SEROLOGICAL DETECTION OF TOMATO SPOTTED WILT VIRUS (TSWV) PROTEINS AND IDENTIFICATION OF COMPONENTS OF TSWV ACQUISITION BY THRIPS

By

MURALIDHAR BANDLA

Bachelor of Agricultural Sciences
A.P. Agricultural University
Hyderabad, India
1980

Master of Agricultural Sciences
Annamalai University
Annamalainagar, India
1982

Submitted to the Faculty of the
Graduate college of the
Oklahoma State University
In partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1997

SEROLOGICAL DETECTION OF TOMATO SPOTTED WILT VIRUS (TSWV) PROTEINS AND IDENTIFICATION OF COMPONENTS OF TSWV ACQUISITION BY THRIPS

Thesis Approved

John L. Sherwood

Thesis Adviser

Jacqueline Fletcher

Carol Bender

Kim Bu

Wayne B Powell

Dean of the Graduate College

ACKNOWLEDGEMENTS

I am grateful to my major advisor and the Chair of my graduate committee Dr. John L Sherwood for his teaching, technical guidance and encouragement. He is my mentor and the skills he taught me are invaluable in my career development. I wish to express my sincere appreciation to the committee members, Dr. Jacqueline Fletcher, Dr. Carol Bender and Dr. Kim Burnham for their guidance and advice.

I also wish to extend my appreciation and gratitude to Dr. Thomas L German, University of Wisconsin, Dr. Diane Ullman, University of California, for her suggestions and her staff, Ms. Leslie Campbell and Ms. Daphne Westcot, for their help. Thanks also goes to Ms. Lisa Myers for lab assistance and help.

My special thanks to my wife Radha and the kids for their love, patience and encouragement during the difficult times. Finally, I would like to thank the Department of Plant Pathology and Division of Agricultural Sciences and Natural Resources for the support for this study.

TABLE OF CONTENTS

LIST OF TABLES

Chapter **Page**

LIST OF FIGURES

Chapter **Page**

NOMENCLATURE

ACMBs	antigen coated magnetic beads
ACP-ELISA	antigen coated plate ELISA
BSA	bovine serum albumin
BYDV	barley yellow dwarf virus
CaMV	cauliflower mosaic virus
CDRs	complementary determining regions
CRS	cellular receptor site
DAS-ELISA	double antibody sandwich ELISA
E-BB	empigen BB
ELISA	enzyme linked immunosorbent assay
GBNV	groundnut bud necrosis virus
GP1	glycoprotein 1
GP2	glycoprotein 2
INSV	impatiens necrotic spot virus
L protein	large protein
L RNA	large RNA
M RNA	middle RNA
N protein	nucleocapsid protein
NFDM	non fat dry milk

NSm	non-structural protein encoded by M RNA
NSs	non-structural protein encoded by S RNA
PBS	phosphate-buffered saline
PLRV	potato leafroll virus
S RNA	small RNA
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAS-ELISA	triple antibody sandwich ELISA
TBS	Tris-buffered saline
TSWV	tomato spotted wilt virus
V	viral sense
VAP	viral attachment protein
Vc	viral complimentary sense

Chapter 1

Tomato spotted wilt virus and scope of the dissertation

Introduction

Tomato spotted wilt disease, first described by Brittleband in 1919 in Australia (4), was determined to be of viral etiology by Samuel et al., in 1930 and the causal agent was named tomato spotted wilt virus (TSWV). By 1980, TSWV had been reported from many parts of the world and had become one of the most economically damaging viruses of agricultural crops (5). TSWV has a host range of at least 550 species of plants in over 75 families that include important vegetable, fruit and ornamental crops (29). Disease symptoms include ring patterns, severe mottling, silvering, wilting, stunting, chlorosis and necrosis of plant tissue that vary with the crop and environment (10).

In nature, TSWV is exclusively transmitted from plant to plant by several species of thrips (Thysanoptera:Thripidae). Among the 20 genera in the family Thripidae, species in the genera *Frankliniella* and *Thrips* transmit the tospoviruses, among which the western flower thrips (WFT), *Frankliniella occidentalis* Perg., is considered to be the most important in the western hemisphere (11). Only when larvae acquire TSWV are the consequent adult thrips capable of transmitting virus in a persistent manner (23). TSWV replicates in its thrips vector (26,28) and transmission of TSWV by adult thrips occurs

intermittently for the duration of their lives. Recent evidence indicates that the virus is retained through overwintering thrips (personal communication from Lloyd Garcia, Department of Entomology, North Carolina State University, Raleigh, NC). As many weeds and crop plants are hosts for thrips and TSWV, infected plants may play an important role in disease etiology (3,6,13).

In the fourth report of the International Committee on Taxonomy of Viruses in 1982, TSWV was indicated as the only member of the tomato spotted wilt virus group. In 1984, based on the genomic organization of TSWV, Miline and Francki (19) suggested that TSWV be considered a possible member of the family Bunyaviridae, a family consisting of about 342 viruses infecting animals. Since that time the data accumulated on the TSWV genome confirms this relationship with the Bunyaviridae, and as a result the genus *Tospovirus* was established within the family Bunyaviridae with TSWV as the type member (9).

Different serogroups of tospoviruses are defined by the serological reactivity to polyclonal antibodies raised against the nucleocapsid protein of TSWV and the sequence homology of S RNA that encodes the nucleocapsid protein. (1,8). Of the 5 serogroups that have been defined (Table 1) the most serious pathogens in North America are TSWV and impatiens necrotic spot virus (INSV). Several other TSWV-like plant viruses, which are serologically distinct, have been reported from different parts of the world. The serogroups are temporary taxa established to accommodate the uncharacterized species that

are serologically reactive (12). It was recommended that, to establish the identity of a species, the serogroup should show N protein sequence with <90% identity to TSWV. It was also recommended that biological characterization based on host range, host specificity and vector specificity should aid in species characterization.

Tospoviruses are spherical, 80-120 nm in diameter, and enveloped with a lipid membrane containing two viral glycoproteins (GPs). Purified virions contain four structural proteins; a 331.5 kDa large protein (L), 78 kDa and 58 kDa glycoproteins (GP1 and GP2) and the 29 kDa nucleocapsid protein (N) (20,25). The genome consists of three linear single stranded RNA molecules: a 8.9 kb large RNA (L RNA), a 4.8 kb middle RNA (M RNA) and a 2.9 kb small RNA (S RNA), each coated with multiple copies of N protein (7,15,16).

The S RNA and M RNA are ambisense, i.e., the RNA encodes one protein in the messenger sense or viral sense (V) and another protein in the viral complimentary sense (Vc). Thus, the ambisense S RNA encodes a 52.4 kDa non-structural protein (NSs) in the V sense and the 29 kDa N protein in the Vc sense (7). The 4.8 kb M RNA encodes the 33.6 kDa non-structural protein (NSm) in the V sense and a 127.4 kDa polyprotein, the precursor for the viral membrane glycoproteins GP1 and GP2, in the Vc sense (15). The L RNA encodes a 331.5 kDa L protein in the Vc sense. The ambisense nature of S and M RNA is unique to tospoviruses and is not found in animal-infecting bunyavirus

in the family Bunyaviridae.

In the family Bunyaviridae, GP1 and GP2 are implicated as viral attachment proteins (VAPs) that bind to surface receptors of the host cells and are involved in maturation and assembly of virions (21,22). However, in tospoviruses the specific roles for the glycoproteins in viral replication or pathogenesis have not been established. NSm was shown to have a potential role in cell-to-cell movement (14). The role of NSs is not known. However, as NSs has the least conserved protein sequence homology among tospoviruses, it may not have a role in genomic replication (27). The L protein has been well studied by Adkins et al. (2) and is implicated in viral genome replication. They documented virion associated replication activity, nuclease and cap snatching activity implicating L protein as the replicase (2).

Despite the availability of several monoclonal and polyclonal antibodies to TSWV, a rapid and specific non-sandwich enzyme-linked immunosorbent assay (ELISA) is lacking for diagnosis of TSWV. An antigen-coated plate ELISA (ACP-ELISA) permits screening of multiple samples in less time with fewer reagents than are required for traditional ELISA. TSWV virions do not bind well to the ELISA plates because of the poor binding of glycoproteins to the polystyrene plates. However, NSs can be used as the target antigen, as it binds to the ELISA plates and is expressed in a concentration in infected plants sufficient to be detected by ELISA. The disadvantages of the ACP-ELISA are lower sensitivity and possible cross-reactivity. In certain instances antibodies

may not bind to the antigen in an ACP-ELISA due to the conformational changes in antigens caused by their binding to the polystyrene. Because monoclonal antibodies have a single affinity for a defined epitope and can be produced in unlimited quantities, monoclonal antibodies selectively developed against sequential as well as conformational epitopes may be advantageous over a polyclonal antibody in ACP-ELISA. By employing novel screening methods, high-affinity monoclonal antibodies can be preferentially produced. Chapter Two in this thesis describes the production of such monoclones against NSs protein and development of an ACP-ELISA for detection of TSWV.

Identification and determination of the relative percentage of viruliferous thrips (thrips that acquired the virus as larvae and thus can transmit the TSWV as adults) from a population of thrips that includes viruliferous and non-viruliferous thrips (thrips that acquire TSWV as adults as a result of feeding on infected plants, and thus cannot transmit the TSWV to a healthy host) would allow vector populations to be used as a predictor of TSWV incidence in a preplanting forecast. Transmission assays or serological assays employing antibodies to TSWV structural proteins have been used to identify viruliferous thrips from the field and greenhouse. Transmission assays have a limited utility in preplant forecasting as it usually takes 2-3 days for symptom development on indicator plants, whereas serological assays overestimate the number of potential transmitters since the non-viruliferous thrips also may test positive. Since the presence of NSs is indicative of TSWV replication, immunological

assays using anti-NSs antibodies can differentiate the viruliferous thrips from non-viruliferous thrips. In Chapter Three the use of the ACP-ELISA in detection of TSWV in its thrips vector is discussed.

The specific mechanisms involved in TSWV acquisition by its thrips vector have not been well studied. The fidelity and specificity involved in virion acquisition is indicative of VAP(s) on the virus and corresponding cellular receptor site(s) (CRS) in the vector. Antibodies can be successfully employed to study and characterize the interaction between VAPs and CRSs. In the family Bunyaviridae, GP1 and GP2 are implicated as VAPs. To establish the role of GPs in thrips acquisition of TSWV and to identify the receptors in thrips, monoclonal antibodies to GP1 and GP2, and their corresponding anti-idiotype antibodies, were produced (Chapters Four and Five)

There are two significant areas of research presented in this dissertation. First, antibodies to NSs are used in ACP-ELISA for rapid detection of TSWV in infected plants and to identify viruliferous thrips. Second the putative role of GPs as VAPs was confirmed and a putative corresponding CRS in thrips was identified. Monoclonal antibodies and anti-idiotype antibodies made against GP1 and GP2 were used to detect the putative CRS in the thrips' midgut. In addition, new methodologies for elution of antigens from sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) gels and production of high affinity antibodies were examined.

References

1. Adam, G., and Kegler, H. 1994. Tomato spotted wilt virus and related tospoviruses. Arch. Phytopath. Plant Protect. 28: 483-504.

2. Adkins, S., Richmond, K., and German, T. L. 1996. Enzyme activities associated with virions of tomato spotted wilt virus. In: International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. C. George Kuo, G. D. Peters and T. L. German, eds. Acta Horticulturae 431:209-218.

3. Bon, W. P., Whitman, H. K., and Black, L. L. 1983. Indigenous weeds as reservoirs of tomato spotted wilt virus in Louisiana. Phytopathology 73:499.

4. Brittlebank, C. C. 1919. Tomato diseases. J. Agric. Victoria 17:231-235

5. Cho, J. J., Mitchell, W. C., Mau, R. F. L., and Sakimura, K. 1987. Epidemiology of tomato spotted wilt virus on crisp head lettuce in Hawaii. Plant Dis. 71:505-508.

6. Cho, J. J., Mau, R. F. L., Gonsalves, D., and Mitchell, W. C. 1986. Reservoir weed hosts of tomato spotted wilt virus. Plant Dis. 70:1014-1017.

7. De Haan, P. 1991. Exploring and exploiting the RNA genome of tomato spotted wilt virus. Ph.D. thesis. Agric. Univ., Wageningen, The Netherlands.

8. De Avilia, A. C., de Haan, P., Kormelink, R., Rsende, R. de O., Goldbach, R. W., and Peters, D. 1993. Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. J. Gen. Virol. 74:153-159.

9. Francki, R. I. B., Fauquet, C. M., Knudson, D. L., Brown, F., eds. 1991. Classification and Nomenclature of Viruses. Pages 450. Rep. ICTV Arch. Virol. 5th ed. Suppl. 2. Springer Verlag, New York.

10. Francki, R. I. B., and Hatta, T. 1981. Tomato spotted wilt virus, pages 491-512. In: Handbook of Plant Virus Infection and Comparative Diagnosis, E. Kurstak, ed. Elsevier/North Holland, Amsterdam.

11. German, T. L., Ullman, D. E., and Moyer, J. W. 1992. Tospoviruses: Diagnosis, molecular biology, and vector relationships. Annu. Rev. Phytopathology 30:315-348.

12. International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. C. George Kuo, D. Peters and T. L. German eds. Acta Horticulturae 431.

13. Kobatake, H., Osaki, T., and Inouye, T. 1984. The vector and reservoirs of tomato spotted wilt virus in Nara prefecturev (Japan). Ann. Phytopathol. Soc. Japan 50:541-544.

14. Kormelink, R., Storms, M., Van Lent, J., Peters, D., and Goldbach, R. 1994. Expression and subcellular location of the NSm protein of tomato spotted wilt virus, a putative viral movement protein. Virology 200:56-65.

15. Kormelink, R., de Haan, P., Peters, D., and Goldbach, R. 1992a. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. J. Gen. Virol. 73:2795-2804.

16. Kormelink, R., Van Poelwijk, F., Peters, D., and Goldbach, R. 1992b. Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs. J. Gen. Virol. 73:2125-2128.

17. Kormelink, R., Kitajima, E. W., de Haan, P., Zuidema, D., Peters, D., and Goldbach, R. 1991. The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structure in infected plant cells. Virology 181:459-68.

18. Law, M. D., and Moyer, J. W. 1990. A tomato spotted wilt-like virus with serologically distinct N protein. J. Gen. Virol. 71:933-938.

19. Miline, R. G., and Francki, R. I. B. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Intervirology 22:72-76.

20. Mohamed, N. A., Randles, J. W., and Francki, R. I. B., 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21

21. Pensiero, M. N., Jennings, G. B., Schmaljohn, C. S., and Hay, J. 1988. Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. J. Virol. 62:696-702.

22. Petterson, R. F. 1991. Protein Localization and Virus Assembly at Intracellular Membranes. Pages 67-106 In: Current Topics in Microbiology and Immunology 170 (Protein Traffic in Eukaryotic Cells). R. W. Compans, ed. Springer-Verlag, Berlin.

23. Sakimura, K. 1962. *Frankliniella occidentalis* (Thysanoptera:thripidae), a vector of the tomato spotted wilt virus, with special reference to color forms. Ann. Entomol. Soc. Am. 55:387-89.

24. Samuel, G., Bald, J.G. and Pittmann, H.A. 1930. Investigations on "spotted wilt" of tomatoes. Ciunc. Sci. Ind. Res. (Australia) Bulletin 44.

25. Tas, P. W. L., Boerjan, M. L., and Peters, D. 1977. The structural proteins of tomato spotted wilt virus. J. Gen. Virol. 36:267-279.

26. Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M., and Custer, D. M. 1993. Tospovirus replication in insect vector cells: immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology 82:1333-1342.

27. Van Poelwijk, F., de Haan, P., Kikkert, M., Prins, M., Kormelink, R., Storms, M., van Lent, J., Peters, D., and Goldbach, R. 1996. Replication and Expression of the Tospoviral Genome. In: International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. C. George Kuo, G. D. Peters and T. L. German, eds. Acta Horticulturae 431:201-208.

28. Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R., and Peters, D. 1993. Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. J. Gen. Virol. 74:341-349.

29. Wijkamp, I. 1995. Virus-vector relationships in the transmission of tospoviruses. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Table 1.

Approved list of established tospovirus species

	Descriptors		
Serogroups	Species	Serological affinity*	N protein sequence@
I	Tomato spotted wilt virus (TSWV)	+	100%
II	Groundnut ringspot virus (GRSV)	+	82%
	Tomato chlorotic spot virus (TCSV)	+	76%
III	Impatiens necrotic spot virus (INSV)	+	55%
IV	Watermelon silver mottle virus (WSMV) & Tospo-to/PBNV-to	+	29%
	Melon spotted wilt virus (MSWV)	+	35%
	Peanut bud necrosis virus (PBNV)	+	30%
	Isolates chry 1 and BR-11t	+	65%
	Isolate BR-09	+	63%
V	Peanut yellow spot virus	+	?

* Serological affinity among the viruses within a serogroup
@ Percentage of homology with TSWV

(Adopted partially from proceedings of the International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops.1996. C. Geroge Kuo, G.D. Peters and T. L. German eds. Acta Horticulturae 431:22)

Chapter 2

Production of monoclonal antibodies to NSs using antigen coated magnetic beads (ACMBs)

Introduction

Diagnosis of diseases caused by TSWV has traditionally been based on serological tests using polyclonal or monoclonal antibodies to the N protein (1,6,9,11,20,24). Several types of serological tests were used, including the precipitation test (4), immunodiffusion test (7), enzyme-linked immunosorbent assay (ELISA) (1,9,20), tissue-blot assay (14), western blot (13), immuno-electron microscopy (2,22), dot-immuno blot (11) and passive-heamaggultination assay (21). Among these, ELISA has been the method of choice for identification of tospoviruses. Only antibody sandwich ELISA, either double antibody sandwich ELISA (DAS-ELISA) or triple antibody sandwich ELISA (TAS-ELISA) can be performed since TSWV particles do not bind well to the polystyrene ELISA plates. Development of serological assays for TSWV was also complicated by the difficulty in isolating virions free of host material (8) and the cross reaction of polyclonal antibodies produced against the whole virion with the other serogroups due to the presence of anti-GP antibodies (3). Specific serological differentiation and detection of tospoviruses can be done using antibodies against a highly purified N fraction (25) or using DAS-ELISA or TAS-ELISA employing anti-N monoclonal antibodies (2,3). Antibodies to non-

structural proteins can also be used for detection of tospoviruses. Among the non-structural proteins, NSs is a more reliable target antigen than the other viral proteins since it is expressed in diseased plants in detectable quantities and is the least conserved protein among tospoviruses. As the presence of NSs is indicative of replication of TSWV, antibodies to NSs may be used to differentiate viruliferous and non-viruliferous thrips. Unlike the whole virions, non-structural proteins can be bound to the polystyrene plates.

Because the ACP-ELISA involves fewer steps and reagents than sandwich ELISA it is economical for screening a large number of samples. The principle disadvantages compared to DAS-ELISA are reduced sensitivity, cross reactivity, and the possible failure of the assay using monoclonal antibodies due to conformation change of antigens when bound to the plate. Most proteins bind to plastic surfaces as a result of hydrophobic interactions between nonpolar protein substructures and the plastic surface, and these interactions are independent of the net charge of the protein. The problem of reduced sensitivity can be avoided by using high-affinity monoclonal antibodies while diluting the infected plant sap.

The most important prerequisite for the successful development of ACP-ELISA is selection of the target antigen for development of antibody. The antigen has to be abundant in infected tissue and should contain epitopes that are both sequential and conformational. High-affinity monoclonal antibodies can

be selected during the screening process by altering the concentration of antigen, the temperature and the time of incubation.

During the process of post-fusion screening, memory-cell derived B-cells producing high affinity antibodies can be efficiently isolated. Broacades-Zaalberg et al. (5) isolated high affinity antibody producing hybridomas using antigen coated erythrocytes. Later, the same technique was used to select high affinity antibody producing hybridoma cell lines by lowering the concentration of coated antigen on erythrocytes (16). Two other investigators used this technique in principle, but erythrocytes were replaced with superparamagnetic polystyrene beads. Antigen-coated magnetic beads (ACMBs) were successfully used in producing high affinity antibodies against thyroglobulin (17) and human chronic gonadotrophin (10). Use of ACMBs facilitate rapid screening compared to the conventional cloning method and also can be used for affinity purification of antibodies or can be employed in immunoassays (12,15,18). ACMBs also have been used in epitope analysis of monoclonal antibodies (17). Antigen can be coated to these uniform superparamagnetic polystyrene beads or covalently bound to the activated beads. Incubating the ACMBs with the hybridoma cells that express the corresponding antibody results in formation of rosettes that can be separated from other cells using a magnet. Thus, ACMBs can be used to enrich antibody producing B-cells from the spleen prior to fusion, to enrich/isolate positive hybridomas after the fusion and as an aid in cloning by isolating the positive hybridomas from non-producers in a positive well after the HAT

selection.

In this study, the utility of NSs-coated magnetic beads in producing high-affinity antibodies to be used in ACP-ELISA to detect TSWV was investigated. The effectiveness of NSs coated polystyrene beads in the isolation of hybridomas secreting an antibody that could be used to detect the bound antigen was evaluated. The NSs antigen was coated at a low concentration to facilitate the isolation of high affinity antibody against a dominant epitope.

Materials and Methods

Plant material and virus isolate

A TSWV isolate (formerly designated TSWV-L) collected from infected tomato on the Hawaiian island of Maui was used in all experiments. *Emilia sonchifolia* (L.) DC. Ex Wight and *Datura stramonium* L. were grown from seed in greenhouses at the University of Hawaii-Manoa and used for maintenance of virus.

Cell culture

Myeloma cell lines and hybridoma cells were cultured in RPMI 1640 medium (Mediatech Inc., Herndon, VA) containing 10% horse serum (Hyclone Laboratories Inc., Logan, UT). After the fusion and until the end of cloning, the hybridoma cells were grown in a medium (special medium) that contained RPMI 1640, 50% myeloma cell line conditioned medium, 15% horse serum, and 2%

Origen's hybridoma cloning factor (Fisher Scientific, Pittsburgh, PA). All the cells were grown at 37 C, 5% CO_2 with 95% relative humidity.

Antigen preparation

A cloned cDNA to the NSs coding sequence was expressed in bacteria (22) and bacterial lysate was a gift from Dr. Thomas L. German, University of Wisconsin. The protein was isolated from sodium dodecyl sulfate (SDS)-polyacrylamide gels in phosphate-buffered saline (PBS) (0.14 M NaCl, 1mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl, pH 7.5). The homogenized gel was centrifuged for 15 min at 10,000 *g*, and the NSs was recovered from the supernatant by a chloroform/methanol/water system (23). The pellet was resuspended in PBS and the protein concentration adjusted to 1 mg/ml. The protein concentration was estimated with Coomassie Plus® Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). Alternatively, the NSs pellet was dissolved in carbonate-bicarbonate buffer and the protein concentration was adjusted to 1 mg/ml and the solution was used for coating the magnetic beads.

Immunization and fusion procedure

Female BALB/c mice were immunized with 50 μg of NSs in PBS emulsified in Freund's complete adjuvant (Sigma Chemical Company, St. Louis). Three subsequent immunizations were given at 10-day intervals using Freund's incomplete adjuvant (Sigma Chemical Co.). After 20 days, a booster dose of 150 μg of NSs without adjuvant was injected. The spleen cells were fused with

myeloma cell line P3X63Ag8.653, 72 hours after the booster dose according to the procedure established by Sherwood et. al. (19) at the Hybridoma Center for Agricultural and Biological Sciences of Oklahoma State University. The fused cells were transferred into T-75 flasks (Corning, Fisher Scientific) containing 50 ml of RPMI 1640 special medium and were cultured for 24 hours. The cells were divided equally into two 50 ml tubes and were spun at 500*g* for 8 min. The cells from one tube were resuspended in 100 ml of special medium with HAT and then transferred into two T75 flasks for ACMBs selection. The cells from the other tube were resuspended into 100 ml of special medium containing HAT and were transferred to 4 x 24 well plates (Corning, Fisher Scientific) at 1 ml per well for subsequent selection and cloning (traditional method).

Selection of anti-NSs hybridomas using ACMBs

Dynabeads M-450 magnetic beads (Dynal Inc., Great Neck, NY) were coated with the antigen. Two forms of beads, either uncoated or tosylactivated, were used. The antigen was coated on the beads as per the manufacturer's protocol except carbonate-bicarbonate buffer, pH 9.6, was used for adsorption of NSs to the beads. This was to mimic the procedure involved in ACP-ELISA plate coating. All the procedures were carried out aseptically and all the solutions were filtered through 0.1μm syringe filters. One ml of beads (4 x 10^8 beads or 30 mg) was coated with 100 μg of NSs for 24 hours at 21 C by end-over-end rotation in a 15 ml conical centrifuge tube (Corning, Fisher Scientific). The beads were then washed 3 times with PBS containing 0.05% Tween-20

(PBST). The beads were then washed once with PBS and then blocked with 5 ml of PBS with 1% horse serum overnight at 4 C. The beads were stored in PBS without any preservatives at 4 C.

The hybridoma cells from the flasks were collected after 10 days (after the HAT selection) into 50 ml conical centrifuge tubes (Corning, Fisher Scientific) and spun at 500*g* for 10 min. The cells were resuspended in cold RPMI containing 10% serum and washed three times with the cold medium. The cells from each tube were then suspended in 5 ml of cold RPMI containing 10% serum and transferred into 15 ml conical tubes. ACMBs at a bead:cell ratio of 1:4 (1×10^6 cells with 4×10^6 beads) were added and incubated at 4 C for 2 h with end-over-end rotation. At the end of the incubation, 8 ml of cold RPMI was added to the tube and the rosettes were isolated by using a MPC-1 magnet from Dynal Inc. The rosettes were resuspended in 10 ml of cold medium (4 C) and then washed five times each with 10 ml of cold RPMI medium. The unbound cells from each wash were collected, centrifuged, pooled, resuspended in 100 ml of special medium containing HAT and then transferred into 4 x 24 well plates at 1 ml/well. The rosettes and the beads were finally resuspended in 10 ml of warm (37 C) special medium containing HAT and transferred into 10 wells in a 24 well plate and screened for antibody production after 7 days. The positive wells were cloned by limiting dilution in 96 well plates (Corning, Fisher Scientific) in RPMI 1640 medium supplemented with HAT and 5% hybridoma cloning factor.

Screening for anti-NSs antibody production

The supernatant from the 4 x 24 well plates from the fusion, the 4 x 24 well plates from unbound cells and the 10 wells from ACMBs, were screened for anti-NSs antibody by ACP-ELISA. All the incubations were at room temperature (21 C) unless otherwise indicated. One gram of TSWV infected *D. stramonium* was ground in 10 ml of PBS-PVP (2% polyvinylpyrrolidone) and the sap either strained through cheesecloth or centrifuged for a short period to remove the plant debris. The sap was diluted 1:10 with PBS-PVP and 100 µl/well was added to the ELISA plate (Nunc MaxiSorp, PGC Scientific, Gaithersburg, MD) and incubated for 2 h on a shaker. The plates were then washed 3 times with PBST and blocked with 1% BSA for 1 h. A 100 µl aliquot of supernatant from each well was added to the ELISA plate well and incubated on a shaker for 2 h. The plates were then washed 3 times with PBST and 100 µl of goat anti-mouse IgG coupled to alkaline phosphatase (Sigma Chemical Co.) diluted in PBST at 0.2 µg/ml, was added to each well and incubated for 2 hours on a shaker. The wells were washed 4 times with PBST and 100 µl of substrate solution (1 mg/ml of *p*-nitrophenyl phosphate disodium in 1 M diethanolamine buffer with 0.5 mM $MgCl_2$ and 0.02% sodium azide) was added to each well. The absorbance was read at 405 nm with a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). Negative controls were BSA coated wells without antigen and with healthy *D. stramonium* sap. Wells coated with BSA were used to blank the machine.

Cloning of positive hybridoma cells

Cells from wells positive in ELISA were immediately transferred to a separate plate and cloned by limiting dilution in 96 well plates (Corning, Fisher Scientific) with special medium containing HT supplemented with 5% hybridoma cloning factor. The cells were observed microscopically for growth and the supernatant from the wells was screened after 10-15 days for the production of anti-NSs antibody. The positive cell lines were expanded and frozen.

Characterization of clones

Clones were isotyped using a murine antibody isotyping kit (Sigma Chemical Co.). No clones were obtained from the ACMBs unbound cells. Among the several clones obtained, two clones each from the ACMBs isolation and the traditional method were further analyzed for their epitope recognition by a simple competitive inhibition of rosette formation. All the incubations were carried out as per the procedure outlined above. In brief, the NSs coated magnetic beads were incubated with the supernatant from each of the four cell lines to block the antigen. The beads, coated with one of the antibodies, were then incubated with the four cell lines and the inhibition of rosette formation was determined qualitatively by examining the beads with a microscope. In addition, the clones were evaluated by ACP-ELISA for reactivity to NSs. Cell culture supernatant from the overgrown cultures was added to plates coated at 0.1 μg/ml of NSs. Also, the secondary antibody conjugate was used at twice the recommended dilution to facilitate the color development at a slower kinetic rate

and the absorbance was recorded at 30 min intervals.

Whole leaf tissue blotting

A whole leaf tissue blotting assay was used to examine the specificity of the anti-NSs monoclonal antibodies. Freshly cut leaves of healthy and TSWV infected D. *stramonium* were frozen at –70 C. The leaves were thawed at room temperature for about 15 min and were blotted dry to remove the excess moisture. Immediately the leaves were sandwiched between two layers of supported nitrocellulose membranes (Optitran™, Schleicher & Schuell, Keene, NH), and four layers of Whatman grade 1 chromatography paper (Fisher Scientific). The sandwich was placed between two 2 mm thick glass plates, and a 15 pound weight was placed on the sandwich. Blotting was performed at room temperature for two hours. One sandwich produced two mirror images of the leaf tissue on two nitrocellulose sheets. The nitrocellulose membranes were soaked in distilled water for 10 min prior to use. All the incubations and washings were performed at room temperature on an orbital shaker. The two nitrocellulose sheets were removed from the sandwich and rinsed in 25 mM Tris buffered saline (0.14 M NaCl, 2.5 mM KCl, pH 8.0) (TBS) for 30 min and then blocked with 5% non fat dry milk (NFDM) in 25 mM TBS for 1 hour. Two monoclonal antibodies, one each from the ACMBs and the traditional cloning method, were used to probe the blots. The blots were incubated in the cell culture supernatants of 1C1A7 and 6B1C1 for 2 hours and washed three times with TBS for 10 min each. The blots were then transferred to rabbit anti-mouse IgG-

alkaline phosphatase (Sigma Chemical Co.) at 0.5 μg/ml in 5% NFDM-TBS for 2 h. The blots were then washed four times with TBS for 10 min each and the color was developed with BCIP-NBT substrate (Sigma Chemical Co.).

Results

Isolation of hybridoma cell lines

Eight hybridoma clones positive to the anti-NSs antibody were established and compared to six clones produced by the traditional method (Table 1). Supernatant from one well with the ACMB selection showed cross-reactivity to the healthy plant sap. Although the traditional method yielded 18 anti-NSs antibody positive wells, eight wells showed cross-reactivity to healthy plant sap. Clones secreting IgM subclass antibody were not taken into consideration. Typically, it took approximately 5-6 weeks to establish stable clones using the ACMBs selection method, compared to 10-12 weeks by the traditional method.

