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The Effect of *Cucumber mosaic virus* 2b Protein to Transient Expression and Transgene Silencing Mediated by Agro-infiltration

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The transient and rapid expression system of a foreign protein *in planta* is a very useful technique in biotechnology application. We have investigated optimum condition of *Agrobacterium*-infiltration technique in which expression level of foreign proteins were maximized without detrimental effects on plants using GFP and *Cucumber mosaic virus* 2b protein, which is known as an enhancer of gene expression and a suppressor of post-transcriptional gene silencing (PTGS). The optimum expression level of both RNA and protein of GFP with minimum leaf impairment was obtained at OD_{600}=0.2 of *Agrobactrium* inocula. The steady-state levels of GFP RNA and protein generally peaked at 3 and 7 days post-infiltration (dpi), respectively. In the presence of 2b, both the magnitude and duration of GFP expression was highly increased and we could detect GFP level until 17 dpi. On the other hands, the 2b-mediated higher accumulation of foreign proteins resulted in the repression of normal leaf growth, possibly due to the limitation of supply of energy or materials required for growth maintenance. Using this *Agrobacterium*-infiltration system with 2b and GFP, we tested a hypothesis for the threshold model of PTGS initiation. Four GFP transgenic lines of *N. benthamiana*, which shows different expression level of GFP were tested to determine the threshold level for PTGS initiation. *Agrobacterium*-infiltration of GFP into those GFP-transgenic plants resulted in the co-silencing of the transgenic GFP. It was found that very low concentration of *Agrobacterium* with GFP and GFP+2b (OD_{600} =0.002-0.02) which could not phenotypically induce an additive GFP expression, was enough to trigger PTGS pathway in all GFP transgenic plants. This strongly indicates that each GFP-transgenic plant should be expressing the transgenic GFP at its own pre-determined level and there was no buffer zone of additive GFP-expression to the threshold. In other words, the PTGS seems to be immediately activated as a self-defensive mechanism if an internal balance of gene expression is broken.

Keywords : agro-infiltration, *Cucumber mosaic virus 2b*, GFP, post-transcriptional gene silencing

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The PTGS is originally recognized as a plant defensive mechanism against plant viruses (Ratcliff et al., 1999; Voinnet, 2001), and the viruses have evolved with various suppressors to inhibit the defensive mechanism through PTGS, so called the counter-defensive strategy (Vionnet, 2001; Vionnet et al., 1999). The PTGS is commonly characterized by the presence of short-interfering (si) RNAs of 21-25 nucleotides. The siRNA are processed from double-stranded (ds) RNA by RNAase III-like enzyme called Dicer (Bernstein et al., 2001; Elbashir et al., 2001; Hamilton and Baucombe, 1999). The siRNA then guides an RNA-induced silencing complex (RISC) for sequence-specific RNA degradation. The CMV 2b protein was reported to be a suppressor of PTGS, and contains a nuclear localization signal (NLS) and the arginine-rich region (ARR) near the NLS. The 2b protein may interfere with the PTGS by affecting transcription factors in the nucleus, and the ARR was demonstrated to be able to bind siRNA or long dsRNAs, thereby interfere with PTGS pathway (Goto et al., 2007; Mayers et al., 2000). Recently, the 2b has been also demonstrated to inhibit directly the endonucleolytic activity of Argonaute1 (AGO1), which is the core component of the RISC complex of PTGS pathway (Baumberger and Baulcombe, 2005; Zhang et al., 2008).

Agro-infiltration on plant leaves can be used for the transient expression of a foreign gene inserted into T-DNA region of binary vector (Fisher et al., 1999). The infiltrated *Agrobacterium* meditates the incorporation of T-DNA region into plant genome, and then foreign genes in T-DNA initiate their expression. The expression level usually peaks

at 2-3 days post-infiltration (dpi), and then the expression level declines rapidly (Kapila et al., 1997; Voinnet et al., 2003). The PTGS was also regarded as a factor involved in the sharp decline of the transient expression system (Johansen and Carrington, 2001). The viral suppressor has been practically applied to enhance the transient expression system of foreign proteins. For instance, the co-expressed p19 protein of tomato bushy stunt virus (TBSV) prevents the initiation of PTGS and allows high level of transient expression of GFP by more than 50-folds in plant (Vionnet et al., 2003). This finding indicated that transient expression in the presence of the viral suppressors may be developed further for a molecular farming of valuable proteins and a rapid system for the characterization of protein function in plant. In this study, the enhanced effect of the 2b protein was investigated by the transient expression system for the biotechnological application. Concurrently a precise condition of *Agrobacterium*-infiltration was examined for the maximum production of foreign proteins. Furthermore, the initiation of the PTGS was analyzed by the additive agro-infiltration with GFP gene into the GFP-transgenic *N. benthamiana*. The GFP-transgenic plants, which express the GFP uniformly throughout the whole plant, were previously developed by the leaf-disc transformation with *Agrobacterium* containing GFP. In that process, the threshold model, which was firstly hypothesized for the regulation of the elevated transgene expression by PTGS (Lindbo et al., 1993), was also examined to verify the initiation of PTGS pathway.

