Engineered GFP as a vital reporter in plants

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Background: The green-fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has recently been used as a universal reporter in a broad range of heterologous living cells and organisms. Although successful in some plant transient expression assays based on strong promoters or high copy number viral vectors, further improvement of expression efficiency and fluorescent intensity are required for GFP to be useful as a marker in intact plants. Here, we report that an extensively modified GFP is a versatile and sensitive reporter in a variety of living plant cells and in transgenic plants.

Results: We show that a re-engineered *GFP* gene sequence, with the favored codons of highly expressed human proteins, gives 20-fold higher GFP expression in maize leaf cells than the original jellyfish *GFP* sequence. When combined with a mutation in the chromophore, the replacement of the serine at position 65 with a threonine, the new *GFP* sequence gives more than 100-fold brighter fluorescent signals upon excitation with 490 nm (blue) light, and swifter chromophore formation. We also show that this modified GFP has a broad use in various transient expression systems, and allows the easy detection of weak promoter activity, visualization of protein targeting into the nucleus and various plastids, and analysis of signal transduction pathways in living single cells and in transgenic plants.

Conclusions: The modified GFP is a simple and economical new tool for the direct visualization of promoter activities with a broad range of strength and cell specificity. It can be used to measure dynamic responses of signal transduction pathways, transfection efficiency, and subcellular localization of chimeric proteins, and should be suitable for many other applications in genetically modified living cells and tissues of higher plants. The data also suggest that the codon usage effect might be universal, allowing the design of recombinant proteins with high expression efficiency in evolutionarily distant species such as humans and maize.

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Background

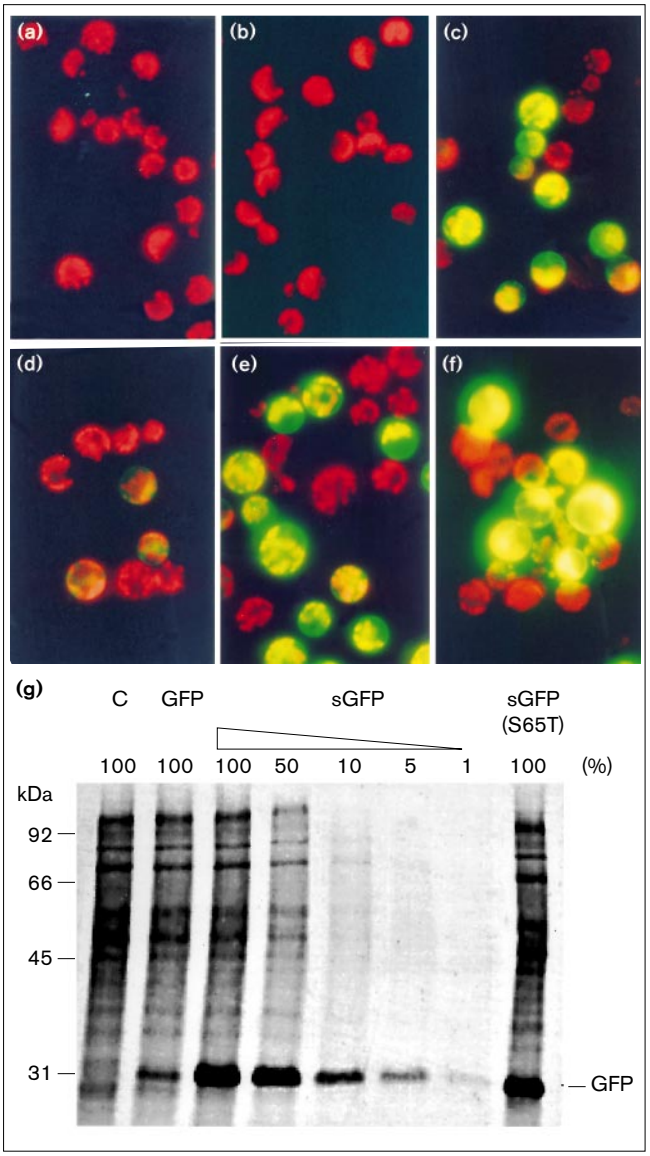
The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has a number of desirable traits as a universal reporter in living cells and organisms [1,2]. Apart from an apparent requirement for molecular oxygen, the formation of the fluorophore appears to be cell-autonomous [1–3]. Direct visualization of gene expression in individual cells is therefore possible without cell lysis and subsequent biochemical analysis, and tissue distortion caused by fixation, staining and section can be avoided. The autocatalytic formation of the chromophore and relative resistance to photobleaching make GFP an attractive fluorescent tag for studying protein interaction, localization and traffic [2–5]. Although the expression of GFP has been demonstrated in *Escherichia coli*, yeast, *Caenorhabditis elegans*, *Drosophila*, mammals and plants [1–15], the broader application of GFP in mammals and higher plants requires higher expression efficiency and fluorescent intensity, especially under blue light, to minimize photobleaching and phototoxicity [2,6]. Here, we have used a modified GFP, and show that it acts as a convenient and sensitive reporter for the visualization of gene regulation, signal transduction and subcellular localization of chimeric proteins in living cells of maize, tobacco, onion and *Arabidopsis*, and in transgenic tobacco plants.

