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Antigenic Analysis of the Coat Protein of *Alfalfa mosaic virus* and its Involvement in Aphid Transmission

H. Massumi[1*], P. Jones[2] and N. Hague[3]

ABSTRACT

Two strains -425 and Y87/47- of Alfalfa mosaic virus (AMV) were propagated in and purified from *Nicotiana tabacum* cv. Samsun NN. Thirty-three AMV specific monoclonal antibodies (MAbs) from two fusions were raised against strain 425. These antibodies were of isotypes IgG1 and IgM. MAbs recognised three types of epitope. Group I did not react with the virus particle surface or viral coat protein of two strains in PTA-ELISA, but they reacted with a 30-kDa structural coat protein of AMV by immunoblot analysis only and were able to recognise cryptotopes. Group II reacted with metatopes of both strains in PTA-ELISA. Group III reacted with a 30-kDa structural coat protein of AMV by immunoblot analysis and in PTA-ELISA for the Y87/47 strain only. Immunoblocking experiments in which suspensions of purified AMV and MAb were offered between parafilm membranes for acquisition by *Myzus persicae* revealed that MAb-2 was effective in blocking (inhibiting) transmission. This result suggests that the epitope which was localised by MAb-2 plays a role in the aphid transmission of AMV.

Keywords: ELISA, Immunoblot, Immunoblocking, Monoclonal antibody, Transmission.

INTRODUCTION

The genus *Alfamovirus*, belonging to the family *Bromoviridae*, has three genomic and one subgenomic RNA molecules. RNA1 (approximately 1.04×10^6 dalton) and RNA2 (0.73×10^6) are monocistronic. RNA3 (0.68×10^6 dalton) is bicistronic (Dore *et al.*, 1991). RNAs1 and 2 of the tripartite genome of this virus encode the replicase proteins P1 (126 kDa) and P2 (90 kDa) whereas RNA 3 encodes the movement protein P3 (32 kDa) and the viral coat protein P4 (24 kDa) (Van Dun *et al.,* 1987; Taschner *et al.*, 1994). The CP is also translated from a subgenomic messenger, RNA4 which is homologous to the 3' terminal 881 nucleotides of RNA3 (Langereis *et al*., 1986). The *Alfalfa mosaic virus* is transmitted by a number of different aphids in a non-persistent fashion. It is also reported to be transmitted by seed (Frosheiser, 1974; Hemmati & McLean, 1977), Cuscuta (Schemlzer, 1956) and pollen (Frosheiser, 1974).

Proteins, such as those of virus capsids, are multi-determinant antigens (Al Moudallal *et al*., 1982) and are made up from amino acid sequences. The monospecificity of MAbs is effective in elucidating the complex antigenic structure of proteins (Al Moudallal *et al*., 1982). It may be used to identify the relationship between viral coat protein and transmission by a vector, i.e. the role of an epitope in transmission.

It has been shown that particle proteins are effective in the transmission of several viruses by their vectors (Harrison and Robinson, 1988). MAbs can be used to detect differences among particle protein epitopes that associate with transmission by the following examples.

[1] Department of Plant Pathology, Shahid Bahonar Kerman University, Kerman, Islamic Republic of Iran.
[2] Department of Plant Pathology, IACR Rothamsted, Harpenden, Herts, AL5 2JQ, U. K.
[3] Department of Agriculture, University of Reading, P. O. Box: 236, Reading, RG6 2AT, U. K.
[*] Corresponding author, e-mail: masoomi@mail.uk.ac.ir

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Massalski and Harrison (1987) reported the production of MAbs by *Potato leafroll virus* (PLRV) and their use to distinguish virus isolates differing in aphid transmissibility. They identified epitopes that depended on the quaternary protein conformation being involved in the passage of PLRV particles from the haemolymph to the salivary glands of *Myzus persicae* (Sulzer). The absence of these epitopes on a poorly transmissible PLRV isolate apparently did not influence virus acquisition or retention by *M. persicae*.

Vector specificity is mainly determined by the ability of luteovirus particles to cross from the haemocell into the cells of the accessory salivary glands. This is supported by the evidence from ultrastructural studies with *Barley yellow dwarf virus* (BYDV) (Gildow and Gray, 1993). In thin sections of the basal lamina or intracellularly in the accessory salivary gland cells in treatments with the specific MAbs no virus particles were observed. However many particles were observed in these tissues in the control treatments. This indicates that MAb binding interfered with virus capsid recognition and penetration of the basal lamina. It is also possible that these MAbs reacted with an epitope which is important for the recognition of a virus capsid by membrane receptors (Torrance, 1995). This paper describes the production of a panel of MAbs to AMV, their reactions with different epitopes, and further investigation of the involvement of surface epitopes in transmission of the virus by *M. Persicae*.

