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How to know when it is time for transmission ?

Martin Drucker, Aurélie Bak, Alexandre Martiniere, Stéphane Blanc

► **To cite this version:**

Martin Drucker, Aurélie Bak, Alexandre Martiniere, Stéphane Blanc. How to know when it is time for transmission?. EMBO Workshop: Green viruses, from gene to landscape, Sep 2013, Hyères-les-Palmiers, France. 129 p. hal-01837538

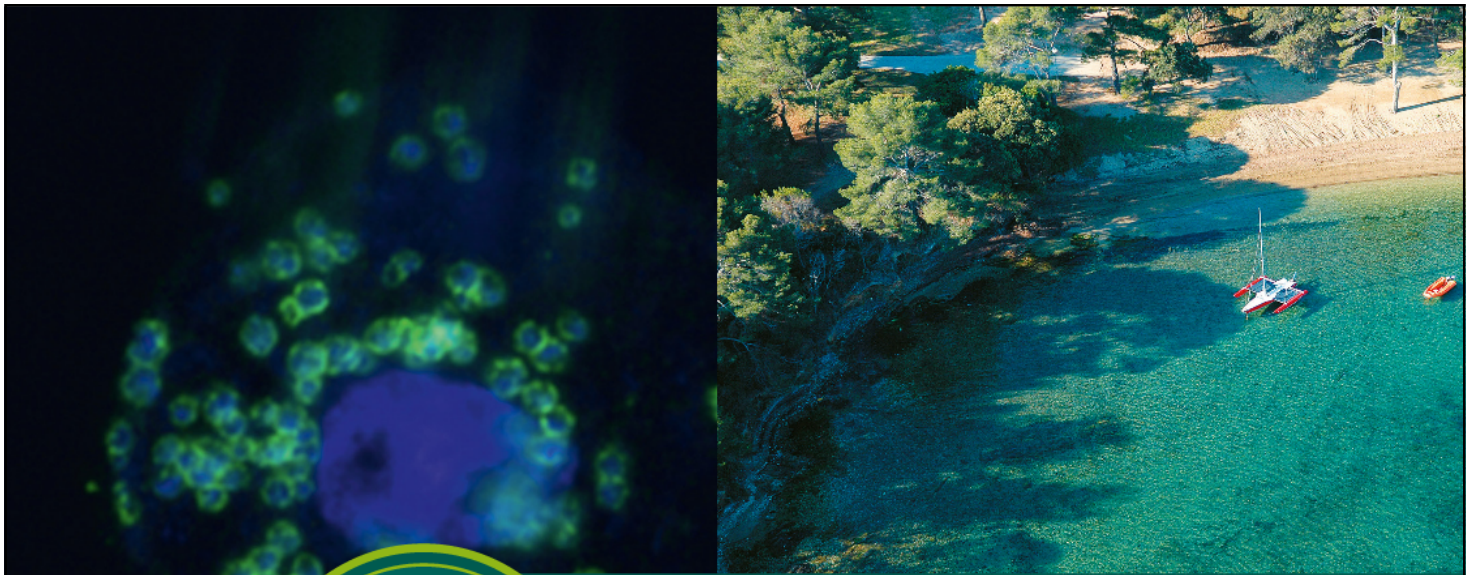
HAL Id: hal-01837538

<https://hal.science/hal-01837538v1>

Submitted on 3 Jun 2020

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**EMBO
Workshop**

The programme for this event
was reviewed and approved by the
EMBO Course Committee.

Green viruses, from gene to landscape

7 – 11 September 2013 | Hyères-les-Palmiers, France

Programme and Abstracts book



EMBO Workshop

Green viruses, from gene to landscape

7-11 September 2013 / Hyères-les-Plamiers, France

Organizers

Isabelle Jupin Institut Jacques Monod, CNRS - Univ. Paris Diderot, Paris, France

Stéphane Blanc INRA, UMR-BGPI, Montpellier, France

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Sponsors



Programme

Saturday, September 7, 2013

Pick-up at the Airport /Railway station

18.00 - 20.00 Registration and welcome get-together
20.00 - Dinner

Sunday, September 8, 2013

08.30 - 08.45 Introduction I. Jupin and S. Blanc

Session 1: Translation, replication and recombination

Chair persons: K. Mäkinen & E. Bejarano

08.45 - 09.15	P. Ahlquist	Protein, RNA and membrane interactions in viral RNA replication
09.15 - 09.45	P. Nagy	Exploring plant virus - host interactions based on yeast model host
09.45 - 10.00	J. Tilsner	Spread globally, replicate locally – a new model for PVX movement
10.00 - 10.15	E. Savenkov	Making sense of nuclear localization: a protein encoded by a cytoplasmically replicating RNA virus acts as a plant transcription factor
10.15 - 10.45	Coffee break	
10.45 - 11.15	A. Miller	Viral RNA parasites of the translation machinery
11.15 - 11.45	A. Simon	tRNA-shaped ribosome-binding 3' translational enhancers: one size does not fit all
11.45 - 12.15	L. Ryabova	Cauliflower mosaic virus (CaMV) TAV interacts with Arabidopsis target-of-rapamycin (TOR) to mediate activation of the TOR signaling pathway towards translation
12.30 - 14.00	Lunch	

Session 2: Intra- and inter-host movement

Chair persons: V. Brault & M. Heinlein

14.00 - 14.30	L. Torrance	Movement strategies of TGB encoding viruses; identification of host factors that interact with TGB1
14.30 - 15.00	C. Ritzenthaler	Grapevine fanleaf virus (GFLV): a virus on the move
15.00 - 15.15	P. den Hollander	Proteomic analysis of the movement tubule – plasma membrane complex of Cowpea mosaic virus
15.15 - 15.30	L. Stavelone	Tyrosine-based signals regulate trafficking of the Cauliflower mosaic virus movement protein in the endomembrane system
15.30 - 16.00	Coffee break	
16.00 - 16.30	J. Ng	Emergent properties of virus-insect vector interactions mediating the whitefly transmission of criniviruses and the implication for control
17.00 - 17.15	N. Bosque-Pérez	Plant viruses alter insect behavior to enhance their spread
17.15 - 17.30	M. Drucker	How to know when it is time for transmission?
17.30 - 18.00	Free time	
18.00 - 20.00	Poster session with drinks (posters corresponding to sessions 1 & 2)	
20.00 -	Dinner	

Monday, September 9, 2013

08.30 - 09.30 O. Voinnet **Keynote lecture:** RNA Biology and viruses

Session 3: RNA silencing

Chair persons: V. Ziegler-Graff & T. Hohn

09.30 - 10.00	J. Burgyan	Diverse functions of RNA silencing in plant-virus interactions
10.00 - 10.15	M. Pooggin	DNA virus interactions with the plant RNA silencing machinery
10.15 - 10.30	J. Milner	The N-terminal domain of Cauliflower mosaic virus protein P6 plays an essential role in suppressing RNA-silencing and innate immunity
10.30 - 11.00	Coffee break	
11.00 - 11.30	Shou-Wei Ding	Gene discovery in the antiviral silencing pathway by forward genetic screens
11.30 - 12.00	J. Carr	Synergistic effects of salicylic acid and host RDRs on virus-induced symptoms
12.00 - 12.15	N. Pumplin	Relocalization of RNA silencing components during virus infection
12.15 - 12.30	F. Jay	A chemical compound screen for in planta enhancers of RNA silencing: mechanisms and potential applications
12.30 - 14.00	Lunch	
14.00 - 18.00	Excursion: boat trip to "Porquerolles Island"	
18.00 - 20.00	Poster session with drinks (posters corresponding to sessions 3 & 4)	
20.00 -	Dinner	

Tuesday, September 10, 2013

08.30 - 09.00 P. Otten **Next generation sequencing lecture:** UHTS sequencing: a pipeline for de novo assembly and identification of viruses infecting plant samples from small RNA data

Session 4: Resistance mechanisms other than silencing

Chair persons: B. Sturbois & P. Palukaitis

09.00 - 09.30	M. Aranda	The amazing diversity of cap-independent translation elements in the 3'-UTR of melon necrotic spot virus (MNSV) and their role in determining the MNSV host range
09.30 - 10.00	C. Robaglia	The TOR pathway and its interactions with viruses
10.00 - 10.15	K. Ishibashi	Structural basis for the coevolution of Tomato mosaic virus and the resistance gene <i>Tm-1</i> at an interface of protein-protein interaction
10.15 - 10.30	I. Livieratos	Pepino mosaic virus TGBp1 interacts with and increases tomato Catalase 1 activity to enhance virus accumulation
10.30 - 11.00	Coffee break	
11.00 - 11.30	S. Dinesh-Kumar	Importance of subcellular compartment specific function of plant NLRs in innate immunity
11.30 - 12.00	F. García-Arenal	Tolerance: mechanisms and role in plant defence to virus infection
12.00 - 12.15	T. Shiraishi	JAX1-mediated resistance as the key to lectin-triggered immunity
12.15 - 12.30	R. Kormelink	The TYLCV resistance gene Ty-1 presents a new class of resistance genes and confers resistance in tomato through enhanced transcriptional gene silencing
12.30 - 14.00	Lunch	

Session 5: Epidemiology, population genetics and evolution

Chair persons: A. Whitfield & H. Pappu

14.00 - 14.30	B. Moury	Preserving the efficiency of resistance genes against viruses from the individual host plant to agricultural landscapes
14.30 - 15.00	D.P. Martin	The emergence and rise of Maize streak virus
15.00 - 15.15	A. Monjane	Synthetically-constructed Maize streak virus adapts to maize via recombination
15.15 - 15.30	R. Almeida	Within plant competition drives differential vector-transmission of pathogen variants
15.30 - 16.00	Coffee break	
16.00 - 16.30	S. Elena	Topography and ruggedness of Tobacco etch potyvirus adaptive fitness landscapes
16.30 - 17.00	D. Fargette	What can be predicted about Rice yellow mottle virus emergence and spread?
17.00 - 17.15	M.L. Caruana	How eBSV polymorphism could enlighten banana evolution?
17.15 - 17.30	A. Varsani	A top-down approach to sampling single stranded DNA viruses in ecosystems
17.30 - 18.00	Free time	
18.00 - 20.00	Poster session with drinks (posters corresponding to sessions 5 & 6)	
20.00-	Dining Cocktail on the beach-side (if wheather permits)	

Wednesday, September 11, 2013

08.30 - 09.00	J. Tait	EMBO Science and Policy lecture: New biotechnologies: innovation, governance and stakeholder interactions
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Session 6: Molecular ecology

Chair persons: C. Malmstrom & U. Melcher

09.00 - 09.30	M. Roossinck	Viruses as partners in plant ecology
09.30 - 10.00	T. Candresse	Contrasted patterns of phytoviral metagenomes in wild and agricultural environments.
10.00 - 10.15	P. Roumagnac	Geo-metagenomics: deciphering the spatial biodiversity of plant viruses associated with the unique fynbos ecosystem of Southern Africa and its neighbouring agro-ecosystem
10.15 - 10.30	C. Malmstrom	Assessing the effects of virus infection on the fitness of wild plants
10.30 - 11.00	Coffee break	
11.00 - 11.30	D. Lindell	Cyanophages are a selective force driving cyanobacterial genome diversification
11.30 - 12.00	C. Suttle	Exploding estimates of viral diversity in the sea has implications for phytoplankton
12.00 - 12.30	Concluding remarks	
12.30	Lunch bags	Bus departure to the Airport /Railway station

Oral presentations

Session 1: Translation, replication and recombination

Chair persons: K. Mäkinen & E. Bejarano

Protein, RNA and membrane interactions in viral RNA replication

Paul Ahlquist^a, Arturo Diaz^b, Bryan Sibert^a, Quansheng Yang^a, Robert Pugh^a, Masaki Nishikiori^a, Kenneth Ertel^a

^a Institute for Molecular Virology, University of Wisconsin - Madison, USA

^b Current address: Salk Institute, San Diego, USA

All positive-strand RNA viruses replicate their RNA in association with rearranged cellular membranes, such as single- or double-membrane vesicles. One of the most frequent types of membrane rearrangements associated with such viral RNA replication are balloon-like vesiculations of the target membrane away from the cytoplasm. Such vesicular compartments, also known as spherules, have long been documented in natural infections by a wide range of positive-strand RNA viruses of animals (alphaviruses, flaviviruses, etc.) and plants (varied Bromoviridae, tymoviruses, etc.) in and beyond the alphavirus-like superfamily.

Brome mosaic virus (BMV) RNA replication, e.g., occurs on endoplasmic reticulum (ER) membranes, in ~60 - 70 nm diameter, invaginated, vesicular compartments. Each such spherule compartment contains 1 to 2 genomic RNA replication intermediates, ~10 viral 2a polymerases, and hundreds of copies of viral RNA replication factor 1a. 1a contributes RNA capping and NTPase/helicase functions to RNA synthesis and directs replication complex assembly: 1a targets ER membranes, induces replication vesicles, and recruits viral genomic RNA replication templates and the viral 2a RNA-dependent RNA polymerase. Recent results are revealing that 1a also recruits host factors essential for vesicle formation and RNA replication, how 1a binds and rearranges membranes, and further facets of 1a-mediated RNA template recruitment. NMR and related approaches show that 1a primarily binds membranes through an amphipathic helix A that inserts into the outer face of ER membranes. Helix A interactions act as a molecular switch, shifting 1a between successive states required for RNA replication complex assembly to control the order and balance of assembly steps. After 1a binding to membranes, 1a self-interactions via both its N-proximal RNA capping and C-proximal helicase domains are required to form the spherule RNA replication vesicles. The 1a helicase domain also interacts with and recruits the viral 2a polymerase. Intriguingly, high levels of 2a polymerase modulate the ultrastructure of 1a-induced membrane rearrangements, presumably by competing with 1a-1a interactions through the helicase domain.

The nodavirus flock house virus (FHV) replicates its RNAs in similar, ~50 nm spherule compartments on mitochondrial outer membranes. Three dimensional imaging by electron microscope tomography and complementary results show that the interior of each FHV RNA replication vesicle remains connected to the cytoplasm through a narrow neck, and contains 1 to 2 RNA replication intermediates and ~120 copies of the sole FHV RNA replication factor, protein A. Protein A has an RNA polymerase domain, essential self interaction domains, and a mitochondrially-targeted transmembrane domain. In early steps separable from RNA synthesis, multiple protein A regions cooperate to recognize defined FHV genomic RNA signals and to recruit these RNAs to mitochondrial membranes. Despite similar replication complex organization, emerging results reveal significant differences in FHV and BMV replication compartment assembly. For BMV, replication protein 1a is the only viral factor required to induce replication vesicles. 1a also recognizes and recruits viral RNA templates into these vesicles in a reaction that requires 1a's NTPase/helicase activity, but viral RNA is not required to form RNA replication vesicles. For FHV, which lacks a viral helicase, RNA replication vesicles form only in the presence of functional viral RNA templates in a reaction that requires active viral RNA synthesis.

Exploring plant virus - host interactions based on yeast model host

Peter Nagy^a, Daniel Barajas^a, Nikolay Kovalev^a, Judit Pogany^a

^aUniversity of Kentucky

Virus-host interaction teaches us many surprising things about both cellular and viral processes. RNA viruses exploit host cells by subverting host proteins, remodeling subcellular membranes, co-opting and modulating protein and ribonucleoprotein complexes, and altering cellular metabolic pathways during infection. To facilitate RNA replication, RNA viruses interact with numerous host molecules via protein-protein, RNA-protein and protein-lipid interactions using molecular mimicry and other means. These interactions lead to the formation of “viral replication organelles”, which produce new viral RNA progeny in host cells. This presentation discusses our current understanding of the role of co-opted host proteins and membranes during tombusvirus replication using yeast as a model host. Genome-wide screens and global proteomics approaches based on yeast led to the identification of ~500 host proteins affecting tombusvirus, a model (+)RNA virus replication. Follow up mechanistic experiments demonstrated that tombusvirus replication depends on many co-opted host proteins, including RNA helicases, chaperones, translation factors. In addition, we find that proteins involved in membrane remodeling are recruited by tombusviruses to build viral replicase complexes. We will discuss novel roles for several host proteins in regulation of viral RNA replication and virus-host interactions.

Spread globally, replicate locally – a new model for PVX movement

Jens Tilsner^a, Olga Linnik^b, Karl J. Oparka^b

^a University of St Andrews/The James Hutton Institute

^b University of Edinburgh

RNA viruses replicate on the cytoplasmic surface of host cell endomembranes. Usually, the establishment of a viral replication complex (VRC) is accompanied by extensive membrane remodelling. These modifications can be mediated by viral RNA polymerases or small accessory transmembrane proteins. Modified membranes serve as scaffolds for oligomeric replication complexes, protective environment, and sites of progeny virus assembly.

Using live-cell and super-resolution imaging of viral proteins and RNA, as well as host cell organelles and cytoskeletal elements, we found that surprisingly, the VRC of the model virus, Potato virus X is organised by its three movement proteins – Triple Gene Block (TGB) 1-3 - which are completely dispensable for replication [1]. The small transmembrane proteins TGB2 and TGB3 induce the formation of densely reticulated endoplasmic reticulum (ER) membranes [2], which accumulate around aggregates of the RNA helicase TGB1. The resulting inclusion body, termed an 'X-body', harbours both non-encapsidated viral RNA and PVX replicase, whilst encapsidated virions accumulate around its perimeter, indicating that the X-body is a virus 'factory' [1].

TGB2 and TGB3 both target plasmodesmata (PD) and modify ER membranes at the openings of the pores in a similar manner to the X-body. These membranous PD 'caps' also harbour replicase and viral RNA, indicating that PVX replication is compartmentalised at PD for movement [3]. TGB1 is required for delivery of coat protein into PD, probably in a complex with RNA. We hypothesize that PVX may move by a co-replicative mechanism. Implications of this model for other plant RNA viruses, and the functions of movement proteins in general will be discussed.

[1] Tilsner J, Linnik O, Wright KM, Bell K, Roberts AG, Lacomme C, Santa Cruz S, Oparka KJ (2012) The TGB1 movement protein of potato virus X re-organises actin and endomembranes into the 'X-body', a viral replication factory. *Plant Physiol* 158, 1359-1370.

[2] Linnik O, Liesche J, Tilsner J, Oparka KJ (2013) Unravelling the structure of viral replication complexes at super-resolution. *Front Plant Biol* 4, 6.

[3] Tilsner J, Linnik O, Louveaux M, Roberts IM, Chapman SN, Oparka KJ (2013) Replication and trafficking of a plant virus are coupled at the entrances of plasmodesmata. *J Cell Biol*, in press.

Making sense of nuclear localization: a protein encoded by a cytoplasmically replicating RNA virus acts as a plant transcription factor

Nina Lukhovitskaya^a, Andrey Solovyev^b, Eugene Savenkov^a

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^b A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119992 Moscow, Russia

Discovery of the mechanisms whereby pathogens subvert the development and defence pathways of their hosts is one of the most fascinating areas of modern biology and medicine. Recent studies have uncovered numerous nucleus-localized proteins encoded by plant RNA viruses. Whereas for some of these viruses nuclear (or, more specifically nucleolar) passage of the proteins is needed for the virus movement within the plant or suppression of host defense, the nuclear function of these proteins is largely unknown. Similar to other RNA viruses, carlaviruses encode their own polymerase and seem to be independent of nuclear functions for replication in the cytoplasm. That is why it was unexpected to discover that a zinc-finger protein p12 encoded by chrysanthemum virus B (CVB, a carlavirus) localizes to the nucleus and act as a plant transcription factor (TF) to upregulate a shoot apical meristem-specific TF termed upp-L (up-regulated by p12). Cross-sections and microscopy revealed cell proliferation in CVB infected leaves as compared to mock inoculated plants. This aberrant tissue structure is referred as 'hyperplasia'. Hyperplasia is a plant virus disease manifestation, which has not been analyzed in detail and very little is known about the mechanism of hyperplasia induction by plant viruses. The ectopic expression of upp-L (induced by p12 expression) in the leaves results in cell proliferation and tissue growth phenotypically manifested as severe leaf malformation, the developmental alterations similar to the symptoms induced by CVB in its host species. The asymmetric changes in the expression pattern of key cell-cycle regulators (down-regulation of cell-division inhibiting E2FC TF and up-regulation of cell proliferation promoting E2FB) suggest that upp-L acts upstream of the core cell-cycle regulators conserved between animals and plants. The induction of upp-L requires translocation of the p12 protein into the nucleus and ZF-dependent specific interaction with the conserved regulatory region in the upp-L promoter. Our results establish the role of the p12 protein in modulation of host cell morphogenesis. We will also provide a model for p12-mediated interference with mitotic cell cycle and endocycle. The identification of the first virally encoded protein that acts as a TF and modulates growth and development of the host represents an important advance and will lead to characterization of a novel type of virus-host interactions via virus-encoded TFs that fine tune host gene expression. Therefore our studies identified a novel nuclear stage of in CVB infection involving modulation of host gene expression and plant development. Whereas it is well established that any RNA virus actively replicating in the cell causes changes in the transcriptome, our study expanded this view by showing that some positive-stranded RNA viruses can directly manipulate host transcription by encoding eukaryotic TFs.

Viral RNA parasites of the translation machinery

Jelena Kraft^a, Mariko Peterson^a, Krzysztof Treder^a, Eugenie Hebrard^b, Véronique Brault^c, Véronique Ziegler-Graff^d, **Allen Miller**^e

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All viruses are parasites of their host's translational machinery. Plant viruses have evolved numerous strategies to gain access to the translation system. These include either cap-independent translation elements (CITE) in the 3' untranslated regions, or possibly the genome-linked proteins (VPg) at the 5' end of viral RNAs that lack a 3' CITE. Both the CITEs and the VPgs bind a subunit of translation initiation factor eIF4F. The efficient Barley yellow dwarf virus (BYDV)-like CITE (BTE) folds into a discrete structure in the absence of protein. It contains three to six helices (depending on the genus) protruding from a central hub. Footprinting studies reveal that a highly conserved helix, and some essential bases around the central hub, are bound by the eIF4G subunit of eIF4F. The N-terminal half of eIF4G, including the PABP and eIF4E binding sites, is not necessary for binding to the BTE or for BTE-mediated translation. An RNA-binding sequence in eIF4G, immediately upstream of the MIF4G domain is essential for binding and function. In contrast, the Panicum mosaic virus (PMV)-like CITE from various Tombusviridae requires the cap-binding eIF4E subunit of eIF4F, which it binds more tightly than any other uncapped RNA. We found that some PTEs function in mammalian translation systems, presumably because of the high structural similarity between mammalian and plant eIF4E. This is the first plant CITE known to function in animals. The polero- and sobemoviruses may initiate translation via VPg-eIF4F interactions that are required for infection. Ploveroviruses appear to lack a CITE and we found that removal of the VPg from the RNA of the sobemovirus Rice yellow mottle virus drastically reduces translational efficiency *in vitro*. In summary, these results reveal just a few of the remarkable and diverse mechanisms by which plant viral RNAs parasitize the translational machinery to initiate a successful infection.

tRNA-shaped, ribosome-binding 3' translational enhancers: one size does not fit all

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Many plant RNA viruses contain elements in their 3'UTRs that enhance translation known as 3' cap-independent translational enhancers (3' CITE). One such 3' CITE is the eIF4E-binding PTE of umbravirus Pea enation mosaic virus (PEMV), which enhances translation at the 5' end in an unknown fashion. We have discovered that a three-way branched element just upstream of this PTE contains a T-shaped, tRNA-like 3-D structure, which binds to 40S, 60S and 80S ribosomes and also engages in a long-distance kissing-loop interaction with a coding sequence hairpin. This kissing-loop T-shaped structure (kl-TSS) differs from a previously identified TSS near the 3' end of Turnip crinkle virus (TCV) that occupies the P-site and only binds to 60S subunits and 80S ribosomes. The TCV TSS is also not associated with any long-distance RNA:RNA interactions, but rather may access the 5' end through subunit joining to form 80S ribosomes. The PEMV kl-TSS does not compete with the TCV TSS for 80S binding, and high-throughput SHAPE of 80S ribosomal RNAs disrupted by kl-TSS binding suggests docking in the vicinity of the A-site. Surprisingly, ribosome binding can occur at the same time as the long-distance RNA:RNA interaction, suggesting that the binding site within the ribosome must allow for the presence of upstream and downstream viral RNA "tails" as well the interacting 12-bp 5' region hairpin. The orientation of the kl-TSS with respect to a tRNA is currently unknown, but one orientation places the sequence involved in the RNA:RNA interaction in the "anticodon" position. Intriguingly, the kl-TSS and prokaryotic ribosome recycling factor (RRF) have similar size and shape, leading to the hypothesis that the kl-TSS is mimicking an RRF and occupying a similar A/P-site location and orientation in the ribosome. Addition of the kl-TSS in trans to a luciferase reporter construct suppressed translation, suggesting that the kl-TSS is required in cis to function. Furthermore, both ribosome-binding and RNA interaction activities of the kl-TSS contributed to translational inhibition in trans. The added kl-TSS was more detrimental for translation than the adjacent PTE, suggesting that the PTE may support the ribosome-binding function of the kl-TSS. These results suggest a model whereby post-termination ribosomes/ribosomal subunits bind to the PEMV kl-TSS and are delivered to the 5' end via the associated RNA:RNA interaction, which enhances the rate of translation re-initiation required to effectively compete with ongoing host protein synthesis.

We have also recently identified a putative TSS in the umbravirus Carrot mottle mimic virus (CMMV), located just upstream from a stable hairpin predicted to engage in a long-distance kissing-loop interaction with a 5' coding region hairpin. Interestingly, all umbravirus 3'CITEs, including the PEMV kl-TSS, the CMMV TSS and the BTE-type elements of TBTV and GRV, are equidistant from a highly conserved hairpin that may also contribute to translation. The discovery of three very different TSS, including two that adopt a common branched structure, suggest that such elements may be in more wide-spread use than currently thought.

Cauliflower mosaic virus (CaMV) TAV interacts with Arabidopsis target-of-rapamycin (TOR) to mediate activation of the TOR signaling pathway towards translation

Lyubov Ryabova^a, Mikhail Schepetilnikov^a

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The viral reinitiation factor TAV from Cauliflower mosaic virus (CaMV) activates the mechanism of reinitiation used to translate the viral polycistronic pregenomic 35S RNA. TAV interacts with the host translation machinery, promoting retention of reinitiation supporting factors—eukaryotic translation initiation factor 3, eIF3 and a host factor, reinitiation supporting protein (RISP)—in polysomes to ensure reinitiation at a further downstream ORF on the same mRNA. The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine-threonine kinase that positively controls translation initiation via phosphorylation of downstream targets in response to integrating signals from growth factors, hormones, energy sufficiency and nutrients. CaMV appears to be the first virus known to interact directly with plant TOR kinase, and exploits the TOR signalling pathway to overcome cellular barriers to polycistronic translation. TAV triggers TOR activation via an as yet unknown pathway but manifested by phosphorylation of S6K1 at T449. Accordingly, TOR-deficient plants fail to promote TAV-activated reinitiation after long ORF translation in plant protoplasts and are resistant to CaMV. Thus TAV binding to TOR is critical for both the reinitiation event and viral fitness. We have shown that RISP is a novel substrate of TOR signalling, and is phosphorylated at S267 in Arabidopsis. According to our data, phosphorylation of RISP is required for complex formation between RISP and TAV as well as to promote TAV-mediated reinitiation in protoplasts. Our current model is that TOR binds to polyribosomal complexes concomitantly with polysomal accumulation of TAV, eIF3 and RISP. Interestingly, TOR, when activated via TAV or in response to the phytohormone auxin, can be uploaded onto polysomes, strongly suggesting that TAV is not required for activated TOR binding to polysomes. Our recent finding suggests that TOR activation supports the phosphorylation status of RISP and another reinitiation factor eIF3h, phosphorylation of which is critical for translation of uORF-containing mRNAs. Our data support a model whereby TOR functions to ensure the phosphorylation state of factor(s) recruited to translating ribosomes to enable reinitiation at a downstream ORF.

Session 2: Intra- and inter-host movement

Chair persons: V. Brault & M. Heinlein

Movement strategies of TGB encoding viruses; identification of host factors that interact with TGB1

Lesley Torrance

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Viruses that encode triple gene block movement proteins (TGB) possess either monopartite genomes, filamentous particles and belong to the *Alpha-* or *Betaflexiviridae*, or are multipartite, have rod-shaped particles and mostly include members of the *Virgaviridae*. All three TGB and capsid proteins (CP) are required for movement of the filamentous viruses (exemplified by *Potato virus X*; *PVX*) whereas CP is not required for cell-to cell movement in the rod-shaped viruses, and in some it is dispensable for long distance movement as well. The functions of the TGB in cell-to-cell movement have been studied in detail over recent years but long distance movement is less well understood.

TGB1 encoded by *PVX* mainly comprises an SF1 helicase domain and has an RNA silencing suppressor function whereas the TGB1 of rod-shaped viruses such as *Barley stripe mosaic virus* or *Potato mop-top virus*; *PMTV*) comprise a C terminal SF1 helicase and a large domain, of disordered structure, at the N terminus (NTD). *PMTV* TGB1 is not known to be a silencing suppressor but the NTD domain plays a role in nucleolar localisation and this activity is associated with long distance movement.

Unlike the other rod-shaped viruses, *PMTV* encodes CP and TGB on separate species of RNA and we have used this property to separate the requirements for movement of virions versus virus RNA- protein complexes. This paper will discuss recent findings including the identification of host factors that interact with TGB1 and their possible roles in infectivity and long distance movement of virions.

Grapevine fanleaf virus (GFLV): a virus on the move

Christophe Ritzenthaler^a, Caroline Hemmer^a, Kamal Hleibieh^a, François Berthold^a, Léa Ackerer^a, Corinne Schmitt-Keichinger^a, Olivier Lemaire^b, Serge Muyldermans^c, Gérard Demangeat^b

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Grapevine fanleaf virus (GFLV) is a nepovirus responsible of a severe grapevine degeneration observed in vineyards worldwide. GFLV is specifically transmitted from grape to grape by the ectoparasitic nematode *Xiphinema index*. GFLV moves from cell-to-cell via plasmodesmata as entire virions through viral encoded tubules that result from the self-assembly of the movement protein (MP). Structurally, GFLV is an icosahedral virus of 30 nm of diameter with a pseudo T = 3 symmetry composed of 60 identical subunits. In recent studies we have been able to resolve the atomic structure of GFLV and identified surface-exposed structural motifs essential for GFLV transmission and movement.

