Construction and evaluation of transgenic tobacco plants expressing the coat protein gene of papaya ringspot virus with different translation leaders

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Abstract. Papaya ringspot virus (PRSV) YK isolate used in this study is a local mosaic strain isolated from Yung-Kang, Tainan, and its genome has been cloned and completely sequenced. A NcoI site before the coat protein (CP) reading frame of PRSV YK was generated by oligonucleotide-directed mutagenesis, and then the CP reading frame with the 3' noncoding region of PRSV YK was ligated with the gus leader sequence from the pGEM vector to create the construct pGCP. To express the CP with a homologous viral translation sequence, the gus leader was replaced by the cDNA sequence corresponding to the 5' region (nt 1-347) of PRSV genome to generate a protein containing 9 kDa polypeptide of PRSV P1 protein fused with the CP, and the construct was designated as pG5CP. In vitro translation from the transcripts derived from pGCP and pG5CP generated protein products of 36 kDa and 45 kDa, respectively. Both proteins reacted with the antiserum to PRSV CP, and the level of 36 kDa protein was higher than that of 45 kDa protein. The CP reading frame with the gus or PRSV 5' leaders was individually subcloned into a Ti binary vector. Transgenic tobacco plants (Nicotiana tabacum L. Havana 423) expressing the PRSV CP gene with the gus leader (GCP lines) or with the viral leader (5CP lines) were obtained by Agrobacterium-mediated transformation. When the transgenic lines were analyzed by western blotting, the protein products of 36 kDa and 45 kDa reacting to PRSV CP antiserum were detected in the GCP lines and 5CP lines, respectively. The presence of the CP gene in the transgenic tobacco was also confirmed by polymerase chain reaction (PCR) using primers specific to the CP gene. Analysis of segregation ratios in the T₂ plants of four GCP lines and four 5CP lines indicated that the CP gene in all of them was nuclearily inherited as a single dominant trait. R₇ and R₈ plants of the four GCP lines and four 5CP lines were inoculated with tobacco etch virus (TEV), potato virus Y (PVY), or pepper mottle virus (PepMoV). The transgenic lines showed significant delay in symptom development and the severity of symptoms was attenuated. The GCP lines expressing the PRSV CP gene by the gus leader accumulated higher levels of CP and showed higher degrees of resistance than the 5CP lines with the PRSV 5' leader. Our results indicate that the homologous viral leader does not enhance CP expression either in vitro or in vivo, nor does it provide better resistance in transgenic tobacco.

Keywords: Coat protein gene; Papaya ringspot virus; Potyvirus; Translation leader.

Introduction

Transgenic plants expressing the coat protein (CP) of a plant virus have been proved resistant to infection by the same or related viruses. The first illustration of CP-mediated resistance was reported by Powell-Abel et al. (1986). In that study the CP gene of tobacco mosaic virus (TMV) was transferred to tobacco, and symptom development was delayed in the transgenic plants when challenged with TMV. Since then, there have been numerous reports demonstrating that transgenic plants with viral CP genes are resistant to virus infection (Beachy et al., 1990; Fitchen and Beachy, 1993). The level of resistance ranged from delay of symptom development to immunity, and the spectrum of resistance to viruses ranged from narrow to broad. The CP-mediated protection has provided an innovative method for control of plant viruses.

Papaya ringspot virus (PRSV) is a member of the genus Potyvirus, the largest group of plant viruses (Reichmann et al., 1992). Gene expression and genetic organization of the virus have been characterized previously (Yeh and Gonsalves, 1985; Yeh et al., 1992). The genome of PRSV contains one large ORF, which encodes a polyprotein of 383 kDa that is proteolytically processed into functional proteins. The destructive papaya ringspot disease caused by the virus is the major factor limiting the wide-scale planting of this fruit tree throughout papaya.
growing areas (Purcifull et al., 1984). In the late 1970’s, PRSV spread throughout Taiwan island and destroyed most of its commercial papaya orchards (Wang et al., 1978). In Taiwan, two major strains of PRSV, the mosaic and will- ing types, were found, with the mosaic considered to be the most prevailing (Chang, 1979). The CP gene of a typi- cal mosaic strain PRSV YK isolated from Yung-Kang, Tainan county, has been sequenced and compared with other strains of PRSV from different geographic origins (Wang et al., 1994). The CP of PRSV is not translated in its final form but processed from the C-terminal part of the polyprotein (Yeh et al., 1992). Therefore, no initiation codon or leader sequence for translation exists in front of the CP reading frame. For potyviral CP genes to be func- tional in plant cells, a leader sequence and an initiation codon must be added for efficient translation and a pro- moter and a terminator are needed for efficient transcription.

