Instituto Juan March
de Estudios e Investigaciones

74 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Plant Viroids and Viroid-Like Satellite RNAs from Plants, Animals and Fungi

Organized by

R. Flores and H. L. Sänger

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INTRODUCTION

H. L. Sänger and R. Flores

Twenty-five years after their discovery, viroids are still the only class of autonomously replicating subviral pathogens whose molecular structure is well defined. The sequences of 27 viroid "species" and of more than altogether 100 sequence variants thereof have been established. They show that viroids are composed by a coat protein-free single-stranded circular RNA chain in the size range between 245-399 bases. In some cases various aberrant forms, generated by sequence deletions and duplications, have been also described. The discovery of viroids has led to the modification of the paradigm established around the turn between the XIX and XX centuries considering viruses as the smallest known biological entities. In fact, viroids are currently the lowest step of the biological scale: they are molecular parasites at the frontier of life.

Viroids were discovered as a consequence of studies aimed at characterizing the agents of some plant diseases initially thought to be induced by viruses. Later on, they have been identified as the etiologic agents of a series of maladies affecting crops of economic importance: potato, tomato, citrus, hop, coconut, grapevine, several subtropical and temperate fruit trees as avocado, peach, apple, pear and plum, and ornamental plants as chrysanthemum and coleus.

Differences between viruses and viroids are not restricted to size but include also other structural and functional aspects. Viroids are circular RNAs with a high degree of secondary structure as a result of about 70% intramolecular self-complementarity. They adopt in most, but not in all cases, *in vitro* rod-like or quasi-rod-like conformations stabilized by elements of secondary and tertiary structure. From a more functional perspective all the available data support the contention that, in contrast to viruses, viroids do not code for any peptide or protein. Therefore, viroids must be replicated by a pre-existing host RNA polimerase(s), and they must elicit their pathogenic effects by direct interaction of their RNA with one or more cellular targets. Viroids can be regarded as parasites of the transcription machinery, whereas viruses are essentially parasites of the translation machinery.

Viroid-like satellite RNAs from plants share some structural properties with viroids: they have a similar size and they exist *in vivo* as circular and linear molecules. However, viroid-like satellite RNAs are not endowed with autonomous replication, and they are functionally dependent on helper viruses by whose coat protein they are encapsidated. Outside the plant kingdom there is so far only one member of this group: the human hepatitis delta agent, a satellite of hepatitis B which is responsible for a fulminant form of hepatitis. The RNA of hepatitis delta virus has also a circular structure and folds into a rod-like conformation. Viroid and viroid-like satellite RNAs also share a common strategy of replication. They follow a rolling circle mechanism with either a symmetric or asymmetric variant, and three main steps: RNA transcription, processing and ligation catalyzed by enzymes with activity of RNA polymerase, RNase and RNA ligase, respectively. One remarkable aspect in this regard is that in some viroid and viroid-like RNAs the RNase activity is, instead of a enzyme, a ribozyme (see below).

More recently three viroid-like RNAs from animal, plant and fungal origin, respectively, have been discovered with a very peculiar property: they have a DNA counterpart. These observations clearly suggest the involvement of a reverse transcriptase activity in the generation of the corresponding DNA forms and, in fact, two of the aforementioned viroid-like RNAs have been found associated with two systems, a plasmid and a virus, encoding reverse transcriptases.

From an evolutionary perspective, viroid and viroid-like RNAs are also very intriguing systems. Their small size, circular structure and, especially, the frequent presence in their strands of ribozyme activities are suggestive indications that they could be molecular fossils of the RNA world which presumably existed on the Earth before the advent of cellular life.

One last point that deserves particular attention, because it has implications far beyond the limits of the specific research on viroid and viroid-like RNAs, is that these small RNAs have been the sources for most of the known synthetic ribozymes. The hammerhead- and hairpin- (or paperclip-) ribozymes have been found in three viroids and in all viroid-like satellite RNAs from plants, in which they catalyze in *cis* the processing of the oligomeric intermediates of the rolling-circle mechanism of replication. Due to their structural simplicity, these two ribozymes are being intensively manipulated to act in *trans* against target RNAs, like those of retroviruses, in what it is one of the most innovative and promising approaches in Biotechnology. Very recently, the first crystal structure of a ribozyme has been elucidated. The analysis of its hammerhead structure has provided insights into the mechanism by which the ribozyme may destabilize a substrate strand in order to facilitate the twisting that ultimately allows cleavage of the scissile bond. Other ribozymes derived from viroid-like RNAs of animal and fungal origin, those from hepatitis delta virus RNA and from the *Neurospora* VS RNA respectively, are also the subject of intensive research.

This Workshop brought together scientist actively engaged in working on viroid and viroid-like RNAs from plants, animals and fungi using molecular approaches. The Workshop provided a unique opportunity for the participants to exchange and share information, to review and discuss new approaches, and to propose directions for future research.

Heinz L. Sänger
Ricardo Flores

Session 1: Structural domains and elements of higher-order structure. RNA rearrangements

Chairperson: Robert H. Symons

RNA-recombination between viroids

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Sequence comparison between different viroids has revealed that some of them exhibit a mosaic of different sequence motifs each of which is characteristic for another viroid "species". This has led to the concept that during viroid evolution RNA recombination between different viroids must have occurred. Direct evidence that this is still taking place today comes from the unique viroid system present in Coleus blumei cultivars. It consists of three GC-rich new Coleus blumei viroids which are named CbVd 1, CbVd 2 and CbVd 3, and whose prototypes are 250, 302 and 362 nucleotides long, respectively. They all contain an identical central region (CR) but its sequence differs from the CRs of all other presently known viroids. This justifies to put the three Coleus viroids into the newly established CbVd group. Except for their CR, CbVd 1 and CbVd 3 are largely unrelated. CbVd 1 exhibits 52% sequence similarity (SS) with hop latent viroid (HLV), and CbVd3 shows 57% SS with grapevine viroid 1B (GVd 1B). CbVd 2, however, is a recombinant viroid that arises by fusion of the right-hand part and the CR of CbVd 1 with the left-hand part of CbVd 3. This new chimeric CbVd 2 exhibits two features not found in viroids heretofore: (i) it is composed of two virtually unchanged parental sequences and, (ii) their two joining points are characterized by a sharply defined boundary.

The dynamics within the "CbVd family" as reflected in the distribution pattern of the three viroids in individual plants was studied over a period of four years. For this purpose their presence in two sets of 50 plants propagated by cuttings from a single mother plant, respectively was analyzed. At the beginning CbVd 1 was present in all plants, about 90% of them contained CbVd 3, 20% harboured CbVd 2, and in 13% of the plants only CbVd 1 and CbVd 2 were found. This pattern changed during the following years of culture in that finally still 100% of the plants contained CbVd 1 and 65% carried the recombinant CbVd 2. But in 50% of the latter plants CbVd 3 had been evidently outcompeted by the novel CbVd 2 offspring. Thus the Coleus viroid system is a naturally occuring kind of missing link which supports the repeatedly proposed concept that RNA recombination has been an important factor in viroid evolution. The possible molecular mechanisms of viroid RNA recombination will be discussed.

Viroid Structures Involved in Transcription, Processing and Pathogenesis

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Replication of the PSTVd-class follows the asymmetric rolling circle mechanism. The (+)strand circular viroid is transcribed by the cellular DNA dependent RNA polymerase II into an oligomeric linear (-)RNA-strand, which is the template for an oligomeric linear (+)RNA-strand. The (+)strand is cleaved to unit length molecules, which are ligated to next generation (+)strand circles. Two specific start sites for the first transcription step were identified, when circular PSTVd was added to a nuclear extract from a non-infected potato cell culture. Both start sites are involved in a specific secondary structure including a GC-rich box. The (-)strand oligomeric RNA forms GC-rich hairpin structures which are essential for replication as reported earlier (1,2). The presence of GC-rich hairpins in plant crude extracts could be confirmed by structure specific oligonucleotide probes. The (+)strand oligomer forms a highly branched structure in the central conserved region which serves as a recognition signal for specific cleavage and ligation, which process is driven by a switch from a tetraloop-motif to a loop E motif (3). A model for an interrelationship of replicability and pathogenicity of different viroid strains was proposed earlier although the primary target of the pathogenic action was not known (4). More recently it was suggested that bending of the pathogenicity determining region in viroids might be the strain discriminating structural feature (5). Such bending could be confirmed experimentally by gel electrophoretic methods, supporting a model of binding a dsRNA-dependent kinase (6). Under *in vivo* conditions, specific crosslinks could be induced, which were formed between viroid and host RNA. In summary, specific viroid mechanisms in transcription, processing and pathogenesis are intimately related to specific viroid RNA stucture of stable and metastable forms of viroids.

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ALTERATION IN BIOLOGICAL ACTIVITY BY HOST SELECTION AND ENGINEERED REARRANGEMENTS OF VIROID GENOMES

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In advance of the description of viroids as an usual class of RNA molecules, the relationship to plant disease was recognized and the unique properties of these agents suspected. The absence of translation of the viroid genome remains a key characteristic of this class of pathogens restricted to plant systems. Thus, the conformation of viroid structures must comprise a deciding factor affecting biological activity. Demonstrated interactions of non-denatured viroid molecules in hybridization reactions (Francis et al., 1995) has expanded the range of potential biologically-active structures beyond the model of minimum free energy rod-like forms.

An interesting collection of 10 viroids have been identified from citrus which include members of the PSTVd, HSVd, and ASSVd families organized into five Groups. Molecular modifications found in citrus exocortis viroid (**CEVd**) from alternate hosts or produced in citrus cachexia viroid (**CCaVd**) by laboratory manipulation result in alterations in the biological activity. Correlation of pathogenicity with specific molecular structures provides an insight into the viroid genome governing the expression of exocortis and cachexia diseases as well as the phylogenetic relationship among citrus viroids.

Long duration infections of CEVd in a hybrid tomato (*Lycopersicon esculentum* X *L. peruvianum*) results in the recovery of two viroid-related species enlarged by either 92 (**CEVd-D92**) or 104 (**CEVd-D104**) nucleotides by repeated sequences in the V and T2 domains. The sequences of the two variants are essentially identical except for the addition of two hexanucleotide strings at the site of initiation of the terminal repeat of CEVd-D104. The -AGCU- tetrad found in the T2 loop of all CEVd variants also marks the site of initiation of the terminal repeated sequences and is repeated in one of the CEVd-D104 hexanucleotides.

Both variants are independently transmissible in the hybrid tomato while CEVd-D92 induces only a very mild petiole necrosis in *Gynura aurantiaca*. Neither variant could be demonstrated to replicate in citron (*Citrus medica*), however, a variant of Citrus viroid IV (**CVd-IV**) was recovered after inoculation with CEVd-D92. Sequences in the V and T2 domains of CVd-IV have homology to CEVd and are very similar to the terminal repeats found in CEVd-D92 and -D104.

Homology and structural properties of a computer derived CVd-IV-like sequence from CEVd suggests a possible relationship between CEVd and CVd-IV.

