Pospiviroids: From Detection to Molecular Pathogenesis

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March 2019

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Acknowledgment

First of all, I would like to thank the funders of this thesis, the Walloon Agricultural Research Centre (CRA-W), the Federal Agency for the Safety of the Food Chain and the Earth & Life Institute of UCL, for giving me their financial support and this research opportunity.

Secondly, I would like to express my gratitude to my promotors, Prof. Claude Bragard and Dr. Bernard Watillon for the great freedom they gave me in my research, their encouragements as well as for their scientific knowledge.

Besides my promotors, I would like to warmly thank the other members of the jury: Prof. Gerhard Steger, Prof. Anne Legrève and Dr. Pascal Gentit, for their insightful comments and encouragements as well as for their questions which incented me to widen the scope of my manuscript.

My sincere thanks also go to the scientific community involved in viroid research for his welcome, encouragements and immense collective knowledge.

This acknowledgment also gives me the opportunity to warmly thank my many colleagues at CRA-W and in partner institutions for their stimulating discussions and research opportunities, their valuable collaboration as well as for the good times spent together.

Last but not least, I would like to deeply thank my family and especially my mother for supporting me throughout this thesis.

Abbreviations:

ADR1: Activated Disease Resistance 1
AGO: Argonaute
ARF: Auxin response factor
BAK1: BRI1-associated kinase
BIK1: Botrytis-induced kinase 1
BKK1: BAK1-like kinase 1
BRI1: Brassinosteroid intensitive 1
CCCVd: Coconut cadang-cadang viroid
CC: Coiled coil
CCR: Central conserved region
CDPK: Calcium-dependent protein kinase
CEVd: Citrus exocortis viroid
CLVd: Columnea latent viroid
CNGC: Cyclic nucleotide-gated channel
CPK: see CDPK
CSVd: Chrysanthemum stunt viroid
CTV: Citrus tristeza virus
DAMP: Damage-associated molecular pattern
DCL: Dicer-like
DEGs: Differentially expressed genes
DRM2: Domains rearranged methyltransferase 2
DSB: Double strand break
dsRNA: Double stranded RNA
EDS1: Enhanced disease susceptibility 1
EFSA: European Food Safety Authority
EPPO: European and Mediterranean Plant Protection Organization
ER: Endoplasmic reticulum
ET: Ethylene
ETI: Effector-triggered immunity
GCN2: General control nonrepressed 2
GLR: Glutamate receptor-like channel
H3K4: Lysine 4 of histone 3
H3K9: Lysine 9 of histone 3
HR: Hypersensitive response
ICTV: International Committee on Taxonomy of Viruses

ISO: International Organization for Standardization
JA: Jasmonic acid
LRR: Leucine-rich repeat
LTR: Long terminal repeat retrotransposons
MAPK: Mitogen activated protein kinase
MDA5: Melanoma differentiation associated gene 5
MeSA: Methyl salicylic acid
NB-LRR: Nucleotide binding leucine rich repeat
NDR1: Non-race specific Disease Resistance 1
NLRC2: Nucleotide-binding oligomerization domain-containing protein 2
NLRP3: NOD-like receptor pyrin domain-containing-3
NOD: Nucleotide-binding oligomerization domain
NPR1: Nonexpresser of PR genes 1
NRG1: N requirement gene 1
PAMP: Pathogen-associated molecular pattern
PARE: Parallel analysis of RNA ends
PCD: Programmed cell death
PCFVd: Pepper chat fruit viroid
PD: Plasmodesmata
PEP: Small peptide elicitor
phasiRNAs: Phased, secondary, small interfering RNAs
PKR: Protein kinase RNA-activated
Pol IV: RNA polymerase IV
Pol II: RNA polymerase II
PoLVd: Portulaca latent viroid
PR: Pathogenesis-related
pro-IL-1β: Pro-interleukin-1β
PRR: Pattern recognition receptor
PSTVd: Potato spindle tuber viroid
PTGS: Post transcriptional gene silencing
PTI: PAMP-triggered immunity
RBOH: Respiratory burst oxidase homolog
RdDM: RNA-directed DNA methylation
RDR: RNA-DEPENDENT RNA POLYMERASE
RIG-I: Retinoic acid inducible gene I
RLK: Receptor-like kinase
RLP: Receptor-like protein

RNAi: RNA interference
ROS: Reactive oxygen species
RSS: RNA silencing suppressor
RT-PCR: Reverse transcription-polymerase chain reaction
SA: Salicylic acid
SAM: Shoot apical meristem
SAR: Systemic acquired resistance
SEL: Size exclusion limit
SERK1: Somatic embryogenesis receptor-like kinase 1
sidRNA: SiRNA independent of DCLs
siRNA: Small interfering RNA
sRNA: Small RNA
TASVd: Tomato apical stunt viroid
TBF1: TL1-binding transcription factor 1
TCDVd: Tomato chlorotic dwarf viroid
TCH: Terminal conserved hairpin
TCR: Terminal conserved region
TCV: Turnip crinkle virus
TE: Transposable elements
TIR: Toll/interleukin 1 receptor
TLR: Toll-like receptor
TMV: Tobacco mosaic virus
TPMVd: Tomato planta macho viroid
UPR: Unfolded protein response
vd-sRNA: Viroid/virus derived small RNA
VIGS: Virus-induced RNA silencing
VirP1: Viroid RNA binding protein 1
VM: Virulence modulating

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1 Introduction

Pospiviroids are plant pathogens, composed only of infectious RNA molecules. They are both latent on many ornamentals and harmful for cultivated plant species like tomato, potato, sweet pepper, citrus or chrysanthemum. Throughout the world, these pathogens cause sporadic outbreaks on susceptible annual crops, especially tomato, while they often remain undetected although widespread on ornamentals.

Since no curative methods can be applied against viroid diseases in crops, main control strategies consist in using prophylactic measures such as using and producing viroid-free plants (Barba et al. 2017), removing diseased plants, disinfect contaminated tools as well as applying quarantine measures (EFSA 2011; Barba and James 2017; Ling 2017). If preventive protection of crops with mild strains are used against viral diseases and although this cross-protection phenomenon has been reported for viroids as well (Niblett et al. 1978; Khoury et al. 1988; Duran-Vila and Semancik, J.S. 1990; Singh et al. 1990), this cross-protection strategy does not seem to have been used commercially on a large scale (Ziebell and Carr 2010). Like for viruses, genetic improvement of susceptible hosts are considered. However, to date, mitigated results have been obtained for viroids (Hammond and Kovalskaya 2017).

Considering that viroid infections can be latent or induce symptoms resembling those of other diseases or abiotic stresses, the use of accurate detection methods is an indispensable prerequisite for most control strategies.

In the EU, still no official detection methods have been designated for the detection of pospiviroids and there is a lack of publically available

comparisons of existing methods. Furthermore, available methods often require two separate tests to allow the detection of all known pospiviroids (Monger et al. 2010; ANSES. 2013; Botermans et al. 2013). In this thesis, a new generic and single detection method has thus been developed and validated according to the international standard ISO/IEC 17025. To assess this new method with already existing ones, an inter-laboratory comparison was organized at the EU level.

As a forward-looking vision of future developments in the field of viroids/viruses detection methods and in an attempt to further close the gaps of fundamental knowledge which still prevent a precise assessment of phytosanitary risks posed by pospiviroids, the use of High-Throughput Sequencing (HTS) was also considered. Indeed, HTS now emerges as a revolutionary universal virus/viroid detection/identification tool that is now more and more regarded as routine detection method regularly applied for viruses and viroids (Massart et al. 2017; Maree et al. 2018). However, since the implementation of HTS with the sole purpose to detect pospiviroids in routine is currently not fit for purpose, in the framework of this work, HTS was rather used to characterize the molecular interactions between one of the most severe isolate of pospiviroids collected on the Belgian territory and tomato.

Indeed, as well as being a promising universal detection method, HTS is a powerful tool to unravel RNA interplay taking place in pathogenesis. Since viroids only consist of RNAs and are known to be degraded by the RNA silencing machinery (Katsarou et al. 2016), HTS of small and total RNAs generated in cognate pathosystems are potentially able to close the persisting gaps of fundamental knowledge which prevent the correct

assessment of phytosanitary risk associated with these pathogens and more generally the management of the associated diseases:

For instance, the discrepancies observed in terms of viroid seed transmission rates (Hammond 2017) is thought to be related to the ability of viroid to invade the germ cells of their hosts (Matsushita et al. 2018). Similarly, the variation observed concerning the insect transmission rate (Van Bogaert et al. 2016) of viroids is only partially explained by viral coinfections and the subsequent encapsidation of viroids in viral proteins (Querci et al. 1997; Syller et al. 1997). This lack of fundamental knowledge about the transmission routes of viroids makes it difficult to assess the actual risk they pose (EFSA 2011). This in turn can cause an increase of management costs for these diseases when strict measures are taken to compensate for the uncertainty of the risk assessment.

Another important practical issue is related to the difficulty in sanitising viroid infected material before its vegetative propagation. Here again, fundamental knowledge is lacking to figure out why high temperature treatment usually applied for viruses does not work as well for viroids (Stace-Smith and Mellor 1970; Hollings and Stone 1970; Paludan 1980; El-Dougdoug et al. 2010) and to understand the variability observed in eradication rates reported in the scientific literature (Barba et al. 2017). If the varying ability of viroids to invade **shoot apical meristems** (**SAMs**) is strongly suspected as the source of variation, the molecular mechanisms underlying this difference remain elusive (Zhu et al. 2001; Qi and Ding 2003; Di Serio et al. 2010; Matsushita et al. 2011).

The very way viroids affect their hosts and lead to the observed symptoms remains obscure. Here again, fundamental mechanisms behind the symptom induction and modulation is still poorly understood and requires further research to provide accurate risk assessments (Owens et al. 2012; Więsyk et al. 2018; Aviña-Padilla et al. 2018).

At the heart of all these underlying fundamental questions lie the defense and counterdefense mechanisms which characterize the interactions between viroids and their hosts. Plants developed several defense systems whose innate immunity and RNA silencing are considered as the two main branches able to target viruses. If it is now well established that viroids are, like viruses, targeted by the RNA silencing machinery, the implication of innate immunity against viroids has only been recently suggested (Zheng et al. 2017). Also, viroids have been found to activate systemic resistance in infected plants (López-Gresa et al. 2016) and to induce the production of **pathogenesis-related (PR) proteins** (Camacho Henriquez and Sänger 1984; Lucas et al. 1985; Granell et al. 1987; Vera et al. 1989; Domingo et al. 1994; Gadea et al. 1996; Tornero et al. 1996; Itaya et al. 2002; Owens et al. 2012) that are more and more considered as a direct consequence of the activation of innate immunity (Mishina and Zeier 2007; Ádám et al. 2018). These findings thus indicate that to understand how viroids interact with their hosts, the components of innate immunity have to be considered as well.

The ability of viroids as unencapsidated and non-coding RNAs to potentially mount an innate immunity response raises other fundamental issues that go beyond the framework of viroid-related research but encompass issues related to phytoviruses, which primarily consist of RNAs, and potentially to any plant self and non-self RNAs. The simple nature of viroids also allows

dissecting the molecular mechanisms underlying plant defense and potentially closing some of the many knowledge gaps which subsist. Moreover, behind this fundamental question of immunity activation is the whole essential challenge of the improvement of the plant resistance to viruses and viroids. Indeed, in the absence of a cure, the selection of resistant plants constitutes the primer sustainable management of these important diseases in crops (Nicaise 2017).

Since diseases are one of the main driving forces of plant evolution (Clay and Kover 1996; Laine et al. 2011), the simple nature of viroids makes them a perfect tool to unfold the molecular mechanisms taking place in plant-pathogen coevolution and to provide a first comprehensive overview of a pathosystem. If viroids are only known to naturally infect plants and seem to only have one phylogenetically related animal counterpart: *Hepatitis delta virus* (Flores et al. 2012), the ability of viroids to replicate in yeast was recently demonstrated (Delan-Forino et al. 2011). Studying the molecular interactions between viroids and their plant hosts is thus also the possibility to assess the specificity and the intricacy of their relationships and gain insight into the uniqueness of their coevolution. This knowledge will enrich our understanding of the evolution of these molecules that some consider as living fossils coming from the RNA world which gave rise to the last universal common ancestor and subsequently to all forms of life on Earth (Diener 2016).

Pospiviroids have several characteristics which make them particularly suited to study all abovementioned fundamental and practical issues. They are taxonomically diverse (Owens et al. 2011) and many genome sequences of pospiviroids are publicly available. Pospiviroids were the first discovered

viroids (Diener 1971; Semancik and Weathers 1972) and have been extensively studied since. They have a large range of hosts and most species multiply and induce symptoms on tomato whose genome has been recently sequenced (Tomato Genome Consortium 2012) and which is easy to handle in a greenhouse.

The present chapter aims at providing readers with an overview of the main outstanding practical challenges regarding pospiviroid and, more generally, viroid disease management that are addressed in this work. To familiarize readers with the fundamental aspects covered, an overview of viroid taxonomic and biological properties focusing on the genus *Pospiviroid* is presented. The main challenges and future prospects in terms of pospiviroid detection are then covered. Similarly, the state of knowledge of plant immunity with a focus on pending questions is provided. This introductory chapter ends up with the main objectives of the thesis. The following chapters then present the research results as they were published in relation to the objectives set out in this introduction. Eventually, in the last chapter, on the basis of the results obtained in the framework of the thesis, an answer is given for each of the objectives and prospective considerations paving the way for future research are provided.

1.1 Regulation and management of viroid diseases

Although the presence of viroids is supposed to be latent in most cases, several viroid species cause severe diseases on economically important crops and three of them are currently considered as quarantine species by most Regional Plant Protection Organizations: Potato spindle tuber viroid (PSTVd), *Chrysanthemum stunt viroid* (CSVd) and *Coconut cadang-cadang viroid* (CCCVd) (EPPO 2019). Economic data on losses due to viroid infections are scarce, however, pospiviroids can cause: yield reduction of potato as high as 97% (Pfannenstiel and Slack 1980), total losses on tomato due to unmarketable fruits (Galindo et al. 1982; Singh et al. 1999) and CSVd infections make chrysanthemum plants unproductive (Randles 2003).

Worldwide, national and regional plant protection organizations set and/or apply rules to avoid the introduction, the establishment and the spread of harmful viroids on their territory.

Although some insects are considered as vectors of some viroid species, their low transmission efficacy and their relative low range (Van Bogaert et al. 2016) make plant material movements the primary sources of introduction and dispersion of viroids in a geographic area.

To avoid large-scale dispersion of viroids and other diseases through vegetative propagation, official and private phytosanitary measures called “certification schemes” are established for economically important crops like: potato, fruit trees and ornamental plants. In these schemes, the initial material used as the source of multiplication has to be carefully checked for these pathogens to allow a safe multiplication of plant material. If infected, sanitation methods have to be applied to obtain viroid free material prior to the multiplication. Unlike viruses which are usually susceptible to high

temperature treatment, viroids are thought to replicate more abundantly in these conditions and thus are recalcitrant to thermotherapy (Stace-Smith and Mellor 1970; Hollings and Stone 1970; Paludan 1980; El-Dougdoug et al. 2010). Although cold treatments have been tested successfully to eradicate viroids (Lizárraga et al. 1980), trials were often performed in conjunction with meristem culture (Paduch-Cichal and Kryczyński 1987; Savitri et al. 2013). These latter techniques rely on the excision of SAMs and its subsequent culture *in vitro* either on a gel supplemented with nutrients and hormones or on a rootstock respectively. The eradication rate achieved by these techniques depends on the size of the shoot excised, the viroid or its variant, the host and its variety, and the growth temperature (Barba et al. 2017).

At a given production area where frequent plant handling is required, mechanical transmission through infected tools constitutes and important way of viroid spreading from initial outbreaks. To prevent this, effective disinfectant has to be applied especially in intensive cropping.

Among viroids, the members of the genus *Pospiviroid* pose in recent years a special challenge for NPPOs. Along with PSTVd, CSVd and *Citrus exocortis viroid* (CEVd) which are known for more than 60 years for their impact on potato, chrysanthemum and citrus, new pospiviroids have been found to be both latent on many ornamentals and harmful for cultivated plant species like tomato and sweet pepper (Singh 2006a; Verhoeven et al. 2008a, b; Luigi et al.2011; Olivier et al. 2011; Olivier et al. 2012).

Throughout the world, these pathogens cause sporadic outbreaks on susceptible annual crops while they often remain undetected although widespread on ornamentals (EFSA 2011). To assess the risk posed by pospiviroids in EU, the link between this potential ornamental reservoir and

the outbreaks on Solanaceae as well as the role of infected seeds in these contaminations have been recently reviewed by the EFSA panel on plant health (EFSA 2011). However, the continuing uncertainty over the role of insects and infected seeds in the spreading of pospiviroids complicated the likelihood assessment of these transmission routes. On the other hand, the panel on plant health concluded that disinfection measures and the accurate generic detection of pospiviroids are among the indispensable tools for the management of these diseases.

To ascertain the absence of these pathogens in plant material, not only for pospiviroids but also in all abovementioned situations, accurate and cost effective detection methods are needed and have to be correctly applied.

Different methods are currently available for the detection of viroids, but international trade requires that standardized detection methods be chosen by authorities to allow the collective recognition of results and acceptance of goods controlled by these means. To this end, the validation of these detection methods according to the international standard ISO 17025 and the comparison of these methods through inter-laboratory comparisons are essential to assess their performance and allow laboratories to select the most appropriate method in their context.

In the EU still no official method has been designated for the detection of pospiviroids. Therefore, the National Reference Laboratories (NRLs) have to carry out extensive performance comparisons of available methods in order to select the most efficient ones. To face the challenges posed by pospiviroids in EU and worldwide, the development and the assessment of generic methods dedicated to the detection and the identification of pospiviroids are thus of primary importance.

1.2 Taxonomic classification of viroids

Viroids are small RNA molecules able to replicate and induce symptoms in plants. Along with PSTVd, the first discovered viroid species (Diener, 1971; Singh and Clark 1971), 31 other viroid species are now officially recognized by the **International Committee on Taxonomy of Viruses** (**ICTV**). Currently, these 32 viroid species are classified into two taxonomic families according to the presence of a **central conserved region** (**CCR**), for the family ***Pospiviroidae***, or **hammerhead ribozymes**, for ***Avsunviroidae***. Within the *Pospiviroidae* the type of CCR and the presence or absence of the two others conserved regions **terminal conserved region** (**TCR**) or a **terminal conserved hairpin** (**TCH**) are used to assign species to the five genera. Members of the *Avsunviroidae*, the presence and type of hammerhead ribozymes together with GC content and solubility in 2 M LiCl are used to assign the four recognized species among three different genera (Di Serio et al. 2014) (Table 1).