Characterization of monoclonal antibodies

Two monoclonals from each technique were selected and compared for their sensitivity to detect NSs. The monoclonal lines produced using ACMB selection were of high sensitivity, and were measured by the rate of increase in absorbance per unit time (Table 2). The performance of clone 6B1C1 was different from that of the other three antibodies in that there was a slower increase of absorbance over time. The absorbance was low at 30 min and

peaked at 60 min, whereas for the other clones the absorbance peaked between 30-45 min. Results of the competitive blocking experiments indicated that antibodies 1G7A2 and 1C1A7 recognized the same epitope (Table 3).

Among the four monoclonals, 1C1A7 was further purified using protein-A affinity chromatography (Pierce Chemical Co.) and the purified antibody was used to investigate the sensitivity limit of ACP-ELISA for detection of NSs. The ACP-ELISA was conducted on gel-isolated NSs, to determine a limit on the amount of NSs that could be detected. Two wells each were coated with one of a series of twofold dilutions of NSs ranging in concentrations from 1000 to 0.122 ng/ml. The wells were read one hour after addition of the substrate. Average absorbance readings at 405 nm for NSs at 0.122, 0.244, 0.488 and 0.976 ng/ml were 0.030, 0.126, 0.226, and 0.436, respectively. A BSA control was used to zero the plate. Absorbance readings at 405 nm for increasing NSs concentration continued to approximately double as the NSs concentration increased to 7.8 ng/ml. At 7.8 ng/ml the absorbance reading at 405 nm after one hour was 2.151. Between 7.8 and 250 ng/ml, the absorbance reading at 405 nm increased about 10% as the NSs concentration was doubled. The absorbance reading for NSs concentrations between 250 and 1000 ng/ml was 3.175. Thus, the lower limit of detection of NSs was about 0.244 ng/ml. The absorbance values obtained in ACP-ELISA over a range of concentrations were typical of standard curves obtained in ELISA.

Tissue Blots

The whole leaf tissue blot probed with monoclonal antibody 1C1A7 (ACMBs) did not show any cross reactivity of the antibody to the healthy plant tissue (Figure 1). Similar results were obtained with the blot probed with monoclonal antibody 6B1C1 (traditional cloning; blot not shown).

Discussion

Antigen-coated magnetic beads can be successfully used for rapid isolation of specific antibody secreting hybridoma cell lines after the fusion of B-cells with myeloma cell lines. This technique generated anti-NSs monoclonal antibodies in less time than was required for the traditional technique. It also offers a rapid and reliable method for generating high affinity antibodies. However, in the present study the affinity of the antibodies to NSs was not estimated. Instead their reactivity was compared by ACP-ELISA, in which the antigen was coated at a concentration of 0.1 μg/ml rather than at the normal concentration of 1-5 μg/ml. The results indicated that the performance of the monoclonal antibodies generated through the ACMBs technique is equivalent to those of antibodies generated by traditional method (Table 2). Horton et al. (10) concluded that clones isolated by ACMBs produced antibodies with higher affinity than those produced by the traditional method. The same observation was also made by Ossendorp et. al. (17). In these studies, the temperature and incubation time during the rosette formation might have facilitated the binding of high affinity antibody producing B-cells to the ACMBs. In the present study NSs

was coated at a concentration of 100 μg/ml of beads, although the capacity of the beads was 600 μg/ml, in order to generate high affinity antibodies to be used in ACP-ELISA. The monoclonal antibodies obtained were used in developing an ACP-ELISA for detecting the TSWV in a plant sample. These antibodies did not react to the healthy plant and did not react with NSs from Impatiens necrotic spot virus. One of the monoclonal antibodies, 1C1A7, could detect 0.244 ng/ml of NSs isolated from SDS-PAGE gels. Whole tissue blots of TSWV infected and healthy plants also demonstrated the specificity and sensitivity of the anti-NSs monoclonal antibodies (Figure 1).

Using ACMBs can result in the rapid isolation of hybridoma cell lines that recognize the bound antigen and thus may be well suited for ACP-ELISA. This technique may offer the advantage of using an antigen that is similarly deformed as it may be when bound to a plate. Since the traditional method produced equally specific monoclonal antibodies, it appears that NSs retains its antigenicity even after binding to the plate. Alternatively, the monoclonals could have been made to the epitopes that are present both in native and denatured conditions. All four monoclonal antibody-producing cell lines also formed rosettes with covalently bound (tosylactivated) magnetic beads. Thus, the use of ACMBs permits selective isolation of high-affinity antibodies in less time with less expenditure than the traditional method. The ACMBs can also be used for epitope analysis of the resulting monoclonal antibodies.

References

1. Adam, G., Lesemann, D. E., and Vetten, H. J. 1991. Monoclonal antibodies against tomato spotted wilt virus: Characterization and application. Ann. Appl. Bio. 118:87-104.

2. Adam, G., and Lesemann, D. E. 1992. Identification and characterization of a new tospovirus isolate from *Anemone coronaria* L. by TAS-ELISA and electron microscopy. Acta Hort. 377:129-140.

3. Adam, G., Peters, D., and Goldbach, R. W. 1996. Serological comparison of tospovirus isolates using polyclonal and monoclonal antibodies. In: International symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. C. George Kuo, G. D. Peters and T. L. German, eds. Acta Horticulturae 431:135-158.

4. Alden, E. 1956. The production of an antiserum against an antigen from an unstable plant virus. MS Thesis, Univ. Illinois, IL, USA.

5. Brocades-Zalberg, O., and Lubbe, F. H. 1984. Methods for the rapid selection of hybrid cells producing specific antibodies of high affinity. Pages 251-265. In: Innovations in Biotechnology. E. H. Houwink and R. R. Van der Meer, eds. Elsevier Science Publishers, Amsterdam.

6. de Avilia, A. C., Huguenot, C., Resende, R. De O., Kitajima, E. W., Goldbach, R. W., and Peters, D. 1990. Serological differentiation of 20 isolates of tomato spotted wilt virus. J. Gen. Virol. 71:2801-2807.

7. Fieldman, J. M., and Noninsegna, J. A. 1968. Antiserum for tomato spotted wilt virus. Nature 219:183-184.

8. German, T. L., Ullman, D. E., and Moyer, J. W. 1992. Tospoviruses: Diagnosis, molecular biology, and vector relationships. Annu. Rev. Phytopathology 30:315-348.

9. Gonsalves, D., and Trujillo, E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

10. Horton, J. K., Evans, O. M., Swann, O. M., and Swinburne, S. 1989. A new and rapid method for selection and coning of antigen-specific hybridomas with magnetic microspheres. J. Immunol. Methods 124:225-230.

11. Hsu, H. T., Wang, Y. C., Lawson, R. H., Wang, M., and Gonsalves, D. 1990. Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific

antibodies. Phytopathology 80:158-162.

12. Johansen, L., Nustad, K., Berg Ierstadvik, T., Ugelstad, J., Berge, A., and Ellingsen, T. 1983. Excess antibody immunoassay for rat glandular kallikrein. monosized polymer particles as the preferred solid phase material. J. Immunol. Methods 59:255-264.

13. Law, M. D., and Moyer, J. W. 1990. A tomato spotted wilt virus-like virus with a serologically distinct N protein. J. Gen. Virol. 71:933-938.

14. Lin, N. S., Hsu, Y. H., and Hsu, H. T. 1990. Immunological detection of plant viruses and mycoplasma-like organisms by direct tissue blotting on nitrocellulose membranes. Phytopathology 80:824-828.

15. Luk, J. M. C., and Lindberg, A. A. 1991. Rapid and sensitive detection of *Salmonella* (O:6,7) by immunomagnetic monoclonal antibody-based assays. J. Immunol. Methods 137:1-8.

16. Myers, C. D., Sanders, V. M., and Vietta, E. S. 1986. Isolation of antigen-binding virgin and memory B cells. J. Immunol. Methods 91: 257-263.

17. Ossendrop, F. A., Bruning, P. F., Van der Brink, J. A. M., and De Boer, M. 1989. Efficient selection of high-affinity B cell hybridomas using antigen-coated magnetic beads. J. Immunol. Methods 120:191-200.

18. Running, Y. W., Christophersen, A. C. 1991. An ELISA-method using magnetic beads as solid phase for rapid quantification of mouse and human immunoglobulins. Hybridoma 10:641-645.

19. Sherwood, J. L., Sanborn, M. R., and Keyser, G. C. 1987. Production of monoclonal antibodies to peanut mottle virus and their use in enzyme-linked immunosorbent assay and dot-immunoblotting assay. Phytopathology 77:1158-1161.

20. Sherwood, J. L., Sanborn, M. R., Keyser, G. C., and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. Phytopathology 79:61-64.

21. Tsuda, S., Natsuaki, K. T., and Tomaru, K. 1992. Passive-hemagglutination assay using specific antibody to tomato spotted wilt virus. Ann. Phytopath. Soc. Japan 58:319-324.

22. Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M., and Contone, F. A. 1993. Tospovirus replication in insect vector cells:Immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology

83:456-463.

23. Wessel, D., and Flugge, V. I. 1984. A method for the quantitative recovery of protein in dilute solutions in the presence of detergents and lipids. Anal. Biochem. 138:141-143.

24. Yeh, S. D., Lin, Y. C., Cheng, Y. H., Jih, C. L., Chen, M. J., and Chen, C. C. 1992. Identification of tomato spotted wilt-like virus on watermelon in Taiwan. Plant Dis. 76:835-840.

25. Yeh, S. D., Chao, C. H., Cheng, Y. H., and Chen, C. C. 1996. Serological comparison of four distinct tospoviruses by polyclonal antibodies to purified nucleocapsid proteins. In: International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. C. George Kuo, G. D. Peters, and T. L. German, eds. Acta Horticulturae 431:122-134.

Table 1.

Comparison of antigen coated magnetic beads versus traditional hybridoma technology for generation of anti-NSs monoclonal antibodies.

Method	No. of wells positives for hybrids	No. of wells positives for anti-NSs antibody	No. of wells reacting to healthy plant sap	No. of clones established	Time (weeks)[@]
ACMBs 1 x 10 wells	10	10	1	8	5-6
Traditional technique 4 x 24	68	18	8	6	10-12

[@]Time from the first screening for anti-NSs antibody through end of cloning and isotyping.

Table 2.

ELISA absorbance values of the four selected anti-NSs monoclonal antibodies taken over 15 min intervals.

Anti-NSs Monoclonal antibody	Absorbance 405 nm			
	15 min	30min	45min	60min
1C1A7	0.348	1.204	2.197	2.832
1C1A3	0.289	1.138	2.078	>3.000
6B1C1	0.068	0.684	1.204	1.896
1G7A2	0.234	0.986	1.833	2.650

1C1A7 and 1C1A3 are obtained through use of ACMBs and 6B1C1 and 1G7A2 are by traditional method

Table 3.

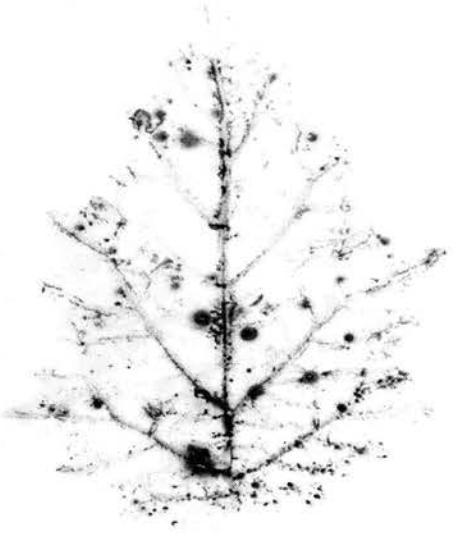
Characterization and epitope analysis by reciprocal competitive blocking of four anti-NSs monoclones

Anti-NSs Monoclonal antibody	Formation of rosettes				
	1C1A7	1C1A3	6B1C1	1G7A2	Isotype
1C1A7	-	+	+	-	IgG2a/κ
1C1A3	+	-	+	+	IgG2a/κ
6B1C1	+	+	-	+	IgG1/κ
1G7A2	-	+	+	-	IgG1/κ

1C1A7 and 1C1A3 were obtained through use of antigen coated magnetic beads and 6B1C1 and 1G7A2 were from the traditional method

Figure 1.

Whole leaf tissue blot of TSWV infected and healthy *Datura stramonium* probed with monoclonal antibody 1C1A7



Infected

Healthy

Chapter 3♣

Use of anti-NSs monoclonal antibody to identify individual viruliferous thrips

Introduction

Tomato spotted wilt tospovirus (TSWV), the type species of the genus *Tospovirus* in the family Bunyaviridae, has a wide host range of at least 550 species of plants in over 75 families that include important and valuable crops (16). The virus is exclusively transmitted in a persistent manner by a number of thrips species of which the western flower thrips (WFT), *Frankliniella occidentalis* Perg., is considered to be the most important (5). Adult thrips transmit TSWV only if the virus is acquired during the larval stage (7,12), with the majority of thrips becoming viruliferous (transmitters) in the second larval stage (16). Even though TSWV replicates in its thrips vector, transmission of TSWV by adults occurs intermittently for the duration of their lives (12,17). Control of TSWV incidence by trying to control thrips with insecticides is ineffective because of the diverse host range of both virus and vector and the prolific reproduction of thrips (3). Yudin et al. (18) indicated that incidence of thrips was an important component of predicting TSWV incidence in lettuce, but without being able to

---

♣ A slightly modified version of this chapter has been published as: Bandla, M. D., Westcot, D. M., Chenault, K. D., Ullman, D. E., German, T. L., and Sherwood, J. L. 1994. Use of monoclonal antibody to the nonstructural protein encoded by the small RNA of tomato spotted wilt tospovirus to identify viruliferous thirps. Phytopathology 84:1427-1431.

identify the number of thrips in a population that are potential transmitters (viruliferous), thrips numbers were not reliable indicators of potential disease incidence. As a result, disease incidence early in the growing season had to be used as a predictor of final disease incidence. Determining the percentage of thrips in a population that are potential transmitters would allow vector population to be used as a predictor of TSWV incidence in a preplanting forecasting system.

Transmission or serological assays have been used to identify viruliferous thrips from the field and greenhouse (1,3,4). Serological assays for detection of TSWV in thrips have used antibodies to TSWV structural proteins. These proteins may be present in the digestive tract of any insect that has fed on an TSWV-infected plant (11). Many of these thrips will never become transmitters; however, the thrips could be positive in a serological assay for detection of virus structural proteins. Including such thrips in estimates of potential transmitters vastly overestimates the percentage of the vector population that could transmit TSWV. Assaying for viruliferous thrips by testing the ability of thrips to transmit TSWV to plants is useful but requires 2-3 days for symptoms to develop on plants after thrips have fed (1). This limits the utility of plant transmission assays for preplant forecasting.

Because TSWV replicates in those viruliferous thrips, detection of a TSWV nonstructural protein (NS) could be used to differentiate thrips that have ingested virus but cannot transmit the virus (non-viruliferous) from viruliferous

thrips. Replication of TSWV in thrips results in readily detectable amounts of the NSs protein encoded by the small RNA, but the NSm protein encoded by the middle RNA is less frequently detected by immnoelectron microscopy (12). In enzyme-linked immunosorbent assay (ELISA) using anti-NSs and anti-N polyclonal serum, Wijkamp et al. (17) detected an increase of both proteins in thrips when the thrips acquired TSWV as larvae. However, ELISA absorbance values for an individual larvae using antiserum to NSs were very low even after amplifying the initial enzyme-substrate reaction. In addition, the polyclonal antiserum produced had to be cross adsorbed prior to use for detection of NSs. Only TSWV viruliferous larvae, not adults were tested in ELISA. These limitations suggest that ELISA with polyclonal antisera may not be suitable for differentiating viruliferous thrips from non-viruliferous thrips. Non-viruliferous thrips would include thrips that have never fed on TSWV-infected tissue and thrips, such as adults, that may have fed on TSWV-infected plants but never become transmitters of the virus.