MATERIAL AND METHODS

Virus

AMV strains 425 and Y87/47 were maintained on *Nicotiana tabacum* cv. Samsun NN plants. The virus was purified from *N. tabacum* cv. Samsun NN as described by Van Vloten-Doting and Jaspars (1972).

Aphids

M. Persicae were obtained from cultures maintained at Rothamsted Experimental Station. Insect transmissions were done using *N. tabacum* cv. Samsun NN infected with AMV strain 425 or Y78/48 as the source of inoculum.

Immunization and Antibody Detection

Two mice (Balb / appnoxinately six week-old females) were immunized with purified AMV strain 425.

200 µg of purified virus was prepared in an equal volume of PBS for immunization by interperitoneal injection on day 1. Mouse 1 received an interperitoneal injection, with the same dose of purified virus, on days 23 and 48. Similar injections were given for mice 2 on days 33, 67 and 92.

Cell Fusion

Four days after the final boosts, the mice were sacrificed and their spleens were removed. The spleen cells were then fused with the myeloma cell line SP2 / 0-Ag 14 (ECACC NO: 85072401) by spinning together in the presence of 50% polyethylene glycol (molecular weight 1500) and 10% dimethyl sulphoxide (99.5% pure) after the method of Kennet *et al*., (1978). All fused cells (hybridomas) were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 20% v/v foetal calf serum (FCS) (DMEM/20) with peritoneal macrophages from young mice (Campbell, 1984).

Hybridoma Production and Antibody Screening

Fused cells were placed in 96-well plates at 37°C with 8% CO_2. For the detection of antibodies the supernatant of the cultures

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was screened by ELISA after cell fusion as well as after cloning. Hybridomas that produced antibodies reacting with AMV were bulked to 24-well plates (Nunclon) and stocks frozen in liquid Nitrogen. Cell lines were cloned twice by limiting dilution (Harlow and Lane, 1988) in DMEM/20 + Fibroblast conditioned media. Subclones were then bulked to 25cm^2 tissue culture flasks (Nunclon) and supernatants were drawn off for screening when growth was confluent and the medium was beginning to show signs of acid production (i.e. turning yellow). All subclones of interest were frozen in a mixture of 90% foetal calf serum and 10% DMSO at –70 ^{o}C in a Cryo Freezing Box (Nalgene) for 24-48 h and then transferred to liquid nitrogen.

Typing of Monoclonal Antibodies

All MAbs were isotyped on Maxisorb (Nunc) microtitre plates, using mouse MAb Isotyping reagents (Sigma Chemical Co. Ltd), following the manufacturers instructions for a capture ELISA.

Plate-trapped Antigen ELISA (PTA-ELISA)

To detect MAbs that recognised epitopes on the virus particle surface or viral coat protein, plate-trapped antigen ELISA (PTA-ELISA) as described by Gallo and Matisoa (1993) was chosen.

Immunoblotting

Proteins were separated by gel electrophoresis in a Mini Protean 11 Slab cell (Bio-Rad) as described by the method of Laemmli (1970). Separated proteins were detected by staining with Coomassie blue R-250. Immunoblotting was carried out as described by Dietzgen and Francki (1988). Nitro blue tetrazolium/phenazine methosulphate/5-bromo-4-chloro-3-indolyl phosphate (Ey and Ashman, 1986) was used as the alkaline phosphatase substrate.

Immunoblocking of AMV Transmission

The culture supernatants from the three different monoclonal antibodies (MAb-1,2 and 22), which recognised the coat protein of AMV (Plate 1), containing 0.5 mg ml^{-1} were mixed at two different ratios (1:1 and 1:20 v:v) with purified virus (5 mg ml^{-1}) and 5% (w/v) sucrose. Purified virus mixed with cell culture media served as a control in these experiments. The suspensions were offered for acquisition between parafilm membranes to one-day old *M. persicae* nymphs as described by Pirone (1964). Around thirty plants were tested per experimental combination and ten nymphs were placed on each test plant and left overnight (14-18 h). After inoculation access, test plants were sprayed with a pyrethroid insecticide and kept in a greenhouse at a temperature varying between 18-24^{o}C for symptom development. After 15 days an indirect-ELISA (Clark and Adams, 1977) was used to check the infection status of all test plants (Table 2).

