Effectiveness and stability of heterologous proteins expressed in plants by *Turnip mosaic virus* vector at five different insertion sites

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Abstract

The N-terminal (NT) regions of particular protein-coding sequences are generally used for in-frame insertion of heterologous open reading frames (ORFs) in potyviral vectors for protein expression in plants. An infectious cDNA clone of *Turnip mosaic virus* (TuMV) isolate YC5 was engineered at the generally used NT regions of HC-Pro and CP, and other possibly permissive sites to investigate their effectiveness to express the GFP (jellyfish green fluorescent protein) and Der p 5 (allergen from the dust mite, *Dermatophagoides pteronyssinus*) ORFs. The results demonstrated the permissiveness of the NT regions of P3, CIP and NIb to carry the ORFs and express the translates as part of the viral polyprotein, the processing of which released free-form proteins in the host cell milieu. However, these sites varied in their permissiveness to retain the ORFs intact and hence affect the heterologous protein expression. Moreover, strong influence of the inserted ORF and host plants in determining the permissiveness of a viral genomic context to stably carry the alien ORFs and hence to support their prolonged expression was also noticed. In general, the engineered sites were relatively more permissive to the GFP ORF than to the Der p 5 ORF. Among the hosts, the local lesion host, *Chenopodium quinoa* Willd. showed the highest extent of support to TuMV to stably carry the heterologous ORFs at the engineered sites and the protein expression therefrom. Among the systemic hosts, *Nicotiana benthamiana* Domin proved more supportive to TuMV to carry and express the heterologous ORFs than the *Brassica* hosts, whereas the protein expression levels were significantly higher and more stable in the plants of *Brassica campestris* L. var. *chinensis* and *B. campestris* L. var. *ching-geeng* than those in the plants of *B. juncea* L. and *B. campestris* L. var. *pekinesis*.

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1. Introduction

The positive-sense ssRNA viral vectors are very effective in expressing heterologous genes in host plants, not only because of their autonomous replication and high levels of expression, but also their possession of amenable genomic contexts to accommodate expressible heterologous ORFs (Porta and Lomonossoff, 2002; Scholthof et al., 1996). The genus *Potyvirus* (the family *Potyviridae*), one of the most important groups of positive sense ssRNA viruses, is the largest group of plant viruses infecting a vast array of crops of economic importance (Hull, 2002; Shukla et al., 1994). A potyvirus genome is about 10 kb long and it contains a single open reading frame (ORF) encoding a polyprotein proteolytically processed into 10 functional proteins by 3 virus-encoded proteases (Riechmann et al., 1992; Shukla et al., 1994; Revers et al., 1999). The proteases P1 and HC-Pro catalyse only their respective C termini (Carrington et al., 1989; Verchot et al., 1991), while the remaining proteolytic sites are processed by NIa protease (Carrington et al., 1993).

Judicious in-frame fusion of a heterologous ORF to a terminal region of a potyviral protein-coding sequence can result in the translation of the heterologous ORF as a part of the viral polyprotein and its subsequent release by proteolytic processing into a fusion protein with a viral protein. If an additional proteolytic site is engineered at the junction, where the heterologous
ORF is connected to the reading frame of the viral protein, a free-form heterologous protein results (Porta and Lomonossoff, 2002). However, in some cases, the expression of a foreign protein by a potyvirus affects virus accumulation and symptom development on hosts (Dolja et al., 1997; German-Retana et al., 2000; Guo et al., 1998). Moreover, the integrity and expression of heterologous ORFs in viral vectors are affected by several factors, including the nature of the heterologous sequence (Choi et al., 2000; Beauchemin et al., 2005), the site of the viral genome used for the insertion (Arazi et al., 2001b, 2002) and the host species employed (Choi et al., 2000).

In the last decade, after the first potyviral vector Tobacco etch virus (TEV) was engineered (Dolja et al., 1992), several potyviruses have been engineered into effective viral vectors for the expression of heterologous proteins in plants (Porta and Lomonossoff, 2002). Most of the alien ORFs are constructed adjacent to the junction between P1 and HC-Pro or Nib and CP by directional insertion. The firstly engineered vector of TEV was developed to express β-glucuronidase (GUS) in a fusion form with HC-Pro, by inserting the GUS ORF at the N-terminal (NT) region of HC-Pro (Dolja et al., 1992). Later, GUS expression in free-form was achieved by introducing a recognition sequence for the Nla protease at the C-terminus (CT) of the GUS ORF (Carrington et al., 1993). The HC-Pro-NT insertion site is also functional for other potyvirus vectors, such as Lettuce mosaic virus (German-Retana et al., 2000), Plum pox virus (PPV) (Guo et al., 1998), Clover yellow vein virus (Masuta et al., 2000), Turnip mosaic virus (TuMV) (Beauchemin et al., 2005), and Zucchini yellow mosaic virus (ZYMV) (Hsu et al., 2004). In addition to the HC-Pro-NT insertion site, the insertion site at CP-NT is also successfully exploited for PPV (Fernandez-Fernandez et al., 2001), Potato virus A (PVA) (Ivanov et al., 2003), TuMV (Beauchemin et al., 2005), Wheat streak mosaic virus (WSMV) (Choi et al., 2000), and ZYMV (Arazi et al., 2001b, 2002). Moreover, the C-terminal region of Nib is also functional in expressing foreign ORFs in PPV and Tobacco vein mottling virus vector (Dietrich and Maiss, 2003). In PVA vector, a mutant generated from a 15 bp-insertion in P3 reading frame retains its ability to infect the plants of N. benthamiana, and Brassica spp. (Tomlinson, 1987). Several infectious clones of TuMV have been generated as useful molecular tools for studying the genetic interactions of TuMV with its hosts (Jenner et al., 2000; Lellis et al., 2002; Tan et al., 2005), the host-range determinants (Jenner et al., 2003; Suehiro et al., 2004), and the viral expression of heterologous ORFs in plants (Beauchemin et al., 2005). In our previous study (Chen, 2006), an isolate of TuMV, designated as YC5, collected from calla lily (Zantedeschia spp. hybrids Black Magic) (Chen et al., 2003) was modified into a vector to express heterologous protein in its hosts (Chenopodium quinoa Wild., N. benthamiana, and Brassica spp.). Insertion in the NT region of HC-Pro, of the GFP ORF or an ORF encoding an allergen from the dust mite Dermatophagoides pteronyssinus Trouessart (Der p 5) resulted in the viral expression of the heterologous protein in host cell milieu (Chen, 2006).

In this study, additional NT regions, such as those of P3, CIP, Nib, and CP of TuMV-YC5 were engineered for insertion of the GFP or Der p 5 ORF. The expression efficiencies of the heterologous ORFs inserted at these sites were analyzed in different host backgrounds. Our results showed that, in addition to the NT regions of HC-Pro and CP, the NT regions of P3, CIP and Nib were also amenable to carry a heterologous ORF to be translated as a part of the polyprotein and processed as free-form protein. However, these insertion sites varied in their permissiveness to retain the inserts intact and hence affected heterologous protein expression. Moreover, the influences of inserted sequences and host plants on the stability and expression levels of the inserted ORFs were also noted.