Rods shaped viruses generally accommodate the production of fluorescently labeled viral particles consisting of fluorescent protein (FP)-coat protein (CP) fusions. In contrast, icosahedral viruses such as GFLV are incompatible with such an approach probably due to steric hindrance that totally prevents virion formation and movement. To circumvent these limitations, we produced single-domain antibody fragments also named Nanobodies that specifically recognize GFLV. When fused to FP and expressed in planta, these so-called chromobodies act as biosensors compatible with the spatio-temporal visualization of viral particles during the different steps of the virus life cycle.

Proteomic analysis of the movement tubule – plasma membrane complex of Cowpea mosaic virus.

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Tubule-guided intercellular transport of plant viruses like Cowpea mosaic virus (CPMV) requires structural modification of the plasmodesma (PD) pore. The native PD connects neighbouring cells and provides plasma membrane (PM) and ER continuity between these cells. Assembly of a viral movement tubule in PD requires removal of PD components such as the compressed ER (desmotubule) and actin spokes, but leaves the PM continuity between cells intact. PD modification and tubule assembly are the result of combined actions of host factors and tubule forming movement proteins (MP) in intimate association with the PM. It can be expected that host proteins required for tubule formation reside in this tubule-PM complex. In infected protoplasts, in the absence of PD, such tubule-PM complexes are formed on the cell surface, i.e. the movement tubule is tightly wrapped by the PM.

We have purified these tubule-PM complexes from protoplasts by immunoprecipitation and analysed the protein content by tandem mass-spectrometry, to identify any host proteins involved in this structure. Seven host proteins were found, including two types of molecular chaperones, i.e. heat shock proteins (HSP) 60, HSP70 and AAA-proteins (ATPases Associated with diverse cellular Activities).

Heat shock proteins 60 were found to be required for correct folding of viral protein complexes. Heat shock proteins 70 (homologues) were shown to interact directly with MPs and are required for Closterovirus PD targeting and intercellular transport. The AAA-proteins function through generation of mechanical force by ATP consumption, and were shown to be required for assembly of viral proteins complexes and intracellular translocation of MPs. Based on their functional association with viral infection, these three protein families were further investigated.

To test their potential role in CPMV infection, members of these protein families were targeted for silencing in *N.benthamiana* plants. Silenced plants were subsequently challenged with CPMV to study the progress of virus infection. Silencing of these host proteins resulted in a reduction of virus spread and virus titre in locally infected leaves. Involvement of these host proteins in tubule-guided plant virus movement is further investigated.

Tyrosine-based signals regulate trafficking of the Cauliflower mosaic virus movement protein in the endomembrane system

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Membrane trafficking is essential in eukaryotic cells as a delivery system for newly synthesized proteins from the endoplasmic reticulum (ER) to reach the plasma membrane (PM) or the tonoplast via intermediate endomembrane compartments. The selective transport of macromolecules between different compartments of the endomembrane system is mediated by small vesicles via adaptor complexes (AP-1–4). The μ subunit of AP complexes is devoted to cargo protein selection via a specific and well characterized interaction with a tyrosin-sorting signal (YXX Φ , where Φ is a bulky hydrophobic residue and X is any amino acid). In plant systems, ER and PM provide membrane continuity between cells through the connections made by plasmodesmata (PD). Virus movement, which requires passage of macromolecules through PD connections, is mediated by one or more virus-encoded MPs with the help of the host cytoskeleton and/or endomembranes. The MP encoded by Cauliflower mosaic virus (CaMV) forms tubules guiding encapsidated virus particle cell-cell transport via an indirect MP-virion interaction. CaMV MP does not require an intact cytoskeleton for both PM-targeting and tubule formation. However, how this and the other tubule-forming MPs targets the PM and form tubules remains to be elucidated. In this study, we examined the three tyrosine-sorting motifs in CaMV MP and showed that each of them interacts directly with subunit μ of an Arabidopsis AP-complex. Fluorophore-tagged MP is incorporated into vesicles labeled with the endocytic tracer FM4-64 and the pharmacological interference of tyrphostin A23, an inhibitor of endocytosis, confirmed that MP traffics in the endocytic pathway. Mutations in the three endocytosis domains revert in the viral context suggesting that vesicle carrier activity is essential for CaMV viability. In our system, mutation of the three tyr-signals blocks internalization of the protein from the plasma membrane to early endosomes, PD localization and tubule formation, but does not prevent targeting of newly synthesized MP to the plasma membrane, at least in the early stages of infection. The evidence we provide that upon mutation of all three YXX Φ -signals MP can efficiently interact with PDL1, a PD protein involved in assembly of CaMV MP into tubules, indicates that this MP mutant is competent to form tubules and its failure to accumulate in PD and to form tubules more probably depends on the inability to target PDs. This suggests that after targeting the plasma membrane (via as yet unknown strategy), MP might use a recycling pathway for specific targeting of PD via constitutive cycling between EE and PD. As constitutive cycling of plasma membrane proteins is blocked by BFA, recycling of MP is supported here by the demonstration that formation of foci (and tubules) is inhibited upon treatment of protoplasts with BFA. Tyrosine-signals can interact with several μ -adaptins and help the same protein to traffic in different compartments of the endomembrane system. We show that CaMV MP interacts with the Arabidopsis μ A-adaptin, which localizes at the trans-Golgi and binds a plant receptor mediating secretion of cargo proteins to the vacuole. Consistently, we show that MP co-localizes in vesicles with the Rab GTPase ARA7, which is resident in prevacuolar late endosomal compartments that mature from TGN to deliver proteins to the vacuole for degradation. TGN is a landing station for endocytosed PM proteins, which if do not enter the recycling pathway, become ubiquitinated and are targeted for degradation. It is conceivable that, to maintain homeostasis, a fraction of the CaMV MP molecules located in large excess at the PM would be not recycled further, and would become part of prevacuolar compartments directed for degradation. Taken together, our results show that CaMV MP traffics in the endocytic pathway and that virus viability depends on functional host endomembranes.

Emergent properties of virus-insect vector interactions mediating the whitefly transmission of criniviruses and the implication for control

James Ng

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Whiteflies of the *Bemisia tabaci* species complex are one of the world's most invasive insect pests that negatively impact plant health and productivity. The risks they pose are twofold. First, their feeding inflicts irreparable damages on plants. Second, their ability to vector devastating plant viruses increases the risk of virus disease epidemics. Members of the genus *Crinivirus* (family *Closteroviridae*) are emerging viruses with an obligate tropism for the phloem tissues of plants they infect, and have developed mechanisms that facilitate their acquisition during phloem feeding, and transmission, by specific whitefly vectors. This phenomenon is exemplified by the transmission of Lettuce infectious yellows virus (LIYV) and Lettuce chlorosis virus (LCV). LIYV is transmitted by biotype A (but not biotype B) of *B. tabaci*, while LCV is transmitted by both the A and B biotypes. The intricacies of these interactions reflect a highly coordinated virus-vector recognition system, which was intractable previously due to the lack of appropriate experimental tools. We have used a combination of strategies, including whitefly transmission, immunofluorescent localization, as well as biochemical and genetic approaches to explore the mechanisms underlying the interactions associated with the specific whitefly transmission of these viruses. Our studies of LIYV have demonstrated that virions are retained specifically in the cibarium or anterior-foregut of viruliferous vector *B. tabaci* biotype A but not in that of the non-vector *B. tabaci* biotype B. In addition, specific virion retention corresponds with successful virus transmission, and is mediated by the LIYV minor coat protein (CPm). Consistent with the hypothesis that specific retention of virions is a pre-requisite for whitefly vector mediated crinivirus transmission, on-going studies of LCV have shown that virions are localized in the cibarium or anterior-foregut of viruliferous vectors of both the A and B biotypes. To further advance our understanding of crinivirus transmission, we have been conducting studies to probe the structure and function of CPm, while also focusing on using the information for the more practical purpose of virus transmission control. To this end, we hypothesized that a competitive inhibitor that mimics the ligand (the CPm) involved in mediating virion retention can compete with the virion for the retention site in the whitefly vector to inhibit virus transmission. To test this hypothesis, we performed studies to: 1) obtain a quantitative estimate of the virion retention (fluorescence) signal phenotype (classified as strong, intermediate, or weak) observed in the foregut of viruliferous whiteflies fed on decreasing concentrations of virions, and 2) test *E. coli* expressed recombinant LIYV CPm (rCPm) or the recombinant major coat protein (rCP) for their ability to function as competitive inhibitors to interfere with LIYV transmission. Results from the first study indicated that the percentage of vector foreguts that contained fluorescent signals was reduced when vectors were fed decreasing concentrations of virions. Correspondingly, among vectors with fluorescently labeled foreguts, the percentage manifesting each of the three fluorescence phenotypes was reduced, suggesting a reduction in the amount of specifically retained virions. These results also allowed us to determine the minimum virion concentrations (10 and 1 ng/ μ l) for use in virus transmission inhibition. Results from the second study indicated that the sequential acquisition of neither rCPm and virions nor rCP and virions inhibited LIYV transmission, whereas the concurrent acquisition of rCPm and virions but not rCP and virions successfully inhibited virus transmission. Considered together, there is clear evidence in support of a common yet unique interactive framework in which the CPm of LIYV, LCV and, most likely, of other criniviruses, as well as yet to be identified vector components recognize one another to determine the success of virion retention and virus transmission by specific whitefly vectors.

Plant viruses alter insect behavior to enhance their spread

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Plant virus infection can alter the suitability of host plants for their insect vectors. Most reports indicate that virus-infected plants are superior hosts for vectors compared to noninfected plants with respect to vector growth rates, fecundity and longevity. Some insect vectors preferentially respond to virus-infected plants compared to noninfected ones, while others avoid infected plants that are inferior hosts. Thus, it appears vectors can exploit changes in host plant quality associated with viral infection. Enhanced vector performance and preference for virus-infected plants might also be advantageous for viruses by promoting their spread and possibly enhancing their fitness. Our research has focused on two of the most important members of the virus family Luteoviridae that infect wheat [Barley yellow dwarf virus (BYDV)], or potato [Potato leafroll virus (PLRV)], and their respective aphid vectors, the bird-cherry oat aphid, *Rhopalosiphum padi*, and the green peach aphid, *Myzus persicae*. Our work has demonstrated that virus infection alters the concentration and relative composition of volatile organic compounds in host plants. We also have shown that nonviruliferous aphids settle preferentially on virus-infected plants and that such responses are mediated by volatile organic compounds. Our recent studies have examined preferences of viruliferous and nonviruliferous aphids for virus-infected or sham-inoculated plants (plants noninfected with virus that are challenged with nonviruliferous aphids). Utilizing diverse bioassays we have confirmed that in both pathosystems nonviruliferous aphids prefer virus-infected plants, while viruliferous aphids prefer sham-inoculated plants. In the wheat- BYDV- *R. padi* pathosystem we have determined that behavioral changes in aphids after virus acquisition are mediated directly by the virus. On-going experiments are examining if such direct virus effects also occur in the potato - PLRV- *M. persicae* pathosystem. A shift in vector preference from infected to noninfected plants following virus acquisition could accelerate the rate of virus spread. Modeling exercises confirm this prediction. Recent research findings will be presented and potential impacts on vector ecology and virus disease epidemiology will be highlighted.

How to know when it is time for transmission?

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Cauliflower mosaic virus (CaMV) uses, like many plant viruses, aphids as the vector for transmission and the non-circulative (or non-persistent) mode as the transmission strategy. This means that virus particles are transported from one host to another attached to the interior of the vector's mouth-parts, which are in the case of aphids the needle-like stylets. For CaMV transmission, a transmissible complex must form that binds to a stylet receptor. This complex is composed of 2 components: the virus particle and the helper component P2, a viral protein that functions as a molecular linker and binds the virus particle to the stylet receptor. P2 and virus particles are largely separated in infected plant cells, with most virus particles localizing to cytoplasmic virus factories (VF) and all of P2 sequestered in a single cytoplasmic viral inclusion, the transmission body (TB). This setup forces the vector to reunite P2 and virus particles during feeding activity, i.e. to amass P2 from TB and virus from VF while the insects test-puncture parenchyma cells. How this is achieved, was an enigma because aphids take up only minute amounts of cytoplasm during the very short (seconds range) test probes and because the TB is hardly accessible to the vector. This is due to its remote location that decreases chances for accidental uptake, and to the fact that a TB as found in the cytoplasm is too big to pass through the stylets.

We solved this mystery recently (<http://elife.elifesciences.org/content/2/e00183>) and uncovered an intriguing phenomenon: the TB dissolves the very moment that aphids insert their stylets into the tissue, and all P2 associates with cortical microtubules throughout the cell. Simultaneously, virus particles are recruited onto microtubules. In this configuration, P2 and virus particles are brought close together and in addition they are distributed homogeneously over the entire cell. This enables efficient acquisition by the vector and hence transmission even after short probing. Remarkably, TB reform after vector departure to be ready for a second round of transmission.

These results open many issues that will be discussed. First of all, the mechanistic details behind the TB reaction are unknown and we have begun unravelling them. Second and more important from a general point of view, our findings suggest that plants perceive aphid activity from the moment of stylet insertion. An objective for the future is to identify the aphid elicitor and the involved pathways. Finally, the results prove that CaMV interferes with the very early plant-aphid interactions to organize transmission. This shows that CaMV is able to respond to cues from the outside world and another question of primordial importance is whether other viruses can do similar, either to achieve transmission or to accomplish other steps of the infection cycle.

Key note lecture

RNA biology and viruses

Olivier Voinnet

ETH Zürich

Session 3: RNA silencing

Chair persons: V. Ziegler-Graff & T. Hohn

Diverse functions of RNA silencing in plant-virus interactions

Jozsef Burgyan

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RNA silencing in plants exists as a defence mechanism against viruses. Plant viruses are inducers as well as target of RNA silencing based antiviral defence. The silencing of RNAs relies on host- or virus-derived 21-24 nucleotide-long small RNA molecules, which are the key mediators of RNA silencing-related pathways. Viral RNAs activate the antiviral RNA silencing generating viral (v) siRNAs, which guide RNA-induced silencing effector complex (RISC) to degrade viral genome. However, we have also observed that viruses are able to harness antiviral silencing for virus benefit controlling host gene expression by v-siRNAs. Plant viruses often cause severe symptoms and damage indicating an efficient counter defensive strategy against the antiviral silencing response. In fact most viruses evolved viral silencing suppressors (VSRs), which are able to suppress the host surveillance. These VSRs often compromise not only the antiviral response but they have significant impact on the endogenous silencing pathways. I will discuss the complexity of RNA silencing in plant-virus interaction and the emerging evidence that viruses use RNA silencing to manipulate host gene expression to modify the cellular environment for the benefit of invading viruses.

DNA virus interactions with the plant RNA silencing machinery

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RNA silencing directed by small RNAs (sRNAs) regulates gene expression and mediates defense against invasive nucleic acids such as transposons, transgenes and viruses. In plants and some animals, RNA-dependent RNA polymerase (RDR) generates precursors of secondary sRNAs that reinforce silencing. Most plant mRNAs silenced by miRNAs or primary siRNAs do not spawn secondary siRNAs, suggesting that they may have evolved to be poor templates for RDR. By contrast, silenced transgenes often produce RDR-dependent secondary siRNAs. We found that massive production of 21, 22 and 24 nt viral siRNAs in DNA geminivirus-infected *Arabidopsis* does not require the functional RDRs RDR1, RDR2, or RDR6. Deep sequencing analysis indicates that these primary viral siRNAs cover the entire virus genome, suggesting that dsRNA precursors of viral siRNAs are likely generated by RNA polymerase II-mediated bidirectional readthrough transcription on the circular viral DNA. Primary viral siRNAs engineered to target a GFP transgene trigger robust, RDR6-dependent production of secondary siRNAs, indicating that geminivirus infection does not suppress RDR6 activity. We conclude that DNA virus mRNAs, which can potentially be cleaved by primary viral siRNAs, are resistant to RDR-dependent amplification of secondary siRNAs. We speculate that, like most plant mRNAs, DNA virus mRNAs may have evolved to evade RDR activity. Our work is funded by Swiss National Science Foundation (31003A_127514) and European Cooperation in Science and Technology (COST, SER No. C09.0176).

The N-terminal domain of Cauliflower mosaic virus protein P6 plays an essential role in suppressing RNA-silencing and innate immunity

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Cauliflower mosaic virus (CaMV), a plant pararetrovirus, encodes a multifunctional polypeptide of 520 amino acids, P6, which is required for a variety of apparently disparate aspects of infection: It acts as a translational transactivator, facilitating the non-canonical translation of downstream open reading frames on the CaMV 35S RNA; it forms motile inclusion bodies that are involved in virus trafficking and it is the main genetic determinant of virus pathogenicity. We have shown that P6 acts as a VSR (suppressor of RNA-silencing), and more recently that it modulates innate immunity by down-regulating SA-responsive signal transduction

To identify the domains of P6 that are responsible for its activities as a pathogenicity effector we infected *Arabidopsis* with CaMV mutants with short in-frame deletions in P6. A mutant CaMV TAV-D3 with a deletion of amino acids 80-110 accumulated at similar titres to wild-type virus but infected plants were asymptomatic (in contrast to WT CaMV which induced severe stunting and leaf distortion). Also, unlike WT CaMV, infection with CaMV TAV-D3 failed to restore GFP expression in an *Arabidopsis* line containing an Amplicon-silenced GFP transgene. Inoculation with a replication-deficient CaMV mutant containing a deletion in the miniTAV domain induced a consistent albeit transient restoration of GFP expression in inoculated leaves, despite this mutant being unable to accumulate either locally or systemically. These results indicate that sequences that are required for VSR activity are present in the N-terminal domain of P6 and are distinct from TAV activity. Symptom expression in CaMV-infected plants may be linked to the ability of the virus to suppress RNA-silencing.

To test the effect of targeted mutations in P6 on its ability to suppress innate immunity, we transiently expressed P6 in *Nicotiana* using agroinfiltration and assayed its ability to suppress the hypersensitive response (HR) induced by an elicitor of cell death and to suppress the PAMP-dependent expression of SA-responsive marker genes. WT P6, when co-expressed in *N. tabacum* with TBSV P19 (which ordinarily elicits a rapid gene-for-gene response), suppressed and delayed the development of necrosis in infiltrated leaf patches. Expression of truncated forms of P6 containing only the N-terminal 112 or 200 amino acids delayed the onset of necrosis with a similar efficiency to WT P6, whereas deletions of all or parts of the N-terminal domain abolished the ability to do so. Agroinfiltration of *N. benthamiana* with constructs expressing WT P6 resulted in a 3- to 10-fold reduction in transcripts levels for PR-1a (a reliable marker for SA-responsive gene expression) compared to empty vector controls. A mutant variant of P6 with a deletion in the miniTAV domain also suppressed PR-1a transcript accumulation but one with a deletion in the N-terminal domain failed to do so. These results indicate that sequences required for the ability of P6 to suppress two markers of innate immunity (cell death and PR gene expression) also localize to the N-terminal domain. We have identified a motif within this domain that is highly conserved across all Caulimoviruses and are testing the effect of replacing individual amino acids.

Our results imply that the activity of P6 as a VSR and its activity as a suppressor of SA-signaling may be driven by a common mechanism that involves the N-terminal domain of the protein. If so, this would provide evidence for cross-talk between RNA-silencing and innate immunity in *Arabidopsis*.

Gene discovery in the antiviral silencing pathway by forward genetic screens

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In response to infection diverse eukaryotic organisms produce virus-derived siRNAs to guide specific virus clearance by the cellular RNA silencing pathway. We show that the antiviral silencing defense of *Arabidopsis thaliana* against cucumber mosaic virus (CMV) requires production and antiviral activities of viral secondary siRNAs in genetic redundant pathways involving DCL4/DCL2, RDR1/RDR6 and AGO1/AO2. We note that the two members of each gene family exhibit distinct antiviral activities, indicating that gene duplication is followed by functional diversification in *A. thaliana*. In addition to amplifying viral siRNAs, we found that RDR1 also mediates production of a novel class of endogenous siRNAs targeting more than a thousand of *A. thaliana* genes, suggesting a new mechanism for RDR1-dependent antiviral activity. Strong genetic redundancy in antiviral silencing may explain why forward genetic screens have not been as successful as in the characterization of the bacteria-induced resistance, for example. I shall describe a forward genetic screen developed in *A. thaliana* to specifically identify new components in antiviral silencing defense using a recently characterized CMV mutant, which uncouples RDR1- and RDR6-dependent antiviral silencing pathways. We identified three previously uncharacterized *Arabidopsis* genes in the defense pathway, which include Virus Resistance Defective 1 (VDE1) encoding a transmembrane protein induced by infection and required for the amplification of both the viral siRNAs and the RDR1-dependent endogenous siRNAs.

Synergistic effects of salicylic acid and host rdrs on virus-induced symptoms

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Salicylic acid (SA) triggers multiple anti-viral mechanisms, including mechanism(s) regulated by a mitochondrial enzyme, alternative oxidase (AOX), which may exert its effects via signaling mediated through changes in levels of reactive oxygen species. However, viruses differ in the extent to which AOX-regulated resistance affects them. Thus, both basal and SA-induced resistance to potato virus X (PVX) can be manipulated by altering the expression of wild-type or mutant versions of AOX in transgenic plants but for tobacco mosaic virus (TMV) the effects are more limited¹. Host RNA-dependent RNA polymerases (RDRs) contribute to antiviral silencing in plants. RDR6 is probably the most important RDR conditioning plant-virus interactions and in *Nicotiana benthamiana* limits the spread of PVX, and prevents infection of the meristem². RDR1 is inducible by SA but although it contributes to basal resistance to TMV, it is dispensable for SA-induced resistance to that virus¹. *N. benthamiana* is a natural *rdr1* mutant but RDR1 activity is restored in transgenic plants expressing *Medicago truncatula* RDR13. Utilizing MtRDR1-transgenic *N. benthamiana* and transgenic plants expressing diminished levels of RDR6 (line RDR6i) we have generated double transgenic lines with defects in AOX-regulated signaling and RDR1- or RDR6-mediated silencing to investigate the relative importance of these factors in SA-induced and basal resistance. We found that the spread of TMV into upper, non-inoculated, leaves is not affected, adversely or otherwise, by the expression of MtRDR1 in *N. benthamiana*, but that recovery from severe TMV disease is. It would appear that the presence of MtRDR1 inhibits severe symptom development by limiting spread of virus into the growing tips of infected plants. Thus, RDR1 may act in a similar fashion to RDR6. Additionally, MtRDR1 expression and SA treatment act additively to promote recovery from disease symptoms in MtRDR1-transgenic plants. Results with RDR6i/Aox double transgenic plants indicate that both systems (AOX-regulated resistance and RDR6-dependent silencing) are equally important in maintaining basal resistance to PVX but that only AOX-regulated signaling plays a role in SA-induced resistance in *N. benthamiana*.

1. Lee et al. 2011 BMC Plant Biol. 11:41 <http://www.biomedcentral.com/1471-2229/11/41>

2. Schwach et al. 2005 Plant Physiol. 138:1842-1852.

3. Yang et al. 2004 PNAS 101:6297-6302.

Relocalization of RNA silencing components during virus infection

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Genetic experiments have established a requirement for the RNA silencing pathway in resistance against viruses, however the molecular mechanism of anti-viral silencing is unknown. In Arabidopsis, anti-viral silencing is mediated by virus-derived small interfering RNA (vsiRNA) processed primarily by Dcl2 and Dcl4 from double-stranded RNA formed during viral replication, from RNA foldback structures, or following amplification by an RNA-dependent RNA Polymerase such as Rdr6. Selected vsiRNAs are then loaded into Ago1 and Ago2 effector proteins, which silence viruses with complementary RNA sequences. Paradoxically, RNA viruses replicate in the cytoplasm, and Dcl proteins are known to localize in the nucleus; The location of vsiRNA processing is unknown, but requires the relocalization of one of these molecules in our models. Additionally, it is not known whether Ago proteins target viral RNA during replication or transcription; silencing viruses during replication requires a mechanism that would recruit Ago to the membrane replication complexes, which vary greatly in localization between viruses. To address these outstanding questions, we created a panel of transgenic lines expressing native promoter, fluorescent protein fusions to the major silencing components, which complement their respective mutant backgrounds (panel includes DCL2/4, AGO1/2, and RDR6). Infection of these plants with different viruses, including Turnip Crinkle Virus and GFP-expressing Turnip Mosaic Virus, combined with confocal imaging of whole tissues has uncovered relocalization of silencing components during infection, and an elegant mechanism that enables dicing of viral RNA.

A chemical compound screen for in planta enhancers of RNA silencing: mechanisms and potential applications

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Plant viruses are only second to fungal pathogens in the extent of damages they incur to crops worldwide, causing critical yield losses both in developed and emerging countries. In facing the ever-increasing food demand, viral disease management continues to rely mostly upon prevention of infection and spread as well as vector control. Efficient plant antiviral treatments are yet to be discovered while transgenic approaches are confronted to public reluctance and substantial design/implementation costs.

In RNA silencing, double-stranded RNA produced during virus replication is recognized by the plant-encoded RNaseIII-like enzymes, Dicer-like 4 (DCL4) and DCL2, and processed into 21-22-nt small interfering (si)RNAs. These incorporate into, and guide Argonaute (AGO) family effector proteins to silence complementary viral RNA at the primary infection sites but also in distant plant tissues owing to the systemic action of siRNAs. Therefore, RNA silencing holds unique features of a true innate immune system because its specificity is not programmed by the hosts but, rather, by generic, structural and nucleotide sequence characteristics of invading viral genomes: in effect, this defense can be interfaced to any potential invader. Further attesting of its importance in antiviral control, plants impaired in production or action of siRNAs are hyper-susceptible to a variety of viruses, and conversely, many viruses have evolved viral suppressors of RNA silencing (VSRs) underlying their virulence and capacity to thrive in their hosts.

We reasoned that priming of RNA silencing by exogenous molecules could provide an alternative and efficient strategy to increase plant tolerance to a broad spectrum of viruses. We explored this possibility by implementing, together with our industrial partner, a medium-throughput screening procedure based on plant RNAi reporter lines developed in our laboratory. I will describe the identification and molecular characterization of a preliminary set of molecules that enhance RNA silencing upon their topical application in planta, and discuss their potential as antiviral compounds.

N

ext Generation Sequencing lecture

UHTS sequencing: a pipeline for de novo assembly and identification of viruses infecting plant samples from small RNA data

Patricia Otten, Loic Baerlocher, Cécile Deluen, Magne Osteras, Laurent Farinelli,

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Illumina ultra high-throughput sequencing (UHTS) uses massively parallel sequencing approach to generate millions of short sequences (<150 nucleotides) from biological samples. Today, on the HiSeq 2500, more than 200 millions paired-reads or reads per lane can be obtained. One human genome can be resequenced at 40x in a 2x100 on a HiSeq 2500 rapid run in less than two days.

End 2006, Fasteris was the first service provider in the world to order an Illumina Genome Analyzer system. Today, our company is equipped with 3 HiSeqs (one HiSeq 2000 and two HiSeqs 2500) and 2 MiSeqs. To address the various biological questions of our clients, we propose a large set of protocols, including Genomic, RNA-SEQ, small RNA, ChiP-SEQ and Mate-Pair protocols. Custom protocols are constantly developed in order to meet specific needs, such as the study of mitochondrial DNA or in case of diagnostic applications.

Nevertheless, all these data are useless without extensive bioinformatics analyses. At Fasteris, we propose standard analyses, including de novo assembly, mapping, comparative expression profiling, peak detection and variant calling. When necessary, we collaborate with external partners to carry out specific analyses, such as genome annotation or large genome assembly. According to our client needs, we also propose custom bioinformatics processes.

In our presentation, we will draw an outline of our activities with an emphasis on our pipeline for the analysis of small RNA data in the case of virus assembly and identification in plant infected samples.

Session 4: Resistance mechanisms other than silencing

Chair persons: B. Sturbois & P. Palukaitis

The amazing diversity of cap-independent translation elements in the 3'-UTR of melon necrotic spot virus (MNSV) and their role in determining the MNSV host range

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Internal ribosome entry sites (IRES) at the 5'-untranslated regions (UTRs) of animal virus RNAs are the most frequently found translation control elements. Unlike animal viruses, many plant viruses (in particular those belonging to the family Tombusviridae) contain cap-independent translation enhancer elements at their 3'-UTRs (3'-CITEs). For Melon necrotic spot virus (MNSV) (genus Carmovirus, family Tombusviridae) we have shown that cap-independent translation initiation of viral RNAs is controlled by a 3'-CITE. By exploring the natural diversity of MNSV, we have identified three different 3'-CITEs in different isolates and showed that at least two of them have been acquired by interfamilial recombination. Interestingly, these different MNSV 3'-CITEs are interchangeable and confer host specificity. Thus, the I-shaped 3'-CITE of most MNSV strains controls translational efficiency of viral RNAs in a eukaryotic translation initiation factor (eIF) 4E-dependent manner. The also I-shaped 3'-CITE of MNSV-264 confers this virus the ability to infect melon plants otherwise resistant to MNSV, as well as non-host species like *N. benthamiana* and *Gomphrena globosa*, functioning in a eIF4E-independent manner. The recently characterized MNSV-N strain contains two 3'-CITEs in its 3'-UTR, one which is structural and functionally identical to the I-shaped 3'-CITE of most MNSV strains, and the other which belongs to a newly identified structural class that we have named V-shaped. This additional V-shaped 3'-CITE of MNSV-N is responsible for its ability to infect resistant melons but not *N. benthamiana* or *Gomphrena globosa*, and is eIF4E-independent. Therefore, our results and those of other authors suggest that the underlying mechanisms controlling host susceptibility to RNA viruses include subtle and very specific interactions between viral 3'-CITEs and host translation initiation factors.

The TOR pathway and its interactions with viruses

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One of the fundamental limit between cellularized life and viruses is the lack of energy generating systems and protein synthesis apparatus encoded by viral genomes. All viruses lacks ribosomes and utilises host translation to produce their own proteins. In cells, protein synthesis is often the most energy consuming process sustaining most , if not all, others cell features. The TOR (target of rapamycin) protein kinase defines a conserved eucaryotic signaling pathway that perceive metabolic energy and regulate growth and survival. One of its main target is the translation apparatus , including translation initiation and ribosome biogenesis. Not surprisingly, numerous viruses target the host TOR pathway to subvert protein translation. In plants, TOR was found required for CaMV translation (see Ryabova's talk). We will review the essentials of the TOR pathway and of its interaction with viruses and show that it is fonctionnally required for the replication of some potyvirus in the Arabidopsis pathosystem.

