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HOST AND VIRAL FACTORS INFLUENCING SYSTEMIC INFECTION AND HOST RANGE OF POTYVIRUSES IN CUCURBITS

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ABSTRACT

HOST AND VIRAL FACTORS INFLUENCING SYSTEMIC INFECTION AND HOST RANGE OF POTYVIRUSES IN CUCURBITS

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This study was directed toward investigation of host and viral factors that influence successful systemic potyvirus infection. On the host side, I studied the mechanism of resistance to zucchini yellow mosaic virus (ZYMV) in two cucumber inbred lines, 'Dina-1' and 'TMG-1', both of which contain a single recessive gene but show different phenotypes when young plants are inoculated on the cotyledons. 'Dina-1' shows a distinct pattern of veinal chlorosis limited to one or two leaves while 'TMG-1' remains virus free. I provide evidence that resistance to ZYMV in 'Dina-1' plants is developmentally regulated, occurs at the level of phloem loading or unloading, and that the amino terminus (NT) of the ZYMV coat protein (CP) is involved in the veinal chlorosis response.

The role of the highly variable NT of the CP also was investigated in systemic infection and host range determination of potyviruses. Chimeric ZYMV infectious constructs were made containing the NT of the CP of watermelon mosaic virus (having overlapping host range with ZYMV), and tobacco etch virus (having non-overlapping host range with ZYMV). Evidence indicated that despite substantial variability in the length and sequence, the NTs of CPs of heterologous potyviruses could facilitate systemic infection of ZYMV in susceptible cucurbit hosts. However, this substitution per se was not sufficient to modify the host range of the virus. The CP-NT from a non-

cucurbit potyvirus triggered a host defense response in cucurbits resulting in recovery from systemic virus infection.

Finally I characterized the interaction of a cucumber poly(A) binding protein (PABP), with the RNA dependent RNA polymerase (RdRp) of ZYMV, and the RdRps other viruses. My data suggest that the carboxy terminal end of the PABP is essential for interaction with the ZYMV-RdRp, but the domains of the RdRp gene involved in this interaction are more complex. The cucumber PABP also interacted with bean common mosaic necrosis potyvirus and cowpea chlorotic mottle bromovirus but failed to interact with certain other potyviruses.

I dedicate this work to the people who I left behind during this journey.

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CC	companion cell
CP	coat protein
CT	carboxy terminus
NT	amino terminus
PD	plasmodesmata
PTGS	post-transcriptional gene silencing
SE	sieve elements

CHAPTER I

REVIEW OF LITERATURE

Potyviruses constitute the largest group of plant viruses and cause economic losses in all major crops of the world (Shukla et al., 1994). In nature, potyviruses are predominantly transmitted by aphids, but they can also be transmitted by mechanical means, and in some cases through infected seeds (Shukla et al., 1994; Ding et al 1992; Maule and Wang, 1996). Potyviral genomes consist of a single positive sense RNA molecule, ca.10kb in size, with a covalently attached viral protein (VPg) at the 5' end and a poly(A) tail encoded in the viral genome at the 3' end (Figure 1). The RNA is encapsidated by ca. 2000 coat protein monomers to form a 750nm long flexuous rod shaped particle (Shukla et al., 1994). The whole genome is translated into a single polyprotein which is subsequently cleaved into nine or more proteins by viral-encoded proteinases (Dougherty and Semler, 1993; Riechmann et al., 1992), many of which are multifunctional (Figure 1).

Functions that have been assigned to the different potyviral proteins are summarized in Figure 1. The P1 protein is a proteinase involved in processing the 5' end of the polyprotein: the second protein HC-Pro, was first named as helper component (HC) for its role in facilitating aphid transmission (Pirone and Blanc, 1996). It was later found to also act as a proteinase (Pro) that cleaves itself at the carboxy terminus. The HC-Pro also has been shown to be involved in symptom expression, genome amplification, and virus

10kb positive sense single stranded RNA

Protein	Function
P1	Proteinase; terminal step in polyprotein processing
HC-Pro	Polyprotein processing; aphid transmission; cell to cell and systemic movement; suppression of gene silencing
P3	Genome amplification
6K1	Unknown
CI	Genome replication; RNA helicase; cell to cell movement
6K2	Membrane localization
VPg	Genome replication; virus movement
NIa	Proteinase; polyprotein processing
NIb	RNA dependent RNA polymerase; genome replication
CP	RNA encapsidation; aphid transmission; cell to cell and systemic movement

Sources: Shukla et al., 1994; Cronin et al., 1995; Rojas et al., 1997; Brigneti et al., 1998; Kasschau and Carrington, 1998; Fernandez et al., 1995; Carrington et al, 1998; Schaad et al, 1997; Nicolas et al., 1997; Dolja et al., 1994, 1995; Rojas et al., 1997.

Figure 1. The potyvirus genome. Individual components of the polyprotein are shown on top of the 10kb genome and their functions indicated.

movement, and more recently it has been found to play a critical role in countering the host defense mechanism of virus induced gene silencing by acting as a suppressor of gene silencing (Shukla et al., 1994; Cronin et al., 1995; Rojas et al., 1997; Brigneti et al., 1998; Kasschau and Carrington, 1998). P3 protein is involved in genome amplification (Meritus et al., 1999). The 6K1 protein has yet to be assigned a function and the 6K2 protein is involved in membrane localization of the virus replicase (Schaad et al., 1997). The cytoplasmic inclusion (CI) protein forms characteristic pinwheel shaped inclusion bodies in the cytoplasm. It has been shown to have RNA helicase activity and also to be involved in cell to cell movement and genome replication (Shukla et al.,1994; Fernandez et al., 1996; Carrington et al, 1998). The VPg is a 21KDa protein covalently linked to the 5' end of the viral RNA and has roles in virus replication, translation and movement (Shukla et al., 1994; Schaad et al, 1997; Nicolas et al., 1997). The NIa protein is the major viral proteinase and processes several proteins at the 3' end of the polyprotein. The NIb is an RNA-dependent RNA polymerase involved in viral replication. Both NIa and NIb derive their names from the formation of inclusion bodies (NI) in the nucleus of infected plant cells (Shukla et al., 1994). Finally the potyviral coat protein (CP) is involved in encapsidation of the viral RNA, aphid transmission, and virus movement (Jagadish et al., 1993; Pirone and Blanc, 1993; Dolja et al., 1994, 1995; Rojas et al., 1997).

The viral proteins described above, allow for successful infection of a host plant via translation and replication in the initially infected cells, cell-to- cell movement through plasmodesmata (PD), and systemic movement of the virus through the phloem (Lucas

and Gilbertson, 1994; Carrington et al., 1996; Lucas et al., 1996). Host resistance responses can interfere with the virus life cycle at one or more of these points. However, the mechanisms of host resistance to viruses in general, and potyviruses in particular, are not well characterized. Host range determinants of potyviruses also are not known.

In this project I sought to address questions concerning mechanisms of resistance to potyviruses, and to examine viral factors influencing systemic infection and host range determination. I characterized resistance to zucchini yellow mosaic virus (ZYMV) at the *zym* locus in cucumbers and studied the role of potyviral coat protein (CP) in systemic infection and host range determination. Therefore, this review will focus on recent literature concerning the role of: 1. Potyviral proteins involved in viral movement, 2. Host resistance to potyvirus infection including resistance to potyviruses in cucurbits, and 3. Host range determination of potyviruses.

Several reviews covering related aspects of plant virus interaction are available. Readers are referred to Fraser (1990) and Provvidenti and Hampton (1992) for genetics of resistance to potyviruses; to Leisner and Turgeon (1993), Lucas and Gilbertson, (1994), Carrington et al. (1996), Lucas et al. (1996), and Soren and Haenni (1996) for plant virus movement; to Revers et al. (1999) for plant potyvirus interaction, and Dawson and Hilf (1992) for host range determination of plant viruses.

Throughout the literature review, references will be made to numerous viruses. A list of abbreviations is shown in Table 1.

Table 1. List of virus abbreviation and their full names used in this text.

Abbreviation	Full name
BCMV	Bean common mosaic virus
BCMNV	Bean common mosaic necrosis virus
BMV	Brome mosaic virus
CMV	Cucumber mosaic virus
CCMV	Cowpea chlorotic mottle virus
LMV	Lettuce mosaic virus
PepMoV	Pepper mottle virus
PRSV	Papaya ring spot virus
PSbMV	Pea seedborne mosaic virus
PVA	Potato virus A
PVV	Potato virus V
PVY	Potato virus Y
SCMV	Sugarcane mosaic virus
SPFMV	Sweet potato feathery mottle virus
TEV	Tobacco etch virus
TuMV	Turnip mosaic virus
TVMV	Tobacco vein mottling virus
WMV	Watermelon mosaic virus
ZYMV	Zucchini yellow mosaic virus

The role of potyviral proteins in cell to cell and systemic movement.

Most plant viruses move cell to cell through plamsodesmata (PD) and long distance through phloem, and so can encounter a number of barriers during the course of movement. PD maintain cytoplasmic continuity between adjacent cells and consist of a central region occupied by an appressed form of endoplasmic reticulum (ER) which is continuous with the ER of adjoining cells (Lucas, 1995). The space between the ER and plasma membrane, called the cytoplasmic annulus, contains a number of proteins and is the site for transport of metabolites and proteins. The number, structure and function of PD may vary between different plant tissues and cell types. Most PD have size exclusion limits of 800-1000da (Tucker, 1982; Robards and Lucas, 1990), which does not permit passage of large macromolecules. Certain plant proteins that traffic through the PD interact with the PD proteins to induce an increase in size exclusion limit (SEL) and facilitate their movement into the sieve element (Balachandran et al., 1997; Beatriz et al 1999).

Plant viral movement proteins (MP) also increase the PD SEL to facilitate their own movement as well as movement of viral RNA and virions (Lucas et al., 1996; Carrington et al., 1996). Potyviruses do not encode a specific movement protein; instead four multifunctional proteins i.e. HC-Pro, CI protein, VPg and CP, have been shown to facilitate cell to cell and long distance viral movement (Revers et al., 1999). Each will be discussed in turn.

HC-Pro has been implicated in both cell to cell and long distance movement, and separate domains of the protein have been shown to be involved in facilitating each type of movement (Rojas et al., 1997; Cronin et al., 1995). In microinjection studies, wild

type HC-Pro increased the PD SEL and facilitated its own movement as well as movement of viral RNA (Rojas et al., 1997). Deletions in the C-terminal part of HC-Pro affected cell to cell movement. A mutation in the central region of HC-Pro, however, inactivated the long distance movement of TEV but had little effect on the genome amplification and cell to cell movement (Cronin et al., 1995). In situ histochemical analysis revealed that the mutant virus was capable of infecting mesophyll, bundle sheath and phloem cells within the inoculated leaves, suggesting that the long distance movement block was associated with entry into or exit from sieve elements. Grafting experiments have shown that HC-Pro is required for both entry into and exit from the phloem (Kasschau et al., 1997). TEV HC-Pro was required in both stock and scion tissues for systemic virus infection to occur.

Rodriguez-Cerezo et al. (1997), observed CI protein of potyviruses associated with connections between plant cells at early stages of infection suggesting that CI might also be involved in the virus movement. Alanine scanning mutagenesis of the CI gene in TEV identified two mutants with substitutions in the N-terminal region of the gene, which amplified to the level of the parental virus but were restricted to single cells in the inoculated leaves (Carrington et al, 1998). Several other CI mutants showed either a slow cell to cell movement or were unable to move long distance through the vasculature. Cells across from an advancing infection front in pea cotyledons infected by PSbMV contained CI and CP, providing further evidence for the role of the CI protein in virus movement (Roberts et al., 1998). In microinjection studies, however, the BCMNV CI protein did not exhibit viral movement protein properties (Rojas et al., 1996). Carrington

et al. (1998), have suggested that the CI protein may direct intracellular translocation of a viral transport complex into plasmodesmata.

The genome linked viral protein (VPg) facilitates long distance movement of the virus. Schaad et al. (1997), made chimeric constructs between TEV-HAT and TEV-Oxnard, two strains of tobacco etch virus (TEV) that show differential response on tobacco cultivar V20. Both strains can replicate and move cell to cell in V20, but only TEV-Oxnard shows systemic infection. Full length cDNA constructs of TEV-HAT containing the CP, HC-Pro or both CP and HC-Pro of TEV-Oxnard were not able to cause systemic infection. However, the chimeric constructs containing the VPg of TEV-Oxnard were able to cause systemic infection thereby suggesting a possible role for VPg in systemic infection. VPg of TVMV-S is responsible for breaking this *va* gene-mediated resistance in tobacco cultivar TN86 which also occurs at the level of movement (Gibb et al., 1989; Nicolas et al., 1997). Virus titer and symptoms produced by TVMV-S in TN86 are identical to those produced on susceptible cultivars. When chimeric constructs were made between the two strains, resistance breaking properties were localized to four amino acids in the VPg gene indicating that this gene is required for virus movement. The *va* gene in tobacco also confers resistance to PVY at the level of movement. A single aa change in the VPg correlated with the resistance breaking in virgin A mutant (VAM) tobacco containing the *va* gene (Masuta et al., 1999).

The potyviral CP is involved in both cell to cell and vascular movement (Dolja et al., 1994, 1995; Rojas et al., 1997). The core portion of the CP is highly conserved among potyviruses while the amino- and carboxy terminal (NT and CT) portions are variable in length and sequence (Shukla et al., 1994). Both NT and CT are exposed on

the surface of virions and are not essential for virion assembly. The conserved core portion is necessary for both encapsidation and cell to cell movement of the virus (Dolja et al., 1995, Rojas et al., 1997). Amino acid substitution in the core region affected cell to cell movement of TEV in tobacco plants (Dolja et al., 1995). In microinjection experiments wild type CP of BCMNV and LMV increased the PD SEL and facilitated transport of viral RNA, while amino acid substitutions in the core region either impaired or abolished cell to cell movement (Rojas et al., 1997).

Deletion of the NT and CT of the CP abolished the systemic movement of TEV (Dolja et al., 1994, 1995). Amino acid substitutions in the NT of the CP have variable effects on virus movement depending upon the particular amino acid being substituted. The conserved DAG motif has been studied most extensively due to its role in aphid transmission (Lopez-Moya and Pirone, 1998; Gal-On et al., 1992; Atreya et al., 1991). When Lys or Arg were substituted for Asp in the DAG motif of TVMV, the mutant viruses failed to move systemically in tobacco plants but replicated and produced virions in the protoplasts (Lopez-Moya and Pirone, 1998). TEV mutants with similar substitutions for Asp in the two DAG motifs in the CP-NT also failed to infect tobacco plants systemically, suggesting that besides its role in the aphid transmission, this motif is also involved in the systemic virus movement (Lopez-Moya and Pirone, 1998). However, substitution of Ala for Thr (DAG to DTG) in the NT of CP of ZYMV-NAA and Gly for Glu (DAG to DAE) in CP-NT of TVMV (Gal-On et al., 1992; Atreya et al., 1991) had no effect on accumulation of the virus in systemic leaves. Both these mutations are associated with the loss of aphid transmissibility and occur when the virus is maintained by rub inoculaton rather than aphid transmission. In each case aphid

transmissibility was regained after the conserved DAG motif was restored (Gal-On et al., 1992; Atreya et al., 1990, 1991). A reduction in the accumulation of PVA was associated with mutation of DAS to DAG in the conserved motif, but there was no effect on the systemic movement of the virus (Andrejeva et al., 1999). Andersen and Johansen, (1998), observed that a single amino acid substitution (Ser 47 to Pro) in the NT of the CP of PSbMV was sufficient to permit systemic spread in *C. quinoa*, suggesting that this region of the CP might also be involved in systemic movement in strain and host specific manner.

Host resistance mechanisms

Plants have evolved a number of mechanisms to protect themselves against virus infection, ranging from complete immunity against virus infection, to restriction of virus movement (White and Antoniw, 1991; Dawson and Hilf, 1992; Pennazio et al., 1999). Phenotypic responses of plants to virus infection vary from systemic symptoms in susceptible plants, to recovery from initial infection, to a hypersensitive response resulting in necrotic or chlorotic lesions at the site of infection, or a lack of symptoms due to failure of the virus to, replicate in or move from the initially infected cells. Certain plants tolerate virus infection without any showing visible symptoms even though the virus is present at detectable levels in systemic tissues. A number of resistance genes have been identified that act at various points in the virus life cycle preventing either virus replication, cell to cell spread, or systemic movement (Fraser, 1990; Provvidenti and Hampton, 1992; Dawson and Hilf, 1992)

Plant protoplasts provide a useful tool to distinguish between resistance genes operating at the level of movement or replication. Inability of the virus to accumulate in the protoplasts of resistant plants suggests that resistance is occurring at the level of replication (or translation) of the viral genome. On the other hand, virus accumulation in the protoplasts of resistant plants that do not show detectable symptoms and virus accumulation in the inoculated leaves indicates a block at the level of cell to cell movement. For example, viral coat protein or RNA could not be detected in the protoplasts of homozygous recessive *sbm-1* peas transfected with the P-1 isolate of PSbMV, suggesting that resistance is occurring at the level of replication (Keller et al., 1998). Similarly, the resistance conferred by the single recessive gene (et^a) in *Capsicum annum* cv. Dempsey to TEV, is due to interference with virus RNA accumulation (Deom et al., 1997). Infection foci were not detected in the inoculated leaves when 'Dempsey' plants were inoculated with TEV-GUS, and northern blot analysis of protoplasts inoculated with TEV RNA did not show virus accumulation. Interference with the accumulation of RNA of PepMoV and TEV-HAT in plants and protoplasts has also been reported in two accessions of *Capsicum chinense* containing the *pvr1* gene.

In other cases virus replication was observed in protoplasts indicating that resistance is occurring at the level of virus movement. For example in the homozymous state, the y^a ($pr2^1$) gene of pepper plants (cultivar Yolo Y) restricts cell to cell movement of PVY (pathotype 0: Arryo et al., 1996). When protoplasts from healthy Yolo Y plants were inoculated with PVY-0 a high percentage of protoplasts (80-85%) showed virus accumulation. PVY-0 was not detected (with ELISA) in the inoculated or systemic leaves, and when protoplasts were prepared from inoculated leaves, only a small number

of protoplasts (0.06%) showed immunoflorescence. The *va* gene in tobacco (*N. tabacum*) appears to restrict cell to cell movement of potato virus *Y* (PVY) and tobacco vein mottling virus (TVMV) (Masuta et al., 1999; Gibb et al., 1989). Both the viruses replicate in the protoplasts of resistant plants. In resistant tobacco cultivar TN86 (containing the *va* gene) TVMV is restricted to a few inoculated cells or small groups of cells in the epidermal strips from inoculated leaves (Gibb et al., 1989). Virus accumulation was not detected in the mesophyll cells by immunostaining or ELISA, suggesting that resistance in TN86 occurs primarily due to restriction of virus cell to cell movement. PVY also was not detected in the inoculated leaves of tobacco cultivar VAM (containing the *va* gene: Masuta et al., 1999). A reduction (ca. 30%) in virus replication in the VAM protoplasts compared to protoplasts from susceptible plants was observed indicating that impairment of replication also contributes to the resistance.

Resistance to long distance movement can occur at the level of phloem loading or unloading resulting in reduced or no virus accumulation in systemic tissues. The structure and number of plamsmodesmata connecting sieve element-companion cells (SE-CC) and those connecting mesophyll cells, differ suggesting that the viruses encounter different levels of barriers in their cell to cell and long distance movement (Leisner and Turgeon, 1993; Lucas et al., 1996; Carrington et al., 1996; Soren and Haenni 1996; Mclean et al., 1997; Sjolund, 1997). Studies with phloem loading from inoculated leaves of several plant species showed that among the cells surrounding sieve elements in the minor veins, vascular parenchyma cells were the predominant cells to become visibly infected with poty- and tobamoviruses (Ding et al., 1998). It was observed that a barrier exists for virus entry into mature smooth walled companion or

transfer cells. Viral proteins (or domains of the same protein) involved in the cell to cell and long distance movement (e.g. CP and HC-Pro) also differ, suggesting different levels of interactions for the long distance vs. cell to cell movement (Carrington et al., 1996; Revers et al., 1999).

