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BARD Project Number: US-2666-95
Evaluating Panel: Plant Protection

Project Title: Identification of potyviral domains controlling systemic infection, host range, and aphid

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Continuation of (Related to) Previous BARD Project:

☐ Yes  ☒ No  Number:

Keywords not appearing in the title and in order of importance. Avoid abbreviations.

Abbreviations used in the report, in alphabetical order: Curcurbits, potyviruses, resistance, molecular biology, vector transmission, systemic infection, symptom development, host range, pathogen-derived resistance

Budget: IS: $ 150,000  US: $ 150,000  Total: $ 300,000

Rebecca Grumet
Signature
Principal Investigator

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**Publication Summary (numbers)**

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Cooperation, briefly explain whether synergistic, complementary or supportive.

Significant collaboration, including a three month visit by an MSU graduate student to the Volcani, allowed for the production of the hybrid ZYMV infectious clones. This collaboration was essential to achieving the objectives of the BARD grant and is recognized in jointly authored publications.
B. Abstract

Potyviruses form one of the largest and most economically important groups of plant viruses. Individual potyviruses and their isolates vary in symptom expression, host range, and ability to overcome host resistance genes. Understanding factors influencing these biological characteristics is of agricultural importance for epidemiology and deployment of resistance strategies. Cucurbit crops are subject to severe losses by several potyviruses including the highly aggressive and variable zucchini yellow mosaic virus (ZYMV). In this project we sought to investigate protein domains in ZYMV that influence systemic infection and host range. Particular emphasis was on coat protein (CP), because of known functions in both cell to cell and long distance movement, and helper component-protease (HC-Pro), which has been implicated to play a role in symptom development and long distance movement. These two genes are also essential for aphid mediated transmission, and domains that influence disease development may also influence transmissibility. The objectives of the approved BARD project were to test roles of specific domains in the CP and HC-Pro by making sequence alterations or switches between different isolates and viruses, and testing for infectivity, host range, and aphid transmissibility. These objectives were largely achieved as described below. Finally, we also initiated new research to identify host factors interacting with potyviral proteins and demonstrated interaction between the ZYMV RNA dependent RNA polymerase and host poly-(A)-binding protein (Wang et al., in press).

The focus of the CP studies (MSU) was to investigate the role of the highly variable amino terminus (NT) in host range determination and systemic infection. Hybrid ZYMV infectious clones were produced by substituting the CP-NT of ZYMV with either the CP-NT from watermelon mosaic virus (overlapping, but broader host range) or tobacco etch virus (TEV) (non-overlapping host range) (Grumet et al., 2000; Ullah et al., in prep). Although both hybrid viruses initially established systemic infection, indicating that the even the non-cucurbit adapted TEV CP-NT could facilitate long distance transport in cucurbits, after approximately 4-6, the plants inoculated with the TEV-CPNT hybrid exhibited a distinct recovery of reduced symptoms, virus titer, and virus specific protection against secondary infection. These results suggest that the plant recognizes the presence of the TEV CP-NT, which has not been adapted to infection of cucurbits, and initiates defense responses. The CP-NT also appears to play a role in naturally occurring resistance conferred by the zym locus in the cucumber line ‘Dina-II. Patterns of virus accumulation indicated that expression of resistance is developmentally controlled and is due to a block in virus movement. Switches between the core and NT domains of ZYMV-NAA (does not cause veinal chlorosis on ‘Dina-I), and ZYMV-Ct (causes veinal chlorosis), indicated that the resistance response likely involves interaction with the CP-NT (Ullah and Grumet, submitted).