Structural basis for the coevolution of *Tomato mosaic virus* and the resistance gene *Tm-1* at an interface of protein–protein interaction

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The *Tm-1* gene of tomato encodes a 754-aa protein that binds tomato mosaic virus (ToMV) replication proteins and inhibits viral RNA replication. *Tm-1* is derived from the wild tomato *Solanum habrochaites*, in which both ToMV-resistant and -susceptible *Tm-1* alleles were found. *Tm-1*^{GCR237} (resistant) and *Tm-1*^{PI390515} (susceptible) have 30-aa differences, among which 16 residues reside within a small region under positive selection (residues 79–112) during coevolution against ToMV (Ishibashi et al. 2012). On the other hand, ToMV mutants that have amino acid substitutions in the helicase domain of the replication proteins (ToMV-Hel) escape from the inhibitory interaction. In this study, we determined a crystal structure of a complex of an N-terminal fragment of *Tm-1* (residues 1–431), which is sufficient for the inhibitory activity (Kato et al., 2013), and ToMV-Hel. The key residues in ToMV-Hel that are changed in resistance-breaking mutants are directly involved in the interaction. The positively selected region of *Tm-1* comprises a flexible loop and an alpha-helix, and is a binding surface with ToMV-Hel. At least 20 amino acid residues of the *Tm-1* protein make direct contact with ToMV-Hel, and 11 of them were polymorphic between *Tm-1*^{GCR237} and *Tm-1*^{PI390515}. Thus, the antagonistic coevolution between a resistance protein and a viral protein has occurred at the interaction interface on both sides.

Ishibashi et al. (2012) PLoS Pathogens, 8(10): e1002975

Kato et al. (2013) Protein Expression and Purification, 89(1): 1-6

Pepino mosaic virus TGBp1 interacts with and increases tomato Catalase 1 activity to enhance virus accumulation

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Various plant factors are co-opted by virus elements (RNA, proteins) and have been shown to act in pathways affecting virus accumulation and plant defence. Here, an interaction between Pepino mosaic virus (PepMV) triple gene block protein 1 (TGBp1; p26) and tomato catalase 1 (CAT1), a crucial enzyme in the decomposition of toxic hydrogen peroxide (H₂O₂), was identified using the yeast two-hybrid assay, and confirmed via an in vitro pull down assay and bimolecular fluorescent complementation (BiFC) in planta. Each protein was independently localized within loci in the cytoplasm and nuclei, sites at which their interaction had been visualized by BiFC. Following PepMV inoculation, CAT mRNA and protein levels in leaves were unaltered at 0-, 3-, and 6- (locally) and 8- (systemically) days post inoculation, however leaf extracts from the latter two time points contained increased CAT activity and lower H₂O₂ levels. Over-expression of PepMV p26 in vitro and in planta conferred the same effect, suggesting an additional involvement for TGBp1 in potexvirus pathogenesis. The accumulation of PepMV genomic and subgenomic RNAs, and expression of viral coat protein in non-inoculated (systemic) leaves were significantly reduced in CAT-silenced plants. It is postulated that during PepMV infection, a p26/CAT1 interaction increases H₂O₂ scavenging, thus acting as a negative regulator of plant defence mechanisms to promote PepMV infections.

Importance of subcellular compartment specific function of plant NLRs in innate immunity

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Plants and animals have distinct evolutionary origins, yet when it comes to defense, they both rely on nucleotide-binding domain leucine-rich repeat (NLR) class of intracellular immune receptors. Understanding the function of NLRs in both animals and plants is a major challenge. For plant NLRs, we know very little about immune receptor complexes that form during pre- and post-pathogen recognition events. Furthermore, we know little about immune receptor complexes spatial distribution, subcellular location, or dynamics during immune signaling.

Emerging evidence points to the importance of subcellular compartment specific function of plant NLRs and immune modulators for successful activation of immune signaling. We study a TIR-NLR protein N that confers resistance to Tobacco Mosaic Virus (TMV) and use it as a model system to understand the molecular mechanisms by which NLRs recognize pathogens and initiate immune signaling. N recognizes TMV in the cytoplasm and undergoes dimerization. Following this event, N associates with the Squamosa Promoter-binding-protein-Like 6 (SPL6) transcription factor in the nucleus to activate successful immune response against TMV. Interestingly, SPL6 is also required for RPS4 NLR-mediated resistance to *Pseudomonas syringae* pv tomato (Pst) bacteria expressing avrRps4 effector. These results point to SPL6 as one of the conserved nuclear components of NLR signaling. We will discuss a model based on these findings wherein the pathogen effector-activated NLR protein complexes are involved in cytoplasmic and nuclear signaling leading to a successful immune response.

Tolerance: mechanisms and role in plant defence to virus infection

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Because infection by virulent parasites has a negative impact on host fitness, hosts have developed defences against parasites. Virulence in parasites and defences in hosts may result in host-parasite co-evolution. In plants, the two major defence strategies are resistance, defined as the host ability to restrict the multiplication of the parasite, and tolerance, defined as the host ability to reduce the negative effects of infection on its fitness. While large efforts have been done to understand plant resistance, tolerance of plants to viruses has received little attention. We have approached the analysis of plant tolerance to viruses using the *Arabidopsis thaliana* and Cucumber mosaic virus (CMV) system because CMV naturally infects wild *A. thaliana* populations with high incidence (1) and, therefore this is a suitable system to address plant-virus co-evolution. Our previous work based on the analysis of the response of 21 wild genotypes of *A. thaliana* to infection by various CMV strains showed that tolerance depended on the host genotype and showed a moderate heritability. Moreover, it demonstrated that the more tolerant *Arabidopsis* genotypes modified their developmental pattern and timing, so that upon infection resources were re-allocated from vegetative growth to increase the production of reproductive structures and seeds (2). Genetic mapping of tolerance determinants allowed the identification of three QTLs for tolerance that co-located with genes known to regulate the transition from vegetative to reproductive growth, which are then good candidate genes for tolerance based on life-history trait modification. As tolerance is a quantitative trait with a moderate heritability that appears associated to changes in the host developmental programme, it can be predicted that its expression will depend on the environment. To approach this question, four *Arabidopsis* accessions largely differing in their degree of tolerance were chosen for further analysis. Their response to CMV infection was analysed in a range of temperature and light conditions. This showed that environmental conditions are involved in the outcome of infection, which varied from high susceptibility to high tolerance. Still, the response pattern over environmental conditions was genetically determined, and differed largely for the accessions previously rated as tolerant and for those rated as susceptible. The role of tolerance in plant defence might be questioned by this strong genotype x environment interaction affecting its expression: if tolerance is effective in only a limited set of conditions, it might have no role in plant-virus co-evolution. To test the possible role of tolerance as a defence mechanism of *Arabidopsis* to CMV in nature, we analysed its variation over wild *Arabidopsis* genotypes, and compared it with the variation in resistance, which can hardly be questioned as a defence mechanism. For this, 12 wild *Arabidopsis* populations were sampled over the Iberian Peninsula and 10 random individuals per population were assayed for resistance and tolerance to two CMV strains. Assays were done in two environmental conditions, simulating a mild or a severe Iberian winter. Results indicated that *Arabidopsis* populations were highly polymorphic for both resistance and tolerance, both traits showing moderate to high heritabilities. In addition, both, resistance and tolerance, depended on the host and virus genotypes and on their interaction, a requirement for a possible role in plant-virus co-evolution. Finally, comparisons of the variation for these traits within and among populations, with that quantified for neutral genetic markers, suggest that both, resistance and tolerance to CMV, are under selection in Iberian *Arabidopsis* population. Overall, these results provided information on the mechanisms of tolerance, including its expression and its putative genetic determinants, and strongly support a role for tolerance in plant defence.

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JAX1-mediated resistance as the key to lectin-triggered immunity

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Plants possess a multilayered defense response, known as plant innate immunity, to infection by a wide variety of pathogens. The primary step is the pattern-triggered immunity (PTI) that perceives pathogen MAMPs to initiate early defense responses. The secondary step is effector-triggered immunity (ETI) that recognize pathogen effectors to activate defense responses usually accompanied by HR. Lectins, sugar binding proteins, play essential roles in the innate immunity of animal cells, but the role of lectins in plants has not been clear. However, recent advances began to uncover the role of lectins in plant innate immunity. LysM-type lectins serve as receptors of bacterial and fungal PAMPs during PTI. Jacalin-type lectin RTM1 is responsible for the resistance to potyviruses. Therefore, further efforts are required to elucidate the nature of such lectin-triggered immunity (LTI).

In this study, we analyzed *Arabidopsis thaliana* JAX1-mediated resistance (JMR) to potexviruses. We initially obtained certain *Arabidopsis thaliana* ecotypes resistant to a potexvirus, plantago asiatica mosaic virus (PIAMV), and found the lectin gene JAX1 similar to the known lectin resistance gene RTM1 is responsible for the resistance. The analyses of JMR indicated that this resistance did not show the properties of conventional resistance (R) protein-mediated resistance and was independent of plant defense hormone signaling. Furthermore, JAX1 was demonstrated to impair the accumulation of PIAMV RNA at the cellular level. Heterologous expression of JAX1 in *Nicotiana benthamiana* showed that JAX1 interferes with infection by other tested potexviruses but not with plant viruses from different genera. On the basis of these characteristics of JMR and their similarity to RTM1, we suggest that LTI can be a general resistance mechanism to plant viruses.

To gain knowledge of viral determinant involved in JMR, we tried to obtain potato virus X (PVX) mutants that can overcome JMR. Despite repeated inoculation tests, PVX could not overcome JMR. Therefore, we employed grafting-mediated inoculation method and obtained some resistance-breaking PVX variants. Sequencing of those variants showed that a single amino acid in the replicase of PVX was responsible for this resistance-breaking property. Reintroduction of the amino-acid substitution to avirulent wild-type PVX was sufficient to overcome the resistance. The residue that determines the resistance-breaking properties was highly conserved among potexviruses. These results suggests the essential role of viral replicase in JMR.

The TYLCV Resistance Gene Ty-1 presents a new class of resistance genes and confers resistance in tomato through enhanced transcriptional gene silencing

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Tomato Yellow Leaf Curl virus and related begomo-geminiviruses are a major threat to tomato production worldwide. Genes from different wild tomato species are introgressed to confer resistance against the virus. Recently we have cloned the Ty-1 resistance gene and shown this gene to be allelic with Ty-3. The genes were found to code for an RNA-dependent RNA polymerase and, although distinct from the known RDR1, 2 and 6 copies, for this reason postulated to confer resistance involving amplification of the siRNA signal. To test this hypothesis, Ty-1 and Ty-3 lines were challenged with TYLCV and the amount of siRNAs and their distribution on the viral genome analysed in comparison to susceptible tomato Moneymaker. While relatively high virus titers were observed in Moneymaker against only low amounts in resistant lines, the amount of siRNAs produced in the latter was quite increased with a consistent and subtle enrichment for siRNAs derived from the CP (V1) and C3 gene in Ty-1 and Ty-3 lines. Genome methylation analysis revealed a relative hypermethylation of the TYLCV CP (V1) promoter region in genomic DNA collected from Ty-1 in comparison to Moneymaker and altogether support the idea that Ty-1 confers resistance against TYLCV through enhanced transcriptional gene silencing. The durability of Ty-1-mediated resistance against geminiviruses will be discussed in light of its role in the RNAi pathway.

Session 5: Epidemiology, population genetics and evolution

Chair persons: A. Whitfield & H. Pappu

Preserving the efficiency of resistance genes against viruses from the individual host plant to agricultural landscapes

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The emergence of virus variants able to circumvent the resistance mechanisms of host plants, i.e. resistance-breaking variants, is a complex phenomenon that involves different evolutionary forces operating at different spatial scales. Resistance breakdown can be decomposed into three major steps: appearance of resistance-breaking virus variants by mutation, accumulation of these variants in plants in competition with wild-type viruses and spread from plant to plant. Resistance breakdown occurs when these three steps are achieved efficiently. If not, the resistance is said durable. Reverse genetics analyses allowed the identification of the mutational pathways responsible for resistance breakdown and the measure of fitness costs associated to these mutations in a number of resistance gene-virus species pairs. However, predicting the consequences of these genetic parameters at higher scales (crop fields, heterogeneous agricultural landscapes) is far beyond our experimental capacities because of the high number of plant-virus combinations to analyze and because of the high number of factors at play during virus emergences. For this purpose, we developed different mathematical models. An analytical model showed that the evolutionary constraint exerted on virus pathogenicity factors (the factors that are recognized by the plant resistance genes) was a good predictor of the durability of the corresponding plant resistance gene. In another approach, simulation models allowed quantifying the respective roles of genetic, demographic and epidemiological factors affecting viral populations during resistance breakdown and estimating what effects on resistance durability could be expected from different leverages available to plant breeders and growers (choice of resistance genes, distribution of resistant and susceptible cultivars among fields, additional control methods acting on epidemics).

The emergence and rise of Maize Streak Virus

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Maize streak virus strain A (MSV-A), the causal agent of maize streak disease, is today one of the most serious biotic threats to African food security. Determining where, when and how MSV-A originated, how it spread trans-continentially and how its pathogenicity in maize changed during and after this spread could yield valuable insights into the emergence of this and other major agricultural pathogens. Similarly, determining where the major extant MSV-A lineages arose could identify geographical hot-spots of MSV evolution. Here we use model-based phylogeographic analyses of hundreds of full length MSV-A genome sequences sampled across Africa to reconstruct a plausible history of MSV-A movements over the past 150 years. We show that since the probable emergence of MSV-A in southern Africa around 1863, the virus spread trans-continentially at an average rate of ~32.5 km/year (95% highest probability density interval, 15.6 to 51.6 km/year). Using distinctive patterns of nucleotide variation caused by 20 unique intra-MSV-A recombination events, we tentatively classified the MSV-A isolates into 24 easily discernible lineages. Despite many of these lineages displaying distinct geographical distributions, it is apparent that almost all have emerged within the past 4 decades from either southern or east-central Africa. Finally, we quantitatively evaluated the symptom phenotypes of 48 representative MSV-A isolates and, through phylogenetic model based trait evolution analyses, demonstrate that during the ~150 years since MSV-A emerged as a maize pathogen there has been a significant increasing trend in the severity of chlorotic symptoms that the virus causes in this host.

Synthetically-constructed Maize streak virus adapts to maize via recombination

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Maize streak virus-strain A (MSV-A; Genus Mastrevirus, Family Geminiviridae), the maize-infecting strain of MSV differs genetically from the otherwise biologically and epidemiologically related grass-infecting MSV-strain B by approximately 11%. MSV-A is believed to have originated when an MSV-B-like movement protein gene and coat protein gene cassette was inserted by recombination into the genome of a MSV-strain F like virus. The progenitor MSV-A is likely to have been selectively favored due to the specific fitness benefits imbued by these MSV-B derived sequences genetic segments. That such “maize-adapted” genetic regions or polymorphisms should exist along the MSV-A genome - and be absent in grass-infecting MSV-B isolates - has been the subject of many evolutionary experiments that seek to identify sites involved MSV adaptation to maize. Previously, we have shown that an experimental scheme involving pairs of reciprocal MSV chimaeras containing whole genes from a maize (MSV-MatA) and grass-adapted MSV (MSV-VW) could efficiently recapitulate the process of MSV adaptation to maize via recombination by reconstituting within progeny viruses all the genes required for a productive infection in maize. Here, we exploit this scheme by synthetically generating a pair of MSVs in which every single nucleotide difference between MSV-MatA and MSV-VW is present within the chimaeras, and is therefore available for recombination and selection in maize.

These synthesized reciprocally chimaeric MSV genotypes were apparently non-infectious in maize. By placing individual genome regions of the synthesized viruses into the genetic background of wt MSV-MatA it was determined that no individual genome region was entirely responsible for the observed lack of infectivity. However, some synthesized chimaeric genes had greater impacts on virulence than others. Accordingly, recombinant viruses with some degree of restored fitness that emerged from mixed infections of the reciprocal chimaeras predominantly contained the least defective genome components of each of the chimaeras. An analysis of these recombinants revealed that most replicated more efficiently than the chimaeric MSVs. Also, among the recombinants we observed differences in pathogenicity profiles in maize.

Although the chimaeric MSV we have synthesized contain the largest amount of disruption to co-evolved nucleotides or amino acid interactions typically found in maize-infecting MSV genomes, our results indicate that the process of recombination can nevertheless restore viral fitness and adaptation to maize.

Within-plant competition drives differential vector transmission of pathogen variants

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Pathogen populations can be highly diverse in both their genetic makeup and ecology, and interactions between distinct pathogen variants can affect disease spread. For vector-borne plant viruses, these interactions are expected to occur during transmission and host plant colonization. Grapevine leafroll-associated virus-3 (GLRaV-3) is an economically important pathogen in *Vitis* ssp., affecting crop quality in grape growing regions worldwide. It is transmitted by several species of mealybugs (Hemiptera, Pseudococcidae) in a semi-persistent manner. GLRaV-3 is genetically diverse, and co-infections of two or more GLRaV-3 variants in one plant are common. Nothing is known about the ecology of the different variants, such as possible interactions during transmission and host colonization, or differences in transmission efficiency by different vector species. It is expected that differences in host colonization, transmission efficiency, and interactions between virus variants influence patterns of disease spread. We tested transmission of two GLRaV-3 variants (I and VI) by two vector species from singly and co-infected hosts. Vector species *Planococcus ficus* transmitted both variants more efficiently than *Pseudococcus viburni*, with a five-fold difference between the two vectors. In single variant inoculations, the two virus variants did not differ in transmission efficiency; overall GLRaV-3 transmission efficiency was similar from singly and co-infected hosts. From co-infected hosts, transmission efficiency of GLRaV-3 variant VI was higher than GLRaV-3 variant I. Furthermore, this pattern held for simultaneous inoculation of individual uninfected plants with both variants from two singly infected source plants. Our results indicate competition between the two virus variants subsequent to inoculation into a new host drive differences in transmission efficiency between variants.

Topography and ruggedness of Tobacco etch potyvirus adaptive fitness landscapes

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RNA viruses are the main source of emerging infectious diseases owed to the evolutionary potential bestowed by their fast replication, large population sizes and high mutation and recombination rates. However, an equally important parameter, which is usually neglected, is the topography of the fitness landscape, that is, how many fitness maxima exist and how well connected they are, which determines the number of accessible evolutionary pathways. To address this question, we have reconstructed a fitness landscape describing the adaptation of Tobacco etch potyvirus (TEV) to a new host, *Arabidopsis thaliana*. Two fitness traits were measured for most of the genotypes in the landscape, infectivity and virus accumulation. We found prevailing epistatic effects between mutations in the early steps of adaptation, while independent effects became more common at latter stages. Results suggest that the landscape was highly rugged, with a reduced number of potential neutral paths and an alternative fitness peak, being the one reached by the evolving TEV population not the global optima.

What can be predicted about Rice yellow mottle virus emergence and spread?

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A recent review entitled « What can be predicted about virus emergence and spread? » concluded that any success in predicting what may emerge is likely to be limited, but that “...forecasting how viruses might evolve and spread following emergence is more tractable (Holmes EC, 2013, *Current Opinion in Virology* 3, 180-184). This statement is tested with Rice yellow mottle virus (RYMV), a plant virus whose molecular epidemiology has been thoroughly studied. We focused on the phylodynamics of RYMV in Africa and opposed two situations: that of RYMV in Madagascar, a well documented island system, which provides a simplified model in which phylogeography of the virus is tractable, to the recent emergence of a new strain around Lake Victoria which illustrates that success in predicting what may emerge in more complex situations is limited.

How eBSV polymorphism could enlighten banana evolution?

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The banana genome (*Musa* sp.) is invaded by numerous badnavirus sequences including those of banana streak viruses (BSVs). Badnaviruses are double stranded DNA pararetroviruses belonging to the family Caulimoviridae with any integration step into the host genome during their life cycle. The majority of viral integrants is mostly defective as a result of pseudogenisation driven by the banana host genome evolution. Conversely, some from BSVs only and named endogenous BSVs (eBSVs), can release a functional viral genome following stresses. All the badnavirus sequences described so far in banana [1] are spread among the three main groups structuring the badnavirus genus diversity [2]. We established that eBSVs are restricted to group 1, have an episomal counterpart, and are present in *Musa balbisiana* (B) genomes only [1;3;4] whereas the others are restricted to group 2 with any episomal counterpart described so far. We elucidated the sequence and the allelic organization for three BSV species (BSOLV, BSGFV and BSI_mV) present in the B genome of the seedy diploid PKW (BB), and developed several PCR and deCAPS markers for a specific eBSV genotyping [3-5]. Then, we investigated the early evolutionary stages of infectious eBSV through the study of their distribution, their polymorphism of insertion and their structure evolution when possible, among the banana B genomes diversity available through a sampling of BB seedy diploids extended to interspecific hybrids exhibiting different levels of ploidy for the B genome. We selected our sampling according to the two areas of sympatry between *M. acuminata* and *M. balbisiana* representing the centers of origin for the most largely cultivated AAB cultivars. One was in India and the other one in East Asia going from Philippines to New Guinea (Perrier et al, 2009). We used the PCR markers and Southern blots analysis to characterize PKW-related eBSV allelic polymorphism and we coded these results in order to create a common dissimilarity matrix with which we interpreted eBSV distribution. As a result, three dendrograms of PKW-related eBSV were constructed for each BSV species using the neighbor joining (NJ) method, as well as one dendrogram resulting from NJ analysis of all three BSV species together. We found that the known phylogeny of banana accessions based on *M. acuminata* genomes can help to elucidate eBSV structural diversity, and that eBSV polymorphisms can shed light on the particularly unresolved question of *M. balbisiana* diversity. We propose for the first time a banana phylogeny driven by the *M. balbisiana* genome. A evolutionary scheme of BSV/eBSV banana evolution is finally proposed.

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A top-down approach to sampling single stranded DNA viruses in ecosystems

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Our current knowledge of viral diversity is heavily biased towards viruses that infect animals, plants and economically important organisms, hence a gross underestimation of the viral biodiversity in ecosystems. Using a combination of sequence independent rolling circle amplification, next generation sequencing and conventional methods our aim is to recover and characterise complete genomes of single stranded DNA (ssDNA) viruses in ecosystems, particular those that may infect plants, fungi and algae, in order to gain insights into virus ecology.

In an attempt to rapidly explore ecosystems for insect-transmitted viruses we have implemented a top down approach using dragonflies which are highly mobile top insect predators, therefore in theory they could act as concentrators by accumulate viruses from their prey. In certain aquatic ecosystems dragonfly larvae can also be top-end predators thereby enabling an indirect sampling of the viral sequence space in these environments. Our pilot work on dragonflies and their larvae has unveiled a variety of novel ssDNA viruses that have some level of similarity to known ssDNA viruses, highly divergent plant-infecting ssDNA viruses and recently the discovery of a novel mastrevirus (family Geminiviridae) in the Caribbean. We postulate that the ssDNA viruses are more widespread in insects than previously recognised and hence top end predators could be used as a viral surveillance tools.

E MBO Science and Policy lecture

New biotechnologies: innovation, governance and stakeholder interactions

Joyce Tait

Innogen Institute, University of Edinburgh

The social science contribution to research on innovative biotechnologies has been dominated by the so-called ELSI approach for two decades, providing mainly a one-way analysis of what innovative technologies will do to or for 'society', with a strong focus on risks and other disadvantages. The Innogen Institute's work on the regulation and governance of life science innovation is based on an interactive analysis that recognises how the approach adopted to regulation and governance of an innovative technology will determine:

- (i) which scientific discoveries are developed as novel products or processes and which are abandoned;
- (ii) how innovative ideas from one area of scientific development need to combine with those from other areas to enable effective application;
- (iii) the benefits and choices available to citizens;
- (iv) the value for money delivered from public investment in basic science;
- (v) how regulatory regimes, their costs and timescales, are key drivers of the above outcomes; and
- (vi) (most important) the innovative capacity of an entire sector and the relative competitive advantage of regions and nations.

Our twin approaches, Analysis of Life Science Innovation Systems (ALSIS) and Adaptive Governance of Innovative Technology (AGIT) have been developed through a series of practical applications over 15 years, covering pesticides, GM crops, pharmaceuticals, crop production and use, stratified medicine, genetic data bases, translational medicine, and synthetic biology. They combine understanding of scientific developments and innovation processes, of policy and regulatory developments and of public and stakeholder perspectives. The outcome of their use is more even-handed in its consideration of both benefits and risks of new technology, adaptive to the needs of rapidly changing innovation environments in areas like synthetic biology, and smarter than previous governance approaches in enabling the development of innovative technology.

Session 6: Molecular ecology

Chair persons: C. Malmstrom & U. Melcher

Viruses as Partners in Plant Ecology

Marilyn Roossinck

Pennsylvania State University

Almost everything we know about eukaryotic viruses comes from studies of disease-causing viruses in humans and their domesticated plants and animals. However, recent work in virus biodiversity is changing our understanding of viruses. In plant virus studies using thousands of individual plant samples we find that most wild plants are infected with RNA viruses that are not causing disease. These plants appear healthy in spite of their viral load. In some cases nearby crop plants are infected with the same viruses, and have serious symptoms and crop losses. At least one acute plant virus appears to have moved from local crops into wildlands, but still does not cause disease in its wild hosts.

About half of the viruses found in wild plants are persistent viruses that infect plants strictly via vertical transmission, and remain in a host through many generations. They are related to viruses of fungi, and there is phylogenetic evidence that transmission has occurred between plants and fungi. This class of viruses has been very poorly studied, but recent plant and fungal genome analyses have shown that portions of some of these viruses are integrated in genomic DNA.

These observations have important implications for why viruses cause disease, and highlight our lack of understanding of viruses in the natural world and their roles in host ecology.

This work was supported by NSF grants EF-0627108 and EPS-0447262, USDA OKLR 2007-01012, The Samuel Roberts Noble Foundation and Penn State University

Contrasted patterns of phytoviral metagenomes in wild and agricultural environments

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The development of novel sequencing techniques (NGS) allows for unprecedented access to viral metagenomes and, in particular, to the yet poorly studied communities of phytoviruses in plant populations. Several strategies have been envisioned to gain access to this phytoviral metagenome, each with advantages and limitations. We are currently running 454 pyrosequencing of purified viral double-stranded RNAs (dsRNAs) in two very different ecological settings, agricultural plots and their immediate environment in south western France and the constrained, paucispecific and uncultivated ecosystem of the Kerguelen Islands, the second most isolated archipelago on earth. The bioinformatics analysis of the sequencing data allow a first evaluation of the diversity and novelty of RNA viruses (single or double-stranded) associated with crops and/or weeds present in such environments. For the study of agricultural sites, repeated samplings (in spring when the crops are implanted and in fall after harvest) allow a first evaluation of seasonal and of inter-annual variations. The results obtained in the Kerguelen Islands demonstrate a very low diversity of single-stranded RNA (ssRNA) viruses, most represented by introduced viruses such as Barley yellow dwarf luteovirus and Cucumber mosaic virus. On the other hand, a large diversity of novel double-stranded RNA viruses was identified, the majority of which belong to three families known to contain plant and fungus infecting agents, the Totiviridae, the Endornaviridae and the recently proposed Amalgamaviridae family. For example, as compared to the 4 Amalgamaviridae species described to date, a partial analysis of the data has allowed the identification of 28 OTUs (Operational Taxonomy Units, defined using as cut-off a 10% amino acid sequence divergence in a short, highly conserved region surrounding the viral RNA polymerase active site). These OTUs likely represent bona fide novel viral species found associated with a wide range of host plants, particularly in the Gramineae. By contrast, a much larger diversity of ssRNA viruses was observed in the temperate agricultural context, with the identification of both known and novel agents belonging to a wide range of viral families (Alpha- and Betaflexiviridae, Bromoviridae, Closteroviridae, Luteoviridae, Potyviridae, Secoviridae, Tombusviridae...). Remarkably, at the sampling intensity used, close to half of the sampled weed species were found to be free of infection by ssRNA viruses. Another observation is that the number of novel ssRNA agents detected remained roughly comparable to the number of known agents, suggesting that in the studied agricultural ecosystem a significant proportion of ssRNA viruses has already been described through classical virology approaches. Simultaneous presence of agents in crops and in neighboring weeds was observed in a few cases, suggesting viral spillover from crop to weeds but not allowing to conclude on a potential reservoir role for the weeds. These first efforts illustrate the potential of these approaches to analyze globally the phytoviral diversity in a given environment as a first step towards the identification of the various drivers shaping this diversity and the resulting viral communities. These approaches have also the potential to improve our understanding of the ecology of viral populations and of their contribution to the functioning of plant communities and ecosystems.

Geo-metagenomics: deciphering the spatial biodiversity of plant viruses associated with the unique fynbos ecosystem of Southern Africa and its neighbouring agro-ecosystem

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Over the past three years we have developed a geometagenomics approach which, because of the sampling design it applies and the depth of sampling it involves, enables the quantitative ecosystem-scale evaluation of spatial variations in, amongst other things, viral demographics, host distributions, and viral gene-flow. The approach is particularly well suited to analysing how viruses respond to ecosystem perturbations. As with the ecogenomics approach of Marilyn Roossink et al. (2009), the geometagenomics approach can precisely link individual sequence reads from bulked mixed sequencing reactions to information on abiotic and biotic conditions of the samples from which the sequences were obtained, the plant hosts from which samples were collected and the spatial arrangement of the samples. However, unlike with the ecogenomics experimental design, where sampling locations are randomly distributed within a reference ecosystem, in a geometagenomics experimental design sampling locations are systematically placed within a predefined grid; the location of which is placed according to available geographic information systems data. This a priori choice of the sampling points allows the identification of reference ecosystems that should be appropriate for determining, for example, the impacts of agriculture on viral demographics and evolution within natural endangered ecosystems or the transmission rates of viruses between wild and cultivated plants.

Our study was conducted in 2010 and 2012 in the Cape Floristic Region, which includes wild areas, including renosterveld shrubland and strandveld shrubland besides wide fertile plains under introduced crops such as barley, wheat and wine. Besides determining the spatial and temporal host distributions of various groups of both known and previously unknown virus species, we compare the virus species richness of the various wild and cultivated sampling locations. Amongst a large number of newly discovered virus species (including many apparently novel single stranded DNA viruses) was one belonging to a new Geminivirus genus that we have tentatively named, Capulavirus.

