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A *TOM1* homologue is required for multiplication of Tobacco mosaic virus in *Nicotiana benthamiana*[*]

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Abstract: The *AtTOM1* gene of *Arabidopsis thaliana* had been shown to be essential for the efficient multiplication of Tobacco mosaic virus (TMV) in *A. thaliana*. In this study, we cloned an *AtTOM1*-like gene from *Nicotiana benthamiana* named as *NbTOM1*. Sequence alignment showed that *NbTOM1* is closely related to *AtTOM1* homologues of *N. tabacum* and *Lycopersicon esculentum* with 97.2% and 92.6% nucleotide sequence identities, respectively. Silencing of *NbTOM1* by a modified viral satellite DNA-based vector resulted in complete inhibition of the multiplication of TMV in *N. benthamiana*. The result suggests that inhibition of *NbTOM1* via RNA silencing is a potentially useful method for generating TMV-resistant plants.

Key words: Tobacco mosaic virus, *NbTOM1*, Virus induced gene silencing, *Nicotiana benthamiana*
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INTRODUCTION

Viral diseases have caused significant yield and quality losses in crop production. Over the past years, transgenic approaches have successfully generated virus-resistant plants (Goldbach *et al*., 2003). An effective method for obtaining resistant transgenic plants is to induce RNA silencing by expressing virus-derived dsRNA in plants (Zhang *et al*., 2005).

A variety of host factors were identified to be involved in the intracellular multiplication of viruses (Lai, 1998; Lee *et al*., 2001; Noueiry *et al*., 2000). Previous studies showed that *AtTOM1* and *AtTOM3* of *Arabidopsis thaliana*, which encode a putative seven-pass transmembrane protein, are essential for the efficient multiplication of a crucifer-infecting tobamovirus (TMV-Cg) (Hagiwara *et al*., 2003; Yamanaka *et al*., 2000; 2002). Two *AtTOM1* homologues, *NtTOM1* and *NtTOM3*, were identified in *Nicotiana tabacum*. Simultaneous RNA interference against *NtTOM1* and *NtTOM3* in *N. tabacum* successfully inhibited the multiplication of Tomato mosaic virus and other tobamoviruses (Asano *et al*. 2005). *AtTOM1* and *AtTOM3* homologues are present in various plant species and their inhibition via RNA interference should constitute a useful method for generating tobamovirus-resistant plants (Asano *et al*., 2005). In this study, we identified an *AtTOM1*-like gene in *N. benthamiana*, named as *NbTOM1* and reported that silencing of *NbTOM1* by a modified viral satellite DNA-based vector results in complete inhibition of Tobacco mosaic virus (TMV) multiplication in *N. benthamiana*.

MATERIALS AND METHODS

Isolation of *TOM1* homologue and construction of gene silencing vector

To identify *TOM1* homologue in *N. benthamiana*, total RNA from *N. benthamiana* leaves was prepared using Trizol reagent (Invitrogen, Carlsbad,

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CA, USA), and applied as a template for first-strand cDNA synthesis using oligo $d(T)_{18}$ and superscript reverse transcriptase (TaKaRa, Shiga, Japan). The *TOM1* homologue fragment was PCR-amplified with the degenerated primers TOM1F (5′ CTTTGTYG TVAAYGGAGTTC 3′) and TOM1R (5′ GTAYTGW GCWGAYACTC 3′) (V=A, C, G; W=A, T; Y=C, T) using the synthesized first-strand cDNA as template. The resulting PCR product was cloned into pGEM-Teasy vector (Promega, Madison, WI, USA) and then sequenced. Based on the fragment sequence, primers TOM1F1 (5′ TCTAGACTATTGTCTTTGG ATTTCACA 3′, restriction site for *Bam*HI is underlined) and TOM1R1 (5′ GGATCCAGGCAATTTT CGCAGGATG 3′, restriction site for *Xba*I is underlined) were designed. The resulting PCR product was digested with *Bam*HI-*Xba*I and inserted into the *Bam*HI-*Xba*I-cut DNAβ vector of Tomato yellow leaf curl China virus (TYLCCNV) (DNAmβ) (Tao and Zhou, 2004) to produce construct DNAmβ:NbTOM1. The infectious clone of DNAmβ and TYLCCNV was constructed previously by the authors' lab (Cui *et al*., 2004; Tao and Zhou, 2004).

Agroinoculation of plants

DNAmβ:NbTOM1 were introduced into *Agrobacterium tumefaciens* strain PGV3850 by electropolation. *A. tumefaciens* cultures containing infectious clone of TYLCCNV and DNAmβ:NbTOM1 were injected into stems of *N. benthamiana* seedlings at six-leaf stage as previously described (Zhou *et al*., 2003). Plants co-inoculated with TYLCCNV and DNAmβ empty vector or water were used as control. Inoculated plants were grown in an insect-free cabinet at a constant temperature of 25 °C with supplementary lighting for 16 h per day.

