Expression of brome mosaic virus-encoded replicase genes in transgenic tobacco plants

Masashi Mori,* Kazuyuki Mise, Tetsuro Okuno and Iwao Furusawa

Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

We introduced replicase genes of brome mosaic virus (BMV) to Nicotiana tabacum cv. Petit Habana (SR1) using two different types of transformation vectors containing cDNAs of BMV RNA 1 and RNA 2. One type (V type) contains cDNA from which complete viral RNAs are transcribed. These RNAs can function as templates for viral replicase. The other type (M type) contains cDNA from which viral RNAs without their 3' non-coding regions are transcribed; these RNAs can only function as mRNA. Viral replicase expressed from the integrated cDNAs in both V and M type transgenic plants can complement an infection by BMV RNA 3.

Brome mosaic virus (BMV) is a plant virus which has a tripartite genome of positive-sense RNAs, designated RNA 1, RNA 2 and RNA 3 in order of decreasing Mr (Lane, 1981). RNA 1 and RNA 2 encode proteins 1a and 2a, respectively, which are thought to be involved in the replication of viral RNAs (Kiberstis et al., 1981; French et al., 1986). RNA 3 encodes protein 3a and the coat protein (Ahlquist et al., 1987). The protein 3a is thought to be involved in cell to cell transport of the virus, and the coat protein is translated from subgenomic RNA 4 which is transcribed from RNA 3 (Miller et al., 1985). RNA-dependent RNA polymerase (RdRp) has been partially purified from BMV-infected plants (Hardy et al., 1979; Bujarski et al., 1982). The RdRp synthesized only negative-strand RNA complementary to genomic RNAs (Ahlquist et al., 1984; Miller et al., 1985). No complete in vitro replication system has yet been obtained, and the mechanism by which BMV RNA is replicated is not completely understood. To identify cis-acting elements for viral RNA replication, it would be desirable to develop an in vivo replication system in which viral replicase is supplied from plants, independent of the viral replication cycle.

We previously constructed plasmids pBB1, 2 and 3 containing full-length biologically active cDNAs corresponding to BMV RNA 1, RNA 2 and RNA 3, respectively (Mori et al., 1991). Infectious transcripts can be synthesized in vitro from pBTF1, 2 and 3, which contain full-length BMV cDNA1, cDNA2 and cDNA3. The inserts were cloned just downstream of a T7 promoter.

The plasmid pBICP35, containing a modified cauliflower mosaic virus 35S RNA promoter with a new StuI site at the initiation site of transcription, was used to construct binary vectors for plant transformation (Mori et al., 1991). Full-length cDNAs of BMV RNA 1, RNA 2 and RNA 3 (i.e. SnaBI–EcoRI fragments of pBB1, 2 and 3) were inserted in StuI/EcoRI-cut pBICP35, creating pBICBR1, 2 and 3, respectively (Fig. 1). A mixture of pBICBR1, 2 and 3 plasmid DNA is infectious for Chenopodium hybridum (Mori et al., 1991). This indicates
that the transcripts synthesized from cDNAs of pBICBR1, 2 and 3 can function as BMV RNAs in transformed plants.

To construct a cDNA of RNA 1 from which the 3' non-coding region had been deleted, pBB1 was digested with XhoI (nucleotide nt 2988) and made blunt with T4 DNA polymerase, and then ligated with EcoRI linkers, creating pBB1(-3). To delete 3' non-coding regions in cDNA of RNA 2, pBB2 was digested with PstI and HindIII (nt 2667) and the resulting fragment was inserted in PstI/HindIII-cut pUC18, creating pBB2(-H). The plasmid pBB2(-H) was digested with HindIII and made blunt with T4 DNA polymerase, and then ligated with EcoRI linkers, creating pBB2(-3). The fragments of SnaBl/EcoRI-cut pBB1(-3) and pBB2(-3) were inserted
in Stu/EcoRI-cut pBICP35, creating pBICBRI(-3) and pBICBR2(-3), respectively (Fig. 1). The constructs pBICBRI(-3) and pBICBR2(-3) contain cDNA of RNA 1 and cDNA of RNA 2 from which 246 and 200 bases in the 3' non-coding regions had been deleted, respectively. Binary transformation vectors pBICBRI, pBICBR2, pBICBRI(-3) and pBICBR2(-3) were mobilized into Agrobacterium tumefaciens LBA 4404 (Ditta et al., 1980). The resulting strains were used to transform Nicotiana tabacum cv. Petit Habana (SR1) (Horsch et al., 1985). Tobacco plants transformed by Agrobacterium containing pBICBRI, pBICBR2, pBICBRI(-3) and pBICBR2(-3) were designated V1, V2, M1 and M2, respectively. We analysed the presence of BMV RNAs and proteins expressed by the integrated cDNAs in transgenic tobacco plants by a complementation test (van Dun et al., 1988).

