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**The *in vivo* mRNA amplification system in
transgenic plants expressing RNA replicon**

Masanori Kaido

1996

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To my parents

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General Introduction

Recently, a number of genes have been transferred into plant cells using various techniques such as Ti plasmid of *Agrobacterium tumefaciens* (Bevan, 1984, Horsch *et al.*, 1985), particle gun (Klein *et al.*, 1992), or electroporation (Fromm *et al.*, 1986). The gene transfer techniques have shed new lights not only on the fields of plant science including plant virology, but also on applied science or agriculture. One of the pioneering works is the production of virus resistant crops (Powell-Abel *et al.*, 1986). Introduction of a viral gene (coat protein gene, replicase gene or movement protein gene, *etc.*) often confers plants the resistance against the virus infection (Lomonossoff, 1995). Some of these crops have already been tested for their antiviral traits in field condition (Tricoli *et al.*, 1995). The next example is breeding of transgenic plants producing desirable proteins. Exploitation of plants for the production of desirable protein is very attractive, since it is inexpensive, can be done in bulk quantities, and requires limited infrastructure. In fact transgenic plants have been used for producing antibodies (Hiatt *et al.*, 1989), growth hormone (Van Dekerckhove *et al.*, 1989), and blood factors (Sijmons *et al.*, 1990). However, yields of those protein products in transgenic plants are often disappointingly low, probably due to lack of strong promoter suitable for high expression of foreign genes in plant cells (Fig. 1). At present time a key determinant for the success in agricultural production of valuable foreign proteins is how to increase protein accumulation in transgenic host plants.

On the other hand, plant virus-based vector systems have been

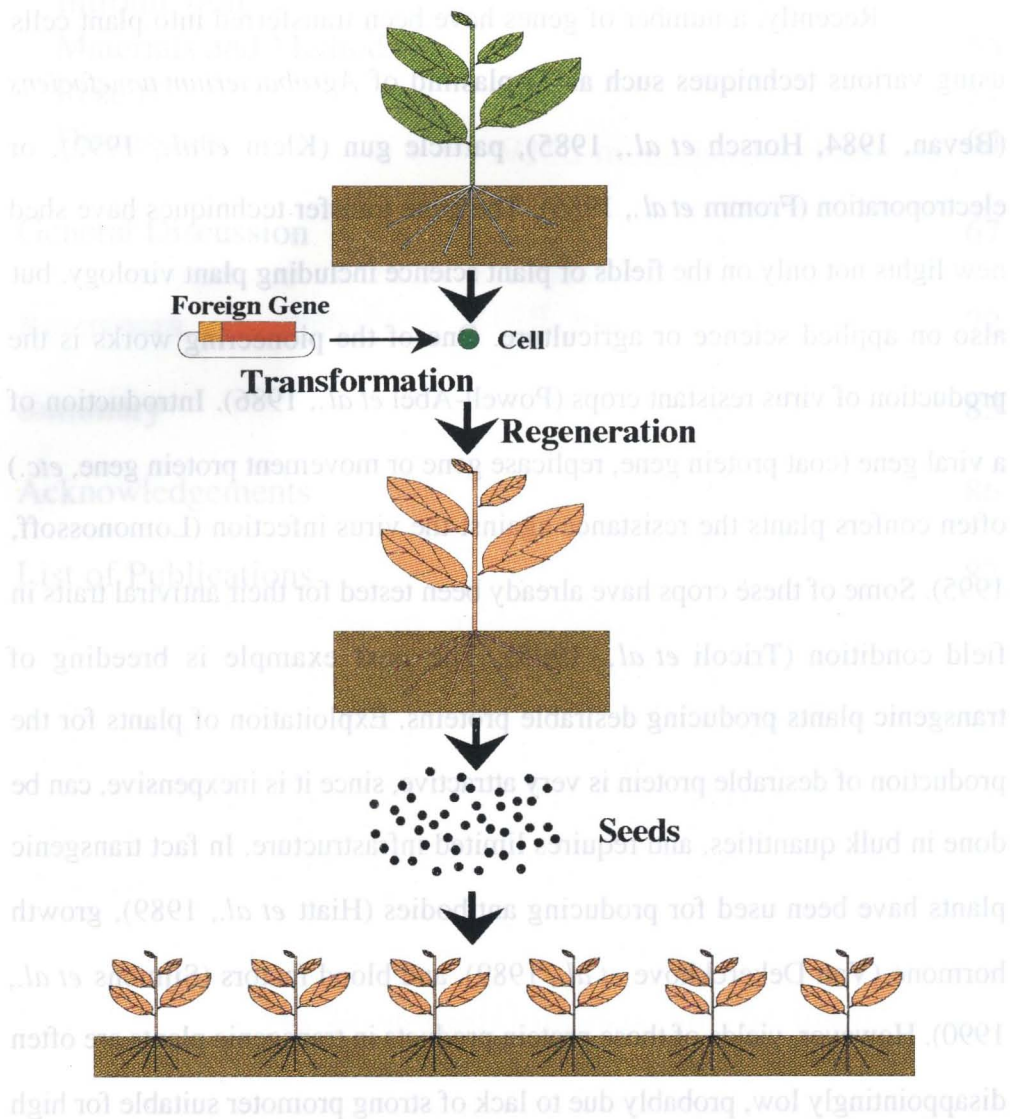


Fig. 1. Schematic representation of 'transgenic plant system' for the production of desired proteins in plant cells. In this system foreign protein accumulates to low level in every cell.
 Colors: red, foreign gene; orange, promoter functional in plant cells; pink, low level accumulation of foreign gene.

developed for the production of desirable proteins. These systems utilize extraordinarily multiplying ability of plant viruses and foreign genes inserted in the viral genomes or substituted with viral genes are multiplied in the host plant cells. However, these vectors also have problems for massproduction of foreign valuable proteins. These mutant viruses have poor or no ability to infect whole plants (Fig. 2, Takamatsu *et al.*, 1987, Dawson *et al.*, 1989, Joshi *et al.*, 1990). Even though the recominant viruses replicate in whole plants (Donson *et al.*, 1991, Kumagai *et al.*, 1993, Hamamoto *et al.*, 1993), the inoculation process will be an obstacle to scale up this system to a farm level.

To develop a more efficient system for high level synthesis of foreign protein in plants, I have combined two systems described above, that is, transgenic plants expressing chimeric viral RNA containing a foreingn gene together with the viral replicase (Fig. 3). This new system is referred to as 'the *in vivo* mRNA amplification system'. The chimeric virus RNA transcribed from transgene is expected to be amplified by the viral replicase, and the amplification is expected to occur in all the cells of the transgenic plants and therefore the accumulation level of foreign gene products should be much higher than that by virus-based vector system in which chimeric virus amplify only in a limited tissue. Another advantage of this system is that it is not expensive compared with the virus-based vector system. In the case that RNA viruses are used for the virus-based vector system, supplying the chimeric viral RNA inoculum is very expensive. Further, it requires much skill to inoculate the chimeric viral RNA to plants. It would also cost much labor to inoculate at the farm level. Taking all these points into consideration, the *in vivo* mRNA

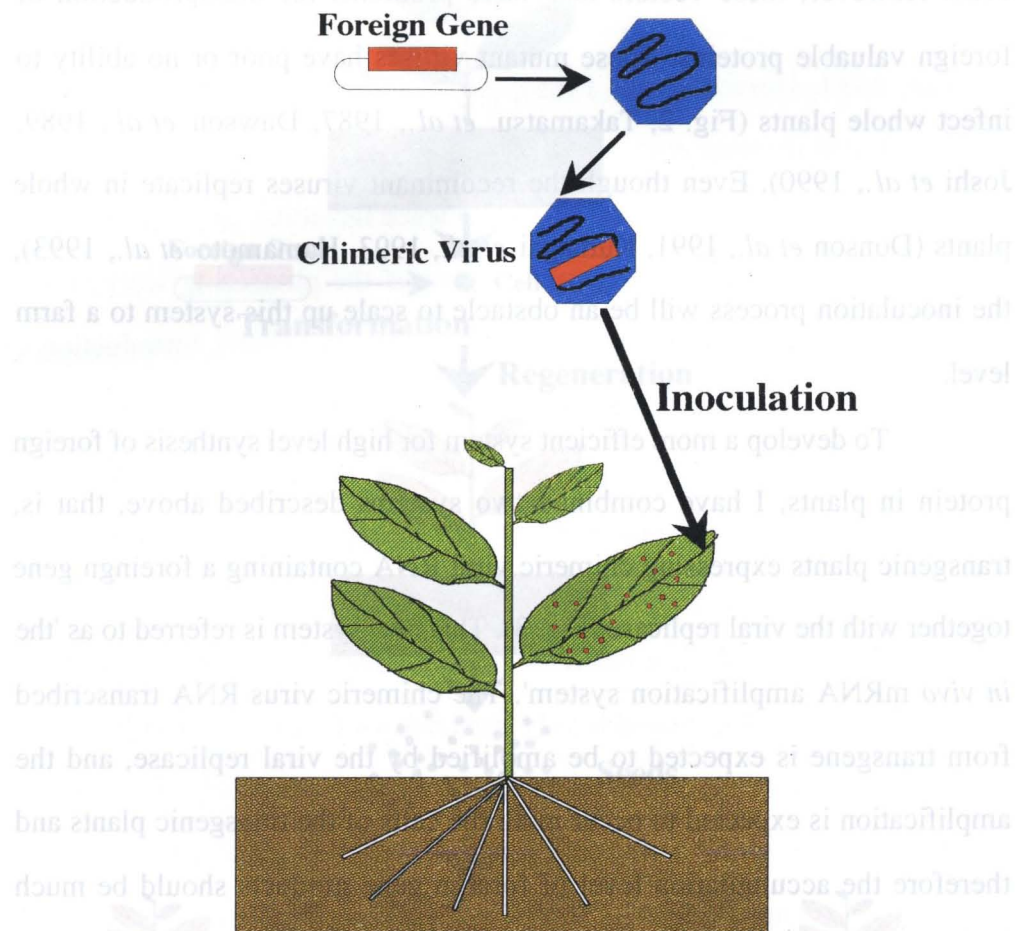


Fig. 2. Schematic representation of 'recombinant virus system' for the production of desired proteins in plant cells. Foreign protein accumulates to high level in primary infected cells.

Colors: red, foreign gene and high level accumulation of foreign protein.

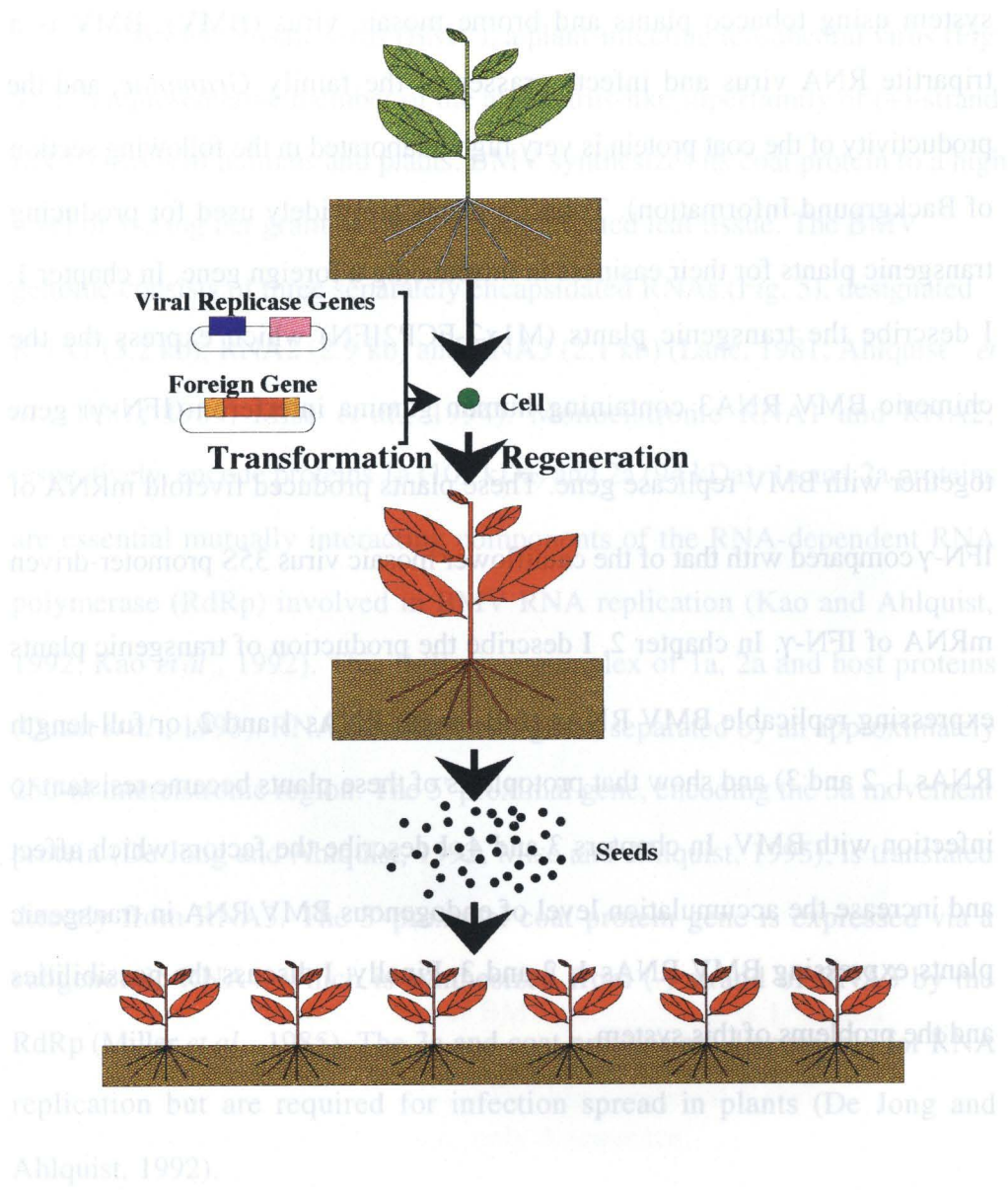


Fig. 3. Schematic representation of the *in vivo* mRNA amplification system. Colors: blue and pink, viral replicase genes; red, engineered foreign gene and high level accumulation of foreign protein.

amplification system is more practical than the systems so far.

In this research I have developed the *in vivo* mRNA amplification system using tobacco plants and brome mosaic virus (BMV). BMV is a tripartite RNA virus and infects grasses of the family *Graminae*, and the productivity of the coat protein is very high (elaborated in the following section of Background Information). Tobacco plants are widely used for producing transgenic plants for their easiness in introducing a foreign gene. In chapter 1, I describe the transgenic plants (M1x2-FCP2IFN) which express the the chimeric BMV RNA3 containing human gamma interferon (IFN- γ) gene together with BMV replicase gene. These plants produced fivefold mRNA of IFN- γ compared with that of the cauliflower mosaic virus 35S promoter-driven mRNA of IFN- γ . In chapter 2, I describe the production of transgenic plants expressing replicable BMV RNAs (full-length RNAs 1 and 2, or full-length RNAs 1, 2 and 3) and show that protoplasts of these plants became resistant to infection with BMV. In chapters 3 and 4, I describe the factors which affect and increase the accumulation level of endogenous BMV RNA in transgenic plants expressing BMV RNAs 1, 2 and 3. Finally, I discuss the possibilities and the problems of this system.

Background Information on BMV

Brome mosaic virus (BMV), a plant-infecting icosahedral virus (Fig. 4), is a representative member of the alphavirus-like superfamily of (+)-strand RNA viruses of animals and plants. BMV synthesizes its coat protein to a high level of 1-2 mg per gram of fresh-weight infected leaf tissue. The BMV genome consists of three separately encapsidated RNAs (Fig. 5), designated RNA1 (3.2 kb), RNA2 (2.9 kb) and RNA3 (2.1 kb) (Lane, 1981; Ahlquist *et al.*, 1981; 1984; Mise *et al.*, 1994). Monocistronic RNA1 and RNA2, respectively, encode proteins 1a (109 kDa) and 2a (94 kDa). 1a and 2a proteins are essential mutually interacting components of the RNA-dependent RNA polymerase (RdRp) involved in BMV RNA replication (Kao and Ahlquist, 1992; Kao *et al.*, 1992). This RdRp is a complex of 1a, 2a and host proteins (Quadt *et al.*, 1993). RNA3 contains two genes separated by an approximately 250-nt intercistronic region. The 5'-proximal gene, encoding the 3a movement protein (De Jong and Ahlquist, 1992, Mise and Ahlquist, 1995), is translated directly from RNA3. The 3'-proximal coat protein gene is expressed via a subgenomic RNA4, which is synthesized from (-) strand of RNA3 by the RdRp (Miller *et al.*, 1985). The 3a and coat proteins are dispensable for RNA replication but are required for infection spread in plants (De Jong and Ahlquist, 1992).

