

# Chapter 19

## Use of GFP to Investigate Expression of Plant-Derived Vaccines

Hong-Ye Li and Mee-Len Chye

### Summary

Plants are low-cost bioreactors for the production of various biopharmaceuticals including oral vaccines. Plant-derived oral vaccines are potentially useful in combating viral infections involving mucosal immunity. Transgenic plants have been generated to successfully produce mucosal vaccines against cholera, hepatitis B, foot-and-mouth disease, and Norwalk virus. As a first step toward the generation of oral vaccines against the severe acute respiratory syndrome coronavirus (SARS-CoV), we have expressed a recombinant S1 protein of the SARS-CoV in transformed tobacco. Since plant transformation and regeneration of stable transformants require considerable time, we initially used a green fluorescent protein (GFP) to tag the antigen in transient expression. GFP was fused to the carboxy-terminus of S1 for expression of S1-GFP to show expression of recombinant S1 by agroinfiltration of tobacco leaves. The GFP tag enables a relatively quick confirmation of antigen expression in plant cells by fluorescent microscopy. Such analysis using GFP that precedes stable plant transformation will enable the rapid screening of multiple constructs to attain optimal recombinant protein expression. Furthermore, this approach determines the subcellular localization of the recombinant protein in plant cells, providing information on optimal subcellular targeting for production in plant bioreactors.

**Keywords:** Autofluorescent protein tag, Plant bioreactors, Plant transformation, Recombinant subunit vaccines, Severe acute respiratory syndrome coronavirus, Subcellular localization.

---

### 1. Introduction

Transformed plants can be easily converted to become effective bioreactors for the production of desired recombinant biopharmaceuticals. To this end, the foreign DNA encoding the relevant recombinant protein can be expressed from the nuclear genome or the chloroplast genome of the genetically engineered plant.

Transgenic plants have already been generated by nuclear transformation to express mucosal vaccines against cholera, Norwalk virus, hepatitis B, foot-and-mouth disease, and severe acute respiratory syndrome, SARS (1, 2). These viral antigens generated in transgenic plants are effective in inducing mucosal and serum immune responses in animals.

In the case of SARS, nuclear-transformed tobacco and tomato expressing the SARS-CoV viral antigens induced immunogenic responses in mice; SARS-CoV-specific IgA increased in mice following oral ingestion of S1-expressing tomato fruits (2). Such edible vaccines deliverable as fruits (e.g., tomato), leaves (e.g., lettuce), tubers (e.g., potato), seeds (e.g., rice or corn), stems, or roots, would obliterate purification procedures required for microbial-derived recombinant vaccines. Further added advantages include the easy storage, transport, and administration by direct ingestion of these oral vaccines. Represented in the form of plant organs (fruits, leaves, tubers, seeds, stems, or roots), these vaccines can be easily grown and distributed, omitting the need for refrigeration in delivery or the requirement for injection by trained health personnel, thereby reducing the costs of immunization programs in developing countries.

Green fluorescent protein (GFP) has been routinely used in laboratories worldwide for tagging proteins in both plant and animal cells. It has been proven easy and convenient to use in transient expression and stable transformation of plant cells. Further, it is pertinent to note that in its application for tagging of viral antigens in plants, GFP can be processed by the ubiquitin-proteasome pathway (3) since ubiquitinated antigens are a prerequisite to processing into antigenic peptides for presentation to the major histocompatibility complex (4).

We describe here several techniques that utilize *Agrobacterium tumefaciens* for the introduction of foreign DNA into plant cells. In nature, *Agrobacterium tumefaciens* is a Gram-negative soil bacterium that transfers and incorporates its transfer-DNA (T-DNA) into the plant nuclear genome. This results in an expression of genes on the T-DNA that will cause crown gall in the infected plant (5). It has been demonstrated in *Agrobacterium*-mediated transformation that following co-cultivation of tobacco BY-2 cells with an *Agrobacterium* harboring a GFP fusion construct, the rate of fusion protein synthesis markedly increased between 24 and 36 h, after which rapid decrease occurred (6). Agroinfiltration is a more recent technique that can be applied to investigate transient expression in plant cells by which an *Agrobacterium* liquid culture is infiltrated into intact plant leaves (7). The feasibility of rapid analyses of foreign protein expression in transiently expressed leaves following agroinfiltration presents a major advantage in its use. The transiently expressed foreign protein is probably derived from transiently synthesized T-DNA

containing the construct that had been mobilized from *Agrobacterium* cells to the tobacco nuclei but has yet to be integrated into the tobacco nuclear genome (8).

