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# A Synthetic Green Fluorescent Protein Gene for Plant Biotechnology

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## Abstract

Since Green Fluorescent Protein (GFP) from *Aequorea* jellyfish emits green fluorescence without any additional substrates or co-factors, it has emerged as a powerful new reporter for use in a variety of organisms. However, improvements such as the alteration of the excitation spectrum and the elimination of a cryptic intron site were necessary for its efficient use in plants. An engineered synthetic GFP with a S65T mutation (replacement of the serine in position 65 with a threonine) in the chromophore has provided up to 100-fold brighter fluorescent signals than the wild-type jellyfish GFP sequence without any toxic effects. The sGFP(S65T) has been widely used for studies such as localization analysis and promoter assays. This sGFP(S65T) is also an ideal candidate for the development of transformation methods for crops and trees in which it has previously proved difficult to obtain transformants via conventional methods. A non-invasive, quantitative detection technique for this GFP has been achieved in conjunction with a fluorescent imaging system.

**Key words:** green fluorescent protein, localization analysis, plant biotechnology, promoter assay, reporter gene, sGFP(S65T), transformation marker, visible marker.

## Abbreviations

sGFP, synthetic green fluorescent protein; S65T, replacement of the serine in position 65 with a threonine.

## Introduction

One of the important objectives of plant biotechnology is the establishment of novel plants with favourable characteristics. A general procedure for the achievement of this purpose is summarized in **Fig. 1**. Needless to say, proteins play essential roles in cellular activities, the genetic information for which is specific for individual species. The possibility of analysing proteins in detail has led to increased understanding of the processes of life. The 2002 Nobel Prize for chemistry was awarded to three persons, including the Japanese scientist, Koichi Tanaka, for their evolutionary methods for the analysis of proteins. It would be useful if proteins could be visualized *in vivo*. Many marker genes have been developed to visualize protein activity, however, substrates are needed in all cases.

Green Fluorescent Protein (GFP; **Fig. 2**) was first discovered by Shimomura et al from *Aequorea* jellyfish (Shimomura *et al.*, 1962) as a companion protein to aequorin in 1962. After thirty years, the crucial breakthroughs came with the cloning of the gene by Prasher et al (Prasher *et al.*, 1992) and the demonstration that expression of the gene in other organisms such as *C. elegans*, Drosophilae, and *E. coli* creates fluorescence. Therefore, the gene contains all the information necessary for the post-translational formation of the chromophore, and no

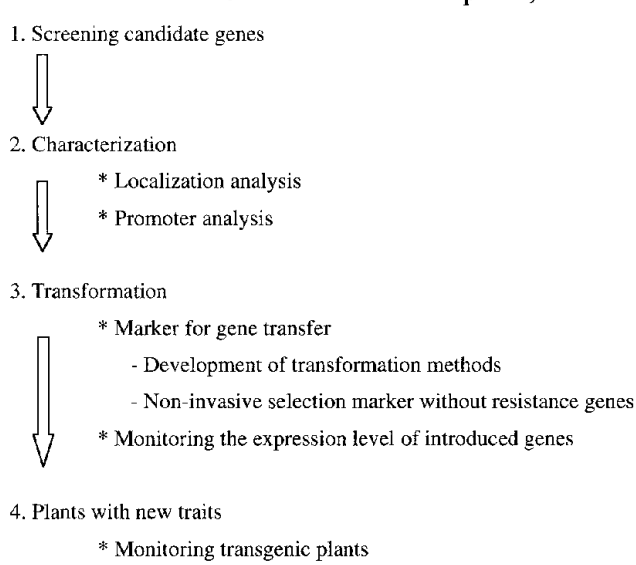



**Fig. 1** Possible contribution of sGFP(S65T) to the establishment of novel plants with favorable traits.

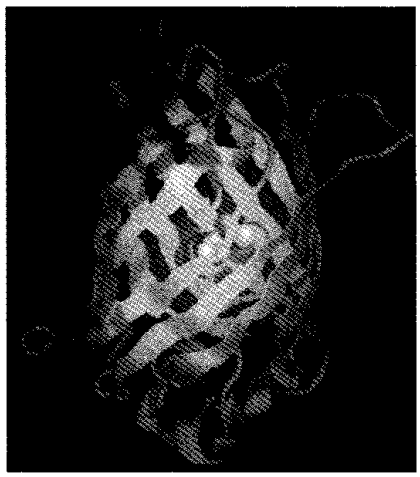


**Fig. 2** Schematic three-dimensional structure of GFP.
The chromophore (space-filling) is located in the center of a barrel-like structure formed by eleven $\beta$-sheets (ribbon).

