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Construction of a Tobamovirus Vector That Can Systemically Spread and Express Foreign Gene Products in *Solanaceous* Plants.

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Abstract

In present-day biology, a facile gene delivery system is required to study gene function in a high throughput way. The use of plant virus vectors that can introduce foreign or extra endogenous genes into plants attracts much attention. It is advantageous in that researchers need only minimal experience and results can be obtained in a short period of time. Here we describe infectious cDNA clones of the tomato mosaic tobamoviruses (ToMV) that were modified for facile insertion of foreign genes and which retained the ability to multiply in plants. This vector, named TocJ, was able to harbor the GFP gene from *Aequorea victoria* and systemically spread in some *Solanaceous* plants. TocJ would be suitable for the overexpression of foreign genes and the analysis of gene functions in various *Solanaceous* plants.

Key words: green fluorescent protein, systemic infection, tobamovirus vector.

Abbreviations

BCIP/NBT, 5-bromo-4-chloro-indolyl-phosphatase /nitrobluetetrazorium; BSA, bovine serum albumin; CP, coat protein; CS, context sequence; DIG, digoxigenin; dpi, days post inoculation; GFP, green fluorescent protein; hpi hours post inoculation; PVDF, polyvinylidene difluoride; sg mRNA, subgenomic messenger RNA; TMGMV tobacco mild green mosaic virus; TMV tobacco mosaic virus; ToMV, tomato mosaic virus; UTR, untranslated region.

Introduction

It has been shown that several viral vectors can be used for the transient expression of foreign genes in plants., such as peptides, proteins like antigens (Porta *et al.*, 1994; Fitchen *et al.*, 1995; Sugiyama *et al.*, 1995; Turpen *et al.*, 1995; Bendahmane *et al.*, 1999) and antibody fragments (Franconi *et al.*, 1999; Hendy *et al.*, 1999; Roggero *et al.*, 2001). This strategy has an impact on basic research and as well as plant biotechnology, since the expression level of foreign proteins is relatively high and rapid accumulation might help to bypass its possible toxicity to plants.

Tobamoviruses multiply rapidly in infected plants and many attempts have therefore been made to modify and utilize their genomes to express foreign genes (Takamatsu *et al.*, 1987; Dawson *et al.*, 1989; Takamatsu *et al.*, 1990b; Donson *et al.*, 1991; Kumagai *et al.*, 1993; Lim *et al.*, 2002). Of the TMV-encoded gene products, coat protein (CP) is the most abundantly expressed. Initially, a tobamovirus vector was constructed by replacing the CP coding region with a foreign gene sequence. These vectors could multiply in infectious leaves and express foreign proteins (Takamatsu *et al.*, 1987). However, they could not spread out to other leaves, because intact CP is necessary for the systemic spread of the virus. To overcome this problem, several approaches were used to express foreign genes with CP expression retained to some extent.

Earlier we reported a ToMV-based vector, where a readthrough 3' context sequence (3'CS) was inserted between the coat protein gene and the foreign gene sequences as an in-frame junction (Hamamoto *et al.*, 1993; Sugiyama *et al.*, 1995). The 3'CS was characterized as a six-base sequence that follows the stop codon for the 126K RNA dependent RNA polymerase (RdRP) gene and permits readthrough of the stop codon (Skuzeski *et al.*, 1991), giving rise to the synthesis of their N' coterminal 183K RdRP. The vector could multiply in inoculated leaves and also spread systemically.

The 3' CS sequence allowed both intact CP and a CP-foreign protein fusion to be produced at a ratio of 20:1.

Dawson *et al.* made a tobamovirus vector that has an extra CP subgenomic mRNA (sgRNA) promoter to drive foreign gene expression (Dawson *et al.*, 1989). They replaced the CP ORF with a foreign gene ORF and then added a second-TMV CP sgRNA promoter and CP ORF. The first CP sgRNA promoter in this TMV-based hybrid vector is an extra one and drives foreign gene expression by being located at the 5' side of the inserted fragment. Initially, this approach faced the problem of genomic instability with a loss of foreign gene sequences as a result of deletion of DNA between duplicated sequences. To overcome this, the vector was improved by using a genome fragment of a heterologous tobamovirus including sgRNA promoter and CP ORF. The new vector, called 30B, showed good stability of the genome organization during multiplication in plants (Shivprasad *et al.*, 1999).

