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By

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ABSTRACT

Cloning and Sequencing of the Helper Component Region of zucchini yellow mosaic virus

By

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Zucchini yellow mosaic virus (ZYMV), a cucurbit potyvirus, is an extremely aggressive pathogen with a devastating effect on cucurbit production worldwide. Towards developing molecular strategies for increasing resistance to ZYMV, a gene necessary for aphid transmission, helper component (HC) was cloned and characterized. Sequence analysis revealed both conserved 5′ and 3′ cleavage sites, resulting in a calculated molecular weight of 52 kd for the ZYMV HC protein. Compared to other sequenced potyviral HC genes, ZYMV differed in conservation towards the amino terminus ("region I"), along with other cucurbit potyviruses. The difference in homology between cucurbit and non-cucurbit potyvirus groups corresponds to a difference with the predominant aphid species vectoring each group. Furthermore, a region I amino acid substitution shown to abolish potyviral aphid transmission is also present in this ZYMV strain, which is non-aphid transmissible. This data supports the hypothesis that region I is an aphid binding domain.

To my mother, Norma, and father, Arturo, whose love and support made this work possible.

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Review of potyvirus biology, regulation of aphid transmission, and zucchini yellow mosaic virus

Objective

One of the areas in which molecular genetic techniques hold the most promise for agriculture is the development of disease resistant plant cultivars. This is exemplified by the development of genetically engineered virus resistances, using cloned viral genes as a novel source of resistance genes (Beachy et al. 1990; Grumet, 1990). By genetically engineering the host so that it expresses a pathogen gene in a way that is detrimental to the pathogen (e.g. at the wrong time, in the wrong amount, or in a counterfunctional form), it is possible to interfere with the normal pathogenic process(Sanford and Johnson,1985; Grumet et al, 1987). Approaches to date have interfered with viral replication by using viral coat protein genes, satellite sequences and antisense sequences. Another possible, but currently untested method, would be to utilize viral encoded genes to interfere with the transmission of virus from one host to the next.

One group of plant viruses for which this approach may be appropriate is the potyvirus group. The potyviruses are the most economically important group of plant viruses, causing approximately 25% of all viral caused crop losses worldwide (Francki et al. 1985; Hollings and Brunt, 1981). One member of this group, zucchini yellow mosaic virus (ZYMV), is very aggressive and pervasive in causing heavy damage to cucurbit crops (Davis and Mizuki, 1986; Nameth et al., 1986; Provvidenti et al. 1984). In order to utilize molecular genetic techniques to engineer resistance to this virus, the cloning and sequencing of genes from ZYMV is needed. Of particular interest is the region of the genome encoding for helper component (HC), a protein that is necessary to facilitate transmission of potyviruses by aphids (Govier and Kassanis, 1974). Efficient aphid transmission has been implicated as a possible reason for the extreme aggressiveness of ZYMV (Lecoq and Pitrat, 1985; Alderez et al., 1985; Alderez, 1985).

In theory, interference with aphid transmission by expressing a heterologous or mutant helper component may be a way to interfere with the infection process. No example of HC-mediated resistance has been demonstrated as of yet. To evaluate this possibility, it is first necessary to further characterize the molecular nature and function of this protein. This thesis describes the cloning and sequencing of the region of the genome encoding the helper component of ZYMV. The sequence is then compared to other known potyviral helper component protein sequences, in order to ascertain how much of the ZYMV helper component is conserved relative to the other potyviruses. Also discussed are the implications any differences might have with regard to host or vector specificity, or HC-virion/ HC-aphid interactions. Future courses of action are also discussed, including what questions will need to be addressed, and ways to accomplish them.

Potyvirus Biology

The largest known group of pathogenic plant viruses is the Potyvirus group; it is surmised that over 100 different members belong to this group (Hollings and Brunt, 1981; Walkey, 1985). The type virus in this group is potato virus Y. Potyviruses have the ability to inflict damage to a wide range of crops around the world; it is estimated that one quarter of the world's viruses that infect plants come from this group (Hollings and Brunt, 1981; Francki et al. 1985). Frequently, potyviruses are present as part of the natural pathogen population, causing chronic reductions in yield and quality (Hollings and Brunt, 1981).

Potyviruses exist as unicomponent, elongated, flexous, filamentous rods 700-900 nm in length and 12-15 nm in diameter. Each viral particle has a coat of approximately 2000 capsid proteins surrounding a positive sense, single stranded RNA of approximately 10 Kb in length (Hollings and Brunt, 1981; Francki et al. 1985). A characteristic feature of this virus is its ability to form aggregates of pinwheel-shaped cytoplasmic inclusion bodies in the infected host (Dougherty and Hiebert, 1980). This is a hallmark of this disease(Hollings and Brunt, 1981).

While it has been demonstrated that potyviruses can be mechanically transmitted, most members of this group are transmitted by aphids in a nonpersistent, noncirculative manner. Other possible members have been shown to be transmitted by seed, mites, or dodder (Hollings and Brunt,1981). Once inside the host plant, the virus is uncoated, although it is not clear whether this event occurs before, during or after cell entry (Shaw et al., 1985). Expression of the viral genome initially results in the synthesis of a large, single polyprotein precursor of approximately 340 Kd in length, in a manner similar to picornaviruses. This was inferred from studies failing to detect any evidence of subgenomic mRNAs in infected tissue; only genome-sized RNAs have been identified (Allison et al. 1986). Furthermore, complete nucleotide sequences of three potyvirus members-tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), and plum pox virus (PPV), all show that only a single open reading frame exists in these viral genomes (Allison et al. 1986, Domier et al. 1986, Lain et al.

Potyviral Polyprotein Map

```
0 Kb  1        2        3        4          5          6          7        8        9      .
  PI  |***HC**|    P3   |       CI       |VPG|   NIa       |       NIb       |CP    AAA
 31Kd      56 Kd  50Kd          71Kd     6Kd   49Kd             58Kd        30Kd
```

<u>Potyvirus gene products</u>

P1 P1 protein--------------------Putative cell to cell movement protein, 5′ protease

HC Helper Component protein------Aphid transmission, 5′protease

P3 P3 protein--------------------?(specific virus-host interactions)

CI Cytoplasmic Inclusion protein ------- ?(RNA helicase, replication, viral/host interactions, long distance transport)

VPg Virally encoded, genome-linked protein--------------RNA stability, replication, infectivity

NIa Small Nuclear Inclusion protein -------3′ protease

NIb Large Nuclear Inclusion protein--------putative RNA-dependent RNA polymerase

CP Coat Protein-------------------Encapsidation of viral RNA, vector transmission, vector specificity

Figure 1- Potyviral genome and polyprotein map

1989). Translation is thought to occur from the 5′ end of the genome, initiating at the end of a leader sequence of approximately 150 bp (Allison et al. 1986). **Figure 1** depicts the potyviral genome and polyprotein map.

Rapid autoproteolytic processing occurs after translation, giving rise to intermediate polyprotein cleavage products (Fig. 2). This is accomplished by three proteinases that are encoded by the genome, and autocatalytically released from the polyprotein. These proteins are known as the nuclear inclusion protein (NIa), the helper component (HC-pro), and the P1 proteinases, and are responsible for the proteolytic events associated with polyprotein processing (Dougherty and Carrington, 1988; Carrington, 1991, in press).

In TEV, the NIa proteinase is 49 kD and catalyzes cleavage at five positions from the carboxy-terminal end of the polyprotein. The second potyvirus proteinase, HC-Pro, appears to catalyze only one proteolytic reaction, an autocatalyic cleavage at its own 3′ carboxyl-terminus (Carrington et al., 1989a). The third protease, PI, catalyzes the remaining cleavage between PI and HC-Pro (Carrington, 1991, in press) As shown in **figure 2**, the HC-Pro resides near the amino terminus of the potyvirus polyprotein and cleaves at a conserved gly-gly dipeptide, resulting in a 87-kD precursor protein in TEV. This protein is further cleaved in vivo into 31-kD and 56-kD products by the 31-kD P1 protease at a conserved tyr-ser dipeptide (Carrington et al. 1991, in press). The 56-kD protein is the HC-Pro (Thornbury et al. 1985). In all, the action of these proteinases result in the cleavage of eight mature proteins from the viral polyprotein.

The positively stranded RNA is also used as a template for negatively stranded RNA, which in turn is similarly replicated to generate additional positively

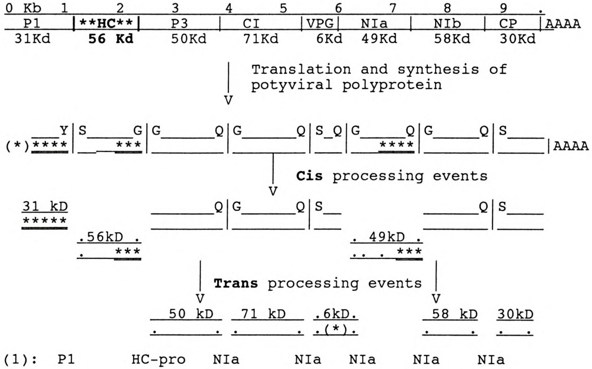


Figure 2- Cleavage products of the TEV potyviral polyprotein

**** : proteolytic domains
(1) : protease responsible for cleavage denoted above

stranded RNA. These (+)RNAs are either translated or encapsidated. Encapsidation is thought to be accomplished in a 5′ to 3′ direction, similar to the potexviruses (Allison et al., 1986).

During the course of infection, viral-encoded proteins aggregate to form pinwheel- or scroll-shaped inclusion bodies in the cytoplasm and/or nucleus. Additionally, some potyvirus infections result in the formation of other types of inclusion bodies (Christie and Edwardson, 1977).

The life cycle of potyviruses is completed by uptake of progeny virions by aphids and transmission to other plants. Aphid transmission requires a virally-encoded protein present in the sap of infected plants, the HC-Pro. It is hypothesized that the HC mediates binding between the aphid stylet and virion; the mechanism has not been defined (Pirone and Thornbury, 1983).

Potyviral Gene Products

Coat Protein (CP)- The coat protein is the major component of the virion, with approximately 2000 units per RNA molecule (Hollings and Brunt. 1981). Its functions include: encapsidation of the RNA, vector transmission, and host specificity. The sizes range from 30-45 kD for different potyviruses, with each virus having only a single, homogeneous type of monomeric subunit. Biochemical analysis reveals that both the N- and C- termini of the protein are exposed to the surface, and removal of these segments by trypsin treatments do not affect the morphological shape or infectivity of the virion (Shukla et al. 1988). In addition, sequence homology among the different potyviruses indicates high variability at the

N- terminus, with the remaining portion highly conserved. The amino-terminal segment is oriented near the surface of the virion, and is responsible for the immunological differences amongst the assorted members of the potyvirus group. The conserved domains may be responsible for protein-protein or protein-RNA interactions (Dougherty et al., 1985).

Large Nuclear Inclusion Protein (NIb)- The large nuclear inclusion protein is one of two virally- encoded proteins which comprise the nuclear inclusion body. It is hypothesized to be a RNA-dependant, RNA polymerase due to the presence of the conserved GDD tripeptide sequence found in all replicases of this type. This includes picornae viruses (eg polio), bacteriophages (eg QB) and plant viruses (Domier et al., 1987).

