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Use of an Attenuated Strain of Tobamovirus for Early Detection of Virus-Induced Gene Silencing

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Abstract

Virus-induced gene silencing (VIGS) can be used to study gene function by mediating sequence-specific mRNA degradation and suppressing the expression of endogenous target genes. We previously demonstrated that the TocJ vector based on the tomato mosaic tobamoviruses (ToMV) was able to multiply, spread systemically and express green fluorescence protein in *Solanaceous* plants. TocJ harbouring fragments of endogenous genes could induce VIGS of the parental gene expression, but also induced viral infection symptoms. In this study, an attenuated strain of ToMV, $L_{11}A$, was used to construct a ToMV vector in order to reduce the virus-induced symptoms. This new vector, named LcJ, was able to spread systemically and mediated VIGS of endogenous genes without visible symptoms. We propose that the use of this attenuated strain for the construction of virus vectors is beneficial for the induction of VIGS.

Key words: attenuated strain, GATEWAY system, tobamovirus, tomato mosaic virus, virus vector, virus-induced gene silencing.

Abbreviations

CP, coat protein; dpi, days post inoculation; GFP, green fluorescent protein; hpi, hours post inoculation; PDS, phytoene desaturase; sg mRNA, subgenomic messenger RNA; TMGMV, tobacco mild green mosaic virus; TMV, tobacco mosaic virus; ToMV, tomato mosaic virus; VIGS, virus-induced gene silencing; wpi, weeks post inoculation.

Introduction

Plant virus vectors harbouring a segment of the host gene sequence can induce silencing of its counterpart endogenous gene in infected plants. This phenomenon is called virus-induced gene silencing (VIGS) and occurs as an RNA-mediated defence mechanism against invading genetic materials in plants (Baulcombe, 1996). The VIGS system has been developed using several different virus vectors. The first demonstration of endogenous gene suppression with VIGS was shown with tobacco mosaic virus (TMV) harbouring a fragment of the phytoene desaturase (PDS) gene (Kumagai *et al.*, 1995). *Nicotiana benthamiana* plants infected with this virus showed photobleaching and accumulation of phytoene. Potato virus X (PVX) and tobacco rattle virus (TRV) vectors were constructed and improved (Ruiz *et al.*, 1998; Ratcliff *et al.*, 2001). The PVX vector was applied for the suppression of cellulose synthase (Burton *et al.*, 2000). The TRV vector has accumulated the most application examples. For example, genes that are related to the *N*-gene hypersensitive response, such as the enhanced disease susceptibility 1 (*EDS1*) gene, have been analyzed (Liu *et al.*, 2002b; Peart *et al.*, 2002). The barley stripe mosaic virus (BSMV) vector can induce VIGS in barley which is a monocot plant (Holzberg *et al.*, 2002). Some DNA viruses can also induce VIGS (Kjemtrup *et al.*, 1998; Peele *et al.*, 2001; Turnage *et al.*, 2002).

Each vector has different characteristics for the host range, spreading speed, virus symptoms. In addition, induction of VIGS by the amplicon system with *Agrobacterium* (Angell *et al.*, 1997), and improvement of virus vectors through DNA shuffling (Toth *et al.*, 2002), small inverted repeats (Lacomme *et al.*, 2003) and satellite RNA inserted with the target sequence (Gossele *et al.*, 2002) have contributed to the improvement of the VIGS system.

Previously, we reported the establishment of the TocJ vector which can harbour foreign gene se-

quences, multiply, spread systemically and express green fluorescence protein (GFP) in *Solanaceous* plants (Hori and Watanabe, 2003), like the 30B vector reported earlier (Shivprasad *et al.*, 1999). Tobamovirus vectors can be a powerful tool for VIGS elicitation taking advantage of the rapid multiplication. We found that TocJ effectively induced VIGS, but also induced severe symptoms in the infected plants. Virus symptoms make it difficult to analyze the phenotype induced by suppression of the target gene.

In this study, we improved the ToMV-based vectors for inducing gene silencing in plants. One of these vectors, LcJ, could elicit VIGS of endogenous plant genes without exhibiting any visible symptoms after infection. Accumulation of the LcJ vector in plants was less than that of the wild type vector TocJ, and the symptoms were invisible. However, LcJ could induce VIGS earlier than TocJ. Most virus vectors for VIGS induction have the problem that the virus symptoms mask the phenotype induced by VIGS. We propose that the use of this attenuated strain as a virus vector is effective for the improvement of virus vectors.

