Viroid-2018

International Conference on Viroids and Viroid-Like RNAs

5-7 July 2018, Valencia, Spain

Polytechnic City of Innovation, Universitat Politècnica de València
Avenida de los Naranjos, 46022 Valencia, Spain

http://www.ibmcp.upv.es/viroid-2018/
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Book of Abstracts

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SUPPORTING INSTITUTIONS

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CONTRIBUTING COMPANIES

Biotest Diagnosticos S.L., http://www.biotestdiagnosticos.es/
Cultek, http://www.cultek.com/
Durviz, http://durviz.com/
Épica, http://epicasl.com/
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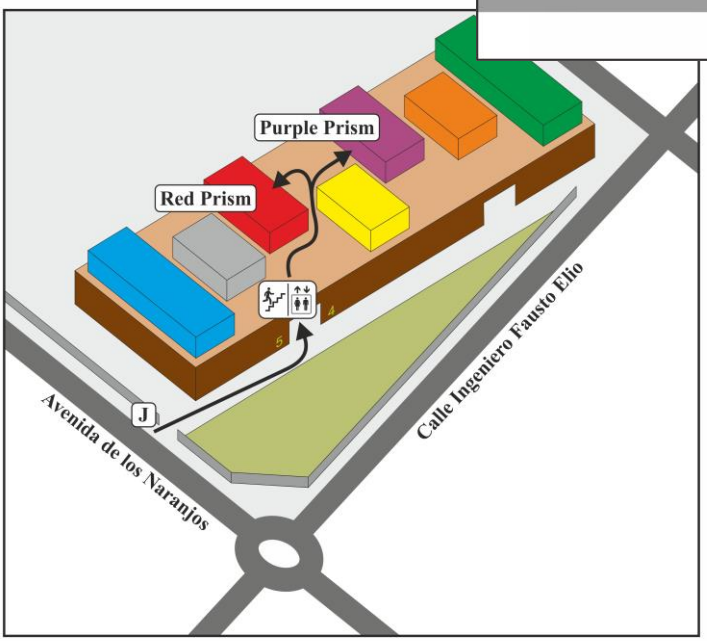
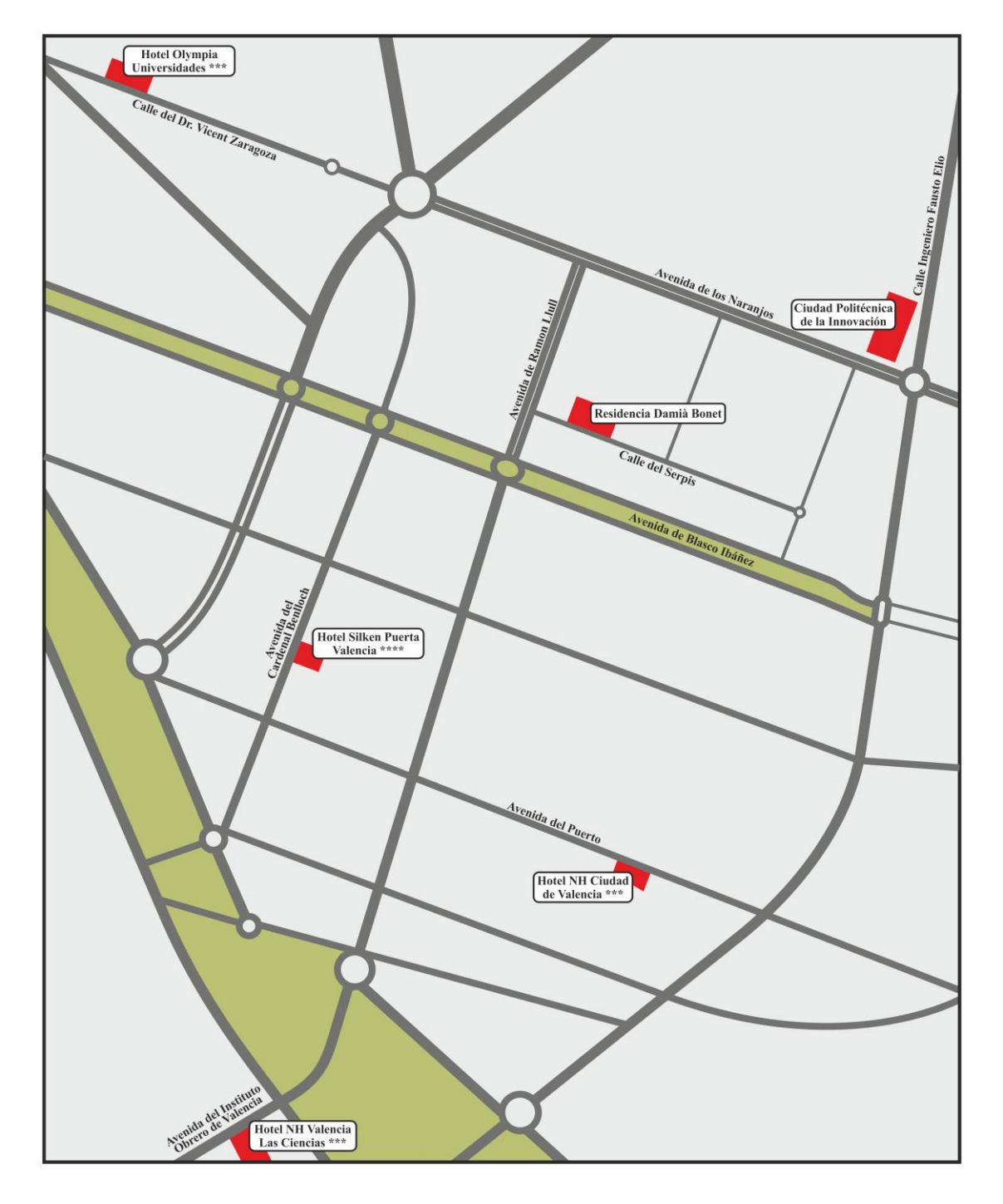
CONFERENCE VENUE

Campus Universitat Politècnica de Valencia, corner between Avenida de los Naranjos and Calle Ingeniero Fausto Elio, Access J, Ciudad Politécnica de la Innovación (CPI), Red Prism.

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Hotel NH Valencia Las Ciencias ***

CONFERENCE PROGRAM

Thursday, July 5th

09:00-10:00. Registration at CPI Red Prism

10:00-10:20. Opening

Chairman: Robert A. Owens

Viroid replication

10:20-10:40. **O1**. How does potato spindle tuber viroid interact with the host machinery for replication? Some recent insights into the mechanism
Y. Wang, J. Qu, S. Ji, A.J. Wallace, J. Wu, J. Jiang, S. Ji, V. Gopalan, Y. Li and B. Ding

10:40-11:00. **O2**. From viroids and ribozymes: RNA back and forth
M.C. Maurel

11:00-11:40. Coffee break and poster session at CPI Purple Prism

Chairwoman: Anna Góra-Sochacka

Host defensive response to viroid infection

11:40-12:00. **O3**. Understanding the interplay between PSTVd and plant defenses
K. Katsarou, N. Kriovrisanaki, I. Bardani, E. Mitta, A. Grigoriadou and K. Kalantidis

12:00-12:20. **O4**. Transcriptome and degradome analysis of the *Citrus exocortis viroid*-tomato pathosystem
T. Olivier, S. Steyer and C. Bragard

12:20-12:40. **O5**. Susceptibility to viroid infection of tomato plant in which RNA silencing key factors –AGO2, RDR6, or DCL2 & 4– is knocked down
T. Suzuki, A. Kasai, M. Fujibayashi and T. Sano

13:00-15:00. Lunch at El Trinquet (Universitat Politècnica de València)

15:00. Bus transfer to Valencia CSIC headquarters in Old Town

Chairman: Ricardo Flores

Viroid transmission and traffic

16:00-16:20. **O6**. Vertical and horizontal transmission of pospiviroids
Y. Matsushita and H. Yanagisawa

16:20-16:40. **O7**. Indirect assessment of viroid functions: chrysanthemum chlorotic mottle viroid-mediated trafficking of foreign mRNA into chloroplasts and the best laid plans of mice and men that often go awry
P. Palukaitis, E. Baek, M.-J. Park and J.-Y. Yoon

Host-viroid interaction and pathogenesis I

16:40-17:00. **O8**. Citrus exocortis viroid causes ribosomal stress in tomato plants
P. Cottilli, B. Belda-Palazón, A. Ferrando, E. Schleiff, I. Rodrigo and P. Lisón

17:00-17:30. Break

Chairwoman: Efthimia Mina Tsagris

17:30-17:50. **O9**. Transcriptomic profiling of *Humulus lupulus* in response to *Citrus bark cracking viroid* (CBCVd) infection
A.K. Mishra, J. Matoušek, D. Mishra, U. K. Killi, A. Kumar, T. Kocábek and J. Jakše

17:50-18:10. **O10**. Identification and characterization of miRNAs in cucumber and their responses to hop stunt viroid (HSVd) infection
Z.X. Zhang, C.J. Xia, W.Y. Hou, Z.F Fan, T. Sano and S.F. Li

18:10-18:30. **O11**. HSVd infection impairs the epigenetic control of repetitive DNA in vegetative and gametic infected cells
M. Castellano, V. Pallás and G. Gómez

18:30. Guided visit to Valencia historical city center

Friday, July 6th

Chairman: Jean-Pierre Perreault

Host-viroid interaction and pathogenesis II

09:00-09:20. **O12**. A yellow mosaic incited by peach latent mosaic viroid: strict association with a single-nucleotide change involved in RNA silencing-mediated cleavage of the mRNA coding for a thylakoid protein
S. Delgado, B. Navarro, P. Serra, P. Gentit, M.A. Cambra, M Chiumenti, F. Di Serio and R. Flores

09:20-09:40. **O13**. CBCVd in hops: what did we learn from its discovery?
J. Jakše, N. Štajner, T. Pokorn, A.K. Mishra, J. Matoušek, T. Guček and S. Radišek

09:40-10:00. **O14**. Effects of potato spindle tuber viroid on the degradome of tomato and *Nicotiana benthamiana*: a comparative analysis
B. Navarro, A. Gisel, P. Serra, R. Flores and F. Di Serio

10:00-10:20. **O15**. *In silico* molecular interaction between PSTVd, TCDVd and some *Solanaceae* species
I. Rekik, O. Ellouze and A. Elleuch

10:20-11:20. Coffee break and poster session at CPI Purple Prism

Chairwoman: Nuria Duran-Vila

11:20-11:40. **O16**. The symptom pattern incited by chrysanthemum chlorotic mottle viroid results from RNA silencing of an enzyme of the Calvin's cycle and from its high mutation rate and population bottlenecks during host colonization
P. Serra, B. Navarro, A. Gisel, F. Di Serio and R. Flores

Taxonomy and new viroid species and sequence variants

11:40-12:00. **O17**.Viroid taxonomy: state of the art and perspectives in the high-throughput sequencing era
F. Di Serio

12:00-12:20. **O18**. Citrus viroid VII, a novel citrus viroid found in Lisbon Lemon in Australia
G.A. Chambers, N.J. Donovan, S. Bodaghi, S.M. Jelinek and G. Vidalakis

12:20-12:40. **O19**. Molecular characterization of apple hammerhead viroid isolates from ancient Italian apple cultivars
M. Chiumenti, B. Navarro, P. Venerito, F. Civita, F. Di Serio and A. Minafra

13:00-15:00. Lunch at El Trinquet (Universitat Politècnica de València)

Chairman: Teruo Sano

Viroid structure

15:00-15:20. **O20**. What's up with the viroid structural compendium?
J.P. Perreault and T. Giguère

15:20-15:40. **O21**. Direct visualization of the native conformation of PSTVd, PLMVd and ELVd at single-molecule resolution by atomic force microscopy
M. Moreno, L. Vázquez, M.A. López-Carrasco, J.A. Martín-Gago, R. Flores and C. Briones

Viroid diseases

15:40-16:00. **O22**. *Citrus bark cracking viroid*: biological properties and impact on Slovene hop production
S. Radišek, T. Guček, J. Matoušek and J. Jakše

16:00-16:20. **O23**. Molecular characterization of potato spindle tuber viroid from dahlia plants in Japan
D. Tsushima and S. Fuji

16:20-17:00. Coffee break and poster session at CPI Purple Prism

17:00-18:00. Meeting of the International Council for the Study of Viroids and Viroid-Like RNAs

21:00. Conference dinner at Balneario Resort Las Arenas

Saturday, July 7th

Chairwoman: Beatriz Navarro

Viroid diagnosis

09:00-09:20. **O24**. Detection of Pepper chat fruit viroid (PCFVd) by reverse transcription loop-mediated isothermal amplification (RT-LAMP)
P. Tangkanchanapas, M. Höfte and K. De Jonghe

09:20-09:40. **O25**. Overcoming the challenges of the *Potato spindle tuber viroid* diagnostic in tomato seeds, leaves and water samples
N.Mehle, P. Kogovšek, N. Rački, T. Jakomin, I. Gutierrez-Aguirre and M. Ravnikar

Viroid biotechnology

09:40-10:00. **O26**. *Citrus dwarfing viroid* reduces citrus apical shoot growth and alters tree hormone profile. Pathogen or an answer to emerging citriculture challenges?
I. Lavagi, C. Lovatt and G. Vidalakis

10:00-10:20. **O27**. An eggplant latent viroid-based platform to produce recombinant RNA in *Escherichia coli*
B. Ortolá, T. Cordero and J.A. Daròs

10:20-11:20. Coffee break and poster session at CPI Purple Prism

Chairman: Peter Palukaitis

These amazing circular RNAs

11:20-11:40. **O28**. The amazing small circular RNAs with hammerhead ribozymes colonizing the genomes of eukaryotes
A. Cervera and M. de la Peña

11:40-12:00. **O29**. Viroids as companions of my professional and personal life
N. Duran-Vila

12:00-12:20. **O30**. Viroids – at the forefront of RNA technology and RNA biology
G. Steger and D. Riesner

12:20-12:40. **O31**. Viroids: reflections on two contentious issues and one unsolved conundrum
R. Flores

13:00-15:00 Lunch at CPI Purple Prism and farewell

POSTERS

Host defensive response to viroid infection

P1. A salycilate hydroxylase involved in the tomato defence against citrus exocortis viroid
S. Minguillón, L. Campos, M. Hernández, I. Rodrigo, J.M. Bellés, V. Conejero, M.P. López-Gresa and P. Lisón

Viroid transmission and traffic

P2. Subcellular RNAi components localization upon PSTVd infection
K. Katsarou, E. Deligianni and K. Kalantidis

P3. Agroinfection methods for evaluating *Chrysanthemum stunt viroid* accumulation and movement in *Chrysanthemum* plants
T. Nabeshima, M. Doi and M. Hosokawa

Host-viroid interaction and pathogenesis

P4. Comparative analysis of gene expression in tomato leaves during development of mild and severe potato spindle tuber viroid infection
A. Więsyk, R. Iwanicka-Nowicka, A. Fogtman and A. Góra-Sochacka

P5. Profiling of microRNAs in PSTVd infected Bulgarian pepper cultivars
D. Ivanova, E. Apostolova, D. Lazarova, N. Tomlekova, G. Yahubyan, V. Baev and M. Gozmanova

P6. Differential gene expression profiles of oil palm seedlings infected with *Coconut cadang-cadang viroid* variants
L.L. Kong, S.S. Thanarajoo and G. Vadamalai

P7. Analysis of gene expression changes in hop plants in response to single and multiple viroid infections using RNA-Seq approach
J. Jakše, J. Matoušek, S. Radišek and N. Štajner

P8. *Avocado sunblotch viroid* interaction with host superoxide dismutase
D. Gobatto, M. Eiras and J.A. Daròs

Taxonomy and new viroid species and sequence variants

P9. Grapevine latent viroid and grapevine hammerhead viroid-like identified by next generation sequencing in an ancient grapevine germplasm collection in Italy
S. Rotunno, A.M. Vaira, D. Marian, F. Di Serio, B. Navarro, A. Schneider, S. Raimondi, L. Miozzi

P10. Occurrence and molecular characterization of *Apple scar skin viroid* infecting pear plants grown in China
N. Hong, H. Zhu, L.P. Wang and G.P. Wang

P11. Application of high-throughput sequencing for the detection of viruses and viroids in apples
R. Bester, S. Malan, H.J. Maree

P12. Transcriptome sequencing reveals novel Citrus bark cracking viroid (CBCVd) variants from citrus and their molecular characterization
Y. Wang, S. Atta, X. Wang, F. Yang, C. Zhou, M. Cao

Viroid structure

P13. Exploring the presence of C^5-methylcytosine in viroid RNAs
B. Navarro, J.A. Daròs and F. Di Serio

Viroid diseases

P14. Selection of AFCVd variants with severe and mild symptom on tomato fruits
T. Suzuki, M. Fujibayashi and T. Sano

