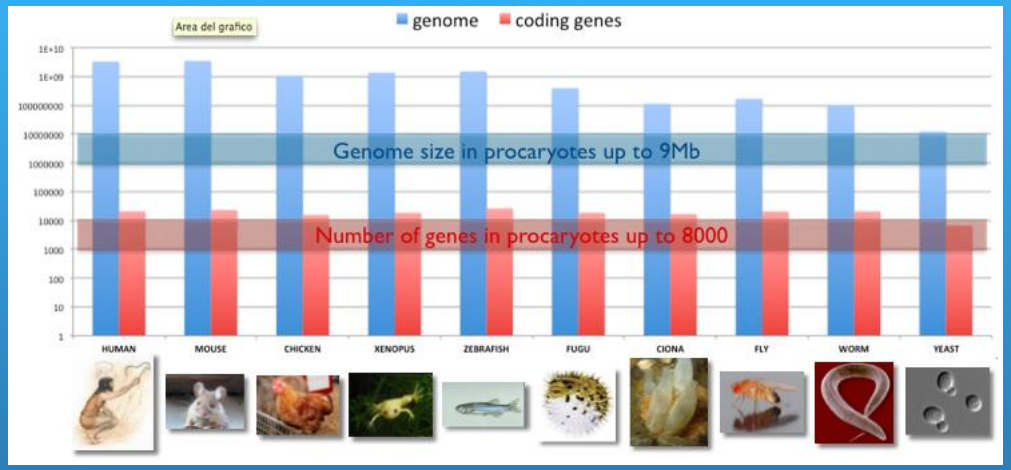
Platform for functional ncRNA analysis of NGS data

Andreas Gisel

CNR – ITB

Bari

The RNA World



non-coding RNA (ncRNA) genes, which produce transcripts that function as structural, catalytic or regulatory RNAs

- ✓ MicroRNA (miRNA) : post-transcriptional regulatory genes
- ✓ PIWI-interacting RNA (piRNA): germline transposon silencing
- ✓ Small interfering RNA (siRNA) : active molecules in RNA interference
- ✓ Small nuclear RNA (snRNA) : includes spliceosomal RNAs.
- ✓ Small nucleolar RNA (snoRNA): most known snoRNA are involved in rRNA modification
- ✓ Long non-coding RNA (lncRNA) : Little is known about them, involved in mRNA regulation

The RNA World

1986

Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5372–5376, August 1986
Biochemistry

Inhibition of gene expression in plant cells by expression of antisense RNA

(chimeric genes/electroporation/plant transformation/transient chloramphenicol

JOSEPH R. ECKER AND RONALD W. DAVIS
Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305

The Plant Cell, Vol. 2, 279–289, April 1990 © 1990 American Society of Plant Physiologists

Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes *in trans*

1990

Carolyn Napoli,[1] Christine Lemieux, and Richard Jorgensen[2]
DNA Plant Technology Corporation, 6701 San Pablo Avenue, Oakland, California 94608

1992

Molecular Microbiology (1992) 6(22), 3343–3353

Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences

Nicoletta Romano and Giuseppe Macino*
Dipartimento di Biopatologia Umana, Sezione di Biologia Cellulare, Policlinico Umberto 1, Università di Roma 'La Sapienza', 00161 Rome, Italy.

whether these are tandemly arranged or located on different chromosomes (Faugeron *et al.*, 1990; Selker, 1990). Pre-meiotic inactivation appears to involve at least two different steps: an initial interaction between homologous sequences followed by sequence modifications, either cytosine methylation as in *A. immersus*, or both methyla-

The RNA World

1993

The C. elegans Heterochronic Gene *lin-4* Encodes Small RNAs with Antisense Complementarity to *lin-14*

Rosalind C. Lee,*‖ Rhonda L. Feinbaum,*‖ and Victor Ambros†
Harvard University
Department of Cellular and Developmental Biology
Cambridge, Massachusetts 02138

Summary

***lin-4* is essential for the normal temporal control of diverse postembryonic developmental events in C. elegans. *lin-4* acts by negatively regulating the level of LIN-14 protein, creating a temporal decrease in LIN-14 protein starting in the first larval stage (L1). We have cloned the C. elegans *lin-4* locus by chromosomal walking and transformation rescue. We used the C. elegans clone to isolate the gene from three other Caenorhabditis species; all four Caenorhabditis clones functionally rescue the *lin-4* null allele of C. elegans. Comparison of the *lin-4* genomic sequence from these four species and site-directed mutagenesis of potential open reading frames indicated that *lin-4* does not encode a protein. Two small *lin-4* transcripts of approximately 22 and 61 nt were identified in C. elegans and found to contain sequences complementary to a repeated sequence element in the 3′ untranslated region (UTR) of *lin-14* mRNA, suggesting that *lin-4* regulates *lin-14* translation via an antisense RNA–RNA interaction.**

Ambros and Horvitz, 1987). *A*
of-function (*lf*) mutation, *lin-4(*
early fates at inappropriately
cell lineage patterns normally
ated at later stages, and the
molts (Chalfie et al., 1981).
heterochronic developmenta
sence of adult structures (su
vulva) and the prevention of
lin-14 null *(0)* mutations ca
that of *lin-4(lf)* and are comple
is consistent with *lin-4* actin
lin-14 (Ambros and Horvitz, 1
mutants skip the expression
cociously execute programs
L3, L4, and adult stages. *lin-*
tions, which cause inappro
late stages of development, r
virtually identical to that of *l*
1987). These observations in
opment a high level of *lin-14*
specifies L1-specific progran
activity in the late L1 specify la
Thus, the normal developmer
cution of L1 programs to later
on the *lin-4*-dependent decre

The temporal decrease in
crease in the level of LIN-14
mally abundant in the nucle

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Cell, Vol. 81, 611–620, May 19, 1995, Copyright © 1995 by Cell Press

par-1, a Gene Required for Establishing Polarity in C. elegans Embryos, Encodes a Putative Ser/Thr Kinase That Is Asymmetrically Distributed

1995

Su Guo and Kenneth J. Kemphues
Section of Genetics and Development
Cornell University
Ithaca, New York 14853

the *ZC22* cDNA may be derived from *par-1*.

Phenocopying* par-1 *in Wild Type via Antisense RNA Injection

Germline transformation rescue with a genomic clone con-

The RNA World

scientific correspondence

Nature **385**, 781 - 782 (27 February 1997); doi:10.1038/385781a0

1997

Plants combat infection by gene silencing

SIMON N. COVEY, NADIA S. AL-KAFF, AMAGOIA LÁNGARA & DAVID S. TURNER

Department of Virus Research, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK e-mail: covey@bbsrc.ac.uk

A Similarity Between Viral Defense and Gene Silencing in Plants

Frank Ratcliff, Bryan D. Harrison, David C. Baulcombe*

Gene silencing in plants, in which an endogenous gene is suppressed by introduction of a related transgene, has been used for crop improvement. Observations that viruses are potentially both initiators and targets of gene silencing suggested that this phenomenon may be related to natural defense against viruses. Supporting this idea, it was found that nepovirus infection of nontransgenic plants induces a resistance mechanism that is similar to transgene-induced gene silencing.

Virus-induced gene silencing (VIGS)

Post transcriptional gene silencing (PTGS)

The RNA World

Cell, Vol. 90, 479–490, August 8, 1997, Copyright ©1997 by Cell Press

Cosuppression in Drosophila: Gene Silencing of *Alcohol dehydrogenase* by *white-Adh* Transgenes Is *Polycomb* Dependent

1997

Manika Pal-Bhadra,* Utpal Bhadra,*
and James A. Birchler†
Division of Biological Sciences
117 Tucker Hall
University of Missouri
Columbia, Missouri 65211

Summary

When two to six copies of a *white* promoter-*Alcohol dehydrogenase* (*Adh*) reporter fusion gene are introduced into the genome, the expression is progressively reduced both in larvae and adults rather than the expected gene dosage effect. In addition, multiple transgenes reduce endogenous *Adh* transcripts, a result that is strongly analogous to "cosuppression" phenomena described in many plant species but which has not been previously observed in animals. Silencing of the *Adh* gene is not influenced by *zeste*-dependent transvection but strongly affected by the *Polycomb* and *Polycomblike* mutations. *Polycomb* and *polyhomeotic* proteins are bound to the chromatin at the sites of the repressed *w-Adh* transgenes.

The RNA World

1997

Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

* *Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA*
† *Biology Graduate Program, Johns Hopkins University, 3400 North Charles Street, Baltimore, Maryland 21218, USA*
‡ *Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA*

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene[1,2]. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression[3,4]. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stochiometric interference with endogenous mRNA and suggesting that there could be a catalytic or amplification component in the interference process.

The Nobel Prize in Physiology or Medicine 2006
Andrew Z. Fire, Craig C. Mello

The Nobel Prize in Physiology or Medicine 2006
Summary | Illustrated Information
Prize Announcement
Press Release
Advanced Information
Popular Information
Nobel Prize Award Ceremony
Andrew Z. Fire
Craig C. Mello

Nobelförsamlingen
The Nobel Assembly at Karolinska Institutet

Karolinska Institutet

English
Swedish

Press Release

2 October 2006

The Nobel Assembly at Karolinska Institutet has today decided to award The Nobel Prize in Physiology or Medicine for 2006 jointly to

Andrew Z. Fire and **Craig C. Mello**

for their discovery of **"RNA interference – gene silencing by double-stranded RNA"**

The RNA World

1999

A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants

Andrew J. Hamilton and David C. Baulcombe*

Posttranscriptional gene silencing (PTGS) is a nucleotide sequence–specific defense mechanism that can target both cellular and viral mRNAs. Here, three types of transgene-induced PTGS and one example of virus-induced PTGS were analyzed in plants. In each case, antisense RNA complementary to the targeted mRNA was detected. These RNA molecules were of a uniform length, estimated at 25 nucleotides, and their accumulation required either transgene sense transcription or RNA virus replication. Thus, the 25-nucleotide antisense RNA is likely synthesized from an RNA template and may represent the specificity determinant of PTGS.

