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## **Restricted virus multiplication in potato plants transformed with the putative replicase component gene of potato leafroll virus**

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### **Introduction**

Genetically engineered resistance to virus infection has been obtained by transformation of plants with wild-type or modified viral genes encoding replicase proteins (reviewed in 2 and 3). Such replicase-mediated resistance proved to be effective against a number of viruses belonging to different taxa. However, replicase-mediated resistance have been tested for viruses that were sap-transmissible, not for those that infect only phloem tissue and are transmitted by insects.

Potato leafroll virus (PLRV) is a member of the genus *Luteovirus*, a group of aphid-transmitted, phloem-limited viruses, and causes severe yield losses in potato worldwide. The viral genome consists of a 5.8kb single-stranded (+) RNA with six major open reading frames<sup>6,8,9,12</sup> (ORFs; Fig. 1). ORF2 possesses the conserved GDD motif of viral replicase.<sup>9</sup> The putative viral replicase was expressed by a -1 frameshift in the region of overlap between ORFs 1 and 2 to produce an ORF1-ORF2 fusion protein.<sup>11</sup>

To determine whether a strategy involving the replicase gene for introducing resistance to viral infection could be effective for PLRV, potato plants were transformed with PLRV ORF2.

### **Materials and Methods**

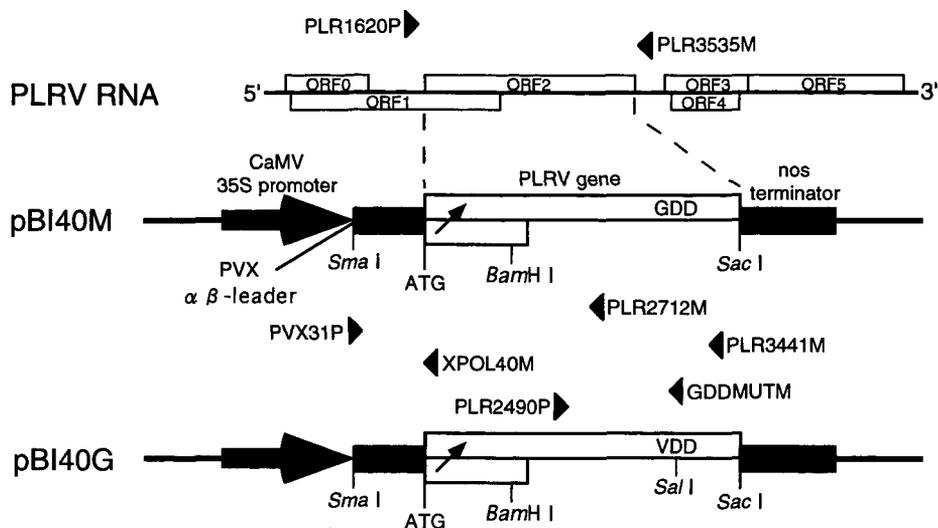
#### **cDNA cloning of PLRV ORF2**

PLRV-J was purified and the genomic RNA was extracted as described previously.<sup>10</sup> The first strand cDNAs that cover ORF2 and its upstream region were synthesized from PLRV-J RNA using Moloney murine leukemia virus reverse transcriptase (BRL; Life Technologies) and an oligonucleotide primer PLR3535M (Table 1). Double stranded cDNAs to ORF2 were then amplified

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**Fig. 1.** Schematic representation of PLRV genomic RNA showing the positions of ORFs and insertion of ORF2 in the plant expression vector pBI40M and pBI40G. ATG start codon was created at beginning of ORF2. Small arrows indicate the position of translational frameshift. Arrowheads indicate the position of primer using in this experiment.

**Table 1.** Primers used in PCR and mutagenesis

Primer	Sense	Sequence <sup>a)</sup>	Position <sup>b)</sup>
PLR1620P	plus	5' <u>CACTTGTGCGACAAGATCTGGGCTGATG</u> 3' <i>Sal</i> I	PLRV 1602-1628
PLR2490P	plus	5' <u>GAGGACCATAAGCTTCTCCAGTG</u> 3'	PLRV 2488-2511
PLR2712M	minus	5' <u>ACAGAACCCGgGCTACCAGTT</u> 3'	PLRV 2702-2722
PLR3441M	minus	5' <u>GCTTGGCAACaAGCTCACGAT</u> 3'	PLRV 3431-3451
PLR3535M	minus	5' <u>GCTGTCGACTCGTGTATGCTTGGCTAG</u> 3' <i>Sal</i> I	PLRV 3511-3537
GDDMUTM	minus	5' <u>AGCATCGTCgaCCATAGCCA</u> 3'	PLRV 3173-3192
PVX31P	plus	5' <u>ATACCCGGGGAAAACTAAACCATACA</u> 3' <i>Sma</i> I	ATACCCGGG+PVX 1-17
XPOL40M <sup>c)</sup>	minus	5' <u>CTCCAGACCGTAATCTTcCATTTGTTAGACTGCTTTTC</u> 3'	PLRV 1644-1660+PVX 67-87