Small Nuclear Inclusion Protein (NIa)- The small nuclear inclusion protein is the major proteinase of the viral polyprotein, responsible for the majority of cleavages. Studies have shown that nuclear inclusion bodies posses proteolytic activity and that this action is inhibited in the presence of antiserum to this protein (Carrington and Dougherty, 1987a), or by site-directed mutations within the protein (Carrington and Dougherty, 1987b). Furthermore, deletion studies indicate that the proteolytic domain is confined to the C-terminal half of the protein (Carrington and Dougherty, 1987b). This domain has been shown to be highly conserved in relation to other NIa proteinases from different potyviruses (Allison, et al. 1986), the 24 kD protein from cowpea mosaic virus (CpMV), and the 3C proteinase of picornaviruses (Domier et al., 1987). There is evidence to show that these proteins belong to the trypsin superfamily of serine proteases by virtue of active site homology (Bazan and Fletterick, 1988).

The NIa proteinase cleaves the polyprotein at five sites (**Fig. 2**). When exogenously supplied proteinase was added to the polyprotein, the junctions between the P3-CI and the NIb-CP were efficiently cleaved in trans(Carrington and Dougherty, 1988). In addition, when synthetic polyproteins containing the junctions connecting the ends of the NIa and VPg proteins with the NIa proteinase were constructed, cleavage also occurred. This suggests that these proteolytic events can also occur in cis. **Fig. 2** illustrates a hypothetical model of the different cis and trans cleavages in the polyprotein. In vitro, these reactions occur rapidly, suggesting that significant levels of polyprotein do not accumulate(Carrington and Dougherty, 1988)

Genome Linked Protein (VPg)- This small protein (6kD in TEV, 24kD in TVMV) is covalently attached to the 5′ end of genomic, viral RNA and confers stability. It may play a role in the initiation of RNA synthesis, as has been inferred from other studies of viruses having similar genome organization (Morrow et al., 1984, and Vartapetian et al., 1984). However, removal of this protein in studies of tobacco vein mottling virus (TVMV) and TEV had no effect on infectivity due to mechanical inoculation or on translation in cell-free systems (Siaw et al. 1985; Hari 1981).

Cytoplasmic Inclusion Protein (CI)- This protein exists as a monomer which aggregates to form diagnostic pinwheel-shaped inclusion bodies in the cytoplasm of all potyviruses. Early in infection, these proteins aggregate in the morphological form of spikes on the plasma membrane. Eventually, these elongate and dissociate into the cytoplasm, floating freely as pinwheel-shaped inclusion bodies (Christie and

Edwardson, 1977). Its functions are unclear, although it has been proposed as a putative RNA helicase.

P3 Protein (P3)- This protein has not been able to be observed in any potyvirus-infected plants; its existence is inferred from sequence data and cell-free translation assays. To date, no function has been conclusively assigned to this protein.

Helper Component (HC-Pro) This protein has been shown to have two functions, one a proteinase, and the other in facilitating aphid transmission. Among the various potyviruses, the carboxyl-terminal half is the most highly conserved (Turpen 1989). Towards the amino-terminus, it becomes less so. The proteinase function was discovered when cell-free translation of synthetic transcripts of the 5′ region of the genome resulted in accumulation of an 87 kD proteolytic product in TEV (Carrington et al., 1989a). Site-directed mutagenesis studies have indicated that this domain resided in the carboxyl-terminal half of the HC-Pro protein, and is no longer than 155 residues from the C- terminus (Carrington et al., 1989a). In addition, microsequence analysis determined the cleavage site to be a gly-gly dipeptide, with the cleavage appearing to act in an autocatalytic manner (Carrington et al., 1989a). In vivo, the 87 kD precursor product is further cleaved into a mature 56 kD HC-Pro and a 31 kD P1 protein. The proteinase responsible has just been identified (Carrington 1991, in press) and the cut site determined in vitro using the wheat germ translation system (Rhoades, 1991, in press). This protease, P1, is the first protein product from the 5′ end of the polyprotein. While the proteinase domain of HC-pro has been shown to reside within the 3′ half of the HC, current studies by C. D. Atreya et al. (1991, in press)

have indicated that an important residue for aphid transmission resides in the N-terminal region.

Based on amino acid homology, the HC-Pro most closely resembles members of the cysteine-type family of proteinases (type member: papain), in which a reactive cys residue approximately 30 a.a. from the amino-terminal domain is followed by an essential his residue approximately 100 a.a. later (Oh and Carrington, 1989). Site directed mutagenesis studies on this protein have confirmed the importance of these residues on HC-Pro activity: deletions of a cysteine a.a. 30 residues from the approximate amino-terminus of the proteolytic domain proved detrimental to activity, as did a his residue 73 a.a. downstream (Oh and Carrington, 1989).

Another function assigned to the HC-Pro is its role as a helper component (HC) for aphid transmission from plant to plant (Thornbury et al., 1985). This will be discussed in further detail in a later section. Briefly, this protein has been shown to be a necessary factor in experiments involving aphid acquisition and transmission of potyviruses (Govier and Kassanis, 1974). Antiserum to partially purified helper component fractions from two different potyviruses, TVMV and potato virus Y (PVY) inhibits aphid transmission of either in a reconstituted assay system (Hiebert et al., 1984). In addition, the viral origin of HC was confirmed when the anti-HC antisera precipitated a discrete set of polypeptides produced by cell-free translation of potyviral RNA (Hiebert et al., 1984). It is hypothesized that the helper component plays a role in the binding of the virion to the aphid stylet, as will be discussed in a later section.

Among the various proteins isolated from potyvirus-infected plants is an amorphous, cytoplasmic inclusion body (CIB) whose function still eludes researchers. Immunological studies have linked this protein to the helper component (DeMeija et al., 1985). Findings show cross-reactivity between antiserum raised to this protein and to the major protein component in active helper component fractions. However, this antiserum has been found to be ineffective against preventing the active helper component from its activity in aphid transmission. In addition, purified CIB protein does not have helper component activity, leading to the speculation that it may be in an unstable or inactive form.

P1 Protein (P1)- This protein is encoded at the amino-terminal end of the open reading frame of the polyprotein. Carrington (1991, in press) has demonstrated that this protein is also a protease, responsible for the autocatalytic cleavage between itself and the HC-Pro. Sequence analysis of P1 shows homology to the 30 kD movement protein of tobacco mosaic virus (TMV), suggesting a role in cell-to cell movement of the virion (Domier et al. 1987). It has yet, however, to be detected in the cells of potyvirus infected plants.

5′ non-coding region- This region, containing the presumptive site of covalent linkage to the VPg protein, has been implicated in the enhancement of RNA translation, analogous to a eucaryotic 5′ cap structure (Carrington and Freed, 1990). Studies show that when this structure is fused to a reporter gene, translation is significantly enhanced, and does not require the presence of a stabilizing/ribosome-binding cap structure; synthetic versions of this region compete

for factors required for protein synthesis in a cell-free translation mix. Among potyviruses, there are two major blocks of homology, termed "Box a" (ACAACAU) and "Box b" (UCAAGCA) (Turpen, 1989). These have been hypothesized to be binding sites for translation factors.

Regulation of aphid transmission in potyviral infections

Of all insect vectors of plant viruses, aphids comprise the largest group. Aphid transmission is divided into three basic types, non-persistent (e.g. stylet-borne), semi-persistent, and persistent (e.g. circulative) (Watson and Roberts, 1939; Sylvester, 1956). The differences between them reflect the time it takes the aphid to acquire the virus during feeding, how soon after acquisition it initiates viral transmission, the length of time it retains the ability to transmit the virus, and the anatomical location of viral retention. By far, the most important in economical terms are viruses transmitted in a non-persistent manner. All aphid transmitted potyviruses are in this category. These viruses are acquired by the aphid soon after the initiation of feeding, are carried on the stylet or other mouthpart, and are transmitted immediately after the aphid inserts its stylet into another plant. In addition, the aphid soon loses (within a few hours) its ability to transmit the virus after leaving the infected plant (Kennedy et al., 1962). Persistent viruses, on the other hand, require a long acquisition feeding time (6-20 hours), and at least a 12-hour latency period before transmission. Aphids retain these viruses throughout their body, in some cases through a moult, and retain the ability to transmit for at least a week. Some persistent viruses have the ability to multiply within the aphid; non-persistents do not (Watson and Roberts, 1939). Semi-

persistent viruses have properties intermediate between these classifications (Sylvester, 1956).

Potyviruses, like another family of non-persistent viruses, caulimoviruses, are not readily aphid-transmissible unless a component from infected plant sap is present before or during aphid acquisition (Govier and Kassanis, 1974; Sako and Ogata, 1981). As discussed above for potyviruses, this factor was found to be a ca. 56 kD protein of viral origin, termed helper component. Its importance to the non-persistent nature of aphid transmission became clear once it was shown that virions attach to the maxillary stylet only on the presence of HC (Berger and Pirone, 1986). Without it, virions accumulate in the gut and are not transmissible. This indicates that HC in some way performs a mediator function in the binding of virion to the aphid stylet, possibly in the form of a "sandwich", with the HC in between the binding sites of the virion and the stylet.

Generally, potyviruses exhibit the highest degree of specificity for their own helper component (Lecoq and Pitrat, 1985). It has been shown, however, that potyviruses are aphid transmissible in the presence of a heterologous HC. Studies in this area have shown that prior access to heterologous HC proteins can either decrease or increase the ability of an aphid to transmit various members or strains of other potyviruses (Katis et al. 1986). The decrease in aphid transmission was hypothesized to be due to a titration effect on the amount of available binding sites on the aphid stylet. Presumably, less space was available for the higher affinity homologous HC to bind its own virion, due to the binding of a heterologous HC (Katis et al., 1986). An increase in aphid transmission was observed in experiments investigating the differences in

transmissibility among three isolates of TEV (Pirone and Thornbury, 1983). These isolates were either highly- (HAT), poorly- (PAT) or non- (NAT) aphid transmissible. In each of these cases, prior access to HC from a heterologous potyvirus, PVY, allowed for successful aphid transmission when aphids were subsequently left to feed on the isolate source plant. In another study, competition assays tested the affinity of a purified HC to mixed populations of its homologous virion and a heterologous virion (Lecoq and Pitrat, 1985). Most often, the aphid transmission rate of the homologous virion was unaffected, while the rate of the heterologous virion became drastically reduced. In one case between watermelon mosaic virus 2 (WMV-2) and zucchini yellow mosaic virus (ZYMV), however, this situation was reversed. When WMV-2 and ZYMV were mixed with WMV-2 HC, the transmission rate of WMV-2 became significantly reduced, while the rate for ZYMV remained unaffected. This suggests that ZYMV may have a competitive advantage (Lecoq and Pitrat, 1985).

