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Protocols

Monoclonal antibody-based triple antibody sandwich-enzyme-linked immunosorbent assay and immunocapture reverse transcription-polymerase chain reaction for *Odontoglossum ringspot virus* detection

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A B S T R A C T

Odontoglossum ringspot virus (ORSV) infects numerous commercially important orchids and causes significant losses worldwide. The coat protein (*CP*) gene of ORSV was cloned and expressed in *Escherichia coli* by using the pET-32a expression vector, and the expression of recombinant protein was confirmed by Western blotting using anti-ORSV antibodies. The recombinant protein was purified using Ni-NTA agarose, and the purified protein was used as an immunogen to produce monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs). Five murine MAbs against ORSV CP were obtained. Among them, two MAbs (6B4 and 1D1) also reacted with TMV CP. The triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) methods using the MAb (8A5) were then developed for sensitive, specific, and rapid detection of ORSV. TAS-ELISA and IC-RT-PCR could detect ORSV in the infected leaf saps with dilutions of 1:10,240 and 1:81,920 (w/v, g mL^{-1}), respectively. TAS-ELISA and IC-RT-PCR detections indicated that ORSV was prevalent in orchids in the Zhejiang Province of China.

1. Introduction

Orchids are one of the most important ornamental plants of the flower industry and are valued for their flowers of different shapes and exotic colors. *Phalaenopsis* and *Oncidium* are the two main orchid genera cultivated in China and exported mainly to Japan, USA, and Europe. Orchids are often propagated by the agamic technique; therefore, they are more susceptible to virus infection than most other crops and their ornamental and commercial values are considerably reduced (Zettler et al., 1990). At present, orchid viruses are widespread in the cultivated orchids worldwide. *Odontoglossum ringspot virus* (ORSV) and *Cymbidium mosaic virus* (CymMV) are the two most prevalent and economically important orchid viruses that are distributed worldwide and infect numerous commercially important orchid genera (Zettler et al., 1990; Wong et al., 1994a; Khentry et al., 2006; Lee and Chang, 2008; Yamane et al., 2008). There is no natural insect vector for ORSV and CymMV transmission and they are transmitted mechanically through infected sap on cutting tools and potting media (Wong et al., 1994a). ORSV causes streak or striped mosaic, diamond mottle, or ringspots on leaves, and sometimes ringspots, color breaking, or distortion on infected flowers (Zettler et al., 1990). ORSV reduces plant vigor and flower quality, which affects the economic value of orchids (Okemura et al., 1984; Pearson and Cole, 1991). However, in some cases ORSV-infected orchids can be symptomless (Zettler et al., 1990). Therefore, the detection of viral infection cannot be made on the basis of symptoms alone. Since virus-free planting material is the primary method for virus control, it is necessary to develop sensitive virus detection methods to produce virus-free orchid plants.

Many diagnostic methods have been developed for the detection of ORSV in orchids: reverse transcription-polymerase chain reaction (RT-PCR) (Ryu and Park, 1995; Seoh et al., 1998; Lee and Chang, 2006; Yamane et al., 2008), real time RT-PCR (Eun et al., 2000), immunocapture RT-PCR (IC-RT-PCR) (Barry et al., 1996), nucleic acid hybridization (Hu and Wong, 1998; Sherpa et al., 2006) and molecular beacon (Eun and Wong, 2000), enzyme-linked immunosorbent assay (ELISA) (Dore et al., 1987; Arunasalam and Pearson, 1989; Hu et al., 1993; Wong et al., 1994b; Lee and Chang, 2008), quartz crystal microbalance (QCM) immunosensors (Eun et al., 2002), immunocapillary zone electrophoresis (I-CZE) (Eun and Wong, 1999), liquid chromatography/mass spectrometry (LC/MS), and matrix-assisted laser desorption-ionization (MALDI) mass spectrometry (Tan et al., 2000). Among these detection methods, ELISA, RT-PCR, and IC-RT-PCR are widely used. ELISA is cost-effective and suitable for the rapid detection of large-scale samples, while RT-PCR and IC-RT-PCR are more suitable for analyz-

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ing viral genome as they are more sensitive as compared to other methods. Preparation of a good qualitative antibody is important to achieve high specificity and sensitivity of ELISA and IC-RT-PCR. Some polyclonal antibodies (PAbs) prepared by using the purified virions have been used to detect ORSV in orchids. However, the PAbs exhibit relatively high background values because of contamination of plant proteins (Elliott et al., 1996). An alternative approach is to use a specific monoclonal antibody (MAb) instead of a PAb. Another alternative is to use PAbs against viral coat protein (CP) expressed in bacterial cells instead of purified virions (Chun et al., 2008; Wu et al., 2009). In this study, the ORSV CP was expressed in *Escherichia coli* using the pET-32a expression system, and MAbs and PAbs against ORSV were produced using the prokaryotic expression ORSV CP as an immunogen. A triple antibody sandwich-enzyme-linked immunosorbent assay (TAS-ELISA) and an IC-RT-PCR assay using the prepared antibodies were developed and used for the detection of ORSV in orchids.

2. Materials and methods

2.1. Virus sources and collection of field samples

ORSV-infected *Phalaenopsis* orchids were identified using antibodies against ORSV, which were purchased from Agdia Inc. (Elkhart, IN, USA). ORSV was isolated from the infected orchids and cultured on *Chenopodium amaranticolor*. The following viruses were characterized and maintained in the author's laboratory: *Tobacco mosaic virus* (TMV), *Cucumber green mottle mosaic virus* (CGMMV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Turnip mosaic virus* (TuMV), *Cucumber mosaic virus* (CMV), CymMV, and *Rice stripe virus* (RSV).

