Strong Fluorescence Expression of *ZsGreen1* in Petunia Flowers by *Agrobacterium tumefaciens*-mediated Transformation

Keun H. Cho, Joo Young Kim, Maria I. Alvarez, Veronica Y. Laux, Lauren K. Valad, and Joshua M. Tester

Department of Environmental Horticulture, University of Florida, Gainesville, FL 32611

Thomas A. Colquhoun and David G. Clark

Department of Environmental Horticulture, University of Florida, Gainesville, FL 32611; and Plant Innovation Center, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611

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ABSTRACT. Fluorescent proteins (FT) have become essential, biological research tools. Many novel genes have been cloned from a variety of species and modified for effective, stable, and strong expression in transgenic organisms. Although there are many applications, FT expression has been employed most commonly at the cellular level in plants. To investigate FT expression at the whole-plant level, particularly in flowers, petunia ‘Mitchell Diploid’ [MD (*Petunia ×hybrida*)] was genetically transformed with seven genes encoding FTs: *DsRed2*, *E2Crimson*, *TurboRFP*, *ZsGreen1*, *ZsYellow1*, *rpulFKz1*, or *aeCP597*. Each gene was cloned into a pHK-DEST-OE vector harboring constitutive figwort mosaic virus 35S promoter and NOS-terminator. These plasmids were individually introduced into the genome of MD by *Agrobacterium tumefaciens*-mediated transformation. Shoot regeneration efficiency from the cocultured explants ranged from 8.3% to 20.3%. Various intensities of red, green, and yellow fluorescence were detected from *TurboRFP*, *ZsGreen1*, and *ZsYellow1*-transgenic flowers, respectively, under ultraviolet light for specific excitation and emission filters. More than 70% of plants established from the regenerated shoots were confirmed as transgenic plants. Transgenic *ZsGreen1* petunia generated strong, green fluorescence in all flower organs of T0 plants including petals, stamens, styles, anthers, and filaments. Most of the chromophores were localized to the cytoplasm but also went into the nuclei of petal cells. There was a positive linear relationship ($R^2 = 0.88$) between the transgene expression levels and the relative fluorescent intensities of the *ZsGreen1*-transgenic flowers. No fluorescence was detected from the flowers of *DsRed2*-, *E2Crimson*-, *rpulFKz1*-, or *aeCP597*-transgenic petunias even though their gene transcripts were confirmed through semiquantitative reverse transcriptase-polymerase chain reaction. T1 generation *ZsGreen1* plants showed green fluorescence emission from the cotyledons, hypocotyls, and radicles, which indicated stable FT expression was heritable. Four homozygous T2 inbred lines were finally selected. Throughout this study, we demonstrated that *ZsGreen1* was most suitable for generating visible fluorescence in MD flowers among the seven genes tested. Thus, *ZsGreen1* may have excellent potential for better utility as a sensitive selectable marker.

Many breeders have focused on the creation of ornamental plants incorporating unique colors in flowers or leaves. Various flower colors have been developed in *Gerbera hybrida*, *Dianthus caryophyllus*, and *Eustoma russellianum* by introducing the recombinant DNA related to the specific pigment biochemical pathways (Elomaa and Holton, 1994). Human eyes sense plant colors by perceiving the reflected or transmitted wavelengths of light ranging from 400 to 700 nm under natural and artificial conditions (Tanaka et al., 2008). The human eye can also sense the fluorescence emission physiologically generated from a chemical energy exchange process. In this

process, shorter-wavelength photons are absorbed by a molecule (excitation), and longer wavelength photons are released (emission) (Marshall and Johnsen, 2017). In contrast, bees (*Apis* sp.), the most important pollinators in nature, do not see primary colors as humans do. Instead, their ability to process ultraviolet light causes them to see a green world as shades of gray, and flowers with brilliant ultraviolet light reflection as strong black or cyan absorption holes against gray foliage (Miller et al., 2011; Papiorek et al., 2016). Some animals generate fluorescence naturally as a means of communication, camouflage, or attraction behavior: a mantis shrimp (*Lysiosquillina glabriuscula*), a swallowtail butterfly (*Papilio nireus*), a jumping spider (*Cosmophasis umbratica*), or a reef coral (*Zoanthus* sp.) (Marshall and Johnsen, 2017). Fluorescent emissions were also announced in plants. For example, pitcher plants (*Nepenthes* sp.) are known to exhibit fluorescence in its flowers and nectar (Kurup et al., 2013). Chlorophyll, lignified cell walls, and vacuolar contents in plants may display very weak auto-fluorescence upon ultraviolet light excitation (Voß et al., 2013). The fluorescence emitted from some plant organs

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K.H.C. is the corresponding author. E-mail: kencho@ufl.edu.

Fig. 1. Fluorescent protein expression in petals of the transgenic petunia was observed through green [GFP (480/510 nm, excitation/emission), **left**] and red [HcRed (590/614 nm), **right**] emission filters under ultraviolet light excitation. Each image was captured using QcapturePro software (Teledyne Qimaging, Surrey, BC, Canada) with the same exposure and RGB conditions. (A) Petunia ‘Mitchell Diploid’ plant as a nontransgenic control plant, (B) *ZsGreen1*-, (C) *ZsYellow1*-, and (D) *TurboRFP*-transgenic petunia flowers were compared for relative fluorescence intensity. The plots were created based on the relative intensity across features (grayscale) in each fluorescent image using plot profile menu of ImageJ software (1.51u; National Institutes of Health, Bethesda, MD).