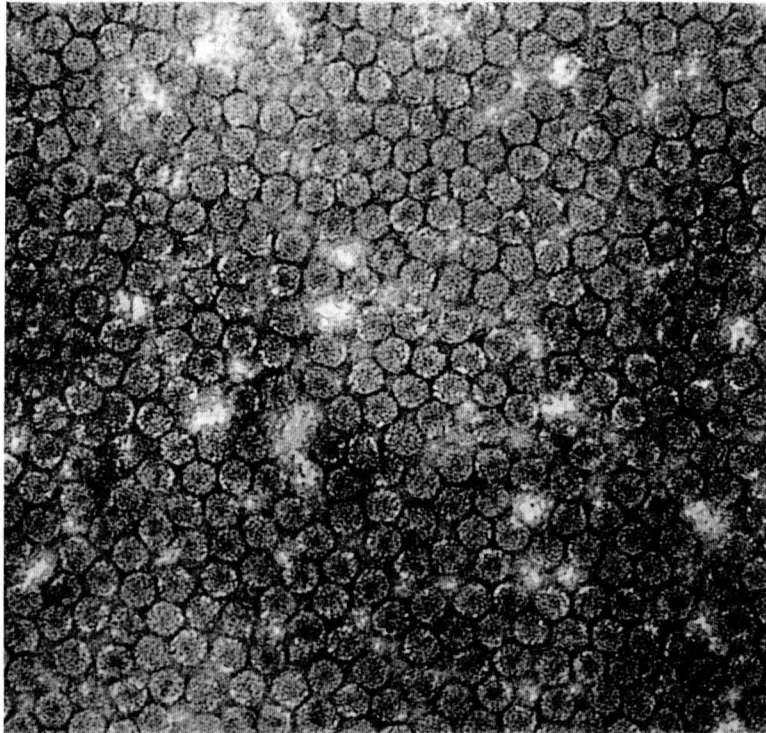


Fig. 4. Electron micrograph of BMV virions.

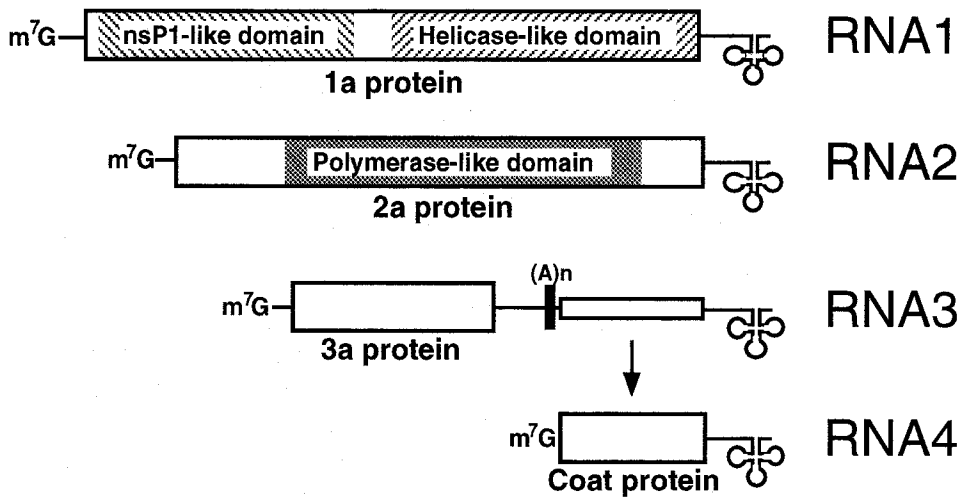


Fig. 5. Schematic map of BMV genomic RNAs 1~3 and subgenomic RNA4.

Symbols: , the clover leaf-like conserved 3' region; m⁷G, cap structure; (A)_n, poly A sequence.

Chapter 1

***In vivo* mRNA amplification system in the transgenic tobacco plants expressing BMV replicase**

INTRODUCTION

Transgenic plants have been used for producing desired proteins such as nuropeptides (Vandekerckhove *et al.*, 1989), blood factors (Sijmons *et al.*, 1990) and antibody (Hiatt *et al.*, 1989). Yields of those protein products in transgenic plants, however, are often disappointingly low. This may be due to lack of a strong promoter suitable for high expression of foreign genes in plant cells. It seems that one of the key determinants for the success in agricultural production of a valuable foreign protein is how to level up the expression of foreign genes in transgenic plants.

To develop a more efficient system for high-level synthesis of foreign proteins in plants, an RNA virus replication system based on RNA-dependent RNA synthesis is very attractive since the replication system at RNA level can apply to amplification of mRNA for a foreign gene in transgenic plant cells and may surmount several impediments encountered in the production of foreign protein in the DNA-directed transcription system. In this chapter I present a new system (an *in vivo* mRNA amplification system) in which DNA-directed mRNA can be amplified *in vivo* by viral replicase expressed from the integrated cDNAs in transgenic plants.

Mori *et al.*, (1992) demonstrated that transgenic tobacco plants (M1x2 plants) expressing BMV RNA replication genes 1a and 2a under control

of the CaMV 35S RNA promoter support RNA-dependent replication of RNA3 in transfected protoplasts. I introduced a cDNA of BMV RNA3 derivative (FCP2IFN, Mori *et al.*, 1993) carrying the human gamma interferon (IFN- γ) gene linked to the 35S promoter into the chromosomal DNA of M1x2 plants and show the replication of FCP2IFN and the production of subgenomic mRNA of IFN- γ in M1x2-FCP2IFN plants.

MATERIALS and METHODS

Plasmid construction

A plasmid pARK22 (kindly provided from Dr. H. Anzai) containing a *bar* gene was digested with *Pst*I, made blunt with T4 DNA polymerase and self-ligated to remove the *Pst*I site, creating pARK22(-Pst). The pARK22(-Pst) was cut with *Eco*RI and made blunt with T4 DNA polymerase, and then ligated to *Pst* I linkers, creating pARK22(E-P). The binary vector pBICP35 containing the 35S promoter and termination signal was partially cut with *Pst*I and digested with *Hind*III. The resulting plasmid was ligated with the *Pst*I-*Hind*III-cut fragment of pARK22(E-P), creating pBICBP35. The fragment of *Sna*BI-*Eco*RI-cut pBB3 (i.e. Full-length cDNA of RNA3, Mori *et al.*, 1991) was inserted into *Stu*I-*Eco*RI-cut pBICBP35, creating pBICBPBR3. The fragment of *Xba*I-*Eco*RI-cut fragment of pBTC2IFN was inserted into *Xba*I-*Eco*RI-cut pBICBPBR3, creating pBICBPCP2IFN.

Plant transformation

pBICBPCP2IFN was mobilized into *Agrobacterium tumefaciens*

(strain LBA4404) using *E. coli* HB101 harboring pRK2013, as described (Bevan, 1984). Transformation of *N. tabacum* cv. Petit Habana (SR1) was carried out using the leaf disc transformation method (Horsch *et al.*, 1985). Transformed shoots and roots were selected by propagation on medium containing both kanamycin and bialaphos and eventually transferred to a glasshouse for production of mature plants.

RNA isolation and Northern blot analysis

RNA was isolated from tobacco plants as described (Mori *et al.*, 1993). The RNA was denatured in formaldehyde/formamide and fractionated on a 1.5 % agarose gel containing 1.8 % formaldehyde and transferred to a BIODYNE membrane. Detection of positive-sense viral RNA was by a ³²P-labeled SP6 transcript from a subclone containing the 200-base *Hind*III (nt 1914)-*Eco*RI 3'-terminal fragment of BMV RNA3 cDNA which is conserved among all BMV RNAs. To detect negative-sense viral RNA we used ³²P-labeled positive-sense RNA of the 200-base 3'-terminal fragment.

Protein analysis

Total protein was extracted from transgenic plants with Laemmli buffer (Laemmli, 1970), and separated on a 15 % polyacrylamide gel containing 0.1 % SDS. Western blot analysis was carried out as described (Mori *et al.*, 1993). Electrophoresed proteins were transferred to an Immobilon-P transfer membrane (Millipore) and probed with antibody against IFN- γ .

RESULTS

Production of transgenic plants expressing both BMV replicase and FCP2IFN

Transgenic tobacco plants (M1x2 plants, Mori *et al.*, 1992) expressing BMV replicase 1a and 2a proteins were transformed with an *Agrobacterium* binary vector (designated pBICBPCP2IFN) which contains full-length cDNA of FCP2IFN (Mori *et al.*, 1993) linked to the CaMV 35S RNA promoter and a *bar* gene as a selection marker that confers resistance to bialaphos (Fig. 6). FCP2IFN is a BMV RNA3 derivative having the IFN- γ gene replacing the coat protein (CP2) gene (Mise *et al.*, 1992) in RNA3. FCP2IFN is able to replicate and produce subgenomic mRNA of IFN- γ in tobacco protoplasts when coinoculated with BMV RNA1 and RNA2 transcripts (Mori *et al.*, 1993).

Expression of BMV replicase and FCP2IFN was tested as described below. Transgenic tobacco plants resistant to both bialaphos and kanamycin were obtained. Integration of cDNAs was confirmed by Southern blot analysis (data not shown). Transgenic tobacco plants expressing both BMV replicase and FCP2IFN were designated M1x2-FCP2IFN plants. F₁ progenies with a different pattern of gene expression were obtained by self-pollination of primary transformants, since both BMV replication genes and cDNA of FCP2IFN in R0 plants are not homozygous. In this study we used three kinds of F₁ progenies expressing replicase alone (M1x2 plants), FCP2IFN alone (designated FCP2IFN plants) and both BMV replicase and FCP2IFN (M1x2-FCP2IFN plants).

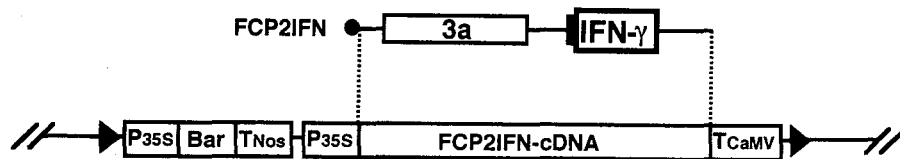


Fig. 6. Schematic representation of binary vector for RNA3 derivative carrying gamma interferon gene (FCP2IFN). ●, cap structure; 3a, 3a gene; ►, T-DNA border sequence; P_{35S}, cauliflower mosaic virus 35S promoter; T_{CaMV}, 35S terminator; Bar, bar gene; T_{NOS}, nopaline synthase terminator. The shadowed box indicates 24 nucleotides coding for the N-terminal amino acids of a coat protein.

Replication of FCP2IFN and production of subgenomic mRNA in transgenic tobacco plants expressing both BMV replicase and FCP2IFN

The expression and replication of FCP2IFN and the production of subgenomic mRNA in M1x2-FCP2IFN plants were analyzed by Northern blotting with strand-specific probes for the conserved 3'-terminal non-coding sequence of the wild type BMV RNAs (Note that these 3'-terminal sequences are not present in viral replicase transcripts expressed in M1x2 plants). The use of a positive strand-specific probe did not give any detectable band in M1x2 plants (Fig. 7, lane 2). However, a band with mobility slower than that of FCP2IFN observed in FCP2IFN-infected tobacco protoplasts was detected in both M1x2-FCP2IFN and FCP2IFN plants (Fig. 7, lane 3 and 4). These bands must be the 35S promoter-driven transcripts with non-viral and polyadenylated sequences at the 3' end. In addition to the 35S promoter-driven transcripts, in M1x2-FCP2IFN plants two more bands with the same mobility as those of FCP2IFN and its subgenomic RNA were observed in FCP2IFN-infected tobacco protoplasts (Fig. 7, lane 4). The replication of FCP2IFN and transcription of subgenomic RNA from FCP2IFN by viral replicase in M1x2-FCP2IFN plants was confirmed by the presence of the negative-strand RNA which comigrated with the negative strand FCP2IFN in FCP2IFN-infected tobacco protoplasts (Fig. 8). These results indicate that in M1x2-FCP2IFN plants BMV replicase initiate negative strand synthesis at a correct site and eliminates the extra nucleotides at their 3' end and leads to replication of authentic FCP2IFN and transcription of subgenomic mRNA from the negative-strand FCP2IFN (Mori *et al.*, 1991, Dzianott and Bujarski, 1989). The

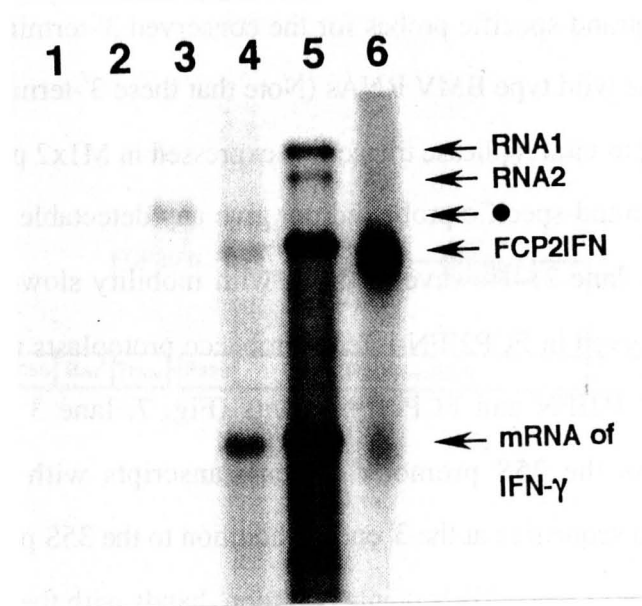


Fig. 7. Northern blot analysis of replication of FCP2IFN in transgenic tobacco plants expressing BMV replicase. Total RNAs were extracted from non-transgenic tobacco plants (lane 1), M1x2 plants (lane 2), FCP2IFN plants (lane 3), M1x2-FCP2IFN plants (lane 4) and tobacco protoplasts inoculated with FCP2IFN together with RNA1 and RNA2 (lane 5). Lane 6 contains 500 pg of in vitro synthesized FCP2IFN and its subgenomic mRNA of IFN- γ . The RNA (5 μ g in lane 1-4, 1.25 μ g in lane 5) was separated on a 1.5 % agarose gel containing formaldehyde, and transferred to a nylon membrane. Positive-sense RNA was detected by a 32 P-labeled *in vitro* transcript complementary to the conserved 3' terminal 200 bases of FCP2IFN and wild-type BMV genomic RNAs. ● indicates the 35S promoter-driven transcripts of FCP2IFN.

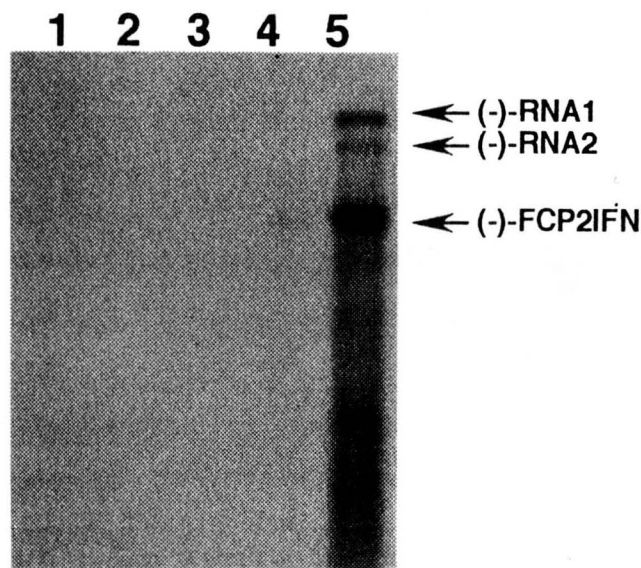


Fig. 8. Northern blot analysis of replication of FCP2IFN in transgenic tobacco plants expressing BMV replicase. Total RNAs were extracted from non-transgenic tobacco plants (lane 1), M1x2 plants (lane 2), FCP2IFN plants (lane 3), M1x2-FCP2IFN plants (lane 4) and tobacco protoplasts inoculated with FCP2IFN together with RNA1 and RNA2 (lane 5). The RNA (10 μ g in lane 1-4, 2.5 μ g in lane 5) was separated on a 1.5 % agarose gel containing formaldehyde, and transferred to a nylon membrane. Negative-sense RNA was detected by a 32 P-labeled positive sense *in vitro* transcript of the conserved 3' terminal 200 bases of FCP2IFN and wild-type BMV genomic RNAs.

accumulation level of the subgenomic RNA was approximately 5-fold larger than that of 35S-driven FCP2IFN and was approximately 0.05 % of the total extracted RNA (data not shown).