jellyfish-specific enzymes are needed. The GFP consists of 238 amino acid residues and the chromophore is a *p*-hydroxybenzylideneimidazolinone formed from residues 65-67, which are Ser-Tyr-Gly in the wild-type protein. The wild-type GFP emits green fluorescence when excited with blue or UV light without any additional substrates or co-factors. The broader application of GFP in higher organisms requires higher expression efficiency and fluorescence intensity, especially under blue light, to minimize photobleaching and phototoxicity. Additionally, eliminating an inadequate splice site (**Fig. 3**) is essential to express functional GFP in plants (Haseloff *et al.*, 1997)

*A synthetic GFP with S65T mutation*

I have previously shown that wild-type GFP signals are detectable in a transient expression system in *Arabidopsis* (Sheen *et al.*, 1995). However, it was concluded that an increase in expression efficiency and quantum yield achieved by blue light excitation would make GFP substantially more useful as a vital marker in plants. First, a synthetic GFP gene with preferred codon usage for higher organisms was made as an sGFP (**Fig. 3**). Then a S65T mutation (replacement of the serine in position 65 with a threonine) was introduced by site-directed mutagenesis to create sGFP(S65T) (**Fig. 3**) according to the observation by Heim et al (Heim *et al.*, 1995). An engineered sGFP(S65T) sequence with codons optimal for high expression of eukaryotic proteins has provided up to 100-fold brighter fluorescent signals than the wild-type jellyfish GFP sequence in plants (Chiu *et al.*, 1996). This S65T mutation additionally causes characteristics such as faster chromophore formation, slower photobleaching, and a single excitation peak ideal for fluorescein isothiocyanate (FITC) filter sets. Moreover, sGFP(S65T) has no toxic effects to plants without the use of any targeting signals (Niwa *et al.*, 1999). At least in rice, sGFP(S65T) is brighter than mgfp5, a thermostable folding mutant (Siemering *et al.*, 1996), under blue light excitation (personal communication from Dr. Ugaki at Tokyo University).

There are three types of sGFP(S65T)-containing vectors (**Fig. 4A**). Two of them originally constructed by Dr. Sheen (pblue-sGFP(S65T)-nos3'KS = sKS and pblue-sGFP(S65T)-nos3'SK = sSK) are promoter-less vectors suited for promoter analysis. The nucleotide sequence between the multiple cloning site of the Bluescript vector and the sGFP(S65T) gene are shown in **Fig. 4B** (Niwa unpublished result and personal communication from Dr. Nonomura at National Institute of Genetics). An unexpected *Eco*RI site was found in the downstream region of the nopaline synthase gene

