STUDIES ON THE INTERACTION OF VIRUSES AND THEIR PLANT HOSTS: TRANSMISSION IN SEED AND SYMPTOM DETERMINANTS

By

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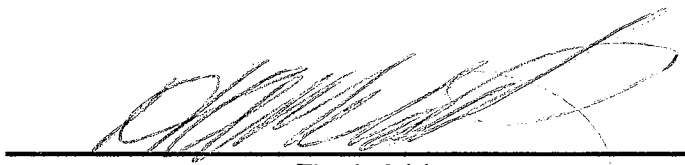

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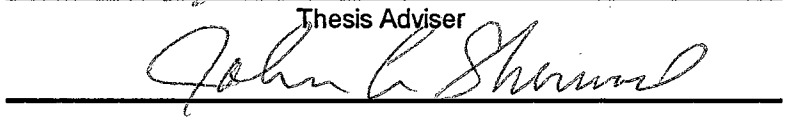

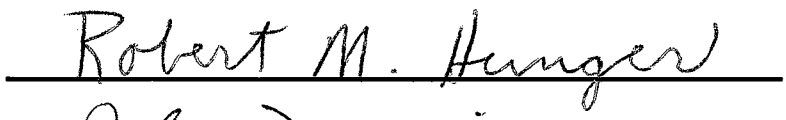

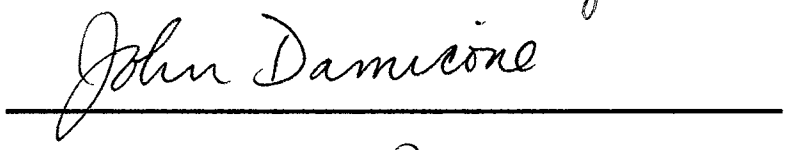

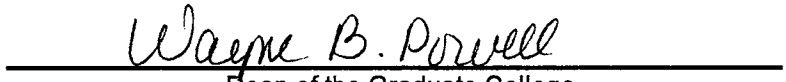

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TABLE OF CONTENTS

Chapter **Page**

1. INTRODUCTION **1**

Literature review 1
References 10

2. TRANSMISSION OF TURNIP YELLOW MOSAIC TYMOVIRUS AND TOBACCO MOSAIC TOBAMOVIRUS IN *Arabidopsis thaliana* AND EVALUATION OF TWO METHODS OF INDEXING SEEDS FOR VIRUS **18**

Abstract 18
Introduction 19
Material and Methods 23
Results 28
Discussion 31
References 36

3. BARRIERS TO SEED TRANSMISSION IN *Arabidopsis thaliana*: THE ROUTE OF TURNIP YELLOW MOSAIC TYMOVIRUS AND TOBACCO MOSAIC TOBAMOVIRUS **48**

Abstract 48
Introduction 49
Material and Methods 51
Results 53
Discussion 55
References 58

4. MAPPING A SYMPTOM DETERMINANT OF COWPEA CHLOROTIC MOTTLE BROMOVIRUS **66**

Abstract 66
Introduction 66
Material and Methods 69
Results 74

Discussion 76
References 80

Appendix

I. CO-INFECTION OF *Arabidopsis thaliana* WITH TURNIP YELLOW MOSAIC TYMOVIRUS (TYMV) AND TOBACCO MOSAIC TOBAMOVIRUS (TMV) ENHANCES SEED TRANSMISSION OF TYMV 89

LIST OF TABLES

Chapter **Page**

CHAPTER 2

Table 1 – Effect of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) on germination of *Arabidopsis thaliana* seed 44

Table 2 – Seed transmission of turnip yellow mosaic tymovirus through successive generations of *Arabidopsis thaliana* by grow-out tests 45

Table 3 - Incidence (%) of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) antigens in embryo, seed coats, intact seed, and seedlings of *Arabidopsis thaliana* by PAS-ELISA 46

CHAPTER 3

Table 1 - Determination of gamete transmission of turnip yellow mosaic tymovirus (TYMV) in *Arabidopsis thaliana* by PAS-ELISA 63

Table 2 - Determination of gamete transmission of tobacco mosaic tobamovirus (TMV) in *Arabidopsis thaliana* by PAS-ELISA 64

Table 3 - Detection of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) in plant parts of the female parents from crosses of *Arabidopsis thaliana* 65

CHAPTER 4

Table 1 - Nucleotide and predicted amino acid changes in RNA 3 of cowpea chlorotic mottle bromovirus type (T) and mild (M) strains 86

Table 2 – Virus phenotypes and symptomatology on cowpea cv. California Blackeye 87

LIST OF FIGURES

Chapter **Page**

CHAPTER 2

Figure 1 – Effect of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) on germination of *Arabidopsis thaliana* seed 47

CHAPTER 4

Figure 1 - Schematic representation of the chimeric RNA 3 of cowpea chlorotic mottle bromovirus (CCMV) 88

NOMENCLATURE

A	adenine
Ala	alanine
AMV	alfalfa mosaic virus group
Arg	arginine
BMoV	blackgram mottle virus (carmovirus tentative member)
BMV	brome mosaic bromovirus
BSMV	barley stripe mosaic hordeivirus
C	cytosine
CB	cowpea cv. California Blackeye
CCMV	cowpea chlorotic mottle bromovirus
cDNA	complementary DNA
CMV	cucumber mosaic cucumovirus
CP	coat protein
cv.	cultivar
ELISA	enzyme-linked immunosorbent assay
Expt	experiment
G	guanine
Germ	germination
HCl	hydrochloric acid
ICTV	International Committee on Taxonomy of Viruses
KCl	potassium chloride
kb	kilobase
kDa	kilodalton
KH_2PO_4	potassium phosphate monobasic
LMV	lettuce mosaic potyvirus
Lys	lysine
mol wt	molecular weight
MP	movement protein
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	sodium phosphate dibasic
NaN_3	sodium azide
Na_3PO_4	trisodium phosphate
NaCl	sodium chloride
OFR	open reading frame
PAS-ELISA	protein-A sandwich ELISA
PBS-T	phosphate buffered saline – Tween

PEBV	pea early browning tobravirus
PSbMV	pea seed-borne mosaic potyvirus
RBSV	raspberry ringspot nepovirus
RT-PCR	reverse transcription polymerase chain reaction
SBMV	southern bean mosaic sobemovirus
T	thymine
TMV	tobacco mosaic tobamovirus
TSV	tobacco streak ilarvirus
TYMV	turnip yellow mosaic tymovirus
TVCV	turnip vein clearing tobamovirus
Val	valine
w/v	weight/volume

CHAPTER 1

INTRODUCTION

Literature review

The interactions between plant viruses and their hosts may lead to anatomical and physiological changes following the general principle of plant pathology that disease results from the interaction of the host, pathogen and environment (Agrios, 1997). After the realization in the past century that the presence of microorganisms in a diseased plant was the cause rather than the consequence of the disease, studies on the mechanisms utilized by pathogens in disease development were initiated. Today, great effort continues in the elucidation of aspects of the host-pathogen interactions that result in disease. Several stages can be individually studied in the complex process of plant disease development, among them pathogen transmission and symptomatology. Relevant aspects of previous studies on seed transmission and symptom induction by plant viruses, reported in the following chapters, are here briefly reviewed.

About 90% of all worldwide food crops are propagated by seeds (Agarwal and Sinclair, 1996) and about 20% of plant viruses are transmitted in seed of at least one host (Matthews, 1991). Despite that only a minority of plant viruses are seed-transmitted, seed transmission is important in both virus ecology and

epidemiology (Agarwal and Sinclair, 1996; Mink, 1993; Strace-Smith and Hamilton, 1988). Seed transmission is the primary means of dissemination for viruses such as barley stripe mosaic hordeivirus (BSMV) (Jackson et al., 1989) and cryptoviruses (Boccardo et al., 1987; Chiko, 1973; Mink, 1993).

The intensive worldwide seed trade fosters seed transmission as one of the most efficient ways viruses may be disseminated, particularly into new areas. Seed-transmitted virus may cause losses by reducing seed viability (Cooper et al., 1984; Hemmati and McLean, 1977; Suteri, 1981), abnormal plant growth (Hicks et al., 1986; McGee, 1995; Stevenson and Hagedorn, 1973; Tu, 1992), and yield reduction (Morales and Castano, 1987; Powell Jr. and Schlegel, 1970; Sdoodee and Teakle, 1988). Virus infected seed may also initiate a disease epidemics by providing secondary inoculum sources, as observed with lettuce mosaic potyvirus (LMV) vectored by aphids. Tolerance as low as zero infected seed in 30,000 is adopted in California, whereas this tolerance is zero in 2,000 seeds in the Netherlands, where the insect vector is less abundant (McGee, 1995).

The presence of a virus in the seed (Sdoodee and Teakle, 1988) or even in the embryo (Jones, 1993; Nolan and Campbell, 1984; Varma et al., 1992) does not always lead to seedling infection, which determines the distinction between a seed-borne virus that is carried in the seed but does not infect the resulting seedling; and a seed-transmitted virus that infects the seedling developed from the virus infected seed (Neegaard, 1979). This is an important distinction for this study, because a

seed-transmitted virus as defined above is referred to by some as a seed-borne virus.

The virus must overcome barriers to virus replication and movement to became established in the seedling. These barriers include anatomical/ biochemical factors in the connection between embryo and maternal tissue (Johansen et al., 1994; Yeung and Meinke, 1993), virus survival in microspores, macrospores or the embryo-sac (Hanada and Harrison, 1971), and limitations on virus replication and/or movement into reproductive tissues (Hampton and Francki, 1992; Hanada and Harrison, 1971; Wang and Maule, 1994). In addition, the virus must not be inactivated during seed maturation (Bailiss and Offei, 1990; Gay, 1969; Uyemoto and Grogan, 1977; Yang and Hamilton, 1974), must resist desiccation during seed storage (Bowers Jr. and Goodman, 1979; Wang and Maule, 1992;) and replicate during seed germination (Johansen et al., 1994). All these events limit virus survival in the embryo and subsequent seedling infection. How the virus overcomes these barriers has been investigated in several host-virus combinations, but no definitive conclusions have yet been made.

Although virus particles may invade any part of the seed (Agarwal and Sinclair, 1996), seed transmission is most closely related to embryo infection (Adams and Kuhn, 1977; Alvarez and Campbell, 1978; Bailiss and Offei, 1990; Crowley, 1957; Gupta and Summanwal, 1980; Mink, 1993; Pesic and Hiruki, 1986; Von Wechmar et al., 1984). Varma et al. (1992) attributed the failure of seed

transmission of blackgram mottle virus (BMoV, a tentative member of the carmovirus group) in *Vigna mungo* to the small amount of virus in the embryonic axis rather than to virus location. Nolan and Campbell (1984) found that healthy seedlings may arise from virus infected embryos because systemic infection is not established.

A virus may enter the embryo either directly and/or indirectly. Direct invasion occurs during embryogenesis by virus from infected gametes (Carroll and Mayhew, 1976b; Hemmati and McLean, 1977) The indirect route of embryo infection occurs by virus from maternal tissue (Carroll, 1972; Hemmati and McLean, 1977; Wang and Maule, 1994) despite the absence of plasmodesmata between maternal and embryo tissues (Caldwell, 1934). Plasmodesmata are considered the route of virus movement from cell-to-cell (Atabekov and Dorokhov, 1984; Deom et al., 1992; Hull, 1989; Maule, 1991). The absence of plasmodesmata linking infected maternal tissue and the embryo (Carroll, 1972; Carroll and Mayhew, 1976a) may explain either the reduced rate or failure of seed transmission when plants are infected at the flowering stage or at a later stage of development (Johansen et al., 1994).

Seed transmission may depend on virus invasion of the floral meristem and then subsequent into the gametophytes and gametes (Bennett, 1969; Carroll and Mayhew, 1976 a,b). Alternatively, Wang and Maule (1994) found that the suspensor might be the route for direct invasion of pea embryos by pea seed-borne

mosaic potyvirus (PSbMV), and thus proposed that the lack of virus movement through the suspensor may be the basis for the lack of seed transmission. Limitation of virus replication/movement may prevent PSbMV from reaching the suspensor before its degeneration and, therefore, be the basis for the failure of seed transmission in the cv. Vedette, in which pollen transmission does not occur (Wang and Maule, 1992). However, this result was challenged by demonstration of PSbMV transmission in the pea cv. Dual by seed from emasculated flowers of healthy plants fertilized with pollen from an infected plant (Johansen et al., 1994).