At the Volcani Center the main thrust was to identify domains in the HC-Pro that affect symptom expression or aphid transmissibility. From the data reported in the first and second year report and in the attached publications (Peng et al. 1998; Kadouri et al. 1998; Raccah et al. 2000), it was shown that: 1. The mutation from PTK to PAK resulted in milder symptoms of the virus on squash, 2. Two mutations, PAK and A TK, resulted in total loss of helper activity, 3. It was established for the first time that the PTK domain is involved in binding of the HC-Pro to the potyvirus particle, and 4. Some of these experiments required greater amount of HC-Pro, therefore a simpler and more efficient purification method was developed based on Ni2+ resin.
C. Achievements

**Main scientific achievements and significance**

This BARD project resulted in the publication of several refereed papers and symposia proceedings that provide an increased understanding of potyviral determinants influencing systemic infection, symptom development, host range, and aphid transmission. The main findings of each subproject and associated publications are listed below.

1. **Analysis of the role of the amino terminus (NT) of the potyviral coat protein (CP) in systemic infection, symptom development and host range.**

**Publications:**


Ullah Z, Grumet R. Localization of Zucchini yellow mosaic virus to the veinal regions and role of viral coat protein in veinal chlorosis conditioned by the zym potyvirus resistance locus in cucumber. Submitted.


Production of hybrid ZYMV infectious clones with substitutions in the variable CP-NT indicated that when the CP-NT came from a non-cucurbit adapted virus (tobacco etch virus, TEV), the plant was able to mount a successful recovery leading to reduced symptoms, virus titer, and virus specific protection against secondary infection. When the CP-NT came from another cucurbit potyvirus (watermelon mosaic virus, WMV), recovery did not occur. These results suggest host recognition of the CP-NT from a non-adapted virus and provide direct evidence for a role of the variable CP-NT in host range adaptation.

The observed recovery phenotype closely resembles phenotypes associated with gene silencing (Ratcliff et al. 1997, 1999; van den Boogaart et al. 1998) and so raises interesting
questions. HC-Pro is known to act as a suppressor of gene silencing (Brignetti et al., 1998; Anandalaskshmi et al. 1998; Kasschau and Carrington, 1998) interactions between the CP-NT and HC-Pro already are well-established for aphid transmission and in-vitro (including work from this BARD project, Peng et al. 1998) and both are involved in long distance virus movement (Dolja et al. 1995; Atreya et al. 1995; Klein et al. 1994; Huet et al. 1994; Cronin et al. 1995; Peng et al. 1998). Future investigations will examine whether the TNT recovery phenomenon is related to gene silencing, and if so, what role the CP-NT plays.

The chimeric viruses also were tested for ability to systemically infect an array of ZYMV local lesion or non-hosts, including those normally infected by TEV or WMV. We did not observe any modification in host range. The failure of the heterologous CP-NTs to extend host range also has implications for risk assessment involving deployment of transgenic virus resistant plants expressing potyvirus CPs.

The CP-NT was also found to play a role in naturally occurring resistance conferred by the zym locus in the cucumber line 'Dina-1'. Analysis of resistance indicated that the zym allele confers a block in long distance movement; the ability to do so varied among ZYMV strains and was influenced by the specific ZYMV CP-NT.

Collectively, these results indicate that the variable potyvirus CP-NT plays a role in host adaptation and can be either a trigger (in the case of a non-adapted CP) or target (in the case of a resistant genotype) for host defenses.

2. Analysis of the role of the helper component protease (HC-Pro) in systemic infection, symptom development and aphid transmission.

Publications
Finding domains in the HC-Pro that are involved in milder symptoms has economical importance for preparing mild virus isolates that may serve for cross protection (see Gal-On, 2000), and for genetic tailoring of infectious clones with mild symptoms that can serve for transient expression in plants of foreign proteins of economical value. The potential for application of mild viruses is immediate and the technology is available. In Israel alone, ca. 1000 ha of watermelons are currently cross protected with naturally found mild ZYMV. Assuming 10% of severe epidemics with total loss in bad virus years, this means a yield loss of 100 ha (ca. 7000 tons with an approximate value of $1 million. Additional work is needed to verify if the domains found in ZYMV HC-Pro are also acting in other viruses.

Finding domains in the HC-Pro that are involved in aphid transmissibility may be useful in the future for expression in plants of deficient HC molecules that will trap virions and render them non-transmissible. Much work, however, is needed to reach this goal.

