J. Fac. Agr. Hokkaido Univ., Vol. 67, Pt. 1 : 1~13 (1997)

Restricted virus multiplication in May Queen potato plants transformed with the coat protein gene of potato leafroll *Luteovirus*

Toru KONDO, Takeshi MATSUMURA*, Noriko TABAYASHI*, Naoko YAMASHITA, Ichiro UYEDA, Tatsuji HATAYA, Haruo SARUYAMA*, Masatoshi TANIDA*, Ikuo KIMURA and Eishiro SHIKATA*

Department of Agrobiology and Bioresources, Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060, Japan

(Received September 27, 1996)

Introduction

Potato leafroll *Luteovirus* (PLRV) infects potatoes worldwide, causing significant economic loss. The virus is transmitted by several aphid species in a persistent manner and the isometric particles are confined in phloem tissue of infected plants. Current control measures are limited to the use of virus-free seed potatoes and the application of insecticides to minimize aphid activity. The use of natural resistant genes for controlling the disease through conventional breeding is difficult because the tetraploid nature of potato cultivars makes analysis and localization of virus-resistant genes impossible. Genetically engineered resistance is needed as an alternative means for reducing damage caused by PLRV infection. Coat protein (CP)-mediated resistance was first reported for transgenic tobacco plants expressing the tobacco mosaic virus (TMV) CP.[15] Resistance against TMV requires an accumulation of CP and does not seem to involve the induction of plant defense mechanisms.[18] However, in other cases of CP-mediated resistance, different mechanisms may be involved.[7]

Resistance to PLRV in transgenic potato and tobacco plants carrying the CP gene has been described previously.[3,4,10,16,20] In all these reports, resistance appears to develop by restricting multiplication of the virus. In the present study, we introduced a CP gene of PLRV into May Queen, one of the major potato cultivars in Japan. We also attempted to reinforce the resistance of transgenic plants by introducing the potato virus X (PVX) 5' non-translated leader ($\alpha\beta$ -leader) sequence, which is known to enhance translation of foreign genes[17,22], upstream of the PLRV CP gene. The resistance of transgenic plants to PLRV at the primary (aphid-borne) and secondary (tuber-borne) stages of infection was also investigated.

*Hokkaido Green-Bio Institute, Naganuma, Yubari-gun, Hokkaido 069-13, Japan

Materials and Methods

Vector construction

cDNA encoding PLRV CP was excised from pUCPLCPSS1[14] by digestion with *Pma* C I and *Sac* I. The fragment was ligated with *Sma* I/*Sac* I -digested pBI121 (Clontech), a binary expression vector, to produce plasmid pBILR1, which contains the sequence encoding PLRV CP, under the control of a cauliflower mosaic virus 35S promoter and the nopaline synthetase gene transcriptional terminator (Fig. 1). The same vector plasmid was used to transform

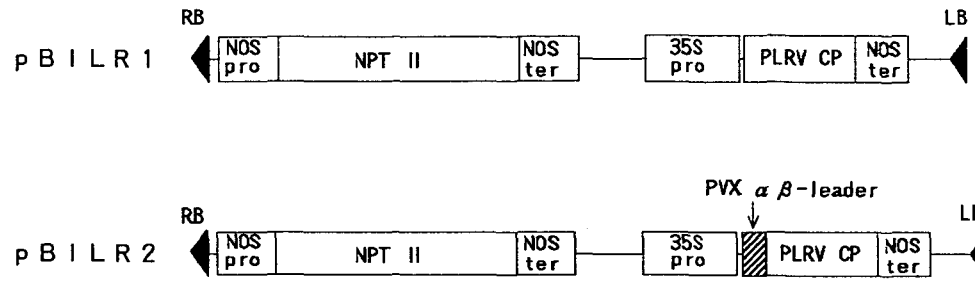


Fig. 1. Schematic representation of two different types of transformation vectors containing the PLRV CP gene. Symbols: RB, T-DNA right border; NOS pro, nopaline synthetase promoter; NPT II, neomycin phosphotransferase II gene; NOS ter, nopaline synthetase terminator; 35S pro, cauliflower mosaic virus 35S promoter; LB, T-DNA left border.

