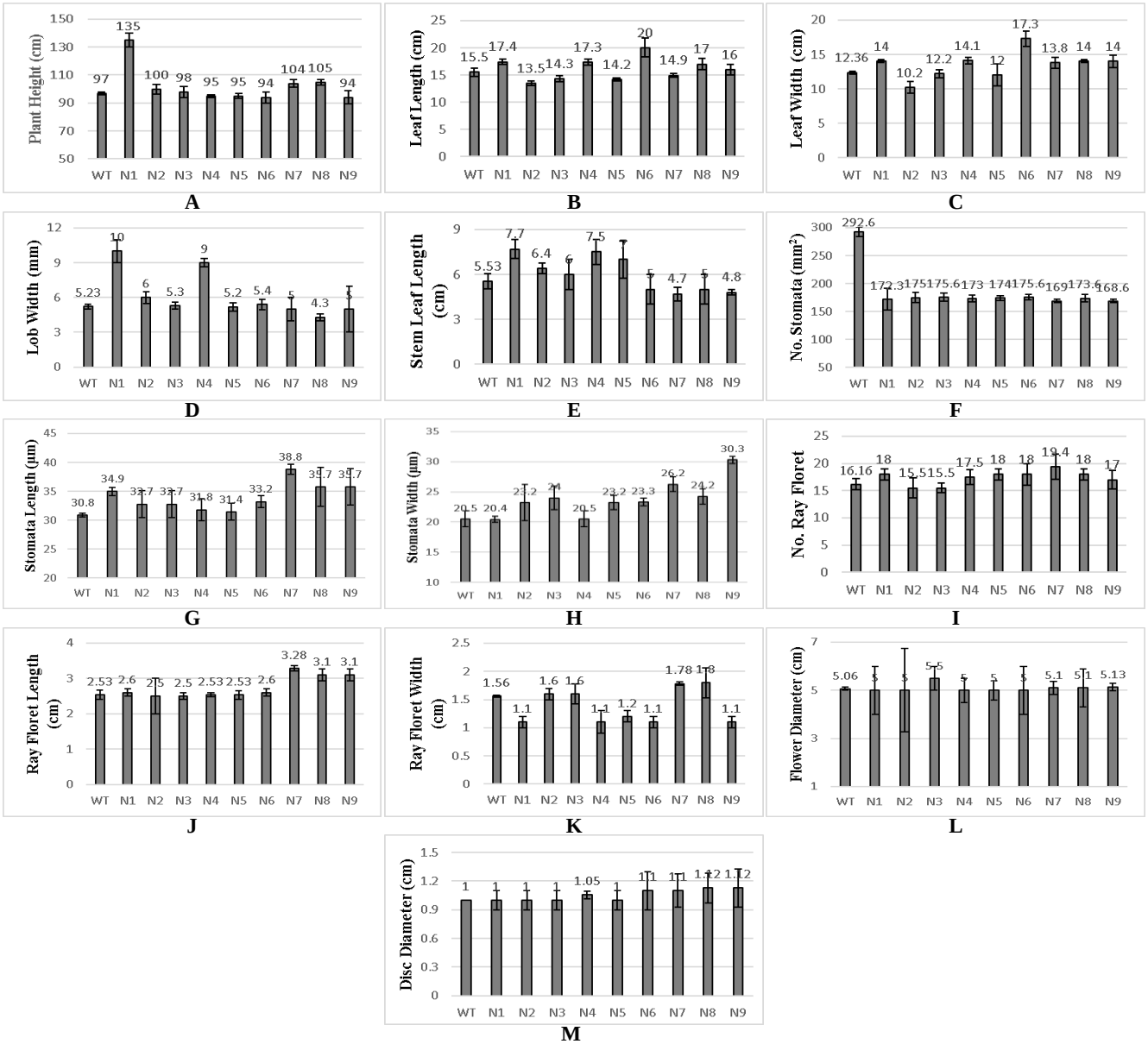

Figure 3. Quantitative characteristics of nine T1 transgenic yellow cosmos plants carrying *npt*II and average of nine wild-type plants with standard deviation, WT: wild type, N: transgenic plant

Table 2. Comparation of phenotypic characteristics of T1 transgenic yellow cosmos and the wild type

