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Detection of geminiviruses from tropical countries by a double monoclonal antibody ELISA using antibodies to African cassava mosaic virus

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Summary — Using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) tests, monoclonal antibodies (mAbs), which were prepared against particles of a Nigerian isolate of African cassava mosaic virus (ACMV), differentiated African geminiviruses (tomato yellow leaf curl virus, TYLCV; ACMV from West and East Africa; and okra leaf curl virus) from those originating in other countries of the world (euphorbia mosaic virus; Indian cassava mosaic virus; and TYLCV (Indian isolate)). All these viruses belong to the whitefly-transmitted geminivirus subgroup III. One mAb reacted with all 7 tested viruses and isolates. For detecting TYLCV from Senegal, it proved necessary to use a reducing agent in extraction buffer. A diagnostic DAS-ELISA was developed. This relies only on the use of mAbs and is useful for large-scale field screening.

geminivirus III / monoclonal antibody / ELISA / African cassava mosaic virus / tomato yellow leaf curl virus

Résumé — **Détection de geminivirus de pays tropicaux par DAS-ELISA utilisant des anticorps dirigés contre le virus de la mosaïque africaine du manioc.** Des anticorps monoclonaux (AcMc) dirigés contre le virus de la mosaïque africaine du manioc («ACMV») (isolat du Nigéria) et utilisés dans un test DAS-ELISA («double antibody sandwich-ELISA») permettent de distinguer (à l'intérieur du sous-groupe III des geminivirus), les geminivirus originaires d'Afrique (ACMV souche Ouest et Est ; virus de l'enroulement jaunissant de la tomate : «TYLCV» ; virus de l'enroulement du gombo) de ceux provenant des autres continents du monde (virus de la mosaïque de l'euphorbe ; virus indien de la mosaïque du manioc ; TYLCV de l'Inde). L'un de ces AcMc réagit avec les 7 geminivirus testés. Néanmoins, la détection du TYLCV (isolat du Sénégal) nécessite un tampon d'extraction spécifique, contenant un agent réducteur. Un test de diagnostic ou de détection utilisant seulement des AcMc, et par conséquent applicable à grande échelle, a été mis au point.

geminivirus III / anticorps monoclonaux / ELISA / virus de la mosaïque africaine du manioc / virus de l'enroulement jaunissant de la tomate

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INTRODUCTION

Cassava mosaic disease (Warburg, 1894) is the most important factor limiting cassava yields in many parts of Africa (Storey and Nichols, 1938) and can be observed almost everywhere on the continent where cassava is grown (Fauquet and Fargette, 1990). The causal agent is transmitted by the whitefly *Bemisia tabaci* (Kufferath and Ghesquière, 1932) and is maintained and carried to new areas in vegetatively propagated planting material (stem cuttings). Cassava mosaic is caused by a geminivirus (Bock and Woods, 1983) first isolated in Kenya under the name cassava latent virus (Harrison *et al*, 1977; Bock *et al*, 1978) and since renamed African cassava mosaic virus (ACMV; Bock and Woods, 1983). It has been detected in Côte d'Ivoire (Walter, 1980), Angola (Sequeira and Harrison, 1982), Nigeria (Adejare and Coutts, 1982) and several other African countries (Harrison and Robinson, 1988; Harrison *et al*, 1991a).

ACMV can be transmitted by inoculation of sap from cassava to *Nicotiana benthamiana* and can be detected by immunosorbent electron microscopy (Sequeira and Harrison, 1982), enzyme-linked immunosorbent assay (ELISA) (Sequeira and Harrison, 1982; Fargette *et al*, 1987) with polyclonal antibodies, and by hybridisation tests with cDNA probes (Roberts *et al*, 1984; Robinson *et al*, 1984). ACMV is placed in the geminivirus subgroup III, whose members have bipartite genomes and whitefly vectors (Francki *et al*, 1991) and are typically serologically related to one another (Roberts *et al*, 1984). These relationships reflect the degree of amino-acid sequence identity (about 70% or more) among the coat proteins of different members (Hamilton *et al*, 1984; Howarth *et al*, 1985).

