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# Production of monoclonal antibodies specific for the recombinant viral coat protein of Apple stem grooving virus-citrus isolate and their application for a simple, rapid diagnosis by an immunochromatographic assay

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A B S T R A C T

A simple and rapid immunochromatographic assay (ICA) for the diagnosis of Apple stem grooving virus (ASGV) in citrus was developed. Nine lines of monoclonal antibodies (mAbs) were produced by immunizing with a recombinant viral coat protein of ASGV as the antigen. According to the competitive-binding ELISA results, the 9 mAbs comprised 2 paratope groups, A and B. After screening for the most effective combination of mAbs, the two lines from different paratope groups (4A12 from group A and 6N31 from group B) were used to create a colloidal gold conjugate and for the test line, respectively, in ICA test plate preparation. The ICA detection using this test plate was accurate for positive and negative samples, and ASGV was detectable to a dilution of 1:2430 for the infected citrus sample. Furthermore, ICA was more sensitive than ELISA for the detection of ASGV isolates in citrus. The simple and sensitive ICA for ASGV provides a straightforward method for diagnosis by non-experts, including nursery workers and growers.

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## 1. Introduction

In citrus, bud union incompatibility occurs when contaminated scions are grafted on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and on its hybrids rootstocks (McClean, 1974). The causal virus was initially identified as Citrus tatter leaf virus (CTLV; Miyakawa and Matsui, 1976). CTLV is currently considered a strain of *Apple stem grooving virus* (ASGV), a definite species of the genus *Capillovirus* of the family *Betaflexiviridae* (Adams et al., 2012). ASGV is widespread among citrus in China (Miyakawa and Ito, 2000). In the United States, ASGV was initially found in Meyer lemon that was introduced from China in the 1900s, and it remains a continuous threat in Florida and California, where trifoliate hybrids became popular rootstocks (Tatineni et al., 2009). Besides citrus, ASGV is widespread in Rosaceae fruit trees, such as apple, pear, apricot, and cherry, and some isolates induce topworking disease in apple trees on such rootstocks as *Malus sieboldii* (Yanase, 1983). Recently, ASGV was also detected in the Kiwifruit (Chinese gooseberry, *Actinidia* spp.) in New Zealand, and ASGV led to the development of symptoms such as chlorotic mottling, mosaic, and ringspots (Clover et al., 2003).

ASGV in citrus is also a serious problem in Japan, where trifoliate orange is used commonly as rootstocks. ASGV is considered one of the most hazardous pathogens of citrus, because it severely affects growth and leads to sudden decline of health in trees. Since there is no vector involved in the transmission, the primary control measure is production and distribution of virus-free nursery trees. To promote this practice, a simple and precise detection method is indispensable. To detect ASGV in citrus plants, biological indexing with citranges (*C. sinensis* (L.) Osb. × *P. trifoliata*) and some herbaceous plants, serological diagnostic techniques such as enzyme-linked immunosorbent assay (ELISA), and gene diagnostic techniques such as RT-PCR and IC-RT-PCR have been developed (Garnsey, 1964; Spiegel et al., 1993; Kawai et al., 1996; Kinard et al., 1996; Marinho et al., 1998; James, 1999; Hailstones et al., 2000; Kirby et al., 2001; Kusano and Shimomura, 1995, 2003; Shimomura and Kusano, 2002; Kusano et al., 2009). Biological indexing is time-consuming and unsuitable for routine screening of infected materials. To perform ELISA, RT-PCR, or IC-RT-PCR, specialized equipments and trained personnel are needed. In contrast, immunochromatographic assay (ICA) is a very simple

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and rapid detection method, which can be performed by almost anyone with minimum instruction. The assay has been applied widely for diagnosis of many human diseases. Recently, it has been used for detecting animal viruses, including avian influenza virus (Peng et al., 2008). The purpose of this study was to develop an ICA kit to detect ASGV from citrus, and make these kits available to under-equipped clinical facilities and also to citrus growers and nursery workers, so that they can detect and discard infected budwoods and nursery trees.

The first step in the development of an ICA kit is preparation of sufficient antigen to produce high-titer specific monoclonal antibodies (mAbs; Kohler and Milstein, 1975). For most plant viruses, the primary choice for antigen is a purified preparation of virus particles. However, ASGV particles have relatively low stability and tend to aggregate with plant debris or with themselves. This aggregation leads to considerable loss of virus particles during the purification process (Sequeira and Lister, 1969) and makes the preparation of purified virus particles extremely difficult. To overcome these difficulties, molecular biology techniques are currently being used to express the genes of interest in heterologous systems and to produce an antigen when required (Targon et al., 1997). By using this method, the coat protein (CP) gene of ASGV was cloned and sequenced (Nickel et al., 2001), allowing for the production of large quantities of recombinant ASGV-CP antigen. In this study, taking advantage of the molecular techniques, sufficient amount of ASGV-CP antigen was prepared. Subsequently, mAbs were produced using the recombinant ASGV-CP antigen and were characterized by analyzing the binding sites and epitopes of the mAbs for ASGV. Finally, the mAbs were used to develop an ICA system, and its usefulness as a simple and rapid diagnostic tool for ASGV was demonstrated using various citrus-infected tissues.

## 2. Materials and methods

This study was approved by the institutional review board, and all the procedures were conducted in accordance with the American Physiological Society's Guiding Principles in the care and use of animals.

