Final Scientific Report

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Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)		1		1

Description Cooperation:

Both PIs closely collaborated by frequent communication through electronic mail and visits to each other's laboratories. Also, the Israeli PI spent a year in the American PI's laboratory for Sabbatical. Constant discussions were held, generating ideas and experiments throughout the project. A significant contribution to current research was exchange of genetic material, including constructs prepared in one laboratory and utilized by the other. These everyday contacts led to several joint publications driven by new discoveries, which were made exclusively due of this increased level

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of collaboration. Both groups freely shared ideas, technologies and materials and the collaboration between the two groups continues. This collaboration will continue with the new grant received, and it will most probably bring better understanding of the life cycle of TYLCV and give us better ways to combat it and the disease it causes.

Patent Summary (numbers)

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Inactivation of SGS3 as molecular basis for RNA silencing suppression by TYLCV V2

The Israeli isolate of *Tomato yellow leaf curl geminivirus* (TYLCV-Is) is a major tomato pathogen, causing extensive crop losses in Israel and in the south-eastern U.S. Yet, little is known about the molecular mechanisms of its interaction with tomato cells. One of the most interesting aspects of such interaction is how the invading virus counteracts the RNA silencing response of the plant. In the former BARD project, we have shown that TYLCV-Is V2 protein is an RNA silencing suppressor, and that this suppression is carried out via the interaction of V2 with the SGS3 component of the plant RNA silencing machinery. This reported project was meant to use our data as a foundation to elucidate the molecular mechanism by which V2 affects the SGS3 activity. While this research is likely to have an important impact on our understanding of basic biology of virus-plant interactions and suppression of plant immunity, it also will have practical implications, helping to conceive novel strategies for crop resistance to TYLCV-Is.

Our preliminary data in regard to V2 activities and our present knowledge of the SGS3 function suggest likely mechanisms for the inhibitory effect of V2 on SGS3. We have shown that V2 possess structural and functional hallmarks of an F-box protein, suggesting that it may target SGS3 for proteasomal degradation. SGS3 contains an RNA-binding domain and likely functions to protect the cleavage products of the primary transcript for subsequent conversion to double-stranded forms; thus, V2 may simply block the RNA binding activity of SGS3. V2 may also employ a combination of these mechanisms. These and other possibilities were tested in this reported project.

Research Activities

During the first funding year we have performed the following experiments and obtained the following data:

1. Cloning and characterization of the tomato ortholog of ASK1

The tomato (*Solanum lycopersicum*) ortholog of ASK1 was cloned from a cDNA library (a gift from S. Tanksley, Cornell University). We used the *Arabidopsis* ASK1-encoding sequences to design and synthesize pairs of degenerated primers which were then used in PCR to amplify and isolate coding sequences of putative tomato ASK1 orthologs. PCR products were cloned, sequenced and analyzed for their interactions with V2 using conventional molecular biology methods. One of the tomato ASK1 orthologs, shown to be biologically relevant, was designated SISK1 (*Solanum lycopersicum* SKP1). The cloned SISK1 will be sequenced and characterized by comparing its amino acid and nucleotide sequences to those of ASK1. Then, its functional interactive activity will be studied with TYLCV-Is V2 and *Arabidopsis* CUL1 in the yeast two-hybrid system, the efficiency of which, for this purpose, has already been established by us.

2. V2 interacts with a cysteine protease that acts as an anti-viral agent

To better understand the molecular pathway by which V2 suppresses RNA silencing, we used the yeast two-hybrid system to screen our tomato yeast two hybrid cDNA library with V2 as bait and search for interactors additional to SGS3. We identified a full-length cDNA clone that encodes a protein product which interacts with V2 in the two-hybrid assay as detected by cell growth in the absence of histidine (not shown). Amino acid sequence analysis of this V2 interactor identified it as a tomato cysteine protease (CYP1) belonging to the papain-like group of cysteine proteases which was first cloned from senescing tomato leaves by Dr. A. Lers at the Volcani Institute (GenBank accession number: AJ003137).

Fig. 1. Schematic diagram of tomato cysteine protease CYP1 and two sub-cloned derivatives. The full-length cDNA of CYP1 consists of 1398 bp coding for a 466 aa-long protein that contains a domain of 113 aa termed granulin at its C terminus. The enzymatically active site resides in the center, between aa 137 and 353.

