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GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF NATURALLY-OCCURRING MULTIPLE POTYVIRUS RESISTANCE IN THE CHINESE CUCUMBER LINE TMG-1

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GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF NATURALLY-OCCURRING MULTIPLE POTYVIRUS RESISTANCE IN THE CHINESE CUCUMBER LINE TMG-1

By

Thanda Wai

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ABSTRACT

GENETIC AND PHYSIOLOGICAL CHARACTERIZATION OF NATURALLY-OCCURRING MULTIPLE POTYVIRUS RESISTANCE IN THE CHINESE CUCUMBER LINE TMG-1

By

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Cucurbit potyviruses cause severe crop losses around the world. The inbred Chinese cucumber line TMG-1 is resistant to three related potyviruses: zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and the watermelon strain of papaya ringspot virus (PRSV-W). The goal of this research was to understand the genetics of this multiple potyvirus resistance by determining the inheritance of resistance to each virus and the relationship of the resistances to each other. Linkage associations with known genetic markers were tested. Physiological studies were performed to examine the spatial and temporal distribution of virus in the resistant line in comparison with the susceptible parent. Tissue-specific differences in expression of the resistances were also investigated.

TMG-1 was crossed with WI-2757, an inbred line that is susceptible to all three viruses. The parents, F_1 (WI-2757 X TMG-1), F_2, and backcross (F_1 X TMG-1 and WI-2757 X F_1) generations were inoculated with virus and their response monitored by symptom expression and ELISA. Direct progeny screening, sequential virus inoculations, and tests of clonally propagated F_2 individuals were performed.

Inheritance of resistance to PRSV-W is due to a single dominant gene, while resistance to ZYMV is conditioned by a single recessive gene. Two independently assorting recessive resistance factors confer resistance to WMV: the first resistance is due to a single recessive gene, while the second results from the epistatic interaction of a single recessive gene from TMG-1 and a single dominant gene from the WI-2757 parent. The two separate resistances to WMV are expressed in different tissues. The resistance that is conferred by the single recessive gene is expressed in the cotyledon and throughout the plant. The resistance that results from the epistatic interaction is expressed only in true leaf tissue.

The ZYMV resistance gene mapped to the same locus as the epistatic recessive resistance gene to WMV. Linkage mapping showed that sex expression (the *F* locus on Linkage Group I) is linked to the cotyledon-expressed WMV resistance, but not ZYMV resistance. Resistance to PRSV is linked to the bitterfree (*bi*) locus on Linkage Group I.

Monitoring of virus spread in each leaf over time indicated a reduced rate of ZYMV and WMV accumulation in TMG-1 relative to WI-2757. The apparent resistance to PRSV-W is likely to be tolerance since high levels of virus were detected despite an absence of symptoms. In summary, TMG-1 has two to three different resistance mechanisms that employ a total of four to five resistance genes to confer resistance to three related potyviruses.

TO ALL THOSE WHO BELIEVED IN ME...

I would like to dedicate this work to all those who have greatly influenced my life. First and foremost, I would like to dedicate this work to the members of my family who have unconditionally supported my academic pursuits from my youth: my mother Mya Thanda Poe, my father U Tha Gyaw Wai, my stepfather James D. Poe, and to my late brother Mra Gyaw Wai and grandmother Daw Mya Sein.

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Viruses

ZYMV	zucchini yellow mosaic virus
WMV	watermelon mosaic virus (also known as watermelon mosaic virus-2)
PRSV-W	papaya ringspot virus - watermelon strain (also known as watermelon mosaic virus-1)

Cucumber Cultivars

TMG-1	Taichung Mau Gua - resistant to ZYMV, WMV, and PRSV
WI-2757	Wisconsin 2757 - susceptible to ZYMV, WMV, and PRSV

Genetic Loci and Phenotypes

bi	bitterfree cotyledon (WI-2757)
F	sex expression (WI-2757 - Female flowers)
Tu	tuberculate or warty fruit/flowers (TMG-1)
ns	numerous spine on fruit/flowers (WI-2757)
Foc	fusarium resistance (WI-2757)
Ccu	scab resistance (WI-2757)
Mpi-1	mannosephosphate isomerase (codominant)
Pgm-1	phosphoglucomutase (codominant)
zymv	resistance to ZYMV
wmv-2	resistance to WMV identified in this study, acts independently and is expressed in the cotyledon and throughout the plant (TMG-1)
wmv-3	resistance to WMV identified in this study, acts epistatically with a dominant gene from WI-2757 to confer resistance in true leaves
Wmv-4	resistance to WMV identified in this study (WI-2757) that acts in concert with a recessive resistance gene in TMG-1
Prsv-2	resistance to PRSV-W identified in this study

Others

ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In order to further the understanding of host-pathogen interactions and to elucidate mechanisms of plant virus resistance by the host plant, it is useful to study resistance phenotypes conditioned by monogenic or oligogenic resistances. To this end, this study addresses questions concerning naturally-occurring resistances to potyviruses that infect cucurbit crops. The inbred, Chinese cucumber line Taichung Mau Gua (TMG-1) is resistant to three, related cucurbit potyviruses: zucchini yellow mosaic virus (ZYMV); watermelon mosaic virus (WMV), formerly known as watermelon mosaic virus 2; and the watermelon strain of papaya ringspot virus (PRSV-W), formerly known as watermelon mosaic virus 1 (Provvidenti, 1985). Resistance to ZYMV was found to be due to a single, recessive gene (Provvidenti, 1987a). The inheritance of resistance to WMV and to PRSV had not yet been characterized. The goals of this research were to study the genetics of inheritance of resistance to these three related viruses, to determine the relationship of the resistances to each other, to examine the genome organization of multiple disease resistances, and to gain some insight into mechanisms of resistance to potyviruses.

1.2 Some Definitions Related to the Study of Resistance Mechanisms

In order to discuss phenomena related to plant virus resistance mechanisms, it is first necessary to define terminology used in this study. Vanderplank (1963) utilized the terms "vertical" and "horizontal" resistance to describe specificity involved in the resistance reaction. Vertical resistance is generally conferred by one to a few genes and exhibits a race-specific response that controls the disease phenotype in a significant manner. In contrast, many genes are believed to condition horizontal, or non-specific resistance. These terms are discussed in more detail below.

Cooper and Jones (1983) proposed terms as discussed by the Virology Group Committee of the Federation of British Plant Pathologists (FBPP). A species that cannot be infected by any biotype of the pathogen is considered immune (i.e. a nonhost). In the past, many investigators (plant breeders, in particular) called a highly resistant cultivar immune. The term "infectible" has been proposed as the opposite of immune. Infectible plants may be "susceptible" or "resistant." A resistant plant is one in which virus infection and/or replication and/or invasion is restricted. This term applies to the cultivar type resistance studied in this thesis. A susceptible plant is one in which virus readily infects and/or replicates and/or invades. A susceptible plant can then be subdivided into "sensitive" or "tolerant" plants, depending on the range of the response exhibited by the host plant. A sensitive plant reacts severely to the pathogen, while a tolerant plant shows few or no symptoms. From the point of view of a plant breeder, a tolerant plant is considered resistant because the pathogen has little effect on quality and yield of the product. However, a plant virologist views tolerance as a form of susceptibility since the host plant suffers from a full-scale viral infection. Tolerance is a difficult term to define since the ability of the plant to suppress disease development may be due to a low level of resistance, i.e. a slight reduction in multiplication and long distance spread.

However, since these terms are not universally used, definitions proposed by Walkey (1985) will be used in this study. The terms defined here apply to genetically controlled vertical, cultivar resistance. Other types of resistance, such as induced (or acquired) resistance and cross-protection are not included in this discussion. A plant is considered to be susceptible if virus can easily infect and multiply within it. Susceptibility may be high or low. A resistant plant is one that suppresses or retards multiplication of a virus or development of pathogenic symptoms. Resistance may be classified as high (extreme), moderate, or low. Tolerance describes the response by the host plant, in which almost normal levels of virus movement and concentration cause mild or virtually no symptoms at all. These terms should be clarified in the future as more research on mechanisms of virus spread and symptom expression are elucidated.

Dawson and Hilf (1992) view virus-host interactions by the extent of virus multiplication and spread and the degree of symptom expression in the host, whether natural or experimental. They observed seven types of virus-host interactions, ranging from "total susceptibility" to "immunity." Plants that suffer "total susceptibility" exhibit marked reductions in growth and yield, and those that do not show obvious symptoms are considered "tolerant," as proposed by Cooper and Jones (1983). They noted varying degrees of susceptibility, in which there is decreased virus replication to reduced or limited virus spread in the host plant. Under laboratory conditions, it is sometimes possible to detect viral replication in protoplasts of plants that are believed to have species resistance (Cheo, 1970; Sulzinski and Zaitlin, 1982; Paje-Manalo and Lommel, 1989; Stenger et al., 1992). In this type of host-pathogen interaction, the resistant plant is called a "subliminal host," i.e. one that is able to support virus replication at the cellular level, but does not permit virus spread or symptom expression (Cheo, 1970).

Cooper and Jones (1983) reserve the term true "immunity" for absolute resistance at the species level, i.e. plants that do not support virus replication even in protoplasts. Holmes (1955) proposed that non-host immunity is conferred by the additive effect of 20 to 40 individual resistances, a concept later called horizontal resistance by Vanderplank (1963). Bald and Tinsley (1967) suggested that non-host resistance is due to the lack of "susceptibility factors" needed by the virus to undergo pathogenesis. It is now generally believed that susceptibility is due to host plant factors that aid in virus multiplication and spread (Fraser, 1990a). The types of resistance discussed here are vertical (Vanderplank, 1963), i.e. resistance phenotypes that result from the action of major gene effects.

1.3 Genetics and Mechanisms of Naturally-Occurring Resistance to Plant Viruses

The topics of host-range determinants of plant viruses and possible correlations between the genetics of resistance and their underlying mechanisms have been reviewed by several investigators (Dawson and Hilf, 1992; Ponz and Bruening, 1986; Fraser, 1987, 1990a, 1990b). According to Fraser (1987; 1990), the major processes involved in virus-host interactions include recognition between virus- and host-encoded factors, signal transduction, and the response. Recognition is probably the main factor involved in nonhost immunity and in cultivar resistance. A defect or lack of a recognition molecule (i.e., reduction or absence of susceptibility) necessary for plant virus multiplication could result in resistance. This type of negative mechanism is most likely to be recessive for a go/no go recognition event and may be considered a passive resistance. In contrast, a positive resistance mechanism proposes that the product of a host resistance gene interacts directly with a virus-encoded function as either a direct inhibitor, or elicits a signal transduction pathway. This type of resistance mechanism is likely to be dominant if recognition is a go/no go event. Both types of mechanisms can sometimes exhibit gene-dosage dependence.

Fraser (1990b) also attempted to correlate dominance and recessiveness of resistance genes with the resistance phenotype expressed. Dominant resistance genes are frequently associated with resistance mechanisms that limit the spread of virus, such as the formation of (necrotic) local lesions. Incompletely dominant or gene-dosage dependent resistances often confer moderate resistance, resulting in partial localization of the virus (such as localization to the inoculated leaf), or a decrease in the overall multiplication of the virus. Some recessive resistance genes appear to confer extreme resistance, such that there are no detectable levels of virus. In summary, plant virus resistance mechanisms exhibit a continuum of processes: (1) mechanisms that involve localization of virus infection tend to be inducible and conferred by dominant genes; (2) those that confer a very high level of resistance tend to be recessive and constitutively expressed; and (3) those that are expressed systemically and affect virus multiplication or long-distance spread tend to be incompletely dominant (i.e., exhibit a gene-dosage effect). These generalizations probably have many exceptions and are meant to provide a general theoretical framework in the study of the genetics of plant virus resistance mechanisms.

1.3.1 Resistance Mediated at the Level of Recognition and/or Signal Transduction

The mode of action of a host plant resistance gene product is known only for a few systems to date. The product of the tobacco N gene elicits a range of reactions that result in a localization type of resistance known as a hypersensitive response. This extremely complex set of reactions reviewed by various authors are not covered here (Dawson and Hilf, 1992; Dumas et al., 1991).

1.3.2 Resistance Targeted at Inhibition of Viral Replication

A host-encoded gene product that directly inhibits plant viral replication is found in the Arlington cowpea (Bruening et al., 1987). A single, dominant gene in *Vigna unguiculata* cv. Arlington codes for a protease inhibitor that specifically inhibits processing of polyproteins of cowpea mosaic virus (CPMV). In an extremely thorough study of the mechanism of resistance, the investigators found cosegregation of the protease inhibitor and resistance to

CPMV-SB. Enzyme inhibition studies were performed in protoplasts of F_2 populations.

Similarly, the *Tm-1* gene on tomato was found to confer resistance to TMV in protoplasts (Motoyoshi and Oshima, 1975, 1977). The *Tm-1* gene inhibits viral replication, but allows TMV to move through the plant (Fraser and Loughlin, 1980). Recent evidence suggests that it may act at the level of replicase inhibition (Watanabe et al., 1988). These two resistances are examples of the positive, active resistance model that acts in a dominant manner, as proposed by Fraser (1990).

Lobenstein and Gera (1990) have identified a 23 kDa protein (called IVR or inhibitor of viral replication) from protoplasts made from tobacco plants that contain the N gene. This protein inhibits replication of several plant viruses and is somehow believed to take part in the localization reaction. Whether this protein is the N gene product, or if is related to the N-gene mediated hypersensitive reaction remains to be seen.

1.3.3 Resistance at the Level of Virus Movement

Since invasion of a host plant by viruses requires both cell-to-cell and long distance transport through the vascular system, recent interest has focused on these processes. While there is a great deal of information on the structure of plasmodesmata (review by Robards and Lucas, 1990), this discussion will concentrate on movement of virus in infected plants (reviews by Hull, 1989; Maule, 1991; Godefroy-Colburn et al., 1991; Deom et al., 1992). After entry of a plant virus into a cell that supports replication, the virus must move cell to cell through membrane-lined plasmodesmata, which are interconnections that provide continuous symplastic connections throughout the tissue until it reaches the vascular system. Viruses have been observed to move long distance through the phloem as virions (Esau and Cronshaw, 1967). Once the virus is in the phloem, it is believed to be carried along the vascular stream with photosynthates to a sink (Agrios, 1988; Leisner et al. 1992). A recent report (Leisner et al., 1992) correlated the movement of cauliflower mosaic virus in infected turnip plants with the flow of photoassimilates both long distance and within individual leaves. The concentrations of both virus and photoassimilates paralleled source (older leaves) - sink (younger leaves) relationships. They also concomitantly decreased basipetally, from the leaf tip to the base.

1.3.3.1 Resistance at the Level of Long Distance Movement

The form in which plant viruses achieve long distance movement is not known. Saito et al. (1990) showed that mutant TMV defective in coat protein assembly is greatly compromised in its ability to spread long distance. Dorokhov et al. (1984) suggested that plant viruses move long distance as informosome-like ribonucleoprotein complexes. Cytological evidence presented by deZoeten and Gaard (1983) showed that pea enation mosaic virus replication complexes may be transported in vesicles. The authors suggested that this type of transport may be common to most or all plant viruses that undergo a dsRNA intermediate and utilize a membrane as a site for replication.

Cytological evidence suggests that resistance may occur at the level of long distance spread in at least two studies. The tobacco masked strain of tobacco mosaic virus first described by Holmes (1934) is either symptomless or produces mild chlorosis. There is a delay in the accumulation of virus in the upper leaves (Ding et al., 1993). This strain was shown to produce less movement protein than a more virulent strain (Watanabe et al., 1987). While the attenuated phenotype had been correlated with reduced replication (Holt et al., 1990), recent cytological evidence suggests that the masked strain has difficulty in accumulating in the vascular cells of the inoculated leaf (Ding et al., 1993).

Resistance to cucumber mosaic virus in resistant pepper lines also appears to be related to a reduction in the efficiency with which virus can invade phloem tissue (Dufour et al., 1989). In resistant varieties, virus spread was limited to cortical cells and a few phloem bundles. It is possible that symptom expression is attenuated if not enough virus is present in young leaves before expansion takes place.

A resistance identified in *Arabidopsis thaliana* ecotype Dijon to infection by turnip crinkle virus also seems to affect long distance virus spread (Simon et al., 1992). There appeared to be little difference in viral RNA accumulation in protoplasts of both the susceptible and resistant ecotypes. In addition, when inoculated, and corresponding opposite leaves of the resistant Dijon ecotype showed accumulation of viral RNA, but further virus spread was not observed. Resistance in the Dijon ecotype appears to be mediated through long distance spread of the virus.

1.3.3.2 Resistance at the Level of Cell-to-Cell Movement

The 30 kDa protein encoded by TMV is believed to be the movement protein (Deom et al., 1987). When this protein was expressed in transgenic plants, molecular exclusion size of the plasmodesmata was increased from 800 to 9,400 daltons (Wolf et al., 1989; Wolf et al., 1991). The role of movement protein in the transport of TMV particles through plasmodesmata has been studied in detail (Citovsky, 1993; Citovsky and Zambryski, 1993). The TMV movement protein has several domains: two that bind single-stranded nucleic acids, a folding domain, a phosphorylation domain, and another domain that allows it to bind plasmodesmata. Passage of movement protein-nucleic acid complexes through plasmodesmata has been observed under EM (Citovsky et al., 1992). These complexes may be analogous to the informosome-like virus-specific ribonucleoprotein (vRNP) particles proposed by Dorokhov et al. (1984) to be involved in both long distance and cell-to-cell movement of plant viruses.