Table 1. Comparison of callus formation, shoot regeneration, and transformation efficiency among the transgenic lines of petunia ‘Mitchell Diploid’ transformed with seven fluorescence genes by *Agrobacterium tumefaciens*-mediated transformation.

Parameters	Fluorescent genes						
	<i>DsRed2</i>	<i>E2Crimson</i>	<i>TurboRFP</i>	<i>ZsGreen1</i>	<i>ZsYellow1</i>	<i>rpulFKz1</i>	<i>aeCP597</i>
Expected fluorescence	Red	Red	Red	Green	Yellow	Blue	Blue
Cocultured explants (no.)	288	285	354	298	482	647	246
Callus formed (no.)	260	260	340	260	438	631	198
Callus formation efficiency (%) ^z	90.3	91.2	96.0	87.2	90.1	97.5	80.5
Regenerated shoots (no.)	41	35	53	33	40	81	50
Regeneration efficiency (%) ^y	14.2	12.3	15.0	11.1	8.3	12.5	20.3
Fluorescence emission ^x	High	0	0	13	10	0	0
	Medium	0	0	5	11	0	0
	Low	0	5	18	11	0	0
	None	41	30	9	8	81	50
Transformation efficiency (%) ^w	0	16.7	73.6	72.7	80.0	0	0

^zPercentage of the callus formed from the total number of cocultured explants on selective medium.

^yPercentage of the shoot regenerated from the total number of cocultured explants on selective medium.

^xFluorescence emission from each flower was visually evaluated and categorized into four intensity levels. Detection was conducted through green [GFP (480/510 nm excitation/emission)], red [HcRed (590/614 nm)], and yellow [YFP (510/560 nm)] emission filters under ultraviolet light excitation with a fluorescence microscope.

^wPercentage of fluorescence-emitting flowers from the total regenerated shoots (putative transgenic lines).

is thought to be a potential visual signal to attract pollinators or engage in bio-communication, but research is still in progress on this topic (Gandía-Herrero et al., 2005).

Since the green fluorescent protein (GFP) was isolated and cloned from a jellyfish (*Aequorea victoria*), it has become an indispensable molecular marker, and several modified versions have been developed (Ckurshumova et al., 2011; Katayama et al., 2008; Matz et al., 1999; Ormö et al., 1996; Shimomura et al., 1962). Synthetic GFPs have been constructed by replacing a serine at position 65 with a threonine (S65T) or a cysteine (S65C), to yield 100 to 120 times brighter fluorescence than wild type GFP under excitation with 490-nm light (Heim

and Tsien, 1996; Pang et al., 1996). Other modified versions, fast folder and thermo-tolerant green variant (FF-GFP) and fast folder thermostable yellow fluorescent protein (FFTS-YFP), have remarkably improved thermostability and folding kinetics for protein expression and functional research (Aliye et al., 2015). Many research groups have attempted to clone GFP-like fluorescent proteins from other species belonging to the genera *Anthozoa*, *Discosoma*, *Aequorea*, and *Trachyphyllia*. These proteins generate a variety of colors: red, cyan, bright green, yellow-green, blue-green, or orange-red (Matz et al., 1999; Voß et al., 2013). *DsRed* was originally cloned from a reef coral (*Discosoma* sp.). Due to the creation of visible precipitates in

Fig. 2. Green fluorescence generated from *ZsGreen1*-transgenic flowers was observed through orange-colored translucent transparent glasses under excitation of a blue light emitting diode. *Petunia* 'Mitchell Diploid' flowers served as a nontransgenic control (**upper**). The plots were created based on the relative intensity (grayscale) across features in the fluorescent image using plot profile menu of ImageJ software (1.51u; National Institutes of Health, Bethesda, MD) (**lower**). The images were photographed by a digital camera (model DMC-LF1; Panasonic, Kadoma, Japan) in a dark room with image's EXIF data of f/3.2, 1/8 s, ISO-800.

cells upon transformation, a nonaggregating mutant, *DsRed2*, became widely used as a red fluorescent protein (Katayama et al., 2008; Matz et al., 1999). *E2Crimson* (a bright far-red fluorescent protein) is a modified version of *DsRed2* and retains the rapid maturation rate, high photostability, high solubility, and low cytotoxicity. *ZsGreen1* (also known as zFP 506) and *ZsYellow1* (also known as zFP538) are bright green and yellow fluorescent proteins, respectively, which were derived from a reef coral [*Zoanthus* sp. (Matz et al., 1999)] and have been modified for high solubility and bright emission (excitation/emission maxima are 496/506 and 528/538 nm, respectively) (Voß et al., 2013; Wenck et al., 2003). *TurboRFP* is a red-orange fluorescent protein (excitation/emission maxima are 553 and 574 nm, respectively) derived from a sea anemone [*Entacmaea quadricolor* (Merzlyak et al., 2007)]. Due to its high photostability and pH stability, *TurboRFP* is more than two times as bright as *DsRed2*. A novel blue chromoprotein, *aeCP597* (absorption most at 597 nm), has been cloned from beadlet anemone [*Actinia equine* (Shkrob et al., 2005)] and carries a chromophore chemically identical to that of the well-studied *DsRed*. The chromoprotein *rpulFKz1* consists of two domains: a Frizzled cysteine-rich domain and a Kringle domain (Bulina et al., 2004). It was discovered in scyfoid jellyfish (*Rhizostoma pulmo*) with complex absorption spectrum peaking

at 420, 588, and 624 nm. Over the past 50 years, fluorescent proteins have been employed as visual, nondestructive reporters for cloning, subcellular localization, tissue-specific gene expression, and tagging enzymes in vivo (Uji et al., 2010; Voß et al., 2013).