Such analysis using *Agrobacterium*-mediated transient expression with GFP-tagged constructs will enable the rapid screening of multiple constructs so that the construct that gives optimal recombinant protein expression can be quickly identified. Upon the identification of such a high-expressing construct, it can then be selected for use in stable plant transformation to generate transgenic plants, a more time-consuming process. This approach by using GFP as an autofluorescent tag can further determine the subcellular localization of recombinant-tagged protein expressed in plant cells, thus providing information for optimal subcellular targeting of such recombinant proteins produced in plant bioreactors.

---

## 2. Materials

### **2.1. Constructs for the Expression of the SARS-Cov S1-GFP Fusion**

1. Plasmid pCRII-S1, a pCRII (Invitrogen) derivative containing the *SARS-CoV S1* cDNA (encoding amino acids 1–658 of the S protein) amplified by the Polymerase Chain Reaction (gift of Dr. L.L.M. Poon and Prof. J.S.M. Peiris, Department of Microbiology, University of Hong Kong).
2. QuikChange Multi Site-directed Mutagenesis Kit with *Pfu-Turbo* DNA polymerase (Stratagene) to obtain an “optimized codon-usage” S1 for cloning into plant transformation vectors.
3. Polymerase chain reaction (PCR) for mutagenesis (50  $\mu$ L): 5  $\mu$ L of 10 $\times$  reaction buffer, 5–50 ng of double-stranded (ds) DNA template, 125 ng of oligonucleotide primer #1 for introduction of nucleotide change, 125 ng of oligonucleotide primer #2 for introduction of nucleotide change, 1  $\mu$ L of dNTP mix (10 mM each), double-distilled water (ddH<sub>2</sub>O) to a final volume of 50  $\mu$ L, Then add 1  $\mu$ L of *Pfu-Turbo* DNA polymerase (2.5 U/ $\mu$ L).
4. *DpnI* and buffers supplied with enzyme.
5. Supercompetent *Escherichia coli* DH5 $\alpha$  cells.
6. Agarose gel electrophoresis reagents and apparatus.
7. GFP gene from plasmid pGFP2 (gift of Prof. Nam-Hai Chua, Rockefeller University, New York).
8. Plant nuclear transformation (binary) vector pGD (9) (gift of Dr. M.M. Goodin, University of California, Berkeley) derived from a commonly used binary vector pCAMBIA-1301.

## **2.2. Agroinfiltration of Tobacco for Expression of the S1:GFP Antigen**

1. Tobacco (*Nicotiana tabacum*) potted plants grown in soil.
2. *Agrobacterium tumefaciens* strain LBA4404 (10).
3. *Agrobacterium* culture medium: yeast extract peptone (YEP) medium (pH 7.0) containing 10 g/L yeast extract, 10 g/L Bacto-peptone, and 5 g/L NaCl supplemented with 10 mM MgSO<sub>4</sub>, 100 µg/mL of streptomycin and, in the case of a derivative harboring pGD, plus 50 µg/mL of kanamycin.
4. Infiltration medium: 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) pH 5.5 plus 10 mM MgSO<sub>4</sub> supplemented with 100 µM acetosyringone (AS, Aldrich).

## **2.3. Analyses of Transiently Expressed Proteins**

### *2.3.1. Laser-Scanning Confocal Microscopy*

1. Fluorescence microscopy or confocal laser-scanning microscope equipped with helium/neon lasers.
2. Image processing software LSM 510 (Zeiss) and Photoshop 6.0 (Adobe).

### *2.3.2. Protein Extraction from Plant Tissues*

1. Liquid nitrogen.
2. Mortar and pestle.
3. Protein homogenizing buffer: 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and freshly add 28 mM β-mercaptoethanol and 2 mM phenylmethanesulfonylfluoride (PMSF, Sigma).

