

## A Novel Selection Method Based on the Expression Level of Green Fluorescent Protein Measured with a Quantitative Fluorescence Imager

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### Abstract

Green fluorescent protein (GFP) has been used extensively as a novel non-invasive reporter in the investigation of issues such as promoter function, transformation marker recognition, and sub-cellular localization analysis. A plant-optimized synthetic GFP modified via the replacement of the serine to threonine at position 65 [sGFP(S65T)] has achieved high levels of expression with no toxic side-effects in many plants. It would be useful to develop a non-invasive selection system based only upon GFP expression levels. A trapping vector was constructed using an sGFP(S65T) and a quantitative fluorescence imaging system was used for non-invasive screening of trapped tobacco cultured cells. Putative trapped lines could be identified using this system. Moreover, the GFP products from the candidate trapped lines could be quantitatively visualized directly via SDS-PAGE using the fluorescence imaging system. We conclude that the non-invasive selection system described here could be a powerful new tool for plant biotechnology.

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**Key words:** fluorescence imaging, green fluorescent protein, non-invasive screening, quantitative detection, reporter gene, trapping.

### Abbreviations

GFP, Green fluorescent protein; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAIL-PCR, thermal asymmetric interlaced PCR.

The production of novel plants with favorable traits is the main goal of plant biotechnology. The introduction of genes into plant genomes is one of the most powerful methods of achieving this. Chemical resistance genes are widely used to distinguish transformed cells from non-transformed ones. Although this method is extremely useful in certain plant systems, it can be difficult to apply it to other systems such as crops and trees. The main reason for this is due to the reduction of regeneration

efficiency on selection medium.

Trapping methods such as gene, promoter, and enhancer traps provide a powerful tool for the analysis of gene function. Although the *uidA* gene, which encodes  $\beta$ -glucuronidase (Jefferson *et al.*, 1987), has been widely used as a trapping reporter in plants (Sundaresan *et al.*, 1995), fixation and treatment with substrates is necessary to detect enzyme activity (Jefferson, 1987; Niwa *et al.*, 1994).

In both cases, distinguishing putative transformed cells from others in a non-invasive and substrate-free manner is the crucial step. GFP from the jellyfish *Aequorea victoria* emits green fluorescence without any additional substrates or cofactors when excited with blue or UV light (Tsien, 1998). GFP has emerged as a powerful new reporter in a variety

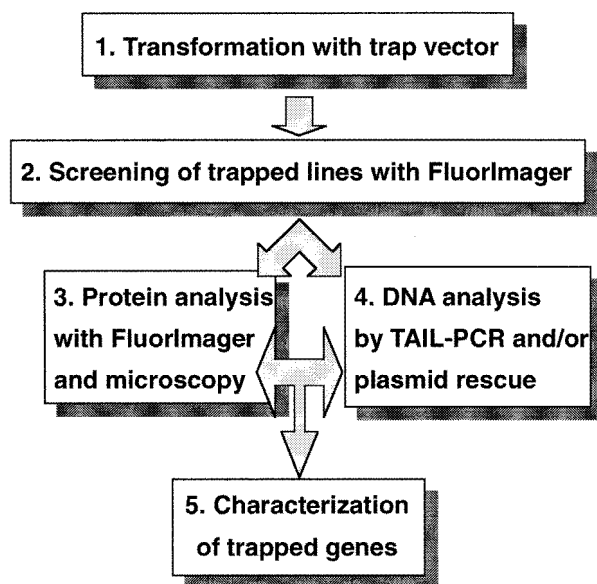


Fig. 1 The trapping method procedure.