<sup>a)</sup>Restriction site underlined. Mismatched base in lowercase letter.

<sup>b)</sup>The numbers correspond to those in the published nucleotide sequence of the PLRV RNA<sup>8)</sup> and PVX RNA<sup>9)</sup>.

<sup>c)</sup>Inserted base in lowercase letter creates the start codon in complementary sense.

from the first strand cDNA using two oligonucleotide primers, PLR1620P and PLR3535M, by polymerase chain reaction (PCR). PCR amplification was performed with *Tth* DNA polymerase (Toyobo) using a three-temperature program (94°C for 1min, 54°C for 2min, 72°C for 3min) for 30 cycles. The amplified cDNAs were cloned in *Sal* I site of a plasmid pUC119, resulting in pPLR2B. The cDNAs were sequenced using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical) or with a DNA sequencer (Model 370A, Applied Biosystems), using dye-labeled primers (Applied Biosystems) and *Tth* DNA polymerase (Toyobo).

### Vector construction

The full length PLRV ORF2 clone pPLR2B was subjected to site-directed mutagenesis using a protocol of Transformer site-directed mutagenesis kit (Clontech). Plasmid pPLR2B was mutagenized with primers PLR2712M and PLR3441M (Table 1) for elimination of *Sma* I and *Sac* I sites without amino acid change, respectively, for further construction in pBI121 described later. This clone was called pPLR2BM. Plasmid pPLR2BM was mutagenized further with primer GDDMUTM (Table 1) to produce pPLR2BG in which the GDD sequence was replaced by VDD.

Since potato virus X (PVX) 5' non-translated leader ( $\alpha\beta$ -leader) sequence is known to enhance translation of foreign genes<sup>14</sup>, it was introduced into upstream of PLRV ORF2. The cDNA fragment was synthesized by reverse transcription and polymerase chain reaction (RT-PCR), using the PVX (kindly provided by Dr. D. Hosokawa, Faculty of Agriculture, Tokyo University of Agriculture and Technology) genome as a template, with two oligonucleotide primers, PVX31P<sup>7)</sup> and XPOL40M which have 5' PLRV ORF2 sequence and 3' PVX  $\alpha\beta$ -leader sequence (Table 1). Five prime half of the PLRV ORF2 fragment was amplified by PCR, using the plasmid pPLR2BM as a template, with two oligonucleotide primers, PLR1620P and PLR2712M. The PVX  $\alpha\beta$ -leader fragment was then fused to the PLRV ORF2 fragment by PCR<sup>13)</sup> with two oligonucleotide primers, PVX31P and PLR2712M. The chimeric gene fragment was cut with *Bam*H I and cloned into *Hinc*II/*Bam*H I-digested pUC119, resulting in pPLR40. A *Bam*H I-*Sac* I fragment of pPLR2BM and pPLR2BG were inserted into *Bam*H I/*Sac* I-digested pPLR40, yielding pPLR40M and pPLR40G, respectively. The plasmids pPLR40M and pPLR40G contained the PVX  $\alpha\beta$ -leader sequence, upstream of the full length PLRV ORF2. Modification in the GDD motif and addition of the  $\alpha\beta$ -leader for the derived pPLR40M and pPLR40G clones were confirmed by sequence analysis. The chimeric genes were excised from the recombinant plasmid with *Sma* I and *Sac* I and inserted into *Sma* I/*Sac* I-digested pBI121 (Clontech), a binary expression vector which contains the cauliflower mosaic virus 35S promoter and the nopaline synthetase gene transcriptional terminator (pBI40M, pBI40G; Fig. 1). The recombinant plant expression vectors were introduced into *Agrobacterium tumefaciens* LBA4404 by a direct transformation method.<sup>1)</sup>

### Potato transformation

Transformation of Potato (cv. May Queen) was carried out using the tuber discs method as described previously<sup>7)</sup>. Transformed shoots were selected by propagation on medium containing kanamycin (50mg/l). Plants transformed with either pBI40M or pBI40G were designated as 40M and 40G, respectively.

