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Genetics

Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA

(tobamovirus/subgenomic promoter/phytoene/antisense)

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ABSTRACT **The carotenoid biosynthetic pathway in higher plants was manipulated by using an RNA viral vector. A cDNA encoding phytoene synthase and a partial cDNA encoding phytoene desaturase (PDS) were placed under the transcriptional control of a tobamovirus subgenomic promoter. One to two weeks after inoculation, systemically infected *Nicotiana benthamiana* plants were analyzed for phytoene. Leaves from transfected plants expressing phytoene synthase developed a bright orange phenotype and accumulated high levels of phytoene. Cytoplasmic inhibition of plant gene expression by viral RNA was demonstrated with an antisense RNA transcript to a partial PDS cDNA derived from tomato. The leaves of the plants transfected with the antisense PDS sequence developed a white phenotype and also accumulated high levels of phytoene. A partial cDNA to the corresponding *N. benthamiana* PDS gene was isolated and found to share significant homology with the tomato antisense PDS transcript. This work demonstrates that an episomal RNA viral vector can be used to deliberately manipulate a major, eukaryotic biosynthetic pathway. In addition, our results indicate that an antisense transcript generated in the cytoplasm of a plant cell can turn off endogenous gene expression.**

Hybrid tobamoviruses have been used previously to express heterologous enzymes in transfected plants (1, 2). In this study, we have developed a new viral vector, TTO1, consisting of sequences from tobacco mosaic virus strain U1 (TMV-U1) (3) and tomato mosaic virus (fruit necrosis strain F; ToMV-F) (4), that was used to deliberately manipulate the carotenoid biosynthetic pathway in *Nicotiana benthamiana*. Since the first committed step in the synthesis of carotenoids in higher plants is the condensation of two geranylgeranyl diphosphate molecules to phytoene, by the enzyme phytoene synthase (PSY) (5), we chose to overproduce this enzyme in transfected plants. We predicted that recombinant, hybrid viruses expressing the PSY gene would be able to alter the biochemical pathway by increasing the level of phytoene.

We were also interested in inhibiting the synthesis of carotenoids by viral delivery of antisense RNA. Although the expression of numerous genes in transgenic plants has been repressed by antisense RNA, the actual mechanism and location of inhibition are not known (6). In the nucleus, antisense RNA may directly interfere with transcription or form duplexes with the heterogeneous nuclear RNA (hnRNA). Alternatively, in the cytoplasm, antisense RNA may form a double-stranded molecule with the complementary mRNA and prevent the translation of mRNA into protein. For our experiment, we chose to inhibit the expression of phytoene desaturase (PDS), a thylakoid membrane-bound enzyme (7), by viral delivery of antisense RNA. Since tobamoviruses replicate solely in the cytoplasm (8), it was uncertain whether the antisense viral constructs would have an effect on endogenous PDS gene expression.

MATERIALS AND METHODS

Plasmid Constructions. An 861-bp fragment (nt 5524–6384) [from ToMV virion RNA (fruit necrosis strain F; ToMV-F)] containing the putative coat protein subgenomic promoter, coat protein gene, and the 3′ end was isolated by reverse transcription (RT)–PCR using ToMV primers 5′-CTCGCAAAGTTTCGAACCAAATCCTC-3′ (upstream) and 5′-CGGGGTACCTGGGCCCCAACCGGGGGT-TCCGGGGG-3′ (downstream) and subcloned into the *Hin*cII site of pBluescript KS(−) (Stratagene): A hybrid virus consisting of TMV-U1 and ToMV-F sequences was constructed by swapping an 874-bp *Xho* I–*Kpn* I ToMV fragment into pBGC152 (2), creating plasmid TTO1. The inserted fragment was verified by dideoxynucleotide sequencing. A partial cDNA encoding PSY was isolated from ripening tomato fruit RNA by PCR using the oligonucleotides: 5′-TATGTATGGTGCA-GAAGAACAGAT-3′ (upstream) and 5′-AGTCGACTCT-TCCTCTTCTGGCATC-3′ (downstream). Approximately 3 × 10^4 colonies from a tomato (*Lycopersicon esculentum*) cDNA library were screened by colony hybridization using a ^{32}P-labeled tomato PSY PCR product. Hybridization was carried out at 42°C for 48 hr in 50% formamide/5× standard saline citrate (SSC)/0.02 M phosphate buffer, 5× Denhardt's solution containing sheared calf thymus DNA at 0.1 mg/ml. Filters were washed at 65°C in 0.1× SSC/0.1% SDS prior to autoradiography. PCR products and the PSY cDNA clones were verified by dideoxynucleotide sequencing. By PCR mutagenesis a *Xho* I fragment encoding tomato PSA was amplified from a *L. esculentum* cDNA and placed under the control of the TMV-U1 coat protein subgenomic promoter by subcloning into TTO1, creating plasmids TTO1/PSY+ and TTO1/PSY−.