P15. Elimination of apple fruit crinkle viroid (AFCVd) during anther development and its depressed propagation in pollen of *Nicotiana tabacum*
J. Matoušek, L. Steinbachová, U.K. Killi, A.K. Mishra, T. Sano, D. Honys and G. Steger

P16. Some pathogenic effects and elimination of hop variants of two pospiviroids, CBCVd and AFCVd, during development of pollen of *Nicotiana benthamiana.*
L. Steinbachová, H. Matoušková, O. Horáková, S. Radišek, D. Honys and J. Matoušek

P17. Identification of the causal agent of coconut tapering disorder in Sri Lanka
L. Perera, M. K. Meegahakumbura, J. W. Randles, Z.X. Zhang and S.F. Li

P18. Presence of HLVd in the collection of hop varieties in Czech Republic
P. Svoboda, J. Patzak, J. Matoušek, I. Malirová and V. Nesvadba

P19. Idetification of *Arabidopsis thaliana* ecotypes of susceptible to viroids
M. Glanowski, P. Moffett

P20. The construction and biological application of infectious citrus viroid clones
C. Steyn, G. Cook, J.T. Burger and H.J. Maree

P21. *Cardamine bonariensis* and *Oxalis latifolia*: potential reservoirs of *Chrysanthemum stunt viroid* in chrysanthemum crops
D. Gobatto, D.A.S. Franco, N. Velasquez, J.A. Daròs and M. Eiras

P22. Identification of vein banding and yellow speckle diseases in Kurdistan province, west of Iran
M. Hajizadeh, G. Ahmadi and V. Roumi

P23. The *in vitro* synthesis of infectious viroid cDNAs
G. Hu, N. Hong, G. Wang, L. Wang and W. Xu

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O1. How does potato spindle tuber viroid interact with the host machinery for replication? Some recent insights into the mechanism

Y. Wang[1], J. Qu[2], S. Ji[2], A.J. Wallace[2], J. Wu[2], J. Jiang[1], S. Ji[3], V. Gopalan[2], Y. Li[3] and B. Ding[2,*]

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Some DNA-dependent RNA polymerases (DdRP) possess RNA-dependent RNA polymerase activity (RdRP), suggesting their evolution from RdRPs that first arose to transcribe RNA templates. This relic RdRP activity is required for transcription of certain noncoding RNAs that regulate gene expression, and for replication of plant viroids and human hepatitis delta virus. Here, we used potato spindle tuber viroid (PSTVd) as a model to uncover cellular factor(s) that regulates RNA-templated replication by DdRP II (Pol II). Pol II-catalyzed replication of PSTVd employs a rolling-circle mechanism that involves the generation of (+)- and (-)-strand RNAs. We report here that the 7-zinc finger (ZF) transcription factor IIIA (TFIIIA-7ZF) from Nicotiana benthamiana, a shorter variant of the DNA template-specific 9-ZF TFIIIA (TFIIIA-9ZF), interacts with Pol II in vivo and is essential for PSTVd replication. Both forms of TFIIIA bind with the (+)-strand, but only TFIIIA-7ZF interacts with PSTVd (-)-strand in vitro and in vivo. Antisense-mediated suppression of TFIIIA decreased PSTVd replication, but overexpression of TFIIIA-7ZF, and not TFIIIA-9ZF, enhanced replication. Interestingly, footprinting assays revealed that TFIIIA-7ZF binds to a region of PSTVd critical for initiating PSTVd transcription. In vitro transcription assay using purified Pol II and TFIIIA variants confirmed a direct role of TFIIIA-7ZF in mediating Pol II transcription using RNA templates. Furthermore, our analysis uncovered critical zinc finger domains of TFIIIA-7ZF in regulating PSTVd binding as well as Pol II transcription using RNA templates. Our results identify TFIIIA-7ZF as a dedicated cellular factor in DdRP-catalyzed RNA-templated replication and shed light on the molecular basis underlying Pol II transcription on RNA templates. This study also highlights both the extraordinary evolutionary adaptation of viroids and the potential of DdRPs for a broad role in cellular processes.

O2. From viroids and ribozymes: RNA back and forth

M.-C. Maurel, F. Leclerc, J. Vergne and G. Zaccai

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A crucial point in early life history is to understand how evolution passed from complex prebiotic chemistry to simple biology. Current cellular facts allow us to follow the link from chemical to biochemical metabolites, from the ancient to the modern world. In this context, the "RNA world" hypothesis proposes that early in the evolution of life, ribozymes were responsible for the storage and transfer of genetic information and for the catalysis of biochemical reactions. Accordingly, the hammerhead ribozyme (HHR), the hairpin ribozyme, and the ribozyme contained in hepatitis-δ virus (HDV) belong to a family of endonucleolytic RNAs performing self-cleavage that might occur during replication. Furthermore, regarding the ultraconserved occurrence of HHR in several genomes of modern organisms (from mammals to small parasites) these small ribozymes have been regarded as living fossils of a primitive RNA world. The existence of contemporary life in extreme conditions, providing habitats for cellular and viral species, encourages us to focus on the activity, persistence and dynamics of RNAs under such conditions. Finally, studying viroids as plausible remains of ancient RNA, we recently demonstrated that they replicate in non-specific hosts, emphasizing their adaptability to different environments, which enhanced their survival probability over the ages. All the results exemplify ubiquitous features of life, notably the plasticity and efficiency of small RNAs, viroids and ribozymes, as well as their diversity and adaptability to various extreme conditions, traits that must have originated in early life history to generate novel RNA populations.

References:

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Delan-Forino C, Deforges J, Benard L, Sargueil B, Maurel M-C, Torchet C. (2014) Structural Analyses of Avocado sunblotch viroid Reveal Differences in the Folding of Plus and Minus RNA Strands. *Viruses*, 6, 489-506.

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O3. Understanding the interplay between PSTVd and plant defenses

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Nuclear replicating viroids rely for most of their infectious cycle to host factors. At the same time it is now clear that the plant responds to viroid infections through a systematic degradation of genomic viroid RNA. Viroids counter balance host defense responses through replication speed but also likely through sub-cellular localization and interaction with specific host factors. We have started to unravel this complex relationship through analyzing the effect of DCL KD lines on viroid infectivity. We have shown that *Pospiviroidae* RNA is mostly processed by DCL4 but this is not necessarily the most efficient anti-viroid silencing pathway. Here we have extended our studies by generating CRISPR DCL KO and by interfering with the miR biogenesis pathway. These plants can serve as important tools to study silencing responses beyond viroid infectivity. In this meeting our results are going to be discussed in the light of the roles suggested for the multiple silencing pathways on viroid biology.

O4. Transcriptome and degradome analysis of the *Citrus exocortis viroid*-tomato pathosystem

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Viroids are the smallest known plant pathogens, consisting of small single-stranded and circular RNA molecules. Although viroids differ from most of plant RNA viruses by the fact that they do not code for any protein, replicate within the nucleus or the chloroplast and form an imperfect dsRNA structure, like RNA viruses, viroids are able to activate and resist RNA silencing and activate systemic acquired resistance (SAR)[1]. As such, viroids offer an opportunity to understand the potential cross-talk between: RNA silencing, R gene-mediated response defense observed in viral infection and SAR. In particular, the elucidation of the eliciting role of these non-coding RNA molecules in SAR is of primary importance to figure out how this universal plant defense mechanism is activated.

Beside their ability to activate and escape plant defenses, the way viroids induce symptoms in some of their hosts remains largely unknown. While post transcriptional gene silencing (PTGS) of some plant coding RNAs initiated by viroid derived small RNAs (vd-sRNAs) has been demonstrated, the role PTGS plays in symptom modulation is not trivial. Yet, the possible roles of protein-viroid interactions or RNA directed DNA methylation (RdDM) induced by vd-sRNAs are still largely unexplored.

In order to understand how plants activate their defenses against viroids and how these pathogens escape these latter while inducing symptoms, the pathosystem consisting of tomato plants cv. Heinz 1706 infected with a severe isolate of *Citrus exocortis viroid* (CEVd) was studied through the lens of the transcriptome and the degradome using high-throughput sequencing technologies. Basically, a dual approach was implemented consisting in: (i) prior *in silico* identification of potential targets of pospiviroids on tomato coding sequences, (ii) the experimental validation of these PTGS and RdDM targets through small RNA and parallel analysis of RNA ends (PARE) sequencing and (iii) the differential gene expression analysis between infected and mock tomato plants by the quantification of PARE and small RNA reads in all known genes.

These analyses allowed for (i) highlighting new tomato PTGS targets, which unravel both counter defense strategies of pospiviroids and plant defense mechanisms, (ii) further understanding the decay pathways of pospviroids and (iii) gaining new insights in the transcriptional shift induced by pospiviroids.

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O5. Susceptibility to viroid infection of tomato plant in which RNA silencing key factors –AGO2, RDR6, or DCL2 & 4– is knocked down

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Viroids are strong RNA silencing inducers, and a large amount of viroid-specific small RNAs (vd-sRNA) accumulate in the infected host cells. Vd-sRNAs generated by the infection of pospiviroid that replicates in nucleus are composed of three majour size classes, 21, 22, and 24 nucleotides, which suggested that multiple dicer-like (DCLs) are involved in the biogenesis. Vd-sRNAs bind to AGO protein and are loaded to RISC complex, then target and suppress the replication of viroid itself or the expression of host genes that include sequences homologous to viroid, which is thought to cause certain disease symptoms. In order to analyze the inter-connection between RNA silencing induced by viroid infection and viroid pathogenesis, we have created transgenic Moneymaker tomato lines in which AGO2, RDR6 or DCL2 & DCL4, key factors of RNA silencing against foreign invaders such as viruses, was knocked down by RNAi-mediated manner. When these transgenic plants were challenged with potato spindle tuber viroid (PSTVd), the accumulation early in infection was significantly higher in DCL2&4-knockdown plant and in RDR6-knockdown plant than that in the control line. In addition, DCL2&4-knockdown plant showed severe leaf curl, chlorosis, and lethal systemic necrosis, which was in marked contrast to the very mild stunting in the control line. On the other hand, in AGO2-knockdown plant, both viroid concentration and symptoms were the same as the control line.

DCLs play a central role in the RNAi pathway and are key components in the biogenesis of small RNAs, called short-interfering-RNA and microRNA. Among the four DCLs described in plants, DCL2 and DCL4 play an important role in the defense against viruses. Published data on the detection of vd-sRNAs in viroid-infected plants suggest that the highly base-paired stem-loop structure of viroids can serve as a substrate for multiple DCLs (Itaya et al., 2001; Papaefthimiou et al., 2001; Itaya et al., 2007; etc.). A more detailed analysis using a series of *Nicotiana benthamiana* DCL-knockdown lines revealed that PSTVd levels dropped when either DCL4 expression alone was suppressed or DCL1, DCL2, or DCL3 was knocked down together with DCL4, which led to a hypothesis that the combined activity of DCL2 and DCL3 is crucial in the defense against PSTVd (Dadami et al., 2013; Katsarou et al., 2016). In DCL2&4-knowckdown tomato plant, in our experiment, the levels of endogenous DCL2 transcript was lower compared to the control line, and conversely the transcription level of DCL1 was significantly higher. The size distribution of PSTVd-derived small RNA in DCL2&4-knockdown plant was significantly changed: the numbers of 21 and 22 nucleotides species was approximately 66.7% and 5% of those in the control line, respectively. Conversely, the numbers of 24 nucleotide species increased by 1100%. The result indicated that tomato DCL2 and DCL4 provide anti-viroid defense in sufficient levels to suppress viroid accumulation early in infection and subsequent development of severe disease symptoms.

O6. Vertical and horizontal transmission of pospiviroids

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Viroids are transmitted through pollen to progeny seeds and healthy mother plants; the infection route from pollen to progeny seeds is called vertical transmission, whereas that from pollen to mother plants is horizontal transmission. Some reports relating to the seed transmission of pospiviroids have been published previously. However, histochemical information on seed infections by viroids is lacking. Therefore, we used *in situ* hybridization to observe the patterns of *Potato spindle tuber viroid* (PSTVd) in shoot apical meristems (SAMs), floral organs, ovaries after fertilization, and mature seed in each developmental stage of infected petunia, (*Petunia* × *hybrida*). In floral organs, PSTVd was present in the reproductive tissues of infected female × infected male and infected female × healthy male but not of healthy female × infected male before embryogenesis. After pollination, PSTVd was detected in the developed embryo and endosperm in all three crosses. These findings indicate that PSTVd is indirectly delivered to the embryo through ovule or pollen during the development of reproductive tissues before embryogenesis but not directly through maternal tissues as cell-to-cell movement during embryogenesis.

These results demonstrated that viroids are indirectly delivered to the embryo through the ovule or pollen during the development of reproductive tissues before embryogenesis, thus establishing vertical transmission. However, the mechanism through which viroids in the pollen tube infect mother plants after pollination and subsequently lead to systematic infection has been unclear. We found that *Tomato planta macho viroid* (TPMVd) was horizontally transmitted by petunia pollen but not PSTVd. Therefore, to elucidate the mechanism by which horizontal transmission of viroid is accomplished, we compared the distribution of TPMVd and PSTVd after pollination. Using tissue-printing hybridization to track the changes in viroid distribution after pollination, we noted that TPMVd was present in petunia stigma, styles, and eventually ovaries, whereas PSTVd was detected in the stigma and upper style but not the ovary. These findings suggest that horizontal transmission of viroids depends on the infection of the lower style and ovary during the elongation of pollen tubes after pollination. Additionally, TPMVd was transmitted horizontally, leading to systematic infection, when we used TPMVd-infected petunia pollen to pollinate the flowers of healthy tomato plants. Fertilization typically does not occur after heterologous pollination and thus likely is not required to accomplish horizontal transmission of viroids.

O7. Indirect assessment of viroid functions: chrysanthemum chlorotic mottle viroid-mediated trafficking of foreign mRNA into chloroplasts and the best laid plans of mice and men that often go awry

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While several members of the viroid family *Avsunviridae* have been shown to either replicate in chloroplasts [1,2] or traffic mRNA into chloroplasts for expression of a reporter gene [3], neither situation has been examined with chrysanthemum chlorotic mottle viroid (CChMVd). Therefore, to examine whether CChMVd sequences were involved in chloroplast localization, we cloned full-length (FL) CChMVd sequences upstream of a reporter gene encoding a modified red fluorescent protein (mRFP) into the agrobacterial vector pROK2 and infiltrated *Agrobacterium tumefaciens* harboring this vector into leaves of *Nicotiana benthamiana*, similar to the assay used to establish the ability of eggplant latent viroid (ELVd) to traffic a reporter gene into chloroplasts [3]. Confocal microscopy was used to show that red fluorescence due to the mRFP was present in both the cytoplasm and chloroplasts. Infiltrated tissues were extracted and protein blotting as well as RT-PCR confirmed the expressing of the mRFP and its mRNA, respectively. Infiltrated chloroplasts were isolated and shown by RT-PCR to contain sequences of both ChCMVd and mRFP mRNA. Additional constructs expressing CChMVd sequences from either nucleotide 125 through 400 and ending at nucleotide 2, or from nucleotides 231 to 372 were tested to delimit the CChMVd sequences involved in trafficking into chloroplasts. While the longer CChMVd fragment showed a cellular distribution of red fluorescence similar to that of FL CChMVd, the shorter fragment showed more mRFP present in the cytoplasm than in chloroplasts, indicating that the sequences 213-372 was not as efficient as either the longer fragment or FL CChMVd in trafficking into chloroplasts. Both viroid fragments and mRFP mRNA were confirmed to be present in isolated chloroplasts by RT-PCR. Since there is no appreciable sequence similarity between ELVd and CChMVd, this suggests that rather than particular sequence, perhaps stem-loop structures in the RNA molecules, possibly involving branches, are required for trafficking into chloroplasts.

Plant virus vectors have been used to express full-length viroid sequences to delimit sequences involved in trafficking into the nucleus [4-6]. A construct based on use of a tobacco mosaic virus vector was used in an unsuccessful attempt to identify sequences that control movement and symptom induction by chrysanthemum stunt viroid in chrysanthemum will be described as a caveat to others.

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O8. Citrus exocortis viroid causes ribosomal stress in tomato plants

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Ribosomopathies are human diseases associated with defects in ribosomal functioning, normally due to a haploinsufficiency of ribosomal proteins or defects on ribosome biogenesis[1]. To date, they have not been described in the plant kingdom. However, several works describe anomalies in plant ribosome biogenesis which are associated with serious developmental alterations[2].

Despite their lack of protein-coding capacity, several lines of evidence have related viroid infections with changes in the translation machinery. In this respect, viroids have been described to provoke alterations in the accumulation of ribosomal proteins or eukaryotic translation factors, to interact with eEF1A or with the ribosomal protein L5, to induce changes in dynamic DNA methylation of ribosomal RNA genes, to produce the over-accumulation of some rRNA-derived sRNAs, or even to trigger degradation of Ribosomal protein S3a-like mRNAs[3-8].