potatoes with a chimeric construct of PVX $\alpha\beta$-leader sequence and PLRV CP gene (pBILR2, Fig. 1). To construct pBILR2, the $\alpha\beta$-leader cDNA fragment was synthesized by reverse transcription and polymerase chain reaction (RT-PCR), using the PVX[9] genome as a template, with two oligonucleotide primers, PVX31P (5'ATACCCGGGGAAAACTAAACCATACA3'; *Sma* I site underlined) and XLR32M (5'TTTAACCACGACCGTACTCATTTTTTAGACTTGCTTT3'). PVX31P corresponds to nucleotides 1-17 of PVX RNA.[9] XLR32M corresponds to nucleotides 3696-3713 of PLRV RNA[12] and 69-87 of PVX RNA. The PLRV CP cDNA fragment was amplified by polymerase chain reaction (PCR), using the plasmid pUCPLCPSS1 as a template, with two oligonucleotide primers, PLCP33P (5'ATGAGTACGGTCGTGGTTA3') and PLCP14M[14] (5'GCTGCAGAGCTCTATTTGGGGTTTTGCAAAGC3'; *Sac* I site underlined). PLCP33P and PLCP14M correspond to nucleotides 3693-3711 and 4299-4319 of PLRV RNA, respectively. The $\alpha\beta$-leader fragment was fused upstream of the CP fragment by PCR[21] with two oligonucleotide primers, PVX31P and PLCP14M. The chimeric gene fragment was cut with *Sma* I and *Sac* I and cloned into *Sma* I/*Sac* I -digested pBI121, resulting in pBILR2. The plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 either by triparental mating using *Escherichia coli* HB101 harbouring pRK2013[5] or direct transformation.[2]

Potato transformation

Potato tubers (cv. May Queen) were peeled and sterilized for 15 minutes in sodium hypochlorite solution (1% chloride concentration), and then rinsed 6 times with sterilized water. Tuber discs were punched out with corkbore (diameter 1cm) and cut into thicknesses of 2-3mm. The discs were inoculated for about 15 minutes with overnight culture of *A. tumefaciens* which was resuspended in Murashige-Skoog (MS) liquid medium containing 3% sucrose. They were then placed in MS medium containing 3% sucrose and 0.8% agar, and incubated at 21°C under 16hr of light. Three days after inoculation, the discs were rinsed with MS medium containing 100mg/l kanamycin and 500mg/l carbenicillin, and then transferred to the MS 0.8% agar medium consisting of the same composition but also containing 0.1mg/l IAA, 0.1mg/l GA_3, 0.1mg/l ABA and 2mg/l zeatin riboside. The discs were placed on a new plate every 2 weeks for shoot formation. When the shoots appeared, they were cut from the callus and transferred to a hormone-free MS agar plate containing 50mg/l kanamycin and 500mg/l carbenicillin for root induction. Kanamycin-resistant plants were subcultured in a selection medium to maintain independent transformants, and to produce plantlets for transferring to soil. All further works with the plants were done in a glasshouse kept at approximately 23°C.

The transgenic lines containing the only PLRV CP gene and chimeric construct were designated as MQ and AB, respectively. Control plants of cv. May Queen were non-transformed but had been regenerated *in vitro* from tuber discs.