Accumulation of human gamma interferon in M1x2-FCP2IFN plants

Immunological analysis using antibody against IFN- γ revealed that IFN- γ with Mrs of approximately 23 kDa and 18 kDa was detected in total protein extract from M1x2-FCP2IFN plants but not in protein from either M1x2 or FCP2IFN plants (Fig. 9). These 23 kDa and 18 kDa proteins may be glycosylated and non-glycosylated IFN- γ , respectively (Mori *et al.*, 1993).

DISCUSSION

The results presented here show that DNA-directed RNA transcripts were amplified and subgenomic mRNAs from the transcripts were produced by viral replicase expressed in transgenic plants. This mRNA amplification system holds potentiality to overcome low production of mRNA by the DNA-directed transcription system. Furthermore, this system is more suitable for mass production of foreign proteins in plants than a direct viral system in which foreign proteins are produced by inoculating plants with a genetically engineered virus carrying foreign genes. These mutant viruses having foreign genes by gene replacement or insertion in viral genome have poor or no ability to infect a whole plant (Takamatsu *et al.*, 1987, Dawson *et al.*, 1989, Joshi *et*

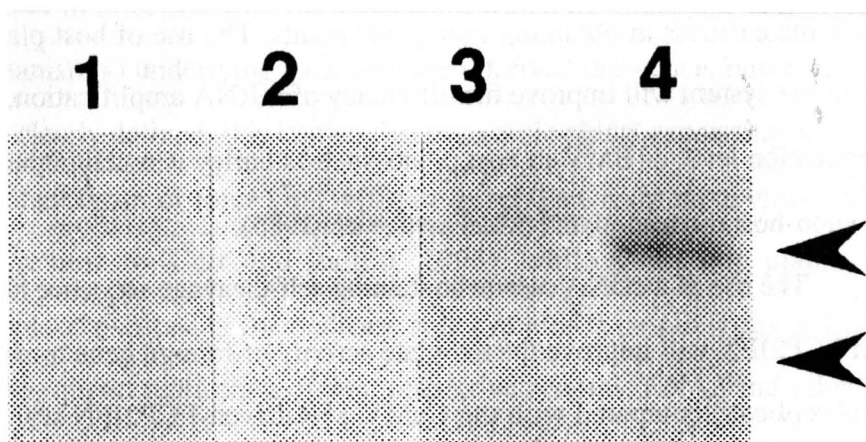


Fig. 9. Immunological analysis of gamma interferon produced in transgenic tobacco plants. Total proteins were extracted from non-transgenic tobacco plants (lane 1), M1x2 plants (lane 2), FCP2IFN plants (lane 3) and M1x2-FCP2IFN plants (lane 4), and separated on 15 % SDS-PAGE. IFN- γ was identified by immunoblotting. The position of IFN- γ is indicated in the margin.

al., 1990). Even though the recombinant virus replicates in a whole plant (Donson *et al.*, 1991, Kumagai *et al.*, 1993, Hamamoto *et al.*, 1993), the inoculation process will be an obstacle to scale up this system to a farm level. The expression level of mRNA in the *in vivo* mRNA amplification system is approximately fivefold greater than that of the CaMV 35S promoter-driven transcripts, however this system has many potentialities for further improvement. In this research tobacco plants (non-host plant of BMV) were used for the easiness in obtaining transgenic plants. The use of host plants of BMV in the system will improve the efficiency of mRNA amplification, since the replication level of BMV in host plants such as barley is much higher than that in non-host tobacco plants (Maekawa *et al.*, 1985).

The use of a cDNA cassette containing self-cleavage sequence at the 3' end of FCP2IFN will improve the template activity of foreign gene transcripts to viral replicase compared with the CaMV 35S driven-FCP2IFN which has heterologous sequences at the 3' end (Dzianott and Bujarski, 1989).

The amplification of mRNA can be controlled at the transcription level in either the foreign gene or viral replication genes if these genes are joined to various promoters instead of the constitutive CaMV 35S promoter. Inducible and organ-specific promoters will open the way to produce the desired proteins at the desired time and desired tissues.

Chapter 2

Inhibition of BMV amplification in protoplasts from transgenic tobacco plants expressing replicable BMV RNAs

INTRODUCTION

Transgenic plants expressing viral genes or their segments become resistant to infection with the virus from which the transgene is derived. The mechanism(s) underlying such pathogen-derived resistance, however, remain to be clearly defined. It is known that transgenic plants expressing either intact or mutant forms of some viral replicase gene(s) are resistant to virus infections (see review by Carr and Zaitlin, 1993), while transgenic plants and/or protoplasts expressing the replicase genes of alfalfa mosaic virus or BMV are able to support replication of their respective genomic RNA3 and subgenomic RNA4 (Taschner *et al.*, 1991; Mori *et al.*, 1992).

In the BMV system, however, repeated experiments have revealed that protoplasts from transgenic plants which express full-length BMV RNA1 and RNA2 (V12 plants) hardly support replication of genomic RNA3 and subgenomic RNA4 (unpublished data), while protoplasts from transgenic plants expressing viral replicase proteins 1a and 2a (M12 plants) consistently supported replication of BMV RNA3 and subgenomic RNA4. Similarly, transgenic tobacco plants (M1x2-FCP2IFN) that express genetically engineered BMV RNA3 carrying the human gamma interferon gene, and BMV replicase component proteins 1a and 2a, produced chimeric RNAs 3 and 4 (Chapter 1.). The accumulation levels of chimeric RNAs 3 and 4 in the plants

was approximately fivefold higher than that of transcripts synthesized under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Chapter 1.). However, the accumulation levels of chimeric RNAs 3 and 4 in the transgenic plants were extremely low compared with those in non-transgenic tobacco protoplasts inoculated with the chimeric RNA3 together with BMV RNAs 1 and 2.

To overcome an impediment in this '*in vivo* mRNA amplification system' and to obtain further insight into the observed suppression of BMV RNA accumulation in V12 transgenic tobacco plants, transgenic tobacco plants expressing various combinations of BMV genomic RNAs or replicase genes were produced and their protoplasts were tested for susceptibility to infection with BMV RNA or cucumber mosaic virus (CMV) RNA. In protoplasts of transgenic plants expressing a set of three full-length genomic RNAs of BMV or both RNAs 1 and 2, the accumulation level of BMV RNA was very low and the protoplasts were resistant to super infection with BMV RNA. This observation sheds new light on virus resistance mechanisms in plants.

MATERIALS and METHODS

Transformation of tobacco plants

V123 plants were constructed as follows: pBICBPBR3 (Chapter 1.) containing a full-length cDNA of BMV RNA3 and the *bar* selectable marker gene, which confers resistance to the herbicide bialaphos, was mobilized into *Agrobacterium tumefaciens* (strain LBA4404) using *E. coli* HB101 harboring

pRK2013 as described (Bevan, 1984). Transformation of V12 plants (Mori *et al.*, 1992) was carried out using the leaf disk transformation method (Horsch *et al.*, 1985). Transformed shoots and roots were selected by propagation on medium containing both kanamycin and bialaphos and explants were eventually transferred to a greenhouse for production of mature plants.

V13 and V23 plants were selected from F₁ progenies of V123 plants by northern blot analysis for the expression of RNAs. V3 plants were produced by transforming SR1 tobacco with pBICBPBR3 as described above.

Integration of BMV cDNAs into the plant chromosome was confirmed by Southern blot analysis (Sambrook *et al.*, 1989). Transgenic and non-transgenic tobacco plants were maintained in a greenhouse controlled at 25 °C.

Preparation of virions from V123 plants

Fifty grams of fresh leaves of 6-week-old V123 plants were powdered in liquid nitrogen and blended in homogenization buffer (0.2 M sodium acetate, 0.01 M ascorbic acid, 0.01 M disodium EDTA, pH 4.8). The homogenate was passed through four layers of gauze and kept at 4 °C for 1 h. After centrifugation at 16,000xg for 10 min, 6% (w/v) polyethylene glycol (PEG) MW 6,000 was added to the supernatant and incubated at 4 °C for 1 h. The precipitate obtained by centrifugation at 16,000xg for 15 min was resuspended in storage buffer (0.1 M sodium acetate, 1 mM disodium EDTA, 1 mM sodium azide, 0.01 M MgCl₂, pH 5.0) overnight at 4 °C. The suspension was cleared of undissolved materials by centrifugation at 7,000xg for 10 min and the clarified solution was layered on storage buffer containing

60% (w/v) sucrose and centrifuged at 64,000xg for 1 h. The virion fraction was dialyzed against a 500-fold volume of storage buffer. The virus concentration was estimated spectrophotometrically using the absorbance value of 5 cm²/mg at 260 nm (Bockstarhlen and Kaesberg, 1965) and stored at 4 °C.

Inoculation of protoplasts with viral RNA

BMV KU1 strain (Mise *et al.*, 1994) and CMV Y strain (Okuno *et al.*, 1993) were used. RNAs were extracted from virions as described (Kroner and Ahlquist, 1992). Protoplasts were isolated from transgenic or non-transgenic tobacco plants at the five- to six-leaf stage as described (Okuno and Furusawa, 1979) and inoculated by the PEG method (Ballas *et al.*, 1987) with viral RNA at a concentration of 1 µg/ml. Following inoculation, protoplasts were incubated at 25 °C for 24 h (Okuno and Furusawa, 1979).

RNA extraction and Northern blot analysis

Total RNA was extracted as described (Chomczynski and Sacchi, 1987). The RNA was denatured in formaldehyde/formamide and fractionated in a 1.5% agarose gel containing 1.8% formaldehyde and transferred to a Hybond-N+ (Amersham) hybridization membrane. A ³²P-labeled SP6 RNA polymerase transcript from pBSPL10 (Mori *et al.*, 1992), containing the 3'-terminal 200-base *Hind*III/*Eco*RI fragment of BMV RNA3 cDNA, whose sequence is conserved among all BMV RNAs, was used to detect positive strand BMV RNAs. Negative strand BMV RNAs were detected by a ³²P-labeled SP6 RNA polymerase transcript from pBSM10 (Mori *et al.*, 1993a) which contains the 3'-terminal 200 base fragment of BMV RNA3 cDNA in the

opposite orientation to pBSPL10. RNA1 and RNA2 lacking the 3' non-coding regions were detected by ³²P-labeled SP6 RNA polymerase transcripts from pBSL1 and pBSL2 (Mori *et al.*, 1992), respectively. CMV RNAs were detected by a ³²P-labeled T7 RNA polymerase transcript from pCY200T, kindly provided by M. Nakayama, containing the 3'-terminal 200-base fragment of CMV RNA3 cDNA, whose sequence is conserved among all CMV RNAs. The accumulation level of BMV RNAs was determined by analyzing northern blots with a digital radioactive imaging system (Fuji Film).

Western blot analysis

Accumulation of BMV CP in V123 plants was analysed by a western blotting method (Mori *et al.*, 1993) using an antiserum to BMV CP.

RESULTS

Production of transgenic tobacco plants expressing BMV RNAs 1, 2 and 3

Transgenic tobacco plants expressing the entire sequence of BMV RNA1 and RNA2 (V12 plants, Mori *et al.*, 1992) were transformed with an *Agrobacterium* binary vector (designated pBICBPBR3) which contains a full-length cDNA of BMV RNA3 linked to the CaMV 35S promoter and a *bar* gene as a selectable marker that confers resistance to bialaphos. Transgenic tobacco plants resistant to both kanamycin and bialaphos were obtained. Integration of cDNAs was confirmed by Southern blot analysis and plants containing a complete set of cDNAs to BMV genomic RNAs were designated V123 plants.

Four lines of V123 plants, thus obtained, did not exhibit any symptoms or show any delay of growth. F₁ progenies of these V123 plant lines were used in subsequent experiments.

Accumulation of BMV RNAs in V123 plants

Viral RNA accumulation in various lines of V123 plants was analyzed by northern blot hybridization using total extracted RNA. Approximately 1 ng of BMV RNAs was detected in 10 µg of total RNA from 6-week-old (five- to six-leaf stage) V123 plants (Fig. 10, lanes 2-4), while approximately 100 ng of BMV RNAs was detected in 10 µg of total RNA from non-transgenic SR1 tobacco protoplasts inoculated with BMV RNA at a concentration of 1 µg/ml (Fig. 10, lane 5). Detection of subgenomic RNA4, which is synthesized from minus-strand RNA3 by BMV replicase (Miller *et al.*, 1985), and detection of minus-strands of BMV RNA1, RNA2 and RNA3 (Fig. 11) indicate that the BMV RNAs transcribed from transgenes by plant DNA dependent RNA polymerase replicated autonomously in the V123 plant cells.

To test the biological activity of BMV RNA transcripts expressed in V123 plants, BMV virions were prepared from leaf samples of the transgenic plants (V123 plant-line 5) and inoculated onto local lesion host plants (*Chenopodium quinoa*) and systemic host plants (barley). Using an inoculum containing the same concentration of BMV virions (50 ng virions/µl), the number of lesions formed on *C. quinoa* leaves was the same order as that of the control inoculation with purified BMV virions isolated from systemically infected barley plants (about 30 lesions/half *C. quinoa* leaf, Fig. 12A). The infectivity of virions from V123 plants to barley was also the same as that of

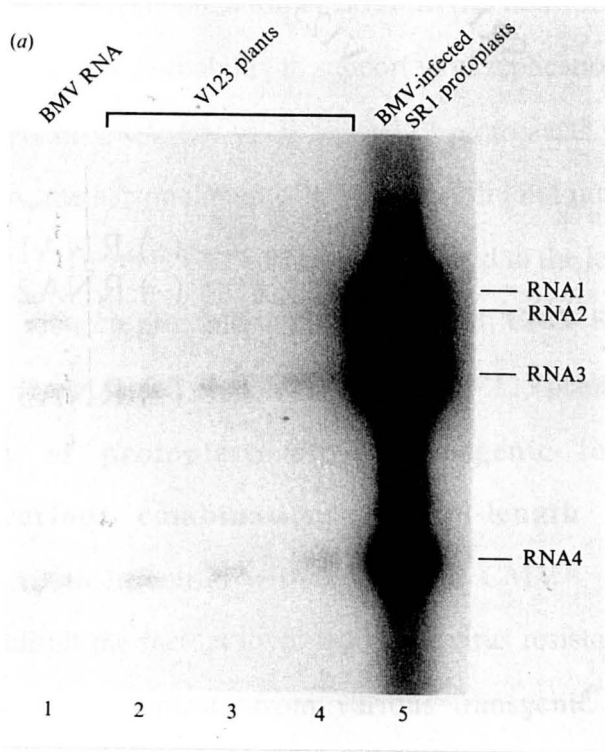


Fig. 10. Northern blot analysis of (+)-strand BMV RNA in V123 plants (lanes 2-4, V123 plant lines 5, 7 and 8) or non-transgenic SR1 tobacco protoplasts inoculated with BMV RNA (lane 5). Lane 1 contains 0.5 ng of BMV RNA extracted from purified BMV virions. Lanes 2-5 were loaded with 5 μ g of total RNA. The blot was probed with a 32 P-labeled *in vitro* transcript complementary to the conserved 3' terminal 200 bases of all four (+)-strands of BMV RNAs.

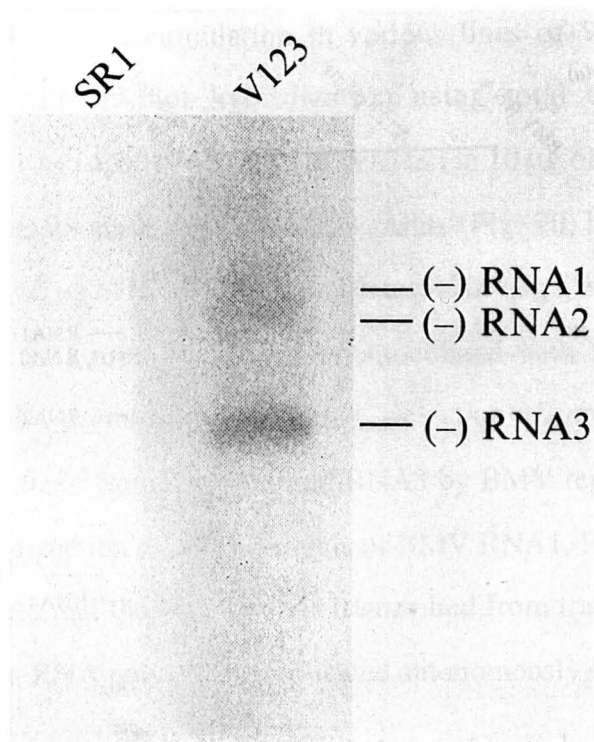


Fig. 11. Northern blot analysis of (-)-strand BMV RNA in V123 and non-transgenic SR1 plant. Both lanes were loaded with 20 μ g of total RNA. The blot was probed with a 32 P-labeled *in vitro* transcript complementary to the conserved 5' terminal 200 bases of (-)-strands of BMV RNAs.

the control inoculation (about 90% of inoculated barley plants showed systemic symptoms, Fig. 12B).