In this chapter, the use of anti-NSs monoclonal antibodies for differentiating viruliferous and non-viruliferous thrips was studied. Monoclonal antibodies can be used in a standard ELISA format without additional amplification. When ACP-ELISA employing anti-NSs monoclonal antibodies developed for diagnosis of TSWV infected plant samples was used to assay individual known viruliferous thrips, a substantial background was observed. Wijkamp et al. (17) cross-absorbed polyclonal antiserum produced to an

expressed NSs protein with acetone-washed powder of healthy thrips to eliminate non-specific background in ELISA and Western blots. Zwitterionic detergents are known to reduce nonspecific binding in ELISA (2,13). Empigen-BB (E-BB), an alkyldimethyl betane, restored antibody binding to meningococcal outer membrane proteins in Western blots when used with either transfer buffer (6) or in primary antibody solution (14). Allen et al. (2) reported that the use of E-BB in the extraction buffer of parasitoid larval antigens from larvae of *Phyllonorycter blancardella* (F.) enhanced the reaction of antiserum to the parasitoid larvae and reduced nonspecific binding to the host larvae in double antibody sandwich ELISA (DAS-ELISA). The use of E-BB in antibody dilution buffer in reducing the nonspecific binding in ACP-ELISA so as to differentiate viruliferous thrips from non-viruliferous thrips was investigated.

Materials and Methods

Plant material and virus isolate

A TSWV isolate (formerly designated TSWV-L) collected from infected tomato on the Hawaiian island of Maui was used in all experiments. *Emilia sonchifolia* (L.) DC. Ex Wight, *Datura stramonium* L., and *Petunia grandiflora* (L.) Cv. Yellow Magic were grown from seed in greenhouses at the University of Hawaii-Manoa and used for maintenance of virus, transmission assays, and acquisition studies as described (11).

Thrips acquisition of TSWV

Thrips acquisition of TSWV was done at University of Hawaii-Manoa in Dr. Ullman's laboratory by Westcot, D. M., as follows. The colonies of WFT were reared on green bean pods (*Phaseolus vulgaris* L.). To obtain viruliferous thrips, late first instar larvae were fed on TSWV-infected *E. sonchifolia* leaves for 48-72 h and then transferred to green bean pods to complete their development into adults in approximately 4-6 days. Virus-free adults were obtained by feeding the larvae on healthy *E. Sonchifolia* leaves prior to transfer to green bean pods. Samples were obtained representing the following groups: (1) larvae fed on TSWV-infected *E. sonchifolia* as larvae and assayed as larvae directly following the 48-72 h of acquisition access feeding; (2) control larvae fed on virus-free *E. Sonchifolia* and assayed as larvae at the same time as group 1; (3) larvae fed on TSWV-infected *E. sonchifolia* and assayed as adults; (4) larvae fed on virus-free *E. Sonchifolia* and assayed as adults; (5) adults fed on TSWV-infected *E. sonchifolia* for 48 h and assayed as adults. Thrips were place in plastic vials containing phosphate-buffered saline (PBS) (0.14 M NaCl, 1mM KH_2PO_4, 8mM Na_2HPO_4, 2.5 mM KCL, pH 7.5) with PVP (2% polyvinylpyrrolidone), and express mailed to Oklahoma State University, Stillwater, for ACP-ELISA.

Purification of monoclonal antibody 1C1A7

The monoclonal antibody 1C1A7 was the source of the antibody used in assay development. The cell line was grown in RPMI1640 (Mediatech,

Inc., Logan, UT) and the supernatant was collected. The immunoglobulin was precipitated from cell culture supernatant with 50% ammonium sulfate and further purified using a protein-A column (Pierce Chemical Co.). The antibody was stored in PBS with 0.02% sodium azide at 1 mg/ml after extensive dialysis against PBS.

Effect of E-BB in antibody dilution buffer

The effect of E-BB (30% a.i.; Albright & Wilson Ltd., Whitehaven, U.K.) and Tween 20 on reducing nonspecific binding in ACP-ELISA was compared by incorporating the detergents in the antibody dilution buffer. The antibody dilution buffer contained PBS, pH 7.5 with 0.1% bovine serum albumin (BSA) with either 0.05% Tween 20 (v/v) or 0.1% (E-BB) (a.i.). Although uses of E-BB concentrations of 0.1-1.0% a.i. have been reported (2,6,14), 0.1% a.i. was used because of ease in handling the viscous material. For detection of NSs by ACP-ELISA in thrips, 20 thrips representing each of five groups as described above were homogenized in 1 ml of PBS-PVP, and wells of ELISA plates (Nunc MaxiSorp, PGC Scientific, Gaithersburg, MD) were coated with 50 μl of each. Ten wells were used for each detergent treatment, and each treatment was replicated twice. Three independently collected thrips samples were coated with 1% BSA. After the samples were incubated overnight at 4 C, the plates were washed three times with PBS containing 0.05% Tween 20 (PBST), and blocked with 75 μl of 1% BSA in PBS for 2 h. Fifty microliters of anti-NSs monoclonal antibody at 0.5 μg/ml in one of the two antibody dilution buffers was added to the

wells and the plates were incubated for 2 h at room temperature. After washing the wells with PBST four times, 50 μl of rabbit anti-mouse IgG-alkaline phosphatase (Sigma Chemical Company, St. Louis, MO.) at 0.2 μg/ml in antibody dilution buffer with the same detergent that was used to dilute the monoclonal antibody was added to each well, and the plates were incubated for 2 h at room temperature. The wells were washed four times with PBST four times, and 50 μl of substrate solution (1 mg/ml of *p*-nitrophenyl phosphate disodium in 1M diethanolamine buffer that contains 0.5 mM $MgCl_2$ and 0.02% sodium azide) was added to each well. The absorbance was read at 404 nm with V_{max} kinetic microplate reader (Molecular Devices, Paulo Alto, CA). The absorbance values at 405 nm were taken at 0.5, 1, and 2 h after addition of substrates to determine the optimum time to read the plate. After finding the optimum time of incubation at room temperature after addition of substrate, the effect of overnight incubation at 4 C after the addition of the substrate on the absorbance values obtained was examined.

Thrips transmission of TSWV

Adult thrips that fed on TSWV infected *E. sonchifolia* as larvae were tested for their ability to transmit TSWV as adults, using a modification of the plant transmission assay developed by Wijkamp and Peters (16). A leaf disc (1 cm diameter) of *P. grandiflora* cv. Yellow Magic (Harris seeds, Rochester, NY) freshly cut with a cork borer was placed in a 1.5 ml microfuge tube along with a piece of Whatman No. 2 filter paper (Clifton, NJ) previously dried over desiccant.

Thrips were starved for 1-2 h and then individually fed on separate leaf discs for 24 h. Thrips were then removed and individually stored in the buffer described above for ACP-ELISA, and the leaf disk was placed on water for the development of localized lesions. Symptoms on leaf disks were visually scored at 24 h intervals for 72 h. Virus-free thrips were included as controls. The experiment was replicated four times with either 25, 50, 90 or 100 adult thrips.

ACP-ELISA of thrips

The ACP-ELISA was used to detect NSs in individual larvae and adult thrips. An individual thrips was ground in 50 μl of PBS-PVP buffer in porcelain mortar with a disposable glass tube, and the sample was placed in a well of an ELISA plate. Negative controls of virus-free thrips also were run. The plates were incubated at 4 C overnight, and the procedure was continued as described above. The antibody dilution buffer with E-BB at 0.1% (a.i.) was used. When the results of plant transmission assay and ACP-ELISA for the same thrips were compared, a positive/negative threshold of absorbance at 405 nm of 0.100 was used. This threshold was based on results obtained from the experiments with groups of thrips and the absorbance values obtained with known virus-free thrips assayed as controls. For the experiments using 25, 50, 90, or 100 thrips, controls of 5, 9, 7 or 24 thrips, respectively, were used. The average absorbance at 405 nm plus four standard deviations ranged from 0.043 to 0.091, so 0.100 was used across all experiments. A two by two *G* test for independence (8) was used to test whether results for identifying viruliferous thrips by plant transmission

assay and ACP-ELISA were independent.

Results

Effect of E-BB in antibody dilution buffer

The use of E-BB reduced the non-specific binding to thrips homogenate tested in ACP-ELISA (Table 1). With E-BB, a ten fold difference in absorbance values was observed between adult thrips fed on healthy plants and adult thrips fed on virus infected plants as larvae. With Tween 20, there was only a three-fold difference in absorbance values between adult thrips fed on healthy plants and adult thrips fed on virus infected plants as larvae. The difference was twenty and five-fold with E-BB and Tween 20, respectively, with larvae fed on healthy plants and larvae assayed after feeding on TSWV infected plants for 48-72 h. Absorbance values in ACP-ELISA for adult thrips on TSWV-infected plants as adults was about twice the value of thrips fed on healthy plants when E-BB was used; indicating small amounts of NSs ingested during feeding were detected. The corresponding values with Tween 20 showed no apparent differences in the absorbance values. Because E-BB effectively reduced the non-specific binding to thrips homogenate tested in ACP-ELISA compared to Tween 20, E-BB was used in the remaining assays.

Effect on substrate incubation time on absorbance values

Reading absorbance values 1h after the addition of substrate permitted differentiation between virus-free thrips and thrips fed on virus-infected

plants as either adults or larvae (Table 2). After 1 h, there was a disproportionate increase in color development in samples of thrips that would not be transmitters compared to samples of larvae or adults that would be transmitters. When after the addition of substrate plates were incubated at 4 C overnight compared to 1 h at room temperature, groups of thrips that could be transmitters versus nontransmitters could be readily differentiated (Table 3). As in the other assays the difference was more apparent when E-BB was used than when Tween 20 was used.

Comparison of ACP-ELISA and transmission assay for detection of viruliferous thrips

Based on the results of the four replications using 25, 50, 90, or 100 thrips, the ACP-ELISA and the plant transmission were similar in identifying viruliferous thrips (Table 4). In a *G* test for independence, the two assays showed close agreement. The *G* test indicated that the results of the two tests were not independent ($G = 97.72$; 1 df: $P < 0.0001$). The two assays were in agreement 92% of the time. The errors were divided: 6% occurred when ACP-ELISA identified thrips as potential transmitters that were not identified as transmitters in the plant transmission assay, and 2% occurred when ACP-ELISA did not detect individuals that transmitted TSWV in the plant transmission assay.

Discussion

ACP-ELISA with monoclonal antibodies to the NSs protein of

TSWV and the Zwitterionic detergent E-BB is an efficient and economical method to screen a large number of thrips for their potential to transmit TSWV. The inclusion of detergents in serological reagents to reduce non-specific binding is common. The development of ACP-ELISA described here was initiated with four monoclonal antibodies produced against NSs with no apparent cross-reactivity to healthy plant tissue in ACP-ELISA. Because of the non-specific binding of antibody to thrips homogenate in ACP-ELISA when Tween 20 was used, only one of the four monoclonal antibodies, monoclonal antibody 1C1A7 used in the study, made possible differentiation between samples of non-viruliferous or viruliferous thrips. However when used with antibody dilution buffer with E-BB all the four monoclonals reported in Chapter One differentiated viruliferous thrips from non-viruliferous thrips. The polyoxyethylene detergent Tween 20 commonly used in serological assays did not minimize the nonspecific binding, which was needed if individual viruliferous and non-viruliferous thrips were to be reliable differentiated.

The effect of function of detergents used in serological assays is not predictable or well understood (9,10,14,15). Zwitterionic detergents improved results obtained in Western blots and ELISA (2,6,14). Wedge et al. (14) concluded that Zwitterionic detergents, like CHAPS and E-BB, enhanced the binding of an antibody to antigen and suggested that these detergents may be employed when antibodies with low or medium titers were used. Vogt et al. (13) and Allen et al. (2) observed that Zwitterionic detergents prevent the non-specific

binding of biotinylated antibody when used in DAS-ELISA with enzyme amplification. Use of E-BB is economical compared to other Zwitterionic detergents and reduced the nons-pecific binding to thrips homogenate in ACP-ELISA. However, because final ACP-ELISA values were not appreciably higher when E-BB instead of Tween 20 was used, it cannot be concluded, as Wedge et al. (14) suggested, that E-BB enhance the reaction between antigen and antibody. Whether E-BB may renature the antigens that are denatured by SDS (14) or reduce the non-specific binding of primary and secondary antibodies (2,13) is unclear. However, use of E-BB clearly reduced the non-specific signal of monoclonal antibodies made to NSs to non-viruliferous thrips compared to Tween 20.

ACP-ELISA with E-BB in the antibody dilution buffer is a rapid and simple assay that differentiates thrips that are non-transmitters from transmitters. This is supported by the close agreement found between the results of ACP-ELISA and plant transmission assay. The greatest amount of error (6%) was in ACP-ELISA identifying thrips as potential transmitters that were not detected in the plant transmission assay. This result is to be expected because plant inoculation by thrips is subject to many biological factors i.e., whether the thrips feed appropriately on the leaf disk or probing time. In contrast, ACP-ELISA was free of this type of variable and NSs was readily detected, thus identifying thrips that could transmit TSWV to *P. grandiflora.*

Error in identifying the potential transmitters that did not transmit in the plant transmission assay may be beneficial because most of the potential transmitters in the population would be detected. Thus, ACP-ELISA estimates of viruliferous thrips are very conservative and valuable for prediction purposes. A 2% error occurred when ACP-ELISA did not detect individuals that transmitted TSWV in the plant transmission assay. This type of error is more serious, because the ACP-ELISA missed the individuals that were viruliferous. This may have occurred for several reasons. Titers of NSs do fluctuate in thrips and may have dropped prior to ACP-ELISA in the few individuals where this error occurred, NSs antigens may have degraded in the thrips during shipments so the protein was not detected by ACP-ELISA, or thrips feeding damage on leaf discs may have been mistakenly scored as positive for TSWV transmission. This type of error involved in a very small percentage of the total number of thrips assayed and should not limit the utility of ACP-ELISA for screening thrips populations for potential transmitters. ACP-ELISA certainly permits screening of individual thrips in less time with less labor than plant transmission assays. Furthermore, ACP-ELISA is economical and less complicated than ELISA that used enzyme amplification systems previously reported to differentiate viruliferous thrips from non-viruliferous thrips (17). Yudin et al., found that disease incidence in lettuce at harvest was significantly associated with early disease incidence and early thrips abundance although thrips numbers could not be used as a reliable predictor of TSWV epidemics. The ability to readily identify viruliferous thrips will allow development of effective models to predict disease prior to crop planting

and permit growers to avoid epidemics and concomitant economic losses.

References

1. Allen, W. R., and Matteoni, J. A. 1991. Petunia as an indicator plant for use by growers to monitor for thrips carrying the tomato spotted wilt virus in greenhouse. Plant Dis. 75:78-82.

2. Allen, W. R., Trimble, R. M., and Vickers, P. M. 1992. ELISA used without host trituration to detect larvae of *phyllonorycter blancardella* (Lepidoptera:Gracillariidae) parasitized by *Pholetesor ornigis* (Hymenoptera:Braconidae). Environ. Entomol. 21:50-56.

3. Cho, J. J., Mau, R. F. L., German, T. L., Hartmann, R. W., Yudin, L. S., Gonsalves, D., and Provvidenti, R. 1989. A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Dis. 73:375-383.

4. Cho, J. J., Mau, R. F. L., Ullman, D. E., and Custer, D. M. 1991. Detection of tomato spotted wilt virus within thrips. Virus-plant interaction of tomato spotted wilt virus. USDA Workshop ARS-87:144-152.

5. German, T. L., Ullman, D. E., and Moyer, J. W. 1992. Tospoviruses: Diagnosis, molecular biology, and vector relationships. Annu. Rev. Phytopathology 30:315-348.

6. Mandrell, R. E., and Zollinger, W. D. 1984. Use of a Zwitterionic detergent for the restoration of the antibody-binding capacity of electroblotted meningococcal outer membrane proteins. J. Immnol. Methods 67:1-11.

