

# Segregation analysis and floral phenotype of T2 transgenic yellow cosmos (*Cosmos sulphureus* Cav.) carrying neomycin phosphotransferase II gene in second generation

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**Abstract.** Muchyiddin MGAA, Sawitri WD, Irsyadi MB, Swandari T, Purwanto A. 2024. Segregation analysis and floral phenotype of T2 transgenic yellow cosmos (*Cosmos sulphureus* Cav.) carrying neomycin phosphotransferase II gene in second generation. *Biodiversitas* 25: 1711-1717. The seed of transgenic yellow cosmos (*Cosmos sulphureus* Cav.) lines harbouring neomycin phosphotransferase II (*nptII*) gene have been obtained from our previous studies. The *nptII* selectable marker gene is commonly used for developing a method to estimate the number of targeted gene integrated into the genome. In order to produce successful clones, it is important to ensure that transgenes are inherited sexually as the dominant trait in T2 progeny. This study aimed to obtain the segregation patterns of *nptII* gene in two populations derived from open- and self-pollinated and evaluated floral phenotypic of T2 transgenic yellow cosmos. The molecular analysis of transgenic yellow cosmos was conducted by PCR and the obtained data was analyzed using a Chi-Square test. The results showed that transgenes were inherited in 1:1 for transgenic and wild-type in both self- and open-pollinated populations. Therefore, it indicated that T2 transgenic plants were still unstable and evolved continuously to segregate independently in the over generation. Surprisingly, the morphological changes were detected in transgenic plants. The mixture of ligulate and tubular types in ray floret was found in some of the flower types in T2 yellow cosmos. In this case, *nptII* gene may diverge in apparently random genome insertion. This insertion would induce morphological changes in the ray floret type of flower since recombination estimates varied among transformation events.

**Keywords:** *Cosmos sulphureus*, *nptII* gene, phenotypic, segregation, transgenic

**Abbreviations:** *nptII*: Neomycin phosphotransferase II, PCR: Polymerase Chain Reaction

## INTRODUCTION

Yellow cosmos (*Cosmos sulphureus* Cav.) is an ornamental plant that originated in South America and has been grown in many tropical countries, including Indonesia. In addition, yellow cosmos as a refugia plant has the potential to produce medicinal compounds such as phenolics, flavonoids, saponin, and natural herbicides (Saleem et al. 2017; Zhou et al. 2018; Respatie et al. 2019; Aftab et al. 2021). However, the valuable compounds are not sufficient for large-scale production. Therefore, genetic engineering is necessary to overcome mass production issues (Bastaki and Cullis 2014; Liao et al. 2018; Anur et al. 2020; Zhu et al. 2020; Kowalczyk et al. 2022). Although information regarding genetic engineering and plant breeding in the yellow cosmos is still limited, techniques for genetic and morphological character transformation have recently been reported.

Genetic engineering in the yellow cosmos was successfully carried out by inserting neomycin phosphotransferase II (*nptII*) selectable marker gene into the plant genome (Irsyadi et al. 2022; Purwanto et al. 2023). Since the Transfer DNA (T-DNA) commonly contains selectable markers to be more feasible for transformant

plant screening, thus this character of *nptII* gene was utilized to develop a method and estimate whether the targeted gene was inserted into the genome over generations (Rashid 2017; Marenkova et al. 2020). In addition, *nptII* can catalyze the phosphorylation of kanamycin antibiotic. Therefore, kanamycin could not inhibit protein synthesis in transgenic plants (Marenkova et al. 2020). The T1 transgenic yellow cosmos harboring the *nptII* gene was generated using in planta method (Irsyadi et al. 2022).

