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Expression, purification, and functional characterization of an N-terminal fragment of the *tomato mosaic virus* resistance protein Tm-1

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ABSTRACT

Tm-1, the protein product of *Tm-1*, a semidominant resistance gene of tomato, inhibits tomato mosaic virus (ToMV) replication by binding to ToMV replication proteins. Previous studies suggested the importance of the Tm-1 N-terminal region for its inhibitory activity; however, it has not been determined if the N-terminal region is sufficient for inhibition. Furthermore, the three-dimensional structure of Tm-1 has not been determined. In this study, an N-terminal fragment of Tm-1 (residues 1–431) as a fusion protein containing an upstream maltose-binding protein was expressed in *E. coli* Rosetta (DE3) cells at 30 °C and then purified. The solubility of the fusion protein was greater when the cells were cultured at 30 °C than when cultured at lower or higher temperatures. The purified N-terminal Tm-1 fragment from which the maltose-binding protein tag had been removed has inhibitory activity against ToMV RNA replication.

Introduction

ToMV[2] is a positive-strand RNA virus, and its genome encodes four proteins of ca. 130 kDa (130 K), 180 kDa (180 K), 30 kDa (movement protein), and 17 kDa (coat protein) [1]. The movement and coat proteins are dispensable for viral RNA replication. Conversely, 130 and 180 K (a read-through product of 130 K) are necessary for ToMV RNA replication and, thus, are referred to as replication proteins [2,3]. After ToMV RNA enters a host cell, the replication proteins are translated from the ToMV genomic RNA. The replication proteins then form a replication complex with the viral genomic RNA and several host factors on host intracellular membranes to replicate viral RNA via negative-strand RNA [4].

Tm-1 is a semidominant resistance gene that inhibits ToMV multiplication [5,6]. *Tm-1* was originally found in the wild tomato species *Solanum habrochaites* S. Knapp & D.M. Spooner, was bred into the cultivated tomato species *S. lycopersicum* L. [7,8], and has since been widely introduced into various tomato cultivars to protect them from ToMV infection. Tm-1 was identified through purification of an *in vitro* inhibitory activity of ToMV RNA replication in a cell extract of a tomato [9]. It binds ToMV replication proteins and prevents formation of the replication complex. Tm-1 consists of 754 amino acids and contains at least two domains: a structurally uncharacterized N-terminal region (residues 1–431) and a TIM-barrel-like C-terminal domain (residues 484–754) (Fig. 1) [9]. The ToMV mutant LT1 encodes replication proteins with amino acid substitutions in their helicase domains that decrease their binding affinities to Tm-1 and, consequently, help resist the effects of Tm-1 [9–11]. As homologs of *Tm-1* are found in other plants, fungi, archaea, and bacteria, *Tm-1* most likely has a primary function other than tobomovirus resistance. However, such function(s) and a three-dimensional structure(s) for Tm-1 or its homologs have yet to be reported.

A truncated form of Tm-1 encoded by a splicing variant that is missing residues 46–263 does not inhibit ToMV RNA replication [9]. A small region of *S. habrochaites Tm-1* (encoding residues 79–112) is positively selected during antagonistic coevolution with ToMV [12]. An Ile → Thr mutation at residue 91 strengthens the inhibitory activity and extends the anti-viral spectrum of Tm-1 [12]. The aforementioned observations suggest that the Tm-1 N-terminal region is important for the inhibitory activity. However, it is not known if the N-terminal region, by itself, is sufficient for inhibition of ToMV replication.

To fully characterize the function of the N-terminal region of Tm-1, both structural and biochemical studies must be performed. For such studies, a purified, stable, and active fragment is necessary. In this study, we expressed, using recombinant DNA techniques, and purified a stable N-terminal fragment of Tm-1 (residues 1–431: referred to herein as Tm-1(431)) and assessed its ability to inhibit ToMV RNA replication.

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[2] Abbreviations used: ToMV, tomato mosaic virus; MBP, maltose-binding protein; DTT, dithiothreitol; DLS, dynamic-light-scattering; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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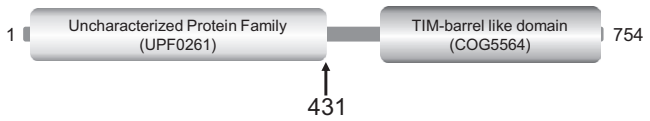

Fig. 1. A schematic of the domain structure of Tm-1 predicted by the NCBI conserved domain database. The position of residue 431 is indicated by the arrow.

Materials and methods

Plasmid constructs

The cDNA encoding Tm-1(431) (Gene ID: 100136895) was PCR-amplified using KOD-Plus polymerase (TOYOBO) and the primers, 5′-CACCATCGAAGGTAGGATGGCAACTGCACAGAGT-3′ (the sequence encoding the Factor Xa-recognition site is underlined) and 5′-TTATTTCGGAGAGATTTCCAAGAATG-3′. The PCR product was purified by electrophoresis through a 1.0% (w/v) agarose gel, isolated using Wizard SV Gel and PCR Clean Up System reagents (Promega, Madison, WI), and inserted into a pENTR/D-TOPO vector to create pENTR-Tm-1(431) using pENTR/D-TOPO cloning kit reagents (Invitrogen, Carlsbad, CA). The Tm-1(431) sequence was confirmed by DNA sequencing. Starting with pENTR-Tm-1(431) and using LR Reaction kit reagents (Invitrogen, Carlsbad, CA), Tm-1(431) cDNA was transferred into a pDEST-mal expression vector [13] to create pDEST-mal-Tm-1(431), which contains a fused maltose-binding protein (MBP) sequence immediately upstream of the Tm-1(431) sequence.