Roossink et al. Ecogenomics: using massively parallel pyrosequencing to understand virus ecology. 2010. Mol Ecol. 19 Suppl 1: 81-8

Assessing the effects of virus infection on the fitness of wild plants

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As a field, plant ecology has developed over the last century with little consideration of the roles of plant-infecting viruses in driving population and community dynamics. Recent documentation of the extent of virus infection in wild plants has increased awareness of the need to quantify virus influence, but studies remain scarce. Here we discuss key issues in the assessment of virus influence on the fitness of wild plants as evidenced in ongoing research with North American native grasses. We consider differences in virus-wild host dynamics that are evident in comparisons of a well-characterized group of crop pathogens and a novel virus endemic to wild hosts. We further consider the roles of host traits and landscape context in shaping the extent and nature of virus influence.

Cyanophages are a selective force driving cyanobacterial genome diversification

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Cyanobacteria from the genus *Prochlorococcus* are extremely abundant in the oceans, as are the viruses (cyanophages) that infect them. How hosts and viruses coexist in nature remains unclear, although the presence of both susceptible and resistant cells may allow this coexistence. We set out to understand the mechanisms enabling long-term coexistence between *Prochlorococcus* and its viruses and the impact this has on genome evolution and diversification. Using an experimental evolution approach we selected for cyanobacteria resistant to infection and used whole genome sequencing to identify the specific mutations that conferred resistance. This revealed mutations primarily in non-conserved, horizontally transferred genes that localize to a single hypervariable genomic island. Viruses have a diminished capacity to attach to the mutants indicating altered cell surface properties. The mutations often imposed a fitness cost to the host, manifested by significantly lower growth rates or a previously unknown mechanism of more rapid infection by other viruses. Many of the growth-impaired strains continued to evolve over time, leading to an improved growth rate and a narrowing of the resistance range. Genome sequencing revealed that these phenotypic changes were due to additional mutations in most of the strains, rather than reversions to the wild-type genotype. These findings indicate that, over time, slow-growing resistant strains are likely replaced in the oceans by newly emerging faster growing cells with altered resistant ranges. Surprisingly, counter mutations in phages that enable them to "reinfect" resistant hosts are rare. Therefore we propose that phages retain sufficient hosts for long-term coexistence through a phenomenon we term "passive host-switching". Combined, our findings indicate that phages are a selective pressure enhancing the diversity of both island genes and island gene content. This diversity emerges as an important genomic mechanism that serves to reduce the effective host population size for infection by a given phage, facilitating long-term coexistence and leading to a complex and dynamic network of interactions between phages and their cyanobacterial hosts in nature.

Exploding estimates of viral diversity in the sea has implications for phytoplankton

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Viruses are the most abundant biological entities on Earth and are major agents of mortality and drivers of global processes. They also likely represent the greatest genetic and biological diversity on the planet. In the oceans there are ~ 10 million virus particles /mL, constituting an estimated 10×10^{30} viruses in the World's oceans. Stretched end-to-end these viruses would span 10 million light years, or further than the nearest 40 galaxies. Genomic and metagenomic data reveal that the genetic diversity of these viruses is enormous and encompasses representatives across a wide-array of virus types including viruses with dsDNA, ssDNA, ssRNA and dsRNA genomes. Virus isolations and genomic and metagenomic analyses reveals that many of these are pathogens of phytoplankton, a group of organisms that is responsible for about half of the primary production on Earth. An exploration of the diversity within viruses infecting phytoplankton reveals not only that the diversity is enormous, but also provides insights into the host organisms infected by these viruses. For example, phylogenetic analysis of the DNA polymerase gene within the Phycodnaviridae, a family of large dsDNA viruses infecting eukaryotic phytoplankton allows one to make inferences about potential host organisms. Similarly, an analysis of RdRP genes in positive-sense ssRNA viruses reveals an enormous diversity of viruses infecting marine protists. Most recently we have been exploring the diversity of ssDNA viruses in the ocean, by purifying and assembling genomes of ssDNA viruses from temperate (Saanich Inlet, 11 samples; Strait of Georgia, 85 samples) and subtropical (46 samples, Gulf of Mexico) marine waters. Eighty-four percent of the sequences had no evident similarity to sequenced viruses; however, 67% of them were similar to at least one other sequence in our dataset. A total of 608 putative complete genomes of ssDNA viruses were assembled, almost doubling the number of sequenced ssDNA viral genomes in public databases. Analysis of these genomes revealed 129 genetically distinct groups of ssDNA viruses, each represented by at least one complete genome, which had no recognizable similarity to each other or to other sequenced viruses. Many of these genetic groups may represent new viral families. Moreover, nearly 70% of the ssDNA sequences had similarity to one of these genomes, indicating that most of the ssDNA sequences in these samples could be assigned to a genetically distinct group. Most of the sequences fell within eleven well-defined gene clusters, each sharing a common gene. Some of these new phylogenetic groups encoded putative replication and coat proteins that had similarity to sequences from viruses infecting plants, suggesting that some of these newly discovered evolutionary groups are likely pathogens of marine phytoplankton.

Posters

Session 1:
Translation, replication
and recombination

Mass-spectrometric analysis of sobemovirus VPg-s reveals an unusual diversity and a novel chemical bond between RNA and a protein

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The 5' ends of single-stranded positive-sense RNA virus genomes may have a viral protein genome-linked (VPg) attached to the RNA over a phosphodiester bond that is formed between the phosphate group of RNA and the hydroxyl group of an amino acid (aa) residue. The aa residues involved in the VPg–RNA linkage have been reported to be either tyrosine or serine. Within one virus genus the aa residue that is used for the bond formation with RNA seems to be highly conserved. Although threonine also contains a hydroxyl group, its use for linking RNA to VPg (or to whatever other protein) has not been reported. Sobemoviruses is an interesting genus of RNA viruses that, despite their small genome size, display remarkable biological differences (for instance host range, transmission strategies, long-distant transport mechanisms, existence of satellites, etc.) between individual species. Sobemoviruses encode also VPg-s. In the current study, a novel mass spectrometry-based approach was taken to determine the aa residues to which sobemoviral genomic RNAs are covalently linked to. We demonstrate that sobemoviruses, differently from all previously characterized virus groups can use different aa residues at different positions for RNA linking. Moreover, we show for the first time that threonine can be utilized for linking RNA to the protein. In addition, we identified the correct termini of VPgs of four different sobemoviruses and discovered several phosphorylation sites in these proteins. The biological significance of these modifications is tested at the moment and will be discussed. Also, we are currently testing whether the aa residues that are used for linking the sobemovirus RNA to VPg are replaceable to some other hydroxyl group containing residue.

Virus interfamilial recombination leads to acquisition of a translation controlling functional RNA element allowing host switch

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The exchange of genetic material by RNA virus recombination may result in dramatic changes in the biological properties of viruses, potentially playing a role in the emergence of new viral pathogens, including resistance-breaking and host-switching strains. Any function in the viral cycle could potentially be targeted by recombination, including translation of viral RNAs. Many plant viruses (in particular those belonging to the family Tombusviridae) depend for translation of their RNAs on functional RNA elements called 3'-UTR cap-independent translation enhancer elements (3'-CITEs). For Melon necrotic spot virus (MNSV) (genus Carmovirus, family Tombusviridae) we have shown that translation of its RNAs is controlled by a 3'-CITE, which functions in a eukaryotic translation initiation factor 4E (eIF4E)-dependent manner for most MNSV strains. In this work we have characterized the MNSV-N isolate, a newly identified resistance-breaking isolate. We found that this virus contains a 55 nucleotide (nt) insertion in its 3'-UTR which was acquired by neat interfamilial recombination with the 3'-UTR of an Asiatic Cucurbit aphid-borne yellows virus (CABYV) isolate. In addition to breaking-down the eIF4E-controlled melon resistance, this isolate seems to be fitter than the other previously described MNSV resistance breaking isolate. By constructing chimeric viruses and assaying their properties, we showed that the recombined sequence is responsible for the MNSV-N ability to break down the eIF4E-mediated resistance. Analysis of the translational efficiency of reporter constructs carrying different control elements, showed that the recombined sequence functions as a 3'-CITE in both, susceptible and resistant plants. The structural and functional characterization of this 3'-CITE showed that it belongs to a new structural class that we have called V-shaped 3'-CITEs, and functions in the absence of eIF4E. In conclusion, this work illustrates that 3'-CITEs consist of modular functional elements that can be transferred among viral species through RNA recombination, interacting differentially with host elements to confer host specificity and assisting host switching.

The formation of multivesicular bodies in Carnation italian ringspot virus infections

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Positive-strand RNA virus replication always occurs in association with rearranged host cell membranes. In infected plants, replication of tombusviruses takes place in membranous structures, known as multivesicular bodies (MVBs) which originate from vesiculation of the limiting membrane of peroxisomes or of the mitochondrial outer membrane. We investigated the mechanism of vesicle formation on mitochondria in Carnation Italian ringspot virus (CIRV) infections. The genome of CIRV consists of a 4.8 kb single-stranded RNA molecule encapsidated in icosahedral particles, containing five open reading frames (ORFs). ORF1- and ORF2-encoded p36 and p95 proteins are essential for viral replication. In particular, p95 contains the conserved motifs of RNA-dependent RNA polymerases and p36 the motifs for viral RNA binding and recruitment to replication sites. ORF 3 codes for the p41 coat protein, ORFs 4 and 5 encode p22, required for cell-to-cell movement of the virus in infected plants, and p19, which is a suppressor of virus induced gene silencing, respectively. The requirements for the formation of MVBs were studied in cells infected by cis-replicating wild type or defective CIRV genomes, and in cells expressing the p36/p95 replicase proteins in the presence or absence of the RNA replication competent DI RNA template. It was shown that MVBs developed in cells transfected with wt CIRV or with defective replication competent genomes expressing only the replicase p36 and p95 proteins. Trans replication of DI RNA supported by the expression of p36 and p95 replicase proteins did not induce MVB formation.

C2 from Beet curly top virus promotes a cell environment suitable for efficient replication of geminiviruses, providing a novel mechanism of viral synergism

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Geminiviruses are plant viruses with circular, single-stranded (ss) DNA genomes that infect a wide range of species and cause important losses in agriculture. Geminiviruses do not encode their own DNA polymerase, and rely on the host cell machinery for their replication. In this work we identify a positive effect of the curtovirus Beet curly top virus (BCTV) on the begomovirus Tomato yellow leaf curl Sardinia virus (TYLCSV) infection in *Nicotiana benthamiana* plants. Our results show that this positive effect is caused by the promotion of TYLCSV replication by BCTV C2. Transcriptomic analyses of plants expressing C2 unveil an up-regulation of cell cycle-related genes induced on cell cycle re-entry; experiments with two mutated versions of C2 indicate that this function resides in the N-terminal part of C2, which is also sufficient to enhance geminiviral replication. Moreover, C2 expression promotes the replication of other geminiviral species, but not of RNA viruses.

We conclude that BCTV C2 has a novel function, probably by restoring the DNA replication competency of the infected cells and thus creating a favourable cell environment for viral spread. Because C2 seems to have a broad impact on the replication of geminiviruses, this mechanism might have important epidemiological implications.

Mapping the determinant for necrotic response in Ourmia melon virus infection of *Nicotiana benthamiana* reveals the plasticity of agro-infectious clones and suggests a new standard for evaluating “infectivity” of cDNA clones: derived infectious sequence.

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We have recently published the assembly of an Ourmia melon virus (OuMV) agroinfectious clones able to reproduce necrotic symptoms on *Nicotiana benthamiana* similar to those obtained inoculating the original virus. During the cloning process we also serendipitously originated a set of clones corresponding to the three RNAs which originated a systemic viral infection only displaying mosaic-mottling later fully recovering in newly emerging leaves. We originated reassortant viruses to identify the virus segment responsible for eliciting necrotic response, and showed that the RNA1, coding for the viral RdRP, carried the determinant for the necrotic response. Then a full length sequence comparison with a wild-type clone for RNA1 revealed a 1 nt deletion in position 600 in a stretch of six cytosine residues in the coding region of the RdRP and 16 nt mutations distributed along the same RNA corresponding to three amino acids changes. A combination of chimeric cDNA clones and site directed mutagenesis demonstrated that the sole responsible for the necrotic response is a 1 nt mutation that results in 1 amino acid change (D to N position 364). Surprisingly some of the chimeric constructs were not infectious. We showed that the progeny RNA that accumulates in the plant is different from that of the two original cDNA, in particular replacing the cytosine deletion present in the mild segment at position 600 and mutating a C residue into G in position 2792 in the 3' UTR region of the necrotic allele. The chimeric clones combining the two variants were not infectious, whereas clones carrying only one of the different mutations could be corrected likely at the transcription step (either in vitro or in vivo in a T7 derived clone). Implications of such discovery are discussed and compared to results obtained inserting a 1 nt deletion in a commonly used agro-infectious clone derived from TMV.

A sensitive method to quantify virion and complementary-sense strands of circular DNA viruses

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Geminiviruses are plant viruses with circular single-stranded DNA genomes transmitted by insects that include emerging and economically significant viruses. Although real-time PCR diagnostic protocols for their detection are available, none of them are able to discriminate between the two strands generated during an infection: the viral strand (VS), encapsidated within virions, and the complementary-sense strand (CS), used as template to generate more viral strands by a rolling circle mechanism. Here we describe a two-step real-time PCR protocol to quantify the amount of VS and CS as well as how many of those strands are arranged as single or double strand. The method was used to quantify VS and CS of begomoviruses Tomato yellow leaf curl Sardinia virus (TYLCSV) and Tomato yellow leaf curl virus (TYLCV) in tomato and *Nicotiana benthamiana*. The results show that the ratio VS/CS is not constant throughout the time of infection, depends on the combination virus-host, and that most CS is restricted to replicative forms of the virus. The protocol presented here is a significant improvement of the techniques in use to quantify circular ssDNA and can help to understand in detail the molecular scenario during replication of any viruses whose genome is made of circular DNA.

Mechanism of mRNA decay suppression by a plant virus

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Viruses use multiple strategies to circumvent the cellular RNA degradation machinery to protect their mRNA from targeting for decay by host mRNA surveillance pathways. To counteract degradation-mediated defense systems, most viruses have evolved strategies that suppress degradation of viral mRNA lacking typical 3'- and 5'-end modifications that are most susceptible to degradation. The most frequent counter-decay strategies of viruses are based on the use of RNA structural features and cis-acting elements that can prevent viral RNA recognition by the cellular surveillance machinery and protect the viral RNA from the action of decay enzyme complexes.

Cauliflower mosaic virus (CaMV) dsDNA genome is amplified via reverse transcription of RNA intermediate in the cytoplasm of infected cells. Two major viral transcripts accumulate in CaMV-infected plant: the terminally redundant 35S pregenomic RNA (35S pgRNA), which covers the whole genome and acts as a polycistronic mRNA for production of viral proteins; and the subgenomic 19S RNA, which codes for transactivator/ viroplasm TAV (ORF VI). Upon infection, as well as upon transient expression of TAV, TAV forms large, cytoplasmic inclusion bodies—so-called viroplasms. In addition to 35S pgRNA, its four spliced variants have been identified, which seem to be important for successful infection. The presence of consecutive open reading frames and exon-exon junctions after the stop codon may act as a powerful trigger for mRNA degradation by NMD. How the 35S RNA escapes the mRNA surveillance system of the host cell still remains an open question.

Our preliminary results indicate that TAV can associate with mRNA decapping complexes or cytoplasmic processing bodies (P-bodies) that are dynamic RNA-protein aggregates containing a number of factors that are essential for mRNA decapping, particularly DCP2, DCP1, and VARICOSE (VCS). Using the *Nicotiana benthamiana* transient expression system, we demonstrated that TAV fused to the red fluorescent protein (TAV-RFP) co-localizes with AtDCP1-GFP, AtDCP2-GFP and AtVCS-GFP. Strikingly, DCP1, DCP2 and VCS, when overexpressed without TAV, are localized within cytoplasmic foci corresponding to PBs, while TAV overexpression results in their re-localization to TAV aggregates. The colocalization of DCP1, DCP2, VCS and TAV prompted us to test whether TAV is located within P-bodies. Using *N. benthamiana* plants transiently expressing Myc-VCS and HA-TAV fusion proteins, we studied their interactions in planta. Indeed, HA-TAV accumulated specifically in the Myc- VCS pellet in *N. benthamiana* plants transiently expressing both recombinant proteins. Our data suggest that TAV perturbs PBs via direct or indirect interaction with the decapping machinery.

Analysis of decapping-deficient *Arabidopsis* mutants revealed that steady state of REV, ATHB8 and SEN1 transcript levels monitored by semiquantitative RT-PCR were at least fivefold higher than in wild type plants. Strikingly, similar to plants defective in the decapping pathway, the steady state levels of these mRNAs were significantly elevated in TAV transgenic *Arabidopsis* plants as compared with the WT plant control, suggesting that TAV can suppress P-body-dependent mRNA turnover in *Arabidopsis*. Taking together, our results indicate strongly that CaMV TAV is involved in complex formation with decapping factors and may participate in suppressing of cellular/viral mRNA degradation, possibly via blocking the decapping machinery. Our results indicate that viruses could also be evading host defense by integration into decapping/ deadenylation/ NMD complexes via interaction with their compounds.

Analysis of the frameshift utilized by potyviruses for PIPO expression

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Potyviruses are the largest group of plant RNA viruses and they are responsible for significant economical losses worldwide. Until recently, the genome of potyviruses was believed to be translated as a single continuous polyprotein. Now, it has been shown that probably all potyviruses contain a small overlapping reading frame in the middle of P3, called PIPO, which is apparently expressed via a ribosomal frameshifting mechanism. In comparison to the rest of the polyprotein, PIPO is in the -1/+2 reading frame. Since its discovery, PIPO has been shown to be involved in virus movement and quantitatively contributing to virulence. The amount of expression is determined by the efficiency and the mechanism of ribosomal frameshifting. Classical and well-described stimulators of programmed -1 ribosomal frameshifting seem not to be present in the genomes of potyviruses, and analysis of conserved sequence motifs suggests instead that a novel +2 shift may be utilized. We are currently investigating the efficiency and direction (-1, +2 or both) of frameshifting in potyvirus-infected cells, and the presence and nature of frameshift stimulators.

Ribosomal protein P0 and P0-containing RNA granules in Potato virus A infection

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Potato virus A (PVA; genus Potyvirus) RNA can be targeted via an action of viral protein genome-linked (VPg) to a specific gene expression pathway which protects viral RNA from degradation and facilitates its translation(1). Acidic ribosomal protein P0 as well as eIF4E/eIF(iso)4E are host factors required by VPg to achieve this (1,2). The positive effect of VPg and P0 on viral translation is reversely correlated with cell-to-cell spread of infection, suggesting that these processes may compete for viral RNA(2). Interestingly, we observed that PVA RNA induces the formation of P0-containing granule structures (POGs). Assembly of POGs doesn't depend on virus replication or movement as PVA RNAs deficient in these functions induce a strong POG-response. PVA RNA co-localizes with POGs. Cytoplasmic RNA granules, like stress granules and processing bodies, play major roles in cellular gene expression. Many animal viruses are known to manipulate these structures to promote synthesis of viral proteins and this may be the case with PVA as well. POGs disappear under conditions promoting VPg-mediated PVA RNA expression, suggesting that the efficient PVA RNA translation counteracts the existence of POGs. We suggest that P0 is an essential host factor required to coordinate PVA RNA functions and to achieve a robust and productive infection.

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2. Hafren, A., Eskelin, K., and Mäkinen K. (2013) "Ribosomal protein P0 promotes Potato virus A infection and functions in viral translation together with VPg and eIF(iso)4E" *Journal of Virology* 87, 4302-4312.

Involvement of membranous structures and plant cell organelles in Tobacco rattle virus (TRV) replication and assembly process.

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Tobraviruses replicate and express their genome in cytoplasm in closely related membranous structures like endoplasmic reticulum. Our research demonstrates participation membranes, vesicular structures in Tobacco rattle virus replication and assembly process in compatible TRV – potato and – tobacco interactions on ultrastructural level. Membranes and vesicular structures formed restricted virus replication and/or capsidation areas in mesophyll and also vascular parenchyma cells. We observed highly connections between inclusions of two types of TRV particles and vesicular areas. Moreover, for the first time for TRV, we demonstrated associations plant cell organelles with replication process – not only ER, but also mitochondria, nucleus and chloroplasts. The ultrastructural analysis demonstrated highly dynamics changes in host mitochondria. The matrix was more electron-opaque, the cristae were irregularly shaped, the intermembrane space was enlarged. In more advanced stages of disorganization the cristae lost their identify and appeared a mass of small vesicles, some of which had a virions contents. The TRV virions of two lengths were plentiful both inside and around cell nucleus. The nucleus formed bleb-like evaginations usually with virions attached to the membranes with patches of electron-dense amorphous material content. Our investigations revealed electron-lucent cytoplasm areas formation in which we observed two types of TRV particles clusters: noncapsidated following to capsidated forms. Immunodetection of TRV-capsid protein considered the connections of nucleus, mitochondria and chloroplast with tobacco rattle virus life cycle and with capsidation process in plant cell infection. The modification of the organelles architecture give rise to the formation of internal vesicles and membrane bodies development, which connected membranes and plant cell organelles in TRV replication and capsidation process.

Host-dependant self-cleavage of P1 protease modulates potyviral replication and antiviral responses

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In several RNA viruses, replication involves the translation of polyproteins, whose processing by protease is a key step for the release of functional subunits. P1 is the first endopeptidase encoded in plant potyvirus genomes; once activated by a still unidentified host factor, it acts in cis on its own C-terminal end, hydrolyzing the P1-HCPro junction. Earlier research suggests that P1 cooperates with HCPro to inhibit host RNA silencing defenses. Using Plum pox virus as a model, we show that although P1 does not have a major direct role in RNA silencing suppression, it can indeed modulate HCPro function by its self-cleavage activity. To study P1 protease regulation, we used bioinformatic analysis and in vitro activity experiments to fine-map the C-terminal catalytic domain. We present evidence that the hypervariable region that precedes the protease domain is predicted as intrinsically disordered, and that it behaves as a negative regulator of P1 proteolytic activity in in vitro cleavage assays. In viral infections, removal of the P1 protease antagonistic regulator is associated with accelerated early replication, greater symptom severity and reduced viral loads. This negative correlation is supported by overaccumulation of host pathogenesis-related protein-2, a recognized marker of systemic acquired resistance. Finally, we suggest that fine modulation of a viral protease activity has evolved to keep viral amplification below host-detrimental levels, and thus to maintain higher long-term replicative capacity.

Interaction of the potyvirus protein HCpro with the cellular microtubule-associated protein HIP2 is required for efficient virus infection in plants

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Microtubules (MTs) maintain the overall shape of non-dividing plant cells by regulating cell wall synthesis and organizing cell organelles and cellular membranes. Here, we provide data on involvement of a MT-associated protein in infection with a potyvirus, Potato virus A (PVA; genus Potyvirus), representing the largest family of plant-infecting RNA viruses. The helper component proteinase (HCpro) of PVA, Potato virus Y and Tobacco etch virus interacted with HCpro-interacting protein 2 (HIP2) of potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) in planta. Interactions of HCpro with HIP2 were localized on MT and MT-intersections in cell cortex in PVA-infected leaves. The results showed that HIP2 is a MT-associated protein homologous to *Arabidopsis* SPIRAL 2 (SPR2). Silencing, complementation and localization experiments demonstrated that SPR2 and HIP2 share similar functions at cortical MTs. Accumulation of PVA was significantly reduced in the HIP2-silenced leaves of *N. benthamiana*, which indicates that HIP2-HCpro interaction supports virus infection. The C-proximal portion of PVA HCpro determined the interaction with HIP2 and was found to contain a stretch of six residues comprising a highly variable region (HVR) in potyviruses. Mutations in HVR reduced fitness of PVA and caused novel necrotic symptoms in tobacco plants. Analysis of gene expression showed that many defence-related genes including ethylene- and jasmonic acid-inducible pathways were induced, whereas genes related to microtubule functions were down-regulated. These results implicate for the first time a role for MTs in potyvirus infection and suggest that HCpro interacts with MTs to impede antiviral defence.

High-throughput sequencing of CMV recombinants

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Recombination is thought to be a major source of virus variability, and may thus play an important role in virus evolution and adaptation to new hosts. Cucumber mosaic virus (CMV), in addition to being an important plant pathogen, has also proved to be a remarkable system for studying recombination. Even under conditions of little or no selection pressure in favor of recombinants, a highly diverse collection of types of recombinant CMV RNAs can be detected by RT-PCR amplification followed by sequencing. We have studied CMV recombination in 1) wild-type plants co-infected with divergent CMV isolates, 2) transgenic plants expressing CMV sequences infected with a divergent CMV isolate, and 3) reassortant CMV isolates created with RNAs from divergent CMV isolates. Under several experimental conditions, the most prevalent recombinant RNAs were ones resulting from precise homologous recombination, which is generally presumed to occur via a copy-choice mechanism. In addition, an imprecise variant of homologous recombination was observed in a GU repeat tract in the 5'-noncoding region of RNA3; this also could occur via copy-choice, but with imprecision in the site of re-initiation within the GU repeat. Three general classes of non-homologous recombination were observed. In some cases, the crossover occurred within short blocks of sequence identity between the donor and acceptor RNAs, and these could be produced by the same general mechanism as the homologous recombinants. We also observed cases in which recombination joined totally unrelated sequences. Among these, an abundant class was composed of recombinants involving CMV RNA5, which is an uncapped RNA corresponding to the 3'-ncr, primarily derived from RNAs 2 and 3. In these recombinants, the 5' end of RNA5 was fused to diverse sites in the viral genome, often creating large insertions or deletions. This repertoire of recombinant types raises interesting questions regarding the mechanisms of recombination.

Genetic recombination of Brome mosaic virus RNAs in agro-infected *N. benthamiana* plants: towards studying the coat protein function(s).

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We have described the debilitating effects of mutations that interfered with the coat protein (CP)-RNA binding on the frequency of recombination between Brome mosaic virus (BMV) RNA3 and sgrNA3a in barley protoplasts (Sztuba-Solińska J, Fanning SW, Horn JR, Bujarski JJ. 2012. Mutations in the coat protein-binding cis-acting RNA motifs debilitate RNA recombination of Brome mosaic virus. *Virus Res.* 170(1-2):138-49). To further study how CP influences BMV RNA recombination in whole plants, the binary vector constructs that carried either wt sequence of BMV RNA3 or RNA3 with two silent marker mutations within the 3a protein ORF were generated, and co-agroinfiltrated on *N. benthamiana* leaves (together with agro constructs carrying wt BMV RNA1 and RNA2 sequences). The accumulation of BMV RNAs in both the infiltrated and systemic leaves, were detected by Northern blotting. To identify RNA3-RNA3 recombination events, the RNA3 3a ORF region was PCR amplified and cDNA products were cloned into pGEM-T-easy vector, and the clones characterized by restriction enzyme digestion. Numerous recombination events were detected among a high number of clones characterized, demonstrating the effectiveness of this system for recombination studies. Current work addresses the effects of CP mutations on BMV RNA recombination in both local and systemic tissues.

Session 2: Intra- and inter-host movement

Genetic dissection of a putative nucleolar localization signal reveals the role of Ourmiavirus coat protein in host specific systemic spread and silencing suppression

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Ourmia melon virus is the type member of a recently characterized plant virus group with a tri-segmented (+)ssRNA genome: RNA1 encodes for the RNA-dependent RNA polymerase (RdRP), RNA2 for the movement protein (MP) and RNA3 for the coat protein (CP). GFP-CP fusion locates into the nucleus and preferentially into the nucleolus. By alanine scanning and deletion mutagenesis, we identified an arginine rich nucleolar localization signal (NoLS) at the N-terminal of CP. In the context of virus infection, we showed that either CP nucleolar trafficking and/or CP specific arginine residues seem(s) to be essential for efficient virus accumulation and *Nicotiana benthamiana* systemic infection without affecting virion formation. In cucurbits, the integrity of the NoLS seemed critical for the plant systemic infection suggesting a possible role of this protein region in determining the host range. Standard tests for identifying a silencing suppressor in OuMV genome failed: nevertheless, a specific defect in virus accumulation of our NoLS mutant could be fully complemented in *Arabidopsis* Dicer mutants: such result is a preliminary indication that integrity of NoLS in the OuMV CP is necessary for its silencing suppression activity during virus replication. We are currently evaluating mutant virus accumulation in Cajal body defective lines of *Arabidopsis* and *N. benthamiana*.

Tomato spotted wilt virus (TSWV) isolates encoding truncated NSs proteins are not transmitted by its thrips vector *Frankliniella occidentalis* and are severely impaired in virus accumulation in adults but not in 2nd-instar larvae

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Tomato spotted wilt virus (TSWV) (Tospovirus, Bunyaviridae) is one of the most agronomically important viruses worldwide. The virus is transmitted between plants by insects in the genera Thrips and *Frankliniella* (Thysanoptera, Thripidae), in a propagative mode. Lack of reverse genetic systems hampers the functional analysis of TSWV genome, but thanks to the selective pressure of the Tsw resistance gene in pepper, we obtained a number of TSWV isolates, carrying a deletion in the NSs coding region. Such deletions caused a recovery phenotype on *Capsicum* spp. and *Datura stramonium*, linked to lack of suppression of silencing activity for the resulting truncated NSs, but in *N. benthamiana* some of the NSs recovery isolates accumulated to wild type level. We show that *Frankliniella occidentalis*, its most efficient vector, fails to transmit TSWV coding for truncated NSs protein. Quantitative RT-PCR and immuno-staining of individual insects detected the mutant virus in 2nd-instar larvae and adults, demonstrating that insects can acquire and accumulate NSs-defective mutants. Nevertheless adults carried a significantly lower viral load, showing for the first time a specific role of NSs in maintaining a high titer of virus in adult thrips. Sequence analyses of reassortant isolates revealed a strict association of lack of transmission to the mutation in the NSs-coding sequence.

Both structural and non-structural forms of the readthrough protein of Cucurbit aphid-borne yellows virus are essential for efficient systemic infection of plants

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Cucurbit aphid-borne yellows virus (CABYV) is a polerovirus (Luteoviridae family) restricted to phloem tissue and strictly transmitted by aphids. Polerovirus cycle in plants implies replication in nucleated cells and translocation over long distances through sieve elements. Polerovirus particles have been observed in plasmodesmata connecting companion cells and sieve elements and also in sap collected from infected plants. Therefore whole virions are supposed to be the long-distance device of poleroviruses. Polerovirus capsid is composed of the major coat protein (CP) and a minor component referred to as the readthrough protein (RT) because it is produced by a bypass of the CP stop codon. Two forms of the RT were reported: a full-length protein of 74 kDa detected in infected plants and a truncated form of 55 kDa (RT*) incorporated into virions. Both forms were detected in CABYV-infected plants. To clarify the specific roles of both proteins in the viral cycle, we generated by deletion a polerovirus mutant able to synthesize only the RT* which still became incorporated into the particle. This mutant was unable to move systemically to non-infected leaves inferring that the C-terminal part of the RT is required for efficient long-distance transport of CABYV. Among a collection of CABYV mutants bearing point mutations in the central domain of the RT, we obtained a mutant impaired in correct processing of the RT that does not produce the RT*. This mutant accumulated very poorly in systemically infected leaves, suggesting that the RT* has a functional role in the systemic spread of CABYV. Interestingly, the full-length RT expressed in transgenic plants was able to complement the deficiency in long-distance movement of the mutant deleted in the C-terminal part of the RT. Taken together, these results infer that both RT proteins are required for a productive movement of CABYV and support the model in which the complete RT, or its C-terminal part, acts in trans on wild-type virions to promote efficient systemic spread of CABYV.

