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Expression of the Tobacco Mosaic Virus Movement Protein Alters Starch Accumulation in *Nicotiana benthamiana*

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***Nicotiana benthamiana* plants were transformed with the movement protein (MP) gene of tobacco mosaic virus (TMV), using *Agrobacterium*-mediated transformation. Plants regenerated from the transformed cells accumulated 30-kDa MP and complemented the activity of TMV MP when infected with chimeric TMVs containing defective MP. These transgenic plants displayed stunting, pale-green leaves, and starch accumulations, indicating that TMV MP altered the carbon partitioning for leaves involved in TMV cell-to-cell movement.**

Keywords: Movement protein, pale-green leaf, stunting, tobacco mosaic virus, transgenic plant

TMV (tobacco mosaic virus) is a well-characterized plant virus with a single, positive-sense RNA genome of 6,395 nucleotides (Holt and Beachy, 1991). Its genome encodes at least four proteins. The 126- and 183-kDa proteins, required for virus replication, are translated directly from the genomic RNA, using the same initiation codon (Pelham, 1979; Ishikawa et al., 1986). The 30-kDa movement protein (MP) and the 17.5-kDa coat protein (CP) are translated from separate 3' coterminal subgenomic mRNAs. MP functions in the cell-to-cell movement of viruses (Deom et al., 1987). CP encapsulates viral RNA and is required for long-distance movement in plants (Takamatsu et al., 1987; Dawson et al., 1988).

Observations of TMV MP made in vivo and in vitro have allowed researchers to assign several functions and characteristics to this protein. To date, four biological activities have been postulated: 1) forming an extended MP-TMV RNA complex (Citovsky et al., 1990, 1992; Waigmann et al., 1994); 2) increasing the molecular size exclusion limit of plasmodesmata (Pd) in inoculated and neighboring cells (Wolf et al., 1989); 3) interacting with cytoskeletal elements to facilitate the transport of the MP-TMV RNA complexes from the cytoplasm to Pd (Heinlein et al., 1995); and 4) interacting with a cell-wall-associated receptor, which then phosphorylates the bound MP, inactivating its ability to dilate Pd (Citovsky et al., 1993).

In this study, we introduced a TMV MP gene into a wild-type tobacco plant (*Nicotiana benthamiana*) via *Agrobacterium*-mediated transformation, and investigated the phenotypic characteristics of the resultant transgenic plants.

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MATERIALS AND METHODS

Construction of the Plant Expression Vector

The TMV MP gene used in this study originated from U3/12-4, a full-length infectious cDNA clone of TMV (Holt and Beachy, 1991). The KpnI-BamHI fragment from U3/12-4 was cloned into the KpnI-BamHI sites of pBluescript KS(+) (Stratagene, USA), creating pBS/TMV-U1 (Fig. 1A). cDNA that contained the TMV MP coding sequence was amplified by polymerase chain reaction (PCR) from the cDNA clone of pBS/TMV-U1 that served as a template. Oligonucleotides (MPN1, 5'-GCGGGATCCGCGGCCGCACATAT GGCTCTAGT-TGTTAAAGGG-3' and MPC1, 5'-CCGCGGCCGC-GAATTCTAT TTAAAACGAATCC-3') were designed with appropriate restriction enzyme sites (underlined) for further cloning into the plant expression vector pMON977 (Monsanto Co., USA). The PCR product resulting from the use of the forward (MPN1) and reverse (MPC1) MP primers was digested with BamHI

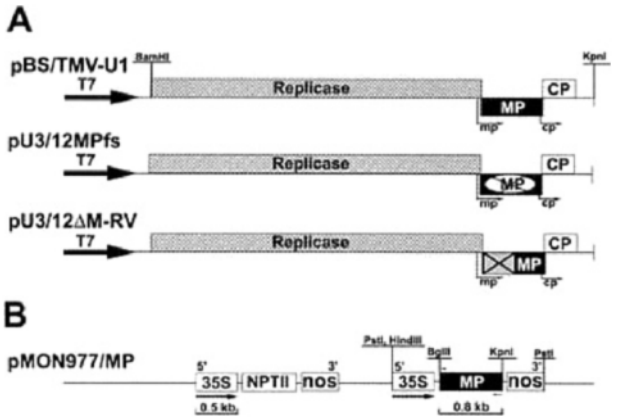

Figure 1. Schematic diagram of constructs used in this study. **A.** Modified forms of full-length TMV-U1 cDNA were constructed to impair MP. Small arrows represent subgenomic promoters. Hatched pattern, replicase; black, MP; open box, CP; x'ed MP, frame-shifted MP; x'ed box, deleted region of MP. **B.** Structure of the pMON977/MP plasmid for expression of TMV MP in *N. benthamiana*. Neomycin phosphotransferase II (NPT II), which is regulated by the CaMV 35S promoter and the 3' end of the nopaline synthase gene, served as a selectable marker for tobacco transformation.