The Citrus Viroid II Group reflect the highly conserved sequence homology of 94% characterized by all members of the Hop Stunt family. Two group II citrus viroids, CVd-IIa and CVd-IIb, while displaying an even greater homology of 97%, are distinguished by the ability of CVd-IIb to induce cachexia disease. Thus, the genome locus controlling disease expression must be specified by a nominal number of nucleotide exchanges from the CVd-IIa sequence. This system has been exploited with the construction of chimeric viroids between CVd-IIa and CVd-IIb as well as site-directed mutagenesis of CVd-IIa to focus on the critical sequences governing biological activity. Structural variations introduced by these manipulations will be discussed.

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Native RNA of the Delta Hepatitis Agent Contains an Idiosyncratic Element of Local Tertiary Structure.

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Like other circular subviral RNA pathogens, the delta hepatitis agent replicates via a rolling circle pathway, generating precursor RNAs which must be cleaved (1,2). In the case of delta RNA, cleavage is mediated by ribozymes (3,4). However, mature genomic RNA does not contain a self-cleavage structure, rather native delta RNA assumes a rodlike conformation (5) in which some of the nucleotides which would otherwise build the genomic ribozyme instead form part of a highly unusual element of local tertiary structure (6). We have explored the evolutionary, biological, and physical properties of this unique structure.

This element occurs in the most highly conserved region of delta RNA. It is one of the features which suggests that the delta genome contains two domains: a conserved "viroid-like" domain (with signals and structures for replication) and a protein-coding domain. The local tertiary structure was originally detected because it is susceptible to UV-induced crosslinking. Evidently, its sugar phosphate backbone is deformed, resulting in a structure which is photoreactive. Interestingly, such deformations are a general feature of protein binding sites in RNA (7). In fact, the delta RNA structural element binds the double-stranded RNA dependent protein kinase (PKR) of mammalian cells in vitro (8) and may bind this, and other cellular proteins, in vivo. In some cases, protein binding may induce a conformational shift, causing the inert rodlike structure to open into an alternative conformation that is competent for replication.

Following UV-crosslinking, the tertiary element is extremely resistant to nuclease digestion. When transcripts containing the UV-crosslink are treated with RNase T1 under conditions which lead to the compete digestion of conventional RNA, the tertiary element survives and can be isolated. RNase T1 fails to cleave at 16 G residues within this structure (9). This nuclease resistance suggests that the element has a high degree of thermal stability and thus that it may be a molecular clamp (as well as a protein binding site). Its melting characteristics were directly tested and found to be

quite surprising: dissociation in 100 mM NaCl/0.5 mM $MgCl_2$ occurred at a *lower* temperature than dissociation in 0.5 mM $MgCl_2$ alone (10). The thermal stability of this element is highly sensitive to the balance between divalent and monovalent cations, suggesting that its formation and disappearance may be influenced by the distribution of positive charges in its surrounding environment.

Since delta replication requires an intact circular template, its survival may depend upon the presence of structures which control the timing of ribozyme formation. The local tertiary element and its flanking helices function in vitro as a ribozyme control element (10) and are likely to play a similar role in vivo (11). Its unusual physical properties suggest that the UV-sensitive element of local tertiary structure in delta RNA may be a molecular clamp with a built-in release mechanism.

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Session 2: Replication: enzymes and ribozymes involved. Editing

Chairperson: Michael M.C. Lai

RNA self-cleavage in coconut cadang cadang viroid: Potential for a role in rolling circle replication.

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Of the 27 viroids characterized so far, three of these constitute the avocado sunblotch (ASBV) group of viroids and both their plus and minus RNAs self-cleave via the hammerhead structure *in vitro*. It is generally assumed that the hammerhead self-cleavage reaction is functional also *in vivo* in its predicted role in the specific processing steps involved in rolling circle replication. Supporting evidence has been provided for ASBV (Daros et al., 1994) as have mutagenesis experiments involving the infectivity of cDNA clones of the 324 nt viroid-like satellite RNA or virusoid of lucerne transient streak virus (Sheldon and Symons, 1993).

In general, limited attempts to find self-cleavage reactions in members of the PSTV-group of viroids have been unsuccessful, with emphasis on potato spindle tuber viroid (PSTV) and citrus exocortis viroid (CEV) within the PSTV-subgroup. An initial report of self-cleavage occurring *in vitro* in a dimeric RNA transcript of PSTV between resides 250-270 in the bottom strand of the central C domain of PSTV (Robertson et al., 1985) was not followed up. In a less direct *in vivo* approach, mutagenesis studies on infectious longer-than-unit-length RNA transcripts of CEV identified a potential site in the upper strand of the C domain which corresponded to G96-G98 of the PSTV sequence (Visvader et al., 1985). However, a more extensive analysis of the approach used indicated that infectivity relied on the ability of monomeric CEV transcripts to form short double-stranded regions of viroid and vector sequences at the junction of the two termini and not to any specific site within the CEV molecule (Rakowski and Symons, 1994).

In view of the hammerhead self-cleavage reaction in the three members of the ASBV-group of viroids, it seems reasonable to predict that RNA-catalysed processing reactions will also be present in the PSTV-group viroids. A conserved feature of members of the PSTV-, ASSV-, and CbV-subgroups is the presence of inverted repeat sequences in both the top and bottom strands of the C domain which allow the potential for two hairpin loops to form. There is also something about this region which is unusual as indicated by the specific cross-linking in PSTV of G98 to U260 on irradiation of the purified viroid with UV-light (Branch et al., 1985). Obviously, the central conserved region in the C domain of these viroids is highly prospective for an RNA catalysed processing site.

In the search for such a processing site, we chose to work with coconut cadang cadang viroid (CCCV) because it is the smallest member of the PSTV-subgroup. The approach has been to prepare cDNA clones to the sequences in the C domain; thus one clone contained the top strand of the C domain connected through a small loop to the bottom and the other was in the reverse orientation. We then explored a wide range of denaturation and reannealing conditions using RNA transcripts

prepared from these clones as well as various divalent and polyvalent cations with analysis for self-cleavage by polyacrylamide gel electrophoresis.

The overall approach was based on our earlier experience with characterizing the hammerhead self-cleavage reaction in vLTSV where success was based on heating and snap cooling the RNA and assembling the reaction mixture on ice before incubation at a higher temperature. No self-cleavage occurred if the solution was brought to room temperature before the addition of Mg^{++} (Forster et al., 1987; Forster and Symons, 1987 a, b). Our conclusion was that there is a range of conformations in such a population of RNA molecules and that only some, if any, may be in a conformation capable of undergoing self-cleavage.

One set of specific conditions for CCCV has identified a reproducible self-cleavage site (about 5% self-cleavage) on the left hand side of the bottom hairpin loop. The site is within a conserved region for members of the PSTV-subgroup and must be considered as a potential *in vivo* processing site. Proving that this site is where processing occurs *in vivo* obviously represents a major challenge.

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Satellite tobacco ringspot virus RNA transiently expressed from a geminivirus-derived vector for analysis of circle formation from extensively-deleted satellite RNA mutants

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A small satellite RNA (sTRSV RNA) is associated with tobacco ringspot virus (TRSV). The most abundant sTRSV RNA is a "monomeric" 359 nucleotide residue linear molecule designated here L-(+)M. The demonstration of circular and of linear, repetitive sequence oligomeric sTRSV RNAs of both polarities in infected tissue, and of self-cleavage by both polarities of sTRSV RNA, supports a symmetrical rolling circle transcription scheme for sTRSV RNA replication. The first, and apparently TRSV-independent, step of this replication scheme is the ligation of L-(+)M to the corresponding circular form, C-(+)M. We prepared an African cassava mosaic geminivirus (ACMV) DNA A plasmid vector with an insert of subdimeric sTRSV cDNA that encodes two sTRSV RNA self-cleavage sites. When such plasmids were introduced into *Nicotiana tabacum* (Xanthi) protoplasts or into *N. benthamiana* plants, L-(+)M accumulated, suggesting that self-cleavage of ACMC transcripts occurred *in vivo*. The unit length wildtype sTRSV RNA sequence between the two self-cleavage sites was replaced by a set of deletion mutants, all of which retained the sequences required for self-cleavage. For the wildtype and most of the mutant constructions, not only L-(+)M but also C-(+)M accumulated. Deletions in three regions of the L-(+)M sequence reduced C-(+)M accumulation substantially. We matched deletions to elements of computer-predicted secondary structure for the wildtype L-(+)M sequence and correlated such elements with reduced or retained C-(+)M accumulation in order to suggest wildtype secondary structure elements that might be deleted without interfering with C-(+)M formation. Predicted secondary structure elements were deleted in stages. The most extensively deleted molecule has 7 and 3 residues, respectively, replacing nucleotide residues 53-211 and 268-350. Each of the two deleted sequence regions was predicted to form a set of three adjacent imperfect stem loops in the wildtype L-(+)M. The extensively-deleted mutant accumulated a greater proportion of C-(+)M in protoplasts than was observed for the wildtype sequence. The secondary structure element best correlated with wildtype or greater levels of C-(+)M accumulation is a helix of the wildtype molecule that incorporates the 3' terminal sequence. A trinucleotide in the 3' region was mutated so as to disrupt and restore, respectively, the calculated helix, which resulted in reducing and restoring, respectively, C-(+)M formation. These results suggest that the 3' stem contributes to the suitability of the small L-(+)M molecules as a substrate for a protoplast RNA ligase and that computed folding of sTRSV RNA may be predictive of sTRSV RNA structure *in vivo*.

Replication and cell-to-cell movement of potato spindle tuber viroid

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Potato spindle tuber (PSTVd) and related viroids appear to replicate via an asymmetric rolling circle mechanism, but the possible role of circular complementary (-)PSTVd RNA as template for synthesis of (+)strand progeny is unclear. Replicative intermediates isolated from infected tissues appear to contain only multimeric linear (-)PSTVd RNAs (1,2), but previous attempts to initiate infection with multimeric (-)RNA transcripts have generally been unsuccessful (3,4). To critically examine the infectivity of (-)viroid RNA, we have developed a ribozyme-based expression system for the production of precisely-full-length (-)PSTVd or (-)tomato planta macho viroid (TPMVd) RNAs that can be readily circularized *in vitro*. Three features of this double ribozyme expression strategy -- the absence of RNA polymerase promoter sequences in the opposite strand of the DNA template, the inability of sequences complementary to the ribozymes to undergo self-cleavage, and the presence of only a 2-nucleotide duplication in the (+)strand sequence -- make the presence of an infectious (+)strand RNA contaminant extremely unlikely. Mechanical inoculation of tomato seedlings with electrophoretically purified (-)PSTVd RNA led to a low rate of infection; parallel assays with the analogous (-)TPMVd RNA resulted in much higher rates of infection. Ribozyme-mediated production of (-)PSTVd RNA in transgenic *Nicotiana benthamiana* plants led to the accumulation of detectable levels of monomeric circular (-)PSTVd RNA and the appearance of large amounts (+)PSTVd progeny, but no circularized (-)PSTVd RNA could be detected in transgenic plants expressing the corresponding (+)PSTVd RNA. Although not a component of the normal replicative pathway, precisely-full-length (-)PSTVd RNA contains all the structural and regulatory elements necessary for initiation of viroid replication.