To explore the effect of viroid infection on the translational regulation, we have analyzed the tomato ribosomal profiles from control and Citrus exocortis viroid (CEVd)-infected leaves, observing that CEVd provokes alterations in global polysome profiles. Interestingly, we have detected the presence of CEVd and its derived VdsRNAs throuhgout the different fractions analyzed, thus indicating that this non-coding RNA pathogen is somehow associated with tomato ribosomes *in vivo*. To study whether the observed alterations in the tomato ribosome profiles could reflect alterations in ribosome biogenesis, we have studied by northern analysis the possible alterations in rRNA processing of the infected tomato plants. Our results suggest that CEVd produces an impairment of the 35S pre-rRNA processing, particularly in the 5' external transcribed spacer. To confirm the observed ribosomal stress caused by viroid, we have studied the induction of *ANAC82*, a transcription factor up-regulated upon ribosomal stress thus leading to growth defects and developmental alterations[9]. In accordance to our previous results, a significant induction of the *SlANAC82* was observed in CEVd-infected tomato plants.

Our results appear to indicate that CEVd provokes defective ribosome biogenesis in tomato, interfering with the translation machinery and therefore causing ribosomal stress.

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O9. Transcriptomic profiling of *Humulus lupulus* in response to *Citrus bark cracking viroid* (CBCVd) infection

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Viroids are the smallest (246–401 nt) self-replicating plant pathogens, consists of a single-stranded, covalently closed, circular and highly structured RNA. Viroid-induced symptoms range from necrosis to less severe developmental disorders, including leaf chlorosis, stunting, flowering alterations and fruit and seed deformations. The identification of viroid-specific small RNAs (sRNAs) in infected plants and the demonstration that viroid genome and/or replicative intermediates are inducers and potential targets of RNA silencing suggested that viroid-induced RNA silencing play an important role in disease development. Recently identified severe hop stunt disease caused by CBCVd (a member of the *Pospiviroidae* family) is one of the most devastating diseases. The CBCVd-infection causes dramatic morphological and anatomical changes, which include leaf epinasty, yellowing, premature flowering and a reduction in cone size, dry root rotting, stunted growth and dieback in hop (*Humulus lupulus* L.). In the present study, we employed CBCVd infecting hop as a system to dissect host interactions with pathogenic noncoding RNAs, using comprehensive transcriptome analyses. In CBCVd-infected hop compared to uninfected plants, 2,948 unigenes were identified to be differentially expressed genes (DEGs), most of which (2,004 unigenes) showed up-regulation. In response to CBCVd infection, several pathways were activated in hop including immune receptor signaling, calcium-dependent protein kinase and mitogen-activated protein kinase cascades as well as prominent genes involved in hormone signaling, hypersensitive responses, protein degradation, metabolic pathways and RNA silencing. The obtained data in our study will contribute for understanding of complexity of host immune systems, which may serve as future targets for improved disease control. Intriguingly, our data and preliminary analysis laid the foundation of involvement of Mediator (Med) complex in viroid pathogenesis. Currently, we are involved in deciphering the role Med-complex in natural mechanism associated with viroid suppression and elimination from male germline (anthers and pollen) using *Nicotiana tabacum* as model plant, which further resolve the long standing question regarding the involvement of Med-complex in viroid pathogenesis mechanism.

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O10. Identification and characterization of miRNAs in cucumber and their responses to hop stunt viroid (HSVd) infection

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MicroRNAs (miRNAs) play important gene-regulatory roles in plant development and stress responses. Some miRNAs have been proven to be critical in virus-host interaction. However, the biological functions of miRNAs in viroid-host interaction remain largely unclear. Here, we used severe and mild strains of hop stunt viroid (HSVd) infected cucumber plants (*cv* 'suyo') at different time points after inoculation to reveal the response of miRNAs to viroid infection by high-throughput sequencing. The results showed that the expression of 39 and 43 miRNAs was altered in severe and mild HSVd infected cucumber plants, respectively. It should be noted that most of these differently expressed miRNAs were identified at 28 days post inoculation (dpi), indicating that HSVd infection altered the expression of cucumber miRNAs mainly at late stage of infection when all the infected cucumber plants had been developed symptoms. Moreover, we analyzed the regulation pathways related to these differently expressed miRNAs and found several conserved known miRNAs in both severe and mild strains infected cucumber plants, including miR397, miR398a, and miR408. They regulate a large number of Cu proteins in plants. Down-regulation of the target of miRNA408 was verified using RT-qPCR and 5' RLM-RACE. Interestingly, altered expression of Cu proteins regulated by miRNA408 could influence photosynthesis. In the previous study, it has been shown that HSVd infection repressed expression of photosynthesis-related genes. Therefore, HSVd infection may regulate the expression of photosynthesis-related genes by miR408. Together, these results reveal that miRNA play some important roles in viroid-host interaction.

O11. HSVd infection impairs the epigenetic control of repetitive DNA in vegetative and gametic infected cells

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Viroids, ancient plant-pathogenic long noncoding-RNAs, have developed a singular evolutionary strategy based on reprogramming specific phases of host-metabolism to ensure that their infection cycle can be completed in infected-cells. However, the molecular aspects governing this trans-regulatory phenomenon, associated to the stress situation induced by viroid-infection, remains elusive. In general, eukaryotic organisms exposed to adverse conditions are impelled to favor a certain degree of transcriptional plasticity to cope with stress. Epigenetic regulation of the genome is a key regulatory mechanism allowing dynamic changes of the transcriptional status in plant-response to stress conditions.

By means of immunoprecipitation assays and bisulfite sequencing of rDNA we show that, in infected plants, Hop stunt viroid (HSVd) recruits and functionally subverts the Histone Deacetylase 6 (HDA6) to promote host-epigenetic alterations of ribosomal RNA (*rRNA*) promoter-regions in vegetative tissues, leading to increasing transcription rates of rRNA. Remarkably, transient overexpression of recombinant HDA6 reverts the hypomethylation status of rDNA observed in HSVd-infected plants and reduces viroid accumulation providing evidence of a functionally link between HDA6 activity and viroid biological efficiency. These results strongly suggest that the host-transcriptional alterations induced as a consequence of viroid-mediated HDA6 recruitment favor spurious recognition of HSVd-RNA as an RNA Pol II template, thereby improving viroid replication.

In addition to the clear alterations in vegetative tissues, HSVd infection is associated with drastic changes in gametophyte development. To illuminate the basis of viroid-induced alterations in reproductive tissues, we have analyzed the cellular and molecular consequences of HSVd infection in the male gametophyte of cucumber plants. Our results indicate that both HSVd mature forms and vd-sRNAs could be recovered from pollen grains of infected cucumber plants. Moreover, viroid accumulation was associated with an increased pollen germination level and heterochromatin decondensation in the generative nucleus. Analysis of DNA methylation in rDNA and TE repeats revealed a significant reduction in the symmetric cytosine methylation context, which was associated with a transcriptional increase of their RNAs, a phenomenon thus far unknown to occur in this reproductive tissue as a consequence of pathogen infection.

In summary, our results reveal that the previously observed epigenetic changes in vegetative tissues are maintained in male gametes, suggesting that these changes will be potentially inherited to the next generation.

O12. A yellow mosaic incited by peach latent mosaic viroid: strict association with a single-nucleotide change involved in RNA silencing-mediated cleavage of the mRNA coding for a thylakoid protein

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Introduction. Peach latent mosaic viroid (PLMVd) (Hernández and Flores, *PNAS USA* 1992) induces distinct phenotypes in its natural host. While most isolates are symptomless in leaves, hence the term latent in the name, some incite peach mosaics (PM) of different severity and a few an extreme albinism (peach calico, PC) (Flores et al., *Mol. Plant Pathol.* 2006). PC-inducing variants have a characteristic insertion of 12-13 nt in one apical loop and accumulate predominantly in symptomatic but not in adjacent green leaf sectors. We have documented that two 21-nt small RNAs (PLMVd-sRNAs) -containing the insertion associated with PC- guide cleavage of the mRNA encoding the chloroplastic heat-shock protein 90 (cHSP90) as predicted by RNA silencing mediated by Argonaute 1 (AGO1), which binds specifically 21-nt sRNAs with a 5'-terminal U, like the two PLMVd-sRNAs holding the PC-associated insertion (Navarro et al., *Plant J.* 2012). Cleavage of the mRNA coding for cHSP90 most likely is the primary molecular lesion causing eventually PC, since this protein mediates biogenesis of chloroplasts from proplastids. To understand how general such mechanism is, we have extended these studies to PM, a question far more difficult because it implies identification of the molecular determinant, which is not associated with any specific insertion, and because in contrast with the well-defined PC phenotype, PM differs in type and severity of symptoms.

Results. After some preliminary studies, we focused on a severe peach yellow mosaic (PYM) expressed in leaf sectors flanked by others green. Full-length PLMVd-cDNAs from both sectors were cloned and sequenced. From two variants, differing in one nucleotide change, we generated dimeric head-to-tail inserts in plasmids, with the corresponding *in vitro* transcripts being then bioassayed in GF305 peach seedlings. While one of the transcripts incited an intense PYM resembling the original field symptom, the other caused only mild alterations, thus supporting the involvement of one specific PLMVd nucleotide in this syndrome. In addition, the differential nucleotide change: i) was preserved in the progeny of the symptomatic but not in that of adjacent green sectors, ii) is absent in a set of PLMVd natural variants that replicate latently, and iii) turned a severe variant into latent when removed, and a latent variant into severe when inserted by site-directed mutagenesis. Analysis of the PLMVd-sRNAs and their potential mRNAs targets in the known peach genome, combined with RNA ligase mediated-rapid amplification of cDNA ends, have revealed that one 21-nt PLMVd-sRNA, with the PYM-associated change and two 5'-terminal Us, guide cleavage of the mRNA coding for a thylakoid protein as predicted by RNA silencing mediated by AGO1. Accumulation of this mRNA is lower in symptomatic than in asymptomatic sectors as shown by RT-qPCR, and some mutants in the gene coding for this protein, involved in photosynthesis, show yellow phenotypes akin to PYM.

O13. CBCVd in hops: what did we learn from its discovery?

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Ten years ago, hop growers in the main growing area, Savinja valley, reported the appearance of severely stunted hop plants. Such plants spread rapidly within hop fields and among farms. Classical and standard molecular diagnostic methods were unable to detect a new pathogen. Therefore, single-step high-throughput parallel sequencing of total RNA and small RNAs from plants with and without symptoms was employed to identify the novel pathogen. Sequences were assembled *de-novo* or mapped to reference viral/viroid genomes, resulting in the identification of a novel sequence of Citrus bark cracking viroid (CBCVd) in the stunted hop plants, never before reported in hops [1]. The new viroid in hops provided a challenge for farmers and authorities to prevent its spread and to eradicate infested plants, as well to develop a fast and reliable method for its detection. A reliable one-step multiplex RT-PCR (mRT-PCR) was developed to simultaneously detect all four viroids which infected hops: Hop latent viroid (HLVd), Hop stunt viroid (HSVd), Apple fruit crinkle viroid (AFCVd) and CBCVd [2]. At least two of the viroids, HLVd and CBCVd, are interesting models for studying host-viroid interactions due to the symptomless infection of the former and the severe stunting disease caused by the latter. Although the RNA molecules are insignificant in length, the pathogenicity of the viroid molecules is still puzzling the scientific community. However, several factors and mechanisms have been proposed for plant hosts, such as transcriptional or post-transcriptional gene silencing (TGS or PTGS), alteration of gene expression, and translocation of proteins. To study possible PTGS mechanisms *in-silico*, prediction of target hop transcripts for HLVd and CBCVd were performed. Prediction models revealed that 1062 and 1387 hop transcripts share nucleotide homologies with HLVd- and CBCVd-derived small RNAs, respectively. Therefore, they could be silenced in the RNAi process. Expression profiles of selected transcripts mainly showed expression fluctuations compared to viroid-free plants, with possible evidence of down-regulation of two transcripts. Additional expression profiles of five pathogenesis-related genes confirmed high expression levels of the four pathogenesis-related genes in HLVd and CBCVd infected plants [3]. To study the response of the hop micro RNA genes to CBCVd infection, we performed identification of 116 miRNAs from the hop genome. Many of them were found to be differentially expressed in response to CBCVd infection. Predicted targets for miRNAs included transcriptional factors that may regulate hop leaf, root and cone growth and development. Quantitative real time PCR analysis of selected targets revealed their negative correlation with their corresponding CBCVd-responsive miRNAs [4]. We believe that efforts to achieve better understanding of the molecular mechanisms of the viroid diseases are needed to initiate novel strategies against these diseases and help to discover genetic resistance.

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O14. Effects of potato spindle tuber viroid on the degradome of tomato and *Nicotiana benthamiana:* a comparative analysis

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Introduction. Viroid-derived small RNAs of 21-24 nt (vd-sRNAs) accumulate in viroid-infected tissues as a consequence of the activity of Dicer-like (DCL) RNases targeting these infectious RNAs or their double-stranded derivatives. vd-sRNAs are incorporated into Argonaute (AGO) proteins, forming the core of the RNA silencing complex, and guide it to cleave viroid and host mRNAs. To further dissect these molecular pathways and to investigate their involvement in viroid pathogenesis, we applied genome-wide approaches based on high-throughput sequencing of sRNAs and degradome analyses. Two different hosts, tomato and *N. benthamiana* —which develop similar symptoms of leaf curling/wrinkling and stunting when infected by potato spindle tuber viroid (PSTVd)— were examined, thus allowing comparative analyses of: i) the mRNAs targeted for cleavage by vd-sRNAs and ii) the effect of PSTVd on the accumulation of miRNAs and their cleaved targets.

Results. Although degradome analyses identified about one hundred tomato mRNAs potentially targeted for degradation by vd-sRNA, data from biological replicates and comparisons of healthy and infected samples reduced the number of true targeted mRNAs —i.e. those identified with high confidence in two infected biological replicates and not in healthy controls— to only eight mostly coding for proteins involved in development. These results were validated by RACE experiments in the three mRNAs examined. Interestingly, RT-qPCR showed that only four of the eight mRNAs actually accumulated at lower levels in the PSTVd-infected tomato, thus showing that cleavage of the mRNAs targeted by the vd-sRNAs is not necessarily associated with the down-regulation of gene expression. When the same degradome analyses were performed with healthy and PSTVd-infected *N. benthamiana* plants, all the identified mRNAs targeted by vd-sRNAs were different from those observed previously in tomato and, besides, they also differed in the molecular pathways they are involved. Therefore, this comparative analysis failed to pinpoint homologous gene(s), targeted by vd-sRNAs, potentially eliciting the common symptoms induced by PSTVd in tomato and *N. benthamiana*. Moreover, no significant modifications in the accumulation of miRNAs and their cleaved targeted mRNAs were detected in the PSTVd-infected tomato and *N. benthamiana* plants with respect to their healthy controls. The implications of these findings on the mechanism of PSTVd pathogenesis will be discussed.

O15. *In silico* molecular interaction between PSTVd, TCDVd and some *Solanaceae* species

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The severity of symptoms depends on the viroid strain (its RNA sequence), host species and cultivar, and environmental conditions, and can affect whole plants or specific organs. In a susceptible host PSTVd can cause a wide spectrum of symptoms, from no symptoms through mild and intermediate to severe and even lethal. The typical severe symptoms are stunting, shortening of stems, severe epinasty and rugosity of leaves, and necrosis of the veins and stems. Mild symptoms primarily appear as subtle stunting and epinasty. In addition to these macroscopic changes, disruption of the plasma membrane and abnormalities of the chloroplast and cell wall have been observed in PSTVd-infected plants. Viroids with minor sequence variations can produce different symptoms in infected plants, suggesting an involvement of vsRNAs in symptom production. Study of the viroid-host interaction has indicated that the mechanism of viroid pathogenesis can be mediated by the viroid genome itself or by viroid genome-derived ss- or dsRNAs that interact with host factors such as proteins or nucleic acids.

In this work we were interested to compare in silico the molecular and genetic interaction between on one side three PSTVd strains (Mild, RG1 and AS1) and TCDVd on the other side Potato (*Solanum tuberosum*), Tomato (*Solanum lycopersicum*), and *Nicotiana tabacum.* The aim of this work is first to identify new genes implied in host pathogen interaction and then to look for the possible interaction of these genes by using bioinformatics tools such as BLASTN et GENEMANIA (http://genemania.org/).

Our results show that different biological, physiological or molecular process in the plant may be affected by viroid infection. The number of the process identified varies from three to seven depending on the host and the pathogen. In the PSTVd genome the homology identified corresponds essentially to the pathogenicity domain or to the terminal left domain, while in TCDVd the homology found correspond to the central domain.

In this work we shows that the genes of the plant involved in the interaction depend on the viroid strain and on the plant species. For example PSTVd- AS1 in tomato may affect genes involved in response to heat. Such as vacuolar protein sorting-associated protein 54, chloroplastic-like which acts as component of the GARP complex (Golgi associated retrograde protein) that is involved in retrograde transport from early and late endosomes to the trans-Golgi network (TGN)? The GARP complex facilitates tethering as well as SNARE complex (Soluble N-éthylmaleimide-sensitive-factor Attachment protein Receptor) assembly at the Golgi (By similarity). It is also probably involved in pollen tube elongation and other polar growth.