Modeling the transport of a viral membrane protein through the early secretory pathway: minimal sequence and endoplasmic reticulum lateral mobility requirements.

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Targeting of the so-called movement proteins (MP) to plasmodesmata is a common feature required for the cell-to-cell movement of plant viruses. The viral MPs, which are predominantly membrane proteins, exploit and interfere with endomembranes and cytoskeletal elements to move within the cell by means of apparently unconventional routes that, in some cases, are dependent on the secretory pathway. In this sense, Melon necrotic spot virus p7B, a type II membrane protein (Navarro et al., 2006), is delivered to plasmodesmata via the Golgi apparatus through a COPII-dependent pathway (Genovés et al., 2012). We previously showed that the hydrophobicity of its transmembrane domain (TMD) was essential in determining not only the insertion of GFP-tagged p7B into the endoplasmic reticulum (ER) membrane but also its further destination (Genovés et al., 2011). However, the TMD by itself was not sufficient to drive the GFP transport beyond the ER, suggesting the presence of additional sorting signals. Here we tracked the movement of GFP-tagged p7B mutants to investigate the relative roles of each extra-membranous domains in the anterograde traffic. From the behavior of these mutants different roles could be assigned to each domain. Truncated mutants and alanine-scanning mutagenesis revealed that Nt mutants gave rise to discrete fluorescence clusters that move along the cortical ER and grouped around Golgi stacks when they were immobilized through actin depolymerisation. In contrast, Ct mutants disrupt ER-Golgi traffic and were homogeneously distributed along the ER. Co-expression studies provided evidence that Nt mutants lose the ability to recruit sar1p, a COPII component, to ER membranes while it increased in Ct mutants most likely interfering with the ER-Golgi transport. Remarkably, a negatively charged residue (D7) and a conserved proline (P10) in the cytoplasmic Nt domain together with a highly conserved lysine (K50) in the luminal Ct domain were critical for the ER export and virus cell-to-cell movement. Fluorescence recovery after photobleaching studies revealed that Ct but not Nt truncation caused a dramatic decrease in the lateral mobility of the protein supporting the idea that the Ct may be involved in a lateral sorting event via the ER. From this data, we propose a model whereby the luminal domain directs the MP to sorting regions in the cortical ER where additional signals in the cytoplasmic face may be necessary for its selective anterograde transport.

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Tomato yellow leaf curl virus (genus Begomovirus, family Geminiviridae) seems not to replicate in its vector, the whitefly *Bemisia tabaci*

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Geminiviruses (family Geminiviridae) are plant viruses with small circular single-stranded DNA genomes encapsidated in twinned particles. This family is divided into four genera according to their host-range, genome structure and insect vector. With more than 200 species, the genus Begomovirus is the largest genus in this family and comprises viruses infecting dicotyledonous plants that are transmitted by the whitefly (Hemiptera: Aleyrodidae) *Bemisia tabaci*. Begomoviruses are an emerging threat worldwide in temperate and tropical regions, causing severe damage to economically important vegetable, root and fiber crops. One of the most devastating diseases caused by begomoviruses is tomato yellow leaf curl disease (TYLCD), caused by a complex of more than ten begomoviruses species, being Tomato yellow leaf curl virus (TYLCV) the most widespread worldwide.

TYLCV is transmitted by *B. tabaci* in a persistent manner and an ample controversy exists about the replication of the virus in the insect vector. Although there are some data supporting the existence of transcription of the viral genes, no experimental evidence is available about the ability of TYLCV to replicate within *B. tabaci*. In the plant cell, TYLCV replication occurs in the nuclei via double-stranded intermediates composed by virion-sense (VS) and complementary-sense (CS) strands. Thus, CS strands are present only when the genomic DNA is replicating. To find out whether TYLCV replicates within whiteflies, a two-step qPCR method for the specific amplification of VS or CS strands was assayed in DNA extracts from viruliferous whiteflies. Time-course of CS and VS accumulation and CS/VS ratio in viruliferous whiteflies did not provide experimental support for TYLCV replication in the insect.

Recombinant TMV-based viruses carrying mutations in conservative Influenza antigen on the surface of chimeric particles provide appropriate model system for studying systemic spread and apical meristem invasion.

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Cell-to-cell transport of plant viruses through plasmodesmata is subject to intensive research, but systemic spread with possible involvement and influence of coat protein (CP) remains understudied. We constructed recombinant viruses TMV-M2e-ser and TMV-M2e-ala based on the cDNA copy of Tobacco mosaic virus (TMV). The CP gene was modified to carry human consensus sequence of conservative ectodomain (M2e) of Influenza A M2 protein that forms tetrameric ion channels in virion. Cysteine residues at positions 17 and 19 were changed to either serine or alanine residues. Viral genomes were cloned between T-DNA borders under control of Actin 2 transcriptional promoter from *Arabidopsis thaliana* and terminator from nopalinsynthase gene in order to use agroinfiltration as the most efficient method for plant inoculations. Both viruses were capable of systemic spread via vascular tissue of *Nicotiana benthamiana*, *Nicotiana excelsior*, *Nicotiana sylvestris* and *Nicotiana clevelandii*. Infections in *Datura stramonium* and *Chenopodium murale* were limited to inoculated leaves. The chimeric particles were stable in plant extracts and during preparation procedures; foreign epitopes were exposed on the surface as shown by immunoelectron microscopy. Reverse transcription of genomic RNA and total RNA from systemic leaves with primers flanking M2e inserts proved genetic stability of TMV-M2e recombinant viruses: sequencing of purified PCR products did not reveal any mutations. Repeated inoculations demonstrated the same pattern of virus-specific proteins as primary infections.

Symptoms of *Nicotiana benthamiana* and *Nicotiana sylvestris* after inoculations with TMV-M2e-ser and -ala mutants differed: growth and development of plants inoculated with TMV-M2e-ser stopped by 10-12 dpi (*N. benth.*) whereas for the ala mutant we observed mild mosaic symptoms and plant development was not impaired. It might be assumed that phosphorylation and/or glycosylation of additional serine residues on the surface of chimeric virions allows invasion into the apical meristem. Morphologically, TMV-M2e-ala and TMV-M2e-ser complexes with primary mouse anti-M2e and secondary gold-labeled antibodies are distinguishable and evidential for putative additional surface charge of ser particles. Chimeric CP-M2e-ala protein accumulated 5 g per 1 kg of fresh material in upper leaves of *N. benthamiana* against 1 g/kg accumulated by CP-M2e-ser protein. Efficacy of ala/ser mutants' replication within inoculated leaves was similar as shown by Coomassie staining of CP-M2e proteins. We analyzed hypersensitive response to the TMV-M2e infections. The sizes of necrotic lesions differed insignificantly which may indicate that M2e epitopes at the C-terminus of CP do not influence cell-to-cell movement. Challenge experiments with ser/ala mutants showed that both recombinant viruses appeared in systemic leaves simultaneously, but their ratio varied from leaf to leaf. Therefore, the spreading rate of recombinant viruses through the phloem is approximately equal and the difference in levels of CP-M2e accumulation might be caused by the processes occurring at the exit from the phloem to mesophyll cells. Chimeric ser/ala particles showed high immunogenicity. Mice vaccinated by these virions were resistant to five lethal doses (LD50) of homologous and heterologous Influenza A/PR/8/34 (H1N1) and A/California/04/2009 (H1N1). Henceforth, TMV-M2e-ala/ser appear to be an appropriate experimental model system convenient for studying poorly known aspects of plant-virus interactions.

Role of myosins in Tobacco mosaic virus movement

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Plant viruses are obligate intracellular pathogens, which depend on host factors to complete their life cycle. Cell-to-cell movement of plant viruses occurs through plasmodesmata (PD), channels that enable intercellular molecular trafficking. Viral movement protein (MP) increases the size exclusion limit of PD to facilitate the passage of virus particles or nucleoproteins. Recently, several studies showed that the intra- and intercellular movement of plant and animal viruses is myosin-dependent, however the exact role of myosins in this processes is not fully understood. Myosins are actin-based molecular motors that drive organelle movements in plants. To gain insight into the role of myosins in plant virus transport, we examined the cell-to-cell and systemic movement of GFP-tagged Tobacco mosaic virus (TMV) upon expression of dominant-negative *Nicotina benthamiana* myosin tails. We found that the overexpression of the dominant-negative forms of three class VIII myosins and two class XI myosins decreases the local and long-distance movement of the virus. This finding is correlated with effects on the targeting of the viral MP to PD as well as on the accumulation pattern of the 126k replicase subunit suggesting a role of myosins in the subcellular targeting of these viral proteins. To gain further insight into the underlying mechanism we are currently investigating whether there are direct or indirect interactions of myosins with the MP and the 126k protein during both transient expression and viral infection. The role of myosins is also tested in *Arabidopsis* using available KO mutants affected in single or multiple class VIII and class XI myosins.

Symplasmic and vascular movement of turnip mosaic virus proceed through membrane-associated viral complexes

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Replication by plant positive-sense RNA viruses induces the formation in the infected cell of elaborate membranous organelle-like platforms that sustain viral RNA synthesis. Turnip mosaic virus (TuMV) infection leads to significant rearrangements of the early secretory pathway. Infection is associated with the formation of at least two distinct types of sub-cellular compartments: a perinuclear globular structure and cortical endoplasmic reticulum (ER)-associated and motile vesicular structures. The viral protein 6K2 has a transmembrane domain (TMD) that is responsible for membrane alterations. The N-terminal soluble tail of the protein has an ER-export signal that is required for cell-to-cell infection. The TMD of 6K2 contains a motif that prevents the fusion of 6K2 vesicles with the plasma membrane on their way to plasmodesmata. The perinuclear globular structure contains ER, Golgi, and COPII coatomers, along with viral RNA and viral replication proteins. These structures are not isolated organelles and are dynamically connected to the bulk of non-modified endomembranes. The 6K2-tagged motile vesicular structures, which also contain viral RNA, are derived from the globular structure and move along transvacuolar ER tubules toward the plasma membrane and dock at plasmodesmata. TuMV infection increases the size exclusion limit of PDs that allows transit into adjoining cells of 6K2 vesicles. Live cell imaging shows that 6K2 vesicles traffic rapidly to the cell periphery and move across the cell wall into adjacent cells. 6K2-tagged and membrane-bound aggregates that contained viral RNA are also found in xylem vessel. We propose a model whereby symplasmic and vascular movement of TuMV is achieved by a membrane-associated viral complex associated with 6K2.

***Frankliniella occidentalis* proteins involved in the interaction with and the response to *Tomato spotted wilt virus* infection of the insect vector**

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Tomato spotted wilt virus (TSWV), the type species of the *Tospovirus* genus, is transmitted by thrips in a persistent propagative fashion. The western flower thrips, *Frankliniella occidentalis*, is one of the most important vectors of TSWV. Once TSWV is acquired by *F. occidentalis*, it journeys through the insect body and replicates in the midgut and salivary gland tissues for transmission to occur. However, the molecular basis of the interactions that result in TSWV entry, replication and spread in the insect vector is largely unknown. The overall goal of this work was to identify thrips proteins that directly interact with TSWV and those that respond to virus infection of the insect vector. Using two-dimensional (2-D) gel electrophoresis, we identified 26 protein spots that displayed differential abundances in response to TSWV infection. From these protein spots, 62% were down-regulated by TSWV infection. Mass spectrometry coupled with Mascot searches resulted in the identification of 37 thrips proteins within the 26 spots. Thirty two of these proteins received gene ontology assignments that revealed biological roles associated with the infection cycle of other plant- and animal-infecting viruses and antiviral defense responses. Furthermore, we have conducted virus overlay assays using 2-D gels to identify larval thrips proteins that directly interact with TSWV. Our experiments revealed that seven unique protein spots specifically interacted with purified TSWV virions. Identification of thrips molecules that are potentially involved in transport, viral replication, and innate immune responses provides new insights into the molecular basis of this interaction. Ultimately, this knowledge has generated a suite of candidate genes that will enable the development of novel strategies to control thrips damage and tospovirus transmission.

Amino acid changes in the N-terminal region of the coat protein facilitate long-distance movement of Plum pox virus and allow it to adapt to new hosts

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Plum pox virus (PPV) is a potyvirus that causes sharka, a severe disease of Prunus trees. In addition to its natural Prunus hosts, PPV can also infect different herbaceous species. Previous studies in our lab with chimeric viruses between PPV-R and PPV-D, two strain D isolates with high sequence similarity but different host specificity, identified pathogenicity determinants related to host adaptation in the N-terminus of the CP. More specifically, chimeras containing a genomic fragment of the 3' region of PPV-D –including the sequence coding for the CP– in a PPV-R backbone were not infectious in Prunus persica, but systemically infected Nicotiana clevelandii and N. benthamiana when specific amino acids (aa) were modified or deleted in a short 30-aa region of the N-terminus of the CP. Here we introduced several of these mutations in both PPV-R/D chimera and PPV-D c-DNA clones to evaluate their effect on PPV infectivity in N. clevelandii and Prunus persica, respectively. In N. clevelandii, the results confirmed that the specific amino acid changes in the N-terminus of the CP increased virus infectivity as they enabled PPV to systemically spread and therefore to adapt to this herbaceous host. Interestingly, these changes that enhanced long distance movement of PPV did not affect its local accumulation in the inoculated tissue. In Prunus, most of these mutations did not significantly affect virus infectivity. However a point mutation and, especially, a large deletion of 30 aa impaired its systemic spread. A dN/dS analysis of CP sequences from public databases showed that, although all the point aa modifications selected in Nicotiana plants were present in field isolates infecting Prunus species, the affected residues are not under positive selection in natural infections. This suggests that these residues may not be contributing significantly to PPV diversification in Prunus hosts. We propose a model in which the N-terminus of the CP is targeted by the host defense machinery in Nicotiana. Mutations in this short CP region allow PPV to overcome the defense response and adapt to this host but sometimes with a trade-off in its ability to systemically spread in other hosts such as Prunus.

Profiling of phloem specific gene expression upon infection by a systemic and a phloem-restricted virus

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Plant viruses exploit the vascular phloem system of their hosts to promote their systemic infection. Several viral factors implicated in virus long-distance movement have been identified, whereas only few plant proteins involved in this process were described yet. Moreover, viruses trigger host gene deregulations that may be part of the plant defence pathways or of an offensive strategy to exploit cellular mechanisms to promote systemic propagation of the virus.

To address this issue, a cell-type specific transcriptomic approach has been undertaken in *Arabidopsis thaliana* with two viruses belonging to different genera: the Potyvirus Lettuce mosaic virus or LMV (filamentous virions, infecting all cell types) and the Polerovirus Turnip yellow virus or TuYV (icosahedral particles, restricted to phloem cells). Despite their differences in virion morphology, genome organisation and tissue tropism, both viruses use the phloem vasculature to traffic over long distances through the host. Transgenic *A. thaliana* plants expressing the GFP under the phloem companion-cell specific AtSUC2 promoter (Imlau et al., 1999) were infected with either LMV or TuYV and used to isolate protoplasts. Fluorescent protoplasts were then sorted by FACS (fluorescence-activated cell sorting) and RNA was extracted and processed by genome-wide micro-arrays (CATMA) or RNA Seq technology (Illumina). Despite variability between experiments related to a late infection time point, a set of up or down-regulated genes was identified for each virus. The number of genes that underwent reprogramming of the phloem cell transcriptome during infection with each virus was low (103 for LMV and 57 for TuYV) and very few common genes were found deregulated by both viruses. For each virus a specific set of co-deregulated genes belonging to the same biological pathway was identified. A cluster of genes involved in sulfur metabolism appeared to be repressed in LMV-infected phloem cells while several genes involved in the glucosinolate metabolic process were elicited by TuYV. The deregulation of the different genes of each pathway was confirmed by quantitative RT-PCR. To assess the potential role of these genes in virus long-distance movement, *A. thaliana* knock-out (KO) mutant lines were selected for the candidate genes and are currently challenged with either virus. As some of the candidate genes belong to small gene families with potentially functional redundancy, production of double or triple mutants is in progress to further test their behaviour upon infection.

Un evolved 5' untranslated region of the Alfalfa mosaic virus RNA 3 suppresses the virion requirement for the viral transport.

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After the start of the infection in a single cell, plant viruses need to invade the adjacent cells, a process denominated cell-to-cell transport, as a previous step to invade the distal parts of the host through the vascular system or systemic transport. The capacity to reach the uninoculated parts of a plant implies that the virus should infect specific cells located at the vascular tissue. In most cases, virus particles are required for this vascular transport. In the present study we have addressed the characterization of viral determinants critical for the long distance transport using the Alfalfa mosaic virus (AMV) model system, which requires virus particle for the systemic transport. AMV is the type member of the Alfamovirus genus within the family Bromoviridae. Its genome consists of three positive RNAs. Monocistronic RNAs 1 and 2 encode P1 and P2 proteins of the RNA polymerase complex, respectively. RNA 3 contains two open reading frames encoding the movement protein (MP) and coat protein (CP), which is expressed through a subgenomic RNA or RNA 4. Previous analysis showed that the AMV MP gene is functionally interchangeable for long and short distance transport by the corresponding gene of viruses belonging to eight genera of the viral family 30K (Sanchez-Navarro et al.; 2006 *Virology* 341: 66-73; Sánchez-Navarro et al., 2010, *J. Virology* 84: 4109-4112). However, the exchange of the Brome mosaic virus MP lacking the C-terminal 48 amino acid residues, generated a chimeric RNA 3 (MPBMV255/CP) defective for long-distance transport (Sánchez-Navarro et al., 2001; *MPMI* 14: 1051-1062). In this study we performed viral evolution experiments, addressed to characterize RNA 3 determinants of the chimera MPBMV255/CP critical for systemic transport. After the seventh passage, we observed systemic infection in all lines. The analysis of the nucleotide sequence revealed that all RNA 3 variants present in the upper parts of the plants contained deletions at the 5' untranslated region (5' UTR). Further analysis of the evolved 5'UTR revealed that this region, in spite of reducing the expression of the MP and drastically the encapsidation of the viral progeny, incremented the cell-to-cell transport. Interestingly, we observed that the modified 5'UTR permits the systemic transport of an AMV mutant defective in virus particles formation. We also observed that the modified 5'UTR incremented the systemic transport of AMV RNA 3 chimeras expressing the MP of Tobacco mosaic virus, Brome mosaic virus, Cucumber mosaic virus or Prunus necrotic ringspor virus. Altogether, these results permit argue the hypothesis that the MP is sufficient to allow the local and systemic transport of plant viruses without the requirement of the virion formation. The evolutive implications of these observations for the cell-to-cell and systemic transport of plant viruses will be discussed.

The G_N glycoprotein of *Tomato spotted wilt virus* (TSWV) inhibits virus acquisition and transmission by thrips.

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Tomato spotted wilt virus (TSWV) is transmitted in a persistent-propagative manner by thrips species, including *Frankliniella occidentalis*. We demonstrated previously that a soluble form of the TSWV G_N protein (G_N-S) binds to *F. occidentalis* midguts and inhibits virus acquisition and subsequent transmission. We hypothesized that G_N-S expressed in plants would reduce virus titer in the vector and the efficiency of virus transmission to plants. To test this hypothesis, we generated transgenic tomato plants expressing G_N-S. We inoculated transgenic tomato plants with TSWV and performed acquisition and transmission assays. The transgenic and non-transgenic tomato plants supported similar TSWV titers when measured by DAS-ELISA. Thrips given an acquisition access period (AAP) on virus-infected transgenic G_N-S tomato plants had significantly lower virus titers and transmission efficiencies than thrips given an AAP on TSWV-infected non-transgenic tomato plants. These results demonstrate that an initial reduction in virus infection of the insect midgut can result in a significant decrease in virus titer and transmission over the life-span of the vector. The transgenic G_N-S plants are a valuable tool for basic studies of G_N-thrips interactions and field experiments examining the utility of the transgenic plants in preventing secondary spread of TSWV.

Use of RNAi-based strategies to interfere with the expression of ribosomal-like and cuticular aphid genes potentially involved in vector transmission of plant potyviruses

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Transmission by homopteran insect vectors is the most common dissemination method of plant viruses. In particular, aphids are vectors of numerous viruses, including potyviruses (family Potyviridae) which are transmitted in a non-persistent manner, with a viral auxiliary factor, the HCPro protein, mediating the reversible retention of virions on the aphid stylet. Using purified HCPro as bait, aphid proteins specifically interacting with the potyviral HCPro of Tobacco etch virus (TEV) have been identified. To functionally validate the involvement of selected candidate genes in virus dissemination, we are exploring RNAi-based strategies to knock down their expression in aphids. Two procedures for RNAi are being evaluated: one is based on feeding insects using artificial diets supplemented with dsRNAs generated in vitro, and the other uses a Tobacco rattle virus (TRV)-based expression vector containing fragments of the targeted genes to induce accumulation of specific siRNAs in planta. After feeding on the artificial diets containing dsRNAs, or on plant tissues producing siRNAs, aphids were analyzed by quantitative RT-PCR to measure accumulation of the targeted gene transcripts. While a highly expressed cuticular protein, RR1Cp2, was drastically reduced in *Myzus persicae* (green peach aphid) after the RNAi treatments, only a limited reduction was achieved in the case of RPS2, a ribosomal-like protein that has lower expression levels than the reference gene, RPL7. These results suggest that the type of gene and its expression level might influence the silencing response. In addition to providing new tools for understanding the mechanisms of virus transmission, the described RNAi methodologies have potential applications in designing novel strategies for virus control based on targeting the expression of specific genes that are essential for transmission in the insect vector.

The fast and the furious – Enhanced movement properties of the Oilseed rape mosaic tobamovirus movement protein

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Our knowledge of tobamovirus intra- and intercellular movement mechanisms is largely based on studies using Tobacco mosaic virus (TMV) as model. TMV cell-to-cell movement depends on the virus-encoded movement protein (MP) and involves interactions of the MP with the ER, plasmodesmata (PD), and the cytoskeleton. However, it remains unknown whether other crucifer-infecting tobamoviruses use similar movement mechanisms for spread. To further explore the movement mechanisms of Arabidopsis-infecting tobamoviruses, we determined the subcellular accumulation patterns of the MP of Oilseed rape mosaic virus (ORMV, also termed Youcai mosaic virus, YoMV) and analyzed the efficiency of this protein to promote virus cell-to-cell movement. Similar to the MPs of TMV and Turnip vein clearing virus (TVCV), ORMV MP localizes to PD and to small, mobile particles in the cell cortex. However, ORMV MP does not share the cytoskeletal and nuclear accumulation patterns shown by TMV MP and TVCV MP, and ORMV cell-to-cell spread is not significantly influenced by treatments to inhibit the secretory pathway, or the cytoskeleton. However, we observed that the ORMV MP accumulates to much lower levels as compared to the MP of TMV; moreover, this protein interacts with the TMV MP and the interaction between the two MPs leads to recruitment of the ORMV MP to the subcellular localizations at which the TMV MP accumulates. This interaction as well as coexpression of ORMV MP with the tombusvirus silencing suppressor P19 or the treatment of the cells with proteasome inhibitor stabilize the protein. This indicates that the accumulation of the ORMV MP is sensitive to silencing suppression and degradation by the 26S proteasome, and that the TMV MP protects ORMV MP from these processes. A chimeric TMV-construct expressing the MP of ORMV instead of its own MP exhibits enhanced cell-to-cell spread, which may be consistent with the strong infectivity of ORMV in Arabidopsis. Taken together, our results highlight specific functions of an MP of an Arabidopsis-infecting tobamovirus and help to better interpret functional genomics and proteomics studies currently undertaken to understand tobamovirus-Arabidopsis interactions.

TMV movement in the context of plant defense responses

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Plant development depends on intercellular communication through cytoplasmic cell wall channels known as plasmodesmata (PD) and involves the cell-to-cell trafficking of macromolecules. Tobacco mosaic virus (TMV) and other viruses use this route to spread their genomes and cause systemic infection. The spread of TMV RNA (vRNA) depends on virus-encoded movement protein (MP) and occurs in a non-encapsidated form, likely through exploitation of the cellular RNA transport machinery. We have developed in vivo tools to investigate transport processes in virus movement at the protein and RNA level, by which we gain evidence for a role of mobile RNA particles that target PD through interactions with membranes and the cytoskeleton. Our studies also focus on the role of small RNAs in virus movement. Interestingly, TMV interacts with the silencing host response in different ways. Whereas the viral 126k/183k replicase acts as a suppressor of RNA silencing, the MP promotes the spread of silencing. Since virus infection produces a unique population of virus and host-derived small RNAs, we are interested to understand whether these small RNAs may play a role in a viral strategy to influence host cell susceptibility with the help of MP. Other parts of our work concentrate on cellular and long-distance signaling responses elicited by the virus. Our aim is to link the cell biology of virus movement to a better understanding of virus-induced defense and signaling responses and thus to attain a more complete picture of mechanisms involved in compatible virus:host interactions. These studies also provide important insights into plant intercellular communication mechanisms and leads for the development of antiviral strategies in crops.

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A trio of viral proteins tune aphid-plant interactions in Arabidopsis

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Virus-induced changes in metabolism affect interactions of plants with insect vectors, such as aphids, leading to suggestions that this might promote transmission of viruses to new hosts. Aphid growth and feeding behaviour was altered on squash and tobacco by infection with cucumber mosaic virus (CMV) but the mechanism(s) underlying this are unknown. We found that in *Arabidopsis thaliana* two CMV proteins, the 2a replicase component and 2b RNA silencing suppressor, induce distinct types of resistance to the aphid, *Myzus persicae*. During CMV infection, the 2a protein activated defensive signalling associated with pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), which caused accumulation of 4-methoxy-indol-3-ylmethylglucosinolate (4MI3M), an aphid feeding deterrent. Feeding deterrence may promote virus transmission by encouraging migration of virus-bearing aphids. Transgenic plants expressing 2b and mutant plants compromised in expression of Argonaute1 (AGO1), a molecular target of the 2b protein, were resistant to aphids. However, this resulted not from feeding deterrence but from increased plant toxicity to the insects. Experiments with double transgenic plants expressing 2b and another CMV-encoded protein, the 1a protein, suggested that 2a-induced feeding deterrence predominates in CMV-infected plants because 1a interferes with 2b-induced aphid resistance. Our results show how an extended viral phenotype (altered host interactions with an insect vector) emerges from the impact of multiple viral proteins on plant defensive signalling.

A novel cuticular protein of homoptera insect mediated transmission of a plant arbovirus

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Numerous viruses can be transmitted in persistent propagative manner by corresponding vector insects. However, the molecular mechanisms enabling virus transmission by vector insects have been poorly understood. Here, we took advantage of unique biological tools, including yeast two-hybrid assay, immunofluorescence, and RNA interference induced by synthesized dsRNA, to investigate the molecular transmission mechanisms of Rice stripe virus (RSV), a plant arbovirus belongs to the genus Tenuivirus, by its insect vector (*Laodelphax striatellus*). The *L. striatellus* cDNA library was screened by RSV pc3 as a bait protein. After screening, we acquired more than 300 positive clones that encoded 114 proteins after sequencing and assembly. To identify the true interaction and eliminate the false positives, seventeen interactors were selected for cotransformation to confirm the interactions. The confirmation results demonstrated that 12 proteins could interact with RSV pc3 in yeast. In a Co-IP analysis, the relative strength of the interaction between interactors from high to low was in the order of RSV pc3 and NCuP, RSV pc3 and Atlatin, RSV pc3 and Jagnual, RSV pc3 and NAC. RSV pc3 and NCuP had the strongest interaction that was 14 times higher than the negative control. This protein interacted with the nucleocapsid N protein, pc3, of RSV in vivo and in vitro. It also colocalized with RSV-pc3 in the cells and hemocytes of *L. striatellus*. The quantity and transmission efficiency of RSV in NCuP dsRNA-injected insects were significantly lower than controls. Pathogens transmitted by insect vector must traverse the insect's hemocoel during their journey from the midgut to the salivary glands, and this obligate migration places them in direct contact with the circulatory and immune systems of the insect. It is possible that this novel cuticular protein binding by the virus may help it escape immune attack in the hemolymph of the vector. Our findings represent an unprecedented advance toward opening the mechanisms enabling viral transmission by definitively clarifying that viruses can use existing vector proteins to protect themselves from degradation in the hemolymph. Thus, identifying these putative vector molecules could lead to new disease control strategies.

Session 3: RNA silencing

Utilising virus-induced gene silencing (VIGS) to dissect cereal plant-fungal interactions

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Virus-induced gene silencing is a powerful tool for down-regulation of expression of target plant genes. It allows moderately high throughput screening of candidate genes of interest, and many different plant genotypes can be tested simultaneously. We have established a Barley stripe mosaic virus-mediated VIGS (BSMV-VIGS) system at Rothamsted Research to allow us to study gene function in wheat and barley. In particular, we have focused on using BSMV-VIGS to identify the defence signalling networks and defence mechanisms causally involved in host responses to infection by the economically important fungal pathogens *Mycosphaerella graminicola* (Mg) and *Fusarium graminearum* (Fg). Fg, the causal agent of the damaging disease Fusarium head blight in wheat, attacks the floral and stem base tissues of cereals and a few non-cereal species, whilst the exclusively foliar pathogen Mg only infects durum and hexaploid wheat. Despite these differences, there are important similarities in the way these two Ascomycete fungal pathogens infect and cause disease in wheat tissue. We are elucidating the role of the chitin (fungal cell wall) recognition machinery in the Mg-wheat interaction, and studying the role of the chloroplast in the tissue specificities of Mg and Fg in wheat. Deep transcriptome sequencing (RNA-Seq) from both interactions has identified many plant genes and pathways that show significant changes in expression levels during different stages of the plant infection process. VIGS will allow us to rapidly pre-screen a number of candidate genes to determine whether they are involved in resistance or susceptibility to Mg or Fg.