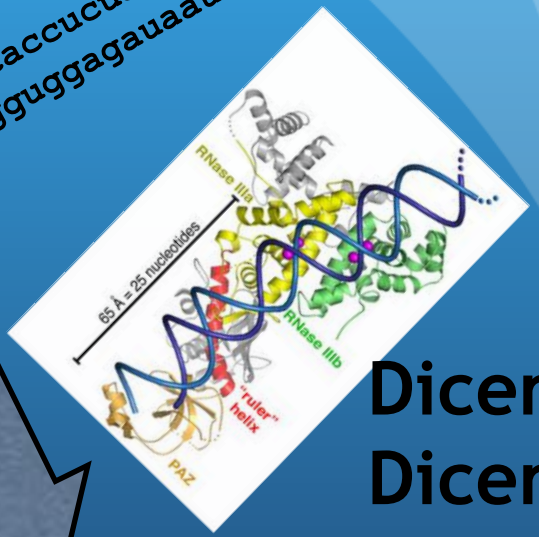
The RNA World

transgene
antisenes RNA

replicating virus

secondary structures

5’ -auugcugaucaccucucuaauauagaugggccccacauauacuggugaggccaccucuauuagagaugucagcaaugug-3’
3’ -uaacgacuaguggagagauuauaucuacccggggguguauaugaccacuccgguggagauaaucucuaccagucguuacac-5’



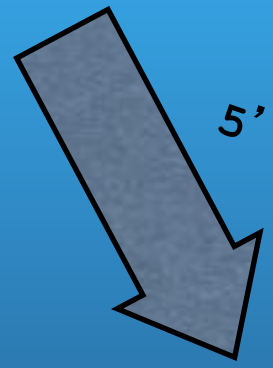
Dicer
Dicer-like

5’ -auagaugggccccacauauac-3’

The RNA World

5’ -auagaugggcccccacauauac-3’

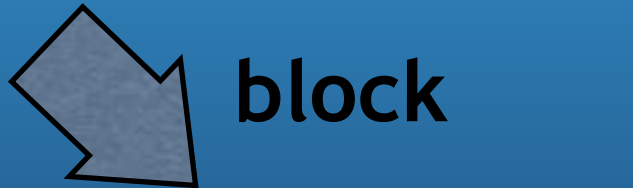
small RNA



RISC

RNA induced silencing complex

block



3’ -uaacgacuaguggagagauuauaucua uaugaccacuccgguggagauaau

cleave

acgacuaguggagagauuauaucuacccgg gguguauaugaccacuccgguggagauaaucucucuaccagucguuacac-5’

The RNA World

Why non-coding RNA?

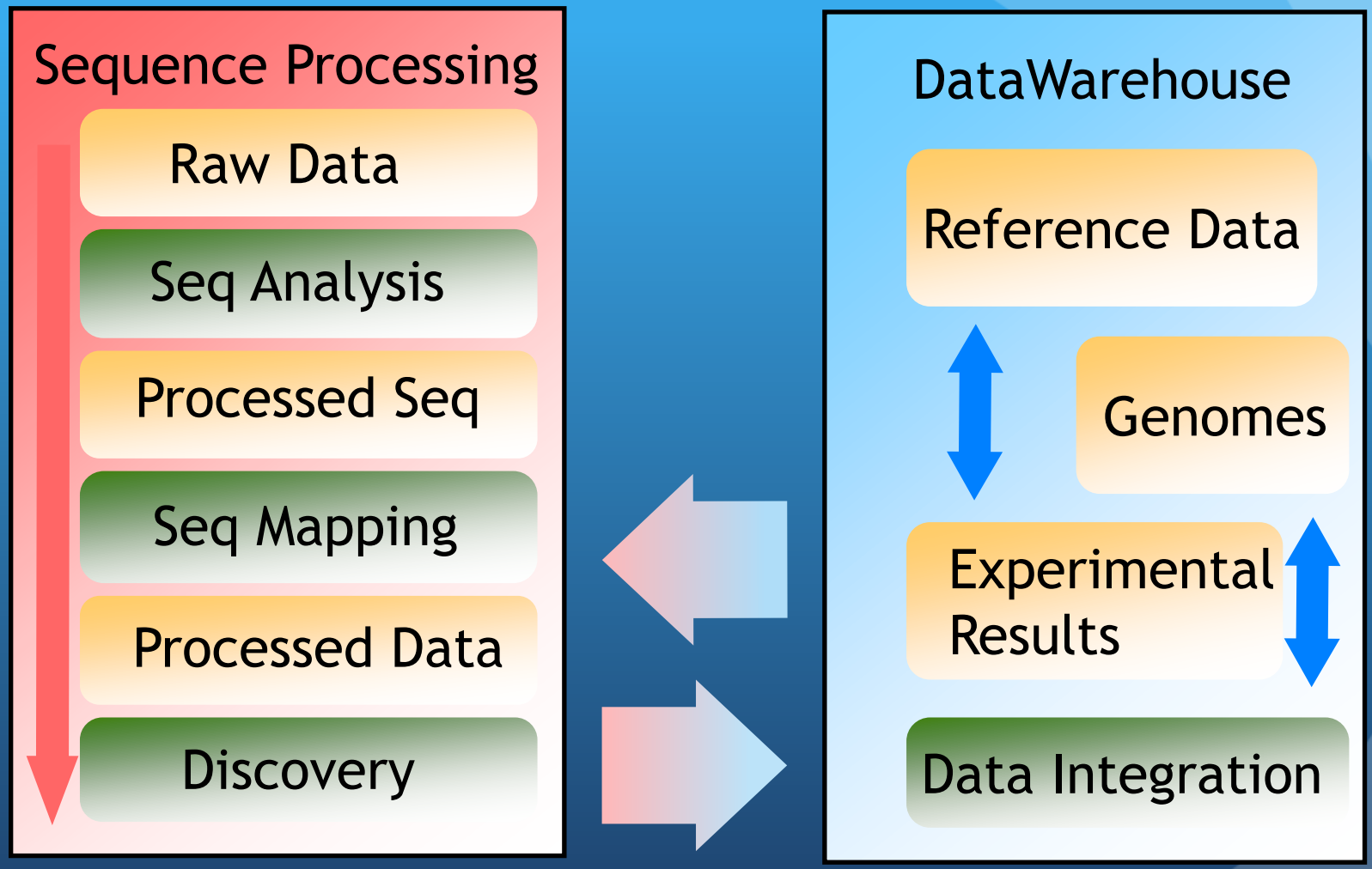
- How many ncRNA genes are there?
- How important are they?
- What functions does a cell ‘delegate’ to RNA instead of protein and why?

To address these questions, new systematic gene-discovery approaches need to be developed that are specifically aiming the discovery ncRNAs

Next Generation Sequencing (NGS) technologies with the vast amount and sensitivity enable such a research.

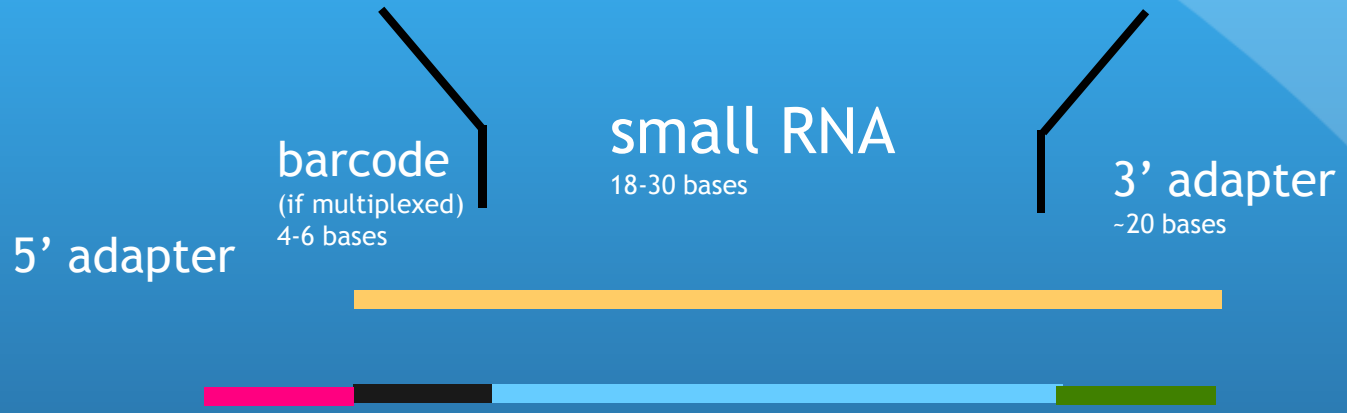
The new spectrum of NGS applications together with the massive amount of data requires a focused investment and development of bioinformatics tools managing and analysing such complex and large datasets to infer biological meaning.