Among whitefly-transmitted geminiviruses, the particles of ACMV (Thomas *et al*, 1986), Indian cassava mosaic (ICMV; Aiton and Harrison, 1989) and okra leaf curl (OLCV; Swanson and Harrison, 1993) viruses have been used to raise monoclonal antibodies (mAbs). In tests with a panel of 17 mAbs to ACMV (Harrison and Robinson, 1988; Harrison *et al*, 1991a) virus isolates from cassava fell into 3 groups on the basis of their patterns of reactions. These groups had different geographical distributions: group A (Africa, west of the Rift Valley), group B (Africa, east of the Rift Valley),

and group C (ICMV; Indian subcontinent). Several of the mAbs to ACMV and ICMV reacted with other whitefly-transmitted geminiviruses infecting a wide range of crop and weed species in many countries in Africa, the Americas, Asia and Europe (Thomas *et al*, 1986; Harrison *et al*, 1991b; Muniyappa *et al*, 1991; Macintosh *et al*, 1992; Swanson *et al*, 1992a, 1992b). Some of the mAbs, in various combinations, were useful for detecting and identifying individual viruses, and others such as SCR20 detected nearly all the viruses.

The mAbs of Thomas *et al* (1986) were prepared to ACMV-JI, an isolate derived by Stanley (1983) from the Kenyan type strain of ACMV. As the plant-breeding program for the improvement of cassava in West Africa necessitated a field test for the diagnosis, and hopefully, the eradication of ACMV, we prepared mAbs to ACMV isolate TMS 30211 from Nigeria. For routine detection of ACMV, a double antibody sandwich (DAS) ELISA relying only on the use of mAbs was developed.

MATERIALS AND METHODS

Viruses

The viruses were maintained at Scottish Crop Research Institute (SCRI, Dundee, UK) at ca 25°C in an insect-proof glasshouse under licence from the Department of Agriculture and Fisheries for Scotland. The following hosts were used: *N benthamiana* for ACMV from West Africa (Nigeria) (ACMV-W) and ICMV from India; *Manihot esculentus* for ACMV from East Africa (Malawi) (ACMV-E); *Lycopersicon esculentum* from tomato yellow leaf curl virus isolates from Senegal (TYLCV-S) and India (TYLCV-I); *Abelmoschus esculentus* for OLCV from Africa (Côte d'Ivoire); and *N benthamiana* for euphorbia mosaic virus (EMV) from America (USA). ACMV-E was maintained in cuttings, TYLCV-S and TYLCV-I by grafting and OLCV by whitefly transmission.

At the INRA research station of Colmar (France) ACMV, isolate TMS 30211 (kindly provided by the International Institute for Tropical Agriculture, Ibadan, Nigeria) and other isolates of ACMV (Kounounguisa *et al*, 1989) were maintained in manually inoculated *N benthamiana* in an insect-proof glasshouse under controlled conditions (18–27°C, 16 h light/day).

Particles of ACMV TMS 30211 were purified according to Kounounguisa *et al* (1989). The purified preparations were stored frozen at –20°C after addition of 0.03% NaN₃.

Preparation of rabbit immunoglobulins

The methods described by Kounounguisa *et al* (1989) were used to prepare rabbit immunoglobulins.