The interaction of V2 with tomato CYP1 and its sub-cloned derivatives (400 and 800, see Fig. 1) was then demonstrated directly *in planta* by fluorescence resonance energy transfer (FRET). YFP-CYP1, CYP1-400 and CYP1-800 were transiently coexpressed with

V2-CFP in tobacco protoplasts, and FRET between these proteins was detected by acceptor photobleaching microscopy. Fig. 2 shows colocalization and FRET between YFP-CYP1 and its fragments and V2-CFP. These data demonstrate that TYLCV-Is V2 interacts with CYP1 within plant cells.

Fig. 2. FRET assay: protein-protein interaction was monitored by FRET microscopy of living *Nicotiana tabacum* protoplasts co-expressing V2-CFP and either YFP-full-length cysteine protease (CYP1), or its YFP-fragments (400 and 800, see Fig. 1). Representative acceptor photobleaching images show CFP (donor) and YFP (acceptor) channels before and after bleaching. After bleaching, CFP fluorescence increased and YFP fluorescence decreased in the bleached areas. All images are projections of several confocal sections.

NOTE: Agrobacterium, studied by the American co-PI is complementing our studies on degradation processes and provides us the tools and knowledge to further proceed with the research described.

3. Agrobacterium induces expression of a plant host F-box protein required for tumorigenicity

Agrobacterium elicits neoplastic growths on many plant species. Moreover, although plants are the natural hosts for Agrobacterium, this microorganism can also transform a wide range of other eukaryotic species, from fungi to human cells. This genetic transformation is achieved by transporting a single-stranded copy of the bacterial transferred DNA (T-DNA) from the tumor-inducing (Ti) plasmid into the plant cell nucleus followed by integration into the host genome. Within the host cell, T-DNA is coated by the bacterial virulence (Vir) protein VirE2, which packages it into a nucleoprotein complex (T-complex). In the T-complex, VirE2 associates with the host VIP1 protein that facilitates nuclear import of the T-complex and its subsequent targeting to and association with the host chromatin.

Before integration, VirF, a bacterial F-box protein exported into the host cell, helps to uncoat the T-DNA from VirE2 and VIP1 proteins. As a part of the Skp1-Cdc53-cullin-F-box (SCF) complex, VirF targets these proteins for proteolysis by directly recognizing VIP1 and promoting proteasomal degradation of both VIP1 and its associated VirE2. VirF itself is not essential for infection of some plant species, but its specific F-box protein function is critical

for the infection process, suggesting that this function may be encoded by the host plant. Furthermore, because SCF complexes play a role in plant defense response against pathogens, F-box proteins would represent attractive candidates for a hypothetical pathogen-induced response factor of which *Agrobacterium* may take advantage to enhance its infection. Here, we identified an *Agrobacterium*-induced *Arabidopsis* F-box protein, VBF, that interacts with the plant ASK1 component of the SCF^{VBF} complex. VBF also recognizes and binds VIP1 and its associated VirE2, forming ternary VBF-VIP1-VirE2 complexes. VBF then acts to destabilize both VIP1 and VirE2 via the SCF^{VBF} pathway. Suppression of VBF expression elevated intracellular amounts of the endogenous VIP1, but rendered *Arabidopsis* largely resistant to *Agrobacterium* tumorigenicity, indicating that, in the infection pathway involving both VIP1 and VBF, VBF functions downstream of VIP1. When expressed in *Agrobacterium* and exported into the plant cell, VBF functionally complemented tumorigenicity of a VirF(-) *Agrobacterium* strain. Thus, *Agrobacterium* subverts a host defense SCF^{VBF} pathway, induced in response to infection, to facilitate this infection.

4. VBF interacts with VIP1 and its expression is upregulated by *Agrobacterium*.

From a 694-member family of *Arabidopsis* F-box protein-encoding genes, five genes may be upregulated by bacterial or fungal infection. We tested each of them for the ability to interact with VIP1 *in planta* using a bimolecular fluorescence complementation (BiFC) assay. Although BiFC has inherent limitations, such as the use of fluorescent tags and relatively high levels of protein expression, it represents one of the best assays for protein-protein interactions and subcellular localization of the interacting proteins *in planta*. Only one tested F-box protein, encoded by *At1g56250*, recognized VIP1 and was designated VIP1-binding F-box (VBF) protein. Initially, the VBF-VIP1 interaction was tested both in *Arabidopsis* and in *Nicotiana benthamiana*, a choice plant for transient gene expression experiments. Fig. 3A, B shows that, in both species, cYFP-tagged VBF interacted with nYFP-VIP1 in the nuclei of living plant cells, resulting in reconstruction of the YFP fluorescence, which colocalized with the nuclear DsRed2 signal. This recognition of VIP1 was specific because it was not observed with cYFP-tagged F-box proteins encoded by the *Agrobacterium*-induced *At3g58890*, *At5g42350*, *At4g02760* (not shown), and *At1g31350* genes (FBX, Fig. 3A, B). Furthermore, we detected no interaction between cYFP-VBF and nYFP-VirE2 (Fig. 3B and not shown), and no differences were observed between the BiFC data in *Arabidopsis* and *N. benthamiana*, allowing us to utilize the latter plant for subsequent experiments.