The time of virus infection in relation to plant development has been cited as the most important factor in viral seed transmission (Agarwal and Sinclair, 1996; Schippers, 1963; Xu et al., 1991). Infection before flowering is considered critical for seed transmission (Couch, 1955; Fajardo, 1930), otherwise the embryo may not be infected. It seems likely, though not always required, that infection before flowering may result in a higher percentage of seed transmission.

Investigations on the genetic control of seed transmission showed a close relationship between seed transmission and virus replication. Hampton and Francki (1992) used pseudorecombinants to determine that RNA 1 may influence seed transmission of cucumber mosaic cucumovirus (CMV). RNA 1 is involved in CMV replication (Hayes and Buck, 1990; Suzuki et al., 1991). Hanada and Harrison (1971) found that seed transmission of raspberry ringspot nepovirus (RBSV) is possibly controlled by RNA 1, which also is involved in virus replication (Greif et al.,

1988). Edwards et al. (1991) and Johansen et al. (1994) found that seed transmission of barley stripe mosaic hordeivirus (BSMV) involves RNA 3 which mediates virus replication (Donald et al., 1993; Jackson et al., 1989; Petty et al., 1990). Edwards (1995) reported that the BSMV genes involved in symptomatology, virus replication, and movement regulated seed transmission in barley. Carroll et al. (1979), however, found that a maternal plant gene controls BSMV seed transmission in barley. Investigating the seed transmissibility of two isolate of PSbMV that are transmitted at rates of 24% and 0.3%, Johansen et al. (1996) found that the frequency of seed transmission was influenced by two regions of the virus genome involved in virus replication and movement. Wang et al. (1997) demonstrated the involvement of the 12kDa gene of pea early browning tobravirus (PEBV) in seed transmission. They showed that deletion of the 12kDa gene significantly reduced virus accumulation in the carpel and ovaries of pea, which are structures in the route of PEBV leading to gamete infection. The 12kDa gene has no assigned function (MacFarlane at al., 1989).

A number of hypotheses have been presented to explain virus transmission in seed produced on infected plants (Bennett, 1969; Caldwell, 1952, 1962; Crowley, 1957; Duggar, 1930). However, none of these hypotheses adequately explain the phenomenon of seed transmission. The mechanism of virus seed transmission may be related to virus replication and/or movement in infected plants, which determines embryo infection. Also, restriction of virus distribution in plant tissue, such as occurs with phloem limited luteoviruses, may be responsible for impairing virus

invasion of the embryo (Jones, 1993).

Upon infection by a virus the plant host may show very diverse external alterations, ranging from unnoticeable to severe symptoms which include plant death (Agrios,1997; Fraser, 1990; Matthews, 1991). The appearance of such symptoms result from molecular interactions between specific molecules of host and virus (Fraser, 1990; Verma, 1991). Identification of the genetic determinants has been facilitated by advances in molecular biology (Daubert, 1988; Fraser, 1990).

Use of resistance is currently one of the most important means for plant disease management. Understanding the molecular interactions between the pathogen and its host constitutes the basic information needed for elucidating the nature of plant resistance to pathogens (Fraser, 1985), and developing this understanding is particularly important in the field of virus disease management due to the limited number of options for controlling viral pathogens (Matthews, 1991). A great amount of research has been conducted in the identification of genes or gene products from both the virus and the host that interact in the process of virus disease (Fraser, 1985; Hagerdorn and Gritton, 1973; Keller et al., 1998; Knorr and Dawson, 1988; Meshi et al., 1988, 1989; Van Loon, 1987).

Information is now available from studies with cowpea chlorotic mottle bromovirus (CCMV), brome mosaic bromovirus (BMV), the alfalfa mosaic virus group (AMV) and CMV (Van Loon, 1987) on virus assembly, symptom expression,

virus accumulation, and transmissibility (Atreya et al., 1990, 1992; Culver and Dawson, 1989; Edwards, 1995; Johansen et al., 1996; Knorr and Dawson, 1988; Perry et al., 1994; Suzuki et al., 1995; Vriend et al., 1981, 1986; Wang et al., 1997; Woolston et al., 1987). Both seed transmission and symptom induction are events that depend on the interaction of the virus with the host and have been investigated at the molecular level. Great progress has been obtained where genetic determinants for seed transmission and symptom induction have been identified in several virus-host combinations (Banersee et al., 1995; Culver and Dawson, 1989; Edwards, 1995; Johansen et al., 1996; Knorr and Dawson, 1988; Lewandowski and Dawson, 1993; Meshi et al., 1989; Neelman et al., 1991; Rao and Grantham, 1995; Shintaku et al., 1992; Suzuki et al., 1995; Tsai and Dreher, 1993; Wang et al., 1997).

This dissertation comprises three studies conducted to investigate the interaction between selected viruses and their plant hosts related to seed transmission and symptom induction. Chapter 2, entitled "Transmission of Turnip Yellow Mosaic Tymovirus and Tobacco Mosaic Tobamovirus in *Arabidopsis thaliana* and Evaluation of Two Methods of Indexing Seed for Virus", reports, for the first time, the transmission of TYMV in seed of *A. thaliana,* and demonstrates the linkage between seed transmission and embryo infection by the virus. The study of virus location in the seed indicated that in systems where the virus infects seed parts other than embryo, data obtained by ELISA in indexing whole seed for virus transmission requires cautious interpretation. Even if the virus is limited to the

embryo, ELISA is still less accurate than grow-out tests. The implications of using ELISA in a seed indexing program for virus-free seed production are discussed. Preliminary results on the effect of co-infection of *A. thaliana* with TYMV and TMV on the incidence of TYMV seed transmission is presented in Appendix I. Double infection of *A. thaliana* plants with TYMV and TMV caused synergistic effect on symptoms induction and increased seed transmission of TYMV by over 125% compared with single infection with TYMV. No effect on seed transmission of TMV was observed.

Chapter 3, entitled "Barriers to Seed Transmission in *Arabidopsis thaliana*: the Route of Turnip Yellow Mosaic Tymovirus and Tobacco Mosaic Tobamovirus", further investigates the conclusion from Chapter 2 that embryo infection is a requirement for seed transmission. The route of TYMV and TMV to reach the seed of *A. thaliana* was determined by crossing healthy and virus infected plants. The results are discussed in light of whether a direct or indirect route is used by the viruses, relating anatomical features from embryogenesis to seed maturation.

In chapter 4, entitled "Mapping a Symptom Determinant of Cowpea Chlorotic Mottle Bromovirus", two CCMV strains are used to investigate the genetic determinant for symptom attenuation. The RNA 3 of the mild strain of CCMV (CCMV-M) was sequenced, chimeric viruses were constructed by exchanging restriction fragments from RNA 3 of the two strains and the genetic determinant of symptom expression located by host inoculation of *in vitro* transcripts. How the

amino acid change in the coat protein affects virus-host interaction is discussed.

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CHAPTER 2

TRANSMISSION OF TURNIP YELLOW MOSAIC TYMOVIRUS AND TOBACCO MOSAIC TOBAMOVIRUS IN *Arabidopsis thaliana* AND EVALUATION OF TWO METHODS OF INDEXING SEED FOR VIRUS

Abstract

The mechanism of transmission of virus in seed was studied in *Arabidopsis thaliana* infected with turnip yellow mosaic tymovirus (TYMV) or tobacco mosaic tobamovirus (TMV). Both TYMV and TMV were detected by serological and biological assays in vegetative and reproductive tissues of *A. thaliana* inoculated at the rosette stage. Both viruses were detected in seed from infected plants, however only TYMV was seed-transmitted. Estimating virus seed transmission by grow-out tests is more accurate than indexing seed by ELISA. This is the first report of TYMV transmission in seed of *A. thaliana*. The incidence of TYMV in seed was higher than in seedlings developed from seed from infected plants. The difference in detecting virus antigen in seeds and actual seed transmission was related to the higher frequency of TYMV or TMV antigen in the seed coat than in the embryo. Virus in the seed coat did not lead to seedling infection. These data support the distinction between TYMV being seed-transmitted and TMV being seed-borne. It was also found that embryo invasion is necessary, but not always sufficient, to lead

to seed transmission and that a barrier may exist under certain conditions that prevents virus from moving from the seed to the developing seedling.

Introduction

About 90% of all food crops worldwide are propagated by seed (Agarwal and Sinclair, 1996) and about 20% of plant viruses are transmitted in seed in of at least one host (Matthews, 1991). Because of the intensive worldwide seed trade, seed transmission is one of the most efficient ways viruses are disseminated, particularly into new areas. A seed-transmitted virus may cause yield reduction by preventing seed germination or inducing abnormal plant growth (Agarwal and Sinclair, 1996). Infected seed may provide the primary inoculum in the early stage of the crop, which later is disseminated by a vector.

Virus may invade any seed part (Agarwal and Sinclair, 1996). However, seed transmission has been most closely related to embryo infection (Adams and Kuhn, 1977; Alvarez and Campbell, 1978; Bailiss and Offei, 1990; Bowers Jr., and Goodman, 1979; Carroll, 1972; Mink, 1993; Pesic and Hiruki, 1986). To become established in the seedling, the virus must overcome barriers to virus replication and movement that limit virus survival in the embryo and subsequent seedling infection. How the virus overcomes these barriers has been investigated in several host-virus combinations, but no definitive conclusions have yet been made (Bennett, 1969; Caldwell, 1952, 1962; Crowley, 1957; Duggar, 1930). The

presence of a virus in seed (Sdoodee and Teakle, 1988), even in the embryo (Jones, 1993; Neacameth and Kobler, 1982; Nolan and Campbell, 1984; Varma et al., 1992), does not always lead to seedling infection. This property distinguishes a seed-borne virus that is carried by seed but does not infect the subsequent seedling; from a seed-transmitted virus that results in an infected seedling from the virus infected seed (Neegaard, 1979).

Most methods to index seed transmission of virus either overestimate or underestimate the actual amount of transmission. The enzyme-linked immunosorbent assay (ELISA) commonly has been used for evaluation of virus incidence in seed (Clark, 1981; Maury et al., 1998). This method does not distinguish seed-borne from seed-transmitted virus, and thereby may overestimate the amount of transmission. Grow-out tests have been more accurate than ELISA in estimating seed transmission (Gillaspie Jr. et al., 1993; Nolan and Campbell, 1984; Pesic and Hiruki, 1986; Spak et al., 1993; Wang et al., 1993). However, there are reports of complete correlation between serology and grow-out tests (Bharathan et al., 1984; Falk and Purcifull, 1983; Hamilton, 1965), primarily when the virus was absent from the seed coat or when only embryo was tested.

Seed indexing does not assure disease control if a vector effectively spreads the virus from a few infected plants grown from an indexed seed lot (McGee, 1995). Thus, resistance to seed transmission would be an effective approach to disease

control and understanding the mechanism of virus seed transmission would facilitate pursuing a genetic solution to manage seed transmission.

Turnip yellow mosaic tymovirus (TYMV) is the type member of the genus Tymovirus, family Bromoviridae, order Mononegavirales (Koenig and Lesemann, 1979; Murphy et al., 1995). There are three well-characterized subgroups: TYMV-1, cauliflower and TYMV-Cd (Block et al., 1987). TYMV-1 and cauliflower are two European subgroups that are distantly related. The Australian TYMV-Cd is closely related to TYMV-1. The type strain of TYMV belongs to the TYMV-1 subgroup. Stock cultures of the type strain consist of a mixture of closely related strains that are impossible to isolate and maintain as a single strain without reversion (Matthews, 1980).

TYMV has icosahedral particles of about 30nm diameter with a positive-sense single-strand genomic RNA of approximately 6.3kb (Keese et al., 1989; Matthews, 1980; Morch et al., 1988). The virus genome encodes for three proteins. The p206 polyprotein is translated from an open reading frame (ORF-206) that comprises over 90% of the total genome (Morch et al., 1988). This polyprotein undergoes autoproteolysis to produce two proteins with calculated molecular weight of 140 and 66 kDa, named p150 and p70, respectively (Bransom et al., 1991) that are essential for virus replication (Weiland and Dreher, 1993). The p69 protein is encoded by the ORF-69 that is almost completely overlapped by the ORF-206. The p69 protein is required for cell-to-cell virus movement (Bozarth et al., 1992;

Bransom et al., 1995). The third protein is a 20-kDa polypeptide that is encoded by a subgenomic RNA (Ricard et al., 1977) and forms the capsid with or without full-length genomic RNA (Higgins et al., 1978; Pleij et al., 1977). In addition to form the virus capsid, this 20-kDa protein is required for efficient long-distance movement of the virus (Bransom et al., 1995).