Details of cooperation

Although this CP work was primarily focused at MSU, significant collaboration allowed for production of the hybrid ZYMV infectious clones. A graduate student supported by the BARD grant, Zakir Ullah, traveled to the Volcani Institute for a period of three months where he initiated work with the infectious clones of Gal-On and Raccah et al. These clones were then generously provided to the Grumet lab to allow for Zakir to complete the various substitutions and perform the biological analyses. Details of the constructions are provided in the Methods sections of the above-mentioned manuscripts. This collaboration was essential to achieving the objectives of our BARD grant and is recognized in the jointly authored publications listed above.

Further interaction resulted from a visit to Israel by R. Grumet as part of the Eucarpia-Cucurbitaceae Meeting 2000. This allowed for discussion of research results and possible future collaborations between the Raccah, Huet, and Grumet laboratories.
SECTION II. TECHNICAL REPORT

A. Background, scientific and agricultural relevance of the project.

Potyviruses form the largest, and one of the most economically important, groups of plant viruses (Shukla et al. 1994). Within the potyvirus group are members that vary in host range from narrow to broad. Moreover, even among isolates of a given potyvirus, there can be variation for symptom expression, host range, and the ability to overcome both naturally occurring and genetically engineered resistance. Understanding of factors influencing these biological characteristics is of agricultural importance for epidemiology and deployment of resistance strategies, including engineered resistance.

Potyvirus infection can be a major factor limiting production of cucurbit crops (cucumbers, squashes, melons, watermelons) in many parts of the world including Israel and portions of the U.S. (Antignus et al. 1989; Davis and Mizuki, 1985; Nameth et al. 1986; Sammons et al. 1989). Among the potyviruses infecting cucurbits, zucchini yellow mosaic virus (ZYMV) is a particularly aggressive virus that has been the subject of extensive biological, molecular, and epidemiological investigation (Desbiez and Lecoq, 1997). ZYMV was first described in Italy and France in 1981 and within a few years had been reported throughout the world (Lisa et al., 1981; Lisa and Lecoq, 1984). Various isolates or pathotypes of ZYMV have been described with differing symptomatology depending on the host genotype (Lecoq and Pitrat, 1984; Greber et al. 1988; Wang et al. 1992). The two isolates that we have worked with most extensively, ZYMV-Ct and ZYMV-NAT/NAA, also differ with respect to symptomatology and host resistance phenotype.

Although potyviruses have been the focus of a great deal of research in the last decade and much has been learned about many aspects of potyvirus biology, the specific potyviral determinants influencing systemic infection, symptom development, and host range are not well defined. Two gene products of likely importance are the coat protein (CP) and helper component protease (HC-Pro). Coat protein is necessary for both cell to cell and long distance movement of the virus (Dolja et al. 1994, 1995; Rojas et al. 1997), and although the HC-Pro was originally defined for its role in facilitating aphid transmission and later for proteolytic activity, it more
Figure 1. Effect of substitutions of the amino terminus (NT) of the ZYMV coat protein gene with the heterologous viruses WMV (WNTFL/P) or TEV (TNTFL/P).

Table 1. Effect of plant age (A) and prior inoculation with ZYMV/TNT hybrid virus (B) on symptoms in squash.

A. Effect of plant age on symptom development.

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<th>Age at time of inoculation</th>
<th>Number inoc</th>
<th>Symptoms 2-3 weeks post inoculation</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>TNT</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>5.0</td>
</tr>
<tr>
<td>17</td>
<td>16</td>
<td>4.2</td>
</tr>
<tr>
<td>50</td>
<td>9</td>
<td>3.7</td>
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</table>

B. Effect of prior inoculation with ZYMV/TNT hybrid virus on infection by ZYMV-NAA/CT or PRSV-W.

<table>
<thead>
<tr>
<th>Age at 2\textsuperscript{nd} inoc.</th>
<th>Number inoc</th>
<th>Symptoms at time of 2\textsuperscript{nd} inoc.</th>
<th>Symptoms 2-3 post 2\textsuperscript{nd} inoc.</th>
<th>Detection by RT-PCR</th>
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<tr>
<td>ZYMV 17</td>
<td>10</td>
<td>5.0</td>
<td>1.5</td>
<td>12/12 3/12</td>
</tr>
<tr>
<td>ZYMV 50</td>
<td>12</td>
<td>1.5</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>PRSV-W 50</td>
<td>13</td>
<td>1.5</td>
<td>4.7</td>
<td></td>
</tr>
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</table>

Data are pooled from two experiments.