### 2.1. Expression and purification of recombinant ASGV-CP

The expression vector of ASGV-CP was constructed as previously described (Magome et al., 1997). RNA from ASGV isolate N297 was used as template for RT-PCR. Total RNA was isolated from young leaves of ASGV-infected Satsuma mandarin (*Citrus unshiu*, Marc.) using the RNeasy plant mini kit (Qiagen, Chatsworth, CA). RT-PCR was performed to amplify the complete CP gene using a forward primer ASGVCPEcRI (5′-AGGAATTCC*ATG*AGTTTGGAAGACGTGCT-3′) with initiation codon (italicized) and a reverse primer ASGVCPSalRV (5′-AGGGTCGAC*CTA*ACCCTCCAGTTCCAG-3′) with termination codon (italicized), corresponding to the N-terminus and C-terminus of CP (underlined), respectively. The PCR product was cloned into a TOPO TA cloning vector (Life technologies, CA, USA) and then subcloned in-frame between the *Eco*RI and *Sal*I sites of the pGEX-5X-3 plasmid. This vector was used to transform *Escherichia coli* BL21 competent cells. The *E. coli* cells were transferred to 8 mL of YT medium with 50 μg/mL ampicillin and cultured at 37 °C overnight. To scale up production, this medium was cultured in 2 L of YT medium (Funakoshi, Tokyo, Japan), including 50 μg/mL ampicillin, at 37 °C for 3 h. When the $OD_{600\,nm}$ of bacterial suspension reached approximately 0.3, IPTG was added to a final concentration of 1 mM, and the suspension was incubated at 37 °C for 2 h. The suspension was centrifuged at 1000 × *g* for 5 min, and the pellet was resuspended with 4% volume of surfactant buffer (B-PER, Pierce, IL, USA). Subsequently, 10 mg/mL lysozyme and 70 U/μL DNase were added to homogenize the *E. coli* cells and incubated at 25 °C for 1 h. After centrifugation (11,000 × *g*, 5 min), inclusion bodies in the precipitate were dissolved in adsorption buffer (20 mM Tris–HCl pH 9.0, 8 M urea) and purified by DEAE-Toyopearl 650 M anion-exchange column chromatography with a linear gradient elution of 0.0–0.6 M NaCl. The fractions with 0.05 or higher $OD_{280\,nm}$ were tested using an ELISA kit for ASGV (Japan Plant Protection Association), and the positive fractions were collected. The buffer was exchanged for storage buffer in the positive fractions (12 mM PBS pH 7.4, 320 mM urea).

### 2.2. Immunization of mice and production of mAbs

Four-week-old female BALB/c mice were injected intraperitoneally 7–9 times with recombinant ASGV-CP suspension (0.2 mg protein), which was emulsified 1:1 with Ribi Adjuvant System (RAS) (Corixa Corporation, WA, USA), at 2-week intervals. After the final injection to mice, splenocytes were isolated from the spleens and fused with the hypoxanthine–aminopterin–thymidine-sensitive mouse myeloma cell line (P3-X16-Ag8-U1) at a ratio of 10:1 using 50% (w/v) polyethylene glycol (Kohler and Milstein, 1975). Hybridomas producing mAbs to ASGV-CP were cloned under conditions of limiting dilution. The cloned hybridomas were cultured in serum-free E-RDF medium (Kyokuto, Tokyo, Japan) with RD-1 (Kyokuto, Tokyo, Japan). The supernatant from the hybridoma culture was dialyzed with 0.02 M phosphate-buffered saline (PBS). The mAbs produced were purified on an immobilized protein G column (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instruction. The purified mAbs were dialyzed with PBS (pH 7.4) 3 times and stored at 4 °C. The concentration of the dialyzed mAbs was measured as absorbance at 280 nm using a spectrophotometer.

### 2.3. Indirect ELISA

All ELISA tests were carried out in 96-well microtiter plates (Thermo Fisher Scientific, Roskilde, Denmark). Titers of ASGV-CP mAbs secreted by hybridoma cells were determined by indirect ELISA, according to a previous report (Kusano and Shimomura, 1995). Aliquots of mAbs adsorbed to a microtiter plate were mixed with alkaline phosphatase (ALP)-conjugated anti-mouse immunoglobulin G (IgG) (KPL, Maryland, USA) at 1:1000 dilution and incubated for 1 h at 37 °C. After washing, 1 mg/mL *p*-nitrophenyl phosphate in 1 M diethanolamine buffer was added as a substrate for ALP and the mixture was incubated for 1 h at room temperature; the A405 value was measured by Labsystems Multiscan MA (Labsystems, Helsinki, Finland).

### 2.4. Competitive-binding ELISA

The mAbs used for detection were labeled with an ALP-labeling Kit-SH (Dojondo Laboratories, Tokyo, Japan), according to the manufacturer's instructions. Experiments on epitope competition of the purified mAbs were carried out using competitive-binding ELISA. The wells were coated with each unlabeled mAb (1 μg/mL), incubated for 1 h at 37 °C, and washed 3 times. After 100 μL of unlabeled mAbs (5 μg/mL) and 100 μL of crude extract (1:30 dilution) of ASGV-infected young citrus shoot were added, the samples were incubated for 1 h at 37 °C; the wells were washed 3 times, followed by incubation overnight at 4 °C with an equal amount of another ALP-conjugated mAbs. After the plate was washed, 100 μL of freshly prepared substrate solution was added; the plate was then incubated for 1 h at room temperature, and the color development was measured at 415 nm with a reference wavelength of 620 nm. The assay was repeated 3 times.

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