Protoplasts were isolated from transgenic plants at the five to six leaf stage as described (Okuno & Furusawa, 1979). Viral RNA 1, RNA 2 and RNA 3 were transcribed from pBTF1, 2 and 3 with T7 RNA polymerase, respectively.

Protoplasts from plants V1-2 and V2-1 were inoculated by means of the polyethylene glycol (PEG) method (Ballas et al., 1987) with RNA 2 plus RNA 3, RNA 1 plus RNA 3, and a mixture of RNA 1, RNA 2 and RNA 3 at a concentration of 1 µg/ml of each RNA; incubation was for 24 h at 25 °C. Similar experiments were carried out for plants M1-4 and M2-1. Accumulation of coat protein in protoplasts was analysed by a Western blotting method (Towbin et al., 1979). As shown in Fig. 2, coat protein was detected in both V1-2 and M1-4 protoplasts inoculated with RNA 2 plus RNA 3, and also detected in both V2-1 and M2-1 protoplasts inoculated with RNA 1 plus RNA 3 (Fig. 2). Meanwhile no coat protein was detected in V1-2 protoplasts inoculated with either RNA 1 plus RNA 3, or RNA 1 plus RNA 2 (Fig. 2). These results indicate that proteins 1a and 2a expressed from the integrated cDNAs were able to complement an infection by inoculation with RNA 2 plus RNA 3 and RNA 1 plus RNA 3, respectively.

To introduce both cDNAs of RNA 1 and RNA 2 into the tobacco nucleus, M1-4 and M2-1, and V1-2 and V2-1 were crossed. We analysed the integration of viral genes in the F1 progeny by a complementation test. As expected, coat protein was detected in protoplasts of the progeny of V1-2 × V2-1 (called V1x2-21) and M1-4 × M2-1 (called M1x2-41) when inoculated with RNA 3 alone.

Replication of BMV RNAs in V1x2-21 or M1x2-41 protoplasts inoculated with RNA 3 alone was analysed by the Northern blot method. The probe for RNAs 1, 2, 3 and 4 was a 32P-labelled SP6 transcript from a subclone, containing the 200-base HindIII (nt 1914)–EcoRI 3' terminal fragment of BMV RNA 3 cDNA which is conserved among all BMV RNAs. Probes for RNA 1 and RNA 2 without 3' non-coding regions were 32P-labell SP6 transcripts from subclones, pBSL1 and pBSL2, containing the XbaI (nt 2616)–HaelII (nt 2082) fragment of BMV RNA 1 cDNA and HaeIII (nt 2490)–HaelII (nt 1953) fragment of BMV RNA 2 cDNA, respectively. Replication of RNA 3 and 4 was detected in both V1x2-21 and M1x2-41 protoplasts inoculated with RNA 3 alone. The amount of RNAs 1 and 2 in M1x2-41 protoplasts did not increase during the 24 h incubation after inoculation with RNA 3 (Fig. 3). These results indicate that RNAs 1 and 2 without 3' non-coding regions transcribed in M1x2-41 protoplasts, cannot be replicated. On the other hand the amount of RNAs 1 and 2 in V1x2-21 protoplasts increased during the 24 h incubation after both inoculation with RNA 3 and mock inoculation (Fig. 3). These results indicate that RNA 1 and RNA 2 transcribed in V1x2-21 protoplasts function as a template for viral replicase and are amplified. There was little difference in the amount of RNA 1 and RNA 2 between V1x2-21 protoplasts inoculated with RNA 3 and mock-inoculated protoplasts (Fig. 3).

In M1x2 plants, truncated viral RNA 1 and RNA 2 transcribed from cDNAs did not replicate. Expression of viral replicase depended on the transcription system of the host plants but not on the viral replication cycle. That is, plants endowed with all trans-acting factors required for replication of viral RNA can support the replication of any foreign RNA as long as the molecule has cis-acting elements required for BMV replication. We also constructed transgenic plants (V1x2-21) expressing BMV RNAs that can replicate. The transgenic plants described here will be useful in the study of the mechanism of viral replication. A V1x2 type plant as well as an M1x2 type could help in the development of a system for the production of foreign proteins in plant cells.

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (03257104) from the Ministry of Education, Science and Culture, Japan.

References


(Received 2 January 1991; Accepted 9 September 1991)