Tobacco mosaic tobamovirus (TMV) is the type species for the genus Tobamovirus, family Togoviradae, order Mononegavirales (Gibbs, 1977; Murphy et al., 1995). TMV has rod-shape particles of 300nm with a positive-sense single-strand genomic RNA of approximately 6.4kb (Zaitlin and Israel, 1975). The virus genome encodes for four proteins (Goelet et al., 1982). The 126-kDa and 183-kDa proteins are translated from the genomic RNA and are necessary for virus replication (Ishikawa et al., 1986) and have been implicated in virus movement (Holt et al., 1990). The 30-kDa and 17.6-kDa proteins are translated from subgenomic RNAs. The former is involved in virus cell-to-cell movement (Deom et al., 1987; Leonard and Zaitlin, 1982; Meshi et al., 1987), while the other is the virus capsid monomer that is involved in other aspects of TMV biology (Dawson et al., 1988) such as long- distance movement (Saito et al., 1990) and symptom expression (Culver and Dawson, 1991).

Arabidopsis thaliana (L.) Heynh. is member of the crucifer family with geographical distribution in Eurasia and North Africa and has been widely introduced elsewhere (Price et al., 1994). It has a life cycle as short as 4 weeks

(Dangl, 1993), with separated vegetative and reproductive phases and a low incidence of outcrossing (Bowman, 1993). *Arabidopsis thaliana* was chosen as the host to investigate seed transmission of TYMV and TMV because of its short life cycle and its capacity to produce a large number of seed under experimental conditions. In addition, both TYMV and TMV reach a high concentration in tissue of infected *A. thaliana*. This study was initiated to evaluate two methods of indexing seeds for virus transmission in an effort to further understand the mechanism of virus seed transmission. Presented here are data indicating that embryo infection is required for seed transmission of TYMV.

Material and Methods

Distribution of TYMV and TMV in tissue of inoculated plants and effect on seed germination

Seeds of *A. thaliana* ecotypes Dijon and La-O (*Arabidopsis* Biological Resource Center, Ohio State University) were sown in disposable plastic pots full of autoclaved potting mixture (LC1 Mix, Sun Gro Horticulture Inc), and the seedlings maintained at 23°C under 16h/8h light/dark cycles. An isolate of TYMV maintained on Chinese cabbage (*Brassica pekinensis* Rubr.) and stored as dehydrated tissue at 4°C was used in this study, along with the common strain of TMV (Rezende et al., 1992; Sherwood and Fulton, 1982) maintained in systemically infected *Nicotiana tabacum* L. Inocula were prepared by grinding infected tissue in phosphate buffer

0.01M, pH 7.0 and was then applied on corundum-dusted leaves using a cotton swab. *Arabidopsis thaliana* seedlings at the rosette stage were mechanically inoculated with either TYMV or TMV. Plants were assayed by ELISA two to three weeks after inoculation. Inoculated plants that were negative by ELISA or showed atypical symptoms of virus infection (probably by spontaneous generation of virus mutations, as observed by Tsai and Dreher (1993) in Chinese cabbage infected with TYMV) were discarded. Healthy control plants were kept separated from infected plants. Mature seed were harvested, air-dried for one to three weeks and stored at 4°C.

Virus distribution in infected plants was determined by infectivity assay and protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA) (Edwards and Cooper, 1985) on samples of: (a) unopened blossoms; (b) opened blossoms; (c) fully developed flowers dissected into sepals, petals, gynoecium, and stamens; (d) siliques (seeds included) at the very beginning stage of development; (e) immature siliques divided into shell and seeds as described above for the flower; (f) mature siliques divided into shell and seeds; (g) root; and (h) stem. Flowers and siliques were dissected under a stereoscopic microscopy with fine tip forceps that were cleaned with 10% (w/v) trisodium phosphate (Na_3PO_4) after handling each organ. Samples were macerated in a white porcelain plate (Fischer Scientific cat # 13-745) with a disposable culture tube (Fischer Scientific cat # 14-9610-25) in phosphate buffered saline-Tween (PBS-T, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 3 mM NaN_3, 0.05% (v/v) Tween-20) + 2% (w/v)

polyvinylpirolidone mol wt 40,000, pH 7.4). Infectivity tests were carried out by mechanical inoculation of each macerated organ on Chinese cabbage or *Nicotiana tabacum* 'Xanthi', hosts for TYMV and TMV, respectively (Matthews, 1980; Zaitlin and Israel, 1975).

Polyclonal antisera specific for TYMV and TMV were produced as previously described (Rezende et al., 1992). Protein-A (Sigma P-3838) in coating buffer (15 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) and protein-A alkaline phosphatase conjugate (Sigma P-9650) in PBS-T were used at 1μg/ml, and p-nitrophenyl phosphatase (Sigma N-2765) was used at 1mg/ml in 1 M diethanolamine, pH 9.8. The assay was carried out in polystyrene plates (Nunc-Immuno Plate MaxSorp Surface), using 100 μl per well for each reagent. Incubations for all steps except the sample step and when the substrate was added were at 37°C for 2 h. The sample step was at 4°C overnight and the p-nitrophenyl phosphate substrate step was at room temperature for 30-50 min. Between each step the plates were washed 3 times with PBS-T for 3 min. Each sample was assayed in three separate wells, which was repeated at least four times. The absorbance was read at 405nm (EIA Reader EL-307, Bio-Tek Instruments, Inc.) with a healthy sample as a blank. The negative threshold for both TYMV and TMV was $\bar{X} \pm 3SD$ (Clark, 1981; Sutula et al., 1986) of the 6 wells of healthy control samples. The ELISA procedure was the same throughout unless otherwise indicated.

The effect of TYMV and TMV infection on seed germination was evaluated by placing 186 to 283 seeds on water dampened filter paper in a Petri dish under ambient light at room temperature and counting the number of seed that germinated after 10, 15, 20, 25, 30 and 40 days. The ungerminated seeds were counted and saved for virus assay by ELISA. A seed was considered to have germinated if cotyledons, hypocotyl and primary root were observed.

Evaluation of two methods for determining seed transmission of TYMV and TMV in *Arabidopsis thaliana*

Seeds collected from TYMV or TMV infected plants were tested for virus infection by grow-out tests and ELISA. A total of 274 seeds were individually assayed by PAS-ELISA for TYMV. To determine the extent of transmission of TMV in seed, 120 groups of 20 seeds and 60 groups of 10 seeds were assayed by ELISA. Grow-out tests were carried out using seed from infected parent plants. Seeds were sown in disposable plastic pots and seedlings grown as described above. Extracts of leaf, flower and silique were assayed by PAS-ELISA. Each plant was tested three times in three separate wells each time. Results from the grow-out tests and ELISA of whole seed were compared to estimate seed transmission. Dry mature seed from plants infected by seed transmission were collected and tested by grow-out tests to determine seed transmission in successive generations.

Location of TYMV and TMV within seeds produced by infected plants

To investigate the location of virus in seed, dried mature seeds produced by ecotypes Dijon and La-O as described above were soaked in distilled water for 24h to soften the seed coat. The embryo was separated from the seed coat following a procedure modified from Meinke (1994) using fine tip forceps and working under a stereoscopic dissecting microscopy. Each seed part was subjected to infectivity and serological assays. For infectivity assay, the macerated seed coat or embryo obtained from TYMV or TMV infected plants was mechanically inoculated in Chinese cabbage and *N. tabacum*, respectively. Groups of seeds produced on healthy or virus infected plants were used as the negative and positive control, respectively. For ELISA of bulk seed, both the embryo and seed coat were surface desinfestated by immersing in 10% Na_3PO_4 solution for 1 min and then washed three times with distilled water. For detection of TYMV, the single seed or seed part was homogenized in 50µl of sample buffer and tested by PAS-ELISA. To detect TMV, groups of 10 or 20 seeds or seed parts were homogenized in 50µl of sample buffer and tested as described above. For ELISA of washed seeds, groups of 100 seeds were placed into microfuge tubes containing either PBS-T or 0.1N HCl (2µl/1 seed) and stirred for 30 min at room temperature. The washing solution was saved and seeds were then rinsed three times with either PBS-T or 0.1N HCl by vortexing 20 seconds and then letting the seeds settle for 40 seconds to facilitate discarding of the supernatant. Both washing solution and washed seeds macerated in PBS-T were tested by ELISA.

Results

Distribution of TYMV and TMV in tissue of inoculated plants and effect on seed germination

The most common symptoms in *A. thaliana* infected with TYMV were mosaic; stunting; delayed flowering, flower abortion; and reduction in size of leaves, flowers and fruits, based on visual observation. Symptoms on ecotype Dijon were milder than on ecotype La-O. The most noticeable symptom of TMV infection was flower abortion. Symptoms of TYMV were more severe when very young seedlings were inoculated. Plant death was observed if TYMV was inoculated at the beginning of the rosette stage. Both TYMV and TMV were detected by ELISA in vegetative (leaves, stem and root), reproductive (sepals, petals, gynoecium, and stamens) and fruit (dry silique shell and seed) tissues of *A. thaliana* ecotypes Dijon and La-O. Virus presence was confirmed by induction of typical symptoms on inoculated Chinese cabbage and *N. tabacum*. No difference in distribution of TYMV was observed in plants infected either by seed transmitted or by mechanical inoculation.

The percentage of seed from TYMV or TMV infected plants that germinated was observed over 40 days. The percentage of germination of seed from TMV infected plants was similar to the percentage of germination of seed from healthy plants for both ecotypes (Table 1). There was a delay in germination of seed from TYMV infected plants of both ecotypes, but by 40 days, the percentage of seed that

germinated from TYMV infected plants versus the uninfected controls was similar (Figure 1).

In single, ungerminated seed from TYMV infected plant virus antigen was detected in 10 out of 15 seeds by ELISA. No difference in absorbance of seed samples from healthy and TMV infected plants was observed when individual seed was assayed for TMV by ELISA.

Evaluation of two methods for determining seed transmission of TYMV and TMV in *Arabidopsis thaliana*

TYMV antigen was detected by ELISA in 877 out of 7,153 seedlings (Table 2). Infected seedlings exhibited severe to mild symptoms such as mosaic, leaf deformation, reduction in growth, reduction in size and number of flowers and siliques. A few infected seedlings were asymptomatic, presumably due to genetic variation among plants. Asymptomatic seedlings infected through seed transmission were also observed by Carroll and Mayhew (1976) in the barley – barley stripe mosaic hordeivirus (BSMV) combination. There was no observed difference in virus distribution in leaves of symptomatic and asymptomatic plants by ELISA. TMV was not detected by ELISA in the 1,606 seedlings tested.

The incidence of TYMV seed transmission was higher in the ecotype La-O than in the ecotype Dijon. There was an increase in the rate of TYMV transmission

through seed produced by plants infected through seed transmission compared to mechanically infected plants (Table 2).

The results from the two methods for determining the frequency of seed transmission indicated that with ELISA on whole seed, the frequency of seed transmission was overestimated compared to results from grow-out tests (Table 3). Hence, ELISA was not an accurate assay to quantify seed transmission of TYMV in *A. thaliana*.

Location of TYMV and TMV within seeds produced by infected plants

The location and incidence of virus and virus antigen in seed was determined by infectivity and serological assays. Seed for this experiment was obtained from mechanically infected *A. thaliana* ecotypes Dijon and La-O. Macerated seed coat or embryo inoculated onto Chinese cabbage produced mosaic symptoms typical of TYMV infection. No TMV infection was observed in *N. tabacum* inoculated with either preparations of seed coat or embryo. Samples of whole seed from TMV infected plants gave necrotic lesions typical of TMV infection, while no infection was recorded from whole seed from healthy plants. In preliminary ELISA experiments with bulk seed samples, TYMV antigen was detected in a single seed or part from a single seed, whereas 10 seeds or seed parts from 10 seeds were needed to detect TMV by ELISA. TYMV was detected in the seed coat, embryo or both, as well as in seedlings originated from seed produced by TYMV infected

plants (Table 3). TMV was detected in samples of groups of seed coats, but not in groups of embryos or in seedlings originated from seed produced by TMV infected plants (Table 3). ELISA was conducted on seed washed in PBS-T or 0.1N HCl. TYMV and TMV were found in both washed seeds and the washing solution from their respective samples. Though both viruses could be detected in PBS-T or 0.1N HCl used to wash seeds, the amount of antigen in the washed seeds did not appear to be significantly altered as ELISA readings of washed and unwashed seed samples were similar, suggesting that TYMV and TMV are predominantly located inside the seed.