The ability of viroids to leave the inoculated cell, initially moving from cell to cell and eventually entering the vascular system, is essential to the establishment of a systemic infection. To investigate the mechanism of viroid cell-to-cell transport, we have monitored the movement of

linear PSTVd RNAs after microinjection into different tomato and tobacco cell types (5). Infectious RNA transcripts were synthesized *in vitro* from a longer-than-unit-length PSTVd cDNA clone, labeled with the nucleotide-specific fluorescent dye TOTO-1, and used for microinjection (6-8). When injected into symplasmically isolated guard cells of mature tomato and tobacco leaves, PSTVd RNA labeled with TOTO-1 remained in the injected cells; in contrast, PSTVd RNA injected into symplasmically connected mesophyll cells moved rapidly from cell to cell. A 1400-nucleotide RNA molecule containing only sequences derived from plasmid pSP64 was unable to move from injected tobacco mesophyll cells, but the addition of PSTVd sequences to this transcript allowed it to move from cell to cell. At the DNA level, cell-to-cell movement of plasmid DNA was also facilitated by the presence of a full-length PSTVd cDNA. We are currently attempting to identify specific sequence or structural motifs responsible for PSTVd movement from cell to cell via plasmodesmata.

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TRANSCRIPTION AND PROCESSING OF HEPATITIS DELTA VIRUS RNA

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The genome of hepatitis delta virus (HDV) is a small (1679 nt) single stranded RNA with a circular conformation and the ability to fold into an unbranched rodlike structure, with about 70% of the bases in Watson & Crick pairing. The transcription of this genomic RNA into RNA seems to use the host RNA polymerase II. This transcription leads to the accumulation not only of genomic RNA, but also an exact complementary RNA, the antigenome, and a polyadenylated mRNA species, which seems to be the template for the translation of the one HDV-encoded protein, the 195 a.a. delta antigen.

The 5'-end of the mRNA maps to a region very close to one end of the rodlike structure. Furthermore, this site is located in the middle of a 3-nt bulge region on the predicted RNA structure. To test the hypothesis that this predicted bulge region acts as site for the initiation of transcription we have used mutagenesis at and around this region. Early analysis of these data suggest that mutations at this bulge can have dramatic effects on the sites at which initiation seems to be occurring.

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Wu, Netter, Lazinski, and Taylor. 1997. Effects of nucleotide changes on the ability of hepatitis delta virus to transcribe, process, and accumulate unit-length, circular RNA. J. Virol. 71: 5408-5414.

Analysis of Artificial and Natural Populations of Mixed Circular and Linear RNAs Using a Novel Two-Dimensional Gel Electrophoresis System

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A novel two-dimensional gel electrophoresis system has been constructed where separation of circular and linear RNAs is achieved via differences in the concentration of the bis-acrylamide crosslinking agent in polyacryalmide gels between the first and second dimension. The ratio of bis-acrylamide to acrylamide increases between the first and second dimension causing the average pore size to decrease. When circular RNAs move from the first to the second dimensions they are selectively retarded causing them to appear as a second diagonal behind the first diagonal of linear RNAs. Both dimensions contain 7M urea to denature the RNA. As there is no difference in the buffer conditions between the first and second dimensions, the two gels can be poured sequentially before the first dimension is run. After the first dimension is run, the gel is removed from the apparatus, the gel is rotated, and then replaced in the apparatus so that the second dimension can be run. This allows the RNAs to remain denatured between the first and second dimension.

A mixed population of circular and linear RNAs of various sizes has been generated to act as size standards for the two-dimensional gel system. Capitalizing on earlier observations by Feldstein and Bruening [Nucleic Acids Res. 21, 1991-1998 (1993)] that small "mini-monomer" RNAs derived from this molecule and containing little more than covalently attached ribozyme and substrate cleavage products are able to efficiently circularize, we have constructed a series of self-circularizing RNAs of precisely-known size. Mixtures of linear and circular RNAs synthesized in vitro and containing 225-1132 nucleotides could be completely resolved using the two-dimensional gel electrophoresis system. Only a single DNA template is required to prepare each pair of circular and linear RNA markers.

Similar analyses of a complex mixture of coconut cadang-cadang viroid RNAs revealed several interesting features. Firstly, there is a relatively large amount of a previously undescribed "fast-slow" heterodimeric RNA species in infected palms. Secondly, there are coconut cadang-cadang viroid RNAs of intermediate size between the fast and slow forms of the viroid present in infected palms. The presence of these RNA species can be explained by evolution of the viroid RNAs as heterodimers, one half of the dimer is wildtype while the other half carries mutations. Once sufficient mutations have accumulated to give a functional viroid, it begins to replicate as a monomer. Lastly, several spots were seen on a two-dimensional gel of a viroid RNA sample which seem to show a species which migrated as a dimer circle in the first dimension and as monomer circles in the second dimension. This implies that the RNA underwent processing within the gel. Experiments to determine if coconut cadang-cadang viroid is capable of undergoing processing in the absence of added protein are underway.

Structure probing of the genomic HDV ribozyme and its 3'- truncates.

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A pseudoknot secondary structure model has been proposed for the HDV ribozyme and the model best fits the experimental data. The RNA folding pathway that leads to the formation of the tertiary structure as well as the interactions within the ribozyme core remain, however, still unknown. In this communication we analyzed the structure of progressively shortened 3'-truncates of the genomic HDV ribozyme. The folded oligomers may be considered as consecutive stages in the process of ribozyme folding.

We synthesized three RNA oligomers: 84-mer corresponding to the 3'-product of the ribozyme and two 3'-truncates: 73-mer and 43-mer. Their structures were probed with single- and double-strand specific nucleases S1, T1 and V1 as well as Pb^{2+} ions that are capable of monitoring RNA flexibility and specific metal ion binding sites.

The results indicate that the P1 and P4 helical segments persist in the ribozyme in the final folded form adopted in the truncated molecules. The formation of the P2 stem is not possible in the truncates, nevertheless, the region that corresponds to the P3-L3 domain shows an ordered structure. The structure is, however, different from that present in the full length molecule. Thus the region spanning the J4/2 junction and 3' terminus causes major rearrangement of the pre-formed P3-L3 secondary structure. We speculate that this region can direct the overall fold of the ribozyme during elongation of the viral RNA.

Session 3: Viroid-host cell interactions

Chairperson: Joseph S. Semancik

A dsRNA-Activated Protein Kinase (PKR) from Tomato Specifically Binds to the Left Half of the Potato Spindle Tuber Viroid

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A protein of ca. 68 kD isolated from tomato (*Lycopersicon esculentum*) has been identified as a plant equivalent of mammalian dsRNA-activated protein kinase (PKR) on the basis of its reaction with monoclonal and polyclonal antibodies specific to human PKR and its characteristic autophosphorylation in the presence of dsRNAs. *In vitro* RNA transcripts, equivalent to (+)-strand, monomeric, potato spindle tuber viroid (PSTVd) or Mexican papita viroid, specifically bind to the tomato enzyme. Experiments with partial PSTVd transcripts showed that binding occurs to the pathogenicity domain-containing left half of PSTVd (nt 1-87, 280-359), but not to the right half (nt 88-279). These results strengthen earlier indications that binding of viroids to host PKR may play an important role in viroid pathogenicity.

SILENCING OF THE WOUND- AND METHYL JASMONATE-INDUCED SYNTHESIS OF PROTEINASE INHIBITORS BY CEVd ACTIVATION OF PLANT DEFENSES

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Due to their sessile condition, plants are inevitably confronted during their life cycle with all sorts of physicochemical and biological agents. To cope with such a diversity of potential aggressions, and aside some specific host-pathogen relationships, plants have followed a reductionistic strategy both in channeling the transduction pathways for different stressing signals and also integrating a broad spectrum of defenses in a multicomponent and polyvalent system of response (1).

Plants have also evolved defense weapons against the attack of insects and herbivores among which, the accumulation of serine proteinases in tomato plants (inhibitor I and II) have been described (2). The signal tranduction pathway for these inhibitors has been proposed as starting by a specific phospholipolysis of the wounded plasma membrane which activates the octadecanoid signalling pathway that leads to the elevation of the level of jasmonic acid (JA) which finally promotes gene expression (3).

Consistent with the idea of convergence of different signalling pathways, Farmer and Ryan (3) had proposed that not only mechanical wounding but pathogens would promote the synthesis of proteinase inhibitors, by damaging the plasma membrane and releasing to the cytosol linoleic and linolenic acid, through specific phospholipolysis, thus activating the JA pathway. Well in contrast to this hypothesis, but consistent with a possible interaction between pathways, is the finding that SA, a signal for activation of plant defenses against viruses and microbial pathogens, inhibits the synthesis of proteinase inhibitors (4, 5). Related to this contention, results obtained in our laboratory indicated that citrus exocortis viroid (CEVd) infection activates the release of these unsaturated fatty acids from the plasma membrane in tomato plants. Also, that the level of SA was considerably enhanced during the infection and the synthesis of a number of defense-related proteins in the host plant, promoted (1). All this prompted us to use the viroid-host interaction as a model system suitable to study interrelations between the pathogenic and the wounding pathways. Now, we present data indicating the prevalence of the pathogenic signal, which blocks the wound response in tomato plants, not only in the case of viroid infection but in two additional infections: those produced by tomato mosaic virus (ToMV) and *Pseudomonas syringae*. The inhibition of the wounding pathway is exerted at the transcription level in two different points (upstream and downstream) the synthesis of JA. We also present results supporting the contention that ethylene and SA are mediators of this inhibition. These results constitute the first biological evidence that there is cross-talk between pathogen- and wound-induced resistance.

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Molecular response to potato spindle tuber viroid infection in sensitive and tolerant tomato cultivars

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Potato spindle tuber viroid (PSTVd) infection causes severe disease symptoms as stunting, leaf malformation and epinasty in the sensitive cultivar "Rutgers" of tomato (*Lycopersicon esculentum* Mill.), whereas the tolerant tomato cv "Goldkugel" shows a practically symptomless response. To unravel the molecular basis of viroid-host interactions in these two cultivars, we applied various experimental strategies including analysis of temporal and spatial accumulation of PSTVd by tissue print and RNA blot hybridization [1], PCR based subtractive hybridization of uninfected and PSTVd-infected tomato cDNA libraries [2], and isolation of PSTVd binding proteins from a tomato cDNA expression library [3].

Temporal and spatial accumulation of PSTVd was slightly different in both cultivars in that the tolerant cv Goldkugel showed a delayed PSTVd accumulation after mechanical inoculation, although the final level of accumulation was comparable in sensitive and tolerant plants [1]. The association of viroid replication with the nuclear transcription machinery [4] prompted us to analyze the effect of PSTVd infection on gene expression in tomato leaves. Library subtraction allowed the isolation of cDNA clones that indicate an altered mRNA accumulation in infected plants [2] The corresponding genes include PR proteins (cathepsin D proteinase inhibitor [5], ß-glucosidase, lipase), ribosomal protein S17, histone variant H3.3 and proteins of the photosynthetic apparatus. Comparison of sensitive and tolerant tomato cultivars revealed a highly complex pattern of the influence of PSTVd infection on mRNA accumulation of these genes. However, our previous assumption that in the tolerant cultivar less genes would be involved in the plant response to viroid infection as compared to the sensitive one, was clearly disproved, indicating that tolerance to PSTVd infection is not simply due to lack or insensitivity of presumptive target compounds of the viroid molecule. Our present work is focussed on the analysis of temporal and spatial patterns of mRNA accumulation in sensitive and tolerant cultivars.