Table 1 : Current taxonomy of viroids according to the ICTV

Family	Genera	Species	Acronym
Avsunviroidae	*Avsunviroid*	*Avocado sunblotch viroid*	ASBVd
	Elaviroid	*Eggplant latent viroid*	ELVd
	Pelamoviroid	*Chrysanthemum chlorotic mottle viroid*	CChMVd
		Peach latent mosaic viroid	PLMVd
Pospiviroidae	*Apscaviroid*	*Australian grapevine viroid*	AGVd
		Apple scar skin viroid	ASSVd
		Apple dimple fruit viroid	ADFVd
		Citrus bent leaf viroid	CBLVd
		Citrus dwarfing viroid	CDVd
		Citrus viroid V	CVd-V
		Citrus viroid VI	CVd-VI
		Grapevine yellow speckle viroid 1	GYSVd-1
		Grapevine yellow speckle viroid 2	GYSVd-2
		Pear blister canker viroid	PBCVd

Cocadviroid	*Citrus bark cracking viroid*	CBCVd
	Coconut cadang-cadang viroid	CCCVd
	Coconut tinangaja viroid	CTiVd
	Hop latent viroid	HLVd
Coleviroid	*Coleus blumei viroid 1*	CbVd-1
	Coleus blumei viroid 2	CbVd-2
	Coleus blumei viroid 3	CbVd-3
Hostuviroid	*Dahlia latent viroid*	DLVd
	Hop stunt viroid	HSVd
Pospiviroid	*Chrysanthemum stunt viroid*	CSVd
	Citrus exocortis viroid	CEVd
	Columnea latent viroid	CLVd
	Iresine viroid 1	IrVd-1
	Pepper chat fruit viroid	PCFVd
	Potato spindle tuber viroid	PSTVd
	Tomato apical stunt viroid	TASVd
	Tomato chlorotic dwarf viroid	TCDVd
	Tomato planta macho viroid	TPMVd

Along with these taxonomic characteristics, the members of both families also differ by the way they replicate: asymmetric vs symmetric pathways, the location of their replication: nucleus vs plastids as well as their secondary structure: rod-like vs more branched structure for *Pospiviroidae* and *Avsunviroidae* respectively.

Members of the family *Avsunviroidae* and those of the genera *Coleviroid* and *Cocadviroid*, from the family *Pospiviroidae*, have a very restricted host range and infect only the species in which they were initially reported and, in some instances, closely related species. Conversely, members of the three other genera of *Pospiviroidae* have relatively broad host ranges. Viroids in the genus *Apscaviroid* infect several woody species while the members of the genera *Pospiviroid* and *Hostuviroid* infect both herbaceous and woody species (Flores et al. 2011).

Presently, two criteria are used to create new viroid species: a sequence identity over the entire genomes of less than 90 % and distinct biological properties, particularly host range and symptoms (Owens et al. 2011). If the genus Pospiviroid now contains nine species, previously, a tenth pospiviroid species was distinguished: *Mexican potato viroid* (MPVd). However, considering the absence of biological differences between TPMVd and MPVd and their sequence similarity (Verhoeven et al. 2011) both taxa were merged and are now considered as TPMVd by the ICTV. Recently, a genetically distinct pospiviroid: *Portulaca latent viroid* (PoLVd) has been identified (Verhoeven et al. 2015a). However, since no biological differences have yet been found with its closest relative IrVd-1, PoLVd does not fulfil all ICTV criteria for species demarcation.

Within the same plant, like viruses, viroid isolates are known to consist of a mixture of genetic variants in which predominant fittest sequences coexist with rare unfit sequences. Such sequences ensemble is often called “quasispecies” (Eigen 1971; Domingo 2002).

The quasispecies is the consequence of the relatively high mutation rate observed in viroid which was estimated using HTS as ranging from 0.005 to 0.00014 for PSTVd (Brass et al. 2017; López-Carrasco et al. 2017) and from 0.0025 to 0.001 for *Avsunviroidae* (Gago et al. 2009; López-Carrasco et al. 2017).

1.3 Specific properties of the Pospiviroidae

1.3.1 RNA structures and sequence singularities

Keese and Symons (1985) proposed to divide the rod-like structure of Pospiviroidae into five domains according to biological functions and sequence homologies: terminal left (TL), pathogenicity-related (P), central domain (C), variable (V), terminal right (TR) (Figure 1).

The terminal left (TL), as mentioned before, is associated with PolII docking and transcription initiation site. TL end of most pospi- and apscaviroids contains an imperfect repeat which allows either a rod-like or a Y-shaped structure (Steger and Perreault 2016). Notably, TL domain contains the two conserved domains: terminal conserved region (TCR) and terminal conserved hairpin (TCH), whose presence allow distinguishing Pospiviroidae genera (Di Serio et al. 2014).

The pathogenicity (P) domain contains an oligupurine/oligopyrimidine pairings stretch which is thermodynamically unstable and consequently called premelting (PM) region (Steger et al. 1984). Since it was observed that slight mutations in PM modulate the pathogenicity of PSTVd, this region was associated with pathogenicity (Schnölzer et al. 1985). Similarly, at the junction of TL and P domains, and partially overlapping with PM, a region called virulence modulating (VM) has been proposed (Riesner et al. 1983). However, it should be noted that since then, other genomic regions have been associated with pathogenicity (Qi and Ding 2003; Zhong et al 2008).

The central (C) domain contains the CCR and it is the most conserved domain of Pospiviroidae. Due to the presence of inverted repeat sequences, the upper strand of CCR (UCCR) is able to form a

thermodynamically stable hairpin called HPI during thermal denaturation. Although HPI is present in all members of Pospiviroidae, its biological function is elusive. If it was first thought that HPI was important for infectivity (Hammond and Owens, 1987), it was later demonstrated that this alternative secondary structure was not essential for PSTVd replication (Zhong et al. 2008). Interestingly, in the center of HPI, is a stretch of 16 nucleotides: GGAKCCCCGGGGMAAC, highly conserved across all genera of Pospiviroidae but whose function is unknown. In the center of the C domain of many pospiviroids lies a particular internal loop which shows homology with the loop E of eukaryotic 5S RNA and whose structural integrity is crucial for replication, especially for transcription supposedly due interaction with the splicing regulator RPL5 (Zhong et al. 2006; Jiang et al. 2018). Zhong et al. (2008) also showed that mutations in CCR were associated with impaired PSTVd replication.

The variable (V) domain is the most variable region flanked with C and TR conserved domain (Keese and Symons 1985). Zhong et al. (2008) showed that mutations in variable domain were associated with impaired PSTVd trafficking. In pospiviroids, V domain hosts one part of the hairpin II (HPII) which associates with its counterpart from the TL domain to form a GC rich and 11-12-nt long helix. This HPII structure was found critical for replication, especially from (-)- to (+)-strands (Loss et al., 1991; Qu et al., 1993; Schröder and Riesner 2002).

The terminal right (TR) domain contains two asymmetric internal loops called RY motifs which were found to be responsible for specific interaction with the bromodomain-containing protein, viroid RNA binding protein (Virp1)(Gozmanova et al. 2003). Furthermore, the RY motif is phylogenetically conserved in several genera of Pospiviroidae and VirP1 was

found to be necessary for replication of both PSTVd and CEVd in *Nicotiana benthamiana* (Kalantidis et al. 2007) indicating a primer role of RY motifs in Pospiviroidae. Additionally, Zhong et al. (2008) showed that mutations in TR domain were associated with impaired PSTVd trafficking. This latter result is consistent with those obtain by Maniataki et al. 2003 in which RY motifs were actually found associated with systemic trafficking.

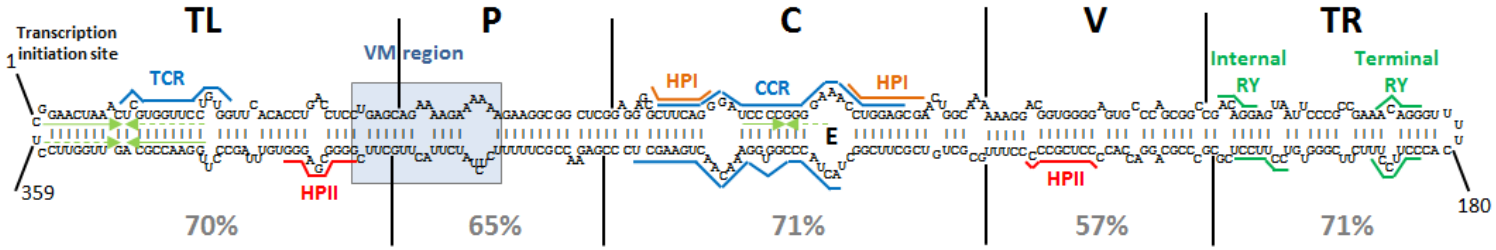


Figure 1: Rod-like secondary structure of the type PSTVd isolate (GenBank reference: NC_002030) showing the five domains proposed by Keese and Symons (1985): terminal left (TL), pathogenicity (P), central (C), variable (V), terminal right (TR). Conserved regions are depicted: terminal conserved region (TCR), central conserved region (CCR), internal and terminal RY, hairpin I and II (HPI, HPII), virulence modulating (VM) region. The transcription initiation site is thought to be located at the left extremity of TL at the position conventionally considered as the first (C1) or the last nucleotide (U359) of the genome. Local inverted repeats are depicted as green arrows. Loop E is shown with the letter E (adapted from Steger and Perreault 2016). Pospiviroid mean percentages of sequence similarity are provided for each of the five domains. These means were computed based on 891 pospiviroid genomes from the eight pospiviroid species able to infect tomato.

1.3.2 Biological cycle

To accomplish a successful infection Pospiviroidae have to be able to (1) enter and exit the nucleus for their replication; (2) pass from cell to cell; (3) enter to vascular tissue for long-distance trafficking to distant plant organs; (4) exit the vascular tissue for systemic infection; and (5) be transmitted to another host.

1.3.2.1 Replication

The right terminal domain which associates with VirP1 (Gozmanova et al. 2003) is thought to induce nucleus importation of PSTVd since the protein contains nuclear localization signals and a bromodomain (Martinez de Alba 2000) and Kalantidis et al. (2007) showed a direct correlation between the expression of VirP1 and infection by PSTVd and *Citrus exocortis viroid*. However, conflicting results suggesting that several other genomic regions can also trigger this movement have been reported (Abraitiene et al. 2008). In this latter study, the upper portion of PSTVd genome and particularly the region involved in HPI/CCR were found to be related to nucleus importation. Another study also showed that PSTVd enters the nucleus in a cytoskeleton-independent manner (Woo et al. 1999).

Once in the nucleus, *Pospiviroidae* are replicated in the nucleoplasm according to an asymmetric cycle where only (+)-circles serve as templates for the synthesis of oligomeric (-)-strands (Rackwitz et al. 1981; Branch et al. 1988; Flores et al. 2009). Unexpectedly, *Pospiviroidae* were found to be replicated by DNA-dependent RNA polymerase II (PolII) rather than RNA-directed RNA polymerases.

Bojic et al. (2012) demonstrated that PolII specifically binds the TL domain. Recent studies showed that, for its replication, PSTVd requires a unique splicing form of transcription factor IIIA (TFIIIA-7ZF) which also binds the TL domain. In agreement with these PolII and TFIIIA-7ZF docking sites, Zhong et al (2008) found the end of TL associated with replication through mutational analysis of PSTVd (Figure 2). Furthermore, Kolonko et al. (2006) identified in vitro a single transcription start site at either position U359 or at C1 that is at the TL hairpin loop of PSTVd. PolII together with TFIIIA-7ZF may require bifurcated conformation of TL to allow replication (Dissanayaka Mudiyanselage et al. 2018) although rod-like structure seems thermodynamically more stable in PSTVd than Y-shape (Dingley et al. 2003). It was also demonstrated that PSTVd directly interacts with the splicing regulator, RPL5, to favor the expression of TFIIIA-7ZF, thereby promoting viroid replication. The ribosomal protein RPL5 was found to bind to central conserved region (CCR) and require loop E motif in CCR (Jiang et al. 2018). In line with these findings, mutations in either loop E (Zhong et al. 2006) or the previous loop located in the CCR (Zhong et al. 2008) strongly impair PSTVd replication (Figure 2). From oligomeric (−)-strand polII synthesizes (+)-stranded oligomers which are cleaved into monomers, presumably by class III RNase (Gas et al. 2008), circularized by DNA ligase I (Nohales et al. 2012), and transported selectively into the nucleolus (Harders et al. 1989; Qi and Ding 2003).

The mechanisms involved in the nuclear exportation remain currently obscure (Ding and Owens, 2003).

1.3.2.2 Cell-to-cell movement

When it comes to cell-to-cell movement, experimental evidence indicates that PSTVd moves through plasmodesmata (PD) and that this movement

might be mediated by a specific sequence or structural motif (Ding et al. 1997). The mechanisms by which viroids, and more generally RNAs, cross PDs are not well understood but these intercellular connections are thought to primarily regulate RNA trafficking by size exclusion limit (SEL) modulation. If small molecules such as growth hormones, minerals, amino acids or sugars can move passively through PD, large molecules like proteins (>1kDa), viral genomes or mRNAs often require an active mechanism for cell-to-cell movement (Zambryski and Crawford 2000). The numbers and SEL of PD vary among different tissues at different development stages. The regulation of SEL is still elusive but two main molecular strategies emerged. Firstly, it is well established that reversible callose (β-1, 3-glucan) deposition regulates PD permeability in response to various biotic and abiotic stresses (Sager and Lee 2014; Liu and Chen 2018). Secondly, although the molecular mechanisms involved remain obscure and controversial, cytoskeleton has also been associated with SEL regulation (Zambryski and Crawford 2000; Su et al. 2010). Furthermore, many proteins like phloem sap proteins and viral movement proteins (MPs) were reported to interact with PD to increase their SEL (Balachandran et al. 1997; Lucas et al. 2006). Su et al (2010) showed for instance that *Cucumber mosaic virus* MP serves actin filaments to increase the PD SEL. To date, how viroids are able to pass through PD is unknown but concerns have been mainly focused on possible interactions with unknown gating proteins. Since disruptions of loops/bulges in the secondary structure of PSTVd led to defective or impaired trafficking (see hereafter) and considering that most RNA loops/bulges are highly structured three-dimensional motifs interacting with other molecules (Leontis et al. 2006), selective viroid-protein interactions depending on viroid genome conformation have been anticipated as the primer mechanism involved in viroid cell-to-cell active

movement (Maniataki et al. 2003; Qi et al. 2004; Zhong et al. 2007; Zhong et al. 2008). However, it has been recently demonstrated that PSTVd derived siRNAs were able to silence callose synthase genes and that this ability, relying on PSTVd-mRNA similarity, greatly affected the accumulation of viroids and the severity of disease symptoms (Adkar-Purushothama et al. 2015).

1.3.2.3 Long-distance trafficking

Like many plant viruses and thousands of plant endogenous RNAs, pospiviroids long-distance trafficking is associated with phloem (Palukaitis 1987; Zhu et al. 2001; Liu and Chen 2018). Mutation analyses showed that several genome positions of PSTVd have been associated with trafficking. Strikingly, most of these positions are located in two defined genomic location:

The first group of trafficking associated positions extends from the right side of TL to the right side of C domain (Figure 2). In this genome stretch, which partially overlaps with VM region, Zhong et al. (2007) found mutations which impair the entry of PSTVd into the phloem. Conversely, in this same region, Qi et al. (2004) showed nucleotide positions associated with defective vascular (bundle sheath) unloading. Takeda et al. (2009) also found positions associated with palisade to spongy mesophyll trafficking. Mutations of the same genomic positions were found to prevent the trafficking of PSTVd in another study (Zhong et al. 2008). Zhong et al. (2008) also found defective trafficking mutations on both sides of the right end of P domain. Remarkably, PSTVd derived siRNAs able to silence callose synthase genes is also located in this genome stretch overlapping the VM region (Adkar-Purushothama et al. 2015).

The second group of positions extends from the right end of the central domain to the right end of the rod-like structure (Figure 2). In this stretch, along with mutations associated with defective trafficking (Maniataki et al. 2003; Zhong et al. 2008), a position showing differential bundle sheath unloading was identified (Qi et al. 2004). Furthermore, since binding motifs of the tomato protein VirP1 are located in the same genome region that trafficking associated positions, it has been suggested that VirP1 could be involved in systemic viroid spread (Maniataki et al. 2003) but no experimental evidence supports this assumption.

When it comes to proteins potentially associated with long-distance trafficking of viroids, Owens et al. (2001) and Gómez and Pallás (2001) reported that one of the most abundant RNA-binding proteins in cucumber phloem, *Cucumis sativus* phloem protein 2 (CsPP2), strongly interacts *in vitro* with *Hop stunt viroid* (HSVd), suggesting the involvement of this protein in viroid phloem transport. Gómez and Pallás, (2004) further showed that CsPP2 effectively bind HSVd in vivo and is associated with the viroid vascular movement. The same study also revealed that CsPP2 presents a double-stranded RNA-binding motif.

Conversely, the 4/1 protein has been found to negatively influence the translocation of PSTVd from inoculated leaves toward young developing leaves (Solovyev et al. 2013). Although the role of 4/1 protein is still elusive, it has been found to bind both RNA and MP of *Tomato spotted wilt virus* (TSWV) (Morozov et al. 2014). Furthermore this protein shows some similarity to alpha-helical domains of myosin-, kinesin-, and ankyrin-like proteins (von Bargen et al. 2001) and potentially contains both nuclear export signal and nuclear localization signal (Morozov et al. 2014).

