INTERNATIONAL BARD WORKSHOP

Induction and suppression of RNA silencing: insights from plant viral infections

March 14-17, 2010, Dan Panorama, Eilat, Israel

R18 K67 Q107 K71 P37 W39 K60 R115 S124 S120 S124 R115 P37 K71 Q107 R18

Silencing

Suppression

Organizers:

Dr. Vitaly Citovsky, State University of New York, Stony Brook

Dr. Yedidya Gafni, ARO, Volcani Center, Israel

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BARD 2010 Workshop
Induction and suppression of RNA silencing: insights from plant viral infections

Program

All talks are held in the Kochav HaYam Hall

Meeting website: http://www.bard-isus.com/ws/silence/silencing.htm

SUNDAY, March 14, 2010

Arriving to Dan Panorama Hotel, Eilat

17:00 Get together, refreshments

18:00 Welcome by the Organizers

18:10 Dr. Edo Chalutz, Head of BARD Foundation
BARD operation and achievements

18:40 Cocktail reception

MONDAY, March 15, 2010

Morning Session (8:30 – 12:20)

Chairperson: Amit Gal-On

8:30 – 9:00
Herman Scholthof, Texas A&M University, USA
Tombusvirus*-plant interactions: RISCy business*

9:10 – 9:40
Henryk Hanokh Czosnek, Hebrew University, ISRAEL
Discovering networks sustaining resistance to* Tomato yellow leaf curl virus *(TYLCV) by silencing genes preferentially expressed in resistant vs susceptible tomato lines

9:50 – 10:20 Coffee Break

10:20 – 10:50
Steve Lommel, North Carolina State University, USA
The* Red clover necrotic mosaic virus *movement protein revealed as a second suppressor of RNA silencing using a new RNA silencing suppression reporter assay

11:00 – 11:30
Guido Sessa, Tel-Aviv University, ISRAEL
A virus-induced gene silencing approach to dissect MAP kinase cascades associated with plant immunity

11:40 – 12:10
Tzachi Arazi, ARO – The Volcani Center, ISRAEL
Effects of spatial and temporal silencing suppression on tomato development

12:20 – 13:00 Short lunch "in-house"

13:15 – 15:15 Underwater observatory

Afternoon Session (17:00 – 19:00)

Chairperson: Hervé Vaucharet

17:00 – 17:30
Alexander Vainstein, Hebrew University, ISRAEL
Reverse genetics of floral scent: application of* Tobacco rattle virus*-based gene silencing in Petunia

17:40 – 18:10
Misha Pooggin, University of Basel, SWITZERLAND
Massive production of 21, 22 and 24 nt small RNAs from the cauliflower mosaic pararetrovirus leader region does not restrict viral infection

18:20 – 18:50
Karen–Beth Scholthof, Texas A&M University, USA
***Growing invisible things: Francis O. Holmes and a biological assay for* Tobacco mosaic virus**

TUESDAY, March 16, 2010

Morning Session (9:00 – 12:10)

Chairperson: Valerian Dolja

9:00 – 9:30
Claude Fauquet, ILTAB - Danforth Center, USA
Intimate relationships between geminiviruses and their satellites and the host gene silencing system

9:40 – 10:20
Chaim Cedar, Hebrew University, ISRAEL
Silence of the genes
KEYNOTE LECTURE

10:30 – 11:00 Coffee Break

11:00 – 11:20
Hervé Vaucharet, INRA, FRANCE
Genetic dissection of small RNA directed regulation in Arabidopsis

11:30 – 12:00
Allison Mallory, INRA, FRANCE
Endogenous suppressors of small RNA-directed regulation

12:10 – 13:40 Lunch

Afternoon Session (13:40 – 17:20)

Chairperson: Henryk Hanokh Czosnek

13:40 – 14:10
Amit Gal-On, ARO –The Volcani Center, ISRAEL
Accumulation of viral-small RNA, micro-RNAs and their targets following ZYMV infection of cucurbits

14:20 – 14:50
Vicki Vance, University of South Carolina, USA
Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing

15:00 – 15:30 Coffee Break

15:30 – 16:00
Gideon Grafi, Ben-Gurion University, ISRAEL
Merging plant response to stress with dedifferentiation

16:10 – 16:40
Shou-Wei Ding, University of California, Riverside, USA
Viral secondary siRNAs in antiviral silencing

16:50 – 17:20
Vitaly Citovsky, State University of New York, Stony Brook, USA
Chromatin-modifying co-repressor complexes in plants

19:30 Gala dinner

WEDNESDAY, March 17, 2010

Morning Session (9:00 – 13:00)

Chairperson: Claude Fauquet

9:00 – 9:30
Ilan Sela, Hebrew University, ISRAEL
A T7-derived nuclear silencing and using the universal vector IL-60 for silencing

9:40 – 10:10
Savithramma Dinesh-Kumar, Yale University, USA
Emerging perspectives on the antiviral innate immune response

10:20 – 10:50 Coffee Break

10:50 – 11:20
Valerian Dolja, Oregon State University, USA
Myosin-dependent dynamics of the plant cell interior

11:30 – 12:00
Yuri Dorokhov, Moscow State University, RUSSIA
Methanol is a volatile signal of plant immunity and intra- and intercellular communication

12:10 – 12:30
Yedidya Gafni, ARO – The Volcani Center, ISRAEL
Suppression of gene silencing in* Tomato yellow leaf curl virus*-Israel

12:40 – 12:55 Concluding remarks by the organizers

13:00 – 14:30 Lunch

Departure

Tombusvirus-plant interactions: RISCy business

Herman B. Scholthof

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Tomato bushy stunt virus (TBSV) is the type species of the *Tombusvirus* genus in the family *Tombusviridae.* TBSV virions are non-enveloped icosahedral T=3 particles of ~33 nm in diameter that encapsidate a positive-sense single-stranded RNA of approximately 4.8 kb. The experimental host range of TBSV is broad with over 120 plant species in more than 20 different families reported to be susceptible at least for local infection. The differences between hosts with regards to requirements for infection have led to the development of TBSV as an attractive model system to obtain insights into host differential processes that govern RNA transport and function in plants, including RNA silencing.