Hybridoma production

For the first fusion experiment, 3–6-week-old BALB/c and B 10 Brown mice were immunized by 3 intraperitoneal injections of ACMV (TMS 30211 virus isolate). Each animal was injected with 75 µg purified virus for the 2 first injections and 100 µg for the third one. The virus was emulsified with an equal volume of Freund's incomplete adjuvant for the first injection and complete adjuvant for the second and third injections. The mice were rested for 10 weeks, and then 2 mice (1 B10 Brown and 1 BALB/c) were given 3 intraperitoneal booster injections with 50 µg virus in saline each on days 5, 4 and 3 before fusion. For the second fusion, 3–6-week-old BALB/c and B 10 Brown mice were immunized by 4 intraperitoneal injections at 2 weeks interval for the first 3 and then 1 month interval for the last injection (60, 45, 50 and 35 µg, respectively). Six weeks later, the mice were given 1 intravenous and 1 intraperitoneal booster injection of 34 and 80 µg virus in saline, respectively. Three days after the second booster injection, the spleens were excised. Cell fusion was performed as described by Al Moudallal *et al* (1982) using the PAI myeloma cell line (Stocker *et al*, 1982). Selected positive cultures were cloned by limiting dilution, using 10^7 thymocytes/ml as feeder cells.

All clones were derived from BALB/c mice cells. The clones were multiplied in pristane-primed BALB/c mice by injection 0.5×10^7 hybridoma cells in 0.5 ml Dulbecco's modified Eagle's medium into the peritoneal cavity. The ascitic fluid was harvested by tapping the peritoneum with a 19-gauge syringe needle, and the cells and debris were removed by centrifugation at 750 *g* for 30 min in a Jouan E96 centrifuge.

Immunoassay used for screening hybridomas

Wells containing growing hybridoma clones were screened for the presence of specific antibodies to ACMV TMS 30211 in both DAS and antigen-coated plates (ACP) indirect ELISA. In ACP-ELISA procedure the microtitre plates (Falcon, Oxnard, CA; Ref 3912) were coated with a purified preparation of ACMV (0.35 µg/ml) in 0.05 M carbonate buffer pH 9.6 (coating buffer) for 2 h. The DAS-ELISA tests were carried out using rabbit IgG raised against ACMV as a first antibody, at 1 µg/ml (2 h), in coating buffer, followed by incubation with the antigen at 0.35 µg/ml in phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T) overnight at 4°C. The rabbit IgG used in this test has been described previously (Kounounguisa *et al*, 1989). In both methods the remaining binding sites on the plastic plates were then saturated by incubation with 1% bovine serum albumin (BSA) in PBS-T. PBS-T

was used as the diluting buffer in the subsequent steps. Hybridoma culture supernatant fluids were diluted 1/3 or 1/5 and an alkaline phosphatase-conjugated affinitypure sheep anti-mouse IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1/3 000 was used as the detecting antibody. The bound conjugate was detected by addition of the substrate, *p*-Nitrophenylphosphate in 0.1 M diethanolamine buffer pH 9.8. Optical density (405 nm) values were measured with a Titertek Multiskan photometer (Flow Laboratories). Absorbance values were considered positive if they exceeded that of the buffer control by a factor of 3.

Labelling of reagent

For biotinylating mAbs, *N*-hydroxysuccinimidobiotin (Sigma) (biotin) dissolved in distilled dimethylformamide (0.2 mg/ml) was added to mAbs (ascitic fluid) diluted at around 1.5 mg/ml, in a 1:5 biotin/mAbs ratio (w/w); after incubating the mixture at 25°C for 2 h, the reaction was stopped by addition of 1 M NH_4Cl (Zrein *et al*, 1986). Alkaline phosphatase-conjugated streptavidin was from Jackson Immuno Research.

Crude extract preparation

Plant extracts prepared at SCRI were ground in mortar and pestle in 0.05 M Tris/HCl buffer, pH 8.0, 2% polyvinylpyrrolidone (PVP), 0.5% Tween 20 (1 g tissue in 10 ml buffer) containing 0.005 M EDTA. A specific buffer for TYLCV was devised through ELISA tests by optimising successive steps including buffer ion, pH, molarity and additives: 0.05 M Tris/HCl, pH 8.5, 60 mM sodium sulfite.