Phenotypic characters	*Cosmos sulphureus*		T-Test	*p*-value
	Wild type (mean±SD)	Transgenic *npt*II (mean±SD)		
Plants height (cm)	97±1.11	102.22±12.9	1.2027	0.2466^{ns}
Leaf length (cm)	15.5±2.41	16.06±2.06	0.5343	0.6005^{ns}
Leaf width (cm)	12.36±2.11	13.51±1.95	0.1916	0.2508^{ns}
Stem leaf length (cm)	5.53±0.47	5.90±1.24	0.8274	0.4202^{ns}
Lob width (mm)	5.23±0.15	6.18±1.97	1.4449	0.1678^{ns}
No. of stomata /(mm^2)	292.67±7.36	186.77±39.76	-7.8557	0.0007*
Stomata length (µm)	30.86±0.30	33.59±2.51	3.2339	0.0051*
Stomata width (µm)	20.5±1.14	23.94±2.99	3.2307	0.0052*
No. of ray floret	16.16±1.22	17.43±1.26	2.1574	0.0465*
Ray floret length (cm)	2.53±0.06	2.74±0.31	2.2259	0.0407*
Ray floret width (cm)	1.56±0.16	1.37±0.31	-0.9568	0.3524^{ns}
Flower diameter (cm)	5.06±0.05	5.12±0.16	0.8360	0.4154^{ns}
Disc diameter (cm)	1.00±0.07	1.05±0.06	1.8388	0.0845^{ns}

Note: (*): significant α: 0.05 of T-test; and ns: non-significant at α: 0.05 of T-test; SD: standard deviation

Table 3. Qualitative characteristics of phenotypic nine T1 transgenic yellow cosmos plants carrying *npt*II and average of nine wild type plants

Plant characteristics	T1 transgenic yellow cosmos plant carrying *npt*ii gene									
	Wild type	N1	N2	N3	N4	N5	N6	N7	N8	N9
Pubescence density	Medium	Medium	Medium	Medium	Medium	Sparse	Medium	Dense	Sparse	Sparse
Leaf type	Board pinnate	Board pinnate	Board pinnate	Board pinnate	Board pinnate	Board pinnate	Board pinnate	Board pinnate	Board pinnate	Board pinnate
Lobs shape	Taper	Taper	Taper	Taper	Taper	Taper	Taper	Taper	Taper	Taper
Upper leaf color	Green	Green	Green	Green	Green	Green	Green	Green	Green	Green
Lower leaf color	Light green	Light green	Light green	Light green	Light green	Light green	Light green	Light green	Light green	Light green
Leaf apex	Deep	Medium	Deep	Deep	Deep	Medium	Deep	Deep	Deep	Deep
Flower type	Daisy	Daisy	Daisy	Daisy	Daisy	Daisy	Daisy	Daisy	Daisy	Daisy
Longitudinal axis	Strong incurved	Strong incurved	Strong incurved	Strong incurved	Strong incurved	Strong incurved	Strong incurved	Strong incurved	Strong incurved	Strong incurved
Collar segment	Absent	Absent	Present	Absent	Absent	Absent	Absent	Present	Absent	Absent
Ray floret type	Ligulate	Ligulate	Ligulate	Ligulate-tubular	Ligulate	Ligulate	Ligulate	Ligulate-tubular	Ligulate	Ligulate
Incisions of apex	Deep	Medium	Deep	Medium	Deep	Deep	Deep	Deep	Deep	Deep
Ray floret color	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Disc color	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange	Yellow-orange

Based on the qualitative characteristics, there was a similarity between the transgenic and wild-type plants (Table 3). The similar characteristics observed were pinnate board leaf, tapered lob shape, green color at the upper leaf, and light green color at the lower leaf. In addition, wild type and transgenic yellow cosmos had daisy type, strong incurved of the longitudinal axis in ray floret. The ray florets had yellow color. Furthermore, disc flowers had a yellow-orange color (Figure 4.E). However, there are several distinct characteristics between transgenic plants and wild type, which include the pubescence density level of nine different transgenic plants. The proportion of pubescence density level was categorized as dense at 11.11% in N7, medium at 55.56% in N1 until N4 than N6, and sparse at 33.33% in N5, N8, and N9 (Table 3). Yellow cosmos had an erect stem and oblong. The branches spread on the top and bottom of the stem. Transgenic plants N2 and N7 had collar segments. It was like ray florets in the transgenic plants that were smaller and shorter than the wild type, denser around the disc flower with a proportion of 22.22% (Figure 4.F-H).

In addition, the plants had mixed ray floret types, such as ligulate and tubular, with a proportion of 22.22% (Figure 4.G). This ray floret type was found in transgenic plants N3 and N7 (Table 3). The tubular ray florets had the same length as the ligulate but were narrower due to their tubular shape (Figure 4.I-J). Furthermore, the Ray floret incisions of the apex have a medium level of 22.22% (Table 4). Also, the type of stomata in transgenic yellow cosmos and wild-type plants were anomocytic. Stomata were found in the upper and lower leaf surface but were dominant in the lower surface for the abaxial type. In addition, the size of the stomata in the transgenic plant was bigger than the wild type (Figure 4.K-L).