The gene arrangement on the TBSV genome includes two 5'-proximal open reading frames (ORFs) that encode replication-associated proteins, an internal coat protein gene, and two 3'-proximal genes of which the longer ORF encodes a ~22 kDa cell-to-cell movement protein (MP). This P22-coding region entirely overlaps another ORF for *p19*, which encodes a highly expressed P19 that has multiple host-dependent biological activities for pathogenicity. P19 from several tombusviruses gained special attention in the past decade because it represents an effective suppressor of RNA silencing with an elegant structure that is designed to specifically bind Dicer-generated 21-nt short-interfering RNAs (siRNAs). We have found that the *in vivo* sequestration of siRNAs by P19 during infection of plants with TBSV is important for its ability to maintain viral load and to invade certain plant species, yet symptoms and infectivity are not strictly correlated to siRNA binding in all host.

A primary mechanistic role for siRNA sequestration by P19 during infection of plants with TBSV is to avoid that these Dicer-products can program an RNA-induced silencing complex (RISC); consequently, RNA silencing is not activated against TBSV. Several chromatography procedures were successfully implemented to provide direct biochemical evidence for a *bona fide* RNA silencing-associated RISC that contains TBSV-derived siRNAs and specifically cleaves TBSV RNA *in vitro* in a novel size-selective manner. The specificity of the *in vivo* RISC-associated antiviral response is evident from our observations that infections of *Nicotiana benthamiana* with *Tobacco rattle virus* (TRV) also yield an *in vitro* active RISC, but in this case against TRV rather then TBSV RNA. Investigations are underway to determine which Argonaute (Ago) proteins of *N. benthamiana* are required for the antiviral RISC activity. For this purpose individual Ago genes are inactivated followed by monitoring the ability of an otherwise silencing-sensitive TBSV mutant to systemically invade these plants.

Discovering networks sustaining resistance to *Tomato yellow leaf curl virus* (TYLCV) by silencing genes preferentially expressed in resistant vs susceptible tomato lines

Assaf Eybishtz, Dagan Edelbaum, Rena Gorovits, and Henryk Hanokh Czosnek

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TYLCV is a whitefly-transmitted geminivirus threatening tomato cultures worldwide. Breeding for resistance to TYLCV has consisted in introgressing resistant traits found in wild tomato species in the cultivated tomato. At least six different loci contributing significantly to resistance have been mapped on tomato chromosomes. However not a single TYLCV resistance gene has been identified so far. The number of loci discovered to date and their specific contribution to resistance led us to postulate that resistance to TYLCV is the result of gene networks that respond to biochemical triggers induced by virus inoculation. To uncover this network, we took advantage of two inbred tomato lines issued from the same breeding program that used *S. habrochaites* as a source of resistance: the TYLCV-susceptible line 906-4 (S) and the TYLCV-resistant line 902 (R).

Postulating that genes involved in the resistance network will be expressed at higher levels in R vs. S plants, we compared the transcriptome of R and S tomato lines, before and after inoculation. We assumed that if these genes are located at important nodes in the network, resistance will collapse upon silencing using the TRV VIGS system. This approach proved fertile. From the ca. 300,000 clones assayed, 69 transcripts were found to represent genes preferentially expressed in R plants. We focused our attention on membrane proteins that may be involved in signal transduction. Silencing of a *Permease I-like protein* gene in R plants followed by virus inoculation led to the appearance of disease symptoms similar to those of infected S plants, and in the early accumulation of large amounts of virus. Silencing of the hexose transporter *LeHT1* gene of R was accompanied by inhibition of growth, and enhanced virus accumulation and spread. In addition, a necrotic response characteristic of programmed cell death (DNA laddering, ROS, JNK expression) was observed along the stem and petioles of infected *LeHT1*-silenced R plants only, but not on infected not-silenced R plants. These results demonstrate that both *Permease* and *LeHT1* are essential for the expression of natural resistance against TYLCV and that their expression correlates with inhibition of virus replication and movement. The phenotype of silenced *LeHT1* constitutes the first evidence for a apoptotic response backing natural resistance to TYLCV in tomato, confirming that plant defense is organized in multiple layers. The role of other R-specific genes is presently under scrutiny.

References:

Eybishtz A, Peretz Y, Sade D, Akad F and Czosnek H (2009) Silencing of a single gene in tomato plants resistant to *Tomato yellow leaf curl virus* renders them susceptible to the virus. *Plant Mol. Biol.* **71**:157-171.