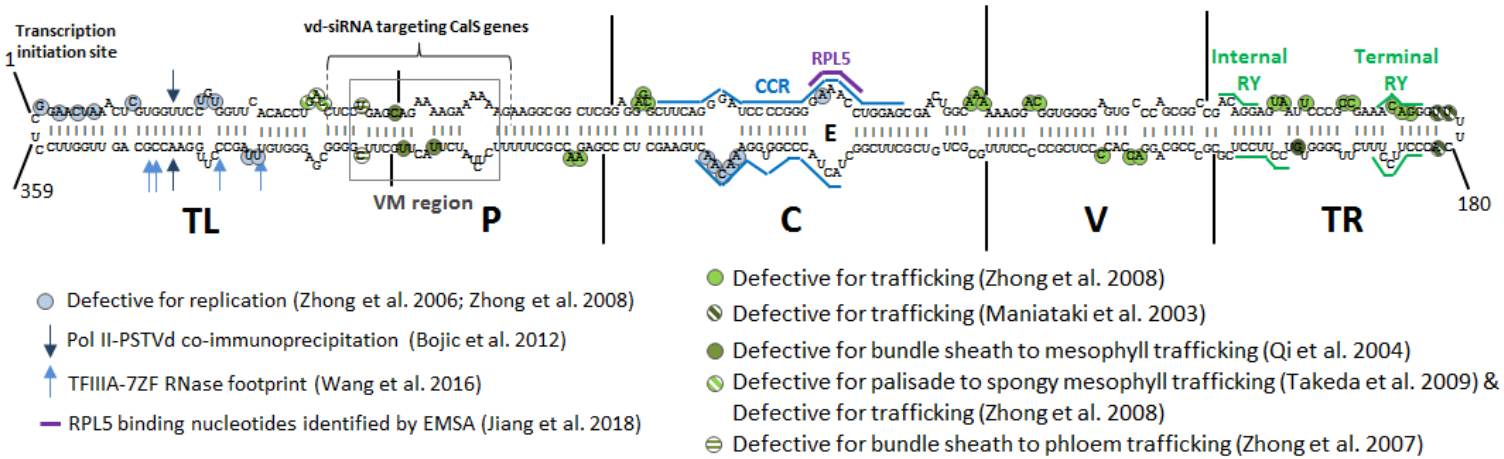


Figure 2: Replication (blue/purple) and trafficking (green) associated nucleotide positions of PSTVd. Circles show nucleotide positions which have been identified by mutagenesis, arrows indicate positions identified by either co-immunoprecipitation of DNA-dependent RNA polymerase II (Pol II) or RNase footprint of TFIIIA-7ZF. Nucleotides which showed modified binding of RPL5 in Electrophoresis mobility shifting assays (EMSAs) are highlighted with purple bars. Central conserved region (CCR) (blue lines), virulence modulating (VM) region (black rectangle) and RY motifs (green lines) are also shown. Vd-siRNA targeting callose synthase (CalS) genes of tomato is delimited with a parenthesis in the upper part of the genome (Adkar-Purushothama et al. 2015).

Trafficking of RNAs in plants is a complex phenomenon which is gaining in importance since RNAs may act as mobile signals to regulate plant development, nutrition allocation, gene silencing, antiviral defense, stress responses and many other physiological processes (Liu and Chen 2018). If some RNAs seem to traffic passively, especially short, stable and abundant ones, evidence exists for an active and selective mechanism. For instance, certain mRNAs may be preferentially transported to specific tissues and it has been revealed that a large number of mobile transcripts harbor a specific motif for phloem transportation called tRNA-like structures (TLSs) (Zhang et al. 2016). In addition, many proteins found in phloem exudates show RNA-binding proteins and some are required to (i) allow the transit of their cognate RNAs through PD (Lucas et al. 1995), (ii) protect RNAs (Cho et al. 2015) (iii) mediate the long-range transport of RNAs (Ham et al. 2009).

1.3.2.4 Spatial distribution of viroids in their host

The analysis of the pattern of systemic trafficking of PSTVd in tomato and *Nicotiana benthamiana* showed that the pospiviroid invade most parts of both plants (Stark-Lorenzen et al. 1997; Zhu et al. 2001; Qi and Ding 2003). PSTVd is thought to be abundant in the phloem and actively multiplying in parenchyma and phloem companion cells (Stark-Lorenzen et al. 1997; Zhu et al. 2001). Notable exceptions of host systemic invasion by pospiviroids are the shoot apical meristems (SAM) and flower organs (excluding sepals) which were found differentially infected, if ever (Zhu et al. 2001).

1.3.2.4.1 SAM invasion and sanitation consequences

Using *in situ* hybridization showed that SAMs of infected tomato and *Nicotiana benthamiana* were seemingly free of PSTVd and TCDVd (Zhu et al.

2001; Qi and Ding 2003; Di Serio et al. 2010; Matsushita et al. 2011). SAMs were also found viroid-free in petunias infected with PSTVd (Matsushita and Tsuda 2014). Using silenced transgenic *Nicotiana benthamiana* (Di Serio et al. 2010) showed that in plants downregulated for RDR6 (see chapter 1.5.1.2), *in situ* hybridization showed that PSTVd successfully invaded SAMs. Zhang et al. (2014b) also revealed that the extent of SAM invasion by CSVd in *Argyranthemum* plants depends on the infected cultivar and that this difference was due a major difference in the number of callose (β-1, 3-glucan) particles deposited at PD in SAM. Matsushita and Shima (2015) also showed that cold temperatures prevent the invasion of SAMs. Because SAMs are often free of viroids and virus, meristem culture are regularly used for viroid/virus eradication. However, in lines with the variable invasion of SAMs observed by *in situ* hybridization, the eradication rate achieved by these techniques depends on the size of the shoot excised, the viroid or its variant, the host and its variety, and the growth temperature. The viroid/host combination is thus a prevalent factor influencing SAM invasion by viroids (Barba et al. 2017).

1.3.2.4.2 Floral organ invasion and seed transmission

Floral organs were also found differentially invaded by pospiviroids (Zhu et al., 2001; Matsushita et al., 2011; Zhu et al. 2002). If pospiviroids infection were found restricted to sepals in several studies using *in situ* hybridization (Zhu et al., 2001; Matsushita et al., 2011; Zhu et al. 2002), Singh (2006b) showed similar titers of pospiviroid in several flower parts of tomato, *N. glutinosa*, *Verbena* sp. and *Impatiens* by using RT-PCR and inoculation of tomato seedlings. Similarly to SAMs, floral organ invasion was found to be dependent on pospiviroid strains (Zhu et al. 2002) and species (Matsushita

et al., 2011) as well as on host species and varieties (Zhu et al., 2001; Matsushita et al., 2011; Zhu et al. 2002; Zhang et al 2014b).

Zhu et al. (2002) showed that the differential invasion of sepals compared to the rest of floral organs was due to specific phloem exit mechanisms that are influenced by both viroid motifs and unknown host factors. Also, floral organs were found protected by RDR6 (Di Serio et al. 2010). In agreement with these differences in terms of pospiviroid presence in floral organs, very variable seed transmission rates have been reported in the literature for several pospiviroid species and host plants, as reviewed by Hammond (2017). For instance, Fernow et al. (1970) reported transmission rates ranging from 0% to 100% using PSTVd infected tomatoes. Obviously, embryos cannot be infected without pospiviroid access to pollen or ovules. In this respect, Matsushita and Tsuda (2014) showed that when present in the petunia floral organs, PSTVd was shown to invade the embryo through the ovule or pollen before embryogenesis. Although seeds can be readily infected by pospiviroids (Matsushita et al. 2018), studies demonstrated good protection of germ cells through low rates of pospiviroid seed transmission (Faggoli et al. 2015).

1.4 Pospiviroid symptomatology

If viroids are thought to multiply latently in many plant species or cause imperceptible symptoms, when present, symptoms caused by viroids are generally similar to those caused by plant viruses, with stunting as the most characteristic (Hadidi et al. 2003).

Pospiviroids species are known to have a large range of plant hosts which encompasses at least 9 taxonomic families: *Amaranthaceae*, *Campanulaceae*, *Chenopodiaceae*, *Cucurbitaceae*, *Compositae*, *Fabaceae*, *Scrophulariaceae*, *Rutaceae* and *Solanaceae*. Among these, the Solanaceae

appears to be the most suitable family for the replication of pospiviroids since many solanaceous species have been described as suitable hosts (Hadidi et al. 2003). Strikingly, tomato has been shown to allow the replication of all known pospiviroid species, except *Iresine viroid 1* (IrVd-1), and produce symptoms. For this reason, tomato was soon considered as a biological indicator for pospiviroids (Hadidi et al. 2003).

Among pospiviroids, CEVd, CSVd and PSTVd are the most characterized species:

Symptoms of PSTVd were first described in 1922 in potato. Growth of infected potato plants may be severely reduced or even cease entirely. However, reduction in growth may also be hardly visible. The stems of infected plants may be smaller, more upright, and produce smaller leaves than their healthy counterparts. Infected tubers may be small, elongated (from which the disease derives its name), misshapen, and cracked. Their eyes may be more pronounced than normal and may be borne on knob-like protuberances that may even develop into small tubers (Owens and Verhoeven 2009).

Symptoms of CEVd were reported since 1948 as a bark scaling disorder called “exocortis disease” affecting trees grown on the trifoliate orange (*Poncirus trifoliate*) (Fawcett and Klotz 1948). Later, other species of the genus *Citrus* were also found affected by CEVd like: ‘Rangpur’ lime (*Citrus limoni*) (Moreira 1959) and ‘Etrog’ citron (*Citrus medica*) (Calavan 1968). ‘Etrog’ citron was adopted as an indicator for the biological indexing of exocortis for its ability to display a variety of symptoms ranging from severe to mild. Typical symptoms on *Poncirus trifoliate* and *Citrus medica* are

severe stunting and bark scaling. Yellow blotching of twigs, leaf epinasty and mid-vein necrosis can also be observed.

Symptoms of CSVd were reported in the early 1950s (Diener and Lawson 1973). It causes light green young leaves, stunting, small leaves and flowers, and reduced rooting ability on Chrysanthemum (Cho et al. 2013).

In tomato, pospiviroid infections first cause growth reduction and chlorosis in the top leaves. Subsequently, this growth reduction may develop into stunting, and the chlorosis may become more severe with the appearance of leaf necrosis (Figure 3 and Figure 4-A, C, D), turning into reddening and/or purpling. In this stage, leaves may become brittle. Generally, this stunting is permanent; occasionally, however, plants may either die or partially recover. As stunting begins, flower and fruit initiation stop (Owens and Verhoeven 2009).

Figure 3 : Symptoms caused by six isolates collected in Belgium from six pospiviroid species on tomato cv. Minibel. Mock inoculated control (Healthy), *Citrus exocortis viroid* (CEVd), *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Columnea viroid* (CLVd), *Potato spindle tuber viroid* (PSTVd) and *Chrysanthemum stunt viroid* (CSVd).

It is generally accepted that viroid replication and symptom development is enhanced as the **temperature** increases above 20°C, at least to 35°C (Singh 1983, 1989). In experimental infections, early onset and increased severity

of symptoms have been demonstrated above 30°C for PSTVd (Harris and Browning 1980) as well as for CEVd (Semancik et al. 1988).

The **amount of light available** is almost as important to symptom expression as the temperature. Symptoms modulation by light intensity was demonstrated for Citrus viroids (Schlemmer et al. 1985), CSVd (Hollings and Stone 1973) and PSTVd (Harris and Browning 1980). **Quality of light** also has an impact on severity of symptoms (Grasmick and Slack 1985).

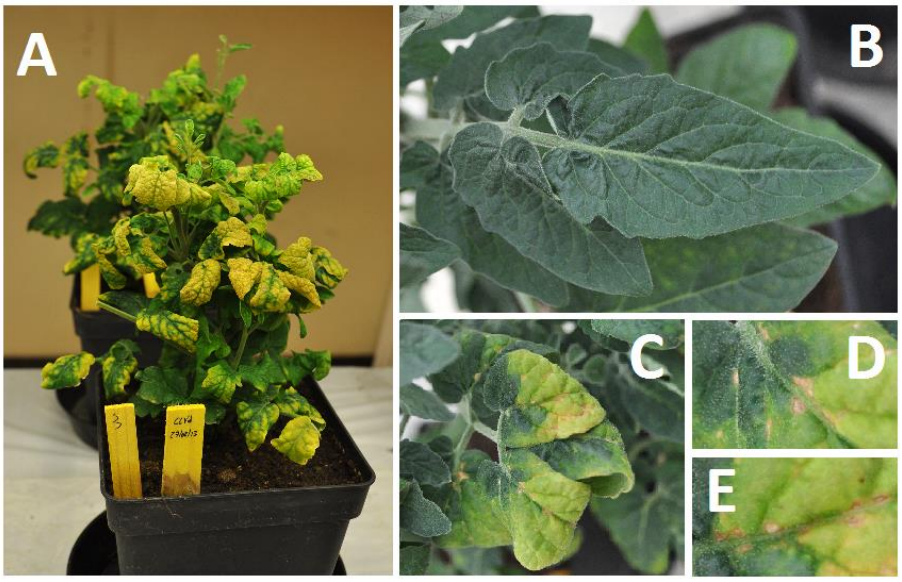


Figure 4 : A) Severe symptoms caused by *Citrus exocortis viroid* (CEVd) on tomato cv. Minibel: plant stunting, leaf chlorosis, downward curl and necrosis. B-E) Leaf closeups showing symptom details of *Potato spindle tuber viroid* (PSTVd) on the same tomato variety: B) mock inoculated plant leaf, C) small chlorotic leaf with epinasty (downward bending), D-E) details of leaf necrosis appearing in symptomatic leaves.

The underlying molecular mechanisms leading to viroid symptom induction and modulation are still largely unknown despite more than 30 years of research efforts. Currently, three main theories, potentially interconnected, are put forward to explain how pospiviroids trigger the first

reactions: (i) the first hypothesizes an interaction between viroids and unknown plant proteins which would initiate symptom development, the two others postulate that viroid-derived small interfering RNAs (vd-sRNAs) trigger the modulation of important protein synthesis through (ii) RNA directed DNA methylation (RdDM) or (iii) post transcriptional gene silencing (PTGS).

Transcriptional gene silencing relying on sequence complementarity between vd-sRNAs and host genome is thus a possible way through which pospiviroids induce symptoms like this was demonstrated for a variant of *Peach latent mosaic viroid* inducing peach calico symptom. Although several PTGS targets of *Potato spindle tuber viroid* (PSTVd) have been identified: PlantWD-40 repeat proteins (Aviña-Padilla et al. 2015), two genes involved in gibberellin or jasmonic acid biosynthesis (Wang et al. 2011a), a chloride channel protein CLC-b-like and a ribosomal protein S3a-like (Adkar-Purushothama et al. 2017), divergences between studies exist and the mechanisms leading to symptoms remain elusive.

The potential induction of symptoms through RdDM was experimentally tested by Wassenegger et al. (1994) who demonstrated that PSTVd cDNA integrated into the genome of *Nicotiana tabacum* becomes specifically methylated upon PSTVd replication. However, no methylation of non-transgenic plant DNA due to viroid infection was demonstrated and the RdDM guide molecules seem to be longer than the 24-nt vd-sRNAs (Dalakouras et al. 2013).

Regarding the protein-viroid interactions, several tomato proteins were found to interact with viroids (Wolff et al. 1985; Werner et al. 1995; Gómez and Pallás et al. 2001; Owens et al. 2001), but although some, like Virp1 (Kalantidis et al. 2007), was found to be essential for their infectivity, the roles they play in symptom modulation are not trivial. Apart from their

interaction with proteins of the RNAi machinery, viroid-derived dsRNAs could directly bind to PRR/disease resistance proteins and trigger PTI/ETI. In mammals, the protein kinase RNA-activated (PKR) is, for instance, known to bind dsRNAs and phosphorylate the translation initiation factor eIF2α, which leads to translation initiation repression. Despite early reports of plant PKR (pPKR) activity in virus-infected plants (Hiddinga et al. 1988; Langland et al. 1995; Chang et al. 1999), no specific kinase could be purified, and the sequence of a putative PKR ortholog is absent from plant genomes (Immanuel et al. 2012). However, the recent discovery of a dsRNA-induced immunity independent of dicer-like proteins (Niehl et al. 2016) offers hope in the search for a plant dsRNAs receptor.

While it is thought that pospiviroids replicate latently in many solanaceous ornamentals, eight species of the genus (Owens et al. 2011) replicate and produce symptoms on tomatoes. This suggests that the induction of symptoms would be independent from the replication and trafficking, and would be rather specific to some susceptible species. However, some mild pospiviroid strains are known to induce very few symptoms on the fast-growing tomato, so that it cannot be excluded that the impact of pospiviroids on slow-growing ornamentals might be difficult to highlight. Conversely, tomato lethal strains of pospiviroids have been identified.

1.5 Detection and identification of pospiviroids

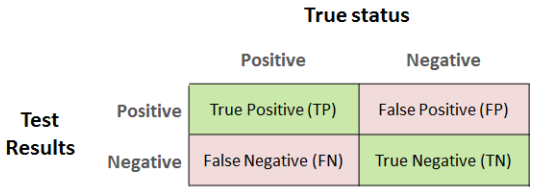
While pospiviroids can be readily detected on a great number and variety of leaf samples (EFSA PLH 2011), the performance of detection methods used in routine laboratories on seed samples is still largely unknown. Different methods are currently available for the generic detection of pospiviroids (Monger et al. 2010; Tiberini and Barba 2012; Torchetti et al. 2012; Botermans et al. 2013; Zhang et al. 2013; Luigi et al. 2014; Olivier et al.

2014), but still no official method has been designated. Therefore, the National Reference Laboratories have to carry out extensive performance comparisons of available methods in order to select the most efficient one on host plant leaves as well as on tomato seeds.

While three methods aiming at detecting pospiviroids already exist (Monger et al. 2010; Torchetti et al. 2012; Botermans et al. 2013), none of them allows a sensitive, specific and selective detection of all pospiviroids in one reaction that can be directly followed by sequencing identification. In the context of the highly globalized trade of plant material, and thus spread of plant diseases, reliable tests are needed to avoid misleading results and potentially high economic losses. Also, fundamental questions regarding pospiviroids distribution within their hosts (invasion of SAM, floral organs or ovules) and more generally the trafficking of these non-coding RNA molecules within plant require sensitive and accurate detection methods to be addressed (Singh 2006b). In terms of epidemiology, the implementation of cost-effective and accurate methods are also essential to assess thoroughly the geographic distribution of pospiviroids, their host range as well as their rate of transmission through seeds and vectors.