Materials and Methods

Vector construction. The pGreen0229 binary vector and the modified GFP4 gene (Hellens et al., 2000) cassette were kindly provided by John Innes Centre (Norwich, UK). The *Eco*RV fragment of GFP cassette (1.4 kb), flanked by CaMV 35S promoter and 35S poly(A), was subcloned into the *Eco*RV site of pGreen0229 to construct pGreen0229-35S:GFP (Fig. 1A). The CMV 2b gene was amplified from CMV-infected pepper by RT-PCR using the primers (5'-ggatccgggttgagcgtgtaaattcc-3',5'-gagctccaatactgccaactc-agctcc-3'), and also inserted at the *Eco*RI site of pGreen0229 under the control of 35S promoter to construct pGreen0229-35S:2b (Fig. 1B). The two recombinant and pGreen0229 (backbone alone) binary vectors were independently transferred into *A. tumefaciens* strain AGL1, with helper plasmid (pJIC) by electroporation (McCormac et al., 1998).

Plants. Wild-type and GFP-transgenic *N. benthamiana* were used for the transient expression by *Agrobacterium*-infiltration. The GFP-transgenic plants were developed by the procedure of leaf-disc transformation with *A. tumefaciens* AGL1 harboring pGreen0229-35S:GFP (Fig. 1). The various GFP-transgenic lines with differential levels of green fluorescence *in planta* were selected, and maintained up to T_2 generation. They were used to investigate the suppressive effect of the 2b protein in the initiation of PTGS pathway.

Agro-infiltration procedure. Three *Agrobacteria* carrying

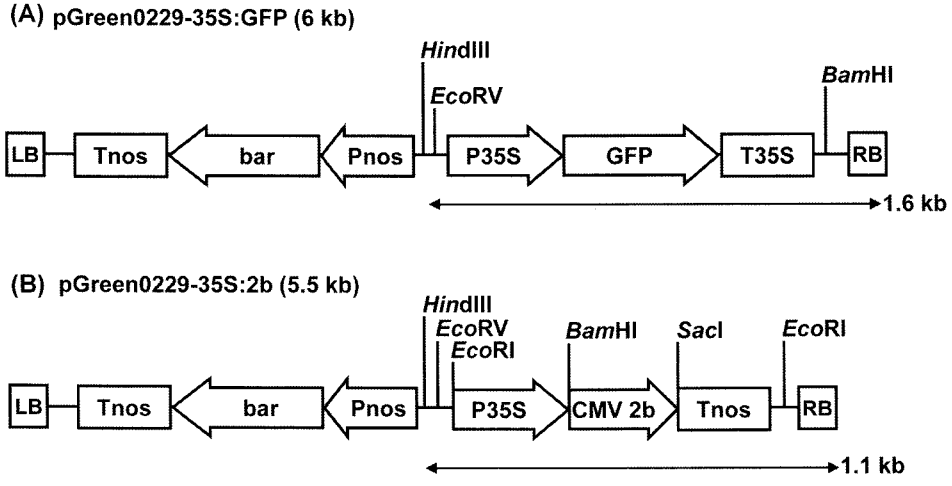

Fig. 1. The schematic representation of T-DNA region of pGreen0229-35S:GFP and -35S:2b binary vectors. The cassette of P35S-GFP-poly(A) (*Eco*RV fragment) was ligated into pGreen0229 at the site of *Eco*RV in multi-cloning sequences (MCS, A). The 2b was cloned from cucumber mosaic virus (CMV) by RT-PCR, and then inserted into pGreen0229 at the site of *Eco*RI of MCS under control of 35S promoter (B). The restriction site of *Hin*dIII was used for Southern blot analysis to investigate the integration pattern of T-DNA in agro-infiltrated leaves. The nucleotide sequence of 2b was previously registered to the GenBank (NCBI) with an accession number of AY827561.

pGreen0229 (mock), pGreen0229-35S:GFP or pGreen0229-35S:2b were commonly grown in LB medium containing 100 mg/l ampicillin, 50 mg/l kanamycin and 5 mg/l tetracycline for 2 days at 28°C. Each of *Agrobacterium* culture was resuspended in the infiltration solution containing 10 mM $MgCl_2$ and 0.1 mM acetosyringone (Voinnet et al., 2003). The concentration of *Agrobacterium* was adjusted by measuring the optical density (OD) at 600 nm.

Southern and northern blot analysis. Total genomic DNA was isolated from the infiltrated leaves using the plant DNAzol® reagent (Invitrogen, USA). Ten microgram of DNA was digested with *Hin*dIII which is the unique site of T-DNA region, and then electrophoresed on a 0.6% (w/v) agarose gel. The DNA was transferred onto Hybond™ N^+ nylon membrane (Amersham Bioscience, UK), and subsequently, pre-hybridized with 20 μg/ml salmon sperm DNA at 65°C in the solution of 5×SSC, 2×Denhardt's solution, 0.1% (w/v) SDS at least for 4 hours. The probes specific to the GFP and 2b genes were labeled with ^{32}P-dCTP (Perkin-Elmer, USA) by random prime labeling system (Rediprime™ II, GE Healthcare, UK). The ^{32}P-dCTP labeled probe was added, and hybridization was performed at least for 12 hours at 65°C. The membrane was subsequently washed with the solution of 1×SSC (10 min), 0.5×SSC 0.1% SDS (20 min), 0.1×SSC 0.1% SDS (20 min) at 65°C.

Total RNA was extracted using Trizol reagent (Invitrogen, USA). Ten microgram of the RNA was fractionated by electrophoresis on 1.2% agarose-formaldehyde denaturing gel, subsequently transferred onto the nylon membrane. The RNA was hybridized with the probes, and washed as described for Southern blot analysis. To visualize the bands, the membrane was exposed to Phosphor imaging screen (Kodak, USA) for 2 to 16 hours and then analyzed by Molecular Imager FX system (Bio-Rad, USA).