Like these, a number of tobamovirus-based expression vectors have achieved success in the expression of foreign genes but in most cases such a tobamovirus vector could challenge only *Nicotiana benthamiana*. This time, we manipulated the ToMV genome to obtain two types of vectors and tried to express GFP as a representative of foreign genes on several *Solanaceous* plants.

Materials and Methods

Plant materials

Nicotiana benthamiana, *Nicotiana tabacum*, *Lycopercicon esculentum*, *Capsicum annuum* var. angulosum, *Cucurbita pepo*, *Perilla frutescens* var. crispa and *Mentha piperita* were used in this study. All plants were maintained at 23 ℃ during a 16 h photoperiod and 8 h dark period.

Plasmid Constructions

Virus cDNAs used in this study were ToMV

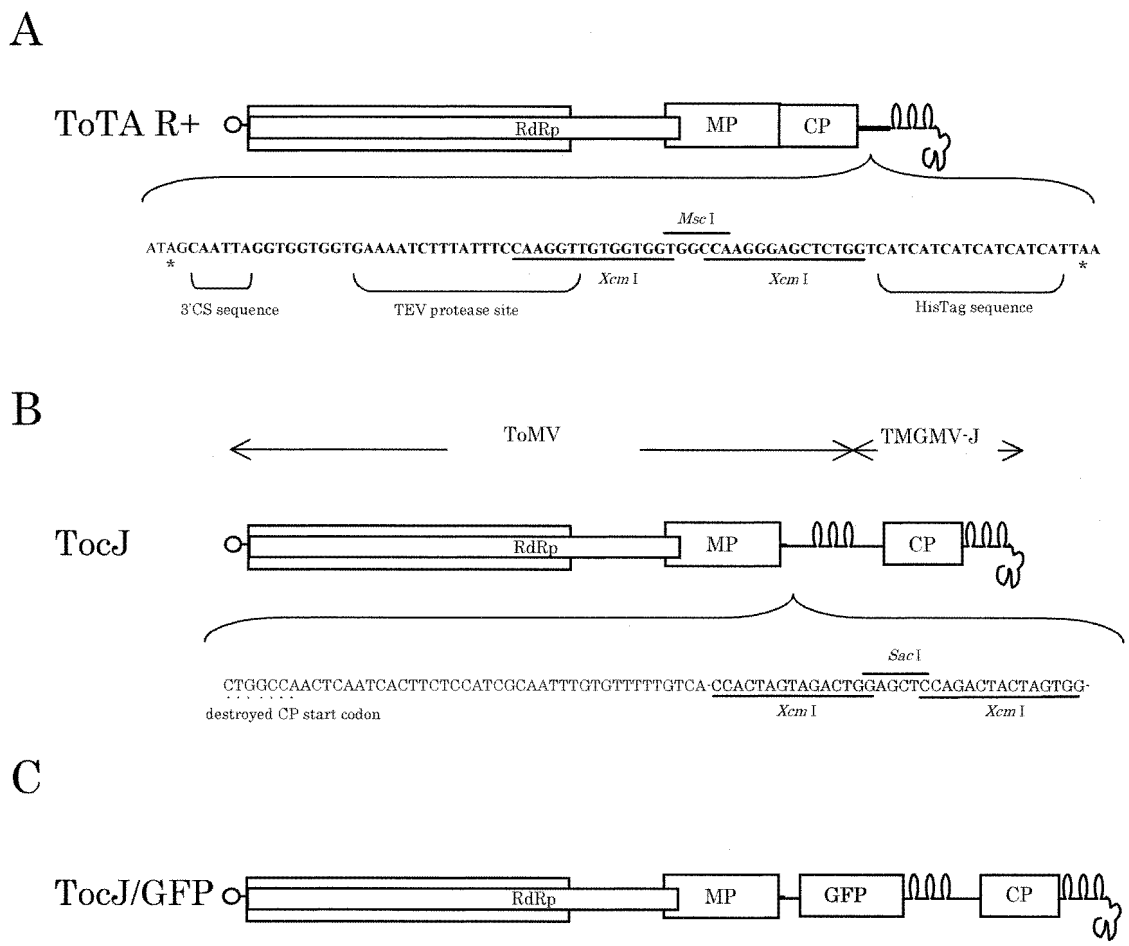

Fig. 1 Schematic represention of vectors. 5'-cap structures are depicted by circles. RNA-dependent RNA polymerase, movement protein (MP) and coat protein (CP) genes are represented by rectangles. The underlined lines indicate inserted restriction sites.
(A) Construct of ToTA R+ vector. The asterisks denote stop codons.
(B) Construct of TocJ vector. Pseudoknot regions derived from ToMV and TMGMV are shown by bulges. The dots in multi-cloning sites (MCS) denote the mutated nucleotides surrounding the start codon of TMV CP. (C) Construct of TocJ/GFP.