Discussion

The virus location in seed appeared to determine the virus transmissibility through seed. TYMV was detected in the embryo and the seed coat of mature seed, while TMV was detected only in the seed coat (Table 3). Thus, seed transmissibility was linked to embryo invasion, as others have proposed (Bailiss and Offei, 1990; Bowers Jr. and Goodman, 1979; Persic and Hiruki, 1986; Wang and Maule, 1994). Except for infection of the embryo, both viruses were similarly distributed in vegetative and reproductive parts of both *A. thaliana* ecotypes. Detection of virus in the reproductive parts, such as seed coat and silique shell was not surprising since those parts are of maternal origin (Meinke, 1994). This is the first report of TYMV transmission in seed of *A. thaliana*, while failure of TMV transmission in seed of *A. thaliana* had been reported by Ishikawa et al. (1991).

Low incidence of seed transmission my result from reduction in seed germination due to virus infection. This hypothesis was eliminated, as the frequency of TYMV infection in germinated and ungerminated seeds assayed by ELISA was similar (Table 3). Hence there was no correlation between the rate of seed germination and viral seed transmission, as has been observed by others (Hampton, 1972; Porto and Hagedorn, 1975; Raizada et al., 1990). These findings indicated that results from grow-out tests are not biased due to effects of virus on seed germination. Additional support to it is provided by the fact that *A. thaliana* usually shows a frequency of spontaneous seed abortion over 0.5% depending on the growth conditions (Meinke, 1994).

The delay in seed germination observed and reduction in growth of TYMV infected seedlings may be related to inhibition of host gene expression by the virus replication in the embryo. The seed coat does not support seedling development, so virus present at this location should not interfere with germination. On the other hand, embryo infection could favor an increase in virus titer prior to seedling tissue development. The cellular machinery of the embryo cells could be directed to support viral replication rather than plant tissue formation, causing delay in the germination. Seed germination and seedling development of TMV infected seed did not differ from seed from healthy plants. A similar observation has been reported in *Arabidopsis* infected by the recently discovered cruficer-infecting turnip vein clearing tobamovirus (TVCV) (Lartey et al., 1997). Hence, virus presence in the seed coat did not appear to affect embryo metabolism associated with

germination.

Similarly, Wang and Maule (1995) found that PSbMV temporarily suppresses host genes expression in pea infected embryo tissue. They showed there was a coincidence of the host transcript-deficient and the virus replication zones. In this phenomenon, called host gene shutoff, virus infected plant cells support massive virus accumulation during the shutoff stage, then the cells recover to provide the condition for virus to be vectored and infect a new host (Aranda and Maule, 1998). Fraser and Gerwitz (1980) observed that host protein synthesis was reduced by up to 75% during early infection of tobacco with TMV and recovered later. They attributed such effect to virus protein synthesis at the expense of those from the host. Whenham et al. (1985) found correlation between reduced tobacco growth and virus content in which host growth rate decreased in the presence of TMV infection. Gene shutoff has been observed in bacterial and animal viruses as well (Rubinstein and Dasgupta, 1989).

Incidence of TYMV antigen in seed was significantly higher than the actual seed transmission of TYMV as determined by the grow-out tests (Table 3). The virus was detected more often in the seed coat than in the embryo, relating low virus incidence in embryo to low seed transmission. This result combined with TMV incidence in seed with no seedling infection may indicate that virus remaining apart from the embryo does not lead to seedling infection. TMV has been found within the seed coat, which can eventually lead to seedling infection by mechanical

inoculation through wounds (Broadbent, 1965; Demski, 1981; Taylor et al., 1961).

Hampton and Francki (1992) observed that virus occurrence in the embryo may exceed the occurrence of seed transmission in several virus-host systems. The results presented here showed that even embryo infection did not assure seedling infection, as the occurrence of TYMV antigen in embryo exceeded the incidence of seed transmission (Table 3). Similar results were obtained by Wang et al. (1993), where overestimation of PSbMV transmission was observed by ELISA using either 30-day-old embryonic shoots or whole seed as compared to grow-out tests. They also observed that healthy seedlings grew from infected embryos. Non-infectious virus, the presence of virus antigen (Varma et al., 1992), and the number, location and activity of infected cells in the seed (Alvarez and Campbell, 1978; Powell Jr. and Schelegel, 1970) may be responsible for these phenomena. Thus, embryo invasion is necessary, but not sufficient, to lead to seed transmission.

The mechanism resulting in *A. thaliana* embryo invasion by TYMV but not by TMV is unknown. It has been found that viruses enter the embryo either directly or indirectly. Direct invasion occurs during embryogenesis by virus from infected gametes (Carroll, Mayhew, 1976; Hemmati and McLean, 1977; Wang et al., 1997). The indirect route occurs by virus from maternal tissue (Carroll, 1972; Hemmati and McLean 1977; Wang and Maule, 1994). After fertilization, the *A. thaliana* zygote undergoes cellular division giving origin to the proper-embryo and the suspensor that supplies the developing embryo with nutrients of maternal origin (Mansfield and

Briarty, 1991). Plasmodesmata interconnect cells of the proper-embryo and connect the proper embryo to the suspensor (Bowman and Mansfield, 1993; Mansfield and Briarty, 1991). This path could be used by TYMV to move from maternal tissue to embryo cells. Embryo development is followed by endosperm absorption, which promotes embryo proximity to the integuments (seed coat precursors). However, no symplastic connection is established between embryo and integuments (Bowman and Mansfield, 1993). Thus, restricted location of TMV in the integuments would not lead to embryo invasion due to an absence of symplastic connection.

The incidence of TYMV seed transmission was higher in ecotype La-O than in Dijon. Milder symptoms and lower incidence of seed transmission in the ecotype Dijon suggest that plant factor(s) may affect the rate of seed transmission. Likewise, variation in the incidence of seed transmission among genotypes has been reported for several virus-host combinations (Bailiss and Offei, 1990; Bowers Jr. and Goodman, 1979; Carroll and Chapman, 1970; Culver and Sherwood, 1988; Ghanekar and Schwenk, 1974; Hemmati and McLean, 1977; Porto and Hagedorn, 1975; Smith and Hewitt, 1938; Tu, 1992; Varma et al., 1992; Wang et al., 1993). Correlation with symptom, host development at the time of infection, the rate of virus inactivation during seed maturation and host susceptibility have been suggested to affect the incidence of seed transmission (Bailiss and Offei, 1990; Hemmati and McLean, 1977; Smith and Hewitt, 1938; Tu, 1992).

The detection of virus antigen in seed may not always be an useful indicator of seed transmission. As for TYMV-*Arabidopsis* combination the ideal condition where the virus is uniformly distributed and highly concentrated in the embryo with no seed coat infection was not observed. Thus, ELISA should be used with caution in indexing seed for virus transmission. Assay of the progeny is generally the more accurate method of determining virus seed transmission, because it overcomes the possible effect of non-infective virus antigen within seed as well the presence of infective virus whose location or titer does not lead to seedling infection. Others have concluded the same in several virus-host combinations (Bailiss and Offei, 1990; Gillaspie Jr. et al., 1993; Nolan and Campbell, 1984; Sdoodee and Teakle, 1988; Spak et al., 1993; Varma et al., 1992; Wang et al., 1993).

Further studies on how TYMV and TMV invade *A. thaliana* seed, what mechanism allows embryo invasion of only a portion of the seed produced by an infected plant, and what prevents TMV to reach the embryo would better clarify the mechanism of seed transmission of virus in plants.

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Table 1 – Effect of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) on germination of *Arabidopsis thaliana* seed [a]

Treatment	Seed tested number[b]	Germ.[c] %	Average %
Dijon healthy			100.0
Expt 1	235	100.0	
Expt 2	205	100.0	
Dijon TYMV infected (SV_1)[d]			98.5
Expt 1	223	99.0	
Expt 2	195	98.0	
Dijon TMV infected			100.0
Expt 1	245	100.0	
Expt 2	199	100.0	
La-O healthy			98.5
Expt 1	254	98.0	
Expt 2	195	99.0	
La-O TYMV infected (SV_1)[d]			97.0
Expt 1	242	98.0	
Expt 2	199	96.0	
La-O TMV infected			98.0
Expt 1	283	97.0	
Expt 2	186	99.0	

(a) Seeds were kept for 40 days in moist chamber.
(b) Seeds for all experiments were from a single seed lot produced by plants infected by seed transmission (SV_1).
(c) Full seedling with cotyledons, hypocotyl and primary root, regardless seedling appearance.
(d) First generation from virus infected seeds.

Table 2 – Seed transmission rates for turnip yellow mosaic tymovirus through successive generations of *Arabidopsis thaliana* by grow-out tests

		Ecotype			
		Dijon		La-O	
Generation[a]		no. tested	% transm	no. tested	%transm
SV_0	Expt 1	511	1.37	101	3.96
	Expt 2	267	2.25	344	9.59
SV_1	Expt 1	587	10.73	477	30.40
	Expt 2	337	3.86	526	25.09
SV_2	Expt 1	923	2.28	843	20.28
	Expt 2	242	2.89	266	27.44
SV_3	Expt 1	425	3.53	410	19.27
	Expt 2	423	2.60	471	20.59

(a) SV_0 = seeds produced by mechanically infected plants.
SV_n = seeds produced by the n-generation of plants infected by seed transmission.

Table 3 – Incidence (%) of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) antigens in embryo, seed coats, intact seed, and seedlings of *Arabidopsis thaliana* by PAS-ELISA

Treatment	Embryo only	Seed coat only	Embryo + seed coat	Whole seed	Seedlings
Dijon TYMV	5.4 (184)[a]	58.2 (184)	10.9 (184)	72.6 (274)	2.6(423)
La-O TYMV	24.5 (184)	12.6 (184)	13.0 (184)	59.5 (274)	20.6(471)
Dijon TMV	0 (180)[b]	100 (180)[b]	0 (180)[b]	100 (120)[c]	0 (846)
La-O TMV	0 (180)	100 (180)	0 (180)	100 (120)	0 (760)

[a] Number of seeds tested in parentheses.
[b] 60 seeds tested as groups of 10 and 120 as groups of 20 seeds.
[c] Pool of 20 seeds.

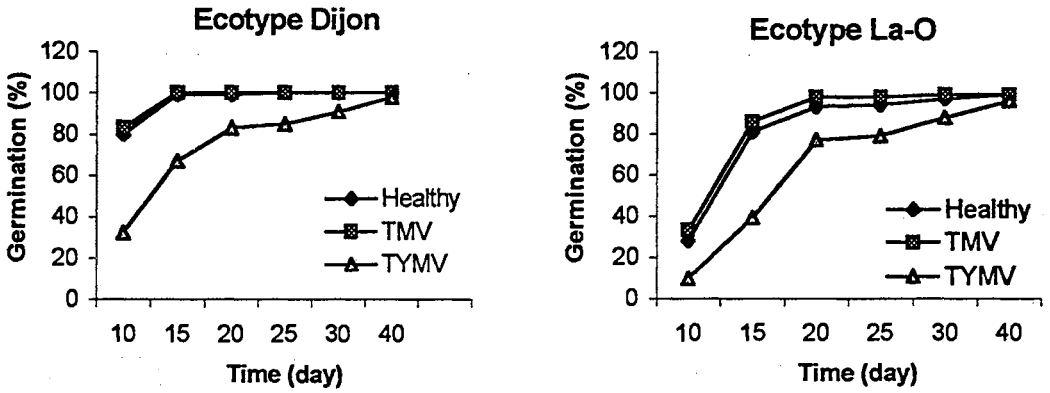

Figure 1 – Effect of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) on germination of *Arabidopsis thaliana* seed

CHAPTER 3

BARRIERS TO SEED TRANSMISSION IN *Arabidopsis thaliana*: THE ROUTE OF TURNIP YELLOW MOSAIC TYMOVIRUS AND TOBACCO MOSAIC TOBAMOVIRUS

Abstract

Turnip yellow mosaic tymovirus (TYMV) is seed transmitted in *Arabidopsis thaliana*, but tobacco mosaic tobamovirus (TMV) is not. Sexual crosses between healthy and virus infected plants were conducted to determine the route of TYMV and TMV leading to virus establishment in the developing seed. The F_1 progeny and healthy female parent were assayed for virus by enzyme-linked immunosorbent assay (ELISA). Comparison of the percentage of infected seedlings from each cross indicated that TYMV could be transmitted from either female or male parent. When TYMV was from the maternal plant, incidence of virus transmission was greater than when virus was from the male parent. On the other hand, the only route possible for TMV to reach *A. thaliana* seed was through direct invasion from maternal tissue. TYMV, but not TMV, was carried by pollen into the embryo-sac. Pollination of flowers on healthy *A. thaliana* with pollen from TYMV infected plants did not result in systemic infection of healthy plants, but TYMV was detected in seed produced on the healthy plants.