Results

Expression of engineered GFP in plant cells

As each GFP molecule represents one fluorophore, high-level expression is important to give good fluorescent signals. Using universal transcription and translation enhancers with strong promoters, we have shown previously that GFP signals are detectable in transient expression systems of monocot maize and dicot *Arabidopsis* [12,13]. However, an increase in the expression efficiency and quantum yield with 490 nm excitation would make GFP substantially more useful as a vital marker in plants. As the preferred codon usage is almost identical between humans and maize, and is compatible with that of other higher plants (such as *Arabidopsis*) [16,17], we tested the expression of a new synthetic *GFP* gene sequence (*sGFP*) with optimal human codons [17] in plant cells. A mutation in the chromophore — the replacement of the serine

Figure 1

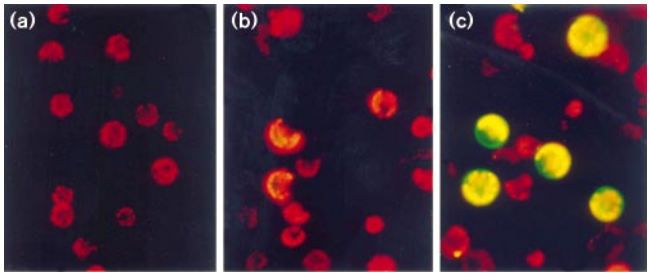


Engineered GFP gives faster and brighter fluorescent signals. Maize mesophyll protoplasts were electroporated with three plasmids expressing **(a,d)** the jellyfish GFP (GFP), **(b,e)** synthetic GFP (sGFP) or **(c,f)** a mutant S65T sGFP (sGFP(S65T)). The transfected protoplasts were observed after (a–c) 4 h or (d–f) 16 h of incubation. **(g)** Synthetic GFP gives a high level of protein expression. Untransfected control (C) and transfected maize protoplasts were labeled with 400 μCi ml^{-1} [^{35}S]methionine for 12 h. Total proteins were solublized in protein loading buffer and separated by 12.5 % SDS-PAGE.

position 65 with a threonine (S65T) — has been shown in *E. coli* to result in enhanced brightness, faster chromophore formation and slower photobleaching [7]. This change was also introduced into *sGFP* by site-directed mutagenesis, to create *sGFP(S65T)*.