2. Materials and methods

2.1. Strategy for creation of insertion sites in infectious cDNA clone of TuMV-YC5

A full-length cDNA clone of TuMV-YC5, namely p35SYC5 (Fig. 1A), with a poly (A) tail of 43 residues, flanked by the 35S promoter and the nos terminator, was constructed by ligating the KpnI-NotI fragment (nt 1938–9875 of TuMV-YC5) released from pTuHUP (possessing nt 1636–9875 of TuMV-YC5, Fig. 1B) with KpnI-NotI-digested p35STu retaining nt 1–1938 of TuMV-YC5 genome preceded by 35S promoter, (Fig. 1B).

To generate TuMV-YC5 vectors possessing insertion sites for heterologous ORFs, the subclones of TuMV-YC5, i.e., p35STu, pTuHUP, pYC5P3, pYC5CIP, YC5Nib, and pYC5ST3 were employed as templates for introducing PCR-based sequence modifications in required contexts. The genomic parts with modifications were used to replace the corresponding wild-type regions of TuMV-YC5, as described in the following sections. An NcoI site was first created individually at the NT regions of HC-Pro, P3, CIP, and Nib by PCR-based mutagenesis (GeneTailorTM, Invitrogen, Carlsbad, CA), as shown in Fig. 1B, for the HC-Pro-NT (as described previously by Chen, 2006). Subsequently, the context NcoI-Nhel-CVYHQA, representing NcoI and Nhel restriction sites, and encoding Nla protease recognition site (CVYHQA, existing naturally between Nib and CP) of TuMV-YC5 was constructed at the initially created NcoI site by PCR-based sequence modification and subcloning strategies.

2.2. Creation of an insertion site at the N-terminal region of P3

The construct pYC5P3 harbouring a cDNA fragment reflecting the nt 1934–4265 of the TuMV-YC5 genome,
possessing the full coding sequence of P3 (preceded by the 3′ partial coding region of HC-Pro and followed by the full coding sequence of 6k1 and 5′ partial coding region of CIP) was used as the template for PCR-based mutagenesis to create an insertion site in the P3-NT. The primers P3sense 5′-ACTACGGTGTCGGAGGGACCATGG
AATGGGAGG-3′ and P3antisense 5′-GGTCCCTCCGACACGGTAGTTTTAAGCTTG-3′ (NcoI site underlined) were employed to create pYC5P3Nco possessing an NcoI site between the codons for the N-terminal 2nd and 3rd amino acids of P3 (Figs. 1C and Fig. 2II). Subsequently, using the primers P3NHNIa 5′-CATTCAATCCATGGACAGCGTATGGTATTTAACGCTTG-3′ (NcoI-NheI sites underlined; nucleotides encoding CVY-HQA double-underlined), and NarSac 5′-CAAAACGAGCTCATTAGCTGATTAGTATGTA-3′ (NcoI-NheI sites underlined; nucleotides encoding CVY-HQA double-underlined), and NarSac 5′-CAAAACGAGCTCATTAGCTGATTAGTATGTA-3′, a 1.7 kbp DNA fragment possessing the full coding sequence of P3 (with the context NcoI-NheI-CVYHQA in its NT region), followed by the full coding sequence of 6k1 and 3′ partial coding region of CIP, was amplified from pYC5P3Nco. After restricting this PCR product with NcoI and NarI (a NarI site was at the 3′ end of the amplified fragment), the product was ligated with NcoI-NarI-digested pYC5P3Nco. This replacement generated pYC5P3NHNIa possessing the context NcoI-NheI-CVYHQA in the P3-NT (Fig. 1C).

Subsequently, the 2.3 kbp KpnI-NarI fragment corresponding to nt 1938–4242 of TuMV-YC5 was released from pYC5P3NHNIa and ligated with KpnI-NarI-digested pTuHUP (containing nt 1636–9875 of TuMV-YC5) to replace the corresponding wild-type genomic region from pTuHUP. This replacement created pTuHUP3NHNIa possessing the NcoI-NheI-CVYHQA context in the P3-NT (Fig. 1C). Finally, the 7.9 kbp KpnI-NotI fragment (nt 1938–9875) released from pTuHUP3NHNIa was ligated with KpnI-NotI-digested p35STu (retaining the 35S promoter-preceded nt 1–1938 of TuMV-YC5) to generate p35SP3NHNIa for inserting heterologous ORF in the P3-NT and expressing free-form foreign protein therefrom (Figs. 1C and 2II).
Fig. 2. Construction of different insertion sites in TuMV-based viral vector. The full-length cDNA of TuMV-YC5 in p35SYC5 possessing a 35S promoter, the modified portions (black triangles) at the N-terminal region of HC-Pro (I), P3 (II), CIP (III), NIb (IV), and CP (V), and the 3′ end nucleotide sequences of TuMV-YC5 followed by a poly (A) tract with a NotI site and nos polyadenylation signal as terminator are schematically represented. Nucleotide and amino acid sequences of the genomic region of the wild-type infectious clone of p35SYC5 and its modified versions with the cloning sites and NIa cleavage site are shown. The arrows indicate the created insertion sites in the TuMV genome. The ORFs of GFP and Der p 5 are directionally inserted at the NcoI/NheI insertion site of TuMV-YC5 vector to express free-form proteins.

2.3. Creation of an insertion site in the N-terminal region of CIP

The strategy for creating heterologous ORF insertion site in the CIP-NT was similar to that described for the P3-NT (Fig. 1C). The construct pYC5CIP (a subclone of pTuHUP) harbouring an insert reflecting nt 2989–4265 of TuMV-YC5 genome representing the 5′ coding region of CIP (preceded by the 3′ coding region of P3 and full coding sequence of 6k1) was used as the template for site-directed mutagenesis. Initial PCR of pYC5CIP with the primer pair Clsense/Clantisense (5′-ACCTACAGTTTATCACCAGAGCCATGGCTCAATGATAT-3′/5′-TGCTGGTGATAAACCCTAGGTGCTGACTGGCC-3′, NcoI site underlined) created pYC5CIPNco possessing an NcoI site between the codons for the N-terminal 1st and 2nd amino acids of CIP (Fig. 2III). From pYC5CIPNco, a 495 bp DNA fragment (nt 3801–4265 of TuMV) possessing the context NcoI-NheI-CVYHQA in the CIP-NT was amplified by PCR with the primer pair CINHNIa/NarSac [(5′-TACCATCAGAGCCATGGCTCAATGATATAGAGGATGACTTGAGCAGAGAATCTC-3′ with NcoI-NheI sites (underlined) and CVYHQA coding seq-
quence (double-underlined)/5′-CAAAAACGAGCTCAGCTGGTCA-TTACTGA-3′). The Ncol-Narl fragment released from the 495 bp PCR product was ligated with Ncol-Narl-restricted pYC5CIPNco to generate pYCS5CIPNHIa that possessed the context Ncol-Nhel-CVYHQa at the CIP-NT.

Subsequently, the 1.25 kbp Sphl-Narl fragment (corresponding to nt 2993–4242 of TuMV-YC5) released from pYC5CIPNHIa was ligated with the Sphl-Narl-digested pTuHUP to generate pTuHUCIPNHIa. Finally, the 7.9 kbp Kpnl-NotI DNA fragment released from pTuHUCIPNHIa was ligated with the Kpnl-NotI-digested p35STu to generate p35S3CIPNHIa possessing a heterologous ORF insertion site in the CIP-NT for expressing free-form foreign protein therefrom (Fig. 2III).