Genomic PCR analysis

The presence of the chimeric PVX $\alpha\beta$-leader sequence and PLRV CP gene in the regenerated potato plants was determined by PCR analysis of genomic DNAs, which had been isolated from individual plants as described by Murray and Thompson.[13] Amplification of the only CP gene and the chimeric gene inserts were carried out using two pairs of oligonucleotide primers, PLCPE1P (5' CGAATTCAATGAGTACGGTCGTGG3') and PLCPE1M (5'CCGAATTC-CTATTTGGGGTTTTGCAA3'), and PVX31P and PLCPE1M, respectively. PLCPE1P and PLCPE1M correspond to nucleotides 3693-3708 and 4302-4319 of PLRV RNA, respectively. PCR amplification was performed with *Tth* DNA polymerase (Toyobo) and 0.5μg of genomic plant DNA using a three-temperature program (94°C for 1min, 50°C for 1min, and 72°C for 2min) for 30 cycles. Aliquots of the PCR products were analyzed by electrophoresis in 1% agarose gel.

PLRV inoculation

Plantlets were propagated by cutting and rooting the stems. Potato plants were inoculated with PLRV by transferring 10 *Myzus persicae* (Sulz.) that had been raised on PLRV-infected *Physalis floridana* (Rydb.). Aphids were placed on leaves of plants approximately 15cm in height for an inoculation access period of 5 days. After 20 or 30 days, leaf tissue was collected from the upper leaves for detection of PLRV.

PLRV detection

The presence of PLRV in the inoculated plants was assayed by enzyme-linked immunosorbent assay (ELISA).[6] When ELISA values of the inoculated plants were as low as those of healthy plants, RT-PCR assay was carried out for more sensitive detection. Tubers of the plants positive for PLRV infection in above assays were retained for evaluating the effects of secondary infection. Tubers were planted after 5 months at 5°C, and virus multiplication in the lower leaves from planted tubers were assayed by ELISA. Statistical significance of ELISA values was determined by the procedure of Duncan's new multiple range tests.[1]

Nucleic acid extraction from leaf tissue and RT-PCR assay were performed according to Hataya *et al.*[8] using two oligonucleotide primers, PLR56KP (5' CCAGAATTCCTCCACCAAGGC3') and PLR3ENDM (5'AGTACTACACAACC-CTGTAAGAGG3'). PLR56KP and PLR3ENDM correspond to nucleotides 5278 -5298 and 5967-5987 of PLRV RNA, respectively. These primers were designed to amplify a 713 nt fragment including the carboxy-terminal portion of the PLRV 56k protein gene and 3' noncoding sequence.[12]

Aphid transmission

Two leaves from each inoculated transgenic and non-transgenic plant were collected one month postinoculation, and used as inocula. Symptoms of PLRV were recorded 2 weeks later.

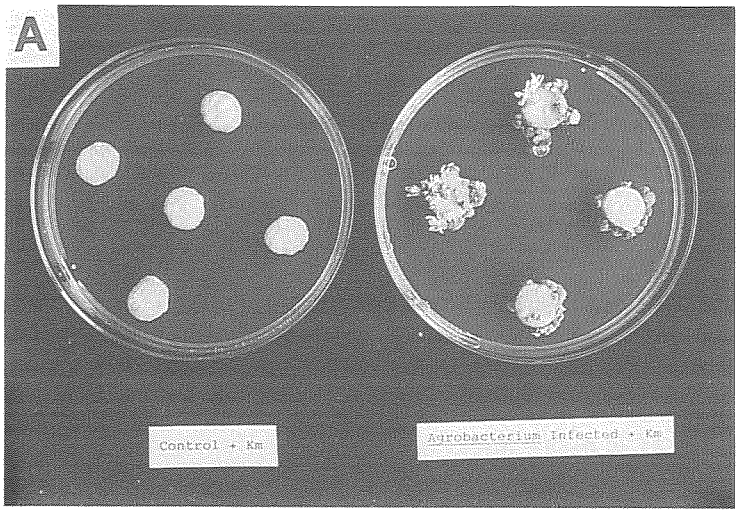
Northern analysis

Total RNAs were isolated from leaf tissues as described by Verwoerd *et al.*[19], and 20μg aliquots were loaded onto a denaturing 1.2% agarose gel containing 0.66M formaldehyde. After electrophoresis, the RNAs were transferred to a positively charged Nylon membrane (Boehringer Mannheim). Hybridization was performed using a digoxigenin (DIG)-labeled RNA probe made from the CP coding region according to Li *et al.*[11] Chemiluminescent detection was performed using a DIG luminescent detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