Eybishtz A, Peretz Y, Sade D, Gorovits R and Czosnek H (2009) *Tomato yellow leaf curl virus* (TYLCV) infection of a resistant tomato line with a silenced sucrose transporter gene *LeHT1* results in inhibition of growth, enhanced virus spread and necrosis. *Planta*, in press. DOI: 10.1007/s00425-009-1072-6.

The *Red clover necrotic mosaic virus* movement protein revealed as a second suppressor of RNA silencing using a new RNA silencing suppression reporter assay

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While a wide variety of assays have been used to identify viral suppressors of RNA silencing (VSRs), a large number of VSRs remain unidentified owing to the fact that VSRs act at various points in the silencing cascade with a varying range of effectiveness. It is not uncommon for these VSRs to have previously described roles in infection and some viruses, such as *Citrus tristeza virus*, encode multiple VSRs. *Turnip crinkle virus* (TCV) is a positive sense RNA virus in the *Tombusviridae* family. TCV lacking its native suppressor, the structural capsid protein (CP), can only move a few cells from the initial site of infection when inoculated onto *Nicotiana benthamiana* before being silenced by the host's RNA silencing defense machinery. This limited movement phenotype forms the basis for a recently devised assay where the delivery of a wide variety of VSRs *in trans* via *Agrobacterium tumefaciens* can restore TCV movement. VSRs tested include the native TCV CP, p19 from *Tomato bushy stunt virus*, the 2b protein of *Cucumber mosaic virus*, HC-Pro from *Tobacco etch virus* and B2 from the insect-infecting *Flock house virus* (FHV). The ability of FHV B2 to complement movement, as well as the inability of any of these suppressors to complement when the TCV p8/p9 movement proteins (MPs) are disrupted suggests that this complementation is due to silencing suppression and is not movement complementation per se. We believe this assay adds yet another tool for the identification of RNA silencing suppressors and in particular may be more sensitive and robust than many assays currently being used.

Red clover necrotic mosaic virus (RCNMV) is a plant infecting bipartite positive sense RNA virus in the *Tombusviridae* family. It has been previously reported that RCNMV suppresses RNA silencing through its viral replication complex, perhaps acting by sequestering endogenous proteins involved in the silencing cascade. Unfortunately, neither of the two replication proteins appears to have a suppression effect without viral replication. Here we present evidence identifying a second mode of RNA silencing suppression employed by RCNMV. We show that delivery of the RCNMV MP *in trans* by either *A. tumefaciens* or through transgenic *N. benthamiana* expressing RCNMV MP can complement movement of a suppressor-deficient TCV. Using this assay and previously constructed alanine scanning mutants of the RCNMV MP, we were able to uncouple its cell-to-cell movement activity from its VSR activity. RCNMV appears to be an excellent model to study VSRs as all identified so far are atypical and appear to act at different steps in the host silencing pathway. Further elucidation of the mode of action of these RCNMV VSRs may reveal additional steps in the silencing pathway.

A virus-induced gene silencing approach to dissect MAP kinase cascades associated with plant immunity

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Mitogen-activated protein (MAP) kinase cascades are fundamental components in signaling pathways of plant immunity activated by disease resistance (R) protein. Despite the large number of MAP kinase kinase kinase encoded in the plant genome, only very few of them have an assigned function in plant immunity. By a functional screen based on virus-induced gene silencing (VIGS) of candidate genes, we identified a novel MAP kinase kinase kinase (*MAPKKKe*) that is required for resistance of tomato plants to Gram-negative bacterial pathogens. Silencing of *SlMAPKKKe* compromised tomato resistance to *Pseudomonas syringae* and *Xanthomonas campestris* strains resulting in the appearance of disease symptoms and enhanced bacterial growth. In addition, silencing of *SlMAPKKKe* in *N. benthamiana* plants significantly inhibited the hypersensitive response mediated by different pairs of *R* genes and corresponding avirulence (*avr*) genes. Conversely, overexpression of *SlMAPKKKe* in *N. benthamiana* leaves caused the activation of pathogen-independent cell death that required SlMAPKKK*e* kinase activity. By epistatic experiments performed by overexpressing *SlMAPKKKe* and suppressing the expression of various MAPKK and MAPK genes, we found that MEK2, WIPK and SIPK are components of a MAP kinase cascade initiated by SlMAPKKKe. These results provide evidence that SlMAPKKKe is a key regulator of cell death associated with plant disease resistance and represents a convergence point of signaling pathways activated by different R/Avr recognition events.

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Effects of spatial and temporal silencing suppression on tomato development

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The identification of miRNAs and other classes of endogenous small RNAs in plants have revealed previously unappreciated posttranscriptional regulatory layers that play critical roles in plant development. Among agricultural crops, tomato has become an important model system, owing to its economic significance, as well as many favorable genetic and agricultural features. However, to date tomato silencing mutants were not identified and hence our knowledge regarding the involvement of this regulatory mechanism in tomato development is limited. To study the roles of silencing in tomato organogenesis, we took advantage of the fact that plant silencing pathways can be efficiently perturbed by viral silencing suppressors. Thus, we have used the pOp/LhG4 transactivation system to transgenically express P19, P0 and P1-HC-Pro silencing suppressors in tomato and inhibit silencing by different mechanisms during leaf, flower and fruit development. Upon activation, silencing inhibited tomato plants displayed various aberrant leaf, flower and fruit phenotypes, depending on the expressed suppressor and its expression profile. These phenotypes are currently analyzed to determine the specific developmental programs and target transcripts that are affected by silencing suppression. Results from current analysis will be discussed.