The ITB ncRNA Platform



Sequence Processing

Small RNA Sequence Analysis

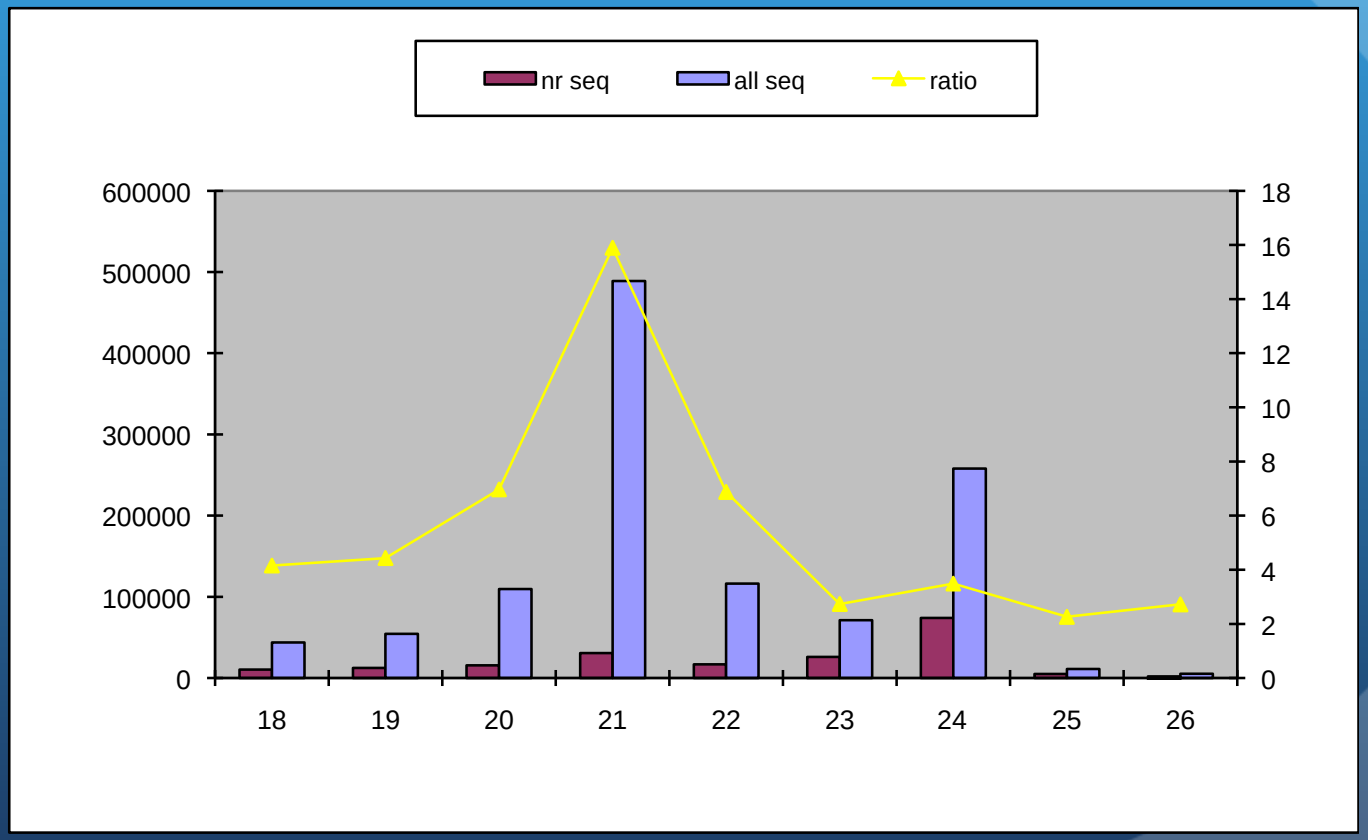


Reads are typically around 50 bases and therefore read into the 3'-adaptor

- ✓ If multiplexed detect barcode sequence and separate experiments
- ✓ Trimming barcode and 3'-adaptor fragment (adaptor sequence not always present since "small RNA" too long ==> discharge!!!!
- ✓ Since sequencing errors tends to be more frequent in 3' part, 3'-adaptor trimming need to accept mismatches

Sequence Processing

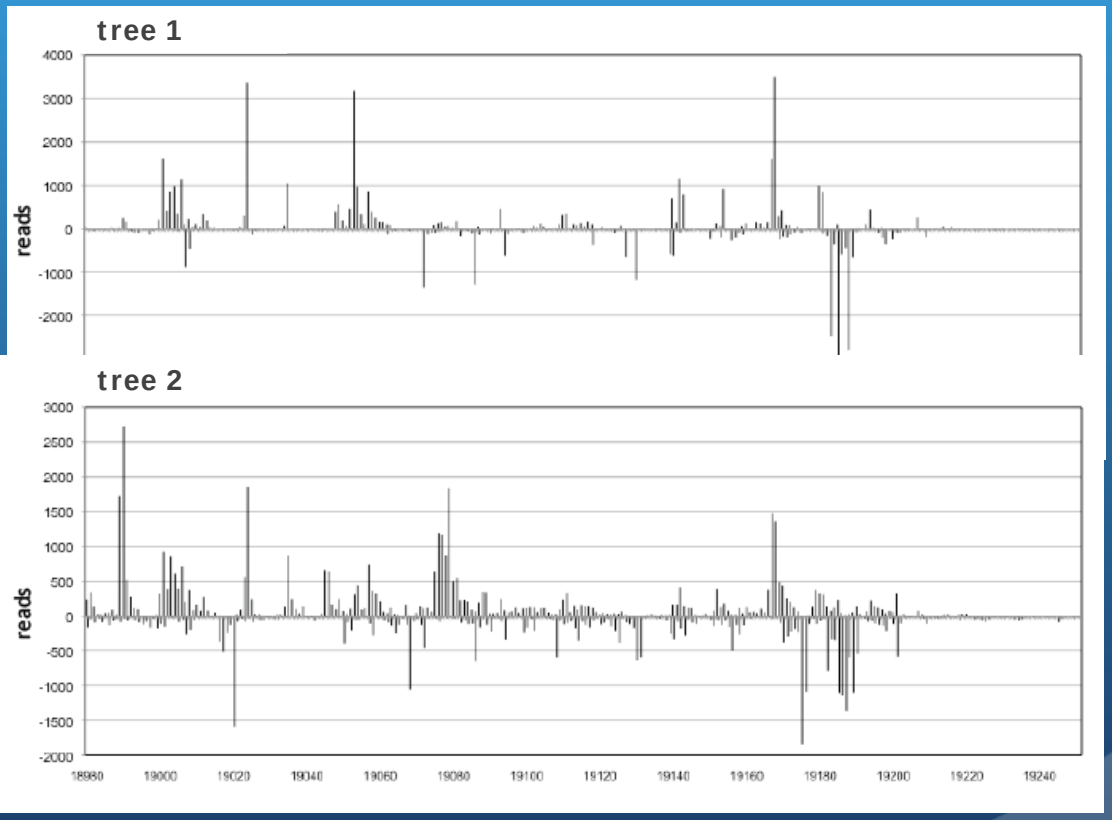
Sequence Analysis



Sequence Processing

Sequence Mapping

BWA mapping on Genome(s)

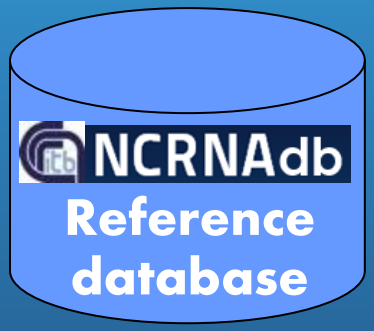


Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics, 25:1754-60

Sequence Processing

Sequence Mapping

BWA mapping on ncRNA Reference



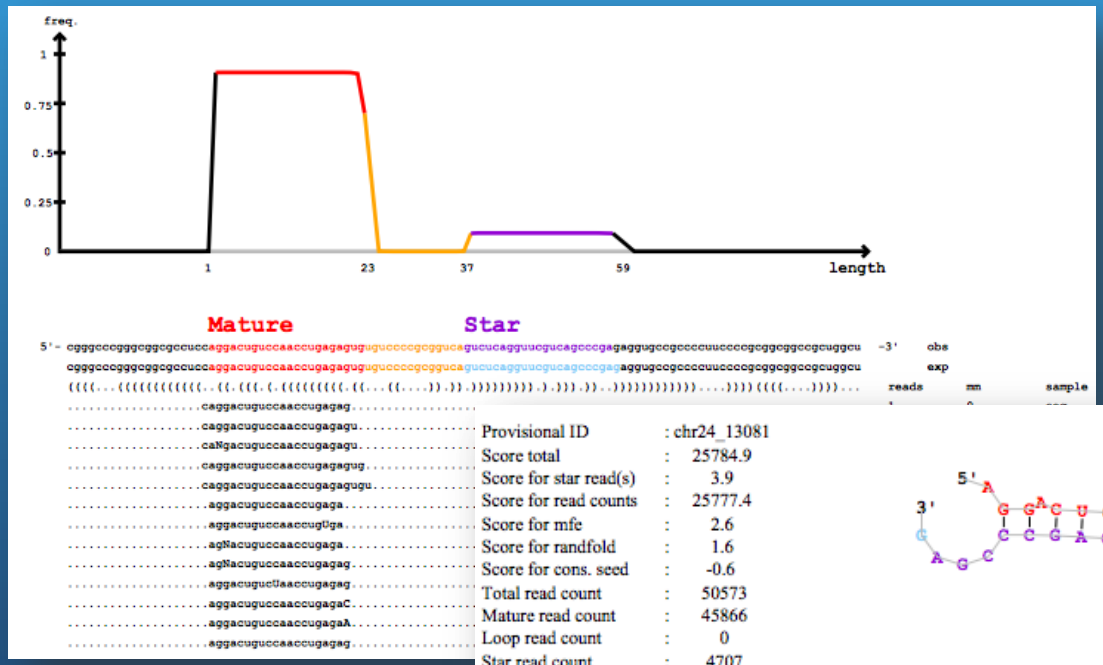

```
1524329 *
 551158 n3435|miRNA|Homo
 452969 n408449|lncRNA|Homo
 452890 n402469|lncRNA|Homo
 452670 n121773|piRNA|Homo
 452589 n203154|pre_miRNA|Monodelphis
 452347 n200513|pre_miRNA|Pongo
 452164 n200465|pre_miRNA|Macaca
 451608 n201030|pre_miRNA|Homo
 451384 n387643|lncRNA|Homo
 411580 n197338|pre_miRNA|Monodelphis
 411074 n201230|pre_miRNA|Pan
 411030 n200698|pre_miRNA|Bos
 410589 n200478|pre_miRNA|Gorilla
 410543 n408444|lncRNA|Homo
 410430 n414739|lncRNA|Mus
 410385 n199475|pre_miRNA|Macaca
 410251 n423547|lncRNA|Mus
 410237 n204227|pre_miRNA|Rattus
 410118 n200501|pre_miRNA|Mus
 409989 n387171|lncRNA|Homo
 409446 n201230|pre_miRNA|Homo
 387835 n200574|miRNA|Macaca
 386659 n204819|miRNA|Monodelphis
 262091 n205000|pre_miRNA|Pan
 261353 n205000|pre_miRNA|Macaca
 252674 n331895|miRNA|Macaca
 252282 n331894|miRNA|Pan
 252115 n200574|miRNA|Homo
 251582 n331896|miRNA|Gorilla
 250300 n200574|miRNA|Gorilla
```

Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. Bioinformatics, 25:1754-60

Sequence Processing

Discovery

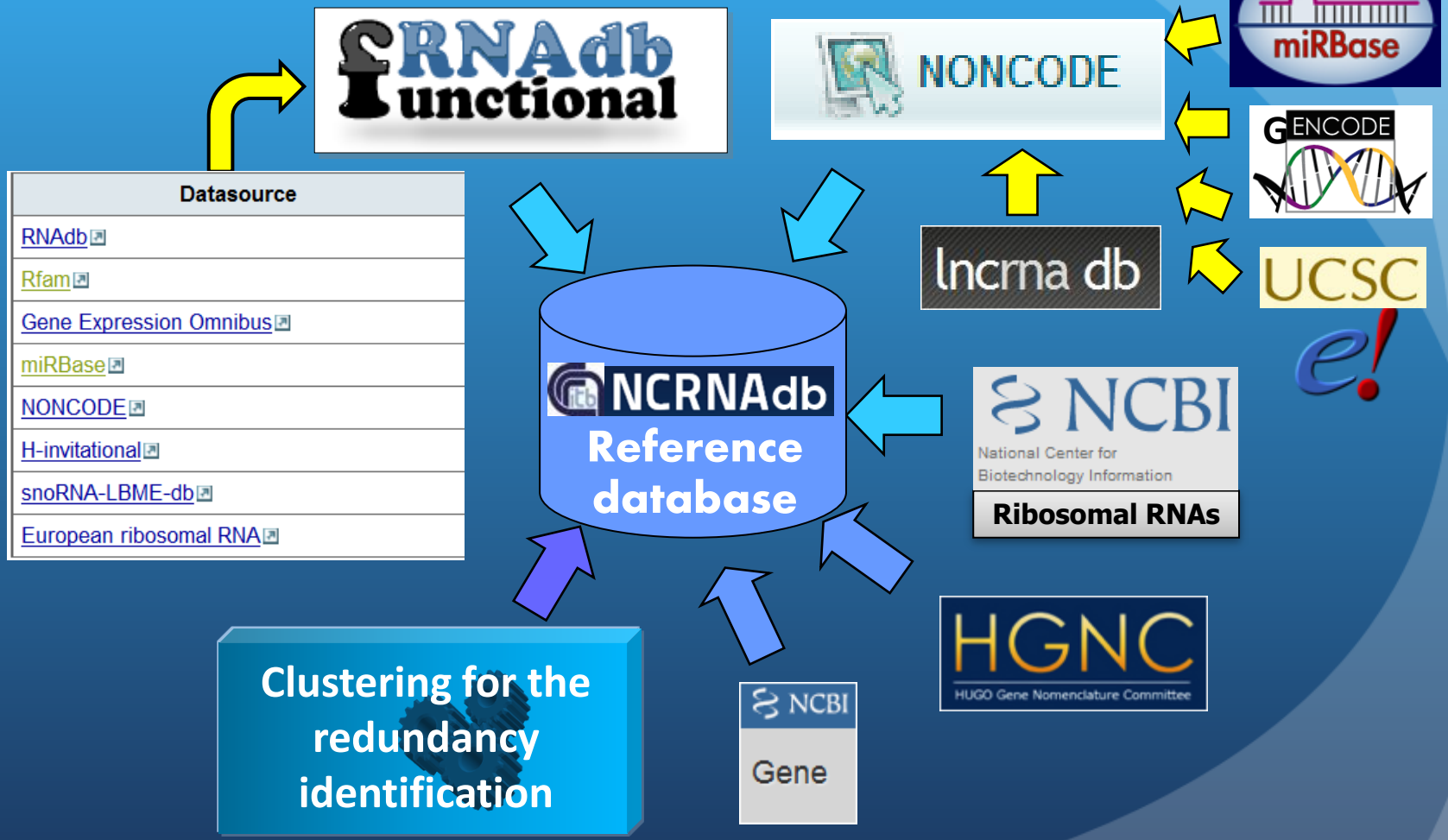
De-novo miRNA discovery using miRDeep2 (animals and plants)



Friedländer, M.R., Chen, W., Adamidi, C., Maaskola, J., Einspanier, R., Knespel, S., Rajewsky, N. 'Discovering microRNAs from deep sequencing data using miRDeep', Nature Biotechnology, 26, 407-415 (2008)