The CP genes of several potyviruses have been trans- ferred to plants with various leaders from plant viruses or other sources. For example, the leader of the nos gene was constructed with the CP gene of bean yellow mosaic virus (BYMV) (Hammond and Kamo, 1993); the leader of gus gene with CP genes of plum pox virus (PPV) (Ravelonandro et al., 1992) and passionfruit woodiness vi- rus (PWV) (Yeh and Chu, 1996); the leader of alfalfa mosaic virus (AIMV) RNA 4 with the CP gene of tobacco vein mottling virus (TMV) (Maiti et al., 1993); the leader of tomato mosaic virus (TMV) with the CP gene of lettuce mosaic virus (LMV) (Dinant et al., 1993); the leader of potato virus X (PVX) with the CP gene of potato virus Y (PVY) (Hefferson et al., 1997); the leader of cucumber mos- saic virus (CMV) RNA 4 with the CP genes of PRSV (Ling et al., 1991; Fitch et al., 1992), watermelon mosaic virus II (WMV II), and zucchini yellow mosaic virus (ZYMV) (Namba et al., 1992); the leader of tobacco etch virus (TEV) with the CP genes of TEV (Lindbo and Dougherty, 1992) and peanut stripe virus (PSV) (Cassidy and Nelson, 1995). In these reports, CP transgenic plants showed various de- grees of resistance to homologous and heterologous potyvirus infection. Comparison between the amino acid sequences of the CPs of several potyviruses revealed that distinct viruses share an average sequence identity of 54% (Shukla and Ward, 1989). The broad resistance to infec- tion by heterologous potyviruses may be due to their high homology in the CP sequence.

In this study, the leader sequence from the gus reporter gene and the PRSV 5’ leader sequence were fused with the PRSV YK CP reading frame to express the gene in plant cells and compare their resistance to potyviral infection in transgenic tobacco plants. These two constructs of the CP gene were introduced into the Ti-binary vector, in which they were flanked with a CaMV 35S promoter and a nos terminator for expression in plant cells. Transgenic tobacco plants expressing the constructed CP genes were generated by Agrobacterium-mediated transformation, and their resistance to infection by heterologous potyviruses was evaluated under greenhouse conditions.

Materials and Methods

Plant Materials

Seeds of tobacco (Nicotiana tabacum L. Havana 423) were surface sterilized by being submerged in 0.1% hypochlorite for 30 min and placed on 2% water agar for germination. The plantlets were grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Leaves from the cultured tobacco plants were used for transformation.

Construction of PRSV CP Gene

Construction of PRSV CP genes with different transla- tion leaders is summarized in Figure 1. Plasmid pYK9D— containing most of the Nb gene, the complete CP gene, and the entire 3’ noncoding region of the genome of a se- vere mosaic type strain from Taiwan, PRSV YK—was ob- tained from previous studies (Wang et al., 1994). Plasmid pBH1121, containing the npt II and the gus genes, was pur- chased from Clontech (Palo Alto, California). The gus gene was moved from pBH121 to the pGEM vector (Promega, Madison, Wisconsin) by Smal and SacI digestion, generating a plasmid pGGUS. The NcoI site was introduced into pGGUS and pTMD9 by in vitro mutagenesis (Taylor et al., 1985 a&b) at the site immediately before the gus and CP reading frames, respectively. The NcoI/SacI fragment of the mutagenized pTMD9 was introduced into the mutagenized pGGUS, resulting in a substitution of the gus ORF by the PRSV CP reading frame with its 3’ noncoding region. The plasmid containing the CP gene with a gus leader was designated as pGGCP. The constructed CP gene with the gus leader was introduced into pBH121 (Clontech) by Smal/SacI replacement to generate plasmid pBGCP in the Ti vector. The CP gene in pBGCP was flanked with a CaMV 35S promoter and a nos terminator for transcription in plant cells (Figure 1).