The validation of detection methods, which consists in assessing their performance, is thus a pivotal step in the development of new cost-effective detection methods and is mandatory for the laboratories which comply with the ISO/IEC standard 17025. However, the requirements concerning validation in this standard can be prone to interpretation. The EPPO pest management standard PM7/98 (EPPO 2010a; EPPO 2014a; EPPO 2018a) proposes a common base of interpretation by specifying the requirements of this latter ISO/IEC standard for plant disease and pest diagnostics. Along with the PM 7/76 (EPPO 2010b; EPPO 2014b; EPPO 2017;

EPPO 2018b), these standards define, and focus on, four main and one minor performance criteria: analytical specificity, analytical sensitivity, repeatability, reproducibility and selectivity, respectively (see Figure 5 for definitions). The PM7/98 also proposes to use three other performance criteria allowing to compare the relative performance of two or more methods: diagnostic sensitivity, diagnostic specificity and relative accuracy (see Figure 5 for definitions).



Analytical sensitivity = Smallest amount of target that can be detected

Diagnostic sensitivity = TP / (TP + FN)

Analytical specificity = Performance of a test with regard to cross-reactions with non-target

Diagnostic specificity = TN / (TN + FP)

Selectivity = The extent to which variations in the matrix affect the test performance (matrix effect)

Relative accuracy = (TN + TP) / (TN + TP + FN + FP)

Repeatability = Level of agreement between replicates of a sample tested under the same conditions

Reproducibility = Ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (time, persons, equipment, location etc.)

Robustness = The extent to which altered test conditions (e.g. temperature, volume, change of reagents) affect the established test performance values (e.g. analytical sensitivity, analytical specificity)

Figure 5: Scheme showing how true positive (TP), true negative (TN), false negative (FN) and false positive (FP) are determined. Definitions of performance criteria defined in EPPO standard PM7/76 and used in PM7/98.

Although PM7/98 allows certain flexibility in terms of validation, the ISO/IEC 17025:2005 requires defining clearly and unambiguously the scope of the tests covered by the laboratory's accreditation by setting out: the targeted organism, the matrix and the method. If full validations are achievable for fixed and restricted scopes, pospiviroids are genetically diverse and have a broad host range making a full validation impossible. So

laboratories implementing pospiviroid detection under accreditation have to make some choices to allow a cost-effective but still accurate validation. Considering this situation, Roenhorst et al. (2018) advocated for allowing partial validation relying on experienced diagnosticians, second distinct test in case of positive samples and appropriate controls at different levels.

In the framework of this thesis, in order to allow a reliable and cost-effective detection and identification of all harmful pospiviroids, a multiplex one-step RT-PCR pospiviroid detection assay that can be coupled with direct sequencing identification was developed and validated according to the EPPO standards. To make the validation of this new test achievable in the context of an ISO/IEC 17025 accreditation, a limited number of pospiviroid strains and host plant species were selected to reflect the most prevalent species/host combinations observed on the Belgian territory. Likewise, in agreement with the EPPO standard PM7/98, bioinformatic assessment of PCR primers was performed based on a maximum of publicly available pospiviroid sequences in order to further validate the analytical selectivity of the test. In this way, the generic detection of pospiviroids in plants using the new detection method was considered as a fixed scope and treated as such in regards to ISO/IEC 17025 and PM7/98 standards. The validation of this new generic detection method is discussed in detail in chapter 3.

When it comes to quality controls, quality assurance is based on adequate use of first-, second- and third-line controls. These controls are essential to monitor: (i) that each test are properly carried out by the inclusion of positive and negative controls (first-line controls), (ii) the competence of the operators/diagnosticians through the analysis of blind samples (second-line controls) and (iii) the overall performance of the laboratories and the

methods they used are in line with other laboratories through inter-laboratory comparison (third-line controls). In the context of phytosanitary risk management, it is worth pointing out that third-line controls are particularly useful since they not only allow the laboratories to benchmark their competence and the accuracy of their diagnostic methods but these controls also help the accreditation bodies and the phytosanitary risk managers assessing the efficiency of their laboratory network.

Despite the importance of third-line controls, the lack of available inter-laboratory comparisons often prevents the laboratories from assessing their overall performance. In the absence of European Reference Laboratories (EURLs) for plant diseases, the accredited laboratories have to find other third-line controls providers. If some individual initiatives of national reference laboratories offered opportunities for foreign laboratories to take part in their inter-laboratory comparisons, the lack of dedicated funding and the scale of the task have so far prevented the establishment of permanent ring test providers meeting the needs of the sector. To mitigate this problem, European laboratories organized themselves through the network Euphresco, hosted at the EPPO, in order to provide in turn some inter-laboratory comparisons. This is the context in which, in the framework of this thesis, a second inter-laboratory comparison dedicated to pospiviroid detection based on PCR technology was organized and, to share the results as much as possible, these latter were thoroughly analyzed and published. These results are discussed in chapter 4.

As a forward-looking vision of future developments in the field of viroids/viruses detection methods and in an attempt to further close the gaps of fundamental knowledge which still prevent a precise assessment of

phytosanitary risks posed by pospiviroids, the use of High-Throughput Sequencing (HTS) was considered. Indeed, HTS now emerges as a revolutionary universal virus/viroid detection/identification tool that is now more and more regarded as routine detection method regularly applied for viruses and viroids (Massart et al. 2017; Maree et al. 2018). In addition, HTS has been largely applied to resolve cognate disease etiology and to allow *de novo* detection/identification of new viruses/viroids (Hadidi et al. 2016). The implementation of HTS in diagnostics thus induces a change of strategy which favors broad spectrum screenings at the expense of specific detection methods. Consequently, the use of this technology in routine lab poses many challenges in terms of validation, accreditation and day-to-day implementation (Maree et al. 2018; Massart et al. 2019) and, compared to PCR based detection methods, HTS requests even more validation flexibility. The ability of HTS to highlight new viruses/viroids also raises important issues in terms of phytosanitary regulation since the pathogenic potential of these newly discovered biological entities are unknown (Massart et al. 2017). Also, HTS can reveal the persistent presence of some viruses/viroids in a given crop so that phytosanitary declaration of 'freedom from viruses' or 'virus free' may be no longer achievable (Maree et al. 2018). Considering that new viruses/viroids can be both parasitic and commensal/mutualistic (Roossinck 2015) and that Koch's postulate may take more than two years to be fulfilled (Di Serio et al. 2018), the use of HTS as a routine diagnostic tool urges the need for the comprehension of the still largely obscure underlying molecular mechanisms of symptom induction of plant viruses and viroids.

Since the implementation of HTS with the sole purpose to detect pospiviroids in routine is currently not fit for purpose, in the framework of this work, HTS was rather used to characterize the molecular mechanisms

of symptom induction of an isolate of *Citrus exocortis viroid* which, together with closely related isolates, induced the most severe symptoms on tomato amongst the pospiviroids collected on the Belgian territory (see figure 3). This HTS characterization is discussed in chapter 5.

1.6 Plant-viroid interaction

Besides the economic and legal importance of viroids, these small RNA molecules constitute the simplest known plant pathosystems and are thus perfect models to dissect the molecular mechanisms underlying plant-pathogen relationships.

Although viroids differ from most RNA viruses by the fact that they do not code for any protein, replicate in the nucleus or in the chloroplast and form imperfect double-stranded RNAs (dsRNAs) structures, like RNA viruses, viroids are able to activate and resist RNA silencing (Wang et al. 2004), also termed RNA interference (RNAi), activate systemic acquired resistance (SAR) (López-Gresa et al. 2016) and recently they ability to mount innate immunity has been suggested (Zheng et al. 2017).

1.6.1 Plant immunity concepts and components

Several concepts have been developed to describe how plants react to pathogens. Currently, out of these theories four different concepts have emerged: PAMP-triggered immunity (PTI), effector-triggered immunity (ETI), systemic acquired resistance (SAR) and RNA silencing, also termed RNA interference (RNAi).

1.6.1.1 Innate immunity

The plant innate immunity is divided into two branches: (i) **PAMP-triggered immunity** (**PTI**) which is the first line of defense consisting of

transmembrane **pattern recognition receptors** (**PRRs**) that respond to slowly evolving **microbial-** or **pathogen-associated molecular patterns** (**MAMPs** or **PAMPs**) and (ii) **effector-triggered immunity** (**ETI**) which acts inside the cell, using **NB-LRR proteins** encoded by most **R genes**. To model the successive steps of the interaction between plants and their pathogens, these two branches have been integrated in the so-called "**zig zag model**" theorized by Jones and Dangl (2006) (Figure 6). This model allows understanding how innate immunity works and modulates the symptoms from highly susceptible plants or compatible plant-pathogen interaction, which leads to plant death, to completely resistant plants (qualitative resistance) which restrict the spread of the disease at the infection point through **hypersensitive response** (**HR**) characterized by a localized **programmed cell death** (**PCD**). Between these two extreme stages, plants show partial resistance, also called quantitative or horizontal or basal resistance varying from the appearance of strong symptoms to inconspicuous symptoms sometimes referred to as tolerance.

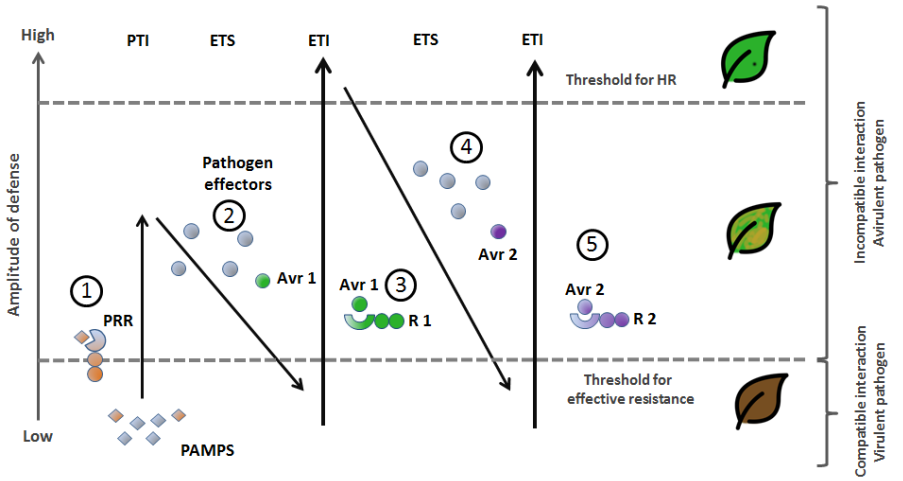


Figure 6: Zigzag model proposed by Jones and Dangl (2006) to conceptualize the plant pathogen interactions according to the involvement of the two branches of innate immunity: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). From bottom left to

top right: **1)** virulent pathogens produce pathogen-associated molecular patterns (PAMPs) and plants unable to recognize these PAMPs may die due to this compatible interaction. However, if plants possess pattern recognition receptors (PRRs) in their cell membranes that are able to recognize PAMPs, the first line of plant defense is triggered: PTI. **2)** Successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS) or incompatible interaction. **3)** One effector considered as avirulent (Avr) (indicated in green) is recognized by NB-LRR protein or R protein (R1 in the scheme), activating ETI. ETI is considered as an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR) which provides a complete resistance. **4)** Pathogen isolates are selected that have lost the green effector, and perhaps gained new effectors through horizontal gene flow (in purple) these can help pathogens to suppress ETI. **5)** Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI.

1.6.1.1.1 PAMP-triggered immunity (PTI)

PTI involves the recognition of conserved, indispensable microbial elicitors called pathogen-associated molecular patterns (PAMPs) by receptors anchored in the plasma-membrane and harboring extracellular PAMP recognition capability that are called pattern recognition receptors (PRRs). The activation of these PRRs results in the triggering of plant defenses which contribute to halt infection before microbe entry into plant cells. Identified PAMPs are important components that are conserved throughout classes of pathogens like: fungal ergosterol, chitin, bacterial flagellin or lipopolysaccharides. Following the same mechanism and beside PAMP produced by pathogens, degradation products of the cell wall resulting from pathogen enzymatic action can also function as endogenous elicitors called damage-associated molecular patterns (DAMPs). These DAMPs molecules released in the apoplast serve as signals to induce innate immunity similar to PAMPs.

The PRR family includes receptor-like kinases (RLK) and receptor-like proteins (RLP). RLK resides in plasma membrane and comprises a putative extracellular ligand-binding domain, a transmembrane domain and an intracellular serine/threonine kinase domain. RLs are similar to RLK but lack

intracellular activation domain, and consequently, they require interaction with adaptor molecules for signal transduction.

Upon the recognition of PAMP by PRR, a complex and still elusive series of reactions are triggered (Figure 7): (i) a rapid phosphorylation of PRR and/or associated kinase like **brassinosteroid insensitive 1** (**BRI1**)-**associated kinase** (**BAK1**), also known as SERK3, and Botrytis-induced kinase 1 (BIK1), (ii) drastic changes in fluxes of H+, K+, Cl_2 and Ca^{2+} ions across the plasma membrane which lead to the elevation of cytoplasmic Ca^{2+} concentration, (iii) changes in cytosolic concentration of Ca^{2+} which is perceived by calmodulin, calcium-dependent protein kinases (CDPKs or CPKs) which allow: (iv) activation of some transcription factors (like WRKY8 WRKY28 and WRKY48), (v) the production of reactive nitrogen intermediates (RNIs) and (vi) the production of reactive oxygen species (ROS) first in the apoplast and then within the cell, (vii) activation of two simultaneous MAPK cascades MKK4/MKK5-MPK3/MPK6 and MEKK1/MKK1/MKK2-MPK4 leading to the activation of transcription factors (like WRKY33) which then activate defense-related genes. Although the way plant manage the interaction between PRRs, calcium channels, respiratory burst oxidase homologues (Rbohs), which produce reactive oxygen species (ROS) in the apoplast, and MAPK cascades is still elusive, several lines of evidence pinpoint the primary role in these processes of heterotrimeric G proteins comprising the highly conserved WD40 repeat-containing protein: Gβ, which regulates a large number of signaling pathways in eukaryotes (Gelli et al. 1997; Zhang et al. 2012; Torres et al. 2013; Aranda-Sicilia et al. 2015; Su et al. 2015; Liang et al. 2016; Miller et al. 2016). The activation of PTI leads the biosynthesis of the stress hormones: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) which are indispensable for both local and systemic acquired resistance (Muthamilarasan and Prasad 2013). SA and JA productions take place in the

chloroplasts and result from the modification of Ca^{2+} concentrations within cells.

1.6.1.1.2 Effector-triggered immunity (ETI)

ETI, also known as R-gene-based immunity, is considered as a second line and intracellular defense able to recognize some specific molecules released by pathogens called avirulent effectors. ETI relies on effector receptors called R proteins which primarily belong to nucleotide-binding leucine-rich repeat (NB-LRR or plant NRL) protein family that are structurally similar to mammalian nucleotide-binding oligomerization domain receptors (NOD-like receptor or animal NLR) (Davis et al. 2011).

Plant NRLs recognize pathogen effectors either through direct interaction or by indirect interactions involving genuine virulence targets of the effector (the guard model) or decoy proteins that the plant has evolved to mimic its respective effector targets (the decoy model). Based on the N-terminal architecture, the NB-LRRs are divided into two subclasses: **coiled coil** (**CC**) motif and **toll/interleukin 1 receptor** (**TIR**) domain. These two subclasses are also referred as **CNLs** for CC-NLRs and **TNLs** for TIR-NLRs.

Upon direct/indirect interaction, NLRs undergo a primary conformational change which is prone to the exchange of ADP/ATP that is linked to the immune receptor. This nucleotide exchange then triggers a second conformational change enabling the NLRs' N-terminus (TIR, CC) to interact with unknown downstream targets.

Although the molecular mechanisms leading to the activation of downstream signaling by NLRs are still elusive, some studies indicate that interactions between several identical NLRs (homo-complex formation) or different NRLs (hetero-complex formation with helper NLRs) are required to trigger an immune response. Also, it has been recently hypothesized that

some NLRs could interact directly with transcription factors in the nucleus to regulate the expression of immunity-related genes (Muthamilarasan and Prasad 2013). If the first step of PTI is well localized in plasma membrane the cellular localizations of NLRs remain vague. Nevertheless, the discovery that NLR-helpers from the **Activated Disease Resistance 1** (**ADR1**) family containing the membrane targeting domain RPW8 like are required for the activation of ETI in viral, bacterial and Oomycetes infections (Cesari 2018) suggest that ETI also has a membrane initial step which could explain the similarity of downstream signaling shared with PTI.

If TNLs and CNLs require different signaling components to activate ETI, defense responses triggered by both types of NLRs have been shown to require ADR1. Interestingly, ADR1 family members are also required for basal resistance and PTI (Bonardi et al. 2011) and they show unusually phylogenetic conservation relative to other plant NLR proteins. Furthermore, transient expression of the CC domains of ADR1 and NRG1, a tobacco homolog of ADR1, induces HR in tobacco plants (Collier et al. 2011). Specificity of TNLs and CNLs signaling thus seems to rely upstream of ADR1. Most CNLs (e.g., RPM1, RPS2, RPS5) require a predicted integrin-like protein termed **Non-race specific Disease Resistance 1** (**NDR1**) (Century et al. 1995; Century et al. 1997; Innes 1998), whereas most TNLs (e.g., RPP2, RPP4, RPP5, RPP21, RPS4) require the nucleocytoplasmic lipase-like protein **Enhanced Disease Susceptibility 1** (**EDS1**) (Parker et al. 1996; Aarts et al. 1998; Wiermer et al. 2005).

Whatever these first NRL activation steps, ETI eventually triggers very similar phenomena as PTI such as: Ca^{2+} cell influx, ROS levels elevation, activation of: MAPK cascade (Thulasi Devendrakumar et al. 2018), WRKY transcription factors, biosynthesis of SA, JA, ET, pathogenesis-related (PR)

genes and cell wall strengthening. Although the immune pathway framework is shared in PTI and ETI, the activation kinetics for ROS production, Ca^{2+} spikes, and MAPK signaling are much more prolonged in ETI compared with PTI (Tsuda et al. 2010; Gao et al. 2013; Tsuda et al. 2013). Recent findings suggest that quantitative differences in the strength and duration of these pathways produce qualitatively different resistance responses and network properties (Cui et al. 2015) which could explain why ETI more frequently leads to **hypersensitive response** (**HR**) (Jones and Dangl 2006) and **systemic acquired resistance** (**SAR**) (Fu and Dong 2013) compared to PTI.