Introduction of the aforementioned genes into plant genomes to create fluorescent flowers that can be detected by humans is an exciting idea, but delivering the concept has been challenging. The development of fluorescent flowers was once thought to be a new model of breeding to appeal to consumers by showing strong and impressive experiences of novel biotechnologies (Mercuri et al., 2002). However, ornamental plants with fluorescence, or even self-luminescent flowers, have not yet to be produced for commercial markets. There have been many attempts to make fluorescent or light-emitting plants. One such attempt was conducted by Ow et al. (1986). They genetically transformed tobacco (*Nicotiana tabacum*) with the gene encoding luciferase from a firefly (*Photinus pyralis*). Once provided with luciferin, the proper substrate, the transgenic plants emitted weak light that could be captured on photographic films. The chloroplast genome of a tobacco was transformed by inserting a *lux* operon cloned from a photobacterium (*Photobacterium leiognathi*) belonging to the order Vibrionales, resulting in autonomously luminescent transgenic plants generating 82×10^6 photons/min (Krichevsky et al., 2010). Kwak et al. (2017) introduced nanoparticles containing firefly luciferase, D-luciferin, and coenzyme-A functionalized chitosan into leaves of watercress (*Nasturtium officinale*), arugula (*Eruca sativa*), and spinach (*Spinacia oleracea*) via a pressurized bath infusion method. As a result, the watercress plants generated a maximum rate of 1.44×10^{12} photons/s during a transient time-lapse. GFP-transgenic african daisy (*Osteospermum ecklonis*) were reported to be appearing distinctly green fluorescence upon ultraviolet light illumination (Mercuri et al., 2002). Recently, strong yellow-green fluorescence was produced by wishbone flower [*Torenia fournieri* 'Crown White' (Sasaki et al., 2014)], transformed with CpYGFP originated from a marine plankton (*Chiridius poppei*). It is now clear that the right choice of vectors, constitutive or spatiotemporal promoters, and translational enhancers for efficient transcript and stable protein expression are vital for strong fluorescence emission in plants.

Despite much research using GFP as a molecular target or marker gene (Chin et al., 2018; Mercuri et al., 2002), there have been few reliable studies reporting development of fluorescent flowers using novel GFP-like fluorescent genes. Therefore, seven fluorescent genes (*DsRed2*, *E2Crimson*, *TurboRFP*, *ZsGreen1*, *ZsYellow1*, *rpulFKz1*, or *aeCP597*) were introduced into petunia plants by *Agrobacterium tumefaciens*-mediated transformation to induce fluorescent expression in flowers. *Petunia* 'Mitchell Diploid' (MD), a model plant renowned for its high transformation capacity (Vandenbussche et al., 2016), was chosen for the present study because it is mostly white with reduced amounts of pigment in the corolla. This reduced the chance of complications that certain petal pigments might interfere with fluorescence by shading excitation (Sasaki et al., 2014). The purpose of this study was to select fluorescent proteins with maximum emission for the development of fluorescent flowers. We also wanted to find fluorescent proteins with potential as useful markers in molecular breeding research.

Fig. 3. Expression of *ZsGreen1* vector for the development of fluorescent petunia flowers. (A) Schematic diagram of the FMV35S:*ZsGreen1*:NOS-T (RB = right border, FMV 35S = figwort mosaic virus 35S promoter, *Xba I* and *Spe I* = restriction enzyme, NOS 3' = NOS terminator, Kan R = kanamycin resistant, LB = left border). (B) *ZsGreen1*-encoded petal showed green fluorescence compared with nonfluorescence in wild-type (WT) petunia 'Mitchell Diploid' (MD). Both stamens (anthers and filaments) and pistils (stigmas, styles, and ovaries) also generated strong fluorescence in transgenic petunia flowers. Weak fluorescence (auto-fluorescence) was emitted from the anthers of MD upon the excitation of ultraviolet light. (C-1 and C-2) Fluorescent protein expression in petal cell of *ZsGreen1*-transgenic petunia (400×). The intact petal from transgenic flowers (C-1) were incubated in 1 M KNO₃ for 5 min, leading to plasmolysis (C-2); scale bar = 30 μm. The pictures were taken through green emission filter [GFP (480/510 nm excitation/emission)] under ultraviolet light excitation.