Susceptibility of V123 protoplasts to infection with BMV and CMV

The low level accumulation of BMV RNAs in V123 plants led us to test V123 protoplasts for their ability to support virus replication by inoculation with BMV RNAs or CMV RNAs. When V123 protoplasts were inoculated with BMV RNA, the accumulation of BMV RNA did not increase 24 h after inoculation (Fig. 13a), while CMV RNA accumulated to the level seen in non-transgenic SR1 tobacco protoplasts inoculated with CMV RNA (Fig. 13b). Similar results were obtained using all four lines of V123 plants.

Susceptibility of protoplasts from transgenic tobacco plants expressing various combinations of full-length BMV RNA1, RNA2 or RNA3 to infection with BMV and CMV

To delimit the factors involved in the virus resistance observed in V123 protoplasts, protoplasts from various transgenic tobacco plants expressing BMV RNA(s) were tested for their susceptibility to infection with BMV RNAs or CMV RNAs. Protoplasts from transgenic tobacco plants expressing full-length BMV RNA1 (V1 plants, Mori *et al.*, 1992), full-length RNA2 (V2 plants, Mori *et al.*, 1992), or full-length RNA3 (V3 plants) were susceptible to infection with either BMV RNAs or CMV RNAs (data not shown).

Protoplasts from transgenic tobacco plants expressing both full-length BMV RNA1 and RNA3 (V13 plants), or both full-length RNA2 and RNA3

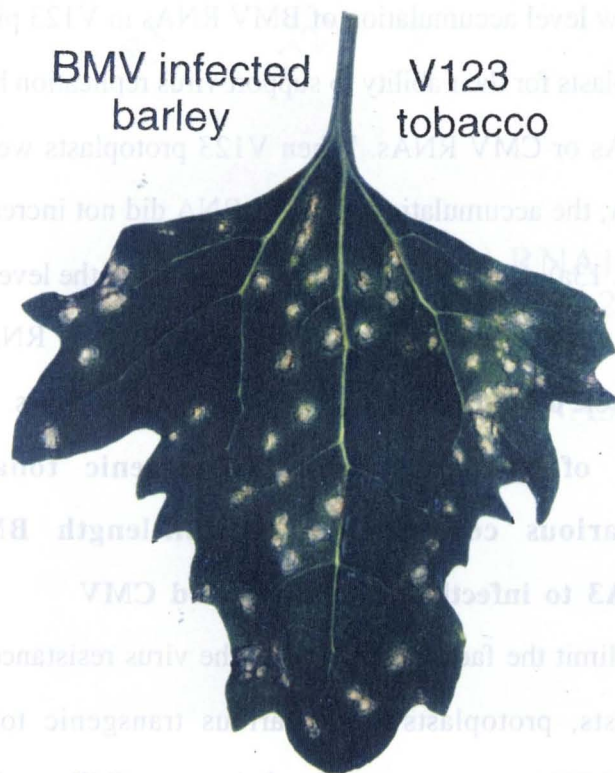


Fig. 12A. Symptoms induced on a half leaf of *Chenopodium quinoa* by BMV virion extracted from V123 plants. 50 ng/ μ l virions extracted from V123 plants or BMV-inoculated barley plants were inoculated on a half leaf of the plant.

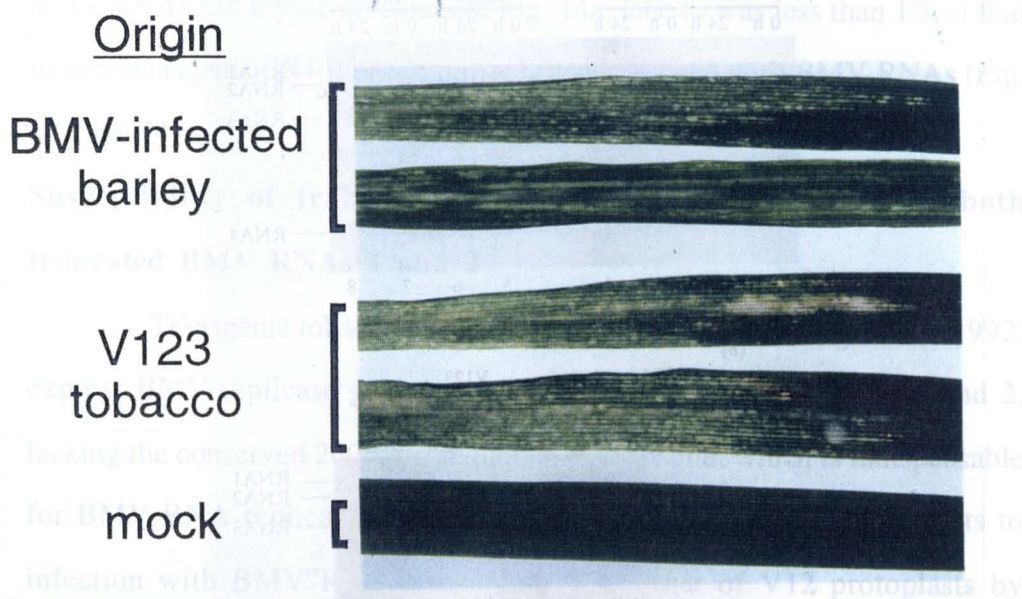


Fig. 12B. Symptoms induced on secondary uninoculated leaves of barley by BMV virion extracted from V123 plants. 50 ng/ μ l virions extracted from V123 plants or BMV-inoculated barley plants were inoculated on a primary leaf of barley plants. mock; mock inoculated with buffer only.

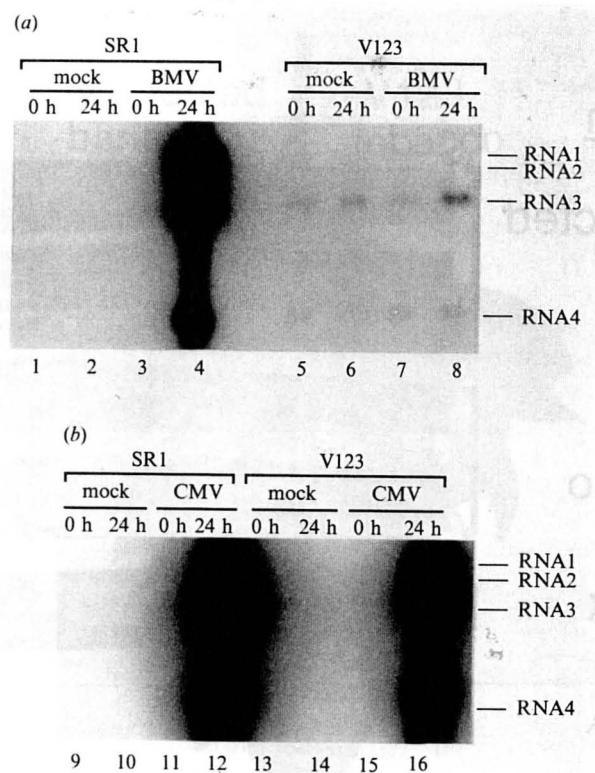


Fig. 13. Northern blot analysis of viral RNAs in V123 protoplasts (lanes 5-8, 13-16) and non-transgenic SR1 tobacco protoplasts (lanes 1-4, 9-12) inoculated with BMV RNAs (a) or CMV RNAs (b). Lanes 1, 2, 5, 6, 9, 10, 13 and 14 contain RNA from mock-inoculated protoplasts. Lanes 3, 4, 7 and 8 contain RNA from BMV RNA-inoculated protoplasts. Lanes 11, 12, 15 and 16 contain RNA from CMV RNA-inoculated protoplasts. Odd- and even-numbered lanes contain RNA from protoplasts 0 and 24 h after inoculation, respectively. All lanes were loaded with 5 μ g of total RNA. The probes used in (a) and (b) were 32 P-labeled *in vitro* transcripts specific for BMV and CMV positive-strand viral RNAs, respectively.

(V23 plants) were also susceptible to BMV and CMV infection (data not shown). By contrast, protoplasts from transgenic tobacco plants expressing both full-length BMV RNA1 and RNA2 (V12 plants) were resistant to infection with BMV RNAs and susceptible to infection with CMV RNAs (Fig. 14). The accumulation level of BMV RNAs in V12 protoplasts inoculated with BMV RNAs (24 h post-inoculation, Fig. 14a, lane 8) was less than 1% of that in non-transgenic SR1 tobacco protoplasts inoculated with BMV RNAs (Fig. 14a, lane 4).

Susceptibility of transgenic tobacco protoplasts expressing both truncated BMV RNAs 1 and 2

Transgenic tobacco plants designated M12 plants (Mori *et al.*, 1992) express BMV replicase genes on truncated forms of BMV RNAs 1 and 2, lacking the conserved 200-base sequence at the 3' end, which is indispensable for BMV RNA replication (Fig. 15). Susceptibility of M12 protoplasts to infection with BMV RNA was compared to that of V12 protoplasts by analysing northern blots with a digital radioactive imaging system. The accumulation level of BMV RNAs in M12 protoplasts was approximately 20 times higher than that in V12 protoplasts and 6 times lower than that in non-transgenic SR1 protoplasts 24 h after inoculation with BMV RNAs (Fig. 14A, lane 4, 8 and 12). The accumulation levels of truncated BMV RNA transcripts in mock-inoculated M12 protoplasts was approximately three times higher than that of viral RNAs in mock-inoculated V12 protoplasts (Fig. 16).

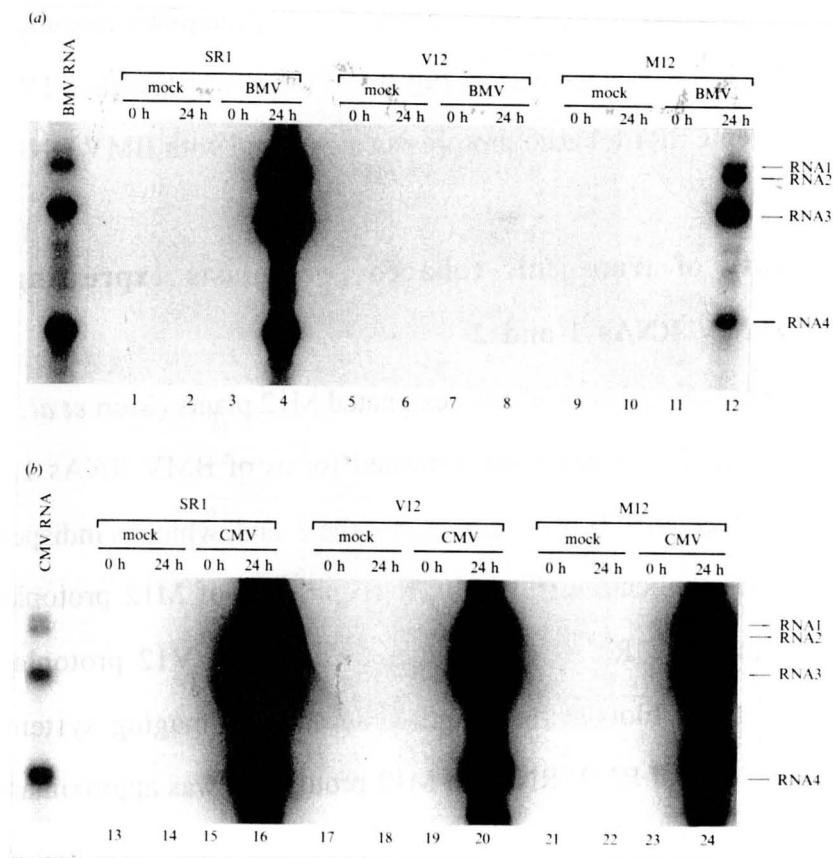


Fig. 14. Northern blot analysis of viral RNA in V12 protoplasts (lanes 5-8, 17-20), M12 protoplasts (lanes 9-12, 21-24) and non-transgenic SR1 tobacco protoplasts (lanes 1-4, 13-16) inoculated with BMV RNA (a) or CMV RNA (b). Lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21 and 22 contain RNA from mock-inoculated protoplasts. Lanes 3, 4, 7, 8, 11 and 12 contain RNA from BMV RNA-inoculated protoplasts. Lanes 15, 16, 19, 20, 23 and 24 contain RNA from CMV RNA-inoculated protoplasts. For other conditions, refer to the legend in Fig.13.

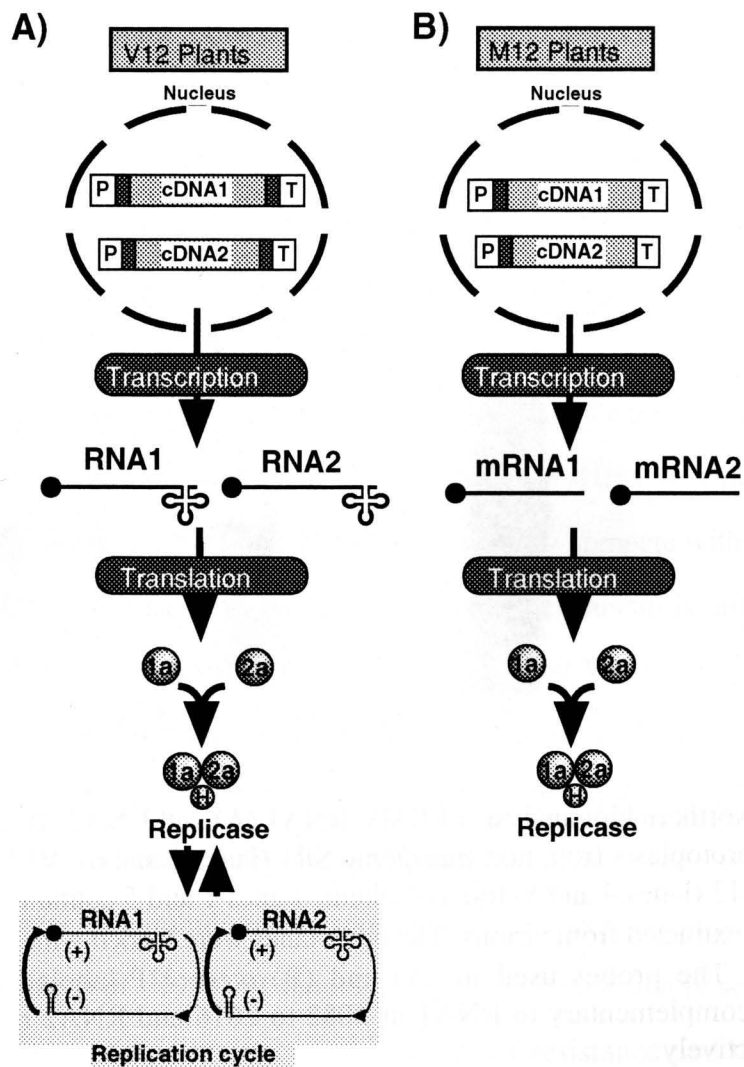


Fig. 15. Schematic representation of V12 and M12 plants. In V12 plants expressed BMV replicase attaches 3' noncoding region of BMV RNA1 and RNA2, thereby replication cycle of BMV RNA1 and RNA2 starts. While in M12 plants truncated BMV RNA1 and RNA2 serve only as the mRNA of the viral replicase genes.

Symbols: P, 35S promoter; T, 35S terminator; 1a, BMV 1a protein; 2a, BMV 2a protein; H, putative host factor involved in the formation of BMV replicase.

