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Molecular characterization of the host-pathogen relationships involved during an infection of GF-305 peach trees by the *Peach latent mosaic viroid* (PLMVd)

Caractérisation moléculaire des relations hôte-pathogène impliquées durant une infection de plants de pêcher GF-305 par le viroïde de la mosaïque latente du pêcher (PLMVd)

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Résumé

Parisi Olivier (2010). Caractérisation moléculaire des relations hôte-pathogène impliquées Durant une infection de plants de pêcher GF-305 par le viroïde de la mosaïque latente du pêcher (PLMVd) (thèse de doctorat). Université de Liège, Gembloux Agro-Bio Tech, Belgique. 131 pages, 12 tables, 22 figures.

Le viroïde de la mosaïque latente du pêcher (PLMVd) est un pathogène mondialement répandu et responsable de pertes (directes et indirectes) relativement importantes au niveau de la culture des pêchers. Cependant, peu de données sont actuellement disponibles en ce qui concerne d'une part le(s) déterminant(s) de pathogénicité de ce viroïde et d'autre part les éventuels mécanismes de résistance des plantes vis-à-vis des viroïdes. L'approche originale de ce travail a été de jeter les bases de cette double caractérisation. Dans un premier temps, le rôle du pseudo-nœud P8, commun à tous les variants du PLMVd actuellement séquencés, a été étudié par mutagenèse dirigée. Dans un second temps, la réponse moléculaire de plants de pêchers infectés par des variants de pathogénicités différentes a été caractérisée par le biais de la cDNA-AFLP. L'objectif principal de cette thèse était d'identifier une voie métabolique éventuellement impliquée dans la résistance des plants de pêcher contre ce viroïde.

Au terme de ce travail, il est apparu que le pseudo-nœud P8 était impliqué soit dans la stabilité du viroïde au sein des cellules infectées soit dans la réplication du viroïde. En effet, le variant inoculé présentant un pseudo-nœud déstabilisé a montré une réplication réduite au cours des douze mois de l'étude. De plus, bien que le viroïde muté soit présent dans les plantes inoculées, aucun symptôme n'a été observé. Il est cependant trop tôt pour déterminer si cette latence apparente est due à une quantité trop faible du viroïde ou bien à une implication du pseudo-nœud dans la pathogénicité du viroïde.

La caractérisation de l'expression des gènes de plants de pêchers infectés par des variants de pathogénicité différente a permis de montrer que le PLMVd réprimait des gènes impliqués dans la photosynthèse et en particulier dans la protection des deux photosystèmes. Cette expression particulière des gènes des plantes infectées peut être mise en relation avec les symptômes de chlorose et de mosaïque s'exprimant au cours d'une infection par le PLMVd. Cependant, nous ne pouvons encore affirmer avec certitude si elle est une cause ou une conséquence de ces symptômes. De même, la cDNA-AFLP a permis de mettre en évidence la répression de protéines de choc thermique (HSPs) dans les feuilles symptomatiques. Ces protéines jouent généralement un rôle dans le repliement des protéines ainsi que dans leur assemblage, leur déplacement, leur stabilisation et leur dégradation. La régulation de leur expression peut donc avoir une grande influence dans les plantes infectées et, peut-être, jouer un rôle dans l'expression des symptômes. De même, le gène codant pour une novel cap-binding protein (nCBP) est apparu sous-exprimé dans les feuilles symptomatiques. Le rôle de ces protéines est encore mal connu mais elles pourraient intervenir dans la régulation de la traduction des ARNm. Leur répression peut donc également avoir un impact important et déstabiliser diverses voies métaboliques. Enfin deux gènes codant clairement pour des protéines de défense des plantes ont été identifiés. Il s'agit d'un gène codant pour un intermédiaire de la thiamine (impliquée dans le déclenchement de la SAR, surexprimé dans les feuilles asymptomatiques) et d'un autre gène codant pour une protéine inhibitrice des polygalacturonases (sur-exprimé dans les feuilles symptomatiques). Le rôle exact de ces protéines dans la protection des plantes vis-à-vis du viroïde n'est cependant pas encore clair.

Ce travail constitue une première étude des relations hôte-pathogène établies durant une infection de plants de pêcher par le PLMVd. C'est également le premier, à notre connaissance à avoir analysé l'expression des gènes de plantes infectées en fonction des symptômes observés.

Summary

Parisi Olivier (2010): Molecular characterization of the host-pathogen relationships involved during an infection of GF-305 peach trees by the *Peach latent mosaic viroid* (PLMVd) (Thèse de doctorat). University of Liege, Gembloux Agro-Bio Tech, Belgium. 131 pages, 12 tables, 22 figures.

The *Peach latent mosaic viroid* (PLMVd) infects peach trees in all production areas. This pathogen is responsible of direct and indirect crop losses. However only a few data are available as regards on one hand the determinant of pathogenicity of this viroid and on the other hand the resistance mechanisms of plants against this pathogen. The original approach of this work was to give the foundation of this double characterization. Firstly, the role of the P8 pseudoknot, present in every sequenced PLMVd, was studied by directed mutagenesis. Secondly, the molecular response of different peach trees infected by different variants was evaluated by the use of the cDNA-AFLP. The main objective of this thesis was to identify a metabolic pathway implicated in the plant defence against the PLMVd.

In the term of this work, it seemed that the P8 pseudoknot was implicated either in the stability or in the replication of the viroid into the infected cells. Indeed, the inoculated variant (with a destabilized pseudoknot) has shown a reduced replication during the cultural season. In spite of the presence of the mutated variant in the plants, no symptom was observed on the peach tree leaves. However, we cannot conclude if this absence of symptom is due to the low viroid quantity either to an implication of the pseudo-knot in the pathogenicity of the PLMVd.

The characterization of the gene expression in the infected peach trees has allowed to highlight that the PLMVd represses genes implicated in the photosynthesis and more specifically genes involved in the protection of the two photosystems. This particular gene expression in the infected leaves was linked to the chlorosis and mosaic induced by the PLMVd. However, we cannot conclude with certitude if these symptoms are a cause or a consequence of this particular genes expression. The cDNA-AFLP has also allowed to identify the repression of genes coding for heat shock proteins (HSPs) in symptomatic leaves. These proteins generally have a role in the protein folding, assembly, translocation, stabilization and degradation. The regulation of their expression may have a great influence in the infected plants and, maybe, play a role in the symptoms expression. The gene coding for the novel cap-binding protein (nCBP) was also identified has repressed in the symptomatic leaves. The biological role of these proteins is unclear but it seems that these proteins act in the regulation of the mRNA translation. The repression of nCBP may thus have an important impact and to destabilize various biological pathways. Finally, two genes implicated in the plant defence were identified. One coding for a polygalacturonase inhibitor (over-expressed in symptomatic leaves) and the other one coding for a thiamine intermediate (involved in the SAR and over-expressed in the non-symptomatic leaves). The role of these proteins in the plant defence against the PLMVd is however unclear.

To our knowledge, this is the first work where the host-pathogen relationship established during a PLMVd infection are studied. This is also the first time were the gene expression is linked to the viroid-induced symptoms.

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List of abbreviations

°C	Celsius degree
ADP	Adenosine di-phosphate
ASBVd	Avocado sunblotch viroid
ATP	Adenosine tri-phosphate
BC	Before Christ
Bt	Bacillus thuringiensis
Cab	Chlorophyll a/b binding protein
CChMVd	Chrysanthemum chlorotic mottle viroid
CCR	Central conserved region
cDNA-AFLP	Complementary -DNAAmplified length polymorphism
CEVd	Citrus exocortis viroid
Cq	Quantification cycle
CsPP2	Cucumis sativus phloem protein 2
Ct	Threshold cycle
CVd	Citrus viroid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide tri-phosphate
ds	double stranded
ELIP	Early light inducible protein
ELISA	Enzyme-Linked ImmunoSorbent Assay
FAO	Food and Agriculture Organization
Ha	Hectare
hpRNA	Hairpin RNA
HSP	Heat shock protein
HSVd	Hop stunt viroid
HTS	Hight throughput sequencing

i.e.	id est
Kg	Kilograms
L	Loop
LAMP	Loop-mediated isothermal amplification
M	Molar
min	Minutes
miRNA	micro-RNA
mRNA	Messenger Ribonucleic acid
MT	Mega Tons
nCBP	Novel cap binding protein
NEP	Nuclear Encoded Polymerase
ng	Nanograms
nt	nucleotides
P	Hairpin
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
PEP	Plastid Encoded Polymerase
PGIP	Polygalacturonase inhibiting protein
PLM	Peach latent mosaic (disease)
PLMVd	*Peach latent mosaic viroid*
Pol II	Polymerase
PPV	Plum pox virus
PR	Pathogenesis-related
PSI	Photosystem I
PSII	Photosystem II
PSI-O	Photosystem I subunit O
PSTVd	Potato spindle tuber viroid

PTGS	Post-transcriptional gene silencing
qPCR	quantitative PCR
RdR6	RNA-dependent RNA polymerase 6
RdRP	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RT	Reverse Transcription
Rz	Ribozyme
s	Seconds
siRNA	small interfering RNA
SSC	Sodium Sulfite Chloride
TCH	Terminal conserved hairpin
TCR	Terminal conserved region
TDF	Transcript derived fragment
TGS	transcriptional gene silencing
THI1	Thiazole biosynthetic enzyme
TMV	Tobacco mosaic virus
US	United States
USA	United States of America
v/v	Volume/Volume

Introduction

Viroids are single stranded covalently closed RNA molecules (ranging size from 246 to 401 nucleotides) that do not encode protein. These RNA molecules contain all the needed informations to complete their life cycle including replication and maintenance in host plants. Viroids belong to a group of non coding RNAs that are able to regulate the host gene expression through means others than encoding proteins of specific functions.

Viroids were discovered by Diener in the ‘70s. During the past thirty years, the sequence and structure of these pathogens were successfully characterized. Initially considered as viral pathogens, Diener was the first to characterize these pathogen molecules as a new group and named it “viroids” due to their particular characteristics close to viruses.

Viroids are considered as the smallest exclusive plant pathogens. They cause diseases on economically important herbaceous or woody crops including some ornamentals. Infection of crops by viroids induces generally important economical lost for the producers. However there are actually no curative control methods against these pathogens.

The *Peach latent mosaic viroid* (PLMVd) belongs to the *Avsunviroidae* family. This 340-351 nucleotides RNA molecule possesses a highly branched secondary structure, replicates in a rolling circle mechanism and is insoluble in LiCl 2M. As the other members of this family, the PLMVd molecule contains a hammerhead ribozyme able to cleave the multimers into monomers during the replication.

The PLMVd infects mainly peach trees. This viroid induces chlorosis and mosaic during severe infections but is often latent in orchards. The PLMVd also induces a decaying of the plants and reduces the commercial quality of the fruits by the appearance of a corky suture in their skin.

In the state of the art, this disease cannot be cured and nothing is known about the host-pathogen relationships.

The Plant Pathology Unit has studied viroids and particularly the PLMVd the past six years in collaboration with the Département de Biochimie (Université de Sherbrooke, Canada) where the structure and the ribozyme of the PLMVd have been a research focus for many years.

This research is a starting point of the comprehension of the plants-viroids relationships established during the infection of peach trees by the PLMVd.

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Chapter I
Literature Review

1. Peach: a contemporary crop

1.1. Taxonomy

The peach [*Prunus persica (L.) Batsch*] belongs to the *Rosaceae* family (subfamily of the *Prunoidae*) with other species often referred as "stone fruits". The subgenus *Amygdalus* contains the commercially important peach and almond. Furthermore, four other species are closely related to peach: *P. davidiana, P. kansuensis, P. ferganensi*, and *P. mira*. The former two are used only as roodstocks. The two others are cultivated: as a shrub in Tajikistan and Uzbekistan (*Prunus ferganensis*) and for the fruit in Tibet, Nepal and northern India (*P. mira*) (Rieger 2007).

Ornamental cultivars of peach are used on a small scale as landscape trees. Fully doubled white flowers, deep red flowers, dwarfism and red-leaved traits have been incorporated in ornamental cultivars (Rieger 2007).

1.2. Cultivars

Thousands cultivars of peach are found worldwide, and far more are grown commercially than is seen with many other tree fruits. This is due to the ease with which peaches are bred. Precocity and homozygosity result in early bearing of uniform fruits, which are edible from almost all seedlings. Many regions have their own breeding programs to produce specifically adapted cultivars. Cultivars popular 30 years ago within a given region have been or are being replaced by newer ones. Thus contrary to other crops no single cultivar is dominant worldwide, or even nationwide. However as Scorza and Okie (1990) point out, several cultivars bred in the U.S.A have been adopted by other countries. Peach cultivars fall into one three major groups:

- Nectarines (simply fuzzless peaches)
- Freestone peaches (the fresh market peaches)
- Clingstone peaches (primarily used for canning).

The terms freestone and clingstone refer to the adherence of the mesocarp (flesh) to the endocarp (pit). This degree of adherence does not affect the canning quality, but firm flesh texture is linked to the clingstone trait. Clingstones retain shape better, have brighter color, and produce clearer juice than freestones when canned. It is unfortunate that clingstones were

not named “firm fleshed” and freestones “melting flesh”, since these names would reflect more clearly the characteristics of the two groups. What confuses the issue even more is that stone free-ness is also function of time of maturation for all types of cultivars, with early ripening cultivars tending to be clingstone and latter ones freestone. Uniformity of flesh color, texture, and flavor are important determinants for freestone peaches. In general, early ripening cultivars tend to be of poorer quality than the mid- or late-season cultivars (Janick, 2005; Rieger 2007).

1.3.Origin, history of cultivation

Peaches are cultivated in china since 4000 years. Actual cultivars derive largely from ecotypes native of southern China which has a climate similar to that of the southeastern United States, a major peach growing region. The silk trading route permits the movement of peaches to Persia (Iran) which is where Europeans thought peaches originated. Peaches arrived in Europe and England in 300 to 400 BC with the help of Greeks and Romans. Finally Peaches were spread to the New World by explorers of the sixteenth and seventeenth centuries. The Portuguese introduced it in South America and the Spaniard into the Northern Florida coast of North America. Native Americans and settlers spread it across North America into Southern Canada, and eventually to California, the major production area in the United States today (Janick, 2005; Rieger 2007).

Improved cultivars were available in the 1800s. They are now grafted onto a variety of rootstocks. As for other crops, peach germplasm went through a narrow bottleneck when large, firm-fruited cultivars descended from “Chinese Cling” and “Shangai” were introduced in the 1850s. Since that time, several active breeding programs have broadened the genetic base of peach, adapting it to regions of production from Ontario to the Guatemalan highlands (Rieger 2007).

1.4.Production

Following statistics of the FAO, the world production of peaches and nectarines was 15,561,206 MT in 2004. These two fruits are commercially produced in 71 countries around the world on about 1.4 million ha. The worldwide average yield is just under 11,200 kg/ha and in the past decade the production has increased of 44 percent. This is largely due to an increase in yield as acreage remained constant. In 1993 the China achieved the first production rank due to a great increase of the production yield since 1980. Prior that, the first production rank was held by the United States and Italy. The production of the 10 first countries is summarized in table 1.

Table 1 : Peach production (in percentage of the worldwide production) of the ten first countries.

Countries	Production percentage
China	42
Italy	13
United States	10
Spain	8
Greece	7
Turkey	3
France	3
Iran	3
Chile	2
Argentina	2

1.5. Botanical description

1.5.1. Plant

The plant is vigorous-growing, but relatively small (1.8 to 3 meters) with a spreading canopy. Trees are lived 15 to 20 years and even less on site with a history of peach cultivation. Leaves are linear with acute tips and finely serrate margins, folded along the midrip, sickle shaped in profile, 5 to 12 centimeters in length (Rieger 2007).

1.5.2. Flowers

Peach flowers are light pink to purplish in color and 2.5 to 4 centimeters in diameter. The color of the inner surface of the hypanthium is indicative of the flesh color: whitish green indicates white flesh and gold indicates yellow flesh. Petals can be large and showy or small and curved margins. Flowers are alone on short peduncles (almost sessile) from lateral buds on 1-year-old wood with usually one to two flower buds per node. Flowers exhibit cleistogamy, pollinating themselves before the opening (excepted for some old cultivars like J.H Hale) (Rieger 2007).

1.5.3. Fruit

The peach fruit is a drupe. The endocarp surrounds an oval large single seed. The flesh corresponds to the mesocarp and the skin to the exocarp. Trees produce some fruits in the second or the third year after planting. Peaches require extensive thinning (approximately 80 to 95 percent of the flowers) for proper fruit size development. Early thinning permits to increase the yield of marketable fruits but is not often practiced because frost is a perennial threat and the thinning before the last frost increases the risk of crop loss (Byers et al. 2003). There are no chemical thinner for peach but ammonium thiosulfate can be used to desiccate blossoms. High-pressure water or ropes dragged through the canopy can physically remove blossoms. Usually the thinning is done by hand 30 to 45 days after full bloom, leaving one fruit per 15 centimeters of one year-old shoot length. Fruits that set the second year are usually removed to promote the growth of the tree. The first commercial crop is generally harvested in the third year, with maximal yield reached during the fifth and six years (Rieger 2007).

1.6.General culture

1.6.1. Soils and climate

For an optimal production and tree longevity, deep and well drained soils are needed. In this point of view, loamy to moderate soils are the best.

Soils previously planted with peaches are avoided because they are proned to the "peach tree short life" syndrome, also known as "peach tree decline" which greatly reduces the productivity. Similarly, nematode attacks result in poor growth and reduce the longevity. Irrigation is beneficial for increasing the fruit size, even in humid climate and is essential on shallow soils or in Mediterranean climates (Rieger 2007).

Frost is a problem because peaches are relatively less cold hardy than other tree fruit species. Furthermore, peaches bloom before apple, pear and cherry. This problem is encountered in almost all growing areas. Hence, they are cultivated in Mediterranean climates and are considered to be warm-temperate in adaptation. Peach breeding has permitted to obtain trees tillable from southern Canada to the tropics and peaches have a wider range of chilling requirement than any other tree species. For example, crops growing in the tropic generally did not require chilling. The peach flower buds tolerate -31.6°C when dormant and the wood is killed just below this level. However open flowers and young fruitlets are killed by brief exposure to -33°C or below. As opposed to apples, peaches do not require cold night to develop red skin color: this is more a function of cultivar and light exposure. Peaches ripen during the summer months but cool as well warm summer temperatures give good fruit quality (Janick, 2005 ;Rieger 2007).

1.6.2. Propagation

Peaches are T-or chip-budded onto seedling rootstocks. In warm climates, pits are planted in fall and seedlings develop enough in spring to be "june-budded", so that scion reaches marketable size at autumn. In northern climates, pits planted in fall are budded in Augustus when scion wood is entering dormancy, and buds are not forced to grow out until the following spring. Furthermore peach roots very well from semi-hardwood cuttings contrary to other tree fruits. This provides an inexpensive method of producing own-rooted trees for high-density orchard (Rieger 2007).

1.6.3. Rootstocks

There are few rootstocks for peach compared to pome fruits, grape and other temperate tree fruits. There are actually no dwarfing stocks available. However, semi-dwarf scions have been produced by crossing normal and dwarf types. These have the potential for reducing tree stature in peach orchards. All rootstocks are virtually grown from seed and are fairly uniform due to the self-pollinating, homozygous nature of peach (Rieger 2007).

1.6.4. Interspecific hybrids of peach

Some hybrids were developed to obtain resistance against pests like nematodes. This is the case of the "Nemaguard" which, as the name indicates, was developed for the resistance against root-knot nematodes (*Melidogyne spp*) (Sherman and Lyrene, 1983). It was often used in California until another nematode (*Criconomella xenoplax*) and limited longevity became problematic. Two other hybrids are often used: GF677 ('Amandier') and GF-655 which are peach-almond crosses from France: they tolerate high pH soils and are useful in calcareous orchards. However they are not popular in the United States (Rieger, 2007).

1.7.Common pests and diseases affecting peaches crops

1.7.1. Insects

Conotrachelus nenuphar are small beetles whose larvae burrow into the fruit flesh causing, in most case, the fruits to fall off. Adult feeding usually induces catfacing or D-shaped brown depressions in the fruit surface. Insecticides are applied against this insect beginning at the petal fall and then continue at 7- to 10-days intervals throughout the spring. This pest is confined to the United States east of the Rocky Mountains and is the major insect pest of peach in the southern United States (OEPP, 1990).

Several species of plant bugs or stinkbugs feed on fruits at various times of the year. Early feeding causes severe catfacing or fruits that have extensive indentations. Some fruits may drop if feeding insects are pervasive. Feeding later in the season induces shallow, corky lesions that induce the elimination of the fruits. Furthermore, the incidence of brown rot is enhanced by feeding lesions late in the season. Insecticides are applied at petal fall to shuck split and as needed throughout the season (Rieger, 2007).

Grapholita molesta lays eggs in shoot tips early in the season and the larvae burrow downward a few inches; wilted and dead expanding leaves at the shoot tips indicate infestation. Later infestations result in fruit loss because the larvae burrow the fruits. Although fruit infestation does not occur until fruits are less than half grown, early season sprays are crucial for controlling this insect. Insecticides are applied at petal fall and shuck split (Bulletin OEPP, 2004).

Platynota stultana and *Archips argyrospila* are, respectively, leaf rollers and fruit tree leaf roller bugs which can cause economic damage to peach in California. These caterpillars use webbing to roll leaves together or onto fruits were they feed. The fruit feeding induces scarring or blemishes which render the fruits unmarketable. Bt and other insecticides can be applied in early summer for omnivorous leaf roller, but dormant oils, with or without insecticides can be applied for fruit tree leaf rollers (Rieger, 2007).

Other borers or mites could be damageable for peach trees and their fruits. Insecticides permit usually their control with efficiency. However, mites have several predators that keep the populations in check and miticides are often used during hot, dry weather in absence of natural predators.

1.7.2. Diseases

Monilinia laxa/ Monilinia fructicola cause one of the most severe diseases of peach in humid, rainy climates. It causes flowers to rot in some years and fruits to rot near harvest. The blossom blight phase is seldom a problem itself but it also signals potential problems with brown rot of ripening fruits later. Fruits previously infected die, shrivel, turn black and hang on the tree. These rotted fruits house spores for next year infection. Removal of these in winter or brown-rotted prior to harvest reduces incidence but is not feasible in large orchards. The only way is to apply fungicide during blooming to control the blossom blight and help to reduce brown rot later. During the summer, fungicide sprays can be applied at 7- or 10-days intervals up to one week prior harvest (EPPO standard, 2004).

Cladosporium carpophilum is another damaging disease. It causes numerous black lesions on the fruit surface nearest the stem end but it causes rarely fruits to drop or rot outright. Lesions can be easily distinguished from bacterial spot by the presence of a yellow halo around them. This disease is almost a cosmetic problem and causes a culling of fruits or a

severe downgrade quality. Fungicide sprays are used to control this pathogen (EPPO standard, 2004).

Peaches are susceptible to several bacterial diseases like bacterial spot (*Xamthomonas campestris pv.pruni*) and bacterial canker induced by *Pseudomonas syringae*. These two pathogens share the characteristic to be difficult to control. The bacterial spot can be controlled by the use of resistant cultivars but for the bacterial canker, the only solution actually available is to maintain the tree healthy from other pathogens and limit drought, waterlogging or freezing injuries (Rieger, 2007).

The most important viral disease in several production areas is the Sharka caused by the *Plum pox virus* (PPV) (Damsteegt et al. 2007). Symptoms of Sharka vary and may be confused with disorders like nutrient deficiencies or injuries caused by pesticides (Celetti et al. 2009). Symptoms can appear on leaves as well as on flowers and fruits. The symptoms vary depending on the peach cultivar, the strain of PPV, the age of the infected tree and the environment. They can be observed only on a few leaves or fruits or they can be expressed throughout the entire plant. In some years symptoms may appear in spring and early summer, but fade or disappear during a period of hot weather only to return again later in the season with moderated temperatures (Celetti et al. 2009).

Leaf symptoms include light chlorotic or yellow rings, spots or blotches, yellow line pattern along veins, veins clearing and leaf distortions. Occasionally peach cultivars with showy flowers may display colour breaking on petals. On peach and nectarine, green fruits symptoms appear as faint mottled yellow lines, patches and rings. As fruit matures and ripens the lines, patches and rings remain yellow surrounded by the normal blush color. Because of the uneven repartition of the virus in within the plant, different fruits on the same infected tree may appear symptom-less while other appear severely diseased. Sugar content may also be affected making fruits less desirable for either fresh market or processing. Finally, infected trees rarely die but frequently become less productive as the disease progresses (Damsteegt et al. 2007; Celetti et al. 2009).

2. The Peach Latent Mosaic Disease

2.1. Biological properties

2.1.1. Generalities

Peach Latent Mosaic (PLM) disease was first reported in France during graft indexing (on GF-305 indicator) of peach germplasm imported from the US and Japan. PLM disease is economically important because it affects the fruit quality as well as the lifespan of trees. It also increases the susceptibility of trees to other biotic or abiotic stresses. The causal agent of the disease is the *Peach latent mosaic viroid* (PLMVd) (Flores et al. 2003).

2.1.2. Host range

The PLMVd is currently detected in peach (*Prunus persica, Batsch*.) and nectarine trees. PLMVd can be graft-transmitted to peach hybrids (almond x peach and plum x peach) but attempts to transmit the viroid to other *Prunus* species were unsuccessful (Flores et al. 2003). However in 1997, Hadidi and colleagues were able to detect the PLMVd in sweet cherry, plum and apricot and wild cultivated pears. However unlike peach, the titer and the distribution of PLMVd in other hosts is relatively low.

2.1.3. Symptoms and economical impact

Symptoms induced by the PLMVd on leaves are rare and non permanent in orchards. Occasionally, alterations on the foliage are observed: (A), blurred chlorotic blotches, yellow-creamy mosaics and in the most severe cases white patterns that may cover most or all the leaf area (named peach calico). All these symptoms were reported previously in US and in Japan and are most likely distinct manifestations of PLMVd infections. Under field conditions, symptoms appear two years after planting infected material. It may include delays in foliation, flowering and ripening. Deformation of fruits that present usually discoloration with cracked sutures (B) and flattened stones (C) and a decrease of the fruit taste, but also bud necrosis, cold and diseases sensitivity, open habit (D) and rapid aging are also observed (Figure 1). More sporadically, pink veins on flowers and wood grooving are presented. In greenhouse conditions, on GF-305 indicators, natural PLMVd isolates can be divided into severe, mild

and latent strains depending on the leaf symptoms that they induce (Flores et al. 2003; Flores et al. 2006).

The main economical impact of the infection by the PLMVd is a decrease of the fruit quality (visual as well as organoleptic). Secondly, the rapid aging of the plants, cold and diseases sensitivity enforced the risk of economical losses (Randles 2003).

Figure 1 : Commonly observed symptoms during a PLMVd infection. (A) foliage alteration, (B) fruit cracked suture, (C) flattened stones and (D) open habit

2.1.4. Transmission

PLMVd is transmissible by grafting and budding but no through the seed. PLMVd has also been experimentally transmitted although at low rate, by *Myzus persicae*. Parallel experiments with *Aphis gossypii* and *Aphis spiraecola* did not provide conclusive results. PLMVd is also transmitted by blades either with purified preparations of PLMVd or contaminated with the viroid by slashing infected plants. This latter result indicates that contaminated pruning tools may play a role in viroid spread in commercial orchards (Flores et al. 2003; Flores et al. 2006).

2.1.5. Geographical distribution and epidemiology

PLMVd was found worldwide in all the peach production area (i.e. North and South America, Asia, the Mediterranean basin…) following results of epidemiology studies. This result may reflect bad nursery habits in the receiving country. The work of Hadidi et al. (1997) has shown the presence of the PLMVd in 55% of samples collected in Europe, Asia, North and South America. The interchange of propagative material of infected peach and nectarine cultivars has certainly been the major factor in the PLMVd epidemiology, particularly considering that this viroid is currently latent (Flores et al. 2003).

2.2.Molecular properties

The PLMVd is a single stranded circular, covalently closed RNA molecule of 335 to 351 nt. The PLMVd belongs to the *Avsunviroidae* family and, as other members of this family, adopts a complex highly branched secondary structure insoluble into LiCl 2M (Figure 2) (Fekih Hassen et al. 2006).