In contrast to the gus leader, the 5’ leader from PRSV was also used for translation of the CP gene. The nucleo- tide sequence of the 5’ end of PRSV HA strain was previ- ously determined by direct RNA sequencing (Yeh et al., 1992). The cDNA clone corresponding to the 5’ end re- gion (nucleotide 1-977) of PRSV HA strain (Yeh et al., 1992) was amplified by polymerase chain reaction (PCR) and cloned into the pBluescript II vector (Stratagene, La Jolla, California). The obtained clone in pHA 1.0 contained 1 kb of the 5’ end region of PRSV. A different construct of the CP gene was generated by substituting the gus leader sequence with the 5’ end region (nt 1-347) of PRSV by Xbal and NcoI digestion, resulting in a plasmid pG5’CP which contained the complete viral leader sequence and the 87 amino acids of the N-terminal part of P, protein of PRSV (Yeh et al., 1992) in frame with the CP reading frame. The PRSV CP gene containing the homologous virus leader se- quence was transferred to pBH121 by Smal/SacI replace- ment in pBH121 vector and designated as pB5’CP (Figure 1).

The two plasmids containing the CP genes, pBGCP and pB5’CP, were mobilized to disarmed Agrobacterium
Figure 1. Construction of PRSV YK CP gene for expression in plant cells. The gus gene in pBI121 was introduced to pGEM vector to generate pGGUS. In vitro mutagenesis was performed to create a Ncol site before the gus and CP reading frames in pGGUS and pTMD9, respectively. The gus reading frame was substituted by the CP reading frame to generate plasmid pGGCP by Ncol/SacI digestion. The gus leader before the CP reading frame was replaced with the PRSV 5' leader from PHA1.0, which contained a cDNA fragment corresponding to the 5' region (nt 1-347) of PRSV RNA, by Xbal and Ncol digestion to generate pG5CP. Two constructs of the CP gene were subcloned in Ti vector pBI121 by substituting the gus gene to generate pBGCP and pB5CP, in which the CP gene was flanked by a CaMV 35S promoter and a nos terminator and coupled with the selection marker npt II gene within the T-DNA borders. The resulting plasmids pBGCP and pB5CP were introduced to Agrobacterium tumefaciens LBA4404 by triparental mating and used for plant transformation.

tumefaciens LBA4404 by the triparental mating method (Rogers et al., 1986). Agrobacterium tumefaciens cells cultured in the LB medium containing 50 mg/l kanamycin and 100 mg/l streptomycin at 28°C for 36 h were used for plant transformation.

In vitro Expression of the CP Gene

To verify the construction, the constructs pGGCP and pG5CP containing the entire CP reading frame with a gus leader and a PRSV 5' leader, respectively, were analyzed by in vitro transcription, in vitro translation, and immunoprecipitation. RNA transcripts were synthesized by run-off transcription using T7 RNA polymerase according to the manufacturer's directions (Stratagene). Approximately 1 μg of linearized DNA templates were used for in vitro transcription. The resulting RNA transcripts were dissolved in 25 μl of RNase-free double distilled water and used for in vitro translation.

Ten μl of transcription products were used in the rabbit reticulocyte translation system and in vitro translation was performed as described by the user’s manual (Stratagene). The products of in vitro translation were labeled with [35S]methionine and analyzed by immunoprecipitation with the antisera to PRSV CP (Yeh et al., 1984) following the procedure described by Dougherty and Hiebert (1980). In vitro translation and immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.
Plant Transformation

Agrobacterium-mediated transformation using leaf disks of tobacco was performed as described by Rogers et al. (1986). Transformsants were selected by MS104 medium (MS basal medium containing 1 ppm BA and 0.1 ppm NAA) containing 300 ppm kanamycin. The shoots regenerated from calli were rooted on the MS medium containing 150 ppm kanamycin. The established plantlets were maintained in a greenhouse for further analyses.