Virus inoculation

TMV:GFP (green fluorescent protein) carrying the *mGFP4* gene was kindly provided by the Sainsbury Laboratory of John Innes Centre. The virus was agro-infiltrated into the sixth true leaf.

GFP image

GFP was detected visually in intact plants with the aid of a hand-held 100 W long-wave ultraviolet lamp (UV products, Upland, CA, USA). Plants were photographed with a Nikon 5000 digital camera and the images were processed using Adobe Photoshop software.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described by Tao and Zhou (2004). cDNA derived from the equivalent of 1 μg of total RNA was used as a template. Primers (TOM1F, TOM1R) that anneal outside the region targeted for silencing were used to ensure that only the endogenous gene transcript was assayed. The elongation factor 1-alpha (*EF-1-α*) served as an internal control for RNA quantity in RT-PCR. The intensities of PCR-generated fragments were analyzed and quantified using Gel-Pro Analyzer 3.1 (Media Cybernetics, Atlanta, GA, USA).

RNA gel blot analysis

Total RNA isolation using systemic leaves and Northern blot analysis were conducted as described previously (Cui *et al*., 2004). Membranes were hybridized with [α-^{32}P]dCTP-labelled probe specific for the TMV movement protein gene. Hybridization signals were detected by phosphorimaging using a Typhoon 9200 imager (Amersham Pharmacia, Piscataway, NJ, USA).

RESULTS AND DISCUSSION

AtTOM1 homologues were found in *Lycopersicon esculentum* (AB193043) *Oryza sativa* (AK066373) and *N. tabacum* (AB193039). Based on the alignment of nucleotide sequences of these homologues, we designed degenerate primers TOM1F and TOM1R. A 0.6 kb cDNA fragment was PCR-amplified with these primers from *N. benthamiana*. Sequence analysis and comparison revealed that the fragment (AM261863, named as *NbTOM1* gene) was closely related to *AtTOM1* homologues of *N. tabacum* and *L. esculentum* with 97.2% and 92.6% nucleotide sequence identities, respectively (Table 1).

To investigate whether *NbTOM1* supports TMV multiplication, we tried to inhibit the expression of *NbTOM1* by virus induced gene silencing (VIGS). For this purpose, the *NbTOM1* fragment were introduced into the TYLCCNV DNAβ-based gene silencing vector (Tao and Zhou, 2004) to generate DNAmβ:NbTOM1. *N. benthamiana* plants were then

Table 1 Nucleotide (bottom left) and amino acid (top right) sequence identities of NbTOM1 with AtTOM1 and TOM1 homologues of *Lycopersicon esculentum* (LeTOM1), *Oryza sativa* (OsTPM1) and *Nicotiana tabacum* (NtTOM1)

	NbTOM1	NtTOM1	LeTOM1	AtTOM1	OsTPM1
NbTOM1	−	97.4	94.3	81.3	74.1
NtTOM1	97.2	−	93.8	77.4	71.9
LeTOM1	92.6	80.3	−	76.0	71.2
AtTOM1	69.5	59.0	52.5	−	71.8
OsTPM1	63.9	80.3	47.2	49.9	−

co-agroinoculated with TYLCCNV and DNAmβ: NbTOM1, TYLCCNV and DNAmβ empty vector or water. Approximately 15 d after co-agroinoculation, plants were challenge-inoculated with the TMV:GFP. Within 3 weeks after challenge-inoculation, all the six tested plants that were co-agroinoculated with TYLCCNV and DNAmβ:NbTOM1 yielded a red signal under UV (Fig.1a), due to non-proliferation of the TMV:GFP clone. In contrast, the control plants yielded a green signal due to the GFP expression accompanying TMV multiplication (Figs.1b and 1c). When *NbTOM1*-silenced *N. benthamiana* plants were challenge-inoculated with Cucumber mosaic virus, the inoculated plant produced severe viral symptoms similar to those in non-silenced control plants (data not shown). These results suggest that the multiplication of TMV is inhibited in the *NbTOM1* silenced plants.