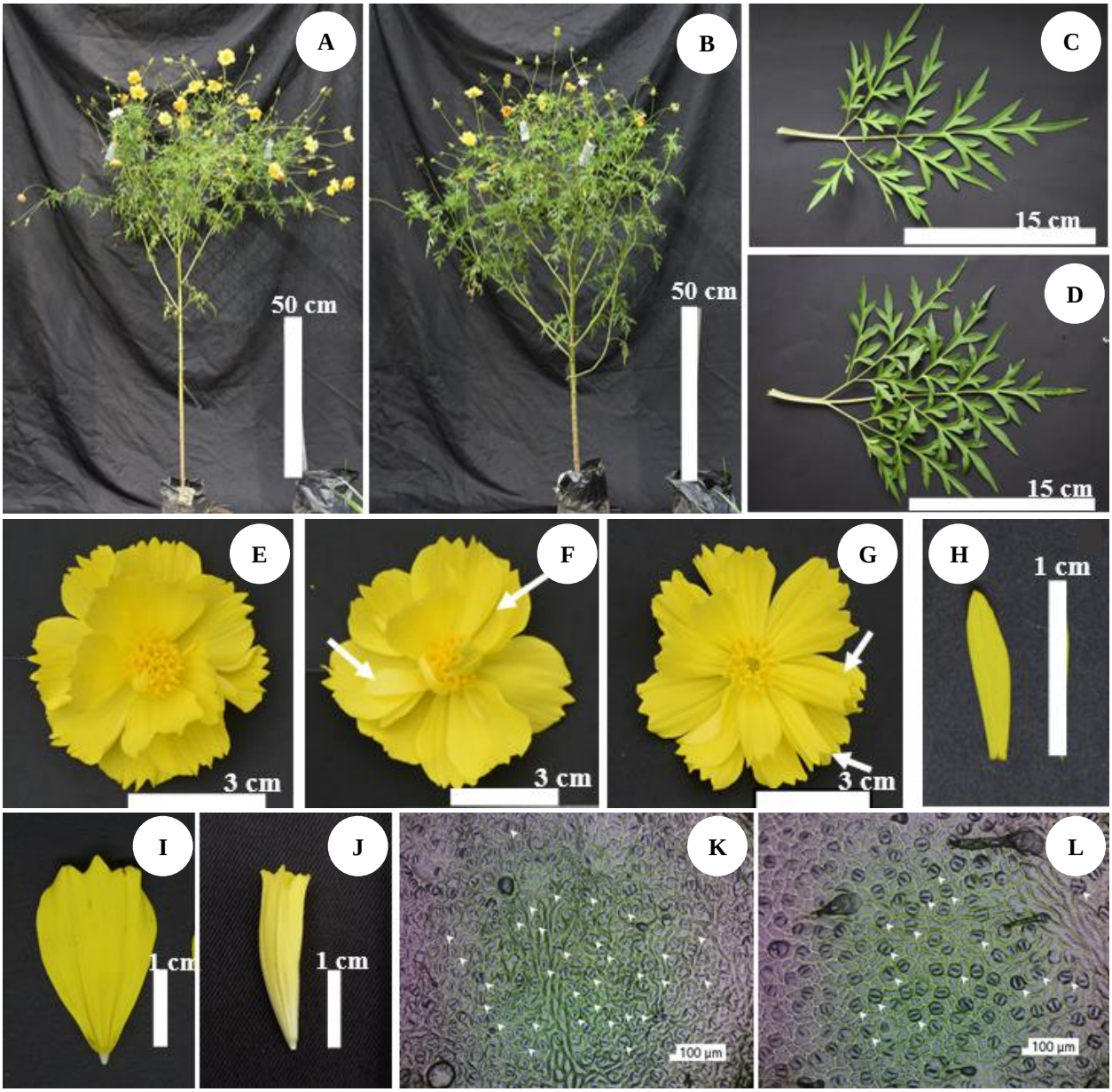

Figure 4. Morphological characteristic organs of wild type and T1 transgenic yellow cosmos carrying *npt*II, A: wild type plant; B: transgenic plant; C: wild type leaf; D: transgenic leaf; E: wild type flower; F: transgenic flower with collar segment; G: transgenic flower with mix ray floret types; H: collar segment organ; I: ligulate ray floret; J: tubular ray floret; K: wild type stomata; L: transgenic stomata in microscope lens 10x

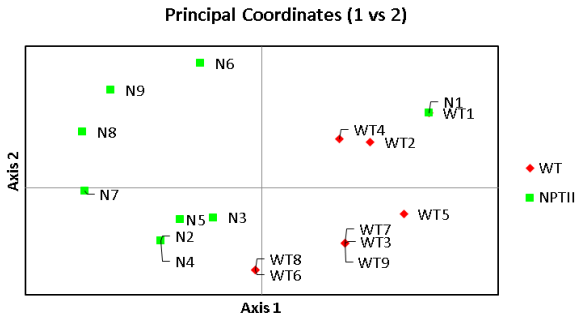

Figure 5. Cluster analysis of qualitative characteristics of T1 transgenic cosmos and wild type on PCoA biplot shows the distribution of nine transgenic cosmos and nine wild-type plants

