# The "resurrection method" for modification of specific proteins in higher plants

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**Abstract** We describe a new method designated "the resurrection method" by which a modified protein is expressed in higher plants in place of the original protein. The modified gene constructed by introducing synonymous codon substitutions throughout the original gene to prevent the sequence-specific degradation of its mRNA during RNA silencing is expressed while the expression of the original gene is suppressed. Here, we report the successful alteration of the biochemical properties of green fluorescent protein expressed in transgenic *Nicotiana benthamiana*, suggesting that this method could be useful for gene control in living plants.
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## 1. Introduction

Reverse genetics, in which a modified gene is expressed in place of the original gene, is a straightforward way to determine the functions of genes and proteins. Gene replacement is routinely achieved in microbes, including yeast, by inserting a mutant transgene into the genome by homologous recombination [1]. Homologous recombination has also been used for targeted mutagenesis in mammals [2]. However, targeted mutagenesis mediated by homologous recombination is still difficult to achieve in higher plants because of the low efficiency of homologous recombination and difficulty in selecting appropriate mutants from among transformants, in spite of the extensive studies undertaken to enhance the efficiency of gene targeting in higher plants, including *Arabidopsis* and cultured rice cells [3–5]. The expression of a mutated protein in place of the original protein has not been routinely achieved in higher plants.

RNA silencing, or RNA interference, is a reliable means of specifically suppressing the expression of a gene of interest in eukaryotes, including higher plants [6]. In the process of RNA silencing, short interfering RNA (siRNA) of 21–26 nucleotides complementary to the target gene is generated and incorporated into the RNA-induced silencing complex (RISC). The siRNA in the RISC then interacts specifically with and degrades the mRNA of the target gene in a sequence-specific manner. As a result, the expression of the protein encoded by the target gene is suppressed.

We established a new simple method to selectively alter the proteins expressed in higher plants using an RNA-silencing-mediated method. In this procedure, designated the "resurrection method", genes are modified to escape sequence-specific recognition and subsequent mRNA degradation by the RISC. These genes, designated "resurrection genes", contain synonymous codon substitutions throughout the coding region to preclude their interaction to the siRNA. Resurrection genes should be expressed in plants in which the original gene has been silenced. Accordingly, the modified protein encoded by the resurrection gene is expressed in place of the original protein in the RNA-silenced plants.

We tested our proposed method using green fluorescent protein (GFP), which is easily detected in vitro and in planta. The expression of GFP in transgenic *Nicotiana benthamiana* plants was silenced by the introduction of a transgene from which is transcribed an intron-spliced hairpin RNA corresponding to the "original" *gfp* sequence. In the GFP-silenced plants, mutated GFP derivatives encoded by resurrection genes were successfully expressed using *potato virus X* (PVX)-based vectors. The significance and potential applications of the resurrection method are discussed.

## 2. Materials and methods

### 2.1. Plants and transformation

The binary plasmid pBICBPdsGFP was created by cloning the *Stu*I–*Hin*dIII fragment of pBICdsGFP [7], encoding an intron-spliced hairpin RNA for *gfp* into the *Stu*I–*Hin*dIII sites of pBICBP35 [8] (Fig. 1A). The transgenic *N. benthamiana* plant G3Nb3, generated using the binary vector pBIG3 (Fig. 1A) and strongly expressing endoplasmic-reticulum-localized GFP (erGFP), was a gift from Dr. Tetsuo Meshi [9]. Transgenic *N. benthamiana* plants in which the expression of erGFP was suppressed were produced by transforming G3NB3 with pBICBPdsGFP using an *Agrobacterium*-mediated method, as previously described [10]. Transgenic *N. benthamiana* smGNb plants, which constitutively express smGFP, were produced by transforming wild-type plants with pBICGFP [7].

