FOURTH JOINT MEETING OF DUTCH AND GERMAN PLANT VIROLOGISTS

March 10 and 11, 2005
WICC, Wageningen

A binational scientific meeting organised under auspices of the

Nederlandse Kring voor Plantevirologie
and the
DPG Arbeitskreis Viruskrankheiten der Pflanzen

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Programme

THURSDAY March 10

08.00 - 08.50 Registration

08.50 - 09.00 Welcome address

Session: Plant virus genome organisation and expression (Chair: Rob Goldbach)

09.00 - 09.20
John Bol: Coat protein enhances translational efficiency of *Alfalfa mosaic virus* RNAs and interacts with eIF4F

09.20 - 09.40
Holger Jeske: Recombination-replication-repair interface of geminiviruses

09.40 - 10.00
Renate Koenig: BNYVV-like particles formed by coat protein expressed from a potexvirus-based vector construct

10.00 - 10.20
Richard Kormelink: TSWV transcriptase *in vitro* prefers cap donors with multiple base complementarity to the viral template

10.20 - 10.50 Coffee/Tea

Session: Virus - Plant Interactions (Chair: Willi Jelkmann)

10.50 - 11.10
Tatjana Kleinow: Interactions of movement protein BC1 and nuclear shuttle protein BV1 of *Abutilon mosaic geminivirus*

11.10 - 11.30
Jan van Lent: Interaction between *Cowpea mosaic virus* movement tubules and the plasma membrane

11.30 - 11.50
Conny Heinze: Symptom variants of *Cucumber mosaic virus* (CMV) are based on more than a single mutation

11.50 - 12.10
Ilona Rolfes: GFP-labeling of mite transmitted *Brome streak mosaic virus* (BStMV)

12.10 - 12.30
Marjolein Snippe: TSWV particle assembly: *in vivo* interactions between the structural nucleoprotein and G1 spike protein

12.30 - 13.30 Lunch (and poster set-up)

Continuation Session: Virus - Plant Interactions (Chair: Willi Jelkmann)

13.30 - 13.50
Christina Wege: Transgenic AbMV DNA B supports mechanical transmission but does not release phloem restriction

Session: New Diagnostic Tools (Chair: Harm Huttinga)

13.50 - 14.10
Nicolette Klijn: Future challenges in the innovation of detection and identification techniques in plant virology

14.10 - 14.30
Frank Rabenstein: Serological and molecular differentiation of *Soil-borne cereal mosaic virus* and *Soil-borne wheat mosaic virus* – two furoviruses occurring on wheat and rye in Germany

14.30 - 15.00 Tea

Session: Host responses: Resistance and RNAi (Chair: Holger Jeske)

15.00 - 15.20
Artur Pfitzner: The allelic resistance genes *Tm-2* and *Tm-22* against *Tomato mosaic virus* differ only in 4 amino acids but recognise two independent domains of the ToMV MP

15.20 - 15.40
Hans Hemmes: Functional analysis of RNAi suppressors of negative strand plant viruses

15.40 - 16.00
Konstanze Dietrich: Occurrence of short interfering RNA in virus-infected Cassava (*Manihot esculenta*) varieties with differential resistance/susceptibility against geminivirus infections

16.00 - 16.20
Simone Ribeiro: The use of RNA silencing for resistance to geminiviruses

16.20 - 16.40
Jörg Schubert: Attempts for genetic improvement of resistance of barley to *Barley yellow dwarf virus*

16.40 - 17.00
Gabi Krczal: Single-chain antibodies against a plant viral RNA-dependent RNA polymerase confer virus resistance

17.30 - 19.00 Posters and Drinks

19.30 - Dinner

FRIDAY March 11

08.30 - 09.00 AK - VK business meeting

Session: New, Emerging and Quarantine viruses I (Chair: John Bol)

09.00 - 09.20
Inas Farouk: Translocation and immunolocalisation of *Watermelon chlorotic stunt virus*, WmCSV, in its vector *Bemisia tabaci* (Genn.)

09.20 - 09.40
Vincent Bijman: Recent developments in Augusta disease of tulip in the Netherlands

09.40 - 10.00
Arjen Werkman: Identification of viroids occuring in tomato and potato, and consequences for testing

10.00 - 10.20
Sharif Barends: NeRNV, a new tymovirus with a genomic RNA having a histidylatable tobamovirus-like 3'end

10.20 - 10.50 Coffee/Tea

Session: New, Emerging and Quarantine Viruses (Chair: Guenter Adam)

10.50 - 11.10
Joe Vetten: Identification and detection of a closterovirus from carrot in Germany

11.10 - 11.30
Ko Verhoeven: A new strain of *Cowpea mild mottle virus* infects French bean in Spain and Morocco

11.30 - 11.50
Hans-Peter Muehlbach: A new bunyaviral-type plant virus is associated with the ringspot disease of European mountain ash (*Sorbus aucuparia L.*)

11.50 - 12.10
Dennis Knierim: Sequence analysis of a serogroup IV tospovirus isolated from *Lycopersicum esculentum* in Thailand

12.10 - 12.30
René van der Vlugt: Natural variation in *Pepino mosaic virus*

12.30 Closing Remarks

12.45 - 13.45 Lunch

Departure

Poster presentations (Thursday, 10th March, 17.30-19.00 hr)

1. Evidence for the occurrence of three nanovirus species infecting faba bean in Ethiopia
   A Abraham

2. Investigations on *Citrus tristeza virus* (CTV) and its occurrence in citrus orchards in arid and semi arid zones of Sudan
   M Abubaker

3. Differentiation of *Cucumber mosaic virus* isolates by hybridisation to oligonucleotides in a microarray format
   G Adam

4. Characterisation of *Cherry leaf roll virus* (CLRV) isolates from different host plants
   S von Bargen

5. Molecular characterization of *Vicia cryptic virus*
   R Blawid

6. A new potyvirus causing flower breaking in *Begonia semperflorens*
   I Bouwen

7. High frequency silencing of multiple targets using a single inverted repeat construct
   E Bucher

8. Construction and agroinfection of a *Potato virus M* full-length clone
   S Flatken

9. Characterisation of proteins involved in translocation of the geminivirus *Watermelon chlorotic stunt virus*, WmCSV, in its vector *Bemisia tabaci* (Genn.)
   A Gadelseed

10. Biological and molecular characteristics of different *Cherry leaf roll virus* (CLRV) isolates
    J Gentkow

11. Large-scale application of real-time RT-PCR for testing *Potato spindle tuber viroid* in potato
    CCC Jansen

12. Multiple infections of commercial Poinsettia
    H Jeske

13. TMV coat protein: Expression, *in vivo* self assembly and mutagenesis
    A Kadri

14. Investigation of development of infection by soil-borne viruses in cereals
    U Kastirr

15. Dutch-German cooperations in evaluating species demarcation criteria for tombus- and tymoviruses
    R Koenig

16. Nuclei instabilities correlated with active rolling circle replication (RCR) and recombination dependent replication (RDR) of Abutilon mosaic virus
    B-J Koo

17. The nucleoprotein gene of *Tomato spotted wilt virus* as a tag protein fusion for easy purification
    and enhanced production of recombinant protein in plants
    C Lacorte

18. Changes in the spectrum of PVY strain groups and their involvement in potato and tobacco diseases
K Lindner

19. Identification and characterization of Tospoviruses from Iran
A Mehraban

20. Detection and Identification of a novel potexvirus infecting allium by paramagnetic beads ssRNA isolation and one tube RT-PCR assay with a new potexvirus genus primer set
R Miglino

21. Characterization and nucleotide sequence of *Grapevine leafroll associated virus*-7 (GLRaV-7)
C Mikona

22. The spread of *Rice yellow mottle virus* in irrigated rice crops
D Peters

23. Identification of viruses in ornamental *Allium* species and control strategies
KTK Pham

24. Molecular and biological characterization of *Beet mild yellowing virus* and determination of the host plant spectrum by agroinfection
D Stephan

25. Epidemiological developments in *Potato virus Y*
M Verbeek

26. Expression analysis of potyviral 6K1 in *Nicotiana benthamiana*
A Waltermann

27. *Alfalfa mosaic virus* detected in true seed of *Solanum kurtzianum* during post-entry quarantine testing
AW Werkman

28. Investigating the mechanism(s) of cross-protection between strains of *Cucumber mosaic virus*
H Ziebell

ABSTRACTS

Oral Presentations

Coat protein enhances translational efficiency of *Alfalfa mosaic virus* RNAs and interacts with eIF4F

JF Bol[1], IM Krab[1] and DR Gallie[2].
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Translation of eukaryotic mRNAs is strongly enhanced by the formation of a closed loop structure due to the interaction between the poly(A)-binding protein bound to the 3'-poly(A) tail and the eIF4G subunit of the eIF4F complex bound to the 5' cap structure. The three plus-strand genomic RNAs of *Alfalfa mosaic virus* (AMV) and the subgenomic messenger for viral coat protein (CP) contain a 5'-cap structure but no 3'-poly(A) tail. Binding of CP to the 3'-end of AMV RNAs is required for efficient translation of the viral RNAs and to initiate infection in plant cells. To study the role of CP in translation, plant protoplasts were transfected with luciferase (Luc) transcripts with 3'-terminal sequences consisting of the 3'-untranslated region of AMV RNA 3 (Luc-AMV), a poly(A) tail of 50 residues (Luc-poly(A)), or a short vector-derived sequence (Luc-control). Pre-incubation of the transcripts with CP had no effect on Luc-expression from Luc-poly(A) or Luc-control but strongly stimulated Luc-expression from Luc-AMV. From time-course experiments it was calculated that CP-binding increased the half-life of Luc-AMV by 20% and enhanced its translational efficiency about 40-fold. The stimulation of translation by CP was cap-dependent and could be reproduced in yeast cells. GST-pull-down assays revealed the binding of AMV CP to initiation factor complexes eIF4F and eIFiso4F from wheat germ. By Far-Western blotting it was shown that this binding occurred through an interaction of CP with the eIF4G and eIFiso4G subunits of eIF4F and eIFiso4F, respectively. The results support the hypothesis that the role of CP in translation of viral RNAs mimics the role of the poly(A) binding protein in translation of cellular mRNAs.

Recombination-replication-repair interface of geminiviruses

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One major driving force of geminivirus evolution is recombination. They may exchange genomic components (pseudorecombination) as a prerequisite for true molecular recombination and may acquire thus new DNA components like DNAs B, beta DNAs, satellite DNAs or even DNAs of different viruses as it has been shown for nanovirus DNA circles. Using an optimized two-dimensional gel electrophoresis in combination with hybridization and electron microscopy we have discovered that the recombinational flexibility is reasoned by a recombination-dependent replication mode (RDR) that is widespread at least among begomoviruses and curtoviruses but does also occur for satellites, beta DNAs and artificial episomes. Compared to complementary strand synthesis (CSR) and rolling circle replication (RCR), which both were found together with RDR, the latter mechanism allows the virus to repair every broken or unfinished DNA intermediate as far as homologous templates are available. Using virus-specific two colour detection, we found out the the frequency by which different geminiviruses enter the same nucleus was surprisingly high in tomatoes and *N. benthamiana*, so that the chance to recombine two viruses is extremely high. Moreover, weeds are ideal cradles for new geminiviruses since they are reservoirs and collect several virus species over years, as exemplified for Sida micrantha-associated geminiviruses. Surveying different combinations of geminiviral and satellite DNAs two strategies can be discriminated between Old World and New World begomoviruses. The first were rather promicous to transreplicate other DNAs, even without a cognate Rep-binding sequence, whereas the latter need the interaction of Rep and Rep-binding sequences and consequently DNAs B were acquired by shuffling of the regulatory region (common region CR) by homologous recombination. The consequences of these findings for efficient resistance breeding and epidemiology will be discussed.