Extracts prepared at IBMC (Strasbourg, France) and the ORSTOM laboratory (Côte d'Ivoire) were made with cassava tip leaves rapidly frozen in liquid nitrogen in a chilled mortar, and ground with PBS-T (1 g tissue to 3 ml buffer) and sterilized quartz sand. The nitrogen step proved essential as otherwise no virus could be detected by ELISA test in the extract. For clarification, the crude extract was centrifuged at 3 000 *g* for 20 min in Jouan E96 centrifuge.

ELISA tests used for diagnosis

MABs were used in indirect DAS-ELISA (*Procedure 1*) as described in Muniyappa *et al* (1991). Microtiter plates were coated with 1 µg/ml γ -globulin from polyclonal antiserum to ACMV-W; infectious sap was prepared as described above; bound viral antigen was exposed to diluted ascitic fluid at a concentration high enough to give a strong reaction with the homologous virus (dilution between 10^{-4} to 10^{-5}), followed by 2 h incubation at room temperature with rabbit anti-mouse globulin alkaline phosphatase conjugate and substrate.

Optical density (OD) readings (A 405 nm) were taken after incubation with substrate for 1 or 2 h at room temperature and again after overnight incubation at 5°C.

Procedure 2 corresponds to direct DAS–ELISA. The mAb was diluted (ascitic fluid dilution 1:1 000) in coating buffer and incubated in microtiter wells for 3 h at room temperature. The viral antigens (crude sap) were diluted in 0.05 M Tris/HCl buffer, at pH 8.0 2% PVP, 0.5% Tween containing 0.005 M EDTA, and incubated over night at 5°C. Biotinylated mAb (dilution 1:10 000) was used to detect captured antigen (2 h at room temperature) and was revealed using streptavidin conjugated to alkaline phosphatase, diluted 1:1 000 in PBS-T containing 1% BSA and 2% PVP (2 h at room temperature) following by the substrate *p*-nitrophenyl phosphate. As in *Procedure 1*, readings were taken after incubation with substrate for 1 or 2 h at room temperature and again after overnight incubation at 5°C. The same mAb was used for coating the plate as second biotinylated antibody.

To detect ACMV in extracts from infected leaves of *N benthamiana*, mAbs were used at various concentrations: ascitic fluids were diluted from 10⁻³ to 10⁻⁴ for mAb 7 and from 5 x 10⁻⁴ to 10⁻⁵ for mAb 11.

Immunoblotting

Western blots tests were carried out as described by Harlow and Lane (1988). Samples of ACMV and TYLCV were extracted from infected leaves and mixed with sample buffer made of 60 mM Tris/glycine, 2% SDS, 100 mM dithiothreitol and 0.01% bromophenol blue.

RESULTS

Characterisation of the mAbs

Only one clone was obtained from the first fusion experiment, the mAb 41, which reacts only in

ACP – ELISA with purified ACMV. Two clones were obtained from the second one, mAb 7 and mAb 11, which react in ACP and DAS – ELISA. Table I indicates the reactions obtained in indirect DAS–ELISA when mAbs 7 and 11 were tested against a range of well-characterised geminiviruses obtained from various geographical areas and various host plants (see list in *Materials and methods*). The pattern of reactions was different for the 2 antibodies. mAb 11 reacted strongly with all tested geminiviruses. By contrast, mAb 7 reacted only with geminiviruses from Africa.

In Western-blot tests, mAbs 7 and 11 gave no reaction with ACMV in infected leaves of *N benthamiana*.

Immunoassays

The results in table I were obtained with virus material extracted in PBS with 0.5% Tween-20 and 2% PVP, pH 7.5. In order to establish if other extraction buffers gave better results, leaves infected with TYLCV were extracted in various buffers. Results obtained in direct DAS–ELISA with mAb 11 conjugated with biotin are shown in figure 1. Similar results were obtained in indirect DAS–ELISA. Low values were reached when standard buffers used for many viruses (Converse and Martin, 1990) and geminiviruses (Harrison *et al*, 1991b) were applied. A specific extraction buffer containing sodium sulfite (see *Materials and methods*) allowed much higher readings with TYLCV isolates from Senegal, Egypt, Nigeria, Burkina-Faso and Sicily. There was a linear relationship between OD (405 nm) values and the dilution of the extract (in a log/log representation; regression coefficient $R^2 = 0.98$). This allowed titration and comparisons of virus concentration in leaf extracts.