The stability of transgene expression in the plant genome is an important factor to investigate, since it provides evidence of consistent transgene expression across generations (Mahajan et al. 2017; Wang et al. 2017; Tong et al. 2021; Lebedev 2022; Sun et al. 2023). The characteristic of transgenic plant populations is that molecular rearrangements will produce unstable transgenic lines (Ren et al. 2017). In most cases, transgenes are usually passed on to offspring according to the theoretical Mendelian ratio in successive generations (Ahuja and Fladung 2014; Ma et al. 2020). Otherwise, non-Mendelian inheritance can occur with a frequency of up to 50% in the population (Yu et al. 2020). On the other hand, the study on the segregation of non-Mendelian inheritance in transgenic

cotton has been evaluated (Hussain et al. 2014). Several factors that cause unstable transgene integration, such as gene silencing and chimeras (Raldugina et al. 2018). Although the presence of the *nptII* gene has been proven in transgenic yellow cosmos, its presence should be stable across generations.

The prerequisite of a stable transgene in plants is the genotype should be homozygous, thus segregation analysis for obtaining homozygous plants was conducted by amplification of *nptII* gene. Yellow cosmos is a cross-pollinated plant (allogamous), therefore to determine the level of gene stability in the second generation, self- and open-pollination were carried out. Self-pollination can be done to produce more homozygous plants, resulting in a higher inheritance. In addition, studying gene stability provides prospects for the success rate of transformation methods in plants. Segregation patterns on the transgenic plants have been reported on *Triticum aestivum* (Fu and Ristic 2010) and *Asparagus officinalis* (Limanton-Grevet and Jullien 2001). However, information about segregation analysis on the T2 transgenic yellow cosmos has not been reported yet.

Morphological characteristics of transgenic plants harboring *nptII* gene have been reported in cotton (Hussain et al. 2014), *Bacopa monnieri* (Largia et al. 2016), *Arabidopsis thaliana* (Rashid 2017), and *Brassica napus* (Raldugina et al. 2018). Variations in the ray floret type have been reported in T1 transgenic yellow cosmos carrying *nptII* gene (Irsyadi et al. 2022). This study aimed to obtain the segregation patterns of the *nptII* gene in two populations derived from open- and self-pollinated and evaluate floral phenotypic of T2 transgenic yellow cosmos. The examination was used by screening the homozygous transgenic cosmos plant population using PCR analysis and morphological observation.

## MATERIALS AND METHODS

### Plants materials

Nine transgenic lines of T1 yellow cosmos harboring *nptII* gene were obtained from Universitas Gadjah Mada, Yogyakarta, Indonesia. These nine lines were obtained from the result of genetic transformation by floral dip (Irsyadi et al. 2022). *Agrobacterium tumefaciens* strain LBA4404 containing binary vector pRI101AN was obtained from Prof. Bambang Sugiharto, Universitas Jember, Jember, Indonesia. Fifteen T2 seedlings from self- and open-pollinated respectively.

### Procedures

#### *Production of T2 progeny*

Nine transgenic lines of T1 plants were self- and or open-pollinated to produce T2 seeds in the greenhouse. Mature seeds were individually collected from T1 plants of self- and or open-pollinated. Then, the seeds of T2 yellow cosmos were planted in soil media and manure with a ratio of 3:1 in polybags for plant material preparation. Wild-type plants were planted separately from transgenic plants and self-pollination was not carried out.

#### *Segregation analysis of T2 progeny*

Segregation analysis of homozygous yellow cosmos line was conducted based on the presence of *nptII* gene by Polymerase Chain Reaction (PCR) with the PCR product of 500 kb. Approximately 500 mg of fresh leaf material from 15 plants of each population (self- and open-pollinated) at second generation transgenic yellow cosmos and control plants were collected from the greenhouse, Faculty of Agriculture, Universitas Gadjah Mada. The genomic DNA was extracted from leaves following CTAB (Cetyl Trimethylammonium Bromide) methods described by Doyle and Doyle (1990) with modifications.