Many other new function and genes has been identified in the different host (tomato, potato and tobacco) as responses to the different strains of PSTVd or TCDVd.

O16. The symptom pattern incited by chrysanthemum chlorotic mottle viroid results from RNA silencing of an enzyme of the Calvin's cycle and from its high mutation rate and population bottlenecks during host colonization

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Introduction. Chrysantemum chlorotic mottle viroid (CChMVd) (Navarro and Flores, *PNAS USA* 1997), a member of the genus *Pelamoviroid* (type member peach latent mosaic viroid, PLMVd), displays the higest mutation rate reported for any biological entity (Gago et al., *Science* 2009). To understand how CChMVd colonizes, evolves and incites disease in its natural host, two natural variants, one with a UUUC tetraloop tightly associated with symptoms and the other with an alternative GAAA tetraloop and asymptomatic (De la Peña et al., *PNAS USA* 1999), were assayed.

Results. As anticipated, the second one induced no phenotype, and RT-PCR, cloning and sequencing of its progeny showed variants with only the GAAA tetraloop. In contrast, the first variant induced early symptoms consisting in adjacent chlorotic and green leaf sectors, with progeny analysis disclosing that variants with the UUUC tetraloop predominate in chlorotic sectors, while in green sectors they coexisted with others having one or two mutations in the pathogenic determinant. Bioassay of the latter mutant variants led to asymptomatic infections (without the UUUC tetraloop in the progeny), showing that one single substitution in this tetraloop abrogates pathogenicity. Therefore, symtomatic and non-symptomatic CChMVd variants display distinct evolutionary trajectories, with the high mutation rate of CChMVd and the ability of some variants to colonize preferentially some leaf sectors and exclude other variants, accounting most likely for the observed results. Thus, CChMVd shows a territorial behaviour, with clearly segregated populations in a single leaf. The finding that a single substitution in the UUUC tetraloop disrupts pathogenicity is consistent with the involvement of RNA silencing. To gain support for this notion, we first examined by deep sequencing the viroid-derived small RNAs (CChMV-sRNAs) generated by RNA silencing: a significant prevalence of those of 21 nt was observed, in line with previous results for PLMVd. On the other hand, the chrysanthemum transcriptome of a cultivar in which CChMVd incites clear symptoms was determined. Combining both set of data we found a 21-nt CChMV-sRNA (containing the pathogenic determinant and with two 5'-terminal Us) potentially targetting for cleavage, as predicted by RNA silencing mediated by AGO1, the mRNA of an enzyme of the Calvin's cycle, the light-independent part of photosyntesis occurring in the chloroplast stroma. RNA ligase mediated-rapid amplification of cDNA ends confirmed that this mRNA was cleaved in the expected site (between positions 10 and 11 of the complementary 21-nt CChMV-sRNA) only in symptomatic sectors. Three additional lines of evidence support that such RNA silencing mechanism mediates the CChMVd-induced primary lesion. First, RT-qPCR showed a reduced level of this mRNA in symptomatic sectors, where the complementary 21-nt CChMV-sRNA accumulates preferentially. Second, a full-length CChMVd mutated so that the mismatch at position 6 in the hybrid between the sRNA and the mRNA was converted into a canonical base-pair, was infectious and incited symptoms more severe than the wild-type. And third, artificially-induced reduction of the expression of the corresponding gene results in chlorotic phenotypes.

O17. Viroid taxonomy: state of the art and perspectives in the high-throughput sequencing era

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Proposed in the early 1990s (Elena et al., *Proc. Natl. Acad. Sci. USA* 1991), the first viroid classification scheme that grouped viroids into species was officially adopted in 1995 by the International Committee on Taxonomy of Viruses (ICTV, https://talk.ictvonline.org). Higher taxa (genera and family) were then proposed (Flores et al., *Arch. Virology* 1998) and nowadays 32 species, five genera and the two families (*Pospiviroidae* and *Avsunviroidae*) have been created by ICTV. As a general rule, ICTV demarcating criteria for viroid species include sequence identity below 90% with respect to members of other viroid species, together with different biological features. While the first criterion is relatively easy to apply, this is not the case for the second, the application of which is generally time-consuming, particularly for viroids i) with restricted or similar host range, ii) inducing latent infections, and/or iii) infecting woody plants. In the last few years, the availability of high-throughput sequencing technologies (HTS) has allowed discovery of several new viroids, especially from latently-infected woody hosts. In the absence of the biological data requested for their proper classification, including autonomous replication and systemic trafficking, several of these viroids are still considered tentative species, a situation that most likely will not change in the short term if not even worsens, as the list of unclassified viroids becomes longer. The strengths and weaknesses of the current viroid species demarcation criteria adopted by ICTV, along with the opportunity of proposing an order, *Viroidales*, as the higher taxonomic rank for viroids, will be discussed.

O18. Citrus viroid VII, a novel citrus viroid found in Lisbon Lemon in Australia

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A novel citrus viroid was discovered following routine biological indexing of a non-symptomatic Lisbon lemon (*Citrus* x *limon* (L.) Osbeck) field tree located in Dareton, New South Wales, Australia, in February 2015. 'Etrog' citrons (*Citrus medica* L.) Arizona 861-S-1, the bioamplification and indicator host for citrus viroids, were graft-inoculated and maintained at 32°C, where they developed epinasty symptoms indicative of citrus viroid(s) infection (Fig. 1). Total RNA extracted from the symptomatic citrons was slash-inoculated into new citrons and tested for all known citrus viroids by conventional RT-PCR with negative results. RT followed by quantitative PCR (qPCR) using a SYBR green assay for the universal detection of citrus apscaviroids [1], gave a 281bp product with a different melting temperature from the known apscaviroids. Sequence analysis showed low similarity with the *Australian grapevine viroid* (AGVd). Overlapping and sequence-specific primers to the 281bp product were designed for the identification of the full genome sequence of the putative circular viroid genome. RT-PCR with the new primers gave a 368 bp product (KX013549-551) from the Lisbon field tree, an adjacent Taylor Eureka lemon tree, and both the graft- and slash-inoculated symptomatic citrons. Sequence analysis showed 52.2% G+C content and 59-59.5% sequence similarity with AGVd (FJ746829). The predicted thermodynamically stable secondary RNA structure was a hairpin with 69% paired nucleotides that contained a terminal conserved region (TCR) and a central conserved region (CCR) with predicted hairpin I (HPI) and hairpin II (HPII) structures, typical of apscaviroids. sPAGE followed by northern blot hybridization analysis of the graft- and slash-inoculated citrons revealed both circular and linear viroid-like RNA forms of the expected size (Fig. 2). Transmissibility and autonomous replication of the viroid-like RNA, were verified by slash inoculation of citrons with a monomeric RNA transcript of 368nt. Four months after inoculation, citrons tested positive for both positive and negative sense RNA molecules identical to the RNA transcript inoculum. Based on the above biological, molecular, and *in silico* analysis (naturally occurring, transmissible, self-replicating, circular, rod-shaped, GC rich, 368 nt, and TCR, CCR containing ssRNA) it has been proposed that this viroid-like RNA is a new species in the genus *Apscaviroid* of the family *Pospiviroidae*, and was tentatively named "citrus viroid VII" (CVd-VII) [2]. CVd-VII meets one of the demarcation criteria for new viroid species (less than 90% sequence identity to other apscaviroids) and further studies are underway to identify distinct biological and molecular properties, for an official proposal to the International Committee on Taxonomy of Viruses (ICTV) [3].

Figure 1. 'Etrog' citrons symptoms [2].

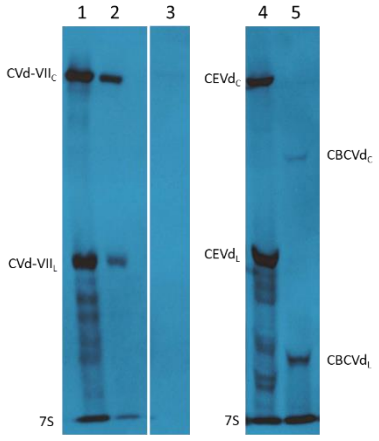

Figure 2. sPAGE analysis of circular (C) and linear (L) forms of viroid RNAs from inoculated 'Etrog' citron. CVd-VII: Citrus viroid VII-368nt (1: graft-inoculated & 2: slash-inoculated), Non-inoculated 'Etrog' citron (3), CEVd: Citrus exocortis viroid-371 nt (4), CBCVd: Citrus bark cracking viroid-284 nt (5) [2].

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O19. Molecular characterization of apple hammerhead viroid isolates from ancient Italian apple cultivars

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Introduction. High-throughput sequencing (HTS) technologies have contributed to the identification of several viroids and viroid-like RNAs, including a small (434 nt) circular RNA containing the conserved domains of hammerhead ribozymes in apple trees grown in China (Zhang et al., *PLoS Pathogens* 2014). Although the possible viroid nature of this RNA was anticipated, the final proof was only recently provided by bioassays confirming its autonomous replication in apple (Serra et al., *Virus Res.* 2018). In the same study, based on *in silico* modeling and co-variation analysis of viroid populations, the two polarity strands of AHVd were predicted to adopt branched conformations. Interestingly, the (+) polarity strand of AHVd, but not its (-) counterpart, was proposed to be stabilized by a kissing loop-interaction, a situation resembling that of peach latent mosaic viroid and chrysanthemum chlorotic mottle viroid (genus *Pelamoviroid*, family *Avsunviroidae*). Besides China, AHVd has been identified only in the cultivar Pacific Gale in Canada (Messmer et al., *Can J. Plant Pathol.* 2017).

Results. In the frame of a project aimed to examine the phytosanitary status of ancient Apulian fruit tree varieties, partially purified dsRNA preparations from several fruit tree species were pooled together and analyzed by HTS (Illumina platform) for the presence of viruses and viroids. By this approach, a single contig of 303 nt sharing high sequence identity with AHVd was identified in one of the sequenced cDNA libraries. RT-PCR with AHVd-specific primers and total RNA preparations from each plant used to prepare the sequenced cDNA library allowed the identification of two apple trees of the local ancient varieties "Mela Rosa Guadagno" (MRG) and "Agostinella" (AG) as the original source plants infected by AHVd, the first report of this viroid in Italy. The natural variability of AHVd viroid populations infecting MRG and AG isolates was studied. With only five changes with respect to the master sequence, AHVd variants from MRG isolate were less variable than those from AG isolate, where polymorphic positions with respect to its master sequence were about fifty. AHVd sequence variants from the two Italian isolates were 97-93 % identical to each other, while they shared lower sequence identity with those from the China and Canada. Interestingly, most nucleotide changes in the Italian AHVd variants did not result in major modifications of the branched secondary structure recently proposed for this viroid by Serra et al. (2018). Interestingly, mutations were found in the two hairpin predicted to form a kissing-loop interaction in the AHVd (+) strand. However, nucleotide rearrangements preserved the potential kissing-loop structure, although stabilized by three instead of four interacting base pairs in the Italian isolates. Altogether, these data showing consistent co-variations preserving both the branched secondary structure and the kissing-loop interaction in AHVd variants of different origins, provide solid support for the major biological role of these structural elements.

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O20. What's up with the viroid structural compendium?

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The subviral pathogens known as viroids are composed of a single stranded and circular RNA genome in the size range of 246-401 nt. The various species known are causing a wide array of symptoms to many plants. An important feature is their non-coding genome. This has for consequence that they depend on their sequence and structure to infect an host. The classification of viroids based only on their sequence was previously shown to be insufficient. To strengthen that classification, we believe that the use of the secondary structure of the viroids is useful. Generally, their structures were predicted with thermodynamics-based RNA folding programs, which were shown to lack precision for RNA longer than 200 nt. Thus the predicted structure of a viroid needs to have more information on its folding to produce accurate models for interpreting any systematic studies.

Following the adaptation of SHAPE probing and computer-assisted structure prediction to the viroid, we have elucidated the structure of at least one sequence variant of all known species: the 4 *Avsunviroidae* members, the 30 *Pospiviroidae* species; 2 viroid-like satellite RNA and 1 not confirmed viroid. There were many significant differences compared to predicted structure in absence of probing data, confirming the importance of this study. In addition to providing a complete compendium of viroid structure, this analysis permitted to ascertain structural motifs that could be important for their biology and classification.

O21. Direct visualization of the native conformation of PSTVd, PLMVd and ELVd at single-molecule resolution by atomic force microscopy

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Atomic force microscope (AFM) has become a relevant nanotechnological platform in the life sciences, allowing structural and dynamic studies of a growing number of biochemical and biological systems. One of the main advantages of AFM over electron microscopy-based techniques is that it offers a 3D surface profile of the imaged sample without requiring any staining or coating, thus minimizing the structural disruption of the biological entity under study. Additionally, it allows the visualization and manipulation of molecular entities across length scales that range from single biomolecules to living cells. As a result, AFM can provide nanometer spatial resolution of RNA molecules of different lengths and structures, as well as of RNA-RNA or RNA-protein complexes.

The compact, rod-like or branched secondary structures of viroid RNAs have been predicted by RNA folding algorithms and further examined using different *in vitro* and *in vivo* experimental techniques. However, direct data about their native tertiary structure remain scarce. Herein, we have applied AFM to image, at single-molecule resolution, different variant RNAs of three representative viroids: potato spindle tuber viroid (PSTVd, the type member of the family *Pospiviroidae*), as well as peach latent mosaic viroid (PLMVd) and eggplant latent viroid (ELVd) that belong to the *Avsunviroidae* (Fig. 1). Our results provide a direct visualization of the native, three-dimensional conformation of single viroid RNA molecules, and highlight the relevance that some elements of tertiary structure play in their stabilization.

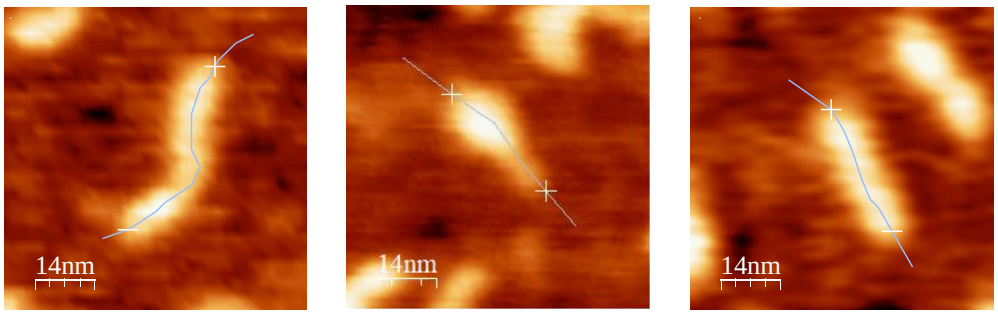

Fig. 1. Examples of single viroid RNA molecules visualized by AFM: PSTVd, PLMVd and ELVd (from left to right).

O22. *Citrus bark cracking viroid*: biological properties and impact on Slovene hop production

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Citrus bark cracking viroid (CBCVd) has been discovered as a causal agent of a highly aggressive disease on hop (*Humulus lupulus*) called »severe hop stunt disease« which was observed for the first time in Slovenia in 2007. CBCVd has a highly negative impact to hop production, since infected plants develop severe stunting in the first year after infection and die in 3-5 years. The spread of CBCVd in hop gardens is extremely rapid, due to the specific agro-technical practices in hop production, which creates ideal conditions for mechanical transmission, and due to the physiological characteristics of hop as a green herbaceous plant. Until now, CBCVd affected more than 100 ha of hop gardens in the vicinity of primary outbreak in Savinja valley, from which half of them was totally eradicated. Strict phytosanitary measures significantly reduce the disease incidence, however majority of infected farms recorded new outbreaks every year. To explore the risk of CBCVd transmission to other cultural plants and to assess the role of weed species as possible CBCVd reservoir, inoculation tests were performed which showed no infections in weeds characteristics for hop fields and no risk for 10 arable and horticultural plants which are frequently grown in Slovenia. In addition CBCVd sensitivity of 32 hop cultivars was tested in 3 years in hop garden where source of infection presents multiple sap inoculations and infected plants planted between sets of tested cultivars. The cultivars showed different level of susceptibility and were classified into three groups: highly sensitive, moderately sensitive and tolerant. CBCVd transmission was studied also on level of possible insect vectors where we focus to major hop pests. Interestingly, the RT-PCR detected 100% presence of CBCVd in two spotted spider mites (*Tetranychus urticae*) which was fed on infected plants; however transmission study excludes this pest as a possible vector. Based on currently known biological characteristics of CBCVd on hop, critical spreading points in hop cultivation were defined which presents basis for disease management strategy.