\textsuperscript{1} Symptoms ranked 0-5 with 5 being most severe.
suggested that the NT of the CP, which is extremely variable in both length and sequence, might be involved in host mediated defense responses and host range determination (Shukla et al. 1994; Xiao et al. 1993; Ullah and Grumet, submitted).

In this portion of the BARD project we sought to further understand the role of the NT of potyviral CPs in facilitating systemic infection and host range determination. Hybrid ZYMV viruses, TNT and WNT (Figure 1), were created by exchanging the NT of the CP of the infectious clone ZYMV-NAA (Gal-On et al. 1992, 1995) with the NT of the CP of either tobacco etch virus (TEV) (non-overlapping host range with ZYMV) or watermelon mosaic virus (WMV) (overlapping, but broader host range), respectively.

Both of the hybrid viruses were infectious on ZYMV-susceptible squash and cucumber plants, indicating that although TEV did not normally infect cucurbits, and has an extremely different CP-NT [the NT of TEV is 29 amino acids (aa) long, while ZYMV is 44 aa with only 13 aa identity to TEV], the TEV NT was sufficient to facilitate systemic infection (Fig. 1A). The heterologous viruses also were aphid transmissible (data in Ullah et al.) indicating that the CP-NT functions necessary for interaction with the HCPro to facilitate aphid transmission also were present.

Interestingly, although the hybrid TNT virus could initially establish infection on squash or cucumber plants (Fig. 1B, lower leaves of TNTFL/P), after 4-6 weeks the TNT-infected plants showed a distinct recovery (upper leaves). Newly developing leaves had progressively less symptoms and reduced virus titer. The loss of symptoms was not due to plant age, the process of initial infection could be repeated again if plants were inoculated at later ages (Table 1A), and the phenomenon did not occur with ZYMV or WNT infected plants which continued to develop increasingly severe symptoms leading to extreme leaf distortion (Fig. 1B). Back inoculations from recovered leaves led to the same process of strong initial infection before recovery, indicating that there was not a change in the virus. Furthermore, although it was possible to establish an infection with ZYMV or TNT on 50 day old plants, secondary inoculation of TNT-recovered plants with ZYMV did not develop symptoms, and virus could only be recovered from ca. 1/4 of the plants by RT-PCR (Table 1B). When inoculated with another cucurbit potyvirus, papaya ringspot virus (PRSV-W), however, the plants became infected and showed typical
Overall, the pattern and nature of recovery resembles classic recovery phenotypes that are typical of certain types of viruses (e.g., nepoviruses, tobraviruses) as well as the recovery phenotype that was first observed for RNA mediated virus resistance in transgenic plants (Ratcliff et al., 1997, 1999; van den Boogaart et al. 1998). In each case recovery has been associated with an induction of post-transcriptional gene silencing. Future investigations will examine whether the TNT recovery phenomenon is related to gene silencing, and if so, what role the CP-NT plays.

The chimeric viruses also were tested for modification of host range and ability to overcome host resistances. Neither the control or hybrid viruses were able to produce symptoms on the resistant cucumber genotype 'TMG-1' or ZYMV-CP expressing transgenic melons (data in Ullah et al.). The hybrid viruses also could not systemically infect several additional ZYMV local lesion or non-hosts (in Ullah et al.). Substitutions of the core or full length coat protein were not infectious (Ullah et al.), likely due to constraints of viral 3' RNA secondary structure necessary for effective replication (Haldeman-Cahill et al. 1998; Majajan et al. 1996).