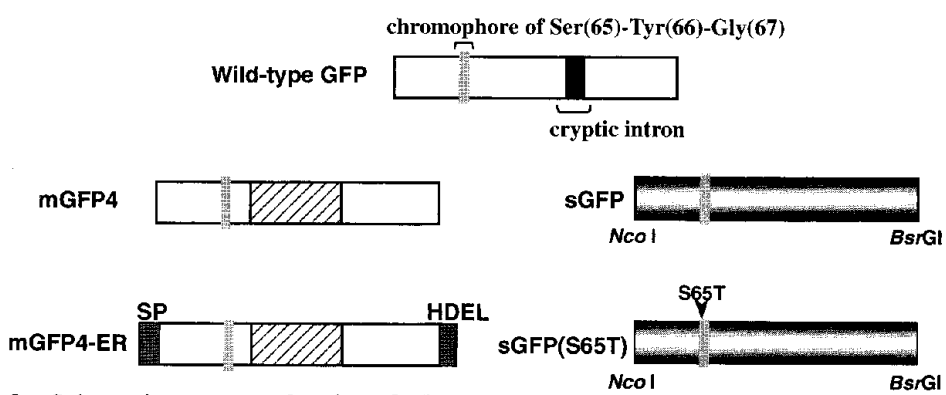
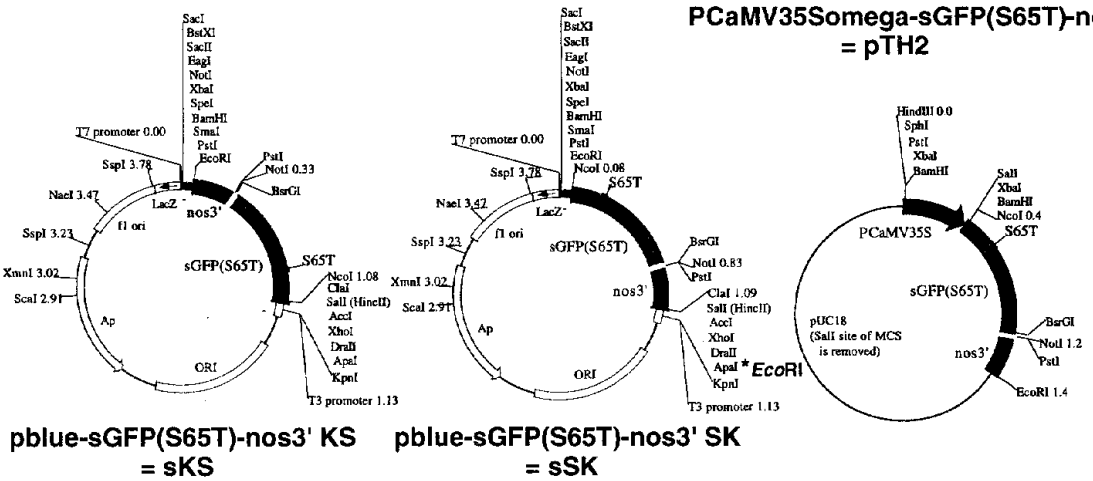



**Fig. 3** Schematic structure of various GFP genes.
Chromophore of Ser-Tyr-Gly and cryptic intron are represented as boxes shown in the figure. *Nco*I and *Bsr*GI restriction sites and the S65T mutation site are indicated at their respective positions. Signal peptide and ER retention sequences are shown as SP and HDEL, respectively.

**A**




**B**

```
                                                        Met Val
TCTAGA ACTAGT GGATCC CCCGGG CTGCAG GAATTC GAT C CC ATG GTG
 XbaI   SpeI   BamHI  SmaI   PstI   EcoRI          NcoI
```

**C**

```
                          1   2   3   4   5   6   7   8   9
                        Met Val Ser Lys Gly Glu Glu Leu Phe
GTCGAC TCTAGA GGATCC ATG GTG AGC AAG GGC GAG GAG CTG TTC
 SalI   XbaI   BamHI NcoI
```

**D**

```
231 232 233 234 235 236 237 238
His Gly Met Asp Glu Leu Tyr Lys  *
CAC GGC ATG GAC GAG CTG TAC AAG TAA AGCGGCCGCCCGGGCTGCAG
                    BsrGI        NotI        PstI
```

**Fig. 4** Physical maps of sGFP(S65T)-containing plasmids (A) and of its sequences (B-D). The synthetic GFP(S65T) gene and polyadenylation signal sequence from the nopaline synthase gene (nos3') were inserted into a modified Bluescript vector in opposite orientations to each other (pblue-sGFP(S65T)-nos3'KS = sKS and pblue-sGFP(S65T)-nos3'SK = sSK). An unexpected *Eco*RI site found in the downstream region of *nos*3' of the sSK vector is represented as **Eco*RI. The sGFP(S65T) gene was driven by the cauliflower mosaic virus 35S promoter (PCaMV35S) with the omega sequence from TMV (PCaMV35Somega-sGFP(S65T)-nos3' = pTH2). The pTH2 is a pUC-derived vector and the original *Sal*I site at the multiple cloning site was removed. Nucleotide sequence and corresponding amino acids and restriction sites of the upstream region of the sGFP(S65T) gene in the sSK vector (B). Nucleotide sequences and corresponding amino acid residues are numbered and restriction sites of N-terminal (C) and C-terminal (D) regions of sGFP(S65T) in pTH2 vector are shown.