Cluster analysis based on principal coordinate analysis

Cluster analysis employing qualitative phenotypic characteristics of nine T1 transgenic yellow cosmos and nine wild-type plants showed that the transgenic plants had different genetic distances from the wild type, except for the N1 plant (Figure 5). At coordinate 1, the percentage of variation was 40.15%, and that in coordinates 2 and 3 were respectively 22.82% and 17.49%. N7, N8, and N9 plants were transgenic plants with the farthest distance from the wild type.

Table 4. The proportion of qualitative characteristics of T1 transgenic yellow cosmos and the wild type

Phenotypic characters	Sub-characters	Proportion characters (%)	
		Wild type	Transgenic *npt*II
Pubescence density	Sparse	33.33	33.33
	Medium	66.67	55.56
	Dense	0	11.11
Leaf type	Board pinnate	100	100
Lobs shape	Taper	100	100
Leaf color	Green	100	100
Leaf apex	Medium	0	22.22
	Deep	100	77.78
Flower type	Daisy	100	100
Collar segments	Present	0	22.22
	Absent	100	77.78
Ray floret type	Ligulate	100	22.22
	Ligulate-tubular	0	77.78
Incisions of apex	Medium	11.11	22.22
	Deep	88.89	77.78
Ray floret color	Yellow	100	100
Disc color	Yellow-orange	100	100

Discussion

The results showed successful *Agrobacterium*-mediated plant genetic transformation using binary vectors pRI101AN containing *npt*II. The *Agrobacterium* strain LBA4404 was used for genetic transformation in the yellow cosmos. *Agrobacterium* strain LBA4404 has the chromosomal background TiAch5, Ti-plasmid pAL4404, opin of octopine, and contains genes for resistance to Rifampicin and Streptomycin. In addition, LBA4404 was more influential on the high-level expression gene in the transgenic plant. Furthermore, It has a low DNA copy number and high-efficiency transformation genetic in plants (Chetty et al. 2013; Yadav et al. 2014; Bahramnejad et al. 2019). LBA4404 strain showed the highest efficiency compared to *Agrobacterium* strains EHA105 and GV2260 in tobacco (Bakhsh et al. 2014). The vector transformation using the cold shock method has been carried out on pCAMBIA2300 into *A. tumefaciens* strain MTCC 431 (Sharon 2017) and *Rhodosporidium toruloides* (Liu et al. 2013).

The application of the floral dip transformation method using binary vector was successfully carried out in yellow cosmos. It indicated that plants could grow and were resistant to Kanamycin, while non-transformant and wild-type plants that did not contain *npt*II would die because they experienced growth disorders. The *npt*II could inhibit protein synthesis and ribosome binding (Davey et al. 2010). The *npt*II is widely used as a selectable marker in genetic transformation. The addition of 50 ppm kanamycin was effective for selecting transgenic cosmos plants, as has also been reported in the selection of transgenic *Glycine max* (Isda 2012) and *Saccharum officinarum* (Fibriani et al. 2019). Molecular analysis was carried out to detect the presence of T-DNA that had been inserted in the plant genome. The target sequences were amplified using the *npt*II gene as a marker selected for Kanamycin antibiotic resistance. The 35S CaMV promoter can express the gene in all plant parts, such as stems, leaves, roots, and flowers. Genetic transformation by floral-dip is faster and simpler to produce transgenic plants. Seeds as genetic material will be more stable than shoots, as reported in *O. sativa* (Rod-In et al. 2014) and *Citrus maxima* (Zhang et al. 2017).

Phenotyping is the process of describing an individual plant based on morphological characters to explain the function of each plant organ (Huh et al. 2014). The results showed similar phenotypes between *npt*II transgenic plants and their wild types. Peña et al. (2017) reported that the transgenic *npt*II plant could not change in main phenotypic characteristics. However, there was a change in the height plant. The transgenic plants that were taller than non-transformants have been reported in the Citrus tree (Pons et al. 2012), Wheat (Peña et al. 2017), *B. monnieri* (Largia et al. 2016; Sarkar and Jha 2021), Tobacco (Marenkova et al. 2021). In addition, the smaller transgenic plant also occurred in *O. sativa* (Lynch et al. 1995).