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**A** pBIG3



pBICBPdsGFP



**B** pTXS.GFP-SN



pTXS.F-smG
(PVX.F-smG)

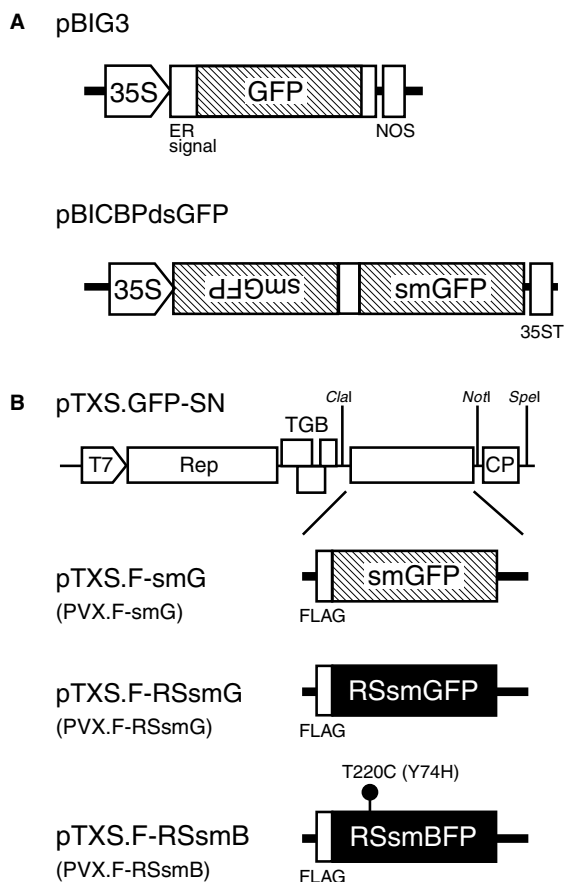pTXS.F-RSsmG
(PVX.F-RSsmG)

pTXS.F-RSsmB
(PVX.F-RSsmB)

Fig. 1. Schematic representation of plasmid constructs. Hatched and closed boxes indicate the coding regions of the original *gfp* and the resurrection *gfp* derivatives, respectively. (A) Transcriptional cassettes of binary plasmids. pBIG3 contains the *gfp* gene with sequences for signal peptides that target the protein to the endoplasmic reticulum (ER). pBICBPdsGFP contains both sense and antisense sequences of smGFP separated by an intron. 35S and 35ST, *cauliflower mosaic virus* 35S RNA promoter and terminator, respectively; NOS, terminator of the nopaline synthase gene. (B) Plasmids for in vitro transcription of infectious PVX vectors. The viruses transcribed from each plasmid are shown in parentheses. T7, phage T7 promoter; Rep, viral replicase; TGB, the viral movement protein genes known as the "triple gene block"; FLAG, the FLAG tag. The location of the base substitution between *gfp* and *bfp* is indicated on the RSsmBFP construct.

### 2.2. PVX vectors encoding GFP derivatives

pTXS.GFP, which encodes a PVX-based vector downstream from the T7 promoter, was a gift from Dr. David Baulcombe [11]. The linker 5′-<u>TCGAC</u>TACGTATAAGAATGCGGCCGC<u>AGCT</u>-3′ (cohesive ends for *Sal*I are underlined), which contains a *Not*I site, was inserted into the *Sal*I site of pTXS.GFP to create pTXS.GFP-SN (Fig. 1B).

The DNA fragment FLAG-smGFP, which encodes soluble-modified GFP (smGFP) with an N-terminal FLAG tag, was amplified from CD3-326 [12] by PCR (Fig. 2). The DNA fragment FLAG-RSsmGFP, which contains synonymous codon substitutions throughout the coding region of smGFP in FLAG-smGFP, was synthesized by PCR using a series of synthetic nucleotides (Fig. 2). The DNA fragment FLAG-RSsmBFP, which encodes the soluble-modified blue fluorescent protein (smBFP) [12] with an N-terminal FLAG tag, was produced by introducing a base substitution (T220C) into FLAG-RSsmGFP using PCR. PCR products encoding FLAG-smGFP, FLAGRSsmGFP, and FLAG-RSsmBFP between *Nar*I and *Not*I sites were amplified from the DNA fragments described above, digested with *Nar*I and *Not*I, and cloned between the *Cla*I and *Not*I sites of pTXS.GFP-SN to create pTXS.F-smG, pTXS.F-RSsmG, and pTXS.F-RSsmB, respectively (Fig. 1B).