(*nos*) terminator of the sSK vector (personal communication from Dr. Kitamoto at Tokyo University). The CaMV35Somega-sGFP(S65T)-nos3' plasmid named pTH2 is a pUC-based vector containing the CaMV35S promoter, the omega sequence from tobacco mosaic virus (TMV), sGFP(S65T), and the polyadenylation signal sequence from *nos*. The pTH2 vector is widely used for localization analysis (**Table 1**) and as a marker for gene transfer (**Table 2**).

*Localization analysis*

Since the sGFP(S65T) gene product shows no-toxic side effects without any targeting signals, it is ideal for cellular and sub-cellular localization analysis. Examples of the use of sGFP(S65T) in this

**Table 1** Intracellular compartments targeted and visualized by sGFP(S65T)

| Subcellular compartment | References for examples |
|---|---|
| Cell-plate | Nishihama *et al.*, 2002; Yokoyama and Nishitani, 2001 |
| Endoplasmic reticulum | Hayashi *et al.*, 2001; Hong *et al.*, 1999; Koizumi *et al.*, 2001; Okushima *et al.*, 2002; Saito *et al.*, 1999; Tamura *et al.*, 2001; Takeuchi *et al.*, 2000 |
| Golgi apparatus | Nebenfuhr *et al.*, 1999; Takeuchi *et al.*, 2002 |
| Microfilament | Chen *et al.*, 2002 |
| Microtubule | Kumagai *et al.*, 2001; Marc *et al.*, 1998 |
| Mitochondrion | Beardslee *et al.*, 2002; Chang *et al.*, 1999; Christensen *et al.*, 2002; Nakazono *et al.*, 2000; Niwa *et al.*, 1999; Okada *et al.*, 2000; Versaw and Harrison, 2002 |
| Nucleus | Chui *et al.*, 1996; Fukaki *et al.*, 2002; Igarashi *et al.*, 2001; Ito *et al.*, 2002; Iwakawa *et al.*, 2002; Kitakura *et al.*, 2002; Kosugi and Ohashi, 2002; Ohta *et al.*, 2000; Okada and Toriyama, 2001; Tamura *et al.*, 2002; Yanagisawa, 2001 |
| Plasma membrane | Chaumont *et al.*, 2000; Ivanchenko *et al.*, 2000 |
| Plastid | Araki *et al.*, 2000; Chang *et al.*, 1999; Chui *et al.*, 1996; Ho *et al.*, 1998; Ishiguro *et al.*, 2001; Isono *et al.*, 1997; Iuchi *et al.*, 2000; Kohchi *et al.*, 2001; Muramoto *et al.*, 1999; Niwa *et al.*, 1999; Obara *et al.*, 2002; Okada *et al.*, 2000; Sitthithaworn *et al.*, 2001; Takechi *et al.*, 2000 |
| Peroxisome | Mano *et al.*, 1999; Mano *et al.*, 2002 |
| Protein body | Personal communication from Dr. Tanaka at Kyoto Prefectural Univ. |
| Vacuole | Mitsuhashi *et al.*, 2000; Morita *et al.*, 2002; Saito *et al.*, 2002; Ueoka-Nakanishi *et al.*, 2000 |

manner include nuclear, plastidic (Chui *et al.*, 1996) and mitochondrial (Niwa *et al.*, 1999) localization studies. **Table 1** summarizes the intracellular localization analysis experiments with sGFP(S65T).

For the purpose of nuclear localization analysis, it should be noted that the monomer size of GFP is small enough to diffuse into the nucleus through nuclear pores. Therefore, the GFP itself localizes not only in the cytoplasm, but also in the nucleus. Of course, if a nuclear localizing signal is fused to sGFP(S65T), most of the green fluorescent signals are detected in nucleus (Chui *et al.*, 1996), however, it is important to eliminate its passive penetration into nucleus. One of the solutions to this problem was achieved by the creation of a GFP-GUS fusion construct to increase the molecular weight of the GFP (Grebenok *et al.*, 1997; Kosugi and Ohashi, 2002). A similar chimeric gene has also been made by Dr. Ugaki at Tokyo University (personal communication).