Three similar constructs were generated by inserting *GFP*, *sGFP* or *sGFP(S65T)* into a plant expression vector with a strong, constitutive promoter (*35SC4PPDK*) and the 3′ *NOS* transcription terminator [12,18]. Plasmid DNA was introduced into maize leaf protoplasts by electroporation. After 3–4 hours, bright fluorescence signals were visible under blue light in 50 % of protoplasts transfected with the *35SC4PPDK–sGFP(S65T)* construct (Fig. 1a–c). This early detection of sGFP(S65T) was due mainly to the enhanced fluorescent signal and rapid chromophore formation induced by the S65T mutation, as shown in *E. coli* [7]. After 15–16 hours of incubation, green fluorescence was detectable in 50 % of all transfected protoplasts, although the fluorescent intensity was much stronger in cells electroporated with the *sGFP* and *sGFP(S65T)* constructs (Fig. 1d–f). As GFP and sGFP have identical amino-acid sequences, the brighter signal from sGFP is likely to be due to a higher level of protein synthesis caused by optimal codon usage, as demonstrated by Haas *et al.* in the preceding paper [17]. To confirm that the selected codon usage gave higher protein expression in maize leaf cells, we examined the amount of ^{35}S-methionine-labeled GFP expressed by the three constructs. As shown in Figure 1g, the amounts of newly synthesized sGFP and sGFP(S65T) were similar and easily detectable without purification, whereas the amount of native GFP was about 20-fold lower.

Figure 2



The expression of *AtCAB2-sGFP(S65T)* is regulated by light in maize leaf protoplasts. **(a)** Untransfected control protoplasts. **(b,c)** Transfected protoplasts incubated (b) in the dark or (c) under light for 20 h.

sGFP(S65T) as a reporter for a weak promoter

As the fluorescent intensity of sGFP(S65T) was dramatically improved, we tested its use as a reporter for a heterologous weak promoter. Activity of the dicot *Arabidopsis CAB2* promoter (*AtCAB2*) [19,20] could be detected when fused to luciferase (LUC) [21–24] and chloramphenicol acetyltransferase (CAT) [25,26] (data not shown), but not when native GFP was used in maize leaf cells [12]. An *AtCAB2-sGFP(S65T)* construct was electroporated into monocot maize leaf cells, and the cells incubated with or without light; after 20 hours incubation, bright fluorescence was only detected in the cells incubated under light (Fig. 2). The sensitivity of sGFP(S65T) as a reporter appeared comparable to that of CAT and LUC. In spite of its lower activity, the *Arabidopsis CAB2* promoter is regulated by light in a similar manner to the maize photosynthetic gene promoters

[18,26–28]. This indicates the presence of a universal light signal transduction pathway in dicot and monocot leaf cells.

sGFP(S65T) as a vital reporter in tobacco protoplasts

We have shown previously that GFP expression can be detected in a maize protoplast transient expression system. In tobacco protoplasts, however, it was difficult to visualize GFP expression from the native *GFP* sequence even with a strong promoter. Maize leaf protoplasts appear to have substantially more synthetic capacity than protoplasts isolated from leaves of a number of plant species [25–28]. To use GFP in leaf protoplasts from tobacco and many other plant species therefore requires a higher level of GFP expression. A construct carrying *35SC4PPDK-sGFP(S65T)* was introduced into tobacco leaf protoplasts by polyethylene glycol (PEG)-mediated transfection [29]. As shown in Figure 3, bright green fluorescence was detected in > 80 % of the protoplasts after 20 hours of incubation (Fig. 3). Weaker signals were also obtained with a similar construct carrying *35SC4PPDK-sGFP* (data not shown). Tobacco leaf protoplasts are very sensitive to hormone treatment and can easily undergo dedifferentiation and regeneration [30]. In combination with the sGFP(S65T) marker, these cells can be used conveniently to study signal transduction and cell-cycle regulation in higher plants [30].