Materials and Methods

Plasmid Construction

TocJ and TocJ/GFP have been described previously (Hori and Watanabe, 2003). A fragment of pLFA1 (Meshi *et al.*, 1986), a biologically active clone of $L_{11}A$, was used to replace the segment of TocJ, TocJ/GFP and TocJ/PDS vectors by *Bst*XI and *Bsp*EI sites to generate LcJ, LcJ/GFP and LcJ/PDS, respectively.

The TogJ vector gained *att*B1, *ccd*B, Cm^r and *att*B2 by inserting the GATEWAY conversion cassette B (Invitrogen) which added *Sac*I sites by PCR (primers used: forward h5′-CGTAGAGCTCATCAACAAGTTTGTACAAAA-3′ and reverse 5′-GTAACGCGTGAGCTCATCAACCAC- TTT GTACAAG-3′) into LcJ at the *Sac*I site. A fragment with *Eco*RV and *Bsu*36I of LcJ vector was replaced with the corresponding segment of TogJ to generate LgJ vector.

Cloning of the GFP gene and PDS sequence

The partial 548 bp ORF of *NtPDS* (corresponding to nt 891-1438 of the *N. benthamiana* cDNA, GenBank Accession No. I23875) was amplified by PCR (primers used: forward 5′-TGGATCCATCTAAAGAATAACGAAATGC-3′ and reverse 5′-TGGATCCTCAGTTTCTGTCAAACCATAT -3′) and inserted into *Xcm*I-treated TocJ, to create TocJ/PDS (**Fig. 1**). The ORF of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was amplified by PCR. The fragment containing the *att*B1 and *att*B2 sequences was amplified by PCR twice (primers used for the first PCR: forward 5′-AAAAAGCAGGCTAAAACAATGAGTAAAGGAGAAG-3′ and reverse 5′-AGAAAGCTGGGTTTATTTGTATAGTTCATC-3′; primers used for the second PCR: forward 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and reverse 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′). This GFP PCR product was recombined into pDONR201 (Invitrogen) using BP CLONASE enzyme. The GFP sequence was further recombined from pDONR201 into LgJ using LR CLONASE enzyme to generate LgJ/GFP.

The partial 193 bp and 100 bp ORF of *NtPDS* (corresponding to nt 1246-1438 and 1339-1438 of the *N. benthamiana* cDNA, GenBank Accession No. I23875) were PCR-amplified using appropriate primers, and then the products were recombined into LgJ to generate LgJ/PDS_{200} and LgJ/PDS_{100}, respectively, as described above (primers used for the first PCR for LgJ/PDS_{200}: forward 5′-AAAAAGCAGGCTAAAGATTGAGCTGAATGAGGATGG-3′ and reverse 5′-AGAAAGCTGGGTAACGTGTTCTTCAGTTTTCGATCAAACCATA-3′; primers used for the first PCR for LgJ/PDS_{100}: forward 5′-AAAAAGCAGGCTAGTGGATATCTTCAAGCTTCTTTT-3′ and reverse 5′-AGAAAGCTGGGTAACGTGTTCTTCAGTTTTCGATCAAACCATA-3′; the primers used for the second PCR for LgJ/PDS_{100} and LgJ/PDS_{200} were the same as those used for the second PCR in the GFP cloning above).

Plant materials

Nicotiana benthamiana plants were maintained at 23℃ with a 16 h photoperiod and 8 h dark period.

In vitro *transcription*

The plasmids of series TogJ and LgJ vectors were linearized with *Mlu*I prior to *in vitro* transcription to allow run-off transcription. The *in vitro* transcription reaction was performed using 1 μg linearized vector DNA, 50 units T7 RNA polymerase (Invitrogen), 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.5 mM GTP, and 0.52 mM m7GpppG (NEB) as a cap analogue in 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$, 25 mM NaCl, and 1 mg ml^{-1} bovine serum albumin (BSA) for 60 min at 37℃.