In addition to HC, viral capsid proteins also play a role in aphid transmissibility. The study discussed above involving the HAT, PAT, and NAT isolates of TEV showed that even though all were transmissible using a heterologous HC, the HAT isolate gave the highest infection rate, followed by the PAT, and then the NAT. Furthermore, HC activity was shown to be greatest from the poorly aphid-transmissible isolate, suggesting that an absence or deficiency of HC was not responsible for differences in transmissibility (Pirone and Thornbury, 1983). In two strains of another non-persistent virus, cucumber mosaic virus, differences in aphid transmissibility have been shown to be associated with differences in their protein coat (Gera et al., 1979). The same has been found with two persistent viruses, pea enation mosaic virus (Hiebert and McDonald, 1973), and barley yellow dwarf virus (Rochow, 1970). In the case for potyviruses, the TEV isolates were found to be distinguishable by monoclonal antibodies to capsid proteins (Dougherty et al., 1985).

Aphid transmission has also been shown to be lost by repeated viral propagation by means of mechanical "rub" inoculation on cotyledons of test plants (Swenson et al. 1964). A NAT strain of TVMV, derived in this manner, was evaluated to test the hypothesis that a specific mutation in the coat protein gene could cause non-aphid transmissibility (Atreya et al. 1990). The coat protein had been implicated in aphid transmission from previous studies; enzymatic removal of the N-terminus of the coat protein results in loss of aphid transmissibility (Salomon, 1989). It has also been shown that this N-terminal domain is exposed to the surface of the viral particle (Allison et al. 1985; Shukla et al., 1988). Studies into the amino acid sequence of the N-terminal domain of this and other NAT strains revealed a tripeptide of DAG which was consistently altered in NAT strains but not in any aphid-transmissible (AT) strains (Harrison and Robinson, 1988; Maiss et al., 1989; Quemada et al., 1990). Further studies have shown that point mutations around this area, especially the G residue, greatly reduced or abolished aphid transmissibility (Atreya, P.L. et al., in press)

Zucchini Yellow Mosaic Virus

Cucurbit species (melons, cucumbers, and squashes), are hosts for three economically important members of the potyvirus group: watermelon mosaic virus (WMV-2), the watermelon strain of papaya ringspot virus (PRV-2), and zucchini yellow mosaic virus (ZYMV). Of the three, ZYMV, is the newest; it is highly aggressive and competitive.

While WMV-2 and PRV-2 have been around since the 1950's, ZYMV was first reported in 1981 in Italy (Lisa et al. 1981). It has since spread throughout the world, causing severe crop losses. (Lisa and Lecoq, 1984). ZYMV causes vein clearing, leaf mottling, and severe stunting and malformation of fruit growth with decreased yields (Davis 1986). It has been described as one of the most aggressive and destructive of all plant viruses and as a serious threat to cucurbit production. A need for resistant cultivars has been emphasized (Davis and Mizuki, 1987).

The extreme aggressiveness of ZYMV has been, in part, attributed to highly successful aphid transmission. Field studies have shown that ZYMV is more successful in establishing infections than WMV-2 and papaya ringspot virus (PRV); natural field spread from inoculated to adjacent uninoculated squash plants was 10-fold greater for ZYMV (Alderz et al. 1985) Given the helper component from WMV-2 and a mixture of WMV-2 and ZYMV virions, infections of ZYMV were established three times more often than were infections in WMV-2 (Lecoq and Pitrat, 1985). These provoked suggestions that ZYMV may have some sort of competitive advantage for aphid transmission.

Therefore, the factors related to aphid transmission may, in part, account for the success of ZYMV. These differences may be reflected in the molecular structure of the HC and/or coat protein genes. In order to understand the factors which enable potyviruses to be successful plant pathogens at the molecular level, the study of the ZYMV helper component would be a good model to work with. By molecularly characterizing the ZYMV helper component, a framework of information will have been made to serve as a basis of later studies dealing with the manipulation of aphid transmission of potyviruses.

The work described herein focuses on the sequence analysis of the ZYMV helper component gene. It is compared to the sequence of other potyvirus helper component genes and the also the helper component gene for cauliflower mosaic virus. Since the HCs of several potyviruses have been shown to be functionally interchangeable (Sako and Ogata, 1981, Lecoq and Pitrat, 1985), areas of conserved sequence might be related to the biological activity of the HC. The comparison of homologous areas of the HC region of ZYMV to the other potyviruses will serve to reinforce or put into question putative regions essential to the biological function of this protein. In addition, if the reason for the extreme competitiveness of ZYMV does indeed lie within the protein sequence of its HC, it may well lie within the areas of high homology within which ZYMV has sequence variation.

CHAPTER 2

Cloning and Sequencing of the Helper Component Region of ZYMV potyvirus

Introduction

In 1981, a new member of the potyvirus family was identified, zucchini yellow mosaic virus (ZYMV). This virus has been shown to be extremely aggressive, and is responsible for a good deal of the virally-related destruction of cucurbit crops (cucumbers, melons, squashes) around the world (Lisa and Lecoq, 1984). ZYMV has been shown to outcompete other potyviruses in establishing infections by rub inoculation (Davis and Mizuki, 1987), natural field spread (Alderez et al. 1985; Alderez, 1987) and aphid acquisition (Lecoq and Pitrat, 1985). In the U.S., as well as in other countries, this virus has been responsible for crop losses of 50-100% (Davis and Mizuki, 1986, Nameth et al., 1986, Provvidenti et al. 1984).

Zucchini yellow mosaic virus, like other potyviruses, is non-persistently aphid transmitted, meaning that the virions are attached to the aphid via the stylet, and are released within a few hours hours of acquisition (Hollings and Brunt, 1981; Kennedy et al. 1962). For potyviruses, aphid transmission requires the presence of a virally-encoded protein, known as the helper component (HC), to facilitate binding between the aphid stylet and the virion (Thornbury and Pirone, 1983) In order for transmission of purified virions to occur, aphids must come into contact with the HC simultaneously or prior to the virions. Contact with purified virions without HC or prior to HC does not allow for aphid transmission (Sako and Okata, 1981). The presence of HC is necessary for the virions to attach to the aphid stylet, specifically the inner maxillary stylet (Berger and Pirone, 1986). Without HC, virions accumulate in the gut and are not transmitted. These studies suggest that the function of HC is to act as

a mediator of binding between the virion and the aphid stylet.

In addition to HC, studies have determined that potyviral aphid transmission is also controlled by the coat protein (CP) (Pirone and Thornbury, 1984). When studies of non-aphid transmissible (NAT) potyviral strains were conducted, some cases were found to be due to the lack of biologically active HC (Kassanis and Govier, 1971; Paguio and Kuhn, 1976; Lecoq, 1976). Other cases were attributed to some other variation within the virion. In these instances, the helper component recovered from these NAT strains was shown to be biologically active, and the coat protein was suggested as the cause of non-transmissibility (Pirone and Thornbury, 1983; Antignus et al. 1989).

The coat protein had been implicated in aphid transmission from other studies; enzymatic removal of the N-terminus of the coat protein results in loss of aphid transmissibility (Salomon, 1989). It has also been shown that this N-terminal domain is exposed to the surface of the viral particle (Allison et al. 1985; Shukla et al., 1988). Studies of the amino acid sequence of the N-terminal domain of a NAT strain of tobacco vein mottling virus (TVMV) were used to test the hypothesis that a mutation in the coat protein could cause non-aphid transmissibility (Atreya et al. 1990). The non-aphid transmissibility of this TVMV strain was attributed to the use of repeated mechanical "rub" inoculations for viral propagation. A previous study demonstrated that the propagation of aphid transmissible viruses in this manner leads to a loss in aphid transmissibility (Swenson et al. 1964). The amino acid sequence of this and other NAT strains revealed a tripeptide of DAG in the N-terminal domain which was consistently altered in NAT strains, but

not in aphid-transmissible (AT) strains (Harrison and Robinson, 1988; Maiss et al., 1989; Quemada et al., 1990). Further studies showed that point mutations around this area, especially the G residue, greatly reduced or abolished aphid transmissibility (Atreya et al., in press)

There is evidence to suggest that the extreme competitiveness of ZYMV is due, at least in part, to highly successful aphid transmission. Studies involving the mixture of potyviruses with HCs other than their own have shown that different HCs exhibit varying degrees of specificity among viruses (Thornbury and Pirone, 1983; Lecoq and Pitrat, 1985). While most viruses are most efficiently transmitted by their own HC, a heterologous mixture of watermelon mosaic virus-2 (WMV-2), ZYMV, and WMV-2 HC established ZYMV infections three times more often than WMV-2. (Lecoq and Pitrat, 1985). Furthermore, field studies have shown that natural field spread from inoculated to adjacent uninoculated squash plants was much greater for ZYMV than for papaya ringspot virus (PRV) or WMV-2 (Alderz et al. 1985).

The importance of HC to the life cycle of ZYMV may lead to a way to interfere with the infection process by using a pathogen-derived resistance approach. Conceivably, aphid transmission may be interfered with by expressing a heterologous or mutant helper component. In order to understand and manipulate the aphid transmission of potyviruses in this way, the molecular basis of the virion-HC-aphid stylet interactions must first be studied. Towards this end, the molecular characterization of the helper component of ZYMV has been undertaken and its amino acid sequence deduced. The resulting sequence was compared to the HC amino-acid sequences of previously characterized potyviruses, tobacco etch virus (TEV), potato virus Y

(PVY), tobacco vein mottling virus (TVMV), and plum pox virus (PPV).

The data indicates that the ZYMV HC sequence shares conservation with the other potyviruses at the central and 3′ end of the region, but differs markedly at the 5′ end. Furthermore, the 5′ end of the HC region has been shown to be important to aphid transmissibility (Atreya, C.D. et al., in press). This study demonstrated that a single amino acid change in this region converted an aphid transmissible potyviral strain to a non-aphid transmissible strain. In support of this, the same amino acid change has been found in this strain of ZYMV, which is also non-aphid transmissible. In addition, recent HC sequence data from other cucurbit potyviruses exhibit similar differences at the 5′ end as this strain of ZYMV (Baker and Grumet, personal communication). Thus, while the majority of the HC region is conserved by all potyviruses, the 5′ end conservation is divided among two groups- non-cucurbits and cucurbits. Interestingly, when the predominate aphid species vectoring the viruses within each group was studied, it was found that all the non-cucurbit potyviruses studied were carried primarily by the Myzus species while the cucurbit potyviruses were carried primarily by the Aphis species. Therefore, in addition to the 5′ end of the HC region being important in aphid transmission, it may also play a role in vector specificity, possibly serving as a domain for aphid binding.

Materials and Methods

Cloning of the 5′ half of ZYMV

a) Virion isolation

ZYMV Connecticut strain isolate (a gift from Dr. R. Davis-Rutgers Univ.) was propagated by rub inoculation on zucchini cotyledons (Cucurbita Pepo cv. Black Jack). Three to five weeks after infection, virions were harvested using the method of Grumet and Fang (1990). Deveined infected leaves (100 g) were homogenized in a blender with a 2:1 (wt:vol) ratio of grinding buffer (0.1 M KPO_4, 10 mM EDTA, 0.1% $NaSO_4$, pH 7.0) and a 1:1 (wt:vol) ratio of organic solvents (50% $CHCl_3$, 50% CCl_4) at 4° C. After filtration through cheesecloth the extract was centrifuged for 15 min at 10,000 x g and the virus was concentrated from the supernatant by precipitating twice with PEG 8000. The first PEG precipitation involved the addition of 1% Triton X-100 (v:v) for 20 minutes at 4° C, then PEG 8000 (8g/100 ml) for one hour at 4° C. After centrifugation for 10 minutes at 5,000 x g, the pellet was resuspended in 1/5 vol Extraction Buffer (EB: 0.1 M KPO_4 pH 7.0, 10 mM EDTA) for 3 hrs. and clarified by centrifugation for 10 minutes at 5,000 x g at 4° C. The second PEG precipitation involved the addition of PEG 8000 (5g/100 ml) and 0.3M NaCl (1.75g/100 ml) for 1-2 hr., and pelleting by centrifugation for 10 minutes at 10,000 x g. The final PEG pellet was resuspended in 1 ml EB overnight at 4° C, and clarified by centrifugation for 10 minutes at 5,000 x g. Na-azide was then added to 1mM.