Reverse genetics of floral scent: application of *Tobacco rattle virus*-based gene silencing in Petunia

Alexander Vainstein, Ben Spitzer, Michal Moyal Ben Zvi, Elena Marhevka, Orit Edelbaum, Naveen Kumar and Moran Farhi

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Flower fragrance is a composite character determined by secondary metabolites of diverse biosynthetic origins. Together with other traits, such as flower color, it is used by plants to lure pollinators and seed dispersers, thereby ensuring plant survival. To enable the exploration of metabolic fluxes and the identification of genes that perform key functions in the production of phenylpropanoids and terpenoids—representing two major metabolic pathways—we employed tobacco rattle virus (TRV)-based gene silencing approach. The lack of studies applying viral vectors to scent research is probably due to the fact that the character is invisible, dynamic, and consists of numerous low-molecular-weight compounds that are detrimentally affected by background noise from nonsilenced cells/tissues. We used anthocyanin pathway suppression as a reporter allowing easily visible identification of silenced flowers/tissues.

Using this virus-induced gene silencing (VIGS) approach for large-scale identification of floral scent genes, we identified and then isolated and characterized a flower-specific MYB-like regulatory genes in petunia named EMISSION OF BENZENOIDS (EOBs). Silencing of EOBII resulted in a reduced level of phenylpropanoid volatile production by flowers. Target genes of EOBII that are involved in the production of phenylpropanoid volatiles and whose expression was reduced in flowers with suppressed expression of EOBII were identified. Based on gene-expression profiling, the levels of phenylpropanoid-pathway intermediates, and the coordinated wide-ranging effect of EOBII on the production of floral volatiles but not on that of anthocyanin, a central regulatory role for EOBII in the biosynthesis of phenylpropanoid volatiles was revealed.

Massive production of 21, 22 and 24 nt small RNAs from the cauliflower mosaic pararetrovirus leader region does not restrict viral infection

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Cauliflower mosaic pararetrovirus (CaMV) is transcribed by Pol II monodirectionally into pregenomic 35S RNA and subgenomic 19S RNA. 35S RNA is reverse transcribed and used as polycistronic mRNA with a 600 nt highly-structured leader. Here, we report that the leader region spawns a massive quantity of 21, 22 and 24 nt viral small RNAs (vsRNAs) in CaMV-infected *Arabidopsis*. These vsRNAs, corresponding to both strands of CaMV genomic DNA, accumulate at levels comparable to the entire complement of endogenous plant siRNAs and miRNAs. The leader region is also transcribed *solo* in both sense and antisense orientations, likely forming the double-stranded RNA precursors of vsRNAs. Genetic evidence shows that all four *Arabidopsis* Dicer-like (DCL) proteins mediate vsRNA biogenesis, whereas the RNA polymerases Pol IV, Pol V, RDR1, RDR2 and RDR6 are not required for this process. CaMV-infected *dcl1 dcl2 dcl3 dcl4* quadruple mutants that only possess a weakened DCL1 activity producing residual levels of 21 nt vsRNAs did not exhibit increased viral DNA accumulation, suggesting that vsRNAs do not restrict CaMV replication. We propose that the CaMV leader region expresses decoy RNAs diverting the silencing machinery from targeting 35S and 19S RNAs and shielding the viral promoters outside of the leader region.

GROWING INVISIBLE THINGS:
Francis O. Holmes and a Biological Assay for *Tobacco mosaic virus*

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In the early 20th century, viruses had yet to be defined in a material way. Instead, they were known better by what they were not—not bacteria, not culturable, not visible with a light microscope. As with the ill-defined 'gene' of genetics, viruses as microbes were substances whose essence had not been revealed. But clarity arrived in 1929 when Francis O. Holmes, a scientist at the Boyce Thompson Institute for Plant Research (Yonkers, NY) reported that *Tobacco mosaic virus* (TMV) could produce local necrotic lesions on tobacco plants and that these lesions were in proportion to dilutions of the inoculum. Holmes' method, the local lesion assay, provided the first evidence that viruses were discrete infectious particles, thus setting the stage for physicochemical studies of plant viruses. The technique was used to monitor the effects of heat and freezing treatments, various chemicals and enzymes, centrifugation, insect transmission, virus titer, serological methods, longevity in vitro, dilution, purifying and concentrating methods including filtration and centrifugation, ultraviolet rays and X-rays using tobacco mosaic and other viruses. In a field where there are few eponymous methods or diseases, Holmes' local lesion assay continues to be used as a primary tool for the study of plant viruses.

Prior to Holmes' assay, "working with viruses was like trying to find a black cat in a dark cellar, with no certain knowledge that the cat was there", quipped F. C. Bawden, an esteemed plant virologist from England. Holmes' local lesion at least allowed for the detection of the cat, although Bawden noted it took a few more years before it was possible to "report a good deal about the shape, size and constitution of the cat". TMV was a success because through Holmes it became visible and an object that became an actor across many boundaries and standardized the work of virology. It also made possible the development of other tools for the job of understanding the nature of the virus.