7. Sakimura, K. 1962. The present status of thrips-borne viruses. Pages 33-40 in: Biological Transmission of Disease Agents. K. Maramorosch, ed. Academic Press, New York.

8. SAS. 1994. JMP Statistics and Graphics Guide, Version 3. SAS Institute Inc., Cary, NC.

9. Sheng, S., and Schuster, S. M. 1992. Simple modifications of a protein immunoblotting protocol to reduce nonspecific background. BioTechniques 13:704-706.

10. Spinola, S. M., and Cannon, J. G. 1985. Different blocking agents cause variation in the immunological detection of protein transferred to nitrocellulose membranes. J. Immunol. Methods 81:161-165.

11. Ullman, D. E., Cho, J. J., Mau, R. F. L., Westcot, D. M., and Custer, D. M. 1992. A midgut barrier to tomato spotted wilt virus acquisition by adult western flower thrips. Phytopathology 82:1333-1342.

12. Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M., and Custer, D. M. 1993. Tospovirus replication in insect vector cells: immunocytochemical evidence that the nonstructural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology 82:1333-1342.

13. Vogt, R. F., Phillips, D. L., Henderson, L. O., Whitfield, W., and Spierto, F. W. 1987. Quantitative differences among various proteins as blocking agents for ELISA microtiter plates. J. Immunol. Methods 101:43-50.

14. Wedge, E., and Svenneby, G. 1986. Effects of the blocking agents bovine serum albumin and Tween-20 in different blocking buffers on immunoblotting of brain proteins and marker proteins. J. Immunol. Methods 88:233-237.

15. Wedge, E., Bryn, K., and Froholm, L. O. 1988. Restoration of antibody binding to blotted meningococcal outer membrane proteins using various detergents. J. Immunol Methods 113:51-59.

16. Wijkamp, I., and Peters, D. 1993. Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. Phytopathology 83:986-991.

17. Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R., and Peters, D. 1993. Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. J. Gen. Virol. 74:341-349.

18. Yudin, L. S., Tabashinik, B. E., Cho, J. J., and Mitchell, W. C. 1990. Disease-prediction and economic models for managing tomato spotted wilt virus disease in lettuce. Plant Dis. 74:211-216.

Table 1

Comparison of the effect of Empigen-BB and Tween 20 in the antibody dilution buffer on detection of TSWV NSs protein in thrips by ELISA.

	Absorbance at 405 nm@					
	Empigen -BB			Tween 20		
Sample	1	2	3	1	2	3
Adults fed on TSWV-infected plants as adults	0.135±0.009	0.180±0.016	0.150±0.010	0.380±0.067	0.450±0.082	0.400±0.073
Adults fed on TSWV-infected plants as larvae	0.920±0.032	0.980±0.051	1.050±0.071	1.200±0.142	1.450±0.126	1.270±0.136
Adults fed on healthy plants	0.095±0.007	0.090±0.012	0.080±0.008	0.495±0.116	0.460±0.091	0.495±0.102
Larvae fed on TSWV-infected plants	1.275±0.041	1.350±0.098	1.875±0.128	1.870±0.195	1.830±0.189	2.235±0.272
Larvae fed on healthy plants	0.075±0.004	0.065±0.008	0.045±0.008	0.400±0.087	0.430±0.076	0.375±0.049

@Mean absorbance values±standard deviation of 20 wells, taken after 1 h.

Table 2.

Effect of Empigen-BB and Tween 20 in the antibody dilution buffer on detection of TSWV NSs in thrips by ACP-ELISA at different times after addition of substrate.

	Absorbance at 405 nm at different times@					
	Empigen -BB			Tween 20		
Sample	0.5	1	2	0.5	1	2
Adults fed on TSWV-infected plants as adults	0.115±0.012	0.180±0.016	0.250±0.017	0.275±0.079	0.450±0.082	0.625±0.086
Adults fed on TSWV-infected plants as larvae	0.450±0.048	0.980±0.051	1.150±0.054	0.850±0.118	1.450±0.126	2.150±0.127
Adults fed on healthy plants	0.055±0.014	0.090±0.012	0.185±0.018	0.280±0.088	0.460±0.091	0.830±0.089
Larvae fed on TSWV-infected plants	0.950±0.087	1.350±0.098	1.650±0.096	0.905±0.196	1.830±0.189	2.650±0.198
Larvae fed on healthy plants	0.040±0.007	0.065±0.008	0.185±0.013	0.240±0.042	0.430±0.076	0.975±0.079

@Mean absorbance values±standard deviation of 20 wells

Table 3.

Effect of incubation in enzyme substrate overnight at 4 C (ON-4C) versus 1h at room temperature (1h-RT) on ACP-ELISA for detection of TSWV NSs protein.

	Absorbance at 405 nm@			
	Empigen -BB		Tween 20	
Sample	1h-RT	ON-4C	1h-RT	ON-4C
Adults fed on TSWV-infected plants as adults	0.180±0.016	0.205±0.014	0.450±0.082	0.395±0.078
Adults fed on TSWV-infected plants as larvae	0.980±0.051	0.985±0.042	1.450±0.126	1.580±0.112
Adults fed on healthy plants	0.090±0.012	0.087±0.011	0.460±0.091	0.380±0.084
Larvae fed on TSWV-infected plants	1.350±0.098	1.470±0.078	1.830±0.189	1.740±0.175
Larvae fed on healthy plants	0.065±0.008	0.075±0.003	0.430±0.076	0.325±0.072

@Mean absorbance values±standard deviation of 20 wells

Table 4.

Identification of TSWV viruliferous thrips by ACP-ELISA using monoclonal antibody to TSWV-NSs and by thrips transmission by *Petunia grandiflora.*

Experimental group of thrips				
	1	2	3	4
Absorbance values (405nm) for thrips positive in ACP-ELISA	0.372 +[a] 0.168 +	0.713 + 0.414 + 0.214 +	1.288 + 1.073 + 1.016 1.010 + 0.859 + 0.808 + 0.689 + 0.674 + 0.629 + 0.411 0.364 + 0.295 0.294 0.270+ 0.263+ 0.216+ 0.134+ 0.131 0.130	3.000 + 2.077 + 2.064 1.728 1.610 + 1.425 + 1.372 + 0.765 + 0.724 + 0.712 0.612 0.521 + 0.421 + 0.421 + 0.412 0.402 + 0.318 0.311 0.299 + 0.299 + 0.289 0.278 0.218 +
No. of thrips negative in ACP-ELISA (A_{405nm}<0.100)	23	47	71	77
No. of thrips positive in plant transmission assay, but negative in ACP-ELISA	1(0.006)[b]	1 (0.059)	2 (0.085,0.000)	1 (0.000)
Total no. of thrips assayed	25	50	90	100

[a] +, thrips was positive in transmission assay to *P. grandiflora*

[b] Absorbance value in ACP-ELISA

Chapter 4

Production of monoclonal antibodies to TSWV glycoproteins using antigens eluted from ChromaPhor® stained gels.

Introduction

The ambisense M RNA (4.8 kb) encodes a 127.4 kDa polyprotein precursor to GP1 and GP2 in the viral complimentary sense and a 33.6 kDa NSm in the viral sense (7). The glycoproteins in the vertebrate-infecting genera of Bunyaviridae are implicated to be involved in maturation and/or assembly of virions and in viral attachment (16). In these genera, the GPs accumulate in Golgi membranes prior to virion assembly. When cloned M RNA segments are expressed in baculoviruses, the GPs are directed to and retained in the Golgi complex (9,12,13,18,20). However heterologous expression of La Crosse virus (LACV) and Rift Valley fever virus GPs results in their accumulation in the plasmalemma in addition to Golgi complex. The function and role of GPs in TSWV infection cycles are not well characterized. The assembly and maturation site for TSWV has not been established in plants (6) or in the thrips vector (25). Ullman et al. (25) immunolocalized the GPs on the membrane associated with the Golgi inside the infected thrips cells. In TSWV infected plants, virions were found in endoplasmic reticulum enclosed in membrane-bound compartments (3,6). Recently Adkins et al. (1) cloned the GPs polyprotein and expressed it in a baculovirus expression system. Their study demonstrated that the proteolytic

processing of the precursor and posttranslational glycosylation of GP1 and GP2 occurs in the absence of other viral proteins. In that study the targeting and accumulation of newly synthesized GPs were observed in the plasmalemma.

GPs are also implicated as the viral attachment proteins (VAPs) in LACV (4,11) and Hantaan viruses (18). In TSWV, the circumstantial evidence and results of immunocytochemical studies suggest that the functional role of GPs may be as VAPs. Envelope-deficient mutants of TSWV obtained through repeated mechanical inoculation on plants (2) are no longer thrips transmissible (27). Ullman et al. (25) demonstrated that TSWV is acquired by thrips through midgut epithelial cells and immunolabelling of midgut cells of thrips larvae revealed TSWV envelope GPs bound to the apical membrane adjacent to amorphous masses of TSWV N protein in the cytoplasm. Based on these observations they proposed that viral GPs might act as VAPs interacting with a cellular receptor in the apical membrane, initiating virus fusion with the apical membrane followed by release of replicative components of the virus in the cytoplasm (24).

Production of specific monoclonal antibodies to GP1 and GP2 is necessary to study the possible role of GPs as VAPs and to identify their corresponding cellular receptor sites (CRS). Isolation of individual GPs from purified virus preparations is necessary for production of antibodies. The most commonly used method is separating the proteins by sodium dodecyl sulfate-

polyacrylamide gels (SDS-PAGE) and then recovering the proteins of interest from the gel. Locating and recovering the proteins of interest from the gel fragments are the two critical and time-consuming steps involved in this process. For locating the proteins, the protein bands are usually visualized by Coomassie blue stain. The principle disadvantage of this technique is that the staining process renders the proteins difficult to recover from the gels. Proteins can be eluted from the gel slices by simply homogenizing the gel pieces (5) or by electroelution (23). The latter technique requires specialized equipment. Nevertheless, the recovery of proteins is relatively low (10). As an alternative, the ChromaPhor® protein recovery method (10) for isolation of GP1 and GP2 antigens for production of monoclonal antibodies was used and is presented in this chapter.

ChromaPhor® stain is a trimethylphenylene dye that stains the proteins green during electrophoresis (10). Protein bands at concentrations greater than 1 μg can be visualized during electrophoresis. The sensitivity of the dye can be enhanced to 100 ng by incubating the gels in a methanol and acetic acid fixing solution. However, once the gels are permanently stained the proteins cannot be eluted. The amount of protein in the band can be determined based on the stain intensity because the dye uniformly binds to proteins.

Materials and Methods

Electrophoresis and elution of GPs

SDS-PAGE (9%) gels were prepared as per the supplier's protocol for the ChromaPhor® system (19), in which Laemmli SDS-PAGE gel buffers are used (8), except that the SDS concentration was 35% of the original concentration. One mm thick mini-gels were made and SDS-PAGE was performed using a mini-PROTEAN II gel system (BioRad, Hercules, CA). Fifteen μg/well (10 well comb) or 100 μg (preparative comb) TSWV was loaded per gel. ChromaPhor® dye (Promega Corporation, Madison, WI) was diluted at 1:1000 (v/v) into the cathode buffer chamber just before electrophoresis. The gels were run until the GP1 and GP2 bands were resolved. For permanent staining, (enhanced method), the gel was incubated in 30% methanol (v/v) and 5% acetic acid (v/v) for 60 min and destained with 10% acetic acid. The GP1 and GP2 bands were cut and stored individually at -70 C. Gel pieces from 10 gels were pooled and ground in liquid nitrogen to a fine powder using a porcelain pestle and mortar. Twenty five ml of PBS-elution buffer (0.14 M NaCl, 1mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl, pH 7.5 with 0.1% SDS) was added to the gel powder and incubated at 37 C for 2 hours. The contents were mixed briefly at 15 minute intervals. The slurry was then transferred into centrifuge tubes and then the pestle and mortar were washed with an additional 5 ml of elution buffer. The tubes were centrifuged at 10,000 *g* for 30 min. The supernatants were then transferred into a fresh centrifuge tube without disturbing the pellet and

centrifuged for another 30 min at 10,000 *g*. Finally the supernatants were dispensed into microfuge tubes at 1 ml per tube and dried to 50 μl in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY) at 37 C. The concentrates were pooled and the ChromaPhor® dye and SDS were removed using a chloroform/methanol/water system (26). The pellet was resuspended in PBS and the protein concentration determined with a Coomassie Plus® Protein Assay Kit (Pierce Chemical Co., Rockford, IL). The protein concentration was adjusted to 0.5 mg/ml. The average yield of GP1 and GP2 was 12.5 μg per gel.

Immunization and production of monoclonal antibodies

Two Balb/c mice were each immunized with 25 μg of GP1 and GP2 emulsified with Titermax adjuvant (Vaxcel Inc., Norcross, CA). Three subsequent immunizations were given at 15 day intervals and after 20 days a booster dose of 100 μg of GP1 and GP2, each without adjuvant, was administered. The spleen cells were fused with P3X63Ag8.653 myeloma cell line 72 h after the booster as per the procedure established at the Hybridoma Center for Agricultural and Biological Sciences, Oklahoma State University, Stillwater, OK (21). The clones were screened by DAS-ELISA. The volume of reagents in the assay was 100 μl and all incubations were carried out on an orbital shaker at 150 rpm. The ELISA plates (Nunc MaxiSorp, PGC Scientific, Gaithersburg, MD) were coated overnight with rabbit anti-TSWV polyclonal serum at 5 μg/ml in carbonate-bicarbonate buffer (coating buffer, pH 9.6). The plates were washed with PBST three times and were blocked with 1% BSA in PBS. One gram of

TSWV-infected *Datura stramonium* was ground in 10 ml of PBST-PVP and the sap was strained through cheesecloth to remove debris. The sap was diluted at 1:10 with PBST-PVP and 100 μl of sap was added per well and incubated for four hours at room temperature. The plates were then washed three times with PBST buffer and then 100 μl of cell culture supernatant was added. Plates were incubated for two hours at room temperature or overnight at 4 C. The plates were washed three times with PBST and the positive wells were identified using antimouse IgG coupled to alkaline phosphatase (Sigma Chemical Company, St. Louis, MO). The cloning of the positive wells was by serial limited dilution. The clones were isotyped and frozen.

Evaluation of monoclonal antibodies

Monoclonal antibodies 1C5d3 (GP1) and 8C2b (GP2) were partially purified from cell culture supernatant with 50% ammonium sulfate precipitation. The pellet was dissolved in PBS and then dialyzed against PBS. The antibody concentration was estimated with a Coomassie Plus® Protein Assay Kit (Pierce Chemical Co.) and adjusted to 1 mg/ml. The utility of these antibodies in capturing and detecting the viral glycoproteins were compared with that of rabbit polyclonal serum made against a membrane glycoprotein fraction of TSWV (a gift from Dr. D. Gonsalves, Cornell University, Ithaca, NY) by DAS-ELISA. Ten wells of ELISA strips (Nunc MaxiSorp, PGC Scientific) each were coated overnight with rabbit anti-GP polyclonal antibody, or with 1C5d3 (GP1) and 8C2b (GP2) at 2 μg/ml in coating buffer (50 mM carbonate-bicarbonate buffer pH 9.6). The

indirect DAS-ELISA was performed as described above except that the captured glycoproteins in polyclonal antibody coated strips were detected by monoclonal antibodies and monoclonal antibody coated strips were detected by rabbit polyclonal antibody. The goat anti-rabbit alkaline phosphatase antibody was obtained from Sigma Chemical Company. Necessary controls were maintained and the wells without antigen were used to blank the plate reader.

The specificity of these two monoclonal antibodies in differentiating TSWV glycoproteins from that of impatiens necrotic spot virus (INSV) (a gift from Dr. Moyer, North Carolina State University, Raleigh, SC) and groundnut bud necrosis virus (GBNV) (a gift from Dr. D. V. R. Reddy, International Crop Research Institute for Semi Arid Tropics, Patancheru, India) were tested by DAS-ELISA using the strips coated with polyclonal antibody to INSV and GBNV (provided by the same sources as the virus preparations).