PCR was conducted using a pair of specific primers for *nptII* gene with sequences of 5'-GTC ATC TCA CCT TGC TCC TGC C-3' and 5'-GTC CGT TGG TCG GTC ATT TCG-3' for *nptII* forward and reverse primers, respectively (Fibriani et al. 2019). The PCR reaction cocktail 12  $\mu$ L mixture consisted of 5  $\mu$ L Powerpol<sup>®</sup> Master Mix, 2  $\mu$ L nuclease-free water, 1  $\mu$ L for each forward and reverse primer (10 mM), and 3  $\mu$ L DNA samples. The PCR reaction was conducted with the temperature of 3 min pre-denaturation at 95°C, 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 1 min extension at 72°C for 40 cycles (PCR T100<sup>™</sup> thermal cycler, Bio-Rad, USA). The presence of *nptII* gene was detected by electrophoresis (Bio-Rad, USA) in 1% agarose gel added DNA staining and 5  $\mu$ L ladder Smobio<sup>®</sup>. The DNA band was visualized in a UV Transilluminator.

#### *Phenotypic of T2 progeny*

Phenotypic yellow cosmos began to be carried out on 3 month old plants. The parameter that has been used for plant phenotyping was morphological observation for flower characteristics based on quantitative and qualitative characters (UPOV 2015). The quantitative characters observed included number of flowers, number of ray florets, ray floret length, ray floret width, flower and disc diameter. The qualitative characters observed included flower head attitude, flower type, longitudinal axis, ray floret type, incisions of apex, ray floret and disc color.

### Data analysis

The segregation pattern of *nptII* gene was analyzed using the Chi-Square test. Quantitative morphological character data were analyzed using Analysis of Variance (ANOVA) and followed by Tukey's HSD test  $\alpha$ : 0.05 and standard deviation using R studio. Qualitative morphological data of T2 transgenic plants (self- and open-pollinated progeny) were analyzed descriptively.

## RESULTS AND DISCUSSION

### **Yellow cosmos progeny development**

The seeds of the T2 population of the transgenic yellow cosmos plant originated from the T1 transgenic plants in the previous study, which carried the *nptII* gene (Irsyadi et al. 2022). The pollination system of each flower of the transgenic yellow cosmos T1 plant was carried out by open- and self-pollination. The seeds were harvested from

the yellow cosmos plant at the physiologically mature stage (2 weeks after pollination). The number of harvested seeds obtained was 128 and 133 seeds from self- and open-pollinated, respectively. Meanwhile, the percentage of germination in self-pollinated seed production also showed lower results compared to seed production from open-pollinated plants (Table 1). Only 15 seedlings were able to grow and DNA samples could be extracted. From each population of transgenic T2 plants, only seven plants carrying the *nptII* gene were obtained from the self-pollinated treatment, and eight plants from the open-pollinated treatment (Table 1). The open-pollinated treatment of wild-type was planted and only DNA from seven samples as a representative of control plants were extracted. However, the wild-type yellow cosmos plants showed higher yields compared to transgenic lines, either open- or self-pollinated transgenic yellow cosmos plants (Table 1). Among the transgenic lines, the open-pollinated plants generate the highest seed production.

### Segregation and integration of *nptII* gene

The T2 plant population derived from nine transgenic lines of T1 yellow cosmos were analyzed for the inheritance of the *nptII* gene. A 500 bp fragment equal to the molecular size of the *nptII* gene was amplified from the total DNA of the transgenic plants (Figure 1). Each population of transgenic T2 plant was performed on 15 plants from each transgenic T2 population of self- and open-pollinated yellow cosmos plants. PCR analysis confirmed that only transgenic plants were detected, not in the non-transformed plants (Table 1). The segregation patterns of T2 populations are shown in Table 2. The PCR positive and negative plants were distinguishable by the presence of the specific bands of the *nptII* gene. A segregation ratio of 1:1 was observed in both self- and open-pollinated population independent events (Table 2). A ratio of 1:1 was observed for independent events which reflects its chimeric nature. These data confirmed the inheritance and integration of the *nptII* gene in the T2 generation.