In summary, 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. Substitution of the CP-NT of ZYMV with a non-cucurbit potyvirus resulted in the induction of a host defense response leading to recovery from the hybrid virus infection, suggesting that variability in the CP-NT has a potential role in host adaptation.

Objective 2. Investigate the role of the ZYMV HC-Pro in symptom development, systemic infection, host range and aphid transmissibility.

In the Volcani Center, the main thrust was aimed at the identifying domains in the HC-Pro that affect symptom expression or aphid transmissibility. From the data reported in the first and second year report and in the attached publications, it is apparent that a major part of the objectives were achieved:

1. The mutation from PTK to PAK resulted in milder symptoms of the virus squash.
2. Two mutations, PAK and ATK resulted in total loss of helper activity.
3. It has been established for the first time that the PTK domain is involved in binding of the HC-
Pro to the potyvirus particle.

4. Some of these experiments required greater amount of HC-Pro, therefore, a simpler and more efficient purification method was developed based on Ni2+ resin.

On the other hand, some additional tasks that were attempted in the study did not yield the expected results or were not successful:

**1. Yeast two hybrid system:**

It is believed that the HC-Pro is functional as a dimer (two molecules of HC-Pro co-associated). Therefore, a construct representing the ZYMV HC-Pro ORF has been prepared. Taking advantage of the experience in of Drs. T. Candresse and O. Le Gall from the Virology laboratory in INRA, Bordeaux, France in preparing two hybrid systems for the HC-Pro of the lettuce mosaic potyvirus, we intended to prepare a dimer made of two HC-Pro molecules, one of the ZYMV and another of the LMV. These experiments did not result in functional helpers. We inserted the HC-pro gene into the pGAD plasmid provided by O. Le Gall (INRA Bordeaux, France), and sent for the constructs for experiments in the O. LeGall laboratory:

a. We amplified the ZYMV HC-pro by PCR: using the infectious full length clone as DNA template, and the following forward primers GAAGCTATGTGAGCGAAGTTGACCACTCT (in italic Spe I site, and in bold the ATG start codon, in underlined is the sequence in P1 at nucleotide position 1052, i.e. 6 amino acid upstream the N-terminal of the HC-pro) and the reverse primer GACTCGAGCTAACCAACTCTGTAATGTTTCA (in italic XhoI site, in bold stop codon, and underlined is the sequence complementary to the 3' end of the HC-pro gene, nucleotide position 2437).

b. we cloned the PCR fragment in the pGEM-T plasmid (Promega).

c. we inserted the HC-pro gene inside the pGAD, using the SpeI and XhoI site, giving rise to pGAD-ZYHC.

"d. We sent this plasmid to the O. Le Gall laboratory in order to study the possible interaction in yeast with their LMV constructs, mostly with the pLexA-LMV HC-pro. But although LMV HC can interact with itself and with the PVY HC (Urcuqui-Inchima, et al, 1999. Virology, 258, 95-99), it did not react with ZYMV HC (O. Le Gall, personal communication).
2. *Expression of an extra HC*

The potyviral HC-Pro is known to have more than one function. In order to assess the ability of complementing functions by two distinct molecules of HC-Pro tailored within the same full length clone, we had the objective to make a construct where the HC-Pro, placed in the natural position (between p1 and p3) was made dysfunctional for aphid transmission, and to add an extra HC-Pro, placed between the Nla and the CP genes. As the extra HC-pro we have chosen that of ZYMV, as well as the one from MDMV. However, these attempts did not produce viable clones, therefore, this line of research has been discontinued.

Figure 2 and Table 2 summarize the features of the “ultimate construct” we aimed to engineer, followed by the more detailed methods and results.