A partial cDNA encoding PDS was isolated from ripening tomato fruit RNA by PCR using the oligonucleotides 5′-TG-CTCGAGTGTGTTCTTCAGTTTTCTGTCA-3′ (upstream) and 5′-AACTCGAGCGCTTTGATTTCTCCGAAGCTT-3′ (downstream). By PCR mutagenesis a *Xho* I fragment containing the partial cDNA encoding tomato PDS (Arg^{208}–Thr^{424}; ref. 9) was placed under the control of the TMV-U1 coat protein subgenomic promoter by subcloning into TTO1, creating plasmid TTO1/PDS−. A unique *Avr* II site was inserted downstream of the *Xho* I site in TTO1 by PCR mutagenesis using oligonucleotides: 5′-TCCTCGAGCCTAGGCTCGCAAAGTTTCGA-ACCAAATCCTCA-3′ (upstream) and 5′-CGGGGTACCT-GGGCCCCAACCGGGGGTTCCGGGGG-3′ (downstream), to create plasmid TTO1A. The partial tomato PDS cDNA was modified by PCR mutagenesis and subcloned into TTO1A be-

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Abbreviations: TMV, tobacco mosaic virus; ToMV, tomato mosaic virus; PSY, phytoene synthase; PDS, phytoene desaturase; RT, reverse transcription.

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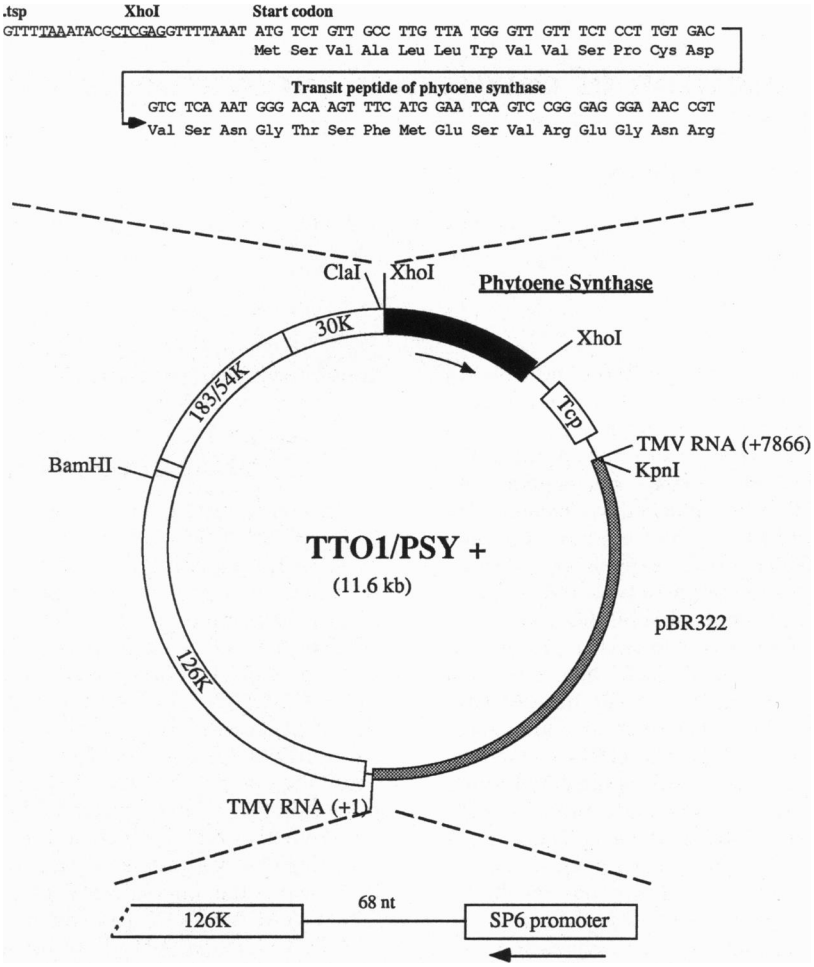