The putative interaction of PSTVd with host proteins was studied by screening a cDNA expression library from PSTVd infected "Rutgers" tomato plants with a digoxigenin labelled PSTVd probe [3]. Among the identified PSTVd binding proteins two ribosomal proteins were of particular interest, because they might be involved in

the nucleocytoplasmic transport of PSTVd RNA [6]. Characterization of protein genes from cv "Goldkugel" that are homologous to the viroid-binding proteins from cv "Rutgers" is currently under way.

Our studies revealed a rather complex pattern of viroid host interaction which involves the regulation of gene expression, nucleocytoplasmic RNA transport and a number of putative target proteins. However, our knowledge of the physiology of PSTVd infected plants is still incomplete, and a good deal of work has to be performed before a unifying concept of viroid pathogenesis can be developed.

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Identification and characterization of a host plant gene and its protein ,which interact with PSTVd RNA in *in vitro* and *in vivo* systems.

Rudolf Sägesser and Emilio Martinez, Elsa Maniataki, Martin Tabler and Mina Tsagris

At first view, viroids are simplistic RNA replicons since they are just composed of a naked single-stranded circular RNA. Despite the extensive knowledge of their structure and their functional domains, much less is known about the primary molecular interactions of viroid RNA with host factors in the plant cell. Except of earlier work, related to interaction of viroids with DNA dependent RNA polymerases and initial biochemical characterization of nuclear complexes containing viroid RNA (rev. in Flores et al. Semin. Virol. 8, 65, 1997), there is so far no sequence information about the host proteins or other cellular constituents, which interact with this unique RNA plant pathogen. We concentrated our efforts in isolation of host proteins, which interact strongly and with high specificity, with PSTVd RNA transcripts. For this purpose, we developed a direct screening method for RNA binding proteins from a cDNA expression library. A test system was established (Sägesser et al., Nucleic Acids Res. 25,3 816, 1997), based on a well known RNA-protein interaction, the interaction between U1 RNA and U1A protein of the human spliceosome. Several parameters of the original protocol (described for DNA binding proteins) were varied systematically, in order to obtain a reliable and specific method. Using this modified protocol, two cDNA libraries, from infected and from non-infected tomato leaves, were screened with an infectious, longer-than-unit-length PSTVd RNA transcript. Two new, unknown plant genes were identified, one of these, preliminary called X1, characterized in more detail. The gene product was found to interact (as tested by independent methods like northwestern assays and the three hybrid system), specifically with PSTVd RNA. We are currently analyzing the specificity of binding of this gene product for different viroids and viroid mutants, as well as its presence in the plant, using a polyclonal antibody. The possible function of X1 in viroid life cycle will be discussed.

RNA-directed DNA Methylation (RdDM) in Higher Plants: A Viroid-Host Cell Interaction that Might Induce Homology-Dependent Gene Silencing

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Previous work in our laboratory has shown that tobacco genome-intergrated PSTVd-specific cDNA becomes *de novo* methylated after autonomous viroid RNA-RNA replication has taken place in these plants [4]. The fact that methylation was only detectable in the viroid cDNA, indicated that homology between the directing RNA and the target sequence is required. If so, one can expect that all genomic sequences that are homologous to viroid RNA might also become methylated in infected plants. Recent studies on RNA-mediated post-transcriptional gene silencing (PTGS) revealed that most of the inactivated genes are *de novo* methylated. Although a DNA/DNA dependent mechanism was originally proposed [1], nowadays the observed methylation is suggested to be directed by the PTGS-inducing RNA [2, 3, 6]. It was found that >80% of homology over a strech of 60 to 130 nt between the 'silencing RNA' and the silenced gene was sufficient for the induction of PTGS. Assuming that the same degree of homology applies to RdDM, viroid RNA should also direct methylation of short genomic sequences. Taking into account that methylation can result in transcriptional gene silencing (TGS), symptom expression in viroid-infected plants might be a consequence of viroid RdDM-induced TGS.

To follow this hypothesis we are looking for the minimal sequence that can be methylated by the viroid RNA. For this purpose viroid cDNAs with a size of 30, 60, 100, and 160 bp have been integrated into the tobacco genome. Subsequently, viroid-infection was initiated in these plants by mechanical inoculation with PSTVd Nt [5]. The detection of a possible methylation pattern within the genome integrated viroid-specific cDNA fragments is currently in progress. In a second set of experiments we are analysing a possible spreading of DNA methylation. Genomic DNAs that were isolated from the previously studied tobacco plants containing methylated viroid-specific cDNAs [4] are treated with bisulfite leading to a conversion of all non-methylated cytosines to uracil. PCR products which had been amplified with primers that specifically bind to the viroid cDNA-flanking regions have now been cloned. Characterization of about 20 sequences revealed a heterologous distribution of 5-methylcytosine residues within different clones indicating a spreading of methylation. However, these results are preliminary and have to be verified.

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Small metastable mRNA specific degradation products and their possible role in *chalcone synthase* cosuppression in *Petunia* plants

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Transgenic *Petunia hybrida* plants wtih a *chalcone synthase A* (*chsA*) coding sequence under control of the *Cauliflower Mosaik Virus 35S* promoter and the *nopaline synthase* (*nos*) 3'UTR sometimes lose endogene and transgene chalcone synthase activity and purple flower pigmentation through posttranscriptional *chsA* RNA degradation. Because both types of *chsA* genes, the endogene and the transgene, are silenced simultaneously this phenomenon is called cosuppression.

We have recently shown, that shorter poly(A)$^+$ and poly(A)$^-$ *chsA* RNAs can be found in these plants and that a 3'end-specific RNA fragment from the endogene is more resistant to degradation. Computer predictions for putative secondary structures of this metastable, accumulating RNA fragment revealed that its termini are located in a region of complementarity within the mRNA between the *chsA* 3'coding region and the 3'untranslated region. Based on these observations we developed a model in which regions of complementarity in RNA molecules permit not only intramolecular but also intermolecular RNA-RNA structures from which an accelerated mRNA specific turnover results. In our model we suggest furthermore that some of the resulting metastable RNA degradation products can act as "effector molecules" in a positive autoregulative RNA degradation cycle by interfering with mRNA processing and turnover. Investigations are in progress to analyse if these "effector molecules" share characteristics with satellite- or viroid-like RNA structures and if they can act as a systemic signal in plant development.

At the DNA level we observed a particular change in the methylation pattern in the 3'region for which metastable RNA fragments were detected. An EcoRII site at the endogene 3'end, which is methylated in leaves but not in petals of wild-type petunias or of transgenic petunias with purple flowers, remains methylated in flowers of transgenic plants that show cosuppression. We are investigating if the above mentioned small RNAs can mediate the maintenance of methylation at this site and if this results in an epigenetically inheritable switch within the *chsA* coding region.

Session 4: Satellite-helper virus and viroid-viroid interactions

Chairperson: George Bruening

Nuclear import and initiation of replication of hepatitis delta virus and potato spindle tuber viroid RNAs.

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Both hepatitis delta virus (HDV) and potato spindle tuber viroid (PSTV) RNAs replicate in the nucleus of animal and plant cells, respectively. We investigated the mechanism of nuclear import of these RNAs. Using a fluorescin-labeled HDV RNA and partially permeabilized HeLa cells, we demonstrated that HDV RNA accumulated only in the cytoplasm. However, upon the addition of hepatitis delta antigen (HDAg), which is the only protein encoded by HDV RNA, the HDV RNA accumulated in the nuclei, indicating that the nuclear import of HDV RNA is mediated by the HDAg. This RNA-transporting activity of HDAg requires both the nuclear localization signal (NLS) and RNA-binding motif of HDAg. Furthermore, the NLS of HDAg interacts with nucleophilin α2, a nuclear importer. Since PSTV RNA does not encode any protein, we examined whether it can enter the nuclei directly. Surprisingly, by itself, it accumulated in the cytoplasm, but in the presence of HDAg, it entered the nuclei, suggesting that HDAg can serve as a nuclear importer for PSTV RNA. Other RNAs, e.g. mouse hepatitis virus RNA, could not be transported into nuclei, even in the presence of HDAg. Furthermore, PSTV RNA could not be transported by hnRNP A1 or polypyrimidine-tract-binding protein (PTB), both of which are nuclear proteins. Thus, HDAg and viroid-like RNAs (including HDV and PSTV) specifically interact with each other. This result also suggests that PSTV RNA interacts directly with the plant cell nuclear importers, since it is imported into the nuclei of plant cells.

Since HDV RNA is unique among viroids in that it encodes a protein, we further investigated whether the mechanism of synthesis of HDAg-encoding mRNA and RNA replication can be distinguished. By using an RNA transfection system, entirely avoiding a cDNA intermediate, we have identified the initiation points of HDAg-encoding mRNA and antigenomic RNA replication. These results reveal a novel mechanism of HDV RNA replication. This result is contrasted with viroid RNA replication.

Replication of barley yellow dwarf virus-RPV satellite RNA: *cis*-acting signals, helper viruses, and host range

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SatRPV RNA depends on RPV barley yellow dwarf luteovirus (BYDV-RPV) for replication and encapsidation[1]. It ameliorates BYDV-RPV-induced disease and reduces virus levels in oats[2]. The replicative intermediates and sequence, with hammerhead self-cleavage structures in both strands, suggest that it replicates via a symmetrical rolling circle mechanism[3]. The (+) strand hammerhead exists in an equilibrium with a slowly (or non-)cleaving conformation that involves base-pairing between stem-loops I and II of the hammerhead[4]. This can form a pseudoknot. To determine roles of these alternative structures in replication we constructed mutants that altered this equilibrium. Mutations that disrupted the base-pairing between stems I and II favored the hammerhead and increased self-cleavage, but decreased replication in oat protoplasts when present in the full-length satRNA sequence. Surprisingly, all mutants replicated to some extent. Double mutants that restored the base-pairing between hammerhead stems I and II reduced self-cleavage and restored efficient replication. To determine whether this basepairing or just slow cleavage *per se* is necessary for efficient replication, replication of a mutant that self-cleaves rapidly but still has potential stem I-II base-pairing is being tested. We propose that the stem I-II base-pairing provides a function other than self-cleavage, such as replicase recognition or ligation.

To identify the factors required for satRPV RNA replication (e.g. replicase) provided by the helper virus or the host, the ability of other viruses and hosts to support satRPV RNA replication was investigated. BYDV-PAV, which has the same host range as BYDV-RPV (grasses only) and related structural proteins but unrelated replicase and protease genes, did not support satRPV RNA replication[1]. In contrast, beet western yellows luteovirus (BWYV), which has more distantly related structural genes and a very different host range (dicots only) but replicase and protease genes that are closely related to those of BYDV-RPV, supported replication of satRPV RNA in *Nicotiana tabacum* protoplasts and shepherd's purse plants[5]. Unlike BYDV-RPV, which encapsidates linear satRPV RNA, BWYV preferentially encapsidated circular satRPV RNA[5]. This may be due to different efficiency of cleavage/ligation of satRPV RNA in the different hosts, or different structural preferences for RNA encapsidated by the viral coat proteins. It appears that the replicase, rather than the structural genes or plant host are the important determinants for ability to support satRPV RNA replication. Next, helper viruses with more distantly related replicases, or with portions of genomes deleted will be tested as helper viruses.