(GenBank accession no. X02144) (Ohno *et al.*, 1984; Meshi *et al.*, 1986) and TMGMV Japanese strain (TMGMV-J, GenBank accession AB078435) (Morishinma *et al.*, 2003). The pTLW3 (Meshi *et al.*, 1986) was the starting plasmid to which modifications were introduced for the construction of ToTA R+ and TocJ vectors. For convenience, the acronym 'p' of the name of the template DNA was deleted in naming each virus vector. **Fig. 1** shows the structures of the ToTA R+ vector (A), the TocJ vector (B) and TocJ/GFP (C). By PCR assisted mutagenesis (Imai *et al.*, 1991) with primers a and b (**Table 1**), one endogenous *Xcm*I site of pTLW3 was diminished by C4394T substitution to make pTLW3 Δ*Xcm*I. The PCR products were self-ligated by the TAKARA blunting kination ligation kit (TAKARA).

Construction of pToTA R+: The segment of [3'CS - tobacco etch virus (TEV) protease site - one *Xcm*I site - spacer *Sac*I site - another *Xcm*I site - His tag site] was inserted into pTLW3 Δ*Xcm*I by PCR with primers c, d and e (**Table 1**). At first, one fragment was PCR-amplified with pTLW3 Δ*Xcm*I and primers c and e. Next, the fragment, primers d and e, and pTLW3 Δ*Xcm*I were used for the next PCR amplification. The PCR product was digested with *Sac*I and self-ligated to get pToTA R+. The final pToTA R+ has an inserted sequence of ATAGCAATTAGGTGGTGGTGAAAATCTTTA-TTTCCAAGGTTGTGGTGGTGGCCAAGGGAG-CTCTGGTCATCATCATCATCATCATTAATG (underlined; *Xcm*I site) between nucleotides 6178 and 6183 of ToMV.

Construction of pTocJ: PCR was performed with pTLW3 Δ*Xcm*I and primers f and g (**Table 1**) to induce substitution of TA-to-GC at nucleotides 6327-8 into pTLW3 Δ*Xcm*I to create *Nae*I site in this position. The product was treated with *Nae*I and self-ligated to get pTLW3 Δ*Xcm*I +*Nae*I. Next, the fragment of nt 5502-6356 of TMGMV-J was amplified with primers h and i which contain *Ngo* MIV (isoschizomer of *Nae*I) and *Mlu*I sites, respectively (**Table 1**). This fragment was treated with *Ngo* MIV and *Mlu*I and inserted into compatibly digested pTLW3 Δ*Xcm*I +*Nae*I. The start codon of ToMV CP ORF was diminished by PCR with primers j and k (**Table 1**). Lastly, the ToMV coat protein sequence (nt 5748-6182) was replaced with a multiple cloning site by PCR with primers l and m (**Table 1**). The PCR products were digested with *Sac*I and self-ligated to obtain pTocJ. The final TocJ has a cloning-sites sequence CCACTAGTA-GACTGGAGCTCCAGACTACTAGTGG (underlined; *Xcm*I site) [one *Xcm*I site - spacer *Sac*I site - another *Xcm*I site] between nucleotide 5747 and 6183 of ToMV. The fragment of nucleotides 5502-6356 of TMGMV-J was joined downstream of ToMV nucleotides 6325 and the following the -GCCGGC- linker sequence.

The ORF of G3 green fluorescent protein (G3 GFP) was amplified PCR with primers n and o (**Table 1**) from LQwt:G fus (Kawakami and Watanabe, 1997). G3 GFP is a spectrometric variant which matches common FITC filter sets (Ogawa *et*