Several cases of resistance to potyviruses occurring at the level of systemic movement have been reported (Murphy and Kyle 1995; Schaad and Carrington 1996; Hinrichs et al., 1998; Mahajan et al, 1998). For example use of an infectious TEV clone engineered to express GUS gene showed that the rate of replication and cell to cell movement was nearly identical in tobacco line V20 showing strain-specific resistance to TEV and the susceptible line Havana 425, however, systemic movement was markedly restricted in the resistant line (Schaad and Carrington, 1996). Immunocytochemical analysis of V20 tissues from infection foci indicated that the block in the long-distance movement was associated with entry into or exit from the sieve elements. Similarly, PVY and TEV-GUS replicated in, and moved cell to cell in a resistant tobacco cultivar containing the RY_{sto} resistance gene, but systemic infection of both viruses was prevented by a resistance reaction that also resulted in necrotic streaks on some cultivars (Hinrichs et al., 1998). In another example, Murphy and Kyle (1995), examined the accumulation and movement of pepper mottle potyvirus (PepMoV) in the resistant *Capsicum annum* L. genotype 'Avelar', using immunotissue blot analysis. Although the viral antigen was detectable in the stem below the inoculated leaves and in the first internode above the inoculated leaves of the resistant genotype, virus was not detected in the first pair of uninoculated leaves, even at 25 days post inoculation. Inoculated leaves also showed reduced accumulation of virus compared to the susceptible control, thus although the

resistance appeared to be operating at the level of movement, intereference with replication may also play a role. Finally, Mahajan et al. (1998) identified a dominant locus conditioning the restricted systemic movement of TEV (*RTM*) in the *Arabidopsis* ecotype Colombia. They proposed that *RTM1* mediates a restriction of long distance movement through a mechanism that differs substantially from those conditioned by the dominant genes associated with gene for gene interaction. Characterization of the gain of susceptibility mutants with *RTM1* suppressed phenotypes revealed that at least two loci *RTM1* and *RTM2* cooperate to condition a restricted TEV movement phenotype (Witham et al., 1999). Interestingly, the predicted amino acid sequence of the *RTM1* gene shows homology to a family of proteins involved in defense against viruses, fungi and insects (Chisholm et al., 2000).

Hypersensitive response (HR) is another type of plant defense observed in response to a large number of viruses (Ponz and Bruening, 1986; White and Antoniw, 1991; Brunt et al., 1996). An HR restricts virus movement from the initial infection foci, and results in the appearance of necrotic or chlorotic local lesions at the site of infection (White and Antoniw, 1991). Local lesions are associated with an induced resistance response and expression of pathogenesis related proteins. Most potyviruses have experimental local lesion hosts (Brunt el al., 1996), but reports of specific hypersensitivity resistance genes for potyviruses are less common. Several wild potato species, and cultivars have been reported to show HR response to PVY, PVV and TEV (Valkonen, 1997; Barker, 1997). For example, when potato cultivars containing the *Nv* gene are graft inoculated with PVV, necrotic lesions and necrotic streaks in the veins develop. The HR response eventually leads to death of the shoot tips and severe necrosis

leading to the collapse of the lower leaves (Barker, 1997). The RY_{sto} gene, which also confers resistance to a number of potyviruses in potato plants restricts movement and accumulation of TEV and PVY through a hypersensitive response-like mechanism (Hinrichs et al., 1998). However, unlike the typical HR for viruses, where individual local lesions are produced, necrotic lesions were observed along the veins when the lower leaf surface was inoculated, but were either absent or their number significantly reduced when the upper surface of the leaf was inoculated. In bean (*Phaseolus vulgaris*), the *I* gene has been demonstrated to confer resistance to a wide range potyviruses through a hypersensitive response (Kyle and Provvidenti, 1993; Scully et al., 1995).

Recovery from successful initial infection is found in response to infection by a number of viruses, including some potyviruses (reviewed by Pennazio et al., 1999). Strong initial symptoms are followed by a gradual reduction in symptom severity and virus accumulation. The recovered plants are resistant to secondary infection by other strains of the same virus or homologous viruses and show phenotypic similarities to post transcriptional gene silencing (PTGS: Matzke and Matzke, 1995; Depicker and Montago, 1997). In transgenic plants showing PTGS, only low levels of transgene RNA could be detected, even though transcription occurs at a relatively high rate. Recent evidence has confirmed that PTGS-like host resistance is a generalized defense mechanism in plants and neither recovery nor plant genome homology is essential for induction of this defense mechanism (Ratcliff et al., 1999). Small species of antisense RNA directed against the transgene RNA in the case of PTGS, and against viral genome in the case of virus induced gene silencing (VIGS) have been observed (Hamilton and Baulcombe, 1999). As a counter defense strategy, a number of diverse plant viruses (e.g. cucumoviruses,

comoviruses, geminiviruses, potyviruses, tobamoviruses) encode proteins that suppress PTGS-like resistance response of host plants against virus infection (Voinet et al., 1999). In potyviruses the HC-Pro has been demonstrated to act as suppressor of PTGS (Brigneti et al., 1998, Anandalakshmi et al., 1998; Pruss et al., 1997; Voinet et al., 1999).

Cucurbit resistance to potyviruses.

In cucurbits, various sources of resistance to potyviruses have been identified (Provvidenti 1985,1987; Gilbert-Albertini et al, 1993; Gibb et al., 1994; Kableka et al., 1997), but in most cases the mechanisms of resistance have not been well characterized. Interestingly, in several cases differences in response have been observed depending on whether cotyledons or leaves were inoculated. The basis for this difference is not yet understood. Resistance to the watermelon strain of PRSV in the muskmelon (*Cucumis melo*) cultivars 'Cinco' and 'Cinbo' occured at the level of movement (Gibb et al., 1994); only 3-7 cells were infected in the inoculated leaves. Cultivar 'Cinco' was highly resistant and only occasionally showed mild symptoms when the cotyledons were inoculated, however, when cotyledons of 'Cinbo' were inoculated mild systemic symptoms were observed but plants remained symptomless when true leaves were inoculated. Resistance to WMV in cucumber (*C. sativus*) line 'TMG-1' involves two or more resistance factors with tissue specific expression, one expressed in both cotyledons and leaves and the other expressed only in the leaves (Wai and Grumet, 1995b). Limited systemic movement of WMV and ZYMV to the first one or two leaves has also been observed in *Cucurbita moschata* line Menina 15 (Gilbert-Albertini et al, 1993) following cotyledon inoculation.

In cucumbers two inbred lines, 'Dina-1' and 'TMG-1', exhibit resistance to an array of potyviruses including ZYMV, WMV, Moroccan watermelon mosaic virus (M-WMV) and the water melon strain of papaya ringspot virus (PRSV-W: Provvidenti, 1987, 1985; Kableka et al 1997; Wai and Grumet, 1995a). Both 'Dina-1' and 'TMG-1' contain a single recessive gene for resistance to ZYMV, but show different responses following cotyledon inoculation. Limited spread of ZYMV was observed when the cotyledons of young 'Dina-1' (ca. 7 days old) plants were inoculated, resulting in a distinct pattern of veinal chlorosis limited to the first or second systemic leaf while the rest of the plant remained symptom free (Kabelka et al., 1997). Virus accumulation was detected in the cotyledons and the chlorotic leaf but not in the upper non-symptomatic leaves. The plants remained symptomless when true leaves were inoculated. TMG-1, however, did not show veinal chlorosis following cotyledon inoculation and systemic spread of the virus to the first leaf was only rarely observed when young plants are inoculated on the cotyledons. Both the genotypes did not show symptoms following true leaf inoculation.

Non-hosts and host range determinants.

Many potviruses have very restricted host ranges, however, several have intermediate host ranges and a few infect a wide range of host plants (Shukla et al., 1994). In non-host species the virus is either unable to translate its genome, replicate in, or move from the initially infected cells. The host range determinants of potyviruses are poorly understood, and it is likely that several genes are involved depending on the nature of block to virus infection (e.g. replication vs. movement). Virus replication has been shown to be the target of non-host resistance against some potyviruses. For example,

Bak et al. (1998) observed that the non-host resistances to PSbMV in tobacco and to PVY in pea operate at the single cell level. Both viruses were unable to replicate in the protoplasts of the non-host plants.

Inability to cause systemic infection also can determine host range, e.g. local lesion hosts of viruses allow for initial virus replication, but prevent systemic spread. Because of the extreme variability in the amino terminal portion of the potyviral CP and its role in systemic movement, it has been suggested that the CP may play a role in host range determination (Shukla et al., 1994; Ward et al., 1994). Xiao et al. (1993) observed a correlation between duplication of amino acids (aa) residues in the amino terminus (NT) of the CP and host range of several isolates of SCMV. Evidence for partial gene duplication in the NT of CP was also found for most potyviruses that had CPs of 287 aa residues or longer (Ward and Shukla 1993; Ward et al., 1994). Based on these observations, Ward et al. (1994), suggested that variability in the CP-NT may have been an important factor in the evolution of new species of potyviruses with new host and vector specificities. However, the role of CP-NT in host range determination of potyviruses has not been tested directly.

Research objectives for this project.

It is obvious from this review of literature that potyvirus-host plant interaction is very complex and requires different levels of interactions between viral and host factors at different stages of infection. Only a few components of this interaction, mainly on the virus side have been identified and numerous questions remain unanswered. For example, how the different viral components interact with each other and with host

components to accomplish multiple tasks is not known. Highly conserved and extremely variable regions in the potyvirus genome have been identified, and functions of several conserved regions have been demonstrated, but the significance of variability is still speculative. Identification and characterization of host factors involved in virus infection have only recently become the focus of plant virus research. In most cases, host resistance mechanisms are not well characterized, and the determinants of host specificity also are poorly understood. The purpose of this dissertation was to address some of these issues using zucchini yellow mosaic virus with the following specific objectives.

Objectives for chapter 1.

Before this project was initiated, the cucumber lines 'Dina-1' and 'TMG-1' were reported as sources of resistance to a number of potyviruses including ZYMV. Different phenotypic responses of the two genotypes to inoculation with ZYMV were observed in our lab (Kableka et al., 1997). 'Dina-1' remained free of symptoms while 'TMG-1' showed veinal chlorosis limited to a single leaf. These two responses were shown to be due to different alleles at the same locus (Kableka et al., 1997). It remained to be investigated why initial systemic infection occurs in 'Dina-1' and why further systemic spread of the virus is then restricted. Is the virus localized to the veinal regions in chlorotic leaves? Why does inoculation of true leaves not result in a similar phenotypic response? Why do certain strains of the virus fail to show veinal chlorosis on 'Dina-1', and what are the viral factors involved in the veinal chlorosis response? These questions and several others formed the basis of the first chapter of my dissertation research.

Objectives for chapter 2.

The role of the amino terminus (NT) of the coat protein (CP) of potyviruses in systemic movement was previously demonstrated, and based on extreme variability in the NT, its role in role in host range determination was suggested (Dolja et al., 1994, 1995; Shukla et al., 1994). However the significance of variability in the CP-NT has not been established and its role in host range determination was not tested directly. In the second part of my dissertation project I focused on further understanding the role of the NT of the CP in potyvirus infection, particularly in facilitating systemic infection and host range determination by making chimeric infectious constructs of ZYMV. The role of the core portion of the CP and the whole CP in host range determination of potyvirses was also investigated.

Objectives for chapter 3.

An understanding of the role of the host factors in facilitating virus infection is one of the main focuses of current virus research. Our lab has initiated projects to identify host proteins interacting with virus proteins in an effort to better understand potyvirus infection in susceptible hosts. We have demonstrated that a poly(A) binding protein (Cs-PABP1) from susceptible cucumber host interacts with the RNA dependent RNA polymerase (NIb) gene of ZYMV (Wang et al., submitted). I worked on an additional project to further characterize this interaction as well as to study the interaction of the Cs-PABP1 with RdRp from an array of other viruses.

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CHAPTER II

RESISTANCE TO ZUCCHINI YELLOW MOSAIC VIRUS AT THE *zym* LOCUS IN CUCUMBER: LOCALIZATION OF VIRUS TO THE VEINAL REGIONS AND THE ROLE OF COAT PROTEIN IN VEINAL CHLOROSIS

ABSTRACT

The *zym* locus in cucumber (*Cucumis sativus* L.) is marked by multiple alleles conferring resistance to zucchini yellow mosaic potyvirus (ZYMV) and is tightly associated (< 1 cM) with resistance to at least three other potyviruses. One allele, zym^{Dina}, from the inbred line 'Dina-1', confers an unusual phenotype that was studied to gain insight into the mechanism of resistance. Following inoculation of cotyledons, 'Dina-1' plants showed a distinct pattern of veinal chlorosis limited to one or two leaves; the rest of the plant remained symptom free. Inoculation of leaves generally did not result in veinal chlorosis or virus accumulation in systemic leaves. Immunoblot analyses indicated that the veinal chlorosis phenotype in 'Dina-1' plants reflected virus distribution within the leaf. Like in susceptible control plants, ZYMV moved from the inoculated coytledons to the predominant sink at the time; however, unlike in susceptible plants, the virus in the sink leaf remained localized to the veinal regions up to 30 days post inoculation. Cotyledon removal experiments indicated that the inability of the virus to spread within

the leaf exhibiting veinal chlorosis, or to establish infection in leaves, was not due to an inability to replicate in the leaves. The pattern of virus accumulation along the veins suggested a block in the ability of the virus to load into or unload from the phloem. Production of chimeric viruses with switches in either the amino- or the central and carboxy-terminal portions of the ZYMV coat protein (CP) showed that the amino terminus of the CP appeared to play a role in the veinal chlorosis response. This is further evidence suggesting that resistance in 'Dina-1' operates at the level of systemic movement as the CP-NT is involved in systemic virus movement. In contrast to the greenhouse, where inoculation of leaves generally did not cause veinal chlorosis, veinal chlorosis was observed with leaf inoculation in the growth chamber. Inoculation of the first, but not the second, leaf resulted in a high percentage of plants showing veinal chlorosis, indicating developmental control over expression of the resistance factor(s). Together these observations suggest that resistance in 'Dina-1' is developmentally regulated and occurs at the level of phloem loading or unloading in the leaves.

INTRODUCTION

Potyviruses form the largest and economically most important group of plant viruses; almost all the major crops of the world are infected by one if not several members of the *Potyviridae* (Shukla et al., 1994). Naturally occurring sources of resistance to potyviruses have been identified in a large number of plant species and can interfere with virus replication, cell-to-cell spread, or systemic infection (Providentii and Hampton, 1992; Carrington et al., 1996, Revers et al., 1999). Examples of interference with replication include the recessive *sbm-1* allele of pea (*Pisum sativum* L.) which

prevents replication of pea seed borne mosaic potyvirus (PSbMV: Keller et al., 1998), and the et^a allele of pepper (*Capsicum annum* cv Demsey) which interferes with accumulation of tobacco etch potyvirus (TEV) RNA (Deom et al., 1997). Interference with the accumulation of RNA of pepper mottle virus (PepMoV) and TEV has also been reported in plants and islolated protoplasts of two *Capsicum chinense* accessions containing the recessive *pvr1* resistance gene (Murphy et al., 1998).

Cell-to-cell movement of potato virus Y (PVY) is restricted by the y^a gene in pepper plants (Arryo et al., 1996) and the *va* gene in tobacco (*N. tabacum*) plants, which also restricts cell to cell movement of tobacco vein mottling virus (TVMV: Masuta et al., 1999; Gibb et al., 1989). The *Arabidopsis* ecotype Bay-0 is resistant to turnip mosaic potyvirus via interference with cell to cell movement (Martin et al., 1998). In the resistant tobacco line V20, long distance movement of TEV is restricted at the level of entry into or exit from the sieve elements (Schaad and Carrington, 1996). Tobacco cultivars containing the RY_{sto} gene also restrict systemic movement of TEV and PVY (Hinrichs et al., 1998) and in *Arabidopsis* two loci, *RTM1* and *RTM2*, cooperate to condition a restricted long distance movement phenotype of TEV (Mahajan et al., 1998; Witham et al., 1999). The *RTM1* protein showed similarity to a group of plant proteins that are implicated in defense against viruses, fungi and insects (Chisholm et al., 2000).

Other types of resistances to potyviruses include hypersensitive reaction response and recovery from initial infection (White and Antinow, 1991; Pennazio et al., 1999). Examples of resistance genes conferring hypersensitive response to potyvirus infection include the *I* gene of beans (*Physiolus vulgaris*) to multiple viruses (Kyle and Provvidenti, 1993; Scully et al., 1995), the *Nv* gene of potato to PVV, (Barker, 1997) and

the RY_{sto} gene of potato to TEV and PVY (Hinrichs et al., 1998). Recovery of plants from initial virus infection is a host defense response resulting in a gradual reduction in symptom severity, virus accumulation, and protection against secondary infection by homologous viruses. The mechanism of recovery of host plants from virus infection show homology to post transcriptional gene silencing (PTGS) and both responses are associated with the appearance of small species of antisense RNA directed against the transgene RNA in the case of PTGS, and against viral genome in the case of virus induced gene silencing (Hamilton and Baulcombe, 1999; Ratcliff et al., 1999; Pennazio et al., 1999).

In a few cases, specific potyviral factors have been shown to play a role in stimulating or overcoming host resistance, presumably through interaction with host resistance factors. For example, the VPg gene of potyviruses is involved in overcoming host resistances operating at the level of replication (Keller et al., 1998) and movement (Nicolas et al., 1997; Schaad et al., 1997; Masuta et al., 1999; Rajamaki and Valkonen, 1999). A single amino acid (aa) substitution (Ser47 to Pro) in amino terminus (NT) of the CP of the NY isolate of pea seed borne mosaic virus (PSbMV), allowed the virus to move systemically in *Chenopodium quinoa* (Andersen and Johansen, 1998). Our studies have shown that the CP-NT of a non-pathogenic potyvirus could induce a resistance response in cucurbits resulting in recovery from systemic virus infection (chapter 3). Solomon (1989) observed cleavage of the NT of the CP of sweet potato feathery mottle virus (SPFMV) in the recovered leaves of *Ipomea nil* plants.

In cucumbers, two sources of naturally occuring resistance to zucchini yellow mosaic potyvirus (ZYMV), have been identified (Provvidenti 1985, 1987; Abul-Hayja

and Al-Shahwan, 1991; Kabelka et al., 1997). Although each is due to a single recessive allele occurring at the same locus, they differ in phenotypic response. One source of resistance comes from 'TMG-1' (zym^{TMG}), an inbred line derived from the Chinese hybrid 'Taichung Mou Gua', that also is resistant to watermelon mosaic virus (WMV), the watermelon strain of papaya ringspot virus (PRSV-W) and Moroccan watermelon mosaic virus (MWMV: Providenti 1985, 1987; Kabelka and Grumet, 1997). The second (zym^{Dina}) has been described in 'Dina-1', an inbred line derived from a Dutch hybrid which is also resistant to above listed viruses, allows for initial infection of cotyledons followed by limited systemic spread (Abul-Hayja and Al-Shahwan, 1991; Kabelka et al., 1997). The *zym* locus is of particular interest because in both 'TMG-1' and 'Dina-1', the *zym* allele appears to be the same as, or tightly linked to genes conferring resistance to MWMV, PRSV-W and WMV (Kabelka and Grumet, 1997; Kabelka et al., 1997; Wai and Grumet, 1995a and b; Grumet et al., in press).