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CITRUS VIROIDS: VARIABILITY, DETECTION AND CONTROL

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Viroids are widespread in citrus hosts. Five distinct viroids and a number of variants have been reported: CEVd (pospiviroid), CVd-IV (cocadviroid), CBLVd and CVd-III (apascaviroids) and HSVd. These viroids are agents of economically important diseases of citrus but are commonly found as latent infections (1,2).

The viroid nucleotide sequence is the only information required for the characterization and classification of viroids (3,4). Although the nomenclature of viroids refers to the symptoms they induce as disease causing agents, the relationship between sequence and pathogenicity still remains unclear.

Plant pathologists need to address the issues of biological characterization (pathogenicity on agricultural crops) and the control of diseases. For the biological and agronomic characterization of citrus viroids, two facts have to be considered: a) citrus are usually infected with several viroids; b) each viroid is present as a population of sequence variants. Therefore, field isolates are not fully characterized unless information about the viroids present and their variability is available.

A number of interactions ranging from interference to synergism have been identified in multiple infections, and will be discussed. Using a set contiguous primers from the upper CCR we generated a DNA library from monomeric viroid RNAs of several field isolates from the five citrus viroids. The SSCP analysis of cloned viroid-DNA allows: a) to estimate the variability of specific field isolates; d) to identify the clones that best represent a given isolate; e) to identify unusual variants. Infectious clones have been constructed to pursue the biological characterization of specific viroid variants.

Using cucumber as a model host for pathogenesis studies, a 28 kDa protein has been identified in symptomless plants infected with CEVd or CVd-IV. This protein can also be induced in uninfected cucumbers by exogenous applications of salicylic acid. This is similar to the response of cucumbers infected with TNV in which the role of a lysozyme/chitinase activity has been identified (5,6). Studies have been conducted to elucidate the implication of viroids in systemic acquired resistance.

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Attempts to Team Citrus Viroids for Horticultural Purposes

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The graft transmissible dwarfing complex # 225T which is used for experimental dwarfing of grapefruit trees in Israel was found to contain at least five different citrus viroids (CVds) with a size range of 284 to 371 nts. Segregation and exclusion of one or more of these viroids was often observed in experimental plots and is presently considered as the main cause of inconsistent dwarfing of trees graft inoculated with a single source of inoculum. Molecular characterization of CVds from some of the locally used dwarfing complexes provided information on the genomic diversity of the citrus exocortis viroid (CEVd) and of the citrus bent leaf viroid isolates and eventually turned to be a useful tool for the tracing the possible source of an unexpected severe reaction of # 225T inoculated grapefruit trees grafted on Rangpur lime rootstock.

The lecture will describe the effects of individual CEVd and CBLVd isolates on the dwarfing of grapefruit trees grafted on Troyer citrange and discuss the main factors that prevented the wide scale commercial application of CVds in newly planted commercial citrus groves.

Other topics to be reviewed include:

(I) Induced tolerance to malsecco disease in CEVd infected Etrog and Rangpur seedlings (with *Z. Solel and N. Mogilner*).

(ii) The effect of CEVd on plant-water relations of Etrog citron seedlings and grapefruit trees grafted on Troyer citrange (with *S. Moreshet, S. Cohen and N. Mogilner*)

(iii) Attempts to map the CEVd sensitivity gene(s) in Tomato- the main problems and the challenge ahead.

(iv) A brief discussion on the natural history of CVds in the Mediterranean basin.

Direct evidence for the existence of non symptomatic strains of chrysanthemum chlorotic mottle viroid and sequence comparison with the reference severe strain

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The causal agent of the chrysanthemum chlorotic mottle disease is a new self-cleaving viroid (CChMVd) of 398-399 nucleotides, which can adopt hammerhead structures in its strands of both polarities and whose predicted secondary structure of lowest free energy is a highly branched conformation (1).

The existence of non symptomatic strains of the same agent (CChMVd-NS) in clones of some chrysanthemum cultivars was postulated previously on the basis of cross-protection bioassays: these strains do not produce recognizable symptoms but they do provide complete protection against the severe strain (CChMVd-S) (2). We have also observed that plants from a clone of the cultivar "Yellow Delaware" remain symptomless after inoculation with the reference CChMVd-S strain. Since this is the cultivar where the CChM disease was initially reported (3), we hypothesized that the plants were infected by a CChMVd-NS strain. To examine this possibility RNA preparations from the plants presumably infected by the CChMVd-NS strain were analyzed by Northern blot hybridization using an RNA probe specific for the CChMVd-S strain: a signal in the position corresponding to the CChMVd-S RNA was observed, providing in this way direct evidence for the existence of a CChMVd-NS RNA.

Several clones of the CChMVd-NS strain were obtained by RT-PCR using two independent pairs of adjacent primers of opposite polarities derived from two separate regions of the sequence of the CChMVd-S strain. Sequencing revealed that the CChMVd-NS variants have the same size, similar sequence, and adopt the same secondary structure of minimum free energy as the variants of the reference CChMVd-S strain (1). Most of the mutations present in CChMVd-NS variants have been previously identified in the CChMVd-S ones, but there are two changes only detected in most of the clones from the CChMVd-NS strain: i) the nucleotide located between the conserved residues CUGA and GA of the hammerhead ribozyme of both polarity strands changes from U to G, and ii) the four nucleotides forming loop 2 of the minus hammerhead ribozyme change from UUUC to AAAG or to AAA. Whether there is a cause-effect relationship between these changes and the pathogenic phenotype remains to be determined. It is possible that the CChMVd-NS strain could be a mixture of pathogenic and latent variants, with the latter being predominant and protecting against the former. Infectivity bioassays with *in vitro* CChMVd-NS transcripts of definite sequences may provide an answer to these questions.

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Session 5: Evolution. Viroid-like RNA systems with a DNA counterpart

Chairperson: Theodor O. Diener

Structure, function and evolution of the *Neurospora* VS ribozyme.

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Neurospora Varkud satellite (VS) RNA contains a ribozyme that is capable of *in vitro* self-cleavage. The cleavage products contain 5' hydroxyl and 2'3' cyclic phosphate termini like those produced by hammerhead, hairpin, and hepatitis delta virus ribozymes; however, the VS ribozyme differs from these others in sequence, secondary structure, and a variety of functional properties, including the ability to recognize and cleave *in trans* a substrate RNA consisting primarily of a hairpin loop.

To investigate the mechanistic basis for the distinctive properties of the VS ribozyme, we have been using multiple, complementary approaches to obtain information about its structure and function. Structure probing experiments have identified many bases and several backbone phosphates that are protected from chemical modification only in the presence of magnesium. Modification interference experiments have shown that many of these protected sites are also required for cleavage activity, suggesting that they may be involved in functionally important tertiary interactions. Four sites of direct metal binding have been identified by phosphorthioate intereference / manganese rescue experiments; modelling suggests that two of these could be near the cleavage site in the active tertiary conformation. Kinetic analyses of mutant ribozymes has identified several non-essential regions and an inhibitory structural element whose removal increases the cleavage rate by about 30-fold. UV crosslinking data combined with a previously-identified loop-loop pseudoknot interaction has allowed us to develop a working model for the relative orientation of all six of the helices in the VS ribozyme.

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Structural studies on the DNA of a plant retroviroid-like element reveal multiple fusions with the DNA of a plant pararetrovirus and the existence of forms with sequence deletions.

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CarSV RNA (Carnation Small Viroid-like RNA) and its homologous DNA, are the two forms of a first retroviroid-like system from plants. CarSV RNA is a 275-nt non-infectious viroid-like RNA, present in certain carnation plants, which contains hammerhead ribozymes in both polarity strands (1). On the other hand, CarSV DNA is organized as a series of head-to-tail multimers forming part of extrachromosomal elements. These elements contain, aside from CarSV DNA, sequences of CERV (Carnation Etched Ring Virus), a plant pararetrovirus of thecaulimovirus family (2), which makes sense since pararetroviruses do not integrate into the host genome. In this study we provide further insights on CarSV DNA as one of the two forms of the retroviroid-like system. Thus, multiple insertion sites of CarSV DNA with respect to the CERV genome have been characterized. The entire genome of the virus seems to be susceptible to integration with CarSV since we have found fusions between both DNAs interrupting all CERV ORFs along with some spacer regions as well. It is noteworthy that all the junctions show, as a common fingerprint, the presence of a short stretch of base pairs existing in both CarSV and CERV DNAs. This suggests recombination events of the copy-choice type mediated by a replicative enzyme with a low processivity, most likely the reverse transcriptase coded by the viral genome. This view is reinforced by the observation that, on the CarSV side, the common sequence of the junction coincides, in most cases, with very strong secondary structure motifs in the CarSV RNA or even with the ribozyme self-cleavage sites. On the other hand, a series of CarSV RNA forms with sequence deletions were previously found (3). Now, we have characterized the corresponding CarSV DNA forms together with a vast array of new DNA forms with sequence deletions with no correspondence among the CarSV RNA species identified to date. Again, very conspicuous secondary structure motifs in the RNA and/or the self-cleavage sites appear associated to the borders of the deleted sequences, which might explain the "jump" or "sliding" of the replicative enzyme. Most interestingly, some of these CarSV DNA forms with deletions, previously found at the RNA level, have been also detected in CarSV-CERV fusions. CarSV DNA is organized as multimers in a head-to-tail manner. Our finding argues that these "shortened" CarSV DNA versions are minor components of the CarSV multimers and replicate along with the complete monomers. The existence of a DNA counterpart may explain the persistence of these RNA forms with sequence deletions, unprecedented among the viroid-like RNAs.

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A Study on the Newt Hammerhead Ribozyme : Association to RNA-Binding Proteins and Intracellular Localisation.

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Hammerhead ribozymes, named after the secondary structure of their catalytic domain, have been identified in subviral plant pathogens, such as certain viroids and virusoids, as well as in the newt (Amphibia, Urodela) cells. The newt hammerhead ribozyme is peculiar, not only because it is the only hammerhead ribozyme found in eukaryotes, but also for many other features.

The catalytic domain of the amphibian ribozyme is part of transcripts made from a highly repetitive DNA family known as Satellite 2. Monomeric and multimeric strand-specific Satellite 2 transcripts have been found in every newt tissue examined (1).

While the catalytic activity of the plant pathogens is functional to their replicative cycle, the biological function of the newt hammerhead ribozyme is presently unknown. Nevertheless, available evidences can be interpreted as suggesting a defined biological function for the newt ribozyme.

First, Satellite 2 transcripts show a high level of sequence and structure conservation in evolutionarily distant urodele species, suggesting that the ribozyme sequences might have been selected during evolution because of a defined function in the cell (2).

Second, the oocyte ribozyme monomers are produced by transcription performed by RNA polymerase II and regulated by the same elements (PSE, DSE) involved in the transcription of snRNAs (3).