Table 1 Oligonucleotides used in this study

	Oligonucleotide names	Sequences[1)]	Nucleotides
a	L-KpnI-cut-Rv	CACCACTCTTTCTCTGaTACCATAAACACGTT	32
b	L-KpnI-cut-F	ATGTTACAACTTTTATCGGTAATACCGTCATCATTG	36
c	T1-RV	ataaagattttcaccaccacctaattgctatGATGCAGGTGCAGAGGTCC	50
d	T2-RV	gagctcccttggccaccaccacaaccttggaaataaagattttcaccacc	50
e	T3-F	gagctctggtcatcatcatcatcatcattaatgATGCATAGGTGCTGAAA	50
f	ToMV3'NaeI+F	ATGTCCGccGGCACGTAAAAAAAGCG	26
g	ToMV3'NaeI+RV	TACGTGccGGCGGACATATATGAACC	26
h	TMGMV-5'-2NaeI	TCGAGgccggcTGTGAAACTCGAAAGGTTCC	31
I	TMGMV-3'MluI	CGTCGacgcgTGGGCCGCTACCCGCGGTTA	30
j	ToMV-CP-ATG->CTG-R	CCTGCctggccaGATTTAATACGAATCAGAATCC	34
k	ToMV-CP-ATG->CTG-F	CCACCctggccaACTCAATCACTTCTCCATCG	32
l	ToMV-chi-clo-F	gactcgagctccagactactagtggATGCATAGGTGCTGAAATATAAAG	49
m	ToMV-chi-clo-R	gtctcgagctccagtctactagtggTGACAAAAACACAAATTGCG	45
n	GFP-for-T-F-3	TATGAGTAAAGGAGAAGAACT	21
o	GFP-for-bam-r	TGGATCCTTATTTGTATAGTTCATCCATGC	30

[1)] Underlined letters indicate restriction sites.
Lower case letters indicate nucleotides in the primers designed to introduce mutations or cloning sites.

al., 1995). The fragment encoding GFP was inserted to ToTA R+ and TocJ TA cloning sites to make ToTA R+/GFP and TocJ/GFP (**Fig. 1**).

In vitro transcription

The vector template DNA was linearlized with *Mlu*I prior to *in vitro* transcription to make a run-off transcription. The *in vitro* transcription reaction was performed using 1 μg linearized vector DNA, 50 unit 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 analog in 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$, 25 mM $NaCl_2$, 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 an *in vitro* transcript using electroporation as described previously (Watanabe *et al.*, 1987). Transcripts derived from 0.5 μg of the template were used for electroporation. Protoplasts were harvested after 15 h culture at 28 ℃.

Firstly transcripts were inoculated to six-week old *N. benthamiana* with carborundum. After 5 days, the 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 sap was inoculated on six-weeks-old *N. benthamiana*, *N. tabacum*, *L. esculentum*, *C. annuum* var. angulosum, *C. pepo*, *Perilla frutescens* var. crispa and *M. piperita*.

Northern blot 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. Then the RNAs were transferred onto Hybond N+ membranes by capillary blotting. After drying at room temperature, the membranes were irradiated with a UV lamp 70,000 micro-J cm^{-2} by UV crosslinker (Stratagene). The fragment of nucleotide 5502-6356 of TMGMV-J was subcloned in a pGEM-Teasy (Promega) vector, to get a pTMGMVprobe. DIG-labeled TMGMV antisense probes were transcribed from the pTMGMVprobe template DNA using a DIG-RNA labeling kit (Roche). The TMGMV probe was hybridized with membranes at high stringency at 68 ℃ overnight. Membranes were washed twice with 2x SSC containing 0.1% SDS for 15 min at 68 ℃, and twice with 0.5x SSC containing 0.1% SDS for 15 min at 68 ℃. The DIG-RNA probe was detected using Anti-Digoxigenin-Ab Fab fragments (Roche) and a BCIP/NBT Membrane Phosphates Substrate (KPL).

Western blot analysis

The total proteins were extracted with PBS buffer using disposable pestles in microcentrifuge tubes. The homogenates were then centrifuged for 10 min at 10,000 *g*. Protein concentrations were determined by Bradford's method. The proteins were separated on 12% polyacrylamide gels containing 1% SDS (Laemmli, 1970) and transferred onto a PVDF membrane (Immobilon; Millipore) using an electroblotting system (BioRad). GFP and TMGMV CP were detected using anti-GFP antiserum (Santa Cruz biotechnogy) and anti-ToMV CP serum (Saito *et al.*, 1989), respectively. The membranes were incubated for 30 min in Tris buffer [50 mM Tris-HCl (pH7.5)] containing 150 mM NaCl and 5% skim milk and treated with respective antibodies for 2 h. Subsequently they were treated with alkaline phosphates-linked goat anti-rabbit antibody (Cell Signaling technology) and BCIP/NBT Membrane Phosphates Substrate (KPL).

