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Quantitative estimation of the effects of propionylshikonin on the binding of TMV RNA and tobacco mRNA to wheat germ ribosome *in vitro*

Shan-Shan Chen [1], Jian Lin [1], Qi-Bin Chen, Yun-Fei Liu, Lei Ma, Chong-Jiu Li, Xiao-Dong Ma *

Department of Applied Chemistry, College of Science, China Agricultural University, Beijing 100193, China

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ABSTRACT

A method involving reverse transcription and real-time polymerase chain reaction (PCR) was developed in this study to detect the effects of the antiviral compound propionylshikonin on the binding of tobacco mosaic virus (TMV) RNA and tobacco mRNA to wheat germ ribosome *in vitro*. TMV RNA–wheat germ ribosome and tobacco mRNA–wheat germ ribosome binding systems were constructed, and the TMV RNA–ribosome and tobacco mRNA–ribosome complexes were isolated from the binding systems using 30% sucrose cushion. The target genes for the quantitative detection of TMV RNA and tobacco mRNA were the TMV coat protein gene and tobacco elongation factor-1α gene, respectively. The designed protocol was efficient for rapid and conclusive determination of the variations in the bound TMV RNA and tobacco mRNA from the complexes with and without propionylshikonin. The inhibition rates, ranging from 26.4% to 63.6%, were detected in the bound TMV RNA with 2–10 μg/mL propionylshikonin in the binding systems. The amount of bound tobacco mRNA did not decrease in the presence of propionylshikonin, indicating that propionylshikonin did not inhibit the binding of tobacco mRNA to wheat germ ribosome. To the best of our knowledge, this is the first study on the interactions among an anti-TMV agent, TMV RNA, and a host using real-time PCR to be reported.

1. Introduction

Tobacco mosaic virus (TMV) is one of the most well studied plant viruses, but it continues to be a major threat in the production of cash crops (e.g., tobacco) and TMV control remains to be a challenge [1]. Since Duggar and Armstrong found in 1925 that pokeweed juice (*Phytolacca acinosa*) could inhibit TMV, numerous other natural and synthetic compounds exhibiting anti-TMV bioactivity have been reported [2–13]. In spite of extensive research efforts [14], only a few chemical agents that can fully protect hosts from TMV infection have been identified. One of the challenges in finding a suitable antiviral agent is the difficulty in inhibiting viral multiplication without causing injury to the host plant, because viruses do not code for proteins but interact with host cells and use their machinery for replication. Yanmei Li et al. [15] found that plants have evolved to produce secondary metabolites with antimicrobial activities to suppress pathogens selectively. Therefore, the selection of natural products with anti-TMV activity is becoming increasingly significant and will potentially play a key role in the chemical control of this virus.

Our previous study on the naturally occurring antiviral activities of several naphthoquinones, which were isolated from the roots of *Lithospermum erythrorhizon* [16], focused on shikonin derivatives. This series of compounds exhibits a wide range of biological activities, including inhibitory activities against human acetyl-coenzyme A acetyltransferase-1 gene and antimicrobial activity [17–22]. In the present work, we found that propionylshikonin (0.02 mM) inhibited TMV infection on *Nicotiana tabacum* L. cv. Xanti nc. at a rate of 67.9 ± 4.5% and that the half-maximal effective concentration (EC_{50}) for its replication inhibition was 0.051 mM (17.6 μg/mL), showing that propionylshikonin is more effective than the commercial antiviral agent 2,4-dioxohexahydro-1,3,5-triazine (Sigma–Aldrich; infection inhibition rate, 56.5 ± 5.9% at 0.02 mM; EC_{50}, 0.322 mM (37.1 μg/mL)) [These contents are stated in Supplementary data].

Previous molecular virology research has characterized the cycle of TMV infection. When TMV infects a plant, TMV particles remove their coat protein (CP) and their RNA binds to the host ribosome and begins to encode at least four polypeptides [23]. Therefore, if an agent can interfere with the binding of TMV RNA to the host ribosome, TMV replication in the host cells in the first phase of protein translation will be directly affected. Current anti-TMV agents only consider actual infection or replication activities and ignore their effects on the host itself [2–13]. The present study hence investigated propionylshikonin's basic mode of action

* Corresponding author. Fax: +86 10 62733084.
E-mail address: dongxm@cau.edu.cn (X.-D. Ma).
[1] Both authors contributed equally to this paper.

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on TMV by constructing TMV RNA–wheat germ ribosome and tobacco mRNA–wheat germ ribosome binding systems *in vitro* and developing a real-time fluorescent quantitative polymerase chain reaction (qPCR) method to detect variations in the bound RNA with and without the antiviral compound. Wheat germ ribosome was selected in this study as it can maximally simulate the tobacco ribosome [24,25]. Propionylshikonin exhibited inhibitory activity against the binding of TMV RNA to wheat germ ribosome, but it did not affect the binding of tobacco mRNA to wheat germ ribosome (Fig. 1).