Materials and Methods

VECTOR CONSTRUCTION AND PETUNIA TRANSFORMATION. The coding regions of *DsRed2*, *E2Crimson*, *TurboRFP*, *ZsGreen1*, *ZsYellow1*, *rpulFKz1*, and *aeCP597* were individually amplified from the construct/vector provided by GloFish LLC (Middleton, WI) using a high-fidelity polymerase (Advantage2; Takara Bio, Mountain View, CA). The primers used for cloning the fluorescent genes are listed in Supplemental Table 1. Each corresponding gene was inserted into the pHK-DEST-OE vector by In-Fusion (Takara Bio) cloning method. The vector was originally constructed in H.J. Klee's laboratory at the University of Florida, which has a constitutive figwort mosaic virus (FMV) 35S promoter and NOS-terminator. After the nucleotide sequence of each plasmid construct was confirmed

with Sanger sequencing service (Genewiz, South Plainfield, NJ), the plasmid was introduced into *A. tumefaciens* strain C58 (ABI) and then cultured on selective medium containing spectinomycin (100 mg·L⁻¹) overnight at 28 °C. The *A. tumefaciens* suspension harboring the pHK-DEST-OE vector with each fluorescent gene was then adjusted to 0.15 of optical density 600 before transformation. MD was used as a wild-type control for all experiments. The transformation was carried out with a modified method described by Jorgensen et al. (1996). Briefly, 5-week-old MD leaves were cut and infected with 10 mL of the *A. tumefaciens* suspension for 15 min on Murashige and Skoog (MS) media containing 1 M galacturonic acid, 200 mM acetosyringon, and 1 mg·L⁻¹ thidiazuron (TDZ). After being placed under dark condition for 2 d, the infected leaves were transferred to new MS media for callus induction (1

Fig. 4. *ZsGreen1* gene transcript levels were analyzed in transgenic petunia flowers of which fluorescence emission was high (line 28), medium (line 2), low (line 22), and non (line 5) under ultraviolet light excitation. Petunia 'Mitchell Diploid' [wild type (WT)] was used as a nontransgenic control plant. (A) Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed different transcript levels among the lines. 18S ribosomal RNA (18S rRNA) was used as a loading control (18 cycles), and 50 ng RNA was used per reaction for all lines. (B and C) *ZsGreen1* gene expression levels were quantified by the $2^{-\Delta\Delta CT}$ method using quantitative PCR in flowers normalizing to the reference gene, elongation factor 1-alpha (*PhEF1α*), or ubiquitin. Data show the relative transgene expression compared with WT (1.0) with standard error of three biological replicates per transgenic line. Asterisks indicate the statistical significance compared with WT (ns, ** = nonsignificant or significant at $P < 0.01$ via Student's *t* test, respectively).

mg·L⁻¹ TDZ, 500 mg·L⁻¹ carbenicillin, and 150 mg·L⁻¹ kanamycin). The calli were then transferred to shooting media and cultured under light condition (16/8 h day/night) for 2 weeks.

The shoots that emerged from the calli were transferred to rooting media. These putative T0 transgenic plants were finally grown in glass greenhouses for later confirmation of fluorescence emission and gene transcript analysis (Supplemental Fig. 1). Callus formation, shoot regeneration, and transformation efficiencies were calculated based on the number of cocultured explants that formed calli and shoots for each transgenic plant.

CONFIRMATION OF FLUORESCENCE EMISSION AND GENE TRANSCRIPT LEVEL. Fluorescence from transgenic flowers was initially checked in the greenhouse through orange-colored transparent acrylic glasses under a blue light-emitting diode [LED (model # WF-501B; UltraFire, Piscataway, NJ)] for excitation. Fully opened flowers [petunia floral developmental stage 7 to 9 (Colquhoun et al., 2011)] of transgenic T0 plants were carefully collected and examined under a fluorescence stereo-microscope (MZ16F; Leica Microsystems, Wetzlar, Germany) through green [GFP (480/510 nm excitation/emission)], red [HcRed (590/614 nm excitation/emission)], and yellow [YFP (510/560 nm excitation/emission)] emission filters without any chemical stains or fixation. Fluorescence emission images were taken to capture the petals, trichomes, and pollen, as well as the flowers, when excited by ultraviolet light. The fluorescent images were photographed with a microscopic camera (Retiga 2000R; Teledyne Qimaging, Surrey, BC, Canada) and combined with QcapturePro software (Teledyne Qimaging). The relative intensities of the fluorescent images (grayscale) were analyzed via plot profiles using ImageJ software (1.51; National Institutes of Health, Bethesda, MD). The petals collected from the transgenic flowers were incubated in 1 M KNO₃ for 5 min, leading to plasmolysis to look at the subcellular localization of *ZsGreen1* fluorescent protein (Serna, 2005).

To analyze expression of transgenes, the flowers were harvested and immediately stored at -80 °C with three biological replicates per T0 transgenic plant. Total RNA was extracted using TriZOL (Thermo Fisher Scientific, Waltham, MA) and treated with TURBO DNase-free (Ambion, Austin, TX). Transcript levels in the transgenic flowers were initially analyzed with semiquantitative reverse transcriptase polymerase chain reaction (sqRT-PCR) using a One-step RT-PCR kit (Qiagen, Valencia, CA). The specific primers for the genes were designed using the Primer3 program (Rozen and Skaletsky, 2000) and are listed in Supplemental Table 1. The 18S ribosomal RNA (18S rRNA) was used as a loading control for the sqRT-PCR analysis. Total genomic DNA was isolated from the flowers of four *ZsGreen1*-transgenic lines (line 02, 05, 22, and 28) and MD plants using a cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) to compare transgene expression. The quantitative polymerase chain reaction (qPCR) primers for the *ZsGreen1*-transgenic petunia (target gene) were designed with PREMIER 5.0 (Biosoft International, Palo Alto, CA) and are shown in Supplemental Table 1. In addition to using ubiquitin as a reference gene for normalization in qPCR analysis, the petunia elongation factor 1-alpha (*phEF1α*, SGN-U207468) was also used as another reference gene. This gene was identified from the Sol Genomics Network [SGN (Fernandez-Pozo et al., 2015)] because it was reported to show consistent expression for the transcriptomic analysis of MD (Mallona et al., 2010). Power SYBR Green PCR master mix (Thermo Fisher Scientific) was used to amplify and detect the products according to the manufacturer's instructions. The