1.6.1.1.2.1 Hypersensitive response (HR)

Hypersensitive response (HR), or pathogen-triggered programmed cell death (pPCD), at the site of infection is often associated with ETI (Chiang and Coaker 2015). Despite the importance of HR in allowing plants to prevent the spread of pathogens and thus providing complete resistance (Kushalappa et al. 2016), the mechanisms leading to pPCD are still poorly understood. Considering the similarity observed between downstream pathways of PTI and ETI, it is hypothesized that HR is the direct consequence of the long-lasting increase of Ca^{2+} concentration within the cell observed in ETI (Grant et al. 2000; Ma et al. 2008; Su et al. 2018) compared to PTI (Ranf et al. 2011; Su et al. 2018). Ca^{2+} is indeed an essential and conserved second messenger in Eukaryotes which activates 6 immunity related CDPKs (CDPK1/2/4/5/6/11) required for ROS and SA production, four of which (CDPK1/2/4/5) being more specifically associated with HR (Gao et al. 2013). It is worth noting that both ROS and SA were also found to be required for HR (Zurbriggen et al. 2010, Huysmans et al. 2017). That is in contrast to JA whose synthesis is thought to also be triggered by an increase in cytoplasmic concentration of Ca^{2+} (Wasternack and Hause 2013)

but which is suppressed by SA and thus does not lead to pPCD (Van der Does et al. 2013). In fact, it was recently demonstrated that, although both JA and SA are produced during ETI, while SA is mainly found at the infection site where HR occurs, JA signaling pathway is activated in the surrounding cells (Betsuyaku et al. 2018).

Despite their central and initial roles in innate immunity and HR, Ca^{2+} flows between the apoplast, the cytosol and the different subcellular compartments, little is known about the ion channels involved in Ca^{2+} signaling related to plant immunity (Moeder et al. 2018). Nevertheless, several **glutamate receptor-like channels** (**GLRs**), especially those of clade 3, are thought to be involved in PTI and **damage-associated molecular patterns** (**DAMPs**) **triggered immunity** (**DTI**) (Kwaaitaal et al. 2011; Manzoor et al. 2013, Mousavi et al. 2013; Farmer et al. 2014; Vincent et al. 2017). Several **cyclic nucleotide-gated channels** (**CNGC**s) have also been associated with immunity. For instance, CNGC11 and CNGC12, localized in the plasma membrane, have suggested being involved in ETI (Moeder et al. 2011) while CNGC2 has been associated with PTI, DTI and ETI (Moeder et al. 2018).

On the other hand, the role of chloroplasts is thought to be central in HR too since: (i) it constitutes a very important source of defense molecules such as ROS, NOI, SA and JA, (ii) light quality and quantity determine the severity of HR which is tightly linked to the light-dependent production of ROS and SA in the chloroplast (Roden and Ingle 2009; Zurbriggen et al. 2009; Mur et al. 2010), (iii) several pathogen effectors have chloroplast localization signals (Guttman et al. 2002), and in some cases suppress immunity (Fu et al. 2007; Jelenska et al. 2007). Consequently, the

chloroplast is considered as a key regulator balancing immunity response and plant growth according to the available light energy (Stael et al. 2014) and thus providing maximum immunity, i.e. HR, at maximum light availability.

Supporting this prime role of calcium, SA and ROS in HR: a positive feedback loop between SA and ROS has been observed (Shirasu et al. 1997) and a calcium binding protein and a calcium regulated ATPase have been identified as markers of HR (Olvera-Carrillo et al. 2015).

In addition, although this phenomenon could be linked to disruption of Ca^{2+} homeostasis, **endoplasmic reticulum** (**ER**) **stress** (see next section on SAR) has been found associated with *Turnip crinkle virus* (TCV) induced HR (Moon et al. 2016).

1.6.1.1.2.2 Systemic acquired resistance (SAR)

ETI is not only involved in defense responses locally but also induces distal reinforcement of the immunity which requires accumulation of salicylic acid (SA) and is characterized by the systemic expression of a set of pathogenesis-related (PR) genes which have been classified in 17 families (Sels et al. 2008) (Figure 7) and whose secretion requires significant enhancement of endoplasmic reticulum (ER) function (Fu and Dong 2013).

This phenomenon called systemic acquired resistance (SAR) is a long-lasting and broad-spectrum induced disease resistance protecting the rest of the plant from secondary infections against pathogenic fungi, oomycetes, viruses, and bacteria. Accumulation of SA is thought to be central in SAR by inducing: (i) a fluctuation in cellular redox state which dissociates NPR1 (nonexpresser of PR genes 1) oligomers and activates subsequently TGAs transcription factors responsible for the transcription of PR genes: PR-1, PR-2, PR-5 and chitinases (Shearer et al. 2012; Fu and Dong 2013) as well as

glutathione-S-transferases (GSTs) (Mueller et al. 2008) and, (ii) a phenylalanine starvation leading to uncharged $tRNA^{phe}$ and the consequent activation of GCN2 (general control nonrepressed 2) kinase able to phosphorylate the translation initiation factor eIF2α (Fu and Dong 2013). Phosphorylated eIF2α then induces a strong reduction in global protein synthesis (Lageix et al. 2008) as well as the translation of the transcription factor TBF1 which, in turn, downregulates genes involved in chloroplast function while upregulating genes involved in defense response (Pajerowska-Mukhtar et al. 2012).

SAR has also been found associated with **ER stress** (Moreno et al. 2012) which is caused by an accumulation of misfolded and unfolded proteins in the ER lumen. ER stress activates a signaling network called the **Unfolded Protein Response** (**UPR**) to alleviate this stress and restore ER homeostasis, promoting cell survival and adaptation (Oslowski and Urano 2011). However, under unresolvable ER stress conditions, the UPR promotes apoptosis. Little is known about how ER stress is activated (So 2018) but disruption in Ca^{2+} homeostasis seems directly linked to this phenomenon (Krebs et al. 2015; Bahar et al. 2016). Remarkably, exogenous SA treatment, viral and bacterial infections were found to induce UPR (Nagashima et al. 2014; Bao and Howell 2017).

Although the nature of the mobile signal for SAR is still under discussion, it has been hypothesized that at least two mobile signals, namely methyl salicylic acid (MeSA), and a complex formed between the lipid transfer protein DIR1 and glycerolipid or lipid derivatives are responsible for the long-distance signal in SAR (Muthamilarasan and Prasad 2013).

Among the still open questions about SAR, is the molecular basis for immune memory in plants which can last for weeks to months, and possibly

even pass to the next generation. The long lasting immune memory phenomenon, often called “priming”, has been attributed to several genetic and epigenetic mechanisms. It has been demonstrated that primed plants showed enhanced MAP kinases: MPK3 and MPK6, whose transcription seemingly depends on NPR1 (Beckers et al. 2009). Several epigenetic modifications were also found correlated with SAR: (i) hypomethylation and hypermethylation in gene-rich regions, (ii) differential methylation of transposable elements, (iii) biogenesis of 21-nucleotides small interfering RNAs (siRNAs) (Dowen et al. 2012), (iv) direct and even transgerational activation of defense related genes like PR1, PR2 or WRKY6, WRKY29, WRKY53 and WRKY70 have been associated with lysine 4 of histone 3 (H3K4) trimethylation or H3K9 acetylation in the promoters of those genes (Alvarez-Venegas et al. 2007; Jaskiewicz et al. 2011; Choi et al. 2012; Luna et al. 2012). Eventually, several proteins from the DNA repair machinery (Durant et al. 2007; Wang et al. 2010; Song et al. 2011) and one protein involved in chromatin remodeling (Kang et al. 2008) were found associated with SAR.

Despite intense research, SAR signaling pathway is still elusive and many questions remain about the priming phenomenon and the transgenerational immune memory.

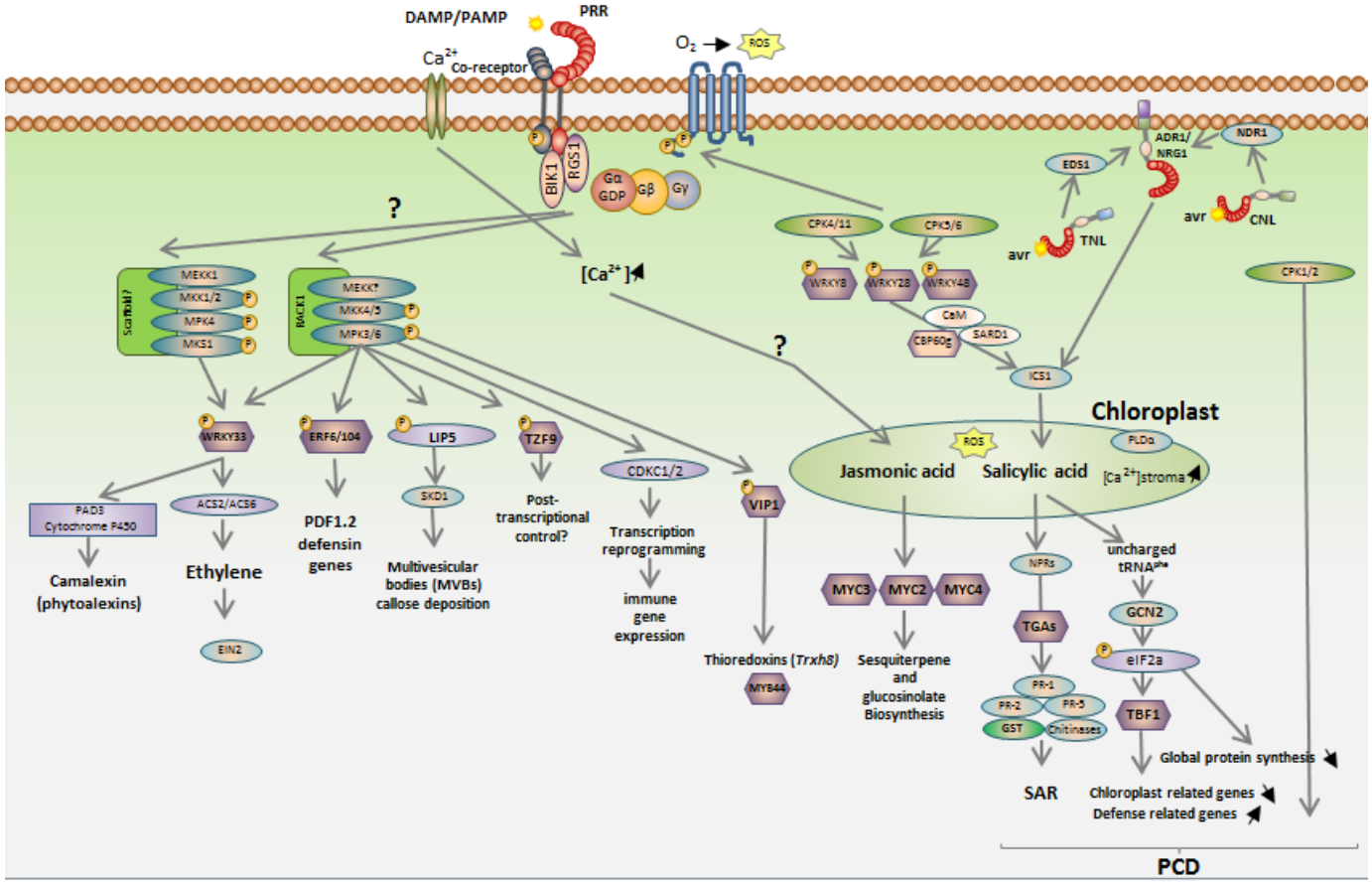

DAMP/PAMP
PRR
Ca2+
Co-receptor
O2
ROS
BIK1
RGS1
Gα GDP
Gβ
Gγ
?
[Ca2+]
?
CPK4/11
CPK5/6
WRKY8
WRKY28
WRKY48
CaM
CBP60g
SARD1
ICS1
avr
TNL
EDS1
ADR1/ NRG1
NDR1
avr
CNL
CPK1/2
Chloroplast
ROS
PLDα
Jasmonic acid
Salicylic acid
[Ca2+]stroma
MEKK1
MKK1/2
MPK4
MKS1
MEKK?
MKK4/5
MPK3/6
WRKY33
ERF6/104
LIP5
TZF9
CDKC1/2
VIP1
PAD3 Cytochrome P450
Camalexin (phytoalexins)
ACS2/ACS6
Ethylene
EIN2
PDF1.2 defensin genes
SKD1
Multivesicular bodies (MVBs) callose deposition
Post-transcriptional control?
Transcription reprogramming
immune gene expression
Thioredoxins (Trxh8)
MYB44
MYC3
MYC2
MYC4
Sesquiterpene and glucosinolate Biosynthesis
NPRs
TGAs
PR-1
PR-2
PR-5
GST
Chitinases
SAR
uncharged tRNAphe
GCN2
eIF2a
TBF1
Global protein synthesis
Chloroplast related genes
Defense related genes
PCD

Figure 7: Simplified scheme of molecular mechanisms of the two branches of plant innate immunity: PAMP/DAMP triggered immunity (PTI/DTI) and effector-triggered immunity (ETI). PAMP recognition receptor (PRR) (in red) together with its co-receptor (dark blue) detect PAMPs or DAMPs leading to the phosphorylation of their intracellular kinase domains (yellow circle with letter P) as well as associated proteins (BIK1, NGS1). These reactions induce the influx of Ca^{2+} from the apoplast in the cytoplasm. The increase of cytosolic concentration of Ca^{2+} leads to the activation of calcium-dependent protein kinases (CDPK). CDPKs allow the phosphorylation of transmembrane NADPH oxidase RBOHD (membrane blue rods) which produces reactive oxygen species (ROS) in the apoplast. The activation of RBOHD seemingly allows the dissociation of G protein heterotrimer (red, yellow and blue circles) which, in turn, activates two MAP kinase cascades (MEKK1, MKK1/2, MPK4, MKS1 and an unknown MEKK, MKK4/5, MPK3/6) cooperating in the phosphorylation of the transcription factor WKY33 leading to the production of the phytohormone ethylene and phytoalexins. The kinase cascade involving MPK3 and MPK6 also activate several transcription factors (purple hexagons) and proteins controlling the expression of immune genes like: defensins, genes involved in callose deposition… On the other hand, the increase cytosolic concentration of Ca^{2+} seemingly induces in the chloroplast: the production of jasmonic acid (JA) and, indirectly through the activation of CDPK4/5/6/11 and other calcium dependent proteins (calmodulin (CaM) and calmodulin binding protein 60g (CBP60g), the production of salicylic acid (SA). JA activates MYC transcription factors involved in the production of sesquiterpene and glucosinolate biosynthesis. SA production induces the activation of NPRs regulatory proteins which interact with TGAs transcription factor controlling the expression of PR genes that are associated with systemic acquired resistance (SAR). SA also enhances the presence of uncharged $tRNA^{phe}$ which activate the GCN2 kinase. GCN2 then activates eIF2α which induces a global reduction of protein synthesis and activate the TBF1. TBF1 represses chloroplast related genes and induces defense related genes. In ETI, defense response is triggered upon direct or indirect recognition of avirulent factors (avr) by two subclasses of NLR (TNL or CNL) (top right). TNLs seemingly depend on the nucleocytoplasmic lipase-like protein Enhanced Disease Susceptibility 1 (EDS1) while CNLs depend on an integrin-like protein termed Non-race specific Disease Resistance 1 (NDR1). Several TNLs and at least one CNL require helper CNLs from ADR1 family (ADR1 or NRG1) to induce SA production through the activation of isochorismate synthase 1 (ICS1). Together with SA production, a starvation of phenylalanine occurs which end up with the inhibition of both protein translation and chloroplast related genes. SA production and CDPK1 and 2 activation are also correlated with programmed cell death (PCD) also known as hypersensitive response. Beside SA and JA production, the chloroplasts also contribute to the production of ROS and are thought to balance immunity response and plant growth according to the available light energy.

1.6.1.2 RNA silencing

RNA silencing, also called RNA interference (RNAi), is a conserved process which allow eukaryotic organisms to repress the expression of specific genes on the basis of sequence complementarities between these genes and double-stranded RNA (dsRNA) structures that are degraded in this process. Plants use RNAi to control transposons, to exert tight control over developmental processes such as flower organ formation and leaf development and as antiviral defense. Two main types of proteins are involved in most RNAi pathways: (i) ribonuclease III-like proteins called Dicer-like (DCL) proteins which cleave dsRNA structures into 21-24-nucleotide small RNA (sRNA) duplexes and (ii) argonautes (AGO) proteins which load these sRNAs and bind messenger RNAs containing sRNA complementary sequences to induce their silencing. Small interfering RNAs (siRNAs) loaded AGO proteins are then able to trigger (i) the production of secondary sRNAs through the cooperation of RNA-Dependent RNA Polymerase 6 (RDR6), which synthesizes a complementary strand of the transcript, and DCL4 and/or DCL2 which cleave the resulting dsRNAs, (ii) the cleavage of the siRNA/transcript duplex through the action of the RNA-induced silencing complex (RISC), a phenomenon known as posttranscriptional gene silencing (PTGS) (iii) a translation inhibition due to the interaction between the RISC and ribosomes on the same transcript, or (iv) the transcriptional gene silencing (TGS) by the specific methylation of DNA cytosines and repressive histone modifications at loci presenting sequence similarities with siRNAs, a phenomenon called RNA-directed DNA methylation (RdDM) (Figure 8).

In dicots, four different DCL proteins have been described with specific roles although three of them DCL2, DCL3 and DCL4 can function redundantly as well (Fukudome and Fukuhara 2017). **DCL1** is thought to be specialized in the production of 21-nt sRNA from non-protein coding genes called microRNAs (miRNAs) which are involved in the developmental regulation of plants through PTGS. Thanks to co-factor proteins and its RNA-helicase domain, which recognizes bulges and terminal loop structures, DCL1 is able to process imperfect dsRNA structures of miRNAs. Due to its higher efficiency on longer dsRNAs (>100bp) (Nagano et al. 2014), **DCL4** is the prime producer of 21-nt sRNA from viral dsRNAs (viral replicative forms) and secondary siRNAs more commonly known as **phased, secondary, small interfering RNAs** (**phasiRNAs**). **DCL2** produces 22-nt sRNAs which are thought to serve as backup for 21-nt and 24-nt sRNAs but also trigger specifically some secondary siRNAs (Parent et al. 2015) and play an essential role in systemic PTGS (Chen et al. 2018). **DCL3** produces 24-nt sRNAs from short dsRNAs (30-40 nt) synthesized by nuclear RNA polymerase IV (Pol IV) and RNA-dependent RNA polymerase *2* (RDR2) in heterochromatic loci (Zhai et al. 2015). 24-nt siRNAs are primarily involved in TGS.