Results and Discussion

Limitations of a vector harboring long foreign sequences between the ToMV CP ORF and 3' UTR

In previous work, we developed ToMV-based 3' CS vectors (Hamamoto *et al.*, 1993). In this work we introduced multiple cloning sites of 90 mer between the ToMV CP coding sequence and the 3' untranslated region (UTR) to construct ToTA R+ (**Fig. 1A**) to insert the foreign sequence. The 3' UTR, located downstream of CP ORF, consists of three pseudoknots followed by a tRNA-like structure (van Belkum *et al.*, 1985; Takamatsu *et al.*, 1990a).

When ToTA R+ was inoculated into BY-2 protoplasts, it was able to replicate and produce viral proteins (data not shown). However, when we inserted GFP sequences into the ToTA R+ vector, the virus could not multiply and produce CP or GFP to a level detectable by western blot analysis in plants or in BY-2 protoplasts (data not shown).

The ToMV CP-3'UTR junction was therefore suitable for efficient expression of short peptides but inadequate for large-size foreign gene sequences. As an alternative method, we adopted a strategy to add an extra sgRNA promoter to the ToMV genome (Shivprasad *et al.*, 1999).

Introduction of an extra subgenomic promoter and 3'UTR into the ToMV genome

A new ToMV-based hybrid replicon vector, TocJ, was created by inserting heterologous sequences from TMGMV-J (Morishima *et al.*, 2003, GenBank accession no. AB078435). The structure of TocJ from 5' to 3' is as follows (**Fig. 1B**). The first 5924 bp of the replicon comes from ToMV, from its original 5' end thorough to the 3'UTR. The original AUG start codon of the coat protein ORF was mutated to CUG. The main part of the coat protein, except for the +45nt leader sequences, was replaced with *Xcm*I restriction endonuclease sites that can generate a TA cloning site, with a 1-nt 3' thymidine overhang feasible for cloning PCR fragments (Kovalic *et al.*, 1991) to allow insertion of foreign sequences. Subsequently foreign sequences would be expressed from the sgRNA promoter (Shivprasad *et al.*, 1999; Grdzelishvili *et al.*, 2000). Following the ToMV 3' UTR, the next 855 bp comes from the 3' sequence of TMGMV, including the CP sgRNA promoter, the CP ORF and the 3' UTR.

To look at its biological activity, TocJ was inoculated into BY-2 protoplasts. RNA samples were collected at 15 h post-inoculation (hpi) for Northern blot analysis. Both TocJ genomic RNA and sgRNAs originating from the ToMV and TMGMV sgRNA promoters were detected as expected (**Fig. 2A**). It was thus confirmed that the introduced sgRNA promoter was functional.

When *N. tabacum* cv. Samsun was inoculated with TocJ, it showed mosaic symptoms after 6 days and SDS-PAGE analysis confirmed TMGMV CP accumulation (data not shown). These data indicated that the TocJ vector could replicate and spread systemically.

It is advantageous that the genomic RNA of virus vectors be encapsulated with CP to form virion particles because of their stability and high infectivity. To test this, protoplasts inoculated with TocJ were homogenized at 15 (hpi) and the sap inoculated onto the leaves of the local lesion host, *Nicotiana tabacum* cv. Xanthi-nc. Crude sap from protoplasts inoculated with TocJ produced as many local lesions as wild type ToMV, while a ToMV mutant with its coat protein replaced with GFP (LΔ C GFP) could not form virion particles nor elicit local lesions (**Fig. 3**). It was confirmed that TocJ progeny could form virus particles in infected tissues.