Movement protein is believed to be inactivated by a plant cell wall-associated protein kinase (Citovsky and Zambryski, 1993). Citovsky et al. (1993) have discovered a developmentally regulated plant cell wall-associated protein kinase that phosphorylates TMV movement protein. The protein kinase activity is found primarily in leaves and is expressed as a function of leaf maturation, i.e. enzyme activity closely follows basipetal leaf development. These discoveries suggest possible mechanisms of resistance to plant virus infection at the level of cell-to-cell spread.

Mutations in the movement protein have been associated with virus accumulation and symptom severity. Since the *Tm-2* gene and its allelic variant *Tm-2*^{2} do not inhibit virus replication in protoplasts, they are believed to cause resistance at the level of virus spread (Motoyoshi and Oshima, 1975, 1977). The *Tm-2* allele has been shown to prevent movement of TMV from infected cells (Nishiguchi and Motoyoshi, 1987). A mutation that allows TMV to overcome the *Tm-2* resistance was found in the 30 kDa cell-to-cell movement protein (Meshi et al., 1988). The allelic *Tm2*^{2} gene is also believed to confer resistance at the level of cell-to-cell spread (Niguchi and Motoyoshi, 1987). Tsai and Dreher (1993) reported that a single amino acid substitution in the turnip yellow mosaic virus movement protein resulted in a fourfold increase in accumulation of viral proteins and a concommitant increase in the severity of symptoms. In summary, it appears that the rates of virus movement, both cell-to-cell and long distance, affect virus accumulation and titer, which in turn can greatly affect symptom expression.

1.3.4 Viral Determinants That Affect Symptom Expression

Genetic engineering of plant virus genomes has been used to study the effects of various changes on symptom expression. Point mutations in Gene VI (unknown function) of cauliflower mosaic virus have revealed virus determinants that affect symptom expression in the host plant (Daubert and Routh, 1990). The role of the coat protein in viral pathogenesis has been examined through site-directed modifications of the TMV coat protein (Saito et al., 1987; Culver et al., 1991; Lindbeck et al., 1992). Similarly, further dissection of viral determinants that affect symptom expression may lead to an understanding of mechanisms by which a host plant expresses resistance to viral infections.

1.4 Possible Use of Non-Host Products to Confer Virus Resistance/Tolerance

Recently, non-host encoded gene products have been found to have inhibitory effects on some plant viruses. It has been reported that high levels of human cystatin C, an inhibitor of cysteine proteases, interfere with autoprocessing of the plum pox potyvirus HCPro and NIa proteases (Garcia et al., 1993). In addition, one of the pokeweed antiviral proteins (PAP) that imparts resistance to tobacco plants from infection by tobacco mosaic virus (TMV) was identified as a ribosome inactivating factor (RIF) using *in vitro* assays (Chen et al., 1993). The mechanism involves removal of a specific adenine base in a conserved loop of the large rRNA. This depurination process prevents ribosomes from binding elongation factors and blocks translation as a result. The protein apparently does not block translation in the host pokeweed plant. The *in vivo* function of this protein and its role in the inhibition of viral replication are not yet understood well. However, such non-host specific antiviral proteins may be useful in the future as genetically engineered resistances.

Little is known about what actually causes symptom expression and modulation in plants. Efforts to utilize genetically engineered plant virus resistance have shed some light on the processes involved in pathogenesis of plant viral infection. Current strategies to confer pathogen-derived plant virus resistance have been reviewed by a number of investigators (Beachy, 1988; Baulcombe, 1989; Grumet, 1990; Scholthof et al., 1993). These strategies include cloning and expression of (1) portions of the viral genome, such as the coat protein or replicase in both the sense and antisense forms; (2) satellite RNAs that have been observed to decrease symptom expression in nature; and (3) other novel approaches such as the use of a capsid-nuclease fusion protein (Natsoulis and Boeke, 1991). Possible mechanisms of coat-protein mediated cross protection have been reviewed (Nelson et al., 1990; Nejidat et al., 1990; Grumet, 1994). Proposed mechanisms of coat-protein mediated cross protection range from preventing uncoating of the incoming virus, competition for replication factors, to interference with cell-to-cell or long distance spread. It is likely that different mechanisms act to confer resistance/tolerance in different virus groups, although similar genetic engineering approaches have been attempted. Carr and Zaitlin (1991) demonstrated that expression of a non-structural sequence (54 kDa protein of TMV) results in markedly decreased levels of virus-specific RNAs in tobacco protoplasts. It is possible that some of the naturally-occurring resistances utilize mechanisms similar to those currently employed by genetic engineering.

1.5 Future Directions in the Study of Resistance Mechanisms

There are several possible resistance mechanisms not yet explored at this time. The role of the cytoskeleton in plant virus replication and movement is not known. The fluidity of the membrane systems may influence the ability of the replication complex to function properly. Inability of a virus replication complex to anchor in the host cell membrane may be involved in some resistances that are expressed only at high temperatures. Certain integral components of membranes that may be involved in recognition of the virus complex may also affect virus replication and movement. Some mechanisms of tolerance may be due to the ability of the host plant to divert nutrient utilization into alternate pathways. The ability to chelate, transport, or utilize micronutrients that are essential cofactors such as iron or sulfur may be altered to allow the plant to function normally. Much work lies ahead to understand plant virus-host interactions and mechanisms that allow the host plant to resist or tolerate the invading pathogen.

1.6 Multiple Disease Resistance and the Concept of a Linkat

Several examples of multiple potyvirus resistance exist in the literature. The most well characterized is the I gene in *Phaseolus vulgaris* cv. Black Turtle Soup 1. The dominant I allele confers resistance to five potyviruses: bean common mosaic virus, blackeye cowpea mosaic virus, cowpea aphid-borne mosaic virus, soybean mosaic virus, and watermelon mosaic virus (Kyle and Dickson, 1988). Another example is in *Pisum sativum*, in which multiple potyvirus resistance is conferred by two tightly linked loci. Five genes that occur as a cluster on chromosome 2 confer resistance to six potyviruses. The temperature-independent genes (*bcm*, *cyv-1*, *pmv*, and *sbm-2*) confer resistance to the NL-8 pathotype of bean common mosaic virus (BCMV NL-8), cl

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clover yellow vein virus (CYVV), pea mosaic virus (PMV), and the lentil strain of pea seed-borne mosaic virus (PSbMV-L1), respectively (Provvidenti, 1987b; Provvidenti, 1990; Provvidenti and Alconero, 1988a). A fifth temperature sensitive gene, *mo*, confers resistance to bean yellow mosaic virus (BYMV) and WMV (Marx and Provvidenti, 1979). A second cluster of five tightly linked genes on chromosome 6 (*sbm-1*, *sbm-3*, *sbm-4*, *wlv*, and *cyv-2*) confer resistance to three strains of PSbMV (standard, lentil, and P4), white lupine mosaic virus (WLMV) and CYVV, respectively (Hagedorn and Gritton, 1973; Provvidenti, 1987b; Provvidenti and Alconero, 1988b). In addition, genes that confer resistance to more than one virus have also been observed in *Solanum* and *Capsicum* (Cook, 1960; Cockerham, 1970).

A possible explanation for the existence of tightly linked loci that confer resistance to several viruses is the concept of a linkat, a cluster of duplicated linked genes that appear to be semi-stable functional units. Demarly (1979) proposed that gene duplications may have occurred that allow for multiple resistance genes at the same locus. This hypothesis is based on trends that he noted in conserved genes, such as those that encode hemoglobins, histones, and ribosomal RNAs. Gene duplications that arise through evolution are maintained in linked clusters because they have coadapted functions. "Antimutator" genes that locally control linkats are proposed to maintain the structure and function of these units. Decreased

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recombination rates are associated with genes within a linkat. Such an occurrence may explain the clustered multiple virus resistance genes on chromosomes two and six in *Pisum sativum* (Kyle and Provvidenti, 1993).

Examination of the molecular genetic structure of disease resistance loci is in progress in tomatoes (Martin et al., 1992; 1993a,b). Yeast artificial chromosome libraries have been made in order to clone the *Tm-2a* and *Pto* loci that confer resistance to TMV and *Pseudomonas syringae* pv. *tomato*. Map-based positional cloning was used to isolate the *Pto* locus. A tightly linked phenotype with *Pto* is resistant to damage by fenthion, an organophosphate insecticide that causes necrotic lesions on plants. Recent evidence has shown that this locus is composed of at least six to seven duplicated regions (Martin, 1993b). Resistance to fenthion damage segregated from resistance to *P.s.* pv. *tomato* after screening more than 1300 F_2 individuals. It appears that the duplicated genes have diverged. Molecular characterization of this locus in currently under study. The results of this work will aid in our understanding of the genetic organization and biochemical reactions involved in plant disease resistance responses. While this study elucidates the mechanism of a bacterial resistance gene, the concept of a linkat seems to apply.

1.7 Sources of Resistance in Cucumber to ZYMV, WMV, and PRSV and the Occurrence of Multiple Potyvirus Resistance in TMG-1

Sources of potyvirus resistance in cucurbits have been reviewed by Provvidenti and Hampton (1992) and Provvidenti (1993; 1989). There are a limited number of resistances to potyviruses in cucumbers. In the Japanese cultivar Kyoto 3 feet long, a single dominant gene confers resistance to WMV (Cohen et al., 1971). The cultivar Surinam Local carries a single recessive resistance gene to PRSV-W (Wang et al., 1984). This resistance appears to limit long distance spread of the virus, as high titers of virus were detected by ELISA in the lower leaves but not in the upper ones. While Provvidenti (1985) determined that a number of Chinese cultivars were resistant to ZYMV, a single plant selection was made from the cultivar TMG-1 that was resistant to three cucurbit potyviruses: ZYMV, WMV, and PRSV. Resistance to ZYMV is due to a single recessive gene in TMG-1 (Provvidenti, 1987). However, the genetic basis of the multiple potyvirus resistance was not characterized.

1.8 Purpose of this Thesis

In this study, inheritance of multiple potyvirus resistance in the inbred, Chinese cucumber line TMG-1 was performed in order to determine (1) the genetics of resistance to each virus; (2) the mode of inheritance, whether the resistance is dominant or recessive; and (3) the relationship of the resistances to each other, i.e. does one gene confer resistance to all three viruses? The mechanisms of resistance were studied by examining the spatial and temporal distributions of each virus in the resistant and susceptible lines. The relationship of the resistances to each other was further examined by attempting to establish linkage markers to each resistance. A physiological study was also undertaken to determine the underlying cause of different segregation ratios obtained when different tissues were inoculated with WMV. A tissue-specific mechanism was found and is further described.

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CHAPTER 2

INHERITANCE OF MULTIPLE POTYVIRUS RESISTANCE IN THE CHINESE CUCUMBER LINE TMG-1

ABSTRACT

The inbred Chinese cucumber line TMG-1 is resistant to three potyviruses: zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and the watermelon strain of papaya ringspot virus (PRSV-W). The genetics of resistance to these viruses and the relationship of the resistances to each other were determined. TMG-1, WI-2757 (a susceptible inbred line), and their F_1, F_2, and backcross progeny were screened for resistance to all three viruses by monitoring symptom expression and virus level using enzyme-linked immunosorbent assay (ELISA). Segregation data indicated that resistance to PRSV-W was due to a single dominant gene. Two independently assorting recessive resistance factors conferred resistance to WMV: the first resistance was due to a single recessive gene and the second resistance resulted from an epistatic interaction between a recessive gene from TMG-1 and a dominant gene from WI-2757. The gene conferring resistance to ZYMV was the same as, or was very tightly linked to, the recessive gene in the epistatic resistance to WMV. ELISA data suggested that the mechanism of resistance to PRSV-W was different from that of ZYMV and WMV.

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INTRODUCTION

Potyviruses cause great economic loss to a wide variety of crops. At least three potyviruses, zucchini yellow mosaic virus (ZYMV) (Lisa and Lecoq, 1984), watermelon mosaic virus (WMV) (Purcifull et al., 1984), and the watermelon strain of papaya ringspot virus (PRSV-W) (Purcifull and Gonsalves, 1984a), cause severe losses in cucurbit crops (Nameth et al., 1985; Nameth et al., 1986; Davis, 1987; Sammons et al., 1989; Perring et al., 1992). Provvidenti (1985) identified resistance to all three viruses in a single plant introduction from the Chinese cucumber cultivar Taichung Mau Gua (TMG-1). Cultivars that are resistant to all three cucurbit potyviruses would be very valuable, especially because of the frequent occurrence of mixed infections (Nameth et al., 1985; Davis and Mizuki, 1987).

Multiple potyvirus resistance could be conditioned by a gene at a single locus, by a cluster of linked genes or by several independent genes. In *Phaseolus vulgaris*, the dominant I allele confers a systemic necrotic resistance at temperatures above 30 C to five potyviruses: bean common mosaic virus, blackeye cowpea mosaic virus, cowpea aphid-borne mosaic virus, soybean mosaic virus, watermelon mosaic virus, and possibly passionfruit woodiness virus (Kyle and Dickson, 1988). To date, it has not been possible to break the linkage among the resistances. *Pisum sativum* has two tightly clustered arrays of multiple potyvirus resistance genes on different chromosomes, as well as the *mo* locus that confers resistance to both bean yellow mosaic virus and WMV (Marx and Provvidenti, 1979; Provvidenti, 1987b ; Provvidenti, 1990; Provvidenti and Alconero, 1988a, 1988b). Other examples of an allele conferring resistance to more than one virus exist in *Capsicum annuum* (Cook, 1960) and in *Solanum* (Cockerham, 1970). In cucumber, several monogenic resistances have been characterized. Resistance to PRSV is controlled by a single recessive gene in the cultivar Surinam Local (Wang et al., 1984), resistance to WMV in Kyoto 3 feet long is due to a single dominant gene (Cohen et al., 1971). Resistance to ZYMV is due to a single recessive gene (Provvidenti, 1987a).

In this work, we sought to determine the genetics of the multiple potyvirus resistance in TMG-1. TMG-1 was crossed with WI-2757, an inbred line that is susceptible to all three viruses. F_1, F_2, and backcross progeny were examined for response to inoculation by each virus. Our data indicate that multiple potyvirus resistance in TMG-1 is conferred by four (possibly five) separate genes.

MATERIALS AND METHODS

Maintenance of Virus Inocula

ZYMV (CT strain, originally supplied in 1987 by R. Davis, Rutgers State University, New Brunswick, NJ), WMV (ATCC PV379), and PRSV-W (ATCC PV380) were propagated in zucchini squash plants (*Cucurbita pepo* cv. Blackjack) (Petoseed Co., Inc.; Saticoy, CA). Cotyledons of one-week old seedlings were lightly dusted with 320-grit carborundum (Fisher Scientific) and mechanically inoculated using sponge plugs. Virus-infected tissue (lyophilized, frozen, or fresh) was macerated in ice cold 20 mM sodium phosphate buffer, pH 7.0, in a pre-chilled mortar and pestle. All non-biological materials were sterilized prior to usage. Virus-infected leaves were harvested to use as inocula sources at the time when symptoms were expressed optimally.

Several tests were performed routinely to verify the identity and purity of each virus. PRSV-W did not cross-react with antibody to ZYMV or WMV, and so was verified to be free of contamination by ELISA (antibody purchased from Agdia) (Clark and Adams, 1977). Since ZYMV-CT and WMV both react with ZYMV antibody, a differential host, *Phaseolus vulgaris* cv. Black Turtle 2, was used (Provvidenti and Gonsalves, 1984b). WMV elicits strong, systemic, mosaic symptoms, while ZYMV only produces red, necrotic, local lesions on the inoculated leaves.

Cucumber Genotypes

The inbred cucumber (*Cucumis sativus* L.) lines TMG-1, resistant to ZYMV, WMV, and PRSV-W, (Provvidenti, 1985) and WI-2757, susceptible to all three viruses, (Peterson et al., 1982) were provided by Dr. Jack Staub (USDA, University of Wisconsin at Madison). The F_1 progeny (WI-2757 X TMG-1) were either self- or sib-pollinated to produce the F_2 generation. Backcrosses were made with both parents: WI-2757 X F_1 and F_1 X TMG-1. In each cross, the source of resistance studied came from the male parent. The inbred line Straight 8 (Stokes Seeds, Inc., Buffalo, NY) was used as an additional control genotype that is susceptible to all three viruses.

Propagation of Rooted Cuttings

Rooted cuttings of TMG-1, WI-2757, and their F_1 and F_2 progeny were made by cutting plants with an ethanol-sterilized razor blade two nodes below the growing tip. After removing the leaf at the lowest node, the cutting was dipped in a fungicide (Captan, Zeneca Agricultural Products, Wilmington, DE) and placed in an 1 1/4 X 1 X 1 1/2 inch rooting cube (Smithers-Oasis; Kent, Ohio). Trays were filled with 2 cm of water from the bottom and the cuttings covered with plastic wrap to maintain high humidity for 5 days. The plastic was peeled back slowly on a daily basis until rootlets emerged through the rooting cubes (approximately 2 weeks). Plantlets were transplanted to wet

Baccto Professional Planting Mix (The Michigan Peat Company, Houston, TX) and allowed to grow for 2 to 3 weeks prior to inoculation with virus.

Experimental Designs

Plants were mechanically inoculated with virus-infected sap at either the cotyledonary stage (ZYMV and PRSV experiments) and/or the true leaf (WMV and ZYMV) stage. Rows of susceptible Straight 8 plants were evenly spaced throughout each experiment in order to detect any possible variation in inoculation technique and symptom expression. For the F_2 experiments, ten rows (10 plants/row) of F_2 individuals were interspersed with five internal control rows. Complete blocks that consisted of inoculated and mock-inoculated TMG-1, WI-2757, and F_1 plants were arranged in either a Latin square or repeating pattern. Backcross experiments (20 to 120 individuals) also contained evenly spaced control rows.