DataWarehouse

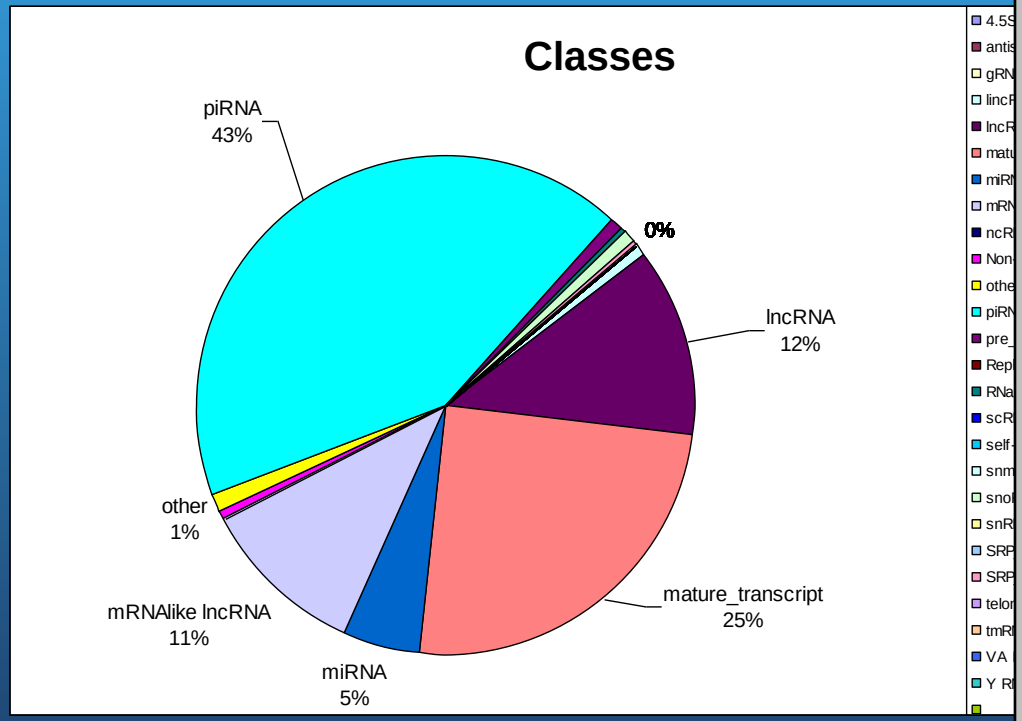
"ncRNADB" Reference Database



DataWarehouse

Some “ncRNADB” Statist

Total sequences: 411552

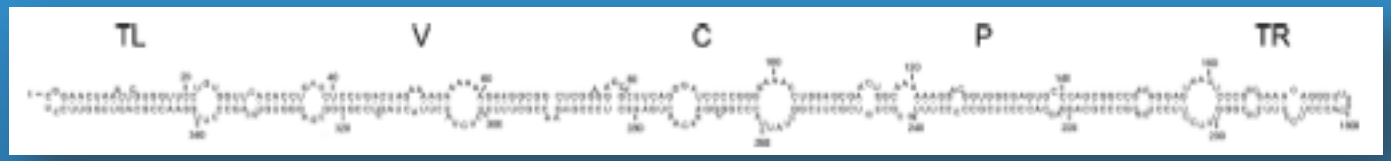


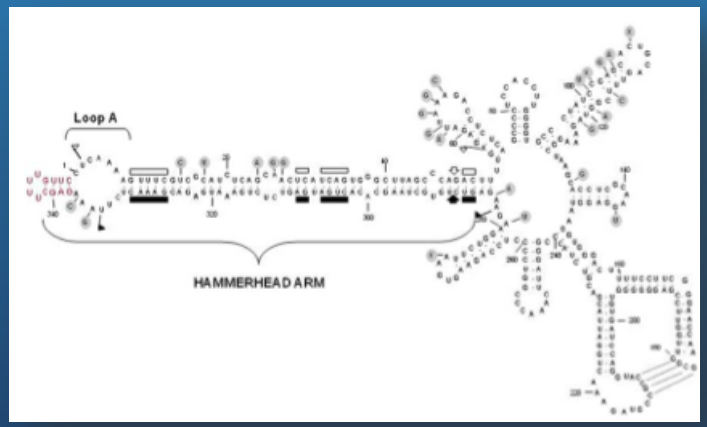
organism	total
Mus musculus	119777
Drosophila melanogaster	102581
Homo sapiens	91286
Rattus norvegicus	66760
Caenorhabditis elegans	4900
Oryza sativa	869
Arabidopsis thaliana	781
Danio rerio	454
Populus trichocarpa	292
Xenopus tropicalis	275
Pan paniscus	274
Caenorhabditis briggsae	271
Pongo pygmaeus	263
Gorilla gorilla	263
Gallus gallus	256
Pan troglodytes	254
Macaca nemestrina	231
Takifugu rubripes	221
Macaca mulatta	221
Bos taurus	211
Tetraodon nigroviridis	209
Saccharomyces cerevisiae	187
Zea mays	165
Monodelphis domestica	159
Lagothrix lagotricha	145

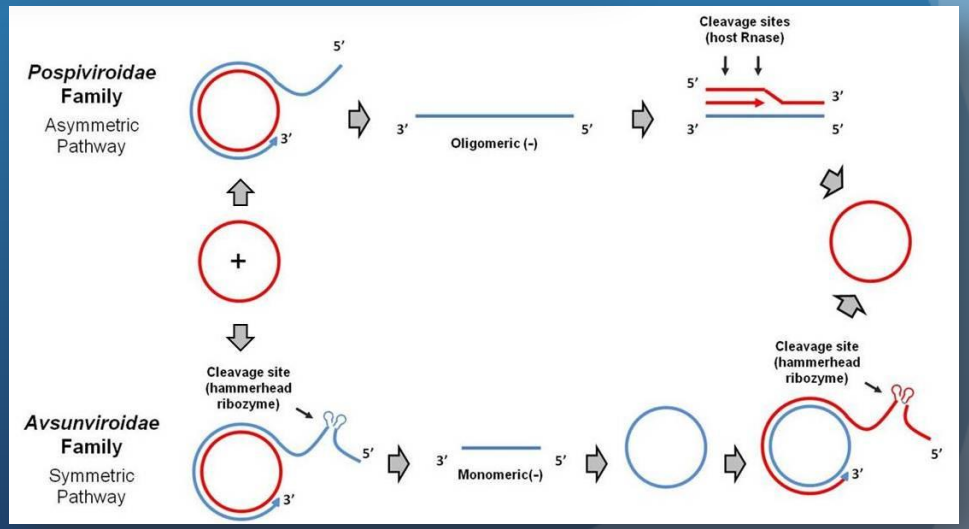
Results

Viroid plant interaction

Viroids ("virus-like") are small circular RNA plant pathogens. They do not code for any protein but can cause heavy symptoms in some ornamental and crop plants.







Results

Viroid plant interaction

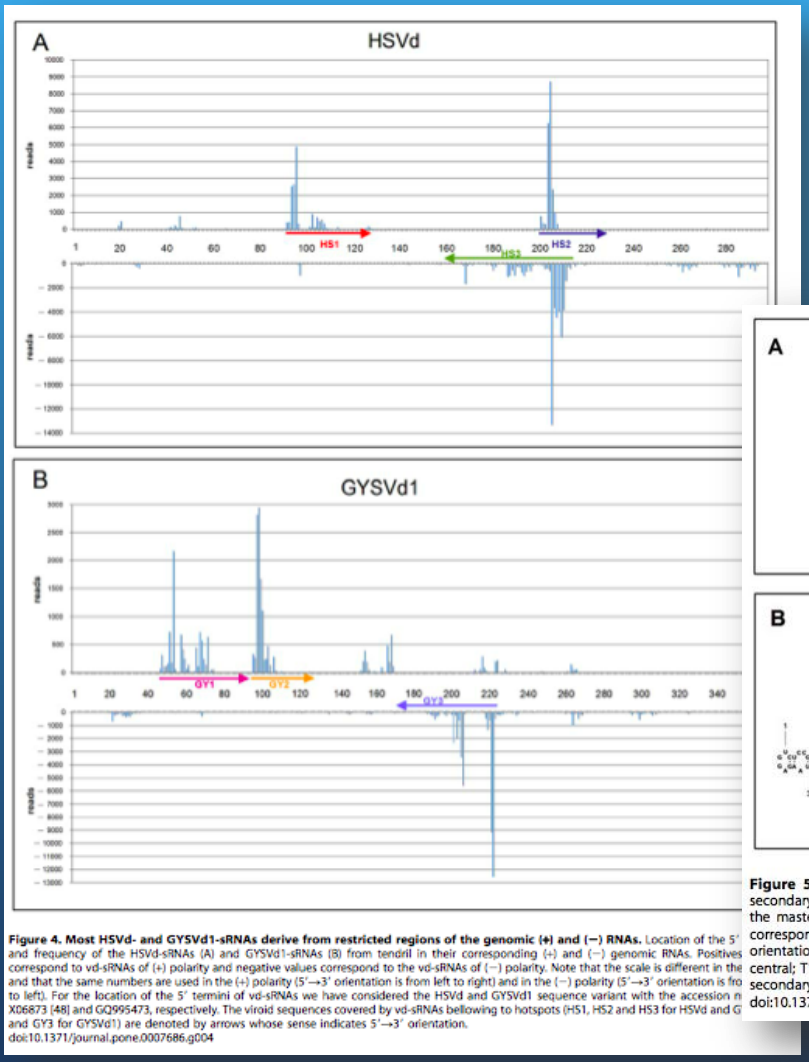


Figure 4. Most HSVd- and GYSVd1-sRNAs derive from restricted regions of the genomic (+) and (−) RNAs. Location of the 5′ and frequency of the HSVd-sRNAs (A) and GYSVd1-sRNAs (B) from tendril in their corresponding (+) and (−) genomic RNAs. Positives correspond to vd-sRNAs of (+) polarity and negative values correspond to the vd-sRNAs of (−) polarity. Note that the scale is different in the and that the same numbers are used in the (+) polarity (5′→3′ orientation is from left to right) and in the (−) polarity (5′→3′ orientation is fro to left). For the location of the 5′ termini of vd-sRNAs we have considered the HSVd and GYSVd1 sequence variant with the accession n X06873 [48] and GQ995473, respectively. The viroid sequences covered by vd-sRNAs bellowing to hotspots (HS1, HS2 and HS3 for HSVd and G and GY3 for GYSVd1) are denoted by arrows whose sense indicates 5′→3′ orientation.
doi:10.1371/journal.pone.0007686.g004

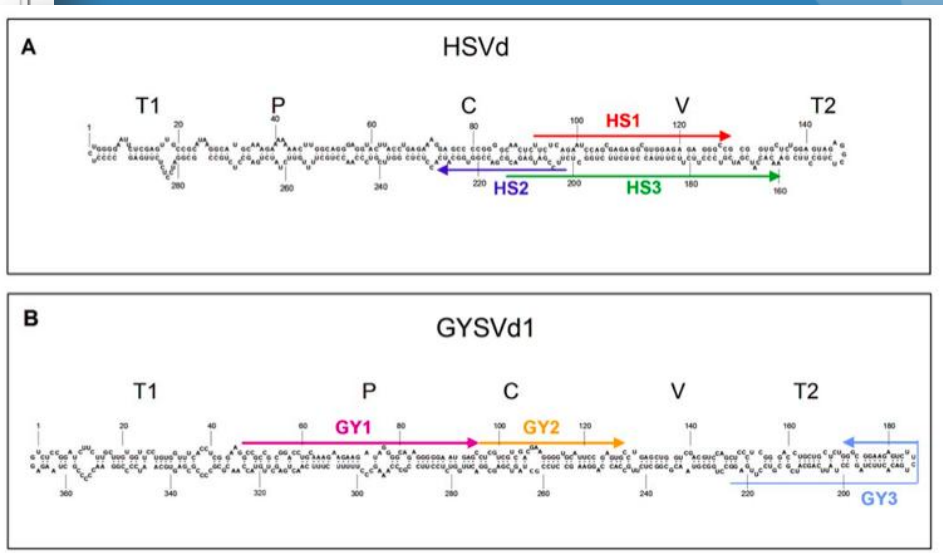
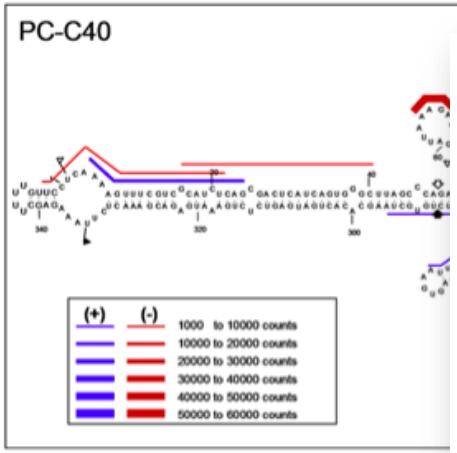