RESULTS

Production of Hybridoma and Antibody Isotypes

Initial screening of 1166 hybridomas derived from two separate fusions revealed 430 which secreted antibodies to AMV, 168 of these hybridomas were subjected to cloning. After two cycles of cloning by limiting dilution, thirty three stable AMV specific hybridoma cell lines were obtained and grown on for mass culture. These MAbs were designated MAb-1 to MAb-33.

Of 33 stable cell lines, 28 were of in the IgG1 subclass and 5 of the IgM class (Table 1).

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Table 1. Some properties and the reaction of different MAbs with two strains of AMV in PTA-ELISA.

Group No	*Antibody No*	*Isotype*	*Derived from mouse No*	Native virus		Coat protein	
				425	Y87/47	425	Y87/47
I	MAb-1	IgM	1	-	-	-	-
	MAb-2	IgM	1	-	-	-	-
	MAb-3	IgM	2	-	-	-	-
	MAb-4	IgM	2	-	-	-	-
	MAb-5	IgM	2	-	-	-	-
II	MAb-6	IgG1	2	32[a]	2	32	4
	MAb-7	IgG1	2	32	8	32	8
	MAb-8	IgG1	2	64	4	32	8
	MAb-9	IgG1	2	32	8	16	32
	MAb-10	IgG1	2	32	8	32	8
	MAb-11	IgG1	2	32	4	16	16
	MAb-12	IgG1	2	64	8	16	8
	MAb-13	IgG1	2	128	32	64	16
	MAb-14	IgG1	2	64	16	64	32
	MAb-15	IgG1	2	64	16	64	16
	MAb-16	IgG1	2	1	0.01	0.01	2
	MAb-17	IgG1	2	64	8	16	16
	MAb-18	IgG1	2	16	4	8	16
	MAb-19	IgG1	2	256	32	32	16
III	MAb-20	IgG1	2	32	4	-	16
	MAb-21	IgG1	2	32	4	-	16
	MAb-22	IgG1	2	32	16	-	16
	MAb-23	IgG1	2	32	4	-	32
	MAb-24	IgG1	2	128	16	-	32
	MAb-25	IgG1	2	64	16	-	32
	MAb-26	IgG1	2	64	16	-	32
	MAb-27	IgG1	2	64	4	-	16
	MAb-28	IgG1	2	64	4	0.01	16
	MAb-29	IgG1	2	128	4	-	32
	MAb-30	IgG1	2	64	8	-	32
	MAb-31	IgG1	2	64	2	-	16
	MAb-32	IgG1	2	64	2	-	16
	MAb-33	IgG1	2	128	4	-	32

[a:] Titre of MAb (in thousands)
(-): No reaction

Reaction of MAbs in PTA-ELISA and Differentiation of AMV Strains

The thirty three MAbs could be assigned to one of three groups (I, II and III) on the basis of their reaction with the AMV virus particle and viral coat protein of two strains of AMV (425 and Y87/47) in PTA-ELISA. The first group (MAb-1 to 5) did not react with the virus particle surface or viral coat protein of two strains. The second group (MAb-6 to 19) recognised the viral particle surface and the viral coat protein of both strains. The third group (MAb-20 to 33) had a reaction with the virus particle of both strains and the viral coat protein of Y87/47, except MAb-28 which had a very low reaction with the 425 coat protein.

In addition, in a PTA-ELISA, the difference in MAbs avidity to the strain 425 was more marked than to Y87/47, so 28 MAbs were able to distinguish the difference between the virus strains (Table 1).

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Investigation of AMV Epitope using MAbs in Immunoblotting Test and ELISA

In the reactivities of MAbs-1 to 5 (group I), the five IgM antibodies which did not recognise the isolated coat proteins and native AMV particles of two AMV strains in PTA-ELISA were further investigated by immunoblotting. These MAbs (group I) recognised the cryptotope of two AMV strains when virus preparations were dissociated in the presence of SDS and β mercaptoethanol. This recognition appears to be specific to AMV because no reactions were detected in similar experiments with preparations of protein extracted from the *Tobacco mosaic virus* (Plate 1). Reactivity of MAbs 6-15 and 17-19 (group II) with coat protein and native virus in both strains was evidence of the existence of metatopes associated with AMV particles. MAb-16 also had a low reaction with both the native virus and coat protein of the two strains. Reactivity of only 13 MAbs (20 – 33, group III) with the native virus, in strain 425, provided evidence for the existence of neotopes associated with AMV particles. In immunoblotting test, these MAbs (group III) appears to recognise a cryptotope, which is exposed on the viral coat protein subunit of both strains.