Results

Gel staining

The visualization of TSWV structural proteins by ChromaPhor® staining (enhanced method) is shown in Figure 1. The apparent molecular weight of GP1 was 95 kDa based on the estimation with the use of 10 kDa protein ladder (Gibco-BRL Life Technologies, Gaithersburg, MD). A faint band with 78 kDa was also seen. The molecular weight of GP2 was 58 kDa.

Production of monoclonal antibodies

The fusions yielded 20 hybridomas positive for GP1 and 23 for GP2, from which four clones for GP1 and six stable clones for GP2 were obtained. The clones were isotyped and monoclonal antibodies 1C5d3 (GP1) and 8C2b (GP2) were compared with rabbit anti-GPs polyclonal antibody (Table 1). Both the monoclonal antibodies identified the target viral glycoprotein from infected plants.

The two monoclonal antibodies, 1C5d3 (GP1) and 8C2b (GP2), were comparable to the rabbit anti-GP polyclonal serum in capturing as well as detecting TSWV glycoproteins (Table 1). The monoclonal antibodies did not react with glycoproteins from either INSV or GBNV in DAS-ELISA.

Discussion

Purification of proteins from SDS-PAGE often involves staining of the proteins by Coomassie blue after electrophoresis. As such, the gels must be subjected to acidic conditions and organic solvents to remove the SDS resulting in precipitation of proteins within the gel matrices. Furthermore, the staining and destaining steps require time. These problems can be avoided by using the ChromaPhor® stain. The recovery of proteins from the gels stained with ChromaPhor® was higher when compared to that from Coomassie blue stained gels. Larson and Shultz (10) obtained yields in excess of 50% for a variety of proteins in which known amounts had been applied to a gel and could recover

protein from bands that contained as little as 2 μg of protein. They also demonstrated that the proteins recovered by this method could be sequenced and used as antigens for producing polyclonal serum.

The TSWV glycoproteins were resolved using ChromaPhor® dye staining and a reduced concentration of SDS. GP1 migration was observed at 95 kDa, which is inconsistent with the 78 kDa previously reported (14,22). However, the GP2 migration was at 58 kDa as reported previously (14,22). The migration of GP1 at 95 kDa was also confirmed using Coomassie blue staining and confirmed by another lab (1). The estimated molecular weight of GP1 in previous reports was based on interpretation of multiple bands and variable migration of the protein in gels (14,15,22). When TSWV is passed through numerous mechanical transmissions, deletions in the GP open reading frame may develop (7). In this study the TSWV was purified from diseased plant material that was recently infected by viruliferous thrips (25). When GP1 was expressed in SF21 insect cells, the protein migrated at a lower molecular weight compared to the migration of GP1 from TSWV virions which was attributed to the patterns of glycosylation which may be different in insect cells than in plant cells (1). Thus, the inconsistent migrations reported previously could be caused by virus being obtained from mechanically inoculated plants and/or differential migration in the gel due to the differences in glycosylation. This is the first report of migration of GP1 at 95 kDa.

The utility of the anti-GP antibodies compared to polyclonal antibodies was estimated by DAS-ELISA. The monoclonal antibodies and polyclonal antibodies were equally sensitive and specific (Table 1). The monoclonal antibodies did not react with either INSV or GBNV in DAS-ELISA. This study demonstrated the utility of the ChromaPhor® elution method in recovering the antigens from the gels. The method offers a quick and easy alternative to the currently available methods. This is the first report of using such a method to elute antigens from gels for production of monoclonal antibodies.

References

1. Adkins, S., Choi, T. -J., Israel, B. A., Bandla, M. D., Richmond, K. E., Schultz, K. T., Sherwood, J. L., and German, T. L. 1996. Baculovirus expression and processing of the tomato spotted wilt virus glycoproteins. Phytopathology 86:849-855.

2. de O. Resende, R., de Haan, P., de Avilia, A. C., Kormelink, R., Goldbach, R., and Peters, D. 1991. Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. J. Gen. Virol. 72:2375-2383.

3. Francki, R. I. B., and Grivell, C. J. 1970. An electron microscope study of the distribution of tomato spotted wilt virus in systemically infected *Datura stramonium* leaves. Virology 42:969-978.

4. Gonzalez-Scarano, F. 1985. La Crosse virus G1 glycoprotein undergoes a conformational change at the pH of fusion. Virology 140:209-216.

5. Hager, D. A., and Burgess, R. R. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: Results with sigma subunits of *E. coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. 109:76-86.

6. Kitjima, E. W., de Avilia, A. C., Resende, R. De O., Goldbach, R. W., and Peters, D. 1992. Comparative cytological and immunogold labeling studies on different isolates of tomato spotted wilt virus. J. Submicrosc. Cytol. Pathol. 24:1-14

7. Kormelink, R., de Haan, P., Peters, D., and Goldbach, R. 1992. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. J. Gen. Virol. 73:2795-2804.

8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

9. Lappin, D.F., Nakitare, G. W., Palfreyman, J. W., and Elliott, R. M. 1994. Localization of Bunyamwera bunyavirus G1 glycoprotein to the Golgi requires association with G2 but not with NSm. J. Gen. Virol. 75:3441-3451.

10. Larson, G. A., and Shultz, J. W. 1993. Applications of the ChromaPhor® protein recovery system. BioTechniques. 15:316-323.

11. Ludwig, G. V., Israel, B. A., Christensen, B. M., Yuill, T. M., and Schultz, K. T.

1991. Monoclonal antibodies directed against the envelope glycoproteins of La Crosse virus. Microb. Pathog. 11:411-421.

12. Matsuoka, Y., Chen, S. Y., and Compans, R. W. 1994. A signal for Golgi retention in the bunyavirus G1 glycoprotein. J. Biol. Chem. 269:22565-22573.

13. Melin, L., Persson, R., Aandersson, A., Bergström, A., Rönnholm, R., and Pettersson, R. F. 1995. The membrane glycoprotein G1 of Uukuniemi virus contains a signal for localization to the Golgi complex. Virus Research 36:49-66.

14. Mohamed, N. A. 1981. Isolation and characterization of subviral structures from tomato spotted wilt virus. J. Gen. Virol. 53:197-206.

15. Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.

16. Nathanson, N., and Gonzalez-Scarano, F. 1994. Bunyaviruses: General Features. Pages 185-192 in: Encyclopedia of Virology. R. G. Webster and A. Granoff, eds. Academic Press, San Diego, CA.

17. Pensiero, M. N., and Hay, J. 1992. The Hantaan virus M-segment glycoproteins G1 and G2 can be expressed independently. J. Virol. 66:1907-1914.

18. Pensiero, M. N., Jennings, G. B., Schmaljohn, C. S., and Hay, J. 1988. Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. J. Virol. 62:696-702.

19. Promega Corporation. 1993. Technical bulletin No. 100:1-14

20. Ruusala, A., Persson, R., Schmaljohn, C. S., and Pettersson, R. F. 1992. Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. Virology 186:53-64.

21. Sherwood, J. L., Sanborn, M. R., and Keyser, G. C. 1987. Production of monoclonal antibodies to peanut mottle virus and their use in enzyme-linked immunosorbent assay and dot-immunoblotting assay. Phytopathology 77:1158-1161.

22. Tas, P. W. L., Boerjan, M. L., and Peters, D. 1977. The structural proteins of tomato spotted wilt virus. J. Gen. Virol. 36:267-279.

23. Tuszynski, G. P., Damsky, C. H., Further, J. P., and Warren, L. 1977. Recovery of concentrated protein samples from sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. 83:119-129.

24. Ullman, D. E., German, T. L., Sherwood, J. L., and Westcot, D. M. 1995a. Thrips Transmission of *Tospoviruses*: Future Possibilities for Management. pages 135-151. In: Thrips Biology and Management. B. L. Parker ed. Plenum Press, New York.

25. Ullman, D. E., Westcot, D. M., Chenault, K. D., Sherwood, J. L., German, T. L., Bandla, M. D., Catone, F. A., and Duer, H. L. 1995b. Compartmentalization, intracellular transport and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. Phytopathology 85:644-654.

26. Wessel, D., and Flugge, V. I. 1984. A method for the quantitative recovery of protein in dilute solutions in the presence of detergents and lipids. Anal. Biochem. 138:141-143.

27. Wijkamp, I. 1995. Virus-vector relationships in the transmission of tospoviruses. Ph.D. Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Table 1.

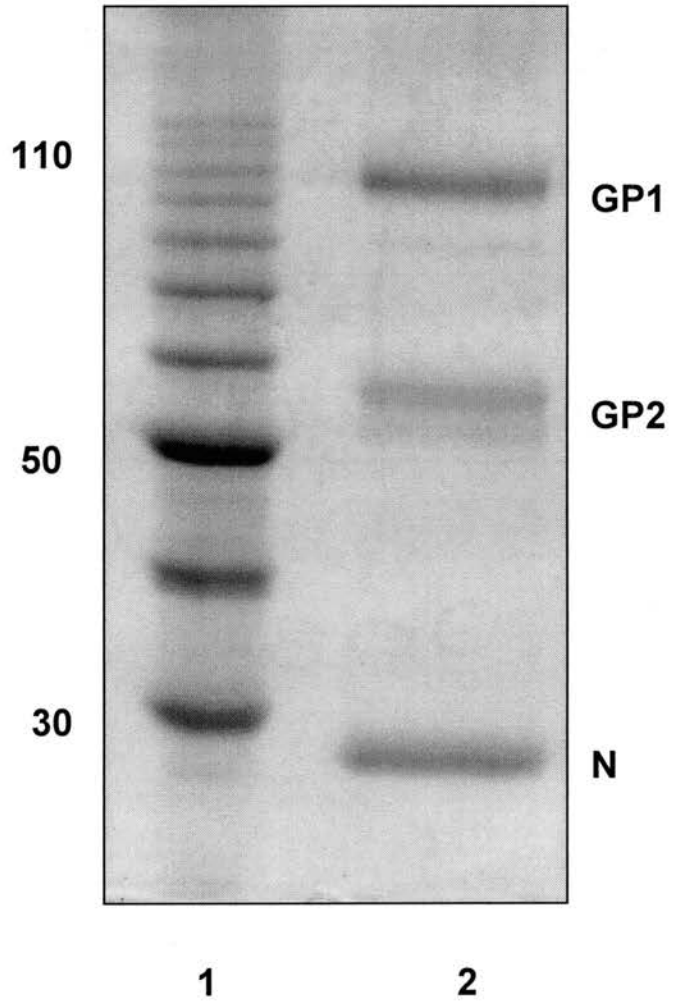
DAS-ELISA absorbance values of monoclonal antibodies in identifying GP1 and GP2 compared to those obtained with polyclonal antibodies.

	Absorbance (A 405nm)*	
Antibodies	Infected *Datura stramonium*	Healthy *Datura stramonium*
Polyclonal	1.103 ± 0.109	0.046 ± 0.017
1C5d3 (GP1)	0.823 ± 0.042	0.026 ± 0.005
8C2b (GP2)	0.696 ± 0.022	0.022 ± 0.006

* Mean absorbance values of 10 wells taken 1 h after addition of substrate. For polyclonal DAS-ELISA monoclonal antibodies 1C5d3 and 8C2b were used as capturing antibody and were coated to the plate at a combined concentration of five μg/ml. For monoclonal antibody DAS-ELISA the polyclonal antibody was used as capturing antibody and coated at five μg/ml.

Figure 1.

Visualization of TSWV structural proteins by ChromaPhor® staining (enhanced method).



(Lane 1) Protein ladder (molecular weight markers) in kDa, (Lane 2) TSWV.

Chapter 5

Identification of a potential cellular receptor site for TSWV in the thrips midgut using anti-idiotype antibodies of anti-GP monoclonal antibodies.

Introduction

The majority of the viruses infecting plants that are transmitted by arthropod vectors are transmitted in a circulative manner. Many of these virus-vector relationships involve a high degree of specificity. The virus has to survive the physiological and biochemical environment inside the insect gut and cross the epithelial cells and other potential barriers to reach the salivary glands. To achieve this passage, viruses have adopted several strategies, of which few are well documented.

The presence of a cellular receptor site (CRS) that binds to the specific viral attachment proteins (VAP) is well characterized for insect pathogenic viruses and other mosquito transmitted animal viruses, i.e., polyhedron-derived baculovirus (19), the *Bacillus thurigiensis crylA*(b) and *crylA*(c) δ-endotoxins (24,39) and western equine encephalomyelitis (WEE) virus (20). No cellular receptors have been identified for insect vectors of plant viruses, although several proteins and transmission factors of virus vector origin have been reported, such as symbionin for potato leafroll virus (PLRV) in aphids

(40), aphid transmission factor of cauliflower mosaic virus (CaMV) (32), the P17 of barley yellow dwarf luteovirus (BYDV) (7) and the P74 protein of beet western yellows luteovirus (BWYV) (5).

The majority of bunyaviruses are transmitted to their vertebrate hosts by arthropod vectors in which they replicate (30). Several studies have demonstrated that viral and host cell membrane proteins interact to mediate virus entry into cells (43). Bunyaviruses are membrane bound and have two glycoproteins (GP1 and GP2) that serve as VAP to interact with cellular receptor sites (CRS) to mediate virus entry. The acquisition of the bunyaviruses by their vectors is well studied with La Crosse virus (LACV). The LACV membrane glycoproteins, GP1 and GP2, interact with host and vector CRS under different circumstances. GP1 binds to cultured vertebrate and mosquito cell lines but not to the vector midgut cells *in vivo*. GP2 binds to cultured mosquito cells and vector midgut cells but not to cultured vertebrate cells (26). It is proposed that GP1 is hydrolyzed by proteases in the mosquito midgut, exposing the GP2 to serve as the VAP.

TSWV is transmitted by both larval and adult western flower thrips (WFT) (*Frankliniella occidentalis*), but only when larvae acquire TSWV are the consequent adult thrips capable of transmitting virus in a persistent manner (29). Although a WFT biotype from the Netherlands has been shown to acquire TSWV during the first larval instar stage (41), biotypes from other localities (e.g.,

Hawaiian islands, United States mainland, Israel) acquire TSWV during both larval instars (36, and Dr. Diane Ullman, personal communication). In TSWV, circumstantial evidence and results of immunocytochemical studies suggest that the membrane glycoproteins serve as VAPs. Envelope-deficient mutants of TSWV obtained through repeated mechanical inoculation on plants are no longer transmissible by thrips, suggesting that the GPs are required for virus acquisition by the thrips (44). The occurrence of an arginine-glycine-aspartic acid (RGD) motif in GP2 (25), a characteristic of cell adhesion molecules, also suggests that the GPs may act as VAPs. Ullman et al. (1995a) demonstrated that TSWV is acquired by thrips through midgut epithelial cells, and immunolabelling of thrips larvae revealed TSWV envelope GPs bound to the apical membrane adjacent to amorphous masses of TSWV N protein in the cytoplasm. Deeper within these cells, the TSWV encoded proteins were compartmentalized in vacuoles, inclusions and vesicles. Based on these observations, they proposed that viral GPs act as VAPs interacting with a cellular receptor in the apical membrane and initiating virus fusions with the apical membrane, followed by release of replicative components of the virus in the cytoplasm. Similar mechanisms were reported for other membrane bound viruses such as HIV (14,33) and certain baculoviruses (19).

Identification and characterization of CRSs is extremely difficult due to their existence in low numbers, the possibility of multiple CRSs and the involvement of intermediate molecules (42). Hence, wherever possible it is

advisable to confirm the identification of a putative CRS by a second approach. Gel overlay assays, also known as virus overlay protein blot assays (VOPBA), and use of anti-idiotypic antibodies (anti-ids), are the two most common techniques used for studying receptors.