### *2.3.3. Western Blot Analysis*

1. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).
2. Separation buffer (3×): 1.125 M Tris-HCl, 0.3% SDS, pH 8.8.
3. Stacking buffer (5×): 0.625 M Tris-HCl, 0.5% SDS, pH 6.8.
4. Stacking gel (5 mL) 5%: acryl-bis solution (40% acrylamide) 0.625 mL, stacking buffer (5×) 1 mL, ddH<sub>2</sub>O 3.375 mL, ammonium persulfate (APS) (30%, prepared fresh) 20 µL, *N,N,N,N*'-tetramethyl-ethylenediamine (TEMED, Bio-Rad) 5 µL.
5. Separation gel (10 mL) 10%: Acryl-bis solution (40% acrylamide) 2.5 mL, separation buffer (3×) 3.33 mL, ddH<sub>2</sub>O 4.17 mL, APS (30%) 20 µL, TEMED 5 µL.
6. Electrode buffer (10×): 250 mM Tris-HCl, 129 mM glycine, 1% SDS.
7. Sample loading buffer (5×): 250 mM Tris-HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol, pH 6.8.
8. Mini-PROTEAN II slab gel unit (Bio-Rad) or comparable apparatus.
9. Rainbow™ colored protein molecular weight marker (Amersham).
10. Hybond-C (Amersham) supported nitrocellulose membranes.

11. Trans-Blot cell (Bio-Rad) or comparable apparatus.
12. Transfer buffer: 25 mM Tris-base, 192 mM glycine, 20% methanol.
13. Primary antibodies against GFP (Clontech) to detect the presence of the GFP fusion recombinant proteins.
14. Secondary antibodies: alkaline phosphatase conjugated anti-mouse IgG (whole molecule) (Sigma).
15. Blocking solution: 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl).
16. TBS with Tween (TTBS): 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20.

---

### 3. Methods

The GFP tag enables relatively quick confirmation of antigen expression in plant cells by fluorescent microscopy. By simple infiltration of *Agrobacterium* cells carrying appropriate gene constructs into tobacco plants leaves, transient expression assays can be performed within 3 days without using expensive instruments or complicated procedures. Two days after agroinfiltration, expression and subcellular localization of the GFP fusion proteins in tobacco leaves can be determined by simple observation under fluorescence or confocal laser scanning microscopy. In addition, RNA and protein can be extracted from the agroinfiltrated leaves for reverse transcription-polymerase chain reaction (RT-PCR), northern blot analysis, and western blot analysis.

#### **3.1. Construction of Vector for Expression of the SARS-CoV S1-GFP Fusion**

The *SARS-CoV S1* cDNA (encoding amino acids 1–658 of the S protein) was amplified by PCR using template derived from total RNA of Vero cells infected with the *SARS-CoV (II)*. Plasmid pCRII-S1 containing the *SARS-CoV S1* cDNA was used as a mutagenesis template for generating derivatives with optimized codon usage for heterogeneous gene expression in plants. Mutagenesis was carried out by PCR with *Pfu-Turbo* DNA polymerase using the QuikChange Multi Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer's specifications.

1. Perform PCR using the following conditions for amplification of derivatives with optimized codon usage: 95 °C for 30 s followed by 12–18 cycles of 95 °C 30 s, 55 °C for 1 min, 68 °C for 1 min/kb of plasmid length.
2. After PCR, add 1 µL of *DpnI* restriction enzyme (10 U/µL) directly to the amplification reaction and incubate at 37 °C for 1 h to digest the nonmutated supercoiled dsDNA.