The leaves have various characteristics that can be used as morphological markers for phenotyping plants. For example, the transgenic yellow cosmos has the leaf shape of a board pinnate divided into five leaflets. The leaf lobes shape is tapper and pointed on the tip. In addition, the upper surface has a green color, but the lower surface has a lighter color, as reported in *C. sulphureus* (Win 2016; Hilmi et al. 2020). Therefore, the characteristics are similar to *C. caudatus* in the same genus (Chan et al. 2017; Purnobasuki et al. 2022).

The stomata characteristics are significant taxonomic tools for plant identification (Tahir et al. 2017). The stomata of yellow cosmos plants were amphistomatic leave. The stomata were found on upper and lower surface leave, but it was found more dominant on lower surface leaves with an abaxial type (Win 2016). The stomata abaxial are irregularly scattered in plants in the class Dicotyledoneae (Montano-Arias et al. 2018). The abaxial epidermis has higher stomata density than the adaxial. It as has been reported in the family Asteraceae, such us *Helianthus annuus*, *Tagetes erecta*, and *Achillea millefolium* (Shahzad et al. 2022). The transgenic and wild-type yellow cosmos plants under study had similar anomocytic stomata. In this stomata type, guard cells are surrounded by neighboring cells whose size and shape are not different from epidermal cells (Grohar et al. 2022). The anomocytic stomata had been reported in *C. sulphureus* (Win 2016), *Heracleum candicans* (Dar et al. 2022), *H. annus* and *T. erecta* (Shahzad et al. 2022). Purnobasuki et al. (2022) reported that *Cosmos caudatus* has anomocytic and anisocytic stomata. The anisocytic stomata were also found in *C. sulphureus* (Tahir et al. 2017).

On the flower, the presence of a collar segment indicates an abnormal reduction in the size of the ray floret. This also occurred in the *npt*II transgenic *B. pilosa* (Wang et al. 2012). On the other hand, an increase in the number of ray florets has been reported in *Pyrus communis* (Freiman et al. 2012). The yellow flower color cosmos in class Asteraceae was controlled by *LUTEIN*, Carotenoid, and *LCYE* genes (Kishimoto and Ohmiya 2006). The LIPLESS and KTANA genes controlled the decrease in flower organ size.

Meanwhile, the increase in organ number and size was controlled by CLV, ULT, WIG, and CYC genes. In addition, changes in the shape of floral organs are controlled by the ETT, PAN, SUPERMAN, and PLURIPETALA genes (Weiss et al. 2005). The coordinate distance between transgenic and wild-type plants shows that the genetic distance is increasingly different based on morphological characteristics (Lukmanasari et al. 2020). A percentage of variation greater than 25% indicates high diversity among individuals or plant populations (Messmer et al. 1992).

In conclusion, genetic engineering is one of the efforts in developing molecular farming. *Agrobacterium* strain LBA4404 was inserted into a binary vector pRI101AN transformed in plants with a transformation efficiency of 73.33% based on total plants resistant to Kanamycin and 10.57% based on total seeds transformed with a band size of 550 bp. The phenotypes of T1 transgenic yellow cosmos were generally similar to that of the wild type. However, there was a decrease in the number and an increase in the size of the stomata. In addition, there was a change in the type of ray floret to a mixture of ligulate and tubular. This study indicated that *the npt*II gene affected the phenotype of the yellow cosmos.

ACKNOWLEDGEMENTS

This study was supported by "*Rekognisi Tugas Akhir*" (RTA) Batch II scheme (5722/UN1.P.III/Dit-Lit/PT.01.05/2022) and funding from Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia. The binary vector and *Agrobacterium tumefaciens* strains were provided by Center for Development of Advanced Science and Technology (CDAST), the University of Jember, Indonesia.

REFERENCES

Aftab N, Saleem K, Khan AHA, Butt TA, Mirza CR, Hussain J, Farooq G, Tahir A, Yousaf S, Zafar MI, Nawaz I, Iqbal M. 2021. *Cosmos sulphureus* Cav. is more tolerant to lead than copper and chromium in hydroponics system. Intl J Environ Sci Technol 18 (8): 2325-2334. DOI: 10.1007/s13762-020-02981-w.

Anur RM, Mufithah N, Sawitri WD, Sakakibara H, Sugiharto B. 2020. Overexpression of sucrose phosphate synthase enhanced sucrose content and biomass production in transgenic sugarcane. Plants 9 (2): 1-11. DOI: 10.3390/plants9020200.

Bahramnejad B, Naji M, Bose R, Jha S. 2019. A critical review on use of *Agrobacterium rhizogenes* and their associated binary vectors for plant transformation. Biotechnol Adv 37 (7): 107405. DOI: 10.1016/j.biotechadv.2019.06.004.

Bakhsh A, Anayol E, Ozcan SF. 2014. Comparison of transformation efficiency of five *Agrobacterium tumefaciens* strains in *Nicotiana tabacum* L. Emirates J Food Agric 26 (3): 259-264. DOI: 10.9755/ejfa.v26i3.16437.