The concentrated viral extract was further purified by centrifugation through a 0-1.2M Cs_2SO_4 step gradient with 30% sucrose in an SW-28 Beckman swinging bucket rotor for 4-5 hours at 100,000 x g at 4° C. The virion band was removed by syringe and diluted in 3X vol EB. Virions were

concentrated by PEG precipitation (5g/100 ml PEG , 1.75g/100 ml NaCl), pelleted by centrifugation for 10 minutes at 5,000 x g, and resuspended in 200 ul EB and 1 mM Na-azide. Sucrose gradients were prepared in the following way: 1.2M Cs_2SO_4 was made in 30% sucrose and further diluted with 30% sucrose to make 0.4M and 0.8M Cs_2SO_4 in 30% sucrose. 9 ml aliquots from each solution were layered in a 38.5 ml Beckman SW-28 tube.

b) Viral RNA extraction

Linear sucrose gradients (7.5-30%) were prepared according to the protocol of Yeh and Gonsalves (1985). Briefly, diethyl pyrocarbonate (DEP) treated solutions of 60% sucrose, 0.6M NaCl, 0.06M Na-Citrate (pH 7.0), and H_2O were prepared, and mixed to create solutions with final concentrations of 7.5%, 15%, 22.5%, and 30% sucrose in 0.15M NaCl, 1 ug/ul bentonite and 0.015M Na-Citrate (pH 7.0). These solutions were layered (8 ml/solution) in a Beckman SW-28 tube and allowed to stand overnight at 4^o C.

Prior to releasing the RNA from the virions, 1 ug/ul of bentonite (an RNAse inhibitor) was added to the purified virion preparation. The virions were then incubated with viral dissociation buffer (0.1M Tris pH 9.0, 1mM EDTA, 1% Sarkosyl, 0.125 ug proteinase K, 1:1 vol:vol) for 20 minutes at room temperature. Dissociated virions were then centrifuged through the preprepared sucrose gradients for 10 hours at 100,000 x g. The viral RNA band (approximately 4 ml from bottom) was collected by syringe in 2 ml fractions and placed in pre-baked, iced Corex tubes. Viral RNA was immediately precipitated with the addition of 0.1 vol DEP treated 2M NaOAc and 2X vol EtOH, overnight at -20^o C. After centrifugation at 10,000 rpm for 15 minutes, pellets were dried and resuspended in 100 ul DEP treated H_20 on ice.

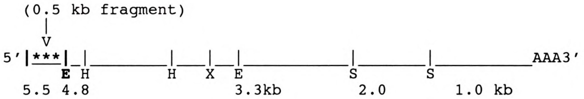


Figure 3- Restriction endonuclease map of clone Z24 (Grumet and Fang, 1990)
E: EcoR1; **H**: HindIII; **X**: XhoI; **S**: SacI
***: position of the 16-mer, "1/2 way Primer"
(5' CTCTCTCCTGGCGGCG 3'), used to clone the 5' of the ZYMV RNA.

c) Primer synthesis

A 0.5 EcoR1 fragment was subcloned from the 5′ terminus of the 5.5 kb Z24 clone of ZYMV RNA (Grumet and Fang, 1990), and partially sequenced; this data was used to design a primer for cDNA cloning (**Fig. 3**). The primer was synthesized at the Macromolecular Structure Facility of Michigan State University.

d) Complementary DNA (cDNA) synthesis

cDNA cloning was based on the method of Gubler and Hoffman (1983) and performed using a kit purchased from Amersham. Double-stranded DNA was then extracted using phenol: $CHCl_3$: isoamyl alcohol (25:24:1), EtOH precipitated, and treated with T4 polymerase for the production of blunt ends. These cDNAs were then blunt-end ligated into SmaI-cut Bluescript KS+ vectors (Stratagene) and transformed into competent, DH5-alpha (rec A-) E. coli cells. All transformants were then prepared for plasmid purification using the STET boiling method (Maniatis et al., 1989) and examined for inserts using the restriction enzyme sites available on the Bluescript polylinker. Clones measuring 3 kb or longer were validated as having an origin in the Z24 0.5 EcoR1 fragment by conventional southern hybridization techniques (Maniatis et al. 1982), using both the 0.5 fragment and the primer as probes.

e) RNA dot-blot hybridizations

The cDNA clones chosen were validated as to their viral origin by dot-blot hybridization to the viral RNAs of the following: ZYMV Connecticut strain, ZYMV CT1, ZYMV CT2 (variants of original CT strain- see Chap.3), ZYMV Pickens strain, ZYMV Orangeburg strain (gift of B. Sammons, Monsanto Agricultural Co.), ZYMV Arkansas strain (gift of H. Scott, Univ. Ark.), and WMV-2.

To prepare viral RNA, virions were harvested from 25 g of infected leaf material from each of the above

strains. After the second PEG precipitation, bentonite was added to 1 ug/ul, and virions were stripped of their coat protein by adding 1 vol RNA dissociation buffer, and allowed to sit at room temperature for 20 minutes. This crude RNA preparation was directly applied to nytran filters, which were pre-wet with 0.4M Tris pH 7.6 + 2X SSC. The filters were then dried and subjected to UV irradiation to covalently bind the RNA. These dot-blots were then subjected to ^{32}P-labelled probe hybridizations as described in Maniatis (1982).

Sequencing and analysis

a) Nested deletion clones for sequencing

Subsequent to identification and subcloning of putative clones which spanned the helper component region, nested deletions were performed to allow for efficient sequencing of the entire 1.5 kb region. Two sets of deletions were made, one originating from the 5' end of the subclone, and the other from the 3' end. Deletion subclones were made using the exonuclease III and mung bean protocol of BRL, which was based on the techniques used by Yanisch-Perron (1985).

b) Sequencing reactions

All sequencing reactions were done using a kit from U.S. Biochemical, with a protocol based on the Sanger dideoxy method of DNA sequencing (Sanger, F., 1981). Double stranded DNA, ^{35}S-dCTP, and Sequenase v.2.0 (Stratagene) were used as reagents. Sequencing gels were made from both conventional 8% acrylamide and 7% "Long Ranger" acrylamide (AT Biochem).

c) DNA sequence analysis

Sequence analyses were performed using DNASIS software (Hitachi Software Engineering).

Results

The yield of virions was 1-5 mg/100g of infected, freshly deveined leaf material. The absorbance ratio (260/280 nm) of the virion isolations ranged from 1.2-1.35 absorbance units. The RNA purified from the sucrose gradients was examined by gel electrophoresis and appeared to be 10 kb in length, the approximate length of full-length RNA. This was then used for cDNA cloning.

Fig. 3 (pg. 25) shows a map of the Z24 clone, comprising the 3′ half of the ZYMV genome. The extreme 5′ end of this clone, a 0.5 kb EcoR1 fragment, was subcloned and sequenced. This information was used to design the primer shown in **Fig. 3**. This primer was 61 bp from the 5′ end of the clone. cDNA clones, ranging from 0.5 to 4.0 kb, were obtained (**Fig 4a**). Three clones, Zy2, Zy4, and Zy149, were found to have the largest insertions and were chosen for further study. These clones had inserts of approximately 3.6 kb. A partial restriction map is shown in **Fig 5**.

To validate the viral origin and relatedness of the clones, each were probed with the terminal 0.5 Eco R1 fragment of Z24 using a dot blot hybridization assay. In addition, the 2.0 kb internal fragment of clones Zy2, Zy4, and Zy149 were subcloned and used as a probe to check for homology to each other and Z24 (**Fig. 4b**). Finally, these probes were used in RNA dot blot hybridizations to check for homology to several strains of ZYMV **(Fig 4c)**. As expected, clones Zy2, Zy4, and Zy149 showed homology to each other, to the 0.5 kb fragment of Z24, and to all strains of ZYMV used.

All three clones were sequenced at their 3′ terminus and found to include the 1/2 way primer sequence and the rest of the overlap with the Z24 clone. This validated

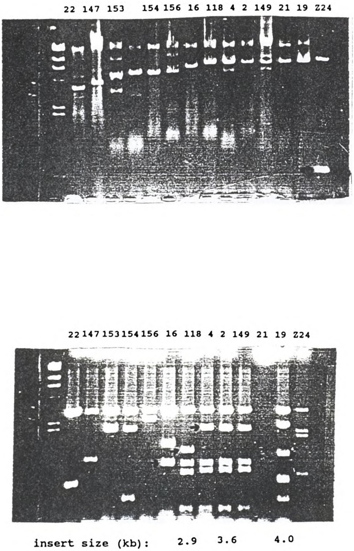
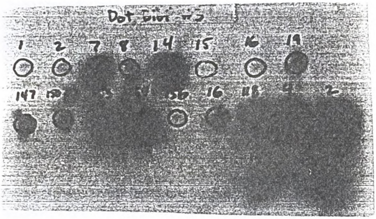


Fig. 4a- Restriction digests of cDNAs containing the largest inserts. Top, DNAs linearized with Bam H1; bottom, DNAs double cut with EcoR1/BamH1.

Probe: 2.0 kb fragment of Zy 149



Probe: 0.5 kb fragment of Z24

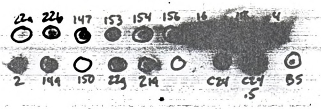
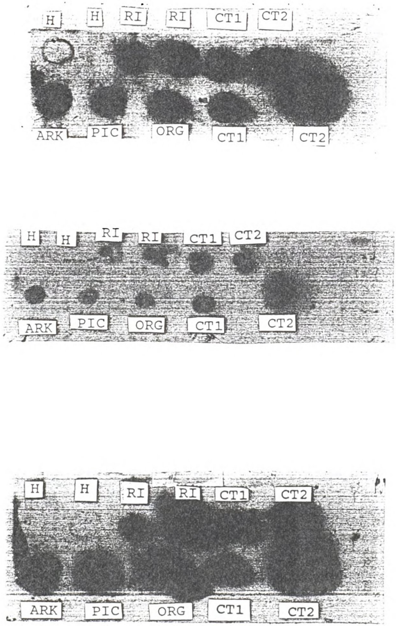


Fig 4b- Dot blot hybridization showing the internal homologies of cDNA clones to each other and Z24. The 2.0 kb E/B fragment of Zy 4 (depicted in fig.4a by arrow)was used as a probe in the top figure, and the 0.5 kb frag. of Z24 in the bottom



Probe

Coat Protein

Z 24

Zy 149
(2.0 kb
fragment)

Fig. 4c-Dot blot hybridizations to whole plant RNA extractions of healthy (**H**) and ZYMV infected plants (**RI**: rub- inoculated ZYMV-CT ; **CT1**, **CT2**: aphid transmitted(AT) ZYMV-CT strains; **ARK**: AT ZYMV-ARK strain; **PIC**: AT ZYMV-PIC strain; **ORG**: AT ZYMV-ORG strain). The probes used originated from clones containing the ZYMV-CT coat protein gene (Grumet and Fang, 1990), Z24, and the 2.0 kb internal fragment from Zy 4.