### 2.3. PVX inoculation

Using Amplicap T7 High Yield Message Maker Kit (Epicentre) according to the manufacturer's instructions, we transcribed the PVX vectors PVX.F-smG, PVX.F RSsmG, and PVX.F-RSsmB from pTXS.F-smG, pTXS.F-RSsmG, and pTXS.F-RSsmB, respectively, after linearizing them with *Spe*I. The purified transcripts (2.5 μg) were inoculated with carborundum onto the sixth leaves of *N. benthamiana* plants approximately five weeks after they had been sown. The plants were incubated under 16 h of light per day at 25 °C for two weeks before analysis.

### 2.4. RNA and protein analysis

Total RNA and total protein were isolated from the 11th leaves of inoculated plants using TRIzol reagent (Invitrogen). Northern blot analysis was carried out as described previously [10] using DIG-labeled probes specific for the PVX sequences, smGFP/erGFP, and RSsmGFP/RSsmBFP. These probes were transcribed from the corresponding DNA fragments, which contained the T7 promoter at the 5′ end and were synthesized by PCR. Western blot analysis was performed using Lumi-Light$^{PLUS}$ Western Blotting Kit (Roche) with anti-GFP (Assay Designs) and anti-FLAG (Affinity BioReagents) antibodies, according to the manufacturer's instructions. The fluorescence of GFP was visually observed by using a 100 W long-wave UV lamp (Black Ray model B 100A, UV products). The fluorescence of BFP and GFP was observed with an LSM410 confocal laser scanning microscope (Carl Zeiss) with 405 and 488 nm lasers and emission filters for 420–480 and 505–530 nm, respectively.

## 3. Results

### 3.1. Construction of resurrection gfp genes

Three constructs encoding GFP derivatives, FLAG-smGFP, FLAG-RSsmGFP, and FLAG-RSsmBFP, were generated. FLAG-smGFP encodes the soluble-modified GFP (smGFP) [12] with an N-terminal FLAG tag (Fig. 2). FLAG-RSsmGFP was constructed to express transcripts that would escape the degradation directed against *gfp*-specific mRNA during RNA silencing by the introduction of synonymous codon substitutions throughout the coding region of smGFP in the FLAG-smGFP construct (Fig. 2). Therefore, the FLAG-smGFP and FLAG-RSsmGFP constructs encode the same protein (Fig. 2). We designated these smGFP genes that were modified to escape silencing "resurrection smGFP (RSsmGFP) genes". FLAG-RSsmBFP is a mutant FLAG-RSsmGFP gene with a single-base substitution, and it encodes smBFP, a spectral variant of GFP, with an N-terminal FLAG tag (Fig. 1B). The RSsmGFP and RSsmBFP genes were designed to avoid some rare codons, such as CGC for arginine, and the motif ATTTA, which is known to destabilize mRNA [13]. We generated the PVX vectors PVX.F-smG, PVX.F-RSsmG, and PVX.F-RSsmB containing the FLAGsmGFP, FLAG-RSsmGFP, and FLAG-RSsmBFP genes, respectively (Fig. 1B).