Figure 5. HSVd-sRNAs and GYSVd1-sRNAs do not cover the same viroid structural domains. Sequence and computer-predicted secondary structure for the (+) strand of the HSVd (sequence variant X06873) (A) and the GYSVd1 (sequence variant GQ995473) (B), corresponding to the master and the consensus variants in the grapevine sequenced viroid populations, respectively. The viroid sequences covered by vd-sRNAs corresponding to hotspots (HS1, HS2 and HS3 for HSVd, and GY1, GY2 and GY3 for GYSVd1) are denoted by arrows whose sense indicates 5′→3′ orientation. The position of five structural domains proposed for PSTVd and closely-related viroids [80] are indicated (P: pathogenic; V: variable; C: central; T1: terminal left; T2: terminal right), although no data on the functional properties of these regions in HSVd and GYSVd1 are available. The secondary structures were obtained by the program Mfold [81].
doi:10.1371/journal.pone.0007686.g005

Results

Viroid plant interaction



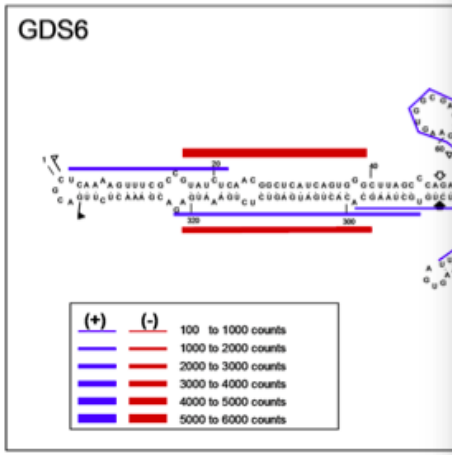


Figure 6. Location and frequency of the 5′ termini of the PLMVd-sRNA
Note that the scale of counts is different in both samples and that only frequen
included. The PLMVd-sRNAs of both polarities are referred to the secondar
thermodynamic, *in vitro*, and *in vivo* data.
doi:10.1371/journal.pone.0007539.g006

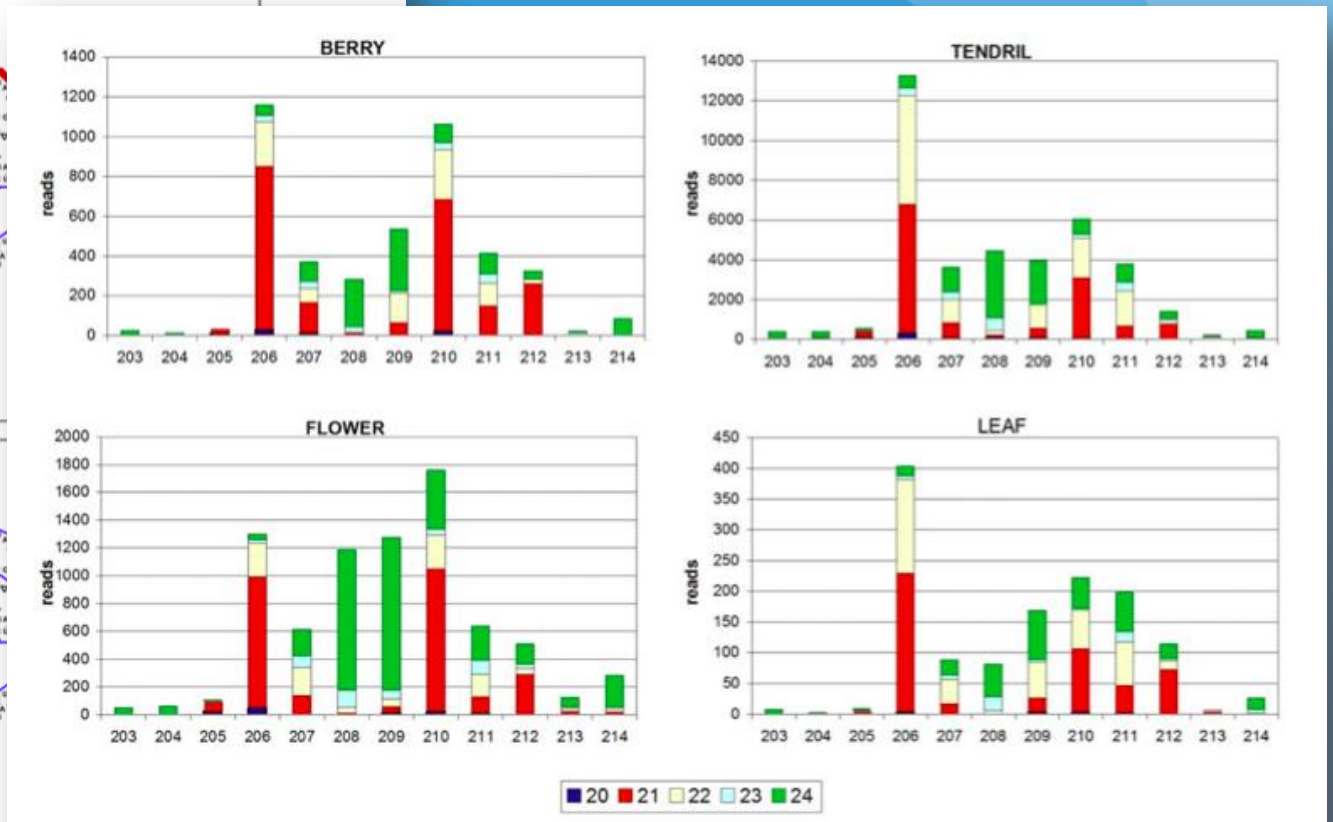


Figure 6. Specific size-classes vd-sRNAs may largely prevail at certain genomic positions. Histograms comparing location of the 5′ termini, frequency and size distribution of (−) vd-sRNAs corresponding to the HSVd hotspot 3 (HS3) and recovered from the different grapevine tissues. Numbers are referred to HSVd sequence variant with the accession number X06873. 5′→3′ orientation is from right to left.
doi:10.1371/journal.pone.0007686.g006

Results

Viroid smallRNA in RDR6 silenced N.benth

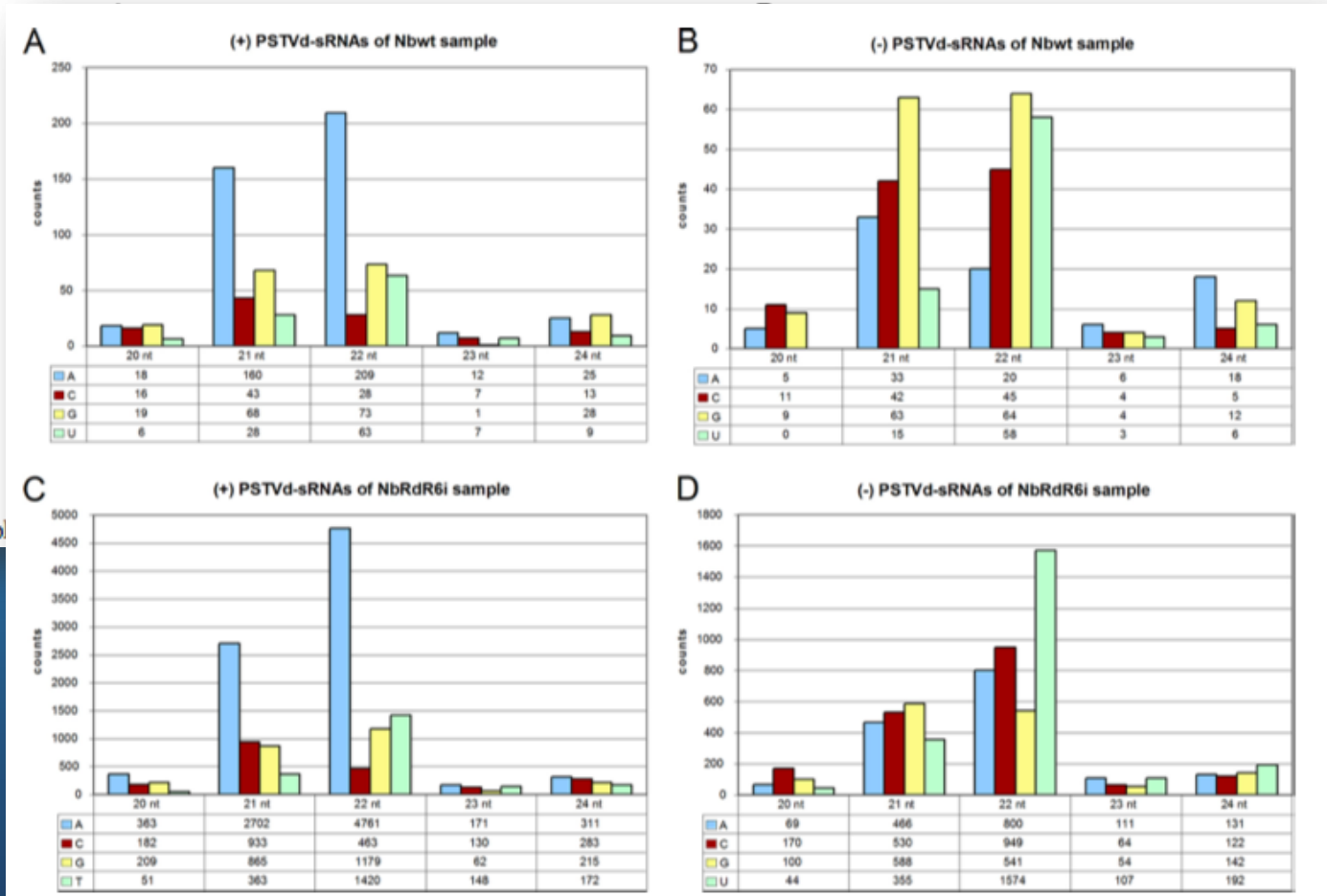


FIG. 6. (unique) pl ...nonredundant ...DR6i samples.