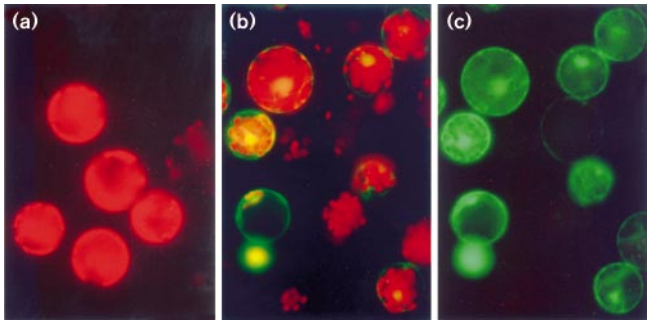
Analysis of organelle targeting in *Arabidopsis*

Many plant proteins have to be targeted to the nucleus and various plastids to serve their proper functions. To visualize such targeting, we used sGFP(S65T) chimeric proteins with either the nuclear localization sequence (NLS) [31] or the plastid transit peptide (TP) [32]. Plasmid constructs were introduced into both living leaves and roots of *Arabidopsis* by DNA bombardment, and the targeting sequences directed the localization of sGFP(S65T) into the nucleus and plastid (Fig. 4b,c,e,f). Without a targeting sequence, sGFP(S65T) accumulated diffusely in the cytoplasm and nucleus (Fig. 4a,d). As no manipulation was required prior to sample observation, the integrity of cell structure and morphology was maintained perfectly. Although plastids in roots and leaves have very different morphologies and functions, the signals and machineries for protein import seem to be similar. The sGFP(S65T) reporter appears to be superior to β-glucuronidase (GUS) fusions [33], which are larger, demand exogenous substrate and infiltration, need cell and tissue fixation, and have leakage problems because the indigo dye generated by enzymatic action often precipitates diffusely. It provides a new and powerful visual tool to study organelle targeting in living cells and to select mutants with abnormal protein localization in intact plants.

Nuclear targeting in onion epidermal cells

Onion skin epidermal cells have recently become a popular system in the study of regulation and sequence requirements for nuclear localization in plants [34]. Constructs expressing sGFP(S65T) and NLS–sGFP(S65T) were bombarded into epidermal cells. Striking signals were observed after 24 hours of incubation, demonstrating the use of this new marker in a simple system (Fig. 5).

Figure 3

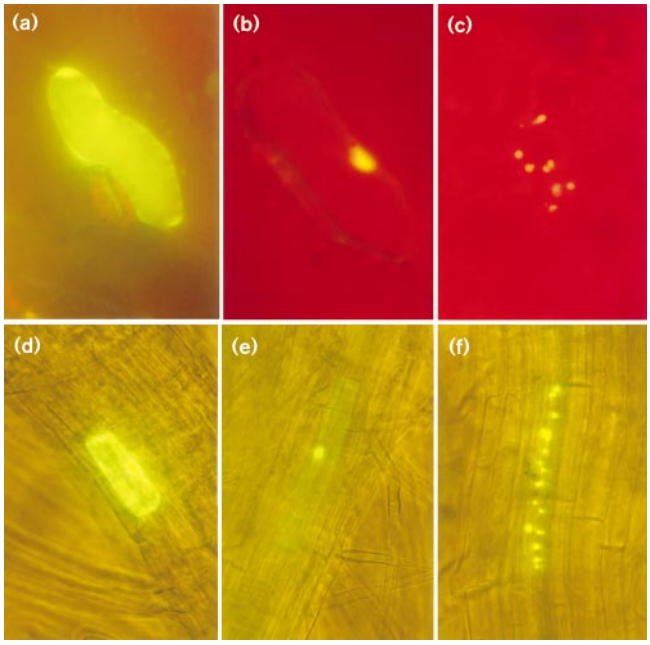


The expression of *35SC4PPDK-sGFP(S65T)* in tobacco mesophyll protoplasts. **(a)** Untransfected control and **(b,c)** transfected tobacco protoplasts after 20 h incubation. In (c)the red autofluorescence of chlorophyll was blocked using a interference filter.