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O23. Molecular characterization of potato spindle tuber viroid from dahlia plants in Japan

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In 2011, Potato spindle tuber viroid (PSTVd) belonging to Pospiviroid family was detected in Akita Prefecture (North Japan). When we surveyed all dahlia leaves (about 5000 stocks) in the field where PSTVd was detected, PSTVd isolates without registration in the database were identified from 7 kinds of dahlia. The newly discovered PSTVd isolates were very similar to the PSTVd-dahlia isolate which had already been found in Japan, and none of the infected dahlia showed any symptoms. PSTVd-infected dahlias in Akita prefecture including plants that having the risk of PSTVd infection were already pulled out and disposed. After those procedure, we had been checked the PSTVd infection with patrol (visual inspection) and molecular biological method (qRT-PCR) regularly, and no infection has been confirmed since then.

Besides, we created a total of 11 sequences of PSTVd-dahlia isolates, and carried out inoculation tests to dahlias and tomatoes. Three months after inoculation, some dahlia plants were clearly infected with PSTVd, but no visible symptoms were observed. However, PSTVd-infected dahlias in our inoculation tests grew poorly one year after inoculation, and didn't root well as we attempted cuttage. Whereas in tomato, PSTVd-dahlia mutants could easily infect without causing noticeable disease symptoms. Mutational analysis of the 11 PSTVd-dahlia mutants by sequencing of cloned cDNAs revealed that four variants obtained mutations after infection and replication in tomato. Here, we dissect the features of PSTVd detected from dahlia, and our results suggested a nucleotides specific to PSTVd-dahlia isolates involved in the latent to mild disease symptoms inducing.

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O24. Detection of *Pepper chat fruit viroid* (PCFVd) by reverse transcription loop-mediated isothermal amplification (RT-LAMP)

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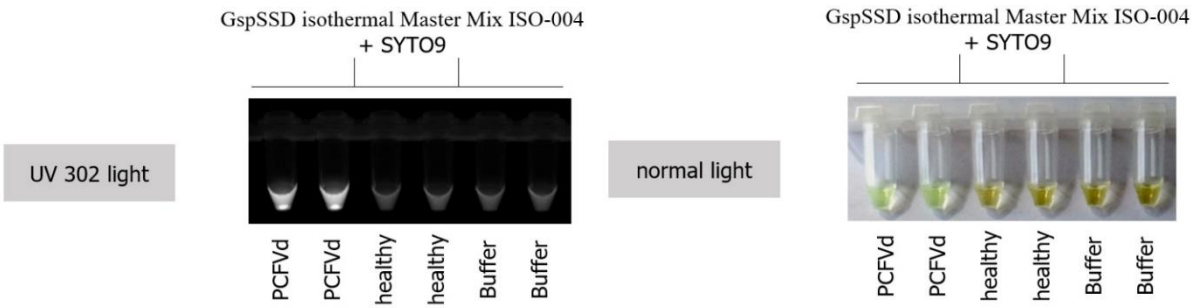
Pepper chat fruit viroid (PCFVd) is one of the most important tomato and pepper diseases causing serious losses, affecting productivity, fruit quality and even international seed trade. In addition, PCFVd experimentally infects eggplant (*Solanum melongena*) and bolo maka plants (*Solanum stramonifolium* Jacq.), a common home-grown vegetable in Southeast Asia which is usually planted near other solanaceous crops. In 2010, this viroid was first found in Thailand in an open-field tomato seed production facility in Khon Kaen province. Subsequently, a survey in 2011 and 2012 revealed that the pathogen was spread widely in numerous tomato seed production areas, including Nong Khai, Chiang Rai, Lamphun and Lampang provinces. Unfortunately, in 2012 the Australian Government Department of Agriculture, Fisheries, and Forestry (DAFF) intercepted PCFVd-infected tomato seed lots imported from Thailand and all the infected tomato seed shipments were destroyed following Australian regulations. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a fast and reliable RNA diagnostic assay, outcompeting distinctly conventional reverse transcription polymerase chain reaction (RT-PCR) in robustness, analytical sensitivity and specificity. In addition, it is also highly cost-effective and the procedure is far less time consuming. In this work, a PCFVd specific RT-LAMP detection assay was developed, based on a set of six primers. PCFVd can be detected within 15 minutes with a sensitivity that is comparable as a probe-based RT-qPCR and 10-100 times higher than the available endpoint RT-PCR methods. No cross-amplification with other viroids and tomato viruses was observed. The diagnostic assay was adapted for on-site applications with a simple plant lysis procedure and SYTO-9, fluorescence dye, for visualization of the results under both visible and UV lights. In the closed-tube reaction mix, positive reactions displayed color change from yellow to lime green under visible light and fluorescence under UV light. The results indicate that this PCFVd RT-LAMP assay will be useful for any in-field application of the method, such as seed certification schemes and quarantine purposes.



O25. Overcoming the challenges of the *Potato spindle tuber viroid* diagnostic in tomato seeds, leaves and water samples

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Potato spindle tuber viroid (PSTVd), as other related pospiviroids, is a serious threat to tomato production. It is seed-borne, very stable outside the host plant, and easily transmissible mechanically. Just one infected plant grown from a single infected tomato seed has the potential to rapidly spread the infection mechanically to neighbouring plants, resulting in a larger outbreak. We have shown that irrigation water can also be a path for PSTVd transmission between plants (1). Therefore, it is essential to monitor for this pathogen through all of the critical points in tomato production and pathogen transmission pathways.

In the contrary to infected plant leaves, PSTVd in irrigation waters and seed samples is usually present at levels below the detection limit of reverse-transcription PCR (RT-PCR) so its detection requires an appropriate concentration step. We have shown that monolithic chromatographic supports (i.e., convective interaction media; CIM) are appropriate for the concentration of PSTVd from water samples (2). PSTVd in tomato seeds can be revealed directly using real-time quantitative reverse-transcription PCR (RT-qPCR). However, RT-qPCR assays that are available are not specific for PSTVd, and therefore sequencing of the RT-PCR product is required for confirmation. Typically, RT-PCR is less sensitive than RT-qPCR, and the confirmatory tests with RT-PCR can be limited and unsuccessful if the PSTVd levels are too low. To improve the sensitivity of PSTVd diagnostic methods, we developed an easy-to-use and efficient concentration method for PSTVd in seed samples (3). This method is based on RNA binding to positively charged anion-exchange resin beads andprovides up to 100-fold more sensitive detection in comparison with procedures without a concentration step.

The detection of PSTVd in symptomatic leaf tissue does not need a concentration step due to high viroid titer. We developed reverse-transcription loop-mediated isothermal amplification (RT-LAMP) procedure for detection of PSTVd in leaf tissue (4). Moreover, we also adapted this assay for direct detection of PSTVd, i.e., without the need to extract the RNA (3). This assay allows rapid detection of PSTVd, in under 30 min, and it may be used on-site for screening of tomato leaves with symptoms.

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O26. *Citrus dwarfing viroid* reduces citrus apical shoot growth and alters tree hormone profile. Pathogen or an answer to emerging citriculture challenges?

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Citrus dwarfing viroid (CDVd) infection of navel orange (*Citrus sinensis* (L.) Osb.) trees on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstock has been previously reported to reduce canopy volume by approximately 50% [1, 2] (Fig. 1). To understand how CDVd reduces canopy volume, a survey of navel orange tree growth was initiated in an experimental block planted in 1998 at the Lindcove Research and Extension Center (LREC) in the San Joaquin Valley, California. We first confirmed that 20 years after planting there was still a statistically significant difference in tree height and canopy volume ($p< 0.01$) between CDVd-infected trees (2.19±0.04 m & 6.63±0.78 m^3, n= 29) and non-infected controls (2.91±0.06 m & 17.28±2.18 m^3, n= 8). Since vegetative growth accounts for most of a tree's canopy volume, we then monitored vegetative apical shoot growth over two seasons in the CDVd-infected and non-infected control trees. We found that the net growth of the apical shoots of CDVd-infected trees (2.60±0.11 cm, n= 232) was significantly lower (reduced by almost 30%, $p< 0.01$) than that of the non-infected controls (3.6±0.27 cm, n= 64). Phytohormones play a key role in tree growth and development. To further understand the physiological mechanisms modulated by CDVd infection, a phytohormone profiling study was undertaken. CDVd-infected trees (n= 3) exhibited a different hormone profile compared to the non-infected controls (n= 3). CDVd-infected trees had higher levels of auxins (active IAA forms) and cytokinins than the non-infected controls in both shoots and roots. CDVd-infected shoots had the only gibberellin (GA) precursor (GA19), and CDVd-infected roots had the only active GA (GA3). CDVd-infected trees had more active abscisic acid (ABA) and precursor of ethylene (ACC) in the shoots and less in the roots than the non-infected controls. The findings of this study indicate that the effect of CDVd on apical shoot growth is likely to include an altered hormonal mechanism to determine the observed dwarfing phenotype. Dwarfed citrus trees are fundamental for high-density plantings, which will be critical to meet the citriculture challenges posed by water shortages, farmland reduction, and labor costs. Dwarfed citrus trees have also the potential to play an important role in the management of the devastating citrus disease Huanglongbing (HLB) by simplifying inspections and chemical control of the psyllid vector, allowing for planting of higher number of trees per land surface unit thus mitigating losses from HLB tree eradication or permitting in ground plantings under protective structures [3]. The term 'Transmissible small nuclear Ribonucleic acids' (TsnRNAs) was used in the past to describe well-characterized viroid RNA species, such as CDVd, that did not induce a disease or crop damage (yield or fruit quality) in specific citrus hosts, but rather acted as regulatory genetic elements modifying tree size [1]. In the absence of a true citrus dwarfing rootstock, the importance of elucidating the TsnRNA-mediated citrus dwarfing mechanism, lies in the potential to develop citrus dwarfing applications that do not require a transmissible agent thus making it easier for regulatory approval and large scale commercial use with reduced risk of cross contamination of untreated orchards.

Figure 1. Non-infected control (L); Citrus dwarfing viroid infected tree (R).

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O27. An eggplant latent viroid-based platform to produce recombinant RNA in *Escherichia coli*

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Viroids replicate through an RNA-to-RNA rolling circle mechanism in which host plant RNA polymerases transcribe viroid strands. After processing, monomeric linear replication intermediates are ligated to viroid mature circular forms by the host DNA ligase 1 or the chloroplastic isoform of tRNA ligase, depending on the viroid family, *Pospiviroidae* and *Avsunviroidae*, respectively (Nohales et al., 2012a; Nohales et al., 2012b). We have recently set up an experimental system to analyze the ligation of *Eggplant latent viroid* (ELVd, *Avsunviroidae*) mediated by the eggplant (*Solanum melongena* L.) tRNA ligase (Cordero et al., 2018). The system is based on co-expression in *Escherichia coli* of a longer-than-unit ELVd transcript and eggplant tRNA ligase. In *E. coli*, ELVd transcripts self-cleave through the embedded hammerhead ribozymes and the resulting monomers with 5'-hydroxyl and 2',3'-cyclic phosphodiester termini are recognized by the co-expressed plant enzyme and circularized. Analysis of ELVd mutants, using this system, has recently shown an unexpected role of ribozyme domain in viroid RNA circularization (Cordero et al., 2018).

One remarkable property of this experimental system is that, despite ELVd does not replicate in *E. coli*, viroid RNAs transitorily accumulate in bacterial cells to amazing amount that largely exceed those of abundant endogenous RNAs, such as the 16 and 23S rRNAs. This is most probably a consequence of the physical interaction between ELVd and tRNA ligase to form a stable ribonucleoprotein complex that is compartmentalized in bacterial inclusion bodies. Interestingly, insertion of RNAs of interest, such as aptamers, extended hairpins and other structured RNAs, into particular positions of the ELVd molecule does not substantially interfere with processing and large accumulation of viroid circular forms. This is the basis of our system to produce large amounts of recombinant RNAs in *E. coli* (Daròs et al., 2018). A cDNA corresponding to the RNA of interest is inserted between positions T245 and T246 of ELVd-AJ536613 cDNA. The transcript that is produced in *E. coli* along eggplant tRNA ligase by a strong constitutive promoter is processed and circularized. Large amounts of chimeric circular molecules in which the RNA of interest is presented on the viroid scaffold accumulate in bacterial cells. These chimeric RNAs can be purified to homogeneity taking advantage of the unique property of viroid circularity. If required, the RNA of interest can be released from the viroid scaffold by an RNase H treatment using flanking oligodeoxynucleotides.

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O28. The amazing small circular RNAs with hammerhead ribozymes colonizing the genomes of eukaryotes

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While circular DNAs are common genomic molecules among most biological entities (viruses, prokaryotes or eukaryotic organelles), circular RNAs (circRNAs) have been historically regarded as unusual nucleic acids in biology. Recent data, however, have confirmed that eukaryotic genes can be expressed through backsplicing as circRNA isoforms with different roles in gene regulation. In our lab, we have discovered that plants and animals have a second natural pathway of circRNA expression, which involves tandem copies of self-cleaving hammerhead ribozymes widespread throughout their genomes. These small (~150-1,000 nt) genome-encoded circRNAs with ribozymes accumulate at high levels in most tissues, and constitute the retrotransposition intermediates of novel forms of non-autonomous retroelements (*retrozymes*). We have found that the genomes of many plants, animals and unicellular eukaryotes have been colonized by diverse families of *retrozymes*, which allow us to draw an evolutionary path for the origin of viroids, viral satellites and other viroid-like circRNAs with ribozymes.

O29. Viroids as companions of my professional and personal life

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Having acquired an *in vitro* tissue culture expertise gave me the opportunity to become a scientist working on viroids. This started as early as in 1979 when Prof. J.S.Semancik was looking for a tissue culture expert to develop an *in vitro* approach to study/characterize the causal agent of the exocortis disease. Having joined his laboratory at UCR, I was able to live first hand a number of events that allowed the characterization of VIROIDS as a new class of disease causing agents.

Since early 1970s when "virus-like" agents were considered as the cause of two diseases (potato spindle tuber and citrus exocortis) their study and further characterization has been linked to the development and use of molecular biology tools. Sucrose density gradient centrifugation and polyacrylamide gel electrophoresis (PAGE) played a critical role in the pioneering studies of PSTVd and CEVd. This was later modified by using PAGE under denaturing conditions, especially as double PAGE (sequential PAGE, return PAGE, two dimensional PAGE), temperature gradient gel electrophoresis,… together with the adaptation to different staining protocols (ethidium bromide, silver, ….). Since then, disease causing agents suspected to be viroids were usually subjected to a number of tests to define: i) RNA or DNA; ii) ssRNA or dsRNA; iii) circular RNA or linear RNA; iv) molecular weight; v) secondary and tertiary structure.

An important even that also took place in the 1970s was "viroid sequencing", actually the first sequence of a full genome. This first RNA sequencing approach has evolved considerably through the use of the evolving nucleic acid approaches: Retrotranscription and DNA amplification, cloning, DNA sequencing and an increasing number of easy to handle sequencing tools,…

The biological characterization of viroids is commonly restricted to assays conducted in greenhouses in many instances using alternative hosts. However, one should not forget that the definition of their role as disease causing agents requires the performance of field assays that in many instances are expensive, not easy to handle and not adequately valued.

By working on research I became aware that "competitiveness" is a quite common way of acting in the scientific world. Since the very beginning I tried to discard this approach and focus on "cooperation" which has given me the opportunity to build interesting relationships and undertake collaboration approaches with students and scientists from different parts of the world. That is how VIROIDS became excellent companions not only in my professional career but also in my personal life.

O30. Viroids – at the forefront of RNA technology and RNA biology

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Viroids were firstly described as the smallest RNA molecules capable to infect plants and to autonomously self-replicate close to 50 years ago. Here we will not review the accumulated knowledge on viroids but highlight methodological development and theoretical progression of RNA technology and basic insights into RNA biology that were induced by research of viroids. Major highlights are:

- the necessity of pure viroid RNA in amounts sufficient for biophysical analysis led to novel chromatography and finally to the plasmid kits, which are the foundation of the company Qiagen;

- the circularity and characteristic structural features of viroids asked for major improvements in quantitative thermodynamic as well as kinetic descriptions of RNA folding processes and showed the functional importance of metastable structures;

- the quantitative interpretation of a viroid's structural feature by NMR spectroscopy led to the detection of two-bond coupling via electron-electron interaction proving unequivocally the covalent nature of H-bonds in biological macromolecules.

That is, several techniques and general features of nucleic acids resulted from basic research on viroids. Specifically, although viroids were the first discovered circular RNA, techniques developed in viroid research will be of help for the much wider spectrum of circRNAs as well as for other non-coding RNAs.

O31. Viroids: reflections on two contentious issues and one unsolved conundrum

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Introduction. Research on viroids has evolved over time. The main focus initially placed on the identification and sequencing of new members of the group (specially those inducing disease), later moved to understanding replication (particularly the templates, enzymes and ribozymes implicated) and more recently, with the advent of RNA silencing, to the role played by this mechanism on pathogenesis. In addition, a constant attention has been paid to the viroid RNA itself, in an attempt to link its apparently simple structure with its remarkable functional properties. Here I will discuss two of these topics and one intriguing observation.