BNYVV-like particles formed by coat protein expressed from a potexvirus-based vector construct

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We have recently determined the nucleotide (nt) sequences of the genomic RNAs of three potexviruses which had been isolated long ago from different species in the Cactaceae and were originally designated as different strains, i.e. BS, CC10 and K11, of *Cactus virus X*. Because serological and molecular studies revealed only rather distant relationships between these viruses, we have proposed to change their taxonomic status and rename them to Zygocactus virus X (ZVX), Opuntia virus X (OVX) and Schlumbergera virus X (ScVX), respectively. The full length cDNA sequence of ZVX RNA was cloned into the 35S promoter-containing vector p35Stupa kindly provided by E. Maiß, Hannover. The cDNA clones obtained consistently produced symptomless local and occasionally also systemic infections in *C. quinoa*. In order to enable insertion and expression of foreign genes an *Asc*I and a *Spe*I site were introduced downstream of the coat protein (cp) promoter and directly upstream of the cp gene. Most of the ZVX cp gene except for its 56 3'-terminal nucleotides were replaced by the corresponding sequence of the ScVX cp gene and by additional 45 nts upstream of the ScVX cp gene which presumably contain the ScVX cp promoter. The expression of foreign genes which are inserted between the *Asc*I/*Spe*I sites is thus directed by the ZVX cp promoter whereas the expression of the ScVX cp gene is directed by its own promoter. Clones, into which the cp gene of *Beet necrotic yellow vein virus* (BNYVV) was inserted, produced local infections in *C. quinoa* and sugarbeet. In the former, BNYVV cp was readily detected by means of ELISA. Immunoelectron microscopy revealed the presence of virus-like particles which were strongly decorated by antibodies to BNYVV. This particle formation is surprising, because in natural infections it is assumed that the cp readthrough protein is necessary to initiate particle assembly.

TSWV transcriptase *in vitro* prefers cap donors with multiple base complementarity to the viral template

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Transcription of segmented negative strand RNA viruses is initiated by cap snatching, i.e. a mechanism in which host mRNAs are cleaved generally at 10-20 nt from their 5' capped end and the resulting capped leaders used to prime transcription of the viral genome. This mechanism has first been decribed for Influenza A virus in the early 80's and since reported for several other viruses. For *Tomato spotted wilt virus* (TSWV), type species of the plant-infecting *Tospovirus* genus within the *Bunyaviridae*, the occurrence of cap-snatching was reported in 1992 and following studies have shown that cap donors require a single base complementarity to the ultimate or penultimate viral template sequence. More recently, the occurrence *in vitro* of "re-snatching" of viral mRNAs, i.e. the use of viral mRNAs as cap donors, has been demonstrated for TSWV. To estimate the relative occurrence of re-snatching compared to snatching of host mRNAs, the use of cap donors with either single, double or multiple complementarity to the viral template was analysed in pair-wise competition in TSWV *in vitro* transcription assays. These analyses have shown a strong preference for multiple-basepairing donors. Similar experiments are currently performed for Influenza A virus transcription initiation. Furthermore, a (first) series of cap-donor mutants has been made to analyse the requirements of cap-donor leader sequences upstream the baseparing residues for TSWV as well as Influenza A virus transcription initiation.

Interactions of movement protein BC1 and nuclear shuttle protein BV1 of *Abutilon mosaic geminivirus*

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Bipartite geminiviruses such as *Abutilon mosaic virus* encodes two proteins responsible for virus movement and pathogenic properties, the nuclear shuttle protein (NSP; syn. BV1) and the movement protein (MP; syn. BC1). Transient expression of green fluorescent protein fusions of MP and NSP in host plant cells has shown that BV1 localizes to nuclei and BC1 to the cell periphery or close to nuclei. By electron microscopy techniques NSP expressed in fission yeast was detected in nuclei whereas MP was localized to protoplasmic leaflets of plasma membranes. Upon co-expression in plants or yeast, BC1 redirected BV1 from nuclei to cell periphery and in sink leaves of host plants, additionally to adjacent cells. BC1 deletion mutants lacking the membrane-binding domain indicated a homo-oligomerization of its C-terminus by yeast two-hybrid and Cyto-Trap analysis. *In-vitro* assembly of double-stranded super coiled DNA with NSP and MP into conspicuous structures was confirmed using electron microscopy and provided evidence for cooperative interaction of MP, NSP and DNA. These results support a model, in which NSP transports viral DNA to the cell periphery, and BC1 acts as a membrane adaptor for NSP-DNA complexes to facilitate cooperative cell-to-cell movement within plants. By use of two-hybrid system, we identified BC1-interacting plant factors, which are currently under investigation.

Interaction between *Cowpea mosaic virus* movement tubules and the plasma membrane

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Cowpea mosaic virus (CPMV) virions traverse the cell wall through nanotubules that are assembled from the viral movement protein (MP). *In planta* these tubules are found exclusively in the plasmodesmal canal. In isolated plant protoplasts, devoid of cell walls and plasmodesmata, a similar polarity of tubule assembly is observed. The MP anchors somehow at the plasma membrane (PM) and from there an outward growing tubule is assembled tightly encased with PM as also occurs within the plasmodesma. Transport tubules are not only formed in protoplasts of host plants, but also in those isolated from non-host plants or even animal cells that express the MP. Obviously, MP interacts with the PM or with PM-associated proteins and such proteins should be conserved in plant and animal cells.
This intriguing interaction between MP and PM was further analyzed by MP-mutant analysis, time-lapse microscopy and FRET/FRAP studies. It has been shown before that prior to tubule formation, MP (-GFP) accumulates in so-called peripheral punctate spots at the PM and it was speculated that these were the nucleation sites for tubule assembly. Time-lapse fluorescent microscopy was performed on protoplasts expressing MP-GFP and showed that the punctate spots are dynamic structures that are immobilized at the PM and that tubules indeed arise from (a sub-population of) these spots.
To investigate the interaction of tubules with the surrounding PM, the diffusion herein of small and large fluorescent PM-associated proteins was examined by fluorescence recovery after photobleaching (FRAP). These studies indicate that tubules made by CPMV MP do not interact directly with the PM, but most likely via a PM intrinsic protein (PIP) or otherwise associated host protein. Using affinity chromatography it was found that purified MP a.o. binds to aquaporin present in the plasmamembrane. Transient expression of MP-YFP and *Arabidopsis* aquaporin P1;4-CFP revealed that these proteins colocalize in punctuate spots on the surface of cowpea protoplasts. Acceptor photobleaching experiments indicate that they also physically interact at these sites, suggesting that aquaporin is used by the virus as a primer for the tubule formation at the plasmamembrane.

Symptom variants of *Cucumber mosaic virus* (CMV) are based on more than a single mutation

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In Asia chili is of high importance for the daily nutrition and grown as local varieties from small holders. Beside other pathogens chili is susceptible to *Cucumber mosaic virus* (CMV), a (+) ssRNA virus with a tripartite genome. Commercial chili varieties with resistance to this disease were not available and therefore resistant varieties have been developed from the Asian Vegetable Research and Development Center (AVRDC, Taiwan). Although some lines showed resistance to many strains, originating in several Asian countries, it was overcome at least by two isolates, CMV-AN from India and CMV-KS44 from Thailand. The responsible genetic determinant was located on RNA 2 by reassortment studies. Chimeric constructs of full-length RNA 2 cDNA clones based on isolate Fny comprising the complete gene silencing suppressor (2b) gene of resistance breaking isolate CMV-AN and partial sequences of 2a gene and 3´ non coding region were able to overcome the resistance in concert with RNA 1 and RNA 3 from the non resistance breaking isolate CMV-P3613. These data suggested that the gene silencing suppressor 2b and/or the 2a protein is responsible for this biological behaviour.
Within the 2a- and 2b-genes two striking mutations between resistant and non-resistant isolates were present. However, site directed mutagenesis did not confirm the significance for these mutations but suggest that other factors are involved for symptom expression. Additional reassortants between different combinations of isolates indicated that also RNA 1 and/or RNA 3 may play a role in symptom determination, and - in some cases - in concert with RNA 2. The symptom expression of reassortants and genetically modified constructs in other host systems were not predictable. These results show that symptom expression is a very complex process depending on synergistic interactions. The results derived from single mutation experiments should therefore not be generalized.

GFP-labelling of mite transmitted *Brome streak mosaic virus* (BStMV)

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Brome streak mosaic virus (BStMV) infects *Poaceae* and is transmitted by eriophyid mites like the other members of the genus *Tritimovirus*: *Wheat streak mosaic virus* (WSMV) and *Oat necrotic mottle virus* (ONMV). For construction of a BStMV full-length cDNA clone (pBStMV-FL) viral RNA was extracted from a French BStMV isolate (11-Cal; BStMV-wt) and cDNA fragments were amplified by RT-PCR. The fragments were cloned in succession under control of an enhanced *Cauliflower mosaic virus* (CAMV) 35S-promoter and its 3'-polyadenylation signal, resulting in pBStMV-FL. This plasmid was transferred into plants by particle bombardment. The virus deriving from pBStMV-FL was able to infect the plant systemically and was detected immunologically. Comparison of the host spectrum of BStMV-FL and BStMV-wt revealed no differences in terms of infected plants and symptom severity. Six species were identified as host plants, at which *Bromus mollis*, *Hordeum vulgare* and *Triticum aestivum* showed obvious virus symptoms. BStMV-FL was labelled with a green fluorescent protein (GFP) at two different positions in the genome. For this purpose the *gfp* gene was integrated in-between P1 and helper component protease (pBStMV-P1/GFP/HC-Pro) and in-between nuclear inclusion body b and coat protein (pBStMV-NIb/GFP/CP). Four weeks after particle bombardment virus symptoms were observed. BStMV-CP and GFP were detected by electroblot immunoassays. Fluorescence of GFP was visualized with a confocal-laser scanning microscope. Therefore, correct proteolytic processing of GFP occurred in BStMV-P1/GFP/HC-Pro as well as in BStMV-NIb/GFP/CP and the released GFPs were functional. In addition, eriophyid mites were collected from the field and used successfully to transmit BStMV. Altogether these findings enable further studies on infection, replication and transport of BStMV-FL and mutants thereof in plants as well as determination of proteins and motifs involved in vector transmission.

TSWV particle asssembly: *in vivo* interactions between the structural nucleoprotein and G1 spike protein

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TSWV (*Tomato spotted wilt virus*) is a member of the Bunyaviridae family. Virus particles are spherical and membrane bound, containing spike proteins that consist of two glycoproteins, G1 and G2. The core contains ribonucleoproteins (RNPs) that consists of genomic RNA tightly associated with the nucleoprotein (N) and small amounts of the viral RNA-dependent RNA polymerase. Enveloped virus particles arise as a result of RNP envelopment with membranes from the Golgi apparatus containing G1 and G2. To investigate *in vivo* interactions between the structural proteins involved in virus assembly, fluorescence techniques (FRET: fluorescence resonance energy transfer and FLIM: fluorescence lifetime imaging microscopy) were employed. To this end, N, G1 and G2 were fused at their N or C-terminus to CFP or YFP. Upon co-expression of N-YFP and N-CFP in mammalian cells, N dimerisation was observed in peri-nuclear aggregates as well as throughout the cytoplasm. The peri-nuclear localisation of N oligomers required actin filaments and microtubules, as demonstrated with the use of inhibitors, which suggested the possible involvement of these cellular elements in TSWV particle formation. Upon co-expression of N-YFP with G2-CFP no FRET was observed, whereas co-expression of N-YFP and G1-CFP did show FRET. Furthermore, G1 was observed to show an altered localisation pattern in the presence of N, i.e. upon single expression of G1 only ER localisation was observed whereas a peri-nuclear accumulation partly overlapping with that of N was observed in the presence of N. Altogether, these results suggest that the actual envelopment of TSWV RNPs' could be triggered by an interaction between N and the cytoplasmic tail of G1.