Five hundred and twenty samples of *Phalaenopsis amabilis*, *Dendrobium*, *Cattleya*, *Cymbidium hyridus*, and *Oncidium* orchids from three farms in the Zhejiang Province of China were collected.

2.2. Cloning and prokaryotic expression of ORSV CP gene

By using the nucleotide sequence of ORSV *CP* gene in GenBank (accession no. EU683879), the forward primer CP-F (5′-CGGGATCC ATGTCTTACACTATTAC-3′, *Bam*HI restriction site is underlined) and the reverse primer CP-R (5′-CGGTCGACTTAGGAAGAGGTCCAAGTA-3′, *Sal*I restriction site is underlined) were designed. The total RNA was extracted from ORSV-infected *Phalaenopsis* orchids using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA). The total RNA was amplified by RT-PCR using the SuperScript™ III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR products were purified using gel extraction columns (Qiagen, Hilden, Germany). The purified PCR product was digested with *Bam*HI and *Sal*I, and then subcloned into the 6× His-tagged prokaryotic expression vector pET-32a (Novagen, Darmstadt, Germany). Sequence analysis was performed to confirm the nucleotide sequence and the open reading frame (ORF) of the *CP* gene in expression vector. The recombinant expression vector pET-32a-ORSV-CP was transformed into *E. coli* BL21 (DE3) cells for the expression of recombinant CP according to the method described by Wu et al. (2009). The recombinant CP was purified using Ni-NTA agarose column, a metal chelate affinity chromatography column, as per the instructions of the manufacturer (Qiagen).

2.3. Sodium dodecyl-polyacrylamide gel electrophoresis and Western blot analyses of the recombinant CP

The recombinant CP was analyzed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described by Wu et al. (2007). Anti-ORSV PAbs (1:1000; Agdia, Elkhart, IN, USA) were used for Western blot.

2.4. Production of PAbs against the recombinant CP

The purified ORSV recombinant CP was used as an immunogen and was injected into three rabbits as described by Wu et al. (2009). The rabbits were bled a week after the 5th immunization, and the antisera were used in TAS-ELISA.

2.5. Preparation of hybridomas and MAbs

The purified ORSV recombinant CP was used as an immunogen and was injected into five BALB/c mice as described by Wu et al. (2010). Hybridomas secreting ORSV CP MAbs were obtained by the method as described by Wu et al. (2009). The titers of MAbs in ascites were determined by indirect ELISA as described by Wu et al. (2010). The isotypes of the MAbs were determined by ELISA using the mouse MAb isotyping reagents according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA). The specificity and sensitivity of the MAbs was determined further by Western blot and antigen-coated plate (ACP)-ELISA analyses using ORSV-infected and healthy plant tissues as the positive and negative controls, respectively. Anti-ORSV IgG was purified from ascites using an immobilized protein-G affinity column (GE Healthcare, Bucks, UK), according to the manufacturer's instructions.

2.6. ELISA

ORSV were detected according to the standard procedures of ACP-ELISA (Jiang et al., 2003). TAS-ELISA for ORSV detection was performed as described by Wu et al. (2009). Briefly, the wells of microtitre plates (Nunc) were coated with the PAbs in 50 mmol L^{-1} sodium carbonate buffer, pH 9.6 for overnight at 4 °C to allow the antibodies to adhere to the wells. The wells were added 5% dried skimmed milk in PBST (0.01 mol L^{-1} PBS, 0.05% Tween-20, pH 7.4) (250 μL/well) and incubated for 1 h at 37 °C to block antibody-free areas of the well. Clarified plant sap samples prepared by grinding leaf tissue in 0.05 mol L^{-1} Tris–HCl, 5 mmol L^{-1} EDTA, pH 8.0 (1 g leaf tissue in 20 mL buffer) were added (100 μL/well) and incubated for 1 h at 37 °C. Negative and positive controls were wells incubated with leaf extracts from healthy leaf and ORSV-infected leaf tissues, respectively. Suitable dilution MAbs were added (100/well) and incubated for 1 h at 37 °C. Goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma) with suitable dilution was added and incubated for 1 h at 37 °C. The alkaline phosphatase conjugate was detected with p-nitrophenyl phosphate at 1 mg mL^{-1} in 10% diethano-lamine buffer, pH 9.8. Each step was followed by four times washing of the plates with PBST. The reaction was stopped with 3 mol L^{-1} NaOH after 30 min at room temperature and A405 was measured with a Microplate Reader Model 680 (BIO-RAD, Hercules, CA, USA). The sample was considered to be positive when its absorbance value was greater than three times that of the negative control.

2.7. IC-RT-PCR

The forward primer (5′-ACCCGTCTAAGCTGGCTTATTTAA-3′) and the reverse primer (5′-TTAGGAAGAGGTCCAAGTAAGTC-3′) were designed by using the conserved nucleotide sequences of ORSV *CP* in GenBank, which were determined based on the alignment of ORSV *CP* RNA sequences using the DNASTAR package (Version 7.0; DNAStar Inc., Madison, WI, USA). IC-RT-PCR was performed according to the method described by Yu et al. (2005). PCR products were recovered, cloned, and sequenced (Jiang and Zhou, 2002).