3. Transform supercompetent *Escherichia coli* DH5 $\alpha$  bacteria using 1  $\mu$ L of the *DpnI*-treated DNA from each reaction.
4. Select clones for extraction of plasmid DNA to verify the change in DNA sequence.
5. The oligonucleotides we used in site-directed mutagenesis are shown in **Table 1**.
6. A total of 13 nucleotide changes were incorporated for an “optimized” S1. Use the optimized S1 for cloning into nuclear transformation vector.
7. Generate plasmid pCV12 (**Fig.1**) or similar nuclear transformation vector for expression of a fusion protein consisting of the SARS-CoV S1 and GFP. This was generated by cloning a 0.8-kb *XhoI-SpeI* GFP fragment from plasmid pGFP2 in the *SalI-XbaI* sites of pCRII-S1 to create an S1-GFP fusion (12). Subsequently,

**Table 1**  
**Oligonucleotides used for site-directed mutagenesis of S1 for codon optimization (reproduced from ref. (12) with permission from the Society for Experimental Biology and Medicine)**

Affected residue	Sequence of primer
R18	5'-GTAGTGACCTTGAC <u>AGAT</u> GCACCACTTTTGAT-3'
T75	5'-GGGTTTCATACTATTAATCAT <u>ACT</u> TTTGGCAACCCTGTCATAC-3'
S113	5'-CCATGAACAACAAGTCACAGT <u>CT</u> TGTGATTATTATTAACAATTCTACT-3'
S169	5'-AGTACATATCTGATGCCTTTT <u>CT</u> CTTGATGTTTCAGAAAAGTC-3'
L209	5'-CCTATAGATGTAGTTCGTGAT <u>CT</u> TCCTTCTGGTTTAAACACTTTG-3'
T247	5'-CAAGACATTTGGGGC <u>ACT</u> TCAGCTGCAGCCTAT-3'
A398	5'-GATGATGTAAGACAAATAGC <u>TC</u> CAGGACAAACTGG-3'
P507	5'-TCTTTTGAACTTTTAAATGCACC <u>T</u> GCCACGGTTTGTGGACC-3'
T509	5'-CTTTTAAATGCACCTGCC <u>ACT</u> GTGTTTGTGGACCAAAATTATC-3'
L597	5'-CTTCATCTGAAGTTGCTGTTCT <u>T</u> TATCAAGATGTAACTGCAC-3'
R620	5'-CAACTCACACCAGCTTGG <u>AGA</u> ATATATTCTACTGGAAACAATG-3'

Nucleotides in italics are mutated; the altered codons are underlined



**Fig. 1.** Plasmid pCV12 that was used for expression of S1 tagged to GFP in plant cells. The plasmid pCV12 is a plant nuclear transformation vector used for the expression of a protein fusion consisting of the SARS-CoV S1 protein fused translationally with GFP. RB and LB represent right and left borders of T (transfer)-DNA for random insertion into the plant nuclear genome; P35S, Cauliflower Mosaic Virus 35S promoter; TNOS, NOS terminator.

a 2.8-kb *Bam*HI-*Apa*I fragment containing the S1 cDNA (encoding amino acids 1–643) and *GFP* cDNA was cloned into the *Bgl*II-*Apa*I sites of a plant binary vector, pGD (12).

### 3.2. Agroinfiltration of Tobacco for Expression of the S1:GFP Antigen

Production of tobacco leaves transiently expressing a protein fusion consisting of the SARS-CoV S1 protein fused with the GFP was carried out using *Agrobacterium*-mediated transformation with plasmid pCV12.