Third, an oocyte ribozyme monomer, as well as its isolated catalytic domain, is able to trans-cleave *in vitro* appropriate oligoribonucleotide substrates (3).

Fourth, the oocyte ribozyme monomer is assembled into a RNP complex which can cleave a synthetic substrate more efficiently than in the corresponding *in vitro* reaction (4).

Based on these data, the newt ribozyme could be conceived as an enzymatic core of a ribonucleoprotein complex, involved in some events of oocyte RNA processing.

To isolate and characterise the proteins that bind to the newt hammerhead ribozyme, an ovary expression library of the newt, *Triturus*, was screened with monomeric transcripts of the ribozyme, according to a recently developed RNA-ligand screening procedure (5). The ability of four selected clones to bind RNA has been confirmed by North-Western analysis.

A first cDNA clone is highly similar, both at nucleic acid and amino acid level, to a cDNA isolated from a rat library. Since the rat protein is not known to bind RNA, the newt clone may represent a new RNA-binding protein, highly expressed in newt oocytes. Its mRNA distribution is tissue-specific, being very abundant in the ovary but absent in every other newt tissue examined.

The second clone appears to represent the newt nucleolin cDNA, that was not isolated previously. Nucleolin is the major nucleolar protein in eukaryotic cells, known to play a role in pre-rRNA transcription and ribosome assembly. By Northern blot analysis, the clone reveals two differently-sized RNA sequences in every newt tissue examined, plus a third band in the ovary.

The characterisation of the remaining two clones, which are not present in databases, is underway.

Reasoning that knowledge of the intracellular distribution of the newt ribozyme could give insights on its possible function, we analysed its localisation by *in situ* hybridisation and Northern blot experiments. Northern blot analysis and *in situ* hybridisation on cell sections showed that the ribozyme is mainly cytoplasmic in the oocytes. *In situ* hybridisation on oocyte spread nuclear content showed that the ribozyme was not present in the snurposomes, subnuclear bodies where the snRNA are localised. The difference to snRNPs was also illustrated by the lack of m_3GpppG cap in the monomeric unit of the ribozyme in oocytes.

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DYNAMIC COMPETITION BETWEEN ALTERNATIVE STRUCTURES IN VIROID RNAs SIMULATED BY AN RNA FOLDING ALGORITHM

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The folding pathways of viroid RNAs were studied using computer simulations by the genetic algorithm for RNA folding. The folding simulations were performed for PSTVd RNAs of both polarities, using the wild type sequence and some previously known mutants with suggested changes in the stable or metastable structures. It is shown that metastable multihairpin foldings in the minus strand replicative intermediates are established due to the specific folding pathway that ensures the absence of the most stable rod-like structure. Simulations of the PSTVd minus strand folding during transcription reveal a metastable hairpin, formed in the left terminal domain region of the PSTVd. Despite high sequence variability, this hairpin is conserved in all known large viroids of both subgroups of PSTVd type, and is presumably necessary to guide the folding of the HPII hairpin which is functional in the minus strand. The folding simulations are able to demonstrate the changes in the balance between metastable and stable structures in mutant PSTVd RNAs. The stable rod-like structure of the circular viroid (+) RNA is also folded *via* a dynamic folding pathway. Furthermore, the simulations show that intermediate steps in the forced evolution of a shortened PSTVd replicon may be reconstructed by a mechanistic model of different folding pathway requirements in plus- and minus-strand RNAs. Thus the formation of viroid RNA structure strongly depends on dynamics of competition between alternative RNA structures. This also suggests that the replication efficiency of viroid sequences may be estimated by a simulation of the folding process.

SEQUENTIAL FOLDING OF PSTVD REPLICATION INTERMEDIATES

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With viroids of the PSTVd-group the rolling-circle-like mechanism of replication is coupled with a structural cycle of the different viroid RNAs. The mature, (+)stranded, circular RNA assumes a thermodynamically optimal, rod-like conformation, which is partially resistent against degradation and optimal for transport and storage. The (+)stranded, oligomeric, linear replication intermediate rearranges kinetically fast from a multi-hairpin structure into a rod-like structure that is an optimal target for the processing to the mature RNA by host enzymes (Baumstark & Riesner, 1997). In contrast, after synthesis the (-)stranded, oligomeric, linear replication intermediate stays for a longer time in a thermodynamical metastable conformation that contains hairpin II as a replication enhancer (Qu *et al.*, 1993).

To verify that model, we have developed an *in vitro*-system for viroid transcription with T7 polymerase (Repsilber, 1993) that allows to vary the rate of RNA synthesis and to analyze in dependence upon time the structures of transcripts by temperature-gradient gel electrophoresis (TGGE). Results of that experimental analysis are interpreted by *SeqFold*, a program that computes the sequential folding of an RNA (Schmitz & Steger, 1997) and simulates the structure-dependent gel-electrophoretic mobility of the RNAs (Rachen, 1997).

As expected, the number of different structures of (-)stranded PSTVd transcripts decreases with increasing time of transcription and incubation; i.e, thermodynamically unfavorable structures formed during transcription rearrange over time into more stable structures. Hairpin II-containing structures are predicted to be part of the structure ensemble, and to migrate at a few but defined positions in TGGE. With mapping by oligonucleotide hybridization we were able to verify that bands at these positions contained structures with the hairpin II element whereas structures migrating at different positions do not contain the hairpin II element.

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POSTERS

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MOLECULAR ANALYSIS OF DOUBLE - STRANDED RNA VIRUS OF AGARICUS BISPORUS AND ASSOCIATED FUNGI

Multisegment dsRNA were detected by agarose gel electrophoresis of preparations purified by CF-11 cellulose chromatography from *Agaricus bisporus, Verticillium fungicola* and *Trichoderma harzianum.* Ethidium bromide-stained dsRNA segments were identified by incubating gels with ribonuclease. The molecular weights of the dsRNA segments of *A. bisporus, V. fungicola* and *T. harzianum* ranged in size from 3.6 kb to 0.78 kb, 3.6 kb to 1.0 kb and 6.3 kb to 0.8 kb, respectively. RT-PCR and sequence analysis showed that at least two dsRNA segments of *V. fungicola* have sequence homology with dsRNA segments of *A. bisporus.*

Genomic diversity among phenotypically different isolates of peach latent mosaic viroid (PLMVd)

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Studies aimed at identifying structural motifs involved in pathogenicity of PLMVd, a self-cleaving viroid able to form hammerhead ribozymes in its strands of both polarities (1), have been carried out. To tackle this question, a RT-PCR amplification approach was followed to generate full-length cDNA clones from several PLMVd isolates that differ in their symptom expression on the peach indicator GF-305. Sequencing of PLMVd variants derived from a severe isolate (D168) and two latent isolates (LS35 and Esc76906) revealed a high number of polymorphic positions in the viroid molecule. Most of the changes were located in a region comprising 2/3 of the molecule which includes the self-cleaving domains although the stabilities of the hammerhead structure and, therefore, the *in vitro* self-cleavage reactions, were not essentially affected. Genetic identity values together with phylogenetic analyses of all the PLMVd sequence variants identified here support their classification into three major groups, each having a characteristic variability pattern. The clustering of the variants does not correlate strictly with the source isolate from which they were derived since two isolates contained sequences belonging to more than one group. These results provide insights into the complex mixtures of molecules which conform each isolate.

Additionally, the knowledge of the sequence of a high number of PLMVd variants has allowed the detection of compensatory mutations that permit long-range interactions between two hairpin loops of the proposed secondary structure of this viroid strongly supporting the presence of a pseudoknot-like element. Different alternatives for this potential element are found for the three groups considered above showing that the distinctions between the members are not limited to their primary structure but also to their higher-order structure.

Evaluation of the infectivity and pathogenicity of particular PLMVd sequences was carried out by inoculating separately a set of PLMVd cDNAs on GF-305 peach seedlings. Interestingly, a differential host response was observed among the PLMVd cDNAs depending on the isolate from which they were derived. Infections induced by cDNA clones obtained from the latent isolates were symptomless. In contrast, some cDNAs clones from the severe isolate induced the appearance of symptoms on the host indicator whereas others could incite both symptomatic and asymptomatic infections. The results of these bioassays parallel the pattern observed in PLMVd natural infections, which are phenotypically stable when caused by latent isolates but exhibit fluctuations in the symptomatology when originated by severe isolates. Therefore, the biological properties of PLMVd isolates may be correlated with both the complexity of the viroid RNA populations and with the presence of specific sequence variants.

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THE USE OF IN VITRO SELECTION STRATEGIES FOR TARGETING OF HAIRPIN RIBOZYMES.

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Replication of certain cicular RNA molecules depends on their ability to undergo specific self-cleavage. It has been shown that *in vitro* this reaction occurs in the absence of proteins. Furthermore the catalytic domain responsible of the reaction has been isolated from several of them and engineered to catalize the trans cleavage of other RNA molecules. It is considered that catalytic RNAs or ribozymes are good candidates for the development of sequence specific gene suppressers and therefore highly specific therapeutic agents. Although current results are very encouraging, it is still necessary to solve several significant obstacles before these molecules may be efficiently used with this meaning. The majority of the studies involving the use of ribozymes so far have been dedicated to get efficient inactivation of a specific gene rather than in developing general-use strategies. So far ribozymes as well as antisense genes have been designed by trial and error and clear rules have not been defined. Only recently, strategies aimed to the identification of good target sites for hammerhead and Group I ribozymes have been reported. Here we present an in vitro selection scheme designed for the rapid and rational identification of accessible target sites within a mRNA molecule for the hairpin ribozyme. The method uses a large pool of putative self-cleaving RNA molecules varying in the sequences of the substrate recognition domain of the hairpin motif, which is linked to the 5' end of the gene to be targeted. It allows the simultaneous selection of both, the most susceptible domain(s) within the RNA target and the most efficient hairpin ribozyme for each particular substrate domain. The scheme has already been successfully tested with single ribozyme sequences. We are currently optimizing the method to be applied to complex pools of ribozyme sequences.

Close structural relationships between two cherry viroid-like RNAs with hammerhead ribozymes

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Cherry chlorotic rusty spot disease is a novel cherry disorder recently observed in Italy and found closely associated with a series of double-stranded RNAs of a putative viral origin and with a viroid-like RNA (cherry small circular RNA1, csc RNA1). In some preparations from symptomatic cherry trees another small circular RNA (csc RNA2) with a slightly higher electrophoretic mobility was also detected, but the low level at which this second viroid-like RNA accumulates did not allow a definitive conclusion on whether or not it was present in all symptomatic samples (1). Molecular characterization of csc RNA1 has shown that it is a molecule of 451 nucleotides able to form hammerhead structures in its both polarity strands which self-cleave *in vitro* as predicted by these ribozymes (2). csc RNA1 contains a direct repeat of 20 nucleotides rich in purines, particularly in A.