The PLMVd presents a hammerhead ribozyme structure on its strand of the plus and minus polarity. The reference sequence (EMBL M83545) has 338 nt consisting in 91 G (25.7%), 87 C (25.7%), 80 A (23.6%) and 80 U (23.6%). Other PLMVd variants with a size comprised between 335 and 342 nt and a high level of punctual mutations have been observed from French isolates D168 (severe), LS35 (latent), Esc76906 (latent) but also from Italian and North American isolates (Flores et al. 2003).

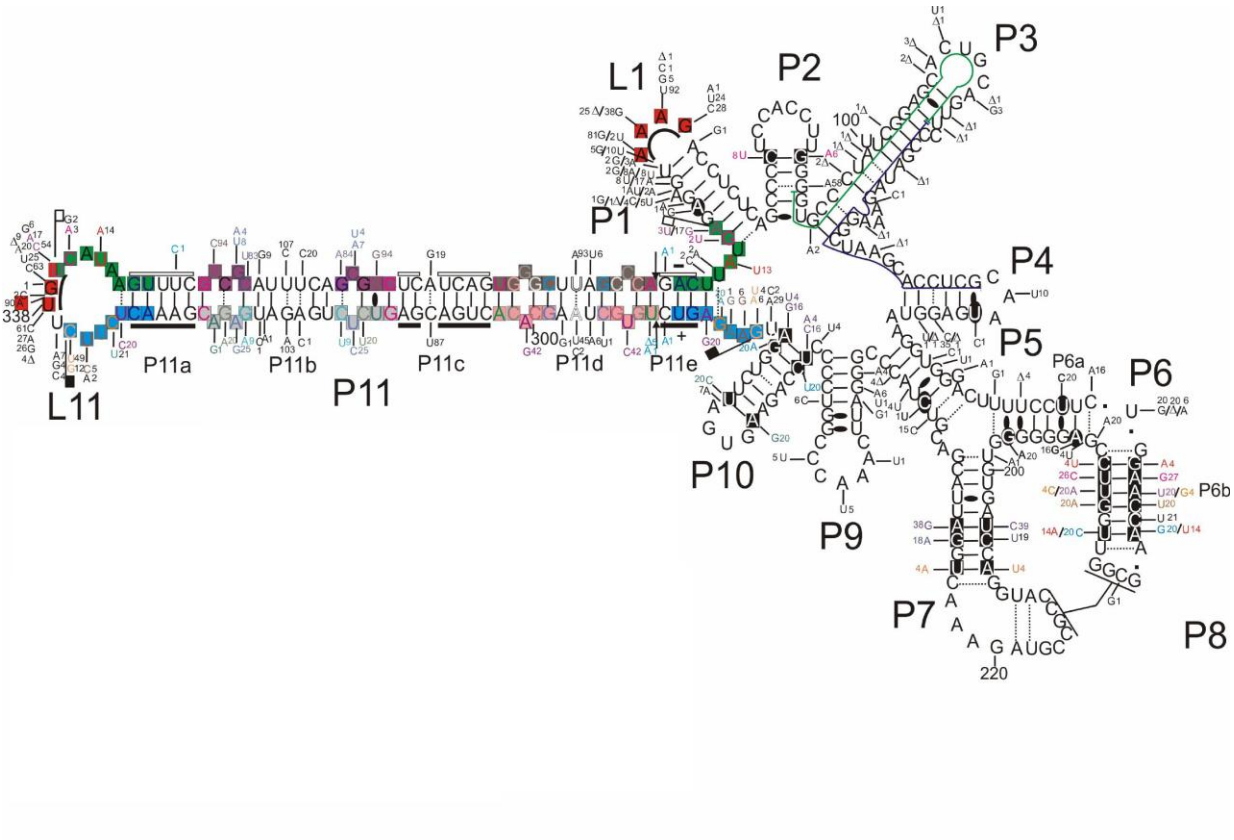
The unequal repartition of the mutations in the PLMVd molecule was demonstrated by Ambros et al. (1998). These authors have suggested that it should be due to three structural constraints limiting the heterogeneity of this viroid:

- the preservation of the hammerhead ribozyme active structure,
- the branched secondary structure,
- a potential pseudoknot interaction between L1 and L11.

Furthermore two structural domains were identified by Pelchat et al. (2000) on American PLMVd variants:

- the left domain, including hairpins P1, P2, P10 and P11 has a highly variable sequence but a conserved secondary structure. The most of the mutations of this domain affects single or double stranded regions. In this latter case, compensatory mutations preserve the secondary structure. This region contains the hammerhead ribozymes required for the PLMVd replication.
- The hairpins P3 to P9 belong to the right domain. This domain could fold in different alternative structures with low sequence variability. These domains have probably a role in the PLMVd life cycle. In consequence, these hairpins suffer selection pressures. This region is maybe involved in the viroid pathogenicity. However this function was not demonstrated.

Figure 2 : Sequences and secondary structure of the PLMVd variants. Most of the mutations found in the 119 sequences are indicated along the secondary structure of the PLMVd reference sequence Ar1 which include a duplication of G at position 258 in this molecule (Beaudry et al., 1995). The frequency of mutations found more than once is indicated in subscript. Base-pairs supported by covariation are squared while those supported by one mutation are in ovals. Nucleotides forming the pseudoknot between L1 and L11 are in red. The symbol Δ indicates a deletion. The symbol + on the left of the RNA molecule indicates the position of 11- to 14-nt insertions found in 6 variants. Regions involved in forming plus and minus polarities in hammerhead structures are flanked by closed and open flags, respectively. The hammerhead consensus sequences of plus and minus polarities are indicated by closed and open bars, respectively. Stems I, II, and III of the plus-polarity hammerhead are in pink, grey, and blue, while those of the minus-polarity hammerhead are in brown, purple, and green. Arrows indicate self-cleavage sites. Continuous and discontinuous lines along the P3 and P4 stems correspond to the positions of the antisense and sense primers, respectively (Fekih Hassen et al, 2006)



A large sequencing, on more than 100 tunisian variants, has permitted to analyze the sequence-structure variations of the PLMVd. Variations appeared predominantly in the regions comprising P1, P6, P7, P9, P10 and P11 stems, as opposed to those including P2, P5 and P8 (Fekih Hassen et al. 2007). An analysis of the sequence covariations has revealed that either, covariations of base-paired residues or nucleotide mutations were detected within the P6a, P6b, P7, P10 and P11 stems. Conversely, only one covariation was found in the P2 stem, and none in the P5 and P8 stems where the sequence seems highly conserved. The selective pressure has maybe preserved them intact. Thus, only P3 and P4 stems might potentially form alternative structures. They provide no covariation-based support for specific structure. The use of primers corresponding to the sequence of these stems may explain the few variations of P3 and P4 stems. The L1 and L11 loops have shown high sequence variability. However,

frequent nucleotide covariations were also observed supporting the formation of a pseudoknot between these loops of the two PLMVd polarities. This pseudoknot could play an important role in the PLMVd stability by closing the replication domain located in the P11 stem (Fekih Hassen et al. 2007).

In this latter study, the analysis of the *in vitro* PLMVd structure has shown that the P7, P9, P10 and P11 stems (with, in some cases, P1 and P6 stems) are the most stable structures. The existence of the P8 pseudoknot was supported by nuclease assay data (Bussiere et al. 2000). A single mutation was observed in all of the sequenced variants supporting that this GC-rich composition is important to preserve its formation. Finally, the variants possessing an insertion in the L11 loop showed various potential structures for this region (Fekih Hassen et al. 2007).

This complete study of the PLMVd sequence-structure has permitted to emit a different definition of the two domains defined by Pelchat et al. (2000). In this new definition, P6 and P7 stems have shown a high level of nucleotide covariations. Moreover, P2 showed less variability suggesting that the right domain is limited to stems P2 to P5 (Fekih Hassen et al. 2007).

Finally, this study on 119 variants has permitted to classify PLMVd variants into two groups. Group I comprises 20 sequences sharing at least 92% homology. Within it two subgroups can be distinguished on the basis of 18 informative positions: I-A (16 variants) and I-B (4 variants). Group II includes 99 variants showing at least 93% homology. This group can also be splitted into three subgroups: II-A (10 variants), II-B (74 variants) and II-C (15 variants) based in 7 informative positions. This group subdivision, applicable to all PLMVd sequences, also supports specific structural features whose implications remain elusive (Fekih Hassen et al. 2007). To conclude on this sequence characterization, it should be noted that group I and group II variants share only 85% of homology which is below the generally proposed 90% to distinguish variants of the same species from different species (Fekih Hassen et al. 2007). However, all other physical and biological characteristics of these variants indicate that they belong to the same species. This suggests that the 90%-homology criterion is not adequate for distinguishing species. Structural similarities, biological properties and ecological niche used for the virus classification should be better criterions for the viroids classification (Fekih Hassen et al. 2007).

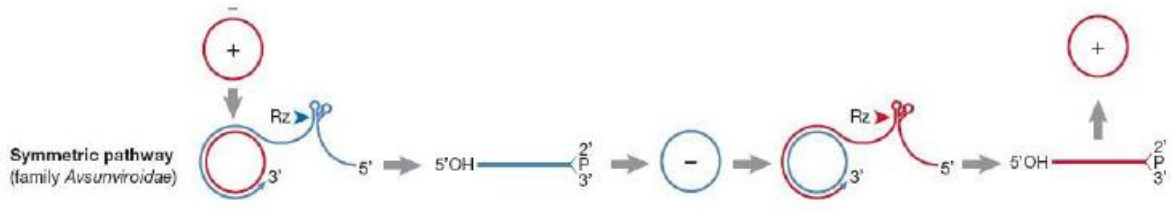
A study of the biological properties of PLMVd variants from different isolates has shown that latent variants induce infections without symptoms. However, the variants from severe isolates induced variable symptoms indicating a mixture of variants (Ambros et al. 1998). Latent and symptomatic isolates (inducing mosaic) are composed by variants whose length is comprised between 335 and 342 nt. However, variants inducing the peach calico have a length comprised between 348 and 351 nt due to an insertion of 12 to 14 nt in the L11 loop (Malfitano et al. 2003).

2.3. Replication

2.3.1. Generalities

The replication of the PLMVd in plants follows a symmetric rolling circle mechanism as presented in figure 3. The PLMVd of the plus polarity (arbitrarily defined) is transcripted by a RNA polymerase in multimers of the minus polarity. These new RNA are then cleaved by the hammerhead ribozyme into monomers which serve as templates to produce the PLMVd of the plus polarity following the same process (Bussière et al. 1999). The polymerase involved in the replication is actually not clearly defined. Some authors stipulate that a nuclear encoded RNA polymerase (NEP) is involved in the replication process. However, for others, a chloroplastic RNA polymerase (PEP, for plastid encoded polymerase) replicates the PLMVd. In another hypothesis, the PLMVd could be replicated by the two polymerases depending on the degree of chloroplast alteration by the infection (Pelchat et al. 2001; Delgado et al. 2005; Rodio et al. 2007).

Figure 3 : Replication of the viroids following the symetric rolling circle mechanism of the *Avsunviroidae*. Rz shows the hammerhead ribozyme (Daros et al. 2006)

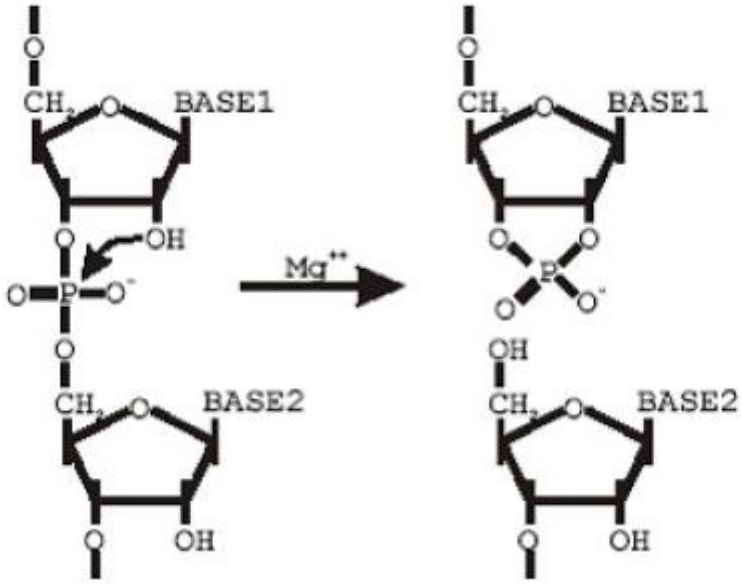


The propagation of the PLMVd following the quasi-species model described by Eigen (1993) was confirmed for the PLMVd. It was effectively shown in two studies that the PLMVd is present in host plants as a population of similar but non identical RNAs (at the molecular point of view). In other terms, in an infected plant, the PLMVd forms a collection of variants (of the same specie) in a dynamic equilibrium and in constant evolution. This appearance of sequence mutations can be explained by the high transcription error rate of the polymerase(s) involved in the PLMVd replication (Ambros et al. 1998; Ambros et al. 1999).

2.3.2. The Ribozyme and the Self-cleavage

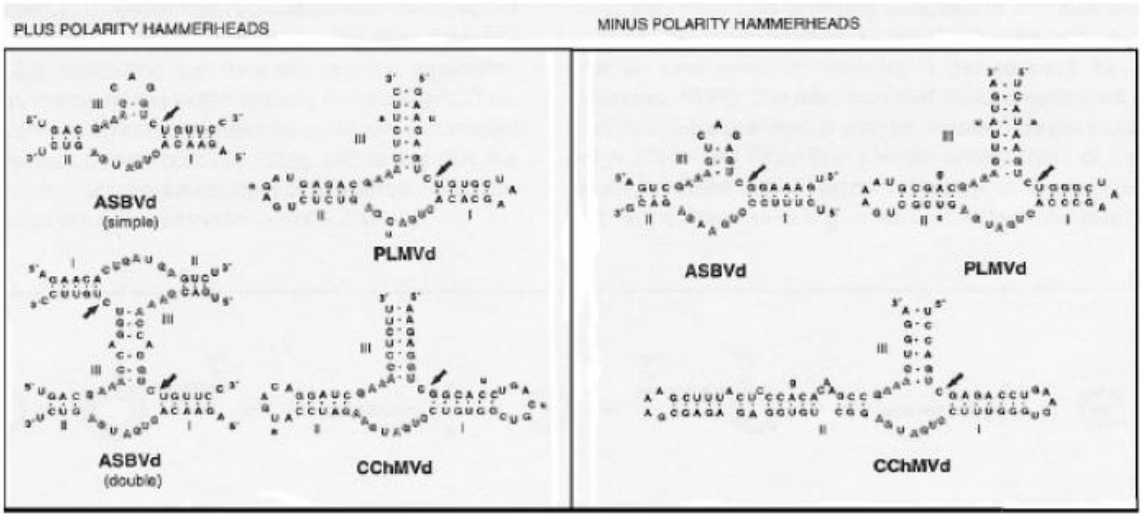
The *Avsunviroidae* can potentially adopt a catalytic structure named hammerhead ribozyme. This structure based on informatics predictions and on sequence co-variations, is conserved in strands of the two polarities of this viroid family (Côté et al. 2003). The hammerhead ribozyme is a small RNA motif formed by three double stranded helixes of non specific sequences surrounding a catalytic core of 11 highly conserved residues. The folding of this structure, in presence of bivalent cations (as the magnesium), leads to the self-cleavage of the RNA at a particular phosphodiester bond with the production of 2',3' cyclic phosphate and 5'hydorxyle ends (Figure 4) (Côté et al. 2003).

Figure 4: Reaction of Self-cleavage (Côté et al. 2003)



The ribozyme structures adopted by the members of *Avsunviroidae* family are slightly different between the species: The *Avocado sunblotch viroid* (ASBVd) can adopt a double stable structure whereas the PLMVd adopts a single stable ribozyme structure highly efficient in the two polarities (Figure 5). This better efficiency leads to the accumulation of the monomeric circular forms in plants infected by the PLMVd (opposed to the ASBVd which accumulates 1 to 8-mer multimeric forms) (Bussière et al. 1999; Côté et al. 2003).

Figure 5 : Structures of the ribozymes of the two polarities in the *Avsunviroidae* family. Arrows show the site of cleavage and roman numerals number the helixes (Côté et al. 2003)



2.4.Detection

In the first time, before the identification of the causal agent of the PLM disease as a viroid, the control of the disease was based on the cross-protection assay performed in "GF-305" peach seedlings grown in greenhouses (Flores et al. 2003; Flores et al. 2006). In this trial, GF-305 seedlings are first inoculated by chip-budding with material from trees to be tested. Approximately two months later they are challenge-inoculated with a severe strain. If the seedlings develop the characteristic symptoms of the severe strain than they were not infected by the first inoculation and the tested plants were healthy. The severe strains are only partially stable and they need to be maintained by periodical inoculations in new indicator plants. The duration of the bioassay is approximately comprised between three and nine months and has enabled the selection and distribution of PLMVd-tested free peach cultivars for the last 25 years (Flores et al. 2003).

The PLMVd can also be detected by polyacrylamide gel electrophoresis and silver staining of leaf or fruit RNA preparations obtained from phenol extraction and chromatography on non-ionic cellulose. However this procedure is not recommended for general use because PLMVd accumulates at very low titer in infected tissues. Molecular cloning of PLMVd has allowed the use of dot-blot hybridization with radioactive and non-radioactive cRNA probes for the detection (Flores et al. 2003).

Since the sequence of the PLMVd is known it is possible to amplify this viroid by RT-PCR using primers derived from the reference sequence. In the 90's several detection methods based on the RT-PCR (like RT-PCR-ELISA and diverse RT-PCR protocols) were developed to detect the PLMVd with more sensitivity. Primers were developed by Fekih Hassen et al. (2006) for the quick and efficient detection of the viroid. This latter test is possible without complex extraction procedure: it requires only a crude sap extraction from leaves with 2X SSC followed by a 100-fold dilution of the extract before the one tube-two step RT-PCR. More recently, the reverse transcription loop-mediated isothermal amplification (RT-LAMP) was developed to improve the sensitivity of the PLMVd detection. This new amplification method is performed under isothermal conditions (60-65°C) with high specificity, efficiency and rapidity. It is characterized by the use of a DNA polymerase with strand displacement activity and a set of four different primers specifically designed to recognize six different regions on the target sequence (Boubourakas et al. 2009). This technique is 100-fold more sensitive than the one-tube two-steps RT-PCR (Boubourakas et al. 2009).

These molecular tools have permitted the simultaneous handling of multiple samples and, additionally, enabled the establishment of the relationship of the PLMVd with specific diseases affecting peach trees on a firm experimental basis. Peach latent mosaic, peach yellow mosaic and peach mosaic diseases, described in France, Japan and US respectively were initially presumed to be induced by the same pathogen based on many common biological characteristics. However data from molecular hybridizations have demonstrated that the PLMVd is only the causal agent of the first two diseases. In contrast, the RT-PCR analysis using PLMVd specific primers showed that PLMVd is not involved in the latter disease which is effectively caused by a virus (Flores et al. 2003).

2.5.Control

There is actually no curative treatment against viroids (and especially PLMVd). However some techniques were developed to regenerate healthy plants form infected ones. From them, thermotherapy (37°C for 45 days) associated with meristem cultures seems efficient. However the best way to preserve orchards and, more generally, cultures from viroids remains the use of healthy planting material (Flores et al. 2003; Flores et al. 2006).

3. Differential gene expression techniques

The visualization of the gene expression is a powerful strategy to characterize the molecular biology of plants. Indeed, the comparison of the expression of mRNA from different genotypes, developmental stages, growing conditions or from healthy and infected plants permits the identification of genes affected by these various factors. One way to characterize the host-pathogen relationships established during a PLMVd infection is to identify the genes affected by the presence of the viroid by the study of their expression in infected leaves. As the transcriptome analysis is an important tool to evaluate the gene expression, several techniques were developed and abundantly used since 20 years (Kuhn 2001).

Ideally a technique for the visualization of the gene expression must respond to the following criteria: (i) visualization of the most of the cellular mRNA, (ii) high reproducibility, (iii) comparison of mRNA from different sources, (iv) easiness and rapidity and finally (v) easy identification and isolation of the gene corresponding to the differentially expressed band in the gel (Bachem et al. 1998).

3.1. The cDNA-AFLP

3.1.1. Introduction

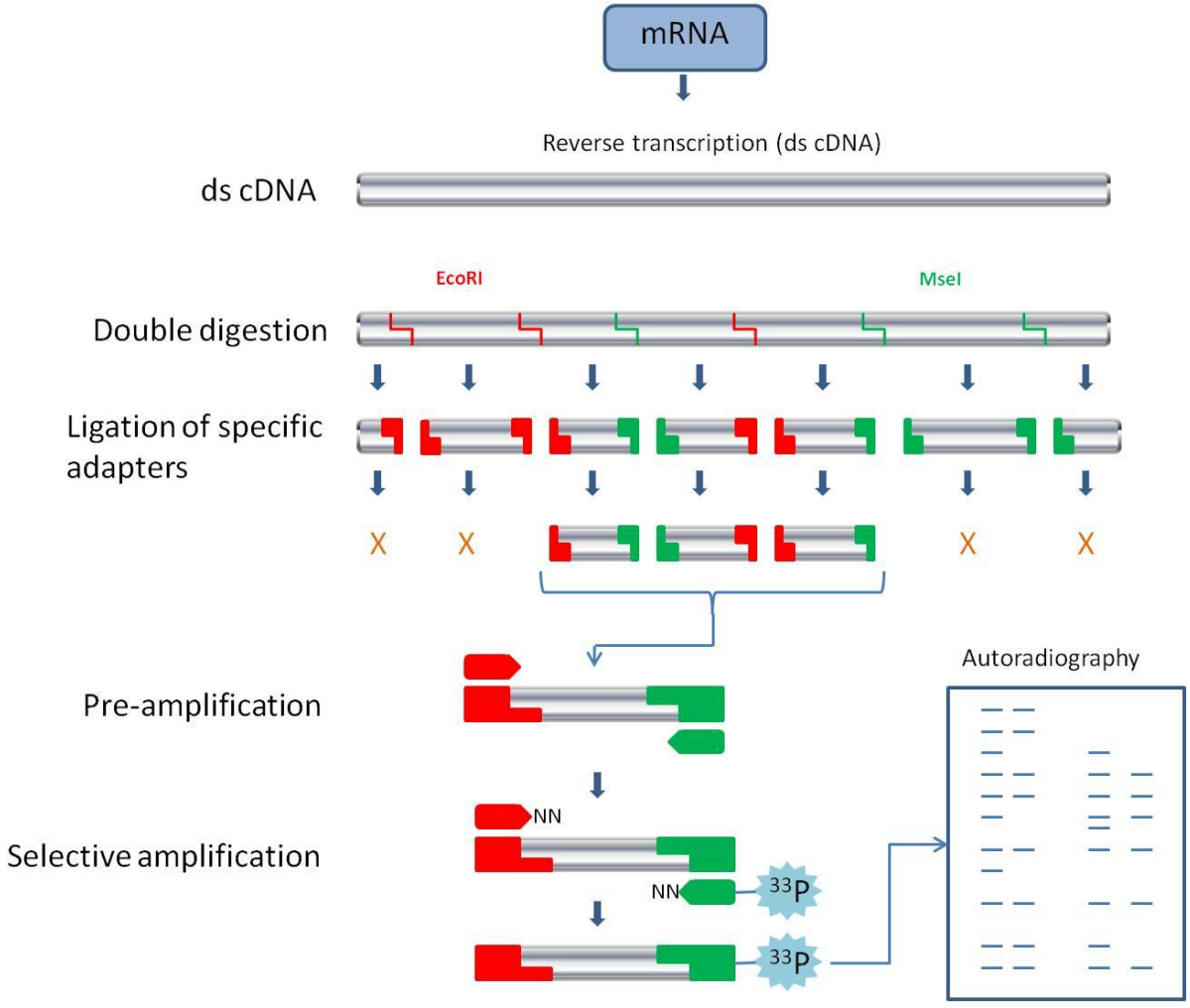
The cDNA-Amplified Length Polymorphism was developed by Bachem et al, (1996) to analyze the expression of mRNA (Massart and Jijakli 2007). Since its first use in 1996, this technique has become a reference for the study of differentially expressed genes (Botton et al. 2008). The technique consists in the use of the RT-PCR technology to compare mRNA populations from different population of cells.

3.1.2. Principle

After the reverse transcription of the RNA, the double stranded cDNA is digested by two restriction enzymes possessing respectively a restriction site of 4 and 6 base pairs. The digested extremities are then ligated with specific adapters. The obtained fragments can be amplified with primers complementary to the adapter sequences. This pre-amplification produces a large amount of fragments which cannot be separated on a polyacrylamide gel.

However, this optional step has the advantage to increase the reproducibility of the results. A second amplification, the selective amplification, is then performed with specific primers. These primers contain the sequence of the adapter and one/two or three nucleotides in their 3' end. This permits the specific amplification of the cDNA fragments possessing these nucleotides after the adapter sequence. Thus a limited number of fragments are amplified which permits their separation on a polyacrylamide gel. To visualize all the possible fragments, amplifications with all combination of specific primers are needed. The radio-labeling of one of the two specific primers increases the sensitivity of the detection and permits the visualization by autoradiography (Figure 6).

Figure 6 : Schematic representation of the cDNA AFLP using EcoRI and MseI as restriction enzymes and radiolabelled with ^{33}P. After the reverse transcription, the ds cDNA is digested with EcoRI and MseI. The ligation of specific adapters allowed the pre/selective-amplification of fragments flanked by the two different adapters. The radiolabelled fragments of interest are the selected after polyacrylamid gel electrophoresis and autoradiography.



3.1.3. Advantages

The main advantage of the cDNA-AFLP is its reproducibility. The amplification in stringent conditions, the use of specific primers and elevated hybridization temperatures increase the reproducibility and limit the false positive results. The cDNA-AFLP is thus considered as a robust, sensitive (permitting the detection of transcripts present in low quantity) and reproducible technique generating only a few false positives (Bachem et al. 1998; Kuhn 2001; Decorosi et al. 2005).

cDNA-AFLP, opposed to other techniques, permits the study of an unknown genome. This technique permits also the study of genes with unknown function (Breyne and Zabeau 2001). Furthermore, the use of all primer combinations permits, in theory, the amplification of all digested cDNA (Donson et al. 2002).

Another advantage of the cDNA-AFLP resides in the obtention of long fragments (>100 bp) which allows a facilitate identification of the genes. Furthermore, the small quantity of mRNA needed (due to the two successive amplifications), is another advantage of this technique (Bachem et al. 1998; Kuhn 2001).

Finally, the cost of the cDNA-AFLP, although higher than the differential display, remains low and makes this technique affordable for relatively small laboratories (Massart 2005).

3.1.4. Disadvantages

The main disadvantage of the cDNA-AFLP is the time consumed for (i) the PCR, (ii) fragment isolation, (iii) the sequencing (shared with the differential display) (Breyne and Zabeau 2001).

Furthermore the false positive results (due to the co-isolation of same size sequences) and the risk to obtain several fragments from one mRNA increase the complexity of the analysis (Lorkowski and Cullen 2004).

Moreover, the repetition of the technique with other restriction enzymes and specific primers is needed to visualize the complete expressed genome (Kuhn 2001). Indeed, if the analysis is performed only with one pair of enzymes, the cDNA which do not possess the restriction site cannot be analyzed (Lorkowski and Cullen 2004).

Finally, the data obtained in different gels cannot be compared as opposed to those obtained with DNA chips (Breyne and Zabeau 2001).

3.1.5. Variations

Some authors have proposed ameliorations to amplify all the reverse transcripted cDNA. The origin of the problem results in the absence of the restriction site (of the six-cutter enzyme) in some cDNAs. The solution proposed by Habu et al, (1997) consisted in the use of only one 4-bases cutter restriction enzyme. This approach permits, in theory, the amplification of all the cDNA but it leads to complex result analysis due to the high number of resulting bands and the presence of more than one fragment for each cDNA (Massart 2005).

3.1.6. Use in plant pathology studies

Several studies have used the cDNA-AFLP to identify differentially expressed genes during plant infections by diverse pathogens like viruses, bacteria, phytoplasma and fungi (Gabriëls et al. 2006; Moser et al. 2007; Wang et al. 2009; Baldo et al. 2010; Wang et al. 2010). These studies were realized to better characterize the host-pathogen relationship, varietal resistances and defense mechanisms involved during an infection.