Western blot analysis. Total soluble proteins were extracted from the infiltrated leaves in a protein extraction buffer (20 mM Tris-HCl pH 8, 5 mM EDTA, 1 mM DTT, Sigma Protease Inhibitor Cocktail). Protein extracts were centrifuged at 15,000 g for 30 min at 4°C and separated by 12.5% SDS-PAGE, and subsequently blotted onto PVDF (Polyvinylidene fluoride) membranes (Amersham Bioscience, UK). Membranes were blocked with 5% skim milk in TBST (Tris-buffered saline solution containing Tween-20) buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 30 min, incubated with anti-GFP polyclonal antibody (Clontech, USA) in TBST buffer (1:1,000 dilution) for 2 hr at 25°C and washed three times with TBST buffer. The membranes were incubated for 2 hr with horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Bioscience, UK) in a 1:5,000 dilution and washed three times again with TBST buffer. The peroxdiase reaction was detected by chemiluminescence method with Super Signal West Dura (Pierce, USA).

Measurement of leaf growth. Leaf growth was monitored to investigate a possible adverse effect, which may be induced during the transient expression of GFP and 2b protein. Three *Agrobacteria* of mock, GFP and 2b were separately cultured, and the final concentration of each *Agrobacterium* was adjusted to OD_{600nm}=0.4. Three *Agrobacterium* inocula (mock, GFP and GFP+2b) were prepared for leaf infiltration. The GFP and GFP+2b indicate the equal mixes of *Agrobacterium* harboring pGreen0229-35S:GFP with that of mock and pGreen0229-35S:2b, respectively. Each *Agrobacterium* inoculum was infiltrated into whole areas of ten different leaves, and the growth of leaves was monitored by measuring both leaf length and width at 0, 3, 5, 7 and 12 days post-infiltration (dpi).

Results

The Optimal condition of Agro- infiltration for transient expression. Wild-type *N. benthamiana* was infiltrated to find out the optimal concentration among various concentrations of the GFP and GFP+2b inoculum ranged from OD_{600nm}=0.04 to 1.6. To compare the enhanced effect of the 2b, the GFP and GFP+2b inocula were infiltrated into the left and right side on the same leaf, respectively and the GFP expressions were photographed at 3, 7, 12 and 17 dpi (Fig. 2). Until 3 dpi, the expressions of the infiltrated zones between with the GFP and GFP+2b looked similar regardless of the presence of the 2b, and the GFP expression strengths above the concentrations of OD_{600nm}=0.04 seemed to be identical. However, at 7 to 12 dpi, the expression only in the presence of the 2b became gradually strong, on the contrary the GFP gradually weakened in the absence of the 2b. It is obvious that the 2b plays a crucial role on the enhancement of co-expressed GFP gene, and the GFP strength was proportionally increased as *Agrobacterium* concentration, however, it was observed to be almost identical beyond the concentrations of OD_{600nm}=0.2. At 17 dpi, the GFP was weakly detected only in the zone with the presence of the 2b, and its strength extremely weakened. As an adverse effect, high concentrations of inocula of OD_{600nm}=0.8 to 1.6 induced the severe disruption on leaf tissue of the infiltrated zones especially infiltrated with GFP+2b. It suggested that the 2b had a detrimental effect on the leaf vitality. Conclusively, the infiltrated zones in the presence of the 2b persistently showed higher and longer expressions of GFP up to 17 dpi than those in the absence of the 2b. Taken together with the leaf vitality of infiltrated