Analysis by Northern blot hybridization of nucleic acid extracts from symptomatic cherry plants showed that probes specific for csc RNA1 gave rise to a signal in the position corresponding to csc RNA2. These results confirmed the close association suspected between csc RNA1 and csc RNA2 and indicated that these two viroid-like RNAs share extensive sequence similarities. Cloning and sequencing of csc RNA2 revealed that this is the case. Both csc RNAs showed almost identical sequences in the hammerhead structures of both polarity strands, a likely reflection of the functional relevance of the self-cleaving domains. However, the two RNAs differed in other regions of molecule: csc RNA2 lacks the 20-nucleotide direct repeat observed in csc RNA1, and presents other deletions with respect to the csc RNA1 sequence. Interestingly, the sequences affected by some of these deletions form hairpin bifurcations in the branched conformation predicted as the most stable secondary structure for csc RNA1; therefore, the most stable secondary structure obtained for csc RNA 2 is considerably less branched and adopts a quasi-rodlike conformation resembling those proposed for some viroid and viroid-like RNAs.

These results suggest that csc RNAs 1 and 2 have derived from a common ancestor molecule mainly through rearrangements events that most probably emerged as a consequence of the low processivity of the RNA polymerase involved in their replication.

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REPLICATION AND EXPRESSION OF SYNTHETIC MINIGENOMES AND SEL-REPLICATING RNAs DERIVED FROM TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS

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To understand gene function and expression in coronaviruses it would be of interest to assemble a cDNA encoding a full length infectious RNA. This has not yet been possible due to the large size of coronavirus genome, with a positive stranded RNA of around 30 kb. To overcome this problem the synthesis of cDNA clones encoding minigenomes that are replicated using a helper virus, or self-replicating RNAs encoding the viral replicase are being performed in our laboratory. These minigenomes are being used to develop a tissue-specific expression system.

Three transmissible gastroenteritis coronavirus (TGEV) defective interfering RNAs of 21, 10.6 and 9.7 kb (DI-A, DI-B and DI-C, respectively) were isolated (Méndez et al, 1996. Virology **217**, 495-507). Dilution experiments showed that the largest DI-A RNA is a self-replicating RNA, thus codes for a functional RNA polymerase and contains the necessary replication signals.

A cDNA complementary to DI-C RNA was cloned under the control of T7 promoter (pDI-C). *In vitro* transcribed DI-C RNA was replicated *in trans* upon transfection of helper virus-infected cells. Rescue of synthetic DI-C RNA was studied by Northern blot analysis using intracellular RNA from different passages: DI-C RNA was transfected to helper virus infected cells and supernatants of these cultures were passed six times. Synthetic DI-C RNA was clearly detected and remained stable from passage 3 to 6 in RNA transfected cultures, but not in the non-transfected ones. Using this rescue system, the minimal sequences required for minigenome replication were analyzed by deleting internal fragments in DI-C cDNA. A collection of 15 DI-C deletion mutants was generated and their ability to be replicated and packaged *in trans* was tested. The sequences required for replication have been reduced to 3.3 kb corresponding to: about 2.3 kb of the ORF 1a, ORF 1b sequences comprising the pseudoknot and a 200 nt stretch from the 3' end ORF 1b, and 700 nt of the 3' end of DI-C RNA. The results suggested that in TGEV there may be two packaging signals located in the 5' and 3' ends of the ORF 1b, or that other restrictions for the minigenome packaging, such as RNA size, restrict its encapsidation.

In the polymerase gene pDI-C contained a 10 kb deletion in gene 1a (from 2.1 to 12.1 kb) and a 1.1 kb deletion in gene 1b (from 16.7 to 17.8 kb). The consensus sequence corresponding to the deleted regions was cloned, and the deletions of pDI-C were replaced to yield a full-length cDNA clone of the TGEV polymerase, pDI-A-21, behind a T7 promoter. The T7 promoter of pDI-A-21 was changed to CMV promoter (pDI-A-21-CMV). The expression of a functional TGEV polymerase is being investigated.

A tissue-specific expression system targeted to the enteric or the respiratory tract of swine has been developed, based on minigenomes derived from DI-C. The expression levels have been optimized by studying the beta-glucuronidase expression using different promoters and flanking sequences. Using a respiratory helper virus (clone C8), a recombinant minigenome expressing the spike gene of a TGEV enteric strain (clone C11) was rescued. These viruses had the ability to infect the enteric tract of swine, indicating the efficiency of the expression system, and the possibility of changing coronavirus tropism by forming pseudorecombinants with the appropriate spike proteins.

Current status of fruit tree viroids research in Italy

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Three years ago, a joint UE project started with the aim to improve knowledges on detection, molecular characterization, epidemiology and elimination of the most important fruit tree viroids: apple scar skin (ASSVd), hop stunt (HSVd), peach latent mosaic (PLMVd) and pear blister canker (PBCVd). In this presentation, we report our activity carried on in the frame of this project, and the current research interests.

1) Comparison of different diagnostic techniques. Bidirectional gel electrophoresis (dPAGE), spot and tissue blot hybridization, with radioactive and non radioactive probes, and RT-PCR techniques were compared to detect the most important viroids in infected fruit tree plants, starting from different matrices collected in different vegetative stages and using different extraction methods.

2) Investigation on the diffusion of fruit tree viroids in Italy. 270 samples (belonging to 68 cvs) of peach, 165 (52 cvs) of plum, 80 (37 cvs) of apricot, 12 (4 cvs) of cherry, 10 (4cvs) of almond, 110 (40 cvs) of apple and 210 (22 cvs) of pear germplasm were investigate to ascertain the presence of PLMVd, HSVd, PBCVd and ASSVd.

3) Characterization of an Italian isolate of pear blister canker viroid. A small RNA species with the structural and functional properties characteristic of viroids was isolated from a pear plant, cv Coscia, showing superficial cracks or splits on bark epidermis of the secondary branches and horizontal deep bark splits on the trunk. Molecular characteristics and sequence analysis showed that the viroid associated to pear plant can be considered a PBCVd isolate. This was the first report about the presence of PBCVd in Italy.

4) Use of *in vitro* micrografting technique to eliminate viroids from infected pear and peach germplasm. Experiments were carried out on pear and peach germplasm infected with PBCVd and PLMVd respectively. Apical meristems (0.2 - 0.3 mm long), taken from infected plants, were cut and *in vitro* micrografted onto pear seedlings *in vitro* cultured. Grafted plantlets were tested either by dPAGE or molecular hybridization assays to verify the effectiveness of the viroid elimination.

5) Current research interests.

- PLMVd was found, during a survey in plum orchards, naturally infecting two plum cultivars located in different areas in Central Italy. The presence of PLMVd was revealed by dPAGE, spot-blot hybridization and RT-PCR assays. The sequence analysis is under way to compare our plum-isolate with other PLMVd isolates already characterized. To verify the transmission of PLMVd-plum isolate to other species, healthy stone fruit germplasm has been grafted with buds collected from PLMVd infected plums.
- Due to the negative results obtained with the traditional tip grafting technique, new experiments are under way to evaluate the possibility to eliminate PBCVd by combining both thermotherapy and tissue culture techniques. At the same time, ASSVd and HSVd infected tissue will be *in vitro* propagated to optimize the eradication methods.

DATA ON THE IMPLICATION OF THE PLASMA MEMBRANE IN THE CEVd -TOMATO INTERACTION

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Based on the increasing evidence supporting the idea that the plasma membrane is involved in signal transduction in both animals and plants, it was anticipated that the plasmalemma would play a critical role in conveying different primary pathogenic signals, including viroids (1). Here it is presented the alterations in the biochemical and biophysical properties of the plasma membrane induced by citrus exocortis viroid (CEVd) infection that may be involved in the pathogenic and defense signalling. i. e: 1) a decrease in the content of oleic, linoleic and linolenic fatty acids; 2) a dramatic increase in lysophosphatidylcholine levels and other phospholipids is detected; 3) these changes are consistent with the enhanced activity of a phospholipase A_2 found and what had been hypothesized (2); 4) also consistent with the changes in the fatty acid composition it has been found a decrease in the fluidity and passive permeability to ions; 5) an increase of the free cytosolic Ca^{2+} seems to be due to a permanent activation of a voltage dependent calcium channel and the impairment of extrusion mechanisms. All These results are discussed in the frame of a commonalty in the design of signal-response strategies of plants to a wide range of environmental stresses.

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A SYSTEM FOR IDENTIFYING PROMOTER ACTIVITY IN THE POTATO SPINDLE TUBER VIROID RNA

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The ability of viroids to replicate autonomously in plants distinguishes them from other subviral pathogens. This property suggests the presence of a viroid RNA promoter which is recognized by host-encoded RNA polymerase. The lack of sequence similarity between viroids and known eukaryotic promoters suggests that host-encoded RNA polymerase possibly recognizes a structure in the viroid RNA which serves as the promoter. To identify such a promoter, four sets of constructs were made with circularly permuted, full-length PSTVd sequences linked to a reporter RNA, the dm strain of the satellite of tobacco ringspot virus (sTRSV). Transcripts obtained from the linear constructs contain the PSTVd RNA in either orientation linked to the non-infectious (-)sTRSV. Because of the paperclip ribozymes on the dm (-) sTRSV, these transcripts cleave accurately and circularize *in vitro*, resulting in the PSTVd RNA being upstream of the greater-than-unit-length dm (-)sTRSV. The promoter within the PSTVd portion of these circular transcripts drives the transcription of (+)sTRSV which is consequently amplified by TRSV. Three-week old *Nicotiana benthamiana* plants were inoculated with TRSV and, then, superinfected with RNA transcripts obtained from the constructs a week later. Four weeks after inoculation, total nucleic acids (TNAs) from *N. benthamiana* were extracted and electrophoresed on a denaturing gel. Bands migrating to the position of the dm sRNA were observed from plants inoculated with the virus plus transcripts from constructs containing circularly permuted, full-length PSTVd of either orientation. On the other hand, no such band was obtained from plants inoculated with the buffer, the virus alone, and the virus plus RNA transcripts from the construct which did not contain PSTVd. To confirm that the bands were indeed derived from dm sRNA and not wild type TRSV sRNA, TNAs were subjected to reverse transcription (RT)-PCR coupled with restriction digestion analysis using *Hinc* II or *Dde* I. The bands obtained were sensitive to *Dde* I but resistant to *Hinc* II indicating that they were indeed derived from dm sTRSV.

Specificity of binding and expression pattern of X1 protein from tomato, the first protein which binds to PSTVd RNA .

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Identification of plant proteins, which show specific binding to PSTVd RNA, will open the way for a step-by step analysis of the molecular mechanism of this unique plant-host interaction. We have used the newly developed method for isolation of RNA-binding proteins (see abstract by Sägesser et al.) and isolated one new tomato gene, preliminary called X1, which specifically interacts with a PSTVd RNA transcript. The primary structure of X1 was compared with sequences in the database, and limited homologies were identified. The X1 gene product was overexpressed in *E.coli* cells and used for preparation of an antibody and in Northwestern assays. A range of viroid and viroid-related RNAs was tested for specific binding, and it was found that X1 binds to the PSTVd subgroup of viroids. We are currently identifying the primary binding site for X1 protein. The specificity of the antibody is under investigation, which will be then used to investigate the presence of X1-viroid complexes in the cell.