FIG. 1. PSY expression vector TTO1/PSY+. This plasmid contains the TMV-U1 open reading frames (ORFs) encoding 126-, 183-, and 30-kDa proteins (126K, 183/54K, and 30K); the ToMV coat protein gene (Tcp); the SP6 promoter, the tomato PSY gene, and part of the pBR322 plasmid. The TAA stop codon in the 30K ORF is underlined. The TMV-U1 subgenomic promoter located within the minus strand of the 30K ORF controls the expression of PSY. The putative transcription start point (tsp) of the subgenomic RNA is indicated with a period.

tween the *Xho* I and *Avr* II sites, creating plasmid TTO1A/PDS+.

***In Vitro* Transcriptions, Inoculations, and Analysis of Transfected Plants.** *N. benthamiana* plants were inoculated with *in vitro* transcripts as described (10). Virions isolated from *N. benthamiana* leaves infected with transcripts were stained with aqueous 2% uranyl acetate, and transmission electron micrographs were taken with a Zeiss CEM902 instrument. RNA was isolated from transfected plants and amplified by RT–PCR using conditions described previously (11) except that the annealing temperature was 60°C instead of 55°C. The oligonucleotides used in the PCRs were as follows: PSY, 5′-CAGCCTTAGATAGGTGGGAA-3′ (upstream) and 5′-GCCTTTCTCTGCCTCATCAA-3′ (downstream); TMV-U1, 5′-TAATCGATGATGATTCGGAGGCTAC-3′ (upstream primer for TTO1A/PDS+, TTO1/PDS−, and uninoculated *N. benthamiana* PDS control); PDS, 5′-GGCACTCAACTT-

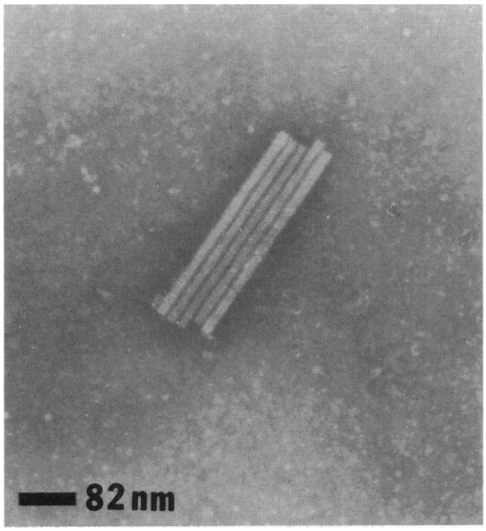

FIG. 2. Electron micrograph of virions from systemically infected leaves of *N. benthamiana* transfected with TTO1/PSY+ *in vitro* transcripts. (Bar ≈ 82 nm.)

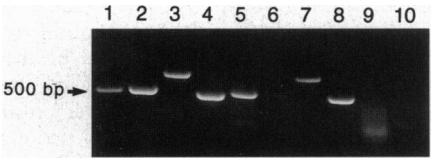

FIG. 3. Amplification of PSY and PDS DNA from plants transfected with *in vitro* transcripts. Lanes 1–4, RT–PCR amplification products from systemically infected leaf transfected with TTO1/PSY+, TTO1/PSY−, TTO1A/PDS+, or TTO1/PDS−, respectively; lanes 5–8, plasmid PCR controls TTO1/PSY+, TTO1/PSY−, TTO1A/PDS+, and TTO1/PDS− respectively; lane 9, uninoculated *N. benthamiana* amplified with PSY PCR primers; lane 10, uninoculated *N. benthamiana* amplified with PDS PCR primers.

FIG. 4. Transfected *N. benthamiana* leaves. Left to right: uninfected, TTO1/PSY+, TTO1/PSY−, TTO1/PDS+, TTO1A/PDS−.

TATAAACC-3′ (downstream primer for TTO1/PDS−), and 5′-CTTCAGTTTTCTGTCAAACC-3′ (downstream primer for TTO1A/PDS+, uninoculated *N. benthamiana* PDS control).