Transgenic AbMV DNA B supports mechanical transmission but does not release phloem restriction

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Strict phloem limitation of the bipartite *Abutilon mosaic virus* (AbMV) in different host plants is accompanied by a lack of mechanical transmissibility. In order to analyse the functionality and thus contribution of movement proteins to tissue restriction, *Nicotiana benthamiana* plants were transformed with AbMV DNA B dimers and challenged by agroinoculation of AbMV DNA A. Several independent plant lines (generations T0 to T3) were capable for replicating and systemically spreading complete bipartite virus genomes, including free DNA B copies. In contrast to reports on other begomoviruses, transgenically expressed AbMV BC1 protein was not able to induce pathogenic symptoms permanently. Different transgenic lines containing functional DNA B copies and expressing BC1 protein indeed developed abnormal leaf phenotypes transiently, but later on recovered and were indistinguishable from wildtype plants. Upon AbMV infection, symptoms were the same in transgenic and wildtype plants, as was the amount of viral DNA accumulating in leaf tissues. Macroscopic and microscopic *in situ* hybridisation revealed that AbMV remained phloem-limited in the DNA B-transgenic plants. Nevertheless, mechanical transmission of the virus had changed: About one fifth of transgenic plants treated with sap from systemically infected wildtype *N. benthamiana* developed full AbMV infections. Thus AbMV DNA B genes BV1 and BC1 can complement for mechanical transmission in inoculated leaves, but in systemically infected leaves fail to support viral invasion of non-phloem cells. Since in mixed infections with CMV, AbMV has been shown to escape from the phloem, some other (perhaps gene silencing-) mechanism may be responsible for AbMV tissue limitation.

Future challenges in the innovation of detection and identification techniques in plant virology

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Since the end of the eighties the strategy of the Dutch government focussed on the reduction of the use of pesticides. As a result less capacity and budget was available for research in the field of plant health. This led to a dramatic loss of expertise, especially in the field of virology. A similar tendency could be observed internationally. Since healthy seeds and planting material are of crucial importance to manage virus diseases, this loss eventually will result in increasing problems caused by viruses. The consequences of the lack of diagnostic methods for potentially harmful viruses and the loss of knowledge and expertise to develop such methods, has to be brought into attention of the responsible decision makers. Therefore coherent research proposals have to be drafted and submitted to national and international governments and organisations. It should be stressed that is necessary to maintain both knowledge and practical expertise in the field of plant virology. New detection and identification methods have to be developed based on reference collection material that, in combination with the classical methods, enable to study the biological and epidemiological aspects of viruses. Such approach will provide a solid base, which is essential to manage actual and future virus problems by prevention and control.

Serological and molecular differentiation of *Soil-borne cereal mosaic virus* and *Soil-borne wheat mosaic virus* - two furoviruses occurring on wheat and rye in Germany

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Rod-shaped virus particles approx. 20 nm in diameter were observed by electron microscopy in leaves of rye and wheat varieties grown in different regions in Germany. Virus isolates were transmitted through infected soil to wheat, rye, and triticale and by mechanical inoculations to *Chenopodium quinoa*. By use of polyclonal antisera to *Soil-borne cereal mosaic virus* (SBCMV) (BBA Braunschweig) and *Soil-borne wheat mosaic virus* (SBWMV) (Univ. of Nebraska, USA) it was neither possible to discriminate the isolates in ELISA and Western blots (WB) nor on the basis of their symptoms on *C. quinoa*. SBWMV (ATCC type strain) and the German isolates 'Eilte', 'Eickeloh' and 'Heddesheim' were propagated on rye variety 'Nikita' for virus purification and antiserum production. Altogether 8 polyclonal antisera (PAS) were raised in rabbits. PAS-45 and PAS-69 produced to the type SBWMV and to isolate 'Heddesheim' showed no or only very weak cross-reactions with the other isolates. By means of specific primer combinations these two isolates were verified in immunocapture RT-PCR as SBWMV whereas all the other isolates were assigned to SBCMV. Two of the four PAS produced to SBCMV reacted in DAS-ELISA, WB and tissue print immunoassay (TPIA) mainly with the homologous virus. However, at high virus concentrations a specific discrimination between SBWMV and SBCMV with PAS was doubtful, particularly by their application in TPIA. Therefore, monoclonal antibodies (MAbs) were produced to both furoviruses. For SBWMV detection a DAS-ELISA based on MAb 4G4 (IgG3, □) was developed. For specific discrimination of all SBCMV isolates MAb 4G11 (IgG2a, □) can be recommended for use in TAS-ELISA. The virus species specific MAbs allow in TPIA an excellent discrimination between both furoviruses and can be applied in large-scale resistance trials. By sequences comparison we assume that the coat protein motifs ATHAY and SIHPF could form specific epitopes for SBWMV and SBCMV, respectively.

The allelic resistance genes *Tm-2* and *Tm-22* against *Tomato mosaic virus* differ only in 4 amino acids but recognize two independent domains of the ToMV MP

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Transposon insertion experiments in tomato plants led to the isolation of the *Tm-2* resistance gene against ToMV.. Sequence analysis revealed that *Tm-2* codes for a 861 aa protein which belongs to the CC-NBS-LRR type of resistance genes. Surprisingly, *Tm-2* contains only 4 amino acid exchanges located in two different domains of the protein in comparison to the allelic *Tm-22*, although sequence analysis of diverse resistance breaking virus strains proved that both resistance genes recognize different parts of the ToMV movement protein. Further investigations on the molecular interaction of these resistance genes with the ToMV MP indicated that Tm-2 and Tm-22 contain two distinct domains for the interaction with the avirulence gene product.

Functional analysis of RNAi suppressors of negative strand plant viruses

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In recent studies we have shown that the NSs protein of *Tomato spotted wilt tospovirus* (TSWV) as well as the NS3 protein of *Rice hoja blanca tenuivirus* (RHBV) suppress RNA silencing in plants.
To further analyse the function of suppressor NS3 in *N. benthamiana* in RNA silencing, expression constructs of these proteins were produced resulting in the production of MBP tagged proteins in bacteria and plants (via A. tumefaciens infiltration). The addition of the fusion partner, included to facilitate pull down experiments, was shown not to interfere with the RNA silencing activity of the proteins. In subsequent experiments deletion mutants were generated. Two conserved domains of the in NS3 protein were deleted. Both NS3 mutant proteins were proven to be expressed, while the RNA silencing suppression activity in plants was lost. Analysis of mRNA and siRNA levels compared to NS3 confirmed these observations and will be discussed. A series of point and deletion mutations in the NSs protein was also produced and analysis will be presented. To further elucidate the biochemical function, *in vitro* RNAi experiments were performed and the function of bacterial purified suppressors examined.
Both TSWV and RHBV are transmitted by insects in the natural situation, and moreover are replicating in their insect vector. To further analyse the possible function of the RNAi suppressor proteins in insects, we set up RNAi (suppression) experiments in *Drosophila* S2 cells using eGFP as reporter. Using this system we were capable to show that S2 cells can efficiently silence the GFP transgene using specific siRNAs, hence RNAi is fully functional. Upon addition of the RNAi suppressor proteins, this effect was completely reversible indicating that NSs and NS3 are efficient RNAi suppressors in both plants and insects.

Occurrence of short interfering RNA in virus-infected Cassava *(Manihot esculenta)* varieties with differential resistance /susceptibility against geminivirus infections

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Geminiviruses are single stranded DNA viruses that replicate in nuclei of infected cells, cause serious disease in cassava. It has been shown that these viruses despite their missing dsRNA phase within their replication can trigger post-transcriptional gene silencing (PTGS) in infected plants. We have therefore studied the accumulation of short interfering RNAs (siRNAs), in resistant and susceptible cassava varieties to correlate the appearance of these RNA species with recovery from infection and level of resistance. Northern analysis of RNA extractions prepared from virus infected cassava, hybridized with viral DNA-A and DNA-B specific probes revealed presence of two classes of virus specific siRNA. All cassava varieties infected with different virus species/strains revealed siRNA accumulation in symptomatic tissues, however hybridization analysis with genomic component DNA-A or DNA-B specific probes indicated that for certain viruses the primary target of PTGS is within DNA-A while for others DNA-B is targeted. Still, high levels of siRNA accumulation

were only found in symptomatic cassava leaves, hence correlated with symptom severity and virus concentration. In the resistant cassava varieties TME 4, TME 3 and TMS 96/1089A that recover from begomovirus infection, siRNA was found only in low concentrations in non-symptomatic plant parts. The recovery in resistant cassava varieties did not depend on the virus species or strain as reported earlier, but rather reflected the resistance status of the plant.

The use of RNA silencing for resistance to geminiviruses

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In recent years, whitefly-transmitted geminiviruses (Genus *Begomovirus*) have become one of the major constraints to tomato production world wide. To evaluate the use of RNA silencing for the control of geminiviruses, we have created a construct containing coding and non-coding (promoter) sequences derived from *Tomato chlorotic mottle virus* (ToCMoV-[BR]), a major problem for tomato cultivation in Brazil. A ~1500 bp fragment containing the 5' end of *rep* gene, the nested AC4 gene, the entire common (promoter) region and the 5' end of the *cp* gene and nested AC5 gene, was PCR amplified and cloned in a GATEWAY donor vector. Subsequent recombination resulted in the fragment being cloned into a GATEWAY a binary vector downstream of the 35S promoter in an inverted repeat array, spaced by an intron. Forty eight transgenic *Nicotiana benthamiana* plant lines harboring the construct were produced and ten were challenged with ToCMoV-[BR]. When the transgenic lines were mechanically inoculated twice, in a ten days interval, most transgenic lines showed delayed onset of symptoms of at least 8 days, but at 45 days post inoculation, viral DNA could be detected in most plants. In two lines (19.3 and 24.2) 50% and 40% of the plants were symptomless and in 20% and 30%, respectively, the virus could not be detected. Northern blot analysis of siRNAs showed the presence of transgene-specific siRNAs and an increase upon virus inoculation. Interestingly this occurred in both resistant and susceptible plants. We are currently investigating to what extent post-transcriptional gene silencing (viral mRNA degradation) or transcriptional gene silencing (viral DNA methylation) are responsible for the observed resistance.

Attempts for genetic improvement of resistance of barley to *Barley yellow dwarf virus*

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In several years barley suffers from dwarfing caused by *Barley yellow dwarf virus* (BYDV). In Germany, PAV is the most spread serotype of this luteovirus. Except tolerance no strongly acting resistance is available and only a limited number of reports exists that resistance was improved genetically applying the principle of pathogen derived resistance.
We tried to use two approaches to improve resistance genetically – expression of RNAi and single chain variable fragment antibodies (scFv). The RNAi-constructs have been based on inverted repeats of the 3'-NTR of BYDV-PAV, divided by an intron. The scFv used in these experiments was directed against the GDD domain of viral RNA-dependent RNA polymerases.
Among approximately 100 barley clones ('Golden Promise') expressing the RNAi only one was identified which showed a stable symptom tolerance still in T3. Resistant genotypes could not be identified.
scFv induced in barley symptom tolerance too, even in T1 generation. The problem consisted in its instability *in planta* – only those clones showed a stable expression which have been based on constructs with a Suc promoter and where the scFv was targeted to the ER (lepB leader, KDEL ER-retention signal). The same holds true for *Nicotiana benthamiana* which revealed delayed virus symptoms after infection with *Potato leafroll virus* and *Potato virus Y*. At the moment homozygous *N. benthamiana* plants are produced to test stability of the scFv-induced resistance.