In response to cotyledon inoculation with ZYMV, 'Dina-1' plants show a pronounced pattern of veinal chlorosis that may provide insight into the mechanism of resistance conferred by the *zym* allele. The veinal chlorosis is restricted to leaf-1 or leaf-2 only, while subsequent leaves remain symptom free. Segregation analyses indicate that the veinal chlorosis is not due to a separate gene from the *zym* resistance gene (Kabelka et al., 1997). Although the the veinal chlorosis phenotype was observed with a number of ZYMV isolates of diverse origin, it is unique to inoculation with ZYMV and was not exhibited in response to inoculation with WMV, MWMV, or PRSV (Kabelka et al., 1997).

In this work we sought to understand the mechanism of resistance to ZYMV mediated by the *zym* resistance alleles in 'TMG-1' and 'Dina-1' and to investigate the role of the coat protein of ZYMV in the resistance response. We provide evidence that the veinal chlorosis phenotype of 'Dina-1' is associated with the accumulation and distribution of virus in the symptomatic leaves and that resistance operates at the level of virus movement. We also show that the NT of the CP is involved in the veinal chlorosis phenotype of 'Dina-1' observed in response to inoculation with ZYMV.

MATERIALS AND METHODS

Plant material, virus stock and inoculation.

Plant material: The three cucumber genotypes used in this study are: the susceptible cultivar 'Straight-8' (W. Atlee Burpee and Company, Warminster, Pa), and the resistant inbred lines, 'TMG-1' and 'Dina-1'. 'TMG-1' and 'Dina-1' were initially provided by Dr. J. Staub (USDA, University of Wisconsin, Madison) and Dr. K. Owens (Seminis Peto Seed Company, Woodland, Calfornia), respectively, and subsequently maintained by self pollination in the greenhouse. Plants were grown in the greenhouse in 15cm clay pots using Baccto soil mix. During winter (October to March) the plants received supplemental light (16h day). Seeds were pre-germinated for 24hr at 30C before sowing. Growth chambers were set at 16hr day, 24C day, 20C night temperatures with an average light intensity of 150 micro Einstein.

Experiments were performed using the Connecticut (Ct) isolate of ZYMV (ZYMV-Ct; Provvidenti et al., 1987; Grumet and Fang, 1990) unless otherwise indicated.

Maintenance of virus stock and rub-inoculation procedures were as described in Wai et al. (1995). Plasmid DNA of the infectious constructs (see below) were prepared using the Wizard mini prep kit (Promega Madison, WI) and directly inoculated onto cotyledons of 7-10 days old plants using the particle bombardment procedure of Gal-On et al. (1995). Infected leaf material (passage 0, P0) was stored at –80F and used to inoculate squash plants (P1) which were then used as a source of inoculum for all subsequent experiments. ELISAs (enzyme linked immunossorbant assay) were performed with leaf discs using the procedure of Wai et al. (1995). Polyclonal antibodies prepared to ZYMV-Ct coat protein were used for both ELISA and immunoblot analyses. Randomized complete block designs were used for experiments when applicable.

Immunoblot analyses.

Leaves harvested for immunoblot analyses were frozen at -80C, thawed at room temperature and used to form a sandwich of three layers of: filter paper; a nitrocellulose membrane (Protran, PH79 pore size 0.1um) prewet with distilled water, the leaf, and a single layer of paper towel. The whole sandwich was placed in a plastic sample bag (Nasco WHIRL-PAK 18oz) and rolled through a pasta machine (Atlas, Italy) using pressure setting 4. Immediately after the run, the membrane was removed from the sandwich, placed on a piece of paper towel and allowed to dry at room temperature. The blotted membrane was then developed using the western blot protocol of Blake et al., (1984).

Making chimeric ZYMV infectious constructs.

The full length infectious clone of ZYMV-NAA was kindly provided by Drs. A. Gal-On and B. Raccah (Volcani Center, Bet Dagan, Israel). The amino terminus (NT) of the CP of the infectious ZYMV-NAA construct was substituted with the CP-NT of ZYMV-Ct (Grumet and Fang, 1990) by cloning the *SacI-MluI* fragment from pCtCPSPX (containing the CP of ZYMV-Ct, Chapter 3) into the infectious full length ZYMV-NAA construct digested with the same enzymes. The core and CT portions of the CP were exchanged between the two constructs using the restriction sites of *MluI* and *AvrII*. *MluI* is a unique restriction site near the start of the core region of the CP of ZYMV (NAA and Ct). *AvrII* is a unique site in the 3' NTR and *SacI* restriction is unique in the NIb gene of ZYMV-NAA infectious construct.

Cotyledon removal experiments.

To test virus replication in the leaves, 7 day old plants were inoculated on their cotyledons. On successive days after inoculation (1-5 dpi), the cotyledons and first leaf (when present) of five plants were sampled for ELISA. The cotyledons were then removed from the stem with a razor blade. Two weeks post inoculation, the first and second true leaves of all the plants were sampled for ELISA. For immunoblot experiments, seven day old 'Dina-1' plants were inoculated on the cotyledons with ZYMV-Ct. Both the cotyledons and one half of the first true leaf were removed from five plants at 3, 4, and 5 dpi and stored at –80F. Ten days post inoculation (after the appearance of the veinal chlorosis symptoms) the intact halves of the first leaves were harvested for immunoblotting as described earlier. Both halves of each leaf were

immunoblotted and probed at the same time. Healthy 'Dina-1' plants were used as negative controls for each experiment while positive controls included plants from which cotyledons were not removed after inoculation.

Sequential inoculation experiments.

'Dina-1' plants were inoculated on the cotyledons with ZYMV-Ct and ZYMV-NAA when seven days old. Two to three weeks post inoculation (depending on growth conditions during individual experiments) one half of leaf-4 or leaf-5 from ZYMV-Ct inoculated 'Dina-1' plants was harvested and used to inoculate two susceptible squash plants. The other half of the leaf was inoculated with ZYMV-NAA at the same time. Healthy 'Dina-1' plants were also inoculated on leaf-4/-5 with ZYMV-NAA. Two weeks later one half of leaf-7 and –8 were harvested and used to inoculate young squash plants. Appearance of symptoms on squash plants was monitored up to 4 weeks post inoculation.

RESULTS

Symptom expression and virus accumulation in the resistant genotypes 'Dina-1' and 'TMG-1'.

When cotyledons of 7-9 day old plants were inoculated with ZYMV, 'TMG-1' plants remained free of symptoms while 'Dina-1' plants showed distinct veinal chlorosis on leaf-1 or leaf-2 approximately 10 days post inoculation (dpi) (Figure 2B). Subsequent leaves of 'Dina-1' remained symptom free. This response was observed with a number of isolates of diverse origin (Kabelka et al., 1997). The susceptible genotype, 'Straight-

8', responded with systemic mosaic symptoms. Two weeks post cotyledon inoculation, 'TMG-1' plants did not show virus accumulation in cotyledons and leaves (Figure 2 and Figure 3), while 'Dina-1' plants showed high virus titer in the cotyledons and the chlorotic leaf (leaf 1 or –2) but not upper leaves, as detected by ELISA (Figure 3). On rare occasions, TMG-1 showed detectable levels of virus in the cotyledons at two weeks post inoculation (data not shown).

Four to six weeks post inoculation (wpi), an additional one or two leaves often showed detectable levels of virus accumulation and mild symptoms in 'Dina-1'. At that time 'TMG-1' also showed virus accumulation in cotyledons and sometimes in the first systemic leaf, indicating that resistance is not completely effective in preventing systemic virus infection. 'Straight-8' showed systemic mosaic throughout the plants, however, symptom severity varied on individual leaves.

When cotyledons of slightly older plants were inoculated (leaf-1 unfolded), 'TMG-1' remained symptom free, while 'Dina-1' plants showed veinal chlorosis on leaf-2 or leaf-3. These results indicated that the specific leaf that developed veinal chlorosis varied with the age of the plant at the time of cotyledon inoculation. The mean leaf position showing veinal chlorosis was 2.43 (n=16) for plants inoculated when 10-12 day old vs. mean leaf position of 1.15 (n=20) for plants inoculated when 7-9 day old. Regardless of whether 7-9 day or 10-12 day old plants were inoculated on the cotyledons, in almost all the cases symptoms remained restricted to a single leaf. In analogous experiments with 'Straight-8', when cotyledons of different ages were inoculated (Figure 4), symptoms and virus accumulation were first detected at progressively higher leaf positions (i.e. in leaves that were newly expanding at the time of inoculation). When 7

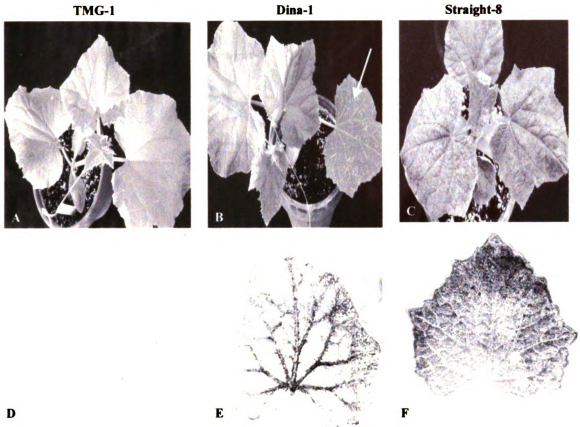

Figure 2. Response of 'TMG-1' (A), 'Dina-1' (B), and 'Straight-8' (C) to inoculation with ZYMV. Plants were inoculated on the cotyledons when 7 days old and pictures were taken 12 days after inoculation. 'TMG-1' remained symptom free, 'Dina-1' showed a distinct pattern of veinal chlorosis on the first systemic leaf, while 'Straight-8' showed systemic mosaic throughout the plant. D., E., and F. Immunoblots of the first systemic leaves of 'TMG-1' (D) 'Dina-1' (E) and 'Straight-8' (F) at 10 days post inoculation, using ZYMV coat protein antiserum. 'TMG-1' did not show detectable levels of virus accumulation, 'Dina-1' showed distribution of virus along the veins while 'Straight-8' showed uniform distribution throughout the leaf. Punch holes in the leaf (F) indicate that smearing of leaf sap did not occur during blotting.

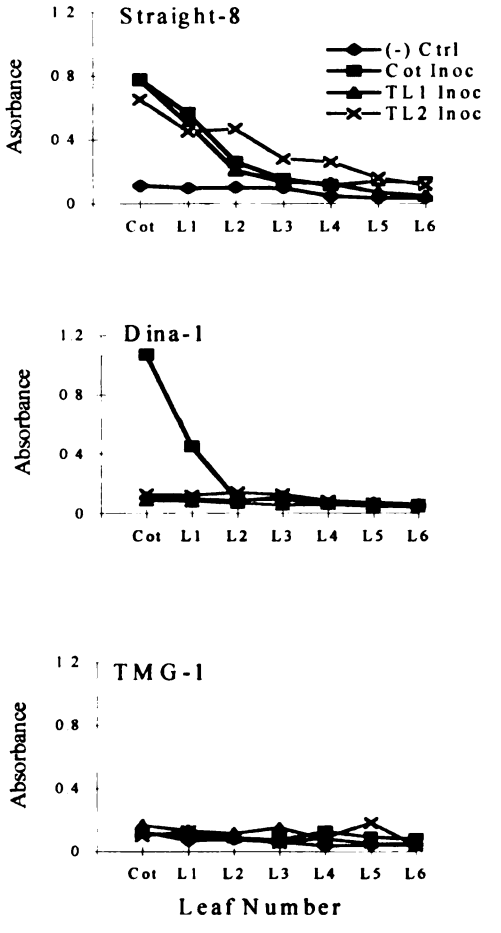

Figure 3. Virus titer in the cotyledons (Cot) and true leaves (L) in 'Straight-8', 'Dina-1' and 'TMG-1' in response to inoculation with ZYMV-Ct. Young seedlings were inoculated on the cotyledons (Cot Inoc), first (TL1) or second (TL2) true leaf. Virus titer was measured by ELISA 14 days post inoculation. The X-axis shows the number of leaves from the base to the top of the plant and the Y-axis shows absorbance at 405nm. Each point represents the mean of 4 replications. The experiment was repeated three times with similar results.

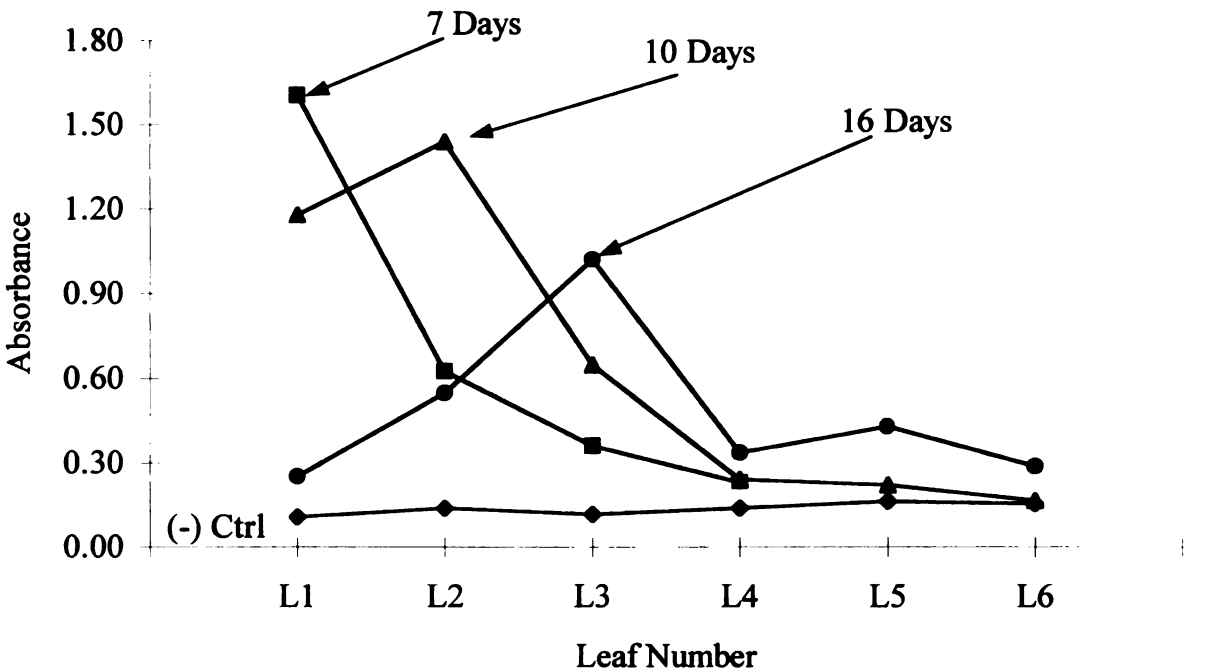

Figure 4. Virus accumulation (as determined by ELISA) in different leaves of 'Straight-8' as affected by plant age at the time of inoculation. Each point represents the mean of 5 replications. The experiment was repeated twice and similar results were observed.

day old plants were inoculated, the virus peaked in leaf-1 (L1) first, when 10 day old plants were inoculated, this peak moved to leaf-2 (L2) and to leaf-3 (L3) when 16 day old plants were inoculated. Together these observations suggest that in both 'Dina-1' and 'Straight-8' the virus moved from inoculated cotyledons to the predominant sink leaf at the time.

The distribution of virus within the first leaf of 'TMG-1', 'Dina-1' and 'Straight-8' was examined by immunoblotting with antiserum to the ZYMV coat protein at 10 dpi (Figure 2B). No virus accumulation could be detected in 'TMG-1' while in 'Dina-1' the virus appeared to be present along the veins. Non-symptomatic leaves of 'Dina-1' did not show virus accumulation (data not shown). 'Straight-8' showed uniform distribution of virus throughout the leaf. These results suggest that the veinal chlorosis pattern in 'Dina-1' plants corresponds to virus distribution within the leaf, and that the symptomless phenotype of 'TMG-1' is associated with the absence of virus accumulation.

If sampled at an earlier time (e.g. 5 days post inoculation) (Figure 5), 'Straight-8' also showed a predominantly veinal pattern of virus distribution, but at 10 dpi there was more uniform distribution, throughout the leaf, indicating virus exit from the phloem and subsequent cell-to-cell spread. 'Dina-1' plants, on the other hand, showed very low levels of virus in the first leaf at 5 dpi and a veinal distribution at 10 dpi that continued up to 30 dpi (Figure 5).

Unlike the response to cotyledon inoculation, when the first or second leaves were inoculated, 'Dina-1' plants remained symptom free and did not show virus accumulation (Figure 3). Occasionally very mild veinal chlorosis symptoms were observed (on leaf-3)

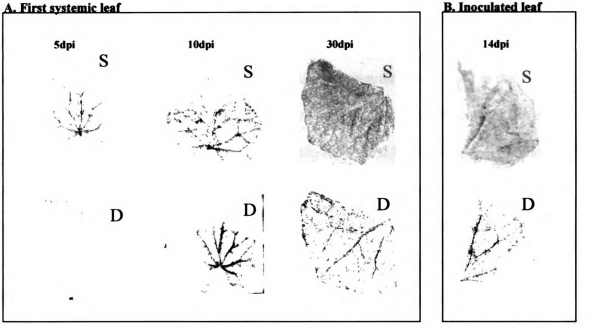

Figure 5. A. Time course experiments showing the accumulation of ZYMV in the first systemic leaf of "Straigh-8" (top) and 'Dina-1' (bottom) leaves at five, ten and thirty days post inoculation. Plants were inoculated on the cotyledons when seven days old.

B. Inoculated first leaf immunoblotted at two weeks post inoculation.

when leaf-1 was inoculated (in the greenhouse: 12%; 8/64), while no symptoms were observed when leaf-2 was inoculated (0/30). 'TMG-1' plants also remained symptom and virus free, while 'Straight-8' plants showed typical systemic mosaic and virus accumulation. Immunoblots showed accumulation of virus along the veins in the inoculated leaves of 'Dina-1' and a more uniform distribution in the inoculated leaves of 'Straight-8' (Figure 5). Virus accumulation in the inoculated leaves of TMG-1 could not be detected with immunoblotting (data not shown). The observed differences in the appearance of veinal chlorosis phenotype following true leaf vs. cotyledon inoculation suggested that the resistance response in 'Dina-1' plants is tissue specific or developmentally regulated. We sought to investigate the nature of the block observed in the true leaves, and the timing of the expression of resistance.

Nature of the block to successful virus infection in the resistant genotypes.

Limitation of veinal chlorosis to a single leaf following cotyledon inoculation in 'Dina-1' plants, localization of virus along the veins, and failure to see symptoms or virus accumulation (Figure 3) with leaf inoculation, raised the possibility that the virus may not be able to replicate in the leaves. The veinal chlorosis phenotype would thus result from the limited supply of virus transported from the cotyledons to the predominant sink leaf. Other leaves either do not receive virus, or import only a limited quantity, which is insufficient to cause the veinal chlorosis phenotype.

To investigate virus replication in the true leaves, the inoculated cotyledons were removed at various time intervals after inoculation, and the plants were scored for veinal

chlorosis and virus accumulation with ELISA and immunoblotting. Although virus titer was not detectable in the cotyledons or leaves at 1, 2, or 3 dpi (Figure 6A), plants from which the cotyledons were removed at 2 or 3 dpi showed veinal chlorosis and high virus titer in leaf-1 (or some cases on leaf-2) at 14dpi (Figure 6B). This indicated that virus replication had occurred in the absence of cotyledons.