**a. Aphid transmissible dysfunctional clone.**

Two mutations have been well described to affect the aphid transmission activity of the HC-pro: namely the mutation ELSC and PAK. We planned to introduce these two mutations within our full length clone. We introduced the ELSC mutation to a HC subclone by site directed mutagenesis according to Kunkel et al. 1987. We checked the clone by restriction mapping. Subsequently we introduced the PAK mutation into this subclone by swapping the gene fragment, from the *Bst*XI site to *Bam*HI as described by Huet et al. 1994. And finally we replaced the HC carrying these 2 mutations within the full length clone driven by the 35S promoter (Gal-On et al. 1996), using the unique *Age*I and *Bam*HI sites. We inoculated this FLC by bombardment as described by Gal-On et al., 1996.

Despite many attempts, this clone was not infectious. Thus we sequenced this double mutated helper, and found out that unexpected rearrangement occurred, which could not be detected by restriction mapping. In particular, frame shifts were present due to a deletion of 27 nucleotides before the ELSC mutation. Therefore we designed a primer to correct the frame shift, and using the original ELSC subclone as a template for our PCR, we engineered the “mutated ELSC” subclone. We sequenced it, confirming the ELSC mutation with no frameshift, and deletion of 9 amino acids upstream (Fig.3).
**Figure 2:** “Ultimate clone”

**TABLE 2: Characteristic of a potyvirus clone expressing an extra HC**

<table>
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<th>Gene</th>
<th>Features</th>
<th>Rationale</th>
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<td>HC pro natural position</td>
<td>Aphid transmission dysfunctional with ELSC and PAK mutation</td>
<td>- the HC should not interact with aphid (ELSC) nor with the CP (PAK)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- it should be active for its other functions</td>
</tr>
<tr>
<td>Extra HC-</td>
<td>His tag at its N-terminal</td>
<td>Selective Nickel purification with no or few “natural HC” not tagged</td>
</tr>
<tr>
<td></td>
<td>- N1a protease sequence upstream the N-terminal of the HC</td>
<td>Cleavage by the N1a protease, only 3 extra aa before the His tag, that should not interfere with the Nickel purification</td>
</tr>
<tr>
<td></td>
<td>- N1a protease sequence downstream the C-terminal of the HC</td>
<td>The HC-pro should self-cleave (having its C-terminus similar to “natural” HC)</td>
</tr>
<tr>
<td>CP-</td>
<td>N1a protease sequence downstream of the C-terminus of the HC</td>
<td>the CP is cleaved by the N1a protease, having a non modified N-terminal</td>
</tr>
<tr>
<td></td>
<td>DAG motif</td>
<td>No interaction with the extra HC</td>
</tr>
</tbody>
</table>
Figure 3: Comparison of the sequence of NAT (not mutated) and mutated ELSC HCs:

NAT: HNNEECGELAAIFCQALFPVVKLSCQTCREKLS
mELSC: HNNEECGELA....................VVELSCQTCREKLS

We introduced this mutation into the full length clone, as it has been reported that deletion (up to 107 aa) in the N-terminus of HC-pro does not affect virus infectivity (Dolja et al, 1997. Virology, 234, 243-252.). This clone was infectious, but interestingly the symptoms were milder than the original full length clone. We checked, by transmission from plant to plant, that this mutant was not aphid transmissible.

b. Engineering a full length clone with an extra-HC-pro gene.

We worked with the HC-pro gene ZYMV, but also of MDMV, which was cloned previously by RT PCR. In order to be able to purify the extra HC-pro we introduced a His tag (6 histidine residue at the N-terminus of the HC-pro). The HC-pro has been tagged with His (Kadouri et al. 1998). Similarly for the MDMV HC-pro, we designed a forward primer containing a sequence coding for the 6 His residue (underlined) followed by the 5’end of the HC-pro gene:

CACCATCACCATCACCATGCAGATCCACAAGCA. After amplification by PCR and cloning in the pGEM-T vector (Promega) these His tagged HCs were subsequently modified by PCR in order to be inserted into the full length clone between the NIb and CP gene. As illustrated in the following figure, the NIa protease is supposed to cleave at the N-terminus of the HC and of the CP (at the NIa cleavage site, in bold in the Fig. 5), and the HC should self cleave at its C-terminus.