Single-chain antibodies against a plant viral RNA-dependent RNA polymerase confer virus resistance

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Crop loss due to viral diseases is still a major problem for agriculture today. We present a strategy to achieve virus resistance based on the expression of single-chain Fv fragments (scFvs) against a conserved domain in a plant viral RNA-dependent RNA polymerase (RdRp), a key enzyme in virus replication. The selected scFvs inhibited complementary RNA synthesis of different plant virus RdRps *in vitro* and virus replication *in planta*. Moreover, the scFvs also bound to the RdRp of the distantly related hepatitis C virus. T_1 and T_2 progeny of transgenic lines of *Nicotiana benthamiana* expressing different scFvs either in the cytosol or in the endoplasmic reticulum showed varying degrees of resistance against four plant viruses from different genera, three of which belong to the *Tombusviridae* family. Virus resistance based on antibodies to RdRps adds another tool to the repertoire for combating plant viruses.

Translocation and immunolocalisation of W*atermelon chlorotic stunt virus,* WmCSV*,* in its vector *Bemisia tabaci* (Genn.)

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To investigate the mechanisms of begomovirus transmission by *Bemisia tabaci*, acquisition and translocation of *Watermelon chlorotic stunt virus*, WmCSV, in the insect was studied. A whitefly transmissible WmCSV isolate from Sudan and 4 non transmissible mutants carrying a single amino acid mutation in the core region of the capsid protein were used for the translocation experiments. *B. tabaci* fed on virus infected watermelon plants were transferred to non host plants for virus discharge and subsequently used to infect watermelons. Transmission was taken as evidence for a functional interaction between virions, the gut membrane and the primary or accessory salivary glands. Fresh, dissected organs from viruliferous whiteflies feeding on wild type or mutant virus were examined by PCR to determine the presence of virus. WmCSV was detected in the midgut, hemocoel and salivary glands of *B. tabaci* for the transmissible and the non transmissible virus mutants. However, in *Trialeurodes vaporariorum* (a non vector of geminiviruses), wild-type WmCSV was detected in the midgut only, thus suggesting that the virus was not capable of crossing the gut wall. In contrast, in B. tabaci, the accessory and/or the primary salivary glands apparently present the significant epithelial barrier to virus transmission. Hence, for the begomovirus WmCSV and its whitefly vector, a pathway similar to the luteovirus / aphid translocation is expected. In immunolocalization studies with organs excised from viruliferous insects carrying wild type or non transmissible virus mutants, a specific labelling with WmCSV antiserum was obtained at the microvilli region of the epithelial cells of the gut wall food canal, indicating for putative virus adsorption sites or, for a putative receptor-mediated delivery to the hemoceol. Respective experiments carried out with primary and accessory salivary glands so far did not indicate for specific adhesion/entry sites into these organs.

Recent developments in Augusta disease of tulip in the Netherlands

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Augusta disease in tulips is caused by *Tobacco necrosis virus* (TNV). The virus is transmitted by zoospores of *Olpidium brassicae.* The host range of this fungus includes many commonly found weeds such as annual blue grass, annual sowthistle, chickweed, dandelion, shepherd's purse and others. Some of these *Olpidium brassicae* hosts are also hosts of TNV.
Damage caused by Augusta disease can be severe in the Netherlands. Disease incidence occurred up to 60% in fields in some years and up to 20 % in flower forcing. There has been a shift in tulip bulb production on sandy soils towards production on heavy clays. Since *Olpidium brassicae* is spread by water and clay soils are more sensitive to standing water, the occurrence and establishment of the disease is enforced. As a common practice in certain areas, bulbs are grown on soils, which have been in pasture for a period of circa 6 years. These pastures are a favourable environment to enhance the spread of *Olpidium brassicae.*
The influence of soil type, plant debris and planting time are examined. The addition of *Pseudomonas* to the bulb dipping bath with fungicides leads to a significant decrease of Augusta. Preliminary results suggest that incorporating Sarepta mustard in the soil might prevent the occurrence of Augusta disease. Several other crops are currently under investigation.
The fungus *Olpidium brassicae* can be detected with the aid of PCR in soils and in closed water supply systems. These methods will be applied in epidemiological studies.

Identification of viroids occurring in tomato and potato, and consequences for testing

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Over the last decades, viroids have been occasionally detected in samples of tomato and potato at the Plant Protection Service in the Netherlands. In tomato the viroid infections concerned commercial greenhouses both in the Netherlands and abroad. In potato all viroid infections have been detected during post-entry quarantine testing of imported breeding material. The viroids found in tomato were identified as *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd), *Potato spindle tuber viroid* (PSTVd) and *Tomato chlorotic dwarf viroid*. All viroids from potato were identified as PSTVd.
The biological characteristics of CEVd, CLVd and PSTVd from tomato were studied by mechanical inoculation of young plants of tomato and potato under greenhouse conditions. Inoculation of tomato resulted in growth reduction as well as distortion and chlorosis of younger leaves. In potato only tubers showed symptoms. Planting these tubers in the field resulted in severely stunted plants, tubers of poor quality and significant lower yields. The severity of the symptoms and yield losses differed between the viroid isolates tested.
Since PSTVd has a quarantine status in the European Union, the question arose whether the status of the other viroids should be reconsidered. Therefore, the risks of CLVd, appearing the most harmful viroid, will be evaluated and used for national and international discussions about the potential risks and future measures on these viroids.
With regard to testing, it is advised to use a broad-spectrum method (e.g. R-PAGE) for initial screening, and subsequent sequence analysis for identification. In cases of outbreaks, when the identity of the viroid isolate is known, and large numbers of samples have to be tested molecular methods (e.g. real-time RT-PCR) are more suitable.

NeRNV, a new tymovirus with a genomic RNA having a histidylatable tobamovirus-like 3'end

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The complete nucleotide sequence has been determined for the genomic RNA of the new *Nemesia ring necrosis virus* (NeRNV) which is widely spread in various ornamental plant species belonging to the *Scrophulariaceae and Verbenaceae*. Judged from its gene content, the folding properties of its 5'untranslated region and from in vitro translation experiments, NeRNV RNA is a typical tymovirus RNA. Its 3'end, however, differs greatly from those of the valine-specific tymoviral RNAs which have been analysed previously. It can be folded into an upstream pseudoknot domain (UPD) and a histidine-specific tRNA-like structure (TLS), a combination which so far has been found only for tobamoviral RNAs. The identity elements found in NeRNV RNA for the recognition by yeast histidyl-tRNA synthetase are more similar to those of yeast $tRNA^{His}$ than the ones found in *Tobacco mosaic virus* (TMV) RNA. As a result NeRNV RNA can be charged with histidine even more efficiently than TMV RNA.

Identification and detection of a closterovirus from carrot in Germany

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Following dsRNA isolation from a stunted and chlorotic carrot plant growing in a seed propagation plot near Bingenheim, Hessen, Germany, a complex pattern of dsRNA bands was obtained. Since one of the dsRNAs had a notably high molecular weight, it was used as starting material for random RT-PCR, cloning and sequencing. Several clones were obtained which shared sequence similarity with viruses of the genus *Closterovirus*. A stretch of about 12,000 nucleotides comprising the complete 3' half of the genome of the carrot closterovirus (CCV) was sequenced and analysed. Based on the *hsp70h* gene, which is commonly used for illustrating relationships in the family *Closteroviridae,* CCV is most closely related to *Beet yellow stunt virus* and *Beet yellows virus*, with which it shares *hsp70h* amino acid sequence similarities of 49% und 48% respectively. Moreover, CCV has a genomic organisation characteristic of the genus *Closterovirus* and, thus, can be confidently assigned to the genus *Closterovirus* of the family *Closteroviridae*. In attempts to develop a serological detection method for CCV, the major coat protein (CP) gene of CCV was expressed in *E. coli* and the resulting protein preparation was used for polyclonal antibody (PAb) production in a rabbit. These PAbs allowed visualisation of numerous closterovirus-like particles by immunosorbent electron microscopy. CCV particles had a normal length of 1600 nm and a width of 12 nm. In Western-blot experiments, the PAbs reacted with a protein band of about 25 kDa which was only present in CCV-infected but not in non-infected carrot plants. PAbs also permitted sensitive detection of CCV in field carrot samples by DAS-ELISA. A reference sample of *Carrot yellow leaf virus* (CYLV) from the Netherlands also gave a strong Western-blot reaction with the PAbs to CCV CP. Therefore, we regard CCV as a German isolate of CYLV, the only known closterovirus infecting Apiaceae.

A new strain of *Cowpea mild mottle virus* infects French bean in Spain and Morocco

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In Spain recently two new virus diseases in French bean (*Phaseolus vulgaris*) have been reported, caused by *Southern bean mosaic virus* (SBMV, Verhoeven et al., 2003) and bean yellow disorder virus BnYDV, Segundo et al., 2004), respectively. During autumn 2003, another disease turned up. Pods of infected plants showed necrotic spots at the time of harvesting, while obvious leaf

symptoms were not observed. The symptoms did not correlate with SBMV, BnYDV or any other virus from Spain. In addition, at the same time similar symptoms were observed in Morocco.
From the symptomatic pods a virus could be transmitted mechanically to *Arachis hypogaea*, *Nicotiana occidentalis*-P1, *Pisum sativum* 'Kelvedon Wonder', *Phaseolus vulgaris* 'Dubbele Witte zonder draad' and *Vigna unguiculata* 'Black Eye'. In addition, it was transmitted to French bean by the tobacco whitefly (*Bemisia tabaci*). Using electron microscopy slightly flexuous filamentous particles with a length of about. 615 nm typical for a carlavirus were observed. In DAS-ELISA both infected plants and viruliferous whiteflies reacted positive with an antiserum to *Cowpea mild mottle virus* (CPMMV). The involvement of a carlavirus was further substantiated by RT-PCR with carlavirus-specific primers (Badge et al., 1996) yielding amplicons of the expected size. By using different primers part of the coat protein gene was sequenced for both a Spanish and a Moroccan isolate. Sequence analysis and comparison of deduced amino acid sequences with gene bank data revealed that both isolates are closely related (identity 99.4%) and show highest identity with the two sequenced cowpea isolates of CPMMV in the NCBI Genbank. The amino acid sequence identities with these cowpea isolates, however, varied between 91 and 95%. Therefore, it is proposed to consider the bean isolates from Spain and Morocco as a separate strain of CPMMV.
To correlate the identified virus to the symptoms in French bean, five isolates were mechanically inoculated onto four bean varieties. In two varieties necrotic spots appeared on the pods for all isolates, while obvious leaf symptoms did not occur in any combination. These results indicate that this new strain of CPMMV caused the necrotic spots on the pods of the originally infected plants.

A new bunyaviral-type plant virus is associated with the ringspot disease of European mountain ash (*Sorbus aucuparia* L.)

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European mountain ash trees (Sorbus aucuparia L.) in Germany suffer from ringspot and mottling symptoms on leaves and from a gradual decay in general. We could isolate double stranded RNA (dsRNA) from symptomatic tissue, which indicates RNA virus infection. Cloning and sequencing of putative viral RNAs allowed the characterization of a new virus associated with the mountain ash disease.
Fractions of dsRNA were extracted by column chromatography. A characteristic pattern of dsRNA of approximately 7 kb, 2.3 kb, 1.5 kb, and 1.3 kb, respectively, was found in leaf samples of symptomatic mountain ash trees from various sites in Germany. No dsRNA was detected in asymptomatic trees.
By random primed reverse transcription, DOP-PCR (degenerate oligonucleotide primed PCR), cloning and sequencing, dsRNA-specific cDNA fragments were obtained. Using 5´-RACE analyses, modified by biotin labelling and magnetic separation, longer cDNAs could be enriched. With our cloning strategy a cDNA of 7.0 kb in length was obtained first. The corresponding RNA harbours one ORF with homology to the RNA dependent RNA polymerase (RdRP) of members of the family *Bunyaviridae*. It shows all conserved sequence motifs of the bunyaviral RdRP and also the typical terminus sequences at its 5´- and 3´-end. Primers derived from the terminus sequences allowed the subsequent identification of three further RNAs of 2.3, 1.6 and 1.3 kb. The corresponding ORFs encode a putative glycoprotein precursor, a putative nucleocapsid protein, and a protein of unknown function. *In situ* hybridization studies using digoxigenin labelled riboprobes for the viral RNA 1 and RNA 3 showed a scattered pattern of virus accumulation in the mesophyll of mountain ash leaves.
The dsRNA pattern, the sequence information and the present image of the viral genome organisation strongly indicate that a new plant RNA virus with similarity to the *Bunyaviridae* is associated with the mountain ash ringspot disease.