and EcoRI and ligated into pMON977. This MP sequence was controlled by the CaMV35S promoter, and was terminated by the nopaline synthase gene (NOS) polyadenylation signal (Kim et al., 2000). The resulting plasmid was denoted as pMON977/MP (Fig. 1B). Following *Agrobacterium* transformation, the TMV MP sequence in the pMON977 vector was verified by sequencing the PCR products generated from the cloning experiments.

Plant Transformation

pMON977/MP and pMON977 were introduced into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell, 1986). Transformed *N. benthamiana* plants were obtained using a standard leaf disc transformation method (An, 1987). The R_0 plants containing pMOM977/MP were selected and self-pollinated to obtain R_1 progeny seeds. Seeds from the R_0 lines were germinated individually on an MS medium (Murashige and Skoog, 1962) that contained 100 μg/mL kanamycin, and the resulting seedlings were transplanted into soil two weeks after germination. These plants were maintained at 25°C/20°C day/night, under a 16-h photoperiod.

PCR and Reverse Transcriptase (RT)-PCR Analysis

Using TMV MP-specific primers (MPN2, 5'-ATGGCTCTAGTTGTT AAAGG-3' and MPC2, 5'-TTAAAACGAATCCGA-3'), we performed PCR to verify the MP sequences in both the transformed *N. benthamiana* lines and their subsequent offspring, as described by Ryu et al. (1998). PCR reactions contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 0.2 mM of each dNTP, 2.5 units *Taq* DNA polymerase (GIBCO BRL, USA), and 100 ng of genomic DNA. The reaction mixtures were taken through 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

For RT-PCR, total RNA was extracted from the leaves, following the method of Ausubel et al. (1999). A first-strand cDNA synthesis reaction was performed with 1-μg samples of total RNA. The reagents were introduced into a Thermoscript RT-PCR System (GIBCO BRL, USA) according to the manufacturer's instructions. RNA samples were primed with oligo$(dT)_{20}$ and the reverse transcriptase reaction was performed at 52°C for 1 h in a final volume of 50 μL. Each 2-μL reaction was used for PCR as described above. RT-PCR products were analyzed on a 1% agarose gel.

Western Blot Analysis

To confirm the expression of TMV MP in transgenic lines, we performed immunoblot experiments with an antiserum generated against an oligopeptide corresponding to a region near the carboxy terminus of MP (Deom et al., 1987). Protein extracts were prepared from the fourth or fifth leaves of mature plants. The leaves were frozen in liquid nitrogen, then ground to a fine powder with a mortar and pestle. Ground samples were resuspended in 2 mL of protein extraction buffer (50 mM Tris-HCl [pH 7.4], 2.5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 2.5 mM DTT) that contained a protease inhibitor tablet (Roche, Germany). The ground slurry was micro-centrifuged at 12,000*g* for 20 min at 4°C. Supernatants were transferred into clean tubes, and the protein concentrations were determined using a Bradford protein assay kit (BioRad, USA), with bovine serum albumin as the standard. Supernatant proteins were separated by 12.5% SDS-PAGE in SDS sample buffer according to the method of Laemmli (1970). These proteins were transferred onto an Immobilon-P PVDF membrane (Millipore, USA), and were incubated first with an anti-TMV MP antibody (1:115 dilution) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, USA) at a 1:2,500 dilution. Protein bands were detected using an ECL kit (Amersham-Pharmacia, UK).