RNA silencing and viroid pathogenesis. Whether RNA silencing mediates the primary viroid-induced lesion leading through an amplification cascade to symptoms, or it is just involved in intermediate-late steps of this cascade, are two distinct issues. Connecting the initial molecular alteration to symptoms demands that they should be early, specific and local (e.g. expressed in sectors rather that in the whole leaf area). An example is peach calico (PC), the albinism incited by certain variants of peach latent mosaic viroid (PLMVd, family *Avsunviroidae)* harboring a characteristic 12-13-nt insertion, while the stunting caused by potato spindle tuber viroid (PSTVd) and some other members of the family *Pospiviroidae* illustrates a late, non-specific, and systemically-expressed symptom. Remarkably, two 21-nt RNAs with the PC-associated insertion accumulating mainly in PC-expressing sectors target for cleavage, as predicted by RNA silencing mediated by Argonaute 1 (AGO1), the mRNA coding for the chloroplastic heat-shock protein 90 involved in the biogenesis of chloroplasts from proplastids, thus establishing a direct and short link between the primary lesion and the phenotypic alteration (Navarro et al., *Plant J.* 2012). Moreover, concordant data for other PLMVd variants inducing a yellow mosaic (PYM) and for chrysanthemum chlorotic mottle viroid (CChMVd, family *Avsunviroidae)* have been obtained: two 21-nt RNAs with the PYM and CChMVd pathogenic determinants (1- and 4-nt substitutions, respectively) target for cleavage, again as predicted by RNA silencing mediated by AGO1, the mRNAs encoding two chloroplastic proteins, some mutants of which result in different chloroses (our unpublished data). Tracing similar connections, if they exist, for members of the family *Pospiviroidae* is a considerably more difficult task due to the kind of the symptoms they induce.

Viroid RNA structure *in silico, in vitro* and *in vivo*. Free-energy minimization provides good estimates for small RNA motifs, but loses accuracy when the size augments. Biochemical and biophysical techniques in solution have inherent limitations resulting from the initial thermal denaturation/renaturation (presumed, but in most cases not proved, to restore the "native" structure) and from the lack of proteins that may stabilize one particular structure. *In planta* approaches, namely SHAPE *in vivo* and co-variations analyses of natural variants in which the 3D structure is preserved, are labor-intensive and not always applicable. A wise conflation of the three approaches, which occasionally do not produce coincidental results, should be applied recalling the Roman dictum: *"in vi(n/v)o veritas"*.

High temperature and increased viroid accumulation. The early observation that PSTVd titer in tomato plants rises with temperature up to 35 °C (Sänger and Ramm, *Adv. Biosc.* 1975), to a good extent then corroborated for other viroids, has remained uninterpreted for more than 40 years. What is the mechanism(s) behind? Increased replication by unwinding the compact viroid RNA secondary structure, thus making it more accessible to RNA polymerases? Decreased activity of degradation routes? Facilitated intracellular, intercellular or long-distance movement? A combination of more that one of these routes?

P1. A salycilate hydroxylase involved in the tomato defence against citrus exocortis viroid

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Salicylic acid (SA; 2-hydroxybenzoic acid) is a plant hormone involved in many processes, playing a central role in plant immunity. SA can be further hydroxylated to form gentisic acid (GA, 2,5-dihydroxybenzoic acid), which has been proposed as a signal molecule complementary to SA. Both phenolic compounds highly accumulate in tomato plants infected with the Citrus Exocortis Viroid (CEVd), and participate in the tomato plant defence against this non-coding RNA pathogen.

Although SA biosynthesis is well known, its catabolism still remains unclear. In *Arabidopsis thaliana*, two salicylate hydroxylases (S3H and S5H) have been described. The S3H enzyme converts SA to both 2,3-dihydroxybenzoic acid (2,3-DHBA) and GA *in vitro*. Arabidopsis *s3h* mutants fail to produce 2,3-DHBA sugar conjugates, accumulate very high levels of SA and its sugar conjugates, and exhibit a precocious senescence phenotype. The S5H catalyzes the formation of GA by hydroxylating SA at the C5 position of its phenyl ring. The *s5h* mutant and *s5hs3h* double mutant overaccumulate SA and display phenotypes such as a loss of susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000.

Our laboratory has identified the putative S5H tomato orthologous (*Sl_Sh*; *Solanum lycopersicum* salicylate hydroxylase). We have studied its activity *in vivo* by expressing *Sl_Sh* in *Nicotiana benthamiana* plants, and we have observed an outstanding reduction of SA levels in the *35S::Sl_Sh* agroinoculated plants, when compared with their corresponding control plants. Besides, *Sl_Sh RNAi* silencing transgenic tomato plants have been generated. Similar to Arabidopsis, these tomato plants displayed an accelerated senescence phenotype. Finally, the tomato *Sl_Sh* RNAi plants were inoculated with CEVd, and *Sl_Sh* expression, the levels of phenolics as well as the pathogen content have been analyzed. Our results appear to confirm the role of *Sl_Sh* in tomato defence against CEVd.

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P2. Subcellular RNAi components localization upon PSTVd infection

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Viroids are infectious long non-coding RNAs inducing important agronomic diseases in a wide plant host range. Since they do not encode for any protein, they use cellular mechanisms in order to infect. RNA silencing has always been proposed as one mechanism that could eventually be manipulated by viroids. In *Nicotiana benthamiana* (*N. benthamiana*) this mechanism consist of three major group proteins: Dicer (DCL) proteins (4 in *N. benthamiana*) which are responsible for dicing recognized double stranded RNA, Argonautes (AGO) proteins (7 in *N. benthamiana*) the effectors of silencing, and RNA-depended RNA polymerase (RDR) proteins (at least 3 in *N. benthamiana*) responsible for the synthesis of secondary siRNAs. Our previous work (Katsarou *et al.*, Plos Path 2016), showed that DCL4 digests PSTVd although the downstream effect on viroid accumulation of this processing is minor compared to the combined effect of DCL2/DCL3 cleavage, suggesting a specific role for each DCL in viroid infectivity. Although, work published last few years by us and other groups have increased our understanding on the interplay of viroids with silencing pathways, there are still important aspects of these interactions that needs addressing. We consider that one important factor might be changes in subcellular localization of the RNAi proteins upon viroid infection. In this work, we are aiming to address this issue by shedding light on the subcellular localization of components of the RNAi mechanism upon infection that could eventually reveal important information about viroid pathogenicity.

P3. Agroinfection methods for evaluating *Chrysanthemum stunt viroid* accumulation and movement in *Chrysanthemum* plants

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Chrysanthemum stunt viroid (CSVd) infects Chrysanthemum (*Chrysanthemum moliforium*) plants and causes serious reduction in cut flower production. In our previous studies, screening program for CSVd-resistance in commercial chrysanthemum cultivars was carried out and several useful resistant genotypes were obtained. In the program, we had applied traditional grafting and leaf primordia (LP)-free SAM culturing as methods for CSVd inoculation [1, 2]. These inoculation method, based on tissue fusion between infected and uninfected tissues, showed high efficiency in infection ratios but establishment of CSVd infection took one or two months. Alternatively, recently we assessed agroinfection method to establish an easy, rapid, and cost-effective method for CSVd inoculation in chrysanthemums.

We constructed binary vectors harboring dimeric CSVd sequences in sense and antisense orientations, named pBIK201:CSVd-2S and pBIK201:CSVd-2AS. Then, we tried to establish agroinfiltration assay in chrysanthemums. In preliminary experiments, we often failed to infiltrate inoculum into greenhouse-grown chrysanthemum leaves, finding that solutions did not penetrate. This difficulty was overcome when we used plants grown *in vitro* and acclimated to incubation conditions. Larger intercellular spaces and surface characteristics such as fewer trichomes or cuticles may have accounted for this infiltration compatibility. We infiltrated *Agrobacterium* EHA105 culture transfected with pBIK201:CSVd-2S and pBIK201:CSVd-2AS into leaves of a susceptible cultivar ‘Piato’. We checked CVSd infection by RT-PCR and northern blotting and found that local infection was established within 3 days and systemic infection within 20 days. CSVd polarities showed no difference in infectivity in this susceptible cultivar. From this result, we concluded that agroinfection methods are useful for inoculating CSVd to chrysanthemum plants.

Currently, we intend to classify CSVd-resistant cultivars [1, 2] by thier mode of resistant mechanisms. Drived by exogeneous promoter, high expression levels of CSVd titers in inoculated tissue was achieved transiently even in CSVd-resistant cultivars. This system enabled us investigating CSVd accumulation and movement in cultivars with differential susceptibility to the CSVd infection.

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P4. Comparative analysis of gene expression in tomato leaves during development of mild and severe potato spindle tuber viroid infection

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Viroids are the smallest known plant pathogen that utilize host factors for efficient propagation and spread through entire plants. Potato spindle tuber viroid (PSTVd) induces a wild spectrum of disease symptoms in infected plants that depends on its RNA sequence, host species and environmental conditions. PSTVd-S23 variant, that differs from the PSTVd-M variant at 9 nucleotide positions located in the P and V domain, induces severe disease symptoms in *Solanum lycopersicum* cv 'Rutgers'. Plants are stunted with curled and epinasted leaves, with chlorosis and necrosis on the stem and veins, while infection by PSTVd-M induces only mild stunting and epinasty. Microarray analysis was applied to compare differences in transcriptome profile of tomato leaves infected with mild and severe variant. The changes were evaluated at four time points corresponding to the subsequent symptom's development stages: (i) the pre-symptomatic, 8 days post inoculation (dpi); (ii) early symptoms, 17 dpi; (iii) full spectrum of symptoms, 24 dpi and (iv) the so-called 'recovery' stage, 49 dpi, when stem regrowth was observed in severely affected plants. In total, 3197 differentially expressed genes (DEGs) were identified during PSTVd infection, and, of these, 3037 were observed in S23 infection while only 934 in M infection. Overall, 160 and 2263 DEGs were specific to mild and severe infection, respectively, while 774 DEGs were observed in both cases. The highest number of DEGs were identified at 17 and 49 dpi upon severe infection and, at 24 and 49 dpi in M infected plants. Depending on the infection stage and PSTVd variant different ratio of up- and down-regulated genes were observed. Moreover, Gene Onthology enrichment analysis revealed differences between overrepresented categories in mild and severe infection. Many genes related to photosynthesis and chloroplast were down-regulated following infection with PSTVd-S23, while in PSTVd-M infected plants only few or no genes were down-regulated. In contrast genes related to jasmonic acid, ethylene, salicylic acid which play major roles in regulating plant defense response to various pathogens were generally up-regulated. Expression of genes related to stress; defense; cell wall; RNA regulation, processing and binding; protein metabolism and modification were also altered during viroid infection.

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P5. Profiling of microRNAs in PSTVd infected Bulgarian pepper cultivars

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Pepper (*Capsicum annuum* L.) is predominantly cultivated vegetable crop worldwide due to rising consumer demands. At the same time, pepper diseases caused by fungi, nematode, bacteria, viruses and viroids impose limitations to crop development and yield. Potato spindle tuber viroid (PSTVd) invades many species of the *Solanaceae* family, including pepper cultivars developing mild symptoms in response to infection.

Two Bulgarian cultivars, Kurtovska kapia (KK, sweet pepper) and Djulunska shipka (DS, hot pepper) were selected to study viroid-host interactions, of which only DS showed a specific phenotype upon PSTVd infection.

Using small RNA high-throughput sequencing, the miRNA profiles of the mock and PSTVd infected samples were compared for each cultivar. At 28 dpi, 32 and 18 conserved miRNAs were differentially expressed in the PSTVd-infected samples of DS and KK, respectively. Of them, six miRNAs- miR162b-5p, miR169e-5p, miR398c-3p, miR408-5p, miR408a-3p, miR482a-3p were validated by RT-qPCR. Taken together, the NGS and RT-qPCR results proved a cultivar-specific dynamism of analyzed miRNAs in response to PSTVd infection.

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P6. Differential gene expression profiles of oil palm seedlings infected with *Coconut cadang-cadang viroid* variants

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Coconut cadang-cadang viroid (CCCVd), the causal agent of the lethal Coconut cadang-cadang disease in the Philippines has been associated with Orange spotting disease (OS) in oil palm where estimated decline in yield was reported to be 25-50% lower than healthy palms (1). CCCVd variants had been characterized from commercial oil palms in Malaysia. These oil palm CCCVd variants had more than 90% sequence similarity with a 246 nt form of CCCVd from coconut (2). Epidemiology of this pathogen and disease is poorly understood, including host pathogen interaction. RNA-Seq analysis to identify differentially expressed genes (DEGs) between healthy and CCCVd oil palm variants infected oil palm seedlings showed that referenced genome assembled into 30,752 annotate genes. Of the 30,752 annotated genes on reference *Elaeis guineensis* genome (EG5), more than 59% of the genes were reliably expressed across both samples. Seventy five (75) unigenes were found to be significantly expressed in CCCVd infected sample relative to the healthy sample using a cutoff of q-value ≤ 0.05, and log2 fold change ≥ 1.5. Thirty three (33) up-regulated and 2 down-regulated genes in CCCVd infected relative to the healthy sample. In addition, 39 genes specific expressed only in CCCVd infected sample and one gene expressed only in healthy seedling. The low number of statistically significant genes is due to the skewed q-value due to noticeable variance between the replicates. Gene Ontology (GO) annotation of these DEGs reveals up-regulated genes assigned to the GO terms of defense response, response to stress, regulation of response to stress and regulation of defense response. These findings will be beneficial for conducting detailed functional analysis of the genes and elucidating the defense mechanisms of oil palm against CCCVd oil palm variant infection.

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P7. Analysis of gene expression changes in hop plants in response to single and multiple viroid infections using RNA-Seq approach

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Four different viroid species infect hop plants: HLVd, HSVd, AFCVd and the recently discovered CBCVd. Different symptoms can be seen on plants infected with these viroids, ranging from presumably symptomless plants in the case of HLVd to complete dieback of plants in the case of CBCVd infection. There is also little information available on mixed viroid infections in hops. Although viroids are tiny non-coding RNA molecules, there is still little information available concerning their footprints on molecular mechanisms of the host. Since they do not code for any protein effectors, they probably interfere with gene expression through RNAi. A powerful tool for studying gene expression on a global scale is available through high-throughput sequencing of RNA or RNA-seq. We have initiated a RNA-seq experiment to address the issue of hop gene expression alterations by single and mixed viroid infections. Viroid-free hop plants of the Slovenian cultivar 'Celeia' were inoculated by bombardment approach using viroid constructs or RNA from natural CBCVd infected plants. Two single and three mixed infected groups of plants were obtained: 1) HLVd, 2) CBCVd, 3) HLVd+CBCVd, 4) HLVd+CBCVd+HSVd, and 5) RNA from natural CBCVd infected plants (HLVd also present). Total RNA was isolated using a Spectrum™ Plant Total RNA Kit and enriched for mRNAs using a mRNA Direct Kit. Strand-specific NGS sequencing was performed on an Ion Proton System and, for each group, three biological replicates were sequenced including control (viroid-free) plants at a depth of ~20 M sequences per replicate. Sequences were aligned to available hop draft genome sequences and more than 80% of sequences exhibited a match to genome. Unaligned sequences were *de-novo* assembled and treated as novel transcripts, not being present in the draft genome. The analysis of DGE was performed by the EdgeR Bioconductor package implemented in the CLC Genomics Workbench tool. The first results obtained in this experiment provide us with a global picture (Fig. 1) of hop gene expression differences after single and multiple viroid infections.

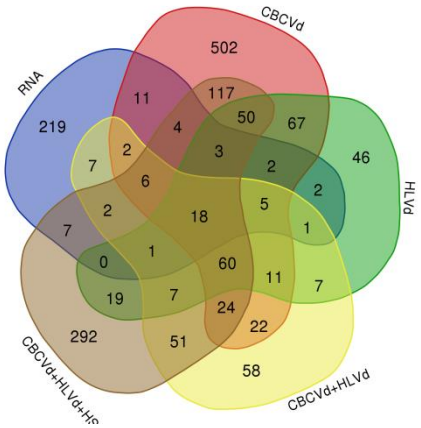

Fig. 1: Venn diagram of DEG in hops (abs fold change ≥ 2.0, P-value ≤ 0.01) for single and multiple viroid-infected plants.