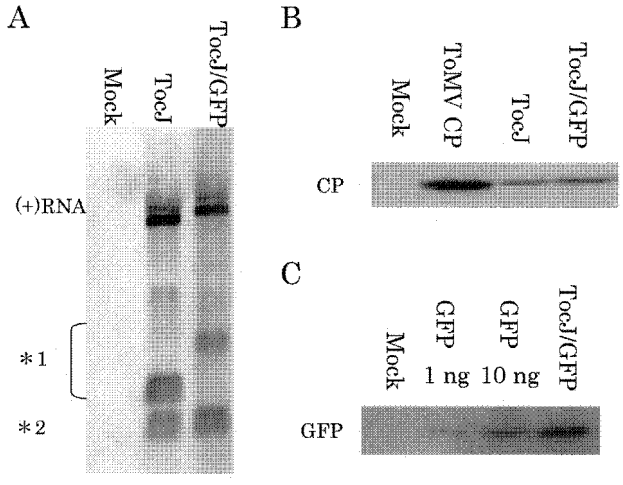

Fig. 2 Multiplication of tobamovirus vector TocJ in protoplasts. (A) Accumulation of RNAs of TocJ vectors in BY-2 protoplasts. Northern blot of total RNA extracted from protoplasts 10^5 cell at 15 hpi was probed with TMGMV (5502 nt-6356 nt) positive-strand RNA-specific probe. *1 denotes sg mRNAs directed by ToMV CP sg mRNA promoter. *2 denotes TMGMV CP sg mRNA.

Western blot analysis of (B) CP and (C) GFP produced in virus-inoculated BY-2 protoplasts 0.25×10^5 cell at 15 hpi. Positive controls were 1ng and 10 ng of GFP.

Expression of GFP by TocJ virus vector in Nicotiana benthamiana

The ORF of the GFP was amplified by PCR and inserted into the *Xcm*I sites of TocJ to create TocJ/GFP (**Fig. 1C**). TocJ/GFP was inoculated into BY-2 protoplasts and RNA was harvested at 15 hpi. Northern blot analysis confirmed that TocJ/GFP could replicate maintaining an intact GFP sequence (**Fig. 2A**). Expression of TMGMV CP and GFP

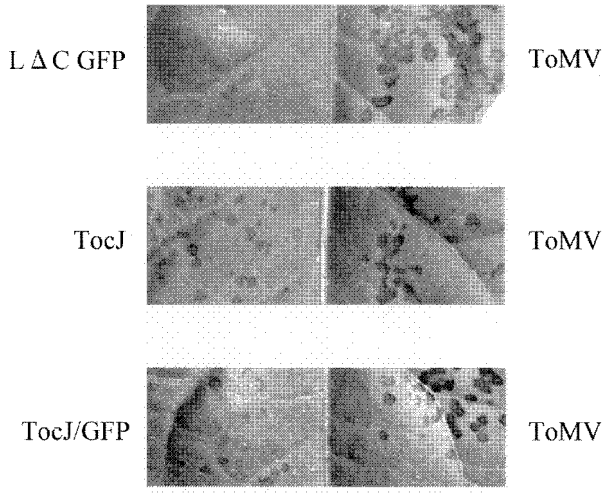

Fig. 3 Local lesions caused by saps extracted from BY-2 protoplasts. A ToMV mutant replaced coat protein to GFP (LΔC GFP), TocJ and TocJ/GFP were inoculated at the left sides of leaves; wild type ToMV was inoculated at the right halves. Photos were taken at 5 dpi.

protein in TocJ/GFP-infected protoplasts was analyzed by Western blot. TMGMV CP was detected in both protoplasts infected with TocJ and those inoculated with TocJ/GFP (**Fig. 2B**). Accumulation of GFP protein in protoplasts inoculated with TocJ/GFP at 15 hpi was estimated to be 125 ng per 10^5 cells (**Fig. 2C**). Extracts of infected protoplasts were inoculated onto leaves of local lesion hosts. TocJ/GFP induced local lesions (**Fig. 3**) as much as TocJ did. It was confirmed that TocJ/GFP formed intact virus particles.

The transcript of TocJ/GFP caused infection in inoculated *N. benthamiana* plants. TocJ/GFP infection produced fluorescent foci visible to the naked eye in the inoculated leaves of *N. benthamiana* by 3 days post-inoculation (dpi) (**Fig. 4A**). GFP fluorescence could be observed in upper, non-inoculated leaves at 7 dpi (**Fig. 4B**) and by 12 dpi, GFP fluorescence had spread more widely (**Fig. 4C**). We analyzed accumulation levels of GFP and CP in these plants by western blot analysis. In TocJ/GFP-inoculated leaves, GFP accumulation increased till 7 dpi, reached a plateau and leveled off at 12 dpi, however the level declined to some extent by 21 dpi. In upper leaves, where the virus moved in systemically, GFP started to accumulate a little at 3 dpi, but its accumulation increased to a high level by 12 dpi. The level seemed to be maintained by 21 dpi (**Fig. 5A**). In contrast to GFP accumulation, it seemed that CP accumulation increased for three weeks after inoculation in both inoculated and systemically infected leaves (**Fig. 5B**). Assuming that its accumulation reflects the degree of virus multiplication, virus multiplication did not always correlate with continuous accumulation of foreign genes. It would be necessary to examine the best time to harvest foreign protein in inoculated tissues in each case.