A favorable reporter for identification of transgenic plants

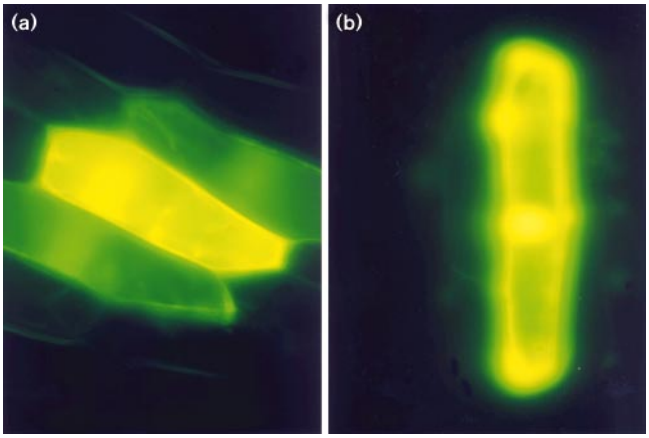
Inefficient expression and low brightness of the native GFP have impeded its use as a visual reporter in transgenic plants. Using *sGFP(S65T)* and *sGFP* (data not shown) fused to the *35SC4PPDK* promoter, transgenic tobacco plants were easily identified by examining directly the leaves of

Figure 4



Organelle targeting in *Arabidopsis*. Constructs carrying **(a,d)** *35SΩ-sGFP(S65T)*, **(b,e)** *35SΩ-NLS-sGFP(S65T)* or **(c,f)** *35SΩ-TP-sGFP(S65T)* were bombarded into *Arabidopsis* (a–c) leaves or (d–f) roots. The expression and localization of sGFP(S65T) was observed after 24 h of incubation.

Figure 5



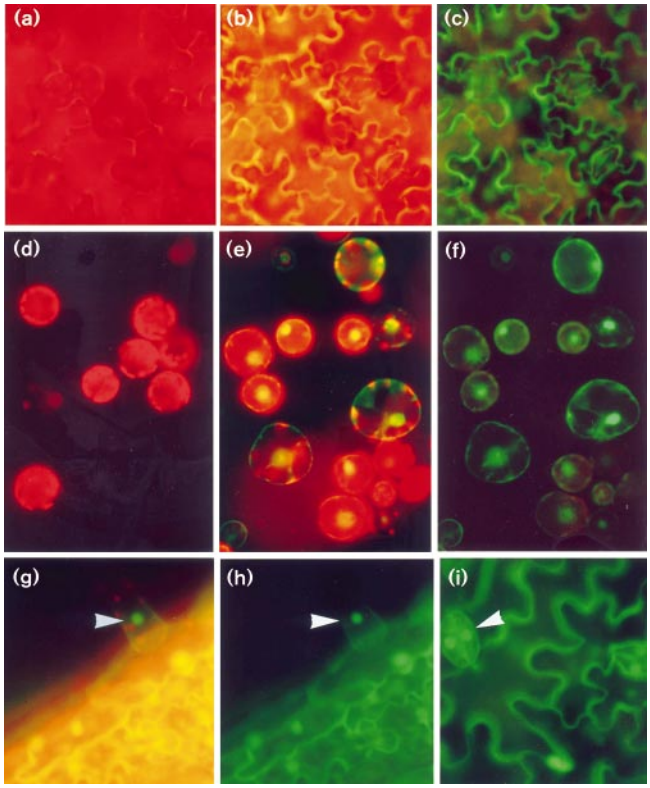
Nuclear localization of sGFP(S65T) in onion cells. Constructs carrying **(a)** *35SΩ-sGFP(S65T)* or **(b)** *35SΩ-NLS-sGFP(S65T)* were bombarded into onion skin epidermal cells. The expression and localization of sGFP(S65T) was observed after 24 hours incubation.

putative transformants under a fluorescence microscope. Untransformed tobacco leaves appeared bright red (Fig. 6a), whereas transformed tobacco leaves showed yellow fluorescence, the consequence of sGFP(S65T) expression (Fig. 6b). The red autofluorescence of chlorophyll was easily blocked by an interference filter (Fig. 6c). This visual identification of plants based on transgene expression is faster, simpler and more reliable than drug selection and polymerase chain reaction (PCR)-based identification. All protoplasts isolated from the leaves of transgenic plants showed green fluorescence, so GFP expression was not limited to a particular cell type (Fig. 6d–f). Most sGFP(S65T) was concentrated around the plasma membrane, and around or in the nucleus. The expression of sGFP(S65T) directed by cell-type-specific promoters will provide a simple and powerful means for sorting and purifying various living cell types from transgenic plants using fluorescent-activated cell sorting [12,13]. It should be noted that a very high level of GFP expression can inhibit the segmentation process in producing transgenic plants (data not shown).