Sequence analysis of a serogroup IV tospovirus isolated from *Lycospericum esculentum* in Thailand

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Tomato plants (*Lycopersicum esculentum*) cultivated at the Asian Institute of Technology (AIT) in Thailand showed symptoms typical for a tosopvirus infection. Serological tests revealed no reaction with an antiserum specific for tospoviruses of serogroup I, II and III (TospoBroadRange; LOEWE® No.07507). However, antibodies specific for tospoviruses of serogroup IV (AGDIA® SRA 61500) strongly reacted with saps of infected plants. The main objective was to determine the nucleotide sequences of the serogroup IV tospovirus. The tospovirus was mechanically transmitted to *Nicotiana benthamiana* and *Lycopersicum esculentum* cv. Lizzy plants. Total RNA was extracted and RT-PCR using primer J13 (CCCGGATCCAGAGCAAT), with conserved eight terminal nucleotides found in all tospovirus RNA termini (underlined) was used together with primers designed from conserved regions of serogroup IV tospoviruses. Six PCR fragments from the RNA-ends were generated, cloned and sequenced. Additional amplification and cloning steps were performed with primers located in the determined regions to complete the sequences. From the M- and L-RNA PCR-fragments of about 4000 nt were amplified with the Phusion™ high-fidelity DNA polymerase (Finnzymes). The L-RNA comprises of 8912 nt and codes for the RNA-dependent RNA-polymerase. Two ORFs are located on the M-RNA (4823 nt) encoding the Nsm protein and the viral glycoprotein precursors (G1/G2) separated by an intergenic region of 433 nt. ORFs coding for NSs- and N-protein were identified on the S-RNA. However, the sequence of the S-segment intergenic region has still to be confirmed. The N-protein showed with 92,7% the highest amino-acid sequence similarity to the recently described Capsicum chlorosis virus (CaCV, AY036058). A sequence similarity of 84,4% was found to *Groundnut bud necrosis virus* (GBNV, NC_003619) and of 85,5 % to *Watermelon silver mottle virus* (WSMoV, NC_003843). According to these findings we consider the new tomato infecting tospovirus from Thailand as an isolate of CaCV, tentatively named as CaCV-AIT.

Natural variation in *Pepino mosaic virus*

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In 1999 *Pepino mosaic virus* (PepMV), a member of the potexvirus genus, was diagnosed in Dutch glasshouse tomatoes. Since then the virus has established itself in Europe and many other countries worldwide. Comparison with the original PepMV, first and last described in 1980 from pepino plants (*Solunum muricatum*) from Peru, showed reproducible biological and molecular differences. These differences lead to the conclusion that the isolates from tomato should be considered a distinct strain of PepMV.
Most tomato isolates appear very similar both in biological and molecular properties. However differences in symptom development and severity on PepMV infected tomato plants in different European countries and different growing conditions have been reported. This has led to some controversy over the phytosanitary status of the virus.
Since 1999 the virus has become widespread in the Netherlands as in some other European countries. This is partly due to the easy spread through infected fruits that contain high concentrations of the mechanically transmitted virus. Generally the virus causes only mild symptoms under Dutch conditions. However, more serious symptoms on fruits and plants have been reported sporadically. To investigate the variability of the virus and possible correlations with presence or absence of symptoms a large number of isolates from different greenhouses were collected and investigated. Detailed comparisons between these isolates, the original tomato and pepino isolates and other deviant PepMV isolates will be discussed.

Poster presentations (Thursday 10th March 17.30 – 19.00 h)

Evidence for the occurrence of three nanovirus species infecting faba bean in Ethiopia

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When 299 symptomatic faba bean samples collected in Ethiopia in 2002 were serologically tested using broad-spectrum monoclonal antibodies (MAbs) to *Faba bean necrotic yellows virus* (FBNYV), 73 (24.4%) samples gave ELISA-positive reactions. Further serological analysis of the positive samples with eight discriminating MAbs revealed contrasting epitope profiles that were categorized into at least three groups designated serotypes A, B, and C. Serotype A appears to be prevalent throughout the country as it was detected in 62 (85%) of the 73 ELISA-positive samples whereas serotypes B and C were found only in few samples from southern Ethiopia. Serology and sequence analysis of the coat protein (CP) and U1 genes of serotype A, B and C isolates indicated that they might represent distinct nanovirus species. Serotype A was similar to the previously described, atypical 'FBNYV' isolate from Holetta, Ethiopia, for which the name Faba bean necrotic stunt virus (FBNSV) has been proposed. CP, M-Rep, U1 and U2 gene sequences of a serotype B isolate resembled those of typical isolates of FBNYV from Egypt and Syria, providing first evidence for the occurrence of FBNYV (*sensu strictu*) in Ethiopia. Serotype C isolates appeared to be serologically most distinct from typical FBNYV isolates. Therefore, all 8 genomic ssDNAs of a representative serotype-C isolate (Eth-231) were sequenced. The individual DNAs of Eth-231 ranged in size from 972 to 1002 nts and had only one major ORF potentially encoding proteins of 12 to 33 kDa. Eth-231 shared overall nucleotide and amino acid sequence identities of only ≤70% and ≤74%, respectively, with FBNYV, FBNSV, and other nanoviruses. Moreover, DNA-C of Eth-231 is very distinct in nucleotide sequence and encodes a Clink protein that lacks the typical LXCXE motif required for cell-cycle regulation. Our data strongly suggests that Eth-231 represents a new nanovirus species for which the name Faba bean yellow leaf virus is proposed.

Investigations on *Citrus tristeza virus* (CTV) and its occurrence in citrus orchards in arid and semi arid zones of Sudan

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Citrus tristeza virus (CTV) often causes quick decline and death, or stem pitting a, reduced vigour and longevity. Yields in susceptible varieties are shortened, hence CTV is considered as a serious threat to the citrus industry worldwide. In Sudan all citrus trees are grafted mainly on sour orange rootstock and this yields a CTV-susceptible combination with scions of sweet orange, mandarin, grapefruit and others. CTV is a serious problem because it is readily transmitted by infected budwood and is also spread by several species of aphids. Up to now, there was no serious work in the diagnosis of citrus viruses occurring in the Sudan by other methods than visual inspection. During the trials to detect CTV in the Sudan a survey was initiated in 2003 and 2004. Fresh leaf material was collected from CTV suspected trees accompanied by tissue printing on nitrocellulose membranes. CTV was detected successfully in thirteen printed samples using a mixture of specific monoclonal antibodies (3DF1 and 3CA5, Plant Print Diagnostics S.L.) originating mainly from orange trees but were collected from different orchards. In two cases also a mandarin and a lime tree respectively reacted positive in this serological assay. In a nested RT-PCR approach starting from RNA, extracted from fresh leaves, from ten samples a specific PCR product was amplified, substantiating the presence of CTV in four trees (three orange, one lime tree), which were presumably tested positive by tissue print. Cloning and sequencing of specific PCR products will authenticate the presence of CTV in Citrus trees in Sudanese orchards.

Differentiation of *Cucumber mosaic virus* isolates by hybridisation to oligonucleotides in a microarray format

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We have aimed for the development of a method necessary for diagnostic chips to detect and differentiate viral nucleic acids in a general way. The microarray system was developed with *Cucumber mosaic virus* (CMV) as model system which consists of serogroups and subgroups. The coat protein genes of 14 different isolates were amplified using Cy-3-labelled generic but species-specific primers. These amplicons were hybridised against a set of five different serotype and subgroup specific 24-mer oligonucleotides bound to an aldehyde-coated glass slide via an aminolinker. The results of the hybridisation revealed that the method allowed a clear differentiation of the 14 different CMV isolates into the serogroupes 1 and 2, and in addition was able to assign 9 out of 10 different serogroup 1 isolates correctly into subgroups 1a and 1b. This differentiation was not possible by RFLP analysis with the restriction enzyme MspI.
The use of amplicons larger than 700 base pairs and their successful differentiation by hybridisation to specific oligonucleotides opens avenues to highly parallel, yet sensitive assays for plant viruses, either after a general PCR amplification or even after in vitro labelling of total nucleic acid extracts.

Characterisation of *Cherry leaf roll virus* (CLRV) isolates from different host plants

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Cherry leaf roll virus (CLRV) is a widespread pathogen of woody plants in Germany and throughout the European community. It has been detected also in a limited number of herbaceous plants, e.g. rhubarb (*Rheum rhabarbarum* L.). CLRV has been reported to be of economical importance in European walnut plantations. In recent years it has been causing reduced yields in cherry trees in the United States, if occurring in mixed infections with *Prunus necrotic ringspot virus* or *Prune dwarf virus*. Sixty-three CLRV isolates from seventeen different host plant species were characterized on molecular and serological level. Phylogenetic analysis of a 280 bp fragment in the 3´ non-coding region of the viral genomic RNA revealed six different isolate groups corresponding to their original host plant species. Also coat protein sequences of CLRV strains were compared leading to similar groupings. Furthermore clustering of CLRV variants based on viral sequence parts, resembled arrangement, based upon serological reactivity of isolates using a set of polyclonal and monoclonal antibodies. Results suggest, that the significant variability of CLRV strains and their association with certain host plant species is due to the natural mode of transmission of the virus by pollen and seed which presumably limits efficient cross-species transmission, leading to rapid genetic isolation and adaptation of CLRV variants to particular host species.

Molecular characterization of *Vicia cryptic virus*

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The nucleotide sequences of the RNA-dependent RNA polymerase (RdRp) and the coat protein (CP) of *Vicia cryptic virus* (VCV) were determined from RT-PCR derived cDNA clones. For this purpose dsRNA was purified from *Vicia faba* cv. "The Sutton" leaves and a RT-PCR fragment of 227 bp was amplified using oligonucleotides according to a published sequence in the GenBank (Y09237) coding for a part of the VCV RdRp. After cloning and verification of this sequence ten additional cloning steps were performed to determine the missing sequence of the RdRp. A primer specific to the non coding region of the dsRNA of VCV RdRp together with random primers were used to obtain the non coding region of the dsRNA with the putative CP region. It was demonstrated that the 5'-ends of the genome are conserved as in many other viruses of the *Partitiviridae*. Seven additional cloning steps were performed to determine the whole CP nucleotide sequence. The 5'- and 3'-ends of the dsRNAs were determined by a modified RACE approach. Sequence analysis revealed one large open reading frame (ORF) for each dsRNA. The larger dsRNA comprises of ~2000 bp with an ORF (nt 93-1940) coding for a putative RdRp with 616 amino acids (aa). The smaller dsRNA consists of ~1800 bp with an ORF (nt 119-1579) encoding a putative CP with 487 aa. Both sequences show high homology on amino acid level to the RdRp and CP of *White clover cryptic virus* (WCCV), respectively. In addition, some conserved motifs observed in RdRps of some ssRNA and dsRNA viruses were also identified in the RdRps of VCV and WCCV.