TocJ/GFP could multiply on several kinds of Solanaceae family plants.

TocJ/GFP virus was also examined for its ability to express GFP in *N. tabacum* (**Fig. 4D-F**), *Lycopercicon esculentum* (**Fig. 4G**) and *Capsicum annuum* var. angulosum (**Fig. 4H**). As a result, GFP fluorescence was detected systemically in these plants. In contrast, when pumpkin (*Cucurbita pepo*), perilla (*Perilla frutenscens* var. crispa) and mint (*Mentha piperita*) plants were inoculated with TocJ/GFP, GFP fluorescence could not detected even at 7 dpi (data not shown). Thus, it appears that TocJ/GFP has a wide host range in the *Solanaceae* family. No visible symptoms were observed in *N. tabacum* and *L. esculentum*, however TocJ/GFP caused senescence, necrosis, leaf curling and dwarfness in nine-week old or older *N. benthamiana* plants, and complete withering in younger, seven-week old plants (data not shown).

Based on these observations of GFP expression, it seems that the TocJ vector could be useful for foreign protein expression. However, due to its rather severe virulence, it may not be suitable for investigating the phenotypes caused by foreign genes in *N. benthamiana*.

The 30B.GFP produced systemic fluorescence later than 19 dpi on *N. tabacum* (Toth *et al.*, 2002). On the other hand our TocJ/GFP produced systemic fluorescence as early as 7 dpi in *N. tabacum*. It seems that the TocJ vector virus may spread more efficiently than the 30B.GFP vector in *N. tabacum*.

Recently Toth *et al.* reported a trial to improve the 30B vector's ability to spread systemically. DNA

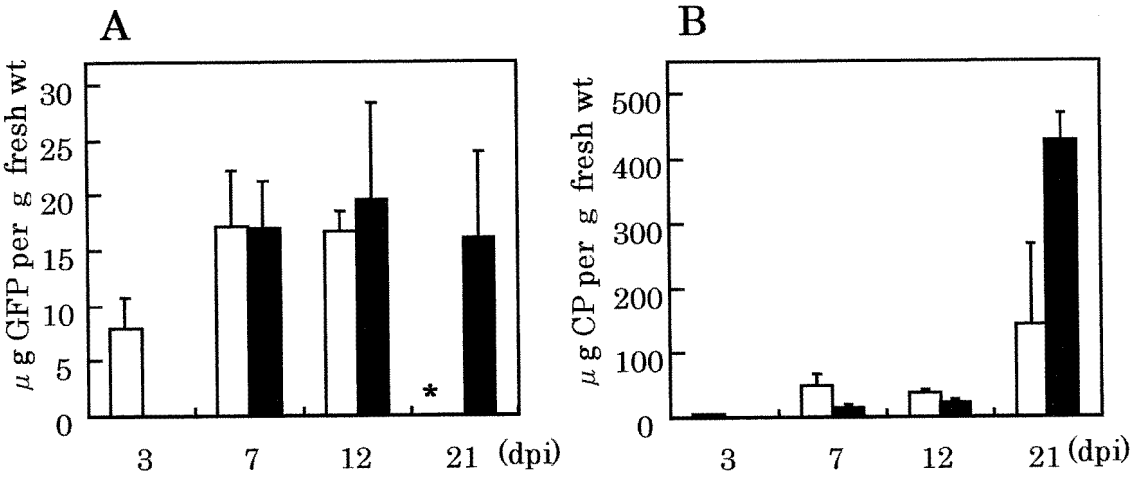

Fig. 5 Accumulations of GFP and CP in *N. benthamiana* inoculated with TocJ/GFP. Open and filled bars indicate accumulation in inoculated leaves, and that in systemically infected leaves, respectively. (A) Accumulation of GFP. The asterisk indicates that GFP level was below the detection limit. (B) Accumulation of CP. The upper 3-4 leaves above the inoculated ones were not present at 3 dpi.