Virus replication in 'Dina-1' plants in the absence of cotyledons was further confirmed by immunoblot experiments (Figure 6C). One half of leaf-1 and the cotyledons were removed at 3dpi, the other half of leaf-1 was removed 7 days later when symptoms appeared. Both the halves of leaf-1 were immunoblotted at the same time using coat protein antiserum. No virus accumulation could be detected in the half leaf removed at the time of cotyledon removal while the other half of the same leaf (sampled seven days later) showed detectable levels of the virus (Figure 6C). Together these results suggested that virus replication in 'Dina-1' plants can occur in the absence of cotyledons, and that the block to systemic spread of virus in 'Dina-1' is occurring at the level of movement.

Nature of leaf specific expression of the resistance response in Dina-1.

The observed response, replication in and movement from the cotyledons, followed by restricted movement of the virus after reaching the true leaf might be due to inherent structural differences between leaves and cotyledons, developmental changes in the host, or a delay due to time required for induction of a resistance response. If the delay in observed resistance following cotyledon inoculation was due to an induced

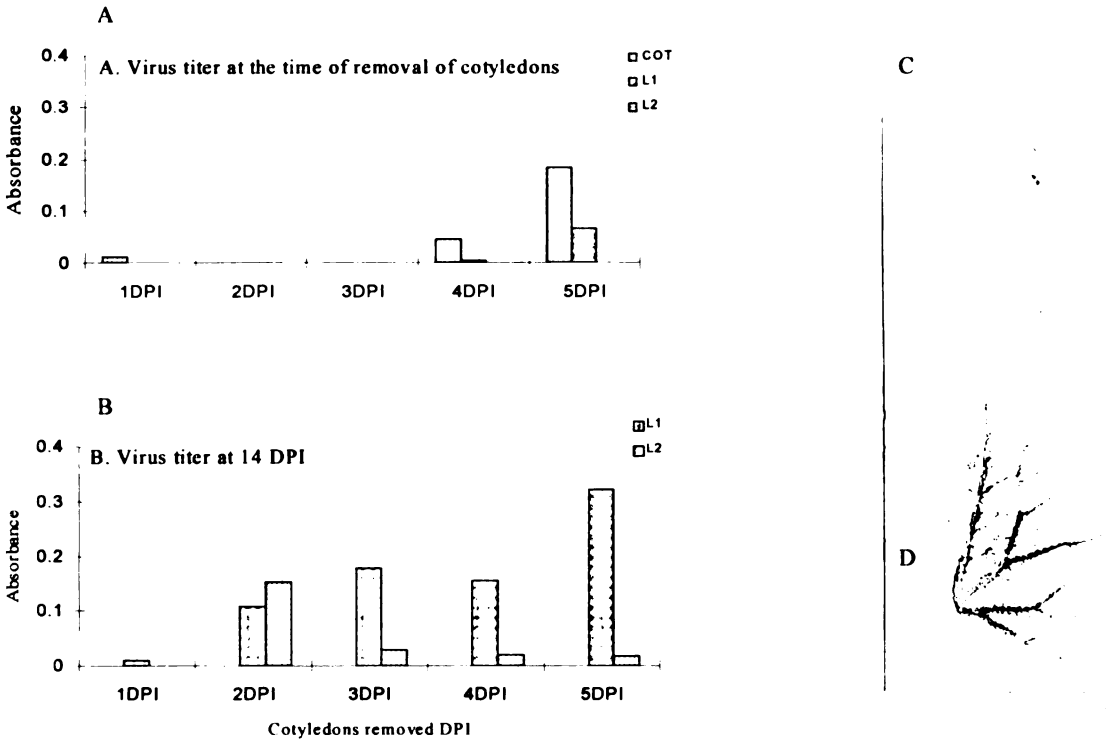

Figure 6. Virus titer in the cotyledons and true leaves of 'Dina-1', at the time of removal of cotyledons (A) and 14 days post inoculation (B). X-axis shows days post inoculation (DPI) at which cotyledons were removed. Y- axis shows absorbance at 405nm. The Data were normalized by subtraction of background ELISA readings. COT = cotyledon, L1 and L2 = leaf-1 and leaf-2 from the base of the plant. Each column represents the mean of five replications. C. and D. Immunoblots showing virus replication in the true leaves of 'Dina-1' plants. Plants were inoculated on the cotyledons with ZYMV-Ct when 7 days old and the cotyledons and one half of the first leaf were removed 3 days post inoculation (C). The other half of the leaf was removed 10 dpi when veinal chlorosis symptoms appeared (D). Both halves of the leaf were immunoblotted at the same time with ZYMV coat protein antiserum. Both the experiments were repeated three times with similar results.

response, a similar pattern of symptom expression would be expected following true leaf inoculation, as was observed with cotyledon inoculation. However, as indicated earlier, when 'Dina-1' plants were inoculated on the leaves, veinal chlorosis generally was not observed suggesting that in the true leaves the resistance mechanism is active prior to inoculation.

Another characteristic of induced resistance is protection against secondary inoculation. This possibility was tested using the NAA isolate of ZYMV (Gal-On et al., 1991, 1992) that did not cause veinal chlorosis or systemic symptoms, but occasionally showed limited systemic movement as detected by ELISA (data not shown), or more frequently by the more sensitive assay of back inoculation on to susceptible squash plants (Table 2). When cotyledons of 7 day old 'Dina-1' plants were inoculated with ZYMV-Ct typical veinal chlorosis symptoms were observed on leaf-1 or leaf-2 at 10 dpi. However, less than 3% (1/ 36 plants) of 'Dina-1' plants, contained ZYMV-Ct in leaf-4 or –5 when tested by back inoculation onto susceptible squash plants (Table 2A). As observed previously, healthy 'Dina-1' plants inoculated with ZYMV-Ct on leaf-4 or –5 did not show veinal chlorosis, and only 2/20 plants (5%) tested had virus in leaf-7 or –8 based on back inoculation. In contrast, following inoculation with ZYMV-NAA on leaf-4 or –5, 72% of the inoculated plants (32/40) showed virus accumulation as detected by back inoculation of squash plants (Table 2B).

To test whether the ZYMV-Ct inoculated plants exhibited induced protection against secondary inoculation, 'Dina-1' plants showing veinal chlorosis on leaf-1 in response to ZYMV-Ct, were inoculated with ZYMV-NAA on leaf-4 or -5 (Table 2C). Half of leaf-4 or –5 was removed for back inoculation to squash (Table 2A) the other

Table 2. Effect of prior inoculation of 'Dina-1' plants with ZYMV-Ct on secondary inoculation by ZYMV-NAA.

Treatment	Veinal Chlorosis		Presence of virus as determined by back inoculation[a]		
	ZY-Ct	ZY-NAA	Leaves tested	ZY-Ct	ZY-NAA
A. Cot inoc	35/36[b]	0/12	L4 or L5	1/36	-
B. L4/5 inoc	0/20	0/40	L7 or L8	2/20	32/40
C. Cot inoc with ZY-Ct followed by L4/L5 inoc with ZY-NAA	as 1A.	-	L7 or L8	0/40	22/40

[a] Half of the leaf from each 'Dina-1' plant was used as an inoculum to inoculated two squash plants and symptoms were monitored for 30 days.

[b] Number of plants showing symptoms/total number of plants inoculated.

half was inoculated with ZYMV-NAA. Two weeks post secondary inoculation with ZYMV-NAA, susceptible squash plants were back inoculated from leaf-7 or leaf-8 of 'Dina-1'. Similar to the results with ZYMV-NAA alone (72%: 32/40), a large percentage of the plants showed systemic infection with ZYMV-NAA after secondary inoculation (52%; 22/40). Thus, prior inoculation with ZYMV-Ct did not prevent subsequent infection by ZYMV-NAA. This provides further evidence against an induced resistance response.

Finally, environmental conditions appeared to influence the appearance of the veinal chlorosis on 'Dina-1' plants following true leaf inoculation. When experiments were performed in the growth chambers instead of the greenhouse, 81% of the plants (13/16) showed veinal chlorosis following the first true leaf inoculation. Inoculation of leaf-2, however, resulted in a greatly reduced number of plants showing veinal chlorosis (19%; 3/16 plants). These observations argue against inherent structural differences in the leaves preventing virus spread, and instead suggest developmentally controlled expression of resistance.

Role of the coat protein of ZYMV in the veinal chlorosis response of Dina-1.

Data presented so far indicated that 'Dina-1' plants interfere with the movement of ZYMV. Since the multifunctional CP of potyviruses is also involved in virus movement (Dolja et al., 1994. 1995; Rojas et al., 1997) and host defense responses (Andersen and Johansen, 1998; chapter 3), we decided to investigate the role of the ZYMV CP in the resistance response of 'Dina-1' plants. The conserved core region of

the CP is involved in cell to cell movement, while the highly variable, surface exposed amino terminus of the CP is essential for systemic movement and is suggested to play a role in host adaptation and host range determination (Dolja et al., 1994, 1995; Shukla et al., 1994). The carboxy terminal (CT) portion of the CP also is essential for long distance movement. This known separation of the cell to cell and systemic movement functions of the CP allowed us to further dissect the potential role of CP in the resistance response of 'Dina-1'.

Chimeric ZYMV-NAA infectious constructs were made containing either the NT of the CP (CtNTFL/P) or the core and carboxy terminal portions of the CP (CtCoreFL/P) of ZYMV-Ct (Figure 7). Both constructs produced systemic symptoms on susceptible squash and cucumber plants. When tested on the resistant genotype 'Dina-1', the chimeric construct containing the NT of CP of ZYMV-Ct (pCtNTFL/P) showed veinal chlorosis on either leaf one or two of approximately 58% (25/43) of the inoculated plants while CtCoreFL/P and ZYMV-NAA inoculated plants remained largely symtomless (Figure 7) and only a few plants showed veinal chlorosis. These results suggest that the NT of the CP of ZYMV plays a role in the veinal chlorosis response of 'Dina-1' plants.

DISCUSSION

Resistance to ZYMV infection in 'Dina-1' plants is controlled by a single recessive allele (zym^{Dina}) that confers an unusual phenotype. When cotyledons of young plants were inoculated with ZYMV, a distinct veinal chlorosis pattern developed on the first or second leaf. Subsequent leaves remained symptom free, and inoculation of leaves generally, did not result in veinal chlorosis. Immunoblot analyses revealed that the

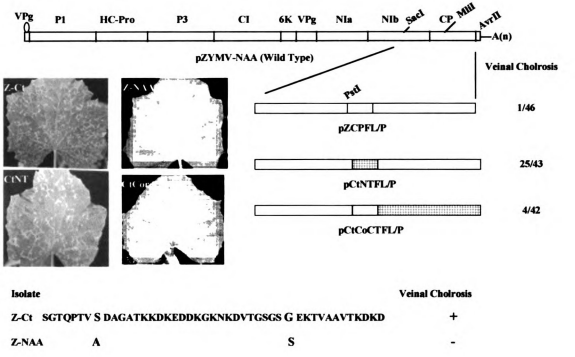

Figure 7. Infectious ZYMV-NAA constructs with the engineered *PstI* site (pZCPFL/P), the NT of the CP of ZYMV-Ct (pCtNTFL/P), the core portion (pCtCoFL/P) of the CP of ZYMV-Ct. All the constructs were infectious on susceptible squash and cucumber plants. The veinal chlorosis response of 'Dina-1' to these constructs is indicated at the right. Comparison of the amino acids sequence of the CP-NTs is shown at the bottom.

pattern of veinal chlorosis reflected the pattern of virus distribution within the leaf. In leaves showing veinal chlorosis, virus accumulation was localized along the veins; non-symptomatic leaves did not show detectable virus accumulation. In susceptible 'Straight 8' plants, virus was initially associated with the veins (at 5 dpi), but by 10 dpi the virus was more uniformly distributed throughout the leaf. In 'Dina-1' plants, the virus remained largely restricted to the veinal regions even at 30 dpi.

Age of the plant at the time of inoculation influenced which leaf showed veinal chlorosis. When older plants were inoculated, veinal chlorosis appeared at progressively higher leaf positions. Analogous patterns showing a relationship between plant age at the time of inoculation and first leaf to accumulate virus, were observed with the 'Straight 8' plants. This suggested that in both 'Straight 8' and 'Dina-1', the virus replicated in the inoculated cotyledons, and, as is typical for virus infection (Leisner and Turgeon, 1993; Andrianifahanana et al., 1997), was then transported to the predominant photosynthetic sink at the time. Consistent with movement from the cotyledons to the predominant sink, the observed pattern of veinal chlorosis and virus accumulation closely resembled the pattern observed for phloem unloading of carboxyflorecein molecules in sink leaves (Roberts et al., 1997). Chlorosis was predominantly observed along class II and III veins, which are the main sites of phloem unloading (Roberts et al., 1997; Oparka and Turgeon, 1999).

Thus, the observed 'Dina-1' phenotype includes initial infection of the cotyledons followed by restriction to veinal regions of the systemic sink leaf, and in most cases inability to establish infection when directly inoculated onto leaves. Localization of virus

to the veinal regions indicated that the virus was either unable to unload from the phloem, or to replicate or move cell to cell following initial unloading in the leaf. Failure to observe the resistance response in the cotyledons could be due to structural differences between cotyledons and leaves, induction of resistance in the leaves, or developmental regulation of the resistance response.

Although an inability to replicate in the leaves could explain the observed veinal chlorosis phenotype (a limited amount of virus is exported from the cotyledons to the leaves), cotyledon removal experiments indicated that ZYMV could replicate in 'Dina-1' leaves. When the cotyledons were removed at 2-3 dpi, detectable levels of virus were not present in the leaves or cotyledons, yet measurable virus accumulation was observed in the leaves one week later. Similarly, in growth chamber experiments, veinal chlorosis symptoms following inoculation of the first true leaf were observed even when cotyledons were removed before inoculation, confirming that the block to systemic infection was not resulting from inability of ZYMV to replicate in 'Dina-1' leaves, and instead indicates a restriction in virus movement.

Several lines of evidence favor a block in long distance movement rather than cell to cell movement. The pattern of veinal chlorosis and localization of virus to the veinal regions suggests a block in phloem unloading, and immunoblots of inoculated leaves that did not lead to subsequent veinal chlorosis, showed accumulation of virus adjacent to the veins. This indicates movement of the virus to the veinal regions, followed by inability to load into the veins. Consistent with this observation, Schaad and Carrington (1996) observed that in the resistant tobacco line V20, which interferes with long distance

movement of TEV, virus accumulation in the inoculated leaves appeared as an apparent tracking along the primary veins.

In cucurbits the phloem is bicollateral in nature (Schmitz et al., 1987). The adaxial phloem consists of sieve elements (SE), and companion cells (CC) separated by a single tracheid and a single vascular parenchyma (VP) cell. Unlike CC of the adaxial phloem, the CCs of the abaxial phloem are modified into intermediary cells, characterized by extensive plasmodesmatal connections with the adjacent bundle sheath (BS) cells. Larger veins contain many more xylem and phloem elements and are associated with ordinary CC (Schaffer et al., 1996). Thompson et al. (1998) observed functional differences between PD connecting mesophyll cells and those connecting mesophyll cells with BS cells in cucumber plants. Using a pseudorecombinant cucumber mosaic virus strain (FFT), they observed that FFT infection was arrested at the BS cells and was not detected in intermediary or other phloem cells suggesting that the BS-phloem interface is the boundary for systemic movement. It is also possible that 'Dina-1' plants restrict movement of ZYMV in the leaves at the BS-phloem interface in an analogous fashion by preventing exit from the phloem when the cotyledons are inoculated or entry into the phloem when the leaves are inoculated.

Further evidence for a block in long distance movement in 'Dina-1' leaves comes from the use of chimeric ZYMV viruses with switches in the coat protein (CP) amino terminus (NT). The conserved core portion of the potyviral CP is involved in the encapsidation of viral RNA and cell to cell movement of the virus while the variable surface exposed N- and C-terminal portions are required for systemic movement (Dolja et al. 1994, 1995; Shukla et al. 1994; Rojas et al. 1997). When the NT of the CP of the

infectious construct of ZYMV-NAA, which did not produce veinal chlorosis on 'Dina-1' plants, was substituted for the respective region of the CP of ZYMV-Ct, the chimeric construct induced veinal chlorosis on 'Dina-1' plants. Substitution of the core and CT portion of the CP did not cause a veinal chlorosis response. This suggests that the CP-NT is involved in the differential response of 'Dina-1' to these two isolates of ZYMV, and further suggests that the block to successful infection in 'Dina-1' is occurring at the level of systemic movement. It appears that the ZYMV-NAA isolate is able to partially escape the block to systemic movement. Like ZYMV-Ct on 'Dina-1' the virus distribution of ZYMV-NAA within the inoculated and systemic leaves shows a veinal pattern (data not shown); however, ZYMV-NAA can be detected in systemic leaves by back inoculation onto susceptible squash plants. Comparison of the amino acid sequence of the CPs of ZYMV-Ct and ZYMV-NAA shows two amino acid differences (Ser7 to Ala and Gly32 to Ser) located in the N-terminal region. Thus it is possible that the CP-NT is involved in interaction with the host resistance factor, and that the amino acid differences between the CP-NTs of these two isolates influences this interaction.

The observation of resistance in leaves but not cotyledons of 'Dina-1' might result from inherent structural differences between the two tissues. On some occasions, however, inoculation of leaf 1resulted in mild chlorosis on leaf 3 in the greenhouse, and in the growth chamber, a majority of the plants showed veinal chlorosis following inoculation of the first leaf. These observations argue against structural differences in the leaves vs. cotyledons. On the other hand, chlorosis was not observed following inoculation of second or third leaves in the greenhouse, and a much lower percentage of

plants in the growth chamber (19%) showed symptoms when the second leaf was inoculated, indicating developmentally regulated expression of the resistance factor(s).

'Dina-1' plants do not appear to exhibit either a classical recovery phenotype or induced resistance. Recovery of susceptible plants from virus infection has been observed for several viruses including potyviruses and is associated with a gradual reduction in symptom severity and protection from secondary infection by strains of the same virus or homologous viruses (Pennazio et al., 1999). We have observed a similar recovery phenotype and protection against secondary virus infection in susceptible cucumber and squash plants in response to inoculation with a chimeric ZYMV containing the amino terminus of the coat protein of a non-cucurbit potyvirus, TEV (chapter 3). The veinal chlorosis phenotype in 'Dina-1' plants, however, differed from virus induced recovery response in susceptible plants in that the recovery was not gradual, symptoms were almost always restricted to a single leaf, non-symptomatic leaves did not show detectable virus accumulation, and were not protected against secondary virus infection. Similarly the failure of ZYMV-Ct inoculated plants showing veinal chlorosis to be protected against subsequent infection by ZYMV-NAA, and the generally constitutive resistance of 'Dina-1' leaves to ZYMV-Ct infection argues against an induced resistance response.

Collectively these results suggest developmental control of a resistance factor that restricts systemic ZYMV infection at the level of loading or unloading from the phloem. This may be contrasted with resistance conferred by the allele in 'TMG-1' plants, zym^{TMG}, which appears to be expressed earlier in development. Although not generally apparent by veinal chlorosis, developmental differences in expression of resistance have been

observed in other cucurbit potyvirus-interactions (Gilbert-Albertini, 1993; Gibb et al., 1994; Wai et al., 1995b). For example when cotyledons of muskmelon (*Cucumis melo*) cultivar 'Cinbo' were inoculated with PRSV-W mild systemic symptoms were observed, but plants remained symptomless when true leaves were inoculated (Gibb et al., 1994). Limited systemic movement of WMV and ZYMV to the first one or two leaves also has been observed in *Cucurbita moschata* line Menina 15 (Gilbert-Albertini et al, 1993). In 'TMG-1' two independently assorting resistance factors to WMV were identified, one expressed in both cotyledons and leaves and the other expressed only in the leaves (Wai and Grumet, 1995b). It will be of interest to determine whether similar mechanisms or resistance gene products are involved in each of these examples.