Intimate relationships between geminiviruses and their satellites and the host gene silencing system

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Geminiviruses are single stranded (ss)DNA viruses infecting hundreds of plants in the world and causing dramatic epidemics in tropical countries. Geminiviruses have a very small genome of about 3kb or 6kb, coding for 4 to 8 genes and are often accompanied with satellites. We have shown previously that geminiviruses are triggering and are subject to the post-transcriptional gene silencing (PTGS) mechanism of their hosts despite the fact that a priori they do not have dsRNA in their cycle. Four PTGS suppressors have so far been characterized from geminiviruses. We also demonstrated that the differential and combinatorial role of 2 of these suppressors were at the origin of synergism between two geminiviruses, ACMV and EACMV, causing a cassava pandemic in Africa. The alphasatellites repress symptoms while betasatellites enhance symptoms of geminiviruses in infected plants and both seems to be important for the epidemiology and evolution of geminiviruses. Some of the Beta-C1 protein from betasatellites have been shown to be PTGS suppressor, and thereby can play a role in diseases where a dual infection of a geminivirus and a betasatellite occurs. Despite the fact that alphasatellites have been shown to be symptom repressors, some have PTGS suppressor capacity as well, leading to the concept that PTGS suppressors may have a more subtile role than anticipated.

Small interfering (si)RNAs derived from controlled genes or viruses are the hallmark of gene silencing activity and deep sequencing of siRNAs allows a more precise description of the interaction between gene silencing and pathogenic molecules. The hosts are capable of controlling, to some extent, both geminiviruses and their satellites and because the response of the plant host is highly specific and stable for every infectious molecule, it is possible to establish characteristic siRNA maps for each of these molecules. Furthermore, the host upon virus infection is highly pro-active by directing synthesis of dsRNA towards all these molecules and/or their messengers, making dsRNA integrate part of a ssDNA geminvirus. All these findings are demonstrating the intimacy of the relationships between geminiviruses and their satellites, to invade their host, cause diseases and evolve. A better understanding of these intimate relationships should allow a better control of the diseases caused by these viruses and their satellites.

Silence of the genes

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DNA methylation represents a general mechanism for gene repression in animal cells. Unlike DNA sequence, however, the methylation pattern is not parentally inherited. Rather, this modification is erased in the early embryo and then re-established anew in each individual. This process takes place according to simple molecular rules that are based on underlying sequence information, ultimately generating a basal epigenetic pattern. This allows the organism to first set up open and closed chromatin structures that define a global gene-expression profile and subsequently make programmed alterations in a cell-type and stage-specific manner during development. Programmed changes in DNA methylation also appear to play a role in cancer. By understanding the molecular logic of this process it may be possible to decipher how this modification influences tumor phenotype.

Genetic dissection of small RNA directed regulation in Arabidopsis

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Small RNAs are involved in a variety of phenomena that are essential for genome stability, development and adaptive responses to biotic and abiotic stresses. Their mode of action also is diverse. They guide DNA elimination during the formation of the macronucleus in protists and heterochromatin assembly in fungi and plants. They target endogenous mRNAs for cleavage and translational repression in plants and animals. They also control the movement of transposable elements at the transcriptional and posttranscriptional level in plants and animals. Last but not least, they protect both plant and animal cells against virus infection through an RNA-based immune system. Here, I will present specificities, redundancies and antagonisms among the various plant small RNA pathways and discuss a new player in PTGS that we have recently uncovered

Endogenous suppressors of small RNA-directed regulation

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Gene expression is regulated by transcriptional and posttranscriptional pathways, which are critical for optimizing gene output and coordinating cellular programs. Through the action of ARGONAUTE (AGO) proteins, small RNA-directed gene silencing plays essential roles during development and virus defense. Although many of the essential components of small RNA biogenesis and action have been identified, the mechanisms that regulate and fine-tune these pathways are not well understood. Through forward and reverse genetic screens, we have uncovered endogenous posttranscriptional gene silencing (PTGS) suppressors that link small RNA generating pathways with general RNA quality control mechanisms. In addition, we show that ZLL/AGO10, one of 10 Arabidopsis AGO, is a negative regulator of AGO1, the major Arabidopsis AGO protein acting in PTGS. Our work points to a regulatory balance between RNA cleavage and translational repression and suggests that during silencing, translational repressive mechanisms can fine-tune small-RNA guided cleavage efficacy.

Accumulation of viral-small RNA, micro-RNAs and their targets following ZYMV infection of cucurbits

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Viral-small RNA (vsRNA) and micro-RNA (miRNA) biogenesis share components of the plant gene silencing mechanism. We are studying the effect of virus infection on small RNA (vsRNA and miRNA) accumulation using mild and aggressive strains of *Zucchini yellow mosaic potyvirus* (ZYMV) by deep sequencing, microarray analysis and qRT-PCR. Potyviruses encode a cytoplasmatic suppressor of gene silencing, HC-Pro, which acts as a suppressor of post transcriptional gene silencing by binding duplex-small RNA. Such binding impairs RNA silencing by sequestration of small RNA, resulting in the inhibition of siRNA-guided RISC assembly. We previously demonstrated that the conserved FRNK motif within the ZYMV HC-Pro is implicated in binding duplex-small RNA. In the mild ZYMV strain, where the FRNK motif is mutated to FINK, the affinity to duplex-small RNA is greatly reduced. We therefore tested whether the differences in duplex-small RNA affinity between aggressive and mild ZYMV strains caused correlated miRNA and miRNA-target accumulation.