Like DCLs, AGO proteins, although they share common structural and biochemical properties, have evolved specialized and diversified functions. The number of AGO family members vary between flowering plant species and, so far, nine functional members have been identified in the plant model *Arabidopsis thaliana*: AGO1, AGO2, AGO3, AGO4, AGO5, AGO6, AGO7, AGO9 and AGO10.

Argonautes family members are usually divided in two main categories. On the one hand: AGO1, AGO2, AGO7 and AGO10 which have slicing

capabilities on target mRNAs and primarily load 21-nt sRNAs (Carbonell et al. 2012; Montgomery et al. 2008; Ji et al. 2011; Zhu et al. 2011) and, on the other hand, AGO4, AGO6 and AGO9 which are associated with RdDM and 24-nt sRNAs (Parent et al. 2012). However, several lines of evidence demonstrate that these two groups at least partially overlap as some argonautes have multiple functions. Moreover, recent studies unraveled new functions of argonautes (Figure 8). Indeed, besides their ability to induce PTGS, translation inhibition and RdDM, AGOs are also able to act as decoys of specific miRNAs and to promote their degradation (Yu et al. 2017), mediates double-strand-break repair (Wei et al., 2012) and seemingly serve as transcription activators (Liu et al. 2017).

AGO1 is one of the best-characterized AGO family members in plants. AGO1 predominantly binds miRNAs produced by DCL1 and has slicer activity that catalyzes sRNA-directed cleavage or translation inhibition of target RNAs together with RISC. The factors determining the choice between mRNA cleavage and translational inhibition by AGO1-miRNA-RISC remains elusive but the ORF target site (Iwakawa and Tomari 2013) and the growth temperature and developmental stage are thought to play pivotal roles in such choice (Von Born et al. 2018). miRNA loaded AGO1 is also able to trigger the synthesis by RDR6 of a complementary strand of the targeted mRNA and the subsequent degradation of the resulting dsRNA into phasiRNAs. Guided by miRNAs and phasiRNAs, AGO1 regulates the expression of genes that are involved in numerous developmental and physiological processes. AGO1 also functions in defense against some viruses upon loading with virus-derived siRNAs (vsiRNAs).

Arabidopsis **AGO2** plays a major role in antiviral defense and has been shown to be required for resistance to a broad spectrum of plant viruses

(Harvey et al. 2011; Jaubert et al. 2011; Wang et al. 2011b). AGO2 binds vsiRNAs targets viral RNAs for cleavage, thereby inhibiting viral replication (Carbonell et al. 2012; Schuck et al. 2013) and cooperates with AGO5 to restrict *Potato virus X* infection (Brosseau and Moffett 2015). Beside its antiviral role, AGO2 is also able to: load some specific miRNA duplexes (Zhang et al. 2014a), facilitate double strand break (DSB) repair (Wei et al. 2012) and mediate de novo DNA methylation by recruiting 21-22-nt siRNAs, which are processed from Pol II/RDR6-dependent dsRNAs by DCL2 and DCL4 (Pontier et al. 2012). AGO2 is also thought to interact with plant immunity by promoting the exocytosis of antimicrobial pathogenesis-related protein PR1 through the translation repression of the SNARE gene *MEMB12* via the binding to miR393b (Zhang et al. 2011).

The expression of Arabidopsis **AGO5** is confined to the somatic cells around megaspore mother cells and was found to be involved in the initiation of megagametogenesis (Tucker et al. 2012).

AGO7 evolved as a highly specific effector of miR390. Together they induce the degradation of TAS3 transcripts into phasiRNAs which target mRNAs encoding two AUXIN RESPONSE FACTORs (ARF3 and ARF4) to allow the proper developmental of lateral organs along the adaxial-abaxial axis (Montgomery et al. 2008).

AGO10 specifically sequesters one group of miRNA165/166 to modulate shoot apical meristem maintenance and establishment of leaf polarity by repressing miR165/166 in Arabidopsis (Liu et al., 2009). Although AGO10 is highly similar to AGO1 in their protein sequences, it differs from its paralog by its restricted expression in procambium, adaxial leaf primordia, and by the meristem and its developmental functions (Moussian et al. 1998; Lynn et al. 1999).

AGO4, AGO6, and AGO9 all bind to 24-nt sRNAs with a 5' A to mediate RdDM with partial functional redundancy and specificity (Havecker et al. 2010; Olmedo-Monfil et al. 2010; Duan et al. 2015; McCue et al. 2015). In the canonical RdDM pathway, the 24-nt siRNAs are primarily derived from the transcripts of Pol IV which serve as substrates for RDR2 (RNA-dependent RNA polymerase 2) production of dsRNAs that are subsequently cleaved by DCL3 and loaded onto AGO4, AGO6, or AGO9. These 24-nt siRNA loaded AGO then recognize nascent PolV transcripts and induce RdDM by recruiting the DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). At the corresponding loci, heterochromatin formation is thought to occur, characterized by DNA methylation induced by DRM2 and repressive histone modifications (Zilberman et al. 2003; Zheng et al. 2007; Matzke et al. 2009; Law and Jacobsen 2010; Olmedo-Monfil et al. 2010; Zhang and Zhu 2011; Pikaard et al. 2012; Matzke and Mosher 2014).

Besides the canonical RdDM pathway, another route called RDR6-RdDM has also been discovered where dsRNA are thought to be cleaved into 21-22-nt siRNAs by DCL4/DCL2 and loaded onto AGO2 (Pontier et al. 2012) and AGO6 (McCue et al. 2012). These two AGO are then able to trigger RdDM supposedly by interacting with PolV nascent transcripts.

Recently, a dicer-independent route for biogenesis of siRNAs able to direct DNA methylation has been discovered (Ye et al. 2016). In this route, Pol II and Pol IV transcripts are thought to associate directly with AGO4 and undergo 3’-5’ trimming to produce “sidRNA” for siRNAs independent of DCLs. sidRNA loaded AGO4 then target loci to direct DNA methylation.

AGO6 shows restricted expression in root and shoot growing points and in the vasculature connecting these regions (Havecker et al. 2010). AGO6 is

thought to interact directly in the Pol V-mediated step of RdDM independently of Pol IV pathway (Eun et al. 2011).

Arabidopsis and maize AGO9 are predominantly expressed in the epidermal layer of the ovule primordium where they bind transposable elements (TE) derived 24-nt sRNAs and regulate the cell fate specification by restricting multiple functional megaspore formation (Olmedo-Monfil et al. 2010). AGO9 is necessary to inactivate a significant proportion of long terminal repeat retrotransposons (LTRs) in the ovule and its predominant TE targets are located in the pericentromeric regions of all five chromosomes of *A. thaliana*, suggesting a link between the AGO9-dependent sRNA pathway and heterochromatin formation (Duran-Figueroa and Vielle-Calzada 2010).

Arabidopsis AGO1 preferentially binds miRNAs with a 5’ U, AGO2 favors siRNAs with a 5’ A, AGO5 has a bias toward sRNAs with a 5’ C, whereas AGO4, AGO6, and AGO9 all bind to 24-nt sRNAs with a 5' A (Havecker et al. 2010 ; Olmedo-Monfil et al. 2010 ; Duan et al. 2015 ; McCue et al. 2015).

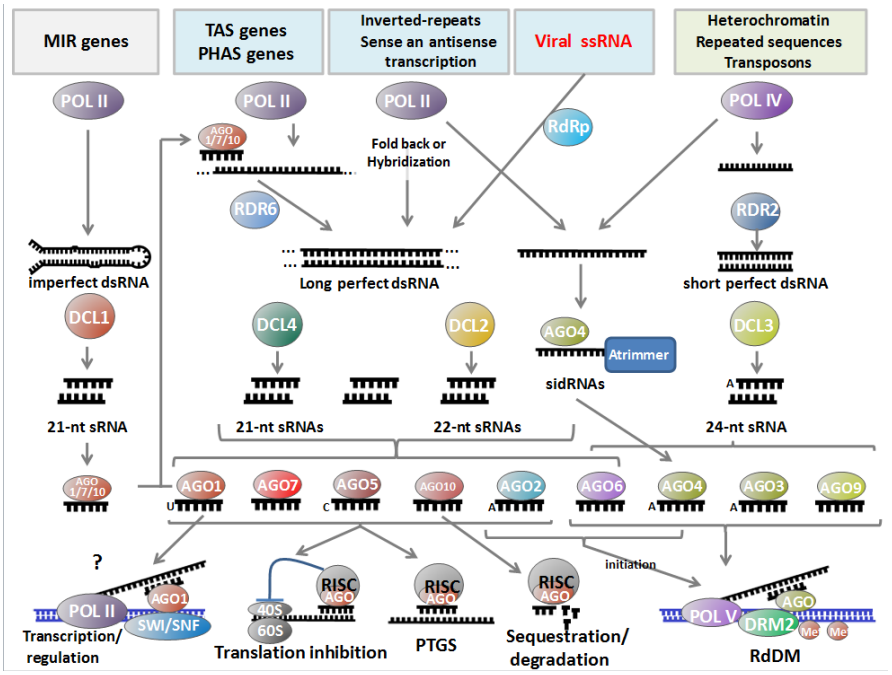


Figure 8: Five plant RNA silencing mechanisms (bottom) resulting from the degradation of endogenous and viral RNA (top) by the four different DCLs producing 21-24-nt siRNAs as well as by Atrimmer nucleases producing sidRNAs (center). Dicer-like (DCL) 1 is involved in the production, form MIR genes transcribed by the polymerase II (POL II), of 21-nt miRNAs which are methylated and primarily loaded in argonaute 1 (AGO1) to either induce translation inhibition, post-transcriptional silencing (PTGS) or the production of secondary sRNAs production which rely on the synthesis of dsRNA by RNA-DEPENDENT RNA POLYMERASE6 (RDR6) and its further degradation by DCL4 and/or DCL2. These latter DCLs also degrade dsRNA structures arising from the fold back of inverted repeat or from the hybridization of sense and antisense POL II transcripts or viral dsRNA replicative forms produced by RNA-dependent RNA polymerases (*RdRP*) from viral single-stranded (ss) RNAs. DCL4 produces primarily 21-nt sRNAs while DCL2 produces 22-nt sRNAs. These sRNAs are able to produce silencing through translation inhibition, PTGS and secondary sRNAs production (phasiRNAs). Plants are also able to produce 24-nt sRNA from short transcripts synthesized by POL IV and RDR2 and degraded by DCL3. These latter sRNAs are then able to trigger the methylation of cytosines in specific genomic regions by the cooperation of sRNA loaded argonaute 4, Pol V and de novo DNA methyltransferase DRM2. While AGO1, AGO2, AGO5, AGO7 and AGO10 are thought to be mainly involved in post-transcriptional gene silencing (PTGS) and translation inhibition, AGO3, AGO4, AGO6 and AGO9 are primarily involved in RNA-directed DNA methylation (RdDM). Additionally, AGO10 is specialized in the sequestration and degradation of specific miRNAs, AGO2 and AGO6 are able to initiate *de novo* methylation using 21-22-nt siRNA, and AGO4 is able to bind sidRNAs that are supposedly produced from RNAs transcribed by Pol II or Pol IV. These sidRNA loaded AGO4s are able to trigger the methylation of cognate genomic regions. Eventually, plant AGO1 seems able to regulate the transcription of genes.

1.6.2 Interactions between plant immunity and viral/viroidal RNAs

Although plant antiviral immunity defense has been initially excluded from the zigzag model, it is now well accepted that plant viruses are able to activate ETI through the direct or indirect interaction between viral proteins, recognized as avirulent factors, and plant NLRs. If several lines of evidence indicate that plant viruses are also able to active PTI and although PTI against viral pathogens has been described in mammalian cells, this PTI activation by plant viruses is still currently hypothetical since neither the receptor of the viral PAMP nor the viral PAMP itself have been identified (Gouveia et al. 2017). When it comes to viroids, recent studies suggest that the innate immunity is also activated but this activation needs to be confirmed and the way these pathogenic RNAs would be able to trigger ETI or PTI is unknown (Owens et al. 2012; Bagherian et al. 2016; Zheng et al. 2017; Więsyk et al. 2018). Since viroids like most of plant viruses consist of RNA structures and since RNAs are able to trigger innate immunity response in mammalian cells, the ability of these RNAs to serve as PAMPs in plants as well is plausible. Recently, this hypothesis was further supported by the finding that double-stranded RNA (dsRNA) and virus-derived dsRNA have been shown to function as viral PAMPs in Arabidopsis and to induce the PTI pathway (Niehl et al. 2016). However, the mechanisms underlying this PTI activation, which will be an important step forward for the sustainable management of viroidal/viral diseases in crops (Nicaise 2017), remains obscure. To help figuring out how virus/viroid RNAs can trigger an innate immunity response, four working models can be envisaged (Figure 9): (i) RNAs are recognized by unknown PRRs to activate PTI (Calil and Fontes 2017; Nicaise 2017), (ii) virus/viroid derived RNAs directly interact with NLRs to activate ETI, (iii) RNA silencing suppressors (RSS) (Nakahara

and Masuta 2014), RNAi machinery components or unknown plant helicases serve as RNA immune sensor and guardee which interact with NLRs to activate ETI. (iv) The fourth model rely on the ETI activation of models ii and iii : ETI activation by RNA-NLR direct or indirect interactions leads to secretion of specific molecules triggering damage-associated molecular patterns (DAMPs) release which are recognized by specific PRRs and their cognate co-receptors to activate DAMP-triggered immunity (DTI) (Calil and Fontes 2017; Nicaise 2017).

First working model: PTI triggered by an unknown dsRNA specific PRR

Eukaryotes possess two important conserved immunity protein domains: **Toll/interleukin-1 receptor** (**TIR**) domains, which function as cell signaling and are widely distributed in animals, plants and bacteria, and **leucine-rich repeat** (**LRR**) domains which are associated with the recognition of a diverse set of PAMPs in plants, invertebrates and vertebrates. Both of these domains are present in other animal transmembrane receptors called **Toll-like receptors** (**TLRs**), some of which being identified as viral nucleic acid receptors like the mammals TLR3, TLR7, TLR8 and TLR9 (Alexopoulou et al. 2001; Hemmi et al. 2002; Lund et al. 2003; Heil et al. 2004) or the Toll-7 of *Drosophila* (Nakamoto et al. 2012). These receptors function as homodimers to allow the activation of downstream signaling pathways upon nucleic acid recognition. Among these receptors, TLR3 is thought to sense long dsRNAs while TLR7 and TLR8/TLR9 need degraded ssDNA and ssDNA/ssRNA respectively to trigger an immune response (Miyake et al. 2018). Although plants PRRs contain LRR as extracellular domain, none of them has been shown to be activated by RNA so far (left side of Figure 9). However, two co-receptors usually associated with PRRs: BAK1 and **BKK1** (**BAK1-like kinase 1**) have been found to slow down the progression of

symptoms caused by RNA viruses in *Arabidopsis* (Yang et al. 2010; Kørner et al. 2013; Nicaise and Candresse 2017). Moreover, *Arabidopsis* mutants for BIK1, a kinase usually associated with PRR, showed increased symptoms in *Plum pox virus* (PPV) infection (Nicaise and Candresse 2017) and mutants *mpk6* and *mpk4* were respectively more susceptible and more resistant to PPV in accordance with the roles of MPK6 and MPK4 in innate immunity (Nicaise and Candresse 2017). However, it should be noted that BAK1, BKK1 and BIK1 are also involved in the signaling of the phytohormone brassinosteroid which could increase the susceptibility of the plant independently of the PTI pathway although the increase PPV susceptibility observed in the mutant *bak1-5* is thought to be mainly due to PTI (Nicaise and Candresse 2017).

The main weaknesses of this model relying on extracellular recognition of viral RNAs are: (i) the absence of evidence showing that viral RNAs could be found in the apoplast and (ii) the absence of plant homologs of animal nucleic acid sensing TLRs (Lewis and Obbard 2014).

Second model: direct interaction between virus/viroid RNAs with NLRs

Although, like transmembrane animal TLRs, plant ETI receptors: TIR-NBS-LRR or TNLs, contain both TIR and LRR domains, no direct interaction between these plant NLRs and viral nucleic acids have been demonstrated so far. However, beside RNA sensing animal TLRs, four intracellular immunity receptors have been found to directly interact with RNA in mammalian:

- The NLR: **nucleotide-binding oligomerization domain-containing protein 2**: **NLRC2** (also known as **NOD2**) has been shown to interact

directly with ssRNAs and activate the production of interferon β (IFN-β) that has antiviral and antiproliferative effects (Sabbah et al. 2009).

- A first helicase containing RNA receptor called **melanoma differentiation associated gene 5** (**MDA5**) (Yoneyama et al. 2005) leads to the activation of interferons α and β (IFN-α/β) and proinflammatory cytokines upon binding to long dsRNAs (Xie 2013; Reikine et al. 2014).
- A second helicase containing RNA receptor called **retinoic acid inducible gene I** (**RIG-I**) (Yoneyama et al. 2004; Yoneyama et al. 2005) leads to the same gene activation than MDA5 but seems to preferentially bind 5'-triphosphate ssRNA as well as short dsRNAs (Xie 2013; Reikine et al. 2014).
- The **protein kinase RNA-activated** (**PKR**) is activated by homodimer formation and autophosphorylation upon binding to long dsRNA (>33 bp). dsRNA-activated PKRs then catalyze the phosphorylation of eIF2α and the activation of NF-κB, leading to the general inhibition of translation as well as transcription of IFN-α/β and pro-inflammatory cytokines (Meurs et al. 1990; Williams 2001).