### 3.2. Expression of resurrection gfp genes in wild-type plants

PVX vectors were inoculated onto wild-type *N. benthamiana* plants to investigate the expression of GFP derivatives. Northern blot analysis using a probe for PVX showed no significant differences in the accumulation of PVX.F-smG, PVX.F-RSsmG, and PVX.F-RSsmB genomic RNAs in the wild-type plants (Fig. 3A). The accumulation of the *gfp* sequence of PVX.F-smG was confirmed on a Northern blot probed for *gfp* mRNA (Fig. 3A). Similarly, the accumulation of the resurrection *gfp* sequences of PVX.F-RSsmG and PVX.F-RSsmB was confirmed on a Northern blot probed with a specific probe

```
                                    FLAG-tag
                         _____
FLAG-smGFP      1'  ATGGATTACAAGGACGACGATGACAAGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTC
                    ******************************* ** ** ***** * ** *** ** ** **
FLAG-RSsmGFP    1"  ATGGATTACAAGGACGACGATGACAAGAGCAAGGGCGAAGAGTTGTTTACCGGCGTGGTG
                    M  D  Y  K  D  D  D  D  K  S  K  G  E  E  L  F  T  G  V  V


               61'  CCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAG
                    ** **  * ** ** * ** ** ** ** ** ** ** ** **     ** ** ** **
               61"  CCTATCTTGGTGGAGCTTGACGGCGACGTGAACGGCCATAAGTTCAGCGTTAGCGGCGAA
                    P  I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E


              121'  GGTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAA
                    ** ** ** ** ** ** ** ** ** **  * **  * ** ** ** ** ** ** **
              121"  GGCGAGGGCGACGCTACTTATGGCAAGTTGACTTTGAAGTTCATCTGTACCACCGGCAAG
                    G  E  G  D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K


              181'  CTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCA
                     * ** ** ** ***** **  * ** ** ** ** ***** ** ******* *****
              181"  TTGCCAGTGCCTTGGCCTACTTTGGTGACCACCTTTTCTTACGGCGTTCAATGTTTTTCT
                    L  P  V  P  W  P  T  L  V  T  T  F  S  Y  G  V  Q  C  F  S


              241'  AGATACCCAGATCATATGAAGCGGCACGACTTCTTCAAGAGCGCCATGCCTGAGGGATAC
                     * ** ** ** ** ***** * ** ** ** ** **    ** ***** ** ** **
              241"  CGTTATCCTGACCACATGAAAAGACATGATTTTTTTAAATCTGCTATGCCAGAAGGCTAT
                    R  Y  P  D  H  M  K  R  H  D  F  F  K  S  A  M  P  E  G  Y


              301'  GTGCAGGAGAGGACCATCTCTTTCAAGGACGACGGGAACTACAAGACACGTGCTGAAGTC
                    ** ** ** ** ***** ** ** ** ** ** ** ** ** ** **  * ** ** **
              301"  GTTCAAGAAAGAACTATTTCTTTTAAAGATGATGGCAATTATAAAACTAGGGCCGAGGTT
                    V  Q  E  R  T  I  S  F  K  D  D  G  N  Y  K  T  R  A  E  V


              361'  AAGTTTGAGGGAGACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGAG
                    ** ** ** ** ** **  * ** **  * ** **  * ** ** ** ** ** ** **
              361"  AAATTCGAAGGCGATACTTTGGTTAATCGTATTGAATTGAAAGGCATTGACTTTAAAGAA
                    K  F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E


              421'  GACGGAAACATCCTCGGCCACAAGTTGGAATACAACTACAACTCCCACAACGTATACATC
                    ** ** ** ** ** ** ** ** ** ** *****  *** ** ** ** **
              421"  GATGGCAATATTTTGGGACATAAATTAGAGTATAATTACAATAGCCATAATGTTTATATT
                    D  G  N  I  L  G  H  K  L  E  Y  N  Y  N  S  H  N  V  Y  I


              481'  ACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTAGACACAACATTGAA
                    ** ** ** ** ** ** ** ** ** ** ** ** *****  * ** ** ** **
              481"  ACCGCTGATAAGCAGAAAAACGGCATTAAGGCTAATTTTAAGATCCGTCATAATATCGAG
                    T  A  D  K  Q  K  N  G  I  K  A  N  F  K  I  R  H  N  I  E


              541'  GATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCT
                    ** ** ** ** **  * ** ** ** ** ** ** ** ** ** ** ** ** ** **
              541"  GACGGCAGTGTGCAGTTGGCTGATCACTACCAGCAGAACACCCCTATCGGAGACGGACCA
                    D  G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P


              601'  GTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAAC
                    ** *  * ** ** ** ** ** ** **  ** *** **  **  *  * ** ** **
              601"  GTTTTGCTTCCTGATAATCACTATTTGAGCACTCAGAGCGCTTTGAGCAAGGACCCAAAT
                    V  L  L  P  D  N  H  Y  L  S  T  Q  S  A  L  S  K  D  P  N


              661'  GAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGC
                    ** ** ** ** ** ***** *  * ** ** ** ** ** ** ** ** ** ** **
              661"  GAGAAAAGGGATCATATGGTTTTTGTTGGAATTCGTTACTGCCGCCGGAATCACTCACGGA
                    E  K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  H  G


              721'  ATGGATGAACTATACAAATAA
                    ***** **  * ** ** ***
              721"  ATGGACGAGTTGTATAAGTAA
                    M  D  E  L  Y  K  !
```