It has been suggested that the HC-Pro induces developmental symptoms by interfering with plant miRNA metabolism, thereby affecting miRNA target gene expression, causing symptoms as such genes are often associated with plant growth and development. In potyvirus-infected plants some miRNA levels are elevated and the complementary strand miRNA* accumulates, while most miRNA* is rapidly degraded in healthy plants. We found that the cumulative level of most miRNAs was maintained following ZYMV infection, with a few exceptions such as miRNAs 160, 168, 171, 390 and 396. In contrast, most miRNA* highly accumulated in ZYMV-infected squash and melon plants.

Considerable differences in miRNA and miRNA* levels were found in a specific subset of miRNAs (159, 160, 166, 390) between plants infected with the severe and attenuated strains, identified by both microarray and deep sequencing. The effects of mild and aggressive ZYMV strains on miRNA target genes such as *AGO1, incurvata-4* and *Auxin Response Factors* will be discussed in the context of viral induction of developmental symptoms. Surprisingly, the HC-Pro^{FRNK} (severe) and the HC-Pro^{FINK} (mild) showed similar suppressor activity in the agroinfiltration assay, and similar vsRNA (21-22 nts) accumulation in ZYMV-infected plants. We identified vsRNA throughout the genome (about 1,100,000 counts), which accumulated preferentially in peaks - “hot spots”. The accumulation peaks in squash infected by the mild and aggressive strains were similar, and peaks did not vary between cucurbit hosts (melon, squash and cucumber). Interestingly, in each peak generally plus and minus vsRNAs in ZYMV-infected plants did not accumulate to the same level. The biological significance of ZYMV-sRNA “hot spots will be discussed.

Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing

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RNA silencing is a highly conserved pathway in the network of interconnected defense responses that are activated during viral infection. As a counter-defense, many plant viruses encode proteins that block silencing, often also interfering with endogenous small RNA pathways. However, the mechanism of action of viral suppressors is not well understood and the role of host factors in the process is just beginning to emerge. Here we report that the ethylene-inducible transcription factor RAV2 is required for suppression of RNA silencing by two unrelated plant viral proteins, potyvirus HC-Pro and carmovirus P38. Using a hairpin transgene silencing system, we find that both viral suppressors require RAV2 to block the activity of primary siRNAs, whereas suppression of transitive silencing is RAV2-independent. RAV2 is also required for many HC-Pro-mediated morphological anomalies in transgenic plants, but not for the associated defects in the microRNA pathway. Whole genome tiling microarray experiments demonstrate that expression of genes known to be required for silencing is unchanged in HC-Pro plants, whereas a striking number of genes involved in other biotic and abiotic stress responses are induced, many in a RAV2-dependent manner. Among the genes that require RAV2 for induction by HC-Pro are FRY1 and CML38, genes implicated as endogenous suppressors of silencing. These findings raise the intriguing possibility that HC-Pro-suppression of silencing is not caused by decreased expression of genes that are required for silencing, but instead, by induction of stress and defense responses, some components of which interfere with antiviral silencing. Furthermore, the observation that two unrelated viral suppressors require the activity of the same factor to block silencing suggests that RAV2 represents a control point that can be readily subverted by viruses to block antiviral silencing and points to the importance of host factors in viral counter-defense.

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Merging plant response to stress with dedifferentiation

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Dedifferentiation signifies the capacity of somatic cells to acquire stem cell-like properties. This process can be induced during normal development and as a response to various stimuli such as pathogen infection and wounding. Dedifferentiation also characterizes the transition of differentiated leaf cells into protoplasts (plant cells devoid of cell walls), a transition accompanied by widespread chromatin decondensation. Transcriptome profiling of dedifferentiating protoplast cells revealed striking similarities with senescing cells; both display a large increase in the expression of genes of specific transcription factor (TF) families including ANAC, WRKY, bZIP and C2H2. Further analysis showed that leaves induced to senesce by exposure to dark display characteristic features of dedifferentiating cells including chromatin decondensation, disruption of the nucleolus and condensation of rRNA genes. Considering that premature senescence can be induced by various stress conditions, our results suggest that the response of plant cells to certain stresses converges on cellular dedifferentiation whereby cells first acquire stem cell-like state prior to acquisition of a new cell fate (e.g., reentry into the cell cycle or death).

Viral secondary siRNAs in antiviral silencing

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In diverse eukaryotic organisms, Dicer-processed, virus-derived small interfering RNAs (siRNAs) direct antiviral immunity by RNA silencing or RNA interference (RNAi). Here we show that in addition to core dicing and slicing components of RNAi, the RNAi-mediated viral immunity in *Arabidopsis thaliana* requires host *RNA-directed RNA polymerase* (*RDR*) *1* or *RDR6* to produce viral secondary siRNAs following viral RNA replication-triggered biogenesis of primary siRNAs. We found that the two antiviral RDRs exhibited specificity in targeting the tripartite positive-strand RNA genome of Cucumber mosaic virus (CMV). RDR1 preferentially amplified the 5'-terminal siRNAs of each of the three viral genomic RNAs whereas an increased production of siRNAs targeting the 3' half of RNA3 detected in *rdr1* mutant plants appeared to be RDR6-dependent. However, siRNAs derived from a single-stranded 336-nucleotide satellite RNA of CMV were not amplified by either antiviral RDR, suggesting avoidance of the potent RDR-dependent silencing as a strategy for the molecular parasite of CMV to achieve preferential replication. Our work thus identifies a distinct mechanism for the amplification of immunity effectors, which together with the requirement for the biogenesis of endogenous siRNAs, may play a role in the emergence and/or expansion of eukaryotic RDRs.