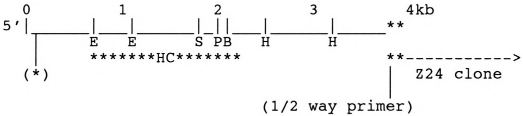


Fig. 5- Partial restriction endonuclease map of Zy 2, 4, and 149. **(*)**: approximate 5' end of all three clones. E: EcoR1; S: SmaI; P: PstI; B: BamH1; H: HindIII. ***__HC__*** : The area denoting the ZYMV-HC region, and the area sequenced.

3′ Terminus

<--------Zy 2, 4, 149
5′ <-ATTGCTTCACTACTATGTAGATCCACATCATAATG
ATTGACTTGTCATCATAGACGAATGCCATGTC
ACAGACAGTGCGACCATAGCTTTCAATTGTGC
ACTTAAAGAATACAATTTTGCTGGCAAATTGA
TTAAAGTGTCTGCAA**CGCCGCCAGGAGAGAG**TGCG
1/2 way Primer Z 24--->

Fig. 6a- Sequence of the 3′ terminus of clones Zy2, 4, and 149. The overlap of the Z24 clone at its 5′ terminus is underlined; the origin of these clones and sequence of the 1/2 way primer are in **bold**.

5′ Terminus

Origin of
Zy Clones: (**149**) (**2**) (**4**)
ZYMV 5′CCAAGAATCAACG**TCAAGCAA**ACGATTT
TGAACGTATTTACAAACAAAGCAATCTA
AACTCTTACAGTATTAAGAAATTCTCCA
ATCACTTCGTTACTTCAGACATACATGG->

Fig. 6b- Sequence of the 5′ ends of Zy 2, 4 and 149. The underlined nucleotides are the conserved bases, designated as "Box B" (see Fig. 6c), that are generally located 40-60 bases from the beginning of the potyviral genome. The origin of the 3 cDNA clones are depicted on top.

Conserved potyviral regions at 5′ terminus

13 TCAAC**ACAACAT**AT 26 39 AATC**TCAAGCA**ATCAAGC 56
Box A Box B

Fig 6c- Conserved sequences, Boxes A and B, located at the extreme 5′ non-coding ends of TEV, TVMV, and PVY (Turpen, 1989)

their origin from the 5′ terminus of the Z24 clone (**Fig 6a**).

Clones Zy2, Zy4, and Zy149 were then sequenced at their 5′ termini **(Fig.6b)**. As depicted, each clone terminated to within a few base pairs of each other. To determine if these clones extended to the 5′ leader sequence, sequence homology at the RNA level was compared to the TEV 5′ terminus (**Fig.6b**). There was homology to the "box B" sequence of the TEV 5′ terminus (40-60 bases from the 5′end; Turpen, 1989), but not to the conserved "box A" region (20-25 bases from the 5′ end) at the extreme 5′ end of the genome **(Fig 6c)**. This suggests that cDNA synthesis proceeded to within 50-100 bases of the 5′ end.

DNA sequencing of the helper component region first involved subcloning the approximate region responsible for this protein. Based on the position of the conserved 3′ gly-gly protein cleavage site in other potyviruses (Carrington et al. 1989a; Oh and Carrington, 1989), it was estimated that the 3′ terminus of the HC gene should reside approximately 2.5 kb from the 5′ end of the virus. In addition, the sizes of the HC proteins from different potyviruses, 52 to 56 kd (Dougherty and Carrington 1988), suggested that the ZYMV HC gene should be roughly 1500 base pairs long (@ MW/[100 MW/a.a.] X 3 bp/a.a.). Based on this information, a unique BamH1 site 2.0 kb from the 5′ end of Zy149 was subcloned and sequenced towards the 3′ end. The conserved gly-gly cut site was found to be about 100 base pairs away, providing evidence that this BamH1 site was near the 3′ terminus of the protein. Fortunately, there was an EcoR1 site 1.5 kb upstream of this BamH1 site. Sequencing was initiated once all the proper subcloning and nested deletions were made. **Fig. 7** shows the positions of all nested deletions spanning the ZYMV HC region.

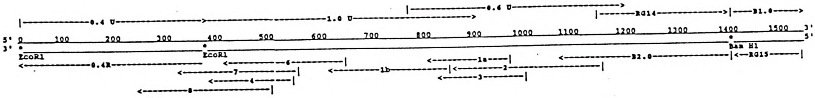


Fig.7- Nested deletions used for DNA sequencing

```
   1  ATTCGTGAGGAAGATATTAAACCAGGTTGTAGTGGGTGGGTGTTGGGCACTAATCATACA     60
   1   I  R  E  E  D  I  K  P  G  C  S  G  W  V  L  G  T  N  H  T     20

  61  TTGACTAAAAGATATTCAAGATTGCCACATTTGGTGATTCGAGGTAGAGACGACGATGGG    120
  21   L  T  K  R  Y  S  R  L  P  H  L  V  I  R  G  R  D  D  D  G     40

 121  ATTGTGAACGCGCTGGAACAGGTGTTATTTTATAGCGATGTTGACCACTAT[TCGTCGCAA    180
  41   I  V  N  A  L  E  Q  V  L  F  Y  S  D  V  D  H  Y [S  S  Q     60
                                                        5'
 181  TCGGAAGTTCAGTTCTTCCAAGGATGGCGACGAATGTTTGACAAGTTTAGGCCCAGCCCA    240
  61   S  E  V  Q  F  F  Q  G  W  R  R  M  F  D  K  F  R  P  S  P     80

 241  GATCATGTGTGCAAAGTTGACCACAACAACGAGGAATGTGGTGAGTTAGCAGCAATCTTT    300
  81   D  H  V  C  K  V  D  H  N  N  E  E  C  G  E  L  A  A  I  F    100

 301  TGTCAGGCTCTATTCCCAGTAGTGGAACTATCGTGCCAAGCATGCAGAGAAAAACTTAGT    360
 101   C  Q  A  L  F  P  V  V  E  L  S  C  Q  A  C  R  E  K  L  S    120

 361  AGAGTTAGCTTCGAGGAATTCAAAGATTCTTTGAACGCAAACTTTATTATCCACAAGGAT    420
 121   R  V  S  F  E  E  F  K  D  S  L  N  A  N  F  I  I  H  K  D    140

 421  GAATGGGGTAGTTTCAAGGAAGGCTCTCAATACGATAATATTTTCAAATTAATCAAAGTG    480
 141   E  W  G  S  F  K  E  G  S  Q  Y  D  N  I  F  K  L  I  K  V    160

 481  GCAACACAGGCAACTCAGAATCTCAAGCTCTCATCTGAAGTTATGAAATTAGTTCAGAAC    540
 161   A  T  Q  A  T  Q  N  L  K  L  S  S  E  V  M  K  L  V  Q  N    180

 541  CACACAAGCACTCACATGAAGCAAATACAAGACATCAACAAGGCGCTCATGAAAGGTTCA    600
 181   H  T  S  T  H  M  K  Q  I  Q  D  I  N  K  A  L  M  K  G  S    200

 601  TTGGTTACGCAAGACGAATTGGACTTAGCTTTGAAACAGCTTCTTGAAATGACTCAGTGG    660
 201   L  V  T  Q  D  E  L  D  L  A  L  K  Q  L  L  E  M  T  Q  W    220

 661  TTTAAGAACCACATGCACCTGACTGGTGAGGAGGCATTGAAGATGTTCAGAAATAAGCGT    720
 221   F  K  N  H  M  H  L  T  G  E  E  A  L  K  M  F  R  N  K  R    240

 721  TCTAGCAAGGCCATGATAAATCCTAGCCTTCTATGTGACAACCAATTGGACAAAAATGGA    780
 241   S  S  K  A  M  I  N  P  S  L  L  C  D  N  Q  L  D  K  N  G    260

 781  AATTTTGTTTGGGGAGAAAGAGGACACCATTCCAAGCGATTATTCAAGAACTTCTTCGAA    840
 261   N  F  V  W  G  E  R  G  H  H  S  K  R  L  F  K  N  F  F  E    280

 841  GAAGTAATACCAAGCGAAGGATATACGAAGTACGTAGTGCGAAACTTTCCAAATGGTACT    900
 281   E  V  I  P  S  E  G  Y  T  K  Y  V  V  R  N  F  P  N  G  T    300

 961  CTACTTGGAGAGAGCATTGAGAAGAAGCCACTCACATCAGCGTGTGTCTCCCAACAGAAT   1020
 321   L  L  G  E  S  I  E  K  K  P  L  T  S  A  C  V  S  Q  Q  N    340

1021  GGAAATTATATACACTCATGTTGCTGTGTAACGATGGATGATGGAACCCCGATGTACTCT   1080
 341   G  N  Y  I  H  S  C  C  C  V  T  M  D  D  G  T  P  M  Y  S    360

1081  GAGCTTAAGAGCCCGACGAAGAGGCATCTAGTTATAGGAGCTTCTGGTGATCCAAAGTAC   1140
 361   E  L  K  S  P  T  K  R  H  L  V  I  G  A  S  G  D  P  K  Y    380

1141  ATTGATCTGCCAGCATCAGAGGCAGAACGTATGTATATAGCAAAGGAAGGTTATTGCTAT   1200
 381   I  D  L  P  A  S  E  A  E  R  M  Y  I  A  K  E  G  Y  C  Y    400

1201  CTCAATATTTTCCTCGCAATGCTTGTAAATGTTAATGAGAACGAAGCAAAGGATTTCACC   1260
 401   L  N  I  F  L  A  M  L  V  N  V  N  E  N  E  A  K  D  F  T    420

1261  AAAATGATTCGTGATGTTTTGATCCCCATGCTTGGCAAGTGGCCTTCATTGATGGATGTT   1320
 421   K  M  I  R  D  V  L  I  P  M  L  G  K  W  P  S  L  M  D  V    440

1321  GCAACTGCAGCATATATTCTAGGCGTATTCCATCCTGAAACGGGATGCGCTGAATTACCC   1380
 441   A  T  A  A  Y  I  L  G  V  F  H  P  E  T  G  C  A  E  L  P    460

1381  AGGATCCTTGTTGACCACGCTACACAAACCATGCATGTCATTGATTCTTATGGATCACTA   1440
 461   R  I  L  V  D  H  A  T  Q  T  M  H  V  I  D  S  Y  G  S  L    480

1441  ACTGTTGGGTATCACGTGCTCAAGGCCGGAACTGTCAATCATTTAATTCAATTTGCCTCA   1500
 481   T  V  G  Y  H  V  L  K  A  G  T  V  N  H  L  I  Q  F  A  S    500

1501  AATGATCTGCAAAGCGAGATGAAGCATTACAGAGTTGGT]GGAACACCAACACAG
 501   N  D  L  Q  S  E  M  K  H  Y  R  V  G ]G  T  P  T  Q
                                           3'
```

Fig. 8- DNA sequence and deduced amino acid sequence of the helper component region of ZYMV-HC. The putative 5' and 3' cut sites of the ZYMV HC are indicated by brackets.