Recently, a study on sensible or resistant apple genotypes to apple proliferation phytoplasma was conducted. It has permitted to highlight differentially expressed genes between resistant and sensible plants. These genes were classified into two groups: (i) the genes implicated in the stress response and (ii) those involved in the electron transfer transport and synthesis of degradation protein (Moser et al. 2007).

Another study has characterized the gene expression in barley 4 and 12 hours after inoculation of *Blumeria graminis*. More than 615 differentially expressed fragments were revealed. From them, 120 interesting bands were sequenced but many of them do not share homologies with genes of known functions. However, it was possible to identify genes implicated in the metabolism and in the pathogen response (Eckey et al. 2004).

Finally a study has highlighted the differential gene expression during hypersensitive response induced by various pathogens. This study has revealed genes coding for the P450 monooxygensase, cellulases, ABC carrier, a serine threonine kinase and also genes implicated in hypersensitive response and other plant defence mechanisms (Cooper 2001).

The cDNA-AFLP was also used for different purposes with the emergence of new control methods against pathogens. In this case the cDNA-AFLP was used to characterize the relationship between pathogens and their biocontrol agents (Massart and Jijakli 2007).

3.2. High-throughput RNA sequencing

Recently developed the High-Throughput RNA sequencing (HTS) technology has led to powerful strategies able to interrogate comprehensively nucleic-acid-based information in a cell with a high resolution and depth (Qui, 2009; Lister 2009). The diversity of applications involving this technology has demonstrated the immense range of cell-processes that can be studied at the base-level. From them, the HTS, can be applied for the study of the transcriptome as an interesting alternative to the other classical technique. It offers the following advantages: (i) quantitative detection of mRNA, (ii) detection of rare transcripts and small RNA, (iii) consist in an open strategy to study the transcriptome. In consequence, this technique cannot be ignored for the future studies of the plant transcriptome and particularly in the study of plant-pathogen interactions.

3.3. Conclusion

After this light review of differential gene expression visualization techniques it appears that the cDNA-AFLP was probably the best adapted technique to characterize the host-pathogen relationship in PLMVd infected peach trees. This technique permits the study of the host-pathogen relationship without knowledge on the studied genome. Furthermore a partial view of the differentially expressed genes will be available with a limited numbers of primer pairs. Furthermore this technique has proved its sensitivity and reproducibility in the past. Finally the cost of the technique is affordable for the Plant Pathology Unit.

4. The Real-Time PCR

4.1. Introduction

The real-time PCR is a largely used technique. In plant pathology studies, it is used to identify and quantify pathogens but also to confirm the expression of genes revealed during a differential gene expression analysis (like cDNA-AFLP).

4.2. Basis

The real-time PCR is based on the detection and the quantification of the amplification products by the fluorescence emission during the amplification cycles. The increase of the fluorescent signal is directly proportional to the amplicons quantity generated during the amplification. By the observation of the fluorescence quantity emitted each cycle it becomes possible to follow the PCR reaction in its exponential phase (where the first significant increase in the quantity of amplicons is correlated with the initial matrix quantity). The cycle from which the product becomes detectable is named threshold cycle (Ct). This point must appear during the exponential phase.

The main advantages of the real-time PCR are its rapidity, the limitation of contamination problems and a higher sensitivity. However the technique is more expensive than the classic PCR for both reagents and the equipment.

4.3. Probes and fluorescence emission

Two types of fluorescent molecules are commonly used for the real-time PCR: molecules which bind double stranded DNA with no sequence specificity (Sybr-Green) and molecules which possess sequence specificity (Taqman probes).

4.3.1. Unspecific molecules

The fluorescence of these molecules increase when they are bound to double stranded DNA formed by the amplification products. This approach has the advantage to do not require the development and the synthesis of a specific probe. Furthermore these molecules are less expensive than the DNA probes.

However, the simplicity of this technique is balanced by the absence of specificity. Every amplified fragments as well as primer dimers produce a detectable signal which induces false positive results. This disadvantage can be limited by an optimal selection of primers and the analysis of melting curves.

The SyBr-Green is the most used molecule. This fluorescent reagent binds the double stranded DNA and emits a fluorescence 1000-fold more important than in solution. Furthermore the SyBr-Green is stable and is not degraded during the PCR cycles.

4.3.2. Specific molecules

Probes specifically bind to a complementary target sequence in the amplification product. The functioning of these probes shares the same basis. The probe is paired with a fluorophore (also named reporter) and a fluorescence absorber (the quencher). In a first time, the quencher is located near the fluorophore and no fluorescence is detected.

The fluorescence emission is based on the spatial separation of the reporter and the quencher after the hybridization of the probe to its complementary sequence. The probes were categorized into two groups following the separation mechanism:

Hydrolysis probes (Taqman and 3'MGB) for which an enzymatic reaction is needed to degrade the probe and release the reporter.

Hybridization probes (Molecular beacon and Scorpion primers) for which a change in the conformation during the hybridization to the target sequence permits the separation between the reporter and the quencher.

Probes ensure a higher specificity and sensitivity of the real-time PCR. However, the cost and the complex design of these molecules constitute a real disadvantage.

Because the SyBr-green permits a good compromise between the cost and the sensitivity, it was chose for this work to confirm the expression of the interesting genes.

4.4. Results analysis

As described below, the amplification and the melting curves as well as the ΔΔCt methodology were used to confirm the gene expression in this work.

4.4.1. Amplification Curves

The fluorescence emitted during the amplification is measured during each cycle and the computer realized the amplification curves for the samples.

4.4.2. ΔΔCt Method

This method permits to correct the experimental variations and determine the relative expression of a gene. The Ct mean (for 3 replicates) is calculated for each gene in the two tested conditions. Next, the difference between the Ct mean of the interesting gene and the endogenous gene is calculated for the two tested conditions (ΔCt). The result gives the normalized gene expression for each condition. Finally, after the calculation of the normalized gene expression differences (ΔΔCt), the relative expression is calculated by the formula:$2^{-\Delta\Delta Ct}$.

4.4.3. Melting Curves

These curves permit to verify the specificity of the amplification. After the PCR steps, the products are slowly dissociated by an increase of the temperature. The first derivative of the fluorescence permits the determination of the melting temperature (Tm) of the PCR product. This Tm is then compared to the theorical Tm of the product to ensure the specificity of the amplification product.

4.5. Uses in plant pathology studies

The real-time PCR allows an absolute or relative quantification of a pathogen specific sequence. It is thus possible to evaluate the importance of an infection or to compare the replication rate of pathogens.

This application of the technique was applied by Zhong et al. (2006) to study the effect of mutations on the PSTVd replication. Mutations were introduced in the loop E of the PSTVd and the mutants were inoculated in *Nicotiana benthamiana*. In the plant inoculated with the variants possessing mutations leading to an abnormal formation of the loop E, no viroidal accumulation was observed with the classical detection technique. Other variants with compensatory mutations preserving the tertiary structure of the loop E, replicate with efficiency in the infecting plants. However, the real-time PCR has permitted to demonstrate

that the accumulation level (in molecule of the minus polarity) was 10 to 100-fold less for the variants with the compensatory mutations and 1000-fold less for the variant with an affected structure of the loop E.

Another example of the use of the real-time PCR in plant pathology studies is given by Ruiz-Ruiz et al. (2009). The authors have quantified the *Citrus leaf blotch virus* (CLBV) in the host pant by reverse transcription real-time PCR (RT-qPCR). This technique was 1000-fold more sensitive than the classic RT-PCR detection method and allowed to detect very small quantities of the pathogen in the plant tissues.

Finally, the real-time PCR technology permits also the study of the plant gene expression in different situations to establish an eventual link between the gene expression and the infection.

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6. Plant-RNA viroid relationship: a complex host pathogen interaction

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Plant-RNA relationship: a complex host-pathogen interaction

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6.1.Abstract :

Viroids are non encapsidated small RNA plant pathogens unable to produce any protein. They are able to infect dramatically a broad range of plants including herbaceous and tree crops. The ways by which viroids are able to induce diseases are actually unknown. However, recent studies have shown that (i) viroids are able to regulate the gene expression of their hosts, (ii) they can modify the host-protein phosphorylation sensibility and (iii) they interact with host-protein implicated RNA trafficking and protein phosphorylation. Moreover during their evolution plants have developed a mechanism able to regulate their gene expression and to degrade exogenous RNAs like viroids: the gene silencing. Unfortunately, this pathway seems, now, also highly implicated in the symptoms development. This review describes studies that are realized since a few years to increase the knowledge about the plants-viroids relationship.

Keywords: Viroids, Plant-interaction, gene silencing, protein interaction, Peach tree disease

6.2.Introduction

Viroids are considered to belong to a group of non coding RNAs that are able to regulate the host gene expression through means other than encoding proteins for specific functions (Qi and Ding, 2003).

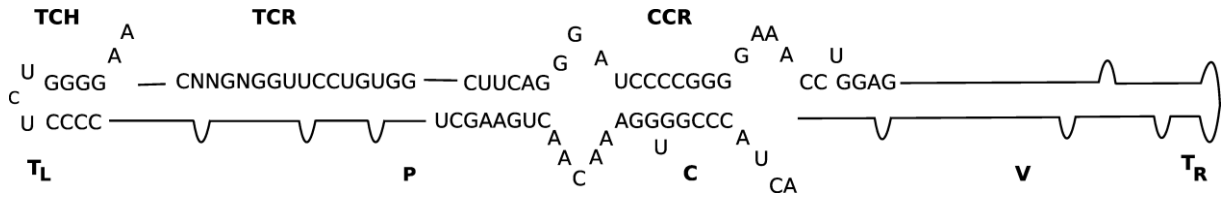
Viroids are the smallest plant pathogens known so far. They only infect plants and cause diseases on economically important herbaceous and woody plants including some ornamentals (Tessitori et al., 2007). Morphological and cytological changes associated with viroid infections are well documented. Typical symptoms are intensified by high temperature and from them, leaf epinasty, chlorosis and stunting (accompanied by a reduction of the root mass) are the most frequent. At the cellular level, the distortion of the cell walls and the plasma membranes are the most visible symptoms (Itaya et al., 2002; Tessitori et al., 2007)

Viroids are studied since the '70s (Diener, 1971) but most studies focused on the primary and the secondary structures of these pathogens, or on the interaction between viroids and plant proteins. These studies have led to a better knowledge about the structure, the conformation, the replication and the pathogenicity of viroids. Unfortunately there is actually a lack of understanding the host-pathogen relationship. This includes the molecular mechanisms of a such relationship, as well as the interactions between viroids and host plant species (Tessitori et al., 2007). However since a few years some studies are undertaken on this research field. A recent review has been published on the interactions between viroids and their hosts and is focused on the replication mechanisms, the structure and the trafficking of the viroids in plants (Ding, 2009). Our review will examine the molecular aspects of the viroid infections in their hosts and particularly the viroid pathogenicity and the plant defence mechanisms.

6.3. Viroids: two families of small plant pathogenic RNAs

Viroids are single stranded small RNA molecules [246-401 nucleotides (nt)]. They are not encapsidated and do not code for any protein (Diener, 1971; Daros et al., 2006). Those molecules possess all the information to complete their life cycle by interacting with host proteins without the need of a helper virus. Viroids can be classified into two families based on biochemical and structural characteristics: the *Avsunviroidae* and the *Pospiviroidae*. The type species of the *Pospiviroidae* family is *Potato spindle tuber viroid* (PSTVd). The *Pospiviroidae* are soluble in LiCl 2M, replicate in the nucleus and some of their functional domains are already identified in their sequence such as a Central Conserved Region (CCR), a pathogenicity domain (P), a Terminal Conserved Region (TCR) and a Terminal Conserved Hairpin (TCH) (Figure 1) (Flores et al., 2004). The sequence of the CCR and the presence or the absence of the TCR and TCH permit to classify the 26 members of this family into five genera (Flores et al., 2004). The PSTVd and by extension the other members of its family adopt *in vitro* (and most-likely *in vivo*) a rod-like secondary structure characterized by alternating double-stranded and single-stranded regions. Mutations, deletions or repetitions observed in the sequence preserve the rod-like secondary structure. This structure has been divided into five structural/functional domains: (i) Central (C), (ii) Pathogenic (P), (iii) Variable (V), (iv) Terminal right (Tr) and (v) Terminal left (Tl). The CCR is localized into the C domain, the TCR and the TCH are within the Tl domain (Keese and Symons, 1985; Flores et al., 2004). Functions have been associated with some of these structural domains: the C domain (and particularly the upper strand of the CCR) is involved in the cleavage and ligation of the multimeric PSTVd RNA intermediates during the rolling circle replication. The P domain is involved in the pathogenicity of the *Pospiviroidae*, probably in interactions with the Tr, Tl et V domains (Gora-Sochacka, 2004).

Figure 1 : Characteristic scheme of the rod-like genomic RNA of the *Pospiviroidae* family with the central (C), pathogenic (P), variable (V), and terminal left and right (TL and TR, respectively) domains. The central conserved region (CCR; genus Pospiviroid), the terminal conserved region (TCR; genera Pospiviroid, Apscaviroid and part of Coleviroid) and the terminal conserved hairpin (TCH; genera Hostuviroid and Cocadviroid) are also shown (modified from Daros et al., 2006)



The *Avsunviroidae* family, whose type species is *Avocado sunblotch viroid* (ASBVd), regroups the four viroids which precipitate in LiCl 2M, replicate in the chloroplast and possess a ribozyme catalytic domain but no Central Conserved Region (CCR) (Flores et al., 2000; Daros et al., 2006). Those viroids have a branched secondary structure formed by hairpins, except the ASBVd which fold in a quasi rod-like secondary structure (Bussière et al., 1996; Flores et al., 2004). To our knowledge, no functional domains (except the ribozyme) have already been identified in their structure. However, De La Pena et al. (1999) have highlighted the importance of four nucleotides in the pathogenicity of the *Chrysanthemum chlorotic mottle viroid* (CChMVd) and Malfitano et al. (2003) have studied the role of a 12-13 nt insertion in the peach calico symptomatology induced by certain *Peach latent mosaic viroid* (PLMVd) variants. The study of CChMVd symptomatic and non-symptomatic variants has shown a loop formed by the nucleotides 82-85 (in the ribozyme region) implicated in the pathogenicity (Figure 2) (De La Pena et al., 1999; De la Pena and Flores, 2002). A substitution of UUUC82-85 by GAAA82-85 induces a change in the pathogenicity from symptomatic to a non-symptomatic variant. This mutation does not affect the quantity of viroids in the plant tissues which confirms its specific implication on the symptoms expression (De La Pena et al., 1999; De la Pena and Flores, 2002). In the case of the PLMVd, isolates inducing an extreme chlorosis (also known as peach calico) possess an hairpin insertion of 12-13nt capped by a U-rich loop at the left of the ribozyme hairpin (Figure 3) (Rodio et al., 2006; Rodio et al., 2007). As for the CChMVd, mutations from U to A in the loop of the hairpin induced a lost of pathogenicity. Only PLMVd variants with this insertion show an important pathogenicity by affecting the plastid transcription and translation (Rodio et al., 2007). Furthermore, the elimination of this hairpin leads to totally non-symptomatic variants without affecting the replication of the PLMVd (Malfitano et al., 2003). These studies are the only ones showing an implication of the sequence/structure of two *Avsunviroidae* in their pathogenicity.

Figure 2: Secondary structure of the lowest free energy of a CChMVd symptomatic variant. Sequences of the plus and minus ribozymes are shown by flags; conserved sequences in most ribozymes are highlighted, self cleavage sites are shown by arrows. Black and white symbols refer to the plus and minus polarity respectively. The nucleotides involved in the symptoms expression are in upper-case. (Modified from De la Pena et al. 1999)

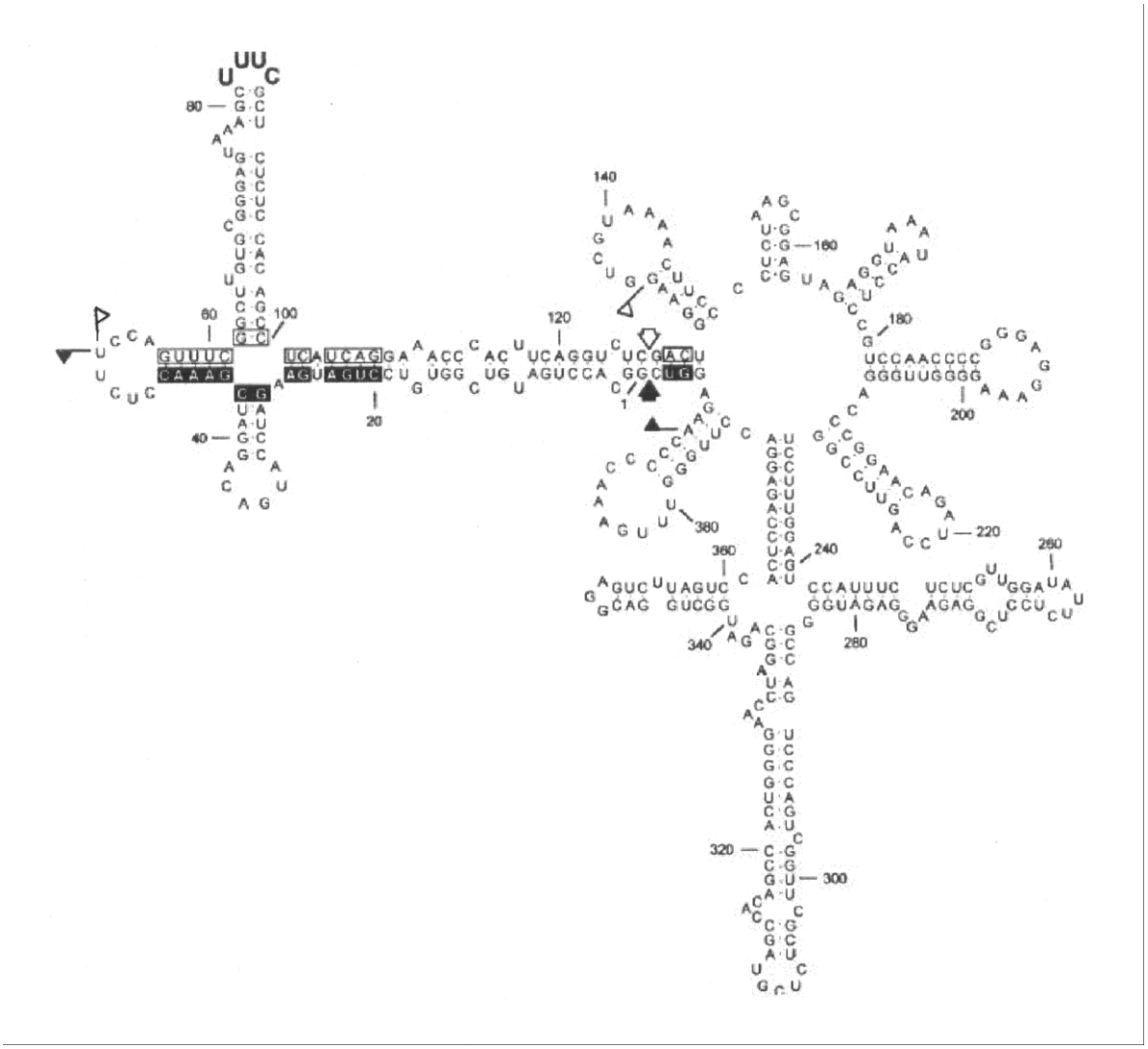
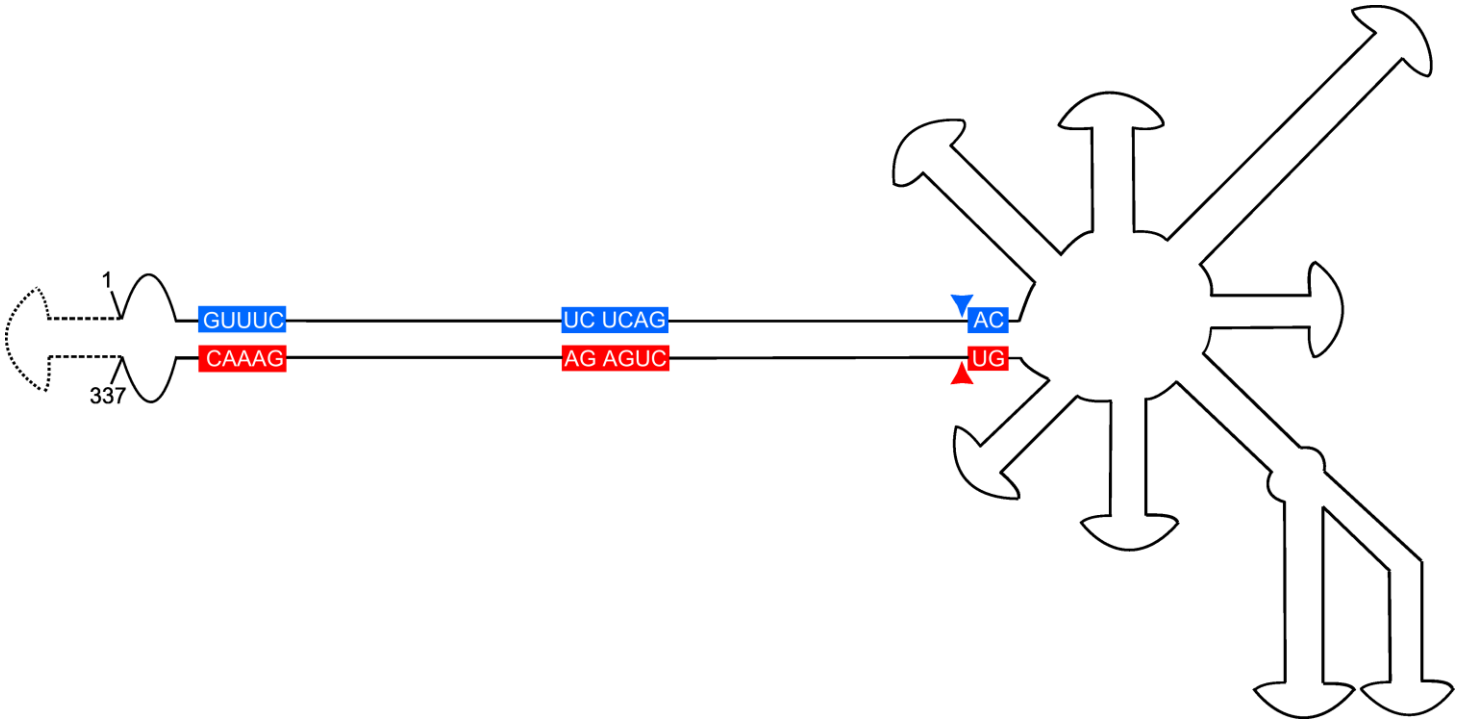


Figure 3: Scheme of the branched genomic RNA of *Peach latent mosaic viroid* (PLMVd; *Avsunviroidae*), in which the sequences conserved in most natural hammerhead ribozymes are shown on a red and blue background for (+) and (–) polarities, respectively, and the self-cleavage sites are indicated by arrowheads. The insertion of 12-13 nucleotides involved in the peach calico symptoms formation is represented by broken lines between nucleotides 1 and 337 (Modified from Daros et al., 2006).



6.4.Molecular host-viroid relationships

For 10 years, several studies have been carried out to characterize the pathogenicity mechanisms involved during a viroid infection. These studies concern essentially the *Potato spindle tuber viroid* (PSTVd) in tomato plants (Hammond and Zhao, 2000; Itaya et al., 2002; Qi and Ding, 2003; Owens, 2007). A recent study on the differential gene expression induced by the *Citrus viroid III* (another *Pospiviroidae*) has shown that this viroid modified the expression of genes involved in plant stress/defence responses, signal transduction, amino acids transport, cell wall structure and RNA silencing suppression. Unfortunately it remains unclear how these non coding small RNA molecules are able to regulate gene expression in their host and how they induce symptoms (Tessitori et al., 2007).

6.4.1. Regulated genes during a viroid infection

Most of the studies are actually made on tomatoes infected by PSTVd due to the easiness of manipulations and the wide knowledge on these two genomes. The PSTVd is certainly the best known viroid concerning the links existing between the sequence, the structure and the pathogenicity. The characterization of domains involved in the replication, the movement and the pathogenicity of this viroid has allowed the study of these mechanisms with a high degree of complexity (Gora-Sochacka, 2004).

Infections by PSTVd can regulate a broad range of genes. Such genes are specifically regulated by the viroid infection while others are also regulated during an infection by the *Tobacco mosaic virus* (TMV) (Itaya et al., 2002). The study has shown that two PSTVd strains (a severe and a mild strain) are associated with the regulation of the expression of some genes in the infected tomato. An over-expression of different intensity was observed for the two strains: infection by the severe strain affects strongly the gene expression (Itaya et al., 2002) compared to the mild strain. The table 1 shows the regulated genes during a severe PSTVd infection. The highlighted tomato genes are involved in defence/stress response (like chitinase and PR genes) as well as in cell wall structure (Cell wall protein), chloroplast function (Cab genes), protein metabolism (like ubiquitin and heat shock protein) and other diverse functions (such as sucrose transporter and ADP/ATP translocator) (Itaya et al., 2002).

Surprisingly, some genes belonging to a family with similar function have shown a different altered expression depending on PSTVd or TMV infection. For example, the Cab

(chlorophyll a/b binding protein) gene was induced by both pathogens, while Cab 10b and Cab 9 were only induced by PSTVd (Itaya et al., 2002).

Due to the difficulties for the growing and the maintenance of woody plant species in greenhouse conditions, fewer studies were carried out on these models. Among them one concerned the differential gene expression analysis on citrus infected by *Citrus Viroid-III* (CVd-III) (Tessitori et al., 2007). This study has led to the identification of some genes with modified expression during the viroid infection. The upregulated identified genes are also known to be involved in environmental stress response (extensin genes), plant defence (ethylene-responsive element binding protein gene), regulation of the gene silencing (Calmodulin-related protein gene), gene expression regulation, developmental processes (Hedgehog interacting protein-like 1) and DNA repair (RecQ DNA helicase gene). The down-regulated genes are also involved in several pathways such as plant defence, salt tolerance and amino acids transport (Tessitori et al., 2007). Table 1 summarizes the observed gene expression regulation during infection by the studied viroids.

Table 1 : Synthesis of observed gene regulation during an infection of tomato plant by PSTVd and Etrog citron by Cvd-III in relation with function. + refers to upregulated genes and – to down-regulated genes (Itaya et al. 2002; Tessitori et al. 2007)

Viroids	Functions	Regulated gene	Regulation
PSTVd	Defence/stress response	Catalase 1	+
		Chitinase	
		PR-1b	
	Cell wall structure	β-1,3-glucanase	+
		Glycine rich protein	
		Cell wall protein	
	Photosynthesis	Cab (Lhcb1-3)	+
		Cab 10b	
		PSI subunit psaL	
	Protein metabolism	Heat Shock Protein 2	+
		Ribosomal protein	
		Ubiquitin (ubi3)	
CVd-III	Defence/stress response	Extensins	+
		Regulator of gene silencing (Calmodulin-	+
		ethylene-responsive element binding protein	+
		β-Galactosidases	-
	Salt tolerance	NHX1	-
	Developmental processes	Hedgehog interacting protein-like 1	+
	DNA repair	RecQ DNA helicase	+
	Gene expression	CONSTANS-like (COL)	+
	Amino Acids transport	Aminoacid permease	-

Both studies demonstrate that the host response during a viroid infection can be specific at the level of the gene expression. Moreover, the study of Itaya et al. (2002) demonstrated that different viroid strains with subtle nucleotide differences (the mild and severe strains of PSTVd only differed for three nucleotides) and different pathogenicity can induce or suppress the expression of common and unique genes in their host but the little differences between the used strains cannot explain, in the present state of knowledge, the major differences in the host gene expression (Itaya et al., 2002). Furthermore, plant responses to PSTVd and TMV infection may share some common mechanisms in addition to their unique features (Itaya et al., 2002). As for the PSTVd study, the CVd-III regulates the expression of genes implicated in several physiological processes but no information is actually available concerning the mechanisms involved in the regulation of the expression of these genes. It has been hypothesized that viroids could interfere with mRNA splicing or with RNA export from the nucleus but the mechanisms involved remain unclear (Tessitori et al., 2007). Finally, the induction of genes encoding for elongation factors, ribosomal proteins, ubiquitins, ubiquitin extension proteins and heat shock proteins during a viroid infection suggests that there are an active synthesis and degradation of proteins as for viral and fungal infections (Itaya et al., 2002). Unfortunately we don't actually know how viroids are able to enhance this protein turnover.