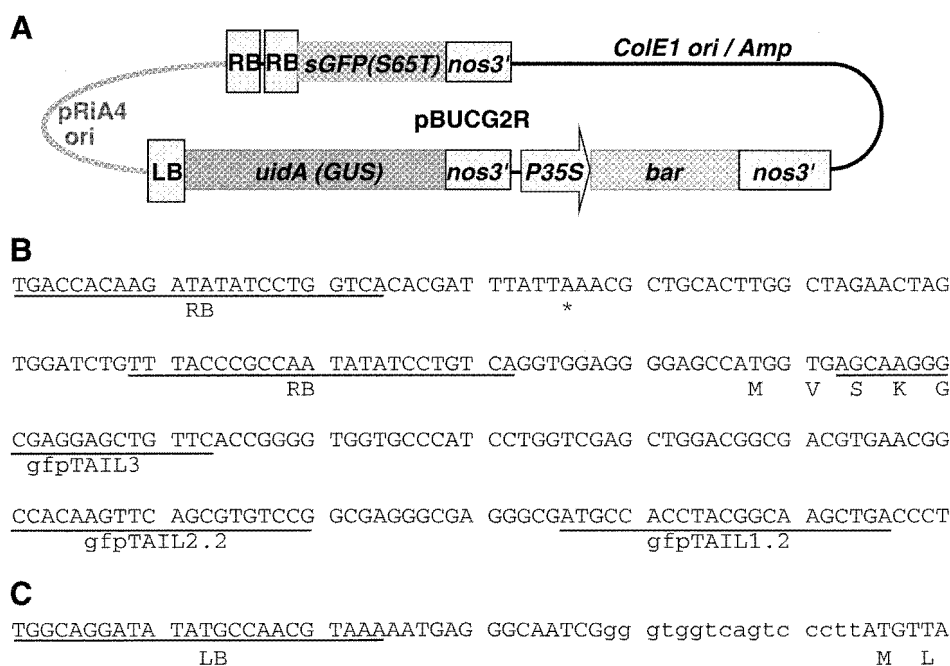


Fig. 2 Structure of test vector pBUCG2R.

The replication origin region of pRiA4, left and first right border (LB, RB), and the glufosinate resistant gene cassette originated from the pDHB321.1 plasmid (kindly provided from Dr. David Bouchez INRA, France). The *uidA* gene (GUS) with a nopaline synthase gene (*nos*) poly (A) signal (*nos3'*) was digested by *EcoRI* treated with T4 DNA polymerase and *SmaI* from pBI221 (Jefferson, 1987) and inserted into the blunted *Clai* sites of the pDHB321.1 plasmid. The replication origin of ColE1,  $\beta$ -lactamase gene (for plasmid rescue) and the sGFP(S65T) with *nos3'* was derived from the CaMV35S-sGFP(S65T) plasmid, the CaMV35S-sGFP(S65T) plasmid is also known as pTH2 (Chiu *et al.*, 1996; Niwa, 2003), by digestion with *BamHI*. The second RB region containing the identical sequence of RB of pTiC58 was constructed using two annealed synthetic oligonucleotides, RBG4in; tcgacagatctgtttaccgccaatatactctgtcaggtggaggtggagc and RBG4out; catggctccacctccacctgacaggatattggcgggtaaacagatctg, treated with *BglII* and *BamHI*. Nucleotide sequences of right (B) and left (C) border regions of pBUCG2R. Two RBs, LB, and the primers used for TAIL-PCR are underlined. N-terminal amino acid sequences of sGFP(S65T) (B) and GUS (C) are shown as a single letter code under the corresponding codon. The in-frame stop codon located in the upstream region of sGFP(S65T) is shown as an asterisk.

of organisms; however, it was initially rarely used in plants because of its toxicity and inadequate splicing (Haseloff *et al.*, 1997). An engineered sGFP(S65T) sequence with codons optimal for high expression of eukaryotic proteins has provided up to 100-fold brighter fluorescent signals (Chiu *et al.*, 1996) than the original jellyfish GFP sequence and was shown to be non-toxic in plants (Niwa *et al.*, 1999). We have already demonstrated non-invasive and quantitative detection of sGFP(S65T)-expressing cells without chemical treatment using a quantitative fluorescent imaging system (Niwa *et al.*, 1999). Here we evaluate whether this system is applicable to selection based only upon the expression level of GFP.

A trapping system with a GFP reporter gene is one of the most suitable ways to evaluate a non-invasive and chemical treatment-free screening

system. Each step of the trapping procedure is shown in **Fig. 1**. Steps No. 2 and 3 are the steps where the quantitative fluorescent imager can be utilized. To construct a test vector, pBUCG2R, the sGFP(S65T) gene was used as a reporter since it is one of the best GFP variants for use in plants (Chiu *et al.*, 1996; Niwa *et al.*, 1999). There are two Right Border (RB) sequences for alternative trapping. If the first RB is used, only the promoter trap lines are selected, because an in-frame stop codon exists in the upstream region of sGFP(S65T) (**Fig. 2B**). The second RB was positioned for gene and promoter trapping. The replication origin from the pRiA4 plasmid was selected because of its high stability in *Agrobacterium*. The *uidA* gene (Jefferson, 1987) is also used as an optional reporter gene at the left border side. Nucleotide sequences of the RB and LB regions of pBUCG2R are shown in **Fig. 2B** and **C**. The test vector pBUCG2R also contains the ColE1 origin and the  $\beta$ -lactamase gene to rescue the adjacent region of T-DNA insertion.

Tobacco BY-2 cells were treated with the *Agrobacterium* harboring the test binary vector and spread on medium supplemented with glufosinate. Glufosinate resistant tobacco calli were analyzed using the GFP imaging system. Three independent lines (S101, M701, and M702) showing a green fluorescent signal were selected (**Fig. 3**) and used for further analysis. To measure the fluorescence intensity of each callus under non-disruptive conditions, quantitative analysis was performed using ImageQuant program. Relative fluorescence intensities of three negative (n1 to n3) and three positive lines are shown in **Fig. 3B**. As can be seen from these results, the expression level of GFP positive lines is clearly higher than that of negative lines. Although these three GFP positive lines did not show GUS expression, we could detect GUS expression in some GFP negative colonies (data not shown). This result indicates that the GUS located at the LB side of pBUCG2R vector is also functional as a trapping reporter.