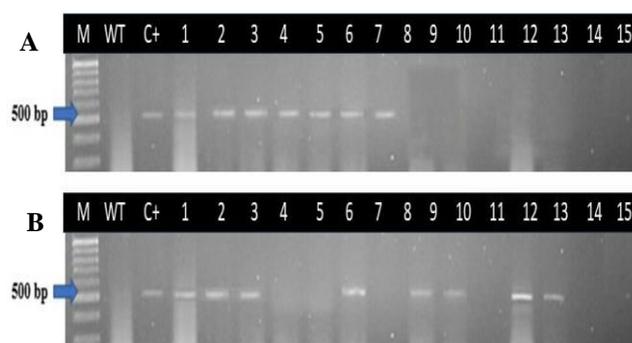
The results showed that the population was still heterozygous, and the *nptII* gene was the single dominant gene. In the T2 population of the transgenic yellow cosmos plants, the ratio pattern was 1:1 (Table 2). It might be caused by the inability of the stamens to pass the transgene on to their offspring. Thus, it suggested that homozygous recessive was increased in the T2 population of yellow cosmos plants. Since the germination of transgenic lines was very low, thus, all analyses were conducted in the limited number of plant samples which was only 15 plants of each treatment.

### Phenotypic characteristics of T1 transgenic yellow cosmos carrying *nptII*

Phenotyping was carried out in 30 populations of T2 plants, which consisted of 15 populations of self-pollinated T2 plants and 15 populations of open-pollinated T2 plants (Table 3). The results showed that average number of flowers and ray florets length have significant differences between wild-type plants and open-pollinated transformant plants, but showed significant differences with self-pollinated plant yields. The highest average number of flowers was

found in wild-type plants of  $40.8 \pm 10.24$  florets compared to transformed plants. The number of ray florets and ray floret length characters were significant difference between wild-type plants and transformant plants. The highest average number of ray florets was found in open-pollinated transgenic plants of  $15 \pm 2.65$  cm. The characters of flower diameter and ray floret width showed that there were significant difference between wild-type plants and open-pollinated yellow cosmos plants, but did not show any significant difference between self- and open-pollinated yellow cosmos plants. Flower diameter showed a significant difference between wild-type plants  $4.47 \pm 1.25$  cm and open-pollinated transgenic plants  $5.14 \pm 0.90$  cm. The highest average was found in open-pollinated transgenic plants with an average of  $5.4 \pm 0.64$  cm for the flower diameter character, and for ray floret width of  $1.59 \pm 0.19$  cm. The ray floret length characters with an average of  $2.46 \pm 0.28$  cm.

The qualitative-based phenotypic character was grouped into self- and open-pollinated transgenic plants (Table 4). The results showed that the flower head attitude of all types of plants has the same type of flower head attitude, flower type, which is upward with a proportion of 100% (Table 4; Figures 2A-B). In terms of flower type, ray floret color, longitudinal axis, and disk color characters, yellow cosmos plants have the same sub-character types between transgenic plants and wild-type plants with a proportion of 100%. In the character of the ray floret type and ray floret arrangement, there are differences between wild-type and transformant yellow cosmos plants. In terms of ray floret type characters, self-pollinated transgenic yellow cosmos plants have ligulate types with a proportion of 13%, and mix ray floret types with a proportion of 33% (Table 4; Figures 2C-F). The self-pollinated transgenic yellow cosmos had 13% ligulate ray floret types, 7% tubular, and 33% mixed ray floret types (Figures 2E-G). In open-pollinated transgenic yellow cosmos plants only had mixed ray floret types with a proportion of 53% (Figure 2F). In the incisions of apex characters, wild-type plants are shallow type with a proportion of 50% and medium with a proportion of 50% (Figures 2H-I). While the proportion of transgenic plants with deep type was 100% for self- and open-pollinated transgenic plants (Figure 2J).