6.4.2. How viroids regulate genes: the gene silencing hypothesis

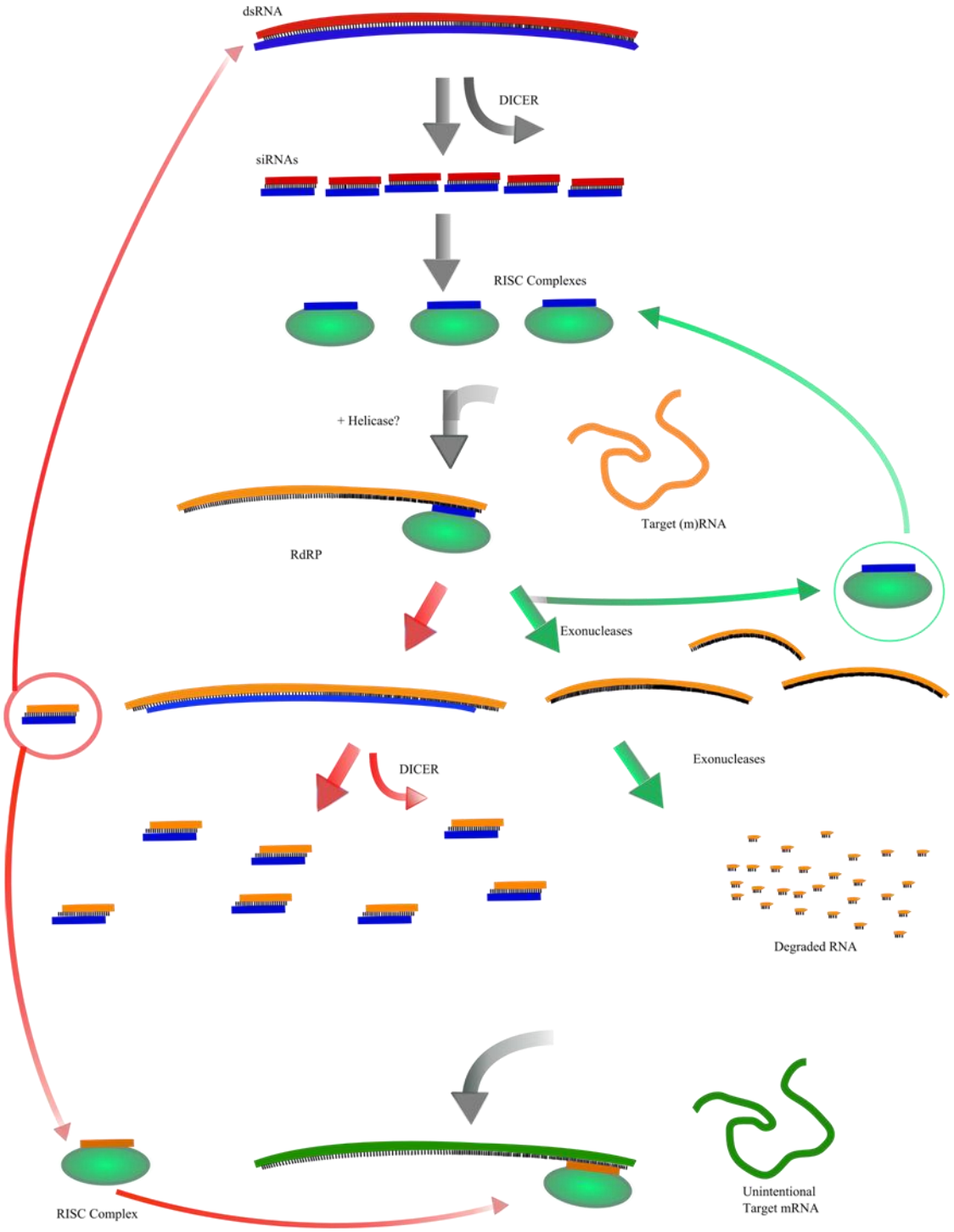
The gene silencing is a cytoplasmic mechanism to regulate gene expression of eucaryote organisms. This phenomenon was first discovered in transgenic plants and named co-suppression or Post-Transcriptional Gene Silencing (PTGS) (Baulcombe, 1996; Jana et al., 2004). It became rapidly clear that PTGS can act against pathogens like viruses or viroids (Angell and Baulcombe, 1997; Jana et al., 2004).

The dsRNA plays an important role uniting the silencing pathway either as a trigger or an intermediate. The gene silencing is characterized by the implication of small RNA molecules (ranging size 21-25 nt long) in the regulation of the gene expression. These small RNAs can be endogenous or exogenous RNAs. The firsts are called micro-RNAs (miRNAs) and are implicated in the normal gene expression regulation during the development. At the opposite, the seconds are called small interfering RNAs (siRNAs) and involved in the plant response against exogenous (pathogenic or not) RNAs. This second pathway will be analyzed.

Exogenous RNAs can induce two mechanisms leading to the silencing of a gene (or to the degradation of its mRNA): Transcriptional Gene Silencing (TGS) and PTGS (Jana et al., 2004).

During PTGS an exogenous double-stranded RNA molecule triggered the mechanism. This molecule is cleaved by the Dicer, a ribonuclease of the RNAse III family, into small (21 and 25 nt long) interfering RNA (siRNA) of both polarities. These RNA species are double stranded and possess two nucleotides long 3' overhangs and 5' phosphates that are the hallmarks of Dicer cleavage products. These two siRNAs classes are produced by two distinct Dicers (Landry et al., 2004) and at least four homologue Dicers were detected in plants. The produced siRNAs are incorporated to the RNA Induced Silencing Complex (RISC) where one of the strands is lost. This new complex targets and cleaves an exogenous (or an endogenous) single stranded (m)RNA showing a good complementarity with these small incorporated RNAs. In plants and other organisms siRNAs can serve as primers (by hybridization on a complementary sequence) for the synthesis of dsRNA by the RNA-dependant RNA polymerase (RdRP). This lead to the creation of more siRNAs, thus amplifying the gene silencing mechanism (Figure 4) (Landry et al., 2004).

Figure 4 Overview of dsRNA-mediated mRNA degradation. dsRNA is cleaved by Dicer into 21- to 23-nt siRNAs. siRNAs are complexed with a large multiprotein complex, the RISC. RISC is thought to unwind the siRNA to help target the appropriate (m)RNA (shown in orange). The siRNA-(m)RNA hybrid is cleaved, releasing the siRNA, and the (m)RNA is degraded by endo and exonucleases. In plants the siRNA can also serve as a template for RdRP using the (m)RNA as a template. Elongation of the siRNA can lead to the production of more siRNAs that could share homology to other genes (shown in green), causing their degradation (modified from Dillin, 2003).



The TGS phenomenon is characterized by the RNA Directed DNA methylation (RdDM) of the promoter sequences. During TGS a dsRNA molecule triggers a dense methylation of the promoter region (leading to the inactivation of this promoter) due to the sequence homology existing between this RNA and the promoter. The length of the target can be as small as 30 nucleotides giving a link between PTGS and TGS. In other terms, the exogenous dsRNA can be degraded on siRNA in the cytoplasm and then induce the methylation of the promoter region by transfer of a signal molecule into the nucleus (Papaefthimiou et al., 2001). The gene silencing (especially PTGS) seems to be common during viral and viroidal infection and is considered as an antiviral immunity in plants (Ding et al., 2004). This is supported by three arguments for infections by plant viruses.

First, viral infections trigger RNA silencing in infected plants that target the viral RNA and all homologous RNA molecules. Detection of viral siRNA of both polarities are effectively common during plant infection (Hamilton and Baulcombe, 1999; Papaefthimiou et al., 2001).

Secondly, plant viruses encode proteins that are able to block the silencing machinery (Li and Ding, 2001). Three types of viral suppressors were identified: HC-Pro, Cmv2b and p25. The first one (encoded by *Potyviruses*) induces a decrease of the 25-nt siRNA quantity but do not block the silencing signal. The Cmv2b (encoded by *Cucumber mosaic virus*, CMV) cannot suppress the silencing when it's established indicating that this suppressor acts at an earlier stage than HC-Pro. It was found that Cmv2b blocks the silencing signal or inhibits the signal-mediated de novo induction of RNA silencing (Li and Ding, 2001). Furthermore, a study has demonstrated that Cmv2b encodes a functional nuclear localization signal. This indicates that the suppression of RNA silencing induced by Cmv2b may occur in the nucleus or that nuclear trafficking is essential for the suppressor activity (Li and Ding, 2001). Finally, p25 (the 25K protein of *Potato virus X*, PVX) suppresses the systemic silencing signal but not the local silencing pathway (Li and Ding, 2001).

Finally plants defective in RNA silencing are often most susceptible to viral infections (or at least as susceptible) than the wild type plants (Ding et al., 2004). This was shown with *Arabidopsis thaliana Heyn, ecotype Wassilewskijia*, and tobacco plants defective or compromise in RNA silencing. The RNA silencing-defective *A. thaliana* was hypersensitive to CMV, but was susceptible as the wild type plant to five other tested viruses (Ding et al., 2004). Tobacco plants were most susceptible to *Tobacco mosaic virus* when deficient in RNA silencing (Ding et al., 2004).

Together these three arguments show that the gene silencing is an antiviral immunity mechanism against plant virus.

The situation should be nuanced during viroid infections. As during viral infections, viroids trigger the RNA silencing pathway in infected plants. Detection of viroidal siRNA of both polarities is also common during plant infections (Gomez et al., 2008). However this immunity mechanism can be bypassed by the viroids without encoding any protein. The explanation of this phenomenon is actually unclear. Several hypothesises were emitted. Viroids should escape the silencing machinery through (1) their cellular localization. The silencing machinery is able to degrade RNA in the cytoplasm but viroids replicate in the nucleus (*Pospiviroidae*) or in the chloroplast (*Avsunviroidae*) so they can escape the silencing mechanism during the critical step of their life cycle. (2) The secondary structure of viroid and especially the highly branched and compact secondary structure of the *Avsunviroidae* could protect these viroids against the silencing enzymes. Furthermore the presence of mismatches in hairpins of the viroid structure restricts the cleavage by the Dicer which needs, preferentially, 19 bp of contiguous dsRNA. (3) Their possible interactions with host proteins leading them inaccessible for the silencing enzymes could play a role on the protection against the silencing machinery (Wang et al., 2004; Landry et al., 2004). Furthermore the gene silencing is not only a defence mechanism against viroids as it seems to be also implicated in the pathogenicity of these disease agents (Wang et al., 2004). Tomato plants expressing an hairpin RNA structure (hpRNA) with a partial PSTVd sequence produce siRNA homologous to the hpRNA and present viroid-like symptoms. However these symptoms are less important than during a PSTVd infection (Wang et al., 2004). Moreover Gomez et al., (2008) has recently demonstrated that Hop stunt viroid-infected Nicotinia benthamiana plants defective for the RNA-dependent RNA polymerase 6 (RDR6) activity (which is an essential enzyme for the PTGS) show a non-symptomatic infection compared to the wild type plants infected by the same viroid strain. This is the first study which clearly links the gene silencing and the symptoms expression for a viroid.

These results could explain how viroids induce symptoms and regulate the host gene expression. Viroids degradation by the host plant defence mechanisms could induce plant damage when viroid siRNAs are homologous to host mRNA or to promoter regions. The plant genes could be regulated and induce typical viroid symptoms. Furthermore it is clear that viroids are also able to induce TGS which can cause symptoms but this pathway was less studied with viroids contrary to the PTGS. However it seems that gene silencing cannot

explain all the induced symptoms. Other interactions should be involved in the gene regulation and/or in the symptoms expression.

6.4.3. Other interactions: protein-viroid interactions

Alone, the gene silencing cannot explain how viroids regulate the gene expression of their host because genes involved in several biological processes appeared over-expressed during an infection (Owens, 2007). Some studies have shown that viroids can interact with host protein but the knowledge on this topic is poor. Only few interactions between PSTVd and tomato plant proteins have been highlighted due to the difficulties to study viroids-proteins interactions. These can be specific like the Dicer-mediated cleavage of the viroid or totally non-specific to the viroid infection like their transport through the vascular system by the phloem lectin PP2 (involved in the long distance transport of RNA molecule through the plant) (Owens, 2007).

The most studied protein-viroid interaction concerns the VIRP1 tomato protein whose binding site is in the right terminal domain of the viroid structure. Martinez de Alba et al. (2003) has demonstrated (by immunoprecipitation of the viroid-protein complex) that PSTVd interacts strongly *in vivo* with VIRP1. This protein possesses a bromodomain which can be implicated in several cellular processes. However the presence of a bromodomain can be considered as a marker of the nuclear localization (at the level of the dynamic chromatin) of the corresponding protein (Martinez de Alba et al., 2003). Moreover some bromodomain-containing proteins have a developmental role in different organisms and viroids, and especially the PSTVd, induce developmental disorders (Martinez de Alba et al., 2003). It is consequently possible that the interaction between the PSTVd and VIRP1 is involved in the symptoms formation (Martinez de Alba et al., 2003). Furthermore, the RNA-directed DNA methylation plays an important role as regulatory or defence mechanisms in plants (Martinez de Alba et al., 2003). The interaction with a bromodomain-containing protein is interesting in this context. PSTVd is able to initiate the methylation of homologous nuclear DNA sequences. It is possible that VIRP1 may play a role in the transmitting of the RNA-directed methylation on all the viroid-homologous sequences. This methylation blocks the synthesis of the corresponding mRNA and could induce the symptoms. The VIRP1 could also have possible roles in the viroid life cycle: (i) this protein could play a role into the viroid trafficking processes into and from the nucleus but also in the all plant and (ii) VIRP1 could

be linked with the Pol II transcription of the PSTVd (Martinez de Alba et al., 2003; Ding and Itaya, 2007).

A second protein viroid interaction has been characterized between the PP2 (Phloem protein 2) and Hop Stunt Viroid (HSVd). It was found (by an immunoprecipitation assay carried out in a phloem exudates of an HSVd-infected cucumber plant) that the phloem protein 2 of Cucumis sativus (CsPP2) forms a ribonucleicprotein complex with HSVd RNA *in vitro* and *in vivo* (Gomez and Pallas, 2004). This interaction permits a long distance transport of the HSVd through the phloem of his host plant (Gomez and Pallas, 2004; Owens, 2007). Up to now, VIRP1 and CsPP2 are the two best characterized protein implicated in the viroid translocation.

Finally, there are some evidences that viroids, especially PSTVd and *Citrus exocortis viroid* (CEVd) are able to regulate the protein phosphorylation. It was demonstrated that PSTVd could stimulate the phosphorylation of a tomato protein associated with double-stranded RNA-stimulated protein kinase activity (Hiddinga et al., 1988; Langland et al., 1995). Incubations of its mammalian homologue with PSTVd strains of various pathogenicity leads to a differential activation (Diener et al., 1993) supporting an implication in the viroid pathogenicity (Langland et al., 1995). More recently Hammond and Zhao, (2000) have characterized a new protein kinase (the PKV protein) whose transcription is up-regulated during a PSTVd infection in tomato. They have found that the level of transcription was higher in the plant infected by a severe or an intermediate PSTVd strain than by a mild strain or in a healthy plant. PKV is similar to cyclic nucleotide-dependent kinase of mammalian implying involvement in the transduction of extracellular signals (Hammond and Zhao, 2000). The modification of the transcription of this gene could have a great influence on the symptoms development. Vera and Conejero (1990) have shown that CEVd is also able to induce and to reduce the *in vitro* phosphorylation of diverse proteins during the infection (after the symptoms appearance). They also noted that those modifications were higher in presence of Mn2+ showing the importance of the Mn2+-dependent protein kinase in the phosphorylation modifications of the host protein. It’s actually unclear how viroids can change the protein phosphorylation but we can afford that these modifications might have a critical incidence on several biological pathways.

6.5. Concluding remarks.

The study of the host-pathogen relationship is very important to develop control methods. Viroids seem able to encounter the plant defence. This shows the importance to have a comprehensive knowledge of the host-pathogen relationship to act efficacy against viroids.

The main studies carried out on viroids concern essentially their structure and their replication. Studies carried out on their interactions with host plants are more recent. Actually viroids are shown to affect the transcription level of genes involved in various functions including defence against pathogen as well as DNA repair, development, stress response and probably other actually not highlighted functions. The same assessment can be made about interactions between viroids and plant proteins with the particularity that there are fewer interactions described than for the gene expression modification (probably due to the chloroplastic localization of the *Avsunviroidae* and the subsequent difficulties for protein isolation limiting the field of these studies to the *Pospiviroidae*). However these studies have given some hypothesises to explain (i) the viroid trafficking through the plant and (ii) the symptoms enhancement by the way of the gene silencing or the protein phosphorylation. More efforts should be made in this research field.

The study of these interactions will help to better understand how these non coding RNA molecules can be so pathogenic and to develop control methods against viroids.

Finally, these small pathogens are probably the best molecules to study the RNA translocation in the plant cells and also to study the plant RNA pathogen evolution. Researches on viroids and viroid-host interactions will support a better understanding of the RNA world.

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Chapter II
Aims and research outlines

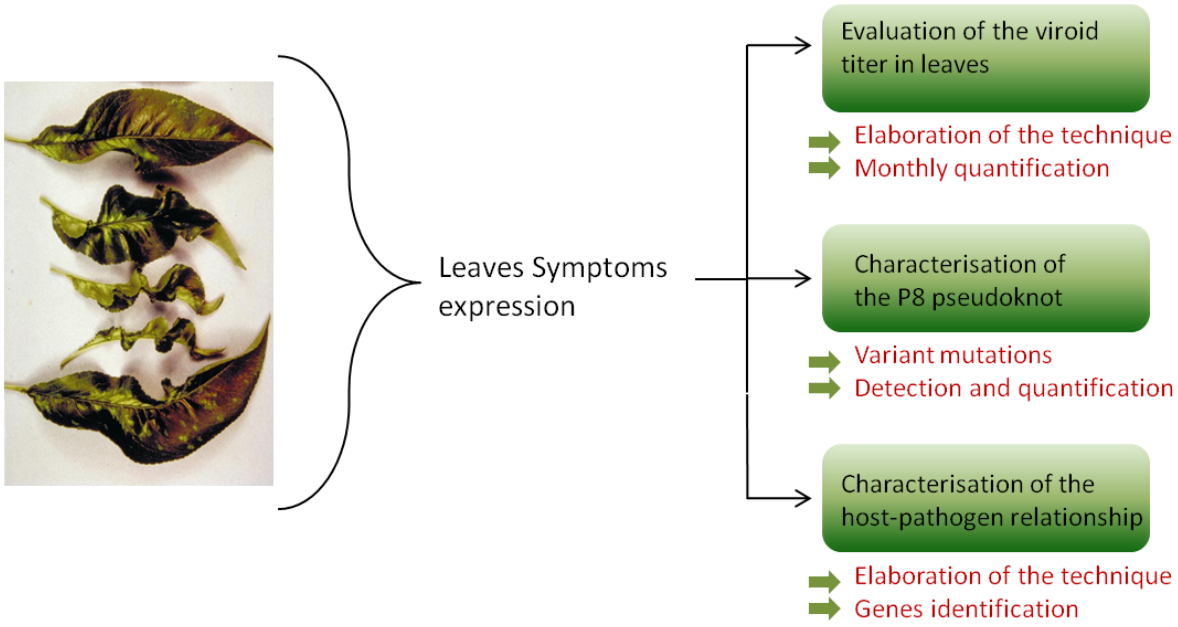
Since their discovery in 1972, the major viroid studies concerned their sequence, structure, replication, pathogenicity and the possible interactions between these aspects. However, during the past decade a great effort was realized to start the characterization of the host-viroid relationships established during an infection. As it infects potato and tomato plants (two well known plant models) and as the *Pospiviroidae* are well characterized, the PSTVd was largely preferred for these studies. This has permitted to identify differentially expressed genes between infected and healthy plants (Itaya et al. 2002). Another study was also realized on citrus infected by the *Citrus viroid III* (another *Pospiviroidae*) (Tessitori et al. 2007). However, no study concerned the *Avsunviroidae* family and their hosts. Furthermore these previous researches did not permit to link observed symptoms to a particular regulation of the gene expression and were unable to identify a control method against these viroids.

We propose a new, interesting and original approach to consider the host-viroid relationship and particularly, the PLMVd-Peach tree pathosystem. It consists in the analysis of the gene expression between infected plants showing symptoms of different intensity (comprising severe chlorosis, mosaic and partial mosaic) to characterize the genes involved in the host-pathogen relationships.

The main aim of this study was to identify plant genes and a viroid structure implicated in the symptoms expression or in the absence of symptoms development. It was thus necessary to dispose of viroid variants of different pathogenicities. These were obtained in previous researches on our laboratory (Parisi 2006; Fekih Hassen et al. 2007) and maintained on GF-305 peach rootstocks in greenhouse conditions.

Specific aims were first to quantify the viroid in peach tree leaves. For this part of the work the quantitative real-time RT-PCR was developed and optimized (Chapter 3.1). Then this technique was applied to characterize the importance of the P8 pseudoknot in the replication and the pathogenicity of the PLMVd *in vivo*. This part of the work was realized in collaboration with the Département de Biochimie de l'Université de Sherbrooke (chapter 3.2). Finally the cDNA-AFLP was used to identify genes implicated in the symptoms development and in the possible plant resistance against the PLMVd. The figure 1 depicted the outline of the study.

Figure 1 : PLMVd leaves symptoms and research outlines of the study on the host-pathogen relationship between peach trees and the PLMVd



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Chapter III
Quantification of the PLMVd in infected plants and determination of the biological role of the P8 pseudoknot

1. Development of a quick quantitative real-time PCR for the in vivo detection and quantification of Peach Latent Mosaic Viroid

1.1. General introduction

Prior to characterize the effect of mutations on the viroid replication and pathogenicity, it was necessary to develop a robust, rapid and sensitive molecular technique allowing the *in vivo* quantification of the PLMVd.

The first step was to design new primers and determine their optimal concentration leading to an amplicon size comprised between 80 and 150 base pairs which ensure an optimal amplification efficiency by real-time PCR.

Next, different PLMVd extraction methods were tested to select the most efficient and rapid technique which does not interfere with the real-time RT-PCR. The extraction with 2X SSC diluted 100-fold before the viroid reverse transcription would be preferred.

Then, the P8 pseudoknot of a severe variant was mutated by the team of Jean-Pierre Perreault (Département de Biochimie, Université de Sherbrooke) from dimeric RNA of the native 151.1 variant. The mutant and the native variant were inoculated on different viroid-free seedlings of GF-305 peach trees in greenhouse.

Finally, the PLMVd quantification was performed for the native and mutated variant to compare their replication in growing leaves. The symptoms development was assessed by visual inspection of the leaves.

The results concerning the development of the real-time PCR technique were accepted for publication:

Parisi Olivier, Lepoivre Philippe, Jijakli M. Haissam. 2010. *Development of a rapid real-time PCR technique for the in vivo detection and quantification of Peach Latent Mosaic Viroid*. Accepted in Plant disease.

Development of a quick quantitative real-time PCR for the in vivo detection and quantification of Peach Latent Mosaic Viroid

Parisi Olivier, Lepoivre Philippe, Jijakli M. Haissam. 2010. Development of a quick quantitative real-time PCR for the *in vivo* detection and quantification of *Peach latent mosaic viroid*. Accepted in Plant Disease

Development of a quick quantitative real-time PCR for the in vivo detection and quantification of Peach Latent Mosaic Viroid

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1.2.Abstract

Viroids are plant pathogens infecting a broad range of herbaceous and tree crops. Among them, the *Peach latent mosaic viroid* (PLMVd) infects mainly peach trees, causing a loss of production with no curative options. Detecting this viroid is thus important for certification procedures aiming to avoid the release of infected material into orchards. Presented here is a complete detection method based on reverse transcription (RT) followed by a quantitative real-time polymerase chain reaction (PCR). New primers were selected and optimal reaction conditions determined for routine application of the method. The technique is 10^5 times more sensitive than the end-point RT-PCR used for PLMVd detection, and permits earlier detection of PLMVd in infected plants. The quick, low-cost extraction procedure used and the quality of the results obtained make this method suitable for routine testing.

Keywords: PLMVd, quantitative real-time PCR, RT-PCR, viroids, technique, peach

1.3.Introduction

Viroids were discovered in the 1970s (7) and classified in two families based on biological, biochemical, and structural characteristics (for a review see 13-14). These families are the *Pospiviroidae*, whose type species is the *Potato spindle tuber viroid*, and the *Avsunviroidae*, whose type species is the *Avocado sunblotch viroid* (ASBVd). Viroids infect various herbaceous and tree species. Most infected plants develop symptoms and can be seriously damaged by viroid infection. There exist no curative options. Therefore viroids are the targets of active detection and eradication procedures.

The *Peach latent mosaic viroid* (PLMVd) belongs to the *Avsunviroidae* family. Like the other members of this family, it folds into a highly branched secondary structure (insoluble in 2M LiCl) and contains a ribozyme catalysing the cleavage of oligomers to monomers during replication (6,8). It infects mainly peach trees and other *Prunus* hybrids (like almond and apricot). Often latent in orchards for several years, PLMVd is transmitted by the cutting and grafting tools commonly used in production areas (14). Currently PLMVd is included in certification programs to avoid its uncontrolled expansion, as described in the EPPO standard PM 4/30(1) (10). Rapid, efficient detection techniques are needed to avoid certification of false-negative material. Currently, end-point RT-PCR (hereafter simply called "RT-PCR") and molecular hybridisation are the most used techniques (16), but unfortunately, the routine use of such detection methods is sometimes insufficient to avoid false negatives (16). Recently, however, a new reverse transcription loop-mediated isothermal amplification (RT-LAMP) was developed, with higher sensitivity than the RT-PCR (3).

Viroid infections are presumed to be systemic, but it has been shown that PSTVd is transported into the sepals and not into the other floral organs, which are purely sink organs (23). This lack of uniformity complicates sampling and the interpretation of negative results in detection campaigns. In addition to being non-uniform, viroid distribution seems to depend on the viroid species concerned. ASBVd is found at higher concentration in symptomatic than in non-symptomatic leaves (18), but to our knowledge, no similar data are available for PLMVd. Finally, the viroid titre also shows peaks and troughs in the course of the growth season (17), so that depending on the sampling date, infected plants may be detected as healthy. A more sensitive detection method could thus be helpful.

Real-time quantitative PCR (hereafter called qPCR) combines the simplicity of PCR amplification with the sensitivity of fluorescence detection during amplification (21). It is widely used for pathogen detection because it allows more sensitive, precise quantification than (RT-)PCR (22). It also gives results more quickly because it does not require post-PCR processing. With some adjustments, it should be applicable to viroid detection. This prospect and the advantages just mentioned have led us to develop an RT-qPCR method for detecting and quantifying PLMVd, based on the use of SYBR green dye.

1.4.Materials and Methods.

1.4.1. Preparation of dimeric PLMVd RNA

Monomeric cDNA of a severe PLMVd variant (GenBank accession no. DQ680690) belonging to PLMVd sequence group II-A (12) was used as template for dimerisation and subsequent inoculations. The monomeric PLMVd cDNA was amplified with the Pfu DNA polymerase (Fermentas, Vilnius, Lithuania). The PCR conditions were: 5 min at 95°C, 35 cycles of 95°C for 30 s, 60°C for 60 s, 72°C for 60 s and a final elongation step at 72°C for 7 min), and the primers (each used at 0.4 µM final concentration) were hPLMVd (5'CCCGATAGAAAGGCTAAGCACCTCG3') and cPLMVd (5'AACTGCAGTGCTCCGAATAGGGCAC3') (11). The amplification products were purified by 1.2% agarose gel electrophoresis and the PLMVd DNA of the expected length was eluted, phosphorylated with T4 polynucleotide kinase (Fermentas, Vilnius, Lithuania), and ligated with T4 DNA ligase (Fermentas, Vilnius, Lithuania). The ligation products were amplified with the High Fidelity PCR enzyme mix from Fermentas (Vilnius, Lithuania). Amplification was carried out with the hPLMVd and cPLMVd primers (PCR cycle: 5 min at 95°C, 35 cycles of 95°C for 30 s, 60°C for 60 s, 72°C for 60 s, and a final elongation step at 72°C for 7 min, the final concentration of each primer being 0.4 µM). Dimers were isolated by electrophoresis through a 1.2% agarose gel. The eluted dimers were cloned into the pCR2.1 cloning vector (Invitrogen, Carlsbad, California, USA) and used to transform chemically competent *E. coli* cells (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. After an overnight incubation in liquid medium with ampicillin, the transformed cells were collected by centrifugation and the recombinant plasmids purified with the GeneJet plasmid miniprep kit (Fermentas, Vilnius, Lithuania). To distinguish the plasmids containing head-to-tail dimers, the dimers were digested with the KpnI restriction

enzyme (Fermentas, Vilnius, Lithuania) and positive clones were sequenced by Macrogen (Seoul, Korea) to confirm the overall orientation of the dimers in the cloning vector. After transcription by T7 RNA polymerase (Fermentas, Vilnius, Lithuania), a dimeric PLMVd RNA molecule with plus polarity was obtained.