Bastaki NK, Cullis CA. 2014. Floral-dip transformation of flax (*Linum usitatissimum*) to generate transgenic progenies with a high transformation rate. J Vis Exp 94: 1-10. DOI: 10.3791/52189.

Chan E, Wong S, Chan H. 2017. Ulam herbs of *Oenanthe javanica* and *Cosmos caudatus*: An overview on their medicinal properties. J Nat Remedies 16 (4): 137-147. DOI: 10.18311/jnr/2016/8370.

Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML. 2013. Evaluation of four *Agrobacterium tumefaciens* strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar Micro-Tom. Plant Cell Reports 32 (2): 239-247. DOI: 10.1007/s00299-012-1358-1.

Dar NA, Raja WY, Tewari D, Bhat ZA. 2022. Pharamocognostic study of roots and aerial parts of less explores *Heracleum candicans* Wall. ex DC. from Betaab Valley, Pahalgam, Kashmir, India. Indian J Nat Prod Resour 13 (3): 1-12. DOI: 10.56042/ijnpr.v13i3.40840.

Davey MR, Sonej JR, Rao MN, Kourmpetti S, Bhattacharya A. 2010. Generation of development of transgenic crop plants: An overview. Transgenic Crop Plants 1 (1): 1-30. DOI: 10.1007/978-3-642-04809-81.

Fatmawati Y, Setiawan AB, Purwantoro A, Respatie DW, Teo CH. 2021. Analysis of genetic variability in F2 interspecific hybrids of mung bean (*Vigna radiata*) using inter-retrotransposon amplified polymorphism marker system. Biodiversitas 22 (11): 4880-4889. DOI: 10.13057/biodiv/d221121.

Fibriani S, Agustien I, Sawitri WD, Sugiharto B. 2019. Genetic transformation and expression of sucrose phosphate synthase mutant in tomato plant. Jurnal Bioteknologi Biosains Indonesia 6 (1): 134-143. DOI: 10.29122/jbbi.v6i1.3341.

Freiman A, Shlizerman L, Golobovitch S, Yablovitz Z, Korchinsky R, Cohen Y, Samach A, Chevreau E, Le-Roux PM, Patocchi A, Flaishman MA 2012. Development of a transgenic early flowering pear (*Pyrus communis* L.) genotype by RNAi silencing of PcTFL1-1 and PcTFL1-2. Planta 235 (6): 1239-1251. DOI: 10.1007/s00425-011-1571-0.

Grohar MC, Rosenfeldt S, Morales M. 2022. Stomatal micromorphology in a complex of Mimosa section Mimosa (Fabaceae). Protoplasma 259l: 203-215. DOI: 10.1007/s00709-021-01655-9.

Handayani E, Irsyadi MB, Alawiyah RLMN, Aris I. 2022. Effect of explants sterilization and plant growth regulators on embryo culture of kepel (*Stelechocarpus burahol*). IOP Conf Ser Earth Environ Sci 985*:* 012016. DOI: 10.1088/1755-1315/985/1/012016.

Hilmi MI, Taryono, Sayekti RS. 2020. Characterization of cosmos (*Cosmos* spp.) accessions from the special region of Yogyakarta and Riau origin. Agrinova 3 (1): 1-5.DOI: 10.22146/a.58345.

Huh YC, Choi HS, Solmaz I, Sari N, Kim S. 2014. Morphological characterization of Korean and Turkish watermelon germplasm. Korean J Agric Sci 41 (4): 309-314. DOI: 10.7744/cnujs.2014.41.4.309.

Irsyadi MB. 2022. Penyisipan Gen SoSPS1 Secara Floral Dip melalui *Agrobacterium tumefaciens* pada Tanaman Kosmos (*Cosmos sulphureus* Cav.). [Thesis]. Universitas Gadjah Mada, Yogyakarta. [Indonesia]

Isda MN. 2012. Optimization of concentration kanamycin in the soybean explants (*Glycine max* L.) to transformation TcPIN gene. Proceeding of Semirata PTN Barat. Universitas Negeri Medan, Medan, 11-13 Mey 2012. [Indonesia]

Jakhar ML, Verma R, Dixit D. 2019. Effect of antioxidant on in vitro degree of browning and culture establishment of Guggul (*Commiphora wightii* (Arnott)): A valuable dessert medicinal plant. J Pharmacogn Phytochem 5: 250-254.

Kadasa NM, Metwali EMR, Soliman HI, Alshehri W. 2021. Creation of borer pests resistance genetically engineering peach (*Prunus persica* L.) plants by cloning cry1Ab gene. Res Sq 148: 465-477. DOI: 10.21203/rs.3.rs-703653/v1.