### PLRV inoculation

Plantlets were propagated *in vitro* cultures, rooted in the test tube with moistened vermiculite, and immediately inoculated with PLRV by aphids as described previously<sup>7)</sup>. PLRV was detected by enzyme-linked immunosorbent assay<sup>4)</sup> (ELISA).

### Genomic PCR analysis

The presence of the PLRV ORF2 cDNA in the regenerated potato plants was determined by PCR analysis of genomic DNAs.<sup>7)</sup> Amplification of the transgene was carried out using two oligonucleotide primers, PLR2490P and GDDMUTM (Table 1). PCR amplification was performed by the procedure described previously<sup>7)</sup> except for the annealing temperature at 54°C.

### Northern analysis

Transcription of PLRV ORF2 was analyzed by Northern blots of total RNA extracts from leaf tissues using specific RNA probes<sup>7)</sup>. Blots were probed with *in vitro* synthesized digoxigenin (DIG)-labeled transcripts complementary to nucleotides 2200–2817 of the PLRV RNA<sup>8)</sup> inserted in pBluescript IISK(-) (Stratagene).

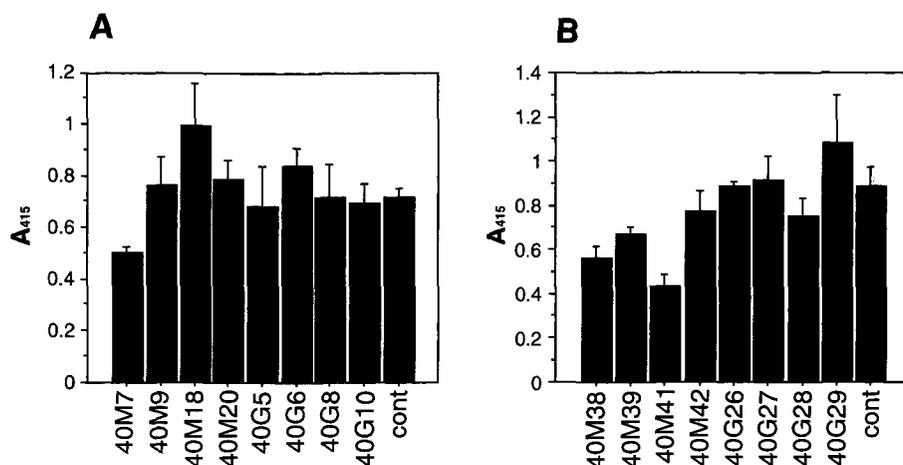
## Results and Discussion

### Sequence analysis

The complete nucleotide sequence of PLRV-J ORF2 will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number AB001894. The insert DNA of a clone pPLR2B is 1882 nucleotides and contains ORF2 encoding 616 amino acids. Alignment of this sequence with homologous sequences from the Scottish<sup>8)</sup>, Netherlands<sup>12)</sup>, Canadian<sup>6)</sup>, and Australian<sup>6)</sup> isolate reveals amino acid sequence identities of 99.0, 96.9, 97.2, and 96.1%, respectively. Typical conserved amino acid motifs of RNA-dependent RNA polymerase and the heptanucleotide motif<sup>9)</sup> of frameshift were identified.

### PLRV resistance

Fifteen 40M and seventeen 40G lines were analyzed for their susceptibility to PLRV. Typical results are shown in Fig. 2. All of the inoculated lines were infected with PLRV. However, the mean ELISA values of some infected transgenic lines, especially 40M7 and 40M41, were apparently lower than those of infected non-transgenic plants (Fig. 2). These results indicated that the infected transgenic lines 40M7 and 40M41 contained apparently lower amount of PLRV than the non-transgenic plants. The same level of reduction of PLRV accumulation in the line 40M7 was confirmed by repeated inoculation experiment (data not



**Fig. 2.** Analysis of the viral accumulation in PLRV inoculated potato plants transformed with the PLRV ORF2. A and B is different experiments. Line (cont) is non-transgenic plants. Ten *Myzus persicae* (Sulz.) that had been raised on PLRV-infected *P. floridana* were placed on leaves of plants approximately 5cm in height for an inoculation access period of 5days. After 40days, upper leaf tissue was collected and tested by ELISA. Plants were classified as uninfected if their ELISA values were not different from healthy control extracts (<0.200). Each bar represents the mean ELISA value ( $A_{415} \pm SE$ ) of infected plants (3-6 plants) of lines tested.

shown).