We are also investigating the inheritance of VIGS in the progeny of virus-inoculated plants. There is significant partial or complete loss of 'foreign' gene sequences inserted into the BSMV vector, particularly during vertical transmission of the virus. However, in cases where silencing is retained in the progeny of BSMV-inoculated plants, the silencing phenotype appears to be stable in the subsequent generation(s). We wish to understand the mechanisms controlling insert stability, and to identify features that are in common between the minimal foreign sequences retained in the VIGS vector and what constitutes the efficient gene silencing trigger. This latter work will aid our on-going strategies to improve the BSMV-VIGS system in order to maximise VIGS insert stability and minimise symptom induction. We envisage that our results may also provide additional insights into the BSMV-host interaction itself.

Reference: Lee et al. (2012) Barley stripe mosaic virus-mediated tools for investigating gene function in cereal plants and their pathogens. *Plant Physiol.* 160, 582-590.

RNA silencing pathways may have a positive effect on Potato spindle tuber viroid infectivity in *Nicotiana benthamiana*

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Viroids constitute the smallest known agents of plant infectious diseases and have often agronomic importance. They are single stranded circular RNAs, classified in two families, and do not encode any protein. Potato Spindle Tuber Viroid (PSTVd) is the type species of Pospiviroidae family, with a size varying from 341 to 361nt depending on the isolate, and replicates in the nucleus in an asymmetric rolling-circle mechanism. PSTVd infection leads to accumulation of abundant viroid small interfering RNAs (vd-siRNAs), capable of triggering RNA degradation of homologous sequences. Yet, PSTVd genomic RNA itself seems to be resistant to siRNA-directed degradation. Sequencing of PSTVd siRNAs after infection indicates that the rod shape-like structure of the mature PSTVd ssRNA, rather than the double stranded RNA (dsRNA) molecules produced during its replication, may serve as substrates for Dicer-like (DCL) enzymes. In order to examine the role of RNA silencing upon PSTVd infection, we produced and characterized *Nicotiana benthamiana* (Nb) knock down lines for the four NbDCL-like enzymes. Subsequently, these lines were infected with the viroid and PSTVd accumulation was estimated. Our analyses showed that infectivity is facilitated by DCL4, but is unaffected by DCL2 and probably DCL3. We also produced and characterized different combinations of DCLi knock down plants by crosses of the original lines. The effect of the combined suppression of DCL enzymes on viroid infectivity will be discussed. Collectively, our results indicate that RNA silencing pathways and/or proteins may be advantageous for viroid infectivity.

Effects of the crinivirus CP-interacting plant protein SAHH on post-transcriptional RNA silencing and its suppression

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In plants, post-transcriptional gene silencing (PTGS) is a sequence-specific mechanism of RNA degradation induced by double-stranded RNA (dsRNA), which is processed into small interfering RNAs (siRNAs). siRNAs are methylated and thereby stabilized by the activity of the S-adenosylmethionine-dependent RNA methyltransferase HEN1. PTGS is amplified by host-encoded RNA-dependent RNA polymerases (RDRs), which generate dsRNA that is processed into secondary siRNAs. To counteract this RNA silencing-mediated response of the host, plant viruses express proteins with silencing suppression activity. We reported the coat protein (CP) of the crinivirus (family Closteroviridae, genus Crinivirus) Tomato chlorosis virus as a suppressor of silencing. In this work we demonstrated that ToCV-CP interacts with S-adenosylhomocysteine hydrolase (SAHH), a plant protein essential for sustaining the methyl cycle and S-adenosylmethionine-dependent methyltransferase activity. This protein was shown to enhance local RNA silencing by contributing to an increased accumulation of secondary siRNAs generated by the action of RDR6. Downregulation of SAHH prevents local silencing but enhanced the spread of systemic silencing. Our results also show that SAHH is important in the suppression of local RNA silencing not only by the crinivirus Tomato chlorosis virus CP, but also by the multifunctional helper component-proteinase HC-Pro of the potyvirus (genus Potyvirus, family Potyviridae) Potato virus Y.

A new role for DCL2 in plant antiviral resistance

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Plants prevent viral infection through RNA silencing. Virus-derived small interfering RNAs produced by Dicer ribonucleases DCL4 and DCL2 guide silencing complexes to viral RNA for its destruction. The current model states that the two Dicers have redundant, but hierarchical functions in antiviral silencing, such that DCL4 is more important than DCL2. We are investigating the alternative hypothesis that DCL4 and DCL2 perform entirely different functions in antiviral immunity: while DCL4 mediates antiviral silencing, DCL2 detects suppression of RNA silencing, and constitutes a sensor for accumulation of viral RNA to activate immune receptors.

Role of plant RNA-silencing pathways in recovery from viral disease symptoms

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Virus-infected plants that initially show disease symptoms may start to develop symptom free new leaves, a phenomenon known as recovery. This phenomenon has indirectly been linked to anti-viral RNA silencing but the proteins involved remain unknown. Here we present a system to study recovery from RNA viral disease in *Arabidopsis thaliana*. Upon infection with the Oilseed rape mosaic tobamovirus (ORMV), *Arabidopsis* plants develop severe symptoms. However, after about 25 days post inoculation first non-symptomatic leaves appear. The recovered leaves still contain virus but viral silencing suppressor activity is absent or only detected in the mid-rib. To identify the pathways involved in recovery, we analysed ORMV infection and subsequent disease symptom formation and recovery in specific *Arabidopsis* mutants. As anticipated, mutants impaired in 21nt siRNA-mediated post-transcriptional gene silencing (PTGS) (*rdr6*, *dcl2*, *dcl4*, *ago1*, *hen1*, *sgs3*) showed delayed or no recovery from symptoms, while mutants with defects in miRNA pathways (*hyl1*, *dcl1*) recovered as wild type plants. Interestingly, mutants of 5'-3' exoribonuclease XRN4 (*xrn4-3*, *ein5*), a known endogenous RNA silencing suppressor, recovered faster than wild type plants. Together these observations emphasize the importance of PTGS in the onset and maintenance of recovery.

Analysis of virus induced leaf developmental abnormalities in tomato

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Virus infected plants display various symptoms with different type and severity and these symptoms can reduce the crop yields enormously. Although the molecular mechanisms behind the virus induced disease symptoms are in the focus of many research groups, however the process still poorly understood. In recent years RNA silencing pathways are very intensively studied. In plants RNA silencing is a natural defence mechanism against invading viruses. During this process the replication intermediers of viruses are diced by plant DCL enzyme to 21-24 nt RNAs (vsiRNA). VsiRNAs incorporates into AGO complex and this complex can cleave further virus RNAs. As a response to this defence system viruses has developed silencing suppressors which repress the plant RNA silencing system. RNA silencing has a role not only in the defence against pathogen infection but also in the regulation of plant endogenous genes. Endogenous small RNAs are involved in developmental processes and regulation of stress response. During virus infection, virus derived small RNAs or RNA silencing suppressor of the virus can interact with host endogenous small RNA pathways and with mRNAs regulated by these pathways and modify their expression. In our experimental system we use two isolates of cucumber mosaic virus (CMV): Nt-CMV causes mosaic symptoms and Sco-CMV causes serious leaf developmental abnormalities (called shoestring symptoms) on tomato. This symptom is very similar to the phenotype of an RNA silencing mutant (rdr6) tomato. The gene product of RDR6 is essential for the production of trans-acting siRNAs. Based on these facts there is a possibility that the virus interfere with the plant endogenous small RNA system or with the expression of leaf polarity genes. We are studying the molecular background of Sco-CMV and try to understand what kind of viral genes and virus derived smallRNAs are responsible for the serious leaf developmental abnormalities and how do they influence the expression pattern of endogenous mRNAs and smallRNAs.

Acknowledgement: This work was supported by OTKA (grant numbers: K-101793 and K-106170).

Reassortment of Beet necrotic yellow vein virus and Beet soil-borne mosaic virus RNAs-1 and -2 indicates a specific interaction of silencing suppressor proteins and RNA-1

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The process of recombination that takes place in RNA viruses corresponds to the formation of chimeric molecules from parental genomes of mixed origin. This process can occur either within a single genomic segment (referred to as RNA recombination) or as exchange of entire genomic segments between multipartite viruses (referred to as reassortment). Both RNA recombination and reassortment require that two or more viruses infect the same host cell.

Multipartite genome, formed by four ssRNAs(+), transmission through the plasmodiophorid *Polymyxa betae*, host range and genome organization are proprieties shared by the two Benyviruses Beet necrotic yellow vein virus (BNYVV) and Beet soil-borne mosaic virus (BSBMV). Recent studies demonstrated amplification and transmission of BSBMV RNA-3 and -4 by BNYVV helper strain. Moreover, in the United States of America, both benyviruses are frequently present in the same cultivated field, infecting the same plant but no natural chimeric forms have been described, from field isolates, so far. The possibility that BNYVV/BSBMV chimeras may be generated has been investigated. Chenopodium quinoa local infection has been carried out using in vitro infectious transcripts of BNYVV and BSBMV RNA-1 and -2 and the behavior of BSBMV/BNYVV chimeras and wild type helper strains has been compared. The wild type combinations Stras12 (BNYVV RNA-1 + -2) and Bo12 (BSBMV RNA-1 + -2) together with the chimera StrasBo12 (BNYVV RNA-1 + BSBMV RNA-2) showed typical chlorotic lesions, while the chimera BoStras12 (BSBMV RNA-1 + BNYVV RNA-2) induced severe necrotic lesions on the leaves, probably due to a hypersensitive response of the plant. The necrosis disappeared when the plant was co-inoculated with BoStras12 together with a viral replicon expressing BSBMV p14, which acts as a suppressor of post-transcriptional gene silencing, but not when BNYVV p14 was employed. Necrotic lesions arose even in *N. benthamiana* plants agroinfectated with BoStras12, both in the agroinfiltrated and not infiltrated leaves. These results evidenced a putative specific interaction between BSBMV p14 and RNA-1, or one of its expressed components, of the virus that requires further investigations and may explain why benyviruses chimeras did not arise in nature so far.

Cocksfoot mottle virus coat protein is dispensable for the systemic infection and suppresses RNA silencing

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Sobemovirus is a small genus of plant viruses with single-stranded positive-sense RNA genome. We have previously reported that P1 of cocksfoot mottle sobemovirus (CfMV) is indispensable for virus movement and accumulation in oats and that it is a suppressor of RNA silencing. Viruses can encode more than one RNA silencing suppressor and the P1 protein has been shown to interact with the coat protein (CP). In the current study we demonstrated that CP of CfMV is an additional RNA silencing suppressor. We also analysed how CfMV CP substitution and deletion mutants move in different host plants because the CP of some sobemoviruses has been reported to be required for systemic virus movement. Surprisingly, all mutant viruses were able to infect the three tested host plants systemically, although usually with reduced accumulation. In addition, the movement of CfMV was characterised with viruses expressing EGFP fused to the C-terminus of CP. EGFP expression was detected in epidermal and mesophyll cells of the inoculated leaves. Although EGFP fluorescence was not detected in upper leaves, some plants displayed CfMV symptoms. Analysis of the upper leaves revealed that the viruses had lost the EGFP sequence and sometimes also most of the CP gene. In conclusion we demonstrated that CfMV CP suppresses RNA silencing and is dispensable for systemic movement. We propose that CP enhances virus accumulation suppressing RNA silencing.

Biochemical properties of the viral suppressor/activator of RNA silencing P1 encoded by the Rice Yellow Mottle virus

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Viral suppressors of RNA interference (VSRs) are remarkable multifunctional proteins that play important roles in the viral cycle as well as in the host cells. In particular, VSRs are key components of viral machineries for counteracting host innate immune responses by targeting different components of the host gene silencing pathways (1, 2). However the biochemical and structural properties that allow VSRs to ensure several functions remain poorly understood. We analyzed the biochemical features of the multifunctional and dual VSR P1 encoded by the rice yellow mottle virus (RYMV) (3-4). In silico analyses of P1 suggested common features with zinc finger proteins. Using proteomic-based mass spectrometry approaches and SDS-PAGE redox shift assays on recombinant P1 protein, we unambiguously demonstrated that P1 reversibly binds two zinc atoms with different strengths (5). A zinc-binding domain mapping using recombinant truncated P1 proteins allowed us to precisely locate the position of zinc atoms in P1. We next found that P1 oxidation by H₂O₂ leads to disulfide bond formation along with zinc release and oligomerization. Zinc release and subsequent conformational changes occurred in an H₂O₂ dose-dependent manner, and was also observed using truncated P1 or other oxidant compounds. More strikingly, zinc release in P1 was found to be reversible, a redox-dependent zinc binding/release change only reported for a limiting number of Zinc fingers and never report for VSR (5). Finally, we found that P1 undergoes complex oligomerization under oxidative conditions in vitro and identified key determinants in responsible for conformational change in P1 structure. Consistent with our in vitro data, complex oxidized oligomeric forms of P1 evolving throughout infection by RYMV were also detected in rice tissues. Our results provide a first link between P1's redox-dependent flexibility and complex P1 patterns in planta. Our current studies aim at analyzing more deeply the redox reactivity of the different domains in P1 in order to decipher how they orchestrate its redox-dependent flexibility. Recent spectroscopy and SDS-PAGE redox shift assays on several truncated P1 proteins already demonstrated that the two zinc binding domains exhibit differential sensitivity towards oxidant molecules, consistent with structural differences suggested by in silico analyses. We are also solving P1 structure using crystallography and NMR to go further in the understanding of P1 structure-functions relationships.

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Geminivirus Rep protein interferes with the plant DNA methylation machinery and suppresses transcriptional gene silencing

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Cytosine methylation is an epigenetic mark that promotes gene silencing and plays an important role in genome defence against transposons and invading DNA viruses. Previous data showed that the largest family of single-stranded DNA viruses, Geminiviridae, prevents methylation-mediated transcriptional gene silencing (TGS) by interfering with the proper functioning of the plant methylation cycle. We describe a novel counter-defence strategy used by geminiviruses, which reduces the expression of the plant maintenance DNA methyltransferases, METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), in both locally and systemically infected tissues.

We demonstrated that the virus-mediated repression of these two maintenance DNA methyltransferases is widespread among geminivirus species. Additionally, we identified Rep (Replication associated protein) as the geminiviral protein responsible for the repression of MET1 and CMT3, and another viral protein, C4, as an ancillary player in MET1 down-regulation. The presence of Rep suppressed TGS of an *Arabidopsis thaliana* transgene and of host loci whose expression was strongly controlled by CG methylation. Bisulfite sequencing analyses showed that the expression of Rep caused a substantial reduction in the levels of DNA methylation at CG sites.

Our findings suggest that Rep, the only viral protein essential for replication, displays TGS suppressor activity through a mechanism distinct from that thus far described for geminiviruses.

Virus-derived small interfering RNAs as pathogenicity factors in Potato virus Y - *Solanum lycopersicum* interactions

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RNA silencing (RS) is a conserved eukaryotic mechanism acting in plants also as antiviral immune system. A successful virus infection requires suppression or evasion of host RS. Small interfering RNAs (siRNAs) are potent RS effectors, and accumulate in plants infected by RNA and DNA viruses as components of this plant immune system, providing target specificity for pathogen RNA post-transcriptional degradation. Virus-derived siRNAs (vsiRNAs, 21-24 nt) are abundant and diverse in infected plants. Although any viral genomic regions can potentially be targeted to generating vsiRNAs, certain regions (also referred to as “hot spots”) are more represented than others in vsiRNA sequenced libraries.

Potato virus Y (PVY, Potyviridae) is an important plant pathogen on solanaceous hosts. The two PVY isolates PVYC-to and PVY-SON41 induce very different disease phenotypes on tomato (*Solanum lycopersicum*), the former being responsible of leaf distortions and the latter inducing null to mild symptoms. In this study, we applied combined *in silico* and molecular biological approaches to identify PVY vsiRNAs putatively able to suppress host mRNAs by sequence complementarity and deriving RS-based suppression, a mechanism that would induce dysfunctional processes in infected host plants. As the final aim of the study, we explored differential expression of specific host-targeting vsiRNA by the two isolates as one of the possible sources of diverse disease phenotype expression.

A computational pipeline was implemented to retrieve 21nt vsiRNAs from PVY isolates, complementary to tomato predicted mRNAs (Solgenomics, ITAG2.3). The comparative study used NCBI blast+ package 2.2. RandFold searched secondary structures in the PVY RNA genome containing putative vsiRNAs sequences identified in previous steps.

Two PVYC-to regions showed potential tRNA-like or microRNA-like secondary structures, not present in the other isolate, possibly accounting for vsiRNAs accumulation “hot spots”. Moreover, we obtained two lists of tomato transcripts perfectly or imperfectly (one or two mismatches were allowed, according to typical microRNA recognition sites) complementary to vsiRNA computed for either of the two PVY isolates, and therefore putative target of vsiRNA-driven RS suppression. Putative targets common to the two isolates were discarded, so to identify a list of genes that could represent targets specific to the aggressive isolate PVYC-to only. Some host transcription factors (e.g. NAC, MYB, TCP, HD-ZIP, MADS-box families) active in vegetative development and leaf morphogenesis were selected for further investigation. Quantitative RT-PCR showed differential expression levels of selected host transcripts upon PVYC-to and PVY-SON41 infections, differing also from healthy plants. For some genes, lower isolate-specific mRNA accumulation suggested RS-driven post-transcriptional regulation, thus apparently confirming the starting hypothesis of a correlation between symptoms and RS of host genes complementary to vsiRNAs. Deep sequencing of vsiRNAs from infected tomato leaves was also performed, and results from this analysis are presented and discussed.

Cauliflower mosaic virus: Silencing and silencing suppression

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The plant pararetrovirus Cauliflower mosaic virus undergoes silencing in plants leading to a collection of siRNAs of all three size classes, whereby all four dicers are involved. Silencing is counter-acted by two types of suppressors, a protein-based one conducted by the N-terminal domain of the transactivator/viroplasm (TAV) protein and an RNA-based one conducted by the viral 8S RNA. TAV interferes with DCL4/DRB4 and as a consequence virus dsRNAs and TAS dsRNAs accumulate.

The 8S RNA coincides with the leader of the 35S RNA and lacks a poly-A tail. It is transformed into double-strand RNA and gives rise to massive amounts of siRNAs, despite of the action of TAV. We consider these siRNAs as decoys diverting RISC complexes and thereby protecting the coding region from si-RNA mediated degradation or translation inhibition. This strategy applies also to RTBV and maybe also to other plant pararetroviruses. 8S RNA it is highly structured and therefore an inappropriate RISC-target. As leader of 35S RNA it is a hindrance for ribosome scanning, that has to be overcome by a special mechanism: "shunting". While this leader seems to be a nuisance and complication for translation, the role of 8S RNA as suppressor seems to justify its evolution.

Molecular characterization of Arabidopsis AGO2 during endogenous and virus-induced RNA silencing

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Most eukaryotes use RNA silencing to regulate gene expression during development and to protect themselves against viruses and transposons. In Arabidopsis, post-transcriptional gene silencing (PTGS) is mediated by small interfering RNA (siRNA) and microRNA (miRNA) duplexes of 21-24bp. Upon their processing from double-stranded RNA (dsRNA) by Dicer-like RNase-III enzymes, selected si/miRNA strands are then loaded into Argonaute (AGO) proteins, which guide silencing via target cleavage (or 'slicing') and/or translational repression. The Arabidopsis genome encodes 10 AGO proteins, among which AGO1 is the main effector of PTGS and antiviral RNA silencing. AGO2 also has been implicated in plant antiviral and antibacterial defense, but the bases of its specificity as well as its endogenous functions and putative targets remain largely unknown. In order to investigate these various aspects, we have undertaken an exhaustive analysis of the biochemical and genetic features of this silencing effector.

Using tobacco BY2 cell lysates we confirmed that AGO2 is a bona fide slicing protein, like AGO1. We have also identified putative AGO2-interacting proteins via immunoprecipitation and mass spectrometry analysis from homogenous Arabidopsis cell cultures. Arabidopsis knockout mutants corresponding to these interactors are being currently tested for their effects on AGO2 biochemical properties and AGO2-dependent responses to various infections. Thirdly, we have taken advantage of the specific loading of AGO2 with miR393* to generate a GFP-based reporter of AGO2 activity in adult Arabidopsis. This reporter will be used in a mutagenesis screen to discover new AGO2 genetic interactors that could also regulate plant antiviral responses.

The results of these analyses are expected to provide a detailed picture of the roles of AGO2 during endogenous and virus-induced RNA silencing processes in Arabidopsis.

Control of episomal and integrated banana streak virus in banana plants is mediated by ptgs and tgs respectively

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Banana streak virus (BSV), the causative agent of banana streak disease, is a plant pararetrovirus belonging to the family Caulimoviridae, genus Badnavirus. The genome of BSV is a circular double-stranded DNA of 7.4 kbp made of three ORFs and like other pararetroviruses replicates via reverse transcription of viral pregenomic RNA (Lockhart, 1990). In the mid-nineties Ndowora et al. (1999), revealed the presence of multiple integrations of Banana streak virus in the genome of banana (*Musa* sp.) whereas this badnavirus does not require integration for its replication. Some endogenous BSV (eBSV) sequences in the *Musa balbisiana* genome are infectious by releasing a functional viral genome following stresses such as those existing in *in vitro* culture and interspecific crosses context. Seedy *M. balbisiana* diploid genotypes (BB) such as Pisang Klutuk Wulung (PKW) harbour such infectious eBSV belonging to three widespread species of BSV (Goldfinger -BSGFV, Imové – BSI_mV and Obino l'Ewai - BSOLV) but are nevertheless resistant to any multiplication of BSV whatever the viral origin. We postulated this resistance is mediated by gene silencing and performed deep sequencing of total small RNAs of PKW using the Illumina ultra-high-throughput technology. We obtained for the first time, experimental evidence of virus-derived small RNA (vsRNA) from eBSOLV, eBSGFV and eBSI_mV by blasting small RNAs against the viral genome counterparts. VsRNAs are systematically enriched in 24-nt class. Interestingly, we showed that hot and cold spots of vsRNA generation do not target similar viral sequences but are directly correlated with the structure of each integration. All together, our findings indicate that eBSVs in PKW are likely silenced at the transcriptional level. In parallel, we deep sequenced small RNAs from Cavendish banana plants (lacking eBSV integrations) infected independently by BSOLV, BSGFV, BSI_mV and 3 other distinct BSV species in order to evaluate silencing-based regulation of episomal virus infection. Abundant vsRNAs were found to be enriched in 21-nt class and most of the hot spots of siRNA production are located in the coding sequences. Our methylation analysis indicated that episomal BSV DNA is not methylated and therefore, is likely silenced only at the post-transcriptional level.

Keywords : banana, silencing, BSV, siRNA

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Combining artificial miRNA-based technology with virus-induced gene silencing in functional analysis assays.

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Virus-induced gene silencing (VIGS) is a well-established reverse genetics technology for assessment of gene functions in plants. VIGS is a transient loss-of-function assay that involves three steps: engineering viral genomes to include fragments of host genes that are targeted to be silenced, infecting the plant hosts and suppressing the target genes expression by post-transcriptional gene silencing (PTGS), the defense mechanism deployed by plants against virus infections. Suppression of specific mRNA accumulation allows correlation between gene silencing and the deriving phenotype, providing clues on gene functions. However, the efficiency of this technology may be affected by various factors, including virus vector properties and susceptibility of plant host species. In several cases, weak and/or non-homogeneous distribution in the plant (or in the single leaf) of VIGS may generate results not fully coherent, particularly in terms of correlation between phenotype and accumulation levels of the specifically suppressed RNA. This often limits the extensive application of the technique to more permissive plant species such as *Nicotiana benthamiana*.

Aiming at increasing VIGS efficiency in functional studies, particularly in key crop species, we produced and tested new constructs using a Tobacco rattle virus (TRV)-based vector in tomato (*Solanum lycopersicum*) and other solanaceous crops. This innovative approach consisted in cloning into the TRV vector a short fragment of a host gene containing at its termini mutations designed for the expression of small interfering RNAs (siRNAs) that mimicked a microRNA (miRNA) structure. The recently developed artificial microRNAs (amiRNAs) technology modifies an endogenous gene silencing mechanism that processes natural miRNA precursors to small silencing RNAs targeting transcripts for degradation. Based on natural miRNA structures, amiRNAs are commonly designed to contain mismatches at specific nucleotides with respect to their target sites. Unlike the conventional amiRNA strategy, where target-specific 21nt small silencing RNAs derive from longer double-stranded RNA (dsRNA) precursors that are processed in the nucleus by DCL1, we designed a vector where amiRNA-like small RNAs are generated when viral intermediate dsRNA forms are targeted by the host PTGS machinery in the cytoplasm. In the viral vector, we inserted mutant sequences designed to contain at both their 5' and 3' termini one or two mismatches at selected positions. Mismatched sequences were computed by the WMD3 web tool (wmd3.weigelworld.org), an algorithm that generates all possible amiRNAs using full-length target gene sequences as input. Short (110nt) amiRNA-like containing sequences were compared for their VIGS efficiency with wild-type sequences, shorter- or longer-sized inserts and inverted-repeat constructs. Upon inoculation of our constructs, VIGS established earlier and more extensively than its wild-type counterpart in tomato, *N. benthamiana* and *N. tabacum*. For instance, suppression of the tomato reporter gene magnesium chelatase (ChII or SU) with the VIGS-amiRNA-like construct produced the typical yellow phenotype earlier and more extensively than its wild-type counterpart, and seven days in advance as compared to the standard TRV-PDS (phytoene desaturase) VIGS vector. Quantitative RT-PCR confirmed the efficiency of our VIGS-amiRNA-like constructs in terms of post-transcriptional suppression of host target mRNAs. Our results are discussed in the light of their beneficial contribution to the functional analysis of genes putatively involved in plant-virus interactions.

Functional analysis of RNA silencing suppressors encoded by flexiviruses

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Viruses encode RNA silencing suppressors to counteract host antiviral silencing. In this study, we analyzed RNA silencing suppressors encoded by flexiviruses. First, we analyzed the RNA silencing suppression ability of multiple virus species of the genus Potexvirus. *Nicotiana benthamiana* plants exhibiting RNA silencing of a green fluorescent protein (GFP) transgene showed reversal of GFP fluorescence when systemically infected with potexviruses. However, the degree of GFP fluorescence varied among potexviruses. *Agrobacterium*-mediated transient expression assay in *N. benthamiana* leaves demonstrated that the triple gene block protein 1 (TGBp1) encoded by these potexviruses has drastically different levels of silencing suppressor activity, and these differences were directly related to variations in the silencing suppression ability during virus infection. These results suggest that suppressor activities differ even among homologous proteins encoded by viruses of the same genus, and that TGBp1 contributes to the variation in the level of RNA silencing suppression by potexviruses. Moreover, we investigated the effect of TGBp1 encoded by plantago asiatica mosaic virus (PIAMV), which exhibited a strong suppressor activity, on the accumulation of microRNA, virus genomic RNA and virus-derived small interfering RNAs. We next analyzed the suppressors encoded by potato virus M (PVM), a member of the genus Carlavirus. In the transient expression assay, the cysteine-rich protein (CRP) of PVM inhibited both local and systemic silencing, whereas TGBp1 of PVM showed suppressor activity only on systemic silencing. Furthermore, to elucidate the roles of these two suppressors during an active viral infection, we performed PVX vector-based assays and viral movement complementation assays. CRP increased the accumulation of viral RNA at the single-cell level and also enhanced viral cell-to-cell movement by inhibiting RNA silencing. However, TGBp1 facilitated viral movement but did not affect viral accumulation in protoplasts. These data suggest that CRP inhibits RNA silencing primarily at the viral replication step, whereas TGBp1 is a suppressor that acts at the viral movement step. Thus, our findings demonstrate a sophisticated viral infection strategy that suppresses host antiviral silencing at two different steps via two mechanistically distinct suppressors.

Through the results from these studies about RNA silencing suppressors encoded by potexviruses and carlaviruses, we suggested that potexviral TGBp1 inhibits both local and systemic silencing while a carlavirus inhibits different steps of RNA silencing using TGBp1 and CRP independently. This may uncover an evolutionary aspect of viral RNA silencing suppressors; acquisition or loss of an RNA silencing suppressor may influence the role of remaining or original RNA silencing suppressor, respectively.

Arms Race Between Viruses and Their Hosts: Structure-Function Studies on the Silencing Suppressor of a Tospovirus (Bunyaviridae, Tospovirus)

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Tospoviruses cause serious diseases in a wide range of field and horticultural crops. The genome of tospoviruses consists of three RNAs, large (L), medium (M) and small (S). The L RNA is organized in negative sense orientation, whereas M and S RNAs are in ambisense. The S RNA codes for a non-structural protein (NSs) in sense direction which has been shown to function as a viral suppressor of gene silencing in plants. While mixed infections involving two distinct tospoviruses in the same plant were reported, it is not known if there is an inter-species complementation between two viruses. We developed and used a system to study such interactions at the molecular level. We used datura (*Datura stramonium*) as a differential host for two distinct tospovirus species, Iris yellow spot virus (IYSV) and Tomato spotted wilt virus (TSWV). Following mechanical inoculation of datura, TSWV causes systemic infection, whereas IYSV infection of datura remains localized to inoculated leaves. We demonstrate that, in a mixed infection, the TSWV NSs is expressed at a much higher level as compared to single infection in inoculated as well as systemic leaves. The systemic symptoms produced by TSWV in the presence of the IYSV were more severe than those caused by TSWV infection alone. Co-infection resulted in the selective movement of only the silencing suppressor (NSs) of IYSV to younger, un-inoculated leaves, suggesting complementation between two distinct tospovirus species. This is the first ever experimental evidence of inter-species interaction facilitating systemic movement of a viral suppressor in an otherwise restrictive host.

Little is known about the structure-function relationships of the tospovirus-coded gene silencing suppressors. To identify regions/sequences critical in suppressor activity, NSs protein sequences of known tospoviruses were compared. In silico analysis showed two conserved regions among 15 known tospoviruses : GKT at positions 191-193 and YL at positions 429-430. Site-directed, point mutations were made to change K192 to A192 (NSs-1), and L430 to A430 (NSs-2). The effects of mutations were evaluated using the agro-infiltration and the GFP silencing system of *Nicotiana benthamiana* line 16c. Our results indicate that the silenced GFP signal in line 16c can be partially restored by the wild-type NSs, while both NSs-1 and NSs-2 lost the ability to restore GFP expression. These results suggest that the conserved GKT and YL regions are essential for the RNA silencing suppressor function of TSWV NSs. We have designed artificial microRNA constructs targeting various regions in NSs and shown proof of concept for resistance to TSWV in transient assays. Identification and characterization of specific sequences of NSs that are critical for its suppressor activity would provide targets for engineering virus resistance that is potentially both broad spectrum and durable.