THE DEVELOPMENT OF ANTI-HLVd ANTISENSE SYSTEMS BASED ON RNA POLYMERASE III PROMOTER ELEMENTS

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Hop latent viroid (HLVd), which occurs worldwide (1) was demonstrated to be deleterious to production in certain hop cultivars in the UK (2) and, therefore, it appears to be of significant theoretical and practical importance. In our previous work, we partly characterized horizontal (3) and vertical (4) distribution of this viroid in Bohemian hops, which were found to be sorely infected with this pathogen. This fact stimulates the research of possible systems of crossprotection against HLVd. Such systems could be based on antisense RNA. During our previous work we analyzed in detail an inhibition of viroid infection by antisense RNA *in vivo* (5). We found that this clear effect of antisense RNA is overcome by some threshold viroid level, probably due to silencing of antisense genes by RNA-directed *de novo* DNA methylation. This methylation appears upon viroid infection (6) and could serve as a feedback to prevent RNA overexpression. We assume that RNA polymerase III-driven antisense genes, such as based on tRNA (7) or 7SL RNA (8) chimeras could increase antisense effect upon plant transformation. For an example, 7SL RNA is very abundant (up to 10000 molecules per single cell (9).

In this study we prepared anti-HLVd minus-sense hammerhead ribozymes (10) targeted against GUC sites (corresponding to nucleotide number 19 and 111 in plus monomeric RNA).The ribozyme antisense arms partly covered computer-predicted loops in minus dimeric transcripts simulating (-)HLVd replication intermediates. Especially ribozyme targeted against GUC in the position 111, called HR57 was found to have ability to cleave up fully the HLVd RNA substrate at low temperature conditions and at molar ratio 1:1. Therefore, this antisense ribozyme was selected for preparation of antisense genes driven by RNA pol.III promoter elements. tRNA ribozyme (162b-long RNA) was prepared by HR57 integration into $tRNA^{tyr}$(7) at the intron position and its activity, was demonstrated *in vitro*. tHR57 gene was successfully transcribed in RNA polymerase III extract from HeLa cells according to procedure (11) and resulting chimeric RNA was found to be stable in plant extracts. A novel 7SL RNA gene was isolated from *A.thaliana* and modified by site-directed mutagenesis with the aim to prepare 7SL RNA ribozyme chimeras. A stability of HLVd target was investigated using a high fidelity RT PCR, TGGE (12)and sequencing methods. A predominant HLVd form isolated from Bohemian hops followed sequence described earlier (1), HLVd quasispecies (about 1.2% from total HLVd) were cloned only after TGGE selection. Sequenced HLVd quasispecies fully retained the ribozyme target sites.

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Complexes containing both polarity strands of avocado sunblotch viroid: identification in chloroplasts and characterization.

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Avocado sunblotch viroid (ASBVd), together with peach latent mosaic and chrysanthemum chlorotic mottle viroids, are the three only members of total of 27 sequenced viroids which can form hammerhead structures in both polarity strands. These ribozymes are functional in vitro and most probably in vivo, and mediate the processing of the oligomeric RNA intermediates generated in viroid replication. ASBVd, the more thoroughly studied of the known self-cleaving viroids, is unusual in other aspects when compared with typical viroids as potato spindle tuber viroid (PSTVd) lacking hammerhead ribozymes: ASBVd accumulates in the chloroplast (1, 2) and its replication is insensitive to α-amanitin (3), as opposed to PSTVd which is found in the nucleus and replicates through a pathway sensitive to the low levels of this inhibitor which characteristically inhibit nuclear RNA polymerase II. These singularities of ASBVd raise interesting questions on its subcellular site of replication: the viroid might replicate in the nucleus by an RNA polymerase I-like activity (or by an unidentified RNA polymerase also resistant to α-amanitin) and then be translocated to the chloroplast, or replication may occur in this organelle and be catalyzed by the chloroplastic RNA polymerase that is insensitive to the inhibitor as well.

To discriminate between these two alternatives, avocado chloroplasts were purified from ASBVd-infected mesophyl protoplasts by a Percoll-based protocol. Analysis of chloroplastic RNAs by non denaturing PAGE and Northern blot hybridization with RNA probes for both polarities revealed the same series of ASBVd-specific bands described previously (4): the subgenomic RNAs, the monomeric and dimeric forms, and two band designated (x) and (y). When these RNAs were treated with RNase in high ionic strength, bands (x) and (y) remained resistant, whereas the rest of viroid and cellular RNAs were degraded. To determine the nature of the RNA components of bands (x) and (y) they were eluted from non-denaturing gels and analyzed by denaturing PAGE and Northern blot hybridization. The major constituents of the untreated (x) and (y) bands were the monomeric circular and linear ASBVd forms of both polarities in similar proportions. Following nuclease treatment the composition of band (y) was unaffected, but only the monomeric linear ASBVd RNAs of both polarities were detected in band (x). Therefore, complex (y) appears to have a compact structure more resistant to nucleases whereas that of complex (x) seems to be more relaxed. Irrespective of their detailed structures the finding that complexes (x) and (y), containing minus ASBVd strands and particularly the monomeric circular form, are chloroplastic is strong evidence supporting the chloroplast as the site of replication of ASBVd.

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IMPRINT HYBRIDIZATION AS A TOOL FOR VIROID DETECTION

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An imprint hybridization method has been designed to detect viroids from infected tissues. Freshly cut tissue pieces are imprinted by firmly pressing the cut surface onto positively charged Nylon or polyvinylidene membranes. The imprinted samples once fixed on the membrane, can be processed immediately or stored. Processing of the imprinted membranes involves: a) preparation of DIG-labeled probes (RNA probes synthesized by *in vitro* transcription or DNA probes synthesized by PCR, using cloned DNA related to specific viroid sequences); b) hybridization of the membranes against the DIG-labeled viroid probes; c) detection of DIG-labeled hybrids (using an anti-DIG-alkaline phosphatase conjugate); d) visualization of the DIG-alkaline phosphatase (using both colorimetric and chemiluminiscence substrate).

In citron, probes for the five viroid groups identified in citrus have been assayed and shown to be highly specific. In addition, a single hybridization using a mixture of the five probes allows a fast screening for viroid-free versus viroid-containing samples. The overall process is simple and can be easily performed by non-specialized personnel including some nurserymen. Membranes containing positive and negative controls and the chemicals needed (including the mixtures of labeled probes) could be easily commercialized as detection kits. Efforts are now being devoted to adapt this imprint hybridization method for the detection of citrus viroids directly from commercial varieties.

In cucumber, this imprint hybridization method has been used in studies of viroid movement and tissue-specific localization. Viroids accumulate mainly in vascular bundles and perivascular fibers. The long-distance movement of the viroids in the infected plants occurs through the vascular bundles, mainly via the phloem. In short-distance movement two mechanisms are involved: a) the division of infected-cells; b) cell-to-cell movement. Both occur in the meristematic tissues and only the second should be important in the tissues developed before the infection.

MOLECULAR VARIABILITY AND RECOMBINATION AMONG ISOLATES OF HOP STUNT VIROID

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Viroids are the smallest known pathogens of higher plants. They exist as single-stranded, circular, highly structured, rod-like RNAs with no protein capsid nor any detectable messenger activity (Diener, 1987; Flores et al.,1997). The RNA molecule of typical viroids contains five structural domains (Keese and Symons, 1985). In general, sequence variation between strains of a viroid is mostly localized in the P (pathogenicity) and V (variable) domains of the molecule. Hop stunt viroid (HSVd) has been found in a wide range of hosts including hop, cucumber, grapevine, citrus, plum, peach, pear (Shikata, 1990) and recently, apricot, almond and pomegranate (Astruc et al., 1996). Sequence variation between HSVd isolates affects all structural domains (Hsu et al., 1994) but little is known on the effect of specific mutations on the pathogenicity or host range of HSVd. Overall sequence homologies (Shikata, 1990) and phylogenetic analysis (Hsu et al., 1994) have indicated that HSVd isolates can be separated into three groups: plum-type (peach, plum and grapevine isolates), hop-type (hop, grapevine, peach and pear isolates) and citrus-type (citrus and cucumber isolates) (Shikata, 1990). The fact that these groups often contained isolates from only a limited number of isolation hosts prompted the suggestion that group-discriminating sequence variations could, in fact, represent host-specific sequence determinants which may facilitate or be required for replication in a given host.

In a effort to further understand the relationships between HSVd and its different hosts, we have cloned and sequenced HSVd variants from eight naturally infected *Prunus* sources, including apricot, peach and Japanese plum. In total, ten molecular variants of HSVd have been identified, nine of which had not been described before. A detailed phylogenetic analysis of the existing HSVd sequences, including the new ones from *Prunus* determined in this work, points towards a reedefinition of the grouping of the variants of this viroid, since two new groups were identified, one of them composed of sequences described here. A bias for the presence of certain sequences and/or structures in certain hosts was observed, although no conclusive host-determinants were found. Surprisingly, our analysis revealed that a number of HSVd isolates probably derive from recombination events and that the previous hop-type group itself is likely the result of a recombination between members of the plum-type and citrus-type groups.

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Expression of enzymatically active Rabbit Hemorrhagic Disease Virus RNA-dependent RNA polymerase in *Escherichia coli.*

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Rabbit hemorrhagic disease virus (RHDV), a member of the *Caliciviridae*, consists of a single plus-stranded RNA genome of about 7.4 kb that has a virus encoded protein VPg attached covalently to its 5' end and is polyadenylated at the 3' end. Viral particles also encapsidate an abundant VPg-linked polyadenylated subgenomic RNA of about 2.2 kb, which is coterminal with the 3' end of the viral genome. The data obtained from the *in vitro* translation and *Escherichia coli* expression studies revealed that the viral RNA is translated into a polyprotein that is subsequently cleaved to give rise to mature structural and nonstructural proteins. Progress on the replication of caliciviruses has been negligible compared with the advances made with the picornaviruses. Because of the lack of a cell culture system for most caliciviruses, such as RHDV, recombinant DNA technology will be important for the production and characterization of viral proteins. Although functional studies have not yet been performed for most of the RHDV nonstructural proteins, the extensive sequence similarities between the RNA-dependent RNA polymerase 3D of picornavirus and the recently described RHDV polyprotein cleavage product p58 indicates that this polypeptide might have a similar role in genome replication.

The rabbit hemorrhagic disease virus (isolate AST/89) RNA-dependent RNA-polymerase ($3D^{pol}$) coding region was expressed in *Escherichia coli* using a glutathione S-transferase based vector, which allowed milligram purification of an homogeneous enzyme with an expected molecular mass of about 58 kDa. The recombinant polypeptide exhibited rifampin and actinomycin D-resistant, poly(A)-dependent poly(U) polymerase. The enzyme also showed RNA polymerase activity in *in vitro* reactions with synthetic RHDV subgenomic RNA in the presence or absence of an oligo(U) primer. Template-size products were synthesized in the oligo(U) primed reactions whereas in the absence of added primer, RNA products up to twice the length of the template were made. The double-length RNA products were double stranded and hybridized to both positive and negative sense probes.

List of Invited Speakers

Workshop on

PLANT VIROIDS AND VIROID-LIKE SATELLITE RNAs FROM PLANTS, ANIMALS AND FUNGI

List of Invited Speakers

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