Secondary Inoculation of Resistant Plants and F_2 Cutting Experiments

To differentiate between possible genetic models for the number of genes conferring resistance to ZYMV and to WMV, experiments were performed in two ways. (1) Clonally propagated sets of genetically identical F_2 individuals were prepared as described above and inoculated with either ZYMV or WMV. Rooted cuttings of TMG-1, WI-2757, and their F_1

progeny were included as controls. Sequential inoculations were performed on F_2 and BC (F_1 X TMG-1) progeny individuals were first inoculated with ZYMV. Those with symptoms were discarded; those without were assayed by ELISA to verify that they were free of virus. In some experiments, they were inoculated with ZYMV a second time to ensure that there were no escapes. Half fully expanded leaves of the virus-free individuals then were inoculated with WMV. Additional inoculated and mock-inoculated control rows were added to experiments that were infected with a second virus.

Data Analyses

Data from individual and combined experiments were analyzed by chi square analysis. The number of times each experiment was performed is included in each table; experiments termed as independent were performed at different times in the greenhouse. Genetic models proposed are the simplest ones that explained the collective data sets.

Scoring of Symptoms

Plants were scored when symptom development was optimal. Symptoms of ZYMV and WMV generally developed within 7 to 10 days after inoculation; symptoms of PRSV-W took 3 to 6 wk. to appear. Susceptibility of an individual plant to virus infection was scored visually and/or by ELISA. Symptoms of cucurbit potyviruses include the presence of mosaic, severe leaf distortion, or rugosity. The rating system used a point scale from 0 to 5, where: 0 = healthy with no evidence of virus; 1 = light mosaic on at least one leaf; 2 = moderate mosaic on one or more leaves; 3 = strong mosaic on one or more leaves; 4 = strong mosaic on several leaves, has spread to terminal leaves, and often accompanied by severe stunting; and 5 = death of the plant due to virus. Many experiments were scored by two people independently. There was agreement to within one point regarding the symptom ratings given to each plant. When assigning a simple classification of resistant or susceptible, any score of 1 or greater (any symptom expression) was classified as susceptible.

Detection of Systemic Virus Spread by ELISA

Virus levels were determined via ELISA. One or two leaves at the half to first fully expanded stage were harvested from each plant and stored at either 4 C or -80 C. Assays were either performed using standard sandwich methods as described by Clark and Adams (1977) or using a modified version of the leaf disk procedure of Romaine et al. (1981). The two methods were verified to give comparable results. At least four or more healthy controls were included on each plate. Healthy and mock-inoculated controls of all the genotypes (i.e. TMG-1, WI-2757, their F_1 progeny, Straight 8, and Blackjack zucchini squash) gave comparable readings. Buffers were prepared according to Clark et al. (1986).

Anti-PRSV-W and some anti-WMV antibody were purchased from Agdia (Elkhardt, IN). ZYMV and WMV were detected with anti-ZYMV (CT strain) polyclonal rabbit IgG antibody (Hammar and Grumet, unpublished). For the sandwich assays, the anti-ZYMV antibody was conjugated with alkaline phosphatase. Absorbance at 405 nm was monitored using an EIA Reader Model EL-307 (Bio-Tek Instruments, Inc., Laboratory Division, Winooski, VT) after reacting with p-nitrophenyl phosphate (Sigma 104 phosphatase substrate). PRSV-W was detected with horseradish peroxidase-labelled conjugate and the substrate o-phenylenediamine dihydrochloride (OPD) supplied by the Agdia kit. Absorbance was monitored at 490 nm.

When performing the leaf disk assays, leaf tissue was sampled with a 6 mm paper hole puncher. Disks were directly placed into ELISA wells along with 200 µl/well coating buffer. Unused wells were filled with buffer. To affix the antigen to the well, samples were either incubated directly or frozen and thawed prior to incubation. Either method worked equally well. Samples were then reacted with 100 µl/well of 1 µg/ml anti-virus-specific antibody in virus buffer at pH 7.4 (Hammar and Grumet unpublished for ZYMV and WMV or Agdia for PRSV-W). Goat anti-rabbit IgG (Sigma Immuno Chemicals A-8025 alkaline phosphatase conjugate) was used to indirectly detect virus-specific antibody at 405 nm as described above.

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RESULTS

When inoculated with PRSV-W, the WI-2757 parent showed mosaic and vein banding symptoms, while the resistant parent TMG-1 grew vigorously and remained symptom-free throughout the life of the plant. The F_1 progeny of WI-2757 X TMG-1 grew vigorously and also did not exhibit symptoms when inoculated with PRSV-W (Table 2.1). The F_2 progeny segregated in a 3:1 (R:S) ratio, the WI-2757 x F_1 backcross progeny segregated in a 1:1 ratio (R:S) ratio, and the F_1 X TMG-1 backcross progeny were all resistant. These data support a model in which a single dominant gene in the TMG-1 parent confers resistance to PRSV-W. Environmental conditions can affect the expression of resistance to PRSV-W. Full expression of resistance to PRSV-W depended on optimal growing conditions for the plants, i.e. high light and warm temperatures. Under cool temperatures and slow-growing conditions, the F_1 progeny showed very mild symptoms (ratings of 1 vs. 3-4 for WI-2757), while the TMG-1 parent remained asymptomatic.

In contrast to PRSV-W, when inoculated with WMV, the F_1 progeny developed symptoms as would be expected for a recessive trait (Table 2.2). F_2 and backcross segregation ratios, however, suggested that more than one gene was involved in the resistance to WMV. Three possible genetic models are presented in Table 2.2. Segregation ratios of resistance in the F_2 population suggest that either of the two independently assorting recessive genes confer

TABLE 2.1 Segregation of Resistance to PRSV-W

Genotype	Number of Plants Resistant	 Susceptible	Expected Ratio (R:S) [a]	χ^2
TMG-1	58	0		
WI-2757	0	52		
F_1	44	0		
F_2 [b]	243	82	3 : 1	0.001 ns
F_1 X TMG-1	75	0	1 : 0	
WI-2757 X F_1 [c]	130	137	1 : 1	0.13 ns

[a] Expected ratios based on a single, dominant gene model.

[b] Data pooled from three independent experiments. Each experiment fits the predicted ratios $\chi^2_{exp1} = 0.003$, $\chi^2_{exp2} = 0.079$, and $\chi^2_{exp3} = 0.003$.

[c] Data pooled from four independent experiments. Each experiment fits the predicted ratios $\chi^2_{exp1} = 0.000$, $\chi^2_{exp2} = 0.042$, $\chi^2_{exp3} = 0.25$, and $\chi^2_{exp4} = 0.008$.

TABLE 2.2 Segregation of Resistances to WMV

	Number of Plants		Genetic Models [a]					
Genotype	R	S	1 gene model		2 gene model		3 gene model	
			R:S	χ^2	R:S	χ^2	R:S	χ^2
TMG-1	41	0						
WI-2757	0	34						
F_1	0	20						
F_2 b	124	167	1:3	47.2**	7:9	0.11 ns	25:39	1.39 ns
F_1 X TMG-1 c	100	55	1:1	6.24*	3:1	8.54*	5:3	0.19 ns
WI-2757 X F_1	0	22	0:1		0:1		0:1	

*,** Significant χ^2 values indicate that the observed data do not support the proposed genetic model: *, $P \leq 0.05$; **, $P \leq 0.01$.

[a] Expected ratios are presented for three different models: (1) resistance conferred by a single, recessive gene; (2) resistance conferred by two independently assorting recessive factors; and, (3) two, separate independently assorting resistances conferred by three genes. The first resistance consists of a single, recessive gene, and the second resistance the result of an epistatic interaction between a single, recessive and a single, dominant gene. See also Table 4A for a further description of the 3-gene model.

[b] Data pooled from three independent experiments. Each experiment fits the predicted ratios for the 3-gene model: $\chi^2_{exp1} = 1.54, \chi^2_{exp2} = 0.029$, and $\chi^2_{exp3} = 0.095$.

[c] Data pooled from three independent experiments. Each experiment fits the predicted ratios for the 3-gene model: $\chi^2_{exp1} = 0.0053, \chi^2_{exp2} = 0.066$, and $\chi^2_{exp3} = 0.019$.

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resistance to WMV in the progeny of TMG-1 and WI-2757. However, the F_1 X TMG-1 backcross screens gave ratios of 5:3 (R:S), instead of the 3:1 (R:S) ratio expected for two independent recessive genes. This result implies the presence of an additional genetic factor. The model that best explains all of these data proposes the presence of an epistatic dominant gene from WI-2757. The first resistance consists of a single recessive gene (proposed genotype: *wmv-2wmv-2*), and the second resistance the result of an epistatic interaction between a single recessive and a single dominant gene (proposed genotype: *wmv-3wmv-3, WMV-4,_*).

Segregation of resistance to ZYMV is summarized in Table 2.3. Consistent with the results of Provvidenti (1987), resistance was conferred by a single recessive gene. We next sought to determine whether the single, recessive gene that confers resistance to ZYMV is also one of the recessive genes that confers resistance to WMV. This possibility was studied using two approaches. In the first set of experiments, the individual members of clonal pairs of vegetatively propagated, genetically identical F_2 plants were inoculated with either WMV or ZYMV. Four possible models are presented: (1) the ZYMV resistance gene is the same recessive gene that independently confers resistance to WMV (*zymv = wmv-2*); (2) the ZYMV resistance gene is the second recessive gene that is involved in

TABLE 2.3 Segregation of Resistance to ZYMV

Genotype	Number of Plants R	S	Expected [a] Ratio (R:S)	χ^2
TMG-1	58	0		
WI-2757	0	57		
F_1	0	42		
F_2 [b]	134	390	1 : 3	0.06 ns
F_1 X TMG-1 [c]	105	115	1 : 1	0.37 ns
WI-2757 X F_1	0	44	0 : 1	

[a] Expected ratios based on a single, recessive gene model.

[b] Data pooled from six independent experiments. Each experiment fits the predicted ratios: $\chi^2_{exp1} = 0.12$, $\chi^2_{exp2} = 0.27$, $\chi^2_{exp3} = 0.52$, $\chi^2_{exp4} = 0.042$, $\chi^2_{exp5} = 0.23$, and $\chi^2_{exp6} = 0.000$.

[c] Data pooled from four independent experiments. Each experiment fits the predicted ratios: $\chi^2_{exp1} = 0.31$, $\chi^2_{exp2} = 0.093$, $\chi^2_{exp3} = 0.085$, and $\chi^2_{exp4} = 0.18$.

resistance to WMV (*zymv* = *wmv-3*); (3) the ZYMV resistance is conferred by the second resistance, in which a single recessive resistance gene from TMG-1 acts in concert with a single dominant gene from WI-2757 (*zymv* = *wmv-3* + *Wmv-4*); and (4) four independently assorting genes confer resistance to WMV and ZYMV (three genes that confer resistance to WMV, and a fourth one that confers resistance to ZYMV). The expected phenotypic and genotypic ratios for these models are presented in Table 2.4.

Models 1 and 3 predict that there would be no individuals that are resistant to ZYMV, but susceptible to WMV. These models are not likely since this class was observed (Table 2.5). Note that Model 3 also can be ruled out because the ZYMV segregation data are not consistent with that of a recessive and a dominant gene acting epistatically. The F_1 X TMG-1 backcross generation segregated 1:1 (R:S) for ZYMV (Table 2.3), and not 1:3 (R:S). Model 4, proposing that the ZYMV resistance is completely independent of the WMV resistances also was not supported by the observed segregation ratios from the clonal pairs experiments (Table 2.5). The model that best fits the observed data predicts that *zymv* is actually *wmv-3*. However, it is not possible to distinguish between this model and the possibility that *zymv* is tightly linked with *wmv-3*.

The second test of the relationship between the resistance to ZYMV and WMV was performed by sequential

Table 2.4 Predicted Genotypes and Phenotypes for Resistance to WMV. A. Summary of Expected WMV Resistance Phenotype Ratios for the Three-Gene Model. B. Predicted ZYMV Phenotypes if Resistance to ZYMV and WMV is controlled by a common gene.

	A			B		
	Expected WMV Resistance Phenotype Ratios			Predicted ZYMV Phenotypes Model		
		Ratios		1	2	3
Genotype	Phenotype	F2	BC	*zymv* = *wmv-2*	*zymv* = *wmv-3*	*zymv* = *wmv-3* + *Wmv-4*
W2__ W3__ W4__	S	27	1	S	S	S
W2__ W3__ w4w4	S	9	1	S	S	S
W2__ w3w3 W4__	R	9	1	S	R	R
W2__ w3w3 w4w4	S	3	1	S	R*	S
w2w2 W3__ W4__	R	9	1	R	S	S
w2w2 W3__ w4w4	R	3	1	R	S	S
w2w2 w3w3 W4__	R	3	1	R	R	R
w2w2 w3w3 w4w4	R	1	1	R	R	S
Total R:S ratio		25:39	5:3	F2: 1:3 BC: 1:1	F2: 1:3 BC: 1:1	F2: 3:13 BC: 1: 3

* Denotes progeny class resistant to ZYMV but susceptible to WMV.

TABLE 2.5 Relationship Between Resistances to WMV and ZYMV

Phenotype		Number of Plants	Predicted Ratios (Numbers) Model			
			1	2	3	4
ZYMV	WMV	Observed [a]	*zymv* = *wmv-2*	*zymv* = *wmv-3*	*zymv* = *wmv-3* + *Wmv-4*	4 independent genes
S	S	93	39 (108)	36 (100)	39 (108)	117 (81)
S	R	42	9 (25)	12 (33)	13 (36)	75 (52)
R	S	14	0 (0)	3 (8)	0 (0)	39 (27)
R	R	29	16 (44)	13 (36)	12 (33)	25 (17)
		$\chi 2$	nd [b]	4.41 ns	nd [b]	17.59 **

Each member of a pair of vegetatively propagated, genetically identical F_2 plants was inoculated with either WMV or ZYMV.

** Significant $\chi 2$ value indicates that the observed data do not support the proposed genetic model: **, $P \leq 0.01$.

[a] Data pooled from three independent experiments.

[b] nd = not determined. These models are rejected due to the presence of individuals in the Z_R/W_S class. χ^2 cannot be determined.

inoculations. F_2 and F_1 X TMG-1 backcross progeny were first inoculated with ZYMV; resistant individuals were then tested for susceptibility to WMV. The results from these experiments (Table 2.6) closely paralleled the results from the clonal pairs experiments. Again, there was a class of individuals that were resistant to ZYMV but susceptible to WMV. These findings make it unlikely that *zymv* is *wmv-2* (Model 1), or that it is equivalent to the epistatic interaction of *wmv-3* and *Wmv-4* (Model 3). Again, a significant chi square value was obtained for the model that the ZYMV resistance is completely independent of the WMV resistances (Model 4). Finally, the hypothesis that *zymv* is the same or very tightly linked to *wmv-3* (Model 2) is supported by these observations. Both experimental approaches led to the same hypothesis.

The relationship between symptom level and virus level was also examined for all three viruses (Table 2.7). TMG-1 showed no symptoms (rating of 0), while WI-2757 exhibited full symptoms (rating of 4). The F_1 progeny showed symptoms in response to ZYMV and WMV but not to PRSV-W. These results reflect the data presented thus far, that the resistances to ZYMV and WMV are recessive, while resistance to PRSV-W is dominant. However, when virus titers were examined, a different scenario was observed. As might be expected, high virus titers were detected in the susceptible WI-2757 for all three viruses and for ZYMV and WMV in the symptomatic F_1 progeny. Very little or no ZYMV and WMV were

Table 2.6 Sequential Virus Inoculation Data: Inoculation of ZYMV-Resistant Plants with WMV.

Genotype	Observed Response to WMV		Expected Ratios in Response to WMV Inoculation							
			Model 1		Model 2		Model 3		Model 4	
			zymv = *wmv-2*		*zymv* = *wmv-3*		*zymv* = *wmv-3* + *Wmv-4*		4 independent genes	
	R	S	R:S	χ^2	R:S	χ^2	R:S	χ^2	R:S	χ^2
F_2 [a]	62	15	1:0	nd [b]	13:3	0.0003 ns	1:0	nd	34:30	22.1**
F_1 X TMG-1 [c]	41	12	1:0	nd	3:1	0.056 ns	1:0	nd	5:3	4.4*

Plants classified as resistant to ZYMV did not exhibit ZYMV symptoms and the upper leaves were free of virus as determined by ELISA immediately preceding the WMV inoculation.

*,** Significant χ^2 values indicate that the observed data do not support the proposed genetic model: *, $P \leq 0.05$; **, $P \leq 0.01$.

[a] Data pooled from two independent experiments. Each experiment fits the expected ratios for Model 2: $\chi^2_{exp1} = 0.13$ and $\chi^2_{exp2} = 0.044$.

[b] nd = not determined. These models are rejected due to the presence of individuals in the Z_R/W_S class. χ^2 cannot be determined.

[c] Data pooled from two independent experiments. Each experiment fits the expected ratios for Model 2: $\chi^2_{exp1} = 0.009$ and $\chi^2_{exp2} = 0.31$.

Table 2.7 Comparison of Symptom and ELISA Data for Young, Fully Expanded Leaves of TMG-1, WI-2757, and Their F_1 Progeny

	ZYMV		WMV		PRSV-W	
	Symptom	ELISA[a]	Symptom	ELISA	Symptom	ELISA
TMG-1	0.0±0.0*	0.028±0.003	0.0±0.0	0.022±0.055	0.0±0.0	1.081±0.324
WI-2757	4.0±0.0	0.582±0.118	4.0±0.0	1.150±0.308	4.0±0.0	1.361±0.312
F_1	3.0±0.0	0.322±0.048	3.3±0.1	0.271±0.033	0.0±0.0	1.320±0.359
Mock-[b] Inoculated	0.0±0.0	0.028±0.003	0.0±0.0	0.015±0.003	0.0±0.0	0.010±0.049

* Each value is the mean of 10 replicate plants ± S.E. (standard error).