1.4.2. Plant material

Ten GF-305 peach trees were grown from viroid-free seedlings and maintained in a greenhouse. Nine plants were inoculated by slashing the stems with a suspension (in 50 mM K_2HPO_4) of the dimeric RNA corresponding to a severe PLMVd variant. One plant remained non-inoculated as a negative control.

1.4.3. Sample preparation

Total RNA extraction

Each sample was prepared by grinding eight leaves (approximately 1 g leaf tissue, precisely weighed) in liquid nitrogen. Of the resulting powder, 400 mg was used for each sample. Total RNA was extracted from this material with 1ml Qiazol Buffer (Qiagen, Hilden, Germany) and resuspended in 50 µl of 0.1% DEPC water according to the manufacturer's instructions. The quality of the total RNA extract was checked by agarose gel electrophoresis and visualization of the ribosomal RNA.

Crude sap extraction

Two protocols of crude sap extraction were compared: one with 2X SSC buffer (20X SSC: 175.3g/l NaCl and 88.2g/l citrate trisodium dihydrate, pH7) and the other with KAJI Buffer (DNAlis, Gembloux, Belgium). Eight leaves (approximately 1 g leaf tissue, precisely weighed) were harvested. Five millilitres of KAJI Buffer or 2X RNAse-free SSC with 1% sodium sulfite as antioxidant was added before grinding. The crude sap was collected and centrifuged for 30 minutes at 12000g (at 4°C to avoid RNA degradation). The supernatant was diluted 100-fold before the one-tube RT-PCR or the separate reverse transcription step of the RT-qPCR.

1.4.4. Reverse transcription step of the RT-qPCR

Reverse transcription of PLMVd (before the qPCR) was carried out on the 100-fold-diluted crude sap with the SuperScript III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions.

1.4.5. RT-PCR

RT-PCR according to (11), with primers hPLMVd and cPLMVd each used at 0.4 µM concentration and Titan one tube RT-PCR system, using a mix of AMV reverse transcriptase and High-fidelity Taq polymerase (Roche Diagnostic, Penzberg, Germany), was used to confirm the infection status of the inoculated plants and as a reference method with which to compare our RT-qPCR. The sensitivity of the technique was evaluated on samples diluted 100-, 1000-, and 10000-fold. Amplification products were electrophoresed through a 1.2% agarose gel with the GeneRuler 100pb plus DNA ladder (Fermentas, Vilnius, Lithuania).

1.4.6. Quantitative real-time PCR

The qPCR was performed on a StepOne real-time thermal cycler (Applied Biosystems, Foster City, California, USA) equipped with the StepOne v.2.1 software. The forward primer used was F-PLMVd and five possible specific reverse primers, selected in conserved PLMVd regions by ClustalW alignment of 119 PLMVd sequences (15), were tested (see Table 1). The five primer pairs amplify PLMVd fragments ranging in size from 50 to 150 bp (Table 1). The F-PLMVd - R7-PLMVd pair was chosen for its high efficiency and low dimerisation potential and the corresponding amplicon was synthesised by Eurogentec (Liège, Belgium) for use as a qPCR standard (standard-F and standard R, see table 1). This standard was diluted in crude extract from healthy tissue so as to take into account the effect of the extract on the efficiency and quantification. The qPCR, performed with the Maxima™ SYBR Green/ROX qPCR Master Mix (using Maxima Hot Start Taq DNA polymerase and final $MgCl_2$ concentration: 2.5 mM) (Fermentas, Vilnius, Lithuania), included the following steps: 10 min at 95°C and 40 cycles of 95°C for 15 s, 60°C for 60 s. To check the specificity of the amplification, a melting curve was added at the end of the qPCR. It included 1 min at 60°C and then a rise by increments of 0.3°C (10 s at each temperature) up to 95°C. Three replicates were run for each sample as recommended for qPCR assay design.

Table 1 : Designed primers for the quantitative real-time amplification of the PLMVd. For each primer pairs the length of the amplicon is comprised between 65 and 120 base pairs. Standard F and Standard R refer to the qPCR standards used for the quantifications. Numerals in brackets refer to the primer position in the PLMVd sequence.

Oligonucleotide name	Oligonucleotide sequence
F-PLMVd	5'-CCTCTCAGCCCCTCCACCTT-3' (72-92)
R2-PLMVd	5'-GCT-TAG-CCT-TTC-TAT-CGG-GAA-G-3' (138-117)
R3-PLMVd	5'-GTG-CTT-AGC-CTT-TCT-ATC-GGG-A-3' (138-119)
R7-PLMVd	5'-CCT-ACC-TTA-CGT-CAT-TGC-G-3' (161-143)
R12-PLMVd	5'CCT-GGG-TTC-TTC-GAC-CGC-TA-3' (197-180)
R14-PLMVd	5'-TCA-CAC-TCC-CCC-TGG-GTT-C-3' (207-188)
Standard-F	5'-CCTCTCAGCCCCTCCACCTTGGGGTGCCCTATTCGGAGCCTGCAGTTCCCGATAGAAAGGCTAAGCACCTCGCAATGACGTAAGGTGGG-3' (72-161)
Standard-R	5'-CCCACCTTACGTCATTGCGAGGTGCTTAGCCTTTCTATCGGGAACTGCAGGCTCCGAATAGGGCACCCCAAGGTGGAGGGGCTGAGAGG-3'(161-72)

1.4.7. Data analysis

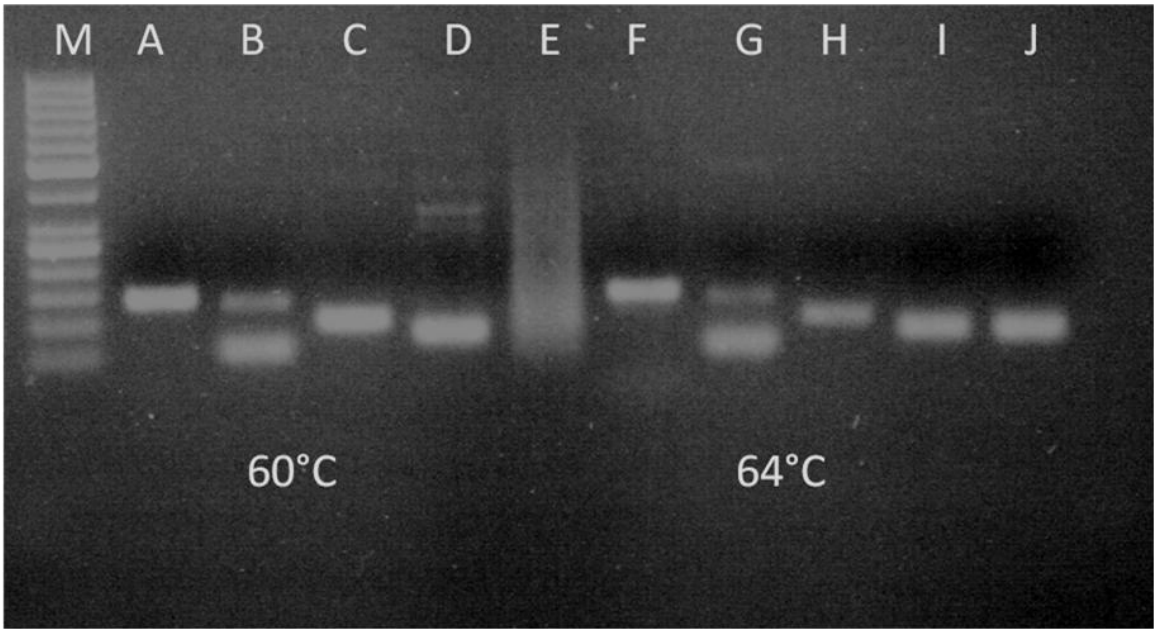
All of the output data were analysed with the StepOne v2.1 software (Applied Biosystems, Foster City, California USA) using the pre-set parameters. The software was used to determine the PCR efficiency and to quantify the DNA in the reaction plate from serial dilutions of the PCR standards. Standard curves were generated for each set of serial dilutions by plotting the quantification cycle (Cq) value against the logarithm of the concentration for the exponential phase of the reaction and fitting a straight line to these data by simple linear regression (5,9,21). The slope of the standard curve was used to determine the efficiency (E) of the PCR: $E=10^{-1/slope}-1$. Optimal PCR efficiency is achieved when a slope of -3.32 is reached (21). The determination coefficient (R^2) was calculated to determine the validity of the linear regression. For detection purposes, a quantification cycle (Cq) of 35 was established as the cut-off for distinguishing positive from negative samples as previously described (19).

1.5. Results

1.5.1. Development of the qPCR on positive controls

To develop the qPCR, we first used dimeric PLMVd cDNA clones to avoid the difficulties of the reverse transcription and the inhibitory effect of total extract. Five reverse primers were selected in conserved regions of the PLMVd highlighted by a multiple sequence alignment of 119 PLMVd sequences. To avoid selecting nonspecific primers amplifying plant DNA or RNA, the five reverse primers (R14, R12, R7, R3, and R2) combined with the forward primer (F-PLMVd) were tested by RT-PCR on crude sap extract from peach tree leaves. Three PLMVd-specific primers were thus identified, yielding amplicons of 65 to 135 basepairs (bp) long, as recommended for qPCR. Primers R12 and R3 appeared nonspecific, as they yielded multiband patterns (Fig 1, B and D). Primers R14 and R7 seemed highly specific (Fig 1, A and C). Finally, the R2 - F-PLMVd pair yielded a smear at 60°C (Fig 1E). A temperature gradient was applied to avoid this smear (data not shown), and amplification was specific at 64°C (Fig 1 J). This primer pair was abandoned, however, as amplification with R14 or R7 was less efficient at 64°C (Fig 1, F and H).

Fig 1 : PCR amplifications performed with the five newly designed primer pairs at 60° and 64°C. M refers to the molecular weight ladder O'GeneRuler 50pb (Fermentas). (A) to (E): use of the reverse primers R14, R12, R7, R3, and R2 respectively at 60°C . (F) to (J): Use of the same reverse primers at 64°C.



A similar, high efficiency was recorded for R14 and R7 (respectively 97.0% and 97.21%), but a slightly higher determination coefficient was obtained with the F-PLMVd - R7 pair (R^2=0.991 vs. R^2=0.984). This primer pair was selected for further optimisations. To determine the lowest concentration allowing optimal efficiency (E) while avoiding primer dimer formation, three primer concentrations were tested in the qPCR: 50 nM, 150 nM, and 300 nM. Best results were obtained at 300 nM (E_{300nM}=97.7% R^2=0.997; E_{150nM}=87.8%, R^2=0.993; E_{50nM}=87.1%, R^2=0.996) (Figs. 2 and 3).

Fig 2 : Optimisation of the F-PLMVd/R7-PLMVd primer pair concentration in the real-time qPCR. Concentration of each primer: 300 nM, 150 nM, or 50 nM as indicated. The standard curves were obtained by plotting Cq values versus the logarithm of the initial quantity of PLMVd cDNA clone. Three replicates were run for each quantity.

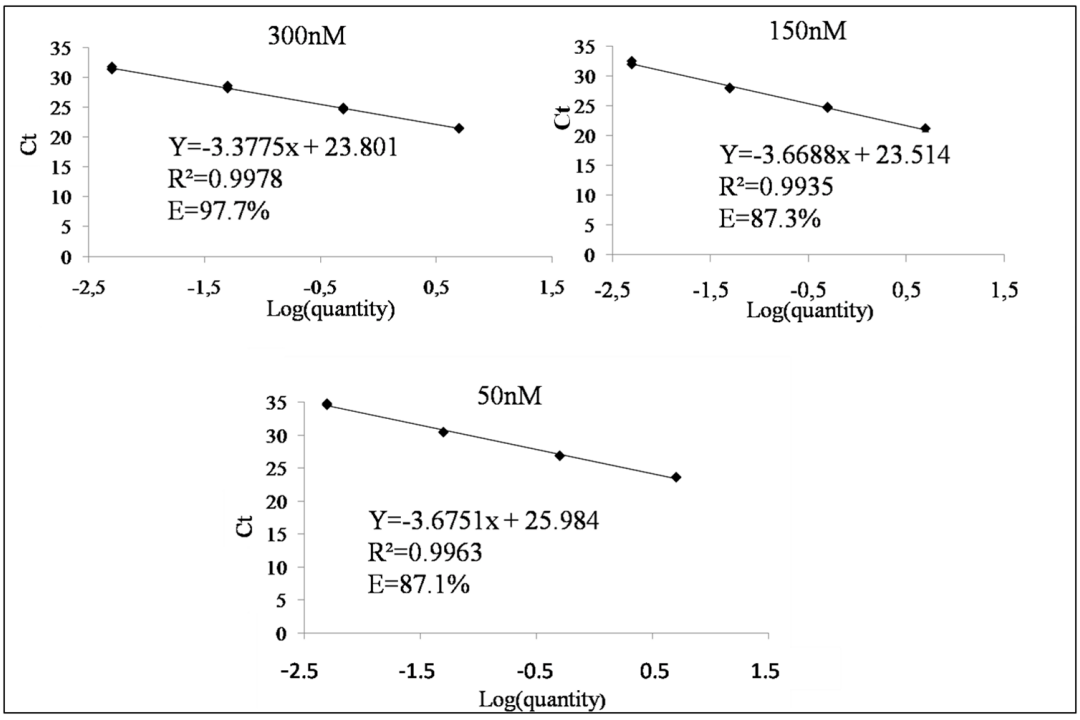
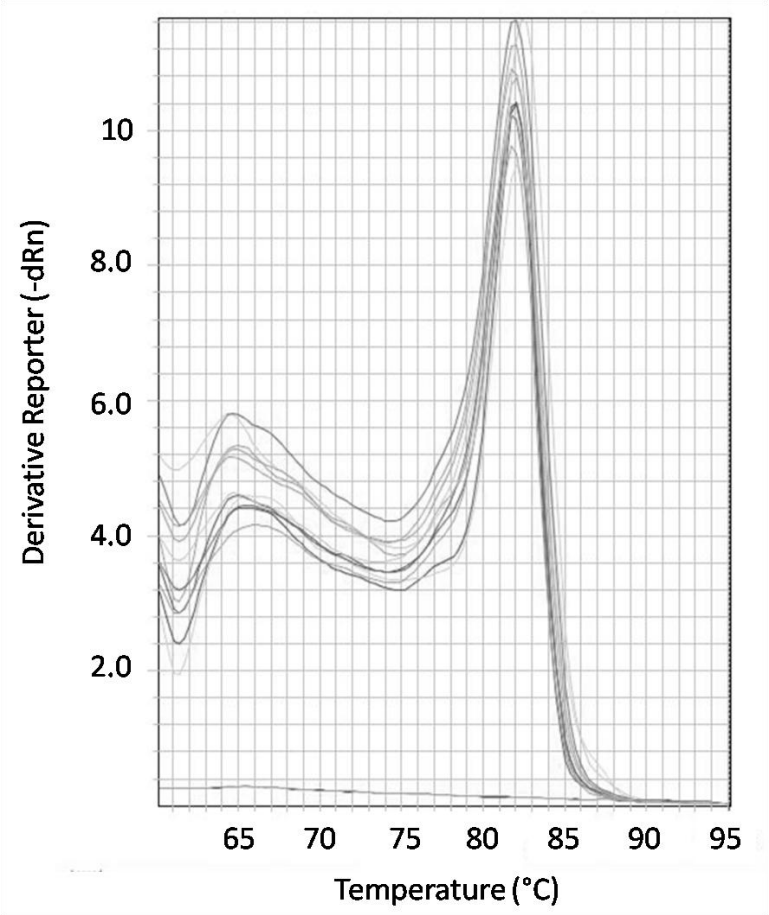


Fig 3 : Melting curve analysis of the amplicon generated with the standard (Fig. 2 A) and no-template control (NTC). The x-axis indicates the temperature and the y-axis the negative first derivative of the normalised fluorescence generated by the reporter during PCR amplification. No peak corresponding to a nonspecific amplicon or primer dimer is observed



1.5.2. Development of the whole procedure, from extraction and RT to qPCR

Selection of the extraction method

A complete test was developed, involving extraction followed by analysis in two separate steps: reverse transcription of PLMVd and quantitative real-time amplification of the fragment defined by the selected primers. Three extraction methods were tested: total RNA extraction and the preparation of crude sap with 2X SSC or KAJI buffer, both of which are commonly used to efficiently detect viroids and fruit tree viruses (1,11). The amplification efficiencies determined by RT-qPCR for these three extraction methods are presented in Table 2.

Table 2 : Influence of the extraction method on the PCR efficiency and R^2 value.

Extraction method	PCR efficiency	R^2
Qiazol	81.5%	0.94
2X SSC	99.0%	0.99
KAJI	88.7%	0.94

The results show that all three extraction methods can be used to amplify the viroid by qPCR. For amounts of extract corresponding to the same weight of leaf tissue from the same tree, the Cq was significantly lower after total RNA extraction than after crude extract preparation (Table 3). This suggests that the total RNA extraction provides more viroid RNA or lacks in components that could inhibit the qPCR reaction. Yet the PCR efficiency and R2 coefficient were higher for samples extracted with the KAJI or SSC buffer (E_{TotRNA}=81.3%, R^2_{TotRNA}=0.94; E_{KAJI}=88.7%, R^2_{KAJI}=0.94 and E_{SSC}=90%, R^2_{SSC}=0.99), and crude sap extraction is easiest to apply for routine use in diagnostic laboratories. A comparison of the two crude sap extraction procedures showed that the better PCR efficiency and lower Cq were obtained after extraction in 2X SSC and 1% sodium sulfite. These results led us to choose this extraction procedure as the most suitable for routine diagnosis in terms of rapidity, viroid recovery, and cost.

Table 3 : Mean of the measured Cq according to the extraction method for three serially diluted samples.

Dilution	Extraction Method		
	Total RNA (mean Cq)	KAJI Buffer (mean Cq)	SSC 2X Buffer (mean Cq)
10^3	17.85 ± 0.6	23.52 ± 0.24	19.84 ± 0.08
10^4	21.16 ± 0.16	28.50 ± 0.67	23.49 ± 0.06
10^5	25.08 ± 0.39	32.93 ± 2.24	26.49 ± 0.08
10^6	28.49 ± 0.28	33.87 ± 1.51	29.79 ± 0.07
10^7	38.91 ± 1.88	36.67 ± 1.21	32.06 ± 0.03

Optimisation of sample dilution

Sample dilution was optimised so as to limit the inhibitory effect of the crude sap in the reverse transcription and subsequent qPCR. A qPCR efficiency between 90 and 99% was obtained with a 100-fold dilution of the crude extract (as recommended previously for the RT-PCR (11)), before the reverse transcription step and the use of 2 µl of the produced cDNA for the amplification (amounting to a total 1000-fold dilution). Detection tests performed on

negative controls (a sample from a healthy plant and a water sample) showed that neither the PLMVd nor any nonspecific amplifiable material was detectable before cycle 35 (chosen as the positive detection limit) under these conditions (Table 4).

Table 4 : Cq mean and standard deviations obtained after the amplification on control samples (water and extract from healthy tissue)

Sample	Cq mean	Standard Deviation
Healthy	35.79	1.61
Water	35.86	0.087

1.5.3. Comparison of our RT-qPCR with the reference RT-PCR.

To compare the sensitivities of the reference RT-PCR and our RT-qPCR, both methods were used to detect the viroid in the same sample. For the RT-PCR, two replicates of three different dilutions were used (1/100 - 1/1000 - 1/10000) (Fig. 4). For the RT-qPCR, expected to be more sensitive, a range of ten-fold dilutions from 10^{-3} to 10^{-7} was tested. The RT-PCR detected PLMVd only in the first dilution, and the RT-qPCR proved to be 10^5 times as sensitive (Table 3; Fig. 4; Fig. 5).

Fig 4 : Sensitivity of the RT-PCR technique. (M) molecular weight ladder; (Bl) blank sample. Letters A to C: sample dilutions 10^4-fold (A, A'), 1000-fold (B, B'), and 100-fold (C, C'). The (') designates the second replicate. The 340-bp amplicon corresponds to PLMVd. The lower band corresponds to primer dimers.

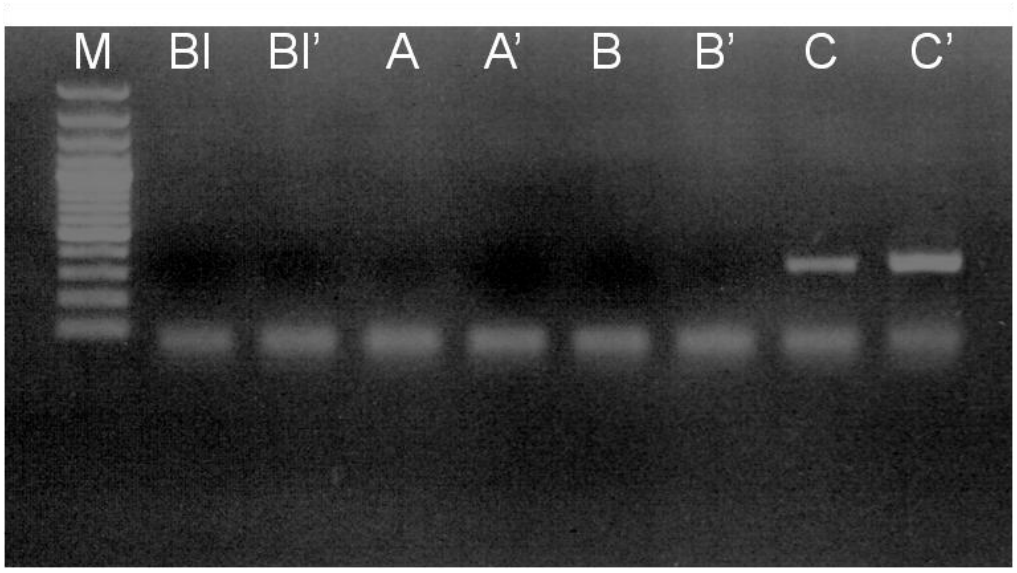
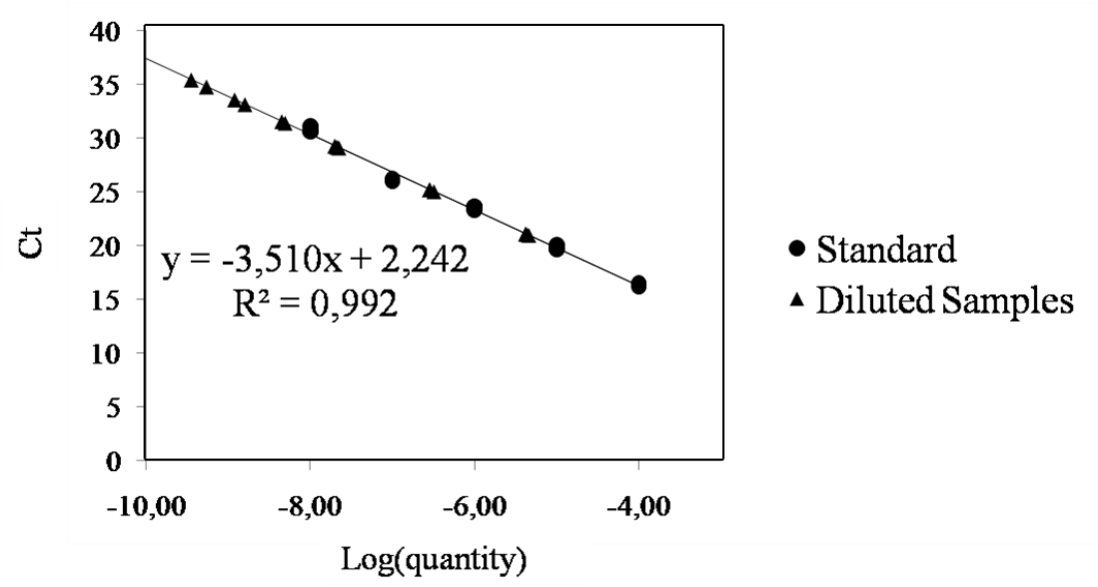
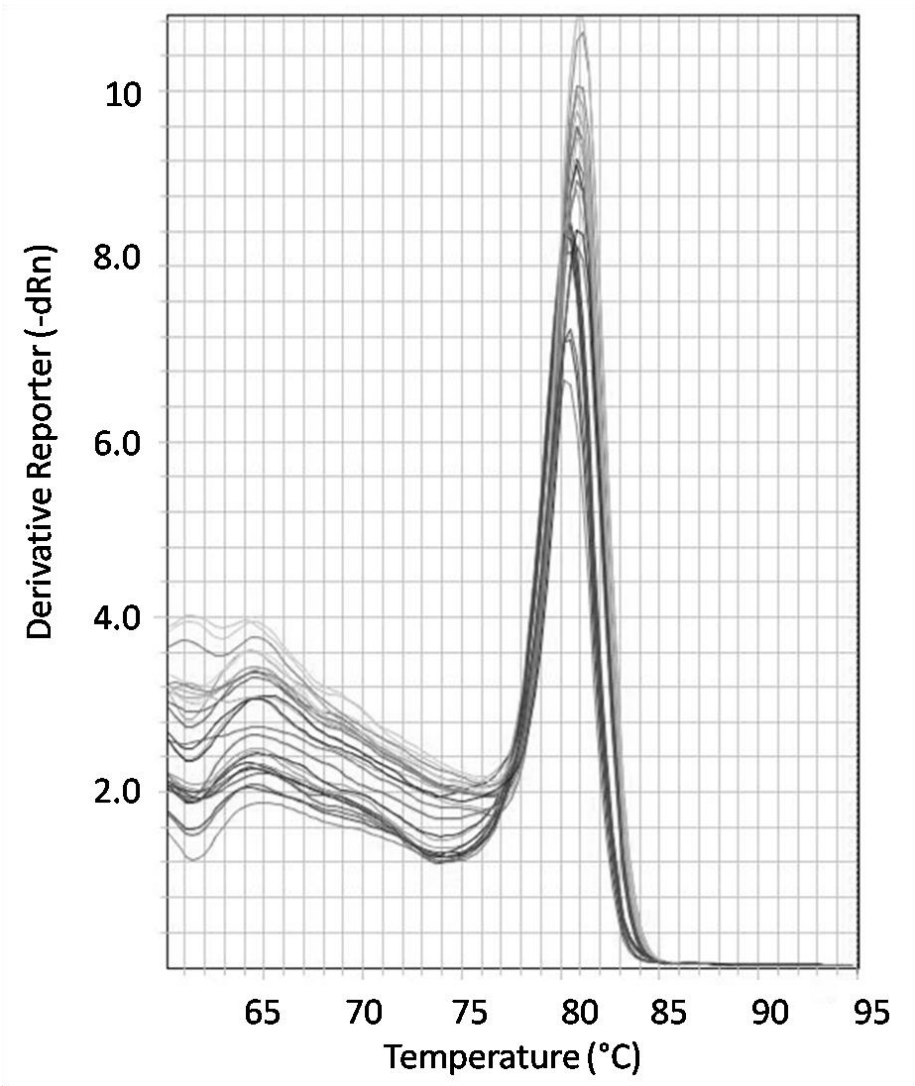


Fig 5 : Standard curve analysis of qPCR sensitivity. The x-axis displays the logarithm of the DNA quantity and the y-axis the measured Cq value. Round points: standards used to calculate the curve; triangles: diluted samples. E: qPCR efficiency.



The standard deviation of the Cq (Cq SD) (calculated for the three replicates of each sample) was near zero for each dilution, showing the repeatability of the technique. The reproducibility of the qPCR was also checked by independent amplifications from the same extract (data not shown). The melting curve confirmed specific amplification of the PLMVd even in the most diluted samples (Fig. 6).