Figure 4

\[
\begin{array}{ccc}
\text{D} & \text{T} & \text{V} & \text{M} & \text{L} & \text{Q} / \text{S} \\
\text{NIb} & \text{His tag} & \text{HC} & \text{CP}
\end{array}
\]

As described in the previous section, we had difficulties to have the infectious aphid transmission dysfunctional clone. Therefore as a preliminary step, we inserted the extra HC (of ZYMV and of
MDMV) in a HC not mutated full length clone. We checked by sequencing that the insertion was correct, and the sequence as expected in figure 2. We named these clones FLC-ZYHC and FLC-MDHC. Unfortunately, these clones were not infectious. At the moment, we do not have more data to explain this failure: if it is of biological or more technical reason.

**Objective 3. Investigate differences between ZYMV isolates that may account for differences in ability to overcome CP-mediated resistance and/or cause altered host range.**

This objective was modified slightly; the differences in host range observed in preliminary studies between the ZYMV-Ct and ZYMV-NAT isolates did not carry over to the infectious clone, ZYMV-NAA. However, ZYMV-Ct and ZYMV-NAA did differ reproducibly in the phenotype caused on the resistant cucumber genotype 'Dina-I'. The mechanism of resistance in 'Dina-I' was studied. Since physiological analyses implicated a block in long distance movement, a possible role of the CP-NT was tested. These experiments are described below.

Within the cucumber germplasm there are at least two sources of resistance to ZYMV, the inbred lines, 'TMG-1' and 'Dina-1' (Provvidenti, 1985, 1987; Abul Hayja and Al-Shawan, 1991). In both cases resistance is due to a single allele that is fully recessive when crossed to susceptible genotypes; however, genetic analyses indicate that the resistance allele in 'Dina-1', which allows for limited viral spread, is dominant to the resistance allele in 'TMG-1' (Kabelka et al. 1997). Each of these genotypes is also resistant to several other potyviruses including WMV, PRSV-W, ZYFV, and MWMV (Kabelka et al. 1997; Gilbert-Albertini et al. 1995; Provvidenti, 1985). Genetic analyses indicate that the multiple potyvirus resistance is either due to the zym allele itself, or to a tightly linked series of alleles (within 1 cM) (Grumet et al. 1999, Kabelka et al., 1997; Kabelka and Grumet, 1997). Thus the zym allele is of both practical value and scientific interest.

'Dina-1' plants exhibit an unusual phenotype which may provide insight into the mechanism of resistance conferred by the zym allele. In response to inoculation of the cotyledons with ZYMV, 'Dina-1' plants develop a distinct veinal chlorosis on the first (or occasionally second) leaf; symptom development is limited to just one leaf, subsequent growth is normal, vigorous, and symptom free. Which leaf developed the veinal chlorosis pattern was dependent on the age of the plant at the time of cotyledon inoculation (data in Ullah and Grumet), suggesting
that the virus was replicating in the cotyledons and moving to the dominant sink a the time. In fact, the observed veinal chlorosis pattern (Fig. 5), which is primarily in class 3 veins, the primary site of phloem unloading, very closely resembles the pattern observed by Roberts et al. (1997) for phloem unloading of the carboxyfluorescein molecule in sink leaves. Tissue blot analysis indicated that the veinal chlorosis pattern reflected distribution of virus within the leaf (Fig. 6A). At 5 days post inoculation (dpi), virus has entered the veins of the first leaf of susceptible cucumber, and at 10 dpi is distributed throughout the leaf. In 'Dina-1', the virus remained constrained to the veinal regions, even at 30 dpi. Although the explanation that replication occurs only in the cotyledons would explain both the nature of the block and timing of expression, we were able to eliminate this possibility based on cotyledon removal experiments (data in Ullah and Grumet).

Veinal chlorosis did not generally occur if leaves were inoculated instead of cotyledons. In the growth chamber, however, veinal chlorosis frequently was observed following inoculation of leaf 1 (13/16 plants showing veinal chlorosis; 81%). In each case, symptoms were limited to a single systemic leaf, generally leaf 3. The frequency of veinal chlorosis following inoculation of leaf 2 dropped to 19% (9/46). These observations showing a difference between cotyledons and leaves in the greenhouse, and leaf 1 and subsequent leaves in the growth chamber, suggest developmental control of expression of resistance.