Infectious Chimeric Viruses and in-Vitro Transcription

We used a functionally defective MP-chimeric virus to determine whether the expressed TMV MP had worked in our transgenic plants. A frameshift mutation in the TMV MP gene was generated by digesting pBS/TMV-U1 with HindIII, filling in the 5' overhang with Klenow polymerase, and self-ligating to reclose. This altered fragment was exchanged for its wild-type counterpart in pBS/TMV-U1 to generate pU3/12MPfs (Fig. 1A). We also used the deletion mutant (Fig. 1A) of the TMV MP gene (pU3/12Δ M-RV; Gafny et al., 1992).

To produce infectious transcripts from pU3/12MPfs and pU3/12Δ M-RV, 1 μg of each plasmid was linearized with KpnI. After removing the overhanging nucleotides by treating them with a Klenow fragment of DNA polymerase I, we conducted in-vitro transcription reactions with T7 RNA polymerase (MEGAscript T7 kit, Ambion, USA). The upper, expanding leaf surfaces of five-week-old plants were mechanically inoculated as described by Szesi et al. (1999). Infected plants were then maintained in a growth chamber at 24°C with a 16/8 h light/dark photoperiod.

Analysis of Starch Accumulation

Transgenic and control *N. benthamiana* seedlings were used for starch-iodine staining. Seedlings were decolorized in 70% ethanol and stained with an iodine-potassium iodide-lactic acid mixture (IKI; stock IKI was 2% iodine and 6% KI, mixed 1:20 with 85% lactic acid), as described by Lindner et al. (1959).

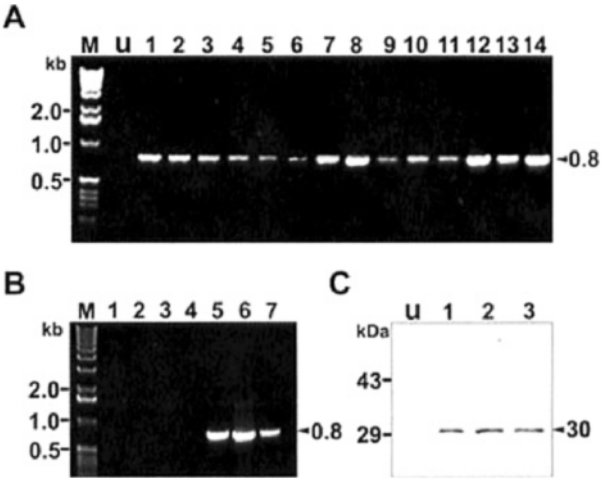

Figure 2. Transgene verification of regenerated plants. **A.** The presence of *TMV MP* in regenerated plants was verified using specific *TMV MP* primers to amplify an 800-bp fragment. Genomic DNA was prepared by a simple and rapid DNA purification method (McGarvey and Kaper, 1991). One microliter of crude preparation was used for PCR, as described under "Materials and Methods." Lane M, molecular size marker, lane U, untransformed control plants; Lanes 1-14, transformed lines Nb2-① to ⑭. **B.** RT-PCR analyses of the expression of *TMV MP* transcript in transgenic *N. benthamiana* lines Nb2-② (Lane 5), -④ (Lane 6) and -⑧ (Lane 7). Lane 1, total RNA from one of the samples (line Nb2-②) was treated with RNase A before its use as a template; Lane 2, ddH_2O was used as template; Lane 3, untransformed control plants; Lane 4, transformed with vector only. **C.** Western blot analyses of the expression of TMV MP in transgenic *N. benthamiana* lines Nb2-② (Lane 1), -④ (Lane 2) and -⑧ (Lane 3). Molecular mass markers are indicated on the left. The arrowhead indicates MP.

RESULTS AND DISCUSSION

Establishment of *N. benthamiana* Transgenic Lines

Following co-cultivation of *N. benthamiana* with pMON977/MP, we were able to isolate 14 independent lines (Nb2-① to Nb2-⑭) on selective media. Using PCR, these lines (R_0) were screened for the presence of the TMV MP sequence on nuclear DNA. All 14 contained the expected single product of approximately 800 bp (Fig. 2A). Among these lines, three (Nb2-②, Nb2-④, and Nb2-⑧) were randomly selected for further study. Those three, containing the MP-specific sequence, were analyzed for transcript expression by RT-PCR analysis (Fig. 2B). The transgenic plants exhibited high steady-state mRNA levels, transcripts of expected sizes, and a two- to threefold difference in the amounts of TMV MP extracted. That the transcripts were of the expected size demonstrated that they had not been deleted or recombined, which is consistent with the association of different levels of MP transcripts with gene copy number in transgenic plants (Duan et al., 1997). In contrast, the control plants contained RNA with no TMV MP-related sequences.