Fig. 3. VBF interacts with VIP1 and is upregulated by Agrobacterium infection. (A) BiFC assay for the VBF-VIP1 interaction in microbombarded Arabidopsis leaves. (B) BiFC assay for the VBF-VIP1 interaction in agroinfiltrated *N. benthamiana* leaves. FBX is encoded by the At1g31350 gene. Free DsRed2 labels the cell cytoplasm and the nucleus and identifies the transformed cells. All images are projections of several confocal sections. (C) Yeast two-hybrid assay for the VBF-VIP1 interaction. The indicated dilutions of cell cultures were grown either in the absence (left panel) or in the presence of histidine (right panel). Lane 1, Gal4AD-VBF+LexA-VIP1; lane 2, Gal4AD-VBF+LexA-FBX; lane 3, Gal4AD+LexA-VIP1, lane 4, Gal4AD-VirE2+LexA-VIP1; lane 5, Gal4AD-VBF+LexA-lamin C; lane 6, Gal4AD-VBF+LexA-ASK1; lane 7, Gal4AD+LexA-ASK1. (D) RT-PCR analysis of the VBF gene expression following inoculation by Agrobacterium. Constitutively-expressed TUBULIN was used as internal control. (E) Q-PCR analysis of the VBF gene expression following inoculation by Agrobacterium. The data represent average values of three independent experiments with indicated standard deviations. (F) Expression of GUS reporter from the VBF promoter in Arabidopsis roots following inoculation by Agrobacterium. (-), (+) indicate mock-inoculated or Agrobacterium-inoculated plants, respectively.

The BiFC data were then confirmed by an independent assay using the yeast two-hybrid system, in which protein interaction is indicated by histidine prototrophy. Fig. 1C shows that VBF interacted with VIP1, and that this interaction was specific because it did not occur with FBX or with lamin C, a known non-specific activator. In positive control, VIP1 interacted with VirE2 (Fig. 3C). Furthermore, as an F-box protein, VBF interacted with ASK1, the Arabidopsis homolog of Skp1 (Fig. 3C, see also below). As expected, neither VIP1 nor ASK1 alone confer histidine prototrophy (Fig. 3C). Under the non-selective conditions, all combinations of the tested proteins resulted in the efficient cell growth (Fig. 3C).

We then demonstrated that *VBF* is induced by Agrobacterium. Using RT-PCR, substantially higher levels of *VBF* transcripts were detected in Agrobacterium-inoculated Arabidopsis tissues as compared to mock-inoculated plants (Fig. 3D). Equal efficiency of the RT-PCR reactions was controlled using *TUBULIN*-specific transcripts. Quantitative real time PCR (Q-PCR) revealed an average of 5-fold increase in *VBF* expression (Fig. 3E). Finally, the activation of the *VBF* promoter by Agrobacterium was demonstrated directly using Arabidopsis plants transgenic for the β -glucuronidase (GUS) reporter driven by the *VBF* regulatory sequences. Fig. 3F shows that bacterial inoculation of these Arabidopsis plants produced substantial GUS activity, detected as blue staining, in the root tissues, known as the

preferred substrate for *Agrobacterium*; no detectable reporter activity was observed in the same plant line in the absence of *Agrobacterium*. Interestingly, similar levels of *VBF* upregulation were observed in plants challenged with *E. coli* (not shown), suggesting that *VBF* represents a gene induced during general plant response to microbial challenge.

5. VBF forms ternary complexes with VIP1 and VirE2 in *planta*. We examined whether VBF, VIP1, and VirE2 can exist in the same complex. To this end, we developed a bridge-BiFC assay in which the BiFC signal is produced when two tagged molecules that do not interact with each other, e.g., VBF and VirE2, are coexpressed with a third molecule, e.g., VIP1, that interacts with each of them and bridges between them. Initially, we tagged VBF with cCFP and VirE2 with nVenus. Fig. 4A shows that coexpression of these proteins produced no BiFC signal. However, when they were expressed together with free VIP1, the reconstructed nVenus/cCFP fluorescence was observed, indicating formation of VBF-VIP1-VirE2 complexes which colocalized with the nuclear portion of the free DsRed2. As the endogenous levels of VIP1 are low, its coexpression is required to enhance detection of BiFC.

