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Establishment and Application of a Fluorescence Polarization Drug Screening System Targeting Tomato Spotted Wilt Virus Nucleoprotein (Post-Print)

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Abstract

A drug screening system based on fluorescence polarization technology was established to target the interaction between the nucleoprotein (NP) of Tomato spotted wilt virus (TSWV) and nucleic acids, and this system was applied for drug screening. Methods: The target gene was cloned into the pGEX-6p-1 expression vector, and heterologous expression of the target protein was performed using an Escherichia coli expression system. A fluorescence polarization-based drug screening system targeting the TSWV NP-nucleic acid interaction was established, and the binding time, DMSO tolerance, variability, and stability of the system were investigated, followed by drug screening. Results: The recombinant plasmid pGEX-6p-1-NP was successfully constructed, and high-quality nucleoprotein was expressed and purified in Escherichia coli. A stable fluorescence polarization-based drug screening system targeting the TSWV NP-nucleic acid interaction was established with a signal-to-noise ratio of 8:1 and a Z-factor of 0.82. Drug screening was performed on 1,000 compounds from a compound library, and one compound with an IC50 of 4.146 mol/L was obtained through preliminary screening. Conclusion: A stable fluorescence polarization screening system was established, which is suitable for screening drugs targeting the NP-nucleic acid interaction. The screened compound provides a reference for the prevention and control of Tomato spotted wilt virus.

Full Text

Development and Application of a Fluorescence Polarization-Based Drug Screening System Targeting Tomato Spotted Wilt Virus Nucleoprotein

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Abstract

Objective: To establish a fluorescence polarization-based drug screening system targeting the interaction between Tomato spotted wilt virus (TSWV) nucleoprotein (NP) and nucleic acid, and to apply this system for compound screening. **Methods:** The target gene was cloned into the pGEX-6p-1 expression vector, and heterologous protein expression was performed using an *Escherichia coli* expression system. A fluorescence polarization assay was developed to target the NP-nucleic acid interaction, and parameters including binding time, DMSO tolerance, variability, and stability were characterized before conducting drug screening. **Results:** The recombinant plasmid pGEX-6p-1-NP was successfully constructed, and high-quality nucleoprotein was expressed in *E. coli* and purified. A stable fluorescence polarization-based drug screening system targeting TSWV NP-nucleic acid interaction was established with a signal-to-noise ratio of 8:1 and a Z-factor of 0.82. This system was used to screen 1,000 compounds from a chemical library, yielding one compound with an IC of 4.146 mol/L. **Conclusion:** The established fluorescence polarization screening system is stable and suitable for screening drugs that target NP-nucleic acid interactions. The identified compound provides a valuable reference for the prevention and control of Tomato spotted wilt virus.

Keywords: Tomato spotted wilt virus; nucleoprotein; fluorescence polarization; drug screening

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Introduction

Tomato spotted wilt virus (TSWV) is a significant agricultural pathogen first isolated in Australia in 1915. The virus is transmitted by insects (thrips) and infects a broad host range (>1,000 species, including both monocots and dicots), causing severe damage to economically important crops such as tomato, potato, lettuce, pepper, cyclamen, and impatiens. TSWV ranks among the ten most important plant viruses identified in recent years.

TSWV belongs to the genus *Tospovirus* within the family *Bunyaviridae*, a large family of negative-sense RNA viruses comprising 350 serologically distinct viruses across five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*. Except for *Tospovirus*, which infects plants, all other

genera are animal viruses capable of infecting humans or livestock. Similar to other bunyaviruses, the TSWV genome consists of three segmented single-stranded RNAs designated S (small), M (medium), and L (large). The S RNA segment encodes the nucleocapsid (N) protein and the silencing suppressor NSs protein; the M RNA segment encodes two surface glycoproteins (Gn and Gc) and a movement protein (NSm); and the L RNA segment encodes the RNA-dependent RNA polymerase (RdRp, or L protein). The NP, RdRp, and genomic RNA assemble into viral ribonucleoprotein complexes (vRNPs) that serve as functional templates for vRNA replication and mRNA transcription. Additionally, TSWV NP interacts with glycoproteins Gn and Gc to participate in virion assembly and with NSm to facilitate cell-to-cell movement, thereby playing a role in spreading the viral genome to neighboring cells [1]. Thus, TSWV NP is a multifunctional protein that performs essential biological functions at various stages of the viral life cycle.