Table 2. Comparison of C_T values of target (*ZsGreen1*) and reference (*EF1 α*) gene in each transgenic line based on the standard curves by qPCR analysis. The gene copy numbers were calculated and estimated by the modified equation (Fletcher, 2014).

Transgenic line ^z	C_T (<i>ZsGreen1</i>)	C_T (<i>EF1α</i>)	X_0 / R_0^y	Estimated transgene copies (no.)
Wild type ^x	30.57	25.37	0.0001	0
28	22.01	26.94	1.3414	1
02	21.31	24.48	0.5271	1
22	23.27	24.12	0.0506	0
05	31.31	20.45	0.0001	0

^zFlowers of the transgenic lines generate variable levels of fluorescence emission and evaluated visually: high (line 28), medium (line 02), low (line 22), and non (line 05).

^y $X_0 / R_0 = 10^{[(C_{T,X} - I_X) / S_X - (C_{T,R} - I_R) / S_R]}$. I_X and I_R are intercepts of the relative standard curves of the target and the reference genes, S_X and S_R are slopes of the relative standard curves of the target and the reference genes, and $C_{T,X}$ and $C_{T,R}$ are the detected threshold cycles (C_T value) of the amplification of the target and the reference genes.

^xPetunia ‘Mitchell Diploid’ as a nontransgenic control plant.

Fig. 5. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed variable transgene transcript level for the petunia flowers transformed with *DsRed2* (1-1 and 1-2), *E2Crimson* (2-1 and 2-2), *rpulFKz1* (3-1 and 3-2), *TurboRFP* (4-1 and 4-2), *aeCP597-NA* (5-1 and 5-2), or *ZsYellow1* (6-1 and 6-2). Petunia ‘Mitchell Diploid’ [wild type (WT)] was compared as a nontransgenic control. 18S ribosomal RNA (18S rRNA) was used as a loading control (18 cycles), and 50 ng RNA was used per reaction in all transgenic lines.

relative transcript levels of the *ZsGreen1* gene in different T0 plants were quantified by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), individually normalizing to the two reference genes with three biological replicates per transgenic line. The transgene copy number of each *ZsGreen1*-transgenic line was estimated by comparing a target gene and a reference gene, PhEF1 α , based on a relative standard curve design (Weng et al., 2004; Yuan et al., 2007). The standard curves were produced by linear regression with logarithm-transformed DNA from the vector construct as a dependent variable and the C_T value as an independent variable resulting from the qPCR (Fletcher, 2014; Zhu et al., 2014).

CONFIRMATION OF STABLE FLUORESCENT PROTEIN ESTABLISHMENT IN T1 PLANTS AND SELECTION OF HOMOZYGOUS T2 INBRED LINES. Seeds were harvested from the self-pollinated flowers of T0 transgenic plants showing high fluorescence emission. Seeds selected from the *ZsGreen1*-transgenic lines were sown in 72-cell plot trays filled with Sunshine potting mix. The seeds germinated inside a growth chamber at 25 °C with a 16-h photoperiod, and the fluorescence emission from the seedlings was immediately examined when the cotyledons

emerged. Four selected lines showed offspring segregation with a 3:1 ratio of fluorescent to non-fluorescent seedlings. T1 seedlings showing fluorescence were then grown in the greenhouse for 3 months and self-pollinated. Seeds of the T2 plants were collected and germinated to select homozygous lines with fluorescent expression in cotyledons of 100% of the seedlings.

Results and Discussion

A limited number of constitutive promoters have been used to drive transgene expression efficiently in plants (Malik et al., 2002; Samac et al., 2004). The commonly used cauliflower mosaic virus (CaMV) 35S promoter confers high levels of GUS expression in leaves and stems, but lower expression levels in flowers and seeds of transgenic tobacco plants (Malik et al., 2002). The FMV 35S promoter has been reported to confer uniform expressions in roots, stems, leaves, and floral organs (Samac et al., 2004; Sanger et al., 1990). Therefore, the FMV 35S promoter was selected for the effective transgene expression in flowers for the present study, and successful fluorescence emission was achieved from the floral organs of some transgenic flowers (Fig. 1). More than 80% of all cocultured explants developed calli, but the shoot regeneration efficiency was variable depending on the trans-