**Figure 1.** Results of amplification and electrophoresis of T2 transformant plant DNA for the *nptII* gene, M: 3kb ladder marker, WT: Wild-Type (negative control), C+: pRI101AN plasmid (positive control). A. 1-15 (self-pollinated T2 plants), B. 1-15 (open-pollinated T2 plants)

## Discussion

The seed of T2 progeny was produced from self-pollination resulting in a low number of seeds and germination rate. This is presumably due to pollination failure or pollen that fails to develop. Yellow cosmos was able to produce 10-17 seeds (Irsyadi et al. 2022), but in this case only a few seeds were produced and the percentage of germination was low (Breygina et al. 2021). In general, transgenes are passed on to their offspring as dominant genes, but this is also possible if transgene inheritance does not match the Mendelian ratio. Several factors influence the segregation abnormality (Rajeevkumar et al. 2015). It might be caused by gamete viability, chromosomal abnormalities, transformation methods, and gene silencing due to rearrangement of transgenes in the plant genome (Tizaoui and Kchouk 2012; Raldugina et al. 2018). Another report showed that the insertion of the *nptII* gene in transgenic plants gives a non-Mendelian ratio due to the presence of chimeras. These results can be interpreted as an indication the plant population consisted of both transformed and untransformed cells the latter being counted higher the proportion of untransformed progeny in the T2 generation. Thus the 1:1 segregation ratio observed for the self- and open-pollinated lineage likely reflects a failure to pass the transformed phenotype through pollen. Previously, in genetic transformation in T1 plants of yellow cosmos, the amplification of the *nptII* gene band showed a 3:1 ratio pattern (Irsyadi et al. 2022). Moreover, gametes lethality causes the process of segregation in meiosis unusual. In addition, iodine stain pollen in the second generation and third derivative with a distorted segregation ratio of 1:1 on *Oryza sativa* (Fu et al. 2000), *Pyrus communis* in seven years (Lebedev 2022), *Malus sieversii* (Luo et al. 2020), and *Prunus domestica* (Ravelonandro et al. 2021). Moreover, segregation of kanamycin resistance can also be caused by transgene transmission that is only through male gametes. This is probably due to an insertional mutation affecting female gametes, such as the segregation pattern in

*Asparagus officinalis* (Limanton-Grevet and Jullien 2001) and transgenic poplar *Populus angustifolia* (Ren et al. 2017).

Morphological parameters have become specific traits in transgenic plants. Characteristics based on qualitative and quantitative are determining morphological transgenic and wild-type plants (Huh et al. 2014; Ali et al. 2018). Transgenic plants expressing the *nptII* gene in yellow cosmos showed some morphological differences, but not completely changes, such as changes in the shape of the flower bands in plants carrying the *nptII* gene in T2 transgenic plants either from self- or open-pollinated with the type of ray floret changing from ligulate to a mixture of ligulate and tubular. This is consistent with previous research that the *nptII* gene affected the morphology of transgenic yellow cosmos plants (Irsyadi et al. 2022). Interestingly, self-pollinated plants that did not carry the *nptII* gene on T2 resulted in a change in the ray floret of the flower to only a tubular type. An increase in the number of flowers, changes in band size, and flower shape are also influenced by several genes, such as the *CYC* gene (Yu et al. 2020). The flower color is controlled by carotenoid, *LUTEIN* and *LCYE* gene (Kishimoto and Ohmiya 2006). Moreover, morphological changes in transgenic plants have been reported on *Triticum aestivum* (Peña et al. 2017), *Nicotiana tabacum* (Marenkova et al. 2021), and *Bacopa monnieri* (Largia et al. 2016; Sarkar and Jha 2021).