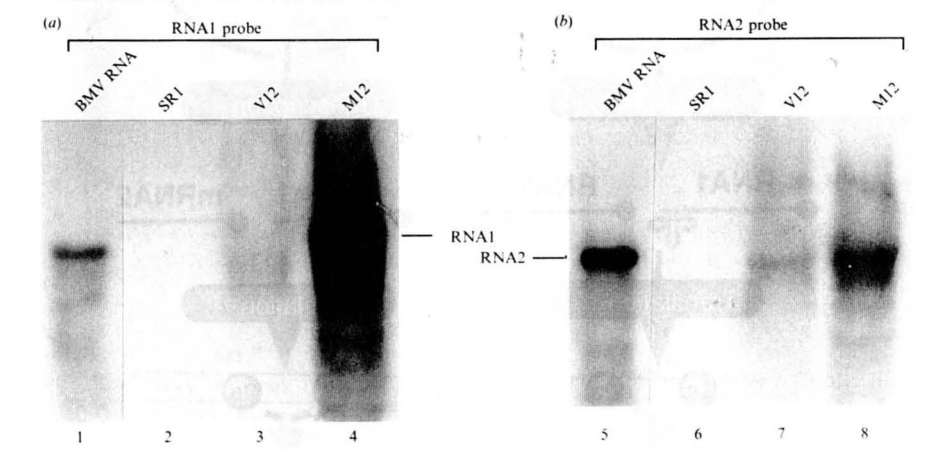


Fig. 16. Northern blot analysis of BMV RNA1 (A) and RNA2 (B) in mock-inoculated protoplasts from non-transgenic SR1 (lanes 2 and 6), V12 (lanes 3 and 7) or M12 (lanes 4 and 8) tobacco plants. Lanes 1 and 5 contain 0.5 ng of BMV RNA extracted from virions. The other lanes were loaded with 20 μ g of total RNA. The probes used in (A) and (B) were 32 P-labeled *in vitro* transcripts complementary to RNA1 nt 2082 to 2616 and RNA2 nt 1953 to 2490, respectively.

DISCUSSION

It has been reported that some of the transgenic plants expressing either intact or mutant forms of viral replicase components exhibit resistance to infection with the virus from which the transgene was derived. Replicase genes mediating viral resistance include the 54 kDa protein gene of tobacco mosaic virus (TMV) (Golemboski *et al.*, 1990), a deleted 2a RNA polymerase gene of CMV (Anderson *et al.*, 1992), non-functional mutant replicases with modified GDD motifs of potato virus X (PVX) (Longstaff *et al.*, 1993) or of ALMV (Brederode *et al.*, 1995). These results suggest that expression of a non-functional replicase component is important for virus resistance in the transgenic plants. The non-functional replicase may compete with challenged virus replicase during virus replication. Such a mechanism is unlikely to be involved in the virus resistance observed in V12 and V123 protoplasts which seem to express fully functional BMV replicase, since the virions obtained from unchallenged V123 plants showed infectivity similar to BMV virions obtained from infected barley leaves. Transgenic plants expressing an intact replicase gene of cymbidium ringspot tombusvirus were also resistant to the virus (Rubino *et al.*, 1993). It should also be pointed out that in several cases of replicase mediated resistance, the degree of resistance and the level of expression of the transgene are not directly correlated (see review by Baulcombe, 1994). So, there may be different mechanism(s) underlying replicase mediated resistance (see below).

Resistance to BMV RNA infection in M12 protoplasts was also evident by repeated experiments, but the level of resistance was extremely

weak compared to that in V12 protoplasts (Fig. 14). M12 protoplasts express truncated BMV RNA1 and RNA2 in which the 3' non-coding regions are deleted. The 3' sequences alone are unlikely to be involved in induction of the strong resistance observed in V12 and V123 protoplasts, since V1, V2, V3, V13 and V23 protoplasts expressing full-length BMV RNA(s) were susceptible to infection with BMV RNA. It should be noted that the truncated BMV RNA1 and RNA2 expressed in M12 plant cells function as mRNAs for BMV replicase proteins 1a and 2a but not as replicons by themselves. So BMV RNA1 and RNA2 expressed in V12 plant cells would be expected to accumulate to higher levels than the non-replicative truncated RNA1 and RNA2 in M12 plant cells. The results obtained in this study, however, showed that accumulation levels of replicable viral RNAs in V12 protoplasts was lower than that of the 35S promoter-driven transcripts in M12 protoplasts (Fig. 16). I speculate that the low level accumulation of RNA1 and RNA2 in V12 protoplasts is caused by mechanisms similar to the mechanism conferring strong resistance on V12 protoplasts to infection with BMV RNA.

These results suggest that expression of autoreplicable viral RNAs by itself, rather than high level accumulation of viral RNAs and/or their encoded proteins, is a crucial factor involved in the induction of strong viral resistance observed in V12 plant cells. RNA1 and RNA2 replicon expression in V12 and V123 plant cells may greatly influence the metabolism of the cells, which could induce unknown response involved in virus resistance.

Such host responses may be triggered by a temporarily high accumulation of RNA1 and RNA2 (Fig. 17). Recently, Lindbo *et al.* (1993) and Dougherty *et al.* (1994) proposed a model to explain the correlation

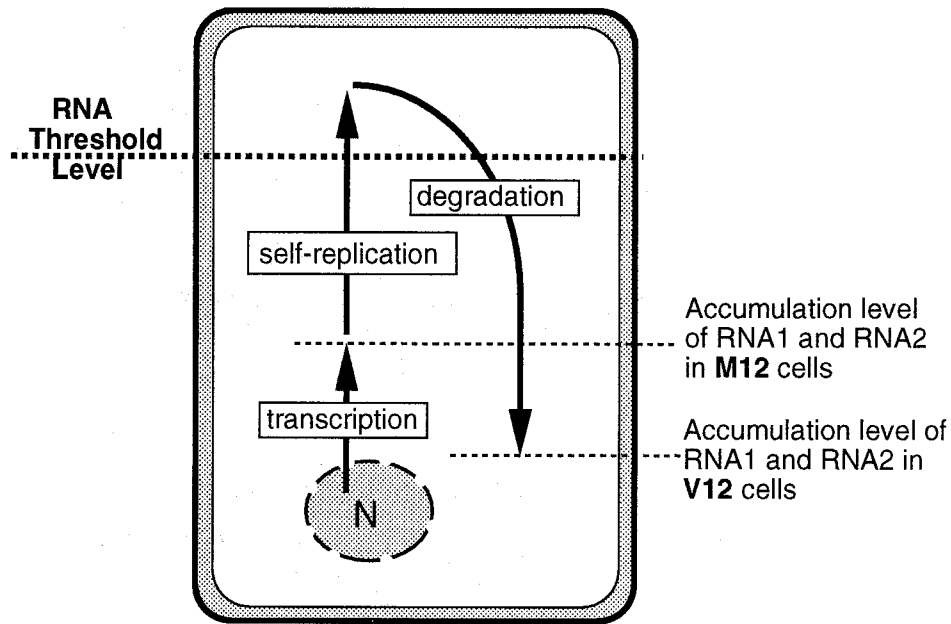


Fig. 17. Schematic representation of the model to explain the lower accumulation of BMV RNAs 1 and 2 in V12 plant cells than that in M12 plant cells. In V12 plant cells BMV RNAs 1 and 2 are supposed to accumulate temporally to the high level by self-replication. Once RNAs 1 and 2 exceed the RNA threshold level (assumed), cytoplasmic mechanisms are supposed to start operating for degradation of the RNAs.

between transgene expression and induction of an antiviral state in the cells of transgenic plants. That is, elevated transgene expression causes a cytoplasmic activity that targets specific RNA sequences for inactivation. Smith *et al.* (1994) suggested that transgene expression over "the RNA threshold level in the plant cell" may trigger the host response. This model may explain the results presented here for 4/4 transgenic lines, as well as the phenomenon known as "gene silencing" in transgenic plants having a transgene homozygous for the silenced locus (Neuhaus *et al.*, 1991; de Carvalho *et al.*, 1992).

Alternatively, it should be noted that tobacco plants transformed with a full-length cDNA of TMV RNA can support TMV replication to the level similar to that in systemically infected tobacco plants (Yamaya *et al.*, 1988). Since tobacco is a host plant for TMV but not for BMV, the strong suppression of BMV RNAs in V12 and V123 protoplasts may be due to a non-host specific response. Induction of virus resistance in V12 and V123 protoplasts may be the result of tobacco cells recognizing the excessive level of BMV RNA and/or replicase as "foreign substance", while TMV, which is highly adapted to tobacco plants was not recognized by the transgenic tobacco. It should also be pointed out that although BMV infects tobacco protoplasts, the virus accumulation level is about seven times lower than that in TMV-infected tobacco protoplasts (Maekawa *et al.*, 1985). Such differences in the potential for virus replication may cause different responses in the transgenic plants.

Chapter 3

Enhancement of the accumulation level of BMV RNAs in transgenic tobacco plants expressing the viral RNAs with auto-cleavable ribozyme sequence

INTRODUCTION

A system in which mRNA of a foreign gene can be amplified *in vivo* by viral replicase expressed from the integrated cDNAs in transgenic plants is the *in vivo* mRNA amplification system (Chapter 1). Although in M1x2-FCP2IFN plants the accumulation level of mRNA of IFN- γ was approximately five-fold higher than that of the cauliflower mosaic virus (CaMV) 35S promoter-driven transcripts, the level was not up to our expectations. In chapter 2 it was shown that protoplasts from transgenic plants expressing replicable BMV RNAs (full-length BMV RNAs 1 and 2 which encode BMV replicase genes) became highly resistant to infection with challenged BMV RNAs. So, the problem of this system is how to overcome the virus resistance induced in the transgenic plants and the resulting suppression of viral RNA or chimeric RNA accumulation.

In this chapter I have improved the efficiency of BMV RNA replication in the viral RNAs expressing plants by introducing auto-cleavable ribozyme sequence (Dzianott and Bujarski, 1988) between the 3' end of the cDNA sequence of BMV RNA and the terminator sequence, since *in vivo* mRNA amplification system, as shown in Fig. 7, transcribed BMV RNAs are supposed to be polyadenylated and since heterogeneous sequences at the 3'

end of BMV RNAs have been reported to greatly reduce the infectivity of the transcripts (Miller *et al.*, 1986, Dzianott and Bujarski, 1989). In transgenic tobacco plants, designated V123R plants, expressing full-length BMV RNAs 1, 2 and 3 with ribozyme sequence at their 3' end, the accumulation level of BMV RNA was enhanced approximately twenty times as much as that in V123 plants without ribozyme sequence. The mechanism of enhanced accumulation of BMV RNAs in V123R plants is discussed.

Methods

Construction of plant transformation vectors

A cDNA fragment of *Sna*BI/*Eco*RI-cut pBB2 (Mori *et al.*, 1991) was inserted into *Stu*I/*Eco*RI-cut pBICBP35 (Chapter 1), creating pBICBPBR2, a BMV RNA2-expressing binary vector containing bialaphos resistance gene cassette as a selectable marker.

pGL2 (Pietrzak *et al.*, 1986) contains hygromycin resistance gene (*Hph* gene) flanked by the CaMV 35S promoter and the terminator sequence. A 1.4 kilobase (kb) *Eco*RV/*Hind*III fragment of pGL2 was inserted into *Eco*RV/*Hind*III site of pBluescript II SK(-) (Stratagene) to create pBSHPH. *Eco*RI site and *Pst*I site within the *Hph* gene in pBSHPH were serially abolished by the method of Kunkel *et al.* (1987) using synthetic oligonucleotides 5' pd-(TTGGGGAATTTAGCGAGAGCC) and 5' pd(CCGC TGTTCTCAGCCGGTCG), respectively, where underlined bases were mutagenized without changing amino acid residues encoded. The *Eco*RV/

HindIII fragment of the resulting plasmid was used to replace the corresponding *EcoRV/HindIII* fragment of pGL2 to create pGL2A. To remove the *PstI* site between the 3' end of *Hph* gene and the terminator sequence, pGL2A was digested with *PstI*, blunt-ended with T4 DNA polymerase treatment and self-ligated. The *XbaI* site between the 3' end of *Hph* gene and the terminator was similarly removed to create pGL2A Δ PX. The pGL2A Δ PX was cut with *SacI* and *HindIII*, made blunt with T4 DNA polymerase, and then ligated with *PstI* linkers, creating pGL2B which has *PstI* site just downstream of the terminator sequence. Synthetic oligonucleotides 5' pd-(AATTATTTAAAT) were annealed and the resulting DNA fragment containing *SwaI* site was introduced into *EcoRI* site just upstream of the 35S promoter of pGL2B. This plasmid was cut with *SwaI* and ligated with *HindIII* linkers, creating pGL2C. A 1.7 kb *HindIII/PstI* fragment of pGL2C was used to replace the *HindIII/PstI* fragment, containing kanamycin resistance gene cassette, of the binary vector pBICP35 (Mori *et al.*, 1991), creating pBICHGP35. cDNA fragment of *SnaBI/EcoRI*-cut pBB3 (Mori *et al.*, 1991) was inserted into *StuI/EcoRI*-cut pBICHGP35, creating pBICHGBR3, a BMV RNA3-expressing binary vector containing hygromycin resistance gene as a selectable marker.

Construction of BMV RNA expression vectors with ribozyme sequence

Synthetic oligonucleotides 5' pd-(AATTCGATACCCTGTCACCGGATGTGT TTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGGACTGTCTGCA GG) and 5' pd-(AATTCCTGCAGGACA-GTCCTGTTTCGTCCTCACGGA

CTCATCAGACCGGAAAACACATCCGGTGACAGGGTATCG) were annealed and the resulting DNA fragment, which contains the ribozyme sequence from the satellite RNA of tobacco ringspot virus (Dzianott and Bujarski, 1988) and an underlined *Pst*I recognition site, was inserted into *Eco*RI site of pUCBR1, pUCBR2, pUCBR3, pBICBR1 (Mori *et al.*, 1991), pBICBPBR2 and pBICHGBR3, creating pUCBR1R, pUCBR2R, pUCBR3R, pBICBR1R, pBICBPBR2R and pBICHGBR3R, respectively, which have the ribozyme sequence between the 3' end of each cDNA sequence and the terminator sequence. The orientation of the inserted ribozyme sequence was checked by double-digestion with *Pst*I and *Sca*I for pUCBR1R and pBICBR1R and with *Pst*I and *Hind*III for the other plasmids.

Plant transformation

V123R plants were constructed as follows: pBICBR1R, pBICBPBR2R and pBICHGBR3R were separately mobilized into *Agrobacterium tumefaciens* LBA4404 using *Escherichia coli* HB101 harbouring pRK2013 as described (Bevan, 1984). Co-transformation of *Nicotiana tabacum* L. cv. Petit Habana (SR1 tobacco) with a mixture of the *Agrobacterium* cultures was carried out using the leaf disk transformation method (Horsch *et al.*, 1985). Transformed shoots and roots were selected by propagation on media containing kanamycin, bialaphos, hygromycin, or various combinations of the two of them. Transgenic plants thus obtained were checked for the expression of BMV RNA(s) by Northern blot analysis (Chapter 2). Transgenic plants expressing BMV RNA1 (V1R plants), RNA2 (V2R plants), RNA3 (V3R plants), RNAs 1 and 2 (V12R plants), RNAs 1 and 3 (V13R plants) and RNAs 2 and 3 (V23R plants) were obtained. V12R plants

and V3R plants were crossed and F₁ progeny resistant to kanamycin, bialaphos and hygromycin were designated V123R plants. All the plants used in the experiments were grown in a greenhouse controlled at 25 °C.

Inoculation tests

Inoculation with BMV-expressing vector plasmids on *Chenopodium amaranticolor* was as previously described (Mori *et al.*, 1991). The number of local lesions were counted 7 days after inoculation. Inoculation of protoplasts with BMV RNA or cucumber mosaic virus (CMV) strain Y RNA, RNA extraction and Northern blot analysis were as described in chapter 2. The accumulation level of BMV RNAs was calculated by analysing each blot with a digital radioactive imaging system (Fuji Film).

RESULTS

Infectivity of the BMV-expressing vectors containing the ribozyme sequence

The ribozyme sequence from the satellite RNA of tobacco ringspot virus was introduced between the 3' end of cDNA sequence and the terminator sequence of BMV-expressing vector, pUCBR1, pUCBR2 and pUCBR3 (Fig. 18). The resulting vectors, pUCBR1R, pUCBR2R and pUCBR3R (Fig. 18) were mixed and tested for their infectivity on *C. amaranticolor*, the local lesion host for BMV. Inoculated at the concentration of 0.1 mg/ml, pUCBR1+2+3 formed 4.8 lesions on half leaves while pUCBR1R+2R+3R formed 18.6 lesions on half leaves (Fig. 19 and 20). When inoculated at the concentration of 1.0 mg/ml, pUCBR1+2+3 formed 18.0 lesions on half leaves while

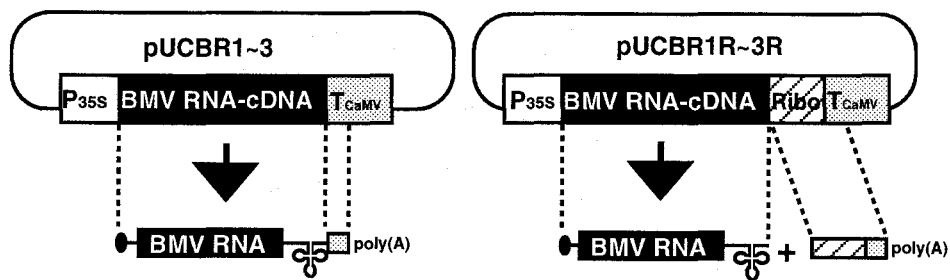


Fig. 18. Schematic representation of BMV-expressing vectors with or without ribozyme sequence (Ribo) and their transcripts. Highly conserved tRNA-like domains that contain *cis*-acting sequence for BMV RNA replication are represented by the cloverleaf structures. A synthetic linker fragment containing ribozyme sequence from satellite RNA of tobacco ringspot virus was introduced between the cDNA sequence of BMV RNA and the terminator sequence (T_{CaMV}). BMV RNAs transcribed from pUCBR1~3 contain non-viral sequences derived from the terminator sequence and poly(A) sequence, while in pUCBR1R~3R, almost all non-viral sequences are supposed to be removed from the 3' end of the transcripts by the ribozyme activity.

pUCBR1R+2R+3R formed 80.7 lesions on half leaves (Fig. 19 and 20). These results indicate that the introduction of the ribozyme sequence increased infectivity of the plasmids by approximately four times on *C. amaltanticolor* leaves.