A new potyvirus causing flower breaking in *Begonia semperflorens*

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Flower colour breaking was found in red and pink flowering cultivars of *Begonia semperflorens*. The leaves of plants with these flower symptoms sometimes showed a vague mottling. After testing plant material in ELISA for the presence of different viruses there was only a positive reaction with a general monoclonal directed against potyviruses. Examination of crude extracts of leaves and flower petals in the electron microscope revealed no virus particles. It was also not possible to transmit a virus mechanically to indicator plants. The extreme low pH of the plantsap (i.e. pH 2) possibly plays a negative role in these experiments. However, the virus could be transmitted mechanically to virus-free *B. semperflorens*. Molecular characterisation was more successful. Total RNA from an infected plant was subjected to a RT-PCR procedure using degenerate potyvirus-specific primers (Van der Vlugt *et al*, 1999). A 653 nt. cDNA-fragment covering the C-terminal part of the viral coat protein (CP) and the 3'non-translated region (3'NTR) was obtained and cloned. The sequence was compared with other viral sequences present in the NCBI database. The CP and 3'NTR nucleotide sequences showed a maximum of 73% and 46% homology with potyvirus sequences present in the database. This suggests that the begonia potyvirus represents a hitherto unidentified potyvirus. For this new potyvirus found in *B. semperflorens* the name Begonia flower break virus is proposed.

High frequency silencing of multiple targets using a single inverted repeat construct

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The use of RNA silencing has become the tool of choice for gene knock-downs in plants and many other organisms. The discovery that double-stranded RNA (dsRNA) is a very good trigger for RNA silencing is the key element in this technology. By arranging transgenes as inverted repeats (IRs), which result in the production of dsRNAs after transcription, it is possible to obtain almost complete repression of the expression of homologous RNAs. So far, due to the high sequence specificity of RNA silencing, this technology was limited to the targeting of single genes only. We here show a new tool that enables the knock-down of at least four genes using a single, relatively small

transgene. We demonstrate this by producing plants expressing a minimal-sized IR cassette containing the sequences of four related tospoviruses. The expression of these cassettes rendered these plants immune against infection with all four viruses. This work shows that by combining a large number of small parts of genes high frequency multiple knock-down constructs can easily be made.

Construction and agroinfection of a *Potato virus M* full-length clone

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The aim of this study was the construction of a *Potato virus M* (PVM; German isolate) infectious full-length clone, which can be used in future to evaluate PVM resistance. Naturally the Carlavirus PVM infects *Solanum tuberosum*. The positive-single-stranded RNA genome of about 8500 nts is encapsidated by coat protein subunits, which form filamentous particles. Total RNA was extracted from PVM infected *Nicotiana hesperis* plants. Synthesis of cDNA with viral specific primers and subsequent PCR revealed two DNA fragments of about 5500 bp and 3000 bp, representing the 5'-end and the 3'- end of the PVM genome, respectively. In a second step, these fragments were cloned in succession into a plasmid, containing an enhanced 35S *Cauliflower mosaic virus* (CaMV) promoter and the CaMV termination signal. Briefly, the larger fragment was cloned downstream of the 35S CaMV promoter, followed by introduction of the smaller fragment. The sequence of the entire PVM German isolate was determined from the full-length clone and compared with sequences of PVM isolates from Russia, Poland and China. The entire cassette consisting of the 35S promoter, the PVM genomic sequence and the termination signal was subcloned into the modified binary vector (pBIN19SN) and electroporated into *Agrobacterium tumefaciens.* Particle bombardment and agroinfection was performed with the PVM full-length clone in *N. hesperis*. In addition agroinfection was done in *Nicotiana benthamiana* and *Lycopersicum esculentum* cv. Lizzy plants to test the infectivity. Plants inoculated by particle bombardment revealed neither virus symptoms nor PVM. However, all test plant species were successfully agroinfected. Three weeks after inoculation virus symptoms were observed in *N. hesperis* and PVM particles were detected by electron microscopy. In addition PVM coat protein was shown by ELISA as well as by Western-Blot and PVM-RNA was amplified by Reverse –Transcriptase – PCR.

Characterisation of proteins involved in translocation of the geminivirus *Watermelon chlorotic stunt virus*, WmCSV, in its vector *Bemisia tabaci* (Genn.)

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Begomoviruses rely on *Bemisia tabaci* vector insects for horizontal transmission. For this process, the virus passages the vector by crossing the midgut and the salivary glands (PSG & ASG) representing epithelial barriers for vector translocation. At each barrier, the transcytosis (endocytosis/exocytosis) event is believed to be based on a receptor-mediated mechanism where vector receptors interact with virions. To investigate the biochemistry of whitefly vector / begomovirus interaction leading to successful virus transmission, proteins extracted from *B. tabaci* insects were reacted with purified WmCSV protein preparations. Upon SDS-PAGE of total *B. tabaci* protein extracts followed by virus overlay assay (far western analysis), five polypeptide species, 63, 51.3, 29.5, 26.3, and 25.7 kDa, were identified specifically reacting with purified WmCSV. None of these proteins reacted with preparations of a purified tombusvirus or with antibodies against the 65 kDa GroEl protein, a protein produced by insect endosymbiotic bacteria often found in *B. tabaci* and also implicated in virus transmission. Similar proteins to those from *B. tabaci*, were also identified in non vector insects (*Trialeurodes vaporariorum* and *Aphis craciphora*), however, the 29.5 and 26.3 kDa protein reactions were only found in *B. tabaci*. Using differential protein purification protocols, soluble and membrane bound whitefly proteins were purified, separated by 2D electrophoresis and analysed in far western assays. Protein spots of the insoluble protein fractions separated by 2D electrophoresis and characterised by a specific reaction in far western analysis with WmCSV virion preparations, were further subjected to in-gel trypsin digestion and analysed by mass spectrometry and MS-MS mass measurement in Q-TOF. Results of the

preliminary protein analysis will be presented.

Biological and molecular characteristics of different *Cherry leaf roll virus* (CLRV) isolates

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Cherry leaf roll virus (CLRV) is a pathogen spread throughout the world. Its hosts are mostly woody plants like cherry (*Prunus avium*), walnut (*Juglans regia*) and elderberry (*Sambucus spec.*) but also some herbaceous plants like rhubarb (*Rheum rhabarbarum L.*) can be infected. As CLRV is being spread mainly through seed or pollen, the natural transmission of the virus is presumably restricted to one host plant species in most cases. CLRV is taxonomically classified within the Family *Comoviridae*, Genus *Nepovirus*, Subgroup C due to its non-enveloped, icosahedral shaped virions which are 28 nm in diameter and its bipartite genome organisation of linear positive-sense ssRNA with a 1,5 kb long non-coding region at the 3'-end of RNA2. Ten isolates of CLRV from different host plants were analyzed by serological and molecular methods. For DAS-ELISA and IC-RT-PCR a polyclonal antiserum against purified CLRV particles of an isolate derived from black elderberry was raised. Using this polyclonal antiserum not all tested CLRV isolates were detectable, confirming the serological divergence of CLRV strains from different woody hosts. Purified virus preparations were analyzed by SDS-PAGE revealing no significant differences in coat protein size. Viral nucleic acids were separated by Agarose-gel-electrophoresis. In native RNA gels some of the CLRV isolates showed slight variations in length of both viral genomic RNAs. To evaluate the genome size of CLRV strains some isolates were separated under denaturing conditions and compared with a RNA standard marker. The RNA1 of CLRV isolates are approximately 8,2 kb long whereas the RNA2 are between 6,7-6.9 kb in length.

Large-scale application of real-time RT-PCR for testing *Potato spindle tuber viroid* in potato

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Recent outbreaks of *Potato spindle tuber viroid* (PSTVd) in potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) in different European countries have highlighted the need for a reliable method appropriate for large-scale testing for this viroid. Currently, methods for the detection of PSTVd can be rather laborious, difficult to reproduce and/or can give false results. The real-time RT-PCR protocol developed by Boonham et al. (Journal of Virological Methods 116, 139-146, 2004) performed extremely well in a recent ring test, comparing different PSTVd detection methods in several laboratories. In addition, real-time PCR technology has proved suitable for high-throughput testing. Therefore, this method was chosen as the starting point for the development of a protocol for the large-scale testing of potato. The initial experiments focused on the specificity of the primers and probes with regard to different isolates of PSTVd and other (pospi-) viroids. Further experiments performed with leaf material concerned sampling position, growing-on temperature and bulking rate. In addition, different grinding and nucleic-acid extraction methods were compared. To monitor false negatives and positives, different controls were included. The final protocol was tested using a hundred samples from the Dutch potato monitoring programme. Future plans include the development of a protocol for direct tuber testing and inter-laboratory ring testing of the protocols.

Multiple infections of commercial Poinsettia

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Commercial Poinsettia (Euphorbia pulcherrima Willd.) are frequently infected by three agents, a beneficial phytoplasm and two viruses which were formerly named Poinsettia mosaic virus (PnMV) and Poinsettia cryptic virus, and classified as tymo- and cryptic virus, respectively. The sequence analysis of both viruses requires a revision of the taxonomy now which may have important implications for quarantine measures in greenhouse propagation of Poinsettia. PnMV is more closely related to marafiviruses than to tymoviruses. The second virus (now named Poinsettia Latent virus, PnLV) showed an unprecedented hybrid genome structure combining the first two thirds of a polerovirus - providing functions for replication - and one third of a sobemovirus coding for the coat protein. The extreme 5' and 3' ends which harbour the putative origins of replication resemble those of poleroviruses. Whereas marafiviruses are transmitted by leafhoppers, sobemoviruses may be spread via soil and watering, a route which would explain the frequent re-occurence of the Poinsettia viruses in plant material that had been tested virus-free before. Under these perspectives much more care should be taken in greenhouses to prevent mechanical dissemination than with the assumption of a cryptic virus. Since PnMV frequently re-occurred together with PnLV - even in quarantine facilities – it may be interesting to search for a genetic dependence of both viruses in future.

TMV coat protein: Expression, *in vivo* self assembly and mutagenesis

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The bi-directional self-assembly of *Tobacco mosaic virus* (TMV) has been studied extensively *in vitr*o. The ability of TMV to assemble *in vitro* and, more important, *in vivo* provides an attractive tool to protect transcripts for a multiplicity of applications. In addition, the resulting virus-derived tubes are used for the production of novel composite materials in nanotechnology. We were able to obtain TMV coat protein expression in *E. coli* and, for the first time, in a eukaryotic yeast-based system. TMV components expressed either in bacteria or in *S. pombe* were able to assemble *in vivo* within the respective cells, even without the presence of the TMV origin of assembly, to give particles heterogeneous in length. Whether these assemblies comprise foreign RNA or not is so far unknown and still has to be determined. Furthermore, site-directed mutagenesis resulted in two types of differently modified TMV rods. Histidine-coated particles should selectively direct the binding of metal ions to their outer surface, while a lateral coat protein mutant (E50Q) exhibits enhanced inter-subunit binding stability in order to produce extremely stable rods, even in the absence of RNA. The latter was able to accumulate to high amounts in plants and produced a distinct phenotype. The histidine-coated particles generated from the mutant C'6xHis were successfully produced *in planta,* too, but behaved abnormally.

Investigation of development of infection by soil-borne viruses in cereals

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Epidemiological investigation has been carried out on soil-borne cereal viruses. Particularly, the impact of environmental factors on the infection progress of the two furoviruses *Soil-borne cereal mosaic virus* (SBCMV) and *Soil-borne wheat mosaic virus* (SBWMV) and the bymovirus *Wheat spindle streak mosaic virus* (WSSMV), all transmitted by fungal vector *Polymyxa graminis*, were studied.
In the case of SBCMV and WSSMV the roots of susceptible accessions were already naturally infected two months after sowing. Starting from this time point the virus spreads into the shoots. Only after another two months the virus can be detected in leaves. It was demonstrated that the infection rate depends on the efficiency of virus transmission by the virus-vector population, the

sowing data, temperature conditions as well as crop species. Rye became earlier infected than wheat and triticale if sown at the same time. Visual inspection of the field revealed that rye plants showed clear symptoms already early in February whereas wheat and triticale plants developed symptoms later in March. Both furoviruses tolerate a broad temperature spectrum under field conditions and once established infection by these viruses is detectable until the harvest time. In contract to this, the propagation of the bymovirus WSSMV seems to be restricted to lower temperatures. Consequently, this virus is detected best at the end of February until beginning of April.
A survey in the cereal growing regions of Saxony-Anhalt and Lower Saxony revealed that there only SBCMV and WSSMV are spread. There rye cultivars appear to be more heavily infected than different cultivars of wheat and triticale.