Inoculation of transcripts to protoplasts and plants

Protoplasts were prepared from *N. tabacum* BY-2 suspension culture cells and inoculated with the

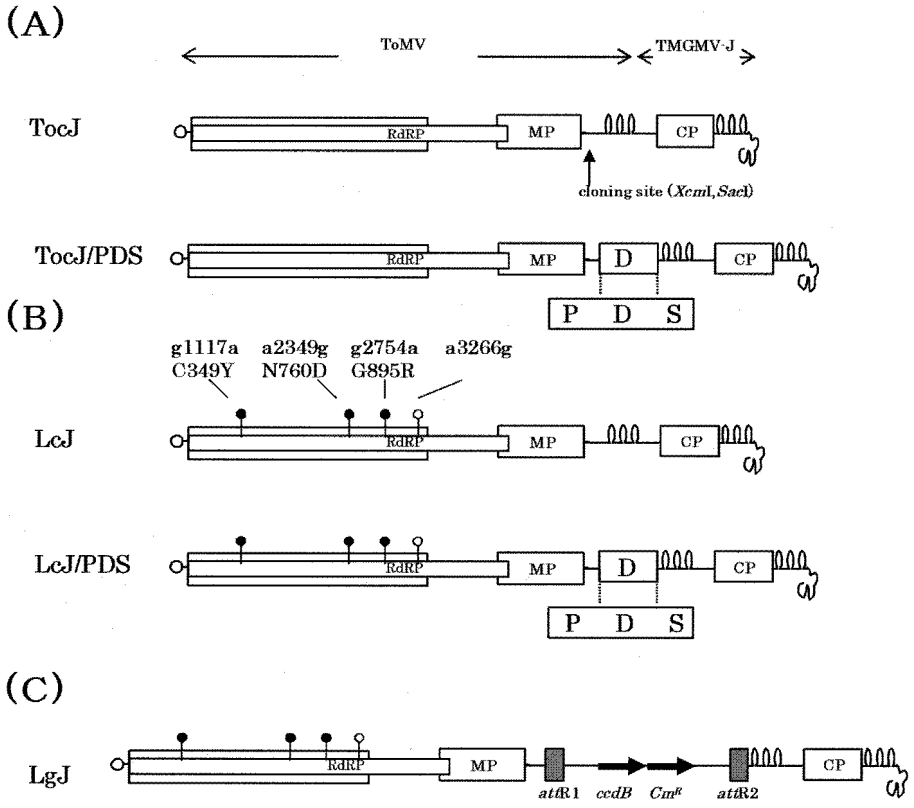

Fig. 1 Schematic represention of the vectors.

The 5′-cap structure is depicted by circles. RNA-dependent RNA polymerase (RdRP), movement protein (MP) and coat protein (CP) genes are represented by rectangles. Pseudoknot regions derived from ToMV and TMGMV are shown by bulges. (A) Construction of TocJ vectors. Partial PDS sequences were inserted into pTocJ using *Xcm*I cloning sites. (B) LcJ vectors. Solid circles indicate amino acid substituting mutations and open circles indicate silent mutations. (C) The LgJ vector contains two recombination sites (*att*R1 and *att*R2) that flank a gene for negative selection, *ccd*B encoding protein toxic to standard *E. coli* strains and the gene for chloramphenicol resistance (Cm^R).

above-mentioned *in vitro* transcripts using electroporation as described previously (Watanabe *et al.*, 1987). Protoplasts were collected 15 h after the culture at 28℃, and used for RNA analysis.

Six-week-old *N. benthamiana* plants were inoculated with the *in vitro* transcripts as described elsewhere (Ohno *et al.*, 1984). After 5 days, inoculated leaves were homogenized in PBS buffer (137 mM NaCl, 8.10 mM Na_2HPO_4-$12H_2O$, 2.68 mM KCl and 1.47 mM KH_2PO_4). The homogenates were then centrifuged for 10 min at 10,000*g*. The homogenates were inoculated on nine-week-old *N. benthamiana* plants.

Northern blotting analysis

Total RNA was purified as previously described (Shirzadegan *et al.*, 1991). Total RNAs were treated with 50% formamide and 2.2 M formaldehyde in MOPS buffer (20 mM 4-morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and separated in 1% agarose gels containing 20 mM MOPS buffer. The RNAs were then transferred onto a Hybond N+ membrane by capillary blotting. After drying at room temperature, the membranes were irradiated with a UV lamp at 70000 $\mu J\ cm^{-2}$ using a UV crosslinker (Stratagene). The tobacco mild green mosaic virus (TMGMV) Japanese strain sequence (5502 bp-6356 bp, GenBank Accession No. AB078435) was subcloned in the pGEM-Teasy (Promega) vector to obtain pTMGMVprobe. A partial PDS sequence (nt 1468-1922, GenBank Accession No. I23875) was subcloned in the pGEM-Teasy (Promega) vector to obtain pPDSprobe. DIG-labelled TMGMV and PDS antisense probes were obtained from pTMGMVprobe and pPDSprobe template DNA, respectively, using a DIG-RNA labelling kit (Roche). TMGMV and PDS probes were hybridized with membranes under high stringency conditions at 68℃ overnight. Membranes were washed twice with 2x SSC containing 0.1% SDS for 15 min each at 68℃, and twice with 0.5x SSC containing