In 2017, the structures of TSWV NP were independently determined by Komoda et al. [2] and by Guo et al. from Nankai University [3]. Komoda and colleagues first reported the crystal structure of TSWV NP in complex with a 25-poly-dT oligonucleotide, representing the first NP structure solved for the *Tospovirus* genus. The structure revealed that NP comprises N-terminal, C-terminal, and core domains, with subunits interacting to form an asymmetric ring-shaped trimer. Similar to nucleoproteins from other bunyaviruses, this trimer contains a positively charged cavity within the ring that binds RNA and plays a crucial role in genome encapsidation. Compounds that interfere with NP-oligonucleotide binding hold promise for controlling TSWV and reducing crop losses.

Various methods are available for detecting protein-nucleic acid interactions, including electrophoretic mobility shift assay (EMSA), DNase I footprinting, AlphaScreen, fluorescence polarization analysis, isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR). However, EMSA, DNase I footprinting, ITC, and SPR involve cumbersome procedures. AlphaScreen requires protein labeling, antibodies, and donor/acceptor beads [4], making it costly. In contrast, fluorescence polarization has long been applied to study interactions between fluorescent molecules and other molecules [5]. This technique requires labeling only the small-molecular-weight ligand, operates in a homogeneous phase without washing steps, and is amenable to automation and rapid screening. It offers lower detection limits (sub-nanomolar range) and enables real-time kinetic monitoring [6-9]. Fluorescently labeled probes exhibit good uniformity, stable properties, and long-term storage capability, ensuring excellent assay reproducibility. As fluorescence polarization is a ratiometric measurement, it is less susceptible to variations in solution color or instrument sensitivity compared to other fluorescence-based techniques, yielding stable and reliable results. Consequently, fluorescence polarization analysis has become the preferred method.

Currently, no specific drugs are available for TSWV control. Only a few commercial agents are used for plant virus diseases, including ningnanmycin, rib-

avirin, physcion, moroxydine hydrochloride, chloroisobromine cyanuric acid, chitosan oligosaccharide, DHT (2,4-dioxohexahydro-1,3,5-triazine), and DADHT (diacetyl-1,3,5-triazine-2,4-dione). Although these compounds show some efficacy against TSWV, none were specifically developed for this virus. NP represents a valuable molecular target for TSWV drug development. In this study, we labeled an oligonucleotide with FITC at its 5' end and employed fluorescence polarization to detect protein-nucleic acid interactions, aiming to identify compounds that disrupt this interaction.

Materials and Methods

1.1 Materials *E. coli* DH5 and BL21(DE3) strains and the pGEX-6p-1 expression vector were maintained in our laboratory. The TSWV NP gene was synthesized by Beijing Augct Biotechnology Co., Ltd. FITC-labeled oligonucleotide (FITC-25T) and unlabeled oligonucleotide (25T) were synthesized by Tianjin Weike Biotechnology Co., Ltd. and General Biosystems Co., Ltd., respectively. Molecular biology reagents were purchased from Thermo Fisher Scientific, protein expression and purification reagents from Sangon Biotech (Shanghai) Co., Ltd., and 384-well black-bottom microplates from Thermo Fisher Scientific.

1.2 Instruments The JN-3000 Plus ultra-high pressure continuous flow cell disruptor was purchased from Guangzhou Juneng Biological Technology Co., Ltd. The protein purification chromatography system (AKTA FPLC) and columns were from GE Healthcare. The Spark 10M multimode microplate reader was from Tecan.

1.3 Buffers for Nucleoprotein Purification

- Lysis buffer: 20 mmol/L HEPES, pH 7.0, 500 mmol/L NaCl, 5 mmol/L DTT
- Ion exchange chromatography (Heparin) buffer A: 20 mmol/L HEPES, pH 7.0, 40 mmol/L NaCl, 5 mmol/L DTT
- Buffer B: 20 mmol/L HEPES, pH 7.0, 1 mol/L NaCl, 5 mmol/L DTT
- Gel filtration chromatography (Superdex 200) buffer: 20 mmol/L HEPES, pH 7.0, 200 mmol/L NaCl, 5 mmol/L DTT

1.4 Expression and Purification of TSWV NP The full-length TSWV NP gene (1-777 bp, GenBank: AB889601.1) was cloned into the pGEX-6p-1 vector. The recombinant plasmid was transformed into *E. coli* BL21(DE3). A single colony was inoculated into LB medium and cultured at 37°C until OD reached 0.6-0.7. Protein expression was induced with 0.5 mmol/L IPTG at

16°C for 16 h. Cells were harvested by centrifugation at 4,500 rpm for 15 min at 4°C, resuspended in lysis buffer, disrupted, and centrifuged at 12,000 rpm. The supernatant was subjected to GSH affinity chromatography. The GST tag was cleaved with PreScission Protease at 4°C overnight. The protein was further purified by ion exchange and Superdex 200 gel filtration chromatography to obtain high-purity target protein. The protein concentration was measured using a NanoDrop 2000, and aliquots were stored at -80°C.