Fig. 4. Formation of ternary VIP1-VBF-ASK1 complexes in microbombarded *N. benthamiana* leaves. (A) Multi-color bridge-BiFC assay. nCerulean/cCFP and nVenus/cCFP signals are indicated in blue and green, respectively; merged image represents overlay of both BiFC signals and DsRed2. Free DsRed2 labels the cytoplasm and the nucleus and identifies the transformed cells. (B) Coprecipitation. Lane 1, GFP-VIP1+His-VBF+HA-ASK1; lane 2, GFP-VIP1+HA-ASK1. Input, the GFP-VIP1+His-VBF+HA-ASK1 sample processed without precipitation.

Next, we replaced the free VIP1 with VIP1 tagged with nCerulean and coexpressed it with nVenus-VirE2 and cCFP-VBF. In this multi-color BiFC, interactions of cCFP-tagged proteins with nVenus- or nCerulean-tagged proteins generate signals of different and specific colors. This approach allowed us to visualize simultaneously both the ternary VBF-VIP1-VirE2 complexes, detected as the nVenus/cCFP BiFC signal, and their constituent VIP1-VBF part, detected as the nCerulean/cCFP BiFC signal, accumulating in the nucleus of the same expressing cell (Fig. 4B).

The formation of the VBF-VIP1-VirE2 complexes was confirmed by coprecipitation. We coexpressed GFP-VIP1 and HA-VirE2 and precipitated them using *E. coli*-produced His-

VBF. Fig. 2C shows that the western blot analysis of the precipitates revealed the presence of all three proteins in the precipitate whereas these precipitates were not detected in the absence of VBF, and no interaction was observed between VBF and a GFP dimer expressed in plant tissues.

During the second funding year we have obtained the following data:

1. V2 is co-localized with CYP1, a papain-like cysteine protease (PLCP) in *planta*

We examined the sub-cellular localization patterns of YFP-tagged V2 coexpressed with CFP-tagged mCYP1 (the mature CYP1, after release of its granulin domain). Fig. 5 shows that, in tobacco protoplasts, mCYP1 was cytoplasmic, accumulating in distinct microbodies. This finding is important because it differentiates tomato CYP1 from its Arabidopsis homolog RD21, which is a vacuolar protein in both its mature and immature forms. V2 also exhibited a cytoplasmic localization (Fig. 5) and largely colocalized with mCYP1 (Fig. 5, light yellow). This colocalization of mCYP1 and V2 is consistent with their ability to interact with each another.

Fig. 5. Subcellular colocalization of mCYP1 with V2. mCYP1-CFP was coexpressed with V2-YFP in *N. tabacum* protoplasts. CFP signal (blue), YFP signal (yellow). Colocalization (light yellow).

2. V2 interaction with CYP1 may inhibit programmed cell death

Proteases play key roles in signaling cascades, regulating a diverse spectrum of cellular events, from defense response to development. Proteases are grouped into several major classes based on the nucleophile they employ for peptide bond cleavage. One of these groups is cysteine proteases, which use a catalytic Cys residue as a nucleophile during proteolysis. Plant genomes encode for approximately 140 cysteine proteases that belong to 15 families of 5 clans. One such group [family C1, clan CA,] is represented by PLCPs. Plants use PLCPs to protect themselves against pests and pathogens; specifically, many cysteine proteases play a role in PCD/HR during defense against pathogen attack.

Fig. 6. CYP1 and mCYP1 population. iCYP1 was expressed with a His tag at its N-terminus and detected with anti-His antibodies. Lanes 1 and 2, duplicates of extracts from cells expressing iCYP1 (36 kDa), which is converted to mCYP1 (28 kDa). Lane 3, recombinant mCYP1.