The differences in developmental expression of the zym^{Dina} locus, and interaction with the ZYMV CP-NT may provide approaches to allow for cloning and further characterization of the *zym* locus. Tight linkage of this locus to resistance against other potyviruses could lead to cloning and characterization of other resistance factors, and thereby provide insights into plant potyvirus interaction and systemic movement in cucurbits.

Future Directions

We have shown in this project that resistance in Dina-1 plants is occurring at the level of phloem loading or unloading, and that the amino terminus of the coat protein plays a role in the veinal chlorosis response. The specific cellular location of ZYMV in the chlorotic veins remains to be determined. Although the viral CP determinant involved in the veinal chlorosis response was identified, other regions of the viral genome

were not tested. Cloning of the *zym* gene from 'Dina-1' or 'TMG-1' plants will be the ultimate target for any future project and will certainly reveal valuable insights into our understanding of the potyvirus resistance in cucumbers.

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CHAPTER III

TESTING THE ROLE OF THE AMINO TERMINUS OF THE COAT PROTEIN IN SYSTEMIC INFECTION AND HOST RANGE DETERMINATION OF POTYVIRUSES

ABSTRACT

The role of the amino terminal (NT) region of the potyviral coat protein (CP) in systemic infection and host range determination was investigated. Chimeric zucchini yellow mosaic virus (ZYMV) constructs were made by substitution of the native CP-NT with the CP-NTs of watermelon mosaic virus (WMV; overlapping host range with ZYMV) and tobacco etch virus (TEV; non-overlapping host range with ZYMV). Both the constructs produced strong initial symptoms on ZYMV-susceptible cucurbit plants and were aphid transmissible. A gradual reduction in symptom severity was observed in squash and cucumber plants infected with TEV CP-NT chimeric virus. Four to six weeks post inoculation the newly developing leaves were either symptomless or showed markedly reduced symptoms. The recovery phenotype was specific to the TEV CP-NT chimeric virus: 1) did not result from a mutation in the virus, 2) provided protection from secondary virus infection in a sequence specific manner, and 3) showed characteristics associated with virus induced post transcriptional gene silencing (PTGS). The chimeric viruses did not overcome naturally occurring resistance to ZYMV in the resistant cucumber line 'TMG-1' or ZYMV coat protein-mediated resistance in transgenic melon plants. The host range of ZYMV also was not affected by these substitutions, and the

chimeric viruses did not systemically infect bean, *N. benthamiana*, or tobacco plants. Together these results show that despite substantial variability in the length and sequence, the NTs of CPs of heterologous potyviruses can facilitate systemic infection of ZYMV in susceptible cucurbit hosts. However, this substitution per se is not sufficient to modify the host range of the virus. The CP-NT from a non-pathogenic potyvirus could trigger a host defense response in cucurbits resulting in recovery from systemic virus infection.

INTRODUCTION

Potyviral coat proteins (CPs) are multifunctional proteins involved in several stages of the viral life cycle. Approximately 2000 CP monomers assemble to encapsidate the 10 kb single stranded RNA genome into long flexuous rod shaped particles (Shukla et al., 1994; Jagadish et al., 1991,1993). Potyviral CPs have been shown to be involved in cell to cell movement through the plasmodesmata (Dolja et al., 1995; Rojas et al., 1997), long distance movement through the phloem (Dolja et al., 1994, 1995), aphid mediated transmission (Pirone and Blanc, 1996), and possibly host mediated defense responses and host range determination (Shukla et al., 1994; Solomon, 1989; chapter 2). The CP is composed of variable amino and carboxy-terminal (NT and CT) regions and a highly conserved core portion (Shukla et al., 1994). Both N- and C-terminal regions are surface exposed and can be cleaved by protease treatment without affecting virion morphology (Shukla et al., 1988; Shukla and Ward, 1989).

The domains of the CP involved in various functions have been identified. The conserved core region is involved in encapsidation of RNA and cell-to-cell movement of the virus (Dolja et al., 1994, 1995; Jagadish et al., 1993). A tobacco etch virus (TEV)

mutant with a substitution of the highly conserved Ser122 residue (S122W) within the core domain was confined to single cells in tobacco plants (Dolja et al., 1995). The cell-to-cell movement defect was restored efficiently in transgenic plants expressing the wild type CP gene. Mutations in the core region of the CP of bean common mosaic necrosis (BCMNV) and lettuce mosaic (LMV) potyviruses affected cell to cell movement in microinjection studies (Rojas et al., 1997). Both the NT and CT of the CP are not essential for the assembly of virions, but are required for systemic movement (Shukla et al., 1988; Jagadish et al., 1991; Dolja et al., 1994, 1995). Deletions of the N- and C-terminal regions affect long distance movement of the virus (Dolja et al., 1994, 1995), and certain amino acid substitutions in the conserved DAG motif in the NT of CP of tobacco vein mottling potyvirus (TVMV) abolished the ability of the mutant viruses to cause systemic infection, but did not affect replication and virion assembly in protoplasts (Lopez-Moya and Pirone, 1998).

The CP in concert with the viral encoded helper component-proteinase (HC-Pro) is also involved in the aphid transmission of potyviruses. The conserved DAG motif in the NT of the CP interacts with conserved motifs in the HC-Pro gene to facilitate aphid transmission (Blanc et al., 1997; Peng et al., 1998; Thornbury et al., 1990). In binding assays, the DAG motif in the CP and the PTK motif in the HC-Pro were shown to be involved in CP-HC-Pro binding (Blanc et al., 1997; Peng et al., 1998). If the virus is maintained by rub inoculation rather than aphid transmission, there is a frequently observed loss of aphid transmissibility that is associated with amino acid substitutions in the CP-DAG motif (Rajamati et al., 1998; Gal-On et al., 1992; Atreya et al., 1990, 1991) or motifs within the HC-Pro (Pirone and Blanc, 1996; Llave et al., 1999; Legavre et al.,

1996, Grumet et al., 1992). Both CP and HC-Pro are also involved in virus movement (Revers et al., 1999), suggesting that the CP-HC-Pro interactions might have implications beyond aphid transmission.

Viral CPs, and in the case of potyviruses, the NTs of CPs may also be involved in interaction with the host leading to resistance responses or host range determination. In *Ipomea nil* plants showing recovery from virus infection Solomon et al. (1989) observed a proteolytic activity that cleaved the NT of the CP of sweet potato feathery mottle potyvirus (SFMV) and we have shown that the NT of the ZYMV CP is involved in the veinal chlorosis phenotype of the resistant cucumber genotype 'Dina-1' (chapter 2). The CPs of brome mosaic (BMV) and alfafa mosaic (AlMV) bromoviruses and cucumber mosaic cucumovirus (CMV), which are also involved in virus movement, have been shown to play a crucial role in host range determination (Mise et al., 1993; Spitsin et al., 1999; Ryu et al., 1998). Potyviruses can vary a great deal in the breadth of their host ranges; some (e.g., tobacco etch virus, TEV) infect only a limited number of plant species while others (e.g., watermelon mosaic virus, WMV) infect a wide variety of species (Brunt et al., 1996; Shukla et al., 1994). It has been suggested that the extreme variability in the NT of the CP might be involved in the host range determination of the virus (Shukla et al., 1994). A correlation between the length of the NT of the CP and the host range of different isolates of sugarcane mosaic virus (SCMV) was observed by Xiao et al. (1993).

In this project we sought to further understand the role of the NT of potyviral CPs in facilitating systemic infection and host range determination. Chimeric ZYMV viruses were created by exchanging the NT of the CP of ZYMV with the respective regions of

the CP of either TEV (non overlapping host range with ZYMV) or WMV (overlapping but broader host range) and tested for infectivity on a range of plant species. Comparison of the NT of the CP of these three potyviruses shows only 13 amino acid (aa) identity (Figure 8). The amino terminus of the CP of TEV is only 29 aa long compared to 44 aa of ZYMV and 46 aa of WMV. For WMV and ZYMV, which have overlapping host ranges, there is only 50% aa identity between the CP-NTs. Our results indicate that despite limited homology, NTs of the CPs of heterologous viruses facilitated systemic infection, but failed to modify the host range of the chimeric viruses. Interestingly, the NT of the CP from the non-overlapping host range virus TEV appeared to play a role in induction of host defense responses.

MATERIALS AND METHODS

Engineering cloning sites into the infectious ZYMV construct.

Two restriction sites (*PstI* and *KpnI*) were introduced in the infectious ZYMV-NAA construct (Gal-On et al., 1991, 1992) to facilitate substitutions in the coat protein region (Figure 9). The *PstI* site was introduced at the NIb-CP junction by PCR amplification (using Vent DNA polymerase with proof reading activity, NE Biolabs Inc. Beverly, MA) of the CP gene and the 193bp of the 3'NTR using NIb-CP junction primer RG81(5'-ATGCTGCAGTCAGGCACCAGCCA-3';(restriction site is underlined) and 3' NTR primer RG40 (5'-AGTGAATTCTCGAGCTTATTCGTGA-3') including an engineered *EcoRI* and a natural *XhoI* site with a single base overlap. The amplified fragment was digested with *PstI* and *XhoI* and cloned into *PstI-XhoI* digested pBlueScript

ZYMV **SG** TQP **TV** A------ **DAG** AT----- **KD** KE **DDK** *GKNK*DVTGS------ *GSSE*

WMV **SG** KE - **TV** ENL--- **DAG** KES-K **KD** AS **DK** *G* *NK*PQNSQVGQ*GSKE*

TEV **SG** ------ **TV** **DAGA** **DAG** K----K **KD** *QK***D** **DK** VAEQ----------

ZYMV ---*KT* VAAVT **KD**---KD

WMV PT*KT*-GT---VS **KD** ----

TEV ----------AS **KD** --RD

Figure 8. Alignment of the amino terminal portions of the CP of ZYMV(NAA), WMV and TEV. Amino acids showing sequence identity among the three viruses are shown in bold letters. Amino acids showing sequence identity between ZYMV and WMV only are italicized.

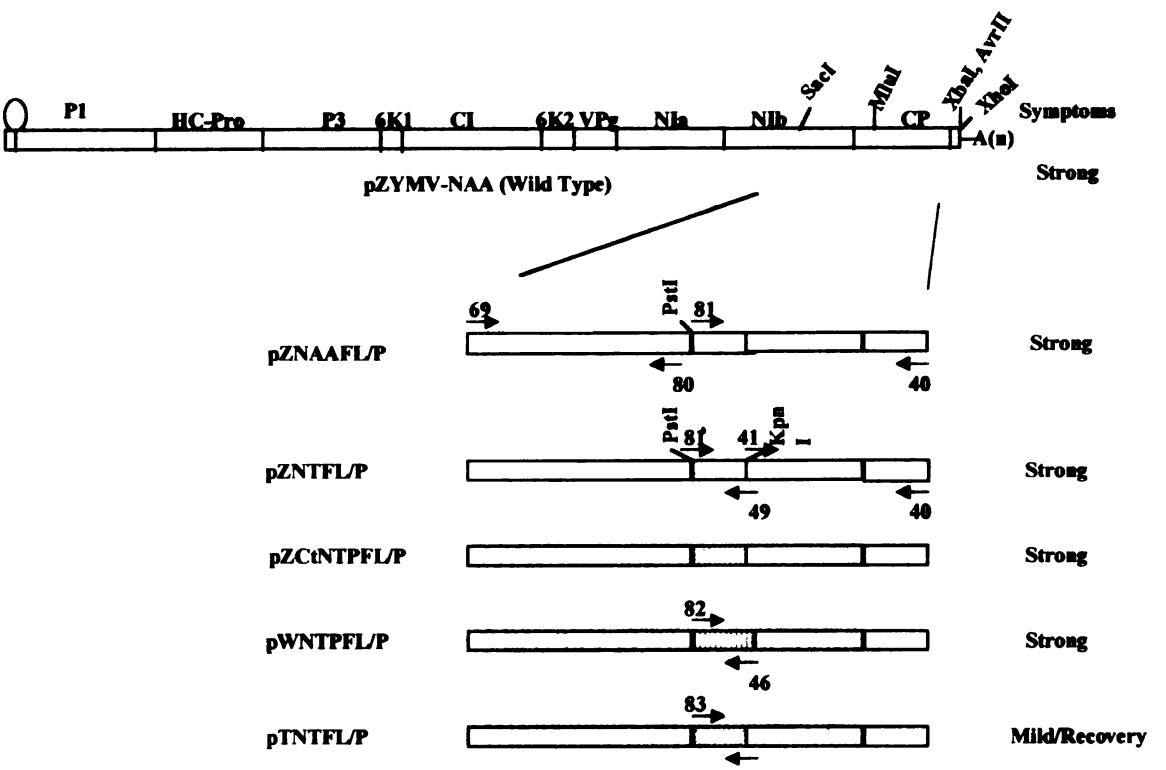


Figure 9. Chimeric constructs of ZYMV-NAA with the introduced changes in the NT of the CP gene and their infectivity on susceptible squash plants. The introduced restriction sites are shown at the top of the control constructs. Shaded areas indicate the NT of the CP swapped with the respective regions of ZYMV-CT (pCTNTFL/P), WMV (pWNTFL/P), or TEV (pTNTFL/P) using the introduced restriction sites.

KS (Stratagene, La Jolla, CA) to produce pZPX. To clone the 1kb 3' fragment of the NIb gene, the forward primer (RG69: 5'-GGTCATACG TATGATGTG-3') priming at position 7280 in the ZYMV genome and the reverse primer (RG80 5'-TGACTGCAGCA TTACAGTGCTCC-3') introducing a *PstI* site at the NIb-CP junction were used. The PCR product was cloned into pZPX digested with *SacI* (a unique restriction site in the NIb) and *PstI* resulting in the construct pZCPSPX. pZCPSPX contained the 2kb, 3' fragment of the infectious ZYMV-NAA construct with an engineered *PstI* site at the NIb-CP junction. The engineered *PstI* site resulted in substitution of two bases (CTCCAA to CTGCAG), but did not change the amino acid sequence.

The *KpnI* site was introduced near the beginning of the core portion of the CP (position 8691: amino acid motif NAGSH) resulting in a substitution from Ser at position 50 in the CP to Thr. The amino acid sequence at this position in the CP of potyviruses is not highly conserved (Shukla et al., 1994). The core and CT portion of the CP (including 193 bp of the 3'NTR) were amplified using RG41 (5'-GCTGGTACCCATGGGAAA ATTGTG-3' including a *KpnI* site) as forward primer (priming at position 8691) and RG40 (3' NTR with *EcoRI* site), and cloned into pUC119 (Stratagene) digested with *KpnI* and *EcoRI* resulting in pZNTKE. The NT of the CP of ZYMV-NAA was amplified using RG81 as forward primer and a reverse primer, RG49 (5'-ATGGGTACCAGCAT TACATCCTTGT-3'), designed to prime 15bp in the core region of the CP (position 8691) and introduce a *KpnI* site. The PCR product was digested with *PstI* and *KpnI* and cloned into pZNTKE resulting in the construct pZNTPKE. The resulting construct pZNTPKE contained the entire CP and an engineered *KpnI* site at the beginning of the

CP core region. pZNTEKP was digested with *PstI* and *AvrII* (a naturally occurring unique restriction 15bp from the CP stop codon), and the CP cloned into pZCPSPX to form pZNTSPX. Both the constructs (pZCPSPX and pZNTSPX) were confirmed by sequencing and restriction enzyme analyses.

Substitutions in the CP region.

The WMV construct used as a template was kindly provided by Dr. Hector Quemada (Quemada et al., 1990). The NT of the WMV CP was amplified by using primers RG 82 (5'-TCTCTGCAGTCAG GAAAAGAAACAGTT-3' with *PstI* site) and RG46 (5'-TTTG GTACCAGCATTTACATCCTT GCT-3' with *KpnI* site) and cloned into *PstI- KpnI* digested pZNTSPX to make in pWNTSPX. The NT of the CP of TEV was amplified from the construct (Allison et al., 1986) using primers RG83 (5'-TATCTG CAGAGTGGCACTGTGGAT-3' with *PstI* site) and RG51 (5'-TGAGGTACCAGCATT AAC ATCCTT ATCCTT-3' with *KpnI* site) and cloned as *PstI-KpnI* fragment into pZNTSPX to form pTNTSPX. No amino acid changes were introduced into the NT of the CP of WMV or TEV by PCR. The entire CP of WMV was amplified by using RG82 as forward primer and RG58 (5'-AACCCTAGGCAGTTT ACCTAGTCTTTA-3') as reverse primer designed to prime 15bp downstream from the CP stop codon and introduce an *AvrII* site. The PCR product was cloned into pZCPSPX resultng in pWCPSPX. The core and CT portion of the CP of WMV was PCR amplified by using RG84 (5'-GTTGGTA CCAAAGGAAAAGAAGTCCCA-3') as a forward primer which introduced a *KpnI* site 15bp in the core and RG58 as a reverse primer and cloned into pZNTSPX to make pWCoSPX.

The CP and the core and CT portions of the CP of TEV were cloned analogously using primer pairs RG 83 (as a forward primer) and RG57 (5'-GAATCTAGAGACACG CAGAAACTATCA-3'), and RG85 (5'-GCTGGTACCTCAGG AACAT TCTC-3' introducing *KpnI* site) and RG57 respectively and resulting in the constructs pTCPSPX and pTCoSPX. The primer and restriction enzyme sites for the full CP and core substitutions are shown in Figure 13. Primer RG57 introduced *XbaI* site 15bp in the 3'NTR, which is compatible with *AvrII*, occurring at the same position in ZYMV (TEV has an internal *AvrII* site in the CP). The full length CP gene of the Connecticut isolate of ZYMV (ZYMV-Ct) was cloned by using primer pair RG81 and RG56 (5'-TAACCTA GGTAGGCGACC-3' priming 15bp from the CP stop codon in the 3' NTR at the naturally occurring *AvrII* site) and resulted in the construct pCtCPSPX. Using the same PCR based approach a second set of WCP, WCore, TCP and TCore SPX constructs were made which contained only the coding sequence of the CP or the core portion of the CP of WMV and TEV. The same 5' primers used for making these constructs while two new 3' primers RG92 (5-AACCCTAGGTAGGCGACCTACC CTTTACTGCGG TGG ACCCAT-3' for WMV) and RG91 (5-GAATCTAGATAGGCG ACCTACCCTTTA CT GGCGGACCCCTAA-3 for TEV) were designed which were complementary to ZYMV in the 3'NTR region and WMV or TEV in the CP coding regions. The templates used for PCR were pWCPSPX and pTCPSPX. All the constructs were confirmed by sequencing and restriction enzyme analyses. Chimeric full length constructs were made by digesting the subclones with *SacI* and *XbaI* (a unique restriction in the 3'NTR) and cloning into the infectious ZYMV-NAA construct.

A chimeric full length construct containing the NT of the CP of ZYMV-Ct was made by digesting pCtCPSPX with *SacI* and *MluI*. *MluI* is a unique restriction site close to the beginning of the core portion of the CP (position 8746). Thus, unlike other NT chimeric constructs, CtNT construct lacked the engineered *KpnI* site. The core portion of the CP of ZYMV-Ct was cloned by using the unique restriction sites of *MluI* and *XbaI*.

Virus Stocks, plant material and growth conditions.