Introduction

Seed transmission is an important feature of both virus ecology and epidemiology. Seed transmission is the primary mean of dissemination for viruses such as barley stripe mosaic hordeivirus (BSMV) (Jackson et al., 1989) and cryptoviruses (Boccardo et al., 1987; Chiko, 1973; Mink, 1993). Seed transmission is one of the most efficient ways viruses may be disseminated into new areas. Seed infection may lead to economical losses due to failure in seed germination (Cooper et al., 1984; Hemmati and McLean, 1977; Suteri, 1981) and abnormal plant growth (Hicks et al., 1986; Steverson and Hagedorn, 1973; Tu, 1992).

Virus particles may invade any part of the seed (Agarwal and Sinclair, 1996). However, seed transmission has been closely related to embryo infection (Adams and Kuhn, 1977; Alvarez and Campbell, 1978; Bailiss and Offei, 1990; Crowley, 1957; Gupta and Summanwal, 1980; Mink, 1993; Pesic and Hiruki, 1986; Von Wechmar et al., 1984). Varma et al. (1992) attributed the failure of seed transmission of blackgram mottle (BMoV, a tentative member of the carmovirus group) in *Vigna mungo* to the small amount of virus in the embryonic axis rather than to virus location. Nolan and Campbell (1984) found that healthy seedlings may rise from virus infected embryos due to virus not establishing a systemic infection. A virus may enter the embryo either directly and/or indirectly. Direct invasion occurs during embryogenesis by virus from infected gametes (Carroll and Mayhew, 1976a; Hemmati and McLean, 1977). The indirect route of embryo

infection occurs by virus from maternal tissue (Carroll, 1972; Hemmati and McLean, 1977; Wang and Maule, 1994).

Seed transmission may depend on virus invasion of the floral meristem and then subsequently of the gametophytes and gametes (Bennett, 1969; Carroll and Mayphew, 1976 a,b). Wang and Maule (1994) showed that virus may enter the developing embryo and that the suspensor might be the route for pea seed-borne mosaic potyvirus (PSbMV) direct invasion of the pea embryo. Limitation of virus replication/movement may prevent PSbMV from reaching the suspensor before its degeneration and therefore be the basis for the failure of seed transmission in the pea cultivar Vedette in which virus is not transmitted by pollen (Wang and Maule, 1992). However, this result was challenged by the demonstration of PSbMV transmission in the pea cv Dual in seed from emasculated flowers on healthy plants fertilized with pollen from an infected plant (Johansen et al., 1994).

The linkage between seed transmissibility and embryo infection, relating the low virus incidence in the embryo to low seed transmission, was shown in *Arabidopsis thaliana* (L.) Heynh. (Chapter 2). Only a portion of the seed produced by infected plants led to virus infected progeny. One hypothesis for this phenomenon is that gametes are responsible for introducing virus into the embryo and not all gametes carry the virus.

Investigation on the route and the barriers to turnip yellow mosaic tymovirus

(TYMV) and tobacco mosaic tobamovirus (TMV) seed transmission was carried out by crossing healthy and TYMV or TMV infected *A. thaliana* and the percentage of infection of the progeny compared to determine whether the viruses reach the seed through the maternal or the paternal gamete. Systemic infection of the maternal plant by virus carried in pollen was also investigated.

Material and Methods

Seeds of *A. thaliana* ecotype La-O (*Arabidopsis* Biological Resource Center, Ohio State University) were sown in disposable plastic pots full of growing medium (Pro Mix BX, Premier Horticulture Inc). The seedlings were maintained in a growth chamber at 25°C under 14h ilumination by using both fluorescent and incandescent light. An isolate of TYMV and the common strain of TMV (Rezende et al., 1992; Sherwood and Fulton, 1982) maintained on *A. thaliana* and stored at -20°C were used in this study. Inocula were prepared by grinding infected tissue in phosphate buffer 0.01M, pH 7.0 and then applying the extract on Carborumdum-dusted leaves using a cotton swab. Seedlings at the rosette stage were mechanically inoculated with either TYMV or TMV. Infection was confirmed by serology and uninfected plants or those with atypical symptoms were removed. Uninoculated control seedlings were kept under the same conditions, but separated from the infected seedlings.

Crosses were carried out by emasculating flowers of the female parent

before bud opening for a three day period, during the flower development stages 10 to 11 (Smyth et al., 1990). All flowers produced subsequently were removed to avoid undesirable crosses. Emasculation was done by removing sepals, petals and stamens using a fine forceps. Each gynoecium was pollinated manually for 5 consecutive days following emasculation by rubbing the stigma with anthers originating from a male parent. Crosses were made with pollen from a healthy male parent to the gynoecium from an infected female parent and vice versa using 10 female parents for each cross. Some plants were allowed to self-pollinate for seed production where both parents were virus infected. Seed was harvested at maturity, air dried for two days, and stored at 4°C. Three to five weeks later seed was sown as described above. All progeny were individually assayed for virus infection by protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA) (Edwards and Cooper, 1985) at flowering. Flower and leaf samples were macerated in a white porcelain plate (Fischer Scientific cat # 13-745) with a disposable culture tube (Fischer Scientific cat # 14-9610-25) in phosphate buffered saline-Tween (PBS-T, 0.14 M NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.05% (v/v) Tween-20) + 2% (w/v) polyvinylpirolidone mol wt 40,000, pH 7.4.

Polyclonal antisera specific for TYMV and TMV were produced as previously described (Rezende et al., 1992). Protein-A (Sigma P-3838) in coating buffer (15 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) and protein-A alkaline phosphatase conjugate (Sigma P-9650) in PBS-T were used at 0.5µg/ml, and p-nitrophenyl phosphatase (Sigma 104-105) at 1mg/ml in 1 M diethanolamine, pH 9.8.

After protein-A incubation, free sites in the sample well were blocked with 5% non-fat dried milk in PBS-T for 60 min at 37°C. The assay was carried out in polystyrene plates (Nunc-Immuno Plate MaxSorp Surface), using 100µl per well for each reagent, except the blocking step that was 150µl . Incubations were at 37°C for 2 h for all steps, except the sample step which was incubated at 4°C overnight, and the step when the p-nitrophenyl phosphate substrate was added the plates which were incubated at room temperature for 10-30 min. Between each step, the plates were washed 3 times for 3 min each with PBS-T. Each sample was assayed in three separate wells. The absorbance was read at 405nm (Tecan Spectra Rainbow, Tecan U.S. Inc.) with a healthy sample as a blank. The negative threshold for both TYMV and TMV was $\bar{X} \pm 3SD$ (Clark, 1981; Sutula et al., 1986) of the 10 wells of healthy control samples. The ELISA procedure was the same throughout unless otherwise indicated.

The percentage of infected progeny within each treatment were compared. For evaluation of pollen transmission, the healthy maternal parent fertilized by pollen from the infected paternal parent also was assayed by PAS-ELISA. The assay was done one week after pollination and repeated three times at intervals of one week.

Results

The incidence of TYMV and TMV in the progeny of the crosses between

healthy and infected parents was determined by grow-out tests and ELISA. The results indicated that TYMV infected progeny were obtained from crosses where one or both parents were infected with TYMV as well as from selfing of infected plants (Table 1). The highest incidence of TYMV in seedlings was observed when both parents were infected. When only one parent was infected, crosses involving an infected female gave a higher incidence than that involving an infected male. These results indicate that TYMV could be derived from both the female and male parents. However the incidence of infected progeny was much higher when the female parent was infected as compared to the incidence when the male parent was infected. Conversely, TMV was not detected in the progeny of either cross (Table 2), indicating that no seed transmission had occurred as previously determined (Chapter 2). TMV was detected by ELISA in the seed coat of seed produced from crosses involving TMV infected female or when both parents were infected with TMV (Table 3). Thus, absence of TMV in the embryo as implied by the lack of seed transmission and the presence of virus in seed coat indicated that seed infection by TMV originated by direct invasion from maternal tissue (Chapter 2).

To better understand the pathway used by both TYMV and TMV to invade *A. thaliana* seed, vegetative, reproductive, and seed tissues of female parent plants were assayed by ELISA. The results showed that TYMV, but not TMV, was present in the flower and seed from the healthy female parent fertilized by pollen from an infected plant (Table 3), indicating that TYMV was carried by pollen into these organs. In addition there was no TYMV or TMV invasion of leaves or silique shells

(Table 3), indicating that no systemic infection of the healthy female parent had occurred. Detection of TYMV in the flower and in the seed, but absence of TYMV in the silique shell (which is of maternal origin), indicated that once TYMV is introduced into the embryo by pollen the virus cannot move to the surrounding maternal tissue.

Discussion

In this study the route of TYMV and TMV to the developing seed of *A. thaliana* was investigated to relate virus movement to the incidence of seed transmission. Through crosses between healthy and virus infected plants it was demonstrated that TYMV, but not TMV, was carried by pollen into the embryo-sac. TYMV carried by pollen led to infection of flower and seed tissues, while maternal vegetative tissue remained virus-free. Seed infection through pollen while the mother plant remains virus-free has been reported for several virus-host combinations (Bennett, 1969; Gilmer and Way, 1960; Hemmati and McLean, 1977; Johanson et al., 1984; Sdoodee and Teakle, 1988). Pollen transmission has not been reported for either TYMV or TMV (Matthews, 1980; Zaitlin and Israel, 1975), while seed transmission of TYMV has been reported (Chapter 2; Hein, 1984; Spak et al., 1993).

Studies have shown that virus can use both a direct and indirect route to infect the embryo (Carroll, 1972; Carroll and Mayhew, 1976a; Hemati and McLean,

1977; Wang and Maule, 1994). The results presented here showed that direct and indirect (gametic) invasion of *A. thaliana* embryo by TYMV may occur. Evidence for indirect invasion (male gamete) comes from virus transmission in seed produced from crossing the healthy female with an infected male plant, where only infected pollen could introduce TYMV into the embryo. On the other hand, TYMV infected the *A. thaliana* embryo either via the female gamete, by direct invasion from maternal tissue or by both, as indicated by making crosses between infected female and healthy male plants. The data presented here do not distinguish between direct or indirect embryo invasion by TYMV in crosses involving infected female plants.

Studies on embryogenesis of *A. thaliana* (Mansfield and Briarty, 1991; Meinke, 1994) showed that cellular division of the zygote gives rise to two cells early after fertilization, which later give rise to the embryo and the extraembryonic suspensor. Nutrients are transported to the embryo from the mother plant through the suspensor. The embryonic cells and the suspensor are interconnected by plasmodesmata, but there is no symplastic connection with the surrounding tissues (Mansfield and Briarty, 1991). Therefore, the symplastic barrier blocks virus movement. The growth of the embryo inside the embryo-sac gives rise to the seed formed by two cotyledons and the embryonic axis. The seed coat originates from the ovule (maternal origin) and protects the embryo. The seeds are enclosed in the fruit (silique), which results from rapid elongation of the gynoecium after pollination (Meinke, 1994), while the ovary of the fertilized flower turns into the silique shell

(also of maternal origin).

The absence of movement of TYMV from the infected embryo to the surrounding maternal tissue when infection occurs by pollen strongly suggests that the absence of symplastic connection between embryo and maternal tissue effectively impairs the direct invasion of virus from maternal origin. This finding confirms the symplastic isolation of the embryonic tissue from the maternal tissue (Mansfield and Briarty, 1991) which is responsible for impairing virus movement from maternal tissue to the embryo and vice versa. Whereas TYMV could be detected in seeds from healthy female plants fertilized by pollen from virus infected male plants, the silique shell (seed excluded) remained virus-free (Table 3), indicating the gametic involvement in embryo invasion. The route of TYMV in the female parent leading to seed transmission is far more effective than the route involving the male counterpart as indicated by the higher incidence of infected seedlings from cross involving the infected female parent (Table 1).