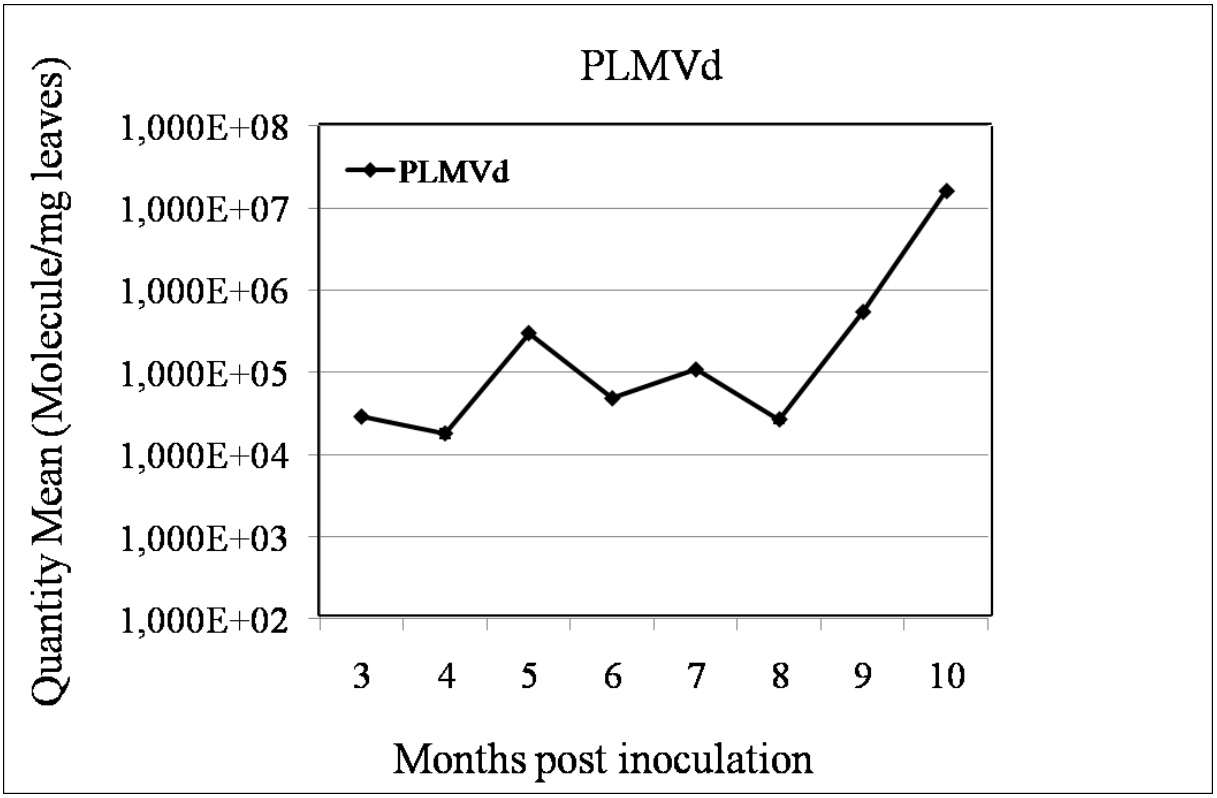
Fig 6 : Melting curve analysis of the amplicon generated with the standard (Fig. 5). The x-axis indicates the temperature and the y-axis the negative first-derivative of the normalised fluorescence generated by the reporter during PCR amplification. No signals of possible aspecific amplicons are present in standards amplifications and in the diluted samples.



1.5.4. Comparison of RT-PCR and RT-qPCR applied to plant samples taken over a 12-month period

To confirm the utility of our RT-qPCR in certification procedures, we performed PLMVd amplifications on extracts from two young peach seedlings inoculated 3 months after emergence. As these seedlings were in active development, we assumed they were actively replicating PLMVd (20). Each month until one year post-inoculation, eight leaves per plant were harvested, frozen, and stored. The reference RT-PCR did not detect PLMVd in the samples harvested until month 8 post-inoculation. With our optimised RT-qPCR, the viroid was already detectable and quantifiable (Cq<35) 3 months post-inoculation (Fig. 7). All the plants were inoculated in June, and the quantity of viroid appeared to remain stable in the greenhouse over the winter. After 8-9 months, with the arrival of spring and increased plant growth, the viroid level increased drastically. During this active replication period the Pearson's correlation coefficient, measuring the strength of the linear relationship between two variables, revealed a significant linear correlation between the viroid titre and the number of weeks of active viroid replication after inoculation.

Fig 7 : Quantification by the optimised qPCR of the PLMVd in leaves harvested three to 12 months after inoculation. Data are expressed in PLMVd molecules per milligramme of leaves



1.6. Discussion

Traditional RT-PCR procedures for detecting PLMVd have clearly shown their limitations in certification programs. Essentially, they are not sensitive enough, and this leads to false-negative results. Problems include primer dimer formation, samples with a low viroid level, and the unequal distribution of PLMVd through the plant. To allow detection of lower viroid quantities and thereby limit the risk of false negatives, we have developed a quick and highly sensitive RT-qPCR.

We demonstrate here the importance of choosing adequate primers and a good extraction method. Use of the primers developed previously yields a fragment that is too long for qPCR (340 nucleotides) (11), there being a risk of reaching saturating levels of fluorescence or of depleting the PCR reagents too rapidly. It was therefore necessary to design new primers. The primers selected in this study allow specific, efficient amplification of an 89-nucleotide fragment suitable for qPCR. The other (recent) sensitive detection protocols of which we are aware use time-consuming total RNA or liquid-nitrogen-based extraction procedures (2-3,19). Here we show that both crude sap extraction methods tested are quite efficient. They are also easy, quick, and relatively cheap, so that the technique can be exploited advantageously by many laboratories. To eliminate the PCR-inhibiting effect of the crude sap (inherent in this type of extraction (2), it is sufficient to dilute the sap 1000-fold, with no need of further purification. Furthermore, by diluting the standards in crude sap from healthy tissue, it is possible to ensure that a negative result really means that the plant is healthy or that the PLMVd concentration is below the detection level of the developed RT-qPCR.

The high sensitivity of this RT-qPCR makes it possible to detect and quantify low-level or early infections of peach trees by the PLMVd. This should help improve the efficacy of certification programs. Moreover, the absence of post-amplification procedures reduces the risk of cross-contamination by comparison with RT-PCR and RT-LAMP (2-3), and permits the use of robotic laboratory handling systems. Finally, the higher cost of the qPCR reagents and the increased manipulation time before amplification are counterbalanced by the accurate results and the possibility of early PLMVd detection.

The technique does not yield absolute quantities of PLMVd in the leaf samples, as it is impossible to recover all of the crude sap after grinding and to evaluate correctly the efficiency of reverse transcription in the presence of crude sap. One can assume, however, that crude sap recovery and the reverse transcription efficiency are the same for all samples

treated in a particular experiment, so that samples can be compared and a picture gained of (i) the infection level or (ii) the replication level of the PLMVd variant in these samples. Furthermore, the quantitative effects demonstrated here are in agreement with the those established by dot blot hybridisation (4). Our results show that PLMVd levels are higher during the spring and summer than during the winter, which may be explained by higher metabolic activity of plants during the growing season and use of plant enzymes for viroid replication (20). Also, we have evidenced a significant correlation between the viroid titre and the number of weeks post-inoculation, as in the case of the citrus viroid CVd III (19). PLMVd thus seems unaffected by plant defense mechanisms during its life cycle in infected plants.

This technique should further the *in vivo* study of PLMVd replication, thanks to its capacity to quantify PLMVd in peach tree leaves quickly and without the need to use dangerous ingredients such as radioactive materials.

Our method should help to avoid the accidental release of infected material into orchards and considerably accelerate the certification of new material (thanks to the reduced incubation time of the plant to be tested). Some of our results, however, suggest that PLMVd is not uniformly distributed in the plant (data not shown), so precautions need to be taken when sampling, especially in the framework of certification. Nevertheless, the high sensitivity of this method should limit the risk of false negatives. Further studies should be carried out to understand the distribution of PLMVd in trees and to optimise sampling procedures.

1.7.Acknowledgements

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2. Determination of the P8 pseudoknot importance for the viroid life cycle

2.1. Introduction

During the last ten years, several studies have permitted to obtain a better knowledge of the PLMVd sequence and structure. These studies have led to (i) the identification of the replication origin, (ii) the characterization of the ribozyme structure, (iii) the elucidation of the determinant of the peach calico pathogenicity, (iv) the establishment of the phylogeny of more than hundred PLMVd variants, (v) the demonstration of the pseudoknot formed by L6-L7 and L1-L11 and (vi) the identification of the global structures adopted by the plus and minus polarity strands of this viroid.

Excepted for the supplementary (and optional) hairpin inducing the peach calico, no PLMVd sequence or structure were implicated in the *in vivo* pathogenicity of this viroid. Moreover, the hammerhead ribozyme appeared to be the single PLMVd structure important for the replication. The situation is different in the case of the *Pospiviroidae* for which the sequence/structure functions are well characterized and permit an easier study of these viroids.

As the function of the hammerhead ribozyme is known, the function of two pseudoknots remained to be elucidated. The P8 pseudoknot seemed a better candidate that the L1-L11 one because (i) its existence was clearly demonstrated and (ii) covariations seemed maintain this structure in all PLMVd sequences previously analyzed. These two arguments have led to the selection and the use of this pseudoknot for our study.

This part of the PLMVd study is dedicated to the determination of the importance of the P8 pseudoknot in the viroid life cycle. Mutations were introduced to destabilize this structure and inhibit its formation. The mutants obtained were inoculated on GF-305 healthy peach trees and their replication and pathogenicity were followed by RT-qPCR and visual inspection respectively.

2.2. Material and methods

2.2.1. Plant and viroid material

Healthy GF-305 peach trees obtained from peach seedlings were grown in greenhouse conditions before their inoculation with PLMVd variants. Ten plants were inoculated in June 2008 with the mutated variant and ten other with the wild type variant. Inoculations were performed with PLMVd dimeric RNA in 50mM KH_2PO_4. Eight to ten leaves were harvested each month from October 2008 to June 2009 and keep frozen at -80°C.

2.2.2. Bacterial strain

The bacterial strain INVαF' belonging to *Escherichia coli* was used for the cloning reactions.

2.2.3. Cloning vector

The vector used for the cloning reactions was the pCR2.1 from the TA cloning kit (Invitrogen, Carlsbad, California, USA).

2.2.4. PLMVd clone

The severe PLMVd variant used in this study was cloned by Fekih Hassen from a sample collected in an Alberta cultivar in Tunisia (Fekih Hassen 2007).

2.2.5. Construction of the dimers

The dimerization of the variant was necessary (i) to obtain the mutated PLMVd at the level of the P8 pseudoknot and (ii) to realize the transcription of the complete sequence of the PLVMd allowing the best inoculation efficiency.

- Purification of the plasmid DNA of *E. coli* using the GeneJet™ plasmid Miniprep kit (Fermentas, Vilnius, Lithuania)

The purification was based on the lysis of the cells followed by the precipitation of the chromosomal DNA and the protein of the bacteria. Several washes have permitted to obtain a large amount of purified plasmids. This purification was realized from transformed colonies growing at 37°C in liquid NZY (5g/l NaCl, 2g/l $MgSO_4$*$7H_2O$, 5g/l Yeast extract, 10g/l NZ amine, pH7) supplemented with 100µg/ml of ampicillin. The purification was realized following the manufacturer's instructions.

- Amplification of the DNA inserted in the recombinant plasmid

The DNA inserted into plasmids was amplified with the Pfu DNA polymerase and the hPLMVd/cPLMVd primers to obtain blunt ended fragments. Furthermore this polymerase possesses a proofreading activity limiting the mutations during the amplification.

The reaction contained: 0.2µM of the two primers, buffer Pfu 1X (Fermentas), 0.2mM of dNTP (Fermentas), 1.25 unit of Pfu DNA polymerase (Fermentas) and 10ng of recombinant plasmid.

The PCR cycle was the following:

- A denaturation at 95°C for 5min,
- 35 cycles each comprising:
  - a denaturation at 95°C for 30s,
  - the annealing at 60°C for 60s,
  - the elongation at 72°C for 60s,
- and a final elongation step at 72°C for 7min

- Purification of the PCR products

The amplification products were migrated in a 1.2% agarose gel and the fragment corresponding to the PLMVd was excised and purified with the QIAEX II Gel extraction kit (Qiagen) following the manufacturer's instructions.

- Phosphorylation of the purified DNA

The phosphorylation of the purified DNA was realized with the T4 polynucleotide kinase (PNK) from Fermentas. This reaction allows the transfer of a γ-phosphate group from the ATP to the 5'-OH group of an oligonucleotide permitting its ligation to another oligonucleotide.

The reaction mixture comprised: Buffer A 1X (Fermentas), 10µM ATP (Fermentas), 10 units of T4 PNK and 1µg of the PCR products.

The mixture was incubated 30 min at 37°C. The reaction was stopped by the addition of 0.5M EDTA pH8. The phosphorylated DNA was then purified with the QIAQuick PCR purification kit (QIAGEN) following the manufacturer's instructions.

- Ligation

This step aimed to produce multimeric DNA from the phosphorylated monomers with the T4 DNA ligase from Fermentas.

The reaction mixture comprised: 1X of ligation buffer, 5% of PEG 4000, 5 units of T4 ligase and 1µg of phosphorylated DNA. It was incubated 1 hour at 22°C. The T4 DNA ligase was inactivated by heating at 65°C for 10 min.

The DNA was extracted by centrifugation (15min, 16100g) with phenol (v/v) and precipitation of the aqueous phase in 2.5 volume of ethanol (EthOh) with 10% of sodium acetate (3M, pH 5.2) at -20°C for 2 hours. The DNA was collected by centrifugation (15 min, 16100g) and washed with 70% EthOh before a final centrifugation (20 min, 16100g). The DNA was air dried and resuspended in 10µl of sterilized distilled water.

- Amplification of the ligation product

The amplification was realized to separate the dimers from the other multimers. The PCR was performed with the High fidelity PCR enzyme mix (Fermentas) and the primer pair hPLMVd/cPLMVd. The reaction was conducted with 0.2µM of the two primers, 1X PCR buffer, 0.2mM of dNTP, 1.75 unit of the enzyme mixture (Taq DNA polymerase and Pfu DNA polymerase) and 100ng of the ligation product.

The used PCR cycle was described previously for the amplification of the PLMVd clones.

- Purification of the dimers

The dimers (+/- 680 base pairs) were purified with the QIAEX II (GIAGEN) gel extraction kit after an agarose gel electrophoresis following the manufacturer's instructions.

- Cloning of the dimers into the vector pCR2.1

The TA cloning system (Invitrogen, Carlsbad, CA, USA) has permitted the direct cloning of the PCR products into the pCR2.1 vector. The needed reactions were realized according to the manufacturer's instructions.

- Verification of the dimer insertion and orientation

The presence and the orientation of the dimers into the cloning vector were controlled by the enzymatic digestion of the plasmid by KpnI and sequencing respectively. The digestion has permitted the selection of the clones inserting two monomers with the same orientation. The selected clones were then sequenced.

The following reaction was carried out for the enzymatic digestion: 0.2 Unit of KpnI restriction enzyme, 1X of KpnI buffer, 1 µg of plasmid.

The mixture was incubated overnight at 37°C. The enzyme was inactivated by heating at 80°C during 20 min.

The digestion profile was observed by migration on a 2% agarose gel.

Following this electrophoresis, the clones containing the well-oriented dimers were sequenced to determine the global orientation of the dimers. The clones permitting to obtain a PLMVd RNA of the plus polarity after transcription were then selected.

The sequencing was realized with the Big Dye Terminator from Applied Biosystems. Two reactions were realized for each sample: forward and reverse.

Compounds	Volume/Quantity
DNA	50ng
M13 Primer (Forward or Reverse)	0.7µl
Big Dye Terminator	2µl
H_2O	5.2µl

The cycle used for the sequencing reaction comprised:

- 25 cycles with:
    - 96°C during 10s
    - 50°C during 5s
- 60°C during 4 min.

2.2.6. Mutated PLMVd variant

The P8-mutated PLMVd was constructed by the laboratory of Jean-Pierre Perreault (Département de Biochimie, Université de Sherbrooke, Canada) from our dimerized severe PLMVd variant. The P8 variant was mutated by directed mutagenesis of the P7 hairpin ($_{212}$CCGC$_{215}$ replaced by $_{212}$AAAA$_{215}$) avoiding the formation of the pseudoknot. The P8 variant was then dimerized.

2.2.7. Transcription of the PLMVd dimers

The transcription was realized with TranscriptAid™ T7 High Yield Transcription Kit from Fermentas following the manufacturer instructions. This transcription with the T7 promoter permits the obtaining of a PLMVd RNA of the plus polarity.

2.2.8. Detection of the PLMVd by RT-PCR

- Primers

The cPLMVd primer is the reverse oligonucleotide corresponding to the nucleotides 91 to 115 of the PLMVd sequence (5'-AACTGCAGTGCTCCGAATAGGGCAC-3'). The hPLMVd primer corresponds to the nucleotides 116 to 140 (5'-CCCGATAGAAAGGCTAAGCACCTCG-3'). These primers were produced by Eurogentec (Liege).

- RT-PCR reaction

The one-tube two-steps RT-PCR was performed with the Titan one Tube RT-PCR system (Roche diagnostics, Penzberg, Germany) following the manufacturer's instructions. The reaction mixture comprised: 400nM of the two primers, 1X buffer, 5mM of DTT, 0.2mM of dNTP, 0.5µl of the enzyme mix and 2µl of crude extract.

The reaction mixture was added after denaturation 5min at 95°C with the reverse primer.

The RT-PCR comprised:

- The reverse-transcription (50°C-1hour)
- A denaturation (95°C-3min),
- 35 cycles comprising:
    - A denaturation (35°C-30 s)
    - The primer annealing (60°C-45 s)
    - The elongation (72°C-45 s)
- A final elongation (72°C-5 min)

2.3. Results

The replication of the P8 variant was assessed by the RT-PCR and the quantitative real-time RT-PCR described in this chapter. Peach tree leaves were harvested from October 2008 to June 2009 and keep frozen to avoid any PLMVd degradation.

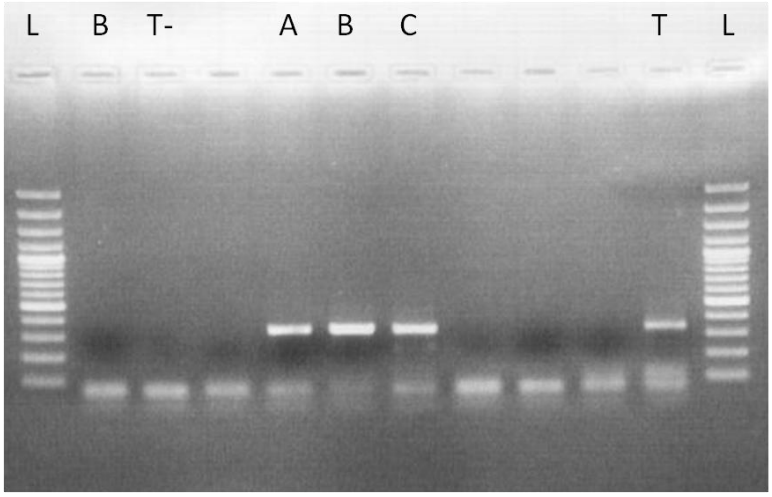
2.3.1. Detection of the PLMVd in the inoculated plant

The RT-PCR was used to detect the inoculated variants from crude extract of peach tree leaves harvested from February 2009 until June 2009 to determine the plants effectively infected. Five plants inoculated by the wild type variant appeared infected by RT-PCR (Table 1, Figure 1). As expected, these plants presented the severe mosaic symptoms induced by the 151.1 variant. The corresponding samples were selected to compare the replication of the P8 variant with the wild type variant. At this time, no amplification product was detectable for P8 by RT-PCR (Table 1). Furthermore, the plants inoculated by P8 did not present any symptoms.

Table 1 : Synthesis of the detection results of inoculated 151.1 and P8 PLMVd variants

Variant	Positive plants	Positivity percentage
Wild-type (151.1)	5	50%
P8	0	0%

Figure 1 : Agarose gel electrophoresis of amplified detection samples. (L) molecular weight ladder GeneRuler 100pb plus DNA ladder; (B) Blank sample; (T-) negative control; (A, B, C) plant samples; (T) Positive control



2.3.2. Quantification of the PLMVd (wild-type and mutated) by RT-qPCR

As the mutations introduced in the PLMVd sequence seemed to have an effect on the PLMVd replication, we have chosen to test if the mutated variant could be detected by our more sensitive RT-qPCR. Furthermore, this approach could allow to obtain a picture of the *in vivo* PLMVd replication along the time.

The RT-qPCR has permitted to detect the mutated PLMVd in 4 plants. One of these positive plants was chosen to be analysed for the complete harvesting period in comparison with the samples coming from a plant infected by the wild-type variant.

The quantifications obtained by the RT-qPCR are given in Table 2 for two tested variants: 151.1.1 and P8.4. All the samples of an infected plant were tested in a single RT-qPCR plate (plate A or B) with 151.1.1 as positive control. All the quantification data (Ct and quantity) were calculated from three technical replicates for each sample. The Table 2 and Figure 2 show that the quantity of the two variants was relatively stable from the autumn to the spring and seems explode from the spring to the summer. Furthermore, the quantity of the P8 variant is always lower than for the 151.1 variant.

The figure 2 shows the evolution of the P8 and 151.1 quantities in plants through the complete harvesting period. This graph shows that the viroid quantity has varied during the season and these variations are similar for the wild-type variant and the mutated one until January. The situation between the two variants became clearly different in February: the variant 151.1 showed, at this time and until the last harvest, an enhanced replication. At the opposite, the quantity of the P8 variant seemed to decrease until May where the replication became more active. The comparison of the viroid quantities between the two variants showed differences from 13-fold to 1000-fold.

Furthermore the variance analysis for the variant 151.1 has revealed that the sampling date is a highly significant factor for the determination of the quantity expressed in molecule/reaction. Finally, the mean analysis (Fisher test) has revealed that the quantities are not significantly different for the samples of October and January but well for the others (Table 3). The same analysis realized for the P8 variant showed that the situation was different for this variant: the calculated quantities are not significantly different between the samples until January (Table 3). Finally, the Pearson’s correlation coefficient, measuring the strength of the linear relationship between two variables, revealed a significant linear

correlation between the viroid titre and the number of weeks of active viroid replication after inoculation.

Table 2 : Quantifications of the inoculated variants

Plate	Infected plant	Harvesting date	Ct mean	Quantity mean (ng/reaction)	Standard deviation	Quantity mean (molecule/ reaction)	Molecule/mg of leaves
A	151.1.1	7/10/2008	32,16	1,322E-09	1,058E-10	2,901E+01	2,901E+04
A	151.1.1	7/11/2008	32,84	8,267E-10	1,126E-10	1,814E+01	1,814E+04
A	151.1.1	24/11/2008	28,77	1,369E-08	1,587E-09	3,005E+02	3,005E+05
A	151.1.1	12/12/2008	31,46	2,204E-09	7,243E-10	4,837E+01	4,837E+04
A	151.1.1	9/01/2009	30,25	4,945E-09	7,301E-10	1,085E+02	1,085E+05
A	151.1.1	28/01/2009	32,29	1,224E-09	2,998E-10	2,687E+01	2,687E+04
A	151.1.1	15/04/2009	27,91	2,476E-08	2,183E-09	5,435E+02	5,435E+05
A	151.1.1	8/06/2009	23,00	7,305E-07	5,084E-08	1,603E+04	1,603E+07
B	P8 4	07/10/2008	34,78	1,114E-09	1,750E-09	2,447E+01	2,447E+04
B	P8 4	07/11/2008	35,79	1,921E-10	1,916E-10	4,217E+00	4,217E+03
B	P8 4	24/11/2008	35,29	2,902E-10	3,168E-10	6,371E+00	6,371E+03
B	P8 4	12/12/2008	37,00	5,898E-11	2,297E-11	1,295E+00	1,295E+03
B	P8 4	09/01/2009	36,43	8,801E-11	3,349E-11	1,932E+00	1,932E+03
B	P8 4	28/01/2009	36,67	7,145E-11	1,352E-11	1,568E+00	1,568E+03
B	P8 4	15/04/2009	39,23	1,212E-11	3,583E-12	2,661E-01	2,661E+02
B	P8 4	08/06/2009	33,61	6,232E-10	2,215E-10	1,368E+01	1,368E+04
B	151.1.1	08/06/2009	23,78	5,666E-07	4,252E-08	1,244E+04	1,244E+07

Figure 2 : Evolution of the viroid quantity (expressed in molecule/mg of leaves) during the cultural season.

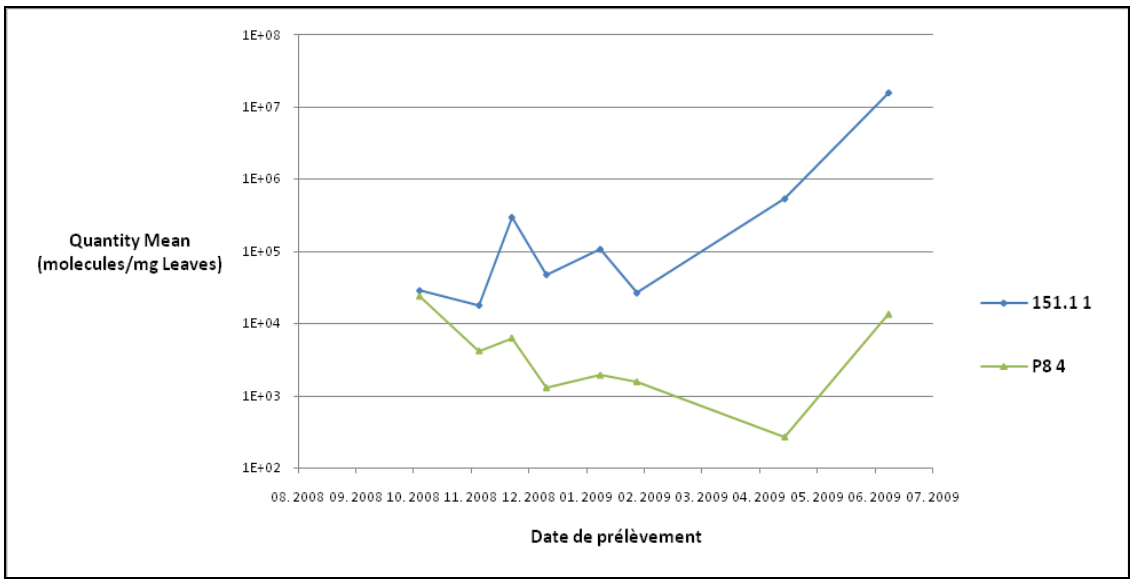


Table 3 : Mean analysis of the viroid quantity along the cultural season. Identical letters in the last column indicates equal

Variant	Sampling date	Molecule/reaction (mean)	Group of equal values
151.1.1	7/10/2008	2,901E+01	B
151.1.1	7/11/2008	1,814E+01	A
151.1.1	24/11/2008	3,005E+02	E
151.1.1	12/12/2008	4,837E+01	C
151.1.1	9/01/2009	1,085E+02	D
151.1.1	28/01/2009	2,687E+01	B
151.1.1	15/04/2009	5,435E+02	F
151.1.1	8/06/2009	1,603E+04	R
P8 4	7/10/2008	2,447E+01	L
P8 4	7/11/2008	4,217E+00	L
P8 4	24/11/2008	6,371E+00	L
P8 4	12/12/2008	1,295E+00	L
P8 4	9/01/2009	1,932E+00	L
P8 4	28/01/2009	1,568E+00	L
P8 4	15/04/2009	2,661E-01	K
P8 4	8/06/2009	1,368E+01	M
151.1.1	8/06/2009	1,603E+04	R

2.4.Discussion

2.4.1. The wild-type variant 151.1

The RT-PCR has shown that 50% of the plants inoculated with the variant 151.1 were effectively infected. This result is in agreement with detection campaigns realized in 2006 (Parisi, 2006). These plants were used as positive and comparison control for the analysis. However, we must note that in the literature 93% of the inoculated plants are positive 9-10 months after the inoculation (Ambros et al. 1998). This difference with our variant could come from any differences (in the sequence or structure) between the used variants leading them easiest to inoculate mechanically in the latter study. Finally, the variability appears rapidly during the replication of the PLMVd and can lead to the dominance of a variant with a sequence slightly different than the inoculated one (Ambros et al. 1999).