CYP1 (AJ003137.1) is a tomato homolog of Arabidopsis RD21, a PLCP which is up-regulated during drought, stress response and senescence. CYP1 is encoded by a single gene located on chromosome 14 and has several distinct domains that include a cys-protease domain, a proline-rich domain, and a C-terminal granulin domain. Similar to RD21, CYP1 accumulates in two active isoforms: the immature isoform, iCYP1, contains a granulin domain, which is cleaved off (not self-catalytically, but by another, yet unidentified protease) in the mature isoform, mCYP1. Indeed, Fig. 6 shows that, within cells, this protein exists as a mixture of the 36-kDa iCYP1 and a smaller, 28-kDa mCYP1; the latter exhibited the same electrophoretic mobility as a “synthetic” mCYP1 produced as a recombinant protein from the mutant *CYP1* gene that lacked the sequence coding for the granulin domain. Although the role of tomato CYP1 in pathogen resistance is still poorly characterized, its potato homolog is known to respond to and become induced during early stages of infection by *Phytophthora infestans*.

3. The F-box protein effector VirF is targeted for degradation through the host ubiquitin-proteasome pathway

The SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complex plays a pivotal role in various biological processes, including host-pathogen interactions. Many pathogens exploit the host SCF machinery to promote efficient infection by translocating pathogen-encoded F-box proteins into the host cell. How pathogens ensure sufficient amounts of the F-box effectors in the host cell despite the intrinsically unstable nature of F-box proteins, however, remains unclear. We found that the *Agrobacterium* F-box protein VirF, an important virulence factor, undergoes rapid degradation through the host proteasome pathway.

To shed light on the mechanism underlying the VirF degradation, we first tested the effect of the proteasome inhibitors, MG132 and lactacystin, on the stability of VirF. Pretreatment of *N. benthamiana* leaves with either proteasome inhibitor prolonged the half-life time of VirF (Fig. 7A, B), suggesting that VirF is degraded through the host ubiquitin-proteasome pathway. Consistent with this notion, exogenous addition of the K0 ubiquitin mutant, which lacks all lysine residues

required for ubiquitin polymerization, delayed the degradation of VirF (Fig. 7C, D). These results prompted us to investigate whether VirF undergoes ubiquitination *in vivo*. For this purpose, Myc-VirF and FLAG-tagged ubiquitin (FLAG-Ub) were coexpressed in *N. benthamiana* leaves. After pretreatment of the leaves with MG132 to enrich the polyubiquitinated VirF species, total protein was extracted and immunoprecipitation was carried out with anti-Myc antibody. Subsequent immunoblot analysis using anti-FLAG or anti-Myc antibody revealed high molecular weight protein species, an indication of polyubiquitination (Fig. 7E). The signal intensity of the high molecular weight species was reduced when the leaves were pretreated with DMSO instead of MG132 before harvesting (Fig. 7F). Together, these results indicate that VirF is polyubiquitinated and targeted for the proteasome-dependent degradation in the host cells.

Fig. 7. VirF is degraded through the host ubiquitin-proteasome pathway. **(A)** Effect of the proteasome inhibitor MG132 on VirF degradation. *N. benthamiana* leaves transiently expressing Myc-VirF were treated with either DMSO (-) or 10 μ M MG132 (+MG132) for 4 hours and analyzed by the cell-free degradation assay. **(B)** Quantification of Myc-VirF degradation described in (A). **(C)** VirF degradation is delayed by the K0 ubiquitin mutant. Recombinant wild-type (WT) or K0 ubiquitin (K0 Ub) (1 μ g/ μ l) was added to protein extracts, and the stability of Myc-VirF was analyzed by the cell-free degradation assay. **(D)** Quantification of Myc-VirF degradation described in (C). **(E)** Ubiquitination of VirF. Myc-VirF and FLAG-ubiquitin were transiently coexpressed in *N. benthamiana* leaves. After pretreatment of the leaves with 10 μ M MG132 for 4 hours, total proteins were immunoprecipitated (IP) with anti-Myc antibody, followed by immunoblotting (IB) with anti-FLAG (left) or anti-Myc antibody (right). Polyubiquitinated VirF species are indicated by bracket [(FLAG-Ub)_n-VirF]. Arrowhead indicates non-ubiquitinated VirF. **(F)** Omission of pretreatment of plants with MG132 reduced the amount of polyubiquitinated VirF. Asterisks indicate IgG heavy and light chains. Molecular mass markers (in kDa) are indicated on the left. Relative polyubiquitinated VirF amounts are shown below the blot. All quantified data are shown as mean of three experiments $\pm$ SEM.