FIG. 8. Biased distribution of the 5′ nucleotide in plus- and minus-strand PSTVd sRNAs. The histograms compare in Nbwt (A and B) and NbRDR6i (C and D) the total counts corresponding to PSTVd sRNAs (20 to 24 nt) of plus polarity (A and C) and minus polarity (B and D) with different 5′ termini.

Results

Host target gene of viroid siRNA

Table 1 Predicted peach mRNAs annotated as transcripts and targeted by PLMVd-sRNAs derived from the PC-associated insertion

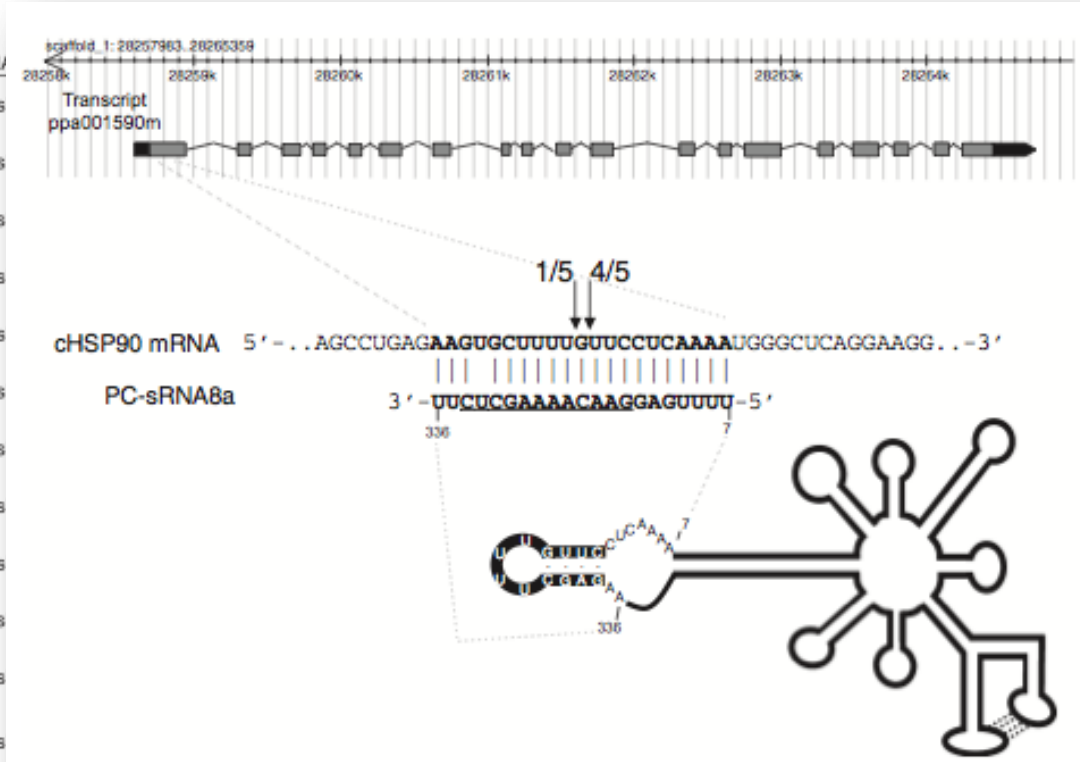
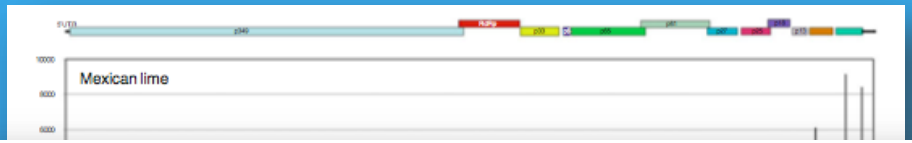


Figure 5. Specific cleavage of peach *cHSP90* mRNA mediated by PC-sRNAs.
Upper panel, predicted *cHSP90* transcript and location of the gene into the peach genome v1.0 (http://www.phytozome.org). Middle panel, *cHSP90* mRNA:PC-sRNA8a duplex (in bold), with the sequence of PC-sRNA8a corresponding to the PC-associated insertion underlined (note that they are of complementary polarity). Arrows mark the predicted and validated cleavage sites by RLM-RACE from five independent clones, with fractions indicating the number of clones producing the same results. Lower panel, schematic representation of the secondary structure predicted for PLMVd variant C40 with nucleotides complementary to PC-sRNA8a highlighted, and those forming part of the PC-inducing insertion on a black background.

sRNA							Chloroplast		
PC-sRNA8a	7	(-)	2	AAGUGCUUUUGUUCCUCAAAA UUCUCGAAAACAAGGAGUUUU	-33.1	1	ppa001590m	Heat shock protein 90. Homolog of *A. thaliana* CR88	asmbl_2300.peachv1pasa0-asmbl_2303.peachv1pasa0	Yes
PC-sRNA8b	7	(-)	169	AAGUGCUUUUGUUCCUCAAAA UUCUUGAAAACAAGGAGUUUU	-30.4	1.5	ppa001590m	Heat shock protein 90. Homolog of *A. thaliana* CR88	asmbl_2300.peachv1pasa0-asmbl_2303.peachv1pasa0	Yes

Results

CTV infected orange plants



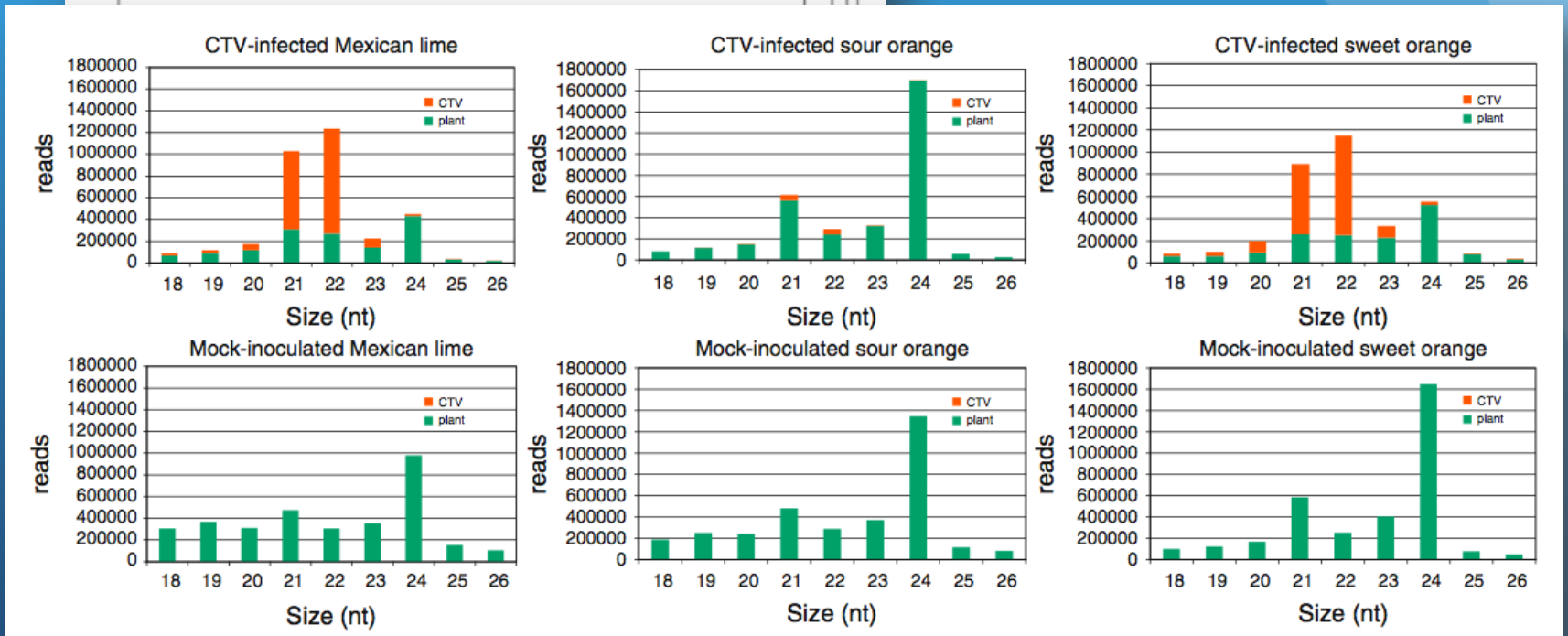


Fig. 1 Size distribution of CTV and plant sRNAs (*orange* and *green*, respectively) in CTV-infected and mock-inoculated Mexican lime, sour orange and sweet orange. The histograms compare the distribution of 18–26-nt total sRNA reads. A minor contamination of CTV-sRNAs observed in the mock-inoculated Mexican lime (representing 0.1% of the total sRNAs of this size) has not been represented

