SUMMARY

Antisera were raised in rabbits against two isolates of Cucumber mosaic virus (CMV), representing groups I (DTL) and II (ToRS). The reactions of these polyclonal antibodies (PAbs) with several CMV isolates were checked, using ELISA, Western blotting and IC-RT-PCR. One of the PAbs against isolate M (group I) enabled detection of most of the CMV isolates tested but failed to recognize isolate P26, originating from lily. A PAb against isolate Wic (group II) reacted also with all isolates tested, showing up to 100-fold improvement in detection sensitivity compared with two other CMV-specific PAbs. The coat protein (CP) of CMV isolates belonging to both groups was detected by Western blotting using M and Wic antisera. The suitability of a mixture of PAbs for immunocapturing CMV and for the sensitive detection of all viral isolates tested by IC-RT-PCR was also shown.

Key words: CMV, serology, polyclonal antisera, ELISA, IC-RT-PCR, Western blotting.

Cucumber mosaic virus (CMV) has a very broad host range and causes economically important diseases of vegetables, ornamentals and agricultural crops (Palukaitis et al., 1992; Palukaitis and García-Arenal, 2003). Control of the virus has focused on producing virus-free propagation material. For this purpose it is necessary to set up efficient virus detection methods. For routine CMV detection, ELISA variants based on polyclonal or monoclonal antibodies (PAbs or MAbs) are widely used, especially when testing large numbers of samples (Haase et al., 1989; Porta et al., 1989; Wahyuni et al., 1992; Hsu et al., 2000). RT-PCR is becoming more and more widely used for detection and identification of virus isolates (Varveri and Boutsika, 1999; Finetti Sialer et al., 1999; Raj et al., 2002). The objective of the present work was to obtain polyclonal antibodies that could recognize a wide range of CMV isolates. The suitability of these PAbs for different detection systems, including ELISA, Western blotting and immunocapture-RT-PCR (IC-RT-PCR) was evaluated.

The CMV isolates used in this study originated from lily (Cas, CB, P26, Simp2), cucumber (J, M), honeysuckle (Wic, WicDS), impatiens (Imp), dahlia (D), red currant (Porz) and tulip (Inz). They were maintained in the virus collection at the Research Institute of Pomology and Floriculture (RIPF), Skierniewice, Poland. CMV isolates from pumpkin (Dn) and cucumber (Og) (kindly provided by Prof. H. Pospieszny, Institute of Plant Protection, Poznań, Poland), as well as isolate Del from delphinium (obtained from Prof. J. Staniulis, Institute of Botany, Vilnius, Lithuania) were also included. On the basis of the reaction with CMV group-specific monoclonal antibodies, isolates Cas, CB, J, and M had been classified as members of group I (formerly defined as serogroup DTL) and isolates D, Dn, Del, Imp, Inz, Og, Porz, Simp2, Wic and WicDS, as members of group II (serogroup ToRS). Isolates of Peanut stunt virus (PSV, provided by Prof. H. Pospieszny), Tomato aspermy virus (TAV, from the RIPF collection) and healthy tobacco plants were used as controls. All virus isolates were maintained in Nicotiana rustica plants in a greenhouse.

CMV isolates M (group I) and Wic (group II), purified according to Tóbiás et al. (1982) with one cycle of differential centrifugation instead of two cycles and final virus purification in 5-30% sucrose density gradient centrifugation, were used for antiserum production.

Standard immunisation consisted of seven series of subcutaneous injections (2-4 injections each time) administered to rabbits, at approximately two week intervals (Harlow and Lane, 1988). Blood samples were collected starting 5 weeks after the first injection. The titres of antisera were measured in F(ab')2-ELISA (Barbara and Clark, 1982) with homologous and heterologous CMV isolates, using serial dilutions of crude antiserum (from 1:1000 to 1:256,000). Results of evaluation and titration of antisera obtained at the end of the immunisation protocol are shown in Fig. 1. The titre of the Wic antiserum was 1:256,000, as checked against CMV M and Wic isolates. The titre of the antiserum raised
232 Serological characterisation of CMV isolates

against M isolate depended on the virus isolate. It was 1:128,000 with the homologous isolate M, and 1:32,000 with the heterologous isolate Wic.

Immunoglobulins were isolated from both antisera using affinity adsorption on a protein-A sepharose CL-4B column. Fractions containing the highest IgG concentration were used to prepare F(ab’)_2 fragments. Immunoglobulins from other fractions combined, adjusted to a concentration of 1 mg/ml, were conjugated with alkaline phosphatase (ALP), following a one-step glutaraldehyde protocol. IgG isolation, preparation of F(ab’)_2 fragments and conjugates were done according to Clark and Bar-Joseph (1984). In order to optimise conditions of CMV detection in F(ab’)_2-ELISA and DAS-ELISA, various dilutions of antisera (1:1000, 1:8000), F(ab’)_2 fragments (1:250, 1:500, 1:1000, 1:2000), immunoglobulins (1:500, 1:1000, 1:2000) and ALP-IgG conjugates (1:500, 1:1000, 1:2000) were tested with samples containing homologous and heterologous isolates of CMV.

Reagents produced from both antisera were then tested against 15 CMV isolates, PSV, TAV, healthy N. rustica and buffer. In several experiments, the same extracts were tested simultaneously with commercially available CMV-specific ELISA kits: CMV-I, CMV-II (Agdia, USA), DTL, ToRS (Loewe Biochemica, Germany) and polyclonal antibodies Cas prepared by Korbin and Kaminska (1998) (Fig. 2).