Fig. 2. Comparison of nucleic acid sequences between the coding regions of FLAG-smGFP and FLAG-RSsmGFP. Both sequences encode the same protein, the amino acid sequence of which is shown below. The bold line indicates the region of the FLAG tag.
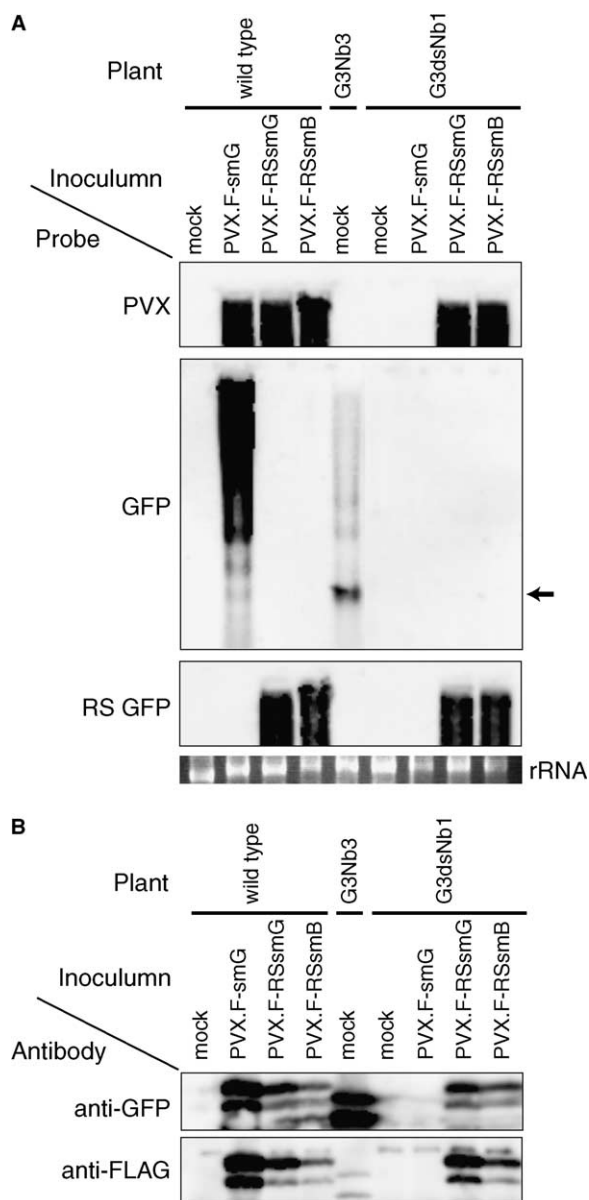
**A**



**B**



Fig. 3. Accumulation of viral RNA and GFP derivatives in *N. benthamiana* plants inoculated with PVX vectors or water (mock). (A) Northern blot analysis of total RNA (2.5 µg). The probes used are shown on the left. (B). Western blot analysis of total proteins. Total proteins extracted from 1 mg of leaves were loaded in each lane. The primary antibodies used are shown on the left.