Drought-inducible gene expression in transgenic plants

To illustrate the use of sGFP(S65T) as a reporter to study signal transduction in intact plants, we generated transgenic tobacco plants carrying sGFP(S65T) controlled by a drought-inducible promoter (*RD29A*) from *Arabidopsis* [35]. The plants were allowed to wilt, to induce the expression of sGFP(S65T). Green fluorescence was clearly visible in many cell types, including the epidermal, mesophyll and guard cells of the leaves (Fig. 6g–i). Striking nuclear accumulation of sGFP(S65T) was observed in guard cells and trichomes. No green fluorescence was detected without the drought treatment. Thus, sGFP(S65T) can serve as an excellent reporter allowing the direct visualization in real time of plant responses to various environmental signals.

Figure 6



sGFP(S65T) as a vital marker in transgenic tobacco plants. **(a,d)** Untransformed control and **(b,c,e,f)** transgenic tobacco plant (a–c) leaves and (d–f) protoplasts were observed using a fluorescence microscope with a FITC filter set without or (c,f) with an interference filter. **(g–i)** Drought-inducible expression of sGFP(S65T) in transgenic tobacco plants. Transgenic plants carrying the *AtRD29A-sGFP(S65T)* construct were allowed to wilt and the expression of sGFP(S65T) on leaf surface was observed (g) without or (h,i) with an interference filter. Bright green fluorescence was most striking in the nucleus of trichome and guard cells as indicated by arrows.

Discussion

The jellyfish GFP is a new reporter with high potential for use in all living cells and organisms. Its use in mammals and higher plants, however, has been limited by inefficient expression, low fluorescence, slow chromophore formation and complex photoisomerization [2]. Several laboratories have made progress recently in improving GFP to be a more versatile and sensitive reporter. These modifications increase the sensitivity of GFP detection, reduce photobleaching and phototoxicity, allow simultaneous analysis of two promoters or two proteins, and offer better matching to standard fluorescence microscopy filter sets [2,3,7,8]. Higher levels of GFP expression are still important to broaden its applications in mammals and higher plants. By combining optimal codon usage [17] and the S65T mutation [7], we have found that sGFP(S65T) gives higher expression levels, faster chromophore formation and more enhanced fluorescent emission by blue light in plant cells. Previously, a universal transcription or translation enhancer

was essential to use the original jellyfish GFP in monocot or dicot cells [12,13]. The detection of GFP in tobacco leaves infected with the tobacco mosaic virus *TMV-GFP* [36] or the potato virus *X PVX-GFP* [9] relied on high copy numbers of TMV and PVX RNA carrying the GFP sequence.

We have shown here that sGFP and sGFP(S65T) can be used to detect the activity of weaker promoters. Furthermore, both work beautifully (without lethal staining, fixation and dissection) in a broad spectrum of transient expression systems, such as electroporated maize protoplasts, PEG-transfected tobacco protoplasts and bombarded *Arabidopsis* and onion tissues. The expression of sGFP and sGFP(S65T) also provides a rapid, simple and non-destructive assessment of transient transfection and stable transformation efficiency. The incorporation of an imaging system should add more precise quantitation power [24].

We have explored the use of sGFP(S65T) as a reporter for light- and drought-inducible gene expression mediated by universal signaling pathways in higher plants. Both responses were conveniently visualized in individual cells. The modified GFP will also be an invaluable reporter for monitoring plant responses to other environmental stimuli, such as pathogens, wounding, touch and stresses, as well as internal physiological, metabolic and developmental activities in living cells and plants. This reporter can serve as a powerful tool, therefore, for elucidating the mechanisms of gene regulation and signal transduction in higher plants.