To characterize the candidate lines at the protein level, SDS-PAGE analysis was performed. **Fig. 4A** shows the direct fluorescence and pre-stained protein marker image of SDS-PAGE gel visualized by the FluorImager. From this analysis, all three lines, including S101, express a monomer-sized GFP. Relative fluorescence intensity of each lane is shown in **Fig. 4B**. There is a good correlation between the quantified value and the amount loaded in the gel. From the sub-cellular localization analysis, the green fluorescence signal was detected in the cytoplasm and the nucleus of S101 cells (data not shown). Since the monomer-sized sGFP(S65T)

with no targeting sequence accumulates diffusely in the cytoplasm and the nucleus, these results indicate that the S101 line is a promoter trapped line.

Further characterization was performed at the DNA level. First, Southern blot analysis was carried out to investigate the number of T-DNA insertions. The result shown in **Fig. 5A** indicates that the S101 line contains a single T-DNA insertion. Then, to analyze the insertion sites of S101 line quickly, TAIL-PCR (Liu *et al.*, 1995) was carried out. The nucleotide sequence of one of the insertion sites from S101 is shown in **Fig. 5B**. The first RB was used in this case and this is to be expected of a promoter trap. Additionally, a TAA stop codon exists in all three frames and a TATA box-like motif was located in the genomic region, therefore this flanking sequence possibly corresponds to the expression of GFP in S101 cells.

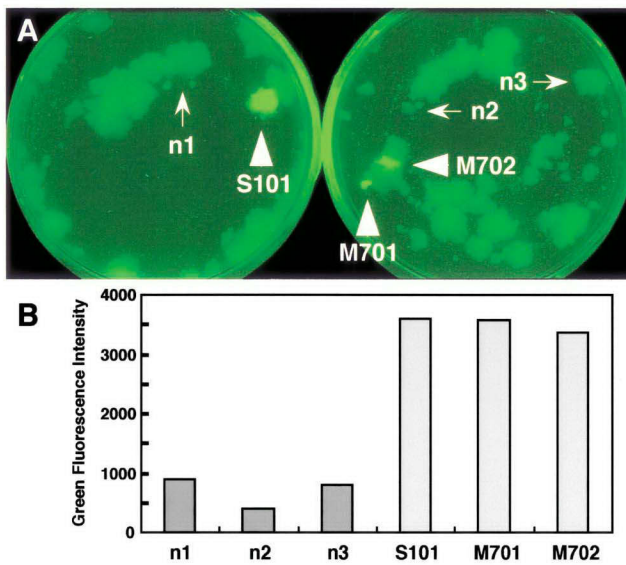
From this experiment, only the monomer-sized GFP expression lines have been successfully selected. This result could represent the possibility that the combined system of the sGFP(S65T) reporter gene and the quantitative fluorescent imaging machine is also useful for drug-free selection of transformed cells. As for the trap vector pBUCG2R, further characterization is necessary to evaluate the potency of the gene trap. The selection system demonstrated here is suited not only to cultured cells but also to individual plants (Niwa *et al.*, 1999) and should be a powerful new tool for plant biotechnology.