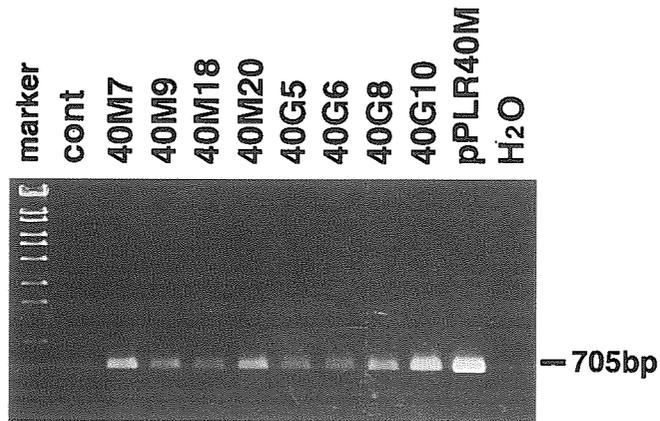
All of 40G lines were highly susceptible to PLRV. Further research with ORF2 protein may elucidate the effect of the glycine to valine substitution.

### Genomic PCR and Northern analysis

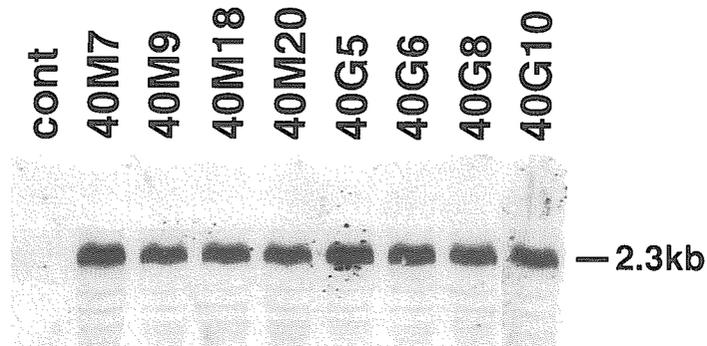
Eight transgenic lines shown in Fig. 2A were confirmed for insertion (Fig. 3) and transcription (Fig. 4) of the transgene. The amount of PLRV ORF2 transcripts was similar in all eight lines examined.

The results of the present investigation show that at least one (40M7) out of eight transgenic potato lines expressing the PLRV ORF2 restricted virus multiplication. The restriction of virus multiplication may have a positive effect on controlling PLRV infection due to the reduced aphid transmission efficiency of PLRV as was evidenced previously<sup>7)</sup>.

The mechanism(s) underlying the restriction of PLRV multiplication in transgenic plants are not yet clear. No correlation between the level of ORF2 transcript and restricted virus multiplication was found in this experiment. In the case of luteoviruses, there also is a possibility of another mechanism for resistance, because of their aphid-transmission and phloem-limited nature.



**Fig. 3.** Detection of transgene in transgenic plants. Agarose gel (1%) electrophoresis of the products of genomic PCR amplifying PLRV ORF2 cDNA. Genomic DNA was isolated from non-transgenic plant (cont) and plants transformed with pBI40M (40M7, 40M9, 40M18 and 40M20) or pBI40G (40G5, 40G6, 40G8 and 40G10). For positive control, cDNA clone (pPLR40M) were used. Marker: *S*<sub>ty</sub> I-digested  $\lambda$  DNA. Position of the PCR product of the expected size (705bp) is indicated at right.



**Fig. 4.** Northern analysis of transgenic plants. Twenty  $\mu$ g of total plant RNA extracted from leaf tissue was electrophoresed in a 1.2% agarose gel containing formaldehyde. The blot was probed with DIG-labeled RNAs complementary to nucleotides 2200-2817 of the PLRV RNA. Position of the mRNA of the expected size (2.3Kb) is indicated at right.

### Summary

Potato plants (cv. May Queen) were transformed with the putative replicase component gene (ORF2) of potato leafroll virus (PLRV) and analyzed for their susceptibility to PLRV. At least one out of eight transgenic potato lines expressing the PLRV ORF2 restricted virus multiplication. The amount of PLRV ORF2 transcripts was similar in all eight lines examined.

### Acknowledgment

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