DNA Extraction and Polymerase Chain Reaction

The presence of PRSV CP gene in the putative transgenic tobacco plants was detected by PCR using primers specific to the CP gene. Total DNAs of the putative transgenic plants or non-transformed plants were extracted following the procedure described by Mettler (1987). One μg of RNAse A-treated DNA was used for PCR as template. The upstream primer HA92, 5'TTCGATGG CGTCCAAAGATGAAAGCT, and the downstream primer HA99, 5'AGGCTGTCAGCCTTATCTG, which reflect nucleotide positions 9256-9271 and 9974-9993 of the PRSV YK sequence (Wang and Yeh, 1997), respectively, were used for amplification. The PCR was performed with periods of 1 min for melting at 94°C, 2 min for annealing at 55°C, and 3 min for synthesis at 72°C for 30 cycles (Saiki et al., 1988). PCR products were analyzed by electrophoresis in 1% agarose gels.

Western Blot Analysis

The expression of the CP in the transgenic tobacco was analyzed by western blotting using the antiserum to PRSV CP (Yeh et al., 1984) as the primary antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson, West Grove, PA) as the secondary antibody. Leaf tissues from three youngest leaves of each putative transgenic plant were homogenized in 4 volume (w/v) of dissociation buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 3% 2-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue). The extracts were heated at 95°C for 5 min and centrifuged at 8,000 g for 3 min for removing plant debris. Total protein of each sample (15 μl) was loaded on 12% gels and separated by SDS-PAGE (Laemmli, 1970), and subsequently transferred to PVDF membranes (Millipore, Bedford, MA). The immunostaining procedure was performed as described by the GUS Gene Fusion System user’s manual (Clontech).

Inheritance Analysis of the Rf Progeny of the PRSV CP Transgenic Plants

The selected transgenic tobacco Rf lines expressing the PRSV CP gene were maintained in greenhouse and self-fertilized to generate Rf seeds. The progenies of transgenic lines containing the CP gene were screened by kanamycin-resistance provided by the nptII gene which was co-transformed with the CP gene. Seeds of Rf transgenic tobacco were surface sterilized and placed on 2% water agar for 4 days in the dark for germination. The germinated plantlets were cultured on the MS medium containing 150 ppm kanamycin. The surviving green plantlets were transferred to soil and grown under greenhouse conditions.

Resistance Evaluation of the Transgenic Plants

Subcloned Rf plants, Rf progenies of transgenic lines expressing the PRSV CP gene, and nontransformant controls at the stage of 5-6 leaves were mechanically inoculated with 1:20 (w/v) dilution of inocula of TEV, PVY and PepMoV (all provided by R. Provvidenti, Cornell University), which were prepared from leaves of virus-infected tobacco in 0.01 M potassium phosphate buffer (pH 7.0), on two fully expanded leaves of each plant. Each inoculum was also applied onto leaves of local lesion host Chenopodium quinoa Wild. The inoculated plants were kept in a greenhouse (20-35°C) and development of symptoms were recorded daily after inoculation.

Results

In vitro Expression of the CP Gene

Protein expression of constructs pGCP and pG5CP were analyzed using in vitro translation and immunoprecipitation, and results are shown in Figure 2. A 36 kDa protein was the major product when the transcript derived from pG5CP was used as template, and a major protein of 45 kDa was detected when the transcript derived from pG5CP was used as template. A 46 kDa protein expressed from endogenous mRNA did not react with the antiserum. Both 36 kDa and 45 kDa proteins were immunoprecipitated with antiserum to PRSV CP. A minor 36 kDa protein was also detected when the transcript from pG5CP was used for translation. This protein also reacted well with the antiserum to PRSV CP and was considered as the protein translated from the second AUG present in the joining Ncol site. The second initiation codon would result in a protein reflecting the entire reading frame of the