Results

Transformation of potato plants

Fifty-one transformants from about 500 tuber discs were obtained. Calli formed in the presence of kanamycin from tuber discs 6-10 weeks after inoculation with *A. tumefaciens*, while all discs that had not been inoculated did not (Fig. 2A). Shoots developed from the calli about 2-4 weeks after their formation. All shoots generated roots in the rooting medium which contained 50mg/l kanamycin. Regenerated shoots from non-transgenic potatoes neither grew in the same medium nor showed root elongation. Transformants showed normal growth and the same appearance as wild ones (Fig. 2B).



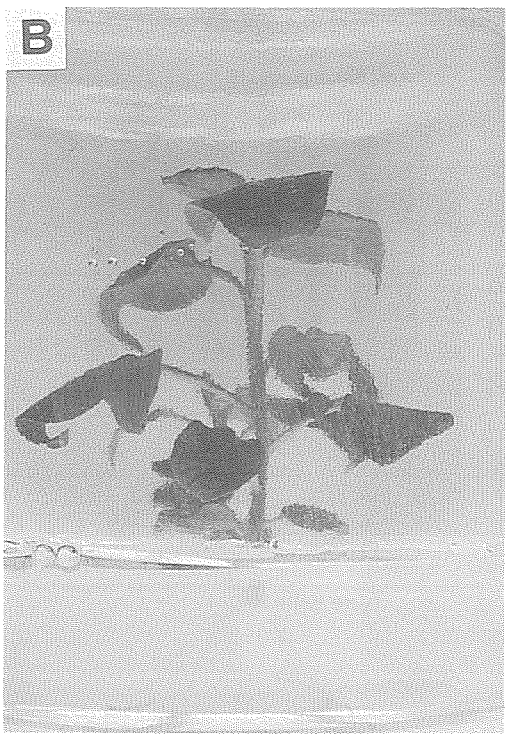


Fig. 2. A. Tuber disc transformation and selection of kanamycin-resistant cells. Tuber discs transformed with pBILR1 are shown on the right, and non-transformed tuber discs are shown on the left. B. *In vitro*-grown transgenic May Queen potato plants.

Genomic PCR analysis

Transgene insertion was identified by genomic PCR analysis (e.g. Fig. 3). A fragment of the expected size of the CP gene insert was amplified in all transformed plants tested. The chimeric gene was amplified and detected only from AB lines, and not from MQ lines.

PLRV resistance at the primary infection stage

Nine transgenic MQ and six AB lines were analysed for their susceptibility to PLRV. Transgenic and non-transgenic plants were inoculated with PLRV by aphids, and viral multiplication was estimated by ELISA since the transgene is not expressed to a detectable level (described below). Some typical data are shown in Table 1. Inoculated plants that showed low ELISA values (0.000～0.200 or 0.000～0.300 depending on experiments) were further investigated for virus infection by RT-PCR assay (e.g. Fig. 4). A specific band of expected size was detected in 9 out of 10 plants tested. Thus, the inoculated plants that showed high ELISA values or were RT-PCR assay positive were considered to be infected with PLRV. The results of these experiments showed that 96～100% of the inoculated plants were infected with PLRV. However, the mean ELISA values