Results

CTV infected orange plants

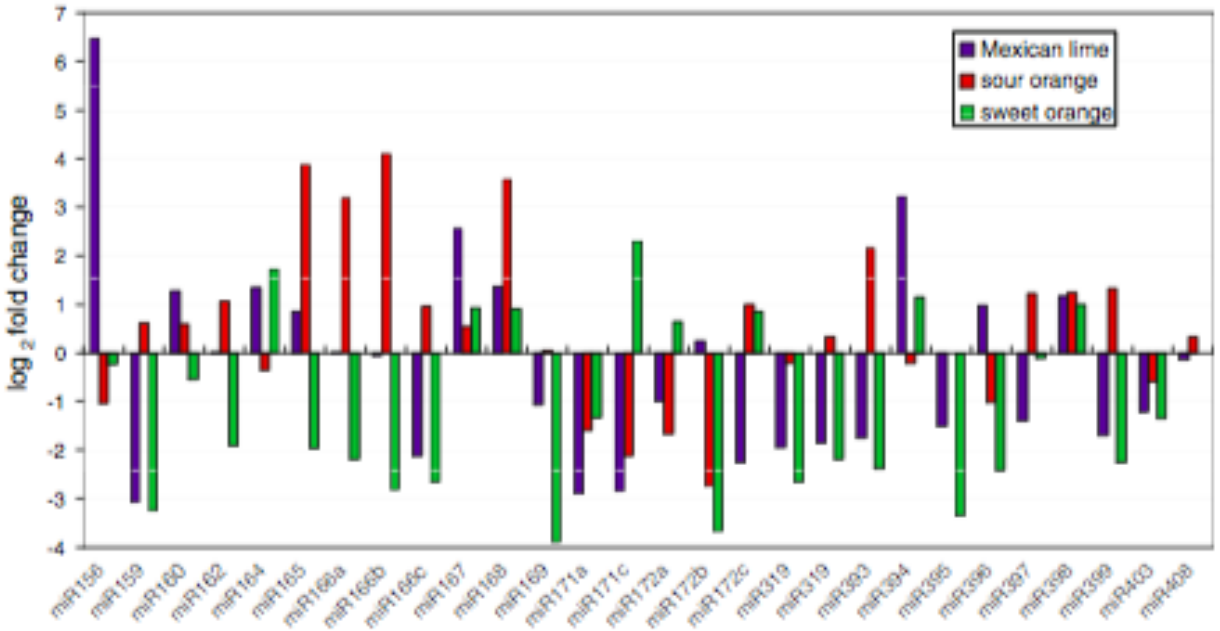
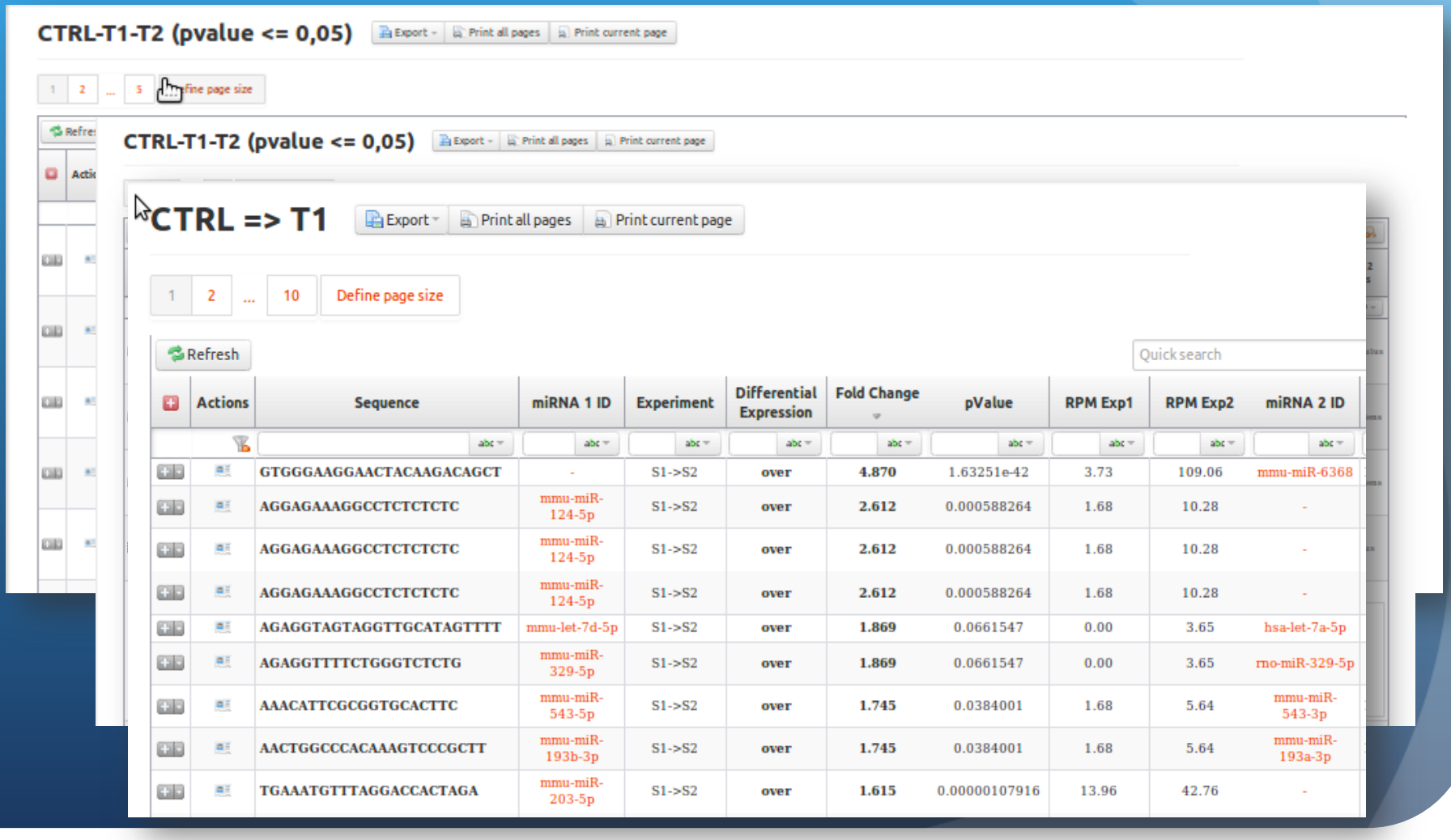


Fig. 8 Effect of CTV infection on the accumulation of specific miRNA from Mexican lime, sour orange and sweet orange. Only miRNAs with more that 10 reads in at least one of the three citrus hosts are considered, and the expression levels of miRNAs are plotted as $\log_2$ of fold change (reads of CTV-infected versus mock-inoculated samples). Some miRNA sequences predicted by comparative analyses (Song et al. 2007) exhibited minor variations with respect to those retrieved from our deep sequencing libraries. In particular: (i) the miR156 and miR393 most frequently sequenced in Mexican lime, sweet and sour orange have an additional nucleotide at the 5′-end when compared with the sequence deposited in the miRbase and predicted, respectively, (ii) the most abundant miR166 and miR172 have their 5′ termini shifted one or two nucleotides downstream with respect to those predicted and deposited in the miRbase, respectively, and (iii) the prevalent miR166 in the three citrus species analyzed is 21-nt long and does not contain the additional 3′-nucleotide proposed in some members of this family (csi-miR166a, csi-miR166b, ctv-mir166, crt-miR166a, crt-miR166b) deposited in the miRbase (see also Supplementary Table S3)

Results

Mouse miRNA fold-change (graphic interface)



Actions	Sequence	miRNA 1 ID	Experiment	Differential Expression	Fold Change	pValue	RPM Exp1	RPM Exp2	miRNA 2 ID
	GTGGGAAGGAACTACAAGACAGCT	-	S1->S2	over	4.870	1.63251e-42	3.73	109.06	mmu-miR-6368
	AGGAGAAAGGCCTCTCTCTC	mmu-miR-124-5p	S1->S2	over	2.612	0.000588264	1.68	10.28	-
	AGGAGAAAGGCCTCTCTCTC	mmu-miR-124-5p	S1->S2	over	2.612	0.000588264	1.68	10.28	-
	AGGAGAAAGGCCTCTCTCTC	mmu-miR-124-5p	S1->S2	over	2.612	0.000588264	1.68	10.28	-
	AGAGGTAGTAGGTTGCATAGTTTT	mmu-let-7d-5p	S1->S2	over	1.869	0.0661547	0.00	3.65	hsa-let-7a-5p
	AGAGGTTTTCTGGGTCTCTG	mmu-miR-329-5p	S1->S2	over	1.869	0.0661547	0.00	3.65	rno-miR-329-5p
	AAACATTCGCGGTGCACTTC	mmu-miR-543-5p	S1->S2	over	1.745	0.0384001	1.68	5.64	mmu-miR-543-3p
	AACTGGCCCACAAAGTCCCGCTT	mmu-miR-193b-3p	S1->S2	over	1.745	0.0384001	1.68	5.64	mmu-miR-193a-3p
	TGAAATGTTTAGGACCACTAGA	mmu-miR-203-5p	S1->S2	over	1.615	0.00000107916	13.96	42.76	-

DataWarehouse

Genomic References

Sequences and Features of:

- ✓ Homo sapiens
- ✓ Mus Musculus
- ✓ Arabidopsis thaliana

ncRNA Projects involved

- ✓ Plant – Viroid interactions in peach tree, grapevine and tobacco
- ✓ Plant – Virus interaction in orange tree
- ✓ Multidrug resistence in dog cell lines
- ✓ Immune response in mouse
- ✓ miRNA driven methylation profile in Arabidopsis

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