![Figure 2. Immunoprecipitation and in vitro translation analyses of transcripts from pG5CP and pG5CP by SDS-PAGE and autoradiography. The transcripts from pG5CP and pG5CP were translated in rabbit reticulocyte lysate and 'S-labeled protein products were immunoprecipitated with the antiserum to PRSV CP.](image-url)
CP, the same as that generated from the gus leader. The results of in vitro translation verified that the two types of leader sequences and the initiation codon were correctly constructed for expression of PRSV YK CP reading frame. Also, the gus leader appeared to have higher translation efficiency than the PRSV leader in vitro.

Expression of the PRSV CP Gene in Transgenic Tobacco Lines

The putative transgenic plants expressing the CP gene from the gus leader were designated as GCP lines and those from the PRSV 5′ leader were designated as 5′CP lines. PCR analysis using primers specific to PRSV CP gene amplified the expected 0.73 kb DNA fragment from four putative GCP transgenic lines GCP-4, GCP-14, GCP-15, and GCP-33; and four putative 5′CP transgenic lines 5′CP-1, 5′CP-2, 5′CP-3, and 5′CP-4 (Figure 3). This fragment was not detected in non-transformant controls (Figure 3).

Western blot analysis was used to examine the expression of PRSV CP in putative transgenic plant lines. A 36 kDa protein was detected in plant lines GCP-4, GCP-14, GCP-15 and GCP-33 (Figure 4A). The 36 kDa protein was co-migrated with the CP from PRSV infected Cucumis meloifera (Figure 4A). A 45 kDa protein was detected in transgenic plant lines 5′CP-1 and 5′CP-4 (Figure 4B), 5′CP-2 and 5′CP-3 (data not shown). The 45 kDa protein reacted with antisera to PRSV CP but in far smaller amounts than the 36 kDa protein from GCP lines (Figure 4A and B). The 36 kDa and 45 kDa proteins detected by the PRSV CP antiserum were not detected in non-transformant controls (Figure 4A and B).

Inheritance and Segregation of the CP Gene in the R, Progeny of the Transgenic Lines

The segregation ratios of R, progenies (resistant vs. susceptible to kanamycin) of the transgenic lines GCP-4, GCP-14, GCP-15, GCP-33, 5′CP-1, 5′CP-2, 5′CP-3 and 5′CP-4 fitted the 3:1 ratio for a single dominant trait (Table 1).

Seeds of R, progeny from transgenic lines GCP-4, GCP-15 and 5′CP-4 were obtained by self-fertilization of R, plants in greenhouse. Homozygous plants of R, progeny were determined by germination of R, seeds in the MS medium containing kanamycin. All the R, plants derived from a R, plant showing resistance to kanamycin represented homozygosity of the CP gene.

Evaluation of Transgenic Plant Lines Inoculated with TEV, PepMoV, and PVY

The inocula of each potyvirus used contained high concentrations of active virions, for 80-130 local lesions per leaf of inoculated C. quinoa were recorded. When the non-transformed plants were inoculated with TEV, they showed symptoms which consisted of prominent vein clearing and severe mottling on systemically infected leaves 8-9 days after inoculation. The infected plants were stunted and leaves with severe symptoms were generally smaller than healthy plants. R, plants of transgenic lines GCP-4, GCP-14, GCP-15, GCP-33, 5′CP-1, 5′CP-2, 5′CP-3 and 5′CP-4 showed attenuated symptoms 2-3 weeks after in-

Table 1. Segregation ratios of the R, progenies from the PRSV YK CP transgenic tobacco lines, which carried the CP gene of PRSV YK with the gus leader sequence (GCP lines) or the PRSV 5′-viral leader sequence (5′CP lines), as determined by the assay for resistance to kanamycin.