[a] Young, fully expanded leaves were macerated in 2 ml of virus buffer. Adjusted ELISA values were calculated by dividing absorbance (at 405 nm to detect alkaline phosphatase conjugated ZYMV and at 490 nm to follow the Agdia horseradish peroxidase conjugated antibody against WMV and PRSV-W) by the fresh weight of each leaf.

b Mock-inoculated controls are an average of TMG-1, WI-2757, F_1, and Straight 8 genotypes. There were no differences among genotypes for control ELISA values.

Leaves were sampled for virus titer at the time of full symptom expression in the susceptible WI-2757 genotype. For ZYMV and WMV, samples were taken at approximately 3 weeks after inoculation; for PRSV-W, samples were taken at approximately 6 weeks after inoculation. Symptom expression was rated on a scale set from 0 to 4, where 0 was assigned to a healthy plant and 4 was given to plants exhibiting severe virus symptoms.

detected in the resistant TMG-1 plants. On the other hand, high levels of PRSV-W were found in the TMG-1 and F_1 genotypes, despite the absence of symptoms.

DISCUSSION

We have examined the inheritance of multiple potyvirus resistance in the TMG-1 cucumber line. Resistance to PRSV-W was due to a single dominant gene; however, there were two separate resistances to WMV that were controlled by a total of three separate genes. The first resistance was due to a single recessive gene. The second resistance was due to the epistatic interaction of a single recessive and a single dominant gene. Resistance to ZYMV was due to a single recessive gene as was published by Provvidenti (1987a). Therefore, the TMG-1 cucumber line has four to five different resistance genes to confer resistance to three related potyviruses (PRSV-W, WMV, and ZYMV).

The gene for resistance to PRSV-W in TMG-1 is not likely to be the same as one of the recessive genes conferring resistance to ZYMV or WMV. The mechanisms of resistance also appear to be different. Little or no virus was detected in the upper leaves of TMG-1 inoculated with ZYMV or WMV. In contrast, the resistance to PRSV in TMG-1 may be more appropriately described as tolerance since in the asymptomatic parent and F_1 progeny, the upper leaves contained virus levels that were comparable to those found in the susceptible WI-2757 parent.

In contrast to the relationship between the resistance to PRSV and ZYMV or WMV, resistance to ZYMV appears to be the same as, or tightly linked with the recessive resistance gene (*wmv-3*) that acts in concert with a dominant gene to confer resistance to WMV. The additional factor encoded by *Wmv-4* may be necessary to confer specificity to WMV vs. ZYMV. It is also possible that *zymv* and *wmv-3* are actually separate, but tightly linked genes. It will be difficult to distinguish between these possibilities unless the resistances segregate from each other, or if the actual genes are cloned and their regulation studied at the molecular level.

Several multiple potyvirus resistance systems have been reported in the literature. The finding that TMG-1 has two independently assorting resistances to WMV is not unprecedented. Kyle and Provvidenti (1987) reported two separate resistances to WMV in *Phaseolus vulgaris*. Similarly, genes located on separate linkage groups in *Pisum sativum* have been found to confer resistance to clover yellow vein virus and pea seed-borne mosaic virus (Provvidenti, 1987b, 1990; Provvidenti and Alconero, 1988a). The possibility that a gene at an apparently non-segregating locus can confer resistance to more than one virus has been reported also in several species including *Phaseolus vulgaris* L. (Kyle and Dickson, 1988), *Pisum sativum* (Marx and Provvidenti, 1979), *Solanum* (Cockerham, 1970), and *Capsicum annuum* (Cook, 1960). It is interesting to note that multiple virus resistance conferred by a single locus has, thus far, been reported only for potyviruses (Kyle and Provvidenti, 1993). Whether the ability to confer multiple virus resistance to potyviruses is a mechanism peculiar to this particular virus group, or whether it is the genetic mechanism evolved by these particular plant species to fight off parasitic infections remains to be seen.

The resistances to PRSV-W and WMV derived from TMG-1 can be contrasted with other resistances reported in the literature. The resistance to PRSV from TMG-1 was dominant, whereas the resistance described in the cultivar Surinam Local was recessive (Wang et al., 1984). The resistance in Surinam Local appears to act at the level of movement, since virus was detected by symptom and ELISA on the first few leaves but not in upper leaves. In contrast, TMG-1 shows no symptoms of PRSV-W, but has high virus titers in the upper leaves. It appears that both the genetics and mechanism of resistance differ between TMG-1 and Surinam Local.

Resistance to WMV in TMG-1 also appears to be different from that reported in the literature. A dominant resistance gene was reported in Kyoto 3 feet long (Cohen et al., 1971), whereas both resistances in TMG-1 are recessive. Since the inheritance appears to be different for the resistances to WMV in Kyoto 3 feet long and TMG-1, they may not be allelic. Tests for allelism have been initiated to determine whether the resistances to WMV and PRSV-W in TMG-1 are at the same loci as those in Kyoto 3 feet long and Surinam Local.

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CHAPTER 3

LINKAGE MAPPING OF MULTIPLE POTYVIRUS RESISTANCES IN THE TMG-1 CUCUMBER LINE

ABSTRACT

The cucumber genotype TMG-1 is resistant to three related potyviruses, zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and the watermelon strain of papaya ringspot virus (PRSV-W). Resistance to ZYMV is due to a single recessive gene (*zymv*), WMV to two recessive resistance factors (*wmv2* and *wmv3 Wmv4*), and PRSV-W to a single dominant gene (*Prsv-2*). Possible linkage relationships between these resistances and various physiological, morphological, electromorphic, and phytopathological markers were studied. TMG-1, WI-2757, (an inbred line susceptible to all three viruses), and their F_2 and backcross progeny were screened for various single gene characters that differ between the two parents. Linkages were detected between resistance to WMV and PRSV-W and traits on Linkage Group I: resistance to WMV was associated with sex expression (*F*) and resistance to PRSV-W was associated with bitterfree cotyledon (*bi*). The proposed sequence on this linkage group is *Prsv-2*, *bi*, *F*, and *wmv-2*. Resistance to WMV was closely associated with ZYMV, but resistance to ZYMV was not linked to bitterfree, suggesting that *zymv* and the second gene conferring resistance to WMV are not located on Linkage Group I.

INTRODUCTION

The inbred, Chinese cucumber line TMG-1 is resistant to three related cucurbit potyviruses: zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and papaya ringspot virus (PRSV) (Provvidenti, 1985). The genetics of resistance to each virus has been characterized. Resistance to ZYMV is due to a single recessive gene (Provvidenti, 1987). A single, dominant gene confers resistance to PRSV (Wai and Grumet, 1994). The inheritance of resistance to WMV is more complex (Wai and Grumet, 1994). The progeny of TMG-1 and WI-2757, a susceptible line, possess two independently assorting recessive resistances. The first resistance is conditioned by a single recessive gene (*wmv2*), while the second resistance results from the epistatic interaction of a single recessive gene from TMG-1 (*wmv3*) and a single dominant gene from WI-2757 (*Wmv4*).

To further understand the relationship of resistances to each other and to try to assign the virus resistance genes to linkage groups within cucumber, linkage relationships with markers were studied. The current morphological linkage map contains fewer than 40 map positions on six linkage groups (Pierce and Wehner, 1990). More recently, Vakalounakis (1992) placed the heart leaf shape marker onto Linkage Group IV and Knerr and Staub (1992) assigned 12 isozymes into four different linkage groups. The isozyme linkage groups have not yet been integrated onto the morphological map assembled by Pierce and Wehner (1990).

The potyvirus resistance genes were tested for associations with both morphological and isozyme markers. Single gene markers that differ between the resistant TMG-1 and susceptible WI-2757 parents were used to establish linkage relationships with resistance to ZYMV, WMV, or PRSV. Our results indicate that resistance to WMV is linked to the *F* locus for sex expression, and resistance to PRSV is linked with bitter cotyledon, the *bi* locus.

MATERIALS AND METHODS

Linkage tests between all the characters described below and resistance to ZYMV or WMV were performed on clonally propagated genetically identical F_2 cuttings as described by Wai and Grumet (1994). Linkage associations between resistance to PRSV-W and bitterfree cotyledon and the sex expression were performed in an F_2 population and the linkage distance between the resistance to PRSV-W and bitterfree cotyledon were estimated from WI-2757 X F_1 backcross populations.

Presence of Cucurbitacins (Bitter Principle). The presence of cucurbitacins was assayed by tasting a small piece of the cotyledon. WI-2757 is sweet (*bibi*), i.e. the absence of cucurbitacin, and TMG-1 and the F_1 (WI-2757 X TMG-1) progeny are bitter (Bi_). A few representative samples were also examined for cucurbitacins by thin layer chromatography (TLC) (Figure 3.1A).

Electromorphic Variants. Two isozymes that differ between the two parents are mannosephosphate isomerase (MPI-1) and phosphoglucomutase (PGM-1) (Figure 3.1B). Samples were prepared and separated by standard starch gel electrophoresis using the protocol of Knerr et al. (1989).

Sex Expression. WI-2757 is a gynoecious cucumber line (*FF*) that only produces female flowers under normal conditions. Male flowers may be induced by exogenous treatments such as silver nitrate. TMG-1 is andromonoecious (*ff*), that is it produces many male flowers and some female flowers under normal conditions.

Spine Number and Tuberculate Fruit. The fruit characters spine number and tuberculate were scored visually. WI-2757 is homozygous recessive for numerous spine (*ns*) and smooth fruit/flower (*tu*); TMG-1 is homozygous dominant for few spine (*Ns*) and warty fruit/flower (*Tu*) (Figure 3.1C).

Resistance to Scab. Conidia of *Cladosporium cucumerinum* were prepared as described by Abul-Hayja (1975) and Vakalounakis (1993). The growing point and young leaves of rooted plants were sprayed to runoff with 4 X 10^5 conidia/ml in distilled water using an artist's airbrush at 10 p.s.i. Plants were incubated in a biotron for 48 h in the dark at 20 C, 100% RH, followed by 4 days at 20 C, 12 h photoperiod, 40 to 50% RH.

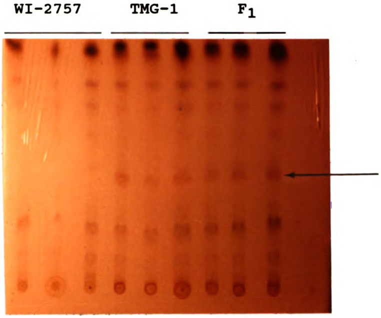

Figure 3.1A. Visualization of cucurbitacins by TLC. The presence (indicated by the arrow) or absence of purported cucurbitacins in the parental and F_1 genotypes was visualized by TLC (Hammerschmidt, unpublished). Cotyledon tissue was homogenized in 2 ml/g fresh weight in chloroform: methanol (1:1, v/v), and then dried. In each set of three lanes, approximately 1/6, 1/3, and 1/2 of an expanded cotyledon were homogenized. Extracts were solubilized in methanol, spotted on a silica gel plate, and developed in a solvent containing 95% chloroform and 5% methanol. The plate was dried, sprayed with a solution containing 3% vanillin solubilized in ethanol and 0.5% (v/v) sulfuric acid, and then baked at 95 to 100 C for 10 minutes. The presence of a band (R_f of 0.49) in TMG-1 and F_1 and its absence in WI-2757 correlate with the bitter and sweet phenotypes.

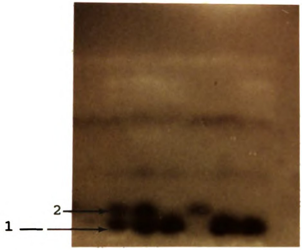

Figure 3.1B Starch gel electrophoresis of isozymes (MPI-1 or PGM-1). Electromorphs are labelled 1 and 2 for fast (lower) and slow (upper) bands.

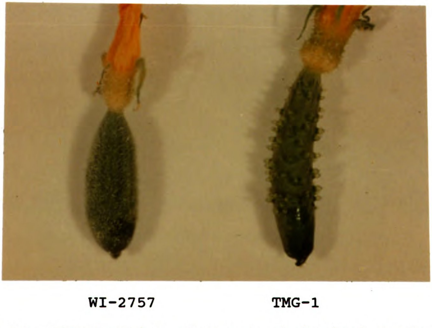

Figure 3.1C Illustration of the morphological markers numerous spine and tuberculate. Female flowers/fruits of WI-2757 have numerous spines and are smooth, whereas those of TMG-1 have few spine and are warty.

Symptoms of susceptible plants ranged from tip maceration to death. WI-2757 carries a single dominant gene to scab (*Ccu*), whereas TMG-1 is susceptible. A comparison of symptom observed in the susceptible TMG-1 is shown in comparison with the resistant WI-2757 (Figure 3.1D).

Resistance to *Fusarium* Wilt. Macroconidia of *Fusarium oxysporum* f. sp. *cucumerinum* were prepared as described by Vakalounakis (1993) and in Appendix D. Rooted plants in rooting cubes were placed into a solution containing 10^5 macroconidia/ml. The inoculum was immediately soaked into the rooting cubes (approximately 12 ml/cube). The plants in cubes then were placed into metal pans filled with sand. The plants were watered daily and maintained in water baths at 28 C for one week until symptoms appeared. Symptoms of susceptible plants ranged from severe wilt to death (Figure 3.1E). WI-2757 is resistant to fusarium (*Foc*); TMG-1 is susceptible.

Resistance to ZYMV and WMV. Half-expanded true leaves were mechanically inoculated with ZYMV and WMV as described by Wai and Grumet (1994). TMG-1 is resistant (*zymvzymv,wmv2wmv2,wmv3wmv3,wmv4wmv4*) and WI-2757 susceptible. The inheritance of resistance is described by Wai and Grumet (1994).

Figure 3.1D The effect of *Cladosporium cucumerinum* (scab) on the susceptible TMG-1 (arrow points to leaf maceration caused by the fungus). Note that WI-2757 is healthy.

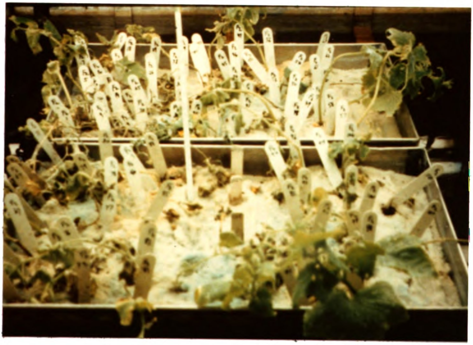

Figure 3.1E The effect of *Fusarium oxysporum* f. sp. *cucumerinum* in an F_2 population. Susceptible plants are severely wilted or dead, whereas resistant plants remain standing and are green.

Analysis of Data

Linkage relationships between single gene characters were analyzed using the LINKAGE 1 (version 3.5) program. Chi square analyses for associations with WMV resistance were calculated using linear models.

RESULTS AND DISCUSSION

The selected markers were easily scored, single gene, homozygous traits of inbred lines that differed between the parents and fit the expected segregation ratios. The following morphological, physiological, disease resistance, and isozyme characters were utilized to map the potyvirus resistance genes: bitterfree cotyledon; sex expression; spine number; tuberculate fruit; isozymes of mannosephosphate isomerase and phosphoglucomutase; and resistance to scab, *Fusarium* wilt, ZYMV, WMV, and PRSV.

Single trait goodness-of-fit tests show that each character used in the analyses fit expected ratios (Table 3.1). There was close agreement between linkage values observed in our study and those already cited in the literature for genes in Linkage Group I and Group IV (Table 3.2, Figure 3.2). The loci for bitterfree and female (sex expression) on Linkage Group I were linked with an estimated map distance of 0.34 vs. 0.37 cM (Fanourakis and Simon, 1987). A linkage association found between the genes encoding the morphological markers for numerous spine and

Table 3.1 Single Trait Goodness of Fit Tests

Locus	Expected Ratio	Observed Ratio and Phenotype		Chi Square Value	P Value
Scab	3:1	72:26	R:S[a]	0.12	0.73
Fusm	3:1	61:25	S:R	0.76	0.38
Male	3:1	76:30	F:f	0.62	0.43
ZYMV	3:1	56:20	S:R	0.07	0.79
WMV	39:25	50:30	S:R	0.08	0.33
Bitr	3:1	80:30	Bi:bi	0.30	0.58
MPI1	1:2:1	28:54:28	LL:LU:UU[b]	0.04	0.98
PGM1	1:2:1	30:55:25	LL:LU:UU	0.45	0.80
SpNo	3:1	30:14	Ns:ns	1.09	0.30
Wrty	3:1	32:12	Tu:tu	0.12	0.73

The following are the genetic designations for the abbreviations above:

Scab = resistance to scab (*Ccu*)
Fusm = resistance to fusarium (*Foc*)
Male = sex expression (*F* is dominant and is strongly female)
ZYMV = resistance to ZYMV (*zymv*)
WMV = resistance to WMV (*wmv-2* or *wmv-3wmv-3,Wmv-4_*)
Bitr = bitterfree cotyledon (*bi*)
MPI1 = mannosephosphate isomerase (*Mpi-1*)
PGM1 = phosphoglucomutase (*Pgm-1*)
SpNo = spine number or numerous spine (*ns*)
Wrty = warty or tuberculate fruit or flowers (*Tu*)

[a] R and S indicate resistant and susceptible classes, respectively.