To determine the number of TMV MP copies in the transgenics, three R_0 plants (from Nb2-②, Nb2-④, and Nb2-⑧) were self-pollinated, and the R_1 progeny were segregated on kanamycin plates. Progeny from Nb2-⑧ segregated into 3:1 (3 kanamycin-resistant : 1 kanamycin-susceptible), which suggests that the MP gene originated from a single genetic locus. The seedling segregation ratio for Nb2-② was approximately 15:1, with all the Nb2-④ seedlings being resistant to kanamycin. This indicates that, for Nb2-②, the MP gene originated from two loci, whereas for Nb2-④, expression was from

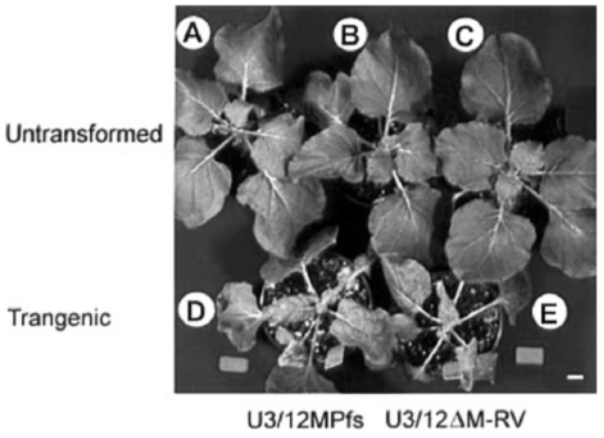

Figure 3. Effects of infectious chimeric virus on TMV MP function. Five week-old untransformed plants (upper) and 6 week-old transgenic plants (lower; Nb2-⑧) were inoculated with the MP-frameshift (pU3/12MPfs) and deletion (pU3/12ΔM-RV) mutant transcripts, and were photographed at 12 d post-inoculation. 'A' was not inoculated; 'B' and 'D' were inoculated with *U3/12MPfs*; 'C' and 'E' were inoculated with *U3/12ΔM-RV*. Bar represents 1 cm.

multiple genetic loci.

The three transgenic lines that produced the MP-specific transcripts (Nb2-②, Nb2-④, and Nb2-⑧) were analyzed for protein expression by western blot analysis (Fig. 2C). Immunoblots revealed that TMV MP accumulated to similar detectable levels in all three lines. To further determine the contribution of MP expression in transgenic plants to viral infection, infectious chimeric viruses were generated by introducing frameshift (*U3/12MPfs*) and deletion (*U3/12ΔM-RV*) mutants into the MP gene (Fig. 1A). As shown in Figure 3, in-vitro transcripts from pU3/12MPfs and pU3/12ΔM-RV were inoculated onto either untransformed or Nb2-⑧ plants. The transcripts of each construct were infectious to transgenic plants, and the viral progeny from *U3/12MPfs* and *U3/12M-RV* were able to spread both locally and systemically. Systemic disease symptoms developed in the MP-expressing plants, but were not detected in the uninfected or untransformed control plants. This infectivity of transcripts from the pU3/12MPfs and pU3/12ΔM-RV clones in MP-expressing plants indicates that MPs were stably expressed and that the *N. benthamiana* cells possessed MP activity.

Overexpression of TMV MP Gene Product Produces Pale-Green Leaves

Morphologies of the transformed *N. benthamiana* plants expressing TMV MP were compared with those of the untransformed controls. A number of those expressing TMV MP (as determined by protein immunoblot analysis) exhibited abnormalities (Fig. 4A). These included pale-green leaves, which first appeared on the older, mature tissues. Untransformed wild-type and empty vector-transformed control transgenic plants grown under the same conditions did not display the pale-green coloring at the same leaf position. To determine whether plants with this phenotype were contaminated with TMV, extracts were prepared from the older leaves. These were then inoculated onto the wild-type tobacco leaves. The treated wild-type plants showed no visible symptoms, suggesting that they were not contaminated with TMV (data not shown). Deom et al. (1990) demonstrated that the largest total amount of MP was consistently detected in the older leaves of tobacco plants that expressed the MP gene under the CaMV35S promoter. Therefore, the pale-green color in older leaves of our *N. benthamiana* might have been due to the accumulation of TMV MP, even though this abnormality had not been reported by Deom et al.. This contrast in the effect of over-expression by TMV MP may have resulted because separate tobacco species were being tested in the two studies: *N. benthamiana* and *Nicotiana tabacum*.