1.5 Establishment and Evaluation of the Fluorescence Polarization Drug Screening Assay The FITC-labeled 25T probe [Figure 1: see original paper] was excited with vertical polarized light at 485 nm. Due to its small size (~7,500 Da), the free 25T probe rotates rapidly in solution, causing depolarization of the emitted light at 535 nm. Upon binding to NP, the protein-nucleic acid complex rotates slowly, preserving the polarization of emitted light [10-11]. The binding between DNA and nucleoprotein was quantified by measuring polarization values (P or mP). Probe concentration, protein concentration, DMSO concentration, and incubation time were optimized, and the Z-factor was used to evaluate assay stability.

Equation 1: Calculation of fluorescence polarization. (S is the intensity of emitted light polarized parallel to the excitation light, P is the intensity of emitted light polarized perpendicular to the excitation light, and G is a correction factor.)

Figure 1: FITC-labeled 25T probe

1.6 Drug Screening The established assay was used for compound screening. In a 384-well plate, 18 L of 2.5 mol/L NP was added, followed by 1 L of 2.5 mol/L fluorescent probe, and finally 1 L of test compound. After incubation at room temperature for 30 min, the polarization value was measured. The positive control (mP) consisted of wells without protein, while the negative control (mP g) consisted of wells without compound. The inhibition rate was calculated as: 1 - (mP - mP)/(mP g - mP), where mP is the polarization value after compound addition.

Results

2.1 Expression and Purification of TSWV NP Following GSH affinity chromatography, ion exchange chromatography [Figure 2a: see original paper], and Superdex-200 gel filtration chromatography [Figure 2b: see original paper], 12% SDS-PAGE analysis [Figure 2c: see original paper] revealed a high-purity target protein at ~28 kDa. The protein was concentrated to 40 mg/mL and stored at -80°C.

Figure 2: Chromatographic analysis and 12% SDS-PAGE of recombinant NP. (a) Ion exchange chromatogram; (b) Size exclusion chro-

matogram; (c) 12% SDS-PAGE analysis

2.2 Optimization of Assay Conditions

2.2.1 Determination of Probe Concentration In a black 384-well plate with a 20 µL reaction volume, the fluorescent probe was serially diluted in protein buffer, with a probe-free well serving as blank control. Parallel polarized light intensity was measured using a Spark 10M microplate reader in FP mode to assess the linear relationship between probe concentration and intensity. Theoretically, polarization values should be consistent across different probe concentrations in protein buffer. As shown in [Figure 3a: see original paper], parallel polarized light intensity exhibited excellent linear correlation with probe concentration across a wide range. However, polarization values increased significantly with decreasing probe concentration, showing substantial fluctuations [Figure 3b: see original paper]. Based on minimal polarization variability, the optimal probe concentration was selected as 125 nmol/L.

Figure 3: Correlation between fluorescent probe concentration and fluorescence intensity. (a) Linear regression analysis between parallel polarized light intensity and probe concentration ($R^2 = 0.9921$); (b) Polarization values at various probe concentrations

Table 1: Average fluorescence intensity and polarization values at various probe concentrations

2.2.2 Determination of Protein Concentration and Incubation Time A saturation curve was generated to determine the dissociation equilibrium constant (K_d) for probe-protein interaction. With the probe concentration fixed at 125 nmol/L, protein concentrations ranged from 20 to 0.075 µmol/L, and polarization values were measured after different incubation times at 25°C. As shown in [Figure 4: see original paper], protein-probe binding reached equilibrium after 10 min at 25°C, with a K_d of 1.23 µmol/L and a maximum polarization value (mP) of 365.40. The optimal protein concentration was selected as 2.5 µmol/L, corresponding to the linear range of 30-90% mP .

Figure 4: Saturation binding curve of labeled 25T to NP. (a) Polarization values increase with protein concentration; (b) Polarization values show sigmoidal change with increasing protein concentration (logarithmic scale)

Table 3: K_d values at different incubation times

2.2.3 Competitive Binding Curve and Incubation Time With probe and protein concentrations fixed at 125 nmol/L and 2.5 µmol/L, respectively, the binding specificity was further evaluated using competition with unlabeled 25T oligonucleotide [12-13]. NP (2.5 µmol/L) and probe (125 nmol/L) were incubated at 25°C for 10 min, followed by addition of varying concentrations of

unlabeled 25T and further incubation for different durations before measuring polarization values. As shown in [Figure 5: see original paper], IC values decreased with prolonged incubation time, stabilizing after 30 min.