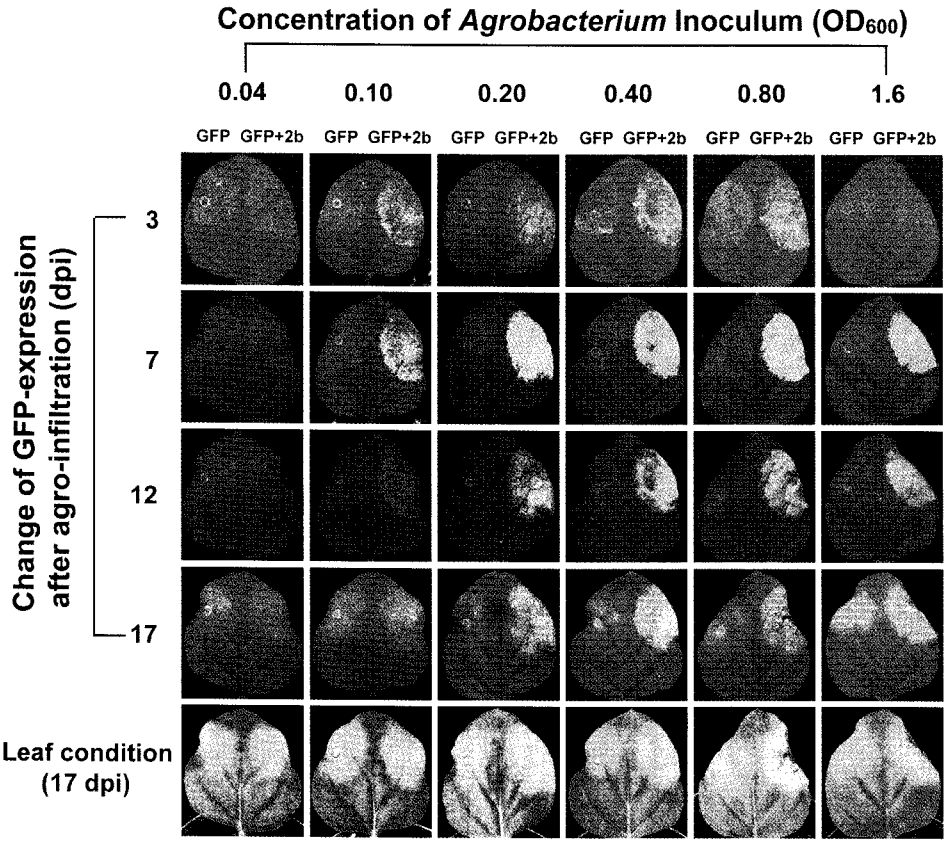

Fig. 2. The change and strength of GFP-expression based on a variety of concentrations of *Agrobacterium* inocula on the wild-type of *N. benthamiana*. The concentration of *Agrobacterium* was measured by spectrophotometer at the wavelength of $OD_{600\ nm}$. The GFP+2b on the right-side top of photos indicates the equal mix of two *Agrobacterium* and pGreen0229-35S:GFP and of pGreen0229-35S:2b. In case of GFP, on the left-side top, the mock *Agrobacterium* of pGreen0229 was equally mixed with that of pGreen0229-35S:GFP to equalize the total number of *Agrobacterium* with that of GFP+2b. The GFP was expressed stronger and maintained longer only in the zones of GFP+2b than in that of GFP alone. In 17 dpi, the tissues, which were infiltrated with presence of *Agrobacterium* containing the 2b gene, were severely destroyed more than with absence of the 2b gene. Each infiltrated leaves was maintained intact to understand a change of GFP expression and photographed at 3, 7, 12 and 17 dpi.

zones, the *Agrobacterium* concentration of OD_{600nm}=0.4 was determined as the optimal concentration to minimize a possible damage and maximize a foreign protein production on the infiltrated leaves until 12 dpi.

Molecular analysis of GFP-expression. The steady-state levels of GFP RNA and protein were analyzed to understand the gene expression change by the 2b in the transient expression (Fig. 3). The GFP RNA level commonly peaked at 3 dpi in the presence or absence of the 2b, and then declined with the passage of time. However, the GFP RNA was highly accumulated in the presence of the 2b, and the RNA was still detected even at 7 dpi in the presence of the 2b (Fig. 3B). It indicated that the 2b induced the high and long-term expression of co-infiltrated GFP gene. On the other hand, the 2b RNA levels were not likely affected by the presence of the GFP RNA and protein. It was because the 2b RNA levels between the zones of GFP+2b and 2b alone were observed to be almost identical. In the GFP protein levels, higher accumulation was commonly observed in the presence than absence of the 2b through the entire period of time (Fig. 3C). Conclusively, the 2b undoubtedly leaded to the higher and longer steady-state levels of GFP RNA and protein. It means that the 2b can be used as an enhancing factor in the agro-infiltrated transient expression system.

Effect of the transient expression to leaf growth. It was confirmed that the compulsory transient expression *via* agro-infiltration generally repressed the leaf programmed growth while it was active (Fig. 3D). The distinctive repression effect on leaf growth was detected from 5 dpi on the leaves infiltrated with the inocula of GFP, GFP+2b and 2b except the mock. The leaves infiltrated with the mock fully grew at 5 dpi, and then entered the stage of senescence rapidly than those with other inocula. In the leaves with the inoculums of GFP alone, the delayed full-growth reached at 12 dpi to the extent that the leaves with the mock had