2.4. Creation of an insertion site in the N-terminal region of Nib

The strategy for creating a heterologous ORF insertion site in the Nib-NT was similar to that described for the P3-NT (Fig. 1C). The construct pYC5Nib harbouring an insert reflecting nt 6112–8438 of TuMV-YC5 genome, representing the major part of 5′ Nib (preceded by the 3′ coding region of VPg and full coding sequence of Nla-Pro), was used as the template for achieving the required PCR-based sequence modifications. Initial PCR of pYC5Nib with the primer pair Nibsense and Nibantisense (5′-CGCGATCTGATCAGCACGACGACGATC-3′ and 5′-GTTTGTGATAGATGCTCCCTGGTTCAG-3′, NcolI site underlined) created pYC5NibNco possessing an Ncol site between the codons for the N-terminal 1st and 2nd amino acids of Nib (Fig. 2IV). Subsequent PCR of pYC5NibNco with the primer pair NibNHIa/Nibdw [(5′-TGGACAAACCGCAGTGGAGAGCGTGGTGGGAG-3′)] amplified a 1.27 kbp product representing nt 7199–8443 of TuMV genome with the context NcoI-NheI-CVYHQA in the Nib-NT. The NcoI-XhoI fragment released from pYC5NibNco was ligated with XhoI-MluI-digested p35S3CPNHNIa, p35SP3NHNIa, p35SCINHNIa, p35SNIbNHNIa, and p35SCP-NHNIa. The resulting constructs possessed the CIP-NT for expressing free-form foreign protein therefrom (Fig. 2V).

2.5. Creation of an insertion site in the N-terminal region of CP

A different strategy involving three successive PCRs with pYC5ST3 (harbouring an insert representing nt 5798–9875 of TuMV-YC5) created the required context Ncol-Nhel-CVYHQa between the codons for the 1st and 2nd amino acids of CP (Fig. 2V). Initial PCR of pYC5ST3 with the primers NbCP-U1 [(5′-TACCATCAAGCATGGTGAGAC-3′)] and NbCP-U2 ](5′-AAGGATCCATGGTGAAGATGATGAGGCA-3′) with Ncol and Nhel sites (underlined) and CVYHQa coding sequence (double-underlined) and MluI-d (5′-AATTCCATGGTGAAGATGATGAGGCA-3′) with MluI site (underlined) amplified a 687 bp DNA fragment. This product served as the template for the subsequent PCR with the primers NbCP-U2 [5′-AAGGATCCATGGTGAAGATGATGAGGCA-3′] and CVYHQa coding sequence (underlined) and CVYHQa coding sequence (double-underlined)] and Mlu-d. The amplified product served as the template for the final round PCR with the primers NbCP-U3 [(5′-CGCGATCTGATCAGCACGACGACGATC-3′)] and CVYHQa coding sequence (underlined) and MluI site (underlined) and Mlu-d. The 817 bp PCR product was end-corrected by AfeI-MluI restriction and the product was used to replace the corresponding region (nt 8644–9412 of TuMV-YC5 genome) in pYC5ST3. This replacement generated pST3C5CPNHNIa possessing the context C′Y′H′Q′A′-Ncol-Nhel-CVYHQa in the CP-NT. The XhoI-MluI fragment (nt 8433–9412 of TuMV-YC5) released from pST3CPNHNIa was ligated with XhoI-MluI-digested p35S5YC5 to generate p35S5CPNHNIa with a heterologous ORF insertion site in the CP-NT for expressing free-form foreign protein therefrom (Fig. 2V).

2.6. Insertion of GFP and Der p 5 ORFs

Construction of the TuMV-HCfuGFP recombinant (derived from p35S5HFuGFP) expressing GFP ORF in-frame fused with HC-Pro or the TuMV-HCFrGFP recombinant (derived from p35S5HCFrGFP) expressing free-form HC-Pro was described previously (Chen, 2006). The other TuMV-GFP recombinants expressing free-form GFP from different insertion sites of TuMV-YC5 were constructed in this investigation. The DNA fragment of GFP ORF was amplified from p35S5HFuGFP (Chen, 2006) using the primers GFP-Nco-u (5′-GGAAGCGGTA-3′) and GFP-Nco-d (5′-ATGGCTAGCTGTGTTTATCACCAGGCA-3′), NcoI site underlined) and GFP-Nhel-d (5′-CCATGGTGAGGCAACGACGACGATC-3′, Nhel site underlined) and Derp-Nco-d (5′-CGCGATCTGATCAGCACGACGACGATC-3′) and Derp-Nhe-d (underlined) amplified a 687 bp DNA fragment. This product served as the template for the subsequent PCR with the primers NbCP-U2 [(5′-AAGGATCCATGGTGAAGATGATGAGGCA-3′)] and CVYHQa coding sequence (underlined) and CVYHQa coding sequence (double-underlined)] and Mlu-d. The amplified product served as the template for the final round PCR with the primers NbCP-U3 [(5′-CGCGATCTGATCAGCACGACGACGATC-3′)] and CVYHQa coding sequence (underlined) and MluI site (underlined) and Mlu-d. The 817 bp PCR product was end-corrected by AfeI-MluI restriction and the product was used to replace the corresponding region (nt 8644–9412 of TuMV-YC5 genome) in pYC5ST3. This replacement generated pST3C5CPNHNIa possessing the context C′Y′H′Q′A′-Ncol-Nhel-CVYHQa in the CP-NT. The XhoI-MluI fragment (nt 8433–9412 of TuMV-YC5) released from pST3CPNHNIa was ligated with XhoI-MluI-digested p35S5YC5 to generate p35S5CPNHNIa with a heterologous ORF insertion site in the CP-NT for expressing free-form foreign protein therefrom (Fig. 2V).