Gel overlay assays have been used to study the putative VAP-CRS interactions of viruses infecting vertebrate cells, such as bovine herpesvirus 1 (42), human cytomegalovirus (1), Sendai virus (17), mouse hepatitis virus (4), visna virus (11,12), murine coronavirus (10,13), choriomeningitis virus (3) encephalomyocarditis virus (23), reovirus (8), and rabies virus (15). With plant infecting viruses, the gel overlay assays has been limited to investigating the relationship of the PLRV with its aphid vector (*Myzus persicae*) (40). A 63 kDa protein produced by the aphid endosymbiont bacteria that specifically bound to PLRV was identified (18). The 63 kDa protein (symbionin) has a protein sequence homology to *groEL* from *E. coli*, a member of the *hsp*-60 family, and thus may act as a chaperon, perhaps protecting the PLRV from degradation (18). A similar gel overlay assay was used to identify an aphid transmission factor which bound to CaMV (31).

Jerne (1974) proposed that antibody production in the humoral immune response might be regulated by the production of anti-antibodies. Now their role in the regulation of the immune system is well defined (reviewed in ref. 38). An idiotope is defined as an autologous antigenic determinant expressed on

immunoglobulin heavy and light chain CDRs that are defined serologically by the binding of anti-idiotypic antibody (16). An idiotype is the set of idiotopic determinants expressed on the variable region of a particular antibody. Jerne (1974) hypothesized that all the antigens recognized by the immune system are represented by the internal images within the immune system. However, the internal image anti-idiotypes can be generated only if the paratope of the antibody is the idiotope. This group of internal image anti-idiotype antibodies represents only a small number of total anti-idiotype antibodies. For example, with anti-tobacco mosaic virus (TMV) anti-idiotype antibodies Urbain et al. (1984) reported that internal image anti-idiotype antibodies account for only 15% of the total anti-idiotype antibodies. These internal image anti-idiotype antibodies will mimic the antigens and can be used as excellent tools in identifying and studying the receptor-ligand interactions when an antibody to a ligand is available. Besides using anti-ids as vaccines, their use as probes to identify cell receptors of infectious agents has been widely reported (9,27,42,45). In addition, the anti-ids are used in the study and characterization of hormone, vitamin, and neurotransmitter receptors (reviewed in ref. 16). The antigenic mimicry of the anti-idiotype antibodies is also used in plant virology. Anti-TMV antibodies were used as antigen to produce anti-idiotype antibodies to demonstrate the antigen mimicry of the anti-idiotype antibodies (38). The investigators showed that anti-idiotype antibodies reacted with all anti-TMV antibodies raised in different animals and demonstrated that injecting the anti-idiotype antibodies back into mouse can produce anti-TMV antibodies. Hu and Rochow (1988) produced anti-

idiotype antibodies against a BYDV monoclonal antibody to identify putative receptors in aphid salivary glands. To demonstrate the specificity and purity of these anti-idiotype antibodies, they raised anti-anti-idiotype antibodies, which reacted to BYDV. They essentially followed the same procedures used by Urbain et al. (1984) to demonstrate the specificity of the anti-idiotype antibodies. Recently, van den Heuvel et al. (1994) used anti-idiotype antibodies to a putative receptor to support the identity of the receptor that they identified by conducting gel overlay experiments. In this chapter, a similar gel overlay assay was performed to identify the CRS in the thrips midgut. The viral GPs were shown to bind to protein from WFT midguts *in vitro* and a preliminary report has been published (2). To confirm the identity of the putative CRS, anti-idiotypic antibodies to the TSWV GPs were produced and used in anti-ids antibody hybridization to thrips midgut extracts on western blots.

Materials and Methods

Maintenance of virus isolate

A TSWV isolate (formerly TSWV-L) collected from infected tomato on the Hawaiian Island of Maui was used and maintained by inoculation with WFT as previously described (37). Infected *Datura stramonium* was used to purify the virus as previously reported (32).

Maintenance of insects

The WFT used in all experiments were maintained on green pods

of *Phaseolus vulgaris* as previously described (36). The greenhouse thrips, *Heliothrips haemorrhoidalis*, were obtained from D. Ullman, University of California, Davis, CA and were identified by D. Ullman. Leafhoppers (*Circulifer tenellus*) were obtained from J. Fletcher and A. Wayadande, Oklahoma State University, and were originally identified by J. K. Bouseman, Illinois Natural History Survey. Aphids (*Schizaphis graminum*) were obtained from J. A. Webster, USDA-ARS, Stillwater, OK, and originally identified by J. A. Webster. These latter insect species were included as negative controls as they transmit a variety of plant viruses and mollicutes but not tospoviruses.

Production of antibodies

TSWV GPs were isolated from TSWV virions using ChromaPhor® stained gels, and monoclonal antibodies to GP1 and GP2 were produced as reported in Chapter 4. Antibodies to either GP1 (1C5d3) or GP2 (8C2b) were used in this study. Rabbit anti-N antibodies and anti-NSs were produced as previously reported (35,36). Monoclonal antibodies to NSs were produced as described earlier in Chapter 2.

Production of anti-ids

Anti-id ascites to monoclonal antibodies 1C5d3 and 8C2b was produced in C3H/Hen mice. The monoclonal antibodies were isolated from culture supernatant by ammonium sulfate precipitation and resuspended in PBS, and the protein concentration was adjusted to 1 mg/ml. A mouse received 8

injection of 100 μg each over a 18 week period. Ascites was induced by injecting 2 X 10^6 S-180 cells (ATCC TIB-66). The ascites fluid was harvested 16 days later and the immunoglobulin fraction obtained by ammonium sulfate precipitation.

Gel overlay assays

Overlay assays with purified TSWV or gel-isolated viral proteins were conducted as described by van den Heuvel (1994). First and second instar WFT larvae, WFT adults, aphids, leafhoppers or greenhouse thrips larvae and adults were ground in 200 μl of hot (90 C) SDS-PAGE sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol) and incubated at 90 C for 5 min. For overlay assays using excised thrips guts, equivalent amounts of excised gut tissue from larvae and from adults were prepared. Thirty μg of each sample was subjected to SDS-PAGE on a minigel electrophoresis chamber (BioRad, Richmond, CA) and then transferred to nitrocellulose membrane. Prestained molecular markers (BioRad) were used to monitor protein separation and transfer.

The nitrocellulose blot was incubated in 5% non fat dry milk (NFDM) in 25 mM Tris buffered saline (TBS) for 60 min at room temperature (RT). For overlay assays with TSWV, virus isolated from approximately 100 g of infected *D. stramonium* was diluted to 10 μg/ml in TBS with 1% NFDM, or macerated infected plant tissue diluted to a final concentration of 0.1 gm/ml in

TBS with 1% NFDM was used. Gel-isolated TSWV GPs were used at 10 μg/ml in TBS with 1% NFDM in overlay assays. Blots were incubated for 3 h at RT or overnight at 4 C on an orbital shaker. Blots were then rinsed three times with TBS for 10 min each and then incubated for 3 h at RT in antibody solution (2 μg/ml in TBS with 1% NFDM). Antibodies to the GPs (1C5d3 and/or 8C2b), N protein or NSs protein were used. Blots were then rinsed three times in TBS and the bound antibody was detected using either goat anti-mouse or goat anti-rabbit alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) followed by BCIP-NBT substrate (Sigma Chemical Co.). Control blots with no virus or viral proteins were also performed.

Western blots were used to detect hybridization between the anti-GPs anti-ids and thrips proteins. The thrips proteins were subjected to SDS-PAGE and then transferred to nitrocellulose blots. The blots were blocked with 5% NFDM and then incubated with anti-ids diluted at 5 μg/ml in TBS with 1% NFDM for 3 h. The bound antibody was detected using avidin DH and biotinylated horseradish peroxidase reagents following the manufacturer's protocol (Vector laboratories, Burlingame, CA).

Results

Gel overlay assays

A 50 kDa thrips protein from extracts of whole insects and dissected midguts selectively binds TSWV membrane GPs (Figures 1-3). Gel

overlay assays involving whole thrips proteins from WFT adults, larvae and isolated virus followed by probing with monoclonal antibodies to GP1 and GP2 revealed a protein with an approximate molecular weight of 50 kDa (Figure 1). A difference in the intensity of the bands from larvae and adults was observed, with only a faint band detected in preparations from adults (Figure 1). This protein was absent in all the non-vector insect species that were included as negative controls. To determine if viral proteins in addition to the GPs were bound to the proteins from the insects, blots were probed with TSWV, TSWV N protein or TSWV NSs protein, followed by antibody to N protein or NSs and the appropriate secondary antibody. Neither the N protein nor the NSs proteins were detected in the assays (blots not shown).

To determine if the 50 kDa protein was detectable in thrips guts, guts from adults and larvae were excised and then assayed by gel overlay. The 50 kDa protein was detected in guts from both adults and larvae, but band intensity was greater in guts from larvae than in those from adults (Figure 2). To determine if the virion GPs alone would bind to thrips proteins, gel isolated GP1 and GP2 were incubated with the blots prior to probing the blots with monoclonal antibodies to GP1 and GP2. As in the virus overlay assays, a 50 kDa band was detected in both thrips larvae and adults, but not in aphids (Figure 3).

Western blot with anti-idiotype antibodies to GP1 or GP2

The observations from the gel overlay assay were confirmed by the

results of western blots with GP monoclonal antibodies anti-ids antibody. The anti-ids of monoclonal antibody 1C5d3 (GP1) and monoclonal antibody 8C2b (GP2) bound to the 50 kDa protein from *F. occidentalis*, but not from *H. haemorrhoidalis* (Figure 4).

Discussion

Based on electron microscopy studies Ullman et al. (1995 a,b) proposed that acquisition of TSWV by thrips is a receptor-mediated process and the GPs serve as VAPs that interact with a corresponding CRS in the thrips midgut. The results presented here support this hypothesis. TSWV GPs bind to a 50 kDa protein in the midgut of the WFT, but not to any protein from non-vector insects including another species from Thripidae. The intensity of the 50 kDa protein was greater in larvae than in adults, an observation which agrees with the hypothesis that receptor abundance is an important determinant of vector competence with membrane bound viruses (20). The proteins that function as CRS may have other physiological functions in the insect (28) and therefore the detection of the 50 kDa protein in the adult WFT might indicate its involvement in other functions. Also, the environment of the midgut in the larvae might mediate binding of GPs to the 50 kDa protein but not in the adult midgut. Proteases and pH play important roles in mediating the endocytosis of membrane bound viruses (6,26,43). It was proposed that symbionin, which has high homology with the *E. coli* heat shock protein *groEL* may chaperone the PLRV inside its aphid vector, *Myzus persicae*. Rabbit anti-*groEL* antibody strongly bound to an approximately

70 kDa protein from both larval and adult thrips, but not to the 50 kDa protein (results not shown). Hence, it is likely that TSWV endocytosis in larvae will be mediated both by the receptor abundance and the environment of the thrips midgut.

The N and NSs proteins from TSWV did not bind to the 50 kDa thrips protein. No binding occurred when anti-GP, anti-N or anti-NSs antibodies were used in gel overlay assays in the absence of viral proteins. The results from the gel overlay assay indicated that a 50 kDa protein interacts with the TSWV GPs and were further supported by western blotting with the GPs monoclonal antibody anti-ids. Since anti-idiotypic antibody to GPs monoclonal antibodies mimic the TSWV GPs, their binding to the 50 kDa protein from thrips midgut provide important supporting evidence that the 50 kDa protein is a cellular receptor. The antigenic mimicry of the anti-idiotype antibodies was also demonstrated with TMV (38) and BYDV (21). A similar approach was used to demonstrate the affinity of PLRV to the symbionin protein in its aphid vector by van den Heuvel et al. (1994).

The present study supports the involvement of a 50 kDa protein as a putative CRS in thrips and of TSWV glycoproteins as VAPs. The interaction between TSWV GPs and thrips midgut proteins demonstrated in this study, strengthens the hypothesis that receptor-mediated events in virus acquisition in thrips midguts involve the viral GPs. This conclusion is consistent with other

accepted models of virus entry for membrane bound viruses, including those proposed for other members of the family *Bunyaviridae* (14,26,33) and certain baculoviruses (19).

References

1. Aldish, J. D., Lahijani, R. S., and St Jeor, S. C. 1990. Identification of a putative cell receptor for human cytomegalovirus. Virology 176:337-345.

2. Bandla, M. D., Westcot, D. M., Ullman, D. E., and Sherwood, J. L. 1995. Thrips midgut proteins that bind to tomato spotted wilt tospovirus glycoproteins, pages 341-349. In: Proceedings of the International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. G. Kuo, G. D. Peters and T. L. German, eds. Acta Horticulturae 431.

3. Borrow, P., and Oldstone, M. B. 1992. Characterization of lymphocytic choriomeningitis virus-binding protein(s): a candidate cellular receptor for the virus. J. Virol. 66:7270-7281.

4. Boyele, J. F., Weismiller, D. G., and Holmes, K. V. 1987. Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissue. J. Virol. 61:185-189.

5. Brault, V., van den Heuvel, J. F. J. M., Verbeek, M., Zieglegraff, V., Reutenauer, A., Herbeck, E., Garaud, E., Guilley, J. C., Richards, K., and Jonard, G. 1995. Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. EMBO J. 14:650-659.

6. Carrasco, L. 1994. Entry of animal viruses and macromolecules into cells. FEBS Letters 350:51-154.

7. Chay, C. A., Gunasinghe, U. B., Dinesh-Kumar, S. P., Miller, W. A., and Gray, S. M. 1996. Aphid transmission and systemic plant infection determinants of barley yellow dwarf luteovirus-PAV are contained in the coat protein readthrough domain and 17-kDa protein, respectively. Virology 219:57-65.

8. Choi, A. H. C. 1994. Internalization of virus binding proteins during entry of reovirus into K562 erythroleukemia cells. Virology 200:301-306

9. Co, M., Gaulton, G. N., Fields, B. N., and Greene, M. I. 1985. Isolation and characterization of the mammalian reovirus type 3 cell surface receptor. Proc. Natl. Acad. Sci. USA 82:1494-1498.

10. Compton, S. R., Stephensen, C. B., Snyder, S. W., Weismiller, D. G., and Holmes, K. V. 1992. Coronavirus species specificity: murine cornonavirus binds to a mouse-specific epitope on it carcinoembryonic antigen related receptor glycoprotein. J. Virol. 66:7420-7428.

11. Crane, S. E., Buzy, J., and Clements, J. E. 1991. Identification of cell

membrane proteins that bind visna virus. J. Virol. 65:6137-6143.

12. Dalziel, R. G., Hopkins, J., Watt, N. J., Dutia, B. M., Clarke, H. A. K. And McConnell, I. 1991. Identification of a putative cellular receptor for the lentivirus visna virus. J. Gen. Virol. 72:1905-1911.

13. Dveksler, G. S., Dieffenbach, C. W., Cardellichio, C. B., McCuaig, K., Pensiero, M. N., Jiang, G. -S., Beauchemin, N., and Holmes, K. V. 1993. Several members of the mouse carcinoembryonic antigen-related glycoprotein family are functional receptors for the coronavirus mouse hepatitis virus-A59. J. Virol. 67:1-8.

14. Freed, E. O., and Risser, R. 1990. The role of the HIV envelope glycoproteins in cell fusion and the pathogenesis of AIDS. Bull. Inst. Pasteur 88:73-110.

15. Gaskta, M., Horvath, J., and Lentz, T. L. 1996. Rabies virus binding to the nicotinic acetylcholine receptor α subunit demonstrated by virus overlay protein binding assay. J. Gen. Virol. 77:2437-2440.

16. Gaulton, G. N., and Greene, M. I. 1986. Idiotype mimicry of biological receptors. Annul. Rev. of Immunol. 4:253-280.

17. Gershoni, J. M., Lapidot, M., Zakai, N., and Loyter, A. 1986. Protein blot analysis of virus receptor: Identification and characterization of the Sendai virus receptor. Biochem. Biophys. Acta. 856:19-26.