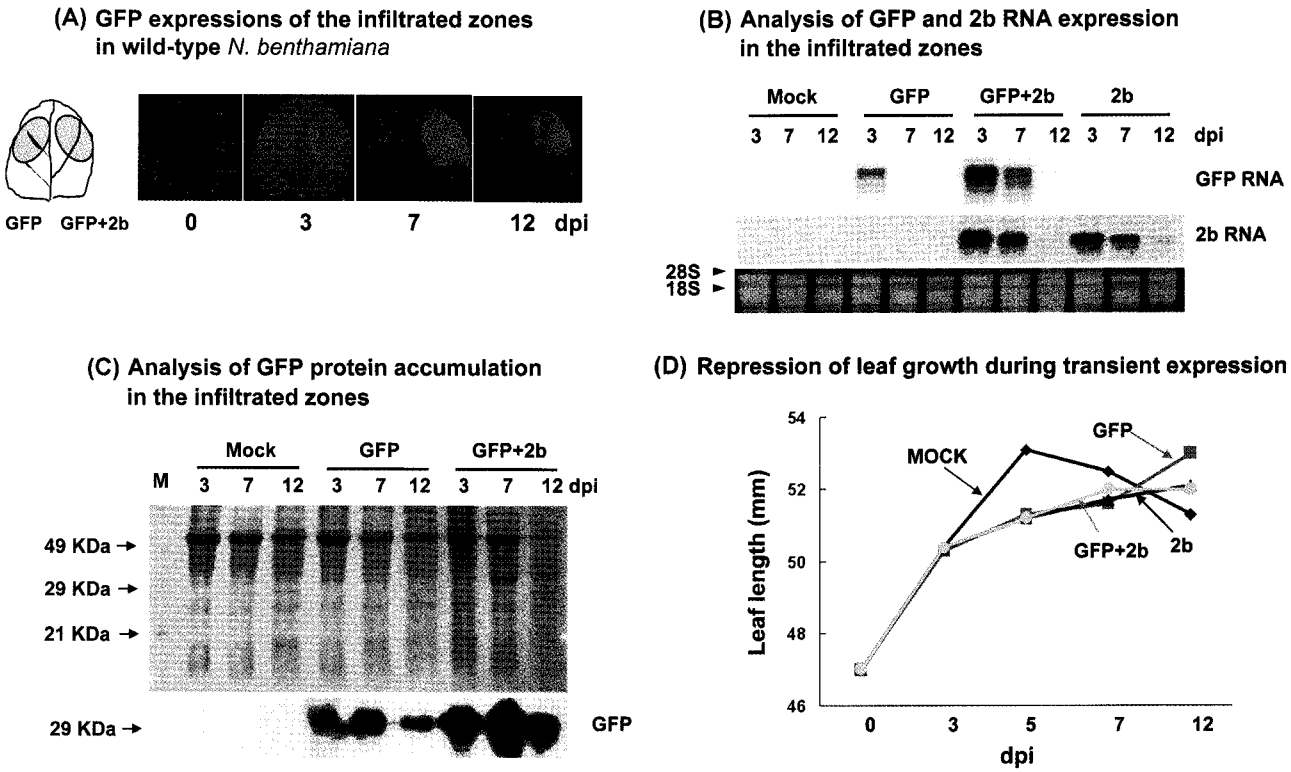

Fig. 3. Effect of the 2b on the transient expression in wild-type *N. benthamiana*. The *Agrobacterium* inocula of GFP and GFP+2b were infiltrated on the left and right sides of leaves, respectively, and the GFP expressions of the infiltrated zones were photographed in 0, 3, 7, and 12 dpi (A). The northern blot analysis of the GFP and 2b RNA in the each infiltrated zones (B). The western blot analysis of GFP protein was performed in the zones of mock, GFP and GFP+2b (C). To examine the effect on the leaf growth, the *Agrobacterium* inocula of the mock, GFP, GFP+2b and 2b were infiltrated into the whole leaf areas of each 10 different leaves, respectively. Leaf growth rate was measured by the length (mm) of the leaves until 12 dpi, and the leaf average lengths for each inoculum were equalized to compare the growth rates each other (D). The mock and M on the Coomassie® blue-stained gel at (C) indicate the *Agrobacterium* inoculum harboring the pGreen0229 backbone vector and the protein size markers, respectively.

reached at 5 dpi. However, the growth repression was persistently maintained up to 12 dpi in the leaves with the inocula of GFP+2b and 2b alone. Such differences of the growth patterns can be explained based on the previous molecular analysis (Fig. 3B). In the leaves infiltrated with GFP alone, the GFP RNA was no more detected at 7 dpi. It indicated that the transient expression was eventually terminated, and that the termination conferred the opportunity upon the infiltrated leaves to return to their normal programmed growth. On the other hands, the leaves infiltrated with GFP+2b and 2b alone were continuously repressed in the growth until 12 dpi (Fig. 3D). It suggests that such the compulsory transient expression seemed to interfere with the plant programmed growth, and that must have converted a great deal of resources available for plant growth to the strong transient expression of foreign proteins. During that process, the 2b must be a leading factor to keep such conversion process stronger and longer. Conclusively, the transient over-expression can be a serious effect on the regular distribution of all the resources for plant growth.

Southern blot analysis of infiltrated leaves. The genomic DNA from the infiltrated leaves was subjected to Southern analysis to identify the integration patterns of the GFP and 2b gene into plant genome. The T-DNA regions of pGreen0229-35S:GFP and pGreen0229-35S:2b commonly have a digestion site of *Hin*dIII at the front of 35S:GFP and 35S:2b cassette (Fig. 1). The digested genomic DNA was theoretically expected to produce numerous sizes of the GFP-genomic DNA fragments over 1.6 kb (35S:GFP) or 1.1 kb (35S:2b) due to the random integrations of multiple T-DNAs into the genomic DNA by a large number of infiltrated *Agrobacteria*. However, the integration pattern was not shown as expected. Moreover, about 3 major bands (the red-colored arrows) were commonly detected in both inocula of GFP and 2b, even though their sizes were different each other (Fig. 4). In fact, the locations of the major bands for 35S:GFP were almost overlapped with those of *Agrobacterium* harboring pGreen0229-35S:GFP (Fig. 4A). It suggested that there might be a possibility for the existence of intact *Agrobacteria* in the infiltrated zone, and their DNAs were also extracted together, and finally