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Chromatin-modifying repressor complexes in plants

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Covalent modifications of histones, such as acetylation, methylation and ubiquitination, are central for regulation of gene expression. Heterochromatic gene silencing, for example, is associated with hypoacetylation, methylation and demethylation, and deubiquitination of specific amino acid residues in histone molecules. Many of these changes can be effected by histone-modifying repressor complexes. Recently, we have described a transcriptional repressor complex from *Arabidopsis thaliana*, which inhibits expression of target genes, such as *FLC*, *LRP1*, and others, by generation of heterochromatic marks in the target chromatin. The complex core is represented by a histone lysine demethylase-like protein KDM1C, and a plant-specific histone methyltransferase, SUVR5. Interestingly, while KDM1-containing repressor complexes have been implicated in histone demethylation, methylation and deacetylation, whether or not they can also mediate histone deubiquitination remained unknown. We identified an *Arabidopsis* otubain-like deubiquitinase OLD1 which directly interacts with the *Arabidopsis* KDM1C *in planta*, and show that both KDM1C and OLD1 are involved in transcriptional repression of the same target genes via histone deubiquitination and demethylation. We also showed that OLD1 binds plant chromatin and has enzymatic histone debubiquitinase activity. Thus, we suggest that, during gene repression, lysine demethylases can directly interact and function in a protein complex with histone deubiquitinases.

A T7-derived nuclear silencing and using the universal vector IL-60 for silencing

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In an attempt to overexpress foreign genes in plants we tried to mimic the expression vector systems based on the activation of the T7 promoter by a T7 polymerase, as is commonly in use in bacteria. Surprisingly, we did not see any expression at all. However, nuclear run-on experiments indicated that the target gene had been transcribed, implicating post-transcriptional gene silencing. However, siRNA corresponding to the silenced gene could not be traced in the cytoplasm and a tobacco RdRP was not involved in the generation of silencing. On the other hand the silenced gene was hypermethylated and a nuclear RdRP and nuclear siRNAs were involved in this type of silencing.

In order to develop a functional genomic tool we attempted to introduce the T&-derived silencing system into the DNA virus: TYLCV. We soon found out that in the way of manipulation we have converted the virus into a plant plasmid (IL60) able to replicate in every hitherto-tested plants including wheat and woody trees. The IL60 has been further developed into a trans-acting platform. The viral intergenic region (IR) carries two opposing promoters. By placing any DNA sequence between these promoters we engendered dsRNA, hence silencing of the inserted sequence. The IL60 served as a mobilizing machinery for the construct to spread and replicate throughout the plant. Endogenous and exogenous genes were thus silenced.

Emerging perspectives on the antiviral innate immune response

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One branch of plant defense system employs specific plant-encoded immune receptors called resistance (R) proteins to recognize specific pathogen-encoded effectors. This defense system relies solely on germ-line encoded molecules, but remarkably, it provides resistance that rivals both the specificity and range of mammalian adaptive immunity.

Plant R proteins share common protein domains with animal innate immunity molecules. These include the TIR (Toll-Interleukin 1 homology region), NB-ARC (Nucleotide binding site typical of Apaf-1, R proteins and CED-4) or NOD (Nucleotide binding oligomerization domain), LRR (Leucine rich repeats) and serine/threonine kinase domains. Despite the structural similarities with animal innate immunity molecules, plant immune receptors recognize specific pathogen effectors while mammalian receptors recognize non-specific microbe associated molecular patterns (MAMPs).

Although several *R* genes have been cloned, the mechanisms of resistance remain elusive. Specifically, how do immune receptors recognize pathogen effector molecules, and how do host cells initiate, mediate, and terminate signaling in resistance? To this end, we are using N immune receptor that confers resistance to tobacco mosaic virus (TMV) as a model system. We will discuss recent advances on innate immune receptor mediated pathogen recognition mechanisms and activation of defense signaling.

Myosin-dependent dynamics of the plant cell interior

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A prominent feature of plant cells is the rapid, incessant movement of the organelles traditionally defined as cytoplasmic streaming and attributed to actomyosin motility. To determine myosin contributions into organelle transport, we have investigated functional profiles of the highly expressed Arabidopsis myosins XI-K, XI-1, XI-I, XI-2, and XI-B that is a closely related paralog of XI-2. The single, double, triple, and quadruple myosin gene knockout mutants were generated and characterized at the cellular and organismal levels. It was found that the myosins, XI-K, XI-1, and XI-2 played partially overlapping roles in the transport of Golgi stacks, peroxisomes, and mitochondria. Inactivation of these myosins in a triple knockout resulted in a virtual arrest of the organelle transport. Interestingly, these myosins are also critical for the ER streaming (collaboration with Hara-Nishimura lab, Kyoto University).

Our analysis of thousands of individual organelles revealed independent movement patterns for Golgi stacks, mitochondria, and peroxisomes indicating that the notion of coordinated cytoplasmic streaming is not generally applicable to higher plants. On the other hand, we proposed that the cytosol dragging caused by the massive movement of the ER strands along thick F-actin bundles provides a best proxy for the traditionally defined ‘cytoplasmic streaming’.