Amplification of Endogenous PDS mRNA. RNA was isolated from transfected plants and amplified by RT–PCR using conditions described previously (11) and the following oligonucleotides: TTO1/*L. esculentum* primers, 5′-TAATCGATGATGATTCGGAGGCTAC-3′ (upstream) and 5′-GGCACTCAACTTTATAAACC-3′ (downstream); *N. benthamiana* PDS primers: 5′-GGCACTCAACTTTATAAACC-3′ (upstream) and 5′-CTCCTTTAATTGTACTGCCA-3′ (downstream).

Isolation and Characterization of *N. benthamiana* PDS cDNA. A partial cDNA encoding PDS (GenBank accession no. U19262) was isolated from *N. benthamiana* leaf RNA by RT–PCR using the oligonucleotides 5′-GGCACTCAACTTTATAAACC-3′ (upstream) and 5′-CTTCAGTTTTCTGTCAAACC-3′ (downstream) and verified by dideoxynucleotide sequencing.

Analytical Procedures. Carotenoids were extracted in methanol and identified on a BioCAD workstation (PerSeptive Biosystems) by their peak retention time and absorption spectra on a 25-cm Spherisorb ODS-1 5-μm reverse-phase column using acetonitrile/methanol/2-propanol (85:10:5, vol/vol) as a developing solvent at a flow rate of 1 ml/min. Absorbance at 286 nm was measured with the BioCAD model 205 detector. A phytoene standard was synthesized according to the method of Davis *et al.* (12).

RESULTS AND DISCUSSION

To manipulate the carotenoid biosynthetic pathway, the open reading frame for PSY and a partial cDNA encoding PDS were placed under the control of the TMV-U1 coat protein subgenomic promoter in both the sense (+) and antisense (−) orientations. Infectious RNAs from TTO1/PSY+ (Fig. 1), TTO1/PSY−, TTO1A/PDS+, TTO1/PDS− were prepared by *in vitro* transcription using SP6 DNA-dependent RNA polymerase and used to mechanically inoculate *N. benthamiana* (10). The hybrid viruses spread throughout the noninoculated upper leaves as verified by transmission electron microscopy (Fig. 2), local lesion infectivity assay, and PCR amplification (Fig. 3). The viral symptoms consisted of the systemic distortion of leaves, plant stunting, and mild chlorosis.

FIG. 5. Transfected *N. benthamiana* plants. (*A*) *N. benthamiana* transfected with TTO1/PSY+. (*B*) *N. benthamiana* transfected with TTO1/PDS−. (*C*) *N. benthamiana* transfected with TTO1A/PDS+. (*D*) *N. benthamiana* flowers: left, uninfected; right, TTO1/PDS−. (*E*) *N. benthamiana* flowers transfected with TTO1/PDS−. (*F*) Uninfected *N. benthamiana* flowers.

Approximately 1 week after transfection, leaves from systemically infected TTO1/PSY+ plants developed a bright orange phenotype that can be a useful, visible marker in the analysis of viral replication and movement (Figs. 4 and 5*A*). By comparison, TTO1-infected plants showed necrotic symptoms similar to those of plants infected with wild-type TMV-U1 (data not shown) and, like plants infected with TTO1/PSY− (Fig. 4), failed to show the bright orange phenotype. Phytoene extracted from *N. benthamiana* transfected with TTO1/PSY+ was quantitated by reverse-phase HPLC, had a retention time similar to that of a phytoene standard, and showed a 10-fold increase over the levels in noninfected plants (Table 1). Because of their severe necrosis, TTO1-infected plants were not assayed. However, an analogous hybrid construct containing a comparable region from TMV-U5 (4), but which did not produce plant necrosis, showed levels of phytoene comparable to those in uninoculated plants.

The expression of antisense RNA to a partial phytoene desaturase (TTO1/PDS−) in transfected plants inhibited the synthesis of carotenoids downstream of phytoene and caused the systemically infected leaves and sepals to develop a white phenotype (Fig. 5 *B, D,* and *E*). This symptom was reproduced in control plants treated with the herbicide norflurazon, a noncompetitive inhibitor of PDS (13). Application of this compound caused a dramatic decrease in leaf carotenoids and chlorophylls and a subsequent accumulation of phytoene. The decrease in photoprotective carotenoids in the leaves may lead to a rapid destruction of chlorophyll by photooxidation. The chloroplasts from TTO1/PDS− transfected plants, when analyzed by transmission electron microscopy, appeared to be structurally normal (data not shown). HPLC analysis of plants transfected with TTO1/PDS− revealed that they accumulated high levels of phytoene that had a similar retention time to phytoene from norflurazon-treated plants (Fig. 6).