Fig. 8 shows the ZYMV HC sequence and deduced amino acid sequence. Since potyviral genomes are expressed as polyproteins that are subsequently cleaved, the 3′ and 5′ termini of the protein must be deduced from protease consensus cut sites (Dougherty and Carrington 1988). In the case of HC, the putative conserved Gly/Gly cleavage site at the 3′ end of this protein (Oh and Carrington, 1989) is also present in ZYMV (**Fig. 8** a.a. #513). The 5′ cleavage site has just been defined as a conserved serine residue approximately 450 amino acids upstream from the conserved 3′ cut site (Carrington, in press). Presumably, the serine residue at at position #58 (Fig. 8) corresponds to this residue. Assuming that these two sites are used in the processing of ZYMV HC, the calculated molecular weight of the protein would be 51.9 kD.

Figure 9 shows the alignment of the HC region amino acid sequences for ZYMV, plum pox virus (PPV; Lain et al., 1989), tobacco etch virus (TEV; Allison et al., 1986), tobacco vein mottling virus (TVMV; Domier et al, 1986), potato virus Y (PVY; Thornbury et al., 1990) and PVC, a non-aphid transmissible strain of PVY (Thornbury et al., 1990). Analysis of conserved amino acids, defined here as any residue shared by at least 4 of 5 potyviruses at a given position, shows that the frequency of conserved residues is highest at the 3′ carboxyl terminus and the central portion of the HC region. At the 5′ amino terminus, however, the frequency of conserved residues diminishes noticeably. To examine these regions of amino acid homology in a quantitative manner, the HC region was subjectively divided into three areas: the 5′ terminus (**Region I**), the central portion (**Region II**), and the 3′ terminus (**Region III**).

Figure 9- Alignment of HC region amino acid sequences for ZYMV, PPV, TEV, TVMV, PVY, PVC (a NAT strain of PVY), CaMV, and CaMV* (a NAT strain of CaMV). Differences between PVC and PVY are indicated on the PVC line

* : conservation in 4/5 strains, ZYMV variable

```
                                                 5′ cleavage site|---->

ZYMV    L V I R G R D D D G I V N A L E Q V     L F Y S D V D H Y|S N   (31)
PPV     F I V R G K H N S I L V D S R C K V S K K Q S N E I I H Y|S D
TEV     F I V R G R S D G M L V D A R A K V T F A V C H S M T H Y|S D
TVMV    F I V R G T C D D S L L E A R A R F S Q S I T L R A T H F|S T
PVY     F I V R G S H E G K L Y D A R S R V T Q S V L N S M I Q F|S N
PVC                                     K       G           V

|--------------------Region I---------------------------------->

ZYMV    S Q S E V Q F F Q G W R R M F D K F R P S P D H V     C K V D H N(64)
PPV     P G K Q F S D G F T N S F M Q C K L R E T   D H Q     C T S D L D
TEV     K S I S E A F F I P Y S K K F L E L R P D   G I S H E C T R G V S
TVMV    G D I F W K G F N A S F Q E Q K A I G L     D H T     C T S D L P
PVY     A D N F W K G L D G N W A R M R Y P S       D H T     C V A G L P
PVC       E               D                                     I D

|--------------------Region I------------------------------->
        *           *                     *   * * *
ZYMV    N E E C G E L A A I A C Q A L F P V V E L S C Q A C R E K L S R(96)
PPV     V E E C G Y V A A L V C Q A I I P C G K I T C L Q C A Q K Y S Y
TEV     V E R C G E V A A I L T Q A L S P C G K I T C K R C M V E T P D
TVMV    V E A C G H V A A L M C Q S L F P C G K I T C K R C I A N L S N
PVY     V A D C G R V A A L T R H S I L P C Y K I T C P T C A Q Q Y A S
PVC                                           E                       N

|--------------------Region I---------------------------->

ZYMV    V S F E E F K D     S L N A N F I I H K D W G S F K E G S Q (126)
PPV     M S Q Q E I R D R F S T V I E Q H E K T V M D N Y P Q F S H
TEV     I V E G E S G E S V T N Q G K L L A M L K E Q Y P D F P M
TVMV    L D F D T F S E L Q G D R A M R I L D V M R A R F P A F T H
PVY     L P V S D L F K L L H K H A R D G L N R L G A D K D R F I H
PVC         A                               S                   V

|--------------------Region I---------------------------->
                *                                               * *
ZYMV    Y D N I F K L I K V A T Q A T   Q N L K L S S E V M K L V Q (156)
PPV     V L A F L K R Y R E L M R V E N Q N Y E A F K D I T H M I G
TEV     A E K L L T R F L Q Q K S L V N T N L T A C V S V K Q L I G
TVMV    T I R F L H D L F D Q R R V T N P N T A A F R E I L R L I G
PVY     V N K F L M A L E H L T E P V D L N L E L F N E I F K S I G
PVC               V
```

```
|---------------------Region I  ---------------------------->
                  * * *
ZYMV      N H T S T H M C Q I Q D I N K A L A K G S L V I Q P E L D L (186)
PPV       E R K E A P F S H L N K I N E L I I K G G M M S A Q D Y I E
TEV       D R K Q A P F T H V L A V S E I L F K G N K L T G A D L E E
TVMV      D R N E A P F A H V N R L N E I L L L G S K A N P D S L A K
PVY       E K Q Q A P F K N L N V L N N F F L K G K E N T A H E W Q V
PVC

|------------------Region I----------------->||------>
                        * * *         * *   *       *
ZYMV      A L K Q L L E M T Q W F K N H M H L T G E E A L K M F R N K (216)
PPV       A S G H L R E L A R Y Q K N R T E N I R S G S I K A F R N K
TEV       A S T H M L E I A R F L N N R T E N M I R G H L G S F R N K
TVMV      A S D S L L E L A R Y L N N R T E N I R N G S L K H F R N K
PVY       A Q L S L L E L A R F Q K N R T D N I K K G D I S F F R N K
PVC

|---------------------Region II---------------------------->

ZYMV      R S S K A M I N P S L L C D N Q L D K N G N F V W G E R G H (246)
PPV       I S S K A H V N M Q L M C D N Q L D T N G N F V W G Q R E Y
TEV       I S S K A H V N N A L M C D N Q L D Q N G N F I W G L R G A
TVMV      I S S K A H S N L A L S C D N Q L D Q N G N F L W G L A G I
PVY       L S A R A N W N L Y L S C D N Q L D K N A N F L W G Q R E Y
PVC             K

|---------------------Region II---------------------------->
            *     *               * *
ZYMV      H S K R L F R N F F E E V I P S E G Y T K Y V V R N F P N G (276)
PPV       H A K R F F R N Y F D V I D V S E G Y R R H I V R E N P R G
TEV       H A K R F L K G F F T E I D P N E G Y D K Y V I R K H I R G
TVMV      A A K R F L N N Y F E T I D P E Q G Y D K Y V I R K N P N G
PVY       H A K R F F S N F F D E I D P A K G Y S A Y E I R K H P N G
PVC                               V

------->|-5’ end of proteolytic domain (Carrington et al.1989)->
                    *   *
ZYMV      T R K L A T G S L I V P L N L D R A R T A L L G E S I E K K (306)
PPV       I R K L A I G N L V M S T N L A A L R K Q L L G E E C I H F
TEV       S R K L A I G N L I M S T D F Q T L R Q Q I Q G E T I E R K
TVMV      E R K L A I G N F I I S T N L E K L R D Q L E G E S I A R V
PVY       T R K L S I G N L V V P L D L A E F R Q K M K G D Y R K Q P
PVC
```

```
|--------------------Region III--------------------------->
                                    *
ZYMV  P L T S A C V S Q Q N G N Y I H S C C C V T M D D G T P M Y (336)
PPV   E V S K E C T S K R G E N F V Y Q C C C V T H E D G T P L E
TEV   E I G N H C I S M R N G N Y V V P C C C V T L E D G K A Q Y
TVMV  G I T E E C V S R K D G N Y R Y P C C C V T L E D G S P M Y
PVY   G V S K K C T S S L D G N Y V Y P C C C T T L D D G S A I E
PVC           R

|--------------------Region III--------------------------->
                                  *
ZYMV  S E L K S P T K R H L V I G A S G D P K Y I D L P A I R G R (366)
PPV   S E I I S P T K N H L V V G N S G D S K Y V D L P T A K G G
TEV   S D L K H P T K R H L V I G N S G D S K Y L D L P V L N E E
TVMV  S E L K M P T K N H L V I G N S G D P K Y L D L P G E I S N
PVY   S T F Y P P T K K H L V I G N S G D Q K F V D L P K G D S E
PVC                                               Y

|--------------------Region III------------------------------->
                      *   *                           *             *
ZYMV  T K M I R D V L I P M L G K W P S L M D V A T A A Y I L G V F H P(399)
PPV   T K T V R D T L V P K L G T W P S M M D L A T A C H F L A V L Y P
TEV   T K F I R D T I V P K L G A W P T M Q D V A T A C Y L L S I L Y P
TVMV  T K R V R D E S V Q K L G K W P S L I D V A T E C A L L S T Y Y P
PVY   T K K V R D M C V P K L G T W P T M M D L A T T C A Q M R I F Y P
PVC

|--------------------Region III------------------------------->
                                                                *
ZYMV  E T G C A E L P R I L V D H A T Q T M H V I D S Y G S L T V G Y H(432)
PPV   E T R N A E L P R I L V D H E A K I F H V V D S F G S L S T G M H
TEV   D V L R A E L P R I L V D H D N K T M H V L D S Y G S R T T G Y H
TVMV  A A A S A E L P R L L V D H A Q K T I H V V D S Y G S L N T G Y H
PVY   D V H D A E L P R I L V D H D T Q T C H V V D S F G S Q T T G Y H
PVC

|--------------------Region III------------------------->
                      *
ZYMV  V L K A G T V N H L I Q F A S N D L Q S E M K H Y R V G | G
(461)
PPV   V L K A N T I N Q L I S F A S D T L D S N M K T Y L V G | G
TEV   M L K M N T T S Q L I E F V H S G L E S E M K T Y N V G | G
TVMV  I L K A N T V S Q L E K F A S N T L E S P M A Q Y K V G | G
PVY   I L K A S S V S Q L I L F A N D E L E S E I K H Y R V G | G
PVC                                               3′ cleavage site
```

Table 1- Conservation of amino acids in the potyviral HC region (refers to Figure 9)

Region I (5′ terminus- a.a. #29- #211)

			{- # of conserved amino acids -}				
	Total	{Conserved[a]	ZYMV	PVY	PPV	TEV	TVMV}
# of a.a	182	38	17	37	36	34	36
% of total a.a.		21%	**9.3%**	20%	20%	19%	20%
% of conserved a.a.			**45%**	97%	95%	89%	95%

Region II (Central region- a.a. #212- #279)

	Total	{Conserved	ZYMV	PVY	PPV	TEV	TVMV}
# of a.a.	68	39	35	38	37	38	37
% of total a.a.		57%	51%	56%	54%	56%	54%
% of conserved a.a.			90%	97%	95%	97%	95%