Plants encode an unusually large number of F-box subunits, making the SCF complexes the largest class of E3 ubiquitin ligases for plants. Therefore, VirF degradation may be induced by a plant SCF complex. To explore this possibility, we generated a plant dominant-negative CULLIN1 (CUL1^{DN}). In a yeast two-hybrid assay, this C-terminal deletion mutant of *Arabidopsis* CULLIN1

(amino acid residues 1 to 420) interacted with ASK1, but not with RBX1, the catalytic core of the SCF complex (Fig. 8A). Similar dominant-negative forms of CUL1 have been used to identify substrates of mammalian SCF E3 ligases. We took advantage of this molecular tool to test potential involvement of a host SCF complex in VirF degradation. FLAG-tagged CUL1^{DN} (FLAG-CUL1^{DN}) and Myc-VirF were coexpressed in *N. benthamiana* leaves, and the protein extracts were analyzed by a cell-free degradation assay. VirF proteins were stabilized in the presence of FLAG-CUL1^{DN} (Fig. 8B, C), suggesting that a plant SCF complex is at least partly responsible for the degradation of VirF in the host cell. So, how does *Agrobacterium* protect its important virulence factor VirF from this host-mediated degradation?

Fig. 8. Mechanism of VirF degradation. **(A)** CUL1^{DN} interacts with ASK1, but not RBX1 in a yeast two-hybrid system. Yeast cells expressing the indicated LexA DNA-binding domain (DBD) fusions and GAL4 activation domain (AD) fusions were grown in the presence (+His) or absence of histidine (-His). TMV MP and TOP1 were used as negative controls. **(B)** Effect of CUL1^{DN} on VirF degradation. Myc-VirF was expressed alone (-) or coexpressed with FLAG-CUL1^{DN} (+CUL1^{DN}) in *N. benthamiana* leaves. The resulting protein extracts were incubated for the indicated time periods. The presence of CUL1^{DN} in the plant extract was confirmed using anti-FLAG antibody (right). **(C)** Quantification of Myc-VirF degradation described in panel B. In all quantifications, relative protein amounts were normalized to the RuBisCo loading control. All quantified data are shown as mean of three experiments $\pm$ SEM. **(D)** VirD5 is necessary for efficient infection of tomato by *Agrobacterium*. Tomato seedlings were inoculated with either wild-type *Agrobacterium* strain or a mutant strain that does not express VirD5 [VirD5 (-)]. Tumorigenicity was quantified as percentage of inoculation sites (50-70 per experiment) that developed a tumor and expressed as mean of three experiments $\pm$ SD. The value of the wild-type control was set at 100%. *, $P < 0.01$.

4. VirF is stabilized by VirD5

We tested the idea that another bacterial effector protects VirF. We focused on VirD5, the function of which within the host cell has been an enigma for many years. To this end, we next examined possible interactions between VirD5 and VirF as well as other and other known exported Vir proteins VirD2, VirE2, and VirE3, in plants using bimolecular fluorescence complementation (BiFC). Only VirF interacted with VirD5 (Fig. 9A). When coexpressed in leaf epidermal cells of *N. benthamiana*, the N-terminal half of yellow fluorescent protein fused to VirD5 (nYFP-VirD5) and the C-terminal half of YFP fused to VirF (cYFP-VirF) led to reconstitution of the YFP fluorescence (Fig. 9A), indicating interaction between the proteins. Similarly, the reciprocal combination (nYFP-VirF and cYFP-VirD5) also restored YFP fluorescence (Fig. 9A). The interacting complexes were observed exclusively in the cell nucleus and colocalized with the

nuclear portion of coexpressed CFP (Fig. 9B), potentially reflecting the nuclear nature of both VirD5 and VirF. No YFP signal was detected when cYFP-VirF was coexpressed with nYFP-VirE3, another *Agrobacterium* effector that is translocated into the host cell, or when nYFP-VirD5 was coexpressed with cYFP-VirE3 (Fig. 9A). In addition, nYFP-VirD5 did not interact with cYFP-VBF (VIP1-binding F-box protein) (not shown), a plant nuclear F-box protein that mimics the molecular function of VirF. We next confirmed the VirD5-VirF interaction in plant cells by an independent approach. CFP-VirD5 and Myc-tagged VirF (Myc-VirF) were transiently expressed in *N. benthamiana* leaves. Myc-VirF coimmunoprecipitated with CFP-VirD5, but not with free CFP (Fig. 9C). In an in vitro interaction assay, glutathione S-transferase (GST)-tagged VirD5 (GST-VirD5) specifically pulled down His-tagged VirF (His-VirF) (Fig. 9D). These observations demonstrate that VirD5 interacts with VirF. This direct binding of VirD5 to VirF may function to stabilize the latter, ensuring that a sufficient amount of the bacterial F-box protein effector is available in the host cell to promote efficient infection. Finally, we showed that VirD5 is necessary for efficient infection of tomato by *Agrobacterium* (Fig. 8D).