Despite early reports of plant PKR (pPKR) activity in virus-infected plants (Hiddinga et al. 1988; Langland et al. 1995; Chang et al. 1999), no specific kinase could be purified, and the sequence of a putative PKR ortholog is absent from plant genomes (Immanuel et al. 2012). Likewise, no homologs of RIG-1 and MDA5 receptors are known in plants. However, these two proteins contain a conserved helicase domain which is thought to have been acquired by grafting from ancestral dicer proteins to allow binding to the viral RNA (Yoneyama et al. 2004; Yoneyama et al. 2005; Sarkar et al. 2008). It is worth noting that **dicers** and their **helicase** domains have seemingly been able to sense viral infections through dsRNA binding for

more than 1.5 billion years and they have been conserved in all major eukaryotic lineages, including plants, fungi, ecdysozoa and vertebrates (Lewis and Obbard 2014). The potential role of dicer helicases as RNA immunity receptors is thus an interesting track to explore to understand RNA sensing in plant and is further developed in the third working model.

Eventually, if no NLRC2 homolog is present in plant, the fact that animal NLRs are able to directly bind RNAs through interaction with their LRR domain and trigger innate immunity suggests that plant NLR could also play the same role in plant innate immunity.

Third working model: indirect interaction between virus/viroid RNAs and NLRs

Beside direct interaction between immunity receptors and RNAs, indirect interactions are also described in mammals and could be considered in plants. For instance, the receptor **NOD-like receptor pyrin domain-containing-3**: **NLRP3** (Jensen and Thomsen 2012), has been found to interact indirectly with viral RNAs through helicases which serve as RNA sensors (Mitoma et al. 2013; Li et al. 2015) (Figure 9). Interestingly, similarly to plant NLR triggered immunity (i.e. ETI), NLRP3 can triggers a cell death known as “pyroptosis”. This inflammation-dependent cell death involves at least two separate signaling cascades: one relying on PRRs detection of PAMPs, which induce the transcription of an inflammatory response signal precursor: pro-interleukin-1β (pro-IL-1β), and the other, depending on NLRP3 itself, which leads to procaspase-1 activation of pro-IL-1β and to secretion of the latter (Li et al. 2015).

Most plant antiviral resistance genes encode typical NLR proteins which specifically recognize viral effectors similarly to what is observed for a large

spectrum of effectors produced by bacteria or fungi (Gouveia et al. 2017). N protein is a well-characterized NLRs conferring resistance against *Tobacco mosaic virus* (TMV) by directly interacting with the helicase domain (known as p50) of TMV replicase. However, N protein activation by p50 does not seem to require viral RNA (Erickson et al. 1999). Another important consideration to note about NLR protein N is its requirement for a NLR-helper: N requirement gene 1 (NRG1), of the ADR1 family (see above), to trigger resistance to TMV. Interestingly NRG1 possesses an N-terminal domain related to the membrane-targeted protein RPW8 which mediates cell death and is probably crucial for the initiation of downstream signaling (Collier et al. 2011). It could be speculated that this interaction between N protein and NRG1 with its potential membrane localization could constitute the missing connection between ETI and PTI although no evidence of this hypothesis has been provided so far (Figure 9). Yet, interaction with NRG1 and membrane PRR co-receptors like BAK1, BKK1 and BIK1 could also be proposed. However, it could be hypothesized that upon indirect or direct recognition of viral/viroidal RNA by NRL and thus ETI activation, DAMPs are released in the apoplast and, in turn, activate DTI/PTI (see fourth model).

If RNAi is considered as an independent pathway of defense beside innate immunity, many links between both systems have been uncovered when it comes to antiviral defense and RNAi components can serve as indirect activators of RNA triggered innate immunity. In plant, as no RNA immunity sensor is known so far, Nakahara and Masuta (2014) proposed a modified zigzag model dedicated to plant viruses where: dsRNAs are considered as PAMPs, Dicer-like (DCLs) proteins replaced the PTI receptors (PRRs) and **RNA silencing suppressors** (**RSS**), specific proteins produced by viruses to inhibit RNAi, substitute pathogen effectors in the traditional zigzag model. In this new model, the RSSs recognized by NB-LRR would be considered as

avirulent factors (Figure 9). The main mechanism for the RSSs appears to be binding with long dsRNA or siRNA duplexes, subsequently inhibiting siRNA biogenesis or RISC formation (Lakatos et al. 2006). Also, several RSSs have been shown to repress or interfere with AGO1: TCV CP, CMV 2b, TBSV P19, PVX P25, Polerovirus P0 and P1 of Sweet potato mild mottle virus (Nakahara, K.S.; Masuta, C. 2014). Interestingly, in many cases these suppressors of silencing also act as avirulent factors which are thought to interact indirectly with R gene products following the ‘‘guard model’’ and resulting in initiation of defense responses against the pathogen (Van der Biezen and Jones, 1998; van der Hoorn and Kamoun, 2008; Zhu et al. 2013). If this evidence demonstrates that viral RSS proteins allow the indirect detection of non-self RNA by plant NLRs, the way viroids, as non-coding RNAs, could trigger innate immunity through interaction between the RNAi machinery components and NLRs is a conundrum.

Considering the possible role of RNAi in (ds)RNA triggered innate immunity, it is worth noting that in mammals, it has been demonstrated that siRNAs are able to activate the innate immunity through PKR, RIG-I or TLR3/7/9 signaling pathways (Meng and Lu, 2017).

If helicase containing receptors like the mammals RIG-I and MDA5 are absent in plant, an interaction between plant NLRs and RNA bound dicer-like protein or argonaute could be considered to explain how viroids could initiate innate immunity. However, Niehl et al (2016) showed that dsRNA PTI elicitation, through SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), was independent of DCLs.

Fourth working model: indirect activation of PTI through DAMPs

According to this working model, the observed interaction between the viral susceptibility and some components of the PTI pathway would be explained by the fact that DAMPs are generated upon infection and consequently activate PTI. It is indeed accepted that plant viruses induce ETI which is associated with pathogenesis-related (PR) protein secretion in the apoplast and specific remodeling of the cell wall (Zavaliev et al. 2011; Stavolone and Lionetti 2017) so that apoplastic DAMPs, like PEP, could be generated (Huffaker and Ryan 2007; Kørner et al. 2013; Yamada et al. 2016). However, the way plants would sense viral infection independently of PTI or ETI and trigger the production of DAMPs in the apoplast is unlikely. A more plausible explanation would be that this DAMP triggered immunity (DTI) activation by RNA would rather constitutes an amplification of initial immunity activation, according to one of the three other working models, than an independent mechanism. This would explain why although PRR co-receptors: BAK1, BKK1 and BIK1 are not strictly required but only modulate the RNA virus susceptibility. It is also worth mentioning that the identity of these putative DAMPs are not known and that Kørner et al (2013) showed that two DAMPs receptors known to perceive the small peptide elicitors (PEPs) produced upon wounding: PEPR1 and PEPR2 are seemingly not involved in antiviral immunity (Figure 9). This RNA triggered immunity amplified by DTI would also resemble what is observed in mammals where both ETI and PTI induce the production of signal molecules (IFNs and ILs) which are released from the infected cell to trigger immunity response through transmembrane receptor binding (Figure 9).

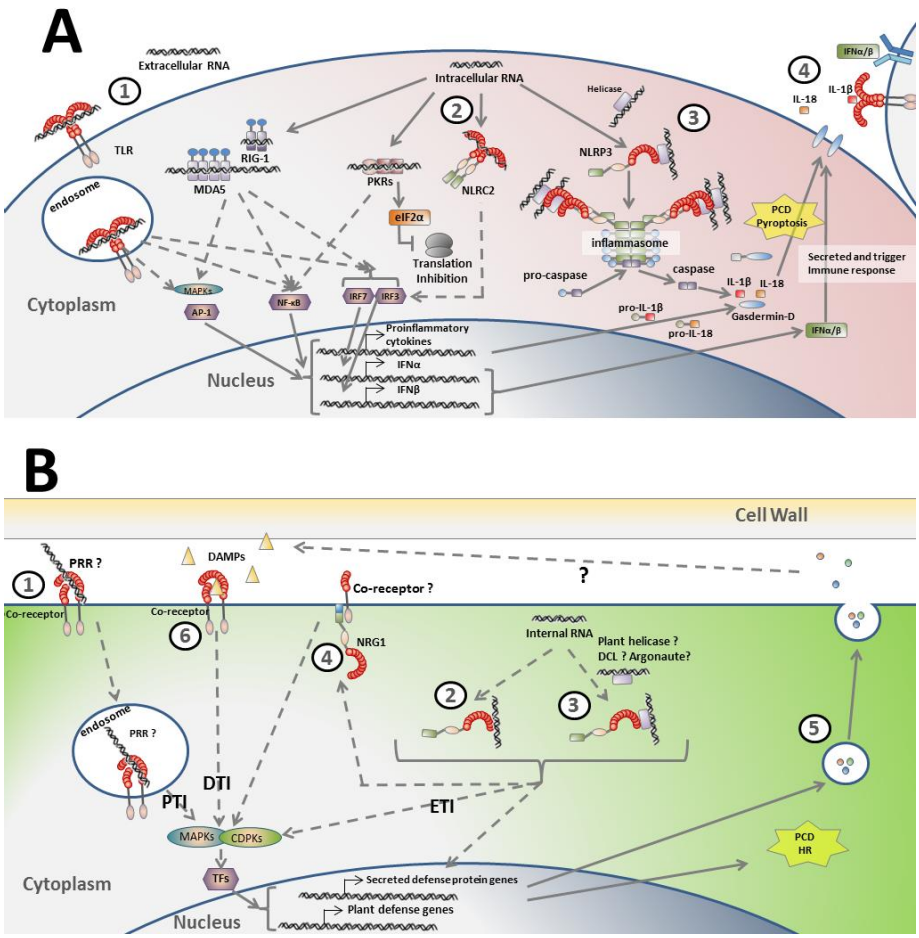


Figure 9: Comparison between human and plant immunity detection of non-self RNAs. **A)** Three fundamental mechanisms through which the human innate immunity detects RNA. **1)** Extracellular RNAs are detected by dimers of plasma membrane or endosomal toll-like receptors (TLRs) through binding to their leucine rich repeat (LRR) domains. RNA activated TLRs then trigger a cascade of reactions ending up with the induction of interferons α and β (IFNα/β) as well as the production of pro-interleukins 1β and 18 (pro-IL-1β/18) which lead to inflammatory response. **2)** Intracellular RNAs directly bind to receptors: (i) helicases containing RNA (MDA5 and RIG-1), (ii) nucleotide binding oligomerization domain containing 2: NLRC2 (NOD2) and induce through a cascade of reactions the upregulation of IFNα/β and pro-IL-1β/18 or IFNβ respectively or (iii) PKRs dimerize upon dsRNA binding and induce IFN production and general translation inhibition through eIF2α phosphorylation. **3)** Intracellular RNAs indirectly bind to NOD-like receptor pyrin domain-containing-3: NLRP3 through the

interaction with human helicases. This interaction allows the oligomerization of NLRP3s which recruits apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) to form the inflammasome. Inflammasome allows the activation of caspase-1 which, in turn, generates active IL-1β/18 and gasdermin-D which trigger a programmed cell death (PCD) called pyroptosis and the secretion of IL-1β/18 leading to inflammatory response. **B)** In plants, how innate immunity is activated by RNA is not known and four models are proposed based on the knowledge of the human innate immunity. **1)** Extracellular pattern recognition receptors (PRRs) detect RNAs and activate co-receptors (BAK1, BKK1 or BIK1) which lead to pattern-triggered immunity (PTI) relying on map kinases cascades (MAPKs), calcium-dependent protein kinases (CDPKs) and transcription factors (TFs). **2)** Unknown NLRs directly detect intracellular RNAs and trigger effector-triggered immunity (ETI) which potentially shares pathways with PTI. **3)** Dicer-like, argonautes, unknown plant helicases serve as guardee in the indirect interaction with NLRs which trigger ETI. **4)** Direct or indirect detection of RNA by NLRs can lead to the activation of plasma membrane related NLR like N requirement gene 1 (NRG1) potentially interacting with PRR co-receptors and subsequently leading to PTI. **5)** ETI activation by RNA-NLR interaction leads to: (i) programmed cell death (PCD) also called hypersensitive response (HR) and (ii) secretion of specific molecules triggering damage-associated molecular patterns (DAMPs) which are recognized by **6)** specific PRRs and their cognate co-receptors to activate DAMP-triggered immunity (DTI).

1.6.3 Interactions between plant RNAi machinery and viruses and viroids

It is now well established that viroid and virus RNA structures are recognized by host ribonuclease III-like proteins, Dicer-like (DCL), and cleaved into 21-24-nucleotides vd-sRNAs (Katsarou et al. 2016; Wang et al. 2012). However, while it is well established that DCL4 and DCL2 play a pivotal role in antiviral defense (Bouché et al. 2006; Garcia-Ruiz et al. 2010), less is known about the specific involvement of DCL proteins in antiviroidal defense. The advent of next-generation sequencing technologies showed that viroid-derived sRNAs accumulate following patterns consisting of variable peaks and valleys along each strand (Bolduc et al. 2010, Diermann et al. 2010, Wang et al. 2011a) but the identity of DCLs involved, the cellular localization of cleavages and the exact viroid RNA substrates of these DCLs are still elusive. Also, the RISC-mediated degradation of viroids and the identity of argonautes involved in this process are still under debate since discrepancies persist on this issue.

When it comes to DCL degradation of viroids, first studies concluded that pospiviroid derived sRNAs were generated mostly from the secondary structure of (+)-strands based on the fact that vd-sRNAs clustered in the two terminal regions of these secondary structures (Itaya et al. 2007; Martín et al. 2007; Machida et al. 2008). These findings thus support that pospiviroids are mainly cleaved by DCL1 in a miRNA-like degradation. This "DCL1" hypothesis was further supported by *in silico* predictions showing that secondary structures of portions and full-length PSTVd genome are consistent with pre-miRNA structures. However, another hypothesis has also emerged suggesting that (+) linear monomeric pospiviroid-RNAs

generated in an alternative pathway during replication are recognized, in a similar way to (pre)tasi-like RNAs, by RDR6, possibly due to the lack of 5'cap or 3' poly(A)-tail, and are used as templates to synthesize phased (vd)dsRNAs that are then incorporated into conventional ta-siRNA biogenesis which involves DCL4 (Gómez et al. 2009). This latter hypothesis is supported by: (i) the structural similarities observed between the nuclear replication of *Pospiviroidae* and ta-siRNA biogenesis and (ii) the dependence of hop stunt viroid-induced symptoms in *Nicotiana benthamiana* on the expression of RDR6 (Gómez et al. 2008). However, it cannot be ruled out that this RDR6/symptom correlation is due to the involvement of RDR6 in the degradation of mRNA targets of vd-sRNAs rather than in the generation of vd-sRNAs. To understand the degradation of pospiviroids, it is also worth mentioning that a significant amount of PSTVd subgenomic copies were found in infected Solanaceae (Minoia et al. 2015).

Recently, a combined activity of DCL2 and DCL3 has been reported to be critical in defending plants from viroid infection since double knock-down *Nicotiana benthamiana* DCL2/DCL3 presented both a reduced production of vd-sRNA and a higher PSTVd titer (Katsarou et al. 2016). Conversely, downregulation of DCL1 and DCL4 transcripts both reduce the titer of PSTVd in *N. benthamiana* plants (Dadami et al. 2013). Since direct biochemical evidence of viroid RNA degradation by specific plant DCLs is lacking and considering that DCLs could also play a pivotal role in the activation or regulation of plant defenses, independently of viroid degradation, the identity of DCLs involved in the degradation of viroids and especially the contribution of DCL1 and DCL4 which both produce 21-nt vd-sRNA remain to be established.

Beside the DCL degradation, it has been hypothesized that the conjugate action of RISC and vd-sRNA loaded argonautes might further degrade viroidal RNAs. If it was first thought that viroids were significantly resistant to RISC-mediated degradation due to their secondary structures (Wang et al. 2004; Gómez and Pallás, V. 2007; Itaya et al. 2007), several lines of evidence indicate that viroids are indeed RISC sensitive (Vogt et al. 2004; Carbonell et al. 2008; Schwind et al. 2009; Serra et al. 2014). In agreement with this latter assumption, Schuck et al (2013) showed that imperfect dsRNA structures can undergo RISC-mediated cleavages which preferentially occur in single-stranded or weakly structured regions known as bulges (Schuck et al. 2013).

Out of the ten different AGO proteins found in plants, AGO1 and AGO2 are known to play a major role in antiviral defense while AGO5, AGO7 and AGO10 can play an additional minor role (Morel et al. 2002; Qu et al. 2008; Takeda et al. 2008; Harvey et al. 2011; Jaubert et al. 2011; Wang et al. 2011b; Schuck et al. 2013). AGO4 has also been found to be involved in antiviral defense against RNA and DNA viruses but through the perturbation of DNA methylation processes (Carbonell and Carrington 2015). For viroids, little is known about the involvement of AGO in their degradation. However, a recent publication showed that viroid derived sRNA are loaded in AGO1, AGO2, AGO3, AGO4, AGO5 and AGO9 and that the overexpression of AGO1, AGO2, AGO4 and AGO5 in infected plants drives a decrease in PSTVd levels, suggesting a targeting of the viroid by vd-sRNAs (Minoia et al. 2014).

If it is well established that viruses have evolved suppressors of gene silencing to allow the successful colonization of their host (Burgyán and Havelda 2011), little is known regarding the strategy of viroid to defeat the

RNAi machinery. Recently, it has been hypothesized that, if viroids are obviously targeted by RNAi, they could also possibly take advantage of RNAi degradation to improve their trafficking by reducing the deposition of callose at plasmodesmata (Adkar-Purushothama et al. 2015).

2 Objectives

Amongst known infectious pathogens, viroids initially discovered by Diener (1971) and subsequently recognized as subviral agents are amongst the most surprising ones. Yet if large progress has been made, there is still a lot of remaining knowledge gaps regarding viroids. As stated in the introduction chapter, a prerequisite to address many fundamental and practical research questions related to pospiviroids is the use of accurate and cost-effective detection and identification methods that are essential to gain a correct overview of pospiviroid distribution within plants, across taxonomic range of hosts and geographic areas. Considering that accurate diagnostics methods are the cornerstone of phytosanitary risk assessments, the first contributions of this thesis were formulated into three applied objectives related to pospiviroid detection and identification:

- Set a one-step and unique RT-PCR generic detection method for the detection of pospiviroids complying with the standard ISO 17025 and meeting the needs of routine European laboratories.
- Compare this generic method to existing detection methods through an inter-laboratory comparison.
- Consider the theoretical potential of HTS as a universal virus/viroid detection technology with regards to pospiviroids detection.