Dutch-German cooperations in evaluating species demarcation criteria for tombus- and tymoviruses

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The VIIIth Report of the International Committee on Taxonomy of Viruses (ICTV) lists biological, serological and molecular properties as species demarcation criteria for tombus- and tymoviruses as well as for many other plant virus genera. In attempts to characterise and classify newly detected tombus- and tymoviruses we have compared the usefulness of the various species demarcation criteria for these viruses. Five new tombusvirus isolates all from the same natural host, i.e. commercially grown statice (*Limonium sinuatum*) from various parts of the world, were more or less indistinguishable in limited host range studies, but by means of immunoelectron microscopy they were readily distinguished and four of them could be assigned to various known tombusviruses. Coat protein sequence comparisons indicated that none of them was exactly identical to one of the previously described viruses. - In the genus tymovirus total nucleotide and coat protein amino acid sequence identities revealed similar groupings as earlier serological studies. The latter, however, tended to suggest much closer relationships than the molecular data and may fail to recognise a new tymovirus as being distinct. Thus, a new tymovirus (provisionally named Nemesia ring necrosis virus) which is widely spread in commercially grown genera in the Scrophulariaceae and Verbenaceae was serologically barely distinguishable from the earlier described *Scrophularia mottle virus.* Molecular studies, however, revealed only rather distant relationships to other tymoviruses. Occasional failures of serology to recognize a new tymo- or tombusvirus as being distinct have been observed also by others. For recognising the distinctiveness of new tymo- or tombusviruses molecular criteria are thus more reliable than host range and serological studies. More or less arbitrarily set borderlines between 'new viruses' and 'strains', e.g. on the basis of percentages of sequence identities, have to be defined for individual virus genera by the respective ICTV study groups.

Nuclei instabilities correlated with active rolling circle replication (RCR) and recombination dependent replication (RDR) of Abutilon mosaic virus

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Geminiviruses replicate their single-stranded DNA genomes through double-stranded DNA intermediates in plant nuclei using the host replication machinery. It has been known for a long time that geminiviruses multiply using a rolling circle mechanism (RCR). In addition, Abutilon mosaic virus (AbMV) as well as other begomoviruses and curtoviruses replicate via a recombination-dependent replication mode (RDR). Nuclei were isolated from AbMV A and B agro-infected Nicotiana benthamiana plants by use of a discontinuous percoll step gradient centrifugation. Every top to bottom fractions were examined by DAPI fluorescence and light microscopy, showing that most of the intact nuclei were present in the 60% percoll fraction. The majority of RCR and RDR intermediates, as analyzed by two-dimensional gel electrophoresis and hybridization, however, were observed in fractions of broken nuclei. No RCR intermediates could be detected in the

fraction of intact nuclei. Similarly, BV1 protein, a movement protein encoded by AbMV B was mainly accumulated in the fraction of broken nuclei, as detected by SDS-PAGE and western blotting, whereas the coat protein (AV1) was found in all nuclear fractions. It seems that nuclei instabilities increased upon viral replication and transport, but not during packaging of DNA into virions. These findings are important prerequisites to set up an in vitro system for run-off replication studies.

The nucleoprotein gene of *Tomato spotted wilt virus* as a tag protein fusion for easy purification and enhanced production of recombinant protein in plants

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Upon infection, *Tomato spotted wilt virus* (TSWV) forms ribonucleoprotein particles (RNPs) that consist of nucleoprotein (N) and viral RNA. These aggregates result from the homopolimerization of the N protein, and are highly stable in plant cells. These properties feature the N protein as a potentially useful novel plant-based protein fusion system. To evaluate this potential, we tested whether the N protein and N protein fusions could be efficiently produced in plants and purified as aggregates, outside the context of viral infection. To this end the N gene was fused to GFP (Green fluorescent protein), either at the amino or carboxy terminus, in a binary vector or in a Potato virus X (PVX) vector. *Nicotiana benthamiana* leaves were infiltrated by *Agrobacterium tumefaciens* transient assay (ATTA) or inoculated with a PVX viral vector. For both expression methods N and N-GFP fusion could be detected by Western blot using antisera against N or GFP. Infiltrated leaves and infected plants expressing N-GFP fusions showed intense fluorescence under UV light. For purification, a standard method used for viral RNPs was used, consisting of two low speed centrifugation steps and one ultracentrifugation on a sucrose cushion. The presence of N and N-GFP fusions after this purification was confirmed by Western blot. Purified N-GFP retained its fluorescence, indicating no major changes in protein structure due to the (mild) purification process. These results show that the homopolimerization properties of the N protein can be used as a fast and simple way to purify large amount of proteins from plants. In further research, the capacity of TSWV N will be tested as an epitope presentation system. As TSWV N assembles in pseudo-RNP structures, it might not face some of the size and conformation constrains of some of the currently available epitope presentation systems.

Changes in the spectrum of PVY strain groups and their involvement in potato and tobacco diseases

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Potato virus Y (PVY) is a member of the *Potyviridae*, a large and economically important plant virus family. The three major strain groups of PVY are PVY^O, PVY^C and PVY^N, differentiated by symptoms on tobacco and potato plants. In addition, there have been two new subgroups of PVY^N, PVY^{NTN} and PVY^NW, first described in 1984 and 1991, respectively. PVY^{NTN} causes necrotic rings on the surface of potato tubers whereas PVY^NW resembles PVY^N in symptomatology on tobacco but has serologically been assigned to strain group PVY^O. During the last 10 years these new PVY^N subgroups have become the prevalent PVY strains in Germany. While in 1997 potatoes were predominantly infected by PVY^O (survey in Bavaria), six years later most of the PVY infections were caused by PVY^N (survey in Germany). More than 90 % of PVY^N infections in 2003 belonged to the PVY^{NTN} subgroup. Results of bioassays also indicated that there has probably been a sharp increase in the proportion of PVY^NW among PVY^O infections, although this could not have been confirmed by molecular-genetic assessments, yet. The increasing importance of the subgroups NTN and NW may be a reason for the decline of PVY resistance of potato varieties since the time period of 1988-93. Based on a susceptibility rating system ranging from grade 1 (no susceptibility) to 9 (very high susceptibility), PVY susceptibility increased on average by two grades per variety during the last 11 to 16 years. Although field resistance of some potato varieties against PVY has also been overcome during recent years, extreme resistance to PVY has been durable so far. On

the contrary, tobacco varieties carrying the va resistance gene have become increasingly infected by PVY in recent years. The PVY isolates that caused the infections on resistant tobacco varieties were shown to belong to the subgroups NTN and NW.

Identification and characterization of Tospoviruses from Iran

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During a survey conducted in two provinces of Iran 5 samples of virus-diseased plant material were collected for further analysis. In all cases large thrips infestations were observed in the outstanding crop. Four samples were collected in Teheran province from tomato, chrysanthemum, gazania and potato, respectively, and one in Gorgan province from soybean. Greenhouse experiments with several indicator plants revealed symptoms typical for tospovirus-infections, and in a few cases tospovirus particles were observed from leaf dip preparations. In subsequent DAS-ELISA experiments none of the samples reacted with TSWV, TCSV, GRSV, INSV, IYSV and WSMV antisera indicating that potential new tospoviruses were involved. Vice versa did none of the known tospoviruses cross react with an antiserum prepared against the Tomato isolate. To further characterize the Iranian tospoviruses, the corresponding nucleocapsid (N) genes were RT-PCR amplified and cloned for nucleotide sequence determination. Alignment of the amino acid sequence of the N protein of all 5 isolates revealed the presence of two tospoviruses with almost 10 % sequence divergence. The samples from tomato, chrysanthemum and gazania were infected with one tospovirus, and soybean and potate with the other. Both tospoviruses could not only be distinguished based on their N protein sequence, but also showed differences in symptomatology on several experimental host plants. For the Tomato isolate the entire S RNA nucleotide sequence has been determined: it contains 3061 nucleotides and shows features characteristic for a tospoviral S RNA. Multiple sequence alignment of the N protein from the tomato isolate with those of other established tospovirus species revealed the highest homology between the Tomato isolate and IYSV (74% identity), indicating that the Tomato isolate should be proposed as a new tospovirus species. Based on the symptoms on tomato fruit the name *Tomato yellow ring virus* (TYRV) is proposed.

Detection and identification of a novel potexvirus infecting allium by paramagnetic beads ssRNA isolation and one tube RT-PCR assay with a new potexvirus genus primer set

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A rapid paramagnetic capture/reverse transcription PCR assay procedure was developed for the detection of viruses from the genus *Potexvirus*.
The Potexvirus ssRNA was selectively isolated from plants sap by streptoavidine coated paramagnetic beads coupled to a biotine labelled *Potexvirus* genus sequence specific primer/probe and then analysed in a one tube RT-PCR assay. An extraction buffer containing cell wall degrading enzymes was used in order to improve the viral detection in bulbs. The RT-PCR assay was performed using a new *Potexvirus* genus specific primer set.
Upstream primer Potex 4 and downstream primer Potex 5 were developed on the sequence of conserved viral replicase–encoding regions.
Ornamental *Allium* plants showing diffuse yellow stripes or slight mottle were investigated for virus infection. ELISA and PCR assay showed the presence of a complex of different viruses consisting of *Potyviruses*, *Tobacco rattle virus*, *Tobacco necrosis virus* and a novel (unknown) *Potexvirus*. Sequence analysis of cloned PCR amplicon obtained with the primers Potex 4 – Potex 5, showed an 83% identity with *Clover yellow mosaic virus*.
Testing the method on primarily infected plants and dormant bulbs of different *Allium* cultivars has verified the robustness for application in the routine detection of potexvirus infections. Detection, performed in microtiter plates, was rapid, specific, contamination free, reproducible and semi-automated.

Characterization and nucleotide sequence of *Grapevine leafroll associated virus*-7 (GLRaV-7)

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Leafroll is a widespread disease of grapevine causing yield losses of economical importance. Filamentous and phloem-restricted viruses of the family *Closteroviridae* are associated with the disease. Up to now 9 serological distinct viruses are described. They are referred to as *Grapevine leafroll associated virus* -1 to -9 (GLRaV-1 to -9) which appear as single or mixed infections in grapevine. The available partial or complete determined nucleotide sequences confirm the serological characterization so far.
GLRaV-7 was isolated as double stranded RNA (dsRNA) from dormant wood of a grapevine of Albanian origin. This isolate referred to as "AA42" displayed a band pattern in gel electrophoresis as known for GLRaV-1 and -3. The first GLRaV-7 specific cDNA clones have been obtained by DOP-PCR. Subsequent cloning was carried out by RT-PCR. Different approaches have been attempted for the determination of the 5'- and 3'- terminal sequences. RACE-PCR with poly(A)-tailing of dsRNA and subsequent RT and PCR with Oligod(T)+anchor and anchor primer, respectively, or RACE-PCR with RT first, subsequent poly(A)-tailing and two polymerase chain reactions with Oligod(T)+anchor and anchor primer, respectively, were carried out. Another attempt with circularization of the viral genome to enable RT and PCR over the 3'/5'-end was abandoned due to no positive results of the control PCR (check if the circularization took place). 14.363 base pairs of the "AA42" nucleotide sequence have been determined so far. Genomic analysis revealed a genome organization as typical for closteroviruses. The putative translation products of the determined ORF sequences were compared to other proteins in the database and showed similarity with translation products of *Little cherry-virus* -1 (LChV-1).
Multiple sequence alignment with "Neighbor-Joining-Tree"-generation based on the amino acid sequence of the HSP70 protein confirmed the close relationship between LChV-1 and GLRaV-7, both unassigned members of the family *Closteroviridae*.
For accurate characterization of the GLRaV-7 genome terminal sequences have to be completed.

