

[*Plant BioTechniques Series* (5)]**Use of Green Fluorescent Protein as a Molecular Tag of Protein Movement *In vivo***

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There are a number of species of proteins in plant cells. In most cases researchers are focusing their interest on one or several proteins and observing what it is or what it is doing for the organism to live. In most cases, researchers raised antisera against the protein of interest. After raising the antisera, they would try to detect the protein(s) using the antisera by immunostaining after fixing the cells.

Recently the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has drawn much attention in biology [1]. It is now widely recognized that GFP is very feasible for use by researchers who are investigating the functions of proteins or looking at the traffic of proteins *in vivo*. No extra special substrate/chemical substances to detect the signal are needed. The protein will not diffuse or penetrate into other places. Direct visualization of gene expression or localization of proteins by fusing with GFP is therefore possible without cell lysis or fixation. One thing we have to do is to fuse the target protein with the GFP protein by molecular recombination technique at the cDNA level. Here we will describe how we manipulated GFP genes to look at the intercellular and intracellular movement of a protein of tomato

mosaic virus (ToMV). The protein is called a movement protein (MP), which functions to move ToMV from one initially-infected cell to surrounding non-infected cells. By using GFP we could observe dynamic shift of localization of the protein [2, 3].

1. Choice of the version of GFP

From the literature one can see that there are a growing number of "plant optimised" GFP at present. Sometimes the selection of a GFP from many variations is important. The optimization is based on different aspects. Please refer to **Table 1**. Amino acid substitutions are shown by one-letter symbols. Some of them are the optimization of codon adapted for plant (plantinized) not for jelly fish so as to be expressed efficiently in plant systems. It is claimed that aberrant RNA processing may interfere with GFP expression studies in plants. The modification of a cryptic *Arabidopsis thaliana* intron sequence gave a so-called Haseloff type GFP.

Mutations that shift light emission and improve strength of fluorescence (*e. g.* S65T; wild type serine at amino acid 65 was substituted by threonine) are now widely introduced [4].

Table 1.

Summary of GFP variants which are modified for use in plant research.

variation name	amino acid substitution	nucleic acid sequence	excitation wavelength (nm)		emission wavelength (nm)	references
			UV	blue		
wild type	—	wt	395	475	508	Gene 111 , 229-233(1992)
S65T	S65T	wt	—	490	510	Nature 373 , 663-664(1995)
mGFP 4	—	Haseloff-type	395	475	508	Trends Genet. 11 , 328 - 329(1995)
G3	S65A/Y145F	Haseloff-type	—	undetermined	undetermined	Ogawa & Umesono, unpublished
cycle 3	F99S/M153T/V163A	random-PCRed	385	488	510	Nature/Biotech. 14 , 315 - 318(1996)
sGFP(S65T)	S65T	plantinized	—	490	510	Cur. Biol. 6 , 325-330(1996)
smRS-GFP	F99S/M153T/V163A/S65T	Haseloff-type	—	488	undetermined	Weeds World 3 , 43-48(1996)
Y66H	Y66H	wt	382	—	447	PNAS. 91 , 12501-12504(1994)
BFP	Y66H/Y145F	wt	380	—	445	Cur Biol. 6 , 178-182(1996)

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Cycle 3 mutant emits somewhat stronger fluorescence than the wt GFP when excited in the UV (395 nm) partly because this GFP has less tendency to form non-fluorescing aggregates [5,6]. However, it is less intense than *e. g.* S65T, when excited in the blue light at 488 nm. If you want to excite with a hand-held UV light, cycle 3 mutant would be best for the purpose. If you plan to do fluorescence microscopy work with FITC-adapted filters or FACS, smRS-GFP [6] or G3-GFP (**Table 1**) would be the best choice. Spectral characteristics of both variants match common FITC filter sets.

It has been documented that S65T has an extinction coefficient of 39,200 M⁻¹ cm⁻¹ at 490 nm allowing that measurement of extinction coefficients are very subject to conditions. Niswender *et al.* reported that for quantitative imaging, 1 μM of cytoplasmic wtGFP is required [7]. The detection limits should be much lower for the bright chromophore variants of GFP with S 65 T mutation.