We cloned a portion of the *N. benthamiana* PDS gene to determine the degree of similarity to the tomato antisense PDS transcript. Nucleotide sequence comparison of 369 bp in the corresponding regions between *N. benthamiana* and tomato PDS (9) indicates that they have 92% identity (Fig. 7). Since the two plant genes have large areas of high homology, cytoplasmic inhibition of the endogenous plant gene by virus-derived antisense RNA could occur through the formation of hybrid, double-stranded RNA molecules which are rapidly degraded. The synthesis of virus-derived antisense PDS RNA in transfected plants resulted in a decrease in endogenous PDS mRNA. The *N. benthamiana* PDS mRNA could not be detected in transfected plants by RT–PCR (Fig. 8, lanes 2 and 4), whereas an expected 490-bp product could be amplified from the virus-derived tomato antisense PDS RNA (Fig. 8, lanes 3 and 4). The PDS mRNA was detected only in the control, noninfected plants (Fig. 8, lane 1).

It was interesting that leaves from systemically infected TTO1A/PDS+ plants (Fig. 5*C*) also accumulated phytoene and developed a bleaching white phenotype. This symptom appeared ≈1 week later than in leaves from the antisense TTO1/PDS− plants. This downregulation may have been caused by direct interference during the translation of mRNA into protein or by duplexes formed between mRNA and virus-derived, negative-strand RNA. There is evidence that inhibition of endogenous genes can occur in transgenic plants containing sense RNA (14–16). Although it has been proposed that this downregulation or "co-suppression" might be caused by the production of antisense RNA by readthrough transcription from distal promoters located on the opposite strand of the chromosomal DNA (17), the actual mechanism is not known.

Table 1. Quantitation of phytoene in leaves of *N. benthamiana* transfected with viral transcripts

Plant	Phytoene, μg (fresh weight)	Fold increase
Untransfected	4.6	1
Transfected with TTO1/PDS−	234.8	51.0
Treated with norflurazon	339.8	73.9
Transfected with TTO1/PSY+	52.4	11.4
Transfected with TTO1/PSY−	1.0	0.2

The phytoene was separated on a Spherisorb ODS-1 column and quantified on a BioCAD workstation (PerSeptive Biosystems). The carotenoid was identified by its spectral properties and similar retention time to an authentic phytoene standard (12).

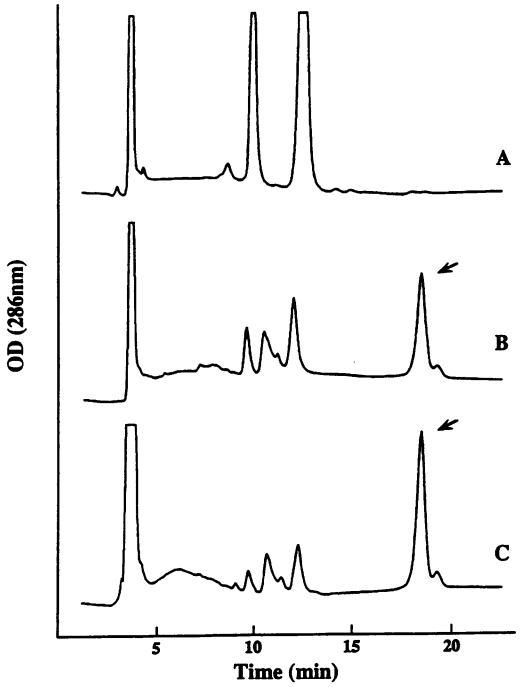

FIG. 6. HPLC separation of carotenoids accumulating in uninfected *N. benthamiana* (trace A), *N. benthamiana* transfected with TTO1/PDS− (trace B), and norflurazon-treated *N. benthamiana* (trace C). Absorbance was measured at 286 nm. Arrows indicate the phytoene peak identified by cross reference to a synthetic phytoene standard (12).