It was demonstrated that TMV occurs in the seed and the silique shell when the female parent is infected but not when the healthy female is crossed with a TMV infected male (Table 3). In the previous study (Chapter 2) TMV was found limited to the seed coat and was absent from the embryo. Both the seed coat and the silique shell are of maternal origin (Koornneef and Karssen, 1994; Meinke, 1994). These observations collectively indicated that the location of TMV within the seed and the maternal origin of the seed coat and the silique shell assure that only direct

invasion from maternal origin is possible. Neither the embryo-sac nor pollen provide the route for TMV invasion of the embryo, as indicated by the lack of seed transmission and absence of TMV from vegetative, reproductive, and seed tissues of *A. thaliana* when the healthy female parent is fertilized by pollen from a TMV infected male (Table 3). This confirms, as reported before (Chapter 2), that the barrier to TMV seed transmission is its inability to reach the embryo. Such a barrier may be virus-specific, since TYMV was detected by ELISA in the embryo of *A. thaliana* double infected by TYMV and TMV (Chapter 2, Appendix I). Hence, both viruses were detected by ELISA in whole seed, yet only TYMV was seed transmitted. TMV reaches the seed coat but remains apart from the symplastically isolated embryo-sac and embryo, blocking seed transmission because embryo infection is essential for seed transmission in this system as it is in most plant-virus combinations (Adams and Kuhn, 1977; Alvarez and Campbell, 1978; Bailiss and Offei, 1990; Crowley, 1957; Grupta and Sammanwal, 1980; Mink, 1993; Pesic and Hiruki, 1986; Von Wechmar et al., 1984). For TYMV, the analysis of the progeny of the crosses (Table 1) indicated virus in either the female or male gamete can result in embryo infection and subsequent seed transmission of virus.

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Table 1 – Determination of gamete transmission of turnip yellow mosaic tymovirus (TYMV) in *Arabidopsis thaliana* by PAS-ELISA[a]

Cross[b]	Replication	Total seedlings	Infected seedlings	Incidence (%)[c]
	1	113	49	43.36
I♀ x I♂	2	107	20	18.69
	3	76	30	39.47
	4	94	27	28.72
Total		**390**	**126**	**32.31**
	1	110	40	36.36
I♀ x H♂	2	161	32	19.88
	3	109	30	27.52
	4	86	25	29.07
Total		**466**	**127**	**27.25**
	1	105	1	0.95
H♀ x I♂	2	88	3	3.41
	3	135	3	2.22
	4	140	3	2.14
Total		**468**	**10**	**2.14**

(a) All F_1 seeds produced by 10 female parents were harvested together. A portion of them was sown in growing medium and seedlings were maintained in a growth chamber. At flowering, seedlings were individually sampled and tested for virus incidence by ELISA.

(b) Crosses carried out by emasculation of flower from female parent and hand pollinated by rubbing anther from male parent. I♀ x I♂ was self-pollinated. Healthy (H) or infected (I) female (♀) or male (♂) parent.

(c) Each seedling was tested in three separated wells.

Table 2 – Determination of gamete transmission of tobacco mosaic tobamovirus (TMV) in *Arabidopsis thaliana* by PAS-ELISA[a]

Cross[b]	No. seedlings tested	No. seedlings infected	Incidence (%)
I♀ x I♂	120	0	0
I♀ x H♂	129	0	0
H♀ x I♂	126	0	0

[a] All F_1 seeds produced by 10 female parents were harvested together. A portion of them was sown in growing medium and seedlings were maintained in a growth chamber. At flowering, seedlings were individually sampled and tested for virus incidence by ELISA.

[b] Crosses carried out by emasculation of flower from female parent and hand pollinated by rubbing anther from male parent. I♀ x I♂ was self-pollinated. Healthy (H) or infected (I) female (♀) or male (♂) parent.

Table 3 – Detection of turnip yellow mosaic tymovirus (TYMV) and tobacco mosaic tobamovirus (TMV) in plant parts of the female parents from crosses of *Arabidopsis thaliana* [a]

	I♀ x I♂[b]		H♀ x I♂		I♀ x H♂	
Organ	TYMV	TMV	TYMV	TMV	TYMV	TMV
Leaf	+	+	-	-	+	+
Flower	+	+	+	-	+	+
Seed	+	+	+	-	+	+
Silique shell[c]	+	+	-	-	+	+

(a) Healthy females fertilized by pollen from infected male parent was assayed by PAS-ELISA one week after pollination and repeated three times with an interval of one week.

(b) Crosses carried out by emasculation of flower from female parent and hand pollinated by rubbing anther from male parent. I♀ x I♂ was self-pollinated. Healthy (H) or infected (I) female (♀) or male (♂) parent.

(c) Seed excluded.

CHAPTER 4

MAPPING A SYMPTOM DETERMINANT OF COWPEA CHLOROTIC MOTTLE BROMOVIRUS

Abstract

The type strain of cowpea chlorotic mottle bromovirus (CCMV-T) produces an intense and extensive chlorosis in cowpea (*Vigna unguiculata* cv California Blackeye - CB), while the attenuated variant (CCMV-M) induces milder symptoms. Infectious RNA transcribed *in vitro* from RNA 3 cDNA clones of both strains was obtained. RNA 1 and RNA 2 from CCMV-T along with RNA 3 from the T and M strains inoculated on CB induced indistinguishable symptoms from those of the parent strains. Comparison of nucleotide sequence of both strains revealed four base changes. *In vitro* transcripts of chimeric CCMV RNA 3 cDNA clones constructed by exchanging restriction fragments containing these changes individually showed that the genetic determinant of symptom expression is located in the 3' portion of the coat protein gene, where the amino acid Ala 151 in CCMV-T is changed to Val 151 in CCMV-M.

Introduction

Cowpea chlorotic mottle bromovirus (CCMV) is a non-enveloped isometric virus with a total genome of 8.4kb formed by three single-strand (+) RNAs (Allison

et al., 1989; Lane, 1979). RNA 1 and RNA 2 are encapsidated in separated particles, while RNA 3 and the subgenomic coat protein mRNA are encapsidated together (Murphy et al., 1995). CCMV belongs to the genus Bromovirus, family Bromoviridae, order Mononegavirales (Lane, 1979; Murphy et al., 1995).

The CCMV type strain genome has been cloned and sequenced (Ahlquist et al., 1981; Allison et al., 1989; Dasgupta and Kaesberg, 1982; Dzianott and Bujarski, 1991; Gunn and Symons, 1980). The RNA 1 and RNA 2, with one open reading frame (ORF) each, encodes for two nonstructural proteins required for virus replication (Kiberstis et al., 1981). The dicistronic RNA 3 encodes for the 3a movement protein (MP) and is the template for the subgenomic mRNA (RNA 4) that encodes for the coat protein (CP) (Dasgupta and Kaesberg, 1982). Both 3a MP and CP proteins are required for systemic infection of cowpea, *Vigna unguiculata* (L.) Walp subsp *unguiculata* (Allison et al., 1990; De Jong and Ahlquist, 1992; Mise and Ahlquist, 1995). The RNA 3 also contains an intercistronic noncoding region which has been established to direct transcription of the subgenomic RNA 4 from the negative strand (Ahlquist, 1994; Pacha and Ahlquist, 1992). The coding regions, the intercistronic noncoding region, and a portion of the 5' and 3' noncoding terminus can be individually deleted with no significant effect on RNA 3 accumulation (Ahlquist, 1994; Pacha et al., 1990), however the terminal sequences of 5' and 3' noncoding regions are required for a normal amount of RNA 3 replication (Pacha et al., 1990). A portion of the intercistronic noncoding region of RNA 3 is required for systemic infection of CCMV in cowpea (Pacha and

Ahlquist, 1992; Pacha et al., 1990). They found that a large deletion in the intercistronic region of CCMV RNA 3 altered the symptoms from a nearly symptomless infection exhibited by the wild type to extensive bright yellow chlorosis induced by the mutant clone inoculated on cowpea, suggesting that change in disease severity was promoted by mutation in a regulatory rather than a protein-coding sequence.

The MP protein has been associated with virus cell-to-cell movement, symptom regulation and host range specificity (Fujita et al., 1996; Mise and Ahlquist, 1995). In addition to virion formatting, the CP protein has been associated with virus long-distance movement (Rao, 1997). The CP of the closely related brome mosaic bromovirus (BMV) has as well been associated with symptom induction and long-distance virus movement (Sacher and Ahlquist, 1989).

Several strains of CCMV have been characterized that induce different symptoms on cowpea (Fulton et al., 1975; Kuhn, 1964, 1968; Kuhn and Wyatt, 1979; Paguio et al., 1988; Sinclair and Backmam, 1989; Walters and Dodd, 1969; Wyatt and Kuhn, 1980). Two strains were used in this study: a mild variant (CCMV-M) obtained by passage through *Phaseolus vulgaris* L. (Kuhn & Wyatt, 1979) and the type strain (CCMV-T) (Kuhn, 1964). The mild strain of CCMV produces less intense chlorosis on cowpea cv California Blackeye (CB) than the type strain. The complete nucleotide sequence of the three genomic RNAs of the type strain have been published (Ahlquist et al., 1981; Allison et al., 1989;

Dasgupta and Kaesberg, 1982; Dzianott and Bujarski, 1991; Gunn and Symons, 1980). The complete nucleotide sequence of RNA 3 of the mild strain is reported.

Reassortments with viral RNAs from the CCMV-T and CCMV-M indicated that the symptom determinant was controlled by RNA 3 (Kuhn and Wyatt, 1979). The objective of this study was to use infectious transcripts derived from cDNA clones of CCMV-T and CCMV-M to identify the molecular variation of RNA 3 responsible for symptomatology on cowpea.

Material and Methods

Viruses, plants, and inoculations

Two strains of CCMV were used. The type strain (CCMV-T) (Kuhn, 1964), and the mild strain (CCMV-M) (Kuhn and Wyatt, 1979). Inoculations were done on cowpea cv California Blackeye, thereafter referred as CB. Purified virus, *in vitro* transcripts derived from viral cDNA, or homogenates from infected leaves were rubbed on Caroborundum-dusted leaves of 8-day-old seedlings and maintained either in growth chambers (14 h light/10 h dark cycle) at 27°C or greenhouse.

cDNA cloning

CCMV-M RNA was isolated from a preparation of purified virus (Wyatt and

Kuhn, 1979). A reverse transcription reaction coupled to polymerase chain reaction (RT-PCR) was used to synthesize and amplify cDNA representing full-length CCMV-M RNA 3. First strand cDNA was generated using the SuperScript Preamplification System for First Strand cDNA Synthesis (Gibco-BRL, Life Technologies) according to the manufacture's instructions. The 3'-primer used was 5'-TGCTCTAGAGGTCTCCTTAGAGATCACC-3', which is complementary to the 3' end of CCMV-T RNA 3 (S. Quan and C.M. Deom, unpublished) and contains a unique 5'-flanking *XbaI* site (underlined). The 5'-primer need to generate cDNA to CCMV-M RNA 3 was 5'-CGGGGTACC**TAATACGACTCACTACTATC**GTAATCT TTACCAAAC-3' (S. Quan and C.M. Deom, unpublished), which contains a T7 promoter (bold) and a unique 5'-flanking *KpnI* site (underlined).

RT-PCR product purified from a 0.8% agarose gel using QIAEX II Gel Extraction kit (QIAGEN Inc.) according manufacture's instructions, and digested with *KpnI* and *XbaI,* was ligated (T4 DNA ligase, New England Biolabs Inc.) into the *KpnI-XbaI* sites of pUC19 (Gibco-BRL, Life Technologies), then was transformed into *Escherichia coli* DH5α (Gibco-BRL, Life Technologies). Colonies were initially selected and screened by restriction enzyme analysis for CCMV-M RNA 3 cDNA.