(Fig. 3A). These results suggest that the synonymous codon substitutions introduced into the *gfp* gene did not affect the stability or replication efficiency of PVX RNA.

Western blot analysis of total proteins extracted from leaves infected with PVX viruses revealed infection-specific bands when they were probed with an anti-GFP antibody (Fig. 3B). The presence of the FLAG tag in GFP derivatives was confirmed by Western blot analysis using anti-FLAG antibody (Fig. 3B). The amounts of FLAG-tagged GFP derivatives were lower in plants infected with PVX.F-RSsmG and PVX.FRSsmB than in plants infected with PVX.F-smG

(Fig. 3B), implying that the mRNA of RSsmGFP and RSsmBFP is translated at a lower efficiency than the original smGFP.

The spectral properties of the GFP derivatives expressed in the wild-type plants infected with PVX vectors were examined by laser scanning confocal microscopy. In wild-type plants inoculated with PVX.F-smG or PVX.F-RSsmG, GFP fluorescence was detected but BFP fluorescence was not (Fig. 4A). In contrast, only BFP fluorescence was observed in wild-type plants inoculated with PVX.F-smB (Fig. 4A). These results confirm the fluorescent properties of the GFP derivatives encoded in the PVX vectors.

### 3.3. Expression of resurrection gfp genes in GFP-silenced plants

We investigated whether GFP was replaced with other GFP derivatives by the RNA silencing-mediated method in transgenic *N. benthamiana* G3Nb3 plants [9] stably expressing GFP with a signal peptide that targets it to the endoplasmic reticulum (erGFP). G3Nb3 plants were transformed with plasmid pBICBPdsGFP (Fig. 1A) to facilitate the expression of intron-spliced hairpin RNA corresponding to the *gfp* sequence. The transformants were visually inspected under UV light to select transgenic lines, including G3dsNb1, in which fluorescence of GFP was not observed. Northern blot analysis revealed *gfp* mRNA in G3Nb3 plants but not in G3dsNb1 plants (Fig. 3A: allow). Strong GFP signals were detected in G3Nb3 plants by Western blot analysis and confocal microscopy, but no GFP signal was detected in G3dsNb1 plants (Figs. 3B and 4A). These results indicate that the erGFP expressed in G3Nb3 was suppressed in the daughter line, G3dsNb1.