<table>
<thead>
<tr>
<th>Plant lines</th>
<th>Number of R, seedlings</th>
<th>Kanamycin-resistant</th>
<th>Kanamycin-susceptible</th>
<th>c2 (3:1)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCP-4</td>
<td>37</td>
<td>6</td>
<td>0.170</td>
<td>0.3-0.4</td>
<td></td>
</tr>
<tr>
<td>GCP-14</td>
<td>34</td>
<td>11</td>
<td>0.008</td>
<td>0.9-1.0</td>
<td></td>
</tr>
<tr>
<td>GCP-15</td>
<td>32</td>
<td>11</td>
<td>0.009</td>
<td>0.9-1.0</td>
<td></td>
</tr>
<tr>
<td>GCP-33</td>
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<td>13</td>
<td>0.177</td>
<td>0.7-0.8</td>
<td></td>
</tr>
<tr>
<td>5′CP-1</td>
<td>19</td>
<td>6</td>
<td>0.013</td>
<td>0.9-1.0</td>
<td></td>
</tr>
<tr>
<td>5′CP-2</td>
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<td>12</td>
<td>0.008</td>
<td>0.9-1.0</td>
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</tr>
<tr>
<td>5′CP-3</td>
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<td>13</td>
<td>0.530</td>
<td>0.5-0.6</td>
<td></td>
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<td>5′CP-4</td>
<td>34</td>
<td>12</td>
<td>0.029</td>
<td>0.9-1.0</td>
<td></td>
</tr>
</tbody>
</table>

*pProbability of goodness of fit was set at a significant level of 0.05.
occlusion (Table 2). In Rₖ plants of line GCP-15, symptom development was significantly delayed in 3 out of 10 inoculated plants, and the other 7 plants remained symptomless for a test period of 42 days (Table 2). The growth of these plants was similar to that of the healthy controls (Figure 5).

When inoculated with PVY and PepMoV, Rₖ plants of GCP and S₅CP lines delayed symptom development for 15-21 days compared to the controls (Tables 3 and 4, respectively). Most of the infected transgenic plants recovered, and newly developed leaves were symptomless. Growth of virus-infected plants in transgenic lines were less affected than the infected non-transformant controls. Plants of line GCP-15 showed the most significant resistance to PVY and PepMoV compared with other transgenic lines, with most of the plants remaining symptomless for a test period of 35 days (Tables 3 and 4). A few plants of line GCP-4 and S₅CP-4 remained symptomless to PepMoV infection (Table 4), indicating these two lines also had a relatively high level of resistance to PepMoV.

Since Rₖ plants of each line were obtained at different times, only 2-4 plants were tested for their resistance to infection by TEV, PVY and PepMoV. A similar delay in symptom development and attenuation in symptom severity were observed in both Rₖ (data not shown) and Rₖ plants. Among them, the Rₖ plants of line GCP-15 had a greater resistance than Rₖ plants of other lines.

**Discussion**

The CP gene of PRSV YK was constructed for expression in plant cells by adding a 35S promoter, a leader sequence, an initiation codon and a nos terminator. Two types of leader sequences, the leader from the commercial gus reporter gene and the PRSV 5’ leader, were introduced...
Table 2. Evaluation of transgenic tobacco lines, which carried the CP gene of PRSV YK with the gus leader sequence (GCP lines) or the PRSV 5'-viral leader sequence (5'CP lines), against TEV infection as determined by symptom development under greenhouse conditions.

<table>
<thead>
<tr>
<th>Plant lines*</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
<th>42</th>
</tr>
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<tbody>
<tr>
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<td>10</td>
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<td>10</td>
<td>10</td>
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<td>8</td>
<td>10</td>
<td>10</td>
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<td>10</td>
</tr>
<tr>
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<td>10</td>
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<td>3</td>
<td>3</td>
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<td>0</td>
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<td>3</td>
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<tr>
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<td>8</td>
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<td>10</td>
</tr>
</tbody>
</table>

*DPI: days post inoculation.
*R, progenies of GCP and 5'CP transgenic lines were used for inoculation. Ten plants of each transgenic line or untransformed control at the stage of 7-15 cm tall were mechanically inoculated with TEV using a 1:20 dilution (w/v) of TEV-infected tobacco tissue as inoculum.