[b] L and U indicate lower and upper bands, respectively.

Table 3.2 F_2 Phenotypic Classes and Their Frequencies in the Cross WI-2757 X TMG-1

Characters	Frequencies:				X^2 (9:3:3:1)	
	(X-Y-)	(X-yy)	(xxY-)	(xxyy)	Value	P Value
Male and ZYMV	41	15	14	6	0.08	0.78
Male and Bitr	51	26	25	4	4.14	0.04
Male and SpNo	21	9	11	3	0.35	0.55
Male and Wrty	24	8	8	4	0.31	0.58
Scab and Fusm	43	15	16	8	0.47	0.49
Scab and Male	57	14	15	12	6.14	0.01
Scab and ZYMV	37	14	17	3	1.22	0.27
Scab and Bitr	52	18	20	8	0.08	0.77
Scab and SpNo	22	7	13	1	1.80	0.18
Scab and Wrty	24	7	11	1	1.16	0.28
Fusm and male	45	17	16	8	0.29	0.59
Fusm and ZYMV	35	12	9	8	2.69	0.10
Fusm and Bitr	43	19	18	6	0.27	0.61
Fusm and SpNo	18	9	11	1	2.72	0.10
Fusm and Wrty	23	7	6	3	0.36	0.55
ZYMV and Bitr	41	16	15	4	0.36	0.55
ZYMV and SpNo	14	5	10	0	3.18	0.07
ZYMV and Wrty	17	4	7	1	0.17	0.68
Bitr and SpNo	22	8	8	6	1.15	0.28
Bitr and Wrty	20	12	10	2	1.75	0.19
SpNo and Wrty	26	6	4	8	9.24	0.002

Table 3.2 (continued)

Characters	Frequencies:						X^2 (3:1:6:2:3:1)	
	(LLX-)	(LLxx)	(LUX-)	(LUxx)	(UUX-)	(UUxx)	Value	P Value
MPI1 and Scab	21	6	39	12	12	8	0.73	0.69
MPI1 and Fusm	14	5	32	10	15	10	2.08	0.35
MPI1 and Male	20	8	34	17	22	5	1.91	0.38
MPI1 and ZYMV	16	4	28	9	12	7	1.57	0.45
MPI1 and Bitr	19	9	42	12	19	9	1.36	0.51
MPI1 and SpNo	7	5	12	6	11	3	1.25	0.53
MPI1 and Wrty	10	2	13	5	9	5	1.19	0.55
PGM1 and Scab	21	6	39	12	12	8	2.35	0.31
PGM1 and Fusm	14	10	32	10	15	5	2.57	0.28
PGM1 and Male	22	7	38	16	16	7	0.34	0.84
PGM1 and ZYMV	12	9	31	7	13	4	4.25	0.12
PGM1 and Bitr	20	10	43	12	17	8	1.66	0.44
PGM1 and SpNo	6	3	18	7	6	4	0.49	0.78
PGM1 and Wrty	7	2	18	7	7	3	0.16	0.92

PGM1 and MPI1:

1	LL,LL	4
2	LL,LU	15
1	LL,UU	11
2	LU,LL	17
4	LU,LU	27
2	LU,UU	11
1	UU,LL	7
2	UU,LU	12
1	UU,UU	6

X^2 (1:2:1:2:4:2:1:2:1)

Value: 4.59 P value: 0.33

Table 3.2 (continued)

Characters	Frequencies						X^2 (39:78:39:25:50:25)	
	(S,LL)	(S,LU)	(S,UU)	(R,LL)	(R,LU)	(R,UU)	Value	P value
WMV and PGM1	10	28	12	10	14	6	2.6	0.41
WMV and MPI1	16	22	12	7	14	9	1.32	1.68

Characters	Frequencies				X^2 (117:39:75:25)	
	(S,X-)	(S,xx)	(R,X-)	(R,xx)	Value	P value
WMV and Bitr	32	24	18	6	4.09	0.13
WMV and Fusm	30	11	18	7	0.16	0.92
WMV and Male	43	7	18	12	7.87	0.01
WMV and Scab	35	12	21	8	0.14	0.84
WMV and SpNo	14	9	9	3	2.98	0.40
WMV and Wrty	17	8	6	4	0.68	0.65
WMV and ZYMV	38	4	14	10	9.44	0.002

The following abbreviations are used:
L = lower, fast-moving band (#1); U = upper, slow-moving band (#2) isozymes.
R = genotypes resistant to WMV, includes w_2w_2 and/or $w_3w_3W_4\_$.
S = genotypes infected with WMV, i.e. $W_2\_W_3\_W_4\_$, $W_2\_W_3\_w_4w_4$, or $W_2\_w_3w_3w_4w_4$.
Scab = resistance to scab (*Ccu*)
Fusm = resistance to fusarium (*Foc*)
Male = sex expression (*F* is dominant and is strongly female)
ZYMV = resistance to ZYMV (*zymv*)
WMV = resistance to WMV (*wmv-2* or *wmv-3wmv-3,Wmv-4_*)
Bitr = bitterfree cotyledon (*bi*)
MPI1 = mannosephosphate isomerase (*Mpi-1*)
PGM1 = phosphoglucomutase (*Pgm-1*)
SpNo = spine number or numerous spine (*ns*)
Wrty = warty or tuberculate fruit or flowers (*Tu*)

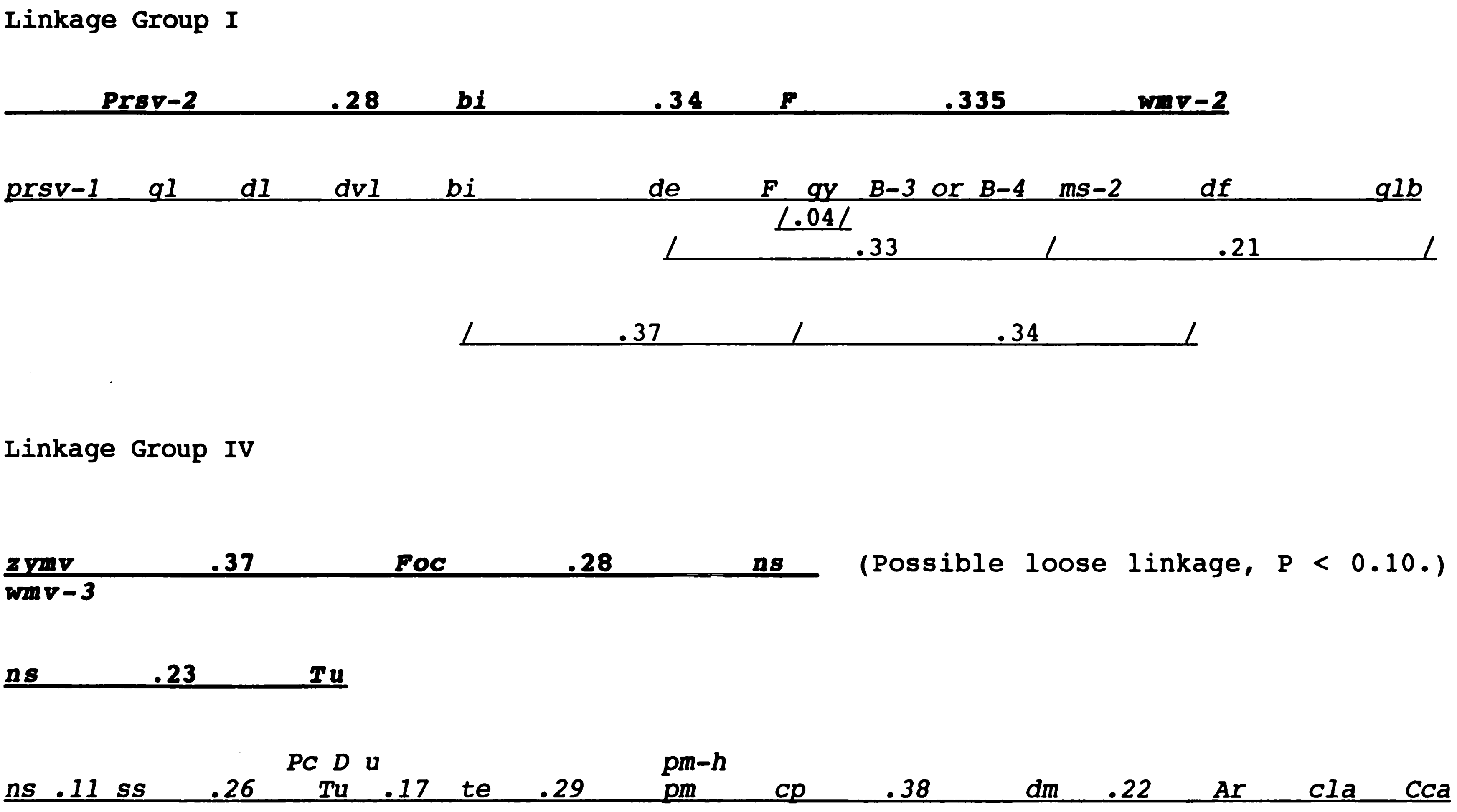

Figure 3.2 Cucumber linkage map for Groups I and IV. Linkages (reported as recombination frequencies) observed in this study (in bold face type) relative to the map published by Pierce and Wehner (1990) for Linkage Groups I and IV.

Figure 3.2 (continued) The genetic loci shown are as follows:

Linkage Group I

prsv-1 = recessive resistance to PRSV-W identified in Surinam Local
gl = glabrous leaves or fruit, i.e. no trichomes
dl = delayed growth - shortening of the hypocotyl and the first internode
dvl = divided leaf
bi = bitterfree cotyledon
de = determinate habit
F = Female, high degree of female sex expression
gy = gynecious
B-3 B-4 = black spine
ms-2 = male sterile
df = delayed flowering
glb = glabrate, stem and petioles are glabrous; laminae slightly pubescent

Linkage Group IV

ns = numerous spine
ss = small spine
Tu = tuberculate or warty fruit
Pc = Parthenocarpy
D = Dull fruit skin
u = uniform immature fruit color
te = tender skin of fruit
pm-h/pm = powdery mildew - hypocotyl; powdery mildew
cp = compact, i.e. decreased internodal length; small flowers
dm = resistance to downy mildew caused by *Pseudoperonospora cubensis*
Ar = resistance to anthracnose caused by *Colletotrichum lagenarium*
cla = resistance to race 1 of anthracnose
Cca = resistance to target leaf spot caused by *Corynespora cassicola*

tuberculate fruit on Linkage Group IV was consistent with the literature (Fanourakis and Simon, 1987). Genes that assorted independently gave very low chi square values. Therefore, the newly observed linkages are likely to be real.

A very strong association was detected between resistance to ZYMV and WMV. This finding supports the proposed genetic model that resistance to WMV is due to two genetic factors (*wmv2wmv2, wmv3wmv3Wmv4_*), one of which (*wmv3wmv3Wmv4_*)is the same or is very tightly linked with the resistance to ZYMV (Wai and Grumet, 1994). Resistance to WMV is also linked to the *F* locus on Linkage Group I. Since resistance is not linked to bitterfree, it is predicted to lie approximately 34 cM to the other side of *F*, near delayed flowering and glabrate. Resistance to ZYMV does not appear to be linked to the *F* locus, suggesting that it is the independent WMV gene *wmv-2*, that is linked to *F*. In addition, there is a possible loose linkage between resistance to *Fusarium* wilt and spine number, between resistance to ZYMV and resistance to fusarium, and ZYMV and spine number, possibly putting *zymv* and *wmv-3* in Linkage Group IV.

In our experiments, the resistance to *Fusarium* wilt from WI-2757 appeared to be recessive. The F_1 progeny died from the disease, while approximately 25% of the F_2 survived the treatment (Table 3.1). In earlier studies, the resistance was reported to be due to a dominant allele

(Netzer et al., 1977; Vakalounakis, 1993). Vakalounakis (1993) has found absolute linkage between resistance to scab and resistance to race 1 of fusarium. A possible explanation to account for this difference is a difference in the disease screen protocol. Our tests were performed with cuttings in rooting cubes and so the plants absorb more inoculum than the standard assay in which roots of young seedlings are dipped into the *Fusarium* inoculum for only a limited period of time. It is also possible that the gene observed in this study is actually a different resistance gene from the ones that have been described by Netzer (1977) and Vakalounakis (1993). In our study, the genes conferring resistance to scab and fusarium not only are dominant and recessive, respectively, but also assort independently. In addition, environmental and epistatic interactions may vary in different genetic backgrounds and influence the results observed.

A separate set of experiments was performed to map resistance to PRSV. The recessive gene that confers resistance to PRSV in the cultivar Surinam Local was reported to be linked with *bi* (Wang et al., 1984). Therefore, we tested for possible cosegregation between our resistance to PRSV with loci on Linkage Group I, *bi* and *F* (Tables 3.3 and 3.4). Our results showed that resistance to PRSV is linked to *bi* at a distance of approximately 28 cM in the WI-2757 X F_1 backcross (Table 3.3). We further tested for linkage with the *F* and *bi* loci in an F_2 population,

Table 3.3 Test for Linkage Association Between Resistance to PRSV and Bitterfree Cotyledon in the backcross WI-2757 X F_1.

Phenotype		Observed[a]	Expected Ratio
R B[b] (TMG-1)		76	1
R b		31	1
S B		31	1
S b (WI-2757)		82	1
χ^2		42.2**	
Single Trait	B:b	107:111	1:1
	χ^2	0.04 ns	
	R:S	107:111	1:1
	χ^2	0.04 ns	

** Deviation from the predicted values for independently assorting genes is significant: $P < 0.01$.

[a] Each experiment fits the predicted single-trait ratio for resistant to susceptible and for bitter to sweet. Data pooled from two independent experiments: $\chi^2 R{:}S_{exp1} = 0.008$, $\chi^2 B{:}b_{exp1} = 0.008$, $\chi^2 R{:}S_{exp2} = 0.25$, and $\chi^2 B{:}b_{exp2} = 0.09$.

[b] R = resistant, S = susceptible; B = bitter, b = bitterfree (sweet).

The estimated map distance for the combined experiments is 28 cM between resistance to PRSV and bitterfree cotyledon (*bi*).

Table 3.4 Tests for Linkage Associations Between Resistance to PRSV (*Prsv-2*) and Genes on Linkage Group I [bitterfree cotyledon (*bi*) and sex expression (*F*)]in an F_2 Population.

A. Test for linkage between *Prsv-2* and *bi*.			B. Test for linkage between *Prsv-2* and *F*.		
(1) Cosegregation between *Prsv-2* and *bi*.			(1) Cosegregation between *Prsv-2* and *F*.		
Phenotype	Observed[a]	Expected Ratio	Phenotype	Observed	Expected Ratio
R B[b] (TMG-1)	75	9	R F (TMG-1)	63	9
R b	12	3	R M	22	3
S B	10	3	S F	23	3
S b (WI-2757)	16	1	S M (WI-2757)	5	1
$\chi^2 = 24.5$** estimated distance = 22.4 cM			$\chi^2 = 0.75$ ns		
(2) Single trait goodness of fit tests.			(2) Single trait goodness of fit tests.		
B:b	87:26	3:1	F:M	86:27	3:1
$\chi^2 = 0.14$ ns			$\chi^2 = 0.03$ ns		
R:S	85:28	3:1	R:S	85:27	3:1
$\chi^2 = 0.003$ ns			$\chi^2 = 0.003$ ns		

** Deviation from the predicted values for independently assorting genes is significant: $P < 0.01$.

R = resistant, S = susceptible; B = bitter, b = bitterfree (sweet); F = female, M = male.

which segregates for all three characters (Table 3.4). In correspondence with the backcross data, we found linkage between *bi* and resistance to PRSV at an estimated distance of 22.4 cM. Because resistance to PRSV was found to be independent of the *F* locus (Table 3.4), we have tentatively placed it to the left of *bi* (Figure 3.2). Since both the dominant resistance to PRSV from TMG-1 and the recessive resistance from Surinam Local were placed on Linkage Group I to the left of *bi*, it will be especially interesting to perform tests of allelism between these two resistances.

In summary, these studies have placed two additional genes *wmv-2* and *Prsv-2* onto Linkage Group I. The proposed gene sequence is *Prsv-2 - bi - F- wmv-2*. In order to further map useful genes, such as those that confer resistance to plant viruses, it will be necessary to develop a more saturated genetic

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CHAPTER 4

TISSUE-SPECIFIC EXPRESSION OF DIFFERENT RESISTANCES TO WATERMELON MOSAIC VIRUS IN CUCUMBER

ABSTRACT

The Chinese cucumber line TMG-1 is resistant to the potyvirus watermelon mosaic virus. The resistance can be conferred by either of two independently segregating factors. To further characterize these resistances, the parents, F_1, F_2, and backcross progeny of a cross between TMG-1 and a susceptible line WI-2757 were monitored for symptom expression and virus accumulation by ELISA. Segregation ratios differed depending on whether cotyledons or true leaves were inoculated. Cotyledon-inoculated backcross progeny segregated 1:1 as would be expected for a single recessive gene, whereas segregation ratios for true-leaf-inoculated progeny were 5 resistant: 3 susceptible. Similar differences were observed between cotyledon and true-leaf-inoculated F_2 progeny. These results suggest that the two resistances are expressed differently: one at the cotyledon stage and throughout the plant, the second only at the true leaf stage. The cotyledon-expressed gene was found to be linked to sex expression (*F*) in Linkage Group I.