genes, ranging from 8.3% (*ZsYellow1*) to 20.3% (*aeCP597*) on selective MS medium (Table 1). Between 33 to 80 individual transgenic T0 plants, per fluorescent gene construct, were transplanted and grown in the greenhouses for the confirmation of fluorescence emission from flowers (Table 1). During the vegetative and reproductive growth period, there were no obvious phenotypic differences between the transgenic plants and MD controls, although no growth parameters were measured. When fluorescence of the transgenic flowers was observed under a fluorescence microscope through GFP, HcRed, and YFP emission filters, considerable differences were observed among the transgenic lines. When excited by ultraviolet light through a GFP filter, *ZsGreen1*, *ZsYellow1*, and *TurboRFP*-transgenic flowers emitted variable intensities of green, greenish-yellow, and yellow fluorescence, respectively (Fig. 1). Approximately 70% to 80% of transgenic T0 plants generated fluorescence from the flowers of *ZsGreen1*, *ZsYellow1*, and *TurboRFP*-transformed petunias (Table 1). On the basis of the plot profile analysis, the highest relative intensity was detected from the *ZsGreen1*-transgenic flowers, which showed 1.5 times more fluorescence intensity than *ZsYellow1*

Fig. 6. *ZsGreen1*-transgenic petunia seedlings generated brilliant green fluorescence (white arrow line) in cotyledons (A), hypocotyl and radicle (B), detected through a green emission filter [(GFP) 480/510 nm, excitation/emission] under excitation of ultraviolet light. The red color of the cotyledon in nontransgenic seedling is chlorophyll *a*-derived auto-fluorescence. These seedlings (T1 generation) were established from seed germination of the self-pollinated T0 transgenic plants.

and *TurboRFP*-transgenic flowers (Fig. 1). The *TurboRFP* transgenic plants were the only ones to generate red fluorescence through the HcRed emission filter under ultraviolet excitation (Fig. 1). Green fluorescence from the *ZsGreen1*-transgenic flowers was clearly observed through orange-colored translucent transparent glasses under excitation of a blue LED light (Fig. 2).

Nontransgenic petunia flowers emitted a very weak, red auto-fluorescence from the stigmas and the bottom parts of the corollas, containing chlorophyll through the GFP filter (Fig. 3B). Very weak emission of green auto-fluorescence was also confirmed from the anthers in nontransgenic petunia through the same GFP filter (Fig. 3B). These fluorescence emissions naturally create bright targets for bees and other pollinators to detect easily (Gandía-Herrero et al., 2005; Miller et al., 2011). No fluorescence emission was detected from *DsRed2*, *Frizzled Kringle*, or *aeCP597-NA*-transgenic flowers. A small number of the *E2Crimson* petunia flowers showed very weak fluorescence through the HcRed emission filter with low transformation efficiency (Table 1). When examined under a high-magnitude-power ($\times 400$) fluorescent microscope, the *ZsGreen1*-transgenic petunia generated very strong, green fluorescence from the reproductive organs: stigmas, styles, anthers, and filaments (Fig. 3B). The green fluorescence emission was also confirmed in the trichomes, pollens, and unopened flowers of the *ZsGreen1*-transgenic petunia (Supplemental Fig. 2). It was difficult to define where the fluorescent proteins were localized in intact cells because the vacuoles fill majority of the petal cells (Fig. 3C-1). Most petal cells are highly vacuolized, and the cytosol is confined to a thin layer at the

periphery of the cells (Serna, 2005). The petals were treated with KNO_3 solution to induce cell plasmolysis, making it easier to distinguish between the cell membrane and cytoplasm. As a result, the *ZsGreen1* fluorescent protein was observed in both the nucleus and the cytoplasm (Fig. 3C-2). GFP can enter nuclear pores because of its small size (27 kDa), so GFP that was not fused to other polypeptides, has been found in both the cytoplasm and the nuclei of transgenic plant, animal, and yeast cells (Hanson and Köhler, 2001). Tanz et al. (2013) also reported that untargeted or free fluorescent proteins are usually localized to the cytoplasm in plant cells but also go into the nucleus due to their size. Therefore, *ZsGreen1* fluorescent protein (26.1 kDa) also appears to be accumulated inside the nuclei of petunia petal cells.

Among the seven transgenes investigated, *ZsGreen1* was the most successful and reproducible, resulting in brilliant green fluorescence emission from the flowers. Of 298 cocultured explants, 33 *ZsGreen1*-transgenic T0 plants were analyzed for fluorescence intensity, which

were then visually evaluated and categorized into four levels (Table 1). One line from each group was selected to compare its transcript and gene expression levels using sqRT-PCR and qPCR analysis, respectively. On the basis of the results of sqRT-PCR analysis, the highest transcript level was shown in line 28, which was categorized into the high fluorescence emission group. As the fluorescent intensity decreased in flowers, the transcript levels also decreased. The quantification results of gene expression using qPCR with genomic DNA showed the same pattern as sqRT-PCR analysis (Fig. 4B and C). A significant positive relationship ($R^2 = 0.88$, $P < 0.01$) between the fluorescent intensity and *ZsGreen1*-transgene expression levels was observed based on the regression analysis (Supplemental Fig. 3). When normalized to the reference genes, *PhEF1 α* or ubiquitin, *ZsGreen1*-transgenic line 28 was identified to show the highest relative quantity of transgene expression. We determined that a single gene was inserted into the MD genome in line 28 and line 02 when estimated by calculation from relative standard curve equations and the thresholding cycles (C_T) of qPCR analysis (Table 2), and subsequent 3:1 segregation in T1 seedlings. When bush monkey-flower (*Mimulus aurantiacus*) was genetically modified with a gene *ZsGreen1* via *A. tumefaciens*-mediated transformation, most of transformed plants (73%) contained one copy of T-DNA (Susić et al., 2014). In our experiments, it was unclear why there was such a large difference in *ZsGreen1* transcript levels between line 28 and line 02, even though both contained a single gene copy (Fig. 4). One likely reason could be that the variability in transgene expression is influenced not only by the copy number of transgene within the integration site