Immunoblocking of AMV Transmission

The three MAbs were mixed at two different ratios with purified AMV. The results of transmission following the mixture of virus with MAb-1 at a ratio of 1:1 and 1:20 were three out of 29 and two out of 30 respectively. In addition, the mixture of virus with MAb-22 at a ratio of 1:1 and 1:20 were two out of 30 and three out of 30 respectively. However, with MAb-2 at both ratios the result was 0 out of 30 (Table 2).

DISCUSSION

The first reports of MAbs raised to AMV were by Halk *et al*. (1984) and Halk (1986) who found that, in the course of immunisation, antibodies are formed which are capable of reacting with corresponding epitopes on the native virus (neo- and metatopes) and with the viral coat protein (cryptotopes). Similar conclusions were

Table 2. The effect on insect transmission of two different MAbs (MAb-1 and MAb-2) in aphid acquisition tests.

No of Mab	Ratio of Virus: MAb (V:V)	Experiment 1	2	3	Total
MAb-1	1:20[a]	2/10[c]	0/10	0/10	2/30
MAb-1	1:1[b]	0/10	1/10	2/9	3/29
MAb-2	1:20	0/10	0/10	0/10	0/30
MAb-2	1:1	0/10	0/10	0/10	0/30
MAb-22	1:20	1/10	1/10	1/10	3/30
MAb-22	1:1	1/10	0/10	1/10	2/30
Purified virus (strain 425) with medium	-	1/10	2/10	1/12	4/32

[a] 1mg virus mixed with 2mg MAbs
[b] 10 mg virus mixed with 1 mg MAbs
[c] Number of plant infected / number of test plant of *N. tabacum* cv. Samsun NN ten aphids per each test plant

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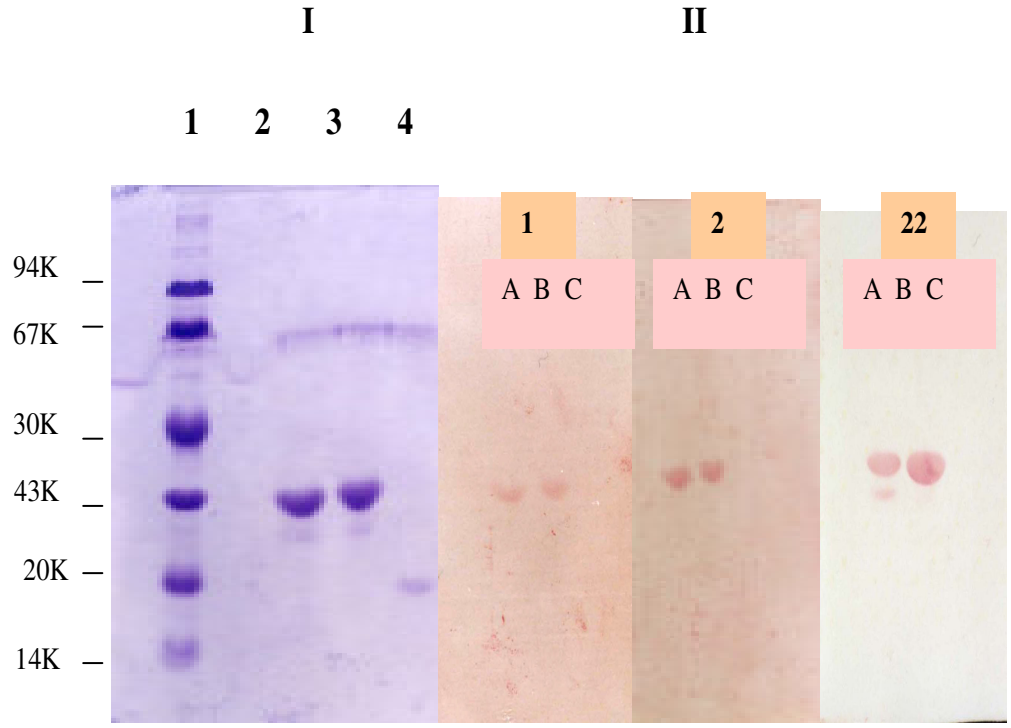