INTRODUCTION

The cucumber cultivar TMG-1 is resistant to several potyviruses including watermelon mosaic virus (WMV) and zucchini yellow mosaic virus (ZYMV) (Provvidenti, 1987; Wai and Grumet, 1994). When crossed with the susceptible genotype WI-2757, two independently assorting resistances to WMV were observed (Wai and Grumet, 1994). The first resistance is due to a single recessive gene (*wmv-2*), whereas the second resistance is the result of an epistatic interaction between a single recessive gene from TMG-1 (*wmv-3*) and a single dominant gene from WI-2757 (*Wmv-4*). The second recessive gene, *wmv-3*, appears to be the same as, or tightly linked with, the recessive gene (*zymv*) conferring resistance to ZYMV. Linkage analyses with these resistances indicated that the resistance to WMV, but not ZYMV, is associated with the *F* locus for sex expression located on cucumber Linkage Group I (Wai et al., 1994). During the course of inheritance and linkage studies, a difference was noted in the response to WMV inoculation depending on whether true leaves or cotyledons were inoculated. This study was initiated to differentiate further between the two resistances to WMV. We found that the two resistances showed different tissue-specific expression; *wmv-2* was expressed in cotyledons and true leaves, the epistatic *wmv3,Wmv4* resistance was expressed only in true leaves.

MATERIALS AND METHODS

Cucumber Genotypes

The inbred cucumber (*Cucumis sativus* L.) lines TMG-1, resistant, (Provvidenti, 1985) and WI-2757, susceptible, (Peterson et al. 1982) were provided by Dr. Jack Staub (USDA, University of Wisconsin at Madison). The F_1 progeny (WI-2757 X TMG-1) were either self- or sib-pollinated to produce the F_2 generation. Backcrosses were made with both parents: WI-2757 X F_1 and F_1 X TMG-1. In each cross, the source of resistance came from the male parent. The inbred line Straight 8 (Stokes Seeds, Inc., Buffalo, NY) was used as an additional control genotype that is suceptible to all three viruses.

Experimental designs and methods for virus maintenance and verification, inoculation, and leaf disk ELISA are as described by Wai and Grumet (1994). To study the different resistances that are expressed in different tissues, cotyledons or true leaves were mechanically inoculated. Linkage relationship between the resistances and sex expression (*F* locus) were determined as described in Wai et al. (1994).

RESULTS

Inoculation of true leaves of F_2 or backcross progeny of WI-2757 X TMG-1 with WMV indicated the presence of two independently assorting resistance factors, one controlled by a single recessive gene (*wmv2wmv2*), the second by the epistatic interaction of a recessive and dominant gene (*wmv3wmv3,Wmv4_*) (Wai and Grumet, 1994). However, other experiments, in which cotyledons rather than true leaves were inoculated, gave different results. When cotyledons of the F_1, F_2 and backcross progeny were inoculated, segregation ratios indicated that resistance to WMV was due to a single recessive gene (Table 4.1). Possible explanations for these differing results include: different environmental conditions when the experiments were performed, different ages of the plants, or tissue-specific expression (cotyledon vs. true leaf).

Upon closer inspection of the susceptible individuals in cotyledon-inoculated experiments, two levels of symptom expression were detected (Table 4.2). In experiments using F_1 X TMG-1 backcross progeny, the individuals again segregated as a single gene trait, 1:1 resistant: susceptible. In one experiment, 1/4 - 1/2 of the susceptible class showed a mild flecking type of mosaic pattern, while the remainder exhibited a more severe mosaic pattern. An approximately 3:1 (susceptible:partially resistant) segregation within the susceptible class would be predicted if the second gene could confer only partial resistance once an infection became established in the cotyledons. In the second experiment, again two levels of symptom expression were observed in an approximately 3:1 ratio. About one quarter of the susceptible class showed symptom spread approximately one tenth of the way down the

TABLE 4.1 Response of Progeny of TMG-1 and WI-2757 to Inoculation with WMV at the Cotyledon Stage.

Genotype	Number of Plants Resistant	 Susceptible	Expected Ratio (R:S) [a]	χ^2
TMG-1	104	0		
WI-2757	0	86		
F_1	0	72		
F_2	81	273	1 : 3	0.74 ns
F_1 X TMG-1[b]	300	291	1 : 1	0.011 ns
WI-2757 X F_1	0	22	0 : 1	

[a] Expected ratios based on a single recessive gene model.

[b] Data pooled from five independent experiments. Each experiment fits the predicted ratios: $\chi^2_{exp1} = 0.65$, $\chi^2_{exp2} = 0.000$, $\chi^2_{exp3} = 0.50$, $\chi^2_{exp4} = 0.000$, and $\chi^2_{exp5} = 0.09$.

leaf, while the remainder ofthe susceptible class exhibited a more extensive mosaic on the leaves. These results gave further evidence for two separable resistances, and led to the hypothesis that the second resistance was not expressed until the true leaf tissue formed.

Table 4.2 Intermediate Symptom Expression by a Subset of the Backcross (F_1 X TMG-1) Individuals Susceptible to WMV After Cotyledon Inoculation.

Resistant (without symptoms)	90	
Susceptible	84	
mild symptoms		20
strong symptoms		64

Data were combined from two experiments.

To differentiate among possible explanations for the different ratios observed in cotyledon and true leaf experiments, sets of experiments were performed where cotyledon only, cotyledon and true leaf, or true leaf only, inoculations were made simultaneously on groups of plants of the same age (two true leaf stage). Again, different segregation ratios were observed depending on whether true leaves or cotyledons were inoculated (Table 4.3). Inoculation of cotyledons alone indicated a single, recessive gene; inoculation of true leaves indicated two resistances. Inoculation of both true leaves and cotyledons

TABLE 4.3 **Segregation of Resistances to WMV Following Inoculation of Either the (A) Cotyledons or (b) True Leaves.**

A Cotyledon inoculation								
Genotype			1-gene model [a] (w_2w_2)		Epistatic 2-gene model $(w_3w_3W_4\_)$		3-gene model $(w_2w_2w_3w_3W_4\_)$	
	R	S	R:S	χ^2	R:S	χ^2	R:S	χ^2
F_2	29	72	1:3	0.56 ns	3:13	5.24*	25:39	4.12*
F_1 X TMG-1 [b]	82	85	1:1	0.024 ns	1:3	50.46**	5:3	12.2**
B Inoculation of true leaf tissue only								
Genotype			1-gene model (w_2w_2)		Epistatic 2-gene model $(w_3w_3W_4\_)$		3-gene model $(w_2w_2w_3w_3W_4\_)$	
	R	S	R:S	χ^2	R:S	χ^2	R:S	χ^2
F_2	34	64	1:3	4.41*	3:13	15.32**	25:39	0.61 ns
F_1 X TMG-1 [c]	132	79	1:1	12.8**	1:3	156.75**	5:3	0.003 ns

Each pair of cotyledon and true-leaf inoculation experiments was performed simultaneously.

*,** Significant χ^2 values indicate that the observed data do not support the proposed genetic model: *, $P \leq 0.05$; **, $P \leq 0.01$.

[a] Expected ratios are presented for three different models: (1) resistance conferred by a single recessive gene; (2) resistance conferred by an epistatic interaction between a single recessive and a single dominant gene; and, (3) two, separate independently assorting resistance factors conferred by three genes. The first resistance consists of a single recessive gene, and the second resistance the result of an epistatic interaction between a single recessive and a single dominant gene.

[b] Data pooled from two independent experiments. Each experiment fits the predicted ratios for the 1-gene model: $\chi^2_{exp1} = 0.33$ and $\chi^2_{exp2} = 0.16$.

[c] Data pooled from two independent experiments. Each experiment fits the predicted ratios for the 3-gene model: $\chi^2_{exp1} = 0.051$ and $\chi^2_{exp2} = 0.019$.

gave the same segregation ratios as when cotyledons alone were inoculated: 21:28 (R:S) (X^2 = 0.73) resistant in the F_1 X TMG backcross progeny. Inoculation of the second true leaf or the eighth true leaf both gave similar results (data not shown). These results suggest that the observed difference is due to the tissue that is being inoculated, and not differences in plant age at the time of inoculation or environmental conditions. These results further indicate that the resistance expressed in the cotyledon is due to the single recessive gene that acts independently (*wmv2*) rather than the epistatic interaction between the recessive and dominant genes (*wmv3*, *Wmv4*) (Table 4.2).

Previous studies had identified a linkage relationship between WMV resistance and the *F* locus for sex expression (Wai et al., 1994). It was now possible to determine which of the two WMV resistances, cotyledon-expressed (*wmv-2*), or true leaf only (*wmv3, Wmv4*), was linked to the *F* locus. The two single traits, resistant vs. susceptible, and male vs. female, gave the predicted 1:1 ratios in the backcross generation (Table 4.4). Analysis of cosegregation indicated a linkage association between the cotyledon-expressed gene and the *F* locus; thus *wmv2* is linked to the *F* locus. These results are consistent with previous obervations (Wai et al., 1994) that: (1) the resistance to ZYMV is due to the same gene, or is tightly linked to the gene that acts in concert with the dominant gene to confer resistance to WMV (*wmv3*), and (2) the resistance to ZYMV is not linked to the

Table 4.4 Test for Linkage of the Cotyledon-Expressed WMV Resistance to the *F* locus in the F_1 X TMG-1 Backcross.

		Cotyledon Inoculation	
Phenotype		Observed[a]	Expected Ratio
R M[b] (TMG-1)		134	1
R F		66	1
S M		82	1
S F (WI-2757)		130	1
χ^2		34.0**	
Single	M:F	216:196	1:1
Trait	χ^2	0.88 ns	
	R:S	200:212	1:1
	χ^2	0.29 ns	

** Deviation from the predicted values for independently assorting genes is significant: $P < 0.01$.

[a] Data from each experiment fit the predicted single-trait ratios for resistant to susceptible and for the number of male to female flowers. Data pooled from two independent experiments: $\chi^2 R{:}S_{exp1} = 0.000$, $\chi^2 M{:}F_{exp1} = 0.61$, $\chi^2 R{:}S_{exp2} = 0.52$, and $\chi^2 M{:}F_{exp2} = 0.02$.

[b] R = resistant, S = susceptible; M = male, F = female.

F locus. Physiological separation of the two WMV resistances also made it possible to map the *wmv2* gene more accurately relative to the *F* locus; the estimated distance is 35 cM.

DISCUSSION

A novel tissue-specific plant virus resistance has been identified. In the progeny of TMG-1 and WI-2757, two independently assorting resistance factors to WMV were identified (Wai et al., 1994). The first resistance is due to the action of a single recessive gene, while the second resistance is conditioned by the epistatic interaction of a recessive gene from TMG-1 and a dominant gene from WI-2757. This work demonstrates that there is differential developmental control of the two resistances. The resistance conferred by the single recessive gene is expressed in the cotyledon and throughout the plant. In contrast, the epistatic resistance is expressed only in true leaf tissue. These experiments cannot distinguish whether it is the recessive factor from TMG-1, the dominant factor from WI-2757, or both that are not expressed until the true leaf stage. We also cannot determine whether the requirement for the epistatic factor from WI-2757 is unique to that genotype or would occur in other crosses with other genotypes susceptible to WMV.

Interestingly, although the resistance requiring recessive gene *wmv3* is not expressed until the true leaf stage, genetic analysis indicates that this gene is the same as, or tightly linked to, the recessive gene *zymv* for resistance to ZYMV (Wai and Grumet, 1994). The resistance due to *zymv* is expressed at the cotyledon stage (Provvidenti, 1987; Wai and Grumet, 1994). Possible explanations for this difference are that an additional factor is necessary for the *zymv* gene product to be able to confer resistance to the related potyvirus WMV or that the two genes are separate, but closely linked.

We have not found other reports of tissue-specific expression of virus resistance. However, standard virus tests usually employ one type of inoculation. In our studies, the first differences appeared when we used vegetatively propagated clonal sets of F_2 individuals to perform direct comparisons of WMV and ZYMV resistance, rather than standard seedling screens (Wai and Grumet, 1994). Developmentally expressed resistances have been described in other systems, for example, in bacteria and fungi, as in the case of resistance to damping off, described to be expressed at the true leaf stage and not in cotyledons. In addition, virus resistance that is only expressed in true leaf tissue is useful, since aphids normally feed on young leaves and not on cotyledons.

Recently, a developmentally regulated plant cell wall-associated protein kinase has been described (Citovsky et al., 1993). This protein kinase was shown to phosphorylate the movement protein of TMV *in vitro*. It is expressed primarily in leaves and increased activity is found as a function of leaf maturation, with the greatest activity found at leaf tips and the least at the base. The protein kinase activity is also correlated with the development of secondary plasmodesmata in mature leaves. Phosphorylation of movement protein is believed to inactivate it (Citovsky and Zambryski, 1993). It is conceivable that a host plant resistance that is expressed only in true leaves may block cell-to-cell movement of the virus. The observations made in this study may be explained by such a model. A subpopulation of plants that exhibit symptoms only at the very base of the leaf correlates well with such a hypothesis. In addition, recent characterization of calcium-regulated protein kinases in zucchini has shown that cotyledons contain the fewest protein kinases (Verhey et al., 1993). This finding also supports a model in which a developmentally regulated protein kinase activity may be involved in a resistance that is only expressed in true leaves. Although we cannot rule out other possible mechanisms, such as reduced rates of replication or inhibition of long distance movement, it would be very interesting to study further whether this tissue-specific resistance acts at the level of cell-to-cell movement.

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CHAPTER 5

SPATIAL AND TEMPORAL DISTRIBUTION OF POTYVIRUS INFECTION IN THE RESISTANT CUCUMBER GENOTYPE TMG-1

ABSTRACT

The inbred Chinese cucumber line TMG-1 is resistant to three related potyviruses: zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and the watermelon strain of papaya ringspot virus (PRSV-W). The genetics of resistance to three viruses is different: ZYMV is due to a single recessive gene, WMV to two recessive resistance factors, and PRSV-W to a single dominant gene. We sought to determine if the resistances also differ in their effect on systemic spread. The kinetics of virus accumulation were studied in the resistant TMG-1 genotype and compared with the pattern in WI-2757, an inbred line that is susceptible to all three viruses. While the spread of PRSV-W is retarded initially, eventually levels of virus detected by ELISA are comparable to those in WI-2757. ZYMV and WMV, however, spread more slowly and do not attain as high a titer in TMG-1 as in WI-2757. More than one resistance mechanism appears to be acting in the resistant TMG-1 cucumber.

INTRODUCTION

The inbred Chinese cucumber line TMG-1 is resistant to three related potyviruses: zucchini yellow mosaic virus (ZYMV), watermelon mosaic virus (WMV), and the watermelon strain of papaya ringspot virus (PRSV-W). The genetics of resistance to the three viruses is different; resistance to the ZYMV is due to a single recessive gene (Provvidenti, 1987), resistance to WMV is due to two recessive resistances, and resistance to PRSV-W is due to a single dominant gene (Wai and Grumet, 1994). There is also a difference in the time that each virus takes to show full symptom expression in susceptible genotypes: approximately seven to ten days for ZYMV, ten to fourteen days for WMV, and three to six weeks for PRSV-W.

Since different genetic mechanisms (e.g. dominant vs. recessive) have been associated with different resistance phenotypes (Fraser, 1990), the resistances in TMG-1 were examined for differences in mode of action. Initial studies suggested that the phenotype of the dominant PRSV-W resistance trait differs from that of the recessive ZYMV and WMV resistances (Table 2.7, Chapter 2). There were high PRSV-W titers in young, fully expanded leaves of the symptomless TMG-1 and F_1 genotypes. In contrast, little or no virus was detected in comparable leaves when TMG-1 was inoculated with ZYMV or WMV. In this study, the kinetics of accumulation of each virus was monitored in the resistant

TMG-1 genotype and compared with the pattern in WI-2757, an inbred line that is susceptible to all three viruses.

MATERIALS AND METHODS

Maintenance of virus inocula, method of inoculation, and detection of virus by the leaf disk method were performed as described by Wai and Grumet (1994). Plants were inoculated at the cotyledonary stage. Virus spread to each leaf position was assayed by indirect ELISA, using the leaf disk method. The experiments for all three viruses were performed simultaneously under identical environmental conditions.

RESULTS

Figure 5.1 shows a comparison of symptoms produced in response to each virus in zucchini squash. Each virus induces distinct symptoms. ZYMV characteristically produces a striping type of interveinal chlorosis, while WMV produces a fine mosaic or mottling. Infection with PRSV-W produces well-defined rugosity. Table 2.7 (Chapter 2) presents data that suggest that the mechanisms of resistance may be different for each virus. TMG-1 plants showed no symptoms in response to each virus. Little or no ZYMV or WMV was detected in the young, fully expanded leaves of TMG-1.

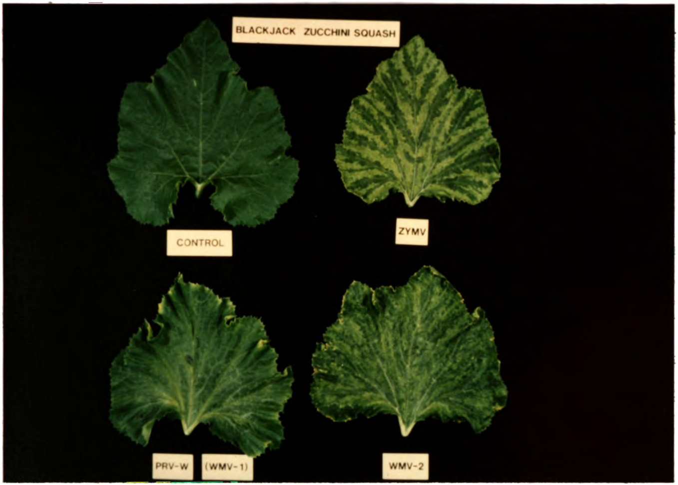


Figure 5.1 Comparison of symptoms produced in response to ZYMV, WMV, and PRSV in zucchini squash. Note that each virus induces distinct symptoms. ZYMV characteristically produces a striping type of interveinal chlorosis, while WMV produces more mottling. Infection with PRSV produces a well-defined rugosity.