Table 3. Evaluation of transgenic tobacco lines, which carried the CP gene of PRSV YK with the gus leader sequence (GCP lines) or the PRSV 5'-viral leader sequence (5'CP lines), against PVY infection as determined by symptom development under greenhouse conditions.

<table>
<thead>
<tr>
<th>Plant lines*</th>
<th>7</th>
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<th>21</th>
<th>28</th>
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<th>42</th>
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<tr>
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<td>GCP-14</td>
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<td>0</td>
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</tr>
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</tbody>
</table>

*The growth stage of plants and the preparation of inoculum were similar to those in Table 2. Ten plants of each line were used for inoculation.

Table 4. Evaluation of transgenic tobacco lines, which carried the CP gene of PRSV YK with the gus leader sequence (GCP lines) or the PRSV 5'-viral leader sequence (5'CP lines), against PepMoV infection as determined by symptom development under greenhouse conditions.

<table>
<thead>
<tr>
<th>Plant lines*</th>
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<th>21</th>
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*The growth stage of plants and the preparation of inoculum were similar to those in Table 2. Ten plants of each line were used for inoculation.
in frame with the CP gene in this study. The ability to generate the correct CP in the two constructs was verified by in vitro transcription, translation, and immunoprecipitation. Accumulation of the CP expressed by the gus leader in calli and leaves of the transgenic plant lines was detectable by western blot analysis. However, in transgenic lines expressing the CP by the PRSV 5′ leader, accumulation of CP was below detectable level except in the plantlet stage (data not shown). The different quantity of the CP between transgenic lines containing the gus leader and the PRSV leader may be due to different translational efficiencies in plant cells. Also, it might be due to the different stability of proteins based on size, conformation, or modified N-terminal structure of CP products.

When the resistance of four GCP lines and four 5CP lines was evaluated under high concentrations of inoculum, GCP-15 showed a high level of resistance. The other three lines, GCP-4, GCP-14 and GCP-33 showed lower levels of resistance as four lines of 5CP transgenic plants. These results were consistent with the previous reports for PRSV (Ling et al., 1991), LMV (Dinant et al., 1993), and PVY (Hefferon et al., 1997), in which plants accumulating detectable or higher levels of the CP showed higher degrees of resistance to virus infection. The results were in contrast to the observations made by Stark and Beachy in SMV (1989) and Namba et al. (1992) in WMV II and ZYMV, in which they showed that transgenic plants exhibiting the greatest protection against infection did not contain the highest level of the CP. These latter observations may explain some discrepancies in our results, such as the failure of the relatively high expressor GCP-14 to show a high level of resistance and a low expressor GCP-33 which did show a good level of resistance. However, both expression levels of CP and degrees of resistance of these two lines are lower than GCP-15.

The transgenic lines with the 5′ leader did not significantly enhance resistance because symptom development was delayed as with the gus leader. Ling et al. (1991) constructed the PRSV CP gene with a leader sequence of CMV RNA 4, but these CP transgenic plants were not resistant to infection by CMV, indicating that the presence of a heterologous viral leader sequence does not confer resistance. This phenomenon was also found in PSTV CP transgenic plants with TEV leader (Cassidy and Nelson, 1995) and PVY CP transgenic plants with PVX leader (Hefferon et al., 1997). In this study, the homologous viral leader sequence was used for expression of PRSV CP, but its expression levels were generally lower than the gus leader both in vitro (Figure 2) and in vivo (Figure 4 A and B). The broad spectrum resistance was highly dosage dependent. The PRSV 5′ leader possibly exhibited lower degrees of resistance since resistance became insignificant when plants were inoculated with higher inoculum dosages (data not shown). In the 5′ leader construct, the CP was fused with 87 amino acids of the N-terminal end of P1 protein. Whether this sequence affects the expression and the resistance provided by the CP sequence needs to be further investigated.