Plate1: (I) SDS – PAGE of AMV coat protein before immunoblotting. The gel was stained with Coomassie blue. Molecular weights of marker protein (M) are given in kilodaltons. Lane 1; Protein markers; Lane 2 purified virus from 425 strain; Lane 3 purified virus from Y87/47 strain; Lane 4 purified TMV as a control. Positions of molecular weight markers are shown on the left. (II) Immunoblotting with purified preparations of 425 strain (A), Y87/47 strain (B) and TMV(C). The blots were probed with antisera (1) MAb-1, 2 and 22.

obtained by Hajimorad *et al*. (1990) and Gallo and Matisova (1993). Hajimorad *et al*. (1990) raised their MAbs by immunisation with a mixture of the five AMV isolates and obtained 15 MAbs with isotypes IgG1 and IgM. Gallo and Matisova (1993) obtained 7 MAbs, of two isotypes (IgG1 and IgG2a) by immunisation of only one AMV isolate. The results obtained here considerably extend those of previous workers, because thirty three mouse MAbs were raised against only one AMV strain (425). In addition, two types of IgG1 and IgM MAbs isotypes were obtained similar to Hajimorad *et al*., (1990).

The properties of the thirty three MAbs specific for AMV were studied in detail. The ability of different monoclonal antibodies to detect epitopes was found to be extremely variable in both PTA-ELISA and immunoblotting tests, despite the fact that the majority of antibodies that recognised those epitopes were located either on the outside or on the inside of coat protein. These MAbs, in different reactions, apparently recognised three types of epitopes. MAbs-1 to 5 the five IgM (group I), reacted with denatured coat protein and were able to recognise cryptotopes. These epitopes seem to be a continuous epitope, because then they were not destroyed when the particles were boiled in SDS-containing buffer. MAbs 20-33 (group III) also reacted with 30-kDa structural coat protein of AMV by immunoblot analysis, which is evidence that they recognise cryptotopes of both strains. However, these MAbs (20-33) in

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PTA-ELISA only reacted with the native virus in strain 425, indicating that they recognised the neotopes associated with the AMV particle. Furthermore these MAbs (in PTA-ELISA) also reacted with native and coat protein (metatopes) of strain Y87/47 (Table 1). The recognition of three types of epitope (crypto-neo- and metatopes) by this group (III) raises the question of whether they were a mixture of MAbs. However, this seems unlikely because they only reacted with antiserum to IgG1 in both ELISA and immunoblotting. In addition, the hybridoma secreting the antibody was obtained after two cycles of limited dilution cloning. Furthermore, Shukla *et al.* (1989) have reported MAbs with the ability to recognise multiple epitopes occurring in potyvirus antigens. MAbs 6-15 and 17-19 (group II) also reacted with metatopes of both strains in PTA-ELISA.

These results along with previous reports (Halk 1986; Hajimorad *et al*., 1990; Gallo and Matisova, 1993) confirm that AMV has a major population of epitopes, in different strains, present either inside or on the outside of coat protein. Antibodies 1-5 or 6-19 are the same isotype and reacted in a similar manner to the two strains and hence it is possible that these antibodies are specific to the same epitopes (or the overlapping epitopes) of the two strains of AMV being tested.

Previous studies of AMV coat protein (Hajimorad *et al*. 1990) have reported the presence of only one band of Mr 30 kDa, which is consistent with the result, described here (Plate 1). It seems likely that proteolytic degradation of preparations was the reason for the presence of a few smaller molecular weight bands observed in some cases. Therefore, these results and those reported by Halk (1986), Hajimorad *et al*., (1990) and Gallo and Matisova (1993) demonstrate that the AMV coat protein contains several epitopes arranged in different conformational structure.

MAbs have been used here for the first time to study the transmission mechanism of AMV. The blocking of aphid transmission or its reduction may occur as a result of MAbs binding with epitopes on the virus capsid which recognise retention sites such as the stylets tips, the food canal and the foregut of the vector mouthparts. AMV transmission was blocked (or probably inhibited) by MAb-2 in two different ratios of 1:1 and 1:20 (virus: MAbs, v:v). However, MAb-1 and 22 had no inhibiting effect as indicated in Table 2. The most prominent of these differences is probably characterised by the effect of a special epitope in transmission that was detected by MAb-2.