However, equally high levels of PRSV-W were detected in young, fully expanded leaves of TMG-1 as were observed in the symptomatic WI-2757 leaves.

Figures 5.2, 5.3, and 5.4 display a comparison of virus spread throughout various stages of infection. Each figure illustrates virus titer at each leaf position in both the resistant TMG-1 and in the susceptible WI-2757. Panel A of each figure shows virus accumulation at week 1, Panel B at week 3, and Panel C at week 6. All three experiments were performed at the same time under identical environmental conditions. Each point represents the average of five replicate plants.

Even during the first week of infection, WI-2757 supports markedly higher levels of virus than the resistant TMG-1 for all three viruses. By week 3, ZYMV and PRSV-W appear to spread to the second leaf in TMG-1; however, virus levels drop off quickly in subsequent leaves. The levels of WMV appear to remain at a constant low level in TMG-1. By week 6, PRSV-W has spread to a higher virus titer in the upper nodes of the resistant plant than either ZYMV or WMV. Figure 5.5 shows a comparison of PRSV-W and ZYMV accumulation in TMG-1 as a percentage of the level in the susceptible WI-2757. Levels of ZYMV sharply drop off after the third leaf, whereas high titers of PRSV-W were detected through leaf 9. The first fully expanded leaf (approximately the third leaf from the apex) was usually

Figure 5.2 ZYMV

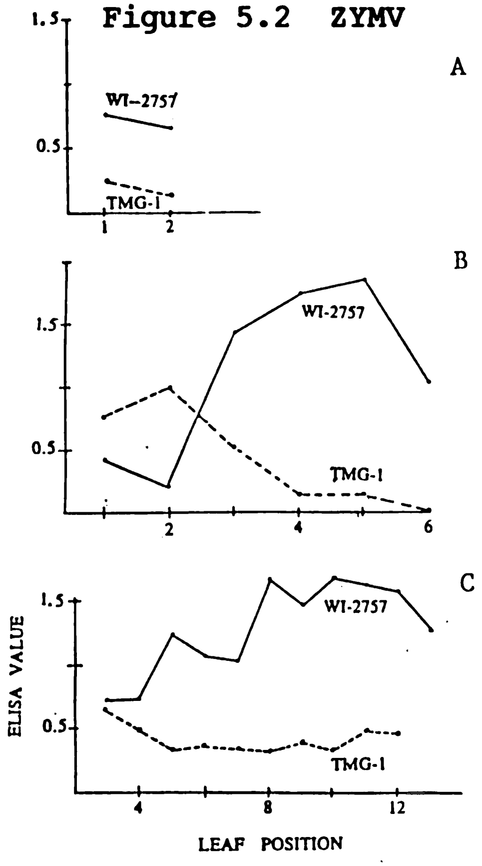


Figure 5.3 WMV

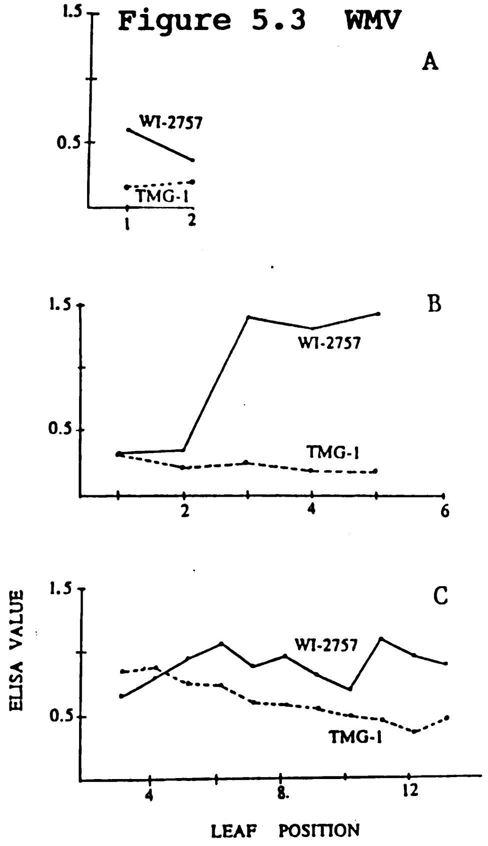
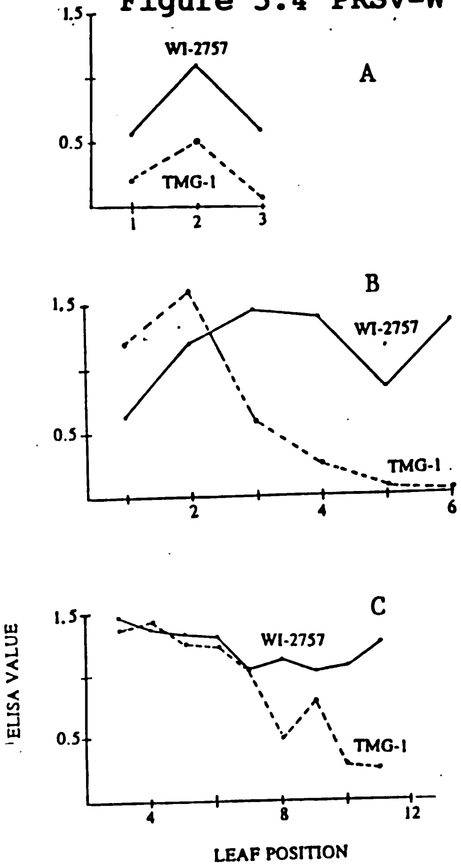


Figure 5.4 PRSV-W



Figures 5.2 (ZYMV), 5.3 (WMV), and 5.4 (PRSV-W). Comparison of virus spread throughout various stages of infection. Each figure illustrates virus titer at each leaf position in both the resistant TMG-1 and in susceptible WI-2757. Panel A of each figure shows virus accumulation at week 1, Panel B at week 3, and Panel C at week 6. All three experiments were performed at the same time under identical environmental conditions. Each point represents the average of five replicate plants. Healthy background levels average at approximately 0.300 in these experiments.

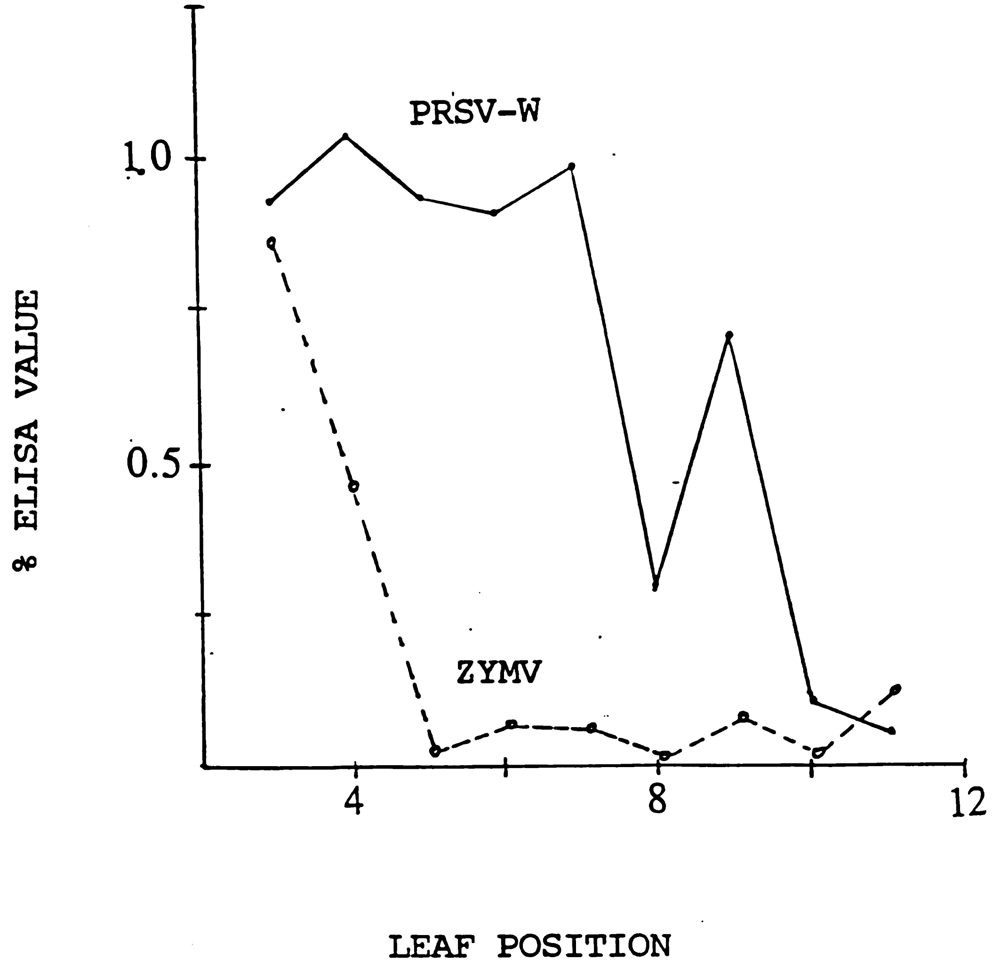


Figure 5.5 Comparison of PRSV-W and ZYMV accumulation in TMG-1 as a percentage of the level in the susceptible WI-2757. In this comparison, the healthy background levels have been subtracted.

sampled. Virus titers in the upper leaves are markedly different for ZYMV and PRSV.

DISCUSSION

Comparison of virus titers in young, fully expanded leaves of TMG-1 (sampled at the time of full symptom expression in WI-2757) suggested that the mode of resistance to PRSV-W may differ from that of WMV or ZYMV. A more detailed analysis over time suggests that a similar response might exist for all three viruses, but with differences in the kinetics of the resistance phenotype. Although PRSV-W accumulation is delayed in TMG-1 relative to the susceptible WI-2757, it appears to move more quickly than either ZYMV or WMV. Moyer et al. (1985) described similar findings in a *Cucumis melo* L. line 91213 that exhibits resistance to WMV. A reduction in accumulation of virus concentration was detected in leaves of the resistant line. The authors called this phenomenon a "suppressive virus resistance." Further characterization of this resistant line by differential temperature treatment of upper and lower leaves suggested that resistance is correlated with reduced movement of virus within leaves, possibly at the level of cell-to-cell movement (Gray et al., 1988).

During the first week of infection, the susceptible WI-2757 supports higher levels of all three viruses. This trend persists over time; accumulation of all three viruses is delayed in TMG-1, indicating that none of the resistance genes confers resistance at the level of complete inhibition of viral replication. A possible reason for high virus titers in the lower leaves in the absence of symptom expression, is that sufficient virus titers do not accumulate in the leaf at the critical time for symptom development. Further experiments would be required to distinguish between resistance mechanisms acting at the level of viral spread versus reduced virus multiplication.

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SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

TMG-1 is resistant to ZYMV, PRSV-W, and WMV. Resistance to ZYMV was due to a single recessive gene, resistance to PRSV-W was conferred by a single dominant gene, and resistance to WMV was due to two independently assorting recessive resistance factors, either one of which may impart resistance to WMV. The first resistance to WMV was due to a single recessive gene and the second was the result of an epistatic interaction between a single recessive gene from TMG-1 and a single dominant gene from WI-2757. The recessive resistance gene to ZYMV (*zymv*) appeared to be at the same locus (either the same, or tightly linked) as the recessive resistance gene that is part of the epistatic interaction. Linkage mapping placed resistance to PRSV-W (linked with bitterfree cotyledon, but not sex expression) and WMV (linked with sex expression, but not bitterfree cotyledon) on Linkage Group I, but at different locations. The two resistance factors to WMV were found to be expressed in different tissues. The single recessive gene was found to be expressed in cotyledon tissue and throughout the entire plant, whereas the resistance that resulted from an epistatic interaction between a single recessive and a single dominant was expressed only in true leaf tissue. The single recessive gene to WMV that is expressed in cotyledon tissue was found to be linked to sex expression. Different mechanisms of resistance were also found to be active in TMG-1. ELISA results showed that resistance to ZYMV and WMV were most likely due to reduced multiplication, whereas resistance to PRSV-W was probably tolerance. A time course study showed that little or no ZYMV or WMV were found in the upper leaves of TMG-1, while high titers of PRSV-W were present in comparable positions.

Several lines of experiments are possible to continue this work. The first would be to perform tests for allelism between the resistances to PRSV-W and WMV in TMG-1 with those described in the literature. The cultivar Surinam Local carries a single recessive gene to PRSV-W and Kyoto 3 feet long has a single dominant gene to WMV. Secondly, further mapping of the resistance genes by RFLP's and RAPD's might uncover closer markers. It may be possible to map the cotyledon-expressed WMV resistance closer to delayed flowering under the appropriate environmental conditions. It may be possible to study mechanisms of resistance by the tissue print method. This method maybe useful in differentiating between resistance that is expressed cell-to-cell vs. long distance spread. The hypothesis that the true-leaf-expressed WMV resistance may act at the level of cell-to-cell movement could be studied by the tissue print method also. Separation of resistances to ZYMV and WMV by creating F_3 and F_4 lines would allow for further dissection of the different mechanisms of resistance. Thus, many interesting questions remain to be investigated in this system.

APPENDICES

APPENDIX A

APPENDIX A

A QUICK AND EASY METHOD TO ISOLATE LARGE QUANTITIES OF HIGH QUALITY POTYVIRAL RNA

One approach to study resistance mechanisms is to determine whether the resistance is expressed at the cellular or at the organismal level. A method to distinguish between these two possibilities is to determine whether viral replication occurs at the same rate and to the same extent in the resistant cultivar in comparison with the susceptible one. This approach was used to elucidate the mechanism of resistance in the Arlington cowpea (Bruening et al., 1987). The investigators found that the resistance mechanism was due to a protease inhibitor.

Potyvirus replication in a protoplast system has been studied by introducing naked viral RNA into the cell via electroporation (Luciano et al., 1987). Luciano et al. used 20 to 80 μg of viral RNA per sample and then tested for increased levels of viral RNAs to determine whether virus replication had taken place. I tried to study virus replication in a cucumber protoplast system using a similar approach.

The standard method of isolating viral RNA involves isolating whole virions in a caesium sulphate gradient by ultracentrifugation, and then isolating RNA in a sucrose density gradient by ultracentrifugation, as was done for

ZYMV by Grumet and Fang (1990). A more efficient method of isolation was needed to obtain the large quantities of viral RNA required to study of virus replication *in vitro*.

When virus was isolated in a caesium sulfate gradient made in phosphate buffer (pH 7.5) and RNA extracted by phenol chloroform extraction, the virus preparations were free from other proteins, but were contaminated with nucleic acids. Different treatments, for example digestion of virions with DNAase I, were tried to remove the host DNA. A report that indicated the use of HEPES buffer could remove contaminating nucleic acids was tested. It was also necessary to develop a method to destabilize the virus coat protein to extract the viral RNA. The standard dissociation buffer involved the use of SDS, which precipitates with the RNA in the ethanol precipitation step. The presence of SDS in RNA preparations was detrimental to cucumber protoplasts. After many trials of varying buffers at different pH, an extremely simple method of purifying virions and RNA without any ultracentrifugation steps was developed. The procedure developed is as follows: isolate virions as decribed by Grumet and Fang (1990), but substitute the basic buffer component with one that contains 10 mM EDTA with 20 mM HEPES at pH 7.0 or 6.5 or 20 mM MES at pH 6.5. The procedure requires that the virus isolation is performed only up to the second PEG precipitation step. All of the cellular debris precipitates out in the pellet, and the remaining supernatant contains pure virions without any associated contaminating nucleic acid. Virus isolated in grinding buffer that consisted of 10 mM EDTA and 20 mM HEPES at pH 7.5 had contaminating nucleic acid on the surface of the virions (Figure A.1, lane 2). It is possible that changing the pH of the phosphate buffer from pH 7.5 to pH 7.0 would also eliminate the contaminants. This possibility was not pursued since the current procedure works well. The procedure recovers almost 90% of the full-length viral RNA from the starting material. An additional check gel showed that approximately one tenth (by visual quantitation) of the total full-length viral RNA is lost in the second pellet and none in the first pellet. An average yield from this procedure would be as follows: approximately 100 g of fresh weight leaf material would yield 2-10 mg of virus (with an absorbance 260/280 ratio of 1.3 to 1.6), from which approximately 2-10 μg of full-length RNA may be isolated by the standard phenol:chloroform extraction method. Yield may be increased by re-extraction of the proteinaceous material at the interface with phenol:chloroform. The main advantage of this procedure is that very large quantities (1 to 2 kg) of leaf material may be processed at one time. In addition, both of the time-consuming and expensive caesium sulfate and sucrose ultracentrifugation gradients have been eliminated.

Several different methods of dissociating virus coat protein work equally well. Stocks may be made 2X, 5X, or 10X of the final concentration. Final concentrations are given below.

(1) Standard RNA prep buffer

100 mM ammonium carbonate, pH 9.0
1 mM EDTA
1% SDS

0.125 ug/ml proteinase K

(2) Tris, EDTA, SDS, Proteinase K

100 mM Tris, pH 9.0
1 mM EDTA
1% SDS

0.125 ug/ml proteinase K

(3) Ammonium carbonate, EDTA, Sarkosyl, proteinase K

100 mM ammonium carbonate, pH 9.0
1 mM EDTA
1% to 1.5% Sarkosyl

0.125 ug/ml Proteinase K

(4) Tris, EDTA, Sarkosyl, Proteinase K

100 mM Tris, pH 9.0
1 mM EDTA
1% to 1.5% Sarkosyl

0.125 ug/ml Proteinase K

(5) Tris, EDTA, Proteinase K (*NO DETERGENT)

100 mM Tris, pH 9.0
1 mM EDTA

1 ug/ml Proteinase K

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All materials used should be RNAase free. Solutions and materials should be autoclaved twice or baked, if possible.