The heterologous CP-mediated protection generally showed lower levels of resistance than homologous CP-mediated protection (Fitchen and Beachy, 1993), and the resistance was usually displayed as a delay of symptom development. Since tobacco is not a host plant for PRSV, the resistance to the homologous virus could not be tested. Protection conferred by heterologous CP sequences has been shown in potyviruses (Stark and Beachy, 1989; Ling el al., 1991) and tomatoviruses (Nejjad and Beachy, 1990). It was suggested that approximately 60% homology in the CP amino acid sequence was sufficient to confer significant structural similarity to provide protection against related viruses (Stark and Beachy, 1989). Although the first 42 nts of the 5′ terminus of the genomic RNA of PRSV showed 80% similarity with those of TEV and PVY (Yeh et al., 1992), the 5CP transgenic plants did not express a high level of resistance to these viruses.

R5 subcloned plants and R5 plants of transgenic tobacco lines were evaluated for broad spectrum resistance, and they showed similar levels of it, indicating that the resistance conferred by the CP gene was inherited as a nuclear trait. Ling et al. (1991) showed that the hemizygosity of the PRSV CP gene in R5 transgenic tobacco resulted in a shorter delay in symptom development. Their observation was similar to the transgenic tobacco plants expressing the CP gene of WMV II or ZYMV that were resistant to infection by other potyviruses (Namba et al., 1992). Stark and Beachy (1989) also showed that plants with hemizygosity for expression of the SMV CP were more resistant to TEV infection. The different degrees of resistance between hemizygous and homozygous plants were not observed in R5 plants in this investigation (data not shown), possibly due to levels of inocula which were 5 to 25 times higher than in other studies, as compared by the local lesion assay on C. quinoa.

In this study, two constructs of PRSV CP gene, containing the gus leader or the homologous PRSV 5′ leader, were transferred into transgenic tobacco. The transgenic lines exhibited broad spectrum resistance to heterologous potyviruses in delaying symptom development and in attenuation of symptom severity. Transgenic line GCP-15, which carried the CP gene with the gus leader, showed a relatively higher level of resistance to infection by the challenge viruses. These two constructs will be transferred to papaya and the ability of CP-mediated resistance against PRSV infection in its natural host papaya will be investigated.

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具備不同前導序列木瓜輪點病毒鞘蛋白基因之
轉基因菸草之構築及其抗病分析

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木瓜輪點病毒 YK 系統為分離自台南縣永康鄉之嚴重寄生型病毒系統，其基因體已被選殖及解序完成。此病毒之鞘蛋白基因及 3' 非轉譯區利用變點突變後接於 gus 基因前導序列，構築成質體 pGGCP。質體 pG5CP 為 pGGCP 上之 gus 基因前導序列被 PRSV 之 5' 片段（nt 1-347，此片段含 P1 蛋白 N 端之 9 kDa 部分）取代後之載體。pGGCP 和 pG5CP 在生體外轉錄及轉譯分析中，分別產生可與 PRSV 之抗血清反應之 36 kDa 與 45 kDa 蛋白，而以前者產生之量較後者為多。此二構築之 PRSV 鞘蛋白基因分別利用農桿菌媒介轉殖至菸草株（Nicotiana tabacum L. Havana 423），轉基因菸草利用西方染色法可觀測到表現良好之 36 kDa 蛋白或較微量之 45 kDa 蛋白。利用聚合 連鎖反應可觀測到 CP 基因存在於菸草之中。分析八個轉基因株系之子代，其分離比顯示 CP 基因均以單一顯性基因形式存在於菸草染色體。四個含有 gus 基因前導序列及四個含有 PRSV 5' 前導序列之鞘蛋白轉基因菸草之 R0 及 R1 世代植株經以 TEV、PVY 及 PepMoV挑戰接種，轉基因植株明顯延緩病徵之表現及減輕病徵之嚴重程度，但含有 gus 基因前導序列之轉基因菸草之抗性優於 PRSV 5' 前導序列之轉基因菸草，此顯示 PRSV 之前導序列並未能增加抗病性。

關鍵詞：木瓜輪點病毒；鞘蛋白基因；馬鈴薯 Y 群病毒；轉譯前導序列。