The newly prepared Wic PAbS detected all CMV isolates tested. These antibodies reacted more strongly than six other PAbS with CMV isolates belonging to group II. This was especially clear for isolates Porz, Simp2 and WicDS, for which the corresponding absorbance values were different by a factor of 3 to 6. Reaction of Wic PAbS with group I isolates was comparable with that obtained with M PAbS, and was stronger than the reactions observed with DTL, ToRS and Cas PAbS. The M PAbS reacted with 14 out of the 15 isolates tested. Homologous CMV group I isolates (Cas, CB, J, M) produced the highest absorbance values among the PAbS tested. For group II isolates, the M PAbS reaction was not so uniform. It was stronger than that of DTL, ToRS and Cas PAbS with CMV isolates Simp2 and Porz, but the reaction with isolates D and Imp was weaker than that observed for other ELISA kits tested. Isolate P26 was not detected with M PAbS.

SDS PAGE was carried out as described by Hill and Shepherd (1972). The viral proteins were transferred to
a Millipore Immobilon-P PVDF membrane using a semi-dry method and developed with antisera M and Wic diluted 1:30,000. Both antisera enabled detection of denatured CP of several CMV isolates belonging to group I and II on Western blots (Fig. 3), although isolates belonging to group II produced consistently stronger signals with antisera Wic, while the bands observed for group I isolates were more pronounced on the blot developed with antiserum M. Differences were observed in CP mobility between isolates of group I and II. The CP of isolates from group II migrated slightly faster in SDS-PAGE than that of group I isolates. These differences were similar to those previously reported by Daniels and Campbell (1992) and Sliwa et al. (2008). However, Edwards and Gonsalves (1983) found that the CP of some CMV-II isolates migrated more slowly in SDS-PAGE than that of CMV-I isolates, attributing these results to the higher number of amino acids in the capsid protein of CMV-II isolates.

The IC-RT-PCR protocol of Candresse et al. (1995) modified by Malinowski (2005) was applied, using a mixture of M and Wic antibodies for coating PCR tubes. Primers CMV1-CMV2 of Wylie et al. (1993) were used for amplification of a CMV CP gene fragment. Reverse transcription and amplification was done using Titan One Step RT-PCR Kit (Roche Diagnostics, Poland), according to the manufacturer's instructions. All CMV isolates tested were amplified in IC-RT-PCR and produced a cDNA fragment of the expected 500 bp size (Fig. 4). This technique was especially useful for detecting isolates P26, Porz, Simp2 and WicDS, which did not react in ELISA with some of the antibodies used in the experiment.

The sensitivity of ELISA and IC-RT-PCR was compared with tenfold serial dilutions (down to 10^-6) of CMV infected-tissue extract (1:50 w/v) in healthy tobacco leaf extract (1:50 w/v). The samples were ELISA-positive in immunosorbent assay with M or ToRS PAbs, only in preparations undiluted, or diluted not more than 100 times. When IgG Wic was used in ELISA, the virus was detected in preparations diluted 10^-2-10^-3. The sensitivity of IC-RT-PCR was 10^-3-10^-6 higher than that of DAS-ELISA. All virus isolates tested were readily detected in extracts of CMV-infected plants diluted 10^-6. These values tally with those of Wylie et al. (1993), de Blas et al. (1994) and Raj et al. (2002).

Serological studies of CMV have shown this species to be antigenically heterogeneous (Kaper and Waterworth, 1981; Palukaitis et al., 1992; Palukaitis and García-Arenal, 2003), which may cause detection problems (Wylie et al., 1993; Hu et al., 1995; Raj et al., 2002). Although several panels of antisera against CMV have been prepared, they vary considerably in their ability to recognize virus isolates (Palukaitis and García-Arenal, 2003). In our DAS-ELISA experiments the set of anti-Wic antibodies utilised detected all CMV isolates tested. In contrast, other ELISA sets failed to detect from one to three of the 15 CMV isolates examined.

IgG to isolate M (group I) reacted better with the homologous than with group II isolates, whereas Wic (group II) PAbs gave comparable ELISA reactions with both CMV groups. Observed differences in antibody specificity were similar to those described by Whayuni et al. (1992) and Yordanova et al. (2002).

The newly prepared antisera detected effectively CMV CP in Western blots. However, the difference between homologous and heterologous reaction observed in Western blotting was more pronounced than in ELISA. In a particular experiment (Fig. 3) isolate M was not detected in the blot with antisera Wic, while the CP band was visible in the blot developed with antisera M. In our previous experiments (Sliwa et al., 2008), the same isolate was detected with M and Wic antisera, although the bands observed for homologous isolate-antisera combinations were stronger than those of heterologous combinations. We speculate that low concentration of the virus may result in completely abolishing the heterologous but not the homologous reaction. Therefore, using both antisera simultaneously for Western blot detection of CMV is advisable.

The concentration of CMV in infected plants is usually high (Kaper and Waterworth, 1981). Therefore ELISA, which is simple, cheap and convenient for large-scale and routine testing, although not as sensitive as PCR, seems to produce satisfactory results in most cases. RT-PCR is becoming more widely used and is
considered to be the most appropriate assay for testing samples in which virus concentration may be exceptionally low (Wylie et al., 1993; Hu et al., 1995). The two antisera, prepared in this study, seem to be suitable for all the techniques we tested. They enabled reliable detection of all CMV isolates used in this study, especially when used together.

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