Fig. 9. VirD5 interacts with VirF in the nucleus. **(A)** In vivo BiFC assay for the VirD5-VirF interaction in *N. benthamiana* leaves. VirE3 was used as negative control. Bar = 20 μm. YFP signal is in green, and plastid autofluorescence is in red. **(B)** Nuclear localization of the VirD5-VirF complexes. The YFP signal is derived from the interacting nYFP-VirD5 and cYFP-VirF. Free CFP is a reference protein that visualizes the cell cytoplasm and the nucleus. YFP and CFP signals are in green and blue, respectively; merged image represents overlay of the BiFC and CFP signals. Bar = 20 μm. All images are single confocal sections. **(C)** Coimmunoprecipitation assay for the VirD5-VirF interaction. CFP-VirD5 and Myc-VirF expressed in *N. benthamiana* leaves were immunoprecipitated (IP) with anti-GFP antibody, followed by immunoblotting with anti-GFP or anti-Myc antibody. **(D)** GST pull-down assay for the VirD5-VirF interaction. His-VirF was detected by anti-His antibody (top). GST and GST-VirD5 proteins were detected by Coomassie blue staining (bottom). Each experiment was performed at least twice with similar results.

During the third funding year we completed the following activities:

1. CYP1 does not affect the RNA silencing suppression activity of V2

We examined the possibility that CYP1 may defend the plant against the invading virus by targeting V2, one of the viral proteins essential for efficient infection. The hallmark of the V2 function is its ability to suppress RNA silencing; therefore, we assayed this V2-induced suppression in the absence and presence of coexpressed CYP1. In this assay, a silencing initiator/reporter GFP is transiently expressed by agroinfiltration in *Nicotiana benthamiana* leaves alone or together with V2, which prolongs the duration of GFP expression in the infiltrated leaf area. Fig. 1 shows that GFP expression was largely silenced at 7 days post inoculation (panel A), and this silencing was not affected by coexpression of CYP1 (panel B). In contrast, coexpression of V2 suppressed silencing, elevating the levels of GFP expression (Fig. 10C). This suppression of GFP silencing by V2 was not affected by coexpression of CYP1 (Fig. 10D). These results indicate that CYP1 does not detectably affect the ability of V2 to suppress RNA silencing.

Fig. 10. *In planta* assay for RNA silencing suppression in the presence and absence of CYP1. GFP fluorescence was visualized at 7 dpi. **A.** GFP infiltration with infiltration solution alone. **B.** GFP infiltration along with CYP1. **C.** GFP infiltration along with V2. **D.** GFP infiltration along with CYP1 and V2 together. Left frames show whole leaf, right show infiltrated zone.

2. V2 does not affect maturation of CYP1

Our western blot analysis of the CYP1 population transiently expressed in tomato leaves (Fig. 11A) revealed that this protein exists in two isoforms with molecular masses of 38 kDa and 33 kDa (lane 1), which were hardly detected in the absence of transient expression (lane 2). Similar isoforms have been reported for the Arabidopsis CYP1 homolog RD21, where the 38-kDa isoform represented the full-length, immature protein and the 33-kDa isoform represented the mature protein with the cleaved-off granulin domain. Thus, the 38-kDa and 33-kDa bands most likely represent the immature and mature forms of CYP1, i.e., iCYP1 and mCYP1, respectively.

Next, we examined whether this CYP1 maturation is affected by the presence of V2. To this end, we transiently coexpressed CYP1 and V2 in tomato leaves, and detected the presence of iCYP1 and mCYP1 by immunoblotting. Fig. 11B shows that both CYP1 isoforms were detected, and their relative abundance was comparable to that observed in the absence of V2 (Fig. 11A). Thus, the V2 does not interfere with CYP1 maturation.

Fig. 11. Western blot analysis of CYP1 isoforms extracted from agroinfiltrated leaves in or without the presence of mature (33 Kd) CYP1. TYLCV V2 protein. **A.** CYP1 was transiently expressed in *N. benthamiana*. Leaf extracts were made three days after infiltration and proteins were separated and analyzed with anti-CYP1 antibodies. iCYP1, immature (38Kd) CYP1. mCYP1.