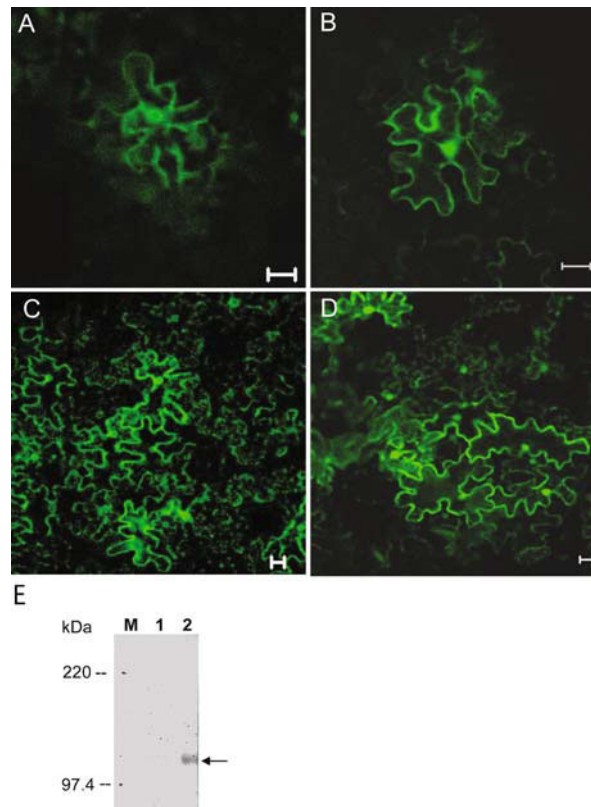
1. Grow *Agrobacterium* LBA4404/pCV12 for infiltration overnight with shaking (200 rpm) at 28 °C in YEP medium supplemented with 10 mM MgSO<sub>4</sub>, 100 µg/mL of streptomycin and, in the case of pGD, plus 50 µg/mL of kanamycin.
2. Subsequently, initiate a new culture by inoculating fresh medium with the overnight culture at 1:10 dilution.
3. After growing for 5–7 h (OD<sub>600</sub> should be ~0.8–1.0), harvest the bacterial cells by centrifugation for 10 min at 1,000 × *g* at 4 °C.
4. Wash the cells twice with 10 mM MgSO<sub>4</sub>, and then resuspend in 10 mM MES (pH 5.5), 10 mM MgSO<sub>4</sub> supplemented with 100 µM AS to form a dilute bacterial suspension with an absorbance of ~0.6 at 600 nm. Keep the suspension for 2–4 h at room temperature before infiltration.
5. Make some tiny wounds using a needle in the region where infiltration will apply. Using a syringe lacking a needle, press the syringe tip on the wounds against the lower side of a tobacco leaf, meanwhile applying counterpressure to the upper leaf, so that the bacterial suspension can be efficiently introduced through the stomata to the leaf lamina. It generally takes a few minutes to do one infiltration. There is no need to wash off the bacterial solution outside the leaves following the procedure. Freshly expanded young leaves are preferentially used for better expression (*see* **Notes 1–4**).

### 3.3. Analyses on Transient-Expressed Proteins

#### 3.3.1. Laser-Scanning Confocal Microscopy

1. Two days after agroinfiltration, the expression and subcellular localization of transiently-expressed GFP fusion proteins can be determined. Representative tobacco leaf epidermal cells are selected and observed by fluorescence microscopy or confocal microscopy.
2. Compare tobacco leaf cells subjected to agroinfiltration using *Agrobacterium* LBA4404 harboring plasmid pCV12 (expressing the S1:GFP-fusion protein) and those agroinfiltrated with LBA4404 harboring pGDG (control expressing GFP alone).
3. Detect fluorescence and collect digital images using a Zeiss LSM 510 inverted confocal laser-scanning microscope equipped with helium/neon lasers or comparable microscope. GFP fluorescence excites at 488 nm, is filtered through a primary dichroic (UV/488/543), a secondary dichroic of 545 nm, and subsequently through BP505-530 nm emission filters to the photomultiplier tube detector.

4. The images can be processed using the LSM 510 software (Zeiss) and Photoshop 6.0 (Adobe) or comparable software. Images of transient expression of S1:GFP antigen in the cytosol of agroinfiltrated tobacco leaves are shown in **Fig. 2**. Expression of the construct with GFP alone was not confined to the cytosol. Nuclear expression is seen because of the accumulation of passive diffusion of GFP into the nucleus (13). The S1:GFP fusion was localized to the cytosol, and also to the periphery of the nucleus as well, implicating that S1:GFP was secreted through the endoplasmic reticulum network. Depending on total experimental conditions, the expression of the target gene at the infiltrated area can vary. Under optimal conditions, almost all cells at the infiltrated area show expression, like in **Fig. 2**. Generally, it should not be difficult to get expression in more than 50% of plant cells at the infiltrated area.



**Fig. 2.** Transient expression of S1:GFP in agroinfiltrated tobacco leaves. Representative tobacco leaf epidermal cells are shown by confocal microscopy 2 days following agroinfiltration of *Agrobacterium tumefaciens* LBA4404 harboring plasmid pCV12 expressing S1:GFP fusion protein (**A, C**) or LBA4404 harboring pGDG expressing GFP alone (**B, D**). Bar represents 20  $\mu$ m. Western blot analysis using antibodies against GFP shows transient expression of S1:GFP in tobacco leaves following agroinfiltration. GFP alone (*lane 1*) or plasmid pCV12 expressing S1:GFP fusion (*lane 2*). Arrow indicates cross-reacting S1:GFP band (calculated size 99.1 kDa). **M**, molecular mass markers. This figure is reproduced from **ref. (12)** with permission from the Society for Experimental Biology and Medicine.