0.1% SDS for 15 min each at 68℃. The hybridized DIG-RNA probes were detected using anti-digoxigenin-Ab Fab fragments (Roche) and a BCIP/NBT membrane phosphatase substrate (KPL).

Results and Discussion

TocJ vectors could elicit VIGS but with severe symptoms

Silencing of PDS leads to the inhibition of carotenoid synthesis, causing plants to exhibit a photobleached phenotype that can easily be detected. Suppression of the PDS gene function has been reported as the test case for gene silencing in many studies employing a variety of molecular tools (Ruiz *et al.*, 1998; Voinnet *et al.*, 2000; Ratcliff *et al.*, 2001; Gossele *et al.*, 2002; Holzberg *et al.*, 2002; Liu *et al.*, 2002a; Lacomme *et al.*, 2003). We inserted a part of the PDS cDNA sequence into our tobamovirus vector, TocJ, to create TocJ/PDS. Nine week-old *N. benthamiana* plants were infected with TocJ/PDS to see whether it could suppress the endogenous PDS gene expression (**Fig. 1A**). The leaves which had already developed before infection showed virus symptoms at two weeks post-inoculation (wpi). Bleached new leaves and stems emerged at 3 wpi and developed during 3-5 wpi. White areas were clearly observed at 5 wpi with virus symptoms (**Fig. 2A**, right). The appearance of the bleached tissues was expected as a result of the VIGS elicitation. Northern blotting analysis was performed to check the accumulation level of PDS mRNA in such leaves. RNA was separately purified from infected green and white leaf areas. It was revealed that accumulation of PDS mRNA was reduced in both the green and white areas of TocJ/PDS infected *N. benthamiana* (**Fig. 3A**). These results suggested that the TocJ/PDS induced gene silencing, or the bleached phenotype, in leaves by targeting endogenous PDS mRNA. However, they also indicated that the level of bleaching would not always predict the level of PDS mRNA. This phenomenon has been similarly shown for any other virus infection (Ruiz *et al.*, 1998). TocJ/PDS infection also resulted in severe virus symptoms like malformation of leaves (**Fig. 2A**, right). The severity of the symptoms was so evident that the symptoms might mask or overestimate the silencing phenotype.

Improvement of the tobamovirus vector by using an attenuated strain as a vector backbone

$L_{11}A$ is a Japanese attenuated strain of ToMV, and was isolated from a virulent L strain of ToMV. It causes barely visible mosaic symptoms in tomato plants in the field, even at later growth stages, and has been used to protect tomato plants from infection with the virulent L strain. $L_{11}A$ has 7 silent and 3 missense substitutions compared with the wild type L strain of ToMV (Nishiguchi *et al.*, 1985). It is likely that these missense substitutions cause attenuation of ToMV (Lewandowski *et al.*, 1993; Hagiwara *et al.*, 2002; Yamamoto *et al.*, 2002). The effectiveness of this virus for use as a vector backbone was tested in a trial to improve the TocJ vector.

A fragment of pLFA1 (Meshi *et al.*, 1986), a biologically active clone of $L_{11}A$, was used to replace the segment of the TocJ vector and introduce the above missense substitutions. This new vector, named LcJ, had a silent nucleotide change of a3266g, and three missense changes of g1117a, a2349g and g2754a leading to amino acid changes C349Y, N760D and G895R, respectively, in the amino acid sequence of replication protein (**Fig. 1B**).

The TocJ and LcJ vectors were separately inoculated into BY-2 protoplasts and their replication abilities were compared. Northern blotting analysis of RNA samples collected at 15 hpi showed that the genomic RNA of the TocJ and LcJ virus vectors accumulated to similar levels (data not shown).