Then, considering that the implementation costs of HTS are not currently competitive for the sole purpose to detect pospiviroids in routine, in the framework of this work, HTS was rather used to characterized the molecular mechanisms of symptom induction of a severe isolate of CEVd collected in Belgium on jasmine nightshade (*Solanum laxum*). The HTS

characterization of a CEVd-tomato pathosystem was used with the goal of answering some of the many fundamental questions contextualized in the introduction chapter that underlie unsolved practical issues that are essential for phytosanitary risk assessment. Also, as one of the simplest, this pathosystem was used to study the activation and the possible interactions between the main defense pathways: PR proteins production, SAR, innate immunity (DTI, PTI and ETI) and RNAi. Regarding these issues, the following research questions were addressed:

- How pospiviroids induce symptoms on tomato?
- How pospiviroids succeed in multiplying and invading plants while they are degraded by RNAi?
- Is RNAi only detrimental to pospiviroids or is there also a benefit resulting from PTGS inactivation of plant defense by viroid derived siRNAs?
- What are the respective roles of DCLs and RISC in pospiviroid degradation?
- Is the innate immunity activated upon pospiviroid infection? If so, how are pospiviroids perceived by the innate immunity and how do they react?
- What are the impacts of RNAi and potential immunity response on the spatial distribution of pospiviroids within plants? What are their consequences in terms of pospiviroid transmission risks and sanitation?

In the light of generated information, the pathogen status of viroids will be questioned and possible consequences in terms of pospiviroid-plant coevolution will be discussed.

3 Generic detection and identification of pospiviroids

3.1 Abstract:

A multiplex one-step RT-PCR aiming at detecting all pospiviroids known to be harmful to cultivated plants has been developed. Specificity, sensitivity, selectivity, repeatability and reproducibility of this test have been assessed in order to fulfill the recommendations of the EPPO standard PM7/98 and provide routine detection laboratories with a cost-effective, easy-to-use and robust pospiviroid detection test. To further understand the epidemiology and ease the management of pospiviroid outbreaks, this RT-PCR diagnostic test can be followed by direct sequencing of the amplicons to identify and characterize the detected pospiviroid isolates.

Thibaut Olivier, Elisabeth Demonty, Frédéric Fauche, Stéphan Steyer. Generic detection and identification of pospiviroids. Archive of Virology 2014, 159(8):2097-102. DOI: 10.1007/s00705-014-1978-6

3.2 Introduction

Although *Potato spindle tuber viroid* (PSTVd), *Chrysanthemum stunt viroid* (CSVd) and *Citrus exocortis viroid* (CEVd) have been known for more than 60 years for their impact on potato, chrysanthemum and citrus, respectively, new pospiviroids have been identified in damaged tomato crops and ornamentals since the 1980s. So far, the genus Pospiviroid comprises ten species, nine of which include viroids that are harmful to tomato and are potentially harmful to potato and sweet pepper: *Tomato planta macho viroid* (TPMVd); *Mexican papita viroid* (MPVd), whose members are closely related to the TPMVd (Verhoeven et al. 2011), *Pepper chat fruit viroid*

(PCFVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Iresine viroid 1* (IrVd-1), *Tomato apical stunt viroid* (TASVd), *Columnea latent viroid* (CLVd), *Potato spindle tuber viroid* , *Chrysanthemum stunt viroid* and *Citrus exocortis viroid* (Owens et al. 2011). IrVd-1, although sharing sequence similarities with the other pospiviroids, is not known to infect tomato (Spieker, 1996a). While three methods for detecting pospiviroids already exist (Monger et al. 2010; Torchetti et al. 2012; Botermans et al. 2013), none of them allows a sensitive, specific and selective detection of all pospiviroids in one reaction that can be directly followed by sequencing identification. In the context of the highly globalized trade of plant material, and thus spread of plant diseases, reliable tests are needed to avoid misleading results and potentially high economic losses. The accreditation process following the ISO/IEC standard 17025:2005 should be the method of choice to address this problem of reliability. However, the requirements concerning validation in this standard can be prone to interpretation. The EPPO pest management standard PM7/98 (EPPO, 2010a) tries to solve this problem by specifying the requirements for plant disease and pest diagnostics. Along with the PM 7/76 (EPPO, 2010b), these standards define, and focus on, four main and one minor performance criteria: analytical specificity, analytical sensitivity, repeatability, reproducibility and selectivity, respectively. In order to allow a reliable and cost-effective detection and identification of all harmful pospiviroids, a multiplex one-step RT-PCR pospiviroid detection assay that can be coupled with identification by direct sequencing was developed and validated according to the EPPO standards.

3.3 Materials and methods

To detect as many strains as possible of the 10 species of the genus Pospiviroid, and to allow their identification by direct sequencing of the

amplicons, a set of existing primers was compared to an alignment of 737 publicly available pospiviroid sequences constructed using BioEdit software (Hall, 1999). Out of this set of primers, three: Pospi1-FW, Pospi1-RE (Verhoeven et al. 2004) and pCLV4 (Spieker, 1996b) were selected and further improved. The analytical specificity of the adapted set of three primers Pospi1deg-FW (5'-GGGAKCCCCGGGGMAAC-3'), Pospi1s-RE (5'-TCAGTTGTWTCCACCGGGT-3'), and pCLV4s (5'-GGGGCTCCTGAGACCGCTC-3') was then studied in silico and experimentally. For the bioinformatics assessment of the analytical specificity, two theoretical threshold criteria were chosen and applied at the same time to calculate the percentage of recognized isolates among targeted pospiviroid sequences as well as non-targeted members of other genera of the family Pospiviroidae. These criteria were as follows: no more than three mismatches between the primer and the targeted viroid region and no mismatch in the last three 3'-end nucleotides of the primer. The analysis showed that almost all strains of all pospiviroid species should be detected (Table 2).

Table 2: Bioinformatic analysis of selectivity. The percentages of isolates for each pospiviroid species fulfilling the two criteria are shown for each of the three primers. The number of sequences used for this analysis is given in the last column.

Target	Pospi1deg-FW	Pospi1s-RE	pCLV4s	Number of sequences
CEVd	97	98		223
CLVd	100		100	78
CSVd	99	100		78
IrVd	100	100		6
PCFVd	100	100		37
PMTVd/MPVd	100	100		14
PSTVd	99	99		250
TASVd	96	100		24
TCDVd	100	100		16

Among the closely related pathogens, only hop latent viroid (HLVd), a member of the genus Cocadviroid, could also be recognized by the primer pair Pospi1deg-FW/Pospi1s-RE in silico. However, as hop is the only known host of HLVd, this potential lack of specificity is not problematic for pospiviroid detection in the matrices tested here. This theoretical pre-assessment was then confirmed experimentally using 18 isolates expected to be detected by Pospi1deg-FW/Pospi1s-RE (Table 3) and two isolates of CLVd (accession number FM995506 and AY3732392) detected by Pospi1deg-FW/pCLV4s. Total RNA was extracted either from 0.1 g of fresh plant leaf tissues or 0.01 to 0.04 g of freeze-dried material using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA extracts were then eluted in a final volume of 50 µL of elution buffer. All nucleic acid extracts were tested by RT-PCR for the presence of pospiviroid RNA using the set of three primers described above. One-step RT-PCR was performed using the kit Titan One Tube RT-PCR (Roche) in a reaction volume of 25 µL following the manufacturer's instructions, with 1 µL total RNA extract. The Pospi1deg-FW, Pospi1s-RE and pCLV4s primers were used at a final concentration of 240 nM. A reverse transcription step was performed at 50 °C for 30 min and terminated at 94 °C for 2 min. PCR amplification immediately followed, with 39 cycles at 94 °C for 30 s, 60 °C for 45 s and 72 °C for 45 s; a final extension of 2 min was performed at 72 °C, and the reactions were then held at room temperature. PCR products were separated by 2 % agarose gel electrophoresis and visualized using GelRed staining on a transilluminator under ultraviolet light. RT- PCR generated two different sizes of amplicons: one of about 195 bp for CEVd, CSVd, IrVd-1, PCFVd, TASVd, TCDVd, TPMVd, MPVd and PSTVd; and one of around 370 bp for CLVd. Prior to the electrophoresis, a 5 µL aliquot of each amplicon was diluted in MilliQ water from 1- to 13-fold to

follow the sequencing recommendations. The sequencing was carried out either by Beckman Coulter Genomics (Bishop's Stortford, United Kingdom) or by Macrogen (Amsterdam, The Netherlands). To check the selectivity, i.e., to avoid nonspecific amplification of plant nucleic acids, two separate experiments using different matrices known to be frequently infected by the targeted pospiviroids were carried out. These matrices were leaves of Solanum lycopersicum, Citrus sp., Petunia sp., Chrysanthemum sp., Solanum jasminoides and Brugmansia sp. These tests showed that no nonspecific bands were observed with the most relevant host species usually affected by pospiviroid infections and that the different matrices used do not affect the RT-PCR (Figure 10).

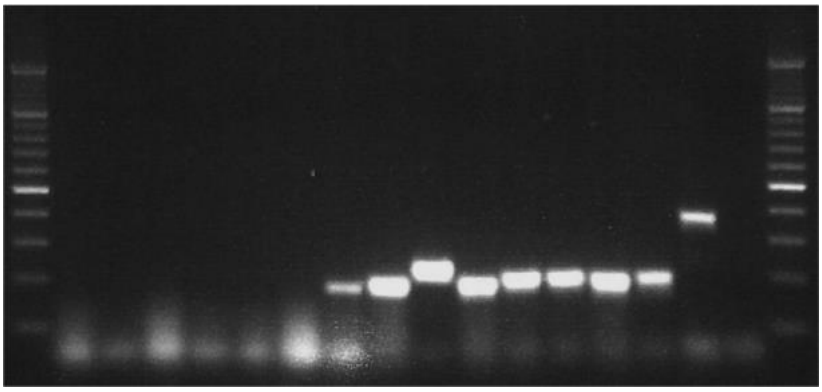


Figure 10: Test of specificity and selectivity with uninfected plants of different species and pospiviroid-infected plants. Uninfected controls, lanes 2 to 7: Solanum lycopersicum, Citrus sp., Petunia sp., Chrysanthemum sp., S. jasminoides, Brugmansia sp. Infected controls, lanes 8 to 16: PCFVd, TPMVd, IrVd-1, TASVd, CEVd, TCDVd, PSTVd, CSVd, CLVd. Water control, lane 17. 100-bp molecular marker, lanes 1 and 18.

Concerning the analytical sensitivity, since the absolute concentration of pospiviroids is not easily determined, relative concentrations obtained by dilutions of naturally infected plant or artificially infected tomatoes/potatoes were used. As two different primer pairs and ten

pospiviroids are involved in the test, this performance criterion was assessed using targets for each primer pair and members of the maximum number of pospiviroid species. The dilutions used were 1-, 10-, 20-, 100-, and 250-fold for CLVd and 1-, 10-, 50-, 100-, 250-, and 500-fold for the other pospiviroids. These preliminary data on analytical sensitivity were further studied during repeatability and reproducibility tests with members of two species amplified by the two different primer pairs, namely PSTVd (accession no. FM998542) and CLVd (accession no. FM995506). The repeatability and reproducibility were checked in blind tests with different dilutions (10-, 100-, 250-, 500-, and 1000-fold dilutions) prepared by diluting RNA extracts from infected tomato plants in an RNA extract from uninfected tomato plants. For reproducibility, tests were carried out at different times, on two thermocycler machines (a calibrated Eppendorf Mastercycler ep gradient and a BIORAD CFX-96) and by two different operators. The assessment of the analytical sensitivity showed a difference in relative detection limits between members of different pospiviroid species, and especially between pospiviroids detected by Pospi1deg-FW/Pospi1s-RE on the one hand and Pospi1deg-FW/pCLV4s on the other hand. According to the compilation of data coming from the tests performed on PSTVd and CLVd (analytical sensitivity, repeatability and reproducibility), the relative detection limit on fresh leaves is estimated under our lab conditions to be 250-fold dilution for PSTVd and 100-fold for CLVd, for which 100 % repeatability and reproducibility (neither false positive nor false negative) were observed. For the other pospiviroids analyzed on their original hosts, detection at 250-fold dilution was possible (Table 3). Although the detection of some pospiviroid infections was still possible below these thresholds, at least up to a 1000-fold dilution for PSTVd, the probability of detection was significantly affected especially for

CLVd. Among the variables studied in the tests of repeatability and reproducibility, multivariate logistic regression, computed using the software R version 2.15.3, showed that operator ($p = 0.02$), pospiviroid species ($p = 0.00$) and dilution ($p = 0.00$) had a significant impact on the results. Neither the thermocycler used nor the date of extraction had a significant influence.

Table 3. Comparison of sensitivity between multiplex and Pospi1-FW/Pospi1-RE uniplex RT-PCR for different dilutions of different pospiviroids

Pospiviroid species	Sequence accession number	Isolate identification number	Species/matrix	Verhoeven	Olivier	Verhoeven	Olivier	Verhoeven	Olivier	Verhoeven	Olivier	Verhoeven	Olivier	Verhoeven	Olivier	Verhoeven	Olivier
				1x	1x	10x	10x	50x	50x	100x	100x	250x	250x	500x	500x	1000x	1000x
CEVd	HG739082	10/0429/VI.2	Solanum jasminoides [a]	++	++			++	++	++	++	++	+	++	+	++	+/-
CEVd	HG739076	11/0171/VI.7	Solanum jasminoides [a]	++	++			++	++	++	++	++	++	++	+	++	-
CEVd	HG739074	08/0250/VI.1	Solanum jasminoides [a]	++	++												
CEVd	HG739073	11/0240/VI.5	Solanum jasminoides [a]	++	++												
CSVd	HG739079	12/0078/VI.2	Argyranthemum frutescens [a]	++				++	++	++	++	++	+	++	+	++	+/-
CSVd	HG739078	10/0809/VI.11	Chrysanthemum L. cv Maribelle Red [b]	++	++												
CSVd	HG739077	10/0809/VI.12	Chrysanthemum L. cv Maribelle Mauve [b]	++	++												
CSVd	HG739080	10/0160/VI	Solanum jasminoides [a]	++	++												
IrVd	HG739075	12/0463/VI.3	Celosia sp.	++	++	++	++	++	++	++	++	++	-	+	-		
MPVd	HG739081	12/0463/VI.4	Solanum lycopersicum [b]	++	++	++	++	++	++	++	++	++	++	+	++		
PCFVd	FJ409044	12/0463/VI.1	Solanum lycopersicum [b]	++	++	++	++	++	++	++	++	++	++	++	++		
PSTVd	FM998542	13/0022/RVI.1	Solanum lycopersicum [b]	++			++				++		++		++		+
PSTVd	AY372400	13/0399/VI.2	Solanum tuberosum [c]	++	++												
PSTVd	X17268	13/0399/VI.3	Solanum tuberosum [c]	++	++												
TASVd	HG739072	11/0238/VI.1	Solanum rantonetti [a]	++	++			++	++	++	++	++	++	++	++	++	++
TASVd	HG739071	11/0197/VI.2	Solanum jasminoides [a]	++	++												
TASVd		11/0240/VI.9	Solanum jasminoides [a]	++	++												
TCDVd	HG739070	10/0326/VI.12	Petunia sp. Surfinia [b]	++	++			++	++	++	++	++	++			++	+/-

a freeze-dried leaves ; b fresh leaves ; c freeze-dried tuber++ intense band ; + less intense band ; +/- very faint band; - absence of band; absence of sign means non tested

To compare the one-step multiplex PCR protocol described here with three common alternative uniplex protocols based on VidFW/RE (Verhoeven et al. 2004) and pCLV4/R for CLVd and Pospi1-FW/RE for the nine other species, comparative tests of analytical specificity and analytical sensitivity were performed with at least one member of each species and for the dilutions previously described. These multiplex/uniplex comparisons showed that RT-PCR based on Pospi1-FW/RE and pCLV4/R primer pairs were able to amplify all of the isolates tested in this study with a good analytical sensitivity. The VidFW/RE primer pair, however, failed to amplify one of the isolates of CLVd (accession no. FM995506) due to mismatches between VidFW and the isolate RNA/cDNA sequence, and this assay was less sensitive than the multiplex RT-PCR for the second isolates of CLVd used in this study. In order to check the ability to discriminate pospiviroids of different species by analyzing the amplicon sequences generated in the one-step generic RT-PCR proposed here, and because BLAST analysis against reference sequences is routinely used in diagnostic laboratories to identify species, an evaluation of this identification method using the PCR product sequences was carried out. This assessment was based on e-values computed by NCBI BLASTN 2.2.28+ (Altschul et al. 1990), which allow the quality of the identification to be quantified as the ratio of the correct-alignment e-value divided by the best wrong-alignment e-value. To this end, first, sections of the ten pospiviroid reference sequences corresponding to the internal part of the Pospi1deg-FW/Pospi1s-RE and Pospi1deg-FW/pCLV4s- RE amplicons were subjected to BLAST search against the NCBI reference sequences for viroids, and the e-value ratios of the first- and the second-best alignments were computed. Second, the Pospi1deg-FW/Pospi1s-RE and Pospi1deg-FW/pCLV4s sequences obtained from the isolates used in this study were also subjected to BLAST search

and compared with the identification based on the entire genome. Direct sequencing of amplicons of both primer pairs gave good-quality sequences, which allowed the correct identification of the detected viroids by BLAST analysis of the consensus amplicon sequences against NCBI viroid reference sequences. The e-value ratios obtained with reference sequences as well as with sequences generated in this validation indicate that the use of our primers in RT- PCR allows the identification of pospiviroids even with the half-genome amplified by Pospi1deg-FW/Pospi1s-RE.

These identifications were confirmed by full genome sequencing using a set of available primers (Önelge 1997; Shamloul et al. 1997; Chung et al. 2001; Verhoeven et al. 2009; Verhoeven et al. 2010). Although direct sequencing of the generated amplicons is a reliable tool for identifying pospiviroids and confirming the infection of a sample, a sequence-based identification can be complicated in the case of mixed infections by different pospiviroids. A lack of sequence quality should then attract the attention of the diagnostician, who should suspect a mixed infection and focus on regulated species by applying a more specific test, such as the protocol of Shamloul et al. 1997, combined with sequencing or the protocol of EPPO for the detection of CSVd (EPPO, 2002). Although a sensitive method based on real-time RT- PCR aiming at detecting all