A 330 bp fragment of the Der p 5 ORF, encoding the group 5 allergen of the house dust mite D. pteronyssinus (Der p 5), was amplified from p35ZYMVDerp5 (Hsu et al., 2004) using the primers Derp-Nco-d (5′-CCATGGTGAGGCAACGACGACGATC-3′) and Derp-Nhe-d (underlined) amplified a 687 bp DNA fragment. This product served as the template for the subsequent PCR with the primers NbCP-U2 [(5′-AAGGATCCATGGTGAAGATGATGAGGCA-3′)] and CVYHQa coding sequence (underlined) and CVYHQa coding sequence (double-underlined)] and Mlu-d. The amplified product served as the template for the final round PCR with the primers NbCP-U3 [(5′-CGCGATCTGATCAGCACGACGACGATC-3′)] and CVYHQa coding sequence (underlined) and MluI site (underlined) and Mlu-d. The 817 bp PCR product was end-corrected by AfeI-MluI restriction and the product was used to replace the corresponding region (nt 8644–9412 of TuMV-YC5 genome) in pYC5ST3. This replacement generated pST3C5CPNHNIa possessing the context C′Y′H′Q′A′-Ncol-Nhel-CVYHQa in the CP-NT. The XhoI-MluI fragment (nt 8433–9412 of TuMV-YC5) released from pST3CPNHNIa was ligated with XhoI-MluI-digested p35S5YC5 to generate p35S5CPNHNIa with a heterologous ORF insertion site in the CP-NT for expressing free-form foreign protein therefrom (Fig. 2V).
with the reagents provided by One-step RT-PCR reaction kit (GeneMark, Taipei, Taiwan, ROC). The primer pairs, HCN-u/HCN-d (5′-ACCCAGAAAGGAAGCA-3′/5′-GATTGTCACGCGGTTAAT-3′), P3N-u/P3N-d (5′-TCCTGACAAACATGCTAATG-3′/5′-TTTCTATTGAGCTCTTCAAC-3′), Cin-u/Cin-d (5′-TGCTCATGTGGTTGGCAG-3′/5′-ACAGGCCTCACTGCTA-3′), Nlb-u/Nlb-d (5′-TACAAGCATCGGACCGTT-3′/5′-TACACTGTTGCTTTTCTGCTA-3′), and CPN-u/CPN-d (5′-TTAAGGACCGTCTAAGA-3′/5′-TTGGCTGTCTGTGAGCCA-3′), were site-specific, respectively, to the sequences flanking the HC-Pro-NT, P3-NT, CIP-NT, Nlb-NT, and CP-NT of TuMV-YC5. The reactions were performed, as described previously (Chen, 2006).

In western blotting, relative yields of GFP and Der p 5 were estimated by comparing to respective standard proteins, the N-NtA affinity column-purified GFP (250 ng per protein band) from ZYM-VGFPlis recombinant infected squash (Hsu et al., 2004), and bacterially expressed Der p 5 (110 ng per protein band) (Hsu et al., 2004). The density of targeted bands was determined based on the Standard Sample Value-smooth Cubic Spline in the Alphalnagmer IS-2200 4.0.1 (Alpha Innotech Corporation, San Leandro, CA). Data were further transformed to the unit of μg protein/g tissue and analyzed by Duncan’s new multiple range test using JMP 5.0 (SAS Institute Inc., Cary, NC, USA).

2.9. Stability assay of TuMV-GFP and TuMV-Derp5 recombinants in host plants

The stability of various recombinants of TuMV-GFP and TuMV-Derp5 were assayed by successive passages of recombinants from infected plant tissues. Recombinant viruses in the infected plants of C. quinoa, N. benthamiana, and Brassica hosts were mechanically transferred to healthy plants at 8–10, 10, and 14–20 days intervals, respectively.

After several passages, the leaf tissues collected from the tested host plants were analyzed by western blotting and RT-PCR with appropriate antisera and primer pairs, respectively. To further monitor the possible deletion of GFP and Der p 5 ORFs from p35SCP GFP and its progeny-infected hosts, the amplified DNA fragments with site-specific primers CPN-u/CPN-d were cloned and sequenced, as described previously (Chen et al., 2003), and the translated amino acid sequences were analyzed by the program of Vector NTI Suite (InforMax, Inc. WI, USA).

3. Results

3.1. Symptoms caused by TuMV-GFP recombinants

The plasmid DNAs of the TuMV-GFP recombinants, p35SHCFuGFP, p35SHCFvGFP, p35SP3GFP, p35SNbGFP and p35SCP GFP, induced chlorotic lesions on the leaves of the local lesion host, C. quinoa 6–7 dpi, similar to those caused by the wild-type p35SYC5 (Table I, Fig. 3A). However, local lesions induced by the p35SP3GFP progeny were conspicuously smaller than those caused by the wild-type p35SYC5.
Table 1
Symptoms of different host plants induced by different recombinants of Turnip mosaic virus (TuMV) carrying the green fluorescent protein (GFP) or the dust mite allergen Der p 5

<table>
<thead>
<tr>
<th>Recombinants</th>
<th>Symptoms of inoculated plants(^a)</th>
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<tbody>
<tr>
<td></td>
<td>CQ</td>
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<tr>
<td>TuMV-35SYC5</td>
<td>CL</td>
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<tr>
<td>TuMV-HCFuGFP</td>
<td>CL</td>
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<tr>
<td>TuMV-HCFrGFP</td>
<td>CL</td>
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<tr>
<td>TuMV-P3GFP</td>
<td>CL</td>
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<tr>
<td>TuMV-CIGFP</td>
<td>PGL</td>
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<tr>
<td>TuMV-NbGFP</td>
<td>CL</td>
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<tr>
<td>TuMV-CPGFP</td>
<td>CL</td>
</tr>
<tr>
<td>TuMV-HCDerp</td>
<td>CL</td>
</tr>
<tr>
<td>TuMV-P3Derp</td>
<td>CL</td>
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<tr>
<td>TuMV-NbDerp</td>
<td>CL</td>
</tr>
<tr>
<td>TuMV-CPDerp</td>
<td>CL</td>
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</table>

CL, chlorotic lesions; PGL, pale-green spots; SP, symptomless; M, mosaic; MM, mild mosaic; Cstu-W, chlorotic stunting and final wilting; M-Stu, mosaic and stunting; Lc-Stu, leaf curl and stunting.

\(^a\) The host plants of TuMV, Chenopodium quinoa (CQ), Nicotiana benthamiana (NB), Brassica campestris var. chinensis (BCC), B. campestris var. ching-geeng (BCCG), B. juncea (BJ), and B. campestris var. pekinesis (BCP), were mechanically inoculated with individual TuMV recombinants. Symptoms were observed 10 dpi for C. quinoa and N. benthamiana, and 14, 20 and 40 dpi for Brassica hosts.

3.2. Expression efficiency of GFP by different recombinants

The GFP expressed by different TuMV recombinants in different hosts was detected by western blotting analyses using GFP antibody. A 79 kDa fusion protein containing HC-Pro (52 kDa) and GFP (27 kDa) was detected from host plants infected with TuMV-HCFuGFP (Figs. 4A, 5A and 6A). The 27 kDa free-form GFP was detected from host plants infected with other TuMV-GFP recombinants, i.e., TuMV-HCFrGFP, TuMV-P3GFP, TuMV-CIGFP, TuMV-NbGFP and TuMV-CPGFP (Figs. 4A, 5A and 6A). The GFP antigenic signal levels in the host plants infected with the TuMV-GFP recombinants were assessed in comparison to a standard GFP antigenic signal (the levels of GFP in hosts infected with TuMV recombinants were considered as reliable indices of GFP expression, since it was on par with that of TuMV CP co-expressed as a part of the viral polyprotein). This assessment revealed wide variation in the levels of GFP in different hosts infected with TuMV GFP recombinants with GFP ORF in different insertion sites (Table 2).