Figure 5: Competitive binding curve by titration with unlabeled probe 25T at different incubation times, keeping labeled probe and NP concentration constant

Table 4: IC values at different incubation times

2.2.4 DMSO Tolerance Since DMSO is the primary solvent for compounds in drug screening, the assay' s DMSO tolerance was evaluated. Different DMSO concentrations (0-10% v/v) were added to the reaction system and incubated at 25°C for 10 min before measuring polarization values. Additionally, with probe concentration fixed at 125 nmol/L, protein was titrated from 20 µmol/L to 0.15 µmol/L in the presence of 0%, 1%, 5%, or 10% DMSO, incubated at 25°C for 10 min, and polarization values were measured. DMSO effects on mP values and saturation curves were used to assess tolerance. As shown in [Figure 6: see original paper], DMSO concentrations up to 10% had no effect on mP or K_d values.

Figure 6: DMSO tolerance of the assay. (a) Effect of DMSO on polarization values; (b) Effect of DMSO on binding curves

Table 5: Polarization values and K_d values at various DMSO concentrations

2.2.5 Assay Stability Evaluation Following optimization, the final screening system components are listed in . The Z-factor is a critical parameter for evaluating assay stability in drug screening; assays with Z-factor > 0.5 are considered stable and suitable for screening [14]. In three separate experiments conducted over three days, negative and positive control groups (30 wells each) were arranged in left, middle, and right sections of 384-well plates. Negative controls (mP g) contained no compound, while positive controls (mP or mP) contained no protein. As shown in [Figure 7: see original paper], the negative control had a mean mP of 327.33 with a coefficient of variation (CV) of 2.9% (<10%), while the positive control had a mean mP of 39.65 with a CV of 17.7% (<20%). The assay achieved a signal-to-noise ratio of 8:1 with a sufficiently large signal window, and a Z-factor of 0.82 (>0.5), demonstrating its suitability for drug screening.

Table 6: Components of the FP assay

Equation 2: Z-factor calculation. (and g denote the means of positive and negative control signals, respectively, while and g denote their standard deviations.)

Equation 3: Signal-to-background ratio

Equation 4: Coefficient of variation (and represent standard deviation and mean, respectively)

Figure 7: Polarization values of negative and positive control groups for Z-factor calculation

2.3 NP Inhibitor Screening Results Using the established screening system, 1,000 compounds from a chemical library were screened. One compound exhibited 90.95% inhibition at 100 µmol/L, with a determined IC of 4.146 µmol/L.

Discussion

12% SDS-PAGE analysis confirmed that NP had the correct molecular weight and high purity, making it suitable for subsequent assay development and screening. When selecting the fluorescent probe concentration for assay development, the principle is that parallel polarized light intensity should be within the linear range and at least 10-fold above background [15], with minimal polarization variability. Based on these criteria, 125 nmol/L was selected. The IC value from competitive binding curves (0.8-1 µmol/L) was similar to the K_d from saturation curves [16], indicating strong affinity between NP and oligonucleotides and demonstrating that fluorescent labeling minimally interferes with binding. Competitive experiments required at least 30 min incubation to ensure equilibrium binding. DMSO tolerance experiments showed that concentrations up to 10% had no effect on mP or K_d values, indicating good tolerance and no interference from compound solvents. The fluorescence polarization-based drug screening system exhibited a negative control CV of 2.9% (<10%), positive control CV of 17.7% (<20%), signal-to-noise ratio of 8:1, and Z-factor of 0.82 (>0.5), demonstrating excellent stability and suitability for high-throughput screening. Using this system, one compound showing >90% inhibition at 100 µmol/L was identified in primary screening, with a confirmed IC of 4.146 µmol/L upon retesting.

Conclusion

This study successfully prepared high-quality TSWV NP using an *E. coli* heterologous expression system and established a fluorescence polarization-based drug screening system. This homogeneous, non-radioactive assay is simple, easy to operate, and applicable to large-scale screening. One compound with an IC of 4.146 µmol/L was identified, providing a lead for subsequent drug design and development and offering a reference for TSWV prevention and control. Furthermore, nucleoproteins from bunyaviruses share structural similarities and interact with viral genomes to mediate encapsidation. This fluorescence po-

larization drug screening system provides a valuable platform for identifying compounds that disrupt NP-genome assembly across the *Bunyaviridae* family.

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