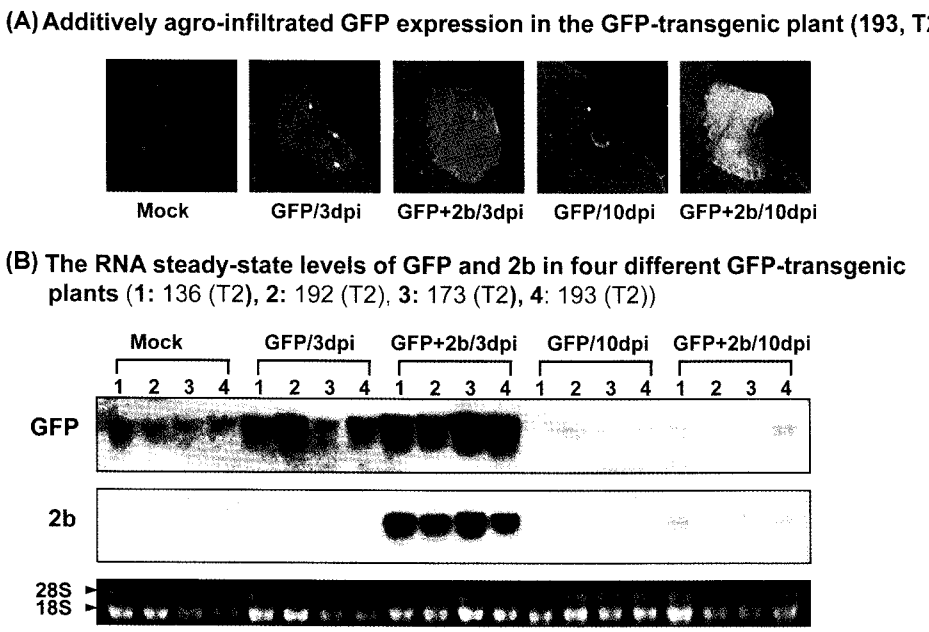

Fig. 4. The over-expression and PTGS suppression mediated by the 2b in the GFP-transgenic plant. Diverse types of GFP-expressing transgenic tobaccos were previously developed and had maintained their pedigree up to T_2 generation. Four different transgenic lines, which expressing green fluorescence uniformly throughout the whole plant, were selected among the population of the transgenic plant lines. The four transgenic lines (T_2 progenies of 136, 192, 173 and 193 line, respectively) were infiltrated with the mock, GFP and GFP+2b inocula, and the 193 lines was representatively photographed under the UV light at 3 and 10 dpi (A). The infiltrated zones of each transgenic lines at 3 and 10 dpi were used for northern blot analysis of the GFP and 2b RNA (B). The GFP RNA gel blot analysis was performed and followed by the 2b RNA analysis with the same nylon membrane.

confounding Southern blot analysis for plant genomic DNA. Substantially, a large amount of *Agrobacterium* was injected into the intracellular spaces of a leaf tissue by agro-infiltration. So, it is too hard to expect the regular pattern of T-DNA integration into plant genomic DNA.

Suppression of PTGS in GFP-transgenic plant. The *Agrobacterium* inocula of GFP and GFP+2b were infiltrated into GFP-transgenic plants, which were previously developed by leaf-disc transformation *via A. tumefaciens* pGreen0229-35S:GFP (Fig. 1A). Four different GFP-transgenic lines (T_2 progenies of 136, 192, 173 and 193 line, respectively), in which the GFP was expressed uniform in whole plant, were selected to verify the suppressive effect of the 2b to PTGS. The green fluorescence commonly grew strong in the additively infiltrated zones with the GFP alone and GFP+2b inoculum at 3 dpi, and then the zone with the GFP alone turned red at 10 dpi (Fig. 5A). This implies that the additive expression by the infiltrated GFP acts as a signal to trigger the activation of PTGS pathway, thereby the transgenic GFP of the transgenic plants became co-silenced with nucleotide sequence homology to

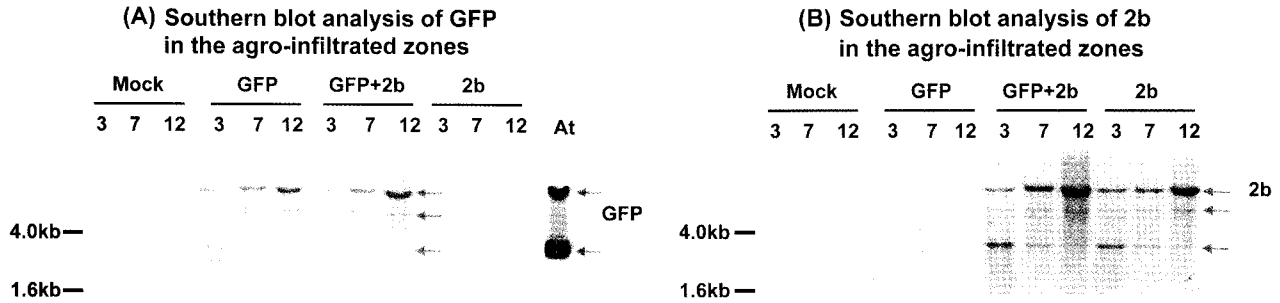

Fig. 5. The Southern blot analysis for the GFP and 2b genes in agro-infiltration leaves. The *Hin*dIII-digested plant genomic DNAs as described in Fig. (A, B) were electrophoresed and transferred onto nylon membrane. The DNA gel blot was performed first with the ^{32}P-labeled GFP probe (A), and then 2b probe (B). The *Hin*dIII-digested DNA from *Agrobacterium* containing pGreen0229-35S:GFP (At on the left of (A)) was also subjected to compare the band pattern with that from the infiltration leaves. The mock indicates the *Agrobacterium* inoculums harboring the pGreen0229 backbone vector. The red-colored arrows indicate the major bands.

the additively infiltrated gene. However, the zone with GFP+2b inoculum strongly maintained the high level of green fluorescence until 10 dpi, and the lesser amount of GFP RNAs were still detected as well as the 2b RNA (Fig. 5B). It was demonstrated once more that the 2b could suppress the initiation of PTGS even in the abnormal accumulation of the excessive foreign proteins. Such suppressive effect to PTGS is recognized to facilitate the persistent accumulation of foreign protein as long as the 2b protein is active.