The relatively low molecular weight of GFP makes it ideal as a fluorescent tag in fusion proteins. Protein sorting, traffic, localization, intracellular fate and extracellular movement can be followed in real time at high resolution, especially with confocal microscopes [4,5,12,14]. Fusion proteins with various fluorescent tags also provide a new way to detect protein–protein interactions or changes in protein conformation *in vivo*. The sGFP(S65T) reporter is a convenient tool for further studies at the single-cell level, and can label cells for functional and physiological analysis of co-transfected genes. It also has potential as a vital marker for enhancer and gene trap screening, investigating recombination and transposition events, fate mapping or cell lineage analysis, tracing chromosome or gene segregation, genetic and molecular mapping, and mutant selection.

Recently, Haseloff and colleagues [14] observed the splicing of a cryptic intron in GFP mRNA which abolished GFP expression in transgenic *Arabidopsis* plants. It is not clear how widespread this phenomenon is in other plant species. Our observation of GFP expression in maize and *Arabidopsis* transient assays suggests that GFP is not, or only partially, spliced in these cells. Splicing is unlikely to occur in maize mesophyll protoplasts as a single polypeptide of the correct size [1,3,9,12] is detected in cells transfected with *GFP*, *sGFP* and *sGFP(S65T)*. Serendipitously, the sequence at the cryptic splice donor site AAAGGTATTGATTT-TAAA was changed to AAgGGcATcGATTTcAAg during the synthesis of sGFP with favored codons, and the cryptic intron in the coding region (400–483) of GFP [14] is therefore eliminated in sGFP and sGFP(S65T).

Conclusions

We have shown that sGFP and sGFP(S65T) are versatile and sensitive reporters in transient expression using maize, tobacco, onion and *Arabidopsis* cells, and in transgenic tobacco plants. These new tools can be used for studies of gene regulation, signal transduction, development and cell biology in higher plants.

Materials and methods

Plasmid Constructions

The construction of the plant expression vector with a strong and constitutive promoter *35SC4PPDK* has been described previously [12,18]. The creation of *sGFP* is reported separately [17]. *sGFP(S65T)* was generated by PCR-based site-directed mutagenesis using two flanking primers: 5′-GCGGATCCATGGTGAGCAAG-3′ and 5′-GGGCGGCC-GCTTTACTTGTA-3′ and two overlapping mutagenesis primers: 5′-GTGACCACCTTCACCTACGGCGTGCAG-3′ and 5′-CTGCACGC-CGTAGGGAAGGTGGTCAC-3′. *GFP*, *sGFP* and *sGFP(S65T)* were amplified using the same flanking PCR primers and inserted into the expression vector between *Bam*HI and *Sma*I sites. Three clones were picked from each construction for initial evaluation. The *Arabidopsis CAB2* [19,20] and *RD29A* [35] promoters were obtained by PCR and fused to the *Nco*I site at the 5′ of *sGFP(S65T)*. Three clones were selected for initial evaluation by transient expression analysis. The primers used were 5′-TGGACTAGAGATTGCCACGTA-3′ and 5′-GGAGGAGA-GAGCCATGGTTGAGGC- GGCCAT-3′ for the *AtCAB2* promoter and 5'-GACCGACTACTAAT- AATAGTAAGT-3′ and 5′-TGTTTGATCCATG-GTCCACCGATTTTT-3′ for the *AtRD29A* promoter. The constructs used for bombardment carried the *35SΩ* regulatory sequence instead of the *35SC4PPDK* promoter [12]. The NLS of SV40 [31] was synthesized (5′-TCGACCATGGCTCCAAAGAAGAAGAGAAAGGT-3′ and 5′-CATGA-CCTTTCTCTTCTTCTTTGGAGCCATGG-3′), annealed and kinased before insertion into the *Sal*I and *Nco*I site of the *35Ω-sGFP(S65T)* plasmid. The TP sequence was obtained from *RBCS1A* (–38 *Bfa*I and *Sph*I +165, blunt ends) [32] and inserted into the blunted *Sal*I and *Nco*I site of the *35Ω-sGFP(S65T)* plasmid. The binary vector used for tobacco transformation was pART27 [37], carrying *35SC4PPDK-sGFP(S65T)* and *AtRD29A-sGFP(S65T)*.