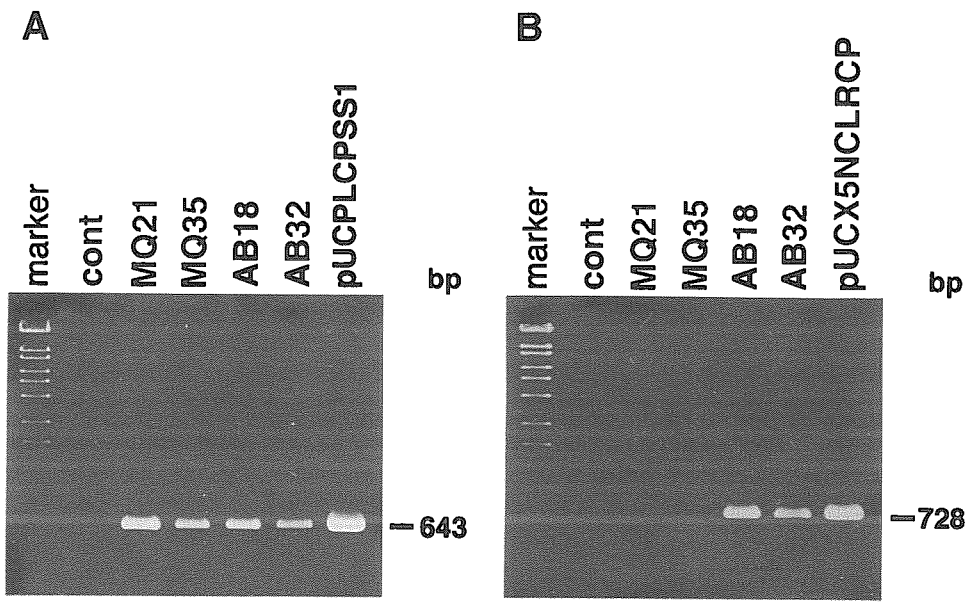


Fig. 3. Detection of transgenes in transgenic plants. Agarose gel (1%) electrophoresis of the products of genomic PCR amplifying PLRV CP gene (A) or chimeric PVX $\alpha\beta$-leader sequence and PLRV CP gene (B). Genomic DNA was isolated from non-transgenic plant (cont) and plants transformed with pBILR1 (MQ21 and MQ35) or pBILR2 (AB18 and AB32). For positive controls, cDNA clones (pUCPLCPSS1 and pUCX5NCLRCP) were used. Marker: *Sty* I-digested λ DNA.

Table 1. Comparison of PLRV accumulation in transgenic and non-transgenic plants at the primary infection stage assessed by ELISA.

	Exp.1		Exp.2		Exp.3	
Line	negative[a)]	mean ± SE[b)]	negative	mean ± SE	negative	mean ± SE
cont[c)]	0/10	0.791 ± 0.061 a	0/5	0.709 ± 0.103 a	0/7	0.708 ± 0.088 a
MQ21	0/10	0.624 ± 0.077 a		NT		NT
MQ35	3/8	0.393 ± 0.039 b	4/10	0.252 ± 0.049 b		NT
AB18		NT[d)]	1/5	0.683 ± 0.170 a		NT
AB32		NT	2/5	0.279 ± 0.085 b	1/8	0.437 ± 0.067 b
healthy		0.180		0.050		0.169

[a)]Number of negative plants/number of inoculated plants. Negative samples had ELISA values below the threshold. Threshold values are <0.200 and <0.300 for Exp.2, and Exp. 1,3 respectively. Negative samples in Exp. 1 and Exp. 2 were further investigated for virus infection by RT-PCR assay. A negative plant in Exp. 3 showed higher ELISA value (0.449) at 40 days postinoculation.
[b)]Average ELISA values (A_{415}) of all infected plants of lines tested in three different experiments are listed. ELISA was performed at 30 days (Exp. 1) or 20 days (Exp. 2 and Exp. 3) postinoculation. Five to ten plants were assayed. Lines sharing a letter are not significantly different from each other at the $P<0.05$ level.
[c)]Non-transgenic control plants.
[d)]NT : not tested.