## Acknowledgements

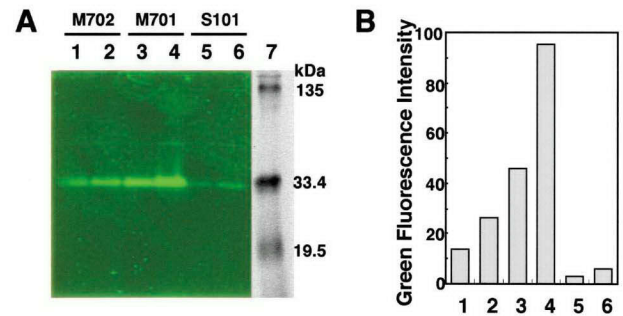
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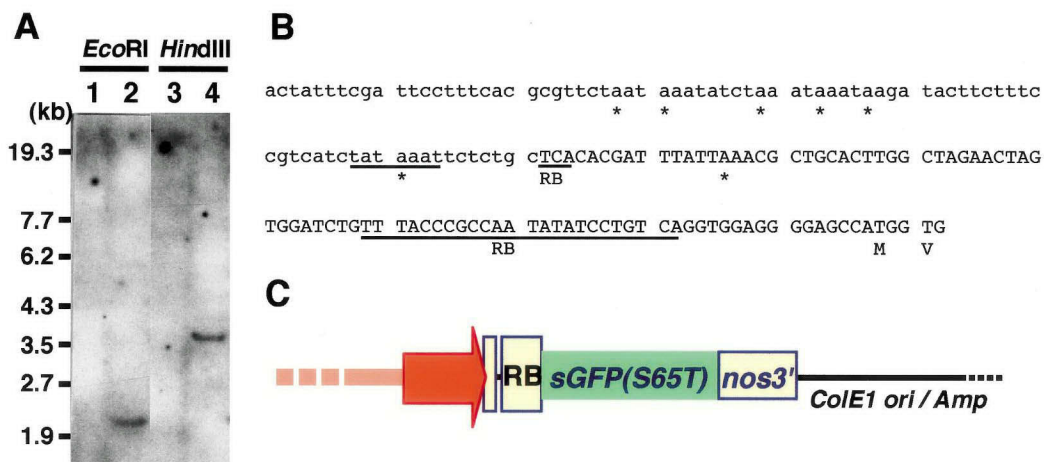
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**Fig. 3** Screening of a trapped line in cultured tobacco cells using a quantitative fluorescent imaging system. (FluorImager SI; Amersham pharmacia biotech)  
 (A) Fluorescent image of cultured tobacco calli transformed with the test vector and visualized with 488 nm excitation. Cultured tobacco cells were co-cultivated with *A. tumefaciens* strain EHA101 (Hood *et al.*, 1986) harboring the test vector pBUCG2R and grown on Linsmaier and Skoog (LS) medium containing 0.4% GELRITE (Merck & Co., Inc.), 0.5 mg ml<sup>-1</sup> carbenicillin-Na, and 4 μg ml<sup>-1</sup> glufosinate-NH<sub>3</sub> (Hoechst). Three GFP-positive (S101, M701, M702) and three GFP-negative colonies (n1 to n3) were selected for quantitative analysis.  
 (B) Relative green fluorescence intensity of GFP-negative and GFP-positive tobacco cells. Each fluorescence intensity was measured with the ImageQuant program.



**Fig. 4** Analysis of GFP-positive lines by SDS-PAGE  
 (A) Visualization of the GFP fluorescence (lanes 1 to 6) and pre-stained protein marker (lane 7). SDS-PAGE analysis was performed according to the method of Laemmli (Laemmli, 1970), using 12.5% running and 3% stacking gel. Proteins were extracted in 100 mM potassium-phosphate buffer (pH7.0) with 1 mM DTT on ice and cell debris was removed by centrifugation at 15000 rpm for 10 min at 4°C. Loading buffer was added to a final concentration of 10 mM Tris-HCl buffer (pH 6.8), 1% SDS, 20% glycerol, 0.05% bromophenol blue (BPB), and 1% 2-mercaptoethanol before loading the gel. Heat treatment was excluded, since GFP fluorescence is sensitive to boiling treatment. Fifteen (lanes 1,3,5) and 30 μg (lanes 2,4,6) of protein extract from each GFP positive line were loaded. After the run, the gel was directly scanned using the FluorImager SI with excitation at 488 nm (Ar laser) and detection with a 515 to 545 nm band-pass filter for GFP and with a 610 nm filter for pre-stained protein markers (Kaleidoscope Pre-stained Standard ; BIO-RAD). Molecular weight of each marker protein is shown on the left side of lane 7.  
 (B) Relative green fluorescence intensity of each lane shown in (A).



**Fig. 5** Analysis of GFP positive lines by Southern blotting and TAIL-PCR.  
 Southern blot analysis of non-transformed (lanes 1 and 3) and S101 line (lanes 2 and 4) hybridized with an sGFP(S65T) gene probe. Ten micrograms of each genomic DNA was digested with *EcoRI* and *HindIII* (A). The insertion regions were amplified by the TAIL-PCR method (Liu *et al.*, 1995) using three specific primers, gfpTAIL1.2 ; tcagctgccgtaggtggcat, gfpTAIL2.2 ; cggacacgctgaactgtgg, gfpTAIL3 ; gaacagctcctgccttctgt, and an arbitrary degenerate W4 primer (Seki *et al.*, 1999). Amplified fragments were used as a template. A sequencing reaction was performed using the BigDye Terminator Cycle Sequencing Ready Kit (PE Applied Biosystems) and electrophoresed on the DNA sequencer ABI PRISM 373 (PE Applied Biosystems). Nucleotide sequence (B) and schematic representation (C) of T-DNA insertion site from S101 line. Tobacco genomic sequence is shown in small letters. The TATA box-like motif and the RB are underlined. The stop codons located upstream region of sGFP(S65T) are shown as asterisks. The putative promoter region is shown as an arrow (C).

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