The results of quantification obtained by RT-qPCR have revealed that the PLMVd quantity varies during the season. The observed evolution seems to follow the growth conditions of the plants. A decrease of the viroid quantity was effectively observed during the winter. From the spring, the viroid quantity has regularly increased following the amelioration of the growing conditions. The literature has already described that the replication of viroids is influenced by the growing conditions (Singh et al. 2003). Our quantifications give a supplementary proof of this impact of the plant physiology to the viroid replication.

However, alone, the reduce growth of the plant cannot explain the decrease of the viroid quantity during the winter. Two hypothesises could give an explaination of this phenomenon. The first one is based on the presence of the gene silencing phenomenon in plant infected by the PLMVd (St-Pierre 2009). The degradation of the viroid by this plant defence mechanism could explain the decrease of the PLMVd quantity in plants during the period of reduce replication. However, as the PLMVd remained in plants, a part of the viroid molecules escaped to this phenomenon by the mean of the sub-cellular localization, the compact structure of the PLMVd or by another unknown mechanism (Landry et al. 2004). The RNA silencing could participate to the PLMVd degradation during the infection but it is clearly compensated by the active replication during the active growth of the plant.

The second hypothesis is based on the unequal repartition of the viroid in plants. Linked to the reduced replication and plant growth during the winter, the PLMVd could be more “diluted” in the sample due the reduced number of young fresh leaves at this time. A study on the precise viroid distribution in the plant should help to better understand this phenomenon.

These two hypothesises could explain the evolution of the viroid quantity along the time. The active growth of the plant and thus the active replication of the PLMVd in every leaves would counterbalance the RNA silencing during the spring and the summer. Finally, other unknown mechanisms, maybe plant defence mechanisms or protein-viroid interactions, reducing the viroid quantity cannot be excluded.

2.4.2. The P8 mutated variant

The RT-PCR appeared unable to detect the P8 variant up to nine months after inoculation. As the reliability and reproducibility of the used RT-PCR was demonstrated (Fekih Hassen et al. 2006), we could conclude that these plant were not infected.

However, in the hypothesis that this variant accumulates at a relatively low rate in the plant tissues, the RT-qPCR, whose higher sensitivity was demonstrated (Parisi et al, 2010), was used to analyse the P8 samples.

In these plants, quantities varying between one molecule per reaction ($2{,}661.10^2$ molecules/mg of leaves) to 24 molecule per reaction ($2{,}447.10^4$ molecules/mg of leaves) were measured. However some of these values are probably imprecise because of their smallness. These quantities are significantly lower than these of the reference sample but they follow the same variation pattern than the quantities of the 151.1 variant (certainly for same reasons described for the 151.1 variant). However, the quantity of P8 is up to 1000-fold lesser than 151.1 during the summer and thus during the active replication of the viroid in the leaves.

These results suggest that the P8 variant, possessing (at the inoculation time) a destabilized pseudoknot, is able to replicate at a relatively low rate leading this variant undetectable by the classic RT-PCR technique. Furthermore these results suggest that the P8 has a role either in the viroid *in vivo* stability or in its replication capabilities. The great or (vital) importance of this pseudoknot for the viroid can be postulated because of the covariations maintaining it in every PLMVd sequenced and is strengthened by our quantification results.

We have also noted that plants presenting a replication of the P8 variant did not express the PLMVd symptoms. However, we cannot conclude if this absence of symptoms is due to an insufficient viroid quantity or if the pseudoknot is also implicated in the viroid pathogenicity.

Finally, we should note that we do not actually know if the progeny of this variant possess already the destabilized pseudoknot or if the effective replication is due to mutations restoring

this structure. A sequencing of the progeny during a complete season will help to elucidate the key of this replication. Moreover, as the growing conditions also influence the replication of the PLMVd, it should be interesting to perform a confirmation of these results in severe controlled conditions.

2.5.Conclusion

The real-time PCR was developed and used to quantify the PLMVd in crude extract of peach tree leaves sampled between the fourth and the twelfth month after the inoculation. This technique appeared, finally, more sensitive than the classical RT-PCR.

Our results have shown that the quantity of viroid varies along the time. The quantity decreases during the winter and increases after January for all of the inoculated variants. The increasing is most probably due to a better replication of the PLMVd after January because of the better plant-growth conditions (Singh et al 2003). Furthermore, the decrease of the viroid quantity may be due to a degradation of the viroid by the RNA silencing and an unequal repartition of the viroid in the infected plants.

The quantification results of the mutated variant seem indicate that the P8 pseudoknot is important for the replication or the stability of the molecule *in vivo* or its resistance to a degradation by the RNAse of the host. However further studies are needed to precisely identify the role of this structure on the life cycle of the PLMVd

Finally, the real-time PCR approach seems adequate to characterize the *in vivo* importance of viroid motifs or structures. It could be used to understand the effect of other mutations on the replication and/or the pathogenicity of the PLMVd. However, optimizations of the sampling method are necessary to avoid any errors in the quantification (or detection) of the viroid.

2.6.Literature cited

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Chapter IV
Identification of genes potentially involved in the host-pathogen interaction

1. Identification of GF-305 Peach tree genes influenced by a *Peach latent mosaic viroid* (PLMVd) infection

1.1. General introduction

The characterization of the host-pathogen relationships is the starting point of the development of efficient strategies to control pathogens.

However, in the case of viroids, and especially for the PLMVd-peach pathosystem, this characterization is highly incomplete, even totally non-existent. This could explain why we are still unable to control these pathogens.

Furthermore, the mean by which the PLMVd induces symptoms on infected leaves as well as the biological pathways altered (or enhanced) by this pathogen were not yet studied. This lack of information makes complicated the control of this pathogen.

The cDNA-AFLP is a powerful middle-throughput technology to characterize the molecular host-pathogen relationships. One of the main advantages of this technique is the possibility to work with non sequenced genome as peach trees.

To characterize these relationships during a PLMVd infection, and to better understand the effect of PLMVd on the symptoms expression, we have applied the cDNA-AFLP on peach tree leaves presenting different symptoms.

Two comparison models were used for this study. The first one has studied the gene expression modification between latent and severe symptomatic areas of an infected plant. The second model has compared the gene expression between leaves presenting a chlorosis and leaves showing a mosaic on two different infected plants.

These results are submitted for publication

Identification of GF-305 Peach tree genes influenced by a Peach latent mosaic viroid (PLMVd) infection

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Identification of GF-305 Peach tree genes influenced by a *Peach latent mosaic viroid* (PLMVd) infection

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1.2.Abstract

The cDNA-AFLP was used to understand, at molecular level, the host-pathogen relationship established during an infection of peach trees by the *Peach latent mosaic viroid* (PLMVd). Peach tree leaves infected by variants with different pathogenicities were harvested after expression of the typical infection symptoms. Two comparison models were used to relate symptoms to transcript-level gene expression: the first compared the expression patterns of symptomatic and asymptomatic leaves of the same plant, while the second compared gene expression levels in a plant displaying severe chlorosis and another showing a mosaic. In these models a total of 493 bands out of 11985 (4.11%) were recognized as differentially expressed. Among them, 13 genes were selected for their involvement in photosynthesis, photosystem protection, plant defence (systemic acquired resistance, polyamine synthesis, polygalacturonase inhibition), mRNA translation, or protein metabolism. The differential expression of 9 of these 13 genes was confirmed by quantitative real-time RT-PCR. The genes confirmed as being differentially expressed between symptomatic and asymptomatic leaves or between leaves showing severe chlorosis or a mosaic suggest that photosynthetic activities are highly disturbed during infection by PLMVd. They further show that peach plants act against this viroid by means of unspecific defence mechanisms like systemic acquired resistance.

1.3.Introduction

Viroids, discovered in the 1970's, are exclusive plant pathogens. These single-stranded RNA molecules that do not code for any protein can induce diseases in several herbaceous as well as tree species (17, 46). Over the past thirty years a great research effort has been devoted to studying the structure and pathogenicity determinants of a few viroid species, such as the *Potato spindle tuber viroid* (PSTVd), the *Citrus exocortis viroid* (CEVd), the *Peach latent mosaic viroid* (PLMVd), the *Avocado sunblotch viroid* (ASBVd), and the *Chrysanthemum chlorotic mottle viroid* (CChMVd) (14, 25). These studies have led to classifying viroids into two families based on their primary and secondary structures, the ribozyme activity, the subcellular localization, the replication mechanism and their solubility in 2M LiCl: the *Pospiviroidae* and the *Avsunviroidae* (14, 25). The *Pospiviroidae* family, whose members have a rod-like secondary structure, is the best-known viroid family. The main species is PSTVd, which infects potato and tomato plants. The fact that these host plants are well characterized has facilitated the study of the *Pospiviroidae* (25), in whose sequences five functional and structural domains related to replication or pathogenicity have been identified (for a review see 14; 29). In contrast, the only functional domains to have been clearly identified to date in the *Avsunviroidae* family, whose members fold into a highly branched secondary structure, are the ribozyme domain (which catalyses cleavage of multimers into monomers during replication) and the replication origin (14, 22). The *Peach latent mosaic viroid* belongs to the *Avsunviroidae* family and infects mainly peach trees. This circular RNA molecule of 338 to 350 nucleotides (nt) causes peach latent mosaic disease (32, 40). Like the other members of the *Avsunviroidae* family, PLMVd possesses a ribozyme (24). Much effort has been devoted to characterizing its replication origin, which seems located near the ribozyme cleavage site in the hammerhead arm (36). One domain of PLMVd has been linked to pathogenicity. It consists of a 12, 13-nt hairpin to the left of the ribozyme region of variants inducing extreme chlorosis (peach calico) (32). The pathogenicity determinant of peach calico is related to the three nt of the ribozyme loop of CChMVd, which have likewise been implicated in the latter's pathogenicity (15, 32). Yet PLMVd variants lacking this hairpin are also pathogenic, so the pathogenicity determinant (or determinants) of *Avsunviroidae*-family viroids remain ill-defined.

The symptoms induced by viroids are well documented. Typical symptoms include stunting, leaf epinasty, and chlorosis. At cell level, one can easily distinguish a distortion of the cell wall, the plasma membrane (Momma and Takahashi 1982; Paliwal and Singh 1981;

Semancik and Vanderwoude 1976; Wahn et al. 1980 cited by Itaya et al. (27)), and also the chloroplasts (Da Graça and Martin 1981; Hari 1980; Lawson and Hearon 1971; Momma and Takahashi 1982 cited by Itaya et al. (27)) and mitochondria (Paliwal and Singh 1981 cited by Itaya et al. (27)). Extreme PLMVd pathogenicity can induce profound chloroplast alterations inhibiting the chromoplast-to-chloroplast transition and leading to the characteristic white/yellow discoloration typical of peach calico (32, 40).

Viroid pathogenicity, symptom expression, and the host response are all poorly understood at the molecular level (46). Concerning the host response, infections induced by viroids cause the accumulation of pathogenesis-related proteins, among which hydrolytic enzymes, subtilisin-like endoproteases, and thaumatin-like proteins have been distinguished. Recent studies have shown that PSTVd regulates the expression levels of protein kinase PKV and of proteins involved in biological processes such as defence and stress responses, cell wall structure, photosynthesis, and protein metabolism (27, 46). Until now no information has been available concerning the response of peach trees to PLMVd infection.

The cDNA-amplified fragment length polymorphism (cDNA-AFLP) method is an interesting technique for studying differential gene expression in various plant species, especially if little sequence information is available (7) as in the case of peach trees. For non-sequenced genomes like that of the peach tree, this gel-based medium-throughput technique makes it possible to analyse gene expression patterns at relatively low cost. Because cDNA-AFLP is based on linker-ligated PCR, whereas differential display is based on arbitrarily primed PCR, the former is a more robust and reproducible method for preliminary detection of differentially expressed transcripts associated with PLMVd infection and symptom expression. With these advantages, cDNA-AFLP combined with validation of the results by quantitative real-time RT-PCR (RT-qPCR) allows highly sensitive quantification of transcript levels for genes involved in the host-pathogen relationship between peach trees and PLMVd.

The aim of this study was to characterize the molecular host response of GF-305 peach trees after PLMVd infection, using cDNA-AFLP to observe gene expression in plants infected by variants of different pathogenicity. Genes showing differential expression were selected for confirmation by quantitative real-time reverse transcription PCR (RT-qPCR).

1.4.Materials and methods

1.4.1. Plant and viroid materials

Three GF-305 peach trees, each inoculated by Fekih Hassen et al. (21) with a different PLMVd variant, were used in this study: one showing a mosaic (variant GenBank reference: DQ680730), another showing severe chlorosis (variant GenBank reference: DQ680704), and one expressing a partial mosaic with symptomatic and latent areas on the foliage (variant GenBank reference: DQ680697). All three plants were kept under greenhouse conditions and their physiological age was the same. Two grams of leaves were collected from each plant, immediately frozen in liquid nitrogen, and kept at -80°C until total RNA extraction.

1.4.2. Total RNA extraction and cDNA synthesis

The QIAzol reagent (QIAGEN, Hilden, Germany) was used to isolate total RNA from 0.4 g frozen tissue. Two biological replicates were used per treatment to obtain a robust estimate of the differential gene expression. Poly(A)$^+$ mRNA was reverse transcribed to double-stranded cDNA with the SuperScript double-stranded cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA), extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and resuspended in water as described in the manufacturer's protocol.

1.4.3. AFLP procedure

The AFLP reaction and polyacrylamide gel electrophoresis (PAGE) of the resulting products were performed as described by Bachem et al. (5) and Vos et al. (48). The cDNA was first digested with EcoRI and MseI for 2 hours at 37°C and then ligated to EcoRI and MseI double-stranded adapters. With the Expand High Fidelity System Enzyme Mix (Fermentas, Vilnius, Lithuania) a preamplification step was performed, involving 15 cycles (94°C, 30 s; 56°C, 60 s; 72°C, 90 s) and a final elongation at 72°C, 5 min with primers corresponding to the EcoRI and MseI adapters and 1/10 template volume. Following this pre-amplification, the product was diluted 10-fold with TE buffer and 5 µl was used for selective amplification with [^{33}P]-labelled selective primers. This amplification consisted of 36 cycles, including 12 touchdown cycles with a gradual reduction of the annealing temperature from 65 to 56°C (-0.7°C per step) and maintenance at 56°C for 24 cycles with an extension step at 72°C for 90 s. All 16 possible primer pairs combining the EcoRI/MseI sequences with one

selective nucleotide were used. Twenty-two other primer pairs with two selective nucleotides were also used (Table 2). Selective amplification products were separated by denaturing electrophoresis in 5% polyacrylamide gels. Finally, the gels were autoradiographed for three weeks at -80°C so as to optimally conserve the DNA bands after migration.

Table 1: 38 Specific primer pairs used for the cDNA-AFLP analysis. The EcoRI primers were ^{33}P-labelled

EcoRI Primers	MseI Primers
E-A /E-T/ E-G/ E-C	M-A
	M-T
	M-C
	M-G
E-A	M-CT
	M-TC
	M-GT
	M-TT
E-T/ E-C/ E-G	M-CT
	M-AT
	M-GA
	M-CA
	M-CG
E-TG	M-AA
	M-GT
	M-TC

1.4.4. Isolation of differentially expressed fragments.

DNA was recovered from frozen gel pieces containing the bands of interest after analysis of the autoradiogram. Fragments excised from the gels were incubated in 100 µl deionized water at 100°C for 15 min before centrifugation at 16100g for 10 min. DNA was precipitated from the supernatantby addition of 10 µl sodium acetate solution (3 M), 5 µl glycogen solution (10 mg/ml), and 230 µl absolute ethanol followed by incubation for 30 min at -80°C. After a 10-min centrifugation at 16100g, the supernatant was discarded. The precipitate was washed with 200 µl of 70% ethanol and resuspended in water before selective PCR re-amplification, performed as described above with 4 µl resuspended template.

1.4.5. Cloning and sequence analysis

The re-amplified DNA was cloned into the pJet 1.2 vector (Fermentas, Marlyland USA) and the constructs obtained were used to transform chemically competent INVαF' *Escherichia coli* cells (Invitrogen Corp., Carlsbad, CA). The cloned fragments (2 clones/fragment) were sequenced by Macrogen inc. (Seoul, Korea). Sequences were analysed with Bioedit software from Tom Hall (Carlsbad, CA, USA). For the homology search, the BLASTX program (available online from the National Center for Biotechnology Information) was used to compare each sequence against all sequences in the non-redundant databases.

1.4.6. Primer design and real-time PCR assay

Primer3 (v. 0.4.0) (available online from the Whitehead Institute for Biomedical Research) was used to design primers for amplification of the selected genes showing differential expression on the autoradiogram. Primer sets were designed to generate amplicons ranging from 150 to 300 bp in length (Table 3). The actin gene was used as an internal control and the sequences of the actin primers were 5'-TATGTTGCCCTGGACTATGACC-3' (for the forward primer) and 5'-AATGAGTGATGGTTGGAAGAGAAC-3' (for the reverse primer), based on a *Prunus sp.* actin sequence. Before the real-time PCR, the primers described in Table 3 were successfully tested after reverse transcription with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA) by amplification with Taq DNA polymerase (Roche Diagnostic, Penzberg, Germany). This amplification consisted of 40 cycles at 94°C, 1 min for denaturation, annealing at 55-60°C for 2 min, extension at

72°C for 1 min, followed by a final extension step at 72°C for 10 min. Gene expression was quantified by real-time PCR with the Maxima™ SYBR Green qPCR Master Mix on a Step One thermal cycler (Applied Biosystems, Foster city, CA, USA) after single-stranded cDNA synthesis as described above. Real-time PCR amplification and detection were carried out over 40 cycles (95°C for 30 s; 56°C for 30 s; 72°C for 1 min). Melting curve analysis was performed to confirm the amplification specificity. The parameters used were 15 s at 95°C, 1 min at 56°C, and 10 s at temperatures ranging from 55 to 95°C (with an increase of 0.3°C at each cycle). The $2^{-\Delta\Delta CT}$ relative expression calculation was performed with the Step One software ver.2.1 for each tested gene. Finally the LinReg software was used to confirm the amplification efficiency of each sample in all reaction plates.

Table 2: Real-time PCR primers derived from the gene sequences used for gene expression confirmation (only the primer sequences for the confirmed genes are shown)

Fragments	Primers	Sequences
4.042.1	Primer Forward	5'-TACCTGGGGACTGAAATGAG-3'
	Primer Reverse	5'-GCAAGAGCATGCAAATTCAA-3'
5.49.1	Primer Forward	5'-GCAGGATGTGTTTGCTCAGA-3'
	Primer Reverse	5'-TCCCATCTGATTTCGACTCC-3'
2.23.1	Primer Forward	5'-TTGAATCTTTGGACCGTTGG-3'
	Primer Reverse	5'-CCAAATAATCCCGTACCATGA-3'
3.067	Primer Forward	5'-AGG-AAG-AGT-TGG-TGG-TGT-GG-3'
	Primer Reverse	5'-TGA-CAT-TAG-GGT-CCA-TGC-AA-3'
4.40.2	Primer Forward	5'-AGG-AAG-AGT-TGG-TGT-GG-3'
	Primer Reverse	5'-TGA-CAT-TAG-GGT-CCA-TGC-AA-3'
5.46	Primer Forward	5'-AAT-TAC-CCG-TGC-AAG-ATT-CG-3'
	Primer Reverse	5'-ACG-CTG-CTC-TTG-TCC-ATC-TT-3'
3.018.1	Primer Forward	5'-CTC-CTA-ATT-GCC-ATG-GTG-GT-3'
	Primer Reverse	5'-ATC-AGA-AGC-ATT-GCC-ATT-CC-3'
5.39	Primer Forward	5'-CCA-CAA-TCT-GGG-TTG-TCT-TG-3'
	Primer Reverse	5'-CTC-TCC-CAC-AAC-CAG-CTC-TC-3'
6.79.2	Primer Forward	5'-GAC-TTT-GCA-GGC-TGA-GGT-TC-3'
	Primer Reverse	5'-AAT-ACT-GAC-CCA-CGC-AGA-CC-3'

1.5. Results

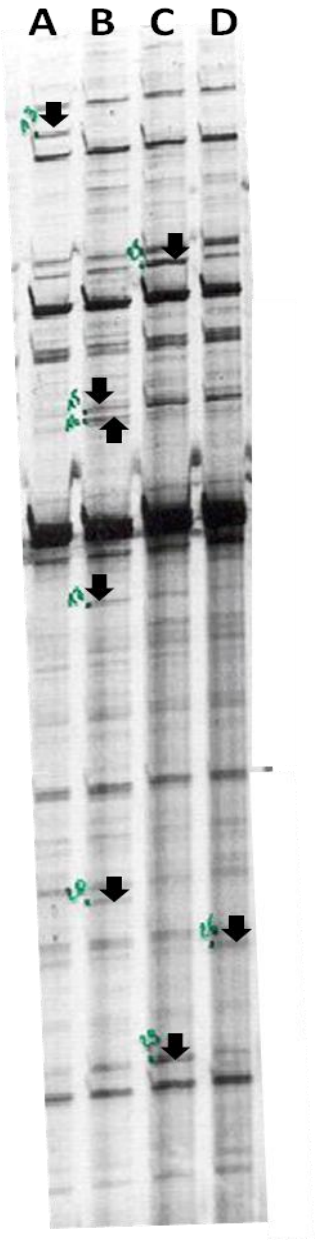
1.5.1. Using cDNA-AFLP and real-time PCR to discover genes regulated during viroid infection

Two comparison models were used to investigate the effect of PLMVd infection on gene expression in peach trees: model 1, including the plant showing the mosaic and that exhibiting severe chlorosis (infected, respectively, by variants DQ680730 and DQ680704), and model 2, including symptomatic and latent leaves of the plant infected by variant DQ680697.

After cDNA-AFLP, 11985 bands were observed: approximately 6057 bands for model 1 and 5928 for model 2. Among them, 239 (3.95%) appeared differentially expressed in model 1 and 254 (4.28%) in model 2 (Figure 1). After careful selection of the differentially expressed bands (easiness of band distinction, strong to moderate differential expression, fragment size exceeding 200 bp, band recovery), the sequence of 93 fragments was obtained. BLASTX was used to screen the UniProt database to find the corresponding gene functions. This analysis revealed 76 plant-gene functions related to chloroplast activities or to other functions such as metabolism and plant defence. Thirteen genes clearly related to important photosynthetic activities, photosystem protection strategies, plant defence, protein metabolism, or mRNA translation were selected for quantitative examination aiming (i) to determine the gene expression level by RT-qPCR and (ii) to better understand the effect of PLMVd on chloroplast activities and the plant response to the pathogen.

To confirm differential expression of the genes selected by cDNA-AFLP, relative quantification of these transcripts was performed by RT-qPCR with SyBr Green dye (Table 1) on a second independent set of samples (actin transcripts were used as an endogenous reference). Differential expression was confirmed for nine of the thirteen genes. Of these, genes 2.23.1 and 3.018.1 are repressed in the severely chlorotic leaves of model 1, seven genes (4.042.1, 5.49.1, 3.067, 4.040.2, 5.46, 6.79, and the just-mentioned 2.23.1) are repressed in the symptomatic leaves of model 2, and one (5.39) is overexpressed in the symptomatic leaves model 2 (Table 1). Among the unconfirmed DNA fragments, one gene displayed an expression pattern opposite to the AFLP result (2.80.1), one displayed no differential expression (5.55), and amplification of the last two fragments (3.011.1 and 3.026.1, corresponding to the same gene) was insufficient to allow determining their precise expression pattern (Table 1). As no conclusion could be drawn regarding these unconfirmed genes, they were excluded from subsequent analyses.

Figure 1: cDNA-AFLP gel analysis. Each band excised for sequencing and real-time PCR analysis is indicated by an arrow and a number. (A,B): samples of model 1 amplified by the primers E-T/M-T; (C,D): samples of model 2 amplified by the primers E-T/M-T.



1.5.2. Similarity to genes related to photosynthesis and photosystem protection

Hypothetical protein sequences were deduced from the reamplified fragments by virtual translation from all six reading frames. These sequences were compared with those of the Uniprot protein sequence database (12). The BLASTX results are summarized in Table 1.

Fragment 4.042, whose downregulation in symptomatic leaves (model 2) appeared slight (1.3-fold) but was nevertheless confirmed by real-time PCR, is related to the gene encoding photosystem I subunit O (PSI-O). This 10-kDa subunit of photosystem I (PSI) was recently discovered in *Arabidopsis thaliana* and is characteristic of higher plants and algae (28). PSI-O seems to be involved in the state 1-state 2 transition between the two photosystems, enabling plants to respond rapidly to changes in illumination. Particularly, PSI-O may be involved in the binding of light harvesting complex II (LHCII) to PSI during the transition. Jensen et al. (28) have shown that PSI-O-deficient plants display a higher PSII excitation pressure than wild-type plants, due to reduction of state transitions. Hence, the capacity to redistribute the absorbed excitation energy between the two photosystems is significantly reduced in plants lacking PSI-O. As our results highlight a down-regulation of the PSI-O gene in symptomatic leaves, we can assume, in agreement with the literature, that the transition is perturbed in these leaves.

RT-qPCR also confirmed repression (2-fold) of the gene corresponding to fragment 5.49.1 in symptomatic leaves of the plant infected by DQ680697 (model 2). This fragment is related to a gene encoding the early light inducible protein (ELIP). ELIPs most probably associate with the PSII and protect it against light excess during light stress or in the early phase of greening of etiolated plants (1, 51). They were first discovered in young greening pea seedlings, and their role was highlighted in mature plants subjected to light stress (1). It was suggested that during this stress, ELIPs act either as chlorophyll pigment carriers or as sinks for excess excitation energy (2, 35, 44). ELIPs are also known to protect the PSII against UVA and UVB light and are induced by salt, wounding, and ozone stress in a time-limited manner (1, 44). This suggests that the primary role of ELIPs is to protect PSII against damage from excessive light. Rossini et al. (41), however, have shown that photoprotection of the PSII by ELIPs is limited or occurs under severe stress conditions, and in the absence of ELIPs, stressed plants seem able to recover. This suggests that other plant processes may compensate for the absence of ELIPs. Both the physiological roles of ELIPs and the mechanisms compensating for their absence are currently unclear. All of our tested plants

grew under the same greenhouse conditions. It seems highly probable that the differential gene expression observed by RT-qPCR was due solely to viroid infection.

Real-time PCR confirmed repression of the gene corresponding to fragment 2.23.1 in both the severely chlorotic leaves of model 1 (10-fold repression) and the symptomatic leaves of model 2 (2-fold repression). This fragment is related to a gene encoding NADH dehydrogenase (also named NDH complex) subunit 6 (Table 1). In plants, NADH dehydrogenase catalyses the transfer of electrons from NADH to plastoquinone (34) and is viewed as a "chlororespiratory" component of the thylakoid membranes (for reviews see 8, 37, 38). As this complex is also known to be abundant in etioplasts, it has been hypothesized that "chlororespiratory" components may act to energize the plastid membrane and favour the synthesis and/or insertion of photosynthetic complexes during the greening process. Yet the absence of any obvious phenotype related to greening in NDH-deficient transformants suggests that this role is not essential (42). The NDH complex also seems to be involved in leaf senescence. Having observed a 30-day delay of leaf senescence in NDH-deficient tobacco plants, Zapata et al. (52) logically postulated that the electron transfer pathway involving the NDH complex and a plastid peroxidase could be involved in programmed cell death. Yet no such phenotype has been reported in the context of other studies on NDH-deficient mutants (42). Furthermore, studies have shown that chlororespiratory pathways may, by increasing the NDH-mediated electron flow, be involved in the adaptation of photosynthesis to conditions such as heat stress and water deficit stress. Furthermore, it seems that the NDH complex and chlororespiratory activities may limit over-reduction of PSI electron acceptors, scavenging reactive oxygen species (ROS) and thus protecting PSI from photoinhibition (42). Endo et al. (20) have shown NADH-dehydrogenase-deficient plants to be more sensitive to photoinhibition than the wild-type plant.