Ago1 duplication of *Nicotiana benthamiana* reveals a miR168-resistant homeolog.

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AGO1 is an important component of the posttranscriptional regulation machinery of gene expression, but also it has a key role in RNA silencing-based antiviral defence. The homeostasis of AGO1 is at least in part coordinated through a feedback mechanism, indeed miR168-guided cleavage of ago1 mRNA ensures an optimal balance of miRNA steady-state levels. The presence of plant viruses may remarkably increase miR168 accumulation in infected tissues but still the reasons of such phenomenon cannot be clearly attributed to either the direct induction of expression of miR168, to a compromised miRNA duplex unwinding or to the induction of AGO1, which in turn has the capacity to stabilize the miR168. *Nicotiana benthamiana* is one of the most studied model plant for plant-pathogen interaction. The recent release of the draft genome sequence may become an instrument to shed new light on additional layers of gene regulation, including ago1 functionalities.

Here we describe the presence of two ago1 homeolog genes in *N. benthamiana*. They differ for several synonymous SNPs and for one 18-nucleotides insertion/deletion (indel), which does not modify the frame of the open reading frame. Importantly, the indel is located just upstream the miR168 target site. A Green Fluorescent Protein (GFP) transient expression system reveals that the indel may determine the expression of the GFP, likely influencing the miR168-guided cleavage of ago1 mRNA. Origin and biological functions with particular implication in antiviral defence of the homeologs ago1 genes will be discussed.

COST FA0806: Plant virus control employing RNA-based vaccines: A novel non-transgenic strategy

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The current virus control methods are limited in number, efficacy and environmental suitability and current EU decisions restrict crop improvement strategies employing transgenic plants. To protect plants against existing and emerging virus diseases new methods are urgently needed. A very promising approach is the exploitation of RNA silencing, a natural, endogenous mechanism in plants that is a sequence-specific process leading to viral mRNA degradation.

COST Action FA0806 brings together several EU labs in order to develop suitable, efficient and cost-effective methods to induce anti-viral silencing in crops by the transient application of dsRNA, siRNAs and/or artificial small RNAs (collectively designated as “RNA-based vaccines”). These vaccines are produced either in vitro or in vivo in large quantities and are applied at laboratory or large scale employing specific delivery machinery.

FA0806 is structured in three Working Groups (WGs), WG1: Development of novel non-transgenic strategies for plant virus control, WG2: Application of novel non-transgenic strategies for plant virus control, and WG3: Socio-economic evaluation of the impact of the novel application methods. In the frame of FA0806, Training Schools and Short Term Scientific Missions provide instruments for scientific exchange and training for early-stage and senior researchers alike. Currently, 61 members from 28 COST countries and eight non-COST members, from Argentina, Australia, China, Mexico, New Zealand, Peru and South Africa participate in the Action.

<http://www.aua.gr/COSTFA0806>

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Properties of post-transcriptional gene silencing suppression proteins of Benyviruses

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Beet necrotic yellow vein virus (BNYVV) and Beet soil-borne mosaic virus (BSBMV) belong to the Benyvirus genus, possess a multipartite genome formed by four ssRNAs(+) and are both transmitted by the plasmodiophorid *Polymyxa betae*. BSBMV and BNYVV are closely related since they possess the same host range, vector and genome organization.

The innate mechanism that plants use to protect themselves against viral infections is called Post Transcriptional Gene Silencing (PTGS). PTGS is triggered by the presence of aberrant RNA or dsRNA generated during the replication of viral genomes and leads to their degradation. To counteract this innate mechanism, viruses co-evolved with their hosts and express viral suppressors of RNA silencing (VSR) that inhibit the PTGS.

In this work we investigated properties of the Benyvirus VSRs. Such VSR consist of cysteine-rich proteins (CRP) of 14kDa expressed by RNA-2 of both BNYVV and BSBMV species used in this study. We demonstrated that p14s have a zinc-finger domain (Znf) able to bind nucleic acids. Agroinfection of *Nicotiana benthamiana* plants demonstrated that these proteins are able to suppress the PTGS downstream of the Dicer proteins action, without interfering with the transitivity. Sequence motifs essential for the nucleolus targeting of the protein (NoLS) and cysteine residues essential for the Znf structure folding, have been also identified.

Both p14s localize in the nucleolus, form homodimers and bind the "coremin" sequence, a stretch of 20 nucleotides present in the RNAs-3 sequence of Benyviruses and required for their systemic spread in the plant. Moreover, the coremin sequence is able to complement defective BNYVV P14 mutants in long distance movement illustrating an obvious link between P14, suppression of RNA silencing activity, RNAs-3 coremin sequence and long distance movement.

Session 4:
**Resistance mechanisms other
than silencing**

Resistance mechanism against LMV in lettuce involving eIF4E, VPg and CI: beyond the tip of the iceberg

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The Potyvirus genus is the largest genus of plant viruses, including Lettuce mosaic virus (LMV) of worldwide distribution in lettuce crops, ornamentals and weeds. To date, the major sources of resistance to potyviruses are components of the eukaryotic translation initiation complex. In particular, recessive allelic lettuce genes *mo11* and *mo1*², used to protect lettuce crops against LMV, were shown to correspond to mutant alleles of the gene encoding the translation initiation factor eIF4E [1]. Our previous work showed that the LMV resistance breaking determinants map not only to the VPg encoding region (the main potyvirus virulence determinant) but also to the C-terminal region of the CI (Cylindrical Inclusion helicase), providing the first example of a potyvirus CI acting as a determinant for eIF4E-mediated resistance breaking [2]. In parallel, during a global survey of the biological and molecular diversity of LMV isolates, we showed that propagation of several non-lettuce isolates of LMV in *mo11* plants is accompanied by a gain of virulence correlated with the accumulation of mutations in the CI C-terminus, while the same isolates were stable when propagated in susceptible lettuce. These results led to the identification of 4 candidate amino acids in the CI C-terminal region, potentially involved in *mo1* resistance breaking.

Mutations at these positions (e.g. A602V, G617S, S621T and A627T) were introduced alone or in combination in a LMV-0 (non resistance-breaking isolate) background. The behavior of these mutants towards *mo1* genes was then analyzed. We hence demonstrated that a single substitution at position 621 in the CI is sufficient to confer the ability to overcome the resistance, as well as the combination of mutations A602V and G617S. However, the resistance-breakdown was not complete: a 100% rate of infection was achieved in susceptible plants for the virulent mutants whereas it varied from 20% to 87% in resistant plants. This partial resistance breakdown was improved by combining mutations at three positions, 602-617-621 or 617-621-627, giving the first example of a gradual mechanism of resistance breakdown. The behavior of the quadruple mutant is still under investigation. Altogether, these data suggest that the complete overcoming of *mo1* resistances requires more than 3 mutations in the CI-Cter: the role of the central region of the CI and the VPg in this mechanism are currently being studied, suggesting an even more complex picture.

In parallel, in order to decipher the mode of action of the resistance, the movement of LMV-0 and its derived GFP tagged-mutants is monitored in susceptible and resistant plants, to determine which viral cycle step is impaired by the *mo1* resistance and restored by the resistance-breaking mutations.

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Ectopic expression of multiple copies of eIF4E and eIF(iso)4E from TuMV-resistant *Brassica rapa* complemented an *Arabidopsis* eIF(iso)4E knock-out line and confused!

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Turnip mosaic virus (TuMV) has been described as the second most important virus affecting vegetable crops worldwide. It has a very wide host range, infecting monocotyledonous and dicotyledonous plants and is transmitted by many different aphid species.

A range of dominant R genes (TuRB01 – TuRB06) conferring pathotype-specific resistance have been identified and mapped in the brassica A genome. More recently, broad-spectrum resistance controlled by a recessive gene (retr01), that is epistatic to a dominant gene (ConTR01) has been mapped in the *Brassica rapa* line RLR22. We have found three copies of the eukaryotic translation initiation factor 4E (eIF4E, one of which is a pseudogene) and three copies of its isoform (eIF(iso)4E) in *B. rapa* line RLR22. retr01 was shown to be coincident with one of the copies of eIF(iso)4E and ConTR01 with one of the other copies of eIF(iso)4E, or one of the copies of eIF4E.

We cloned two copies of eIF4E and two copies of eIF(iso)4E (including the candidates for retr01 and ConTR01) from a resistant *B. rapa* RLR22 plant that was homozygous for all copies of eIF4E and eIF(iso)4E and transformed them into the *Arabidopsis* eIF(iso)4E knock-out line Col-0::dSpm. Following mechanical inoculation and aphid transmission of the CDN 1 isolate of TuMV to the transformed *Arabidopsis* plants, all copies of eIF4E and eIF(iso)4E complemented the knock-out line. Plants developed severe TuMV symptoms and high levels of virus were detected in leaves. TuMV-inoculated, untransformed Col-0::dSpm plants were completely resistant, showing no symptoms and no detectable virus infection. Complementation of the Col-0::dSpm was very confusing in that it indicated that when ectopically expressed in the *Arabidopsis* knock-out line, TuMV was able to use the retr01 allele of eIF(iso)4E. To add to the confusion, TuMV also appeared to be able to use both of the ConTR01 allele candidates and the other copy of eIF4E.

Phenotyping of *B. rapa* populations segregating for the RLR22 alleles of these genes, clearly showed TuMV could not use any of them in this species. In an attempt to understand these conflicting results, we sequenced the VPg from TuMV-infected, complemented plants, however, this only revealed a single synonymous mutation relative to wild-type CDN 1 TuMV. We have also studied the expression of the different copies of eIF4E and eIF(iso)4E in the TuMV-resistant *B. rapa* RLR22 plant line, a susceptible *B. rapa* plant line and the transformed *Arabidopsis* plants, in an attempt to explain the disparity between results in *Arabidopsis* and *B. rapa*.

Diversity, genetic structure and selection of the L resistance gene to tobamoviruses in populations of the wild pepper, *Capsicum annuum* var. *glabriusculum* in Mexico.

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Host-pathogen co-evolution is a central question in Biology as it is at the root of pathogen emergence, host switch and host range expansion, and the composition and dynamics of ecosystems. However, contrary to agroecosystems, evidences for plant-virus co-evolution in wild ecosystems are almost non-existent. To understand plant-virus co-evolution, we propose to use as a model system the wild pepper *Capsicum annuum* var. *glabriusculum* (chiltepin), which is the wild ancestor of the domesticated *C. annuum*. Wild populations of chiltepin are found in the tropical dry forests of Mexico (1), where they are exploited for their small pungent fruits. Their cultivation by traditional local farmers has recently started in several regions. A large collection of samples representing wild and cultivated chiltepin populations in Mexico has been obtained and characterised on the basis of microsatellite marker variation (2). Tobamovirus resistance in *Capsicum* spp. is controlled by the L gene. The different alleles of this gene (from L1 to L4) confer resistance to different Tobamovirus pathotypes (from P0 to P1,2,3) according to the gene-for-gene model. The L resistance gene has recently been cloned and characterised as a single-gene locus encoding a CC-NB-LRR protein, which targets the coat protein (CP) of tobamoviruses (3). This recognition initiates signalling pathways leading to resistance by hypersensitive response (HR). In the absence of the resistance gene (L+ allele), or in the absence of L/CP recognition, the virus is not detected by the host resistance system, which results in a successful infection. L-gene resistance is only expressed at temperatures below 28°C, except for allele L1a, which is thermoresistant. L1a recognises pathotypes P0 and P1 at 24°C, and only the former one at 30°C (4).

In this study, we assessed the L gene frequency in different chiltepin populations, and we confirmed the strict correlation between the presence of the L gene and a resistance phenotype. Interestingly, the frequency of L gene was significantly higher in the wild populations than in the cultivated ones, which suggests that the pre-domestication of this species has not focused on Tobamovirus resistance. The analysis of more than 100 full-length L gene sequences showed a high variability, even if most of the sequences were closed to the L1 and L1a alleles. A strong geographical structure of polymorphisms at this gene was observed, which could correspond to local adaptations of chiltepin populations toward various Tobamovirus pathotypes (i.e., P0 and P1 are recognised by L1 and L1a, respectively) and/or different environmental conditions (i.e., the high temperature-tolerant L1a allele and other related alleles were more frequent in the warmest regions of Mexico). Finally, we identified several co-variations and sites under positive selection, mainly located in the domain involved in the CP recognition. Interestingly, one co-variation was detected between two sites under positive selection and discriminating L1 and L1a alleles, which could correspond to a major adaptive signature of the L gene in Mexico. The possible role of these sites in virus recognition at different temperatures is being analysed by protein co-expression assays. Altogether, these results suggest that the L resistance gene is a target for selection by virus infection, which could support the hypothesis of Tobamovirus-chiltepin co-evolution potentially influenced by environmental conditions.

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Cloning of the *Arabidopsis rwm1* resistance gene reveals a role for a chloroplast phosphoglycerate kinase in Watermelon mosaic potyvirus infection.

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To find novel plant factors required for potyvirus infection, we developed the Watermelon mosaic virus (WMV)-*Arabidopsis thaliana* pathosystem. Phenotypic screening and genetic analysis identified the *rwm1* gene, responsible for recessive resistance to several strains of WMV in the Cvi-0 accession. The *rwm1*-mediated resistance acts at an early stage of infection by impairing virus RNA accumulation in initially infected leaf tissues. Subsequent map-based cloning delimited *rwm1* to a 114- kb region on chromosome 1 containing 30 annotated genes. Positional and functional candidate gene analysis predicted that *rwm1* encodes a chloroplast-targeted phosphoglycerate kinase, cPGK2, an evolutionary conserved enzyme with a key role in carbon metabolism. Sequence analysis revealed a single amino acid substitution localized in the N-terminal region of the cPGK2 protein that correlates with virus resistance. We further proved by virus-induced gene silencing of an orthologous gene in *Nicotiana benthamiana* that cPGK is required for efficient WMV infection. Functional validation in *Arabidopsis* using stable genetic transformation is currently underway. In conclusion, the findings from this work open a challenging research area to provide novel insights on plant resistance mechanisms and opportunities to promote the genetic control of plant virus diseases.

Méthodes de sélection ou d'obtention de plantes résistantes aux potyvirus et/ou potexvirus. Ouibrahim L. and Caranta C. Brevet PCT/055196 déposé le 28/09/2012

Tomato spotted wilt virus cell-to-cell movement protein (NSM) triggers a hypersensitive response in *Nicotiana benthamiana* transformed with the functional Sw-5b resistance gene copy

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Although the Sw-5 gene cluster has already been cloned for more than ten years ago, and Sw-5b identified as the functional gene copy that confers resistance to Tomato spotted wilt virus (TSWV), its avirulence (Avr)-determinant still has not been identified. While earlier *Nicotiana tabacum* SR1 plants transformed with a copy of Sw-5b were totally immune and did not reveal any visual response upon challenging with TSWV, here we show that *N. benthamiana* transformed with Sw-5b does give a rapid hypersensitive-like response (HR) on leaves and stem tissues. Using these plants, the TSWV cell-to-cell movement protein (NSM) was identified as the Avr-determinant using a PVX-replicon expression system, but not when using the non-replicative, high-level expression vector pEAQ-HT, even in combination with other TSWV genes. HR was induced in Sw5b-transgenic *N. benthamiana* after agroinfiltration with a functional cell-to-cell movement protein (NSM) from a resistance inducing (RI) TSWV strain (BR-01), but not with NSM from a Sw-5-resistance breaking (RB) strain (GRAU). The discrepancy in results between the use of a viral replicon vs non-replicative expression vector indicated that the sole expression of NSM apparently is not sufficient to trigger the resistance response. Furthermore, the HR induced in Sw-5b-transgenic *N. benthamiana* after NSM expression from a replicon did appear weaker when compared to its induction after a challenge with TSWV-RI virus. A co-expression of TSWV NSM RI with other viral (non)structural proteins, NSS and N, did not improve this response. This is the first biological demonstration that Sw-5 mediated-resistance is being triggered by the NSM cell-to-cell movement protein of TSWV. The HR response triggered by NSM and only when expressed from a viral replicon is discussed in light of its involvement in viral cell-to-cell movement.

A linear signaling pathway formed by three MAPKKKs leading to systemic necrosis induced by a potexvirus in *Nicotiana benthamiana*

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Plantago asiatica mosaic virus (PIAMV) induces systemic necrosis accompanied by several hypersensitive response (HR) hallmarks in *Nicotiana benthamiana*. In previous studies, necrosis induced by PIAMV depends on SGT1, RAR1, and mitogen-activated protein kinase (MAPK) cascade components, MAPKKK α and MEK2. However, the signaling pathway leading to systemic necrosis remains poorly understood. The MAPK cascades are associated with responses to various abiotic and biotic stresses such as plant pathogens. MAPK cascades consists of three components namely MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. Of these three types of kinase, the MAPKKKs exhibit the most divergence in the plant genome. Their great diversity is assumed to allow MAPKKKs to regulate many specific signaling pathways in plants despite the relatively limited number of MAPKKs and MAPKs. In this study, we cloned three novel MAPKKK genes from *N. benthamiana*: NbMAPKKK β , NbMAPKKK γ , and NbMAPKKK ϵ 2. Transient overexpression of full-length NbMAPKKK β or NbMAPKKK γ or their kinase domains in *N. benthamiana* leaves induced HR-like cell death associated with hydrogen peroxide production. In addition, knockdown of NbMAPKKK β or NbMAPKKK γ expression using tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) significantly suppressed the cell death induced by PIAMV. Furthermore, in epistasis analysis of the functional relationships among three MAPKKKs including NbMAPKKK α performed by combining transient overexpression and VIGS, silencing of NbMAPKKK α suppressed cell death induced by the overexpression of the NbMAPKKK β kinase domain or of NbMAPKKK γ , but silencing of NbMAPKKK β failed to suppress cell death induced by the overexpression of NbMAPKKK α or NbMAPKKK γ . Silencing of NbMAPKKK γ suppressed cell death induced by the NbMAPKKK β kinase domain but not that induced by NbMAPKKK α .

These results demonstrate that in addition to NbMAPKKK α , NbMAPKKK β and NbMAPKKK γ also function as positive regulators of the cell death induced by PIAMV. Furthermore, these three MAPKKKs form a linear signaling pathway leading to the PIAMV-induced cell death; this pathway proceeds from NbMAPKKK β to NbMAPKKK γ to NbMAPKKK α .

The immunity regulator BAK1 contributes to resistance against diverse RNA viruses

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The plant innate immune system detects potential biotic threats through recognition of microbe-associated molecular patterns (MAMPs), or danger-associated molecular patterns (DAMPs), by pattern recognition receptors (PRRs). A central regulator of pattern-triggered immunity (PTI) is the BRI1 associated kinase 1 (BAK1) which undergoes complex formation with PRRs upon ligand binding. Although viral patterns inducing PTI are well known from animal systems, nothing similar has been reported for plants. Antiviral defense in plants is rather thought to be mediated by posttranscriptional gene silencing of viral RNA, or through effector-triggered immunity, i.e. recognition of virus-specific "effectors" by resistance proteins. Nevertheless, infection by compatible viruses can also lead to the induction of defense gene expression, indicating that plants may recognize viruses through PTI as well. Here we show that PTI, or at least the presence of the regulator BAK1, is important in antiviral defense of Arabidopsis plants. Arabidopsis bak1 mutants show increased susceptibility to three different RNA viruses during compatible interactions. Furthermore, crude viral extracts, but not purified virions, induce several PTI marker responses in a BAK1-dependent manner. Overall, we conclude that BAK1-dependent PTI contributes to antiviral resistance in plants.

Host responses to virus infection in *Arabidopsis thaliana* are affected by environmental conditions in a genotype-dependent manner.

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Plants have developed a variety of mechanisms to compensate for the cost of biotic and/or abiotic stresses. Upon parasite infection, host may respond differently, which may be categorized into different strategies [1]. While some are well characterized, tolerance has received less attention. Tolerance can be defined as a compensation of the negative effects of parasitism in the host fitness, often associated to modifications of life history traits. Life-history theory makes predictions for the adjustment of resource investment by organisms, based on the notion that trade-offs exist between resources allocated to different fitness components: growth, reproduction and survival [2]. Thus, parasite infection may modify pre-defined resource distribution and consequently induce plastic modifications to the host. Models for evolution of resource allocation predict that parasitized organisms will allocate more resources to reproduction, subtracting them from those dedicated to growth and survival [2]. Our group tested these predictions in the plant-parasite system of *Arabidopsis thaliana* and the generalist virus Cucumber mosaic virus (CMV). It was shown that plastic modifications upon CMV infection were substantial compared to mock inoculated plants [3]. More specifically, resource allocation modification upon infection and life-history responses was different depending on the allometric features of *Arabidopsis* genotypes and two groups were distinctively significant, with group1 genotypes being on average more tolerant than those of group2 [3]. Since tolerance is a quantitative trait based on phenotypic plasticity upon virus infection, the optimal amount of resources allocated to each of these components may be corrected according to environmental conditions in order to maximize the organism's fitness. Hence, tolerance might be environment-dependent. To test this hypothesis, four *Arabidopsis* ecotypes (two belonging to each previously-described groups), were inoculated with CMV and grown in similar conditions with the exception of two factors: the light intensity [50 or 250 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, respectively named low light (LL) and high light (HL)], and the temperature [17°C, 22°C and 27°C]. First, we compared the plant-metrics of mock-inoculated plants within the different environmental conditions tested. Overall, the factor 'light intensity' had a much greater impact on the plant architecture and developmental schedule, than the factor 'temperature'. Interestingly, when looking at the total Biomass (BM), plants of all four ecotypes performed their best and their worst in the same environments. The most extreme performance loss was observed for plants of group2 with a 97% variation in the total BM, while such variation was only of 70% for plants of group1, underlying a better tolerance to abiotic stresses for that latter group. The effect of CMV infection on plant-metrics was not as severe as that of light or temperature. Although fine-tuned, response to virus infection was extremely different among allometric groups and environmental conditions and phenotypic outcome was genetically determined. While plants of group2 displayed a limited type of response to the infection, plants of group1 showed a wider range of reaction to virus multiplication, spanning from over-compensation, to tolerance and sensitivity. Such observation seems to correlate with the ability of plants of group1 to exhibit a bigger plasticity in their life cycle and phenotypic response upon stresses. The most representative data will be presented to illustrate the numerous changes observed due to biotic and abiotic factors and their combination. Our current goal is to identify the genetic determinants of virus-tolerance in *Arabidopsis thaliana*, and to further characterize regulation mechanisms involved in its pathway.

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Correlation between single nucleotide substitutions in the coat protein gene of Pepino mosaic virus and symptom induction in tomato

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Pepino mosaic virus (PepMV) is considered one of the most important viral diseases of tomato. It has been shown that the rate of PepMV molecular evolution is significantly higher than the rates recently reported for other plant RNA viruses. Four different genotypes, with an intergenotype RNA sequence identity ranging from 78% to 95% have been distinguished so far: Peruvian (LP), European (EU), American 1 (US1) and Chilean 2 (CH2). The CH2 genotype has become dominant in the viral population in Europe. The shift in the prevalent genotype of PepMV from the EU to CH2 genotype has occurred also in North America since early 2010. Single nucleotide differences between isolates from the same genotype have been shown to affect symptom development and aggressiveness. Therefore, the characterization of new PepMV pathotypes is important for understanding PepMV disease emergence. Recently, the presence of a new pathotype of PepMV, which causes severe yellowing symptoms on tomato plants, has been reported. The phylogenetic analysis based on full length genomes revealed that the “yellowing isolates” grouped within the CH2 strain, sharing 99-100% overall nucleotide and amino acid sequence identity with non-yellowing CH2 isolates. No recombination events were detected. Single nucleotide polymorphism analysis indicated the presence of one point mutation, occurring in position 155 of the coat protein (CP), as unique to the yellowing isolates. Selection pressure analysis using the HyPhy and PAML programs, based on CP sequences of different PepMV isolates, showed that the CP is evolving mainly under the action of purifying selection operating on several amino acid sites, thus supporting its important functional role during PepMV infection. Codon number two was found to be under positive selection by five out of six analytical methods used in PAML and HyPhy. Codon 155 was determined to be under positive selection pressure by only one out of the six analytical methods. The variants with the identified point mutation in codon 155 of the CP, which results in replacement E155K, displayed different biological properties compared to other CH2 isolates. Indeed, in contrast to previously described isolates, these isolates infect two varieties (out of 16 tested) of *Solanum tuberosum* inducing similar symptoms to those observed in tomato. They also infect a wide range of tomato varieties displaying yellowing symptoms irrespective of environmental conditions. Several weeks after the beginning of infection, the yellowing symptoms gradually disappeared and the plant apical leaves became green again. The sequencing of several clones of the CP gene from PepMV populations sampled at the green top of the plants resulted only in “mild-type” sequences (with E155). This finding suggests that codon 155 could be directly involved in PepMV symptoms in tomato and that, during the course of infection, the yellowing isolates may revert towards mild-type sequences. Further research will be performed to elucidate the role of point mutations in the genome of PepMV.

Identification and characterization of host genes implicated in potexvirus resistance in solanaceae.

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Plants are continually challenged by various pathogens. The Rx gene confers an extreme resistance against common strains of PVX (potato Virus X) 1. In this biological system, the recognition step involves an indirect interaction between the RX protein and the PVX capsid protein (the elicitor) 2. In the RX/PVX pathosystem, the molecular cascade and the mechanisms conferring the resistance remain unclear. However it has been shown through different strategies, that RX1 protein could interact directly with RanGAP2 and that RX accumulation involves other proteins like HSP90, SGT1 or RAR1 3-6.

In order to identify host proteins implicated in Rx-mediated resistance, a yeast two hybrid strategy has been performed using as bait, a minimal fragment of the PVX CP that has been identified through serial deletions as done before for other Potexvirus coat protein 7. This minimal fragment has been shown to be sufficient to elicit the defense reaction when transiently expressed in wild type or expressing Rx-Nicotiana. The preys consisted of cDNA libraries from wild type or Rx-expressing Nicotiana infiltrated with the coat protein of PVX.

In the general purpose of finding host proteins implicated in virus resistance in Solanaceae, another virus the Pepino Mosaic Virus (PepMV), a very agronomic important pathogen of tomato, has been exploited using the same strategy. The coat protein and the triple gene block proteins (TGBp1, TGBp2 and TGBp3) have been used as baits to screen cDNA libraries from tomato plants inoculated with an avirulent strain of PepMV.

The screening of more than 10 millions of clones has led to the identification of several potential interactors. For PVX, a transcription factor and a chloroplastic protease have been studied in more details. These interactions have been confirmed in yeast, in vivo by BiFC and their implication in Rx resistance has been pursued through transient overexpression. The results are in agreement with their implication in a general resistance mechanism against viruses. For PepMV, seven interactors have been selected for upcoming functional validation experiments such as transient gene silencing (VIGS- viral induced gene silencing) or over/under-expressing lines. The Tomato TILLING platform held at URGV is also currently exploited for validation purpose.

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Mechanisms underlying potexvirus-induced systemic necrosis in plants

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Systemic necrosis is the most destructive symptom induced by plant viruses. To reveal mechanisms underlying the development of systemic necrosis, *Plantago asiatica* mosaic virus (PIAMV)-*Nicotiana benthamiana* pathosystem was established. In this pathosystem, Li1 isolate of PIAMV systemically infects, and induces systemic necrosis, in *N. benthamiana*. In contrast, Li6 isolate systemically infects the plant asymptotically. The systemic necrosis induced by Li1 was associated with programmed cell death (PCD) and biochemical features characteristic of hypersensitive response (HR). Using virus-induced gene silencing, we revealed that the necrosis caused by Li1 infection was dependent on SGT1, RAR1, and downstream mitogen-activated protein kinase (MAPK) cascade. Moreover, our results suggested that viral multiplication is partly restrained even in systemic necrosis induced by viral infection, and that this restraint requires SGT1 and RAR1 but not MAPK cascade. Transient and inducible expression of PIAMV-encoded proteins indicated that the necrosis-eliciting activity resides in RNA-dependent RNA polymerase (RdRp), and the necrosis was induced in an RdRp dose-dependent manner. Surprisingly, necrosis-eliciting activity resides in the helicase domain (HEL), but not in the polymerase domain (POL) that contains amino acid 1154 (aa 1154), pathogenicity determinant between Li1 and Li6 isolates. This necrosis-eliciting activity was also observed in HEL encoded by Li6. These results suggest that necrotic symptoms induced by PIAMV infection depend on the accumulation of a non-isolate specific elicitor HEL, whose expression is indirectly regulated by aa 1154 that controls replication.

A new source of resistance in potato to Potato virus Y

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Potato virus Y (PVY) is the most important viral pathogen of potato worldwide and causes significant economic damage in related crops such as pepper, tomato and tobacco. PVY is spread by aphids, particularly *Myzus persicae*, where the transmission occurs very rapidly (within minutes of the aphid starting to probe the plant) so that insecticides sprayed onto the plants cannot take effect fast enough to prevent virus inoculation. An additional important route of PVY transmission is through vegetative propagation of infected plants, so that for potato production it is important that tubers used for commercial planting are free from PVY infection. A few sources of dominant resistance to PVY have been reported in wild species of potato; these are Rysto from *Solanum stoloniferum*, Ryadg from *S. tuberosum* spp. *andigena* and Rychc from *S. chacoense*, and have been mapped to the potato chromosomes XII, XI and IX, respectively. Thus far, none of these resistance genes have been precisely mapped or cloned.

The James Hutton Institute, Dundee curates the Commonwealth Potato Collection, a large germplasm collection of wild and cultivated potato species. We have screened our collection of *S. tuberosum* group phureja (Phureja) accessions by mechanical inoculation to identify individuals with resistance to PVY. We found that 28 of 39 Phureja accessions were completely resistant to PVY (strains PVYo, PVYC, PVYN and PVYNTN) and also to PVA. In six accessions, PVY infection was restricted to the inoculated leaf, and five accessions were completely susceptible to PVY infection. Phureja is diploid making mapping of traits more straightforward than with cultivated (tetraploid) potato, and the potato genome sequence is derived from a Phureja clone (DM). JHI has a number of Phureja populations derived from controlled crosses between resistant and susceptible clones. Testing of progeny of several crosses between resistant and susceptible lines suggested that the Phureja resistance could be due to a single, dominant gene. PCR markers published for Rysto and Ryadg did not associate with resistant plants suggesting that the Phureja resistance is different to the previously known resistances. Using DArT, SNP and other genotype analysis of segregating populations we have mapped PVY resistance to a region of chromosome IX not previously associated with known virus resistances. However, more detailed genetic analysis suggests that resistance is influenced by a second locus on chromosome IV. To complement this work we are using a GFP-tagged PVY to examine the effect of this resistance on the early stages of virus replication and potential movement in inoculated leaves.