2. Materials and methods

2.1. Preparation of materials

2.1.1. RNA extraction and determination of purity and concentration

N. tabacum L. cv. was cultivated at a greenhouse in China Agricultural University. The common strain of TMV (U1) was propagated and maintained in this systemic host as well as purified using the method developed by Gooding [26]. TMV RNA was extracted from the purified virus rods using the phenol–chloroform method as described by Fraenkel-Conrat et al. [27]. Tobacco leaf total RNA was extracted from healthy leaves with TRIzol Total RNA Reagent (Tiangen Biotech, Co., Ltd., Beijing, PR China). The concentrations and purities of RNA were determined using a micro-UV spectrophotometer (Nano-Drop® ND-100 Spectrophotometer). The purities were judged by the value of the A_{260}/A_{280} ratio and further examined using 1.0% denatured agarose gel electrophoresis.

2.1.2. Wheat germ ribosome

The commercial Wheat Germ Extract Kit (Promega Corporation, USA) was used to construct both TMV RNA–wheat germ ribosome and tobacco mRNA (part of tobacco leaf total RNA)–wheat germ ribosome binding systems *in vitro* [24,25]. The wheat germ extract contains the cellular components necessary for protein synthesis (tRNA, ribosomes, initiation factor, and elongation factor). The extract was treated with micrococcal nuclease to destroy endogenous mRNA and thus minimize background translation; this step maximally simulates binding in host cells.

2.1.3. Propionylshikonin

Propionylshikonin was synthesized according to the studies of Sojin An [22] and Byung-Zun Ahn [28], whose mass spectroscopy (MS) as well as ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data were the same as previously reported spectra [29].

2.2. Construction of TMV RNA–wheat germ ribosome and tobacco mRNA–wheat germ ribosome binding systems

The *in vitro* binding systems were established based on the instructions in the Promega Wheat Germ Extract Kit as well as on the methods of Kairat Madin and Seiichi Yokoe [24,25]. Actidione (a protein translation inhibitor targeting peptide transferase) was added to the cell-free protein translation system; once ribosome and RNA formed a complex in the 5′ end, actidione deterred further protein translation, keeping it in the recognition or binding phase, rather than in the elongation phase. In total, 10 μg of TMV RNA or tobacco mRNA (excessive amount to guarantee adequate binding) was bathed in water at 67 °C for 10 min to unfold the secondary structure and then instantly cooled down on ice. A reaction mixture containing 25 μL of Promega Wheat Germ Extract, 80 μM amino acid standard solution, 75 mM potassium acetate, 100 μM actidione, and 40 U of RNase inhibitors was then added. RNase-free distilled deionized H_2O (ddH_2O) was added to the final volume of 50 μL, which was subsequently mixed and incubated at 25 °C for 30 min.

2.3. Isolation of TMV RNA–ribosome and tobacco mRNA–ribosome complexes and their corresponding excessive free RNA

The eukaryotic cell ribosome, which is composed of two subunits (60S and 40S), has a molecular weight of 3.9–4.5 × 10^3 kDa. However, TMV RNA contains 6395 nucleotides with a molecular weight of 2.1 × 10^3 kDa, which is smaller than that of the RNA–ribosome complexes. Therefore, sucrose cushion was used to achieve centrifugal separation according to the different sedimentation coefficients of the RNA–ribosome complexes and free RNA. In total, 0.5 μg/mL ethidium bromide (EB) was added to the

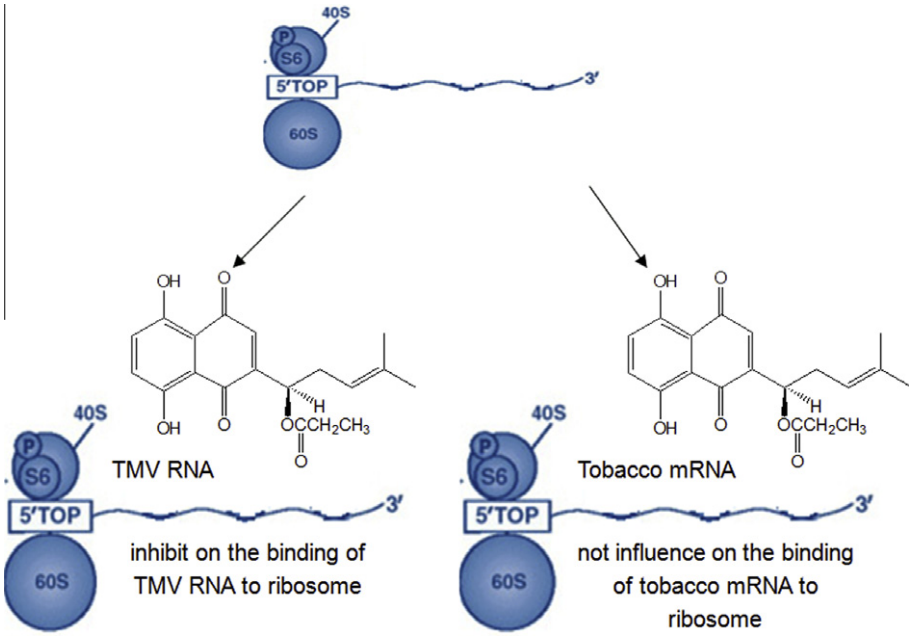

Fig. 1. Basic mode of action of propionylshikonin against TMV.