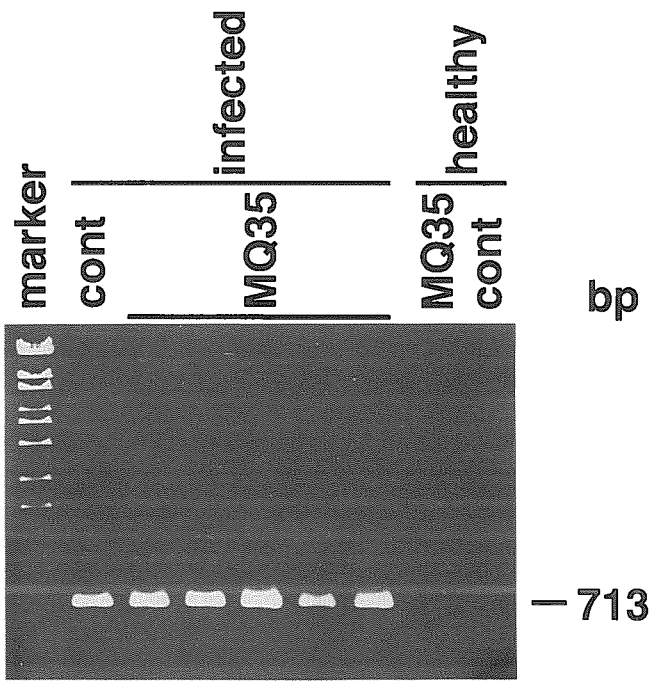


Fig. 4. RT-PCR assay of transgenic plants. Agarose gel (1%) electrophoresis of RT-PCR amplified cDNA products of PLRV. Marker: *Sty* I -digested λ DNA.

of some infected transgenic lines were apparently lower than those of infected non-transgenic plants. Statistical analysis of the ELISA values indicated that the infected transgenic lines MQ35 and AB32 contained a significantly lower amount of PLRV than the non-transgenic plants showing a significant level of PLRV resistance at the primary infection stage. The ELISA value of line AB32, containing the $\alpha\beta$-leader sequence, was not significantly different from that of line MQ35 (Table 1, Exp.2).

Aphid transmission

Significantly reduced virus accumulation in the transgenic plants will be expected to lower the efficiency of transmission. Therefore, plants with primary infection were used to determine the role of transgenic plants as a source of PLRV transmission by aphids. In the first test, three aphids from each line were transferred to a single *P. floridana* plant after an acquisition access period of 3 days. The virus was not transmitted to five test plants from the infected MQ35 plant, while only one test plant out of five was infected from the infected non-transgenic plant (data not shown). To increase the efficiency of transmission, the number of aphids and acquisition access period were increased to 20 and 4 days, respectively, in a following test (Table 2). The aphid transmission rate of PLRV from the infected transgenic plant, which showed lower ELISA values than the non-transgenic plant, was apparently lower than that from the infected non-transgenic plant.

PLRV resistance at the secondary infection stage

From the results of experiments on primary infection, four transgenic lines (shown in Table 3) were selected for analysis of PLRV infection in tubers produced by infected transgenic plants. All tubers of the selected plants were planted and the emerging shoots were tested by ELISA and RT-PCR assay.

Table 2. Transmission of PLRV by *Myzus persicae* from transgenic and non-transgenic plants previously inoculated with PLRV.

Line	ELISA value[a]	Transmission efficiency[b]	
		Leaf 1[c]	Leaf 2[c]
cont[d]	1.033	2/3	5/5
MQ35	0.303	0/2	3/5
AB32	0.317	0/3	1/5
healthy	0.145	0/1	0/2

[a]ELISA value of inoculum plants at 20 days postinoculation.

[b]Transmission efficiency is determined as the number of infected plants out of the total number of inoculated plants, as was determined by symptom occurrence. Twenty aphids from each line were transferred to a single *Physalis floridana* plant after they had an acquisition access period of 4 days. Inoculation access period was 5 days.

[c]Inoculum leaf was collected one month postinoculation.