Production of transgenic tobacco plants expressing BMV RNAs 1, 2 and 3 which are linked to the ribozyme sequence

The ribozyme sequence was introduced into BMV-expressing binary vectors and then SR1 tobacco plants were transformed with various combinations of these *Agrobacterium* binary vectors. After crossing, five lines of V123R plants, containing a set of cDNAs of BMV genomic RNAs were obtained. These plants did not exhibit any symptoms or show any delay in growth as V123 plants. These five V123R plant lines were used in subsequent experiments.

Accumulation of BMV RNAs in V123R plants

Viral RNA accumulation in various lines of V123R plants was assayed by Northern blot analysis and compared with that in V123 plants. Approximately 10 ng of BMV RNAs was detected in 10 μ g of total RNA from a fully expanded leaf of 6-week-old (five- to six-leaf stage) V123R plants while approximately 0.5 ng of BMV RNAs was detected from a leaf of corresponding position of V123 plants (Fig. 21). The above results indicate that the introduction of the ribozyme sequence increased the accumulation level of BMV RNAs in the transgenic plants by approximately twenty times.

Susceptibility of V123R protoplasts to infection with BMV and CMV

Despite the increase of the accumulation level of BMV RNAs in V123R

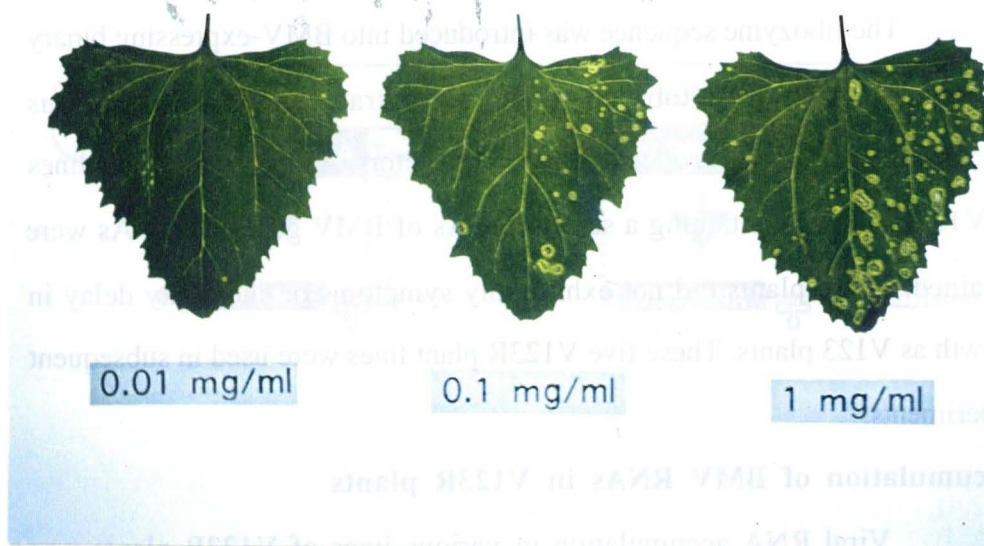


Fig. 19. Infectivity of BMV expressing vectors with or without the ribozyme sequence on *Chenopodium amaranticolor*. The left half of the leaves were inoculated with pUCBR1+2+3. The right half of the leaves were inoculated with pUCBR1R+2R+3R. Concentrations of plasmid solution are indicated. The photograph was taken at 7 days after inoculation.

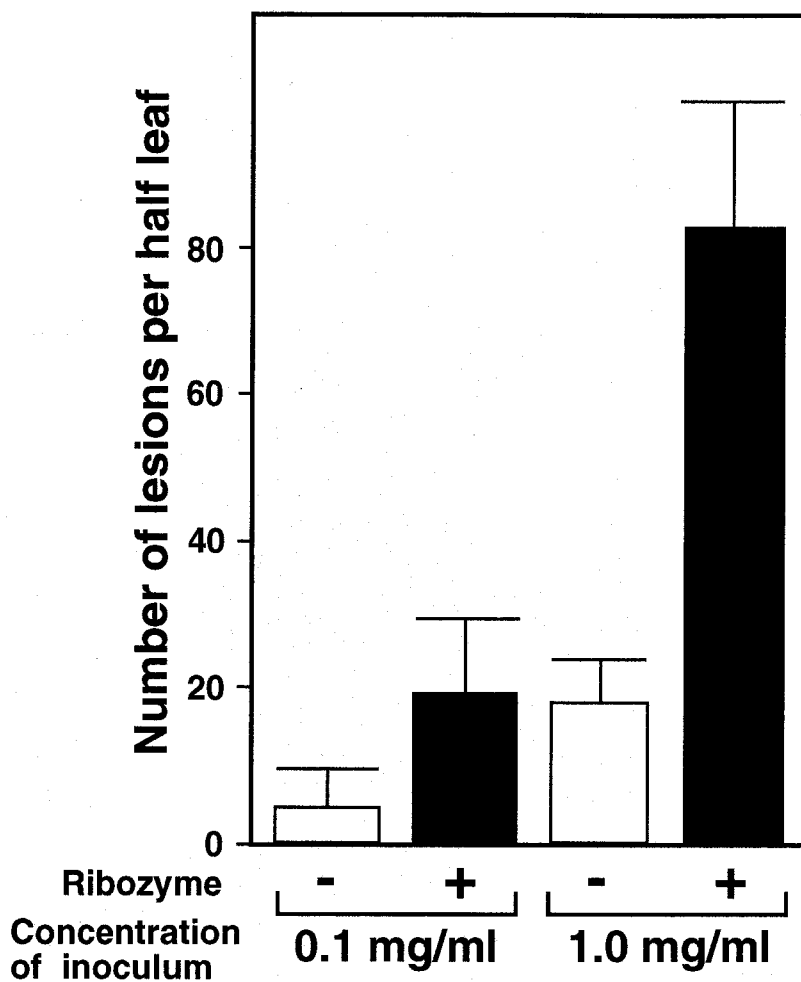


Fig. 20. Infectivity of BMV expressing vectors with or without the ribozyme sequence.

Half-leaves of *C. amalanticolor* were inoculated with either pUCBR1+2+3 or pUCBR1R+2R+3R at the concentration of 0.1 mg/ml or 1.0 mg/ml. Five half leaves for each concentration were used and the average number of local lesions with the standard deviation is shown. Open columns; pUCBR1+2+3; Closed columns; pUCBR1R+2R+3R.

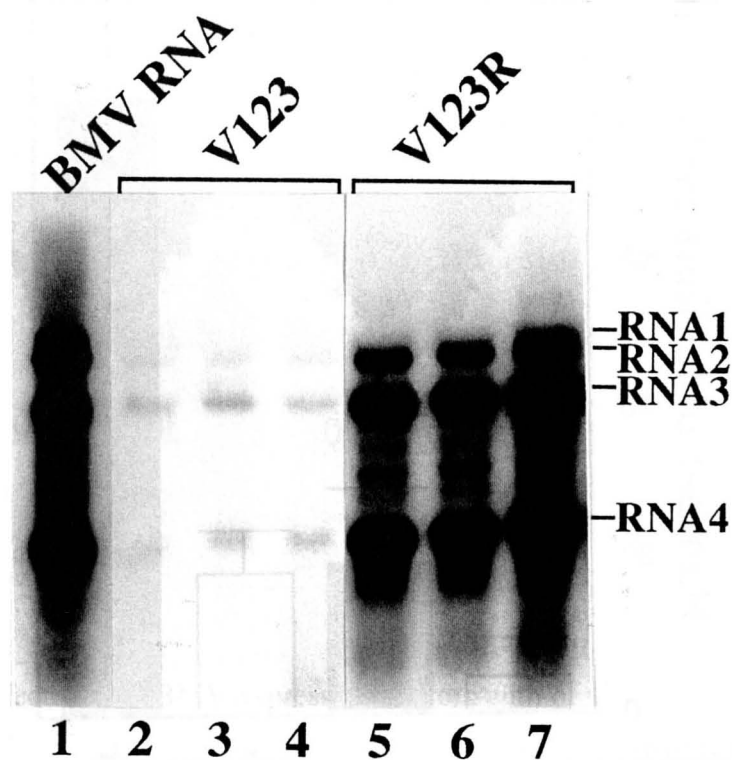


Fig. 21. Northern blot analysis of BMV RNA in V123 plant lines 5 (lane 2), 7 (lane 3) and 8 (lane 4) and V123R plant lines 52x41 (lane 5), 51x33 (lane 6) and 49x36 (lane 7). Lane 1 contains 10 ng of RNA extracted from BMV virions. Lanes 2~7 were loaded with 10 μ g of total RNA. The blot was probed with 32 P-labelled *in vitro* transcripts complementary to the conserved 3' terminal 200 bases of all four positive strands of BMV RNAs.

plants, the level was still 10 to 20 times lower than that in the BMV-infected protoplasts prepared from non-transgenic SR1 tobacco. So, we tested V123R protoplasts for their ability to support viral RNA accumulation after inoculation with BMV RNA or CMV RNA. When V123R protoplasts were inoculated with BMV RNA, the accumulation of BMV RNA did not increase 24 h after inoculation (Fig. 22),

while CMV RNA accumulated to the level of that in non-transgenic SR1 tobacco protoplasts inoculated with CMV RNA (data not shown). Similar results were obtained using all five lines of V123R plants.

DISCUSSION

The results presented here showed that the accumulation level of BMV RNAs can be enhanced in transgenic plants expressing the viral RNAs with the more accurate 3'-terminal sequences by introducing the ribozyme sequence between the 3' end of cDNA sequence and the terminator sequence. The 3'-terminal tRNA-like structure common to all four BMV RNAs contains all of the signals required for initiation of viral RNA replication and deletion or addition of heterogeneous sequence greatly reduced their template activity (Miller *et al.*, 1986). Dzianott and Bujarski (1989) developed an *in vitro* transcription system that utilized self-processing to adjust 3' termini in transcribed viral RNAs. Recently, this system has been applied to BMV RNA transcription from the plasmids in yeast (Quadt *et al.*, 1995). Tobacco plants transformed with the cDNA cassettes including the CaMV terminator sequence (Fig. 18) are supposed to express BMV RNAs bearing heterogeneous

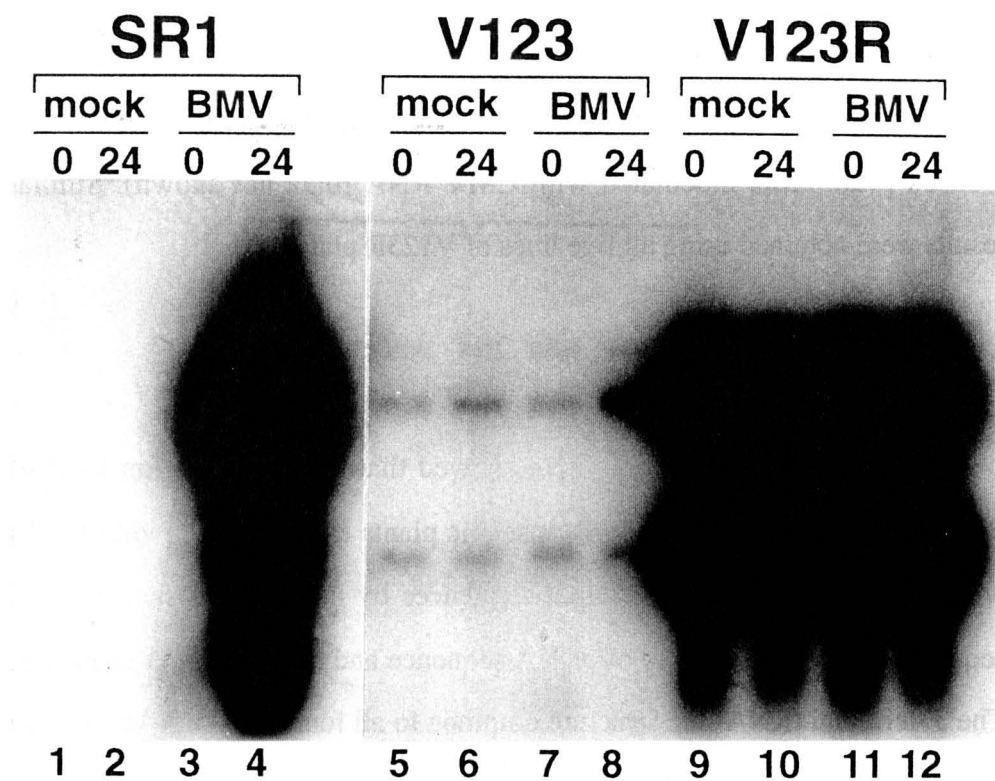


Fig. 22. Northern blot analysis of viral RNAs in non-transgenic SR1 tobacco protoplasts (lanes 1~4) and V123-line 5 protoplasts (lanes 5~8) and V123R-line 52x41 protoplasts (lanes 9~12) inoculated with BMV RNA. Lanes 1, 2, 5, 6, 9 and 10 contain RNA from mock-inoculated protoplasts. Lanes 3, 4, 7, 8, 11 and 12 contain RNA from BMV RNA-inoculated protoplasts. Odd- and even-numbered lanes contain RNA from protoplasts 0 and 24 h after inoculation, respectively. All lanes were loaded with 10 μ g of total RNA. The probes used were 32 P-labelled *in vitro* transcripts specific for BMV positive strand RNAs.

sequences over 200 bases, roughly estimated from the result presented in Fig. 7 of chapter 1, and this might have interfered with effective replication of BMV RNAs in V123 plants. So, I have introduced the same ribozyme sequence as Dzianott and Bujarski (1988) to the BMV RNA-expressing vectors and preliminarily tested their infectivity onto *C. amaranthicolor*, a local lesion host for BMV, and obtained approximately four times higher infectivity than those without the ribozyme sequence (Fig. 19). This result led me to apply it to the *in vivo* mRNA amplification system and I observed approximately twenty times higher accumulation of BMV RNAs in V123R plants than that in V123 plants (Fig. 21).

Although V123R plants achieved approximately 20 times higher BMV RNA accumulation than V123 plants, it should be pointed out that the resistance against the infection with BMV RNA is still valid in V123R cells, for V123R protoplasts inoculated with BMV RNA did not support further BMV RNA accumulation (Fig. 22). Smith *et al.* (1994) proposed a mechanism of virus resistance induced in transgenic plants; transgene expression over the RNA threshold level in the plant cell may trigger the host response that targets specific RNA sequences for degradation (Lindbo *et al.*, 1993, Dougherty *et al.*, 1994). BMV RNA incorporated into V123R protoplasts by inoculation might have been degraded by such a mechanism. Higher level of accumulation of BMV RNA detected in V123R plants than in V123 plants may result from higher level replication of BMV RNA, reflecting that transcribed and trimmed BMV RNAs with ribozyme sequence immediately start to replicate and accumulate before the resistance response starts operating.

Results presented here show that supplying the more accurate viral

RNA in transgenic plant cells enhances the accumulation level of the viral RNA. Trials to increase the accumulation level of BMV RNAs in the transgenic plants of V123 or V123R would contribute to understanding the resistance mechanism(s) induced in transgenic plants expressing viral RNA.

Chapter 4.

Wound-enhanced viral amplification in transgenic plants expressing BMV genomic RNAs

INTRODUCTION

In chapter 3, I have introduced ribozyme sequence into the transgene and produced transgenic tobacco plants (V123R plants) expressing a set of BMV genomic RNAs having more accurate 3' terminal sequence than those in V123 plants. Although viral RNA accumulation has been increased to some extent in V123R plants, these plant cells still showed resistance against infection with BMV and suppressed endogenous viral RNA accumulation (Chapter 3).