To generate first strand cDNA a 25μl mixture of 5μg of viral RNA and 20 pmol of the 3' primer in DEPC treated water was heated denatured at 70°C for 10 min and immediately transferred to 50°C. The mixture was added to equal volume of 42°C prewarmed mixture to give to the 50-μl reaction a concentration of 50 mM

KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 1 mM DTT, and 200 units of SuperScript II Rnase H$^-$ Reverse Transcriptase (Gibco-BRL, Life Technologies). After incubation at 50°C for 50 min the reaction was terminated by heating at 70°C for 15 min and chilling on ice. Following brief centrifugation, RNA was digested using 2 units of *E. coli* RNAse H by incubation for 20 min at 37°C. cDNA was amplified by PCR in a 100-µl reaction mixture containing 1 µl of first strand cDNA, 50 pmol of each 3' and 5' primers, 50 mM KCl, 20 mM Tris-HCl (8.4), 0.2 mM dNTPs, and 2.5 units of Turbo *Pfu* polymerase (Gibco-BRL, Life Technologies). PCR were performed with the following parameters: 2 min at 94°C, 30 cycles of 45 s at 94°C, 90s at 50°C and 5 min at 72°C then 72°C for 15 min.

Five clones were transcribed *in vitro* as well as plasmids containing full-length cDNA of CCMV-T RNA 1 and RNA 2 (pCCT1and pCCT2; S. Quan and C.M. Deom, unpublished). To prove the infectivity of the clones, *in vitro* transcription was performed. The *Xbal*-linearized plasmid were used as the template to obtain full-length capped transcripts following manufacture's instructions (RiboMAX Large Scale RNA Production Systems-T7, Promega) with addition of RNA cap structure analog (New England Biolabs Inc.).

Inoculum prepared with 5 µg of each capped transcript of RNA 1 (cT1) and RNA 2 (cT2) from CCMV-T and RNA 3 (cM3) from CCMV-M was used to inoculate Carborumdum-dusted primary leaves of 8-day-old CB seedling. After inoculation, the leaf surface was rinsed by spraying distillated water. As controls, seedlings

were inoculated with distillated water (mock), 20 μg of purified CCMV-T, 20 μg of purified CCMV-M, or CCMV-T RNA 1-3 transcribed *in vitro.* The seedlings were kept either in a growth chamber at 27°C under 16/8h light/dark cycles or greenhouse. Infection was evaluated by the symptoms that developed and confirmed either by western blot analysis or protein-A enzyme-linked immunosorbent assay (PAS-ELISA) (Edwards and Cooper, 1985).

In vitro transcription

Infectious transcripts were synthesized *in vitro* from *Xbal*-linearized plasmids containing full-length cDNA of CCMV-M RNA 3 (construction described here) and CCMV-T RNAs 1 and 2 (pCCT1 and pCCT2; S. Quan and C.M. Deom, unpublished) using RiboMAX Large Scale RNA Production System-T7 (Promega) following manufacture's instructions with addition of 3 mM of RNA Cap Structure analog (New England Biolabs Inc.).

Sequencing

Two clones (pCCM3-4 and pCCM3-5) were selected for sequencing. Four subclones were constructed by deleting sequence between *KpnI/MfeI* or *MfeI/XbaI* restriction sites to make them suitable for sequencing. Protruding ends were filled with T4 DNA polymerase (New England Biolabs Inc.) followed by religation of the blunt end with T4 DNA ligase (New England Biolabs Inc.). The subclones were

transformed as described above. The resulted subclones pCCM34MX, pCCM34KM, pCCM35MX, and pCCM35KM were sequenced using the primer pair M13-20 forward and M13-48 reverse (Molecular Genetics Facility, The University of Georgia) and the sequences aligned and compared to the known sequence of CCMV-T RNA 3 (Allison et al., 1989).

Chimeric construction

Five unique restriction sites (*KpnI, MfeI, BsiWI, HpaI, and XbaI*) were used for constructing chimeric viruses. Clones CCMV-T RNA 3 (pCCT3) and CCMV-M RNA 3 (pCCM3) were enzymatically digested and the restriction fragments exchanged between them to give eight RNA 3 chimeric viruses. Ligation, transformation, *in vitro* transcription, and inoculation of these chimeric viruses were done as described above. Four to six cowpea seedlings were coinoculated with transcripts from each chimeric RNA 3 cDNA clones and CCMV-T RNA 1 and RNA 2 cDNA clones from *in vitro* transcripts prepared as described above. Each experiment was repeated at least three to five times with three independently synthetized *in vitro* transcripts.

The spatial and temporal distribution of both strains of CCMV within systemically infected cowpea was investigated by ELISA. The procedure for ELISA was as described before (Chapter 2), using polyclonal antibodies specific for the strain CCMV-R, which react similarly with CCMV-T and CCMV-M.

For spatial and temporal virus distribution 8-day-old cowpea seedlings were inoculated at the distal portion of each primary leaf with preparation of known concentration of purified virus. The whole plant was harvested at 6h, 12h after inoculation, then five other samples with one-day interval. The samples were obtained as following: distal, medium and proximal portion of the inoculated leaf; apical, medium and basal portion of the stem; and root. Two leaf discs (75mm) was removed from each half-leaf lamina. From stem and root the sample consisted of a portion about 1cm long. Samples were ground in 500μl of buffer as described before (Chapter 2). Each sample was tested in four repeated wells (Immulon 2, Dynatech Laboratories, Inc.).

ELISA was also used for estimation of the relative concentration of each chimeric virus. Systemically infected, fully expanded leaflet of cowpea was harvested 12 days after inoculation to represent each plant. The samples were prepared as described above, with each samples tested in four repeated wells with 6 repetitions represented by individual plant. A standard curve generated by four samples with different concentration of purified virus was used as a control.

Results

cDNA cloning

Five full-length cDNA clones of CCMV-M RNA 3 (pCCM3) were obtained.

Infectious transcript from those clones gave typical attenuated symptoms in cowpea CB when coinoculated with transcripts from RNA 1 and RNA 2 of cDNA clones of CCMV-T. Transcripts from CCMV-T RNA 1, 2 and 3 cDNA clones were inoculated onto CB as a control. The symptoms induced by the transcripts from all five clones (pCCM3) were indistinguishable from those of CCMV-M purified virus, while symptoms induced by transcripts from the CCMV-T cDNA clones were indistinguishable from those of CCMV-T purified virus.

Sequencing

Sequencing alignment of the subclones pCCM34KM and pCCM35KM, and pCCM34MX and pCCM35MX using the sequence analysis software MacDNASIS (Hitachi Software) showed 100% similarity between them. Comparison between CCMV-M RNA 3 (reported here) and CCMV-T RNA 3 (Allison et al., 1989) sequences showed four nucleotide changes (Table 1; Figure 1). Two changes in the 3a MP gene, one change in the intercistronic noncoding region and two changes in the CP gene. Analysis of the predicted amino acid sequence showed that one out of the three nucleotide changes was silent (Table 1). There was a change from Lys to Arg in the 3a MP gene at position 286, and in the CP gene Ala changed to Val at position 151 (Table 1). There was also a reduction of 6 As in the internal poly(A) tract of the intercistronic noncoding region. The number of bases in the poly(A) tract among CCMV strains normally varies from 35 to 45 (Ahlquist, 1994).

Mapping the mild symptoms determinant of CCMV-M

Chimeric cDNA were generated between cDNA clones of CCMV-T RNA 3 and CCMV-M RNA 3 (Figure 1). RNA transcripts derived from each chimeric cDNA clone were inoculated onto cowpea plants together with transcripts from cDNA clones of CCMV-T RNA 1 and RNA 2. All chimeric RNA 3 that contained the majority of the CP gene and 3' untranslated region of CCMV-M RNA 3 (*HpaI – XbaI* region) induced mild symptoms on cowpea that were indistinguishable from those induced by CCMV-M purified virus (Table 2). Similarly, all chimerics RNA 3 that contained the majority of the CP gene and 3' untranslated region of CCMV-T RNA 3 (*HpaI – XbaI* region) induced severe symptoms on cowpea indistinguishable from those induced by CCMV-T purified virus (Table 2).

The only difference between RNA 3 cDNA clones of CCMV-M and CCMV-T in the *HpaI – XbaI* region is the occurrence of 1813 C in RNA 3 of CCMV-T and a 1807 U in CCMV-M. This single nucleotide change results in an Ala151 → Val substitution in the CP of CCMV-M. This single nucleotide change at position 1813 of CCMV-T RNA 3 is involved in determining symptom phenotype in cowpea.

Discussion

Viral determinants have been identified for seed transmission (Edwards, 1995; Hampton and Francki, 1992; Johansen et al., 1996; Wang et al., 1997),

symptomatology (Banersee et al., 1995; Culver and Dawson, 1989; Knorr and Dawson, 1988; Rao and Grantham, 1995; Shintaku et al., 1992; Suzuki et al., 1995), and vector specificity (Atreya et al., 1990, 1992; Hanada and Harrison, 1971; MacFarlane et al., 1996; Perry et al., 1994; Woolston et al., 1987). Here an attenuated strain (Kuhn and Wyatt, 1979) was cloned, sequenced and the sequence compared to the type strain (Kuhn, 1964) in an attempt to identify changes in the viral genome responsible for symptom attenuation on CB. Following identification of nucleotide and amino acid changes potentially responsible for symptom attenuation, chimeric viruses were constructed by exchanging restriction fragments between infectious cDNA clones.

Sequence analysis of RNA 3 clones of CCMV-T and CCMV-M revealed that a single base change (C 1813 to U 1807) in the CP gene, with consequent change in the amino acid from Ala 151 to Val was responsible for the change in the symptomatology on CB from severe to mild. The effect of modifications in the CP on symptom induction has been reported for several plant-virus combinations (Bancroft et al., 1972; Culver and Dawson, 1989; Dawson et al., 1988; Heaton et al., 1991; Neelman et al., 1991; Petty and Jackson, 1990). However, how these changes actually modify disease symptoms is unclear.

CCMV long-distance movement is a component of the virus infection cycle that depends on the production of stable particle through virion assembly (Rao, 1997). To assembly nucleoprotein, the CCMV coat protein interact with the

genomic RNA through its basic N-terminal arm formed by 25 amino acid residues (Van der Graaf et al., 1991, 1992; Vriend et al., 1981, 1986), which constitute a highly basic region containing 6 Arg, 3 Lys, and no acidic residues (Rees and Short, 1982). It was suggested that the primary driving force for nucleoprotein assembly originates from the interactions between the positive side chains of the Arg and Lys residues present in the N-terminal arm and the negative phosphate groups of the RNA (Argos, 1981; Vriend et al., 1981). Amino acid changes in the CCMV CP would introduce conformational alteration in the polypeptides, which would affect symptom induction (Culver and Dawson, 1989) or alter virus stability or movement (Flasinski et al., 1997). However, the amino acid change observed in the coat protein reported here is located in the opposite extremity of the polypeptide.

The disease symptoms induced in the host by the virus infection are closely related to the age at which the host tissue is invaded by the virus. It is generally accepted that early virus invasion during morphogenesis is required for normal symptom development otherwise the symptomatology may be attenuated (Bos, 1978; Fraser, 1987). Flasinski et al. (1997) found that a single amino acid change in the CP of the closely related BMV delayed virus movement in *Chenopodium hybridum* L. Symptom severity has been correlated with higher virus accumulation in systemically infected leaves as a result of more efficient virus movement within the host (Tsai and Dreher, 1993). On the other hand, that the milder strain of cauliflower mosaic caulimovirus (CaMV) accumulates more than the severe strain.

Suzuki et al. (1995) indicated that virus assembly, virus transport and symptom expression of CMV on tobacco were affected by a single amino acid change in a specific site of the CMV coat protein that altered coat protein structure. A mutation in the RNA 3 intercistronic non coding region of CCMV resulted in reduction in virus accumulation and RNA 4 transcription in cowpea . Along with those changes, the symptoms induced by the mutant were more severe than those induced by the wild type CCMV. Here, data from ELISA on spatial and temporal distribution of CCMV-T and CCMV-M in cowpea indicated that by three days after inoculation both viruses strains are found in inoculated and non-inoculated leaves, stem, and root. Estimation of the relative virus concentration by ELISA showed a slightly higher accumulation of CCMV-M than CCMV-T (also observed among the chimeric virus). Therefore, both strains have similar pattern of accumulation and movement within systemically infected cowpea CB, indicating that those virus features are likely not responsible for the mild phenotype of CCMV-M. The relative virus concentration determined here by ELISA agrees with data obtained by Kuhn and Wyatt (1979) using spectrophotometric analysis of purified virus.

Another possibility would be that the change in symptom is driven by direct interaction at the nucleic acid level rather than by protein interaction as found by Pacha and Ahlquist (1992). This is the case of viroid and satellite RNA where no protein is produced (Visvader and Symons, 1985). A common aspect between the mutation reported by Pacha and Ahlquist (1992) and that reported here is their direct effect on coat protein. These results demonstrate that modification in the

CCMV coat protein gene can affect symptom production.