[d]Non-transgenic control plants.

Table 3. Comparison of PLRV accumulation in transgenic and non-transgenic plants at the secondary infection stage assessed by ELISA.

Line	values of primary infection[a]	mean ± SE[b] Exp.1	Exp.2
cont[c]	high	1.612 ± 0.135 a	0.956 ± 0.087 a
MQ21	high	1.756 ± 0.134 a	NT
MQ35	low	NT[d]	0.815 ± 0.063 ab
AB18	high	NT	0.885 ± 0.102 ab
AB32	low	NT	0.665 ± 0.041 b
healthy		0.084	0.072

[a]The results of Table 1. are summarized.

[b]Average ELISA values (A_{415}) of all infected shoots of lines tested in two different experiments are listed. ELISA was performed at 20 days (Exp.1) or 30 days (Exp.2) after emergence. Nine to twenty plants were assayed. Lines sharing a letter are not significantly different from each other at the $P<0.05$ level.

[c]Non-transgenic control plants.

[d]NT: *not tested.*

ELISA values of the selected lines are summarized in Table 3. Lines MQ21 and AB18, that had no detectable restriction of virus multiplication during primary infection, showed the same response to virus multiplication. Although MQ35 showed some degree of restriction of virus multiplication during primary infection, it showed the ELISA value which were not statistically different from non-transgenic plants during secondary infection. AB32 showed lower ELISA values than non-transgenic plants at a statistically significant level. However, the effect was not as clear as that during primary infection. As a secondary symptom, many shoots of all transgenic lines clearly exhibited leaf-roll symptom.

Expression of the PLRV coat protein gene in transgenic plants

Two transgenic MQ lines and six AB lines were analysed by Northern blots for expression of PLRV CP gene transcripts. A single transcript of approximately 800 nucleotides was detected in all transformed plants tested (e.g. Fig. 5). The accumulation of the transcripts differed among transgenic lines carrying the same construct. There was no correlation between the level of transcripts accumulation and resistance to PLRV. The levels of transcripts accumulation showed no direct relationship with the two CP gene constructs.

All eight transgenic lines were analysed for possible accumulation of PLRV CP using ELISA, which can detect as little as 3ng/ml purified virus. However, none of the transgenic plants accumulated detectable levels of CP.

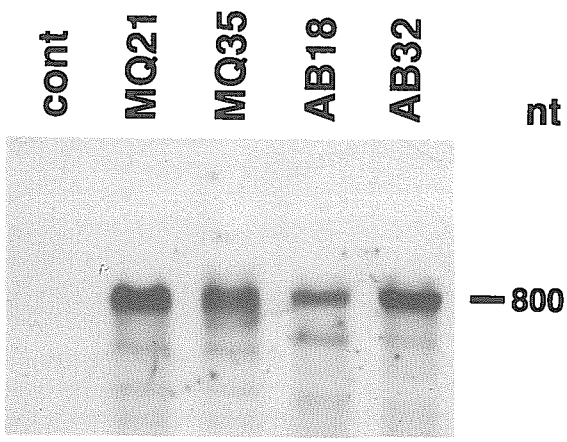


Fig. 5. Northern analysis of transgenic plants. Twenty μg of total plant RNA extracted from leaf tissue was electrophoresed in a 1.2% agarose gel containing formaldehyde.

Discussion

We have successfully transformed potato cv. May Queen with the PLRV CP gene. The inoculation assay in this study showed that none of the potato lines transformed with the two constructs became immune to PLRV infection, but some lines restricted the virus multiplication only at the primary infection stage. At the secondary infection stage, the virus restriction was found to be less effective. These results suggest that the mechanism which restricts virus multiplication is dose-dependent. Therefore, the higher the virus concentration in the inoculum, the less effective virus restriction will be. The restricted virus multiplication may have a positive effect on controlling PLRV infection due to the reduced aphid transmission efficiency of PLRV as was evidenced in this study.