As mentioned above, in general, the intensity of the 27 kDa free-form GFP antigenic signal detected from host plants infected with TuMV-GFP recombinants matched with that of the co-expressed TuMV CP (Figs. 4A; 5A and 6A). However, in C. quinoa, infected with TuMV-GFP recombinants with GFP ORF inserted in P3-N, CIP-N, and NIB-N, the GFP antigenic signal was significantly lower than that of the co-expressed TuMV CP (Fig. 4A). Intriguingly, though the recombinant, TuMV-P3GFP induced chlorotic spots on the inoculated leaves of B. campestris var. pekinesis, GFP and
Fig. 3. Symptom development in Turnip mosaic virus (TuMV) host plants infected with different TuMV-GFP and TuMV-Derp5 recombinants. (A) Local lesion types caused by the TuMV-GFP recombinants on plants of Chenopodium quinoa. The constructs of p35SHCFrGFP, p35SHCFuGFP, p35SNIbGFP, and p35SCPbGFP caused chlorotic lesions similar to that caused by wild-type TuMV-35SYC5 vector (YC5); smaller chlorotic lesions were caused by the p35SP3GFP construct (P3); pale-green lesions were caused by the p35SCIGFP construct (CIP). (B) Symptom types on systemic host Nicotiana benthamiana infected with the different recombinant viruses. The recombinants derived from the constructs of p35SHCFuGFP (HCfu), p35SHCFrGFP (HCfr), p35SNIbGFP (NIb), and p35SCPbGFP (CP) induced symptoms of mosaic and wilting, similar to those caused by wild-type TuMV-35SYC5 vector (YC5). Plants infected by the virus progeny derived from p35SP3GFP (P3) showed chlorotic stunting 10–14 dpi, and wilting after 20 dpi. Symptoms of mosaic but not wilting were caused by the virus progeny derived from the constructs of p35SCIGFP construct (CIP). (C) Symptom types on systemic host N. benthamiana infected with the TuMV-HCDerp recombinant derived from the construct of p35SHCDerp (HC) induced chlorotic stunting 10–14 dpi, and finally wilting 20 dpi; infected with the other different TuMV-Derp5 recombinant viruses derived from the constructs of p35SP3Derp (P3), p35SNIbDerp (NIb), and p35SCPbDerp (CP), plant developed symptoms of mosaic, followed by wilting, similar to those caused by wild-type TuMV-35SYC5 (YC5); lane Mock, the buffer-inoculated plants.

TuMV CP were not detected in the systemic leaf tissues (Fig. 6A).

3.3. Stability of GFP expression by different TuMV recombinants

Temporal and across-the-generation (viral) stability of GFP expression was monitored for 6 months by (serial) western blotting analyses of hosts after initial inoculation and also after every successive trans-inoculation of active viral progeny on new host individuals. As evidenced by the results, the effective and sustained expression of GFP was dependent upon the site for insertion of the GFP ORF in viral genome and it was profoundly influenced by the host species (Table 3). Stable GFP expression from all insertion sites, except CP-NT, was evident in the plants of the local lesion host C. quinoa and the systemic host N. benthamiana throughout the period of study (equivalent to 21 and 18 passages, respectively). Among the Brassica...
hosts, *B. campestris* var. *chinensis* and *B. campestris* var. *chingeeng* supported TuMV expression of GFP for 4.5–6 months (equivalent to 11–13 passages), depending upon the site for insertion (*Table 3*). The TuMV expression of GFP in plants of *B. campestris* var *pekinensis* and *B. juncea* were very unstable, as evidenced by the undetectability of expression within 1–4 passages (*Table 3*).

### 3.4. Symptoms caused by TuMV-Derp5 recombinants

The TuMV-Derp5 constructs p35SHCDerp, p35SP3Derp, p35SNlbDerp, and p35SCPDerp, proved infectious to plants of the local lesion host, *C. quinoa*, inducing chlorotic local lesions, 6–7 dpi, similar to that caused by the wild-type TuMV-35SYC5 (*Table 1; Fig. 3A*). However, inoculation of the plants of *C. quinoa* or the systemic hosts with the construct p35SCIDerp did not result in symptom development (further analysis of p35SCIDerp-inoculated host individuals by RT-PCR and Western blotting did not evidence infection; data not shown). The virus progenies (collected from single lesions of *C. quinoa*) TuMV-P3Derp, TuMV-NIbDerp, and TuMV-CPDerp (derived from p35SP3Derp, p35SNlbDerp, and p35SCPDerp, respectively), induced systemic wilting on plants of *N. benthamiana* 7–9 dpi (*Table 1; Fig. 3C*); TuMV-HCDerp (derived from p35SHCDerp) induced chlorotic stunting 10–14 dpi and finally wilting 20 dpi. On plants of *Brassica* hosts, TuMV-NIbDerp and...
Fig. 6. Western blotting of the expression of GFP and Der p 5 ORFs in plants of *Brassica* hosts infected with different TuMV-GFP and TuMV-Deep5 recombinants. The individual extracts from equal amount (0.15 g) of leaf tissues were separated in a 12% acrylamide gel, transferred onto a PVDF membrane, and immuno-detected with the 1000×-diluted antiserum to GFP (Hsu et al., 2004), the 100×-diluted antiserum to Der p 5 (Hsu et al., 2004), or the 1000×-diluted antiserum to TuMV-YC5 (Chen et al., 2003). Leaf tissues of *B. campestris* var. chinensis, and *B. campestris* var. chin-geeng were collected at 14 dpi; and *B. juncea*, and *B. campestris* var. pekinensis collected at 20 dpi. (A) Detection of GFP. Lane M, protein markers; lane CK1, the positive control of the purified GFP by Ni-NTA affinity column from ZYMV-GFPHis recombinant infected squash (upper panel) (Hsu et al., 2004) and wild-type TuMV-35SYC5-infected individual host tissues (lower panel); lane HCFu, GFP fused with HC-Pro protein; lane HCFr, free-form GFP; lanes P3, CIP, NIb, and CP, the GFP ORF was inserted in the TuMV-GFP recombinants at the N-terminal regions of P3, CIP, NIb and CP, respectively; lane Mock, the buffer-inoculated plant. (B) Detection of free-form Der p 5. Lane M, protein markers; lane CK2, the positive control of bacteria-expressed Der p 5 (Hsu et al., 2004) (upper panel) and wild-type TuMV-35SYC5-infected individual host tissues (lower panel); lanes HC, P3, NIb, and CP, the Der p 5 ORF was inserted at the N-terminal regions of HC-Pro, P3, NIb, and CP, respectively; lane Mock, the buffer-inoculated host plant.
Table 2

<table>
<thead>
<tr>
<th>Insertion</th>
<th>Relative yield of expressed GFP and Der p 5 in different hosts (as protein band)</th>
<th>Der p 5</th>
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<td>Relative yield of expressed GFP and Der p 5 in different hosts (as protein band)</td>
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In western blotting analysis, the 12 kDa Der p 5 antigen was detected from the plants of *C. quinoa* individually inoculated with the TuMV-Derp5 constructs, p35SfChDerp, p35SP3Derp, p35SNibDerp, and p35SCPderp (Fig. 4B), but not from those inoculated with p35SCIlderp. However, the Der p 5 antigenic signal intensity was weaker in plants inoculated with p35SP3Derp or p35SNibDerp. Likewise, while Der p 5 antigen was detected also from the plants of *N. benthamiana* individually infected with the viral recombinants TuMV-HCDerp, TuMV-P3Derp, TuMV-NibDerp, and TuMV-CPDerp (derived from respective plasmid constructs through *C. quinoa*) the antigenic signal intensity was weaker in plants infected with TuMV-P3Derp or TuMV-NibDerp (Fig. 5B). In *Brassica* hosts, Der p 5 antigen was detected from the plants infected with TuMV-HCDerp, TuMV-P3Derp or TuMV-CPDerp. However, while the antigenic signal was much weaker in plants infected with TuMV-P3Derp, the same was not detected from plants infected with TuMV-NibDerp (Fig. 6B). An immunosignal (very close in molecular weight to Der p 5) was detected also from TuMV-NibDerp-infected plants of *Brassica* hosts, was understood to arise from a possible cross-reactivity of the antibody with a host polypeptide, since the same signal was evident also in mock-inoculated control individuals.