ZYMV and chimeric virus stocks were maintained in the growth chamber in sthe usceptible squash (*Cucurbita pepo*) cultivar Midas (Willhite Seeds Incorporated, Poolville, TX). TEV was maintained on the tobacco (*Nicotiana tabacum*) cultivar Burley. Growth chambers were set at 16/8 hrs 24/20C day/night conditions and an average light intensity of 150 μEinstein. Experiments were conducted in the greenhouse using a randomized complete block design and the plants received supplemental light during winter months (October-April). Numbers of replications in each block varied from experiment to experiment and are indicated in the results section. Plant genotypes used in this study were: resistant cucumber (*Cucumus sativus*) line 'TMG-1' (Provvidenti, 1987, Seeds provided by J. Staub, Univ. of Wisconsin), susceptible cucumber cultivar 'Straight-8' (Burpee Seed Co, Warminster, PA), CP transgenic melon (*Cucumus melo*) line 207 (Fang and Grumet, 1993), susceptible melon cultivar 'Hales Best Jumbo' (Hollar Seeds, Rocky Ford, CO), Black turtle-II beans (*Phaseolus vulgaris*: Provvidenti and Gonsalves, 1984), *Nicotiana benthamiana*, and *Chenopodium amaranticolor*. All the experiments were repeated two to three times except the

experiment with the CP transgenic melon plants, which was done once with 5 replications due to limited supply of seeds.

Plant inoculations.

Plasmid DNA of the full length constructs was purified using the Wizard plasmid miniprep system (Promega, Madison, WI) and directly inoculated onto the plants using the particle bombardment method of Gal-On et al, (1995). After the plants showed systemic symptoms, young symptomatic leaves were harvested and stored at –80°F as passage 0 (P0) stock. All the tissue used for subsequent inoculation was either from the original P0 plants or from stock (P1) plants inoculated with P0 inoculum. Plants were rub inoculated on leaves or cotyledons dusted with carborundum using inoculum prepared from young symptomatic leaves ground in 0.02M Phosphate buffer (PH 7.0). For back inoculations from beans, *N. benthamiana*, and tobacco plants, inoculated or systemic leaves were harvested from each inoculated plant, ground separately in phosphate buffer, and used to inoculate two susceptible squash plants.

PCR, RT-PCR and ELISA tests of the inoculated plants.

PCR amplifications were run for 25 cycles using Vent DNA polymerase (NE Biolabs). For RT-PCR, total RNA was extracted from inoculated and healthy plants using Trizol RNA extraction reagent (Gibco BRL Grand Island, NY) according to the manufacturer's instructions. Two μg of total RNA was used for first strand cDNA synthesis using AMV or MMuLV reverse transcriptase in a 20μl reaction mixture. Five μl of the first strand cDNA reaction mix was used as a template in 50μl PCR reaction using Vent DNA polymerase and virus specific primers for 35-40 cycles. The resulting

PCR products were run on a 0.8% agarose gel. ELISAs were performed according to the leaf disc procedure as described by Wai and Grumet (1995). Tissue printing was described in chapter 2. Plants were scored for symptom severity on the five youngest leaves on a scale of zero to five: 0=symptom free, 5=highly symptomatic, including severe mosaic and laminar distortion.

Aphid transmission.

Aphid (*Myzus persicae*) cultures were maintained on tobacco plants in the growth chamber. Using a soft camel hair brush, the aphids were collected in a petri dish and allowed to feed on virus infected squash leaves for 2-3 minutes. After acquisition feeding, 8-10 aphids were transferred to individual healthy squash plants (2 weeks old) and given a minimum of two hours of inoculation feeding time. All the aphids were then hand removed; the plants were transferred to growth chambers and sprayed to kill any escaped aphids.

RESULTS

Tests of the CP-NT chimeric constructs on susceptible squash and cucumber plants.

The chimeric ZYMV-constructs containing the NT of the CP of WMV (pWNTFL/P) and TEV (pTNTFL/P) were infectious on susceptible squash and cucumber plants (Figure 9). Symptoms appeared 5-7 days after inoculation on plants inoculated with the control or pWNTFL/P constructs, while a delay of 1-3 days was observed when susceptible plants were inoculated with pTNTFL/P. The inoculated plants showed strong systemic symptoms at 2-3 weeks post inoculation with all the constructs. Thus despite

limited sequence homology, the heterologous NTs were able to facilitate systemic infection.

Interestingly, 4-6 weeks after inoculation there was a gradual reduction in symptom severity on TNTFL/P infected susceptible squash and cucumber plants. Newly emerging leaves were either completely free of symptoms or showed very mild symptoms, while older leaves showed strong symptoms, comparable in severity to the control constructs (Figure 10). The recovery phenotype was associated with reduced virus titer in the younger leaves (Figure 11). Plants inoculated with the control constructs or pWNTFL/P developed increasingly severe symptoms throughout the course of the experiments: the newly formed leaves were severely distorted, had significantly reduced leaf lamina (Figure 10), and showed high virus accumulation (Figure 11).

The observed recovery in TNTFL/P inoculated plants does not appear to be caused by the developmental changes in the host associated with the age of the plant. When squash plants were inoculated with TNTFL/P at different developmental stages strong symptoms were observed 2-3 WPI irrespective of the age of the plant at the time of inoculation (Table 3A). The recovery also was not due to a loss of infectivity of the virus. Although virus titers were reduced in the recovered leaves, when these recovered leaves were used as an inoculum source to infect squash plants, typical symptoms were again observed (data not shown).

When the recovered leaves from TNTFL/P inoculated squash plants were inoculated with ZYMV (strain NAA or Ct), the challenge virus was not able to produce strong symptoms up to six weeks post secondary inoculation (Table 3B). Control plants of the same age inoculated with ZYMV showed typical symptoms 2-3 WPI (Table 3A).

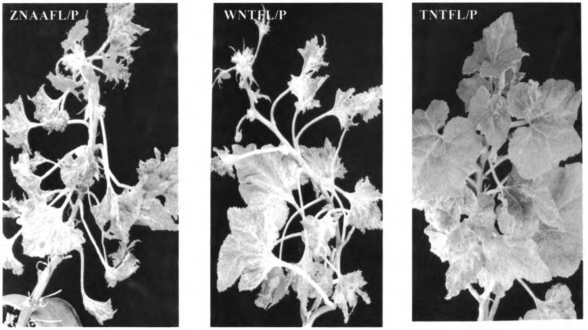


Figure 10. Symptom expression on susceptible squash plants 6 weeks post inoculation. Wild type ZYMV-NAA (left) and the chimeric construct containing the NT of the CP of WMV (center) showed increasingly strong symptoms. However, the chimeric ZYMV construct containing the NT of the CP of TEV (right) showed a marked reduction in symptom severity.

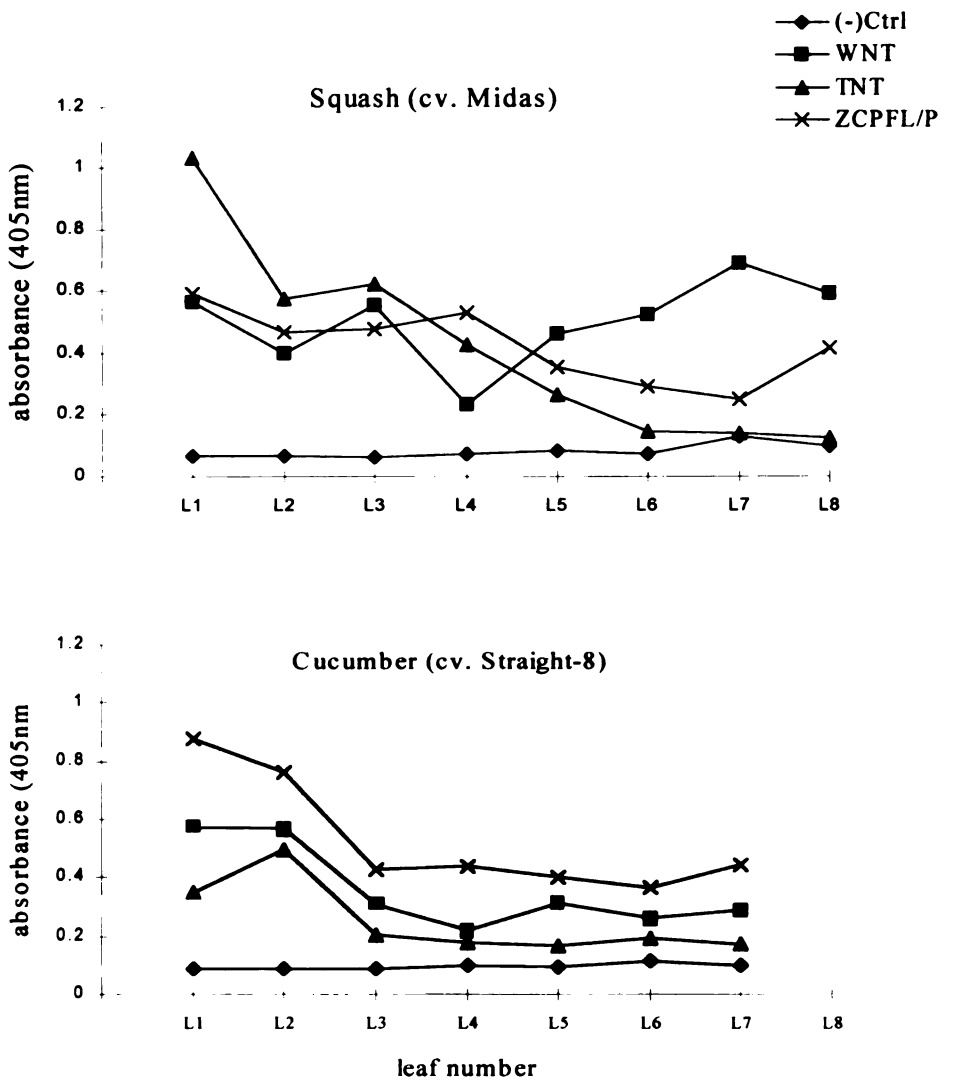


Figure 11. Virus titer (as determined by ELISA) in susceptible squash and cucumber plants at 30 days post inoculation (DPI). The top 7 or 8 leaves were sampled for ELISA from 6 plants for each virus. The X-axis shows leaf number from base to the top of the plant. The Y-axis shows absorbance at 405nm. Each point represents a mean of 6 replications. Experiments were repeated two times, each with 6 replications and similar results were observed.

Table 3. Effect of plant age (A) and prior inoculation (B) with ZYMV/TNT hybrid virus on symptom development in squash plants.

A. Effect of plant age on symptom development.

Age at Inoc	Number Plants Inoc.	Symptoms 2-3 weeks post inoculation[a] TNT	NAA/CT
7	20	5.0	5.0
17	16	4.2	4.7
50	9	3.7	4.1

B. Effect of prior inoculation of squash plants with TNT hybrid virus on infection by ZYMV or PRSV. Cotyledons were inoculated with TNT when 7 days old.

Age at 2^0 inoc.	Number Plants Inoc	Symptoms at time of 2^0 inoc.	Symptoms 2-3wks post 2^0 inoc.	Detection by RT-PCR TNT	ZYMV
17-ZYMV	10	5.0	1.5		
50-ZYMV	12	1.5	1.7	12/12	3/12
50-PRSV	13	1.5	4.7		

Data are pooled from two experiments.

[a] Symptoms were given a ranking from 0-5 with 5 being the most severely symptomatic.

RT-PCR on the recovered plants inoculated with the two viruses showed the presence of the TNT hybrid CP in most of the plants (12/12 plants tested) but fewer plants (3/12) showed the presence of ZYMV RNA, as determined by presence of the TEV-CP NT or ZYMV-CP NT sequences, respectively. These results suggest that ZYMV could not establish a successful infection in the recovered plants after secondary inoculation. The observed protection was virus specific, and secondary inoculation with the watermelon strain of papaya ring spot virus (PRSV) on the recovered leaves produced strong systemic symptoms (11/13 plants tested). The above recovery phenotype and protection against secondary inoculation was also observed when the non-recovered pTNTFL/P inoculated plants were inoculated with ZYMV-NAA as early as ten days after initial inoculation (Table 3B).

The chimeric viruses containing the NT of the CP of WMV or TEV also were aphid transmissible: 10/12, 12/12 and 7/12 plants showed systemic infections 10-14 days post-aphid inoculation with ZNAAFL/P, WNTFL/P and TNTFL/P viruses, respectively. The NT of the CP of WMV and TEV in the hybrid viruses contained the DAG motif known to be involved in the aphid transmission of potyviruses (Pirone and Blanc, 1996). Aphid transmission of potyviruses requires an interaction between CP and HC-Pro (Peng et al., 1998; Blanc et al., 1997). These results show that the NTs of the CPs of WMV and TEV were capable of interaction with the ZYMV HC-Pro to facilitate aphid transmission.

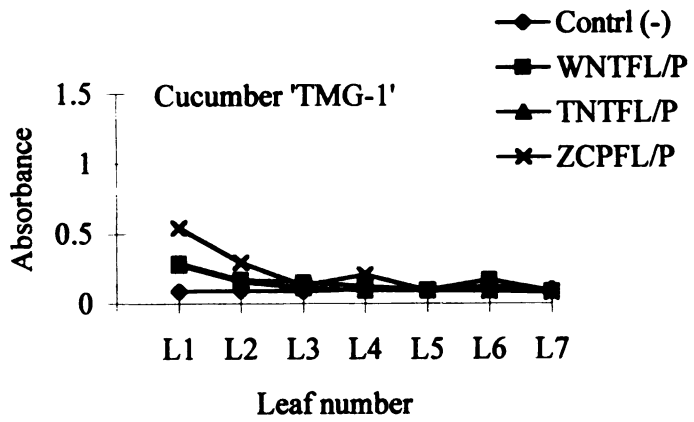
Tests on cucumber and melon plants with natural or genetically engineered resistance to ZYMV.

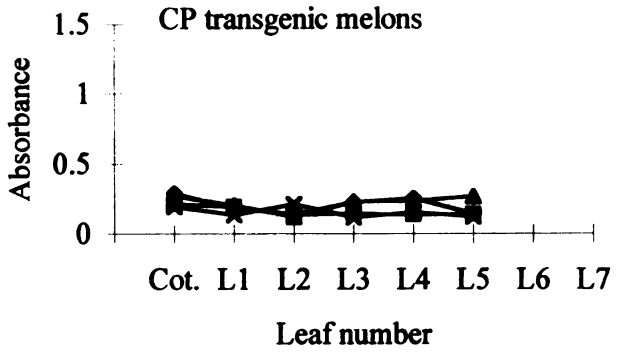
The chimeric viruses were tested on the cucumber genotype, 'TMG-1', possessing the recessive *zym* resistance allele (Provvidenti, 1987; Kabelka et al., 1997) and on transgenic melons engineered for resistance with the ZYMV CP gene (Fang et al., 1993). Neither the control nor the chimeric viruses were able to produce symptoms on the resistant cucumber genotype 'TMG-1' or CP transgenic melons. Virus accumulation could only be detected in the first one or two leaves of, TMG-1, at 4 weeks post inoculation (Figure 12). All the viruses showed severe symptoms on the susceptible melon cultivar, 'Hales-Best Jumbo'. These results indicate that the NT of the CP of WMV or TEV does not affect the resistance response of 'TMG-1' or ZYMV CP-transgenic melon plants to ZYM-NAA.

Tests on local lesion-hosts or non-hosts of ZYMV.

The chimeric viruses also were tested for infectivity on several additional local lesion or non-hosts including: Black Turtle-II beans (*Phaseolus vulgaris*), *Chenopodium amaranticolor, Nicotiana benthamiana* and tobacco (*N. tabacum*). ZYMV produces local lesions on Black Turtle-II bean and *Chenopodium amaranticolor* plants but does not infect *N. benthamiana* and tobacco (Brunt et al., 1996; Provvidenti and Gonsalves, 1984). WMV is reported to systemically infect black Black Turtle-II beans (Provvidenti and Gonsalves, 1984) and *N. benthamiana* (Brunt et al., 1996), while TEV produces local lesions on *C. amaranticolor* and systemically infects *N. benthamiana* and tobacco plants (Brunt et al., 1996).

Figure 12. Virus titer (as determined by ELISA) in resistant cucumber genotype 'TMG-1', transgenic melon line 207 expressing the CP gene of ZYMV-Ct in 'Hales Best Jumbo', and susceptible commercial cultivar 'Hales Best Jumbo' (bottom) 26 days after inoculation. Each point represents the mean of 5 replications. Cot = cotyledon, L1-to L5 represents leaf number 1 to -5 from the base of the plant. The 'TMG-1' and CP transgenic melon plants did not show symptoms while 'Hales Best Jumbo', plants showed severe foliar symptoms and only three to four leaves could be sampled for ELISA.


Contrl (-)
WNTFL/P
TNTFL/P
ZCPFL/P
Cucumber 'TMG-1'
Absorbance
1.5
1
0.5
0
L1 L2 L3 L4 L5 L6 L7
Leaf number


CP transgenic melons
Absorbance
1.5
1
0.5
0
Cot. L1 L2 L3 L4 L5 L6 L7
Leaf number

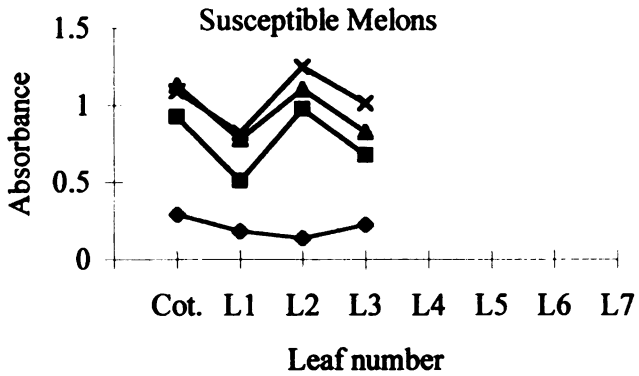

Susceptible Melons
Absorbance
1.5
1
0.5
0
Cot. L1 L2 L3 L4 L5 L6 L7
Leaf number

Table 4A. Summary of the inoculation experiments of various constructs using infected leaves of squash (for ZYMV-derived constructs) or tobacco (for TEV) as a source of inoculum onto squash, *C. amaranticolor* (C. amarant.), bean *N. benthamiana* (N. benth) and tobacco.

	No. of plants showing symptoms/ No. plants inoculated									
	Squash[a]	C.amarant.		Beans		N. benth.		Tobacco	
Construct	Sys.[b]	Local	Sys.	Local	Sys.	Local	Sys.	Local	Sys.
ZYCPFL/P	12/12[c]	3/7	0/7	18/26	0/52	0	0/30	0	0/9
TEV	n.t.	n. t.	n.t.	n. t.	n.t.	0	13/13	0	18/18
WNTFL/P	12/12	5/7	0/7	15/26	0/54	0	0/32	n. t.	n.t.
TNTFL/P	10/12	7/7	0/7	17/26	0/38	0	0/40	0	0/39

Table 4B. Back inoculation of susceptible squash plants from inoculated and systemic leaves of bean, N. benthamiana or tobacco plants

	Source of inoculum					
	Bean		N. benthamiana		Tobacco	
Virus	Inoc. L	Sys. L	Inoc. L	Sys. L	Inoc. L	Sys. L
ZYMV	5/7	0/34	2/22	0/27	0/19	0/13
WNTFL/P	6/7	0/36	1/14	0/26	n.t.	n.t.
TNTFL/P	5/7	0/20	3/22	0/36	0/15	0/26

[a] Squash plants did not show local lesions;
[b] Sys. = systemic, Inoc. = inoculated, and L=leaf, n.t. = not tested
[c] Number of plants showing symptoms on squash plants/ number used as inoculum source.