The threshold level of PTGS. The additive infiltration of GFP into the GFP-transgenic plants clearly induced the GFP co-silencing by the nucleotide sequence-specific degradation. In order to find out the existence of a buffer zone or threshold level before the PTGS is initiated, firstly five different concentrations of *Agrobacterium* inocula containing GFP alone and GFP+2b with the OD_{600nm}= 0.0125 to 0.2 were infiltrated into the GFP-transgenic plants (T_2 progenies of line 193, Fig. 6A). Although the infiltrated GFP was additively expressed plus the transgenic GFP until 4 dpi, such additive GFP RNA was believed to trigger the initiation of PTGS. Subsequently, all the concentrations of *Agrobacterium* inocula in the absence of the 2b led to the entire co-silencing of GFP in the transgenic plants at 8 dpi. However, in the presence of the 2b the GFP expression was maintained high until 8 dpi without the co-silencing (Fig. 6A). Secondly, four different transgenic plants, which consisted of two strong (T_2 progenies of 105 and 136) and two weak (178 and 181) GFP-transgenic plants, were infiltrated with the much lesser concentrations (OD_{600nm}=0.0002 to 0.2) of *Agrobacterium* inocula (Fig. 6B). The common feature was the co-silencing of transgenic GFP at 7 dpi in the absence of the 2b due to the

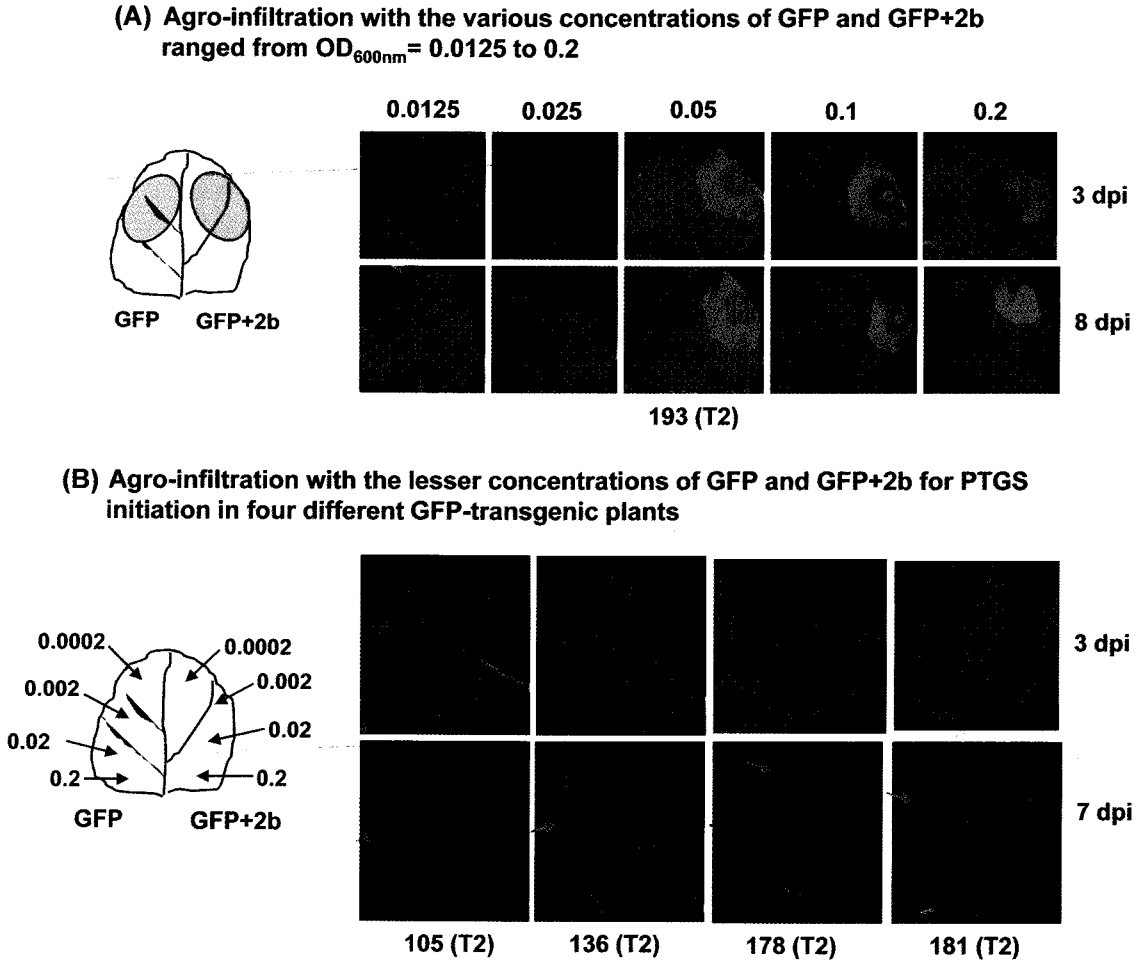

Fig. 6. Analysis of the threshold model for PTGS initiation with the GFP-transgenic plants. The T_2 progenies of transgenic line 193 were infiltrated with *Agrobacterium* inocula (GFP and GFP+2b) of the various concentrations ranged from OD_{600nm}= 0.0125 to 0.2 (A). The T_2 progenies of four different transgenic line 105, 136, 178 and 181 were infiltrated with *Agrobacterium* inocula (GFP and GFP+2b) of the various concentrations ranged from OD_{600nm}=0.0002 to 0.2 (B). The red and yellow-colored arrows indicate the infiltrated zones where the transgenic GFP became co-silenced. The schematic diagrams on the left of photos indicate the sites and the concentrations of each *Agrobacterium* inocula.