Region III(3′ terminus- a.a. #280- #461)

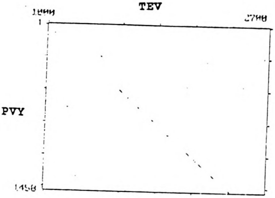
	Total	{Conserved	ZYMV	PVY	PPV	TEV	TVMV}
# of a.a.	182	87	79	86	81	82	81
% of total a.a.		48%	43%	47%	44%	45%	44%
% of conserved a.a.			91%	99%	93%	94%	93%

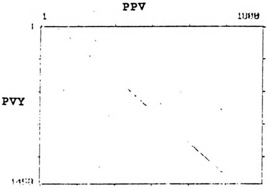
a: A conserved amino acid is defined as 4 of the 5 potyviruses examined in a given amino acid at a particular position

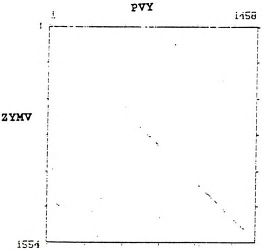
Region I, which begins at the putative 5′ cleavage site of the HC region (a.a. #29, Carrington et al., in press), contains 182 residues, 38 of which are conserved (21%). As seen in **Table 1**, this is the region containing the lowest frequency of conserved amino acids. Most of the potyviral

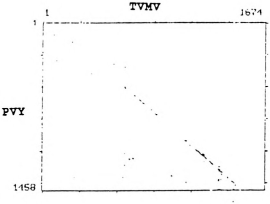
HC sequences contain the majority of conserved residues, except one- ZYMV. While the other potyviruses share in no less than 34 out of 38 conserved residues (90%), ZYMV shares in only 17 (45%). This abrupt departure from conservation of ZYMV relative to the other potyviruses ends at the conserved region FRNK (a.a. #212),where ZYMV regains the same frequency of shared amino acids relative to the other potyviruses. At this juncture, Region II begins, spanning from a.a. #212 until a.a. #279, the point at which Carrington et al. (1989) had determined the 5′ end of the proteolytic domain of the HC to be. Region II contains 68 residues, 39 of which are conserved (57%). In this region, ZYMV shares in 35 of the 39 conserved residues (90%), only slightly less than the other potyviruses, which range from 37-38 shared residues. Region III comprises of the entire proteolytic domain as defined by Carrington et al. (1989). This region contains 182 residues, 87 of which are conserved (48%). ZYMV shares in 79 of these conserved residues (91%), again just slightly less than the others.

Figure 10 presents HARR Plots depicting indicating regions of nucleic acid homology between all the potyviral HC regions. The data shows that all the potyviral HC regions are homologous to one another at the 3′ half, while the 5′ halves show varying degrees of homology, with ZYMV showing the least homology within this half. This is in agreement with the data at the amino acid level.


TEV
1800
2700
1
PVY
1458


PPV
1
1000
1
PVY
1458


PVY
1
1458
1
ZYMV
1554


TVMV
1
1674
1
PVY
1458

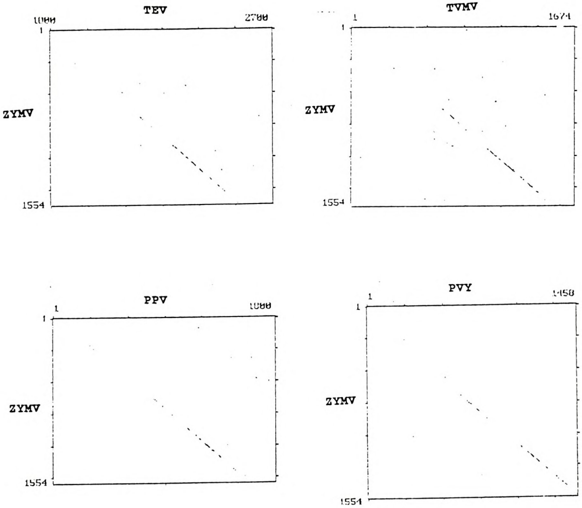


Fig. 10- HARR plots showing nucleic acid homology between various potyviruses

Discussion

The longest of the cDNA clones were shown, by sequence analysis of the conserved 3′ G-G cleavage site (Carrington et al., 1989a) and the putative 5′ Y-S cleavage site (Carrington et al., in press), to contain the ZYMV HC region (**Fig. 8**). The combined length of the Z24, the clone spanning the 3′ half of the ZYMV genome, and Z149, the longest of the cDNA clones, measured 9.1 kb. Relative to the lengths of other potyviruses, which range from 9.4 kb (TVMV; Domier et al., 1986), to 9.7 kb (PPV; Lain et al., 1989), the combined ZYMV cDNAs would appear to be full-length. Indeed, the 5′ termini of Zy2, Zy4, and Zy149 revealed the presence of the conserved "Box B" region (Turpen, 1989) from the potyviral leader sequence (**Fig. 6b**). The "Box B" conserved region is found approximately 40-60 base pairs from the 5′ potyviral terminus. Since all three clones terminate in the same area, there may be some type of secondary structure preventing the reverse transcriptase from reading the beginning of the leader sequence. One project underway is to complete the cloning of the rest of the ZYMV virus using a primer homologous to the 5′ end of the Zy149 clone. The three clones, Z24, Zy149 and the extreme 5′ end could then be combined to produce a full-length infectious transcript.

Figure 8 shows the DNA sequence of the ZYMV-CT HC region and its corresponding proposed amino acid sequence. Included is sequence information upstream and downstream of the gene. Both the conserved 5′ Y-S cut site (Carrington, in press) and the 3′ G-G cut site (Carrington et al., 1989a) are present. The number of amino acids in the ZYMV HC is 456. Its molecular weight is calculated to be 52 kd, a value within the size ranges determined for other potyviral HC proteins(50-56 kd, Carrington et al.,

1989a; Dougherty and Carrington, 1988, Hiebert et al., 1984; Raccah et al.,1984; Thornbury et al. 1985).

Assuming the hypothesis that HC acts as a mediator molecule binding the virion to the aphid stylet is correct, the functions of the HC protein are three-fold: it acts as a protease, binds to the coat protein, and binds to the aphid. Since the HCs of several potyviruses have been shown to be functionally interchangeable (Sako and Ogata, 1981; Lecoq and Pitrat, 1985), areas of conserved sequence in the HC region may be related to the biological activity of HC. The protease domain has been assigned to the 3′ carboxyl region, within 180 amino acids from the 3′ terminus (Carrington et al. 1989). This region, designated **Region III** here, is well conserved among all the potyviruses. As seen in Table 1, 48% of the residues in this region are conserved, with the frequency of conservation high (>90%) among all published potyviral HC sequences.

Region II, the central region, is also well-conserved among all published potyviral sequences, with 57% of the residues in this region is shared by at least 4 out of 5 potyviruses. This region has a carboxyl domain upstream from the 5′ terminus of the proteolytic domain (region III), and an amino terminal domain 3′ from the FRNK sequence. Since potyviruses have been shown to be aphid transmitted with heterologous HCs (Lecoq and Pitrat, 1985), it is conceivable that a conserved region exists within the HC protein which allows for common binding to the virion. In addition, it is known that potyviruses can be transmitted by several aphid species (Alderez, 1987; Kennedy et al., 1962; Pirone and Harris, 1977; Van Hoof, 1980). Then, a conserved HC region may serve as a common binding domain to aphid stylets. As all potyviral sequences contain a high frequency of shared amino acids

in Region II, perhaps this region serves as a common binding site to either the coat protein, aphid stylet, or both.

Region I, which comprises the 5′ terminus of the HC region, differs from Regions II & III in that it has a relatively low percentage of conserved amino acids (21%). Moreover, ZYMV is significantly lower relative to the other potyviruses in the frequency of these conserved residues (**Table 1**). The significance of this may be understood by examining the function ascribed to this region. Recent studies show that this region plays an important role in aphid transmission. Comparative sequence analysis of the HC region of PVY and an HC-defective, non-aphid transmissible strain (PVC), showed two conserved amino acid differences in the 5′ half of the HC region. One was a K->E substitution at a.a. #83, and the other a I->V at #259 (depicted in **Fig. 9** in **bold type**). Further mutational analysis studies by Atreya, C.D. et al. (in press), has shown that the K->E substitution at a.a. #83 of Region I results in a loss in aphid transmissibility.

In further support for this hypothesis, the strain of potyvirus used for this study, ZYMV-CT, is non-aphid transmissible, and has the K->E and I->V amino acid substitutions at positions 84 and 259 respectively. By coincidence, the mechanism by which both PVC and ZYMV-CT are hypothesized to have become non-aphid transmissible is the same: both strains were repeatedly propagated by mechanical "rub" inoculations, a technique shown to select for non-aphid transmissibility (Swenson et al., 1964). Conceivably, the lack of transmissibility could be due to changes in the CP, HC, or both. The CP of this strain, however, contains the conserved tripeptide DAG (Grumet and Fang, 1990), which has been shown to be altered in the CPs of other non-aphid transmissible potyviral strains

(Atreya, P.F. in press). Clearly, it is of interest to determine whether the HC of ZYMV-CT is biologically active; this will be addressed in the Addendum. So far, sequence analysis suggests that, due to the K->E and I->V substitutions found in Region I of the ZYMV-CT HC, the HC is probably inactive.

Given that Region I is important to aphid transmissibility, there may be differences between the way by which ZYMV is aphid transmitted relative to the other potyviruses that explains the divergence of shared amino acids in this region. Recent data indicates that ZYMV-CT is not alone in differing from the other potyviruses in the type and frequency of residues which are conserved in the 5′ terminus. It has been noted at a recent meeting (R.Grumet, personal communication) that two other strains of ZYMV, ZYMV-FL and ZYMV-CA, and papaya ringspot virus type W (PRV-W) share virtually the same sequence in the HC region as does ZYMV-CT, even with the homology differences at the 5′ end (C. Baker, personal communication) Because the published sequences are not available at this time, amino acid comparisons are not depicted in **fig. 9**. However, it appears there are two groups of potyviruses that share sequence similarity in Region I of the HC: the cucurbit potyviruses (ZYMV and PRV-W) and the non-cucurbit potyviruses (PVY, PPV, TVMV and TEV). Interestingly, there is a correlation between these two groups and the predominate aphid species which vectors the potyviruses in each group. The cucurbit potyviruses are mainly carried by the Aphis species (Alderz, 1987), while the other group, which have either solanaceous (TEV, TVMV, and PVY) or prunus (PPV) hosts, are primarily carried by the green peach aphid, Myzus persicae (Van Hoof, 1980; Kennedy et al., 1962; Pirone and Harris, 1977). Thus, perhaps Region I may have a role in defining vector specificity.

The amino acid change at position 83 (K->E), for both PVC and ZYMV and at 81 (C->V), for ZYMV, when examined more closely, may shed some light as to the mechanism of aphid binding. Both these changes occur in a conserved cysteine-rich region. Robaglia et al. (1989) has found that similar regions found in the HC sequence of the tobacco veinal necrosis strain of PVY, PVYn, are similar to sequences found in proteins known to form "zinc fingers", and may be possible metal binding sites. Indeed, in vitro activity of HC is known to require Mg^{++} ions (Raccah and Pirone, 1984). If this conserved cys-rich region is a metal binding site, a change from a basic amino acid to an acidic amino acid and/or a change from an essential cys to a nonpolar amino acid may render the site inactive. These sites are prime candidates for in vitro mutagenesis once an infectious in vitro full-length transcript for ZYMV can be produced.