Assessment of Der p 5 level in the host plants infected with TuMV-recombinants in comparison to a standard Der p 5 antigenic signal revealed the relative yield of Der p 5 in different hosts infected with different TuMV-Derp5 recombinants. In host plants infected with TuMV-HCDerp and TuMV-CPDerp, significantly higher levels of Der p 5 were noticed, in comparison to that infected with TuMV-P3Derp or TuMV-NibDerp (Table 2).

In any host, the level of Der p 5 expression by TuMV-recombinants in comparison to a standard Der p 5 antigenic signal revealed the relative yield of Der p 5 in different hosts infected with different TuMV-Derp5 recombinants. In host plants infected with TuMV-HCDerp and TuMV-CPDerp, significantly higher levels of Der p 5 were noticed, in comparison to that infected with TuMV-P3Derp or TuMV-NibDerp (Table 2). In any host, the level of Der p 5 expressed by TuMV-P3Derp or TuMV-NibDerp did not match with the level of TuMV CP (Figs. 5B and 6B).

3.6. Stability of Der p 5 expression by TuMV recombinants

The stability of Der p 5 expression was monitored for 6 months by (serial) western blotting analyses of hosts after initial inoculation and also after every successive trans-inoculation of active viral progeny on new host individuals. Of the TuMV-Derp5 recombinants, TuMV-CIDerp was not retrievable from TuMV-CPDerp caused symptoms, 7–9 dpi, similar in severity to wild-type TuMV-35SYC5. However, TuMV-HCDerp induced mild mosaic symptom on plants of *Brassica* hosts, 14 dpi, strikingly similar to that induced by the TuMV-HCfGFP. This was different from the mosaic or mosaic and stunting induced by the other recombinants (Table 1). Delayed symptoms developed on plants of *B. juncea* and *B. campestris* var. *pekinesis* infected with TuMV-HCDerp and TuMV-P3Derp. However, unlike the mosaic, which remained mild on plants of *B. juncea* beyond 40 dpi (Table 1), the slight leaf curl noticed on plants of *B. campestris* var. *pekinesis* (until 14–16 dpi) became aggravated into severe leaf curl and stunting 20 dpi (Table 1).
inoculation at 8–10, 10, and 14–20 days intervals, respectively. Host plants

<table>
<thead>
<tr>
<th>Number of mechanical passages of recombinant</th>
<th>Host plants</th>
<th>GFP inserted at NT region of</th>
<th>Der p 5 inserted at NT regions of</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>HC-Pro</td>
<td>P3</td>
</tr>
<tr>
<td>CQ</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
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<td>NB</td>
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<td>18</td>
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<td>BCC</td>
<td>13</td>
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<td>BCCG</td>
<td>13</td>
<td>11</td>
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<tr>
<td>BJ</td>
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<tr>
<td>BCP</td>
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<td>0</td>
<td>1</td>
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a The plants of Chenopodium quinoa (CQ), Nicotiana benthamiana (NB), Brassica campestris var. chinensis (BCC), B. campestris var. ching-geeng (BCCG), B. juncea (BJ), and B. campestris var. pekinensis (BCP) were mechanically inoculated with individual TuMV recombinants. Leaf tissues collected from C. quinoa 8–10 dpi, from N. benthamiana 10 dpi, from B. campestris var. chinensis and B. campestris var. ching-geeng 14 dpi, from B. juncea and B. campestris var. pekinensis 20 dpi, were used for the analysis of the expression of GFP and Der p 5 by western blotting.
b The viral progenies from infected plants of C. quinoa, N. benthamiana, and Brassica hosts were successively transferred to healthy plants by mechanical inoculation at 8–10, 10, and 14–20 days intervals, respectively.
c The ORFs of GFP and Der p 5 were individually inserted at different insertion sites in the TuMV vector, including the N-terminal regions of HC-Pro, P3, CIP, Nib and CP.
d “-“: not detectable.
e The expression of GFP ORF by fusion to the HC-Pro protein was consistent for 13 passages on the plants of B. campestris var. chinensis, while that of free-form GFP was consistent for 11 passages.

3.7. Analysis of integrity of GFP and Der p 5 ORFs inserted in NT regions of TuMV

To understand the structural fate of GFP and Der p 5 ORFs in active TuMV recombinants, RT-PCR analyses were carried out on host plants, after initial infection and also after several successive passages. From sequence data, RT-PCR products of GFP ORF in different NT regions were expected to yield 902–960 bp products. However, in the initial RT-PCR analyses, apart from the expected full-length products, shorter fragments were also amplified (Fig. 7A). Sequencing of those shorter products revealed their coding potential for a partial GFP polypeptide starting with the first 2 amino acids, followed immediately by the stretch of amino acids 122–239 (data not shown). The precision of deletion from GFP ORF, irrespective of the TuMV insertion sites, imply a possible sequence and/or a secondary structural peculiarity of GFP ORF that may favor specific deletion. RT-PCR after several passages revealed several larger deletions from GFP ORF, as evidenced by the shorter products (Fig. 7A). For instance, RT-PCR profiles of p35SCPGFP-infected plants of C. quinoa, B. campestris var. chinensis, and B. campestris var. ching-geeng, after 11 successive passages, showed obvious deletion in GFP ORF (Fig. 7A). Sequencing of several shorter RT-PCR products and multiple alignment of their potential translates revealed occurrence of deletions from the central portions of GFP ORF, the extent of which varied among the hosts (Fig. 8). In plants of C. quinoa and B. juncea, two discontinuous regions representing the amino acids 27–146 and 167–199 of GFP ORF were deleted. However, in the plants of B. campestris var. chinensis, the deletion was of the region representing the amino acids 23–196. In the plants of B. campestris var. ching-geeng, a minor deletion in GFP ORF rendered it devoid of a short fragment encoding the amino acids 23–86 (Fig. 8).

Likewise, from the hosts plants infected with TuMV recombinants bearing Der p 5 ORF in different NT regions, RT-PCR products of 522–581 bp were expected. In initial RT-PCR analyses, the expected full-length products were amplified from the plants of C. quinoa and N. benthamiana infected with every TuMV-Derp5 construct. The full-length products were amplified also from the Brassica host plants infected with every TuMV-NibDerp construct, except from those infected with TuMV-NibDerp (Fig. 7B), suggestive of deletion of complete or major portion of the Nib-NT inserted Der p 5 ORF from the recombinant virus on (or briefly after) infection. Sequencing and sequence alignment of RT-PCR products from the host plants after certain number of passages revealed deletion of significant parts of Der p 5 ORF in the NT regions of HC-Pro, P3, and CP. Within five successive passages of TuMV-CPDerp progeny on the plants of B. campestris var. chinensis and B. campestris var. ching-geeng, the part of the ORF encoding the amino acids...
25–111 was found deleted (data not shown). As early as the second passage of TuMV-P3Derp on the plants of *B. campestris var. chinensis*, the part of the Der p 5 ORF encoding the amino acids 4–90 of Der p 5 was found deleted (data not shown).