In this chapter, I have searched factors that may enhance the replication of BMV RNAs in the transgenic plants, V123R. Various factors have been known to alter the pattern of gene expression in plants. These include nutrients, ions, drugs, stresses, and physical parameters such as pressure and temperature. Among those, for example, wounding by chewing insects or other mechanical injury through which plant viruses invade plant cells, activates the expression of a set of genes in leaves of several plant families (Green and Ryan, 1972; Brown and Ryan, 1984; Ryan, 1990). To test whether wound stress affects virus resistance observed in V123R plants, I investigated the effects of wound stress and MeJA, which is known to be induced in the plant cells by wound stress, on BMV RNA replication in transgenic plants. Consequently, it was shown that wounding and MeJA treatment enhanced virus amplification in the transgenic plants expressing

BMV genomic RNAs.

MATERIALS AND METHODS

Transgenic plants

Transgenic tobacco plants used in this study were V123R plants and V12R and M12 plants (described in chapter 2).

Stress treatments

Five- to 7-week-old transgenic tobacco having 7-9 leaves were used for wounding. The surface of the leaves 7 and 8 of transgenic plants, was rubbed with a mixture of distilled water and carborundum (600 mesh), and rinsed with distilled water immediately after rubbing. Total RNA was extracted from the wounded leaves 3, 7 and 10 days after rubbing.

For MeJA or SA treatment, 7-week-old transgenic tobacco plants having 8-10 developed leaves were used. Leaf discs (approximately 4 cm²) were punched out from leaves 9 and 10, and floated on a solution containing 50 µM MeJA (Wako Biochemicals, Osaka, Japan) or 50 µM sodium salicylate (Nacalai Tesque, INC., Kyoto, Japan) at 25°C with 16-h illumination per day. Total RNA was extracted from the leaf discs 0 and 3 days after the MeJA or SA treatment.

Analysis of the accumulation level of BMV RNAs in the transgenic plants

Total RNA extraction and Northern blot analysis were as described in chapter 2. The accumulation level of BMV RNAs was determined by analysing

Northern blots with a digital radioactive imaging system (Fuji Film).

RESULTS

Effects of wound treatment on the accumulation level of endogenous BMV RNAs

It is known that gene expression in plants is commonly activated by various stresses, including drought, pathogens, and wounding. Here, I investigated effects of wound treatment on the accumulation level of BMV RNA in V123R plants. Total RNA was extracted 0, 3, 7 and 10 days post wound treatment (hereafter referred to as dpw), and endogenous BMV RNA accumulation in three plant lines of wounded V123R plants was assayed by Northern blot analysis. Approximately 60 ng of BMV RNA was detected in 10 µg of total RNA from 7-dpw V123R plants (Fig. 23). Similar results were obtained for all three lines of V123R plants tested (Fig. 23B). In time course experiments, the accumulation level of positive strand BMV RNAs in wounded V123R plants reached maximum level at 3 dpw and did not change so much until 10 dpw (Fig. 24A). In contrast, V12R plants allowed increase in the accumulation of BMV positive strand RNAs during the first three days, followed by drastic decrease by 7 dpw (Fig. 25A). On the other hand, the accumulation level of negative strand BMV RNAs in either V123R or V12R plants increased 3-fold during the first three days and declined intensively by 7 dpw (Figs. 24B and 25B).

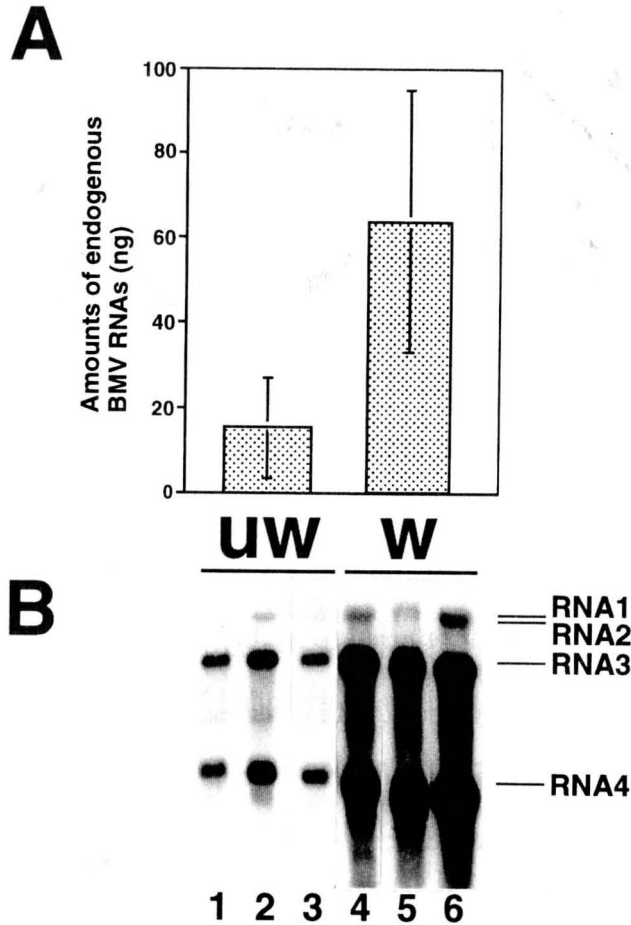


Fig. 23. Effects of wound treatment on the accumulation of endogenous BMV RNAs in V123R plants. (A) A bar graph shows the accumulation level of BMV RNAs in unwounded and wounded V123R plants. The length of the dotted column represents the mean value with the thin line of the standard deviation of the ^{32}P radioactivity described below from ten or more plants in each of three independent plant lines, such as that shown in panel B. The value was determined by comparing the ^{32}P radioactivity in the RNA 1+2+3+4 band of samples with that of the known amounts of BMV virion RNAs (see B) (B) Northern blot analysis of BMV RNA in unwounded (uw: Lanes 1-3), and wounded (w: Lanes 4-6) plants. Total RNA was extracted from plants 7 days after wound treatment. All the lanes were loaded with 10 μg of total RNA. The blot was probed with ^{32}P -labelled *in vitro* transcripts complementary to the conserved 3' terminal 200 bases of all four positive strands of BMV RNAs.

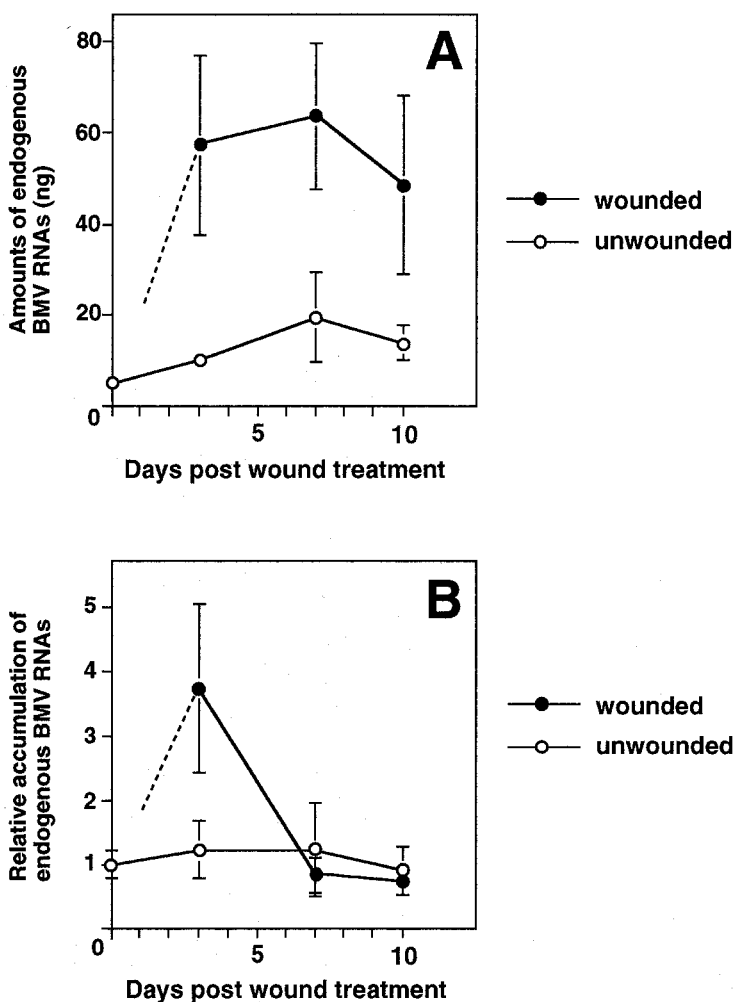


Fig. 24. Time course of the accumulation level of endogenous BMV RNAs in wounded V123R plants. The accumulation level of positive (A) and negative (B) strand BMV RNAs in V123R plants is shown. Total RNA was extracted from plants 3, 7 and 10 days post wound treatment. The value in (A) was the mean from ten or more plants and determined by comparing the ^{32}P radioactivity in the RNA 1+2+3+4 band of samples (10 μg of total RNA) with that of the known amounts of BMV virion RNAs. The value in (B) was the mean from relative values compared with the value of unwound plants at 0 day. The vertical lines indicate the standard deviation. The probes used in (A) and (B) were ^{32}P -labelled *in vitro* transcripts specific for positive and negative strand BMV RNAs, respectively.

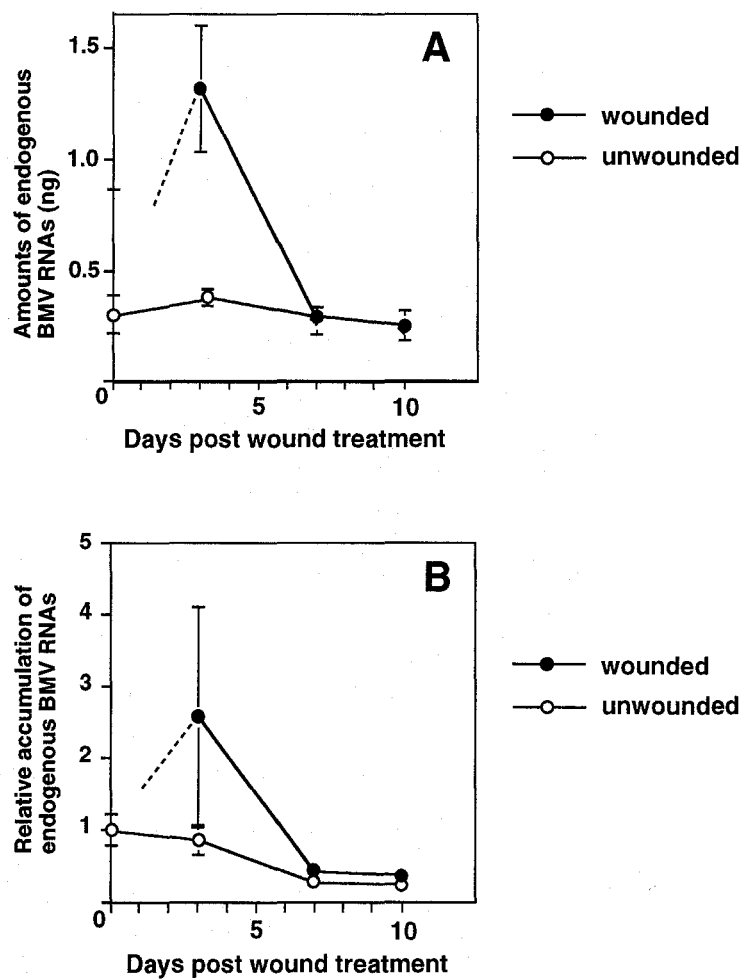


Fig. 25. Time course of the accumulation level of endogenous BMV RNAs in wounded V12R plants. The accumulation level of positive (A) and negative (B) strand BMV RNAs in V12R plants was determined, as described in the legend to Fig. 23.

Enhancement of viral RNA replication by wound treatment

Mori *et al.* (1992) have previously produced M12 plants expressing truncated BMV RNAs 1 and 2 lacking 200 bases of the 3' non-coding region indispensable for BMV RNA replication. In these plants truncated BMV RNAs 1 and 2 function only as mRNA of viral replicase genes (Fig. 15). To know whether wound-enhanced accumulation of BMV RNAs as above was due to the activation of plant transcription system and/or of viral RNA replication itself, I examined the accumulation level of truncated BMV RNAs 1 and 2 in M12 plants after wound treatment. Wound treatment did not enhance the accumulation level of the truncated BMV RNAs 1 and 2 in M12 plants but rather suppressed (Fig. 26). In contrast, the accumulation level of BMV RNAs in wound-treated V12R plants at 3 dpw was higher than that at 0 dpw (Fig. 26).

Effects of MeJA or SA treatment on the accumulation level of endogenous BMV RNAs

Recent reports indicate that MeJA is a stress-related compound, and is induced in response to wounding. Since wounding enhanced the accumulation level of BMV RNAs in V12R plants (Fig. 26), I have examined whether MeJA enhances the accumulation level of BMV RNAs in V12R and/or M12 plants. In leaf discs of V12R plants treated with MeJA the accumulation level of BMV RNAs 1 and 2 largely increased, while such an enhancement was not observed in M12 plants treated with MeJA (Fig. 27). Water-treated leaf discs of V12R plants also showed the enhanced accumulation level of RNAs 1 and 2 (Fig. 27), probably because wounding by cutting the leaves activated replication of

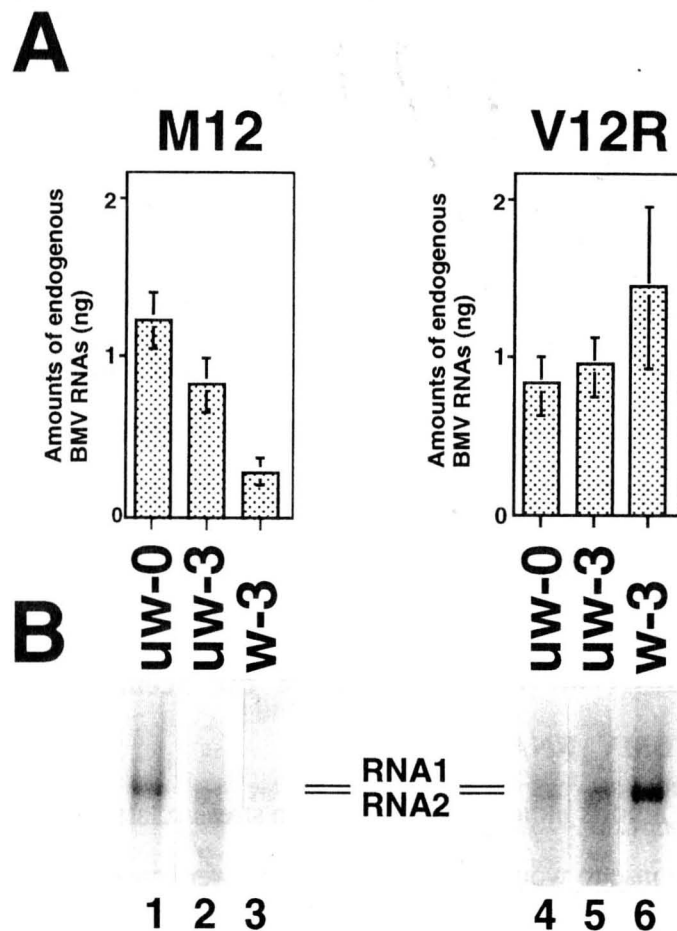


Fig. 26. Effects of wound treatment on the accumulation of endogenous BMV RNAs in M12 and V12R plants. (A) A bar graph shows the accumulation level of BMV RNAs in unwounded (uw) and wounded (w) V123R plants, as described in the legend to Fig. 23. Note that the scale of the histograms in this panel is expanded compared with that in Fig. 23. (B) Northern blot analysis of BMV RNA in unwounded (Lanes 1, 2, 4 and 5), and wounded (Lanes 3 and 6) plants. Total RNA was extracted from plants 0 (Lanes 1 and 4) and 3 (Lanes 2, 3, 5 and 6) days after wound treatment. Other features are as described in the legend to Fig. 23.