P8. *Avocado sunblotch viroid* interaction with host superoxide dismutase

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Viroids consist of an infectious circular, single-stranded, non-protein-coding RNA, completely dependent on host cell factors for replication, processing, intra- and intercellular movement and pathogenesis. Aiming to identify host proteins that interact with viroid RNAs during the infectious process, we analyzed avocado (*Persea americana* Mill.) leaves infected with *Avocado sunblotch viroid* (ASBVd), the type species of the genus *Avsunviroid* in the family *Avsunviroidae*. Leaves of infected avocados (approximately 10 g) were placed on ice with the abaxial side facing up and were exposed to UV light (254 nm) for three hours using a UV cross-linker apparatus. RNA-protein complexes were purified according to Daròs and Flores (2002). Purified ASBVd-protein adducts were separated by PAGE in 5% denaturing gels and eluted by diffusion. After elution, preparations were analyzed in the Proteomics Laboratory of the Universitat de València (SCSIE), ProteoRed, Spain. Proteomics analysis by HPLC followed by mass-spectrometry showed the presence of a protein similar to *Arabidopsis thaliana* [Cu-Zn]2 superoxide dismutase (SOD; emPAI 0.21) and *Oryza sativa* subsp. *Japonica* [Cu-Zn] SOD (emPAI 0.22). This strategy was previously used to identify two chloroplastic RNA binding proteins, PARBP33 and PARBP35, which facilitate hammerhead-mediated self-cleavage of the multimeric ASBVd transcripts. SOD is an enzyme that catalyzes the dismutation of the superoxide radical into oxygen and hydrogen peroxide, an important reaction to defend cells from oxidative damage. The enzyme is found in the cytosol of virtually all eukaryotic cells, where it binds Cu and Zn. Changes in the level of this enzyme are common when cells are exposed to different types of stresses, both abiotic and biotic. ASBVd replicates and accumulates in the chloroplasts of infected cells, although viroid molecules likely traverse the cytosol to move cell to cell, having the opportunity to interact with the host Cu-Zn-SOD. Further work is needed to confirm this interaction by different approaches and to understand its role in the infectious process.

Reference:

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P9. Grapevine latent viroid and grapevine hammerhead viroid-like identified by next generation sequencing in an ancient grapevine germplasm collection in Italy

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Up to now, nearly 70 different viruses are known to infect grapevines, about half of which are associated with major diseases. By contrast, only six grapevine-infecting viroids and a viroid-like RNA, which is likely a novel viroid, have been identified. Although most of the viroids generally do not elicit severe symptoms in grapevines, some of them are the agent of diseases in certain environmental conditions or in combination with certain viruses. Moreover, some of grapevine-infecting viroids may cause severe diseases in other crops. While the identification of the five grapevine viroids dates back to the end of the 900s, Grapevine latent viroid (GLVd) and Grapevine hammerhead viroid-like RNA (GHVd) were identified only recently thanks to the application of next-generation sequencing (NGS) technologies.

NGS technologies have, indeed, revolutionized the biodiversity studies and are going to deeply impact on the diagnostic protocols. NGS-based metagenomics approaches are particularly useful to investigate the plant associated viromes, offering a unique opportunity to reveal the presence of all viral agents in the investigated plants, including still unknown entities. In this work, we used an NGS-based approach in order to characterize the virome associated to a grapevine germplasm collection located in Grinzane-Cavour (Piedmont, Northern Italy), comprehending a wide diversity of current and ancient grapevine varieties. The collection includes around 400 grape cultivars from North-Western Italy, as well as regional, national and international varieties used as references, and it is among the largest and richest in materials neglected, endangered to disappear.

NGS of cDNA libraries of small RNAs from about one third of the accessions in the germplasm collection was performed, and the virome was reconstructed using an *ad hoc* bioinformatics pipeline. Results were then validated by other independent molecular methods. Thanks to this approach, viruses routinely present in the grapevines, along with recently discovered viruses and viroids, and viral entities never described before in Italy were identified. In particular, we detected for the first time in Italy the two recently discovered viroids, GLVd and GHVd. According to our data, GLVd presence is limited to few plants in the collection, originally from extra-European territories, i.e. Armenia, Georgia, Uzbekistan and North America, whereas GHVd is more widely distributed among the grapevine accessions, including extra-European, European and Italian grapevines.

P10. Occurrence and molecular characterization of *Apple scar skin viroid* infecting pear plants grown in China

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To understand the infection statues and molecular characteristics of *Apple scar skin viroid* (ASSVd) in pear trees grown in China, a total of 443 pear samples were collected from eight orchards located in different provinces. Meanwhile, 32 apple samples were collected from two apple orchards adjacent to pear orchards to have a look for molecular characteristics of ASSVd from the two-host species. All sampled pear trees were asymptotic and some apple trees showed typic scar skin symptoms on fruits. Total RNA from these samples was extracted and subjected to RT-PCR tests for ASSVd using a set of primers to amplify the full genomic RNA of the viroid.

Amplicons of the expected size (329-335 bp) were obtained from 75 pear samples and 21 apple samples, accounting for 16.9% and 65.6% of tested samples, respectively. Products from 37 pear samples and 13 apple samples were cloned and 2-5 clones from each product were sequenced. Totally, 140 cDNA sequences of ASSVd isolates with sizes ranging from 329 bp to 335 bp were obtained. It was found that ASSVd haplotypes from pear trees were more varible in sizes than those from apple trees (Fig. 1). Among ASSVd haplotypes from pear trees, the most prevelent haplotypes were 330 nts, accounting for 44.6%. Whilst, the most prevelent haplotypes from apple trees were 331 nt, accounting for 43.6%, and 334-nt and 335-nt haplotypes were absent in tested apple trees. Size and sequence variations were also found within some isolates. ASSVd sequences from tested pear sanples shared 90.7-100 % identities with each other and 87-99% identities with other ASSVd sequences available in GenBank.

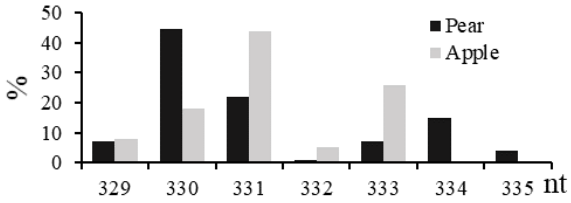

Figure 1

In the phylogenetic analysis of ASSVd sequences obtianed in this study and referred from GenBank database showed that all ASSVd sequences grouped into two groups. Multiple alignments revealed that ASSVd isolates in group I and group II differed at 15 nucleotide sites (Table 1). As previous reported results, the secondary structure of the central conserved region of ASSVd genomic RNA was very stable. However, the left-termial (TL) region and pathogenic (R) region differed between menbers in the two phylogenetic groups. Sequence variation between the two groups resulted in the variations of loop sizes and positions as indicated by two representive sequences of KFLS and BYH (Fig. 2).

Table1 The divergent nucleotides between groups I and II

Site	3	4	42	44	46	47	179	180	256	257	258	282	283	287	288
GI	G	T	G	T	G	A	T	C	G	C	C	T	C	G	A
GII	T	A	T	A	A	T	C	T	T/A	T	T	C	T	T	G

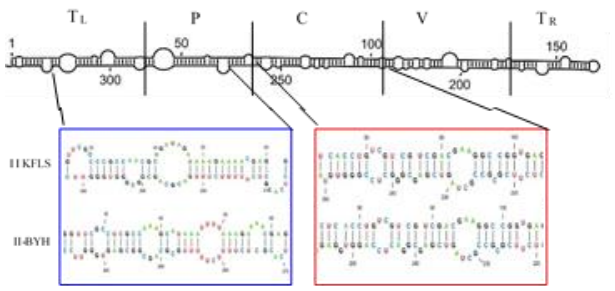

Figure 2

Acknowledgements This study was financially supported by the program for Key International S&T Cooperation Projects (grant no. 2017YFE0110900) and the Fundamental Research Funds for the Central Universities with program (grant no. 2662016PY107).

P11. Application of high-throughput sequencing for the detection of viruses and viroids in apples.

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Introduction

The use of High Throughput Sequencing (HTS) to determine the virome of a sample has been shown to be a reliable approach for a variety of crops. In this study, an RNA-Seq approach was followed to construct the viromes of apple trees. Samples with known disease profiles were used in this study.

Methods

High quality total RNA was extracted from leaf petioles and midribs using an adapted CTAB method (White et al., 2008). Samples used as positive controls for ELISA and RT-PCR, were used for the HTS study. RNA was assessed for quality and shipped to Macrogen (Korea) for ribo-depletion, library construction and sequencing on an Illumina platform.
Residual adaptors and low quality bases were trimmed from raw read data. Paired reads that were retained after trimming were used for *de novo* assembly. Assemblers were compared for their effectiveness to assemble virus and viroid contigs.

Results

On average 16 million read pairs for each sample were retained after quality trimming and filtering. Assembled contigs were subjected to BLAST searches against the NCBI non-redundant DNA databases to identify viruses and viroids in samples. Expected viruses (ASPV, ASGV and ACLSV) as well as apple hammerhead viroid were detected.

Discussion

In this study we were able to detect viruses and viroids in apple samples using HTS of ribo-depleted total RNA. This confirms that HTS is a valid method for both known and unknown pathogen detection in fruit trees.

Acknowledgements

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P12. Transcriptome sequencing reveals novel citrus bark cracking viroid (CBCVd) variants from citrus and their molecular characterization

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Citrus bark cracking viroid (CBCVd), previously called *Citrus viroid IV*, belongs to the genus *Cocadviroid* within the family *Pospiviroidae*. CBCVd has been identified as an important causative agent in citrus and hops. In this study, we obtained the full-length genomes of different variants of all detected citrus viroids from Pakistan through transcriptome sequencing. Different CBCVd variants were first found in Pakistan. These newly discovered Pakistani CBCVd variants were provisionally called "CBCVd-LSS" for their low sequence similarity (80.9%–88.9%) with the CBCVd RefSeq sequence (NC_003539). The two most predominant CBCVd sequences from Pakistan had the closest identity, 90.6% and 87.9%, with two CBCVd sequences isolated from hops. Identification and molecular characterization of CBCVd from citrus in Pakistan and China were also reported. The length of CBCVd from China ranged from 282 to 286 nucleotides, while that of the one from Pakistan ranged from 273 to 277 nucleotides. Based on genetic diversity and phylogenetic analysis, two main CBCVd clades were identified. CBCVd sequences from Pakistan, China, and other countries were further divided into six sub-clades. Sequence alignment revealed some nucleotide changes between these sub-clades, and analysis indicated that several mutations could significantly affect the primary and secondary structure of the viroid. Our results indicated that the CBCVd sequences from Pakistan and China were significantly different with respect to genome and secondary structure and Pakistan might be one of the independent geographical origins of CBCVd worldwide.

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P13. Exploring the presence of C^5-methylcytosine in viroid RNAs

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Nucleotide modifications in RNAs were initially thought to be mainly restricted to the stable and highly abundant transfer and ribosomal RNAs (tRNAs and rRNAs). In these RNAs, modifications have been mapped by technically demanding studies that support their possible structural and/or functional roles. More recently, the improvement of RNA bisulfite sequencing protocols, originally developed for identifying C^5-methylcytosine (m^5C) in DNA, unveiled the widespread occurrence of m^5C in many cellular coding and non-coding RNAs, thus highlighting that the previous reports on the limited presence of this modified nucleotide in cellular RNAs other than rRNAs and tRNAs were mainly due to technical limitation. Information on whether viroid RNAs contain m^5C is poor and dates back to early RNase fingerprints analyses of bisulfite-treated viroid RNA preparations (Domdey et al., 1978, *Nucleic Acids Res.* 5, 1221-1236). These studies were performed mainly to determine the secondary structure of potato spindle tuber viroid (PSTVd) genomic RNA. Therefore, they did neither provide information at single-nucleotide resolution nor on the presence of m^5C in viroid replication intermediates, particularly viroid RNAs of the complementary (-) polarity. To conclusively answer the question regarding the presence of m^5C in either polarity strand, a protocol based on RNA bisulfite sequencing was developed to test at single-nucleotide resolution the viroid highly-structured RNAs, whose base-paired cytidines are particularly resistant to bisulfite conversion. This protocol was then applied to explore the presence of m^5C in the (+) and (-) strands of PSTVd and avocado sunblotch viroid (ASBVd), which are members of the type-species of the families *Pospiviroidae* and *Avsunviroidae*, respectively. Results of these analyses support the absence of m^5C in both strands of PSTVd and ASBVd.

P14. Selection of AFCVd variants with severe and mild symptom on tomato fruits

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Apple fruit crinkle viroid (AFCVd), a tentative member of the genus *Apscaviroid*, family *Pospiviroidae*, has a narrow host range and is known to infect apple, hop, and persimmon as natural hosts. In our study, tomato, cucumber, and wild hop have been identified as new experimental herbaceous hosts for AFCVd. Foliar symptoms were very mild or virtually undetectable, but infected tomato produced small, cracked, and distorted fruits which are similar to those on apples infected with AFCVd.

After infection in tomato, cucumber, or wild hop, AFCVd isolate from hop raised adaptive mutations in the nucleotide. Major variants in tomato, cucumber, and wild hop differed in 10, 8, and 2 nucleotides respectively from the predominant one in the inoculum. The major variants in tomato and cucumber were almost identical, and the one in wild hop was very similar to the one in the cultivated hop.

Detailed analyses of the host-dependent sequence changes that appeared in a natural AFCVd from hop and selected infectious cDNA clones from hop, tomato, and persimmon after transfer to tomato revealed that the major variant in tomato emerged by selection of a minor variant present in the inoculum (i.e., hop) followed by 1-2 host-dependent *de novo* mutations. Comparison of the secondary structures of major variants in hop, tomato, and persimmon after transfer to tomato suggested that maintenance of stem-loop structures in the left-hand half of the molecule is critical for infection.

In the process to transfer a natural AFCVd isolate from hop to tomato, two host adaptive mutants AK6-2 and AK6-T2 with different nucleotide sequence were successfully selected. Then, their infectious cDNA clones were established, and, by adding another infectious cDNA clone HS1 selected similarly from AFCVd persimmon isolate, a total of three infectious RNAs transcribed in vitro were inoculated into tomato for the comparison of pathogenicity. As a result, fruits infected with AK6-2 and AK-T2 became small, cracked, and distorted, and depressions and yellowing appeared along the strip line, but those infected with HS1 exhibited only mild discoloration. In addition, among those infected with AK6-2 some showed more severe symptoms. The nucleotide sequence analysis revealed that severe one (TS) was different in 3 nucleotides from normal one (TM), and structures of 33rd and 34th loops on the predicted secondary structure can be changed. Analysis on the mechanism of fruit symptom development by AFCVd are now underway by focusing on these 3 nucleotides.

P15. Elimination of apple fruit crinkle viroid (AFCVd) during anther development and its depressed propagation in pollen of *Nicotiana tabacum*.

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The haploid male germline of higher plants plays a vital role in plant fertility and crop production through the generation and transport of male gametes, double fertilization, seed production and finally through effective mechanisms of the elimination of parasitic nucleic acids from pollen. Some viroids—parasitic, non-coding single-stranded, circular and highly structured RNAs—can be either pollen-transmissible (spread via pollen) or eliminated from developing pollen, depending on plant-pathogen combinations. There is currently no information about molecular interactions of viroids within pollen but it can be assumed that viroid RNA interacts specifically with the metabolism of developing male gametophyte involving small RNAs. Here we are describing infection of a pollen model plant, *N. tabacum* with a hop isolate of apple fruit crinkle viroid (AFCVd; 368–372 nts), which is a member of the family *Pospiviroidae*, genus *Apscaviroid.* Tobacco infection of AFCVd was achieved by RNA inoculation using the biolistic method. AFCVd propagated to high levels in tobacco leaves, while it was dramatically suppressed in immature anthers of tobacco, as well as in young microspores and in differentiating pollen, where this strongly depressed propagation reached its maximum on the fifth stage when vegetative pollen cell is filled with starch and the generative nucleus is spindle-shaped. AFCVd retained its original sequence in tobacco and showed stably an excess of (+) to (-) strands while propagating in pollen. Although AFCVd caused a moderate pathogenic effect on somatic tissues of *N. tabacum* it did not impair plant flowering characteristics and did not increase the level of abortive pollen, which reached <5% in infected and healthy samples, as detected using fluorescence microscopy of DAPI-stained mature pollen. Thus, it follows from our results that there is strong mechanism(s) that keep viroid elimination and propagation levels depressed in pollen. Pollination with infected pollen confirmed no transfer of AFCVd by seeds in the *N. tabacum* model. Analysis of natural mechanisms associated with viroid suppression and elimination from the male germline using NGS, transcriptome, degradome and protein profiling are in progress. Tobacco transgenotes are in preparation to analyze specific changes possibly induced by AFCVd expression in developing pollen using pollen-specific promoters.

This work was supported by the bilateral Czech and German Science foundations project GAČR 18-10515J and DFG STE 465/10-1.

P16. Some pathogenic effects and elimination of hop variants of two pospiviroids, CBCVd and AFCVd, during development of pollen of *Nicotiana benthamiana.*

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Viroids are single-stranded, circular RNA molecules in the range of 250–400 nucleotides that replicate autonomously in infected host plants and although they do not code for any protein, they promote specific diseases on susceptible and sensitive plants. Citrus bark cracking viroid (CBCVd) and Apple fruit crinkle viroid (AFCVd) are both members of *Pospiviroidae* that are listed as pathogens newly adapted to hop (*Humulus lupulus*, L.) causing detrimental effects and significant losses in the yield of this important crop. High adaptability of CBCVd and AFCVd is the reason why we initiated analysis of possible transmissibility of these parasitic RNAs through the haploid male gametophyte, pollen and their possible impact on pollen development. In our experiments we found that both pospiviroids infect *N. benthamiana*, which was used as common model species for detailed analysis of pollen. Individual pollen developmental stages in *N. benthamiana* were characterized microscopically (using DAPI fluorescent dye to visualize nuclei and Iodine staining for starch detection) in correlation with the particular flower developmental stage.