As expected, all ZYMV derived constructs produced systemic symptoms on squash (Table 4A). When *C. amaranticolor* and Black Turtle-II bean plants were inoculated, local lesions were observed on the inoculated leaves with both the control and chimeric viruses; however, none of the viruses produced systemic symptoms (Table 4A). Susceptible squash plants back inoculated from the inoculated leaves of Black Turtle-II beans, showed symptoms for all the constructs, but no symptoms were observed on plants back inoculated from systemic leaves (Table 4B). No local or systemic symptoms were observed for any of the ZYMV-derived constructs on *N. benthamiana* or tobacco plants. Back inoculated squash plants from the systemic leaves of *N. benthamiana* or tobacco did not show symptoms (Table 4B). A limited number of squash plants (ca. 10%) back inoculated from the inoculated leaves of *N. benthamiana* showed symptoms. These results suggest that the NT of CP of WMV or TEV is not sufficient to allow ZYMV to cause systemic infection in *C. amaranticolor*, Black Turtle-II beans, *N. benthamiana*, or *N. tabacum*.

Core and whole CP substitutions.

We also investigated the effect of substitution of the core and carboxy terminus (for simplicity will be referred to as core) and the full length CP of WMV and TEV, in facilitating movement of the chimeric viruses (Figure 13). Substitutions also were made with the Connecticut isolate of ZYMV (ZYMV-Ct). Bombardment of squash plants with the original ZYMV-NAA construct gives 50-70% infection, on average. A comparable percentage of the control constructs carrying the core regions (CtCoFL/P) or the entire CP (CtCPFL/P) ZYMV-Ct showed symptoms 5-7 days after particle bombardment with

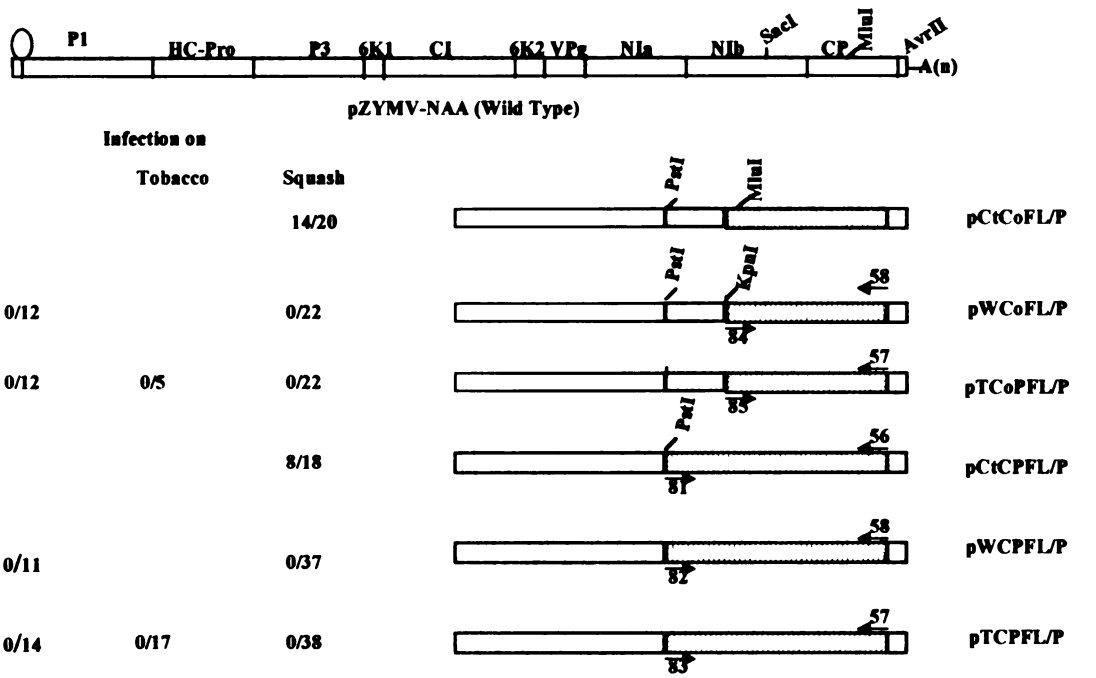


Figure 13. Hybrid ZYMV-NAA constructs containing the core and carboxy terminus (Co) of the coat protein of ZYMV-Ct (CtCoFL/P), WMV (WCoFL/P) or TEV (TCoFL/P) and their infection on squash plants. Hybrid viruses containing the entire CP of ZYMV-CT, WMV and TEV are pCTCPFL/P, pWCPFL/P and pCTCPFL/P respectively. Introduced restriction sites are indicated at the top of the control constructs. Numbers on the left indicate the number of plants showing symptoms/number of plants inoculated by particle bombardment with full-length cDNA constructs.

the full length cDNA constructs (Figure 13). The chimeric viruses containing the core or the entire CP of WMV (WCoFL/P, WCPFL/P) and TEV (TCoFL/P TCPFL/P), however, were not infectious on any of the plants tested including squash, *N. benthamiana*, and tobacco (Figure 13). We were also unable to detect symptoms (up to two months after inoculations), or virus titer with ELISA, tissue prints, RT-PCR, or back inoculation (data not shown) in these plants. To account for possible effects of a foreign 3' NTR on replication, a second set of constructs were made that retained the entire 3' NTR of ZYMV and only the coding sequences of the CP or core region were substituted with the respective regions WMV or TEV. These constructs were also not infectious on squash, *N. benthamiana* or tobacco plants (data not shown).

DISCUSSION

The NT of the potyvirus CP, which is essential for systemic movement and aphid transmission of the virus, is highly variable in length and sequence (Shukla et al., 1994; Dolja et al., 94, 1995; Atreya et al., 1990; Blanc et al., 1997). Our results show that despite substantial variation, the CP NTs from heterologous potyviruses can facilitate systemic infection of chimeric ZYMV viruses in ZYMV-susceptible cucurbits. This was observed for chimeric viruses with CP-NTs from viruses having both overlapping (WMV) and non-overlapping (TEV) host ranges with ZYMV. The NT of the CP of ZYMV is 44 amino acids (aa) long, compared to 46 aa of WMV and only 29 aa of TEV. There are only 13 aa that are identical among the CP NTs of the three viruses, six of which occur in KD pairs and three of them form the DAG triplet shown to be involved in

aphid transmission (Blanc et al., 1997; Atreya et al., 1990). This suggests that only a few key amino acids in the NT of the CP are required to facilitate systemic movement of potyviruses, even in species that are not typically hosts. Previous studies have shown Asp in the conserved DAG motif to be critical for systemic movement. Substitution of Lys or Arg for Asp in the DAG motif of TVMV and TEV affected systemic movement of the virus (Lopez-Moya and Pirone, 1998). However, mutations in the second and third positions of the DAG motif are frequently observed in association with aphid transmission and do not affect systemic virus movement (Atreya et al.,1990, 1991; Gal-On et al., 1992; Andrejeva et al., 1999).

The ZYMV CP-NT chimeric viruses were also aphid transmissible. Since successful aphid transmission requires interaction between the CP-NT and HC-Pro (Blanc et al., 1997; Flasinski and Cassidy, 1998; Peng et al., 1998), this indicates that the features necessary for CP - HC-Pro interaction to facilitate aphid transmission were present in the heterologous CP-NTs. In binding assays, the conserved DAG motif in the NT of CP and the PTK motif in the HC-Pro gene have been shown to be involved in this interaction (Blanc et al., 1997; Peng et al., 1998). A sequence of 7 aa (DTVDAGK) in the NT of the CP was sufficient for binding HC-Pro (Blanc et al., 1997). The chimeric viruses used in this study contained the amino acids TV and DAG in different contexts in the NT of the CP, but were all aphid transmissible suggesting flexibility in this motif for interaction with the HC-Pro gene of ZYMV. These observations are also consistent with previous studies showing facilitation of aphid transmission by heterologous helper components (Atreya and Pirone, 1993; Hobbs and McLaoghlin, 1990; Lecoq and Pitrat, 1985; Sako and Ogata, 1981). Mixing experiments using purified virions from one virus

and HC-Pro from a different virus resulted in aphid transmission, although some combinations were less effective than others (Sako and Ogata, 1981; Lecoq and Pitrat, 1985; Hobbs and McLaoghlin, 1990). For example, HC-Pro from ZYMV facilitated aphid transmission of WMV and vice versa, but the ZYMV and WMV HC-Pro were less effective in facilitating transmission of the less closely related PRSV-W (Lecoq and Pitrat, 1985). Similarly, aphid transmission of the NAT isolate of TEV was facilitated by the aphid transmissible (AT) isolate of PVY, but not by WMV-AT (Simons, 1976). A chimeric TVMV construct containing the HC-Pro gene of ZYMV also was aphid transmissible (Atreya and Pirone, 1993).

Although the TNT chimeric virus produced strong symptoms at the initial stages of infection, 4-6 weeks post inoculation the plants showed a marked recovery characterized by a progressive reduction in symptom development and virus titer, and a virus-specific resistance to secondary inoculation. The reduction in symptom severity was not a result of plant age or mutation of the infecting virus, and was unique to the TNT chimeric virus; the parent ZYMV and the chimeric WNT viruses caused increasingly severe symptoms over time. This indicates that the NT of the CP could be the target of host defense responses and that the variability in the NT has a role in host adaptation. The role of the NT of potyviral CP in host adaptation and resistance responses has previously been suggested (Shukla et al., 1994, Xiao et al., 1993, Solomon, 1989, chapter 2). A single aa substitution (Ser47 to Pro) in the NT of the CP of the NY isolate allowed PSbMV to produce systemic symptoms in *C. quinoa* (Andersen and Johansen, 1998) suggesting that the NT of the CP is involved in the localization of the virus to the inoculated leaves in *C. quinoa* in a host specific manner. Evidence for the

role of the CP-NT in inducing host defense responses also comes from the response of the resistant cucumber genotype 'Dina-1' to inoculation with chimeric ZYMV infectious constructs (chapter 2). Unlike ZYMV-Ct, ZYMV-NAA does not produce veinal chlorosis symptoms on 'Dina-1' plants. When the NT of CP of the infectious ZYMV-NAA construct was substituted with CP-NT ZYMV-Ct, the resulting chimeric construct produced veinal chlorosis on 'Dina-1' plants. Substitution of the core region of the CP between the two virus strains, on the other hand, did not result in veinal chlorosis. In another example of recovery from potyvirus infection, a protease activity associated with the cleavage of the NT of the CP of SPFMV was observed in *Ipomia nil* plants (Solomon, 1989). Another, not mutually exclusive, possibility is that the eventual recovery observed with the chimeric TNT virus reflects sub-optimal interaction between the heterologous CP-NT and other viral or host proteins thereby allowing host defenses to be more effective. The TNT virus was slower in establishing initial infection, which could have resulted from this sub-optimal interaction.

Interestingly, the above described recovery phenotype also shows striking resemblance to recovery of infected plants from infection by a number of viruses including potyviruses (reviewed by Pennazio et al., 1999) and to recovery responses observed in transgenic plants expressing RNA mediated protection. Recent studies with several types of viruses (e.g. Tobra-, Caulimo- and Nepoviruses) have shown that the natural recovery phenotype is associated with induction of a post transcriptional gene silencing (PTGS) like resistance response which leads to specific degradation of the viral RNA (Al-Kaff et al., 1998; Covey et al., 1997; Ratcliff et al., 1997, 1999; Hamilton and Baulcombe, 1999). As a counter-strategy, many viruses encode specific proteins, which

suppress PTGS-like resistance of the host (Brigneti et al., 1998; Anandalaskshmi et al.1998; Kasschau and Carrington, 1998; Voinnet et al., 1999). In potyviruses P1-HC-Pro acst as a suppressor of PTGS (Brigneti et al., 1998; Kasschau and Carrington, 1998; Voinnet et al., 1999). Klein et al., (1994) observed a recovery phenotype in tobacco plants associated with certain mutations in the P1 or HC-Pro genes of TVMV. The mutated viruses caused strong initial symptoms on tobacco plants, however, by 25dpi the newly emerging leaves were almost symptomless suggesting that these mutations might interfere with the ability of the virus to suppress PTGS.

Unlike HC-Pro, CP has not been directly implicated in suppression of PTGS. It will be of interest to determine whether the observed recovery associated with the TNT chimeric virus infection is a result of a PTGS like resistance response. Interaction between CP and HC-Pro has been well documented for aphid transmission (Blanc et al., 1997; Peng et al., 1998) and due to the overlapping functions of these proteins, e.g., both proteins appear to be involved in cell to cell and long distance viral movement, it is conceivable that the two proteins may interact at other stages in the viral life cycle. Andrejeva et al., (1999), have suggested that potyvirus HC-Pro and CP have coordinated functions in virus host interactions. They observed that the effects on virus accumulation and movement caused by simultaneous mutations made in the HC-Pro and CP genes of PVA were different from the expected 'sum' of phenotypic changes observed following mutation of only one gene at a time.

The NT hybrid viruses did not overcome naturally occurring resistance to ZYMV in the cucumber line 'TMG-1'. This is not entirely surprising as 'TMG-1' is also resistant to WMV (Provvidenti, 1985, 1987; Kabelka and Grumet, 1995), and TEV is a

non-pathogen of cucumbers. Interestingly, however, the chimeric viruses also did not overcome the ZYMV CP mediated resistance. Although the full length CP confers a high level of protection against ZYMV infection (Fang and Grumet 1993; this study), the core portion of the CP is not sufficient to provide resistance (Fang and Grumet, 1993). This suggests that although the NT is required for the high level of transgenic CP mediated resistance, which is virus specific and not RNA mediated (Grumet et al., 1995, 1998), the resistance does not depend on the NT of the CP of the incoming virus. Interestingly, the recovery phenotype associated with the CP-NT of TEV also did not appear to be targeted against CP-NT in a sequence specific manner, and was effective to protect against secondary infection by ZYMV-NAA and ZYMV-Ct. Together these results suggest that the CP-NT could be involved in triggering host resistance responses targeted against other parts of the virus genome.

Substitution of the CP NT did not appear to alter the host range of ZYMV. We were unable to detect local or systemic infection in *N. benthamiana* and tobacco plants or systemic infection in *C. amaranticolor* or *P. vulgaris* plants after inoculation with ZYMV or the chimeric viruses. The failure to observe systemic infection by the chimeric viruses in the local lesion hosts suggests that the host defense responses in these plants leading to virus localization were not dependent on the specific CP-NT. Only a limited number (ca.10%) of *N. benthamiana* plants showed the presence of virus in the inoculated leaves. The inability of ZYMV and chimeric viruses to cause local infection in *N. benthamiana* and tobacco suggest impaired replication or cell-to-cell movement of the virus in these non-hosts.

Although the chimeric constructs containing the CP or core region of the Connecticut isolate of ZYMV were infectious on squash plants, when squash, *N. benthamiana*, or tobacco were inoculated with the chimeric ZYMV constructs containing the entire CP, or the core portion of the CP of TEV or WMV, no symptoms were observed. This might be due to a number of reasons related to the functions of the core of the CP functions, such as encapsidation or cell-to-cell movement, or more general factors influencing virus viability such as the ability to replicate or processing of the polyprotein. Although we can not rule out encapsidation or cell-to-cell movement, transencapsidation among potyviruses has been observed in several systems (Simons 1976; Bourdin and Lecoq, 1991; Lecoq et al., 1993) and the cell-to-cell movement mediated by the potyvirus CP did not appear to be responsible for host specificity in micro injection studies (Rojas et al., 1997). The bean common mosaic necrotic virus (BCMNV) moved cell-to-cell and increased plasmodesmata SEL in a non-host (lettuce) and facilitated the movement of LMV CP RNA in *N. benthamiana* (Rojas et al., 1997). Successful proteolytic processing also does not appear to be a problem. The infectious CP-NT chimeric viruses used the same hybrid CP protease cut sites as did the CP or core substituted constructs, indicating that they were processed properly. However, the CP coding sequence and the 3' NTR have been shown to be co-adapted for genome amplification through a requirement for base pair interactions leading to complex secondary structures (Haldeman-Cahill et al., 1998; Mahajan et al., 1996). Chimeric constructs in which only the coding sequence of the CP or core region was substituted (leaving an intact ZYMV 3' NTR) also were not infectious (data not shown). Comparison of the nucleotide sequence of the three viruses used in this study shows

considerable variability in the 3'NTR and the carboxy terminus of the CP raising the possibility that the chimeric constructs might be replication defective due to defective secondary structure in this region.

Our results showed that the CP-NTs from heterologous potyviruses could facilitate systemic infection of ZYMV in susceptible cucurbit hosts, but this substitution was not sufficient to modify host range of the chimeric viruses. Substitution of the CP-NT of ZYMV with a non-cucurbit potyviruses resulted in induction of a host defense response leading to recovery from the chimeric virus infection, suggesting that the variability in the CP-NT has a potential a role in host adaptation.

Future Directions.

Our data revealed that heterologous NT from the CP of a non-cucurbit potyvirus could induce (or fail to suppress) a resistance response to the chimeric ZYMV in cucumber and squash plants. The recovery phenotype was similar to the phenotype associated with virus induced gene silencing. Further characterization of this recovery response is important. It will also be useful to know if CP-NTs from other non-cucurbit potyviruses could induce a similar resistance response and this will help us better understand the nature of this resistance response. Identification of the domains within the CP-NT involved in host recovery from chimeric virus infection will also be very useful in further understanding the recovery response, and this could have practical implications in engineering transgenic plants expressing viral coat proteins.

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CHAPTER IV

CHARACTERIZATION OF THE INTERACTION OF THE CUCUMBER POLY(A) BINDING PROTEIN WITH THE REPLICASE GENES OF VIRUSES

ABSTRACT

Deletion studies were performed to identify the domains involved in the interaction between a cucumber poly(A) binding protein (PABP) and the RNA dependent RNA polymerase (RdRp) gene of zucchini yellow mosaic virus (ZYMV) in the yeast two hybrid system. Deletions of 47 amino acids (aa) from the carboxy terminus of the cucumber PABP resulted in weak interaction with ZYMV RdRP, while deletions of 143 aa completely abolished the interaction, suggesting that the C-terminus of the PABP is involved in interaction with the RdRp. Deletions from both the amino and carboxy termini of the RdRp gene abolished the interaction in yeast, suggesting that a large portion of the RdRp protein is required for the interaction. Interaction of the cucumber PABP was also studied with the RdRp proteins from other viruses. Bean common mosaic potyvirus and cowpea chlorotic mottle bromovirus RdRp genes interacted strongly with the cucumber PABP protein, and the RdRp gene from poliovirus showed weak interaction. However, RdRps of watermelon mosaic and tobacco vein mottling potyviruses did not interact with the cucumber PABP.

INTRODUCTION

Successful systemic infection by a pathogen depends on compatible interactions between the pathogen and its host; this is particularly true in the case of viruses, which have extremely small genomes and limited protein coding capacity. Several host proteins have been shown to be involved in replication of RNA viruses, either as components of the viral replication complex, or by binding directly to the viral genome (reviewed in: Lai, 1998; Strauss and Strauss, 1999). For RNA viruses, a majority of the factors found in association with the viral replicase, the RNA dependent RNA polymerase (RdRp), are subverted from the host RNA-processing and translation machinery. For example, elongation factor EF-1α and different subunits of eIF3 have been found in association of replicase complexes of an array of bacterial, plant, and mammalian viruses such as Qβ phage, brome mosaic virus, tobacco mosaic virus, vesicular stomatitis virus, measles virus and poliovirus (Lai, 1998; Strauss and Strauss, 1999).

Potyviruses also have a small genome, and presumably interact with a number of plant proteins for successful infection. The 10kb genome of potyviruses, has a genome linked viral protein (VPg) covalently attached to the 5' end and a poly(A) tail at the 3' end. The entire genome is translated into a single polyprotein which is subsequently cleaved into at least nine proteins by viral encoded proteases (Shukla et al., 1994). To better understand host-potyvirus interaction, a project was initiated in the lab to identify host factors interacting with the RNA dependent RNA polymerase (RdRp) gene of zucchini yellow mosaic virus (ZYMV).