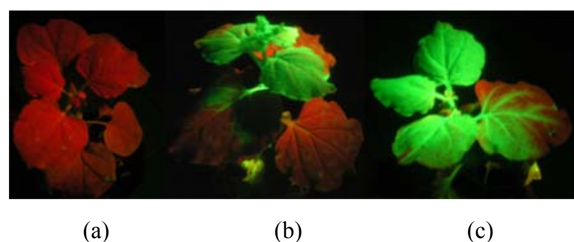

Fig.1 Effects of the knockdown of *NbTOM1* expression on TMV accumulation in *N. benthamiana*. Plants were pre-inoculated with TYLCCNV and DNAmβ: NbTOM1 (a), TYLCCNV and DNAmβ empty vector (b) and water (c), respectively, and then challenge inoculated with the TMV:GFP clone 15 d after pre-inoculation. The *NbTOM1* silenced plant appear red due to non-proliferation of TMV:GFP (a). The *NbTOM1* non-silenced control plant appear green due to GFP expression resulting from TMV:GFP accumulation (b, c). Photographs were taken under UV light 3 weeks after challenge inoculation

To assay the mRNA levels from the endogenous *NbTOM1* gene, we performed semi-quantitative RT-PCR. The results obtained revealed that the level of *NbTOM1* mRNA was reduced by more than 80% in the systemic *NbTOM1* silenced *N. benthamiana* plants compared with the control plants. In contrast, the level of mRNA of *EF-1-α*, which served as an internal control for RNA quantity in RT-PCR amplification, was similar in *NbTOM1*-silenced and the control plants (Fig.2). These results suggest that *NbTOM1* mRNA is a target of VIGS.

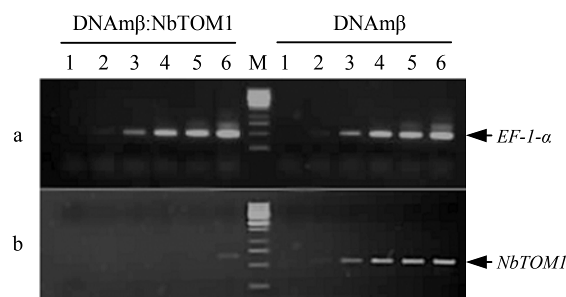

Fig.2 Semi-quantitative RT-PCR analysis showing the effect of VIGS with DNAmβ:NbTOM1 in *N. benthamiana*. Lanes 1~6 correspond to products from PCR cycle Nos. 15, 18, 21, 24, 27 and 30. Lane M represents size markers. (a) PCR products for *EF-1-α* derived from *N. benthamiana* co-agroinfected with TYLCCNV and DNAmβ:NbTOM1 (left) or with TYLCCNV and DNAmβ empty vector (right). Gene *EF-1-α* served as an internal control for RNA quantity in RT-PCR; (b) PCR products of *NbTOM1* from *N. benthamiana* co-agroinoculated with TYLCCNV and DNAmβ: NbTOM1 (left) or with TYLCCNV and DNAmβ empty vector (right)

To further monitor the level of TMV multiplication, total RNA was isolated from leaves and analyzed by Northern blot hybridization using a probe specific for TMV. Hybridization results revealed a complete inhibition of TMV multiplication in *NbTOM1* silenced plants, correlating well with the results from the corresponding GFP expression assays (Fig.3).

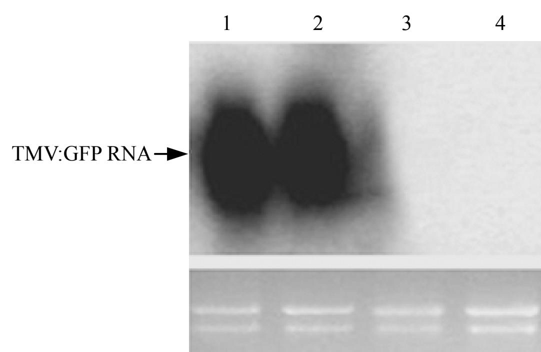

Fig.3 Northern blot analysis of TMV accumulation in *NbTOM1* silenced and non-silenced *N. benthamiana* plants at 18 d post-inoculation with TMV:GFP
Lanes 1 and 2 show viral RNA from *NbTOM1*-non-silenced plants; Lanes 3 and 4 show viral RNA from individual *NbTOM1*-silenced plants. Lower panels show RNAs stained with ethidium bromide

The discovery of host factors, including proteins, membranes, and nucleic acids that are implicated in viral infection cycles is providing fundamental clues about the molecular bases of viral susceptibility. As a host factor, *AtTOM1*, which is associated with the TMV-Cg replication complex, supports efficient TMV-Cg multiplication in *A. thaliana* (Yamanaka *et al*., 2000). In this report, we characterized an *AtTOM1*-like gene from *N. benthamiana* (*NbTOM1*), and demonstrated that *NbTOM1* plays an essential role in TMV multiplication. Our results are consistent with previous reports.

We have demonstrated that the VIGS-induced knockdown of *NbTOM1* confers strong TMV resistance in *N. benthamiana*. As *AtTOM1* homologues have been reported in several kinds of plants (Asano *et al*., 2005), similar strategies might be applicable to a wide range of economically important plants. Compared with conventional transgenic approaches, VIGS offers advantages for generating virus-resistant plants because it does not rely on stable plant transformants.

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