LcJ/GFP, an LcJ-based virus with a full-length GFP sequence inserted, was constructed from TocJ/GFP (Hori and Watanabe, 2003) using the same pLFA1 fragment to evaluate the systemic spreading ability of the LcJ vector and the amelioration of the virus-induced symptoms. In *N. benthamiana* plants, LcJ/GFP caused fluorescent GFP spots of similar sizes and similar fluorescent intensities at 3 dpi to TocJ/GFP (data not shown). In both plants, GFP fluorescence was detected systemically by 6 dpi. The plants inoculated with TocJ/GFP showed severe symptoms at 12 dpi, whereas LcJ/GFP caused no visible symptoms (data not shown). Thus, it was concluded that the LcJ vector with foreign sequences inserted could spread systemically without visible symptoms.

In order to see whether LcJ could induce VIGS, LcJ/PDS, an LcJ-based virus with a part of the PDS sequence inserted, was constructed in a similar way by exchanging the replication protein gene fragment of pLFA1 with that of TocJ/PDS. LcJ/PDS induced the bleached phenotype in *N. benthamiana* at 2 wpi, one week earlier than TocJ/PDS which induced a similar phenotype. Furthermore, LcJ/PDS did not show any visible symptoms at 3 wpi (**Fig. 2B**, right), when TocJ/PDS infection caused symptoms (**Fig. 2B**, middle). Northern blotting analysis showed that PDS mRNA accumulation was de-

Fig. 2 Several plants infected with ToMV-based virus vectors.
(A) Mock (left)-and TocJ/PDS (right)-inoculated *N. benthamiana* plants at 5 wpi.
(B) Mock-, TocJ/PDS- and LcJ/PDS-(left to right) inoculated *N. benthamiana* plants at 3 wpi.
The white area in mock plant is a flower.

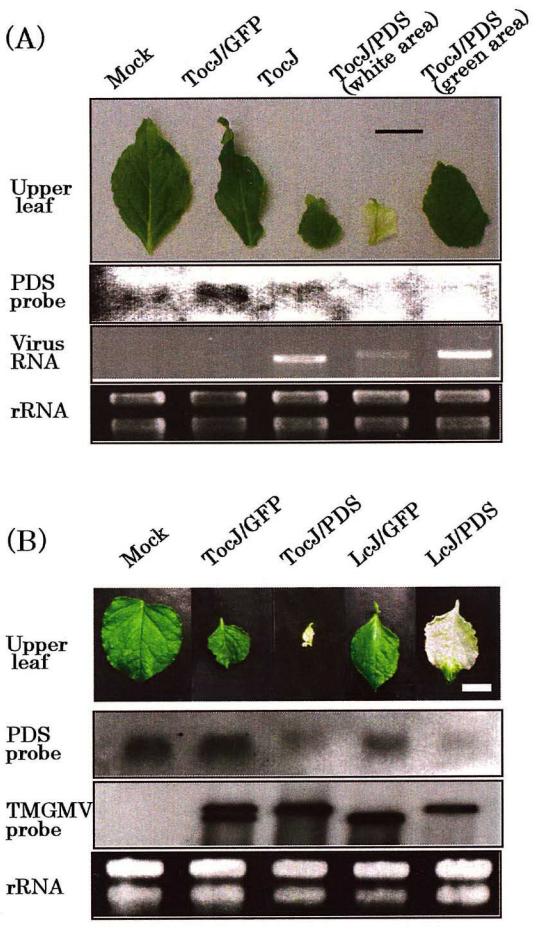

Fig. 3 Virus RNA, and accumulation of PDS mRNA.
(A) A part of the upper leaves above the inoculated ones used for RNA preparation of (left to right) mock, TocJ/GFP, TocJ, TocJ/PDS (white leaves), and TocJ/PDS(green leaves). The RNA samples were taken at 3 wpi.
(B) A part of the upper leaves above the inoculated ones used for RNA preparation of (left to right) mock, TocJ/GFP, TocJ/PDS, LcJ/GFP, and LcJ/PDS in systemically infected leaves. The RNA samples were taken at 3 wpi. The virus RNA and rRNA bands are stained with ethidium bromide. The PDS and TMGMV probe panels are the results of northern blotting analysis. The major genomic RNA is shown in the virus RNA and TMGMV probe panels.