**Table 2.** Segregation analysis of T2 progeny yellow cosmos

Total plants tested	Segregation analysis		X <sup>2</sup>	Df: 1; α: 0.05; X <sup>2</sup> : 3.84
	+ <i>nptII</i> gene	- <i>nptII</i> gene		
<b>Self-pollinated population</b>				
15	7	8	0.66	1:1
<b>Open-pollinated population</b>				
15	8	7	0.66	1:1

**Table 1.** The T2 transgenic yellow cosmos populations

Treatment	No. of seed harvested	No. of seedling	Percentage of seedling (%)	No. of DNA extraction	+ <i>nptII</i> gene	- <i>nptII</i> gene
Wild-type (open-pollinated)	191	94	49.21	7	0	7
Self-pollinated (transgenic plants)	128	30	23.43	15	7	8
Open-pollinated (transgenic plants)	133	59	44.36	15	8	7

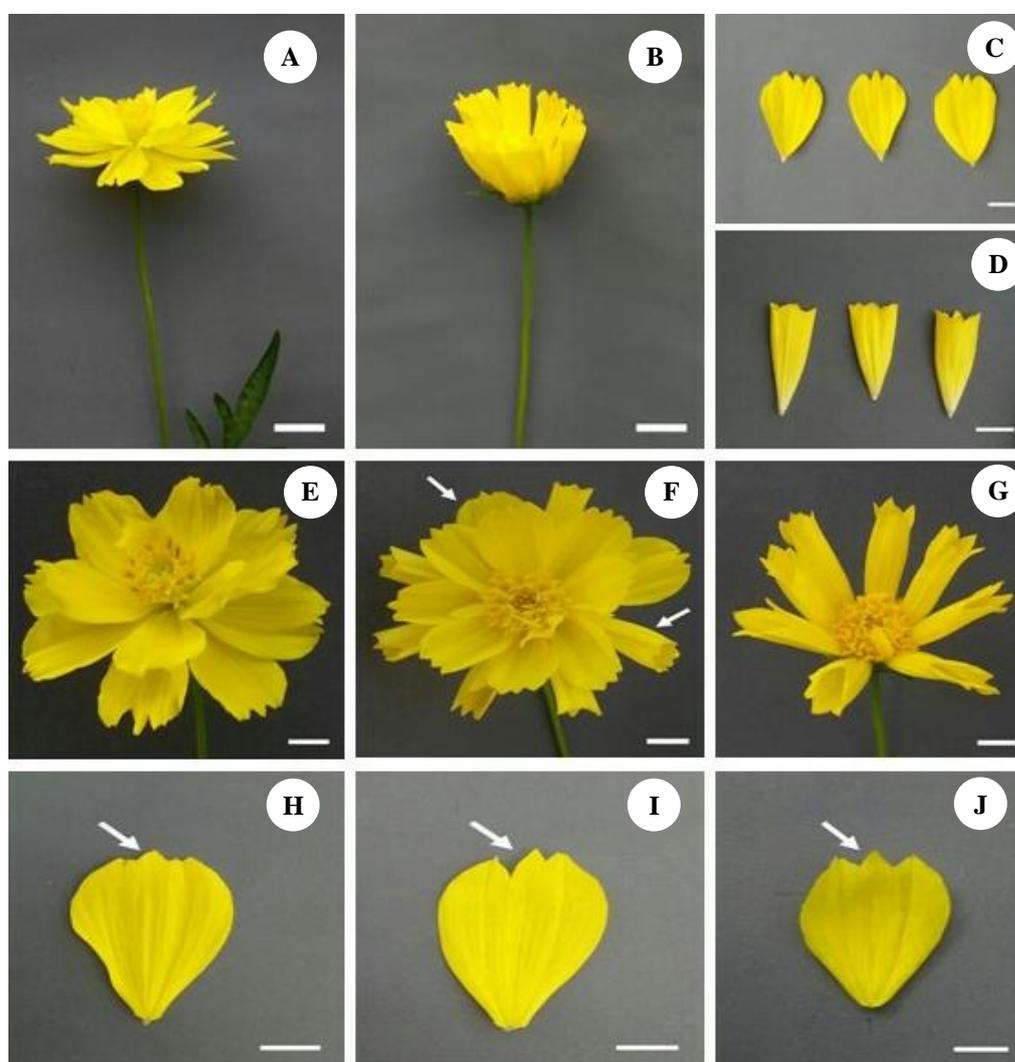
**Table 3.** Comparison of quantitative phenotypic characters of 15 T2 self-pollinated transgenic plants, T2 open-pollinated transgenic plants, and an average of 15 wild-type plants