We then investigated the expression of GFP derivatives from PVX vectors in G3dsNb1. In G3dsNb1 plants inoculated with PVX.F-smG, no viral RNA was detected by Northern blot analysis when probes for PVX and *gfp* were used (Fig. 3A). In these plants, no band corresponding to FLAG-smGFP or erGFP was detected by Western blot analysis (Fig. 3B), and no GFP fluorescence was detected by confocal microscopy (Fig. 4A). In contrast, in G3dsNb1 plants inoculated with PVX.F-RSsmG or PVX.F-RSsmB, efficient accumulation of viral RNAs was shown by Northern blot analysis with probes for PVX and resurrection *gfp* (Fig. 3A). Western blot analysis revealed the accumulation of FLAG-smGFP and FLAG-smBFP in these plants, respectively, as observed in the wild-type plants. These results indicate that the suppression of erGFP expression in G3dsNb1 is due to sequence-specific RNA degradation by RNA silencing targeted to the "original" *gfp* sequence, and show that the resurrection *gfp* genes encoded by PVX.F-RSsmG and PVX.F-RSsmB escape this sequence-specific RNA degradation. The mobility of the bands was slightly lower than that of erGFP expressed in G3Nb3 plants or the reference GFP, which is probably attributable to the N-terminal FLAG tag (Fig. 3B). Fluorescence corresponding to GFP or BFP was observed by confocal microscopy in G3dsNb1 plants inoculated with PVX.F-RSsmG or PVX.FRSsmB, respectively (Fig. 4A). Similar results were obtained with two other lines of plants in which the expression of erGFP was stably suppressed. The fluorescence of erGFP in G3Nb3 plants was mainly localized to the outer boundaries of cells (Fig. 4B). In contrast, in G3dsNb1 plants inoculated with PVX.F-RSsmG, some patches of GFP fluorescence were observed
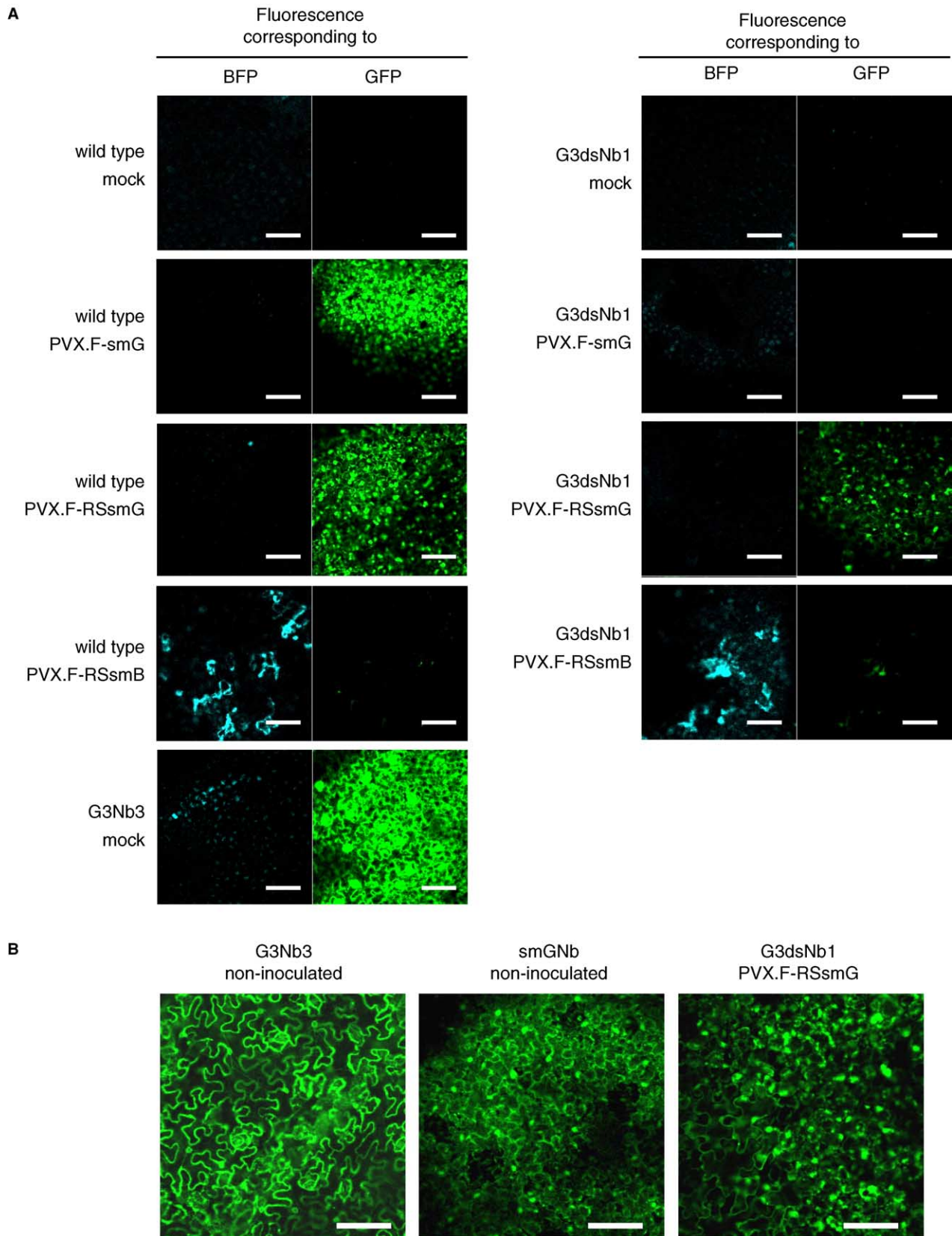
Fig. 4. Confocal laser scanning microscopy to detect the fluorescence of BFP and GFP in the upper leaves of *N. benthamiana* plants inoculated with PVX vectors. Bars = 100 μm. (A) Micrographs of wild-type and transgenic plants inoculated with various PVX vectors or water (mock). The plants and PVX vectors used are shown on the left. (B) Comparison of intracellular GFP distributions among G3Nb3, smGNb, and G3dsNB1 infected with PVX.F-RSsmG.