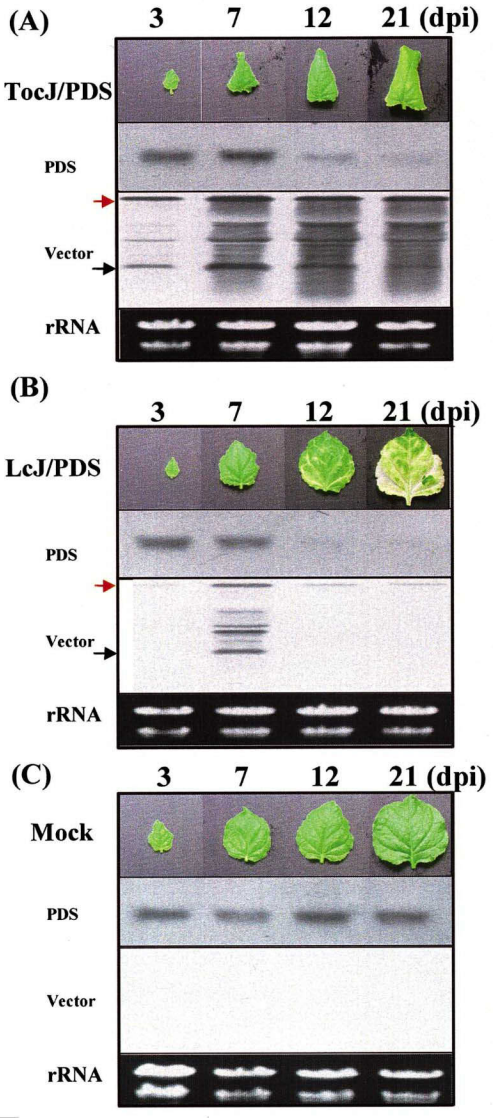

Fig. 4 PDS mRNA and virus RNA accumulation change with VIGS.
The fifth or sixth leaves above the TocJ/PDS-(A), LcJ/PDS-(B) and mock-(C) inoculated leaves at 3, 7, 12 and 21 dpi, from which RNA was extacted. The PDS and Vector panels are the results of northern blotting analysis with the PDS and TMGMV probe, respectively. Red and black arrows, respectively, indicate the virus genome RNA and virus CP subgenomic RNA. The rRNA observed after ethiduim bromide staining.

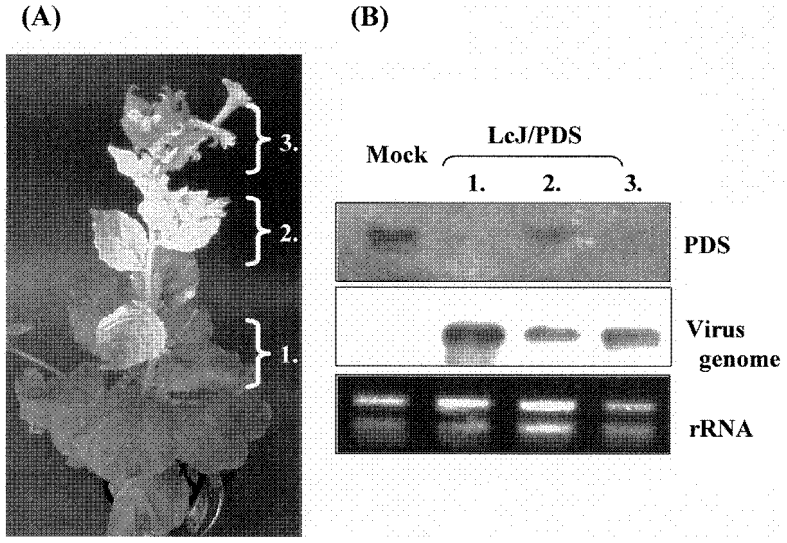

Fig. 5 Reversion of the VIGS bleaching phenotype.

(A) *N. benthamiana* plant inoculated with LcJ/PDS after 5 wpi. Tissue 1 represents the lower green leaves which were fully developed at the inoculation. Tissue 2 represents the white areas which were fully bleached. Tissue 3 represents upper most mottled leaves.

(B) The rRNA panel shows rRNA stained with ethidium bromide. The PDS and vector panels are the results of northern blotting analysis with the PDS and TMGMV probe, respectively. The major genomic RNA is shown in vector panel. RNA samples from Tissue 1 - 3 (1 - 3) and mock plants were taken at 3 wpi.

creased in plants inoculated with LcJ/PDS to a similar level to that in plants infected with TocJ/PDS at 3 wpi (**Fig. 3B**). The data suggest that the LcJ vector is advantageous for the induction of gene silencing, in terms of the lack of severe symptoms and the efficacy of suppression of endogenous gene function.