but also by the chromatin status (heterochromatic or euchromatic). Additionally, transgene expression could be affected by nearby regulatory sequences, such as enhancers or silencers (Dolgova and Dolgov, 2019). Although the results of the sqRT-PCR analysis showed that every fluorescent gene was transcribed into RNA (Fig. 5), no fluorescence emission was detected from the *DsRed2*, *rpulFKz1*, *E2Crimson*, and *aeCP597* transgenic flowers. Because the nucleotide sequence of each construct was verified using Sanger sequencing (Sanger and Coulson, 1975), it was unlikely that the plasmids contained mutations disrupting full-length protein synthesis. There were brilliant red or blue fluorescence emissions from concentrated plasmid cultures for the fluorescent genes (data not shown). Other possible reasons may be due to translational errors or detection problems with filters. These could also be issues with sequestration or degradation of petunia for efficient excitation and emission. (Sasaki et al., 2014). Zhou et al. (2005) mentioned that leaf chlorophyll and leaf age negatively affected the detection of fluorescence in rice (*Oryza sativa*), medicago (*Medicago truncatula*), and arabidopsis (*Arabidopsis thaliana*).

Through seed germination tests of T1 generation, it was confirmed that the cotyledons, hypocotyls, and radicles of *ZsGreen1*-transgenic petunia generated strong green fluorescence, detected through GFP filters under excitation of ultra-violet light (Fig. 6). The cotyledons and hypocotyls of nontransgenic seedlings exhibited a red auto-fluorescence under the GFP filter due to chlorophyll *a* (Chl *a*) content (Sasaki et al., 2014). Fluorescence confirmation of the T1 progeny indicated a stable expression of the fluorescent protein in cells. It also suggested that the *ZsGreen1* gene could be a strong visual marker for the selection of homozygous lines in T1, T2, and even T3 generations if genetically cloned without mutation. *ZsGreen1* can allow for an easily screened genetic pipeline (Supplemental Table 2).

In conclusion, transgenic petunia flowers emitting strong green fluorescence were successfully generated using the *ZsGreen1*-pHK-DEST-OE construct (Fig. 3A). *TurboRFP*-transformed petunia also generated red fluorescence in petals but requires further modification to increase the intensity. Sasaki et al. (2014) reported that the use of an enhancer such as the 5'-untranslated region of the alcohol dehydrogenase gene of arabidopsis could promote a massive accumulation of fluorescent protein in wishbone flowers. Sasaki et al. (2014) also recommended the translational enhancer sequence, Ω (omega), from the tobacco mosaic virus as an additional tool for efficient transcription. It was noted that correct detection methods should be developed to lower the natural auto-fluorescence from plant tissues and maximize the fluorescence emission. Throughout the screening procedures of homozygous lines, it was observed that *ZsGreen1* can be a strong visual marker because of its stable expression in cotyledons, hypocotyls, and radicles of seedlings at early stages. *ZsGreen1* has been widely used in transgenic bacteria, fungi, mouse, and mammalian cells but rarely used for plant transformation (Susić et al., 2014). It was reported that the *ZsGreen1* gene was expressed in callus tissue, but the expression intensity gradually decreased during further plant development when transformed under CaMV 35S promoter (Susić et al., 2014). Here, we demonstrated that transgenic petunia successfully emitted strong, green fluorescence in whole-flower organs using pHK-DEST-OE vector containing FMV 35S promoter. As a reporter gene, *ZsGreen1* also proved superior to EGFP gene

because it was brighter than EGFP in bush monkey-flower (Susić et al., 2014). Whereas *ZsGreen1* was reported to generate stronger relative brightness (1.02) than GFP (1.0), *ZsYellow1* was much weaker (0.38) than GFP (Matz et al., 1999). This work may prove useful for developing fluorescent flowers with the FMV promoter and novel fluorescent gene, *ZsGreen1*, via DNA recombination techniques.

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Supplemental Fig. 1. Regeneration of transgenic plants after *Agrobacterium tumefaciens*-mediated transformation with fluorescent genes. (A) Cocultured explants formed calli. (B) The calli of a putative *ZsGreen1*-transgenic petunia generated a high level of green fluorescence (white arrow line). This image was taken with orange transparent glasses for emission under excitation of a blue light emitting diode (450 nm). The red color in the leaves and callus is chlorophyll *a*-derived auto-fluorescence. (C) Shoots were initiated from the calli on selective medium containing kanamycin (750 mg·L⁻¹) and carbenicillin (500 mg·L⁻¹). (D) Rooted transgenic plants were transplanted into potting soil and grown for later confirmation of fluorescent protein and transgene expression in flowers.

Supplemental Fig. 2. Strong green fluorescence emission was detected in unopened flowers, trichomes, and pollens of *ZsGreen1*-transgenic petunia through a green emission filter (GFP, 480/510 nm, excitation/emission) under the excitation of ultraviolet light.