Van den Heuvel *et al*. (1991) reported the inhibiting effect of simultaneous acquisition of MAbs and purified PLRV on virus transmission by *M. persicae*. After the simultaneous acquisition of MAbs and PLRV, six of nine MAbs tested had an inhibition effect of between 13-32% on the virus transmission. MAbs are also used to investigate the relationship between aphid-transmission and surface epitopes of the capsid protein of PLRV (van den Heuvel *et al*. 1993). Epitopes on the surface of the aphid species-specific transmission of BYDV isolates interacts specifically with a receptor site on the salivary gland membrane of the aphid. MAbs recognised these epitopes that are located at the BYDV virion surface, using a triple antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Rizzo and Gray, 1992). Two MAbs cross reacted with specific epitopes and this may be important for transmission of *African cassava mosaic virus* by *Bemisia tabaci*(Gennadiuf) (Thomas *et al*., 1986). There is a relationship between aphid transmission of *Potato virus A* (PVA) and reactivity with MAb A5B6 (Andreeva *et al*., 1994). This MAb reacted in ELISA and an immunoblotting assay with non-transmissible isolates, but responded weakly or not at all with transmissible isolates.

The result (Table 2) clearly indicated the potential of MAb-2 to bind to an epitope on the AMV coat protein and block transmission. Because the number of infected plants was zero after using this antibody in transmission, the experiments

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show the reduction in transmissibility of the virus by the aphids (Table 2). Van den Heuvel *et al*. (1991, 1993) applied MAbs in the transmission of *Potato leaf roll virus*H (PLRV) to find out whether a specific epitope was involved in aphid transmission. After the simultaneous acquisition of MAbs and PLRV, six MAbs considerably reduced virus transmission between 13 and 32%. In comparison to AMV the transmission of PLRV (using efficiently transmitted isolates) with aphids is very high (70%) which may have been the reason for its non-transmission (blocking), when mixed with an antibody.

It is concluded that the site on the coat protein of AMV which has a function in aphid transmission, recognised by MAb-2, may indicate a relation between the virus and aphid.

It seems that there are few epitopes which are involved in the transmission of AMV and MAb-2 reacted with only one of them. However the lack of transmission (Table 2) has the effect of blocking specific epitopes on the viral capsid by MAb-2, coupled with its low aphid transmission of AMV (Pirone and Megahed, 1966).

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بررسی خاصیت آنتی ژنی پوشش پروتئینی ویروس موزائیک یونجه و نقش آن در انتقال ویروس توسط شته سبز هلو (*Myzus persicae*)

ح. معصومی، ف. جونز و ن. هوگو

چکیده

به منظور بررسی نقش پوشش پروتئینی ویروس در انتقال توسط شته سبز *Myzus persicae* دو سویه ٤٢٥ و Y٨٧/٤٧ ویروس موزائیک یونجه در گیاه توتون رقم سامسون تکثیر و خالص سازی گردیدند. سپس علیه سویه ٤٢٥ این ویروس تعداد ٣٢ نوع آنتی بادی مونوکلونال تهیه شد. آنتی بادیهای حاصل از نوع ایزوتایپ های IgG1 و IgM بودند و بر اساس واکنش در مقابل سویه های یاد شده به سه گروه عمده تقسیم شدند. گروه اول هیچگونه واکنشی در تست الیزا نشان ندادند اما واکنش آنها در وسترن بلات پوشش پروتئینی این ویروسها مثبت بود. گروه دوم در تست الیزا با هر دو سویه واکنش نشان دادند. گروه سوم با وسترن بلات پوشش پروتئینی هر دو سویه واکنش نشان داده اما در تست الیزا تنها قادر به واکنش در مقابل سویه ٤٢٥ بودند. برخی از این آنتی بادیها با ویروس خالص شده (سویه ٤٢٥) مخلوط گردیدند و شته‌ها به مدت یک دقیقه از سوسپانسیون ویروس (حاوی ٥ در صد سوکروز) از طریق غشاء پارا فیلم مورد تغذیه قرار گرفتند اما از بین این آنتی بادیها فقط آنتی بادی شماره ٢ قادر به ممانعت از انتقال ویروس توسط این شته بود. نتایج حاصل از این بررسی بیانگر آن است که اپی توپ تشخیص داده شده به وسیله این آنتی بادی احتمالا در انتقال ویروس دخیل است.

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