3. General characterization of plant proteolytic system: high turnover of F-box proteins via proteasomal degradation pathway

As an example of an F-box protein, we selected the VirF protein of *Agrobacterium* that functions within plant cells. F-box proteins are intrinsically unstable. To support this notion, we analyzed the stability of VirF by a cell-free degradation assay. Protein extracts were prepared from *N. benthamiana* plants transiently expressing Myc-VirF. In these plant extracts, VirF protein amounts rapidly declined over 30 min; in contrast, Myc-VirD2, an unrelated control protein, was stable over 30 min, indicating that VirF destabilization in plant extracts is specific (data not shown).

To shed light on the mechanism underlying the VirF degradation, we first tested the effect of the proteasome inhibitors MG132 and lactacystin on the stability of VirF. Pretreatment of *N. benthamiana* leaves with either proteasome inhibitor prolonged the half-life of VirF (Fig. 12A, B), suggesting that VirF is degraded through the host ubiquitin/proteasome pathway. Consistent with this notion, exogenous addition of the K0 ubiquitin mutant, which lacks all lysine residues required for ubiquitin polymerization, delayed the degradation of VirF (Fig. 12C, D). These results prompted us to investigate whether VirF undergoes ubiquitination *in vivo*. For this purpose, Myc-VirF and FLAG-tagged ubiquitin (FLAG-Ub) were coexpressed in *N. benthamiana* leaves. After pretreatment of the leaves with MG132 to enrich the polyubiquitinated VirF species, total protein was extracted and immunoprecipitation was carried out with anti-Myc antibody. Subsequent immunoblot analysis using anti-FLAG or anti-Myc antibody revealed high molecular weight protein species, an indication of polyubiquitination (Fig. 12E). The signal intensity of the high molecular weight species was reduced when the leaves were pretreated with DMSO instead of MG132 before harvesting (Fig. 12F). Together, these results indicate that VirF is polyubiquitinated and targeted for the proteasome-dependent degradation in the host cells.

Fig. 12. VirF is degraded through the host ubiquitin-proteasome pathway. **A.** Effect of the proteasome inhibitor MG132 on VirF degradation. *N. benthamiana* leaves transiently expressing Myc-VirF were treated with either DMSO (-) or 10 μ M MG132 (+MG132) for 4 hours and analyzed by the cell-free degradation assay. **B.** Quantification of Myc-VirF degradation described in (A). **C.** VirF degradation is delayed by the K0 ubiquitin mutant. Recombinant wild-type (WT) or K0 ubiquitin (K0 Ub) (1 μ g/ μ l) was added to protein extracts, and the stability of Myc-VirF was analyzed by the cell-free degradation assay. **D.** Quantification of Myc-VirF degradation described in (C). **E.** Ubiquitination of VirF. Myc-VirF and FLAG-ubiquitin were transiently coexpressed in *N. benthamiana* leaves. After pretreatment of the leaves with 10 μ M MG132 for 4 hours, total proteins were immunoprecipitated (IP) with anti-Myc antibody, followed by immunoblotting (IB) with anti-FLAG (left) or anti-Myc antibody (right). Polyubiquitinated VirF species are indicated by bracket [(FLAG-Ub)_n-VirF]. Arrowhead indicates non-ubiquitinated VirF. **F.** Omission of pretreatment of plants with MG132 reduced the amount of polyubiquitinated VirF. Asterisks indicate IgG heavy and light chains. Molecular mass markers (in kDa) are indicated on the left. Relative polyubiquitinated VirF amounts are shown below the blot. All quantified data are shown as mean of three experiments $\pm$ SEM.

Cooperation

A significant cooperation was implemented throughout the entire project period. The Israeli and the US PIs were in constant contact by mail and phone and several visits to each other labs were arranged. Also, a 14 month visit (Sabbatical) of the Israeli PI to the American lab was a great contribution to the project and a significant period for establishing the ways and approaches that will be taken in the continuing BARD project beginning December 2013.

The good cooperation between the two PIs also led to organization of a BARD workshop on the subject of their research ("Gene silencing and plant pathogens", 2010, Eilat, Israel).

Beside these, contacts and discussions, the collaborating labs exchange genetic material, i.e., expression constructs, this greatly facilitated experiments by both groups.

Close cooperation between the PIs and significant findings of this project were instrumental in generating another successful BARD-funded research by the same groups as well as establishment of an applied research program headed by Prof. Gafni aimed at preventing geminiviral infection *in planta* and funded by the Chief Scientist of the Ministry of Agriculture of Israel.

List of publications

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