The spread of *Rice yellow mottle virus* in irrigated rice crops

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Rice yellow mottle virus (RYMV), a sobemovirus, is endemic in Africa, south of the Sahara, and occurs mainly in irrigated rice ecosystems. Infected plants give yellow or orange discoloured rice leaves, a delay in plant growth and reduced tillering. These symptoms result in lower yields, but early infected plants give the highest losses as they fail to set fruit. RYMV is primarily known as a beetle-transmitted virus. A scattered distribution of infected plants and the occurrence of small infected spots might be the result of RYMV spread by beetles. However, various infection patterns can with difficulty be explained by beetle transmission. Small infected spots occur next to field-wide spots (20 m or more in diameter). Completely infected crops are found next to completely healthy crops. Infected plants can be found on untided-land along roadsides, levees and at corners. Severe infections can occur in parcels in which cows have been housed for the night in the contra-season. These observations stimulated us to search for other mechanism by which RYMV could spread in the field. Transplantation of seedbeds with a limited infection resulted in a drastic increase of infected plants in the field. This number increased again sharply 3 to 4 weeks after transplantation due to wind-mediated leaf contact and root-released virus from infected plants as shown in field and greenhouse experiments. Weeding, application of fertilizers may also enhance the number of infected plants. Some infections are caused by cows and donkeys occasionally fouraging on rice, and by grass rats gnawing plant on tided land. Mowing an infected crop, and fouraging stubble fields enhance the inoculum which can infect the next seedbed by virus released from infected plants when plowed down and other mechanisms such as beetles and rats. Some severe seeded infections can occur when frequented by cows. The spread and epidemiology of RYMV as deduced from our results will diagrammatically be presented.

Identification of viruses in ornamental *Allium* species and control strategies

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In *Allium giganteum*, the most important ornamental onion grown in the Netherlands, a virus disease occurs showing symptoms of light green to yellow stripes on the leaves, a smaller inflorescence and/or a twisted flower stem. In earlier research the disease was associated at first with 'Onion mosaic' virus (Onion yellow dwarf virus?) and afterwards with increased concentrations of Shallot latent virus (SLV). From 15 stocks grown in different areas, plants with and without symptoms were tested in ELISA for the presence of SLV, Leek yellow stripe virus (LYSV) and potyviruses. SLV was present in all plants tested. In all plants showing symptoms of stripe mosaic a potyvirus was detected which was absent in symptomless plants. The amino acid sequence of the (partial) coat protein of this virus showed less than c. 60% homology with related potyviruses, among which potyviruses known to occur in *Allium* species. As name for this new potyvirus we propose Ornamental onion stripe mosaic virus (OOSMV). In plants with severe stripe mosaic, often in combination with necrotic streaks, LYSV was present as third virus in addition to SLV and OOSMV. OOSMV was also found to be associated with (stripe) mosaic in other ornamental onions (4 out of 7 species and 4 out of 15 cultivars tested). Probably, resistance to OOSMV occurs in some species (e.g. *A. jesdianum* and *A. hirtifolium*) and hybrids, which offers perspectives for resistance breeding. In stocks containing less than c. 6% OOSMV, the virus can be controlled by rogueing. In some cases pyrethroid sprays will be helpful in virus control.

Molecular and biological characterization of *Beet mild yellowing virus* and determination of the host plant spectrum by agroinfection

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The *Beta vulgaris* infecting Polerovirus *Beet mild yellowing virus* (BMYV) is one causal agent of the yellowing disease complex in sugar beets. Like all members of the family *Luteoviridae* BMYV is primarily limited to the vascular tissues of its hosts and transmitted by aphids in a persistent manner. The total nucleotide sequence of a German Polerovirus isolate (BMYV-IPP) was determined and used to include BMYV in the phylogenetic system. For molecular and biological characterisations a BMYV full-length cDNA clone ($BMYV_{fl}$) was constructed. Briefly, four cDNA fragments were combined to a full-length clone under control of an enhanced CaMV 35S-promoter and a ribozyme. The full-length clone was subcloned into the modified binary vector pBIN19 and after electroporation into *Agrobacterium tumefaciens* strains ATHV and LBA4404 plants were agroinfected. $BMYV_{fl}$ systemic infections were successfully established by agroinfection in *B. vulgaris*, *Nicotiana benthamiana*, *N. clevelandii*, *Capsella bursa-pastoris* and *Lamium purpureum*. Additionally it was shown that BMYV is replicating in epidermal and mesophyll cells of agroinfiltrated leaves to a high extend. Agroinfection did not lead to an extension of the BMYV host plant spectrum compared to that previously described in the literature and mainly determined by aphid transmission of BMYV. Immunological investigation of agroinoculated plant tissues of *N.tabacum* cv. 'Xanthi', *N. occidentalis*, *N. rustica* and *Chenopodium capitatum* revealed local virus infections in infiltrated mesophyll cells. However, in these plant species no systemic BMYV infection could be detected. Moreover in some plant species like *N. glutinosa* or *N. edwardsonii* neither infected plant tissue in agroinfiltrated leaf areas nor a systemic BMYV spread was detectable. Provided that $BMYV_{fl}$ reaches the phloem tissue in all tested plant species the method of $BMYV_{fl}$-agroinfection is a easy to use procedure to determine local and systemic infectable, local but not systemic infectable or non-host plants of BMYV.

Epidemiological developments in *Potato virus Y*

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Potato virus Y (PVY) is still responsible for important losses in ware potato production and seed potato quality. In the Netherlands a control system is in place that is based on monitoring virus infections in the field and flight data of a selected group of aphids. From these data foliage destruction dates are determined in order to prevent tuber infection. Of the three PVY strains, PVY^O (old), PVY^C (common) and PVY^N (new), PVY^N is generally considered to predominate. However, recent research revealed that PVY^O and PVY^C are more present than generally assumed. Based on earlier research the green peach aphid (*Myzus persicae)* is considered the most efficient vector of PVY. As determined from trap data, the population of *M. persicae* appears to decrease in the Netherlands, however, problems with (primary) PVY infections persist. No data are available on the relative efficiency of PVY transmission of different field isolates (clones) of M. persicae or the role of other aphid species.
To investigate whether efficiencies of different clones of the major vector of PVY are of the same order or not, potato fields were sampled for *M. persicae* and strains of PVY. Transmission experiments were carried out with the wild and the laboratory clones of *M. persicae* from potato to *Physalis floridana* with two virus strains: PVY^N and PVY^O.
A great variability was observed between the aphid clones in transmission efficiency. For example: two clones were not able at all to transmit PVY^O, whereas one clone infected *P. floridana* with PVY^N for 100%. These results clearly indicate a relationship between the origins of *M. persicae* and its capability to transmit *Potato virus Y*.

Expression analysis of potyviral 6K1 in *Nicotiana benthamiana*

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Plum pox virus (PPV) belongs to the genus *Potyvirus* within the family *Potyviridae*. The viral genome is of positive polarity and contains one large open reading frame (ORF). Translation of the ORF results into a polyprotein of approximately 355 kDa. Cleavage of the polyprotein into mature viral proteins has been shown by *in vitro* cleavage assays or the detection of proteins *in vivo*. One of the products of proteolytic processing – the 6K1 polypeptide – has to our knowledge not yet been identified *in vivo*. In this study we report the detection of the potyviral 6K1 as a polypeptide of 6 kDa in virus-infected plants. For the detection of 6K1, a polyclonal antibody was produced which recognized *E. coli* expressed PPV-6K1 specifically. However, the antibody did not react with extracts of PPV-infected *Nicotiana benthamiana*. Therefore, a mutant of PPV was constructed (PPV-His6K1) by insertion of 7xHis residues into the N-terminal part of 6K1 facilitating Ni-affinity purification of expressed 6K1. The infectivity of PPV-His6K1 was tested on *N. benthamiana* and coat protein (CP) levels were measured by western blot analysis. Plants infected with PPV-His6K1 expressed similar levels of CP and showed symptoms comparable to PPV-infected ones. For the detection of 6K1 *in vivo, N. benthamiana* were infected with mutant PPV-His6K1 or wild-type PPV as negative control. Thirteen days post inoculation, extracts from infected plants were subjected to affinity chromatography. With the 6K1-antiserum, a protein of ~6 kDa was detected in purified extracts of PPV-His6K1-infected *N. benthamiana* but not in PPV-infected ones. The result was confirmed using an antibody directed against multiple His residues. The identified polypeptide of ~6 kDa suggests that the potyviral 6K1 is efficiently processed from the polyprotein *in vivo*. Nevertheless, the role of the mature 6K1-polypeptide during viral life cycle still remains to be investigated.

Alfalfa mosaic virus detected in true seed of _Solanum kurtzianum_ during post-entry quarantine testing

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Each year about sixty *Solanum* seed accessions from the Dutch-German gene-bank are tested for the presence of quarantine viruses as part of the annual seed reproduction programme. This testing is obliged by the legislation of the European Union (EU) for all *Solanum* species entering the EU. This legislation also applies to previously imported untested true-seed accessions present in EU gene-collections. In spring 2003 for one accession of *Solanum kurtzianum* virus symptoms appeared upon mechanical inoculation of test plants, indicating an infection by *Alfalfa mosaic virus* (AMV). The presence of this virus was confirmed by DAS-ELISA using AMV-specific antibodies. Testing of the remaining twenty-three plants of this accession revealed no further infections, thus demonstrating that the rate of seed transmission was low. The infected plant was destroyed, although AMV is not listed as a quarantine organism in the EU. However, since true potato seed is used in the initial stages of potato production, there is a high risk that viruses may become widely spread. Finally, this finding emphasizes the importance of test plants as a tool for post-entry quarantine testing, since this method enables the detection of viruses apart from those under specific testing.

Investigating the mechanism(s) of cross-protection between strains of _Cucumber mosaic virus_

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The type species of the genus *Cucumovirus*, *Cucumber mosaic virus* (CMV), infects more than 1,000 plant species, including many economically important cucurbit crops. Direct control of CMV infection or control of its aphid vectors in the field has been difficult or impossible to achieve. An alternative control strategy may be the use of cross-protection, the phenomenon in which mild or attenuated strains of viruses protect plants against infection with severe strains of the same virus. It is thought that in most cases post-transcriptional gene silencing (PTGS) is the mechanism behind cross-protection. We have used an engineered mutant of Fny-CMV lacking the *2b* gene, a suppressor of gene-silencing (Fny-CMVΔ2b) in cross-protection assays. This mutant successfully cross-protected against the parental wild-type strain Fny-CMV in tobacco and *N. benthamiana*. Using RT-PCR to distinguish between both strains, no Fny-CMV could be detected in systemic leaves in most cases of cross-protected plants. However, in one experiment the presence of Fny-CMV could be detected in systemic leaves arguing against the hypothesis that Fny-CMVΔ2b induces gene-silencing and hence the degradation of viral RNA from the parental strain. Currently, work is underway to look for the presence of small interfering RNAs, markers for the induction of PTGS, in cross-protected tobacco plants.
m species entering the EU. This legislation also applies to previously imported untested true-seed accessions present in EU gene-collections. In spring 2003 for one accession of *Solanum kurtzianum* virus symptoms appeared upon mechanical inoculation of test plants, indicating an infection by *Alfalfa mosaic virus* (AMV). The presence of this virus was confirmed by DAS-ELISA using AMV-specific antibodies. Testing of the remaining twenty-three plants of this accession revealed no further infections, thus demonstrating that the rate of seed transmission was low. The infected plant was destroyed, although AMV is not listed as a quarantine organism in the EU. However, since true potato seed is used in the initial stages of potato production, there is a high risk that viruses may become widely spread. Finally, this finding emphasizes the importance of test plants as a tool for post-entry quarantine testing, since this method enables the detection of viruses apart from those under specific testing.

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