Table I. Reaction strengths (OD (405 nm)) after overnight incubation with substrate (4°C) in indirect DAS–ELISA test, with geminiviruses from various host plants and from different geographic origins (see list in *Materials and methods*) extracted in PBS with 0.5% Tween-20 and 2% PVP, pH 7.5.

Antibody	Virus						
	ACMV-W	ACMV-E	ICMV	TYLCV-S	TYLCV-I	OLCV	EMV
mAb 7	3	3	0	1	0	3	0
mAb 11	3	3	3	2	3	3	2

Reaction strength classification: 0: OD < 0.15; 1: OD = 0.15–0.60; 2: OD = 0.60–1.8; 3: OD > 1.8.

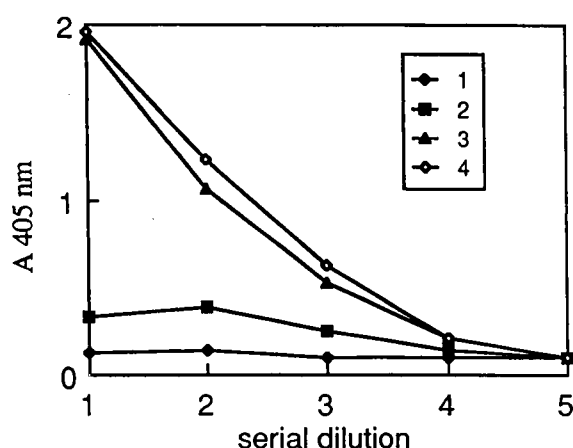


Fig 1. OD (405 nm) values in direct DAS-ELISA test with mAb 11 (see *Materials and methods*) for a 5-fold dilution series of TYLCV-S-containing sap extracted in: (—○—) PBS with 0.5% Tween-20 and 2% PVP, pH 7.5; (—■—) 0.05 M Tris/HCl, 0.005 M EDTA, pH 8.0, 2% PVP and 0.5% Tween; (—▲—) 0.05 M Tris/HCl, pH 8.5, 60 mM sodium sulfite; (—◇—) as for (3) + 1% mercaptoethanol.

When mAb 11 was conjugated with biotin and used in direct DAS-ELISA (see *Materials and methods*), ACMV-W was readily detected at dilutions of 1:1 000 and TYLCV-S up to 1:25 of the crude extracts (table II). However, the sensitivity of direct DAS-ELISA tests was lower than that of indirect DAS-ELISA tests, by a factor of 10 for ACMV and 1:25 for TYLCV. Coating the plates with mAbs gave values comparable and sometimes higher than those obtained when coating with polyclonal serum. For instance, in one set of experiments with TYLCV-containing extracts in direct DAS-ELISA, values were 1.65 with the

mAbs and 0.95 with the polyclonal antibodies, whereas background reactions with healthy sap were similar (0.12 and 0.13, respectively). In both tests however, the background values were higher than those obtained in indirect DAS-ELISA (0.12 vs 0.07).