Stunting and Pale-Green Leaves Are Associated with Starch Accumulation

After the R_0 lines were self-pollinated and germinated on an MS medium with 100 μg/mL kanamycin, the seedlings were transplanted into soil. Growth of the transgenic plants was significantly stunted compared with the control (Fig. 4, B and C). Duan et al. (1997) had reported that significant amounts of starch accumulated in the leaves of transgenic tobacco plants expressing the Geminivirus MP gene, and that this starch accumulation caused alterations in leaf texture and color. They also showed significantly increased stunting in several R_1 lines and their subsequent progeny, compared with the control. In our study, stunting of the transgenic plants may also have resulted from starch accumulation. To confirm this, we used a starch-iodine test to compare the amount of accumulation in the three R_1 lines (Nb2-②, Nb2-④, and Nb2-⑧) versus that found in the young, untransformed plants. As shown in Figure 4D, transgenic plants showed starch accumulation, which was consistently detected in their older leaves. We suggest, therefore, that TMV MP has a pleiotropic effect on leaf physiology (including starch accumulation) that hinders plant growth.

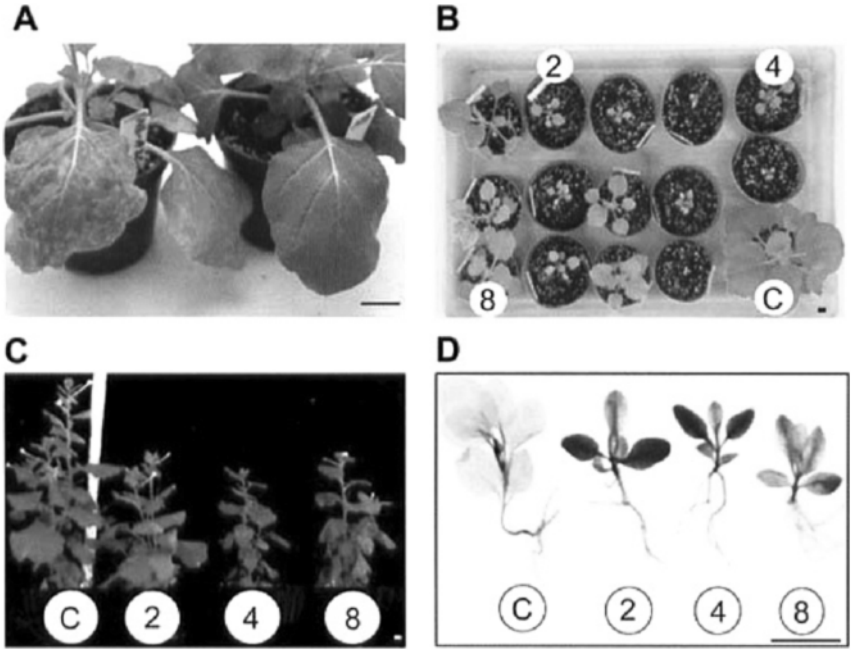

Figure 4. Phenotypes and starch accumulation of transgenic plants constitutively expressing TMV MP. Phenotypes including stunting and pale-green leaves can be seen in the transgenic plants. **A.** The R_0 plant morphology of a five-week-old representative transgenic TMV MP plant Nb2-③ (left) shows pale-green leaves. **B.** Five-week-old representative transgenic TMV MP plants (Nb2-① to ⑭) and a normal wild-type plant of the same age are shown (ⓒ). ②, ④, and ⑧ indicating the three selected transgenic lines Nb2-②, -④, and -⑧. **C.** The whole-plant morphology of 13-week-old R_1 plants shows stunting in the three selected transgenic lines Nb2- ② , - ④ , and - ⑧ . **D.** Starch-iodine test for starch accumulation in three-week-old plants. The transgenic lines show starch accumulation in old leaves and the stem (② , ④ , and ⑧), while untransformed control plants (ⓒ) do not show starch accumulation. Bars represent 1 cm.

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