### 4. Discussion

Most of the genomic regions and the mature proteins of potyvirus are involved in genome amplification or other essential functions (Haldeman-Cahill et al., 1998; Urcuqui-Inchima et al., 2001; Kekarainen et al., 2002). Therefore, when a viral genome is genetically engineered for expressing a heterologous protein, judicious selection and appropriate manipulation of insertion sites is necessary to avoid unintended negative effects (Mushedgean and Shepherd, 1995). Several potyviral vectors have been successfully developed to express heterologous proteins by inserting the ORFs mostly in the N-terminal region of HC-Pro (Beauchemin et al., 2005; German-Retana et al., 2000; Guo et al., 1998; Hsu et al., 2004; Masuta et al., 2000) or in the N-terminal region of CP (Aracez et al., 2001b, 2002; Beauchemin et al., 2005; Choi et al., 2000; Fernandez-Fernandez et al., 2001; Ivanov et al., 2003). Recently, the N-terminal region of P1 (of PVA) has also been demonstrated as the third effective insertion site in potyviral vector for the expression of GFP ORF in plants of *N. benthamiana* and *N. tabacum* (Rajamaki et al., 2005).

In the present study, we investigated the permissiveness of the TuMV genome to stably carry GFP or Der p 5 ORFs in the context encoding the NT regions of HC-Pro, P3, CIP, Nlb,
Fig. 8. Alignment of the deduced amino acid sequences of the GFP ORF expressed via the TuMV-CPGFP derived from p35SCPGFP in different host plants of Turnip mosaic virus (TuMV) after several successive passages. Plant tissues were collected at the 13th passages for the Chenopodium quinoa (CQ); at the 12th passages for the Brassica campestris var. chinensis (BCC) and B. campestris var. ching-geeng (BCCG); and the 4th passages for the B. juncea (BJ) for analysis by reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR amplified DNA fragments were cloned and sequenced, and the translated amino acid sequences were aligned by the program of Vector NTI Suite (InforMax, Inc. WI, USA). GFP ORF containing 239 residues is used as a template; dots represent the deleted amino acids; the remained amino acids identical to the residues of GFP are marked.

and CP and express the respective translate as a part of the viral polyprotein, the correct processing of which releases the heterologous protein in host cellular context. To appraise the effectiveness of the expression of GFP and Der p 5 from NT regions of P3, CIP and NIb, the proteins were also expressed, in parallel, from NT regions of HC-Pro and CP known for their effectiveness in expressing heterologous proteins. The TuMV recombinants were checked in different host backgrounds to assess the potential of host plants to support the expression of heterologous ORFs by TuMV. However, the efficiency and stability of expression of heterologous ORFs from the presently characterized sites varied. Moreover, the efficiency and stability of expression of an ORF from a particular context was dependent upon the particular ORF and the host plant employed. Though GFP expression from NIb-NT was weaker than that from CP-NT, it was stronger than that from HC-Pro-NT in several of the hosts, as evidenced by higher level of GFP. Though GFP ORF was expressible also from the P3-NT and CIP-NT contexts, the level of expression was lower than that from other insertion sites, in majority of the hosts (Table 2). Though Der p 5 was effectively expressed from the CP-NT and HC-Pro-NT in all the host plants employed, its expression from the P3-NT, NIb-NT and CIP-NT was either poorer or below an immunodetectable level, depending upon the host plants. The NIb-NT proved to be the least effective site for Der p 5, as evidenced by the protein’s very low level in the infected plants of C. quinoa and N. benthamiana and its undetectability from the plants of Brassica hosts (Table 2). Der p 5 expression from the CIP-NT was not assessable, for the inoculation of the plants of the local lesion host C. quinoa or the systemic hosts by the plasmid construct p35SCIDerp did not result in infection.

The recombinants carrying either GFP or Der p 5 ORF in the CP-NT induced disease symptoms comparable to the wild type, in timing and severity, suggesting that the heterologous protein expression from the CP-NT context did not affect TuMV assembly and functions, probably because the amino acid residues changed in the CP-NT were restricted to the virion surface (Shukla et al., 1994). Similar situation was known also from
and Der p 5 ORFs in majority of the hosts (Table 2). Reduction also functionally important for virus replication (Fernández et al., 1997), and CP (Dolja et al., 1994, 1995). Moreover, CIP is involved in virus movement (Carrington et al., 1992; Kekarainen et al., 2002) essential for viral genome functions as an RNA-dependent RNA polymerase (Riechmann et al., 1992).

The P3-NT provided poor support for the expression of GFP and Der p 5 ORFs in majority of the hosts (Table 2). Reduction or delay in symptom development was noticed from host plants infected by TuMV-P3NT recombinants. Especially, in the plants of *B. campestris var. pekinensis*, the systemic infection ability of TuMV-P3GFP recombinant was severely retarded. This could be attributed to the changes in a few amino acid positions in the N-terminus of P3 protein. Potyvirus P3 protein has been shown to play roles in the replication of PVA (Merits et al., 1999), accumulation of TVMV (Klein et al., 1994), and the spread of TuMV in the hosts such as *B. oleracea* L. and Japanese radish, *Raphanus sativus* L. (Suehiro et al., 2004). In a parallel investigation, we have also noticed that only the P3-NT of TuMV-YC5 was not suitable to express a conserved motif of tospovirus 1 protein (data not shown).

When expressed from certain TuMV contexts, in certain host backgrounds, the conspicuously lower level expression of the heterologous GFP and Der p 5, in comparison to that of the down-stream co-expression of TuMV CP as a part of the viral polyprotein, was quite intriguing. This discrepancy (that can be understood, if the profiles of RT-PCR and western blotting performed after the first passaging are scrutinized) was pronounced in *C. quinoa* in the expression of GFP or Der p 5 from the NT regions of P3, CIP and NiB (Figs. 4 and 7), while similar tendency was evident also in other host backgrounds (Figs. 5–7). This was suggestive of selective post-translational (or co-translational) degradation of the heterologous proteins, when they were expressed from certain TuMV genomic contexts. This host discrimination against the heterologous protein molecules, in a viral genomic context-dependent manner, could not be explained except by stressing the minor differences in the N-terminally acquired extra amino acids (the Nla protease cleavage site preceded the native N-terminus by a few amino acids, as shown in Fig. 2). Except for their N-terminal variations, the translates were identical in their sequences and hence, also in structures, irrespective of their translation from any TuMV context. The heterologous proteins expressed from the HC-Pro-NT, P3-NT and CP-NT possessed, respectively, serine, glycine and alanine, while those expressed from the CIP-NT and NiB-NT possessed threonine as their N-terminal amino acids. As elucidated by the great deal of studies conducted on the N-end rule phenomenon, the N-terminal residue of a protein could determine its metabolic stability, since ubiquitilation of a protein to be degraded is subsequent to the specific binding of the protein by N-recognins. A protein’s possession of a destabilizing N-terminal residue (an essential major component of protein’s intrinsic degradation signal or N-degron) renders it susceptible to N-recognin binding and subsequent ubiquitilation and degradation by the 26S proteasome (Varshavsky, 1997; Varshavsky et al., 1998; Pickart, 2000). An earlier study which identified threonine as a destabilizing residue, when it was the N-terminal residue of the *Photinus pyralis* luciferase, expressed transgenically in *N. tabacum* (Worley et al., 1998) tends to explain the presently noticed lower stability of GFP and Der p 5 with threonine N-terminus. If the poor stability of GFP and Der p 5 was dictated by the N-end rules of the host plants studied, appropriate manipulation of TuMV CIP-NT and NiB-NT could improve their effectiveness to express heterologous proteins therefrom. However, the N-terminal glycine being considered as a stabilizing residue (Varshavsky, 1996, 1997; Varshavsky et al., 1998), the lower stability of the glycine-N-terminus GFP expressed
from P3-NT sounds incongruous. Hence, the fact that, despite overall consensus, the N-end rules of different organisms exhibit considerable variations in the identity of N-terminal amino acids potential to act as stabilizing/destabilizing residues (Varshavsky, 1996) should be considered. Overall, our data stress the necessity for considering the features of N-end rule pathway operating in the host system, when the viral genome is manipulated for heterologous protein expression.