Our results agreed with those obtained by other researchers[3,10,16,20] on three points: (1) Potatoes transformed with the CP gene of PLRV were infected with PLRV by aphids, but virus multiplication was restricted, (2) transgenic CP mRNA was readily detected by Northern analysis, and (3) CP was not detected by ELISA (and was difficult to detect by Western analysis) in uninoculated transgenic plants. Little or no accumulation of transgenic CP indicates that the virus resistance mechanism observed by us and others is different from CP-mediated resistance described by Reimann-Philipp and Beachy.[18] Van der Wilk *et al.*[20] hypothesized that the protection of transgenic plants expressing the PLRV CP gene is based on interference of viral multiplication at the RNA level. Barker *et*

al.[4] showed that resistance to PLRV replication in transgenic tobacco plants was broadly related to the amount of PLRV CP transcript detected. Although we were unable to detect a correlation between the amount of CP transcript extracted from whole leaves and resistance to PLRV, if resistance is affected by transgene, the CP transcript may contribute to resistance. Recently, however, Presting *et al.*[16] obtained several highly resistant lines that were derived by transforming potato plants with the control construct carrying only vector sequences and the NPT II gene. They suggested that the most likely cause of this virus resistance is somaclonal variation. More research is needed to clarify the mechanism underlying the restriction of PLRV multiplication in transgenic plants.

In this study, we attempted to reinforce the resistance of transgenic plants by introducing the PVX $\alpha\beta$-leader sequence upstream of the PLRV CP gene. Pugin *et al.*[17] showed that, in the presence of the $\alpha\beta$-leader in front of the ATG initiation codon of the potato virus Y CP gene, all the tested plants accumulated large amounts of the coat protein, whereas the coat protein was not detected among the plants carrying the cassette without the $\alpha\beta$-leader. Additionally, in the presence of the $\alpha\beta$-leader, the PVY CP mRNA steady-state content was 10 times higher than that without the $\alpha\beta$-leader. However, our results showed that transgenic CP was not detectable by ELISA, and no direct relationship was observed between the levels of CP transcripts and the CP gene constructs. These results indicate that the presence of the $\alpha\beta$-leader alone is not a sufficient condition for effective expression of protein and mRNA or PVX $\alpha\beta$-leader sequence was not effective for PLRV CP gene expression.

Our results in this study showed introduction of PLRV CP gene to potato cv. May Queen, one of the major potato cultivars in Japan, are promising for obtaining some degree of resistance to PLRV. Furthermore, it was shown that restriction of virus multiplication led to reduction of aphid transmission efficiency. In order to obtain more complete resistance, improvement of the promoter and 5' non-translated sequence, or the introduction of an additional resistant gene will be needed.

Summary

Potato plants (cv. May Queen) transformed with two constructs of the coat protein (CP) gene of potato leafroll virus (PLRV) were produced and analysed for their susceptibility to PLRV. One construct contained only the PLRV CP gene, while the other contained the chimera of potato virus X $\alpha\beta$-leader sequence fused to the PLRV CP gene under the control of the cauliflower mosaic virus 35S promoter. CP transcripts were readily detected by Northern analysis, but CP was not detected by the enzyme-linked immunosorbent assay in transgenic plants. One each from the two constructs of transgenic lines showed restricted virus

multiplication at the primary (aphid-borne) infection stage. However, at the secondary (tuber-borne) infection stage, restriction was found to be less effective. Based on these results, the restriction of virus multiplication mediated by CP gene appears to be effective when the inoculum dose is as low as that in a case of aphid inoculation.

Acknowledgement

This research was supported by Grant-in-Aid for Cooperative Research No. 06556008 from the Ministry of Education, Science and Culture, Japan, and a grant from the Hokkaido Foundation for the Promotion of Scientific and Industrial Technology. We thank the Research Center for Molecular Genetics, Hokkaido University for the use of their facilities.

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