It is possible that the competitiveness of ZYMV for aphid transmission may lie in the specific amino acid changes in the highly conserved regions. These changes may cause the HC to bind better to the virion or to its aphid vector. A way to study this would be to generate a full-length infectious transcript of ZYMV and generate site-directed mutations to screen for mutants deficient in aphid binding or virion binding. In this way, the putative binding domains will be able to be mapped. Another possibility is to create "pseudo-recombinants", using regions of DNA from an AT isolate of ZYMV and splicing it into the homologous DNA region of the NAT isolate. This is readily feasible, since there are convenient restriction sites which can cut out the majority of the HC.

CHAPTER 3

Addendum

One area of interest in the study of potyvirus biology is to determine the protein domains responsible for aphid transmission. Past studies have determined that potyvirus aphid transmission is in part controlled by two viral-encoded proteins, the helper component (HC) and the coat protein (CP) (Pirone and Thornbury, 1984). When studies of non-aphid transmissible (NAT) potyviral strains were conducted, some cases were found to be due to the lack of biologically active HC (Kassanis and Govier, 1971; Paguio and Kuhn, 1976; Lecoq, 1976). Other cases were attributed to some other variation within the virion. In these instances, the helper component recovered from these NAT strains was shown to be biologically active, and the coat protein was suggested as the cause of non-transmissibility (Pirone and Thornbury, 1983; Antignus et al. 1989).

The coat protein had been implicated in aphid transmission from other studies; enzymatic removal of the N-terminus of the coat protein results in loss of aphid transmissibility (Salomon, 1989). It has also been shown that this N-terminal domain is exposed to the surface of the viral particle (Allison et al. 1985; Shukla et al., 1988). Studies of the amino acid sequence of the N-terminal domain of a NAT strain of tobacco vein mottling virus (TVMV) were used to test the hypothesis that a mutation in the coat protein could cause non-aphid transmissibility (Atreya et al. 1990). The non-aphid transmissibility of this TVMV strain was attributed to the use of repeated mechanical "rub" inoculations on the cotyledons of test plants for viral propagation. A previous study demonstrated that aphid transmissible viruses propagated in this manner leads to the loss in aphid transmissibility (Swenson et al. 1964). The amino acid sequence of this and other NAT strains revealed a tripeptide of DAG in the N-terminal domain which was

consistently altered in NAT strains, but not in aphid-transmissible (AT) strains (Harrison and Robinson, 1988; Maiss et al., 1989; Quemada et al., 1990). Further studies have shown that point mutations around this area, especially the G residue, greatly reduced or abolished aphid transmissibility (Atreya et al., in press)

The Connecticut (CT) strain used in our laboratory was found to have an unaltered DAG region in the coat protein (Grumet and Fang, 1990). However, upon aphid transmission assays, the CT strain was found to be either non-aphid transmissible or extremely delayed in exhibiting symptoms when compared to other strains. Therefore, the site of alteration causing this non-aphid transmissibility is at a location other than the DAG region of the coat protein. It could be at another region within the coat protein or within the helper component. This addendum outlines a series of experiments to locate the site of these alterations.

The following ZYMV strains were used as a control for aphid transmission: Orangeburg (ORG), Pickens (PIC) and Arkansas (ARK). These strains exhibited symptoms within 2-4 weeks following aphid transmission assays (**Table 2**). Test zucchini plants aphid inoculated with the CT strain, on the other hand, usually died within 4-5 months without exhibiting symptoms. Fortunately, two plants which managed to survive past this time showed symptoms at 6 months. Previous to these studies, this CT strain was propagated for many generations by rub inoculation, while the other strains were propagated by aphid transmission. It may well be that the CT strain had lost the ability to aphid transmit because of this, and

TABLE 2

Aphid transmission of various ZYMV strains

ZYMV Strain	Symptom Onset	Description of Symptoms
ARK	2-4 wks	extremely severe, malformed, spiny leaves, stunted growth
PIC	2-4 wks	severe leaf mosaic and chlorosis leaves distorted
ORG	2-4 wks	severe leaf mosaic, chlorosis, slight enations, leaves distorted
CT	6 mo.	least severe mosaic and chlorotic symptoms, leaves distorted
CT-1	2-4 wks	least severe mosaic and chlorotic symptoms, leaves distorted
CT-2	2-4 wks	least severe mosaic and chlorotic symptoms, leaves distorted

that, somehow, some variant of the strain had multiplied enough to infect the plant by six month's time. The variant CT strains collected from the two plants were labelled CT1 and CT2. Upon subsequent aphid transmission, both CT1 and CT2 showed classic CT symptoms 2-4 weeks after initial infection.

Since the ZYMV-CT strain contains the conserved DAG region in the coat protein, locating the site of the variance could lead to an additional area of significance concerning aphid transmissibility. It is conceivable that this variance could be occurring within the coat protein or the helper component. For this question, a set of mixing experiments involving the isolation of the virions and helper component from both the AT CT1 and CT2 strains and NAT CT strain is proposed (**fig. 10**).

The main idea behind these experiments is to determine which component, the virion or the HC, is responsible for causing the deficiency in aphid transmission in the NAT CT. For example, if there is a variation in the coat protein, the mixing of the NAT virion with the AT helper component should not allow the aphid to successfully transmit the virus, while the mixing of the NAT HC with the AT virions should produce normal aphid transmission. Fig. 9 outlines the experiment.

Methods

1) **aphid feeding:** Aphids should be starved 3-4 hrs. before being allowed to feed on a 20% sucrose solution containing the above mixtures. Feeding should occur through a parafilm-covered sachet, with 15-min. acquisition times. Aphids should be allowed to feed on healthy zucchini plants for 2-4 hrs. before being killed mechanically and removed. Plants are then sprayed to ensure complete eradication of aphids.

2) **virion isolation:** Virions should be isolated in a similar fashion previously described in materials and methods

MIXING EXPERIMENTS

Source		Isolations
Non-Aphid Transmissible	----->	virion
CT strain	----->	HC-enriched plant sap (HC)
Aphid-Transmissible	----->	virion*
Revertants (CT1 & CT2)	----->	HC-enriched plant sap (HC*)
Aphid-Transmissible	----->	virion-c
Control (ZYMV-ARK)	----->	HC-enriched plant sap (HC-c)

aphid feeding solutions -->	rub inoculation & aphid transmission-->	results RI	AT	
A. Controls				
1) virion + HC		Inf.	No inf.	
2) virion* + HC*		I	I	
3) virion-c + HC-c		I	I	
4) virion, virion*, or virion-c		I	NI	
5) HC, HC*, or HC-c		NI	NI	
B. Mixes			Virion	HC
6) virion + HC*		I	NI	I
7) virion + HC-c		I	NI	I
8) virion* + HC-c		I	I	I
9) virion* + HC		I	I	NI
10) virion-c + HC		I	I	NI
11) virion-c + HC*		I	I	I

Figure 11- Mixing Experiments to determine source of variation in a revertant form of a non-aphid transmissible ZYMV-CT strain

3) **helper component enriched plant sap:** Crude HC-enriched plant sap should be made by methods similar to Sako & Ogata (1981). Briefly, this includes grinding 3g of 3-4 wk. old infected leaf tissue in 10 ml 0.3M K_2HPO_4 pH9; centrifugation at 3,500 x g for 10 min.; centrifugation at 150,000 x g for 3 hr.; use upper supernatant as virion-free, HC extract.

If the variation is occurring within the helper component protein, then HC would be inactive, rendering the mixes with both the virion* and virion-c non-aphid transmissible. Meanwhile, HC* would show symptoms when mixed with the NAT virions. The opposite would occur if the source of the variation resided within the virion coat protein. If the variation is occurring on both the virion and HC, then both the NAT virion and NAT HC mixed with the complementary HC-c or virion-c would not show aphid transmission.

Once the region of this variation has been identified, mutants can be made from either the coat protein or the helper component from the AT strain to identify the exact location of the mutation. In addition, the area in question can be sequenced in both the NAT and AT revertant strains to reveal the nature of the base pair differences.

Another issue facing investigators in the field of potyviral aphid transmission involves the exact mechanism by which helper component acts. Since it has been demonstrated that HC is responsible for the virion's ability to bind to the aphid stylet (Berger and Pirone, 1986), the next question which must be answered is to the location on the virion and/or aphid stylet HC binds to. With this work, it should now be possible to clone a functional HC protein; in fact, a primer has been made (**fig. 12**), which includes a restriction site exactly at the 3' cut site, for direct cloning into an expression vector. The cloning of a functional HC gene can lead to

```
                         gly-gly cut site
                                |
                                v
     ZYMV 5' A T T A C A G A G T T G G T G|G A A C A C C A A C A C A 3'
RG 14 primer*    T A C A G A G T T G G T G  G T A C C C C A A C A C A
                                                  ^
                                   Kpn restriction site
```

Fig.12- Primer at the 3′ terminus of the ZYMV-CT HC with a Kpn restriction site. *Note: RG14 primer made is complementary to the one above:

RG14: 5′ TGTGTTGGGGTACCACCAACTCTGTA 3′

deletion analysis studies and to the production of HC protein (functional or mutant) for binding studies. In addition, antibodies may also be raised specifically against the HC protein and be used for a number or purposes. Finally, large scale HC purification can be attempted using the anti-HC proteins and immuno-affinity columns.

Once a quantity of functional HC protein is made, studies to find physical evidence for the binding of HC to the virion can be undertaken. For example, protein-interactions can be investigated through size chromatography. The HC can be shown to bind to either the virion or to individual coat protein subunits if, subsequent to the mixing of HC and virion/ or coat protein, there is evidence of a third, larger fraction comprising of the bound proteins in addition to the individual fractions. These fractions can be visualized through PAGE. In a different experiment, the HC can be immobilized for a binding assay to virions and/or aphid stylets. Anti-HC protein can then be used to inhibit binding. If HC acts as a type of "sandwich" between the two, then anti-HC should inhibit binding to the virion and the stylet individually, or together. In addition, anti-coat protein (CP) can be utilized to ascertain whether HC is binding to any exposed RNA portion of the virion or to other exposed proteins, such as the vPG. If anti-CP does not inhibit binding, this conceivably could be happening. Furthermore, there is circumstantial evidence that HC may bind to RNA. According to one method of purification of TEV HC, the final step in this process involved HC binding to an oligo-dT affinity column, leading to speculation that the HC may be binding to the RNA (Thornbury et al. 1985). To study this, gel retardation assays involving the binding of the RNA with the HC can be performed. Again, a

third, larger band representing the binding of RNA to the HC would be evidence of the interaction.

For future experiments, mutant HC can be constructed that are deficient in binding to either the virion or aphid stylet. These can then be inserted transgenically into plants to test for protection against the aphid spread of this disease. Theoretically, the titration effects of utilizing the available binding sites will prevent the functional HC from performing properly.

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