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Table 1 - Nucleotide and predicted amino acid changes in RNA 3 of cowpea chlorotic mottle bromovirus type (T) and mild (M) strains

Gene with Change	Position		Base change		Amino acid change	
	T	M	T	M	T	M
3a MP	913	913	C	U	Silent	
3a MP	1098	1098	A	G	Lys	Arg
Intercistronic region	1291	1291	C	A	Untranslated	
CP	1813	1807	C	U	Ala	Val

Table 2 – Virus phenotypes and symptomatology on cowpea cv. California Blackeye

Inoculum[a]	Phenotype[b]	Symptom
T virus	T1T2T3	Severe
M virus	M1M2M2	Mild
pCCT	cT1cT2cT3	Severe
pCCM	cT1cT2cM3	Mild
pTMKM	cT1cT2cT3ch	Severe
pMTKM	cT1cT2cM3ch	Mild
pTMMB	cT1cT2cT3ch	Severe
pMTMB	cT1cT2cM3ch	Mild
pTMBH	cT1cT2cT3ch	Severe
pMTBH	cT1cT2cM3ch	Mild
pTMHX	cT1cT2cT3ch	Mild
pMTHX	cT1cT2cM3ch	Severe

(a) See text for inoculum identification.
(b) Capital letters denote the type (T) or mild (M) strains from which RNA 1-3 came from; low case (c) denotes RNA from *in vitro* transcription; (ch) denotes chimeric RNA 3.

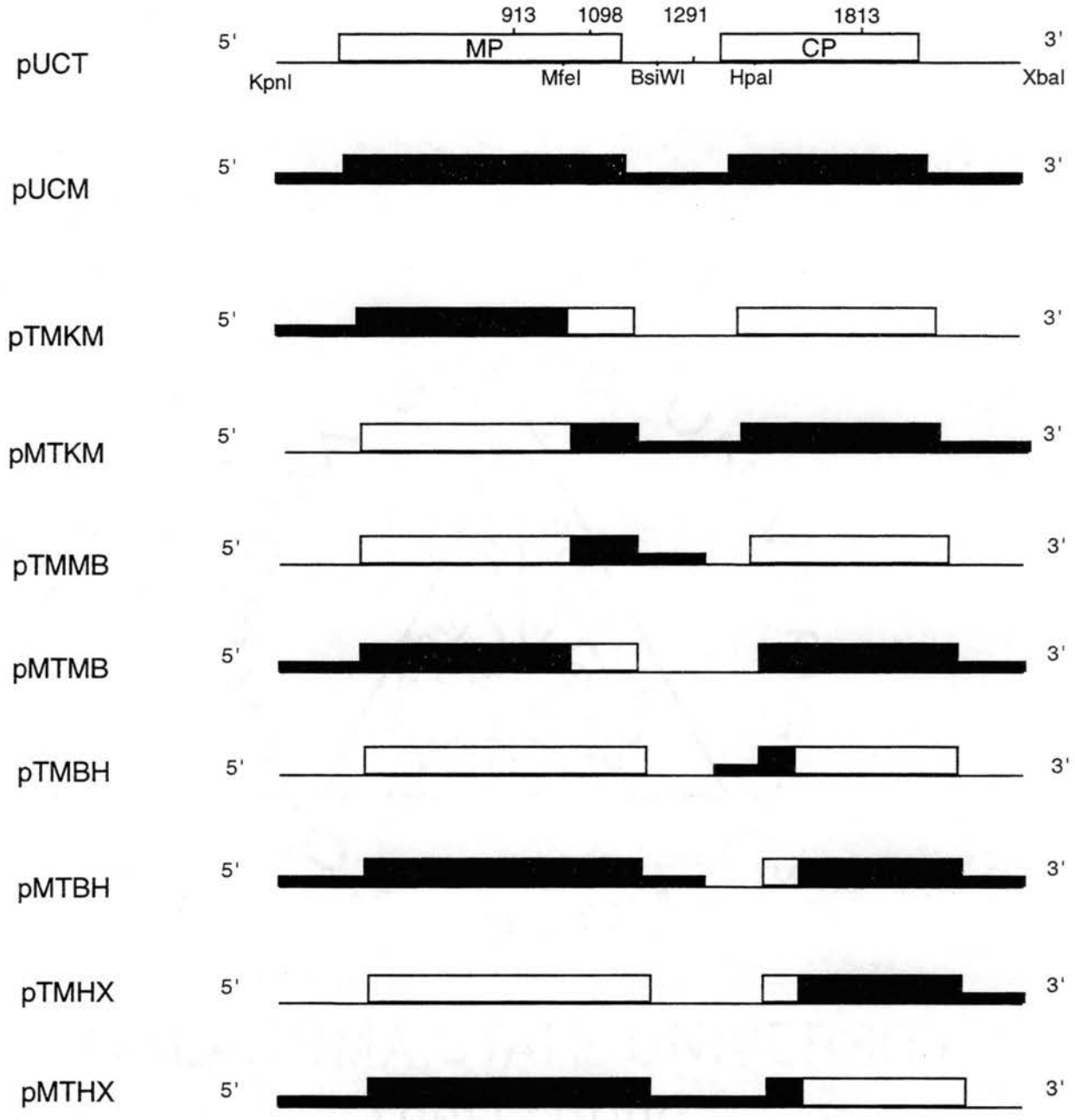

Figure 1 - Schematic representation of the chimeric RNA 3 of cowpea chlorotic mottle bromovirus (CCMV).

(A) pUCT is the type strain CCMV genomic RNA 3 cDNA.
(B) pUCM is the mild strain CCMV genomic RNA 3 cDNA.
(C) Chimeric RNA 3 cDNA representing the exchanged restriction fragments between the strains T and M. Number at the top represents the position of the base changes. The restriction enzyme sites are indicated at the bottom.

APPENDIX I

CO-INFECTTION OF *Arabidopsis thaliana* WITH TURNIP YELLOW MOSAIC TYMOVIRUS (TYMV) AND TOBACCO MOSAIC TOBAMOVIRUS (TMV) ENHANCES SEED TRANSMISSON OF TYMV

Studies on the mechanism of seed transmission reported in Chapter 2 indicated that turnip yellow mosaic tymovirus (TYMV), but not tobacco mosaic tobamovirus (TMV), was transmitted in seed of *Arabidopsis thaliana* (L.) Heynh. Seed transmission of TYMV was linked to embryo infection, as there was similar distribution of both TYMV and TMV within *A. thaliana*, except in the seed. TMV was limited to the seed coat, but TYMV infected both the embryo and the seed coat.

The finding that seed transmission of TMV is blocked by the inability of the virus to invade the embryo led to investigate whether co-infection of *A. thaliana* with TYMV and TMV would affect the incidence of seed transmission. Here, preliminary results are presented and briefly discussed.

The methodology adopted was as described in Chapter 2, except that *A. thaliana* ecotype La-O were inoculated with both TYMV and TMV by inoculating TYMV following by TMV 10 min later on leaves other than those inoculated with TYMV. Seedlings originated from seed produced by *A. thaliana* co-infected with

TYMV and TMV were assayed by grow-out tests.

Arabidopsis thaliana singly infected with either TYMV or TMV gave typical symptoms of each virus, while plants doubly infected with both TYMV and TMV showed severe mosaic, stunting and flower abortion. No difference in virus distribution was observed in both vegetative and reproductive tissues of plant singly or doubly infected with TYMV and TMV.

An increase of over 125% in the incidence of transmission of TYMV was observed (Table A, for incidence of seed transmission with TYMV single infection see Table 1, Chapter 3). There was no effect on TMV transmission is seed (see Table 2, Chapter 3). This latter observation assures there was no encapsidation of TMV-RNA in TYMV protein.

Table A – Incidence of seed transmission in *Arabidopsis thaliana* ecotype La-O co-infected with turnip yellow mosaic tymovirus and tobacco mosaic tobamovirus as determined by grow-out tests[a]

Expt	Seedlings Tested	TMV Transm No. of infected Seedlings	%	TYMV Transm No. of infected seedlings	%
I	108	0	0	76	70.4
II	142	0	0	99	69.7
Average	**125**	**0**	**0**	**88**	**70.1**

[(a)]Seedlings were tested by PAS-ELISA (as described in Chapter 2) for TYMV and TMV infection.

Although embryo invasion is required for transmission of TYMV in *A. thaliana* seed, healthy seedling may rise from TYMV infected embryos (Chapter

2). This finding indicates that either the virus location or the virus concentration in the embryo likely affects seedling infection, as has been proposed by others (Jones, 1993; Nolan and Campbell, 1984; Varma et al., 1992). To test these hypotheses, *A. thaliana* seed from TYMV infected plant either singly or dually infected with TMV was dissected into the embryo and the seed coat, and the macerated part assayed by PAS-ELISA (Table B).

Table B – Incidence (%) of turnip yellow mosaic tymovirus (TYMV) in embryo, seed coat, and seedling of *Arabidopsis thaliana* from TYMV and tobacco mosaic tobamovirus (TMV) infected plants as determined by PAS-ELISA

Treatment	Embryo only[a]	Seed coat only[a]	Embryo + seed coat[a]	Seedling
TYMV	0	33.3	58.9	31.1[b]
TYMV + TMV	1.1	22.2	75.5	70.1[c]

(a) Average of three replications of 30 seeds each.
(b) Average of two replications of 113 and 107 seedlings each
(c) Avarege of two replications of 108 and 142 seedlings each.

There was a closer approximation between incidence of embryo infection with TYMV and the incidence of seedling infection when seeds were produced in dually infected *Arabidopsis* plants than when produced by singly infected with TYMV (Table B). It indicates that virus concentration in the embryo either favored its detection by ELISA or its transmission through seed.

Related and unrelated viruses can simultaneously infect a common host, which may result in interactions that effect host symptoms and/or virus replication (Kassanis, 1963). One virus may bring about antagonistic, additive or synergistic

effects. These interactions have been studied in regards to the effect on host reaction (Anjos et al., 1992; Barker, 1987; Carr and Kim, 1983; Cohen et al., 1988; Costa, 1969; Hamilton and Nichols, 1977; Hoffmann et al., 1998; Kuhn and Dawson, 1973; Pio-Ribeiro et al. 1978), virus accumulation (Barker, 1989; Cohen et al., 1988; Dodds and Hamilton, 1972; Hamilton and Dodds, 1970; Hoffmann et a., 1998; Jones and Mitchell, 1986; Kuhn and Dawson, 1973; Vance, 1991), insect transmission (Elnagar and Murant, 1978; Waterhouse and Murant, 1983), and seed transmission (Kuhn and Dawson, 1973; Wang and Maule, 1997).

It is not known why co-infection of *A. thaliana* with TYMV and TMV increased the incidence of TYMV seed transmission. The observation that not all infected embryos produced infected seedling (Table 3, Chapter 2) indicates the importance of the quantitative aspect for seed transmission. Interaction between TYMV and TMV may have promoted a higher concentration of TYMV in the embryo tissue and enhanced seed transmission. Increase in virus titer in a host co-infected by unrelated viruses has been reported in several virus-host combinations (Barker, 1989; Cohen et al., 1988; Hoffmann et al., 1998; Jones and Mitchell, 1986; Vance, 1991; Vance et al., 1995). Kuhn and Dawson (1973) found an increase of 57% in southern bean mosaic sobemovirus (SBMV) transmission in seed from cowpea dually infected with cowpea chlorotic mottle bromovirus (CCMV) compared with singly infected plants. There was no correlation between virus concentration in the maternal plant tissue and seed transmission. Indeed, a decrease in BSMV concentration was observed in dually

infected cowpea (Kuhn and Dawson, 1973).

Another hypotheses would account for the increase in the number of *A. thaliana* embryo infected by TYMV. Verma (1991) reported a relationship between virus-encoded factors and infection of an otherwise non-host plant. Hamilton and Nichols (1977) found that co-infection of barley with TMV and bromegrass mosaic virus induced systemic infection by the otherwise locally infecting TMV. Similarly, Costa (1969) reported systemic infection of cotton seedlings by the locally infecting tobacco streak ilarvirus (TSV) when co-inoculated with anthocyanosis virus. Thus, TMV could have incidentally facilitated embryo invasion of TYMV by increasing the number of infected embryos and enhancing seed transmission.

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