### 3.3.2. Western Blot Analysis

To confirm the transient expression of S1:GFP in tobacco leaves following agroinfiltration, western blot analysis was carried out using antibodies against GFP. Total proteins were extracted from tobacco leaves infiltrated with plasmid pGDG expressing GFP alone or plasmid pCV12 expressing the S1:GFP fusion.

1. Harvest plant tissues and immediately freeze in liquid nitrogen. Grind using a mortar and pestle in protein homogenizing buffer. Use about 10 mL of ice-cold homogenizing buffer per 1 g of tissue.
2. Boil ground tissue at 100 °C for 10 min, centrifuge at 14,000 rpm for 5 min, and transfer the supernatant into a new Eppendorf and store at -20 °C. Determine the protein content.
3. Denature the protein samples in 1× SDS sample loading buffer by heating at 95 °C for 5 min before loading onto the gel. Separate protein samples (20 µg) by SDS-PAGE using a 10% gel using a Mini-PROTEAN II slab gel unit or comparable apparatus.
4. Use Rainbow™ colored protein molecular weight markers in gels destined for transfer to Hybond-C membrane.
5. Electrophoretically transfer proteins to Hybond-C membrane from SDS-PAGE gel using a Trans-Blot cell at 50 V for 1.5 h in cold transfer buffer. A piece of Hybond-C membrane, cut to the size of the gel and wetted with distilled water, was placed on top of the gel without inclusion of any air bubbles. The gel and the membrane were then sandwiched between two pieces of Whatman No. 3 paper that were also cut to the size of the gel and were presoaked in transfer buffer (*see Note 5*).
6. For detection using anti-GFP antibodies in western blot analysis, incubate the blot for 1 h at room temperature (RT) in blocking solution with gently shaking.
7. After washing the blots twice for 10 min in TTBS, then incubated for 2 h at room temperature in monoclonal anti-GFP antibodies diluted 1:5,000 in TTBS with gentle agitation.
8. Wash the blot twice for 10 min with TTBS, followed by incubation in anti-mouse IgG alkaline phosphatase diluted 1:30,000 in TTBS for 1 h at room temperature with gentle shaking.
9. Wash in TTBS four times for 10 min, and stain the blot in freshly prepared color development solution at room temperature until the reacting protein bands are visible.
10. Stop the reaction by washing the membrane twice for 5 min in water. Results of western blot analysis using antibodies against GFP of total plant proteins extracted from tobacco leaves infiltrated with plasmid pCV12 (expressing the S1:GFP fusion) are shown in **Fig. 2e** (lane 2 shows an expected band of calculated size 99.1 kDa, demonstrating that the S1:GFP fusion protein was successfully expressed).

---

## Notes

1. The technique is suitable for leaves or petals. *Agrobacterium* has to be introduced into the leaves through the stomata or via wounding. In transient gene expression, the levels in expression may be age dependent and may differ in leaf tissues at varying stages of development. Generally, the highest level of transient expression is observed in tissues that contain recently expanded leaf cells.
2. Agroinfiltration can also be used in conjunction with vacuum infiltration on either detached or attached plant leaves. With the use of detached leaves, the leaves should be best incubated on wet filter paper rather than on solidified MS medium following agroinfiltration since incubation on MS often gave lower expression levels (8).
3. The pH of the *Agrobacterium* resuspension can be within a range of pH 5.0–5.8. We usually use pH 5.5. The concentration of *Agrobacterium* cells is important for infiltration. The optimal concentration of the *Agrobacterium* suspension is  $OD_{600} = 0.4\text{--}0.6$ . The *Agrobacterium* culture is grown to log phase and then resuspended to  $OD_{600} = 0.4\text{--}0.6$  for better expression. If the bacterial cells are out of log phase, the efficiency could be much decreased.
4. Acetosyringone is usually added in the agroinfiltration medium to increase the efficiency. It has been demonstrated in Chinese Cabbage that agroinfiltration can be improved by adding hydrogen peroxide (14). Since the plasmid vector for agroinfiltration does not necessarily carry an antibiotic-resistance selectable marker gene, a smaller vector can be considered for use to enhance T-DNA transfer in plant cells by agroinfiltration.
5. Make sure there are no air bubbles between nitrocellulose membrane and gel or else certain regions will not be transferred to the membrane. Alternatively, the blot can be assembled under transfer buffer to avoid trapping bubbles.