Field tests

Indirect DAS-ELISA using mAb 11 has been successfully used for screening cassava plants for the presence of ACMV. The ORSTOM field cassava collection in Côte d'Ivoire and the glasshouse collection at the INRA research station of Colmar, which include varieties from several countries, were both tested in this way. Forty-one isolates were tested and yielded OD (corrected for background) ranging from 0.6 to 2.8 (Côte d'Ivoire: 13 isolates; Kenya: 8; Nigeria: 8; South American Continent: 4; India: 3; Madagascar: 3; Togo: 1; Central African Republic: 1). This test has also been successfully used (OD > 1.5) with the sodium-sulfite-containing buffer to detect geminiviruses in tomato leaf samples from Senegal, Egypt, Nigeria, Burkina-Faso and Sicily, in laboratories located in these countries or in laboratories in Europe that had received samples from there. In Egypt, these mAbs have also been successfully used to assess the presence of a suspected whitefly-transmitted geminivirus in the ornamental plant *Althea rosea* (Malvaceae) which exhibited symptoms close to OLCV and which might be a natural reservoir of this virus.

Table II. OD (405 nm) values obtained in direct DAS-ELISA and indirect DAS-ELISA with ACMV-W and TYLCV-S extracts in a 10-fold dilution series for ACMV and 5-fold dilution series for TYLCV and mAb 11.

Virus	Serial dilution					Healthy
	1	2	3	4	5	
ACMV	1/1	1/10	1/100	1/1 000	1/10 000	
direct DAS-ELISA	> 2	> 2	> 2	0.53*	0.11	0.08
indirect DAS-ELISA	> 2	> 2	> 2	> 2	0.51	0.06
TYLCV	1/1	1/5	1/25	1/125	1/525	
direct DAS-ELISA	> 2	1.32	0.48*	0.19	0.11	0.12
indirect DAS-ELISA	> 2	> 2	1.74	0.61	0.17*	0.07

The first dilution (1/1) is the crude extract prepared as described in *Materials and methods* (1 g leaf in 10 ml extraction buffer). * Last dilution giving a reading more than twice the background reaction obtained with healthy sap.

DISCUSSION

The ELISA results indicate that the 2 mAbs (11 and 7) that we have prepared with ACMV-W, recognize 2 different conformational epitopes, which are not preserved when the viral protein is denatured by treatment with SDS and dithiothreitol in Western blot tests. They also indicate that mAb 11 is directed against an epitope shared by 7 viruses or isolates from various hosts and locations, whereas the epitope recognized by mAb 7 is restricted to the 4 viruses or isolates from Africa. The contrasting patterns of reactions between the 2 mAbs suggest a geographical variation of the epitope profile, a feature already reported for other mAbs directed towards geminiviruses (Harrison and Robinson, 1988) and viruses from other groups (Matthews, 1990).

Any large epidemiological, sanitation or resistance program in a developing country implies a large number of tests and requires unrestricted access to inexpensive immunological reagents. Using monoclonal instead of polyclonal antibodies in the coating stages of the DAS-ELISA tests will overcome the difficulty linked to limited amounts of polyclonal antibodies. Direct DAS-ELISA using mAbs as captured antibodies and second biotinylated antibodies is sufficiently sensitive for the routine detection of ACMV and TYLCV. However, a more sensitive detection of the viruses can be achieved by indirect DAS-ELISA. The nature of the extraction buffer is also critical for optimal virus detection and must be adjusted for each geminivirus studied. For extracting TYLCV-S, for instance, it is necessary to use the reducing agent sodium sulfite, which presumably stabilizes the virus structure and maintains the antigenic reactivity in the ELISA test. Similar results have been described for tobacco leaf curl virus (Macintosh *et al*, 1992).

The 2 mAbs described in this article allowed the detection of whitefly-transmitted geminiviruses from various plants and from various geographical origins. They have also been successfully used to assess virus concentrations in plants and to search for natural reservoirs of the viruses. Furthermore, using mAb 11 in immunoblotting, one of us has recently detected geminiviruses in the following host plants in Egypt: tomato, okra, cucumber, *Datura stramonium*, *Cucurbita pepo*, and *Althea rosea* (Gosselin and Thouvenel, unpublished results). These results confirm the ability of mAbs 11 in the detection of geminiviruses in various plant species. However,

these tests must be complemented by other information such as symptomatology, transmission and host range when full identification of the virus is needed.

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