Qualitative phenotypic characters	<i>Cosmos sulphureus</i>			F-value	p-value
	Wild type	Transgenic self-pollinated	Transgenic open-pollinated		
Number of flowers	40.8±10.24a	25.87±14.26b	30.73±11.40ab	5.558	0.00722**
Number of ray florets	12.8±1.19b	15±2.65a	15.11±2.47a	4.917	0.0121*
Flower diameter (cm)	4.47±1.25b	5.14±0.90ab	5.4±0.64a	3.506	0.0391*
Disc diameter (cm)	1.17±0.20a	1.17±0.14a	1.11±0.17a	0.584	0.562 <sup>ns</sup>
Ray floret length (cm)	1.93±0.48b	2.34±0.38a	2.46±0.28a	6.555	0.00333**
Ray floret width (cm)	1.26±0.26b	1.467±0.37ab	1.59±0.19a	4.833	0.0129*

Note: means±SD followed by the same letter in the same rows were not significantly different according to HSD  $\alpha = 0.05$ , (\*\*): Very significant  $\alpha = 0.01$  of HSD test, (\*): Significant  $\alpha = 0.05$  of HSD test

**Table 4.** The proportion of qualitative characteristics of T2 transgenic plants based on the presence and absence of transgenes with an average of 15 wild-type plants

Qualitative phenotypic characters	Sub-characters	Proportion characters (%)				
		Wild type	Self-pollinated		Open-pollinated	
			+ <i>nptII</i> gene	- <i>nptII</i> gene	+ <i>nptII</i> gene	- <i>nptII</i> gene
Flower head attitude	upward	100	100	100	100	100
Flower type	daisy	100	100	100	100	100
Ray floret type	ligulate	100	13	13	0	0
	tubular	0	27	40	53	47
	ligulate and tubular	0	7	0	0	0
Longitudinal axis	strong	100	100	100	100	100
Ray floret arrangement	single	0	0	6.67	0	0
	double	100	46.67	46.67	53	47
Ray floret color	yellow	100	100	100	100	100
Disk color	yellow	100	100	100	100	100
Incisions of apex	shallow	50	0	0	0	0
	medium	50	0	0	0	0
	deep	0	100	100	100	100

**Figure 2.** Morphological characters of T2 transgenic flowers: A. Growth character of upright flowers on ligulate flowers (non-transformant and transformant (open- and self-pollinated)), B. Growth character of upright flowers on tubular flowers (transformant - self-pollinated), C. Ligulate ray floret (non-transformant and transformant (open- and self-pollinated)), D. Tubular ray floret (transformant open- and self-pollinated), E. Transgenic flower with ligulate ray floret types (non-transformant), F. Transgenic flower with mix ray floret types (transformant open- and self-pollinated), G. Transgenic flower with tubular floret types (transformant self-pollinated), H. Shallow incision of apex (non-transformant and transformant), I. Medium incision of apex (non-transformant and transformant), J. Deep incision of apex (non-transformant and transformant), Bar: 1 cm

In conclusion, transgenes were inherited in a 1:1 ratio for transgenic and wild-type in both self- and open-pollinated populations. It indicated that T2 transgenic plants were still unstable and evolved continuously to segregate independently in the over generation. The morphological changes were detected in transgenic plants, while it was not detected in wild-type. The mixture of ligulate and tubular type in ray floret was found in some of the flower types in T2 yellow cosmos. Although *nptII* gene generally did not cause alterations of the main phenotypic changes, in our case, *nptII* gene may diverge in apparently random genome insertion. This insertion would induce morphological changes in the ray floret type of flower since recombination estimates varied among transformation events.

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