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PDS1-Nb  CTGACGAGCTTTCGATGCAGTGCATCTTGATTGCTTTGAACAGATTTCTT  50
         ||||||| ||||| |||||||||||| ||||| || |||||||| ||||||
PDS-Le   CTGACGAACTTTCAATGCAGTGCATTTTGATCGCATTGAACAGGTTTCTT  50

PDS1-Nb  CAGGAGAAACATGGTTCAAAAATGGCCTTTTTAGATGGTAACCCTCCTGA 100
         ||||||||||||||||||||||||||||||||||||||||| ||||||||
PDS-Le   CAGGAGAAACATGGTTCAAAAATGGCCTTTTTAGATGGTAATCCTCCTGA 100

PDS1-Nb  GAGACTTTGCATGCCGATTGTGGAACATATTGAGTCAAAAGGTGGCCAAG 150
         |||||||||||||||||||| ||||| ||||||||||||||||||||||
PDS-Le   GAGACTTTGCATGCCGATTGTTGAACACATTGAGTCAAAAGGTGGCCAAG 150

PDS1-Nb  TCAGACTAAACTCACGAATAAAAAAGATCGAGCTGAATGAGGATGGAAGT 200
         ||||||| |||||||||||||||||| ||||||||||||||||||||||
PDS-Le   TCAGACTGAACTCACGAATAAAAAAGATTGAGCTGAATGAGGATGGAAGT 200

PDS1-Nb  GTCAAATGTTTTATACAGAATAATGGCAGTACAATTAAAGGAGATGCTTT 250
         ||||| ||||| ||| || | | || ||| |||| | ||||||||||||
PDS-Le   GTCAAGAGTTTTATACTGAGTGACGGTAGTGCAATCGAGGGAGATGCTTT 250

PDS1-Nb  TGTGTTTGCCACTCCAGTGGATATCTTGAAGCTTCTTTTGCCTGAAGACT 300
         ||||||||| |||||||||||| || |||||||| |||||||||||||||
PDS-Le   TGTGTTTGCCGCTCCAGTGGATATTTTCAAGCTTCTATTGCCTGAAGACT 300

PDS1-Nb  GGAAAGAGATCCCATATTTCCAAAAGTTGGAGAAGCTAGTGGGAGTTCCT 350
         ||||||||| |||||||||||||||||||||||| |||| ||||| |||
PDS-Le   GGAAAGAGATTCCATATTTCCAAAAGTTGGAGAAGTTAGTCGGAGTACCT 350

PDS1-Nb  GTGATAAATGTCCATATAT                                369
         ||||||||||| |||||||
PDS-Le   GTGATAAATGTACATATAT                                369
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FIG. 7. Nucleotide sequence comparison of *N. benthamiana* leaf PDS (PDS1-Nb) and tomato PDS (PDS-Le). The nucleotides are aligned to maximize sequence similarity. The tomato PDS sequence encodes amino acids Asp^{294}–Ile^{415} (9).

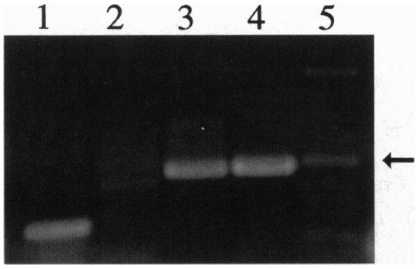

FIG. 8. Expression of PDS from plants transfected with *in vitro* transcripts of TTO1/PDS−. Lane 1, RT–PCR amplification products from uninoculated *N. benthamiana*; lanes 2–4, RT–PCR products from systemically infected plant transfected with TTO1/PDS−, amplified with *N. benthamiana* PDS primers (lane 2), *L. esculentum* PDS primers (lane 3), or both *N. benthamiana* and *L. esculentum* PDS primers (lane 4); lane 5, 1-kb ladder used as molecular size marker (BRL). Arrow indicates the 452-bp TTO1/*L. esculentum* PDS RT–PCR product.

To our knowledge, the dramatic increase in levels of phytoene in transfected plants is the first evidence that a eukaryotic biosynthetic pathway can be deliberately manipulated by a cytoplasmic, autonomously replicating, RNA viral vector. The inhibition of the expression of a specific enzyme (such as PDS) by viral RNA will be helpful in elucidating biochemical pathways and may reveal the cellular location and mechanism behind antisense regulation. In addition, our results suggest that other vectors, such as the sindbis virus (18), could be developed for cytoplasmic inhibition of gene expression in mammalian cells.

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