The fifth and sixth leaves above the leaves inoculated with TocJ/PDS and LcJ/PDS were compared in detail at 3, 7, 12 and 21 dpi. LcJ/PDS induced bleaching along the leaf vein from 12 dpi (**Fig. 4B**). Bleached areas had expanded widely by 21 dpi. Unlike LcJ/PDS, TocJ/PDS did not induce bleaching of the leaves at this position, but did induce severe malformation of the leaves (**Fig. 4A**). The upper leaves above those used in **Fig. 4** showed severe bleaching with severe malformation (data not shown).

The levels of the PDS mRNA and virus RNA were analyzed by northern blotting analysis. In both plants inoculated with TocJ/PDS and LcJ/PDS, the level of PDS mRNA remained the same until 7 dpi. It started to decline at 12 dpi to 21 dpi compared with mock-inoculated plants. Accumulation of PDS mRNA in plants inoculated with LcJ/PDS was decreased to a lower level at 12 dpi than that in TocJ/PDS-inoculated plants. The level of the subgenomic RNA and virus genome of LcJ/PDS was always lower than that of TocJ/PDS. Both TocJ/PDS and LcJ/PDS showed a maximum accumulation of the subgenomic RNA and virus genome at 7 dpi ahead of the PDS mRNA decrease. In both cases, virus accumulation reduced along with the PDS mRNA decrease. It is likely that the induced VIGS worked as a virus defence system as well.

Currently, the mechanism of the attenuation of symptoms is unknown and the reason why LcJ/PDS induced bleaching earlier than TocJ/PDS is unclear. Recently, it was reported that $L_{11}A$ failed to suppress PTGS (Kubota *et al.*, 2003), and this fact would explain that LcJ vector induce VIGS earlier than TocJ vector. We could confirm that the lower virus accumulation of LcJ/PDS was still enough to elicit VIGS without severe virus symptoms. These data indicate that the LcJ vector has advantages of the attenuation of symptoms and the promptness of VIGS induction. Most plant virus vectors have the problem of causing severe symptoms concurrently with VIGS induction. In this report, we overcame the virulence of ToMV vectors by using an attenuated strain without loss of the ability to induce VIGS. It is commonly assumed that the accumulation of target mRNA over a certain threshold would induce gene silencing (Meins, 2000). Although the attenuated virus vector showed less virus RNA accumulation than the wild type virus, it could spread systemically. In this time, it caused no virus symptoms but was easily sufficient for VIGS induction. Currently, trials to create attenuated viruses *in*

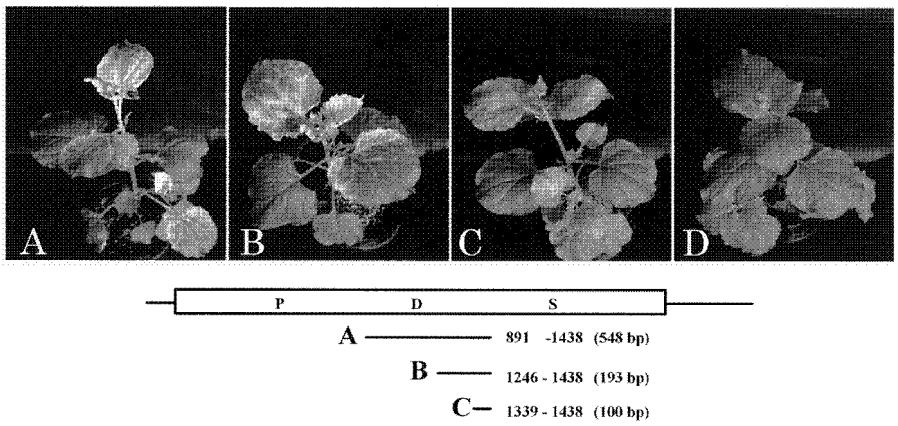

Fig. 6 Degree of bleaching with different lengths of PDS transgenes.
N. benthamiana plants inoculated with (A) LcJ/PDS (548 bp), (B) LgJ/PDS_{200} (193 bp), (C)LgJ/PDS_{100} (100 bp) and (D) mock after 3 wpi.

vitro have been successful (Hagiwara *et al.*, 2002; Kurihara and Watanabe, 2003). We expect that the use of the attenuated strain with other virus vectors will also substantially improve its applicability.