Expression of Tm-1(431)

Escherichia coli Rosetta (DE3) cells were transformed with pDEST-mal-Tm-1(431) and grown in Luria-Broth medium, 50 μg/ml ampicillin, 34 μg/ml chloramphenicol to an A_{600} of ~0.5. After the culture had been held in ice water for at least 15 min, expression was induced by addition of isopropyl-1-thio-β-galctopyranoside (final concentration, 10 μM). Cells were cultured for an additional 16 h at 30 °C unless indicated otherwise and then centrifuged at 5,000*g* for 15 min at 4 °C. The cell pellet was suspended in sonication buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2 mM 2-mercaptoethanol), disrupted by sonication, and centrifuged at 27,000*g* for 30 min at 4 °C.

Purification of Tm-1(431)

The clarified lysate was subjected to MBPTrap HP chromatography (GE Healthcare Bio-Sciences, Piscataway, NJ), and the bound proteins were eluted in sonication buffer containing 20 mM maltose. Next, the sample was chromatographed through HiLoad 26/60 Superdex 200 pg (GE Healthcare Bio-Sciences, Piscataway, NJ) in 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2 mM 2-mercaptoethanol at 4 °C. After pooling the fractions containing the fusion protein (MBP-Tm-1(431)), it was concentrated by MBPTrap HP chromatography and then treated with Factor Xa (Merck Millipore, Billerica, MA) for 16 h at 20 °C. Tm-1(431) was separated from the MBP tag and intact fusion protein by HiLoad 26/60 Superdex 75 pg chromatography (GE Healthcare Bio-Sciences, Piscataway, NJ) with 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol (DTT) as the eluent. Tm-1(431)-containing fractions were pooled and chromatographed through MBPTrap HP to remove any remaining MBP. The flow-through fraction was subjected to HiLoad Q HP chromatography (GE Healthcare Bio-Sciences, Piscataway, NJ), and Tm-1(431) was eluted using a 150–500 mM NaCl linear gradient in 20 mM Tris–HCl, pH 8.0, 1 mM DTT at 4 °C.

Dynamic-light-scattering (DLS) measurements

DLS intensities were measured with a Zetasizer Nano ZS instrument (Malvern Instruments, UK) at 15 °C [14]. Data acquisition and manipulation were performed using Zetasizer Nano software and an alpha-value of 0.01 that was a function of the fitting sensitivity of the raw data. The data are reported as the mean value of five replicates. The polydispersity (*Pd*) value was calculated using Eq. (1), where *SD* is the standard deviation of the distribution and $D(H)$ is the experimental mean hydrodynamic diameter obtained from the DLS measurements.

$$Pd(\%) = \frac{\mathrm{SD}}{D(H)} \times 100 \quad (1)$$

Inhibition of in vitro ToMV RNA replication by Tm-1(431)

In vitro replication of ToMV RNA was performed essentially as described [9,15]. Briefly, wild-type (WT) ToMV and LT1 RNA (800 ng each) were individually translated in 100 μl of a membrane-depleted, evacuolated BY-2 protoplast lysate-based translation mixture at 23 °C for 1 h. Portions of the translation mixture (14 μl) were mixed with 1 μl of 50, 200, or 1000 ng purified Tm-1(431) or 1 μl buffer, then with a membrane preparation (5 μl), and incubated at 15 °C for 1 h. Next, a substrate mixture for RNA replication containing [α-^{32}P]CTP was added to each sample, and the mixtures were then incubated at 23 °C for 1 h. ^{32}P-labeled RNA was purified by phenol extraction and ethanol precipitation, and subjected to gel electrophoresis (2.4% (w/v) acrylamide and 8 M urea). Gel RNA bands were detected by autoradiography.

Results

Optimization of MBP-Tm-1(431) expression conditions

The soluble and insoluble lysate fractions, and the whole lysate, before and after induction, were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the proteins were visualized with Coomassie Brilliant Blue staining (Fig. 2A). Substantial amounts of MBP-Tm-1(431) were found in the soluble and insoluble fractions of the lysate, with ~30% of the protein found in the soluble fraction when the cells were cultured at 10 °C (Fig. 2A). MBP was, therefore, a useful carrier for expression of soluble Tm-1(431). Even though MBP-Tm-1(431) was found in the soluble fraction, it was not immediately obvious if it was correctly folded. In general, low-temperature cultivation of *E. coli* often promotes the correct folding of a protein [16] by preventing inclusion-body formation [17–19]. Culture at low-temperature seems especially useful for proteins expressed in large amounts [20]. Therefore, as noted above, we first expressed MBP-Tm-1(431) at 10 °C. Although MBP-Tm-1(431) was soluble after chromatography through Superdex 200 pg, most of it eluted in the void volume (Fig. 2B, top panel), which suggested that it had aggregated. A small peak of MBP-Tm-1(431), as assessed by SDS–PAGE and western blotting (data not shown), eluted a little earlier than expected (theoretical molecular mass, ~100 kDa). We then tested the effect of the cultivation temperature. When MBP-Tm-1(431) was expressed at higher temperatures, i.e., 15, 23, 30, and 37 °C, its yield in the chromatographically retained fraction increased as the temperature increased until the temperature was somewhat higher than 30 °C (Fig. 2B). Induction at 30 °C for 16 h was, therefore, the best expression condition found.

Purification and characterization of Tm-1(431)

The purification protocol for Tm-1(431) is shown in Fig. 3A. The cell lysate containing MBP-Tm-1(431) was first subjected to maltose affinity (MBPTrap) chromatography (eluent: 20 mM