18. Hogenhout, S. A., Verbeek, M., Hans, F., Houterman, P. M., Fortass, M., van der Wilk, F., Huttinga, H., van den Heuvel, J. F. J. M. 1996. Molecular bases of the interactions between luteoviruses and aphids. Agronomie 16:167-173.

19. Horton, H. M., and Burand, J. P. 1993. Saturable attachment sites for polyhedron-derived baculovirus on insect cells and evidence for entry via direct membrane fusion. J. Virology 67:1860-1868.

20. Houk, E. J., Arcus, Y. M., Hardy, J. L., and Kramer, L. D. 1990. Binding of western equine encephalomyelits virus to brush border membrane fragments isolated from mesenteronal epithelial cell of mosquitoes. Virus Research 17:105-118.

21. Hu, J. S., and Rochow, W. F. 1988. Anti-idiotypic antibodies against anti-barley yellow dwarf virus monoclonal antibody. Phytopathology 78:1302-1307.

22. Jerne, N. K. 1974. Towards a net work theory of the immune system. Annals of Immunol. 125C:373-379.

23. Jin, Y. -M., Pardoe, I. U., Burness, A. T. H., and Michalak, T. I. 1994. Identification and characterization of the cell surface 70-kilodalton sialoglycoprotein(s) as a candidate receptor for encephalomyocarditis virus on human nucleated cells. J. Virol. 68:7308-7319.

24. Knight, P. J. K., Knowles, B. H., and Ellar, D. J. 1995. Molecular cloning of an aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CrylA(c) toxin. J. Biol. Chem. 270:7765-7770.

25. Kormelink, R., de Haan, P., Peters, D., and Goldbach, R. 1992. The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. J. Gen. Virol. 73:2795-2804.

26. Ludwig, G. V. Israel, B. A., Christensen, B. M., Yull, T. M., and Schultz, K. T. 1991. Role of La Crosse virus glycoproteins in attachment of virus to host cells. Virology 181:564-571.

27. Okenhouse, F. C., Klotz, W. F., Tandon, N. N. and Jamieson, G. A. 1991. Sequestrin, a CD36 recognition protein on *Plasmodium falciparum* malaria-infected erythrocyte identified by anti-idiotype antibodies. Proc. Natl. Acad. Sci. USA 88:3175-3179.

28. Rossmann, M. G. 1994. Viral Cell recognition and entry. Protein Science 3:1712-1725.

29. Sakimura, K. 1962. The present status of thrips-borne viruses, pages 33-40. In: Biological Transmission of Disease Agents. K. Maramorosch, ed. Academic Press, New York.

30. Schmaljohn, C. S., and Patterson, J. L. 1990. Bunyaviridae and their Replication:Part II. Replication of Bunyaviridae, pages 1175-1195. In: Field's Virology. B. N. Fields and D. M. Knipe, eds. Raven, New York.

31. Schmidt, I., Blanc, S., Esparandieu, P., Kuhl, G., Devauchelle, G., Louis, C., and Cerutti, M. 1994. Interaction between the aphid transmission factor and virus particles is a part of the molecular mechanism of cauliflower mosaic virus aphid transmission. Proc. Natl. Acad. Sci. USA 91:8885-8889.

32. Sherwood, J. L., Sanborn, M. R., Keyser, G. C., and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. Phytopathology 79:61-64.

33. Stein, B. S., Shantharaj, D. G., Lifson, J. D., Penhallow, R. C., Bensch, K. G., and Engleman, E. G. 1987. pH independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell 49:659-668.

34. Ullman, D. E., German, T. L., Sherwood, J. L., and Westcot, D. M. 1995a. Thrips Transmission of *Tospoviruses*: Future Possibilities of Management, pages 135-151. In: Thrips Biology and Management. B. L. Parker ed. Plenum Press, New York

35. Ullman, D. E., Westcot, D. M., Chenault, K. D., Sherwood, J. L., German, T. L., Bandla, M. D., Catone, F. A., and Duer, H. L. 1995b. Compartmentalization, intracellular transport and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. Phytopathology 85:644-654.

36. Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M., and Custer, D. M. 1993. Tospovirus replication in insect vector cells: immunocytochemical evidence that the nonstructural protein encoded by the S RNA if tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology 82:1333-1342.

37. Ullman, D. E., Cho, J. J., Mau, R. F. L., Westcot, D. M., and Custer, D. M. 1992. A midgut barrier to tomato spotted wilt virus acquisition by adult western flower thrips. Phytopathology 82:1333-1342.

38. Urbain, J., Slaoui, M., Mariame, B., and Leo, O. 1984. Idiotypy and internal images. Pages 15-58, In: Idiotypy in biology and medicine, Academic Press, New York.

39. Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H., and Bulla, L. A. Jr. 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thruringiensis*. J. Biol. Chem. 270:5490-5494.

40. van den Heuvel, J. F. J. M., Verbeek, M., and van der Will, F. 1994. Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by *Myzus persicae*. J. Gen. Virol. 75:2559-2565.

41. Van de Wetering, F., Goldbach, R., and Peters, D. 1996. Tomato spotted wilt tospovirus ingestion by first instar larvae of *Frankliniella occidentalis* is a prerequisite for transmission. Phytopathology 86:993-999.

42. Varthakavi, V., and Minocha, H. C. 1996. Identification of a 56 kDa putative bovine herpesvirus 1 cellular receptor by anti-idiotype antibodies. J. Gen. Virol. 77:1875-1882.

43. White, J. M. 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. 52:675-697.

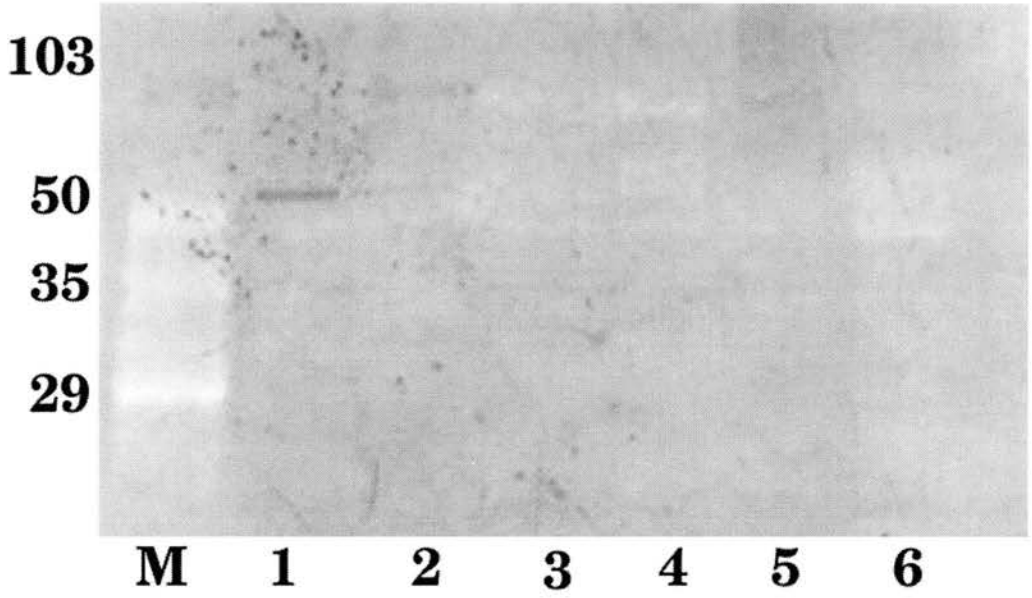
44. Wijkamp, I. 1995. Virus-vector relationships in the transmission of tospoviruses. Ph.D. Thesis, Wageningen Agricultural University,

Wageningen, The Netherlands.

45. Xue, W., and Minocha, H. C. 1993. Identification of the cell surface receptor for bovine viral diarrhea virus by using anti-idiotypic antibodies. J. Gen. Virol. 74:73-79.

Figure 1.

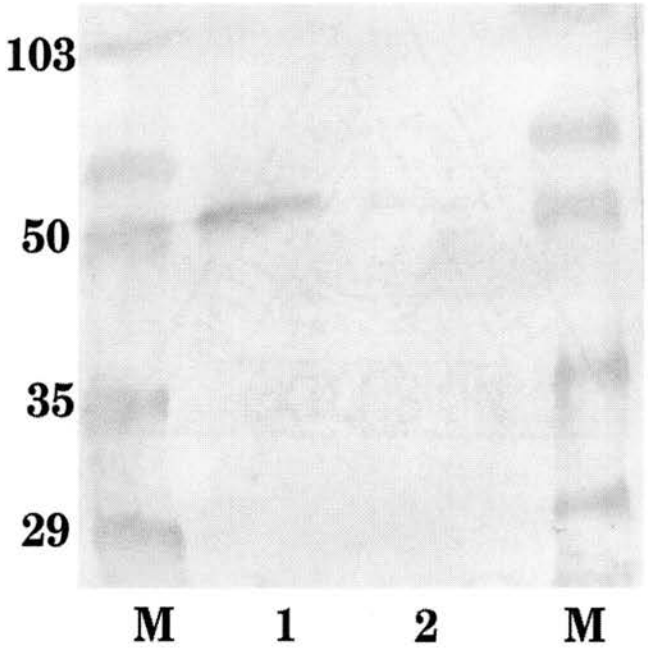
Gel overlay assay of whole insect proteins with partially purified TSWV as the overlay and probed with anti-GPs monoclonal antibodies



(Lanes 1 and 2) larvae and adults of WFT *Frankliniella occidentalis*, (lanes 3 and 4) larvae and adults of *Heliothrips haemorrhoidalis*, (lane 5) adult aphids of *Schizaphis graminum* and (lane 6) adult leafhoppers of *Circulifer tenellus*. Molecular weights markers in kDa.

Figure 2.

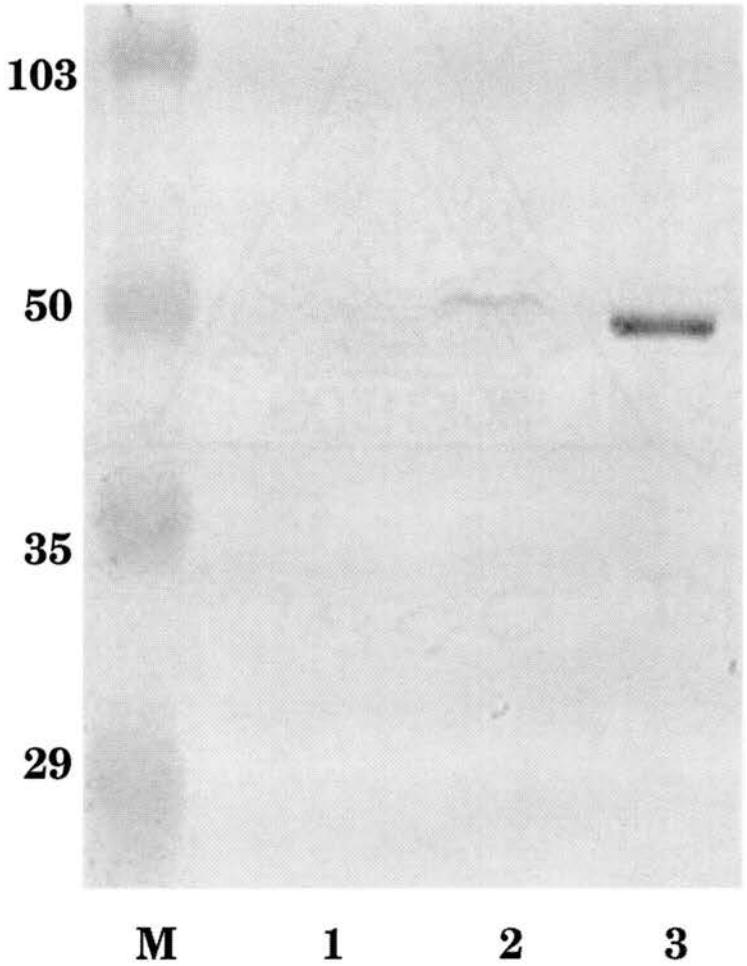
Gel overlay assay of excised midgut proteins from *Frankliniella occidentalis* with partially purified TSWV as overlay and probed with anti-GPs monoclonal antibodies.



(Lane 1) larvae and (lane 2) adults. Molecular weight markers in kDa.

Figure 3.

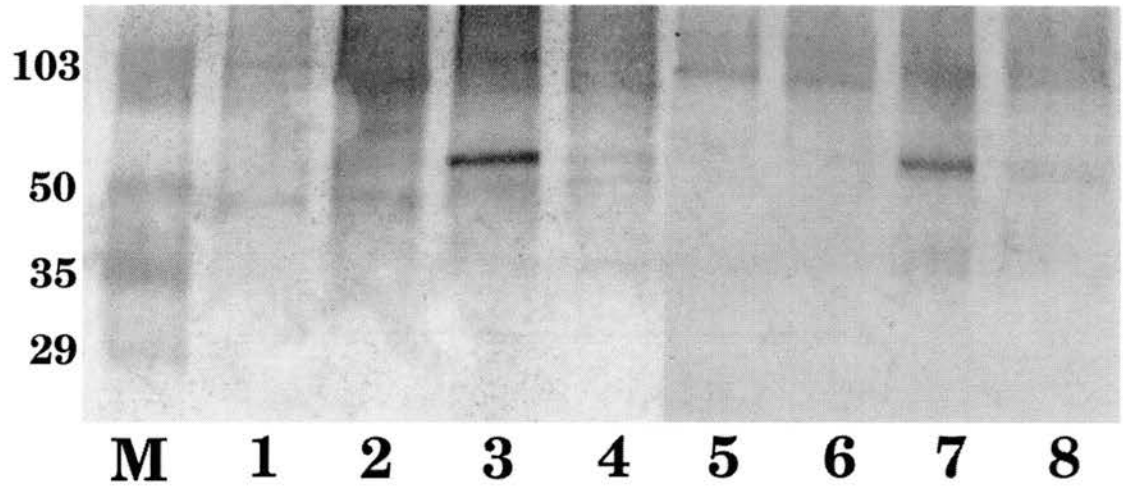
Gel overlay assay of whole insects from *Frankliniella occidentalis* and *Schizaphis graminum* with gel isolated TSWV-GPs as the overlay, probed with anti GPs monoclonal antibodies



(Lane 1) *S. graminum* adults (lanes 2 and 3) *F. occidentalis* adults and larvae. Molecular weight markers in kDa.

Figure 4.

Western blot of *Heliothrips haemorrhoidalis* and *Frankliniella occidentalis* with anti-idiotypic antibody to TSWV GP1 or GP2.



(Lanes 1 and 5) larvae and (lanes 2 and 6) adults of *H. haemorrhoidalis*, (lanes 3 and 7) larvae and adults (lanes 4 and 8) of *F. occidentalis*. Molecular weight markers in kDa.

VITA

Muralidhar Bandla

Candidate for the Degree of

Doctor of Philosophy

Thesis: SEROLOGICAL DETECTION OF TOMATO SPOTTED WILT VIRUS (TSWV) PROTEINS AND IDENTIFICATION OF COMPONENTS OF TSWV ACQUISITION BY THRIPS

Major Field: Plant pathology

Biographical

Education: Graduated from Andhra Pradesh Agricultural University, Hyderabad, India with Bachelor of Science in Agriculture in June, 1980; received Master of Science in Plant Pathology from Annamalai University, Annamalai Nagar, India in Nov, 1992. Completed the requirements for the Doctor of Philosophy with a major in Plant Pathology at Oklahoma State University in December, 1997.

Experience: Worked as Assistant Research Specialist, Rice Research Station, Maruteru, India from 1983-84. Worked as Agricultural Finance Officer at Corporation Bank, Mangalore, India from 1984 to 1989. Employed by Oklahoma State University as Lab Manager, Hybridoma Center for Agricultural and Biological Sciences from 1991 to 1996.

Professional Memberships: American Phytopathological Society; Institute for Laboratory Animal Research (ILAR)