Reversion of VIGS

In *N. benthamiana*, the bleached phenotype was most evident in the sixth to eighth leaves above the leaves inoculated with LcJ/PDS at 5 wpi (**Fig. 5**, tissue 2). The leaves were immature at the time of inoculation and fully developed at 3 wpi. In the upper most leaves, some parts of the leaves were green and some were white, thus forming a mottled tissue (**Fig. 5**, tissue 3).

We isolated RNA separately from the lower green leaves which were fully developed at the inoculation with LcJ/PDS (**Fig. 5** tissue 1), white leaves which were fully bleached (tissue 2) and upper mottled leaves (tissue 3). The virus genome appeared to be accumulated most in green areas, less so in mottled areas and least in white areas (**Fig. 5**). Analysis of the mock-inoculated plants showed that the accumulation of PDS mRNA was almost equal and did not depend on the growth stage or tissues (see **Fig. 4C**). In all tissues of LcJ/PDS-inoculated plants, accumulation of PDS mRNA was less than that in mock-inoculated plants (**Fig. 5B**). It was unexpected that the white areas retained the highest level of PDS mRNA accumulation, while the level of PDS mRNA was low in the green and mottled leaves.

It is puzzling that most of the bleached tissue retained the highest level of PDS mRNA. We speculate that, together with the result of **Fig. 4**, the bleached phenotype was triggered even by low PDS activity, but most importantly, at the early stage of development, and then the leaves expanded to their full size with bleaching (tissue 2). At 3 wpi, LcJ/PDS virus was strongly suppressed along with PDS mRNA, and then it was likely that the level of PDS mRNA recovered to close to the original level in the white areas at 5 wpi. Consequently, since many LcJ/PDS virus multiply in tissue 3 and reinforce the VIGS again, the newly developed tissue showed mottling. These results indicated that LcJ escapes VIGS to some extent, and continues to partially suppress the PDS mRNA. Another tobamovirus vector, 30B, is able to effectively escape VIGS, and continue partial suppression (Hiriart *et al.*, 2003). It differs from the TRV vector, which could induce strong but temporal VIGS (Ratcliff *et al.*, 2001). Our results indicate that the LcJ vector has the same unique character as 30B, and is effective for phenotype analysis by VIGS.

Use of the Gateway system to insert long foreign sequences into tobamovirus vectors

We further improved the vectors by introducing Gateway Cloning Technology (Invitrogen, CA, USA). Gateway Cloning Technology allows high efficiency cloning in a short time by an *in vitro* recombination reaction. This method has previously been applied to some plant virus vectors (Liu *et al.*, 2002a, b). We induced the gateway cassette into the *Sac*I site of LcJ to generate a new vector, LgJ. From this, the recombination site of LgJ can be replaced with an insert with approximately 100% efficiency by reaction with LR CLONASE enzyme. After recombination, *att*B site sequences of about 24 bp remain at both ends of the subcloned insert.

We checked the influence of these additional *att*B site sequences on the VIGS induction by LgJ vectors. LgJ/PDS was constructed and inoculated to *N. benthamiana* plants. Reduction of PDS mRNA and appearance of the bleached phenotype

were observed to almost the same level at the same stages as those observed with LcJ/PDS (data not shown). This indicated that the attB site sequences did not have an adverse effect on the VIGS induction. The feasibility of using the LgJ vector will contribute significantly to the functional analysis of plant genes.

Whether LgJ vectors with shorter insert lengths could elicit VIGS was investigated. LgJ/PDS_{100} and LgJ/PDS_{200} harbour partial PDS sequences of 100 bp and 193 bp long, respectively (corresponding to nt 891-1438 and 1246-1438 of *N. benthamiana* cDNA, respectively). LcJ/PDS, LgJ/PDS_{200} and LgJ/PDS_{100} were separately inoculated to *N. benthamiana* plants. None of the plants showed any symptoms, and all developed new bleached leaves at 2 wpi. However, each virus induced a different severity of bleaching (**Fig. 6**). At 3 wpi, LgJ/PDS_{100} caused only light green areas (**Fig. 6C**). LgJ/PDS_{200} caused more vivid bleaching, although it was a little more restricted in area than LcJ/PDS, which contained a 548 bp PDS sequence (**Fig. 6A, B**). However, LgJ/PDS_{200} still caused sufficient strong bleaching for phenotypic observation. These results indicate that the degree of the phenotype is proportional to the insert length. Insert lengths of more than 200 nt are desirable for induction of VIGS and detection of the phenotype using LgJ vectors.

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