2. Construction of virus mutants containing of movement protein-GFP fusion proteins

Four primers were prepared to construct ToMV MP : GFP fusions (**Fig. 1**). Primer pglyGFP includes a *SacI* site (lowercase; the site is not found in the ToMV full-length cDNA clone), sequences directing the synthesis of polyglycine (underlined) and followed by N-terminal sequences from GFP; AGTCAAagact-cATTCTGGGTGGTGGTGGTTATGAGTAAAG-GAGAAGAACT. Primer G2+3 includes a *BstEII* site (lowercase; unique site in the ToMV full-length

cDNA clone) and antisense GFP C-terminal sequences; AAGggttaccTTATTTGTATAGTTCATCCATGCCATG. High-fidelity PCR [8] to amplify G3 GFP sequences were performed with the two above primers and G3 GFP plasmid DNA as a template. The products were restricted with *SacI* and *BstEII* overnight.

Primer L392EcoRV has a sequence of TACGGAT-CATTGACATATgatatcGGA, which is colinear with ToMV genomic sequences [9] and covers an *EcoRV* site (lowercase) located at n.t. 392. Primer L2-*SacI* has the sequence TACATgagctcATCCGCGACCGACGTCTCG, which has an antisense sequence of ToMV and is designed to introduce a *SacI* site (lowercase) just after amino acid 260 th of the MP and to fuse MP and GFP in the same reading frame. A fragment encompassing MP and upstream genomic sequence was made by high-fidelity PCR [8] using two primers, L392EcoRV and L2-*SacI*, and wild type ToMV cDNA (pTLW 3)[8] as a template. The PCR products were restricted with *SacI* and *AccIII* (n. t. 3759; another unique site in the ToMV full-length cDNA clone).

The *AccIII* (n. t. 3759)-*BstEII* (n. t. 5799) fragment in pTLW3 was replaced with *AccIII/SacI* restricted PCR fragments containing the MP gene and a *SacI/BstEII* restricted GFP fragment. The resultant virus LQwt : Gfus virus would produce a MP truncated at the C-terminus by 4 amino acids and fused with G3 GFP, but lacked an intact CP (**Fig. 1**).

3. Observation of GFP localization

(1) Observation of whole plants

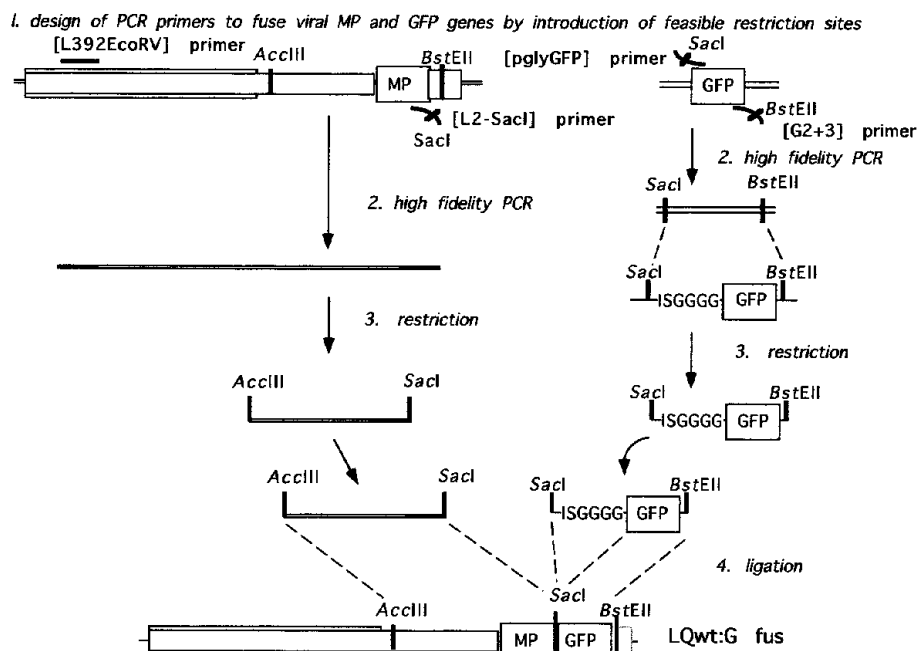


Fig. 1 Scheme of construction of virus expressing MP : GFP fusion proteins.

Erratum

S. Nakamura, T. Iwai, R. Honkura, M. Ugaki, M. Ohshima and Y. Ohashi, **Four Chitinase cDNAs from *Chenopodium amaranticolor***, *Plant Biotechnology*, **14**, 85-86 (1997).

The sentence, "A polyclonal antibody against CAM 18 was used as the.", in legend of Fig. 2 in page 86 should become "A polyclonal antibody against CAM18 was used as the primary antibody."