The prominent symptoms were observed in *N. benthamiana* after inoculation with AFCVd and CBCVd either by the biolistic method or by leaf infiltration of *A. tumefaciens* strains bearing dimeric infectious vectors of these hop viroids. According to our RT-qPCR data, AFCVd and CBCVd viroid propagate to high levels in immature anthers of *N. benthamiana*, while very low levels or traces of these parasitic RNAs were quantified in mature *N. benthamiana* pollen. Accordingly, mature pollen of infected *N. benthamiana* showed no apparent morphological defects. However, the rare occurrence of mature pollen with three nuclei was noted in CBCVd infected tobacco plants in spite of tobacco has naturally bicellular mature pollen. Our results showed strong elimination of both viroids from infected tissues on the level of pollen and no seeds transmissibility if pollination was made using pollen from viroid-infected plants. Viroid elimination from developing *H. lupulus* pollen we detected also for HLVd earlier (Matoušek, J. *e.a.* Biol.Chem.389:905,2008) and for CBCVd infecting hop in recent experiments. This suggests strong mechanism(s) preventing AFCVd and CBCVd transmission through pollen. To stimulate expression of viroid in *N. benthamiana* pollen, the plants were transformed using *A. tumefaciens* - mediated transformation with infectious plant vectors bearing dimeric AFCVd and CBCVd cDNAs. Transformed plants showed strong morphological symptoms including defects in pollen development. A large number of aborted pollen (34% and 62% in AFCVd and CBCVd transformants, respectively) together with increased occurrence of young immature pollen grains (8% and 15% in AFCVd and CBCVd transformants, respectively) were found in cracked anthers of opened flowers in comparison to control plants (3,9% aborted pollen and 0,3% young immature pollen). Moreover, the rare occurrence of pollen with malformed nuclei or even tricellular pollen was found in transformed tobacco plants.

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P17. Identification of the causal agent of coconut tapering disorder in Sri Lanka

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Over the last few decades, a debilitating disorder of coconut palms, named as 'tapering disorder', which reduced the productivity of palms and eventually lead to death of palms has been found widespread causing important economic losses to coconut growers in Sir Lanka. Disorder affected coconut palms showed sudden or gradual tapering of the stem near the crown, shortening and scorching of leaves, drastic reduction in the size of nuts and yield and finally the death of the palm. The time taken from appearance of the first symptoms to the death of the palms varies from about six months to one year or greater (Fig 1). To identity its causal agent, RNA-seq was done for the tapering disorder affected coconut palms following by bioinformatics analysis. Young leaves of four disorder affected coconut palms were collected in Sri Lanka in 2017 and pooled for sequencing. More than 20 million raw reads were obtained. Removal of adaptors of the raw reads and low quality of the data resulted in about 19 million clean reads that were *de novo* assembled into contigs using Trinity. Blastn analysis of the obtained contigs showed that one contig of 659 nt matched to citrus bent leaf viroid (CBLVd) (AB006736) with 99% sequence similarity and one contig of 305 nt matched to citrus exocortis viroid (CEVd) (HQ667138) with low sequence similarity. The contig of 659 nt contained more than two units of the genome of CBLVd, indicating the presence of replication intermediates of CBLVd in diseased coconut palms. It should be noted that the contig of 305 nt only contained the central conserved region (CCR) of the genus *Pospiviroid*, implying the existence of a novel pospiviroid in the tapering diseased coconut palms. However, the association of CBLVd and the possible novel pospiviroid with coconut tapering disease is still unclear. In the near future, large-scale surveys and bioassays will be performed. In a word, CBLVd and a possible novel pospiviroid were detected in tapering diseasded coconut palms using RNA-seq. This supplies a new clue to identify the causal agent of this emerging disorder in Sri Lanka.

Fig 1. Symptoms of coconut tapering disorder affected palms.

P18. Presence of HLVd in the collection of hop varieties in Czech Republic

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The hop plant, *Humulus lupulus* L., is a dioecious perennial species, and only female cones are used for beer brewing. Hop as a perennial and vegetative-propagated crop is endangered by viruses and viroids. Hop latent viroid (HLVd) from the group *Pospiviroid* is widespread in hops (MATOUSEK et al. 1994, PETHYBRIDGE 2008, SEIGNER et al. 2014, ZIEGLER et al 2014). Hop Research Institute Co., Ltd., has one of the largest collection of hop varieties in the world. In 2017, the presence of HLVd by dot-blot molecular hybridization in hop cones was evaluated in this collection. In total 126 varieties and genotypes from 20 countries were evaluated (Australia, Belgium, Canada, Czech Republic, France, Germany, Netherlands, Japan, South Africa, Yugoslavia, Caucasus, Lithuania, New Zealand, Poland, Russia, Slovenia, Spain, England, Ukraine, USA).

All samples analysed were positive from weak to strong infection, except the Smoot Cone from New Zealand and 4 new varieties from the Czech Republic, which were negative for the HLVd presence. For many varieties and countries of origin, this is the first information about presence of HLVd. Further monitoring and occurrence of HLVd will be the subject of further research.

Acknowledgement:

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P19. Identification of Arabidopsis thaliana ecotypes of susceptible to viroids

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Viroids are single-stranded, circular, highly structured, non-coding RNAs. They replicate in either the nucleus or in chloroplasts through a rolling circle mechanism. Viroids cause disease in economically important plants including tomato, hops, chrysanthemums etc. However, certain avenues of research on viroid infection mechanisms have been hampered by a lack of a model host that is amenable to genetic analysis and that presents genetic variation in susceptibility. Indeed, the model plant *Arabidopsis thaliana* has never been reported to allow systemic infection by viroids. A number of studies have shown that different ecotypes of *Arabidopsis thaliana* show differing susceptibility to different viruses. We hypothesised that like viruses, natural variation in viroid susceptibility might exist in *Arabidopsis*. To this end, we have infected 80 ecotypes of *Arabidopsis* with a mix of three viroids and scored plants for infection based on symptoms and northern blotting for viroid RNA. Preliminary results indicate that, although viroid susceptibility is not common in *Arabidopsis*, we have nonetheless identified several susceptible ecotypes. Interestingly, the symptoms of infected plants differ widely between ecotypes, ranging from severe to asymptomatic. A summary of our findings will be presented, along with ongoing strategies to identify components involved in *Arabidopsis*-viroid interactions.

P20. The construction and biological application of infectious citrus viroid clones

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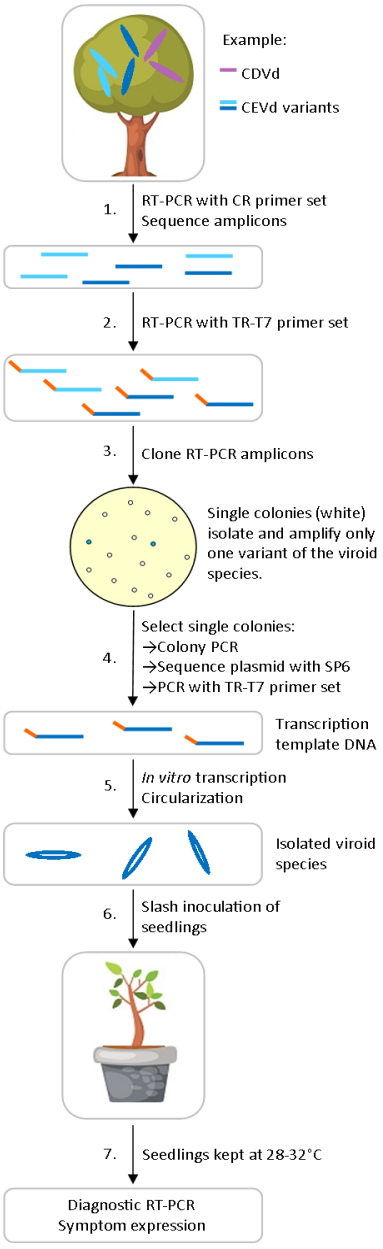

Introduction. The isolation of single citrus viroid infections from naturally infected citrus is challenging since they are commonly found in combination with various other pathogens.

Methodology. Complete genomes of seven citrus viroid species were cloned via the following steps (see Figure left):

1. Viroid species sequence determination using Conserved Region-primers to allow for amplification of all strain variants;
2. Full genome amplification using Terminal Region-T7-primers containing a leading T7 promoter sequence to amplify one specific strain variant only;
3. Cloning of amplicons (full viroid genome with a leading T7 promoter sequence);
4. Sequence confirmation and PCR preparation of transcription templates using Terminal Region-T7-primers;
5. *In vitro* transcription and enzymatic circularization;
6. Slash-inoculation to 6 month old 'Etrog' citron (*Citrus medica*) seedlings;
7. Seedlings kept at 28-32°C before transfection confirmation and symptom evaluation.

Results and Discussion. All transfections could be confirmed via RT-PCR one month post inoculation, but interestingly, typical CEVd epinasty symptoms were only observed after 14 months. Sanger sequencing of CEVd indicated that two variants were present - the original strain and a variant with three point mutations. One of these point mutations has previously been described to alter the symptom expression (Murcia et al. 2011). It is also known that the variant population is influenced by the citrus host type (Bernard et al. 2009).

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P21. *Cardamine bonariensis* and *Oxalis latifolia*: potential reservoirs of *Chrysanthemum stunt viroid* in chrysanthemum crops

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Chrysanthemum stunt viroid (CSVd), genus *Pospiviroid*, is easily transmitted by mechanical inoculation, foliar contact, grafting and contaminated cutting tools. Symptoms induced by CSVd in susceptible chrysanthemum varieties include malformations, epinasty, colour breaking, reduction in number and size of flowers and shortening of internodes, which induces plant dwarf. However, in many situations it does not induce visible symptoms, facilitating its spread and maintenance in the field. CSVd has a wide host range, including species of economic importance and weeds from different botanical families. In a phytosociological study carried out in chrysanthemum fields in the State of São Paulo, Brazil, and in the Region of Rionegro (Antioquia), Colombia, it was observed that different weeds were present in all types of crop systems (cut and pot). Despite intensive weed control in the chrysanthemum crops, in Brazil, *Cardamine bonariensis* Pers. (Brassicaceae) has been found throughout the whole production cycle, and it was observed in all cultivated areas in São Paulo State, Brazil. This species, originating in Europe, was probably introduced in Brazilian crops through the informal exchange of plants and ornamental seedlings. In order to evaluate the susceptibility of *C. bonariensis* to CSVd, seedlings were mechanically inoculated with a Brazilian CSVd isolate. Symptomless plants of *C. bonariensis* were also collected in chrysanthemum crops and tested for the presence of CSVd. We did not detect CSVd in the *C. bonariensis* plants from the field. However, the viroid was mechanically transmitted to this species, according to results from RT-PCR with CSVd specific primers at 30 days after inoculation. CSVd did not induce apparent symptoms in this host. During the survey carried out on chrysanthemum crops in the Region of Rionegro, we observed that plants of clover, *Oxalis latifolia* (Oxalidaceae), were widespread in the fields, and many of them showed symptoms of mosaic and leaf distortion. This weed, originating in Mexico, is worldwide distributed, since it adapts easily in tropical and temperate climates. Plants of clover showing mosaic were collected and submitted to RNA extraction, RT-PCR, and the DNA amplified products with expected size were sequenced. Sequences were aligned and compared with other CSVd variants deposited in databases. Six Colombian CSVd isolates from clover sequenced here (MF359712, MF359713, MF359714, MF359715, MF359716, MF359717) showed high nucleotide identity with other CSVd variants deposited in the GenBank. To date, this is the first report of CSVd infecting naturally clover (*O. latifolia*) and experimentally *C. bonariensis*. These results point to the epidemiological importance of these weeds, which can be considered potential reservoirs of CSVd in the field.

P22. Identification of vein banding and yellow speckle diseases in Kurdistan province, west of Iran

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Grapevine yellow speckle viroid 1 (GYSVd-1), *Grapevine yellow speckle viroid* 2 (GYSVd-2) and *Grapevine fanleaf virus* (GFLV) are world widespread. Grapevine yellow speckle viroids able to induce yellow speckle (YS) and the simultaneous infection with GFLV with leads to increased symptoms called vein banding (VB). The presence of YS and VB were surveyed in Kurdistan province during August to September 2015 and 36 grapevine leaf samples with YS, VB and non-symptomatic were collected and tested by multiplex RT-PCT. Results of multiplex RT-PCR showed that 22, 21 and 7 samples were infected GYSVd-1, GYSVd-2 and GFLV, respectively. All seven vines were infected by GFLV; they also were infected by at least GYSVd-2. Notably, 5 out 7 of the vines which infected by GFLV, GYSVd-1 and/or GYSVd-2, VB symptoms were observed (Fig. 1; A and B) suggesting that the presence of GFLV with grapevine viroids not sufficient to express the VB symptoms. Probably various other factors, including climate conditions (Salman *et al*., 2014), sequence variability of viroids (Polvika *et al*., 1996; Salman *et al*., 2014) and virus and/or viroids titer (Szychowski *et al*., 1995), are involved in their symptoms appearance. We also observed VB and YS symptoms in vines which infected only by GYSVd-2 (Fig. 1; C and D). The ability of GYSVd-2 to induce mild VB symptom by grapevine viroids has been already reported (Martelli *et al*., 2014). In conclusion, these data showed that vineyards in west of Iran were infected by VB and YS diseases. Also, our data show that in Iran grapevine yellow speckle viroids can become more damaging as they elicit VB in vines simultaneously infected by GFLV that is widespread and by GYSVd-1 and/or GYSVd-2.

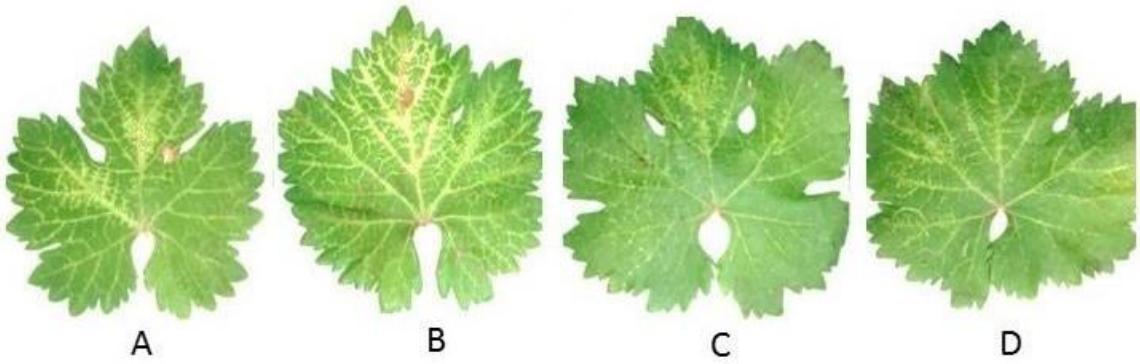

Fig 1. A; Representative leaf of *Vitis Vinifera* infected by GFLV, GYSVd-1 and GYSVd-2 showing VB symptoms. B; Representative leaf of *V. Vinifera* infected by GFLV and GYSVd-2 showing VB symptoms. C; Representative leaf of *V. Vinifera* infected by GYSVd-2 showing YS symptoms. D; Representative leaf of *V. Vinifera* infected by GYSVd-2 showing VB symptoms.

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P23. The *in vitro* synthesis of infectious viroid cDNAs

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The synthesis of infectious nucleic acids *in vitro* is an important strategy in elucidating the pathogenicity and virulence of viroids and their variants. In this study, monomeric RNAs (M-RNAs), monomeric cDNAs with cohesive ends (M-DNAs-coh), and dimeric cDNAs (D-DNAs) of the *Citrus excortis viroid* (CEVd) and M-DNAs-coh of the *Peach latent mosaic viroid* (PLMVd) were constructed. The infectivity analysis indicated that the D-DNAs, regardless of their orientation in the vectors and the mono-DNAs-coh were infectious, while the M-RNAs had no infectious activity. The cyclization, the DNA template concentration required for infectivity, the biological function of the transcribed RNAs, and the viroid titer accumulation over time were also assessed for the CEVd infectious DNAs. Both inoculations of the D-DNAs and M-DNAs-coh resulted in similar dwarf symptoms characteristic of a CEVd infection; there was no dwarf difference related to the orientations of the D-DNAs inserted in the vectors. It is worth noting that the mechanical inoculation was successfully performed via stems or woody barks instead of via leaves, which will be helpful for a bioassay on woody index plants or on the plants with leaves that are unsuitable for an inoculation. This study will contribute useful information regarding the synthesis of viroid infectious nucleic acids via cDNA to elucidate their pathogenic mechanisms or other bio-features.

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