in the inner regions of cells (Fig. 4B). This distribution of GFP fluorescence in the G3dsNb1 plants inoculated with PVX.F-RSmG resembled that in transgenic *N. benthamiana* plants constitutively expressing smGFP (Fig. 4B). This indicates that the intracellular localization of FLAG-smGFP expressed from PVX.FRSmG differed from that of erGFP expressed in G3Nb3 plants.

## 4. Discussion

In eukaryotic molecular biology, application of RNA silencing has been mostly limited to simply suppressing gene expression. On the other hand, the new technique described here utilizes RNA silencing as a means of modifying the selected protein. Recently, there have been several reports on gene replacement mediated by RNA silencing in mammalian systems [14–16]. In those studies, synonymous codon substitutions were introduced into only a few codons in the target region of the siRNA to allow it to escape RNA silencing. However, genes targeted to escape RNA silencing in plants and *Caenorhabditis elegans* should be modified throughout the length of the gene, as described in this study, because RNA silencing spreads from the silencing initiator region to the adjacent 5′ or both 5′ and 3′ regions of the target gene in these organisms [6,17].

By the resurrection method, we successfully replaced the erGFP expressed in transgenic *N. benthamiana* with GFP derivatives that had different biochemical properties: the presence of a FLAG tag, different intracellular distributions, and different spectral properties. This method can therefore potentially alter the properties of proteins expressed in higher plants. Because RNA silencing has been widely and routinely used to knock down the expression of specific genes in plants [6], the simple concept of the resurrection method should provide a rapid and easy means by which to alter various proteins selectively and to investigate gene function in a wide range of plant species.

The translational efficiency of the resurrection genes RSmGFP and RSmBFP was lower than that of the original *gfp*. This suggests that some factors, such as GC content, codon usage bias, and secondary structure of mRNAs, reduced the translational efficiency of these genes. On the other hand, it is possible that the expression of resurrection genes will be more efficient than that of the original genes when the expression of the latter is relatively low in vivo. Codon redundancy may allow the design of various resurrection genes that encode the same protein but that are expressed with different efficiency.

The resurrection method has many potential applications. The transient expression of resurrection genes quickly and easily replaces the original protein in gene-silenced plants, as demonstrated in this study. When a resurrection gene is integrated into a chromosome of a silencing-induced plant, it must stably replace the original gene. The use of appropriate promoters to control resurrection genes may make it possible to express the modified proteins ectopically or inducibly in the silencing-induced plants. Proteins essential for the growth of plants, the silencing of which is lethal for the plant, can be modified using the resurrection method by introducing the resurrection gene before the induction of silencing, to avoid the interruption of the expression of essential proteins.

The resurrection method could simultaneously replace the expression of multigenes that contain common sequences targeted by siRNA, with the expression of a single resurrection gene. This could be especially useful for modifying the proteins of polyploid plants, including important crops, because the modification of these plants is quite difficult by homologous recombination or genetic disruption by insertion. The resurrection method could also be useful for expressing a protein ectopically when its expression is suppressed by RNA silencing induced by endogenous microRNA [17]. It may also be possible to functionally complement the silenced gene by expressing the same protein.

In conclusion, the new resurrection method should be useful not only for the basic analysis of protein function but also in practical breeding in a wide range of higher plant species.

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