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

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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 x hybrida)] was genetically transformed with seven genes encoding FTs: DsRed2, E2Crimson, TurboRFP, ZsGreen1, ZsYellow1, rpsFKz1, 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, stigmas, 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-, rpsFKz1-, 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 glutriuscula), a swallowtail butterfly (Papilio nireus), a jumping spider (Cosmophus 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
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; Ormò 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...
DsRed2 fluorescent protein) is a modified version of Katayama et al., 2008; Matz et al., 1999).

became widely used as a red fluorescent protein (Katayama et al., 2008). DsRed2 two times as bright as Zoanthus reef coral [ZsYellow1 (Vo et al., 2013; Wenck et al., 2003)]. The chromoprotein consists of two domains: a Frizzled cysteine-rich domain and a Kringle domain (Bulina et al., 2004). It was discovered in scyphoid 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; Voβ 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), artichokes (Eruca sativa), and spinach (Spinacia oleracea) via a pressurized bath infuision method. As a result, the watercress plants generated a maximum rate of 1.44 × 10^10 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. 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 (gray scale) 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.
Materials and Methods

Vector construction and petunia transformation. The coding regions of DsRed2, E2Crimson, TurboRFP, ZsGreen1, ZsYellow1, rplFKz1, 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 mM galacturonic acid, 200 mM acetosyringone, 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. 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.
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. 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 using 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 (pEFlαα), 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).

![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⁻ΔΔCT method using quantitative PCR in flowers normalizing to the reference gene, elongation factor 1-alpha (PhEF1αα), 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).](image-lang)
relative transcript levels of the ZsGreen1 gene in different T0 plants were quantified by the 2−ΔΔCT 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, PhEF1α, 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 resulting from the amplification of the target and the reference genes.

**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.

Table 2. Comparison of C\textsubscript{T} values of target (ZsGreen1) and reference (EF1α) 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 | C\textsubscript{T} (ZsGreen1) | C\textsubscript{T} (EF1α) | \(X_0 / R_0\) | Estimated transgene copies (no.) |
|----------------|-----------------------------|--------------------------|----------------|---------------------------------|
| Wild type\textsuperscript{a} | 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 |

\textsuperscript{a}Flowers 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).

\(X_0 / R_0 = \frac{1}{T} \left[ \frac{S_X - (C_{T,X} - I_X)}{S_X - (C_{T,R} - I_R)} \right] \). \(I_X\) and \(I_R\) are intercepts 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 (\(C\textsubscript{T}\) value) of the target and the reference genes.

\(\text{Petunia ‘Mitchell Diploid’ as a nontransgenic control plant.}\)

**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 transgenes, 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.
No fluorescence emission was detected from Kringle vacuolized, and the cytosol is confined to a thin layer at the majority of the petal cells (Fig. 3C-1). Most petal cells are highly proteins were localized in intact cells because the vacuoles fill mental Fig. 2). It was difficult to define where the fluorescent excitation (Fig. 1). Green fluorescence from the ZsGreen1 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 (Gandia-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 (×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 KNO₃ 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, PhEFlα 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 (CT) 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.

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 α-derived auto-fluorescence. These seedlings (T1 generation) were established from seed germination of the self-pollinated T0 transgenic plants.
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, pOupFKz1, E2Crimson, and aceCP597 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 other possible reasons may be due to translational errors or plasmid cultures for the fluorescent genes (data not shown). Other possible reasons may be due to translational errors or plasmid cultures for the fluorescent genes (data not shown). 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 ultraviolet 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 autofluorescence 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** |                        |                                        |
| DsRed2                | Forward                | 5'-TCTAGAATGGCCCTCCTCCGAGAGC3'         |
|                       | Reverse                | 5'-ACTAGTGCAGAGGCAGATCTGAGG-3'         |
| E2Crimson             | Forward                | 5'-TCTAGAATGGGAGAGCAGAGAAGC3'          |
|                       | Reverse                | 5'-ACTAGTGCAGAGGCAGATCTGAGG-3'         |
| TurboRFP              | Forward                | 5'-ACCAATTTTCTAGGAGAGCAGATCTGAGG-3'    |
|                       | Reverse                | 5'-GCTCGGATCCACTAGTTAGTAGGTGGATGGAT-3' |
| ZsGreen1              | Forward                | 5'-TCTAGAATGGCCAGGAGGAGC3'             |
|                       | Reverse                | 5'-ACTAGTGCAGAGGCAGATCTGAGG-3'         |
| ZsYellow1             | Forward                | 5'-ACCAATTTTCTAGGAGAGCAGATCTGAGG-3'    |
|                       | Reverse                | 5'-GCTCGGATCCACTAGTTAGTAGGTGGATGGAT-3' |
| rpulFKz1              | Forward                | 5'-ACCAATTTTCTAGGAGAGCAGATCTGAGG-3'    |
|                       | Reverse                | 5'-GCTCGGATCCACTAGTTAGTAGGTGGATGGAT-3' |
| aeCP597               | Forward                | 5'-ACCAATTTTCTAGGAGAGCAGATCTGAGG-3'    |
|                       | Reverse                | 5'-GCTCGGATCCACTAGTTAGTAGGTGGATGGAT-3' |
| **sqRT-PCR primers**  |                        |                                        |
| DsRed2                | Forward                | 5'-AGGACGCGCTGCTTAC3'                  |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| E2Crimson             | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| TurboRFP              | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| ZsGreen1              | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| ZsYellow1             | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| rpulFKz1              | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| aeCP597               | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| **qPCR primers**      |                        | Target and reference genes              |
| ZsGreen1              | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |
| PhEF1-alpha           | Forward                | 5'-ACGGGACGCGCTTAC3'                   |
|                       | Reverse                | 5'-TGCTGACGCGCTTAC3'                   |

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

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

* T0 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.

*y T1 generation petunia were established from the seedling of self-pollinated T0 transgenic plant.