(1) Add at least 50 ug of virus to the tube in at least 100 μl of total volume with buffer.

(2) Add bentonite to at least a final concentration of 1 ug/ml.

(3) Use dissociation buffer of choice.

(4) Add Proteinase K.

(5) Leave on bench top for 20 minutes.

(6) Extract three times with equal volume of phenol:chloroform:isoamyl (50:48:2), pH 6.5 to 8.0.

(7) Add NaOAc to a final concentration of 0.3 M if using the Tris dissociation buffer. It is not necessary to add if ammonium carbonate is used.

(8) Add 2.5 to 3 volumes of 95% to 100% ethanol. Spin down precipitate RNA. Verify RNA by standard gel electrophoresis.

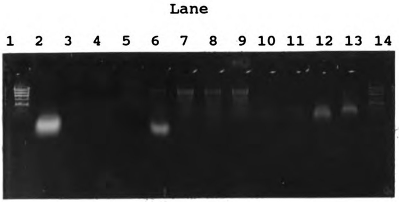

Figure A.1 Visualization of the purity of ZYMV RNA isolated under different conditions. The different pH indicated are the pH of the grinding buffer used to isolate virions. Samples were separated on a 0.6% agarose gel, pre-run with ethidium bromide. The gel was run for 12 minutes at 150 volts and 65 mAmps.

Lanes 1 and 14 are Lambda Hind III markers;

lanes 2 - 5 are isolated whole virions:
2 is HEPES + EDTA at pH 7.5
3 is HEPES + EDTA at pH 7.0
4 is HEPES + EDTA at pH 6.5
5 is MES + EDTA at pH 6.5

lanes 6 - 9 are apparent full-length viral RNAs isolated by the standard phenol / chloroform method
6 is HEPES + EDTA at pH 7.5
7 is HEPES + EDTA at pH 7.0
8 is HEPES + EDTA at pH 6.5
9 is MES + EDTA at pH 6.5

lanes 10 and 11 were treated with RNase at 37 C, 15 min.
10 is HEPES + EDTA at pH 7.0
11 is MES + EDTA at pH 6.5

lanes 12 and 13 were treated with DNase at 37 C, 15 min.
12 is HEPES + EDTA at pH 7.0
13 is MES + EDTA at pH 6.5.

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Lanes 2 and 6 show that isolation of virus in the HEPES + EDTA buffer at pH 7.5 brings along contaminating nucleic acids. On the other hand, virus that was isolated in buffers at pH 7.0 or pH 6.5 were free of these contaminants (lanes 3, 4, 5, 7, 8, and 9). MES was chosen as an alternate buffer because the buffering capacity of HEPES is not optimal at pH 6.5. Lanes 10 and 11 confirm that the nucleic acids that were isolated are sensitive to RNase treatment, and, therefore, are probably viral RNAs. Digestion with DNase (lanes 12 and 13) does not eliminate the nucleic acids. The degradation observed is probably due to contaminating RNases that are frequently found with DNases.

In summary, the virus and viral RNA extraction procedures presented here allow for isolation of good quality viral RNAs that are free from nucleic acid contamination. Both ultracentrifugation steps have been eliminated, so that large quantities of leaf material can be processed at greatly reduced expenditure of time and materials.

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Luciano, C.S., Rhoads, R.E., Shaw, J.G. 1987. Synthesis of potyviral RNA and proteins in tobacco mesophyll protoplasts inoculated by electroporation. Plant Science 51:295-303.

APPENDIX B

Appendix B

TMG-1 IS RESISTANT TO APHID TRANSMISSION OF ZYMV AND PRSV-W

In Chapter 1, resistances to ZYMV and PRSV-W were characterized via rub inoculation of cotyledons and/or true leaves. However, the question arises whether TMG-1 also confers resistance to aphid transmission of these viruses, since transmission of these viruses in nature occurs by aphids.

Aphids (*Myzus persicae* Sulz.) were maintained on tobacco plants (*Nicotiana tabacum* L. cv. Burley) in a growth chamber. Plants for aphid transmission experiments were grown to the one to two-leaf stage. Aphids were harvested by tickling their underside using an artist's paintbrush. The insects were collected in Petri plates and sealed with parafilm until feeding time (1-2 hours). Aphids were then placed on detached symptomatic zucchini leaves and allowed to feed for at least 1 minute. Those that crawled off the leaves were killed. Ten aphids were placed on each plant and allowed to feed for 2 to 3 hours. Visible aphids were removed using the artist's paintbrush and squashed by hand. Plants were then sprayed to drip with 5 ml/gallon 50% Malathion Emulsifiable Concentrate (Meijer, Inc., Grand Rapids, MI) in a chemical hood, allowed to dry overnight and sprayed again the next day. Plants were placed in a random arrangement and grown in a growth chamber set at a constant temperature of 26 C and 16 hours of light.

In each experiment, 10 plants each of the resistant TMG-1, susceptible WI-2757, and their F_1 progeny were planted, 2 plants/6 inch plastic pot. Two plants of each genotype were mechanically inoculated as controls, the remaining eight plants were inoculated by aphids. Plants were monitored for symptom expression daily. Full symptoms took 2 to 3 weeks to appear for both ZYMV (Pickens and CT2, aphid transmissible strains as described by Bada and Grumet, unpublished) and PRSV-W.

Table B.1 Resistance of TMG-1 to aphid-transmitted ZYMV

	Exp't 1 - ZYMV-Pickens No. Plants Symptomatic / No. Inoculated		Exp't 2 - ZYMV-CT2 No. Plants Symptomatic / No. Inoculated	
Genotype	Rub Inoc.	Aphid	Rub Inoc.	Aphid
TMG-1	0/2	0/8	0/2	0/8
WI-2757	2/2	8/8	2/2	5/6
F_1	2/2	8/8	2/2	8/8

Table B.2 Resistance of TMG-1 to aphid-transmitted PRSV-W

	Exp't 1 - PRSV-W No. Plants Symptomatic / No. Inoculated		Exp't 2 - PRSV-W No. Plants Symptomatic / No. Inoculated	
Genotype	Rub Inoc.	Aphid	Rub Inoc.	Aphid
TMG-1	0/2	0/8	0/2	0/8
WI-2757	?/2	8/8	2/2	8/8
F_1	0/2[a]	0/8[a]	0/2[b]	0/8[b]

? Symptoms were not clear.

[a] F_1 plants were stunted in growth, in comparison with the normal hybrid vigor they exhibit.

[b] Mild symptoms were observed on lower leaves of F_1 plants.

These experiments demonstrate that TMG-1 is resistant to ZYMV and PRSV-W when transmitted by aphids. The F_1 progeny showed symptoms when inoculated with ZYMV, as was observed for mechanically inoculated F_1 plants. This result supports the previous finding that resistance to ZYMV in TMG-1 is recessive (Chapter 2). In contrast, the F1 progeny showed little or no symptom in response to aphid-inoculation with PRSV-W. This finding is consistent with the observation that resistance to PRSV-W in TMG-1 is dominant (Chapter 2). Therefore, the resistances to ZYMV and PRSV-W in TMG-1 are effective in protecting the plant in a natural infection situation.

APPENDIX C

Appendix C

CUCUMBER SCAB DISEASE SCREEN PROTOCOL

Disease:
Scab, *Cladosporium* rot, spot rot, spotting of cucumber, leaf blight, fruit, gummosis.

Pathogen:
Cladosporium cucumerinum Ellis and Arth.

Culture Description:
On potato dextrose agar (PDA) hyaline when young, but greenish to black with age.

Microscopic Description:
Mycelium: septate and pigmented. Spores: conidia mostly one-celled, some septate; colored; oblong (4.1-5 X 15.2-18.8 microns); borne on short branched dark conidiophores.

Source:
M.J. Havey, Department of Horticulture, University of Wisconsin, 1575 Linden Drive, Madison, WI 53706.

Relative Stability:
No races reported. No loss of pathogenicity after 10 years of periodic transfer on PDA.

Variants:
Sectoring occurs to give non-sporulating culture.

Storage and Retrieval:
Store at 4 C on PDA for 3 months or on PDA under sterile minieral oil for 1 yr. For retrieval aseptically remove a piece of mycelium, place on PDA, and incubate at 20 C.

Inoculum Increase:
Add a few mls of sterile distilled water to a PDA slant culture. Scrape with a sterile bacteriological loop to dislodge spores. Aseptically remove a loopful of spores and streak over entire surface of PDA slant Incubate at 20 C.

Inoculum Preparation:
Add a few mls of distilled water to a 3-5 day old scab culture grown on a PDA slant. Scrape with sterile bacteriological loop to dislodge spores. Filter through a single layer of cheesecloth to remove mycelial fragments.

Quantification:
Method 1: Count spores with a hemacytometer. Adjust spore concentration to 4 X 10^5 spores/ml with distilled water.

Method 2: Count spores. Adjust concentration to 2 X 10^5 spores/ml.

Method 3: Dilute with distilled water until water until spore suspension is light green in color when held to the light.
Check percent germination of spore suspension on water agar after 24 hr.

Inoculum Distribution and Delivery:
Method 1: Spray hypocotyl of plant in cotyledon stage with inoculum (4 X 10^5 spores/ml) using an airbrush.

Method 2: Using a Pasteur pipette, place a 0.01-0.03 ml droplet of inoculum (2 X 10^5 spores/ml) on cotyledon when cotyledons are just expanded. Inoculating cotyledons beyond this stage may give a resistant reaction on susceptible plants.

Method 3: Spray inoculum (light green in color) on growing point and young leaves of plants in cotyledon stage up to the fifth leaf stage. Some growing points of resistant plants may be damaged using this method.

All seedlings should be marked, by punching the tip of the cotyledon with a Pasteur pipette, at inoculation so late germinating, uninoculated seedlings can be distinguished from resistant plants.

Host:
Cucumis sativus L., cucumber.

Source of resistance:
Wisconsin SMR18.

Differentials - Controls:
Susceptible check - Straight 8. Resistant check - Wisconsin SMR18.

Growth of Host:
Cucumber seeds are sown in steam sterilized coarse grade vermiculite in wooden flats (52 X 36 X 7 cm). Each flat contains 10 rows, 25 seeds/row. Resistant and susceptible checks are sown in row 6. The flats are placed on a heated germination bench. Vermiculite temperatures of 32 C ensure rapid and uniform germination. Newspaper on top of the flat prevents cooling by evaporation. Newpaper is removed when seeds germinate. If inoculating plants in the true leaf stage, transplant 2 wk old seedlings to steam sterilized soil in 4 inch plastic pots. Soil composed of sand:peat: field soil:field compost (1:1:1:1). Fertilize plants in 4 inch pots once/wk.

Tissue Age:
Methods 1 & 2: Inoculate plants when cotyledons are just expanded.

Method 3: Spray inoculum on plants in cotyledon stage up to the fifth leaf stage.

Postinoculation Environment:
Incubate plants at 20 C in the dark for 48 hr at 100% relative humidity. If leaves become watersoaked, leaf tissue on older plants may collapse and resistant plants appear susceptible. After incubation, grow plants at 20 C. Warmer temperatures can cause a resistant reaction on susceptible plants.

Disease Response:
Plants are rated as susceptible or resistant 5-7 days after inoculation.
Method 1: The hypocotyl of susceptible plants is girdled or has sunken necrotic lesions. Resistant plants show no reaction or, rarely, a faint watersoaked lesion.

Method 2: A sunken lesion develops on the cotyledon of susceptible plants. Resistant plants show no reaction or a glossy spot where inoculum was applied.

Method 3: The growing point of susceptible plants is killed and young leaves have necrotic lesions. Resistant plants remain healthy, though some damage to the growing point may occur.

Disease Rating Scale:
Hypocotyl tissue
0 = no symptoms
1 = blisters on hypocotyl
3 = restricted tan lesion on hypocotyl
5 = small tan lesion(s) on hypocotyl
7 = large sunken lesion(s) on hypocotyl
9 = dead

Cotyledon tissue
0 = no symptoms
1 = glossy spot
3 = glossy spot with necrotic flecks
5 = necrotic flecks surrounded by chlorosis
7 = small necrotic lesions surrounded by chlorosis
9 = large sunken necrotic lesion
10 = dead due to inoculum runoff onto growing point or hypocotyl

Growing point
0 = no symptoms
1 = growing point damaged, cotyledons no symptoms
3 = growing point dead, cotyledons no symptoms
5 = growing point dead or damaged, cotyledons lesions
7 = growing point dead or damaged, one cotyledon dead
9 = dead

Multiple Inoculation:
Methods 1 and 2: Simultaneous inoculation with anthracnose, downy mildew, angular leaf spot, or bacterial wilt.
Subsequent inoculation with cucumber mosaic virus (CMV) and powdery mildew.
With Method 2, scab resistant seedlings inoculated with *C. cucumerinum* and *Colletotrichum orbiculare* (anthracnose) had fewer lesions and less disease severity than seedlings inoculated with *C. orbiculare* alone.
Method 3: Previous inoculation with anthracnose, downy mildew, angular leaf spot, bacterial spot, bacterial wilt, CMV, and powdery mildew.

Saving Host:
Method 2: Both resistant and susceptible seedlings can be transplanted to steam sterilized soil.
Methods 1 and 3: Only resistant plants survive.

Some of the information in this handout was obtained from:

Abul-Hayja, Z.M. 1975. Multiple disease screening and genetics of resistance in cucumber. Ph. D. thesis. University of Wisconsin-Madison. 149 pp.

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APPENDIX D

APPENDIX D

CUCUMBER *FUSARIUM* WILT DISEASE SCREEN PROTOCOL

Disease:
Fusarium wilt of cucumber, cucumber wilt, foot-rot of cucumber.

Pathogen:
Fusarium oxysporum (Schlect.) Synd. and Hans. F. sp. *cucumerinum* Owen.

Culture Description:
On potato dextrose agar (PDA) white mycelium, purple pigment usually develops with age.

Microscopic Description:
Mycelium: septate. Spores: macroconidia-crescent shaped; multiseptate; microconidia-single celled; oval chlamydospores - resting structure; thick cell wall.

Source:
American Type Culture Collection; 12301 Parklawn Drive, Rockville, MD 20852. Races 1 through 3 available.
M. J. Havey, Department of Horticulture, University of Wisconsin, 1575 Linden Drive, Madison, WI 53706. Race 2 available.

Relative Stability:
Three races reported. Probably loses pathogenicity after periodic transfer on PDA.

Variants:
No information.

Storage and Retrieval:
Store in sterile soil at 4 C for several years. Store on PDA or PDA under sterile mineral oil for 3 months. For retrieval, aseptically remove soil or mycelium, place on PDA, and incubate at 24 C.

Inoculum Increase:
Place a piece of mycelium in 50 ml of potato dextrose broth (PDB) in a 125 ml Erlenmeyer flask. Shake on a rotary shaker 26-30 C for 3-6 days.

Inoculum Preparation:
Comminute fungus and PDB in Waring blender at low speed for 30 sec. 2 times. Centrifuge at 3,000 rpm for 10 min. to wash the spores. Discard supernatant. Resuspend pellet in distilled water.

Quantification:
Count spores with a hemacytometer. Check percent germination of spore suspension on water agar after 24 hr.

Inoculum Distribution and Delivery:
Fill metal pan (50 X 29 X 11 cm) with silica sand. Weigh sand. Need 10^5 spores per gram sand. Calculate ml of concentrated inoculum needed per pan. Mix inoculum with deionized water to 1,500 ml volume Add to sand. Mix thoroughly in a large mising pan. Return sand to metal pan. Make 8 X 1.5 cm furrows. Plant 20 seeds per row. Row 5 contains resistant and susceptible checks.

Host:
Cucumis sativus L., cucumber.

Source of Resistance:
Wisconsin SMR18.

Differentials - Controls:
Susceptible check Straight 8. Resistant check Wisconsin SMR18. Differentials for races:

Race	1	2	3
MSU 8519	S	R	R
MSU 441034 (Chipper)	S	S	R
PI 390265	R	S	R

R = resistant, S = susceptible.
Include cucumber cultivar Ashley to check pathogenicity of race 3.
Reference for races: Armstrong, G.M., J.K. Armstrong, and D. Netzer. 1978. Pathogenic races of the cucumber - wilt *Fusarium*. Plant Disease Reporter 62:824-828.

Growth of Host:
See inoculum distribution and delivery section. Water with tap water or fertilizer daily. Photoperiod 12 hr. light, 12 hr. dark.

Tissue Age:
Seeds are sown in infested sand. See inoculum distribution and delivery section.

Postinoculation Environment:
Place pans in Wisconsin temperature tanks. Sand temperature 28 C. No light first 2 days to prevent drying.

Disease Response:
Plants are rated on a scale from 0 to 9 three weeks after inoculation.

Disease Rating Scale:

0 = no symptoms
1 = hypocotyl browning, no wilt, no stunting
3 = cotyledon lesion, no wilt, no stunting
5 = slight wilt, stunted
7 = severe wilt, stunted
9 = dead

Plants rated 0 are classified as resistant, 1 or 3 as intermediate, 5, 7, or 9 as susceptible.

Multiple Inoculation:
No information. Experiments planned.

Saving Host:
Using this method, resistant and possibly intermediate plants can be transplanted to steam sterilized soil.

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