Strikingly, we have also demonstrated that the myosins shape the tracks they run on. Myosin inactivation in the multiple knockouts had a profound effect on the formation and architecture of F-actin bundles in root hairs and some epidermal cells. Because the ER network is shaped by actin cytoskeleton, the myosin-dependent changes in F-actin also affected ER distribution.

At the cellular level, progressive myosin elimination in the multiple knockouts resulted in a gradual reduction in cell sizes. Conspicuously, the extent of this reduction was dependent on the cell type. Whereas the size of the guard cells was essentially unchanged even in the quadruple knockouts, the root hairs were ~10-fold shorter in the multiple knockouts compared to those in the wild type. The sizes of other cell types exhibited intermediate size reduction phenotypes. Although the cell morphologies in the knockouts were largely unchanged, abnormal branching phenotypes of the root hairs and trichomes were observed in particular quadruple mutants.

Myosin elimination has also affected an overall plant stature and development with differential effects on distinct plant organs. In particular, plant heights and leaf rosette diameters were ~2.5-fold smaller in quadruple mutants that also exhibited a substantial delay in the onset of flowering. In contrast, myosin inactivation had only a moderate effect on the root growth.

In conclusion, we have identified class XI myosins that are responsible for organelle trafficking and dramatically redefined a textbook staple concept of cytoplasmic streaming. We have also revealed a novel function of myosin motors in shaping the actin cytoskeleton.

We have demonstrated that the actomyosin motility is required for the optimal diffuse growth of the most cell types, and is critical for the polarized growth of the root hairs. Collectively, interference with the myosin-dependent processes at the cellular level results in profound defects in plant growth and development indicating that the myosins are among the major determinants of plant size and productivity.

Methanol is a volatile signal of plant immunity and intra- and intercellular communication

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In higher plants pectin methylesterase (PME), a ubiquitous enzymatic component of the plant cell wall, is an efficient enhancer of virus- and transgene-induced gene silencing (VIGS and TIGS) via activation of siRNA and miRNA production. Furthermore, the tobacco PME interacts with the movement protein (MP) of *Tobacco mosaic virus* (TMV), suggesting that PME may be involved in cell-to-cell movement of plant viruses. We suggested that the PME-generated methanol earlier described by R. Boyle as the “sowrish spirit” (1661) may play role of a volatile signal molecule in plant resistance to pathogens and plant-plant communication. Our experiments showed that PME transgenic tobacco appearing dwarfing, increased methanol generation and enlarged cell size compromised nuclear transport of the GFP:NLS reporter accompanied by resistance to TMV and *Ralstonia solanacearum*, bacterial pathogen exploiting the export into host nucleus its protein effectors of type III secretion system.

Next, we developed an assay system, in which a PME transgenic plant or nontransgenic traumatized plant (as “emitters”) placed in a hermetically closed 20 l desiccator together with intact plant (“receiver”). The gas chromatography and mass spectrometry analysis showed in plant box significant accumulation of methanol vapor which was responsible for changes of protein nuclear transport and resistance to TMV and *R. solanacearum* in neighboring plant “receiver”.

To reveal genes responsible for described effects of “emitter” on “receiver” we used subtractive hybridization approach and isolated more than 300 genes up-regulated by methanol. “Receiver” immunity was accompanied mainly by activation of genes responsible for cell-to-cell communication [beta-1.3-glucanase and non-cell autonomous pathway protein (NCAPP)], and RNA silencing (Dice-like 1). In contrast to examined volatile organic compounds (VOCs) responsible for plant-plant communication such as methyl salicylate, methyl jasmonate, ethylene, cis-3-hexinol, methanol induced specifically activity of NCAPP gene.

We isolated NCAPP gene promoter using *N. benthamiana* chromosome walking and transgenic plants expressing GUS gene under control of NCAPP promoter were constructed. The comparative effects methanol and VOCs on GUS expression in transgenic plants will be discussed.

Results of co-agroinjection of NCAPP gene with TMV MP:GFP or GFP:NLS indicating on important role of methanol induced NCAPP in mechanism of plant cell intra- and intercellular communication will be presented as well.

Suppression of gene silencing in *Tomato yellow leaf curl virus*-Israel

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Tomato yellow leaf curl geminivirus (TYLCV) is a major tomato pathogen, causing extensive crop losses. We identified a TYLCV protein, V2, in an Israeli isolate of the virus, which acts as a suppressor of RNA silencing. Specifically, V2, but not other TYLCV proteins, inhibited RNA silencing of a reporter transgene GFP. The suppressor activity of V2 was confirmed under the original TYLCV promoter, and the promoter's activity was studied.

While hunting for the host-cell targets of V2 in a tomato cDNA library, we found that V2 interacts directly with the host protein SISGS3, which is the tomato homolog of the *Arabidopsis* SGS3 protein, known to be involved in the RNA-silencing pathway. The interaction of V2 with SISGS3 was demonstrated directly *in planta* by fluorescence resonance energy transfer (FRET) microscopy, which allows the detection of protein interactions within living cells as well as a determination of the subcellular localization of the interacting proteins.

More recently we found that V2, which contains an F-box motif, interacts with ASK1, a homolog of Skp1 that represents the SCF protein machinery for targeting of substrate proteins to proteasomal degradation. These observations indicate a possible mechanism for suppression of RNA-silencing activity that involves targeted degradation of SlSGS3 by V2 via the SCF pathway. An alternative mechanism by which V2 might interfere with the interaction between RDR6 and SGS3 also will be discussed.