Bsa-ngs combined strategy for faster identification of micro-tom tomato genes affecting pvx resistance

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The Potato Virus X (PVX) resistance gene, Rx, was cloned in *Solanum tuberosum*. Rx encodes a CC-NBS-LRR like protein and its presence leads to extreme resistance (ER) upon PVX infection (Bendahmane et al., 1999). The Rx gene was introduced in the Micro-Tom cultivar genome, leading to PVX resistance. An EMS-mutagenized Rx Micro-Tom population was produced and challenged with a partially breaking strain of PVX. Five mutants with a clear surviving phenotype were selected. Genetic and molecular characterizations led to the identification of five independent mutations: 3 are recessive and 2 are dominant. None of the mutations are localized on the Rx gene (Sturbois et al., 2012).

To identify one of the dominant mutants, named #1179, a BSA-NGS strategy was developed using a segregating population from the #1179 X Rx cross. The F2 progeny was phenotyped after inoculation with the partially breaking PVX strain. Two pools were created according to their resistance or their susceptibility. The goal of this approach is to detect differential SNP between the resistant and the susceptible pools. After DNA extraction, these pools, the parental lines and the WT Micro-Tom were sequenced by HiSeq Illumina®. All the reads were mapped to the reference genome of tomato (The Tomato Genome Consortium, 2012). CLC Bio software was used to detect SNP(s) corresponding to #1179 mutation. After subtraction of common SNP from the #1179-R pool, potential candidate genes which may be linked to PVX resistance will be identified.

The same approach will then be pursued to find out candidate genes for the #2135 recessive mutation.

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How does broad-spectrum resistance to Turnip mosaic virus work in *Brassica rapa*?

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The potyvirus Turnip mosaic virus (TuMV) is a major constraint on the cultivation of a wide range of plant species worldwide. It causes significant economic losses in Brassica species such as Chinese cabbage (*Brassica rapa*), which is one of the most important vegetable crops in the world. The *B. rapa* line RLR22 has broad-spectrum resistance to TuMV, which is undefeated; most described sources of resistance are isolate/strain specific. Most characterised plant virus resistances are dominant traits. In contrast, more than half of the resistance genes discovered for plant viruses in the Potyvirus genus are recessive and based on mutations in plant eukaryotic translation initiation factor 4E (eIF4E), or its isoform eIF(iso)4E (Robaglia and Caranta, 2006). These resistances are mostly strain-specific and are overcome by mutations in the VPg viral protein (that has been shown to interact with eIF4E, or eIF(iso)4E) (Robaglia and Caranta, 2006).

Segregation following a cross between RLR22 and the TuMV-susceptible R-o-18 line of the closely related *B. rapa* ssp. *trilocularis* (Roxb.) Hanelt. (yellow sarson) revealed the resistance was due to a recessive gene, *retr01* (coincident with a copy of eIF(iso)4E) that was epistatic to a dominant gene, *ConTR01* (coincident with one of the other copies of eIF(iso)4E, or one of the copies of eIF4E) (Rusholme et al., 2007). As the susceptible parent in the original cross, R-o-18, was a different sub-species to RLR22 (*B. rapa* var. *pekinensis*, Chinese cabbage), the genetic inheritance of resistance was also investigated in crosses with Chinese cabbage lines. Segregation ratios were consistent with those predicted for the single recessive gene (*retr01*). We have discovered that the highly sought after broad-spectrum resistance to TuMV is due to a novel, recessive, natural mechanism, based on the mis-splicing of eIF(iso)4E in *B. rapa*. This results in a range of eIF(iso)4E splice variants, the most common of which appears to be non-functional for the virus. *B. rapa* has three eIF4E genes and three eIF(iso)4E genes. The inability of TuMV to access multiple copies of eIF(iso)4E in Chinese cabbage and the broad-spectrum of the resistance, suggest it may prove to be durable.

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BiP proteins targeted to the endoplasmic reticulum regulates HRT-mediated hypersensitive response by modulating expression and protein stability of HRT in *Nicotiana benthamiana*

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Arabidopsis HRT, a typical CC-NB-LRR type disease resistance (R) protein, induces the hypersensitive response (HR) when recognizing the corresponding effector protein, which is the coat protein of turnip crinkle virus (TCV-CP). Here, co-expression of HRT and TCV-CP induced a typical HR in *Nicotiana benthamiana*. To explore genes involved in HRT/TCV-CP-mediated HR in *N. benthamiana*, we screened by virus-induced gene silencing (VIGS) about 450 ESTs that were found as putative defense-related genes from pepper microarray experiments. VIGS of one particular gene, encoding a pepper luminal binding protein precursor (CaBiP), which belongs to a heat shock protein 70 (HSP70) protein family targeted to the endoplasmic reticulum (ER), greatly reduced HRT/TCV-CP-mediated HR just like silencing of HSP90 or SGT1. Moreover, protein level of ER-resident HSP70 increased during HR progression. However, cell death induced by non-host bacterial pathogen *Pseudomonas syringae* was enhanced, and the amount of ER-resident HSP70 was decreased unlike HR condition. Due to the feature of HSP70 as a chaperone, the level of HRT proteins was determined. Surprisingly, both expression and protein stability of HRT were greatly reduced in the BiP-silenced plants. In mammals, BiP was known as a master regulator of signal transducer in unfolded protein response (UPR) following ER stress. Treatment of tunicamycin, a known UPR elicitor, inhibited HRT-mediated HR and also accumulation of HRT protein, but enhanced accumulation of ER-resident HSP70. These results indicate ER-targeted BiP proteins and possibly N-glycosylation are important for R protein-mediated HR by modulating the status of R proteins rather than UPR-induced cell death.

Multiple resistance pathways elicited by TMV in N gene tobacco

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Hypersensitive resistance to tobacco mosaic virus (TMV) in tobacco is conferred by the N gene, which elicits the best characterized plant virus resistance response. Resistance involves multiple responses activated by phytohormones, although those activated by salicylic acid (SA) are the best characterized. At least three responses that inhibit the infection of TMV are activated by SA, through independent pathways: alternative oxidase (AOX), a mitochondrial enzyme involved in regulating reactive oxygen species; pathogenesis related (PR) proteins, synthesized in response to SA via the regulator NPR1; and RNA-dependent RNA polymerase 1, encoded by the gene RDR1. Another pathway, involving the synthesis of an inhibitor of virus replication (IVR), was shown to be independent of SA, as was the pathway involving the transcription factor (TF) ERF5. We found that ERF5 is upstream of and regulates IVR production. By contrast, expression of different PR genes is regulated by the TFs MYB1, WRKY and TGA. The kinetics of gene expression of AOX1, PR1, RDR1 and IVR were examined in tobacco plants by real-time PCR, as were the kinetics of gene expression of ERF5 and MYB1. The plants examined included wild-type NN tobacco, nn tobacco expressing mutants of the N gene that no longer restricted TMV to the inoculated leaf, and NN tobacco silenced for expression of ERF5, MYB1, or both genes. The results indicate complex regulation of expression of the various defense genes, with ERF5 and MYB1 affecting the expression of each other, even though they are in different pathways. In addition, the SA-independent TF ERF5 interacts directly both with its end-product, IVR, and with the SA-dependent TF MYB1, as determined in yeast two-hybrid system and by bimolecular fluorescence complementation. Both TFs are distributed subcellularly between the tonoplast membrane and the nucleus. A model for the effects of the TFs ERF5 and MYB1 on the expression of the defense effector genes (AOX1, PR1, RDR1 and IVR) will be presented.

**Session 5:
Epidemiology, population
genetics and evolution**

DNA satellites associated with monopartite and bipartite begomoviruses in the New World

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Begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are plant ssDNA viruses that are transmitted by the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae). Begomoviruses cause serious diseases in economically important crops, mostly in tropical and subtropical regions. Two types of DNA satellites associated with these viruses have been described: betasatellites and alphasatellites, both half the size of the helper virus components. Betasatellites are associated with monopartite begomoviruses from the Old World and are dependent on them for replication, movement in plants and *B. tabaci* transmission. Betasatellites consist of an A-rich region, a region that is conserved among all betasatellites, and a single ORF. Alphasatellites, associated with Old World and New World begomoviruses, contain a single ORF coding for a replication-associated (Rep) protein with similarity to those of nanoviruses and an A-rich region. Unlike typical satellites, alphasatellites are capable of self-replication in host plants but require a begomovirus for movement within the plant and for insect transmission.

In screening wild plant species collected in Cuba and Venezuela for the presence of begomoviruses, we identified circular ssDNA molecules associated with viral infection, half the size of betasatellites and alphasatellites. Two classes of DNA satellite-like molecules were differentiated associated with bipartite begomoviruses infecting malvaceous species in Cuba and a monopartite begomovirus infecting *Merremia dissecta* (Convolvulaceae) in Venezuela, respectively. Although all of these molecules share some genetic features (they do not code for any ORF, contain an A-rich region, and share a conserved region of variable length with betasatellites), they seem to be only distantly related.

Spatial Dynamics of Genetic Exchange in a Multipartite Plant Virus

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Multipartite plant viruses have multiple genome components which are individually encapsidated within separate coat proteins. This strategy, combined with vector mediated transmission, may lead to high rates of reassortment, whereby discrete genome components get exchanged among different strains. Reassortment can have important epidemiological implications, and may lead to the emergence of highly virulent strains or facilitate adaptation to novel hosts. As multiple genetically distinct strains must infect the same host plant for reassortment to occur, rates of reassortment may differ among locations as a result of spatial variation in disease incidence and local differences in genetic variation. Identifying potential geographic 'hotspots' of reassortment where novel viral lineages are likely to arise could be important for informing surveillance efforts. However, spatial variation in the extent to which reassortment shapes the evolutionary dynamics of plant viral populations has not been assessed. I will discuss my analyses of spatial variation in reassortment in Cardamom Bushy Dwarf Virus (CBDV), a multipartite nanovirus which infects large cardamom, *Amomum subulatum*, in sub-Himalayan regions of Northeast India, Nepal and Bhutan.

Wheat dwarf virus and a diverse set of luteoviruses infect both grasses and cereals

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Both wild and cultivated grasses are commonly infected with different viruses. However, the infected grasses are often symptomless or the symptoms are difficult to discriminate from those induced by other biotic factors or abiotic stress. The virus infections may still affect the composition of grasslands and infected grasses can act as reservoirs for infecting cereals. We have used sequence analyses as part of attempts to understand the ecology of viruses infecting grasses and cereals.

Barley yellow dwarf virus (BYDV)/Cereal yellow dwarf virus (CYDV) constitute a group of viruses in the family Luteoviridae that infect grasses and cereals. They are occurring throughout the world and it is probably the most widely spread group of plant-infecting viruses. BYDV/CYDVs have a genome of single-stranded (+) RNA and they are classified into several species (BYDV-PAV, BYDV-MAV, etc.), which are transmitted by different aphids in a persistent manner. The different virus species can be difficult to discriminate using serological methods such as ELISA, and the high viral diversity is only now beginning to be revealed. In a survey of BYDV/CYDVs in cereals and grasses from different regions of Sweden and Estonia, we have detected a high incidence of viruses and sequence analyses have revealed infection with BYDV-PAV, BYDV-MAV, BYDV-PAS, BYDV-OYV, BYDV-GPV and BYDV-RMV. The four latter species were found for the first time in Sweden and BYDV-GPV for the first time outside China. Interestingly, several species of BYDV/CYDV could be found in the same field and with up to three species in the same plant. Different viruses could be detected in forage grasses and cereals growing next to each other, suggesting that the forage grass was not the virus source for the infection in cereals. Additional studies are required to determine the host and aphid vector specificities of the different BYDV/CYDV genotypes. BYDV-OYV has previously only been described as a single isolate from an oat plant in Latvia. We could now find isolates of BYDV-OYV infecting Triticale and *Festuca pratensis* in different parts of Sweden. The first complete genome sequencing of BYDV-OYV shows that it is related to viruses within the genus Luteovirus, but sequence comparisons reveal that it is distinct from the other species and should tentatively constitute a new species.

Another type of virus infecting grasses and cereals is Wheat dwarf virus (WDV; genus Mastrevirus; family Geminiviridae). This virus has a genome of single-stranded circular DNA and it is transmitted in a persistent manner by leafhoppers of the species *Psammotettix alienus*. WDV is distributed throughout Europe and Asia, where it causes yield reductions in wheat and barley. In our studies to identify virus reservoirs for cereal-infecting viruses, WDV was detected using ELISA at a low frequency in randomly sampled ryegrass plants, and infection could also be confirmed by PCR. Nucleotide sequence analyses revealed that the ryegrass isolates were closely related to WDV isolates previously characterized from wheat in Sweden. Infection tests using WDV isolates from wheat resulted only in low infection rates and low virus titers in different ryegrass species and cultivars, while wheat plants were very susceptible. The results suggest that WDV may persist at low levels in ryegrass and other grasses.

Detection of plum pox virus by AmplifyRP® based on recombinase polymerase amplification

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Plum pox virus (PPV), a member of the genus Potyvirus in the family Potyviridae, causes the most destructive viral disease known as plum pox or Sharka disease of stone fruit species including apricot, cherry, peach and plum. As an important regulated pathogen, detection of PPV is thus of critical importance to quarantine and eradication of the disease. Agdia Inc. has recently developed an isothermal detection of PPV by AmplifyRP® based on recombinase polymerase amplification. In AmplifyRP®, DNA fragments (amplicons) specific to the RNA genome of PPV are produced at a single constant temperature without thermal cycling and the results are recorded with a portable fluorescence reader in real-time or on high-affinity detection strips. The whole test from sample preparation to result can be completed in as little as 30 minutes. The AmplifyRP® for PPV detects all seven currently known strains (PPV-C, PPV-D, PPV-EA, PPV-M, PPV-Rec, PPV-T, or PPV-W) of PPV. The detection of PPV by AmplifyRP® is not only rapid and simple but also as highly specific and sensitive as conventional PCR.

European nanoviruses: Identification of three new species and new DNA components

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Viruses of the genus *Nanovirus* (family *Nanoviridae*) predominantly infect legumes, are persistently transmitted by aphids, and have unusually small virions (17-20 nm) and genome segments (~1 kb). The nanovirus genome is typically formed by a set of eight circular single-stranded (ss) DNAs, each of which is individually encapsidated. The genomic DNAs are referred to as DNA-R, -S, -C, -M and -N, which code for master Rep (M-Rep), structural (capsid), cell-cycle link, movement and nuclear shuttle proteins, respectively. Three other nanovirus DNAs (DNA-U1, -U2 and -U4) encode proteins of unknown functions. Nanoviruses occur in the Old World but, until recently, were not known from Europe, apart from the sporadic occurrence of Faba bean necrotic yellows virus (FBNYV) in southern Spain. By characterizing viruses of pea crops in Germany in 2009, we discovered an undescribed nanovirus. Its eight DNA components were sequenced, and clones thereof served to reconstitute an infectious and aphid-transmissible nanovirus. Since this virus differed from known nanoviruses by ~40% in overall nucleotide sequence we regarded it as a new nanovirus species and named it pea necrotic yellow dwarf virus (PNYDV). Using a selection of broad-spectrum monoclonal antibodies for the analysis of about 100 symptomatic pea plants collected in Austria, Hungary, Serbia and Sweden in 2010, >50% of these plants were shown to be infected by a nanovirus. Epitope profiling and sequence analysis suggested that the vast majority of the infections are caused by PNYDVs, very similar to the German isolate of PNYDV. The high incidence and severe symptoms of PNYDV in some of the countries suggest that this virus emerges as important production constraint for organically farmed pea crops in south-eastern Europe. Furthermore, both epitope profiling and sequence analysis indicated the existence of two additional nanovirus species in Europe, which differ in capsid protein amino acid sequences from PNYDV and other nanoviruses by > 40%. These new species are currently represented by (i) a pea isolate from Austria (AT15) and (ii) a pea isolate from Sweden (SE153) and a nanovirus isolate from a *Medicago lupulina* plant in Austria (AT3). First genetic analysis of AT3 and SE153 suggests that they are sufficiently similar to be regarded as host-adapted and/or geographic variants of the same nanovirus species. Two strikingly distinct variants of DNA-U2 differing also from all other known DNA-U2 components were also encountered in both AT3 and AT15. Although the significance of this finding is enigmatic, AT3 and AT15 are the first nanovirus isolates from which two functionally equivalent DNAs have been detected. In addition to a genetically diverse range of undescribed paraRep-encoding DNAs ('alphasatellites') that were found associated with AT3, AT15, SE153, and PNYDV isolates, we identified a small (497 nts) DNA component (DNA-X) from an Austrian isolate of PNYDV (AT1). Its sequence lacks a major ORF but shares the common regions I (stem loop) and II with the genomic DNAs of PNYDV, thus possessing the domains required for replication initiation by the M-Rep protein of PNYDV. DNA-X could be maintained by aphid transmission and was detected in 9 of 16 geographically separated PNYDV isolates from Austria. Therefore, it may represent a new type of satellite DNA associated with nanoviruses. Our data provide evidence that nanoviruses are more widespread in the Old World than originally thought and exhibit new features in genome organization and association with satellite DNAs.

High level of inter-species recombination amongst dicot-infecting mastreviruses and phylogeographic analysis towards identifying the origin of their most recent common ancestor

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Viruses of the genus *Mastrevirus* (family Geminiviridae) are transmitted by leafhoppers and infect either mono- or dicotyledonous plants. The dicot-infecting mastreviruses have, until recently, been an under-investigated group of viruses. They have been identified in Australia, south and north-east Africa, the Middle East and India, although their geographic range likely is much more extensive than this. Dicot-infecting mastreviruses have been recorded mainly from pulses, particularly chickpea and lentil, but have also been identified in other hosts including tobacco and sugarbeet. We have sequenced a large number of full-length dicot-infecting mastreviruses from across the known geographical host range of these viruses, including regions for which sequences were not previously available (Turkey, Iran, India and Eritrea). The genomes of all available dicot-infecting mastreviruses were analysed for genetic diversity, recombination and possible geographic origins of these viruses. Global genome analysis revealed that the diversity of dicot-infecting mastreviruses in Australia is greater than that found in the rest of the world. Further, the detected inter-species recombination events outnumbered intra-species recombination events in contrast to what we have observed in monocot-infecting mastreviruses. A phylogeographic analysis of the dicot-infecting mastreviruses indicates that the most recent common ancestor of the currently known dicot-infecting mastreviruses is likely nearer Australia than the any sampled region and we provide a plausible scheme for spread of the dicot-infecting viruses to their present locations.

The impact of RNA silencing on the evolution of a small viral genome

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Plants have evolved a small RNA pathway for anti-viral defense involving DCL2 and DCL4 as primary double-stranded (ds) RNA processing enzymes, and AGO1 and AGO2 as its main effector proteins. The system is also amplified through the action of plant-encoded RNA-dependent RNA Polymerases including, chiefly, RDR6 and RDR1. As a counter-defense to antiviral silencing, viruses have developed viral suppressor of RNA silencing (VSRs). For example, the P38 capsid protein from Turnip crinkle virus (TCV) contains an AGO-hook domain that binds to AGO1 and AGO2 and prevents their loading with virus-derived small interfering (siRNAs) produced by the host DCL2 and DCL4. Various steps of the (+)ssRNA virus replication participate to the production of both intra and inter molecular dsRNA molecules that serve as precursors to the biogenesis of antiviral siRNA. This suggests that a selective pressure is exerted onto viral genomes by RNA silencing, which possibly shapes the nucleotide sequence, secondary structure and amino acid content of viral RNAs and proteins, including by the positive selection for key residues involved silencing suppression by VSR.

To estimate the extent to which RNA silencing indeed constrains viral genome evolution, we studied changes incurred to the small TCV genome in recurring infection experiments conducted in parallel over ten generations in wild-type *Arabidopsis* plants and in mutant plants simultaneously deficient in DCL2, DCL4 and DCL3 function (*dcl234* mutants). Combined analyses involving en masse viral genome re-sequencing, siRNA profiling and host transcriptome studies of the WT- versus *dcl234*-evolved viral lineages will be presented and their results discussed.

Around the World in 30 Years: The Phylogeography of Tomato spotted wilt virus and Iris yellow spot virus

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Research on the spread of human and animal viruses throughout the world's population is one of the most active areas in current evolutionary biology. Studies have tracked the movement of viruses based on information about the routes and frequency of human and animal global transport. The same cannot be said of plant viruses, yet crop plants are frequently exported and imported between countries and are of vital importance to the global economy. Within the phytopathogenic viruses of economic significance are various RNA negative virus species, members of the Tospovirus genus. Tomato spotted wilt virus (TSWV), the type species of the genus, is considered the second most important plant virus and is, on its own, responsible for over a billion dollars in crop losses. Like TSWV, many tospoviruses infect a large range of hosts, including many crop species of high economic value. Given the significance of tospoviruses, we have undertaken an extensive analysis to elucidate the timescale and route of spread of two tospovirus – TSWV and Iris yellow spot virus (IYSV). We retrieved all publicly available nucleoprotein (N) sequences, for TSWV and IYSV, with known sampling dating and location from the GenBank. Multiple sequence alignments were conducted using MUSCLE as implemented in SeaView (V. 4.4.0). The final datasets resulted in a total of 523 sequences for TSWV (Length = 777 nt, sampling interval: 1989–2010) and 82 sequences for IYSV (Length= 828 nt, sampling interval: 1996–2009). Phylogeographic analyses of TSWV and IYSV datasets were performed by ancestral reconstruction of discrete states in a Bayesian statistical framework implemented in BEAST. The maximum clade credibility (MCC) trees were generated using the program TreeAnnotator and were visualized using the program FigTree. The MCC trees were then compared to the levels of export and import of a specific crop species – tomatoes for TSWV and onions for IYSV – between the countries present in the trees. For both IYSV and TSWV, migration intensified beginning in the mid-1980s, correlating with various trade liberalization policies, however our phylogeographic analysis demonstrated that the patterns of spread for TSWV and IYSV were quite distinct. In the United States, TSWV circulates primarily within the country, and all sequences appear to stem from a single introduction; IYSV, on the other hand, is traded back and forth between the US and various countries in the Americas, Asia and Europe. There are also similarities between the trade routes of both viruses: in Europe the countries of Italy, Serbia and France present a heavy traffic of both viruses, whereas Spain presents a more isolated, internal trade, much like TSWV in the US. The general lack of sequences and, more specifically, the lack of proper host plant and country identification of the isolates, hinders the formation of a more complete view of tospoviruses' trade routes. However, our research presents many hypothetical transmission routes for TSWV and IYSV across the globe, serving as a basis for further molecular/evolutionary studies and possible changes to government trade control measures, particularly those concerned with plant viruses.

Endogenous Plant Pararetroviral Sequences in Natural and Managed Landscapes: Epidemiology and Evolution

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The central mountain ranges of Mexico are home to the greatest diversity for genus *Dahlia*. First described in 1791, the genus has 35 recognized wild species in addition to the cultivated forms, known as either *D. pinnata* or *D. variabilis*. Majority of these wild dahlia species were found in the Mexican mountain ranges. Two distinct caulimoviruses, *Dahlia mosaic virus* (DMV), and *Dahlia common mosaic virus* (DCMV), and an endogenous plant pararetroviral sequence (DvEPRS, formerly known as DMV-D10) were reported to be associated with dahlia mosaic, a serious disease affecting cultivated dahlia (*D. variabilis*). To better understand the incidence of these pararetroviruses, selected wild *Dahlia* species in their natural habitats from west - central Mexico were tested for the three caulimoviruses. Virus species-specific primers and PCR were used followed by cloning and sequencing of the amplicons. Results showed that the wild dahlia species in their natural habitat contained DMV-D10. Viral sequences were found in 91% of the samples (n=56) representing four different wild species. Genetic diversity studies were performed using a dataset of 7 full-length EPRSs isolated from cultivated and wild *Dahlia* spp. Assessment of all open reading frames (ORFs) using phylogenomic and population genetics approaches showed that genetic diversity of EPRSs occurring in dahlia is very diverse. Phylogenetic analyses showed that EPRSs formed one clade, indicating a lack of clustering by geographical origin and no divergence due to source (cultivated vs. wild). Population genetic analyses found negative selection for all ORFs, with the replicase region more variable than other ORFs. Recombination events were found that provided evolutionary evidence for genetic diversity. The discovery of plant pararetroviruses in wild dahlia species in their natural habitats suggests a possible emergence, co-existence and co-evolution of pararetroviruses and their host plants.

Cassava virus characterization and study of their interactions in plants affected by Frogskin Disease in Latin America

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Cassava (*Manihot esculenta* Crantz.) is the third most important source of calories for human nutrition in the tropics and viral diseases continue to limit cassava yields worldwide. Viruses infecting cassava in Africa are associated to significant yield losses and epidemic outbreaks, e.g. Cassava Brown Streak Disease (CBSD). In Latin America viruses infecting cassava are associated to mild, temporal and/or localized disease outbreaks, which are relatively easy to control by positive selection as in the case of Cassava Frogskin Disease (CFSD). Nevertheless, in both cases when these diseases occur, yield reductions are significant, limiting farmer's incomes. We observed that previously reported cassava viruses are no longer frequently detected in cassava grown in Colombia and after using a combination of biological, serological, molecular and metagenomic approaches, a different set of viruses was detected in cassava from Latin America affected by CFSD. These viruses occur more frequently in mixed infections in severely diseased plants but they are not always associated to disease symptoms in leaves or roots when they occur in single infections. Genome characterization revealed the presence of novel virus species belonging to families Alphaflexiviridae, Luteoviridae, Reoviridae and Secoviridae and thus they are likely transmitted by different vector families. The low transmission of CFSD and its efficient control by positive selection suggests an inefficient vector for this disease, which could be related to the requirement of mixed infections and the presence of different vectors for severe disease to occur. In addition, the symptomless nature of single-infections in cassava could explain temporal outbreaks due to distribution of unnoticed contaminated material. Sequence data analysis indicates regional variability and also re-assortment events. Using real-time PCR and indicator plants we are now studying virus interactions in disease development in single and mixed infections and their role in CFSD. We expect that the study of cassava virus biology, distributions and epidemiology will help prevent the occurrence of emergent viral diseases in this crop.

Session 6: Molecular ecology

Deriving ecological and evolutionary insights from broad sequencing of non-cultivated plants

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The last decade has changed the way we perceive viruses, particularly those of plants. We now appreciate that viruses are not exclusively pathogens, but can have other effects, sometimes beneficial, on host and vector populations. Next generation sequencing has enabled deep sequencing of all nucleic acids associated with single plants resulting in multiple virus discoveries. It has made possible also broader exploration, such as of many plants in a biome, by using barcoding of sequences. We now realize that plant virus diversity is many-fold greater than we had imagined, complicating the taxonomic classification and biosecurity regulation of viruses detected only by sequence segments from metagenomic analyses. Combining the power of deep sequencing with ecological breadth of coverage is a challenge. Two examples of extracting information from broad, but shallow, sequencing derived from the Plant Virus Biodiversity and Ecology project of the Tallgrass Prairie Preserve (TPP) in the Great Plains of the US will be presented. (1) Diversity in populations of the TPP tymovirus, *Asclepias asymptomatic virus* (AsAV), found at high titer and prevalence in the green milkweed *Asclepias viridis*, was explored. Rarefaction analysis suggested that 15 to 50% of the residues of the AsAV genome are capable of variation. Pairwise nucleotide diversities between pairs of viral sequences obtained from milkweed plants were narrowly distributed around 3.5%. However, nucleotide diversities between viral sequences from non-milkweed plants were more broadly distributed, suggesting roles of host defenses and adaptation in shaping viral populations. (2) Many plants of the TPP contain sequences related to Southern tomato virus. Network analysis was used to assign these sequences to multiple distinct viruses. These and other examples demonstrate that network analysis can be a highly useful tool in viral ecological investigation and taxonomy.

Antarctic viromics: Is permafrost thaw in the Antarctic 'seeding' water bodies with their preserved microbial communities?

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The Antarctic continent is the most physically and chemically extreme environment to be inhabited by microorganisms, where climatic conditions and lack of liquid water hamper life. Yet it still supports diverse microbial communities including algae. Over the past 5 years a few attempts have been made to explore various viromes, initial reports suggesting high viral abundance in the pristine Antarctic Lakes and a few studies have recently characterised various bacteriophages in the Dry Valley soils. Nonetheless, the overall dynamics of virus ecology of the Antarctic remain a total mystery.

The impacts of climate change are evident in the Antarctic, especially in the McMurdo Dry Valleys. Thawing of permafrost is resulting in the destabilization of paleo-lake deposits that have preserved algal mats for thousands to tens of thousands of years. It is currently unknown whether these melting sedimentary deposits are seeding the streams, ponds, and lakes, releasing the preserved microbial communities. The downstream impacts on the microbial ecology of the Dry Valley lakes, especially viral dynamics and evolution is also unknown. In order to address this question we have decided to use circular single stranded DNA viruses (ssDNA) recovered from algae collected from a paleo-lake deposit that has been frozen as permafrost since the last ice age in the Garwood Valley as indicators using metagenomic approaches coupled with conventional techniques to recover full ssDNA viral genomes. Our hypothesis is that if the stream, ponds and lakes in the dry valleys are being seeded by viable microbial communities, the ssDNA viruses recovered from the permafrost preserved algal mats should be similar to those found in the water bodies with certain viral species sharing high sequence identity. If not, we would clearly see the marked difference in the ssDNA viral community structure and high sequence diversity. Through our preliminary investigations, we have recovered some diverse circular ssDNA viruses from algal mats recovered from retreating glaciers in the Garwood Valley. These preliminary results will be discussed in the context of the viral diversity from our first pilot run together with some of the complexities of viral metagenomics.

De novo reconstruction of plant RNA and DNA virus genomes from viral siRNAs

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In antiviral defense, plants produce massive quantities of 21-24 nucleotide siRNAs. Here we demonstrate that the entire genome sequences of DNA and RNA viruses as well as viroids give rise to viral siRNAs and can therefore be reconstructed by deep sequencing and de novo assembly of viral/viroid siRNAs from infected plants. Thus, such 'siRNA omics' (siRomics) approach is applicable for universal diagnostics of known and emerging viral and viroid diseases and for virus diversity studies. We also show that bioinformatics analysis of viral/viroid siRNAs allows to identify a consensus master genome and its microvariants which compose the viral or viroid quasi-species and thereby correct potential cloning errors when constructing the wild type infectious clone.

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