Despite the lack of veinal chlorosis following leaf inoculation in the greenhouse, immunoblot analysis of the inoculated leaves of 'Dina-1' plants showed accumulation of virus along the veins (Fig. 6B) suggesting cell to cell movement from primary infection foci, but failure to load into the veins. Further evidence for a block in long distance movement comes from the use of chimeric ZYMV viruses utilizing the ZYMV-NAA isolate that does not cause the veinal chlorosis phenotype, but does exhibit limited systemic movement. When the NT of the CP of ZYMV-Ct, which differs from ZYMV-NAA at two amino acid positions, and is required for long distance movement (Dolja et al. 1994, 1995), was substituted into the infectious ZYMV-NAA construct, the chimeric virus caused veinal chlorosis on ca. 60% (25/43) of the cotyledon-inoculated plants (Fig. 5). Substitution of the core, which is involved in cell to cell movement (Dolja et al., 1994; Rojas et al. 1997), did not cause veinal chlorosis. These results suggest that
Figure 5. Veinal chlorosis on first systemic leaf of resistant 'Dina-I' plants following cotyledon inoculation with ZYMV-Ct and effect of substitutions in the amino terminal and core portions of the coat protein on the veinal chlorosis phenotype.
Isolate Veinal Chlorosis

Z-Cl  SGTQPTV S DAGATKK KDDEDKGNK DVTS GS G EKTVAAVT KDKD +
Z-NAA  A  -

pZYMV-NAA (Wild Type)

Veinal Chlorosis

pZCPFL/P

pCtNTFL/P

pCtCoCTFL/P
Figure 6. A. Tissue print analysis of first systemic leaves of susceptible 'Straight 8' (S) and resistant 'Dina-1' (D) plants following cotyledon inoculation with ZYMV. First leaves of the 'Dina-1' showed veinal chlorosis. B. Inoculated leaves of 'Straight 8' and 'Dina-1' plants.
the CP-NT plays a role in the veinal chlorosis response and further suggests that the block to infection in 'Dina-1' occurs at the level of systemic movement. Thus it is possible that the CP-NT interacts with the host resistance factor, either by interfering with long distance movement (e.g., phloem loading or unloading), and/or by recognition of the virus. The differences in developmental expression of the $zym^{Dina}$ locus, and interaction with the CP-NT may provide approaches to allow for cloning and further characterization of the $zym$ locus.

4. (New objective) Identification of host factors interacting with potyviral proteins.

We produced a cucumber cDNA library for yeast two hybrid analysis and screened for host proteins interacting with ZYMV CP and the RNA-dependent RNA polymerase (RdRp). We demonstrated, by both yeast two-hybrid interaction and in-vitro expression and binding, a novel interaction between the RdRp and poly-(A)-binding protein (PABP) (Wang et al., in press). This is the first report of interaction between a viral RdRp and host PABP, and is of particular interest as replication of the minus strand is initiated at the 3' poly A tail. Future studies will examine the biological significance of this interaction.
Literature Cited


Ullah Z, Grumet R. Localization of Zucchini yellow mosaic virus to the veinal regions and role of viral coat protein in veinal chlorosis conditioned by the zym potyvirus resistance locus in cucumber. Submitted.


Wang X, Ullah Z, Grumet R. Interaction between Zucchini yellow mosaic potyvirus RNA-dependent RNA polymerase and host poly (A) binding protein. Virology. Accepted for publication.

List of Publications and Abstracts Resulting from the BARD Grant

Publications (manuscripts for each of the following are attached to the report):


Ullah Z, Grumet R. Localization of Zucchini yellow mosaic virus to the veinal regions and role of viral coat protein in veinal chlorosis conditioned by the zym potyvirus resistance locus in cucumber. Submitted.


Theses


Abstracts