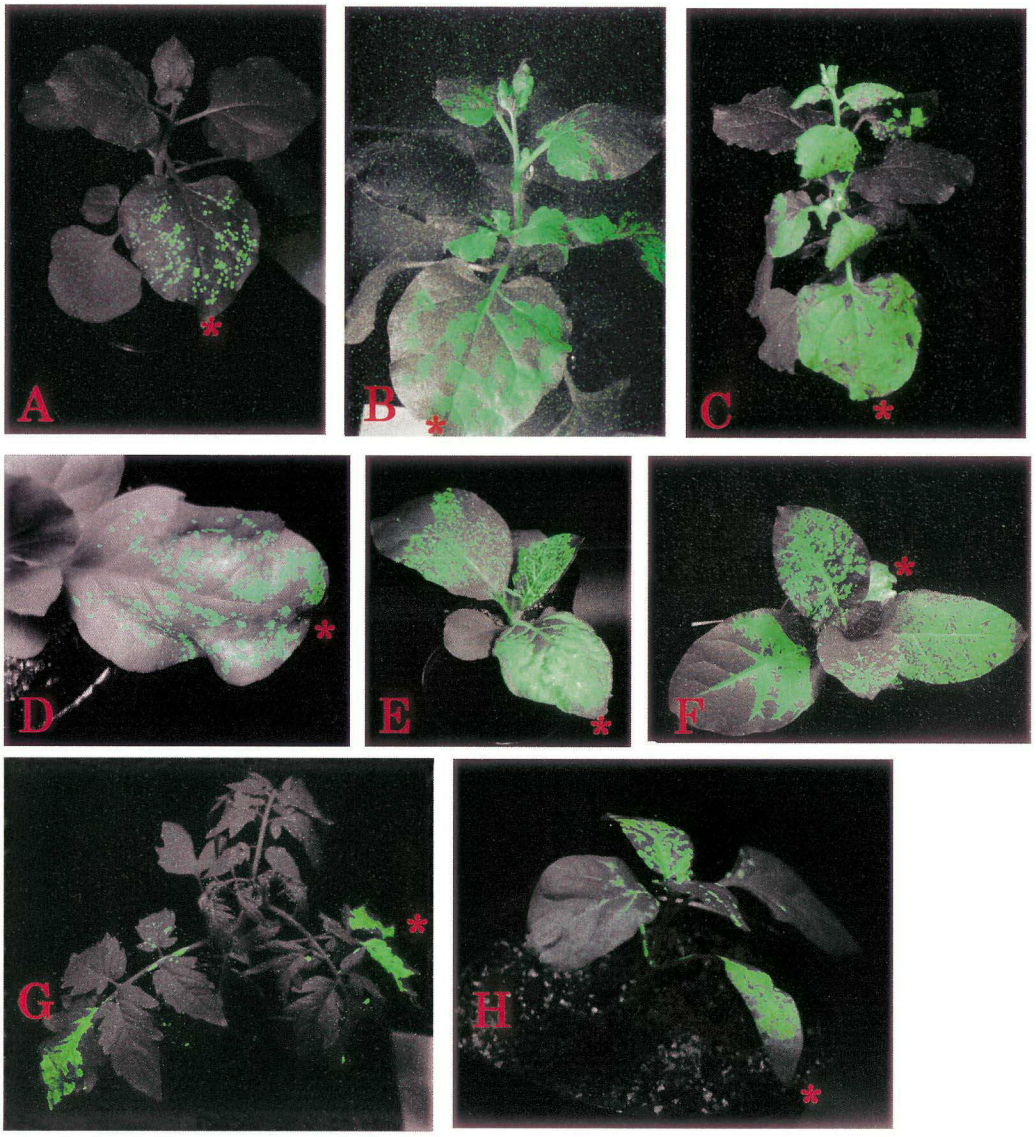

Fig. 4 TocJ/GFP inoculated plants.
(A-C) *N. benthamiana* with TocJ/GFP at (A) 3 dpi, (B) 7 dpi and (C) 12 dpi.
(D-F) *N. tabacum* cv. Samsun with TocJ/GFP at (D) 3 dpi, (E) 7 dpi and (F) 12 dpi.
(G) *L. esculentum* with TocJ/GFP at 10 dpi.
(H) *C. annuum* var. angulosum with TocJ/GFP at 7 dpi.
Two images are merged, an image of GFP fluorescence under a mercury lamp with GFP plus filter set (Leica) and an image under visible light. The asterisks indicate inoculated leaves.

shuffling improved the movement and host range properties of the 30B vector (Toth *et al.*, 2002). Likewise it is still possible that we can improve our TocJ vector with better properties.

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