Protoplast transient expression

Maize seedlings were grown in the dark for 11–12 days before illumination for 16–18 h as described [25,26]. The preparation, electroporation and incubation of the maize mesophyll protoplasts were as described [25,26]. Transfected protoplasts were incubated at 23 °C for 4–20 h to allow the accumulation of the GFP, sGFP and sGFP(S65T). The labeling and analysis of GFP with ^{35}S-methionine have been described [12]. The protocols for tobacco mesophyll protoplasts preparation, PEG transfection, and incubation were similar to those used for carrot protoplasts described previously [29], with some modifications. Healthy and expanded tobacco SR1 leaves were cut to about 2 cm^2 and digested in an enzyme solution consisting 1.2 % Cellulase R10 and 0.4 % Macerozyme R10 in K3 medium [38] with 0.4 M sucrose for overnight in the dark at 23 °C. Protoplasts were collected by floating. Plasmid DNA carrying *35SC4PPDK-sGFP(S65T)* (20 μg) was added to 0.25 ml freshly isolated tobacco protoplasts (10^6 ml^{-1}) in 0.4 M mannitol, 20 mM $CaCl_2$, 5 mM MES, pH 5.7. An equal volume of 40 % PEG 4000 in 0.4 M mannitol and 100 μM $Ca(NO_3)_2$ (brought to pH 10 using KOH before autoclaving) was added immediately, mixed well and incubated for 10 min at room temperature. The mix was diluted with 4 ml K3 medium containing 0.3 M sucrose. The transfected protoplasts were incubated in the dark for 20–24 h before being photographed.

Tissue Bombardment

Tissues from *Arabidopsis thaliana* (Columbia) were prepared as described previously [12]. Onion epidermal cell layers were peeled and placed inside up on the MS plates [12]. Plasmid DNAs of appropriate fusion genes (0.5 μg) were introduced to *Arabidopsis* leaves and roots using a pneumatic particle gun (PDS-1000/He; BIO-RAD). The condition of bombardment was vacuum of 28 inch Hg, helium pressure of 1550 or 1800 psi for *Arabidopsis* and 1100 or 1300 psi for onion, and 6 cm of target distance using 1.1 μm of tungsten microcarriers. After bombardment, tissues were incubated on the MS plates for 24 h at 22 °C. Samples were observed directly or transferred to glass slides.

Tobacco transformation

Stable transformation was performed based on the established protocol using tobacco SR1 leaves [39].

Fluorescence microscopy

The fluorescence photographs of maize mesophyll protoplasts were taken using a Zeiss Universal microscope equipped with epi-fluorescence condenser III RS and a FITC filter set comprising exciter filter (BP 450-490), chromatic beam splitter (FT 510), and barrier filter (LP 520), and Kodak Ektachome *Elite* 400 color film. The optimal exposure time was 30 sec. The light source was provided by a HBO 50 W high-pressure mercury bulb. The fluorescence photographs of tobacco protoplasts and tissues were taken using a Leitz DM-R microscope through epifluorescence filter set I3, which contains an excitation filter with band pass of 450–490 nm, RKP 510 dichromatic mirror, and 520 nm long pass filter. The microscope is also equipped with an interference filter that can be used to block the red autofluorescence from chlorophyll. The light source was provided by a 100 W high-pressure mercury bulb. *Arabidopsis* tissues were observed with Olympus fluorescent microscopy (AH2-RFL) with a filter set providing 455–490 nm excitation and emission above 515 nm.

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