Keshavareddy G, Kumar ARV, Ramu VS. 2018. Methods of plant transformation- A review. Intl J Curr Microbiol Appl Sci 7 (7): 2656-2669. DOI: 10.20546/ijcmas.2018.707.312.

Kishimoto S, Ohmiya A. 2006. Regulation of carotenoid biosynthesis in petals and leavesof chrysanthemum (*Chrysanthemum morifolium*). Physiol Plant 128: 436-447. DOI: 10.1111/j.1399-3054.2006.00761.x.

Kuluev BR, Knyazev AB, Lebedev YP, Chemeris AV. 2012. Morphological and physiological characteristics of transgenic tobacco plants expressing expansin genes: AtEXP10 from arabidopsis and PnEXPA1 from poplar. Russ J Plant Physiol 59 (1): 108-117. DOI: 10.1134/S1021443712010128.

Largia MJV, Satish L, Johnsi R, Shilpha J, Ramesh M. 2016. Analysis of propagation of *Bacopa monnieri* L. from hairy roots, elicitation and bacoside: A contents of Ri transformed plants. World J Microbiol Biotechnol 32 (8): 1-11. DOI: 10.1007/s11274-016-2083-7.

Liu Y, Koh CMJ, Sun L, Hlaing MM, Du M, Peng N, Ji L. 2013. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene RtGPD1 and development of genetic transformation method by

dominant selection in oleaginous yeast Rhodosporidium toruloides. Appl Microbiol Biotechnol 97 (2): 719-729. DOI: 10.1007/s00253-012-4223-9.

Lukmanasari P, Purwantoro A, Murti RH, Zulkifli. 2020. Similarity level of *Nepenthes* spp. based on the qualitative characters. Jurnal Ilmu Pertanian 5 (1): 140-149. DOI: 10.22146/ipas.55728.

Lynch PT, Jones J, Blackhall NW, Davey MR, Power JB, Cocking EC, Nelson MR, Bigelow DM, Orum TV, Orth CE, Schuh W. 1995. The phenotypic characterisation of R2 generation transgenic rice plants under field and glasshouse conditions. Euphytica 85: 395-401. DOI: 10.1007/978-94-011-0357-2_48.

Marenkova TV, Kuznetsov VV, Deineko EV. 2021. Features of expression of foreign genes in complex insertions in transgenic tobacco plants with a mosaic pattern of nptii gene expression. Russ J Genet 57 (3): 319-328. DOI: 10.1134/S1022795421030108.

Marenkova TV, Sidorchuk YV, Kusnetsov VV, Deineko EV. 2020. Effect of changes in genome ploidy on the mosaic character of nptii gene expression in epialleles of the transgenic tobacco line Nu21. Russ J Genet 56 (2): 204-212. DOI: 10.1134/S1022795420020088.

Mayavan S, Subramanyam K, Jaganath B, Sathish D, Manickavasagam M, Ganapathi A. 2015. Agrobacterium-mediated in planta genetic transformation of sugarcane setts. Plant Cell Rep 34 (10): 1835-1848. DOI: 10.1007/s00299-015-1831-8.

Messmer MM, Melchinger AE, Boppenmaier J, Herrmann RG, Brunklaus-Jung E. 1992. RFLP analyses of early-maturing european maize germ plasm - I. Genetic diversity among flint and dent inbreds. Theor Appl Genet 83 (8): 1003-1012. DOI: 10.1007/BF00232964.

Montano-Arias S, Camargo-Ricalde S, Grether R. 2018. Stomatal complexes of twenty four species of Mimosa (Leguminosae) occurring in Mexico. Acta Bot Mex 122: 97-107. DOI: 10.21829/abm122.2018.1260.

Peña PA, Quach T, Sato S, Ge Z, Nersesian N, Dweikat IM, Soundararajan M, Clemente T. 2017. Molecular and phenotypic characterization of transgenic wheat and sorghum events expressing the barley alanine aminotransferase. Planta 246 (6): 1097-1107. DOI: 10.1007/s00425-017-2753-1.

Pons E, Peris JE, Peña L. 2012. Field performance of transgenic citrus trees: Assessment of the long-term expression of uidA and nptII transgenes and its impact on relevant agronomic and phenotypic characteristics. BMC Biotechnol 12 (41): 1-15. DOI: 10.1186/1472-6750-12-41.

Purnobasuki H, Rakhashiwi GA, Junairiah, Wahyuni DK, Putra RE, Raffiudin R, Soessilohadi RH 2022. Morpho-anatomical characterization and DNA barcode of *Cosmos caudatus* Kunth. Biodiversitas 23 (8): 4097-4108. DOI: 10.13057/biodiv/d230830.