Supplemental Fig. 3. *ZsGreen1*-transgenic corollas showed different levels of fluorescence intensities depending on the lines (**A** and **B**). The plots were created based on the relative intensity (grayscale) across features in the fluorescent image using plot profile menu of ImageJ software (1.51u; National Institutes of Health, Bethesda, MD). (**C**) A linear regression ($R^2 = 0.8784$, $P < 0.01$) showed a significantly positive relationship between the relative fluorescence intensity and *ZsGreen1*-transgene expression level based on the quantitative polymerase chain reaction analysis.

Supplemental Table 1. Oligonucleotide primers used in this study

Fluorescent gene	Primer	Sequences
PCR primers for In-Fusion cloning		
<i>DsRed2</i>	Forward	5'-TCTAGAATGGCCTCCTCCGAGAAC-3'
	Reverse	5'-ACTAGTGCAGGAACAGGTGGTGGC-3'
<i>E2Crimson</i>	Forward	5'-TCTAGAATGGATAGCACCGAGAACGT-3'
	Reverse	5'-ACTAGT GCTATTGGAAAAAGGTGATGCC-3'
<i>TurboRFP</i>	Forward	5'-ACCAATCCTTTCTAGAATGAGCGAGCTGATCAAGG-3'
	Reverse	5'-GCTCGGATCCACTAGTTCATCTGTGCCCCAGTTTG-3'
<i>ZsGreen1</i>	Forward	5'-TCTAGAATGGCCCAGTCCAAGCAC-3'
	Reverse	5'-ACTAGT TCAGGGCAAGGCGGAGC-3'
<i>ZsYellow1</i>	Forward	5'-ACCAATCCTTTCTAGAATGGCCCACAGCAAGCAC-3'
	Reverse	5'-GCTCGGATCCACTAGTTTAGTGATGGTGATGGTGAT-3'
<i>rpulFKz1</i>	Forward	5'-ACCAATCCTTTCTAGAATGGCTAAGGCTATCGTGG-3'
	Reverse	5'-GCTCGGATCCACTAGTTTAGTATTGAGTATTGCCC-3'
<i>aeCP597</i>	Forward	5'-ACCAATCCTTTCTAGAGCTTCATTGGTTACCGAGGATATG-3'
	Reverse	5'-GCTCGGATCCACTAGTTTAGTGATGTCCAAGCTTTGATGG-3'
sqRT-PCR primers		
<i>DsRed2</i>	Forward	5'-AGGACGGCTGCTTCATCTAC-3'
	Reverse	5'-TGGTGTAGTCCTCGTTGTGG-3'
<i>E2Crimson</i>	Forward	5'-CTTACATCAAGCACCTGCTG-3'
	Reverse	5'-GTGACCACCACCCTTCAACT-3'
<i>TurboRFP</i>	Forward	5'-ACCGTGAACAACCACCACTT-3'
	Reverse	5'-GATGATGCAGCCGTTCTGG-3'
<i>ZsGreen1</i>	Forward	5'-GACCAAGGAGATGACCATGAAG-3'
	Reverse	5'-TGGACTCGTGGTACATGCAG-3'
<i>ZsYellow1</i>	Forward	5'-CGTGAGCGTGAAGGAGAACT-3'
	Reverse	5'-CTGCCACTTCTGGTTCTTGG-3'
<i>rpulFKz1</i>	Forward	5'-TGGCTAATGGTGAATGCAAA-3'
	Reverse	5'-CAACGCAACCTTGATTCTCA-3'
<i>aeCP597</i>	Forward	5'-CGAGCTTGCAGGGAAATAAC-3'
	Reverse	5'-ACTTGCCTTGTCTGCCTTT-3'
qPCR primers		
<i>ZsGreen1</i>	Forward	5'-GTGAGCGTGGAGGAGAACTG-3'
	Reverse	5'-TCCCAGTTGTCGGTCATCTT-3'
<i>PhEF1-alpha</i>	Forward	5'-CCTGGTCAGATTGGAAACGG-3'
	Reverse	5'-CAGATCGCCTGTCAATCTTGG-3'

Supplemental Table 2. Germination rate and fluorescent protein expression from T2 generation seedlings of *ZsGreen1*-transgenic petunia.

Transgenic line (T0) ^z	T1 generation ^y	Germination rate (%)	Germinated seeds (no.)	Fluorescent seedlings (no.)	Nonfluorescent seedlings (no.)	T2 generation
28	1	N/A	—	—	—	—
	2	75	15	15	0	Homozygous
	3	N/A	0	—	—	—
	4	80	16	16	0	Homozygous
	5	10	2	2	0	Homozygous
	6	0	0	—	—	—
	7	100	20	20	0	Homozygous
	8	N/A	—	—	—	—
34	1	N/A	—	—	—	—
	2	25	5	5	0	Homozygous
	3	N/A	—	—	—	—
	4	N/A	—	—	—	—
	5	95	19	14	5	3:1
	6	20	4	3	1	3:1
	7	100	20	20	0	Homozygous
	8	60	12	9	3	3:1

^zT0 generation petunia were selected among the transgenic lines showing high expression of fluorescent protein in flowers. Three transgenic lines having offspring segregation with a 3:1 ratio of fluorescent to nonfluorescent seedlings were then identified.

^yT1 generation petunia were established from the seedling of self-pollinated T0 transgenic plant.