---

## Acknowledgments

We thank Prof. J.S.M. Peiris and Dr. L.L.M. Poon (Department of Microbiology, University of Hong Kong) for gift of plasmid pCRII-S1, Prof. N.-H. Chua (Rockefeller University, New York) for plasmid pGFP-2, and Dr. M.M. Goodin (University of California, Berkeley) for plasmids pGD and pGDG. We are grateful to the Society for Experimental Biology and Medicine for permission to use previously published figures that had appeared in **ref.** (12).

## References

1. Walmsley, A.M. and Arntzen, C.J. (2003) Plant cell factories and mucosal vaccines. *Curr. Opin. Biotechnol.* 14, 145–150.
2. Porgrebnyak, N., Golovkin, M., Andrianov, V., Spitsin, S., Smirnov, Y., Egolf, R., and Koprowski, H. (2005) Severe acute respiratory syndrome (SARS) S protein production in plants: development of recombinant vaccine. *Proc. Natl. Acad. Sci. USA* 102, 9062–9067.
3. Dantuma, N.P., Lindsten, K., Glas, R., Jellne, M., and Masucci, M.G. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin “proteasome-dependent proteolysis in living cells”. *Nat. Biotechnol.* 18, 538–543.
4. Kloetzel, P.M. (2004) Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPPII. *Nat. Immunol.* 5(7), 661–669.
5. Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol. Mol. Biol. Rev.* 67, 16–37.
6. Yoshioka, Y., Takahashi, Y., Matsuoka, K., Nakamura, K., Koizumi, J., Kojima, M., and Machida, Y. (1996) Transient gene expression in plant cells mediated by *Agrobacterium tumefaciens*: application for the analysis of virulence loci. *Plant Cell Physiol.* 37, 782–789.
7. Schob, H., Kunz, C., and Meins, F., Jr. (1997) Silencing of transgenes introduced into leaves by agroinfiltration: a simple, rapid method for investigation of sequence requirements for gene silencing. *Mol. Gen. Genet.* 256, 581–585.
8. Kapila, J., De Rycke, R., Van Montagu, M., and Angenon, G. (1997) An *Agrobacterium*-mediated transient gene expression for intact leaves. *Plant Sci.* 122, 101–108.
9. Goodin, M.M., Dietzgen, R.G., Schichnes, D., Ruzin, S., and Jackson, A.O. (2002) pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. *Plant J.* 31, 375–383.
10. Hoekema, A., Hirsch, P.R., Hooykas, P.J.J., and Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir and T-region of the *A. tumefaciens* Ti plasmid. *Nature* 303, 179–180.
11. Poon, L.L.M., Wong, O.K., Luk, W., Yuen, K.Y., Peiris, J.S.M., and Guan, Y. (2003) Rapid diagnosis of a coronas associated with severe acute respiratory syndrome (SARS). *Clin. Chem.* 49, 953–955.
12. Li, H.Y., Ramalingam, S., and Chye, M.L. (2006) Accumulation of recombinant SARS-CoV spike protein in plant cytosol and chloroplasts indicate potential for development of plant-derived oral vaccines. *Exp. Biol. Med.* 231, 1346–1352.
13. Gorlich, D. and Mattaj, I.W. (1996) Nucleocytoplasmic transport. *Science* 271, 1513–1518.
14. Shin, D.-I. and Park, H.-S. (2005) Transient expression in Chinese Cabbage by hydrogen peroxide-aided agroinfiltration. *Agric. Chem. Biotechnol.* 4, 229–230.