Temporal and across-the-generation (of virus) stability of expression of heterologous ORFs in recombinant viruses is an important criterion in terms of cost effectiveness and overall labour intensiveness of the strategy of viral expression of economically important proteins in plants. Batch-wise western blotting analyses of host individuals trans-inoculated successively at regular intervals with viral progenies obtained from previously infected individuals revealed the temporal and across-the-generation expression pattern of GFP and Der p 5 from NT regions of different TuMV genes in different host backgrounds (Table 3). These results stressed the importance of careful selection of viral genomic contexts for insertion of heterologous ORFs as well as host plants, depending upon the insert of interest. Though several promising combinations of heterologous ORF, viral insertion site and host plants ensuring consistent heterologous protein expression throughout the period of viral passageing on hosts were evident, there were also unproductive combinations. In the latter category, the heterologous protein expression was diminishing through successive passages of viral progeny on host individuals, as evidenced by decline in antigenic signal intensity in (serial) western blotting analyses performed after each trans-inoculation (data not shown). The heterologous ORFs used in the present study seem to vary in certain undetermined molecular characteristics, by which the TuMV genetic system clearly discriminated between them, irrespective of the site of insertion. When compared to Der p 5 ORF, the GFP ORF was expressed more efficiently from the majority of the insertion sites (Table 2). Moreover, as described above, the support extended by the host plants to the virus to express the heterologous ORFs was biased in favour of GFP (Table 3). For GFP expression from any of the TuMV contexts studied, C. quinoa and N. benthamiana extended support throughout the period of investigation. Among the Brassica hosts, B. campestris var. chinensis and B. campestris var. chin-geeng, extended full or considerable period of support to GFP expression, depending upon the site from which the GFP ORF was expressed. However, the support extended by plants of B. juncea and B. campestris var. pekinensis for GFP expression was very transient. For Der p 5 expression, C. quinoa extended temporary to full period support, while N. benthamiana extended very transient to considerable period of support, depending upon the context. The support extended by Brassica hosts for Der p 5 expression was still shorter in duration.

To understand the underlying cause of the loss of TuMV expression of GFP/Der p 5 from certain contexts, under certain host backgrounds, the structural integrity of the GFP and Der p 5 ORFs in various TuMV genomic contexts were checked by RT-PCR analyses. The RT-PCR analyses were done on the infected host plants, after initial inoculation and also after several successive passages of viral progenies on host individuals. The results evidenced deletions from GFP and Der p 5 ORFs, the extent and frequency of which varied between the ORFs, depending upon the TuMV contexts and the host plants (Figs. 7 and 8). We noticed that the deletions, which were understood to occur during the course of viral replications, were exclusively from within the heterologous ORFs, without affecting the flanking viral sequences (Fig. 8). The phenomenon of selective deletion from heterologous ORFs inserted in viral genome, while maintaining the integrity of viral genome sequences was reported earlier by several groups. The selective deletion from within heterologous ORFs inserted in HC-Pro sequence of PPV (Guo et al., 1998), CP-NT sequence of WSMV (Choi et al., 2000), and HC-Pro-NT and CP-NT of TuMV-UK1 (Beauchemin et al., 2005) are a few examples. However, there are cases wherein the inactivation of foreign ORFs by selective deletion of foreign inserts is imprecise. Deletion of parts of the HC-Pro-NT together with portions of foreign ORFs inserted in this context was reported from TEV vector (Dolja et al., 1993) and Beet yellow virus (closterovirus) (Dolja et al., 1997). The instability of foreign ORFs may be dependent upon the length of the foreign sequence (Dolja et al., 1993) or the sequence characteristics per se (Choi et al., 2000; Beauchemin et al., 2005). Insertions could alter the viral genome structure and organization, ranging from minor accommodative changes to serious alterations (Scholthof et al., 1996) to the extent of causing breakdown of viral structure and organization. The deletion-mediated elimination of inserts from the viral genome may be governed by a molecular mechanism largely involving viral recombination (Dolja et al., 1993; Guo et al., 1998; Van Vloten-Doting et al., 1985).

To summarize, the viral mechanism of deletion-mediated elimination/inactivation of heterologous ORFs discriminated between foreign inserts. There was a modulation of this viral mechanism by the host factor, as evidenced by the host-dependent variation in the structural stability of the ORFs and hence their expression. Also, the results hinted at the possibility for post-translational degradation of heterologous proteins based on the N-end rule of the host. Hence, in the efforts to express heterologous proteins in plants, the present report stresses the necessity for considering three equally important criterions, i.e., the nature of the ORF, the viral insertion site manipulated and the host plant employed. However, beyond the insert-viral insertion site-host triangle, there may be several factors to be considered, depending upon special requirements. For instance, from economic point of view, the yield of the protein of interest is a primary criterion. If insertion of a heterologous ORF in a certain relatively less permissive viral genomic context ensures high yield of the protein from a temporarily cooperative host within a short period of time, such combination may be opted. In the present study, deletion of segments from heterologous ORFs was faster for those constructed at CP-NT than those at HC-Pro-NT. This may not be surprising, since the N-terminal domain of CP is the most variable region in the potyviral genome, varying significantly in both length and sequence among isolates of a single virus, and that this region is commonly the site of recombination between potyvirus isolates or species (Shukla et al., 1994). However, prior to the deletion of the insert, the yield of the
expressed GFP or Der p 5 via this insertion site was relatively higher than via the other insertion sites in most of the host plants. In the special cases, such as dust mite allergen Der p 5, since the viral expression in plants is intended for immunotherapeutic oral administration of Der p 5 to improve the immunotolerance of patients to dust mite allergen, edibility of the plant hosts may be a desirable criterion. The biosafety concern raised when we expressed Der p 5 by ZYMV in zucchini squash (Hsu et al., 2004) and our subsequent switching-over to TuMV-Brassica system (Chen, 2006) (with the recombinant construct TuMV-HCDerp used in this study) is worth considering.

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