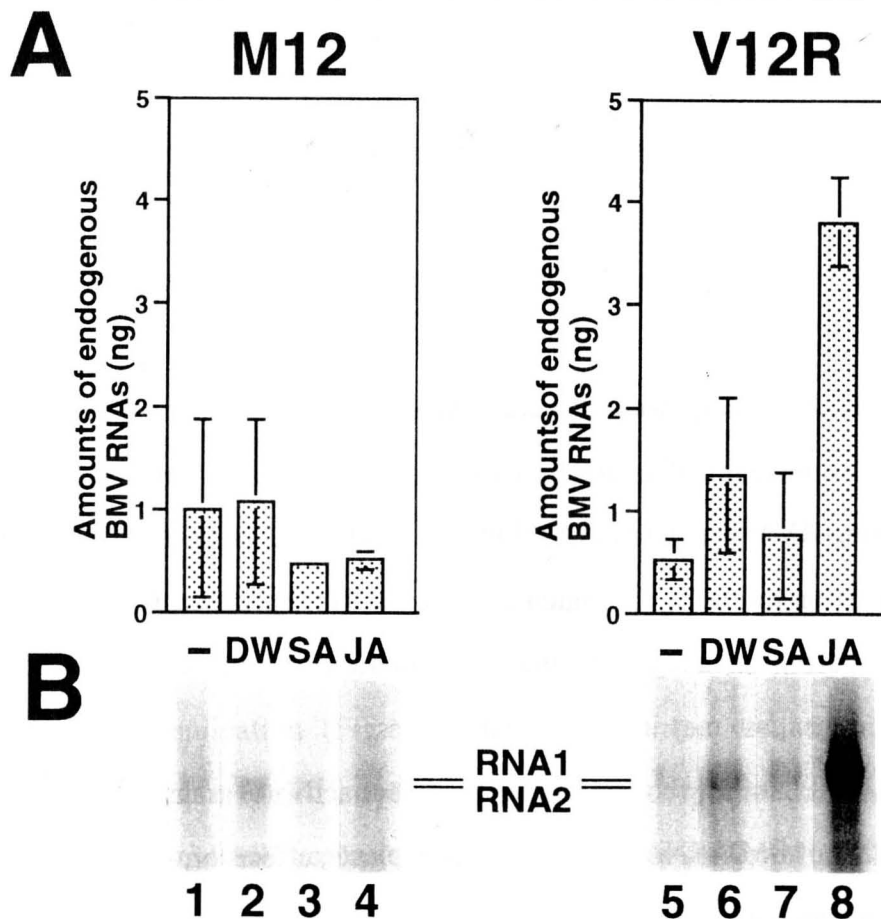


Fig. 27. Effects of SA and MeJA treatment on the accumulation of endogenous BMV RNAs in M12 and V12R plants. (A) A bar graph shows the accumulation level of BMV RNAs in variously stressed M12 (Lanes 1-4) and V12R (Lanes 5-8) plants, as described in the legend to Fig. 23. (B) Northern blot analysis of BMV RNA from unstressed plants (-: Lanes 1 and 5), and from the leaf discs floated on distilled water (DW: Lanes 2 and 6) in the presence of 50 μ M SA (SA: Lanes 3 and 7) and of 50 μ M MeJA (JA: Lanes 4 and 8). Total RNA was extracted from plants 3 days after stress treatment. Other features are as described in the legend to Fig. 23.

BMV RNAs 1 and 2 as described above. Since it is known that MeJA and SA function antagonistically, I have tested SA for the effect on viral RNA replication in V12R plants. The accumulation level of viral RNA in leaf discs of either V12R or M12 plants treated with SA was smaller than that in water-treated leaf discs (Fig. 27).

DISCUSSION

In this study wound treatment has been shown to enhance the accumulation level of endogenous BMV RNAs in the transgenic plants expressing BMV genomic RNAs (Fig. 23). Such wound-enhanced viral RNA accumulation might be correlated with the fact that plant viruses need to be introduced into cells by mechanical inoculations or with the aid of vector insects; either case may induce wound stresses.

To examine whether wound treatment activates transcription and/or replication of BMV RNAs in the transgenic plants, effects of wound treatment on the accumulation level of endogenous viral RNA were compared between M12 and V12R plants. Indeed, only in V12R plants wound treatment enhanced the accumulation level of endogenous BMV RNAs (Fig. 26). Reduction of BMV RNA accumulation in M12 plants after the wound treatment may result from the altered metabolism of the cells that enhances viral RNA degradation and/or inactivates the 35S promoter-driven transcription. Since such metabolic changes are supposed to occur in V12R plants as well as in M12 plants, the enhancement of BMV RNA accumulation in wounded V12R plants would be

due to the activation of viral RNA replication and/or the suppression of virus resistance that inhibits viral RNA replication, since one of the characteristic differences of viral RNA transcripts expressed in V12R plants from those in M12 plants is whether they are replicable or not.

Time course experiments in wounded plants showed continuous accumulation of positive strand BMV RNAs in V123R plants and transient accumulation of positive strand BMV RNAs in V12R plants and negative strand BMV RNAs in both V123R and V12R plants (Figs. 24 and 25). These results suggested that positive strand BMV RNAs in V123R plants might be protected from attack of the viral resistance by the encapsidation with CP that is encoded on RNA3 and further indicated that effects of wound stress on BMV RNA replication were transient.

It is known that MeJA is generated in many organs of plants, especially after wound treatment (Creelman *et al.*, 1992). Since wound stress enhanced BMV RNA replication (Fig. 26), I expected that wound-associated compounds, such as MeJA, may enhance BMV RNA replication in transgenic plants expressing BMV genomic RNAs. Comparative studies using M12 and V12R plants showed that MeJA also is one of the factors which activate BMV RNA replication in V12R plants. The results may suggest a novel role for MeJA to amplify viral RNA. MeJA might induce activators and/or suppress inhibitors of BMV RNA replication, since MeJA is generally known to function as an inducer (Parthier *et al.*, 1987) or a repressor (Weidhase *et al.*, 1987; Reinbothe *et al.*, 1993) for certain gene expressions.

It has been well documented that SA is an endogenous signal for the activation of certain plant defence responses and for the establishment of

enhanced disease resistance (Yalpani and Raskin, 1993; Ryals *et al.*, 1994). In contrast, recent experiments have demonstrated that SA inhibits the synthesis of the proteinase inhibitor induced by jasmonic acid (JA) (Doares *et al.*, 1995). Seo *et al.* (1995) suggested that JA could inhibit SA biosynthesis in transgenic plants expressing small GTP-binding proteins. These reports demonstrated that both JA and SA are potent inducers of plant defense responses and gene activation, and function antagonistically. I examined the effects of SA treatment on BMV RNA amplification in transgenic plants expressing viral RNA, and showed that SA treatment decreased the accumulation level of endogenous BMV RNAs in either M12 or V12R plants (Fig. 27). SA treatment might suppress the endogenous JA level in V12R and M12 plants. The results also indicated that enhancement of BMV RNA replication in the transgenic plants is associated with the alteration of gene expression induced by MeJA, but not by SA.

The results in this chapter showed that wound stress and MeJA treatment activated the replication of endogenous BMV RNAs in the transgenic plants. The elucidation of the mechanism will not only contribute to the production of the desired proteins in the *in vivo* mRNA amplification system, but also open the way to understanding the mechanism of the replication of plant virus RNA.

General Discussion

A new departure in the *in vivo* mRNA amplification system described in chapter 1 is the amplification of mRNA by replication, not by transcription. I have expected that extraordinary ability of BMV replicase would bring about the much higher productivity of foreign gene products in the transgenic tobacco plants. Although the accumulation level of mRNA of IFN- γ in M1x2-FCP2IFN was fivefold higher than that of CaMV 35S promoter-driven transcripts, it was not up to our expectation. The results obtained in chapter 2 show that the accumulation of endogenous BMV RNAs in transgenic plants expressing replicable BMV RNAs is suppressed by the virus resistance induced in the transgenic plant cells. These data lead to the speculation that the accumulation of chimeric BMV RNA3 is suppressed in M1x2-FCP2IFN plant cells, for chimeric BMV RNA3 is replicated by the viral replicase. These results present the basic and serious problems of the *in vivo* mRNA amplification system.

On the inactivation of transgene expression in transgenic plants, the phenomenon called homology-dependent gene silencing has been reported (Jorgensen, 1992, Finnegan and McElroy, 1994, Matzke and Matzke, 1995). Homology-dependent gene silencing is supposed to be the defense system of plant cells against overexpression of a gene. Two different mechanisms have been proposed to explain these phenomena, a transcriptional inactivation through either *de novo* methylation or heterochromatin formation (Meyer *et al.*, 1993, Neuhuber *et al.*, 1994) and a post-transcriptional process such as RNA

turnover (de Carvalho *et al.*, 1992, Dehio and Schell, 1994, Meins and Kunz, 1994)). The *in vivo* mRNA amplification system presented here may be influenced by such mechanisms, leading to low level accumulation of the desirable protein. The former mechanism, however, seems not to be applied to the case. Even though the transgenes and/or their promoters are *de novo* methylated, the chimeric BMV RNA once transcribed before the methylation would be amplified by BMV replicase and therefore the system is supposed to be hardly influenced by a transcriptional inactivation.

It is probable that activation of the transgene RNA turnover is concerned in the low level accumulation of the desired protein in the *in vivo* mRNA amplification system. Recently Lindbo *et al.* (1993) have proposed a model to explain the virus resistance induced in transgenic plants expressing viral genes from a viewpoint of homology-dependent gene silencing; resistance to tobacco etch virus (TEV) in transgenic tobacco plants expressing the TEV coat protein transgene is due to increased accumulation level of TEV-specific RNA resulting from transcription of the transgene plus the replicating TEV genome in the cytoplasm. A key experiment showed that, even though the coat protein transgene was transcribed at approximately the same rate in resistant and sensitive plants, steady-state levels of transgene RNA were significantly lower in the former than in the latter. This links the antiviral state with posttranscriptional degradation of the transgene RNA and TEV genomic RNA. They also have suggested that an RNA-dependent RNA polymerase derived from the genome of host plant cells could synthesize short complementary segments of an RNA that had accumulated to intolerably high levels in the cytoplasm. The short duplex RNAs formed on the target RNA would then be a

substrate for double-strand RNases (Lindbo *et al.*, 1993). This model was supported by other studies using transgenic tobacco plants expressing the coat protein gene of potato virus Y (Smith *et al.*, 1994), the replicase gene of potato virus X (Mueller *et al.*, 1995) and TEV 6- and 21-kDa open reading frames (Swaney *et al.*, 1995). This model could explain the correlation between the low level accumulation of the transgene RNA and the strong virus resistance induced in the transgenic plants. Although any direct evidence on the acceleration of sequence-specific RNA degradation in the transgenic plant cells has not yet been reported, to date this degradation model is most reliable to explain RNA-mediated virus resistance.

Although the *in vivo* mRNA amplification system has encountered an obstacle by the defense system of the transgenic plant cells, the results obtained in chapter 3 and 4 show that it could be possible to surmount and achieve high-level accumulation of the desired protein by breaking the virus resistance. Although the precise features of the virus resistance mechanism underlying have not yet been clarified, trials to break the virus resistance would contribute to understanding of the mechanism. Using the chimeric viral RNA constructed between suppressed and resistance-breaking strains of cucumber mosaic virus (CMV), Hellward and Paulkaitis (1995) showed that the resistance against CMV induced in the transgenic tobacco plants expressing a part of the replicase gene of CMV targets the viral RNA, not the protein, and that sequence-specific RNA turnover is not a sole mechanism of the virus resistance. Results obtained in chapter 4 strongly suggest that the unknown genomic features concerning to suppress the viral RNA amplification in the transgenic plants exist. From the results that wound treatment and MeJA treatment enhanced the accumulation

level of BMV RNA in the transgenic plants (Fig. 23 and Fig. 27), these genomic features are supposed to be located in and influenced by the wound signal transduction pathways (Seo *et al.*, 1995). Wound stress induces the expression of mitogen-activated protein kinase and afterwards JA and MeJA are induced. JA and MeJA are known to play a critical role in the plant defense system against pathogen attack by inducing proteinase inhibitor II (Peña-Cortés *et al.*, 1995). The results obtained in chapter 4, however, indicate that MeJA helps the viral RNA replication in the transgenic plants. The elucidation of unknown features of the materials, such as JA and SA, in the wound signal transduction pathways may help the *in vivo* mRNA amplification system to work more effectively.

Instead of the constitutive CaMV 35S promoter, using inducible promoters, for example, a heat shock promoter and a hormone-inducible promoter might be effective to improve the amplification level of the viral RNA in the transgenic plants. Efficient virus replication and propagation usually depend on stage and growth condition of the host plants. So, cultivating the transgenic plants in the conditions that do not allow the expression of viral RNA could lead the plant reaching to the best condition for the virus replication without inducing the virus resistance response, and then transferring them to the condition allowing the viral RNA expression might have the transgenic plant cells realize the best environment for the virus replication. On the other hand, controlling BMV RNA replication using the mutant BMV which can replicate at 25°C but not at 32°C (Kroner *et al.*, 1990) might also be effective to the amplification of the virus in the transgenic plants.

Finally, the transgenic plants presented here provided the useful

materials for basic studies in virology and plant biology. To achieve the desired level of accumulation of the desired protein in the *in vivo* mRNA amplification system, more profound knowledge and understanding on the virus replication system and the virus-host plant interaction would be essential.

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Summary

Chapter 1.

I have constructed transgenic tobacco plants (M1x2-FCP2IFN plants) expressing BMV replication genes and BMV RNA3 derivative (FCP2IFN) carrying the human gamma interferon (IFN- γ) gene. In M1x2-FCP2IFN plants the RNA3 derivative expressed from the integrated cDNA was replicated and subgenomic RNA (i.e. mRNA of IFN- γ) was produced by BMV replicase. The accumulation level of the mRNA of IFN- γ was approximately 5-fold higher than that by cauliflower mosaic virus 35S RNA promoter. In addition IFN- γ accumulated in M1x2-FCP2IFN plants.

Chapter 2.

Transgenic tobacco plants (V123 plants) expressing a set of full-length brome mosaic virus (BMV) genomic RNAs from the cauliflower mosaic virus 35S promoter were produced. The accumulation level of BMV RNAs in V123 plant cells was approximately 1% of that in non-transgenic tobacco protoplasts inoculated with BMV RNAs. The level of BMV RNA in V123 protoplasts did not increase after inoculating the protoplasts with BMV RNAs, while V123 protoplasts supported the accumulation of cucumber mosaic virus (CMV) RNAs to a level similar to that in non-transgenic tobacco protoplasts after inoculation with CMV RNA. Such BMV-specific-resistance was also observed in protoplasts from V12 plants expressing full-length BMV RNA1 and RNA2, both of which are required and sufficient for BMV RNA replication. On the

other hand, protoplasts from M12 plants, expressing truncated BMV RNA1 and RNA2 in which the 3' 200-nucleotides required for BMV RNA replication were deleted, exhibited weaker resistance to infection with BMV RNA than V12 protoplasts, although the accumulation level of truncated BMV RNA1 and RNA2 in M12 protoplasts was higher than that of BMV RNA1 and RNA2 in V12 protoplasts. These results suggest that expression of BMV RNA replicons is involved in the induction of resistance, rather than high level accumulation of BMV RNAs and/or their encoded proteins.

Chapter 3.

We have previously reported that protoplasts from transgenic tobacco plants, V123 plants, expressing a set of genomic RNAs of BMV become resistant to infection with BMV RNA, and the accumulation level of BMV RNAs in the transgenic plants is very low. To improve the efficiency of BMV RNA replication in transgenic plants, we have introduced auto-cleavable ribozyme sequence of the satellite RNA of tobacco ringspot virus between the 3' end of cDNA sequence of BMV RNA and the terminator sequence in each of the expression vectors for BMV RNAs 1, 2 and 3. Inoculation with a mixture of these plasmids induced about four times as many local lesions on *Chenopodium amaranticolor* leaves as those with plasmids without the ribozyme sequence. Subsequently, we obtained transgenic tobacco plants, V123R plants, containing a set of BMV cDNAs whose 3' end is immediately followed by the ribozyme sequence. The accumulation level of BMV RNAs in these plants was about twenty times higher than that in V123 plants, which do

not have the ribozyme sequence. Protoplasts from V123R plants, however, did not support further accumulation of BMV RNAs after challenge inoculation with BMV RNA, suggesting that resistance response against BMV infection is still valid in V123R plant cells.

Chapter 4.

Wound stress increased the accumulation level of endogenous BMV RNAs in V123R plants by 4-fold when compared to that in unwounded plants. Methyl jasmonate (MeJA), a wound-associated compound, also enhanced viral RNA accumulation in the transgenic tobacco plants expressing both of complete BMV RNAs 1 and 2, but did not in the plants expressing BMV RNAs 1 and 2 lacking a part of the 3' non-coding region indispensable for replication of the viral RNAs, suggesting that either treatment enhanced viral RNA replication rather than transcription of the viral RNAs from chromosome DNA. These results demonstrate a new role for MeJA to amplify viral RNA.

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List of Publications

1. MORI, M., ZHANG, G., KAIDO, M., OKUNO, T. & FURUSAWA, I. (1993). Efficient production of human gamma interferon in tobacco protoplasts by genetically engineered brome mosaic virus RNAs. *Journal of General Virology* **74**, 1225-1260.
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