additively infiltrated GFP. However, a peculiar appearance was observed from the two zones of the concentrations (OD_{600nm}=0.0002 and 0.002). In those zones, the additive GFP-expression was not detected at 3 and even 7 dpi regardless of the presence or absence of the 2b. It was likely that such the concentrations of infiltrated GFP were extremely too low to show the additive GFP-expression. Nevertheless, the transgenic GFP at the zones of the concentration of OD_{600nm}=0.002 became definitely co-silenced at 7 dpi (indicated by the yellow-colored arrows in Fig. 6B). Even in the weak GFP-transgenic plants (T_2 progenies of 178 and 181), the GFP co-silencing was also observed. It indicated that even a tiny concentration, even though it did not give rise to the additive GFP-expression, resulted in the co-silencing of transgenic GFP by PTGS. Considering the results from the concentration of OD_{600nm}= 0.2, the threshold model could be logically valid. The additive GFP-expression might have exceeded a threshold level which a transgenic plant allowed. Thereby, the PTGS was finally triggered by such aberrant GFP-expression. However, in the concentrations of OD_{600nm}=0.002 and 0.02, PTGS was triggered unless the additive GFP-expression was detected. Especially, the concentration of OD_{600nm}= 0.002 was recognized as the extremely low concentration not to show a trace of the additive GFP-expression even in the presence of the 2b. It suggested that the existence of a threshold level or buffer range covering the additive GFP-expression appeared questionable before PTGS was initiated. Based on the results (Fig. 6B), the GFP-transgenic plant must be allowed to express its transgenic GFP at the accurately pre-determined level. And if the balanced level is disturbed by even a trace of additive GFP, PTGS seems to be immediately activated to eliminate the aberrant RNA as a self-defensive mechanism.

Discussion

The agro-infiltration has been recently accepted as a simple and rapid technique for expressing foreign proteins in plant cells. Although the GFP in this study was used as the reporter to evaluate the enhancing effect of the 2b for the excessive protein expression, other useful proteins can be also applicable in this system. To achieve such the expression, the 2b should be expressed simultaneously together with the useful genes. The correct function of the 2b protein has not been fully understood so far. The recent reports postulated that the 2b should not be only an effector of the transcription factor in the nucleus (Goto et al., 2007), but also an inhibitor of the endo-nuleolytic activity of the RISC complex of PTGS pathway in the cytoplasm (Zhang et al., 2008). It is certain that these postulated functions should contribute to the excessive expression of foreign genes. According to the screening results of *Agrobacterium* concentration for transient expression (Fig. 2), it should be necessary to previously discover an optimal concentration minimizing the detrimental effect to leaf vitality. The integration pattern of *Agrobacterium* T-DNA into plant genome has been identified in some previous reports (De Buck et al., 2000; Abdal-Aziz et al., 2006). Many transgenic plants were revealed to contain the vector backbone sequence together with intact T-DNA sequence, and even more some of them contained the entire sequence of vector backbone (Kim et al., 2003). It proved that the possibility for the integration of entire vector with T-DNA region was quite higher than expected. To decrease the co-transfer of the backbone sequences, additional left border (LB) sequences were inserted close to the original LB (Kuraya et al., 2004). It was believed that the co-transfer might be certainly derived from the inefficient termination at the LB sequence of T-DNA region. Practically, a large number of infiltrated *Agrobacterium* can separately transfer their T-DNA and backbone sequences into diverse sites of plant genome, so that it might be impossible to identify a regular pattern of T-DNA insertion.

The PTGS has been primarily recognized as a defensive mechanism for an aberrant gene expression, for instance, viral RNA amplification in the plant cytoplasm. Two signals have been proposed for the PTGS initiation in plants. The first one is a dsRNA which is produced by read-through transcription of the inverted repeat (IR) arrangement of multiple transgenes. The second is the excessive RNA transcripts beyond a threshold level. Such signals have been reported to trigger efficiently the initiation of PTGS (Meins, 2000; Schubert et al., 2004; Vaucheret et al., 2001). That is, the PTGS seems to be a self-defensive and sophisticated mechanism to control the status of gene expression against the aberrant RNAs. The concept of 'the threshold level' is generally regarded that there should be a certain extent of the buffer range covering the additive RNA transcripts before switching to the PTGS. Taking account of the GFP-silencing (Fig. 4 and 6A) in the absence of the 2b, the co-silencing of transgenic GFP by agro-infiltration might be caused by the additive GFP transcripts that exceed threshold levels which each transgenic plants differently allowed. However, the extremely low concentrations of *Agrobacterium* of OD_{600nm}=0.002 were also revealed to trigger unexpectedly the PTGS even though it did not apparently induce the additive GFP-expression on the infiltrated zones (Fig. 6B). This finding is the first report to demonstrate that neither the buffer range nor the threshold level exists in the PTGS initiation. A new hypothesis should be developed to elucidate that finding. That is, the transgenic plant itself is likely destined to express its transgenes at 'the accurately pre-determined

level' by certain endogenous factors in plant, although further researches should be needed to figure out such factors. In that point of view, the PTGS can be regarded as a internal balancing system for the gene expression, so it can be activated by a trace of aberrant RNA transcripts.

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