A leaf cDNA library was constructed from the susceptible cucumber (*Cucumis sativus*) host 'Straight-8', and screened for interaction with the ZYMV RdRp gene using the yeast two hybrid system (Wang et al., submitted). Interestingly, a poly(A) binding protein (PABP) was repeatedly isolated in these screens. This interaction was also confirmed in subsequent *in vitro* binding experiments.

Potyvirus replication occurs in the cytoplasm and proceeds by copying of the plus strand to minus strand followed by copying back to the plus strand (Martin and Garcia, 1991; Schaad et al., 1997). Presence of a poly (A) tail is essential for replication of picornaviruses such as poliovirus, encephalomyocarditis virus and rhinovirus (Cui et al., 1993, Todd and Semler, 1996; Todd et al., 1997; Agol et al., 1999) and recent evidence suggests it is also necessary for potyvirus replication (Tacahashi and Uyeda, 1999). A study of the interaction of the viral RdRp gene with the host PABP could therefore be of fundamental importance in understanding replication of plant viruses.

In this project I attempted to characterize the interaction the ZYMV RdRp protein with the cucumber PABP by identifying the domains of the two proteins involved in this interaction. The interaction of the PABP with polymerase genes from other viruses was also studied. Deletion of the carboxy terminal 150aa of the PABP abolished interaction with the ZYMV-RdRp protein; however, the components of the RdRp protein involved in the interaction were more complex. The cucumber PABP also interacted with the replicase genes from bean common mosaic necrosis potyvirus (BCMNV) and cowpea chlorotic mosaic bromovirus (CCMV), but failed to interact with certain other viruses including some members of the potyvirus group.

MATERIALS AND METHODS

ZYMV-RdRp and Cucumber PABP deletions.

All the PABP deletions were made from NI8 (Figure 14 B), the longest cDNA obtained from the two hybrid screen of Wang et al (submitted). NI8Δ300 was amplified by PCR, using primer pair RG110 (5'-AAA*GAATTC*GGCTTTGTAAATTTTGAG-3': forward: position: 5' end of NI8) and RG157 (5'-TCT*CTCGAG*CAAATGTAGAAC CTCAGT-3': reverse, position 1801) introducing *EcoRI* and *XhoI* sites (underlined), respectively. Position numbering is based on the full length *Cs-PABP1* gene (Wang et al., submitted). The PCR product was cloned into the yeast two hybrid vector, pADGAL4 (Stratagene) as an *EcoRI-XhoI* fragment, resulting in the construct pADNI8Δ300. NIΔ*Mlu* was amplified with RG110 and RG115 (5'-GTA*CTC GAGCAT GCGAGCTC*AAAG GTACAGGCTGC TGG-3': Position 1476 near *MluI* site and introducing *XhoI*, *SphI*, and *SacI* sites at the 3'end). The PCR product was cloned as an *EcoRI-XhoI* fragment into pADGAL4 to form pADNI8Δ*Mlu*, and into pBlueScript to form pBSNI8Δ*Mlu*. pBSNI8Δ*Mlu* was then digested with *SphI*, and religated to form pBSNI8Δ*Sph*. The *EcoRI-XhoI* NI8Δ*Sph* fragment was then subcloned to pADGAL4 to form pADNI8Δ*Sph*. pADNI8ΔSac was made by digesting pADNI8Δ*Mlu* with *SacI* to drop the 418 bp and religating the plasmid band.

All the RdRp deletions were made from the full-length RdRp clone (Figure 14). RdRpΔ1, Δ4, Δ5, Δ6, Δ7, Δ8 were amplified by PCR using primers as indicated in Figure 14; all the PCR products were inserted into the yeast two hybrid vector pBDGAL4 as *EcoRI-SalI* fragments. The sequences of primers RG100, 101, 121, 125, 126, 127 were as follows, restriction sites are underlined: 5'-AGA*GTCGAC*CCTGA C TTTCTCAAGC-

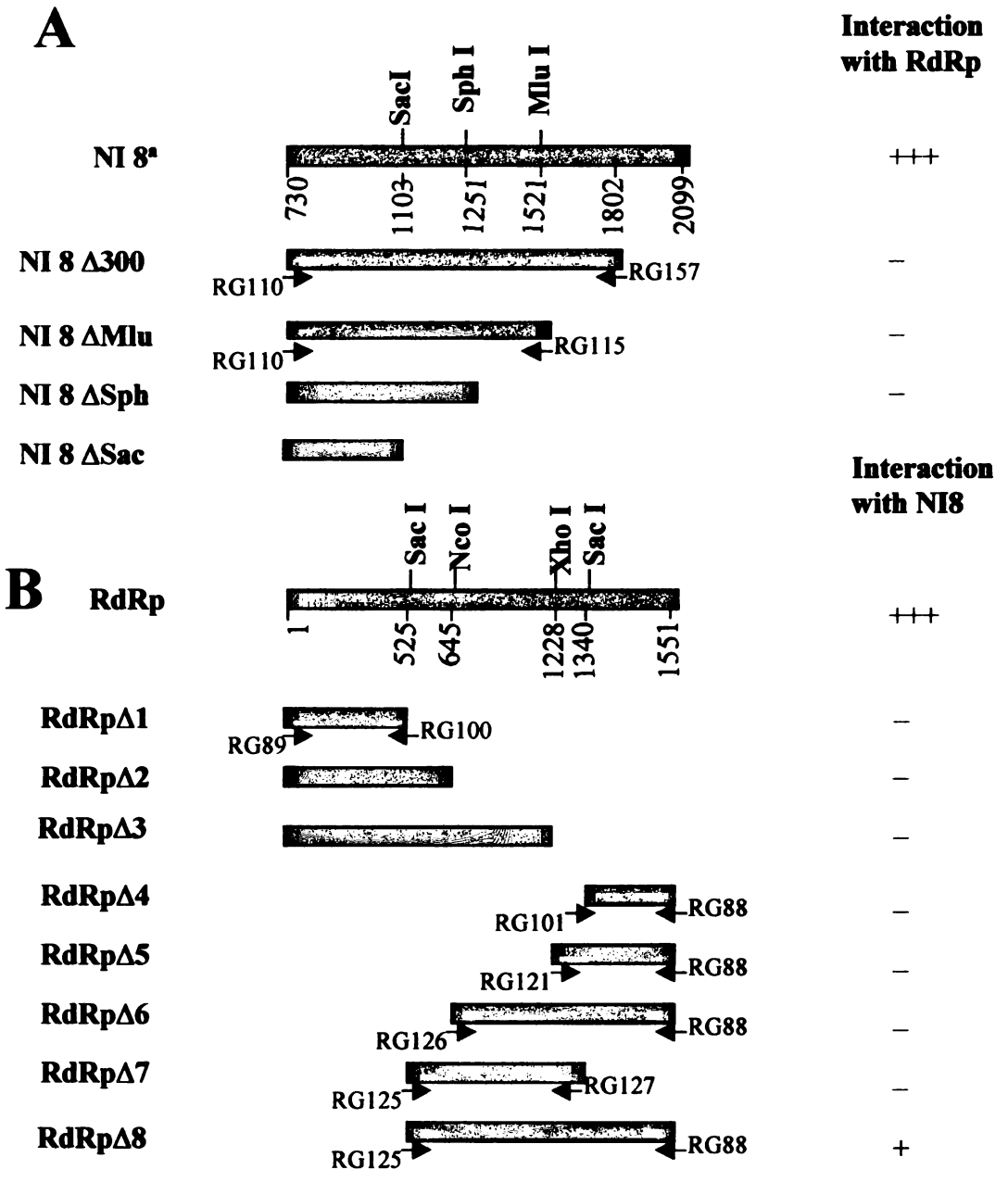

Figure 14 Deletion analysis to determine regions in PABP and RdRP responsible for the interaction. A. Different sizes of cDNA NI 8 were amplified by PCR or generated by restriction enzymes digestion. All the deletions were tested against full-length RdRp in the yeast two-hybrid system. B. Different sizes of RdRp was amplified by PCR or generated by restriction enzymes digestion. All the deletions were tested against NI 8 in the yeast two-hybrid system. Positive interactions are shown by + sign. [a] Positions of NI8 were marked according to the full-length sequence.

3', 5'-CCG*GAATTC*TGCGCTGCGAT GATT-3'; 5'-ACT*GAATTC*CT CGAGAAAGAG AGAAT-3'; 5'-GTG*GAATTC*CCAA TTCTTGCTCCTGA-3'; 5'-TTA *GAATTC*GAG CTCAGGCCGCTT-3', 5'-TTC*GTCGA* TCTCGAGTTTTGGAGTG-3'. RdRpΔ2 was generated as follows: pBDRdRp was digested with *NcoI*, filled in with Klenow fragment, and digested with *EcoRI*. This RdRp fragment was ligated to pBD, which was digested with *SalI*, blunted, then cut by *EcoRI* to form pBDRdRpΔ2. pBDRdRpΔ3 was produced by cutting pBDRdRp with *EcoRI* and *XhoI*, and then subcloning to *EcoRI-XhoI* digested pBDGAL4.

Cloning of the RdRp genes from other viruses.

The infectious construct of tobacco vein mottling virus (TVMV) was provided by Dr. Pirone (Univ. of Kentucky). The RdRp gene was amplified from this construct using the primer RG133 (5-AA*GAATTC*CAAGGGGAGAAGCGAAAA-3) that primed at the 5'end of the RdRp and introduced an *EcoRI* site, and primer RG134 (5'-AA*CTCGAG* TGTATCACTTTGA AATCTCAC-3') which introduced a *XhoI* site at the 3' end. The PCR product was digested with *XhoI* followed by partial digestion with *EcoRI* (TVMV RdRp gene has an internal *EcoRI* site) and cloned into pBDGAL4 cut with *EcoRI* and *SalI* (compatible with *XhoI*). The CCMV (provided by Richard Allison, Michigan State University) 2a gene was cloned analogously using the primer pairs RG122 (5'-GC*GAA TTC*ATGTCTAAGTT CATT CCAG-3': forward) and RG124 (5'-GC*CTCGAG*TTA TTTAGAAAGGGTC TTAC-3': reverse). Watermelon mosaic virus RdRp gene (Quemada et al., 1990) was amplified using the primer pair RG145 (5-GCA*GA ATTC*AG

CAGAAAGGAAAGATG-3': forward) and RG146 (5'-TTT*GTCG AC*TTGTA AAGACACTGATTC-3': reverse). The PCR product was cut with *SalI* followed by partial digestion with *EcoRI* and cloned into the bonding domain vector cut with the same restriction enzymes. BCMNV RdRp was cloned analogously by using the primer pair RG151 (5'-GAA*GAATTC*GGTACCAGCAAGAAGGATAGATGG-3': forward) and RG152 (5'-TTC*GTCGAC*CCTAGGTTGTGTTGACACGGATTC-3': reverse). RG151 and RG152 also introduced two useful cloning sites; a *KpnI* (RG151) site adjacent to the *EcoRI* site and an *AvrII* site (RG152) next to the *SalI* site. All the clones were verified by restriction enzyme analyses and sequencing.

The BMV 2a gene (provided by Richard Allison, Michigan State University) was amplified using primer RG142 (5-ATC*GAATTC*ATG TCTTCGAAAACCTGG-3': forward) and primer RG143 (5-AAC*CTCGAG*TTTCAGATCA GAGGG CT-3': reverse). The PCR product was cut with *EcoRI* and *XhoI* and cloned into pBDGAL4 cut with EcoRI and SalI. The 3D gene of poliovirus was cloned by digesting the construct pJG3D prey (provided by Dr. Karla Kiukegaard, Stanford university) with *EcoRI* and *XhoI* to drop the 3D gene, and cloned into pBDGAL4 cut with *EcoRI* and *SalI*.

Yeast transformation and study of the interaction between NIb and Cs-PABP1.

Yeast transformation, growth media, and X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) filter assays were performed following the supplier's procedures (Stratagene). To test the effect of deletions in the cucumber PABP on interaction with ZYMV-RdRp, yeast cells were transformed with pBDRdRp (binding domain vector containing the wild type ZYMV RdRp gene), and each of the PABP deletions in

pADGAL4. The transformants were plated on SD (synthetic dropout) medium without leucine (L), tryptophan (T), and histidine (H: SD-L-T-H). As a control for transformation efficiency, transformants were also plated on SD-L-T. Colonies that grew on selection medium were restreaked onto SD-L-T-H, transferred onto nitrocellulose membrane (Schleicher & Schuell), and assayed for expression of β-galactosidase activity (*Lac Z*) by X-Gal filter assay. Colonies that survived medium without histidine and turned blue in the X-Gal assay were considered as positives. Deletions in the RdRp gene were tested similarly by transforming competent yeast cells with *Cs-PABP1* in the pADGAL4 vector and the RdRp deletions in the pBDGAL4 vector.

To test the interaction of the RdRp genes of different viruses with the cucumber PABP, competent yeast cells were transformed with pBDGAL4 containing the RdRp gene of the respective virus and pADGAL4 containing the *Cs-PABP1* gene. As a control cells were also transformed with pBDRdRp constructs alone. The transformants were plated on appropriate plates and tested for interaction as described above.

RESULTS AND DISCUSSION

Identification of the interacting domains of the cucumber PABP and ZYMV-RdRp.

To identify the domains of the CsPABP and the ZYMV NIb genes responsible for interaction in the yeast two hybrid system, a series of deletions were made in both proteins. The deletions made in the PABP are shown in Figure 14. When 47aa were deleted from the carboxy (C-) terminal end (pADNI8Δ300), a very weak interaction was observed as measured by the lower intensity of the blue color after X-gal test compared to the wild type construct and the longer time required for the color to appear (four hours

vs. overnight). Deletions of 143aa from this end of the protein (pADNI8Δ*Mlu*) completely abolished the interaction, suggesting that the C-terminal end of the PABP is critical for interaction with the ZYMV RdRp protein in the yeast two hybrid system. The smallest interacting PABP cDNA obtained from the two hybrid screen (NI 351) encodes the last 130 amino acids, further suggesting that the carboxy terminal conserved (CTC) domain might be involved in the interaction with RdRp (Wang et al., submitted).

The C-terminal third of the PABP protein is not as highly conserved among PABPs as the amino terminus, and functions of the C-terminus are less well defined. At the C-terminus, there is a 71 amino acid region (amino acids 553-624) that is conserved among *Arabidopsis,* wheat, and cucumber. This CTC (C-terminal conserved) domain was also found within other characterized PABPs (e.g. yeast, vertebrate) and has been implicated in homodimerization and efficient poly (A) binding (Kuhn and Pieler, 1996). The N terminal two-thirds of the predicted PABP contains 4 RRMs (RNA recognition motifs), which are found in PABPs from all sources, including yeast and animals (Le et al., 1997) and directly interact RNA (Kuhn and Pieler, 1996). The two smaller cucumber cDNAs (NI439 and NI359) lacked the RRMs, suggesting that the RRMs are not involved in binding with ZYMV RdRp (Wang et al., submitted).

To identify the domains of the ZYMV RdRp involved in the interaction with the Cs-PABP1, a total of eight deletions were made in the ZYMV RdRp protein (Figure 14B). Deletions from both the amino and carboxy termini of the RdRp abolished the interaction in yeast. Only one protein with 156 aa deletions from the 5' end showed mild interaction. Although we cannot rule out the possibility that certain deletion products were unstable, it may be that a large portion of the RdRp is necessary for the interaction

with PABP. RdRps, like other types of DNA and RNA polymerases, have been shown to consist of finger-palm-thumb domains resulting in intramolecular interactions between the amino- and carboxy-terminal portions of the molecule (O'Reilly and Cao, 1998; Lesburg et al., 1999) and such interactions also may be important for association with the PABP. Similar problems in assigning functions to specific RdRp domains were observed with the tobacco etch potyvirus (TEV) RdRp. Loss of interaction between the TEV RdRp and the NIa protein were observed with both amino and carboxy terminal deletions of the TEV RdRp, and nuclear localization capacity was eliminated by deletions from either terminus and by small insertions at several positions in the protein (Li and Carrington, 1993; Li et al., 1997).

Interaction of the cucumber PABP with RdRp genes from other viruses.

Replication of the potyvirus positive sense ssRNA genome is initiated at the 3' end by the RdRp protein to make a minus sense RNA. Since the potyvirus genome contains a poly(A) tail at the 3' end, interaction of PABP with ZYMV RdRp raised the possibility that this interaction might have implications for virus replication. To investigate if the PABP has a more general role in virus replication, the interaction of the cucumber PABP with the RdRp of other viruses containing a poly(A) tail (the potyviruses: WMV, TVMV, and BCMNV, and the Picornavirus, poliovirus), or viruses without poly(A) tail (Bromoviruses; BMV and CCMV) were studied (Table 5). Among the potyviruses tested, the cucumber PABP interacted with the RdRp gene of BCMNV, but not with the WMV and TVMV RdRps. This is intriguing because WMV is more closely related to ZYMV and infects cucurbits, while BCMNV is more distantly related

to ZYMV and does not infect cucurbits. Interestingly, the cucumber PABP also showed mild interaction with the 3D gene of poliovirus. Poliovirus, like potyviruses, belongs to the *Picornavirus* super group, and it also contains a poly(A) tail at the 3' end.

Cowpea chlorotic mottle virus 2a protein also showed strong interaction with the cucumber PABP protein; however the interaction with the closely related BMV 2a protein could not be confirmed as the 2a gene from this virus also turned on the two reporter genes, in the absence of the PABP. CCMV lacks a poly(A) tail suggesting that the functional significance of PABP-RdRp interaction may not depend on the presence of a viral poly(A) tail.

The significance of interaction between the PABP and viral RdRp and its effect on the viral life cycle remain to be determined. It is possible that this interaction plays more than one role in virus infection or has different roles in the infection of different viruses. A number of possible roles for the cucumber PABP interaction with ZYMV RdRp can be proposed. For example, it is possible that the association of the RdRp with PABP interferes with the PABP polymerization (Kuhn and Pieler, 1996) and so

Table 5. Interaction of the poly(A) binding protein with viral RdRp proteins.

Source of RdRp gene	Interaction with poly(A) binding protein	
	Growth on (-) His	X-gal test
ZYMV	++	++
BCNMV	++	++
TVMV	-	-
WMV	-	-
BMV	?	?
CCMV	++	++
Polio virus	-	-

might facilitate removal of PABP from the poly(A) tail. Another possibility is that the RdRp sequesters PABP from binding to hnRNA in the nucleus, inhibiting RNA processing. Recent studies have shown that viral-induced shut down of host protein synthesis, which is thought to facilitate viral infection by increasing accessibility of host factors for viral purposes, can be mediated at least in part, by sequestration or cleavage of PABPs (Piron et al., 1998; Chen et al., 1999; Joachims et al., 1999). In the case of potyvirus, inhibition of host gene expression and virus-mediated mRNA degradation has been observed to occur in a reversible manner during pea seed borne mosaic virus (PSbMV) infection (Wang and Maule, 1995; Aranda et al., 1996; Aranda and Maule, 1998). Such an observation would not be inconsistent with sequestration of PABP. During potyvirus infection, RdRp is expressed in large quantities and can accumulate in the nucleus as an inclusion body (NIb). Interestingly, the interaction between influenza A NS1 and human PABPII, which takes place in the nucleus and results in mRNAs with poly(A) tails that are too short to allow for export, occurs via the carboxy terminus of PAPBII (Chen et al., 1999).

Further experiments will need to be performed to determine the biological significance of the RdRp-PABP interaction.

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