Rai GK, Rai NP, Kumar S, Yadav A, Rathaur S, Singh M. 2012. Effects of explant age, germination medium, pre-culture parameters, inoculation medium, and selection regime on *Agrobacterium*-mediated transformation of tomato. In Vitro Cell Dev Biol Plant 48: 565-578. DOI: 10.1007/s11627-012-9442-3.

Rashid A. 2017. Comparison of a kanamycin versus hygromycin resistance gene in transgenic plant selection of *Arabidopsis thaliana* L. Adv Cell Sci Tissue Cul 1 (1): 1-2. DOI: 10.35841/Cell-Science.1000101.

Respatie DW, Yudono P, Purwantoro A, Trisyono YA. 2019. The potential of *Cosmos sulphureus* flower extract as a bioherbicide for *Cyperus rotundus*. Biodiversitas 20 (12): 3568-3574. DOI: 10.13057/biodiv/d201215.

Rod-In, Sujipuli W, Ratanasut K. 2014. The floral-dip method for rice (*Oryza sativa*) transformation. J Agric Technol 10 (2): 467-474.

Saleem M, Ali HA, Furqan MA, Uzma S, Ammara S, Irshad I. 2017. Chemical characterisation and hepatoprotective potential of *Cosmos sulphureus* Cav. and *Cosmos bipinnatus* Cav. Nat Prod Res 33 (6): 897-900. DOI: 10.1080/14786419.2017.1413557.

Sarkar S, Jha S. 2021. Effects associated with insertion of rol genes on morphogenic potential in explants derived from transgenic *Bacopa monnieri* (L.) Wettst. Plant Cell Tissue Organ Culture 146 (3): 541-552. DOI: 10.1007/s11240-021-02092-5.

Setiawan A, Teo C, Kikuchi S, Sassa H, Kato K, Koba T. 2020. Centromeres of *Cucumis melo* L. comprise Cmcent and two novel repeats, CmSat162 and CmSat189. Plos One 15: e0227578. DOI: 10.1371/journal.pone.0227578.

Shahzad A, Iqbal S, Kayani S, Shafiq T, Zafar M, Naeem M, Yasmin H. 2022. Anatomical and palynological investigation within flora of family Asteraceae. BioRxiv: 1-23. DOI: 10.1101/2022.04.22.489184.

Sharon K. 2017. Cloning of HVA22 homolog from *Aloe vera* and preliminary study of transgenic plant development. Intl J Pure Appl Biosci 5 (6): 1113-1121. DOI: 10.18782/2320-7051.6033.

Subramanyam K, Chinnathambi A, Thaneswari RM, Ganapathi AAS, Manickavasagam M, Ganapathi A. 2015. Highly efficient *Agrobacterium*-mediated in planta genetic transformation of snake gourd (*Tricosanthes cucumerina* L.). Plant Cell Tiss Organ Cult 123: 133-142. DOI: 10.1007/s11240-01508214.

Tahir MA, Sarwar R, Safeer S, Hamza I, Khan MF. 2017. Anatomical variations in stomatal attributes of selected species of family Asteraceae. Commun Plant Sci 7 (2017002): 110-114. DOI: 10.26814/cps2017002.

UPOV. 2015. Cosmos Cav. www.upov.int/edocs/upov.

Wang CK, Hsu SY, Chen PY, To KY. 2012. Transformation and characterization of transgenic *Bidens pilosa* L. Plant Cell Tiss Organ Cult 109: 457-464. DOI: 10.1007/s11240-011-0110-9.

Weiss J, Delgado-Benarroch L, Egea-Cortines M. 2005. Genetic control of floral size and proportions. Intl J Dev Biol 49: 513-525. DOI: 10.1387/ijdb.051998jw.

Win NW. 2016. Anatomical characteristics of *Cosmos sulphureus* Cav. from family Asteraceae. Meral Portal 1-19.

Yadav S, Sharma P, Trivastava A, Desai P, Shrivastava N. 2014. Strain specific Agrobacterium-mediated genetic transformation of *Bacopa monnieri*. J Genet Eng Biotechnol 12: 89-94. DOI: 10.1016/j.jgeb.2014.11.003.

Zhang Y, Zhang D, Zhong Y, Chang X, Hu M, Cheng C. 2017. A simple and efficient in planta transformation method for pommelo (*Citrus maxima*) using *Agrobacterium tumefaciens*. Scientia Horticulturae 214: 174-179. DOI: 10.1016/j.scienta.2016.11.033.

Zhou G, Guo J, Yang J. 2018. Efect of fertilizers on Cd accumulation and subcellular distribution of two cosmos species (*Cosmos sulphureus* and *Cosmos bipinnata*). Intl J Phytoremediat 20: 930-938. DOI: 10.1080/15226514.2018.1448362.