1.5.3. Similarity to genes related to plant defence, protein metabolism, and mRNA translation

Fragment 4.040.2 shows high homology (E value=$1e^{-56}$) with the *Citrus sinensis* gene encoding the thiazole biosynthetic enzyme (THI1), while fragment 3.067 shows high homology with the THI1 gene of *Alnus glutinosa* (E value=$4e^{-71}$). This gene appeared overexpressed (1.8- and 1.5-fold for 4.040.2 and 3.067, respectively) in asymptomatic leaves during viroid infection. THI1 is known to play a role in thiamine biosynthesis, but the complete pathway of thiamine biosynthesis in eukaryotes remains to be elucidated (26). THI1 homologues are the only known enzymes to take part in thiazole biosynthesis in a diversity of organisms, both unicellular (archaea and fungi) and highly organized (plants) (26). Thiamine is a known activator of plant disease resistance (3). It is notably involved in inducing systemic acquired resistance (SAR) against a broad range of pathogens in treated plants (3, 4). The observation that asymptomatic leaves overexpress an enzyme involved in thiazole synthesis is thus consistent with a potential role of thiamine in plant protection against viroids.

Fragment 5.39, which is overexpressed 3.6-fold in symptomatic leaves of model 2, shows high homology (E value=$2e^{-20}$) with the *Prunus mume* gene encoding polygalacturonase-inhibiting protein (PGIP). PGIPs are plant proteins known to contribute importantly to plant defence against fungi by preventing their penetration into cells (13, 39). These proteins interact with fungal polygalacturonases to inhibit their effect on the plant cell wall (16). Studies have shown that different PGIPs are produced during an infection, reflecting different degrees of plant resistance or sensitivity towards fungi (13, 16). PGIPs can also be induced by wounding and by the use of elicitors like jasmonate (13).

Fragment 5.46, which is 2-fold overexpressed in asymptomatic leaves, is highly homologous (E value=$4e^{-99}$) with the *Saussurea medusa* gene encoding the cytosolic heat shock protein 70 (HSP70), while fragment 6.79, which is 14-fold overexpressed in the same leaves, is highly homologous with a gene coding for an HSP binding protein (Table 1). HSPs are responsible for protein folding, assembly, translocation, and degradation in normal cellular processes (50). They also act to stabilize proteins and membranes, and they can assist protein refolding under stress (10). In plants, *hsp* gene expression is frequently induced in response to heat stress or viral infection (9, 10, 50). HSP70 proteins are known to interact with a wide range of co-chaperone proteins that regulate their activity or assist the folding of specific substrate proteins (50). In particular, HSP70-family proteins have essential functions in preventing aggregation and assisting the refolding of non-native proteins under stress and

normal conditions (50). They are also involved in protein import, translocation (also of viral proteins), and degradation and in controlling the biological activity of folded regulatory proteins (9, 50).

Fragment 3.018.1 shows high homology (E value=$2e^{-32}$) with the novel cap-binding protein (nCBP) gene of *Arabidopsis thaliana*. Expression of the gene appeared repressed 3-fold in severely chlorotic leaves (model 1) and 1.6-fold in the symptomatic leaves of model 2. Cap-binding proteins, also called eukaryotic initiation factors 4E (eIF4E), specifically recognize and bind the m^7G functional group found at the 5'-ends of most eukaryotic cellular mRNAs (43). Binding of eIF4E is the first step in the assembly of several initiation factors on the mRNA, before binding of the 40S ribosome, which leads to translation (11, 43). In addition, characterization of the nCBP of *A. thaliana* has revealed novel properties (43), such as a higher affinity for tm^7G and a lower rate of translation initiation than eIF4E (43). These features could make repression of the gene deleterious for the plant.

Table 3: cDNA-AFLP gene fragments displaying similarity to other known proteins and relative fold changes in expression of the genes in the two tested comparison models, as determined by quantitative RT-PCR. A value <1 corresponds to repression in the severely mosaic leaves of model 1 or in the symptomatic leaves of model 2. The gel expression pattern of the band corresponding to the gene is represented by a minus for a repression in the severely mosaic leaves of model 1 or in the symptomatic leaves of model 2

cDNA fragment	TDF size (bp)	Related known protein	Organism	Related accession number	Blastx score	Gene expression fold change		Gel expression pattern	
						Model 1	Model 2	Model 1	Model 2
4.042.1	402	Photosystem I subunit O	*Arabidopsis thaliana*	NP_563815	$7e^{-39}$	Not tested	0.74 ± 0.2	-	-
5.49.1	427	Early light inducible protein	*Populus Trichocarpa*	XP_002312552	$4e^{-45}$	Not tested	0.5 ± 0.1	-	-
2.23.1	245	NADH dehydrogenase subunit 6	*Vitis vinifera*	YP_567131	$3e^{-20}$	0.1 ± 0.05	0.5 ± 0.05	-	-
2.80.1	386	Oxygen evolving enhancer protein 2	*Solanum tuberosum*	CAA67696	$2e^{-43}$	Not tested	0.47 ± 0.1	+	+
3.011.1	200	Chlorophyll a/b binding protein	*Panax ginseng*	AAB87573	$1e^{-16}$	Not tested	0.06 ± 0.2	-	-
3.026.1	200	Chlorophyll a/b binding protein	*Panax ginseng*	AAB87573	$1e^{-16}$	0.19 ± 0.15	Not tested	-	-

cDNA fragment	TDFsize (bp)	Related known protein	Organism	Related accession number	Blastx score	Gene expression fold change		Gel expression pattern	
						Model 1	Model 2	**Model 1**	**Model 2**
3.067.1	507	THI1	*Alnus glutinosa*	Q38709.1	$4e^{-71}$	Not tested	0.673 ± 0.2	-	-
4.040.2	407	THI1	*Citrus sinensis*	O23787.1	$1e^{-56}$	Not tested	0.55 ± 0.1	-	-
5.46.1	682	HSP70	*Saussurea medusa*	AAV97978.1	$4e^{-99}$	Not tested	0.50 ± 0.2	-	-
3.018.1	263	nCBP	*Arabidospis thaliana*	NP_197312.1	$2e^{-32}$	0.317 ± 0.1	0.6 ± 0.1	-	-
5.39.1	219	PGIP	*Prunus mume*	AAV33432.1	$2e^{-20}$	Not tested	3.6 ± 0.2	+	+
6.79	662	HSP binding protein	*Ricinus communis*	XP_002515568.1	$3e^{-99}$	Not tested	0.07 ± 0.02	-	-
5.55.1	355	S-adenosylmethionine decarboxylase	*Malus x domestica*	BAC55113.1	$8e^{-30}$	1 ± 0.2	Not tested	+	+

1.6.Discussion

1.6.1. PLMVd infection and photosynthesis

We have detected by cDNA-AFLP and confirmed by real-time PCR the simultaneous repression of three genes involved in photosynthesis or photosystem protection. PSI-O, ELIP, and NADH dehydrogenase subunit 6 contribute jointly to maintaining photosynthesis in plants exposed to excessive light or oxidative stress.

According to the findings of Jensen et al (28), the slight repression of PSI-O (involved in state transition) observed in symptomatic leaves probably causes reduction of the electron flow through both photosystems. Yet Jensen's observations on *Arabidopsis thaliana L. Heyn cv. Columbia* suggest that PSI-O repression in peach trees is probably not involved in mosaic development, but rather in delayed flowering (a delay of 5 to 7 days is commonly observed in peach trees during PLMVd infection as well as in *A. thaliana* during PSI-O repression) (28, 31). PLMVd induces several symptoms likely to be related to perturbed photosynthesis: (i) growth reduction, (ii) early plant decay, and (iii) mosaic expression as described during PLMVd infection (23). All of these symptoms might be due to a decreased photosynthetic capacity caused by a lack of PSI-O, but it remains to be elucidated if they are a cause or a consequence (or both) of PSI-O repression.

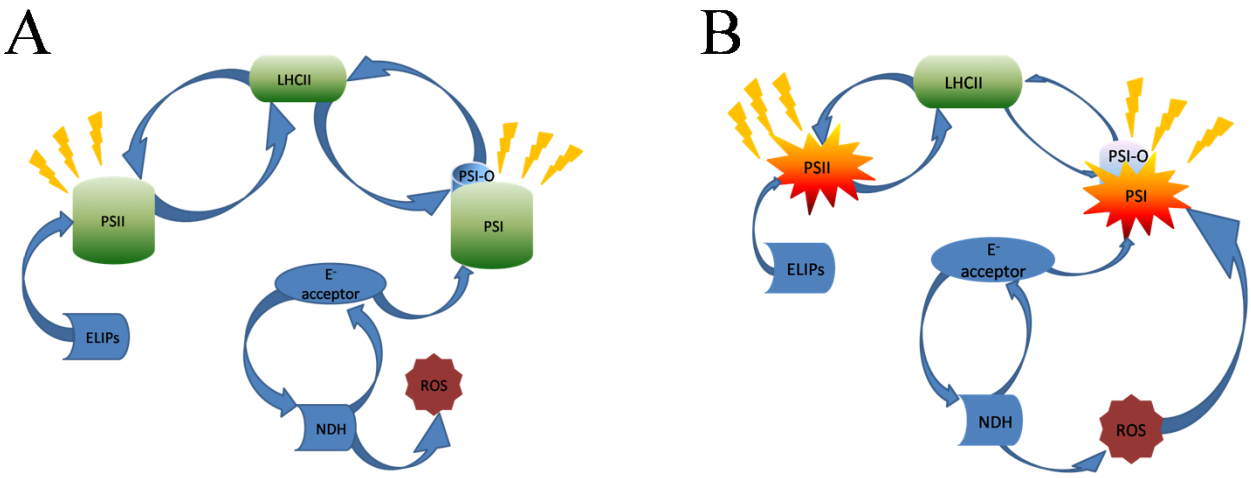
PSI-O repression could lead, because of a reduced electron flow, to an increased excitation pressure on the PSII, which generally causes photoinhibition. Yet ELIPs are known to be overexpressed during this deleterious phenomenon (41). Although the exact role of these proteins is unclear, it has been proposed that ELIPs may play their photoprotective role under conditions of severe stress such as those encountered during PSI-O repression. It should be noted, however, that ELIPs were underexpressed in symptomatic PLMVd-infected leaves. This repression might explain why PLMVd (i) seems to perturb considerably the photosynthetic apparatus and (ii) induces chlorosis or mosaics. The PSII might be less protected in PLMVd-infected than in healthy plants, where ELIP expression is relatively high. The failure of PSII protection mechanisms might induce the destruction or at least the destabilization of PSII and explain the observed symptoms of the infected plants.

One might expect the observed downregulation of NADH dehydrogenase subunit 6 to repress the activity of the entire NDH in symptomatic leaves. According to the literature, however, this repression might not be sufficient to induce symptoms (42). On the basis of the described role of the NDH complex in photoprotection and in the synthesis or protection of

photosynthetic complexes (20, 42), the lack of NDH is likely to induce symptom development by reinforcing the negative effects of PSI-O and ELIP repression.

We have thus shown that PLMVd infection of peach trees represses three genes involved in state transitions or in the protection of PSII. The repression of NDH dehydrogenase further suggests that PSI might also be challenged during infection. In other words, both major components of the photosynthetic apparatus would seem to be less protected and thus more vulnerable to light in infected plants, even under normal growth conditions. Normal light conditions such as those used here could be harmful to plants in which all of these genes are repressed, because this repression could reduce the capacity of the plants to regulate photosynthesis and to redistribute the absorbed light energy. A schematic model of the possible action of PLMVd on photosynthesis is proposed in Figure 2. According to this model, in the absence of PLMVd infection, PSI-O, ELIP, and NDH are able to protect both photosystems, the first two proteins providing protection against light fluctuations or excess light and the third affording protection against ROS. The system remains balanced and permits normal growth of the plant without any disorder (Figure 2A). When PLMVd infects the plants, all three genes are underexpressed and the corresponding protein levels are low. Repression of PSI-O increases the excitation pressure on PSII (LHCII being unable to shift the energy balance to PSI). This "artificial" excess of light, combined with decreased ELIP protection, amounts to severe stress and a decreased capacity of the plant to counteract photoinhibition of PSII. Photoinhibition leads to production of ROS, which damage both PSII (the possibility of repair being reduced by photoinhibition) and PSI, the latter being rendered more sensitive to ROS by the low level of NADH dehydrogenase (Figure 2B). In our model, the observed symptoms thus appear as a consequence of repression of these three genes. Yet we cannot be sure that the repression is not a consequence, rather than a cause, of symptom expression.

Figure 2 : Model describing possible consequences of viroid infection on plant photosynthesis. (A) In healthy plants, ELIPs and PSI-O protect the photosystems against excess light and the NDH complex scavenges ROS. (B) During PLMVd infection, repression of PSI-O limits binding of LHCII to PSI, inducing increased excitation pressure on PSII. Repression of ELIP gene expression also limits protection of PSII against light damage, while repression of NADH dehydrogenase subunit 6 (NDH) decreases protection of PSI against reactive oxygen species (ROS).



1.6.2. Effects of PLMVd on plant defence and metabolism

Heat shock proteins are involved in multiple pathways. In particular, HSP70-family proteins participate in the protein life cycle (folding, assembly, translocation, and degradation), protein stabilization, and protein refolding under conditions of stress (50). Repression of these proteins is likely to perturb protein metabolism and hence plant biological pathways, causing enhancement of symptom expression. In addition to the HSP and the HSP-binding protein mentioned here, our cDNA-AFLP analysis revealed relative overexpression of several other HSPs in asymptomatic leaves (and thus relative repression in symptomatic leaves, data not shown), but for lack of sufficient information, we can draw no conclusions regarding their involvement in PLMVd infection. In another study of the genes induced and repressed by PSTVd, certain HSPs were found to be induced during infection (27).

In both models, the present study has highlighted repression of nCBP in symptomatic leaves. All nCBPs studied to date have a higher affinity for mRNA M^7G and promote a lower translation rate than other cap-binding proteins, but their precise biological role remains unclear (6, 43). Repression of nCBP could thus increase mRNA translation and the protein content of infected cells, causing destabilization of several biological pathways (43). According to the proteins/pathways affected, the transcriptome might also be altered. The effects of nCBP repression might be exacerbated by simultaneous repression of key HSPs

(through the presence of unfolded proteins or a reduced protein degradation rate), thus possibly explaining the greater severity of symptoms in leaves where this dual repression is observed. This is, to our knowledge, the first observation of HSP repression associated with nCBP repression in severe symptomatic viroid infection.

The role of PGIP in the PLMVd-host interaction remains unclear. PLMVd does not encode any proteins (18). Polygalacturonases are not produced in this case, and not involved in the host-pathogen relationship. To our knowledge, furthermore, PGIP has never been mentioned in a study on viroid-host interactions. It is also unclear why peach overexpresses a polygalacturonase inhibitor in symptomatic leaves during viroid infection. It could be that infecting viroids nonspecifically stimulate defence pathways involving these enzymes. The study of Itaya et al. (27) has shown that viroids induce nonspecific responses during infection (such as responses observed during viral infections). Clearly, further study is needed to understand completely the interactions established during viroid infection.

Although viroids induce both specific and nonspecific responses during infection, infected plants have seemed so far to be unarmed against viroids. Plant protection against viroids is indeed currently limited to eradication of infected plants. The present fundamental study of the peach-PLMVd relationship was prompted by the need for new control strategies. One result obtained here suggests that enhancement of systemic acquired resistance, a nonspecific plant defence mechanism effective against some viruses, fungi and bacteria, might be a pretty good alternative strategy for controlling PLMVd. We refer here to the observed overexpression of THI1, the gene encoding the thiazole biosynthetic enzyme, in asymptomatic leaves. Thiazole being an intermediate in thiamine biosynthesis, THI1 overexpression might result in an increased level of the SAR-priming molecule thiamine (although this remains to be demonstrated). SAR is generally triggered by pathogen-induced cell death, occurring in local lesions that can spread over the entire plant (3). If SAR is indeed triggered by and active against PLMVd, it remains necessary to elucidate the underlying mechanisms. Nevertheless, SAR being a hypersensitive response that does not require induction by a pathogen (47) and is inducible by other agents (e.g. certain chemicals, (3, 4), enhancing this pathway could be the future of plant protection against viroids.

As in many other gene expression studies, it has not been possible here to determine how PLMVd regulates expression of the identified genes. Several hypotheses were proposed and tested but none was verified (data not shown). One hypothesis envisaged is that repression might be due to gene silencing (25). Gene silencing has been proposed as a hypothetical

immunity mechanism against RNA pathogens (19), but viroids must escape the gene silencing machinery, since they are continuously present in infected plants. It has even been proposed that viroids might exploit gene silencing to induce their symptoms (18, 30, 33, 49), rendering highly damageable for the plant what at first glance appeared as an elegant strategy against these uncontrollable pathogens. As PLMVd is known to induce this defence-related phenomenon in infected plants, St Pierre et al. (45) performed a sequence-based analysis to determine whether the siRNAs produced by PLMVd might be able to repress the genes identified here. Unfortunately, none of the siRNAs sequenced was sufficiently homologous to the known sequences of these genes (Parisi. O, Dubé. A, Perreault. JP, and Jijakli.M.H, unpublished data). Furthermore, to us it seems improbable that the viroid would be able to cause repression by trans-cleaving homologous sequences, because (i) the probability of finding the homologous mRNA and the PLMVd together should be low and (ii) the homologous mRNA region would have to be relatively unfolded to be accessible to the ribozyme. How PLMVd exerts its pathogenicity is currently unclear. The sequencing of viroid hosts and advanced host-pathogen studies should contribute greatly to determining the pathogenicity mechanisms of viroids.

1.6.3. Concluding remarks

Our results suggest that PLMVd infection destabilizes PSII and PSI by repressing genes involved in either the state transition or PSII/PSI protection. Repression of these genes could imaginably induce all the symptoms observed during a PLMVd infection: chlorosis, mosaic, delayed flowering and foliation. PLMVd-induced alteration of protein metabolism, furthermore, is likely to increase the severity of the symptoms. Lastly, infected plants seem to act against PLMVd by means of nonspecific defence mechanisms, with a possible involvement of SAR. The efficacy of this protection remains to be studied, but enhancing it may be a way to counter these pathogens. This paper is a first step towards assessing the influence of PLMVd infection on peach gene expression. It provides a basis for further study, needed in order to gain a more complete view of PLMVd infection in peach trees.

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Author-Recommended Internet Resources

Bioedit Software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html)

Blastx (http://www.ncbi.nih.gov/BLAST)

Primer3 (http://frodo.wi.mit.edu/

Chapter V
Conclusions and Perspectives

The knowledge of the interaction between the host and the pathogen during its attack should be a pre-requisite to the elaboration of an efficient and sustainable control method. As we are actually unable to control viroid diseases by curative methods, the study of such interaction is necessary to elucidate the relationship established during an infection and to target the weakness of the pathogen. Several studies concerning host-response during viroid infections were realized the past ten years. They concerned mainly the PSTVd and the CVd III, two viroids belonging to the *Pospiviroidae* family. The studies on the PSTVd have shown that this viroid modified the plant gene expression at the same time in a specific and non specific way: some genes appeared affected by the PSTVd as well as by the TMV whereas the expression of other genes were modified only by the presence of the viroid. Despite these studies, the way by which these viroids induced or repressed the genes of an infected plant as well as the control methods against these pathogens remain unclear.

Such studies were not undertaken within the framework of the *Avsunviroidae* family yet. It results probably from the lack of data concerning the determinant of pathogenicity of these viroids but also from the difficulty to realize such studies on non model genome. This is particularly the case of the PLMVd, infecting exclusively peach trees, for which pathogenicity determinants are unknown (excepted for the peach calico symptomatology).

Finally, the response of the host may vary depending on the severity of the pathogen. In this context, it is also important highlight the pathogenicity determinants or at least the important structures for the viroid life cycle. Such determinants were identified for the members of the *Pospiviroidae* family but also for the CChMVd (an *Avsunviroidae*) and a hairpin inducing a severe pathogenicity (the peach calico) was also discovered for the PLMVd. However for this latter viroid the sequence or the structure inducing the common chlorosis or mosaic is unknown. The identification of the pathogenicity determinant of the PLMVd would be of great asset for the further characterization of the host-pathogen relationship.

This study was thus articulated on these two aspects of the infection of peach trees by the PLMVd. In the first part of the work (i), we have (a) developed the molecular tools needed to (b) characterize the importance of the P8 pseudoknot in the PLMVd life cycle. The second part of the research (ii) was focused on the study of the host-response to PLMVd variants of different pathogenicities.

(i) a. The first part of this study concerned the implication of the P8 pseudoknot in the replication and, eventually, in the pathogenicity of the PLMVd. We have thus developed a RT-qPCR technique able to detect and quantify small viroid quantities. This detection method appeared to be 10^5 times more sensitive than the end-point RT-PCR commonly used. Furthermore, our quantification results have also shown that the viroid quantity varies during the cultural season with a peak during the summer. The lowest quantity during the winter. This shows clearly that the replication capacity of the PLMVd is linked with the growing conditions of the plants. Finally some of our results have suggested that the PLMVd is not uniformly distributed in the plants. In consequence, care should be taken during the sampling especially for certification campaigns.

(i) b. The new, sensitive, developed RT-qPCR technique has permitted to follow the replication of the PLMVd mutated variant. It has consisted in a variant mutated in the loop P7 to destabilize and inhibit the formation of the P8 pseudoknot, always present in all sequenced PLMVd variants. The viroid replication was followed from 3 to 12 months after inoculation. This evaluation of the viroid replication was realized by RT-PCR and RT-qPCR. The results have shown that the mutated variant was undetectable by the RT-PCR. However our developed RT-qPCR has shown that the viroid was able to replicate but its quantity was always lower than the native variant. This suggests that the P8 pseudoknot has effectively a role in the viroid *in vivo* stability or in the replication capabilities. Furthermore, this importance of the P8 pseudoknot is strengthened by the presence of covariations maintaining the pseudoknot in every sequenced PLMVd variants. Finally, as the replication of the mutated variant seems more important during the summer, we cannot exclude that mutations have restore the pseudoknot folding.

Perspectives: The RT-qPCR developed in our study could be, after some optimizations, used in the programs of certification of peach trees. From them the optimization from the two tubes protocol (with the reverse transcription and the qPCR in two separate tubes) to a one tube protocol seems necessary (i) to avoid the risk of contamination and (ii) simplify the samples handling. Furthermore, the study of the PLMVd repartition in the plants is needed to

avoid false negative results during certification/detection campaigns and the subsequent release of infected material in orchards. The achievement of these prospects will lead to one of the most sensitive PLMVd detection and quantification technique.

The role of sequence/structural viroid motifs begins to be understood. However this knowledge actually lacks for the PLMVd, and more generally for the *Avsunviroidae* family. Our results have suggested that the P8 pseudoknot is important for the PLMVd life cycle (by the mean of the stability or replication). However its precise role needs to be elucidated. Studies on mutated variants will improve the knowledge about the viroid sequence/structural motives. Viroids are regularly qualified as models for the RNA biology. To understand how they interact with plant proteins, they induce their pathogenicity and which sequences/structures are necessary to complete their life cycle is of great importance to qualify the mechanisms involved in the RNA biology and the evolution of these last molecules. In consequence well characterized mutated variants could be used also to evaluate the importance of the mutation in the viroid-plant interaction. However as suggested by our results, it seems necessary that such a characterization should be performed in controlled conditions to avoid the effect of non optimal growing conditions on the viroid replication.

(ii) The second part of this work was focused on the characterization of the host response during a PLMVd infection. The cDNA-AFLP technology was used to dispose of an open strategy (without *a priori*) to visualize the gene expression of infected peach trees. This strategy allowed the identification of peach genes that may be responsible of the symptom expression and/or the plant defence. To our knowledge, this is the first report concerning the characterization of the host response during a PLMVd infection with variants of different pathogenicities. Some of the genes identified in our study might have an implication in the plant defence because they are involved in unspecific defence pathways. Others might be implicated in the symptoms expression because they take part to the photosynthesis activities, the protein metabolism and the mRNA translation.

The enhanced plant defence mechanisms identified in our study are relatively unspecific. The first, and most interesting, identified mechanism is the systemic acquired resistance probably enhanced by the over-expression of the thiazole biosynthetic enzyme, an intermediate of the thiamine, in the non-symptomatic leaves. This plant defence mechanism is generally triggered by "pathogen-induced cell death" and lead to the production of pathogenesis-related proteins and reactive oxygen species (ROS) which permit the control of pathogens (at least bacteria, viruses and fungi). To our knowledge this defence pathway was never observed during a viroid infection and its efficiency against these pathogens needs to be confirmed. The second gene product related to a plant defence mechanism highlighted by our study is the Polygalacturonase inhibiting protein (PGIP). This protein inhibits the polygalacturonase of fungi and characterizes the sensitivity or the resistance of the plants against these pathogens. However as the PLMVd does not produce any protein, the role of the PGIP in the host-response against the PLMVd is unclear. Moreover, it is the first time that this protein is identified during a PLMVd infection, although, the PSTVd is also known to induce defence proteins acting against fungi (PR-1b and chitinase). Maybe these responses are completely unspecific and useless to protect plants in the case of the viroid infections.

Our results have also highlighted a repression of genes implicated in the mRNA translation (nCBP) and in the protein metabolism (HSPs). The repression of these genes in the most symptomatic leaves could explain these enhanced symptoms by a deregulation of multiple biological pathways. This is the first time that the simultaneous repression of these genes is observed during a viroid infection. The consequence of this particular gene expression is thus undetermined and subject to hypothesizes. Further studies on this simultaneous repression could probably enlighten its precise consequences.

Furthermore our results have permitted to elaborate a model of the photosynthesis perturbation during a PLMVd infection. Three key genes implicated in the photosynthesis activities and photosystems protection were effectively highlighted during our study. These genes have the particularity to maintain the photosystems function during stress conditions (by the

regulation of the light absorption, the energy distribution and the elimination of ROS). However they appeared repressed in the most symptomatic leaves during a PLMVd infection. We can thus suppose that their repression induces the destabilization of the two photosystems because of the presence of ROS and a relative light excess (as well as during normal light conditions). These results are the first where symptoms during a PLMVd infection can be linked with a particular gene expression.

Finally, we assume that our study has revealed a limited numbers of genes potentially implicated in the host response during the infection of peach trees by the PLMVd. This limited number is the well known limitation of the cDNA-AFLP. This middle-throughput technique which, in theory, can give a complete picture of the gene expression in a pool of cells, is progressively replaced by high-throughput RNA sequencing since a few years which appeared most powerful than the cDNA-AFLP. In consequence, this technique cannot be ignored for the future studies of the plant transcriptome and particularly in the study of plant-pathogen interactions

Perspectives: It would be interesting to confirm the implication of the genes identified in the defence of the infected trees against the PLMVd. This could be done by treating plants with thiamine or a chemical homolog of thiamine (like TMP), known to activate the SAR, during a PLMVd infection. This assay should be conducted with plants infected by variants of different pathogenicity to evaluate the exact effect of the thiamine (and thus the SAR) on the PLMVd. Furthermore a screening of the peach cultivars may help to find plants resistant or at least tolerant to the PLMVd. The SAR enhancement capabilities could be a marker of such cultivars. Finally the PGIP involvement in the plant defence against the viroid needs to be confirmed, for example by studying the effect of the repression of the gene coding for this protein during a viroid infection.

The precise effect of the repression of the genes implicated in the photosynthesis and/or in the photosystems protection needs also to be confirmed. This could be done by using a silencing strategy targeting these genes in viroid-free plants. This effect of the PLMVd on the photosystems

could be evaluated also in various peach cultivars to determine if tolerant plants exist.

Another contribution may come from the fragments that show no similarities with the databases. Their isolation constitutes a first step on the host-pathogen relationships characterization. Maybe some specific mechanisms involved during the PLMVd infection could be identified by their mean.

Moreover, it should be interesting to link the study of the host-pathogen relationship with sequence/structural studies of the PLMVd. For example, the use of mutated variants, in pseudoknot regions (as those used in this study) or in other regions (following the progress of the characterization of the PLMVd), could give important informations on how these regions play a role in the viroid-plant interaction. These studies would give a global view of the interaction between the viroid sequence/structure and its *in vivo* behavior.

Our study gives a fundamental knowledge about a part of the mechanisms involved during a PLMVd infection. However at the state of the art, it is greatly improbable that one of these pathways could be used to manage this infection, excepted, maybe, the way of the SAR but further investigations of this strategy are needed to confirm this possibility. Nevertheless we are convinced that further characterization coupled with functional analyses of the genes identified here will give a better understanding of the established relationships in this pathosystem.