Macromolecular trafficking within the sieve element-companion cell complex, phloem unloading, and post-phloem transport were studied using the sGFP(S65T) under the control of the *AtSUC2* promoter (Imlau *et al.*, 1999). Plasmodesmata of the sieve element-companion cell complex, as well as plasmodesmata into and within sinks, allow trafficking of the 27-kD non-phloem GFP protein.

*Transformation marker*

Transformation has been performed with selection markers that can distinguish transformed from non-transformed cells via the observation of different growth rates on selection medium (**Fig. 5**). Although this method is powerful and efficient in certain well technically-developed model plants, it is sometimes very difficult to apply to various plants such as crops. The main reason for this is the decrease in regeneration efficiency due to the effects of chemicals used for selection. Analysis of the expression of the marker genes within the candidate cells and plants is also necessary. Since I

**Table 2** Plant and other species successfully used

| Species | References for examples |
|---|---|
| *Arabidopsis thaliana* | Chui *et al.*, 1996; Kinoshita *et al.*, 2001; Niwa *et al.*, 1999 |
| Barley (*Hordeum vulgare*) | Cho *et al.*, 2002; Gubler *et al.*, 2002 |
| Bean (*Vicia faba*) | Marc *et al.*, 1998 |
| (*Glycine max*) | Personal communication from Dr. Furutani at Kyoto Prefectural Institute of Agricultural Biotechnology |
| Fern (*Adiantum capillus*)[1] | Personal communication from Dr. Kanegae at Tokyo Metropolitan Univ. |
| Lily (*Lilium longiflorum*) | Personal communication from Dr. Tanaka at Yokohama City Univ. |
| Maize (*Zea mays*) | Chang *et al.*, 1999; Chui *et al.*, 1996; Yanagisawa, 2001 |
| Moss (*Physcomitrella patens*) | Hara *et al.*, 2001; Kabeya *et al.*, 2002 |
| Onion | Chui *et al.*, 1996 |
| Petunia (*Petunia hybrida*) | Personal communication from Dr. Tanaka at Institute for Advanced Technology, Suntory Ltd. |
| Poplar | Personal communication from Dr. Sakai at Kyoto Univ. |
| Rice (*Oryza sativa*) | Hagenbeek *et al.*, 2000; Hasizume *et al.*,1999; Jang *et al.*, 2002; Okushima *et al.*, 2002; Personal communication from Dr. Morita at Kyoto Prefectural Univ. |
| Spiderwort (*Tradescantia reflexa*) | Personal communication from Dr. Takumi at Kobe Univ. |
| Sweet potato (*Ipomoea batatas*) | Personal communication from Dr. Mistukawa at Toyota Central R&D Labs., Inc. |
| Tamarix (*Tamaricaceae Tamarix*) | Personal communication from Dr. Yoshida at Taisei Co. |
| Tea (*Camellia sinensis*) | Niwa unpublished results |
| Tobacco (*Nicotiana tobacum*) | Chen *et al.*, 2002; Chui *et al.*, 1996; Kohchi *et al.*, 2001; Okada and Toriyama, 2001; Yang *et al.*, 2001 |
| (*Nicotiana sylvestris*) | Kobayashi *et al.*, 2001 |
| Torenia (*Torenia fournieri*) | Personal communication from Dr. Kobayashi at Osaka Univ.; Personal communication from Dr. Tanaka at Suntory Ltd. |
| Verbena (*Verbena hybrida*) | Tamura *et al.*, 2002 |
| Wheat (*Triticum aestivum*) | Madin *et al.*, 2000, Personal communication from Dr. Yoshida at Hokkaido National Agricultural Experiment Station |
| Yeast (*Saccharomyces cerevisiae*) | Nakamura *et al.*, 1997; Versaw and Harrison, 2002 |
| Fungus (*Aspergillus nidulans*), | Valdez-Taubas *et al.*, 2000 |
| (*Aspergillus niger*) | Gordon *et al.*, 2000 |
| (*Aspergillus oryzae*) | Personal communication from Dr. Gomi at Tohoku Univ. |
| (*Magnaporthe grisea*) | Balhadere *et al.*, 2001; DeZwaan *et al.*, 1999 |
| Human | Kuruto-Niwa *et al.*, 1998 |

[1] Growth was severely affected in stable transformants

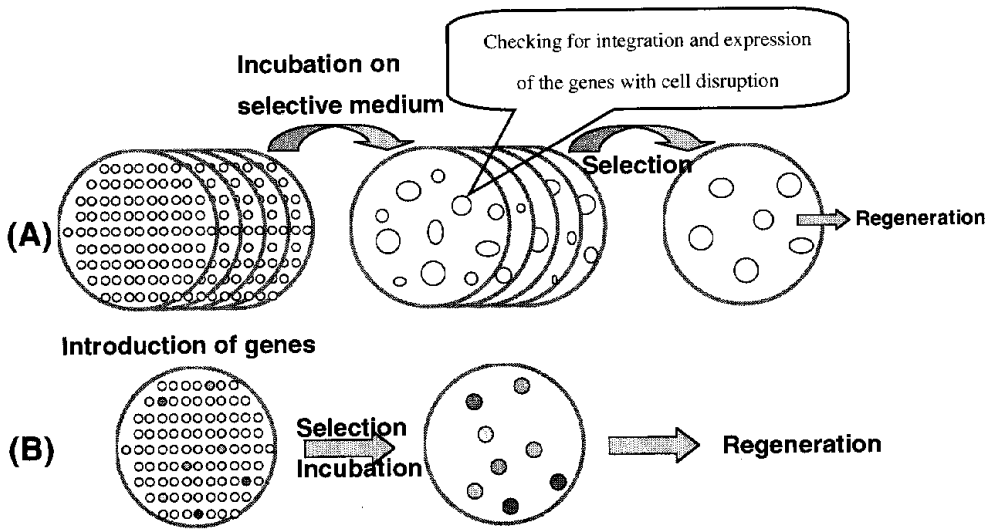



**Fig. 5** Comparison of conventional transformation procedures using a selectable marker (A) and the novel method using the directly visible sGFP(S65T) (B).

already mentioned that the sGFP(S65T) produces a very bright signal without any toxicity, it therefore has the potential to resolve these difficulties. In the case of cultured tobacco cells, GFP-positive cells could be distinguished from non-transformed cells under non-selective conditions (Niwa *et al.*, 1999). The sGFP(S65T) gene is ideal as a non-invasive visualization marker for both transient systems and stable integration into chromosomes. It has also been used to develop novel gene delivery methods. The sGFP(S65T) gene is also used in a MAT vector system for generating marker-free transgenic plants (Sugita *et al.*, 2000; Endo *et al.*, 2002).

As the 35S promoter in the pBI vector (Jefferson, 1987; Jefferson *et al.*, 1987) directs expression of the GUS gene in both *Agrobacterium* and plants, it is therefore impossible to distinguish the GUS signals originating from the transformed plants from those originating from *Agrobacterium*. Therefore it is important to introduce an intron into the GUS gene in this vector (Ohta *et al.*, 1990). GFP will localize to both the nucleus and the cytoplasm regardless of the promoter used. In the case of sGFP(S65T) driven by the 35S promoter of pTH2 however, there seems to be no need to introduce an intron as the gene does not seem to be functional in *Agrobacterium*, or at least only weakly functional (Niwa unpublished result; personal communication from Dr. Sugaya at Saitama University). The solely nuclear localization of this GFP additionally provides evidence that the GFP expression can be attributed to the transformed plants as opposed to that of *Agrobacterium*. In the case of sGFP(S65T) driven by the 35S promoter of pBI, GFP expression does occur in *Agrobacterium*, however again it is possible to distinguish GFP expression attributable to the transformed plant cells from that attributable to the *Agrobacterium* from the nuclear localization of the GFP. However, the introduction of an intron into the GFP gene would eliminate the possibility of expression of GFP in *Agrobacterium*, and thus ensure that all GFP expression could be attributed to the transformed plant cells. The sGFP(S65T) gene containing such an intron has been constructed by Dr. Mitsukawa at Toyota Central R&D Labs., Inc. (personal communication). Reasons as to why the 35S promoter of pTH2 is dysfunctional in Agrobacterium is not known, although there are two differences between the 35S promoter of pTH2 and that of pBI vectors. The 35S promoter of pTH2 is shorter (**Fig. 4**) than that of pBI vectors and contains the omega sequence from TMV.

*Detections of sGFP(S65T)*

Because the spectrum of sGFP(S65T) is almost identical that of FITC, fluorescence microscopy and confocal laser scanning microscopy is adequate for the detection of GFP fluorescence for analysis at the cellular level. When the sGFP(S65T) gene is used for plant biotechnology such as transformation marker and promoter analysis, the non-invasive detection system at the tissue and whole plant level is essential. The FluorImager (**Fig. 6**), a fluorescent

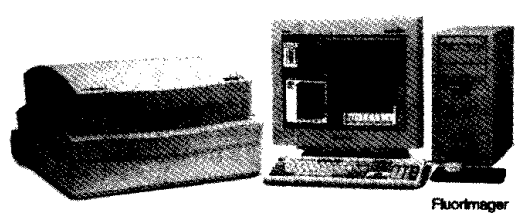



**Fig. 6** The FluorImager, used for non-invasive quantitative detection of sGFP(S65T).

imaging system, is suited to this purpose. The measurement of organ-specific expression of sGFP(S65T) has been demonstrated (Niwa *et al.*, 1999). Homozygous plants could be distinguished from heterozygous plants and fully fertile progenies could be obtained from the analyzed plants. In the case of *Arabidopsis*, about 3000 seedlings can be quantitatively detected at any one time without any lesions.

Once the post-translational maturation of chromophore takes place, sGFP(S65T) can be directly detected after SDS-PAGE using systems such as the FluorImager (Niwa unpublished results) and UV trans-illuminator. Immunological detection of sGFP(S65T) has been achieved by both polyclonal and monoclonal antibodies from MBL (Medical and Biological Laboratories Co., Ltd. Nagoya, Japan; unpublished results).

*Performance of sGFP(S65T)*

The use of sGFP(S65T) has been attempted in a wide variety of plants. Plant species in which its use has been successfully applied are summarized in **Table 2.** They include model plants, crops, and trees. Moreover, the sGFP(S65T) is also used in fungi, yeast, and human cultured cells. In the case of *Adiantum capillus*, a transient expression system has proved successful, however, the growth of the ferns was severely affected for stable transformation (personal communication from Dr. Kanegae at Tokyo Metropolitan University). It is not known whether this observation is specific to sGFP(S65T) or general phenomenon of GFP. The nature of GFP, which absorbs blue light as an emission light may influence the growth of ferns.

The sGFP(S65T) has been used to develop a highly efficient cell-free and robust protein synthesis system using wheat embryos (Madin *et al.*, 2000). Heat-shock response was quantitatively detected under the control of HSP18.2 promoter (Matsuhara *et al.*, 2000). Moreover, activities of promoters such as sulphate transporters from *Arabidopsis* (Takahashi *et al.*, 2000; Yoshimoto *et al.*, 2002), *FIE* (Kinoshita *et al.*, 2001) and *MEA* (Yadegari *et al.*, 2000), rice cytochrome c (Jang *et al.*, 2002), rice actin and barley endosperm-specific hordein (Cho *et al.*, 2002), *Bra r 1* from *Brassica* (Okada and Toriyama, 2001) and NCR derived from soybean chlorotic mottle virus (Fukuoka *et al.*, 2000) have been visualized using sGFP(S65T).

*Future aspects*

To date, when plants contain metabolites for auto-fluorescence, whose spectrum is close to or overlapping with GFP fluorescence, for example the roots of tobacco, mature pollens, and stigma of pistil, it is impossible to distinguish the GFP signal from the background fluorescence. A Multifluorescence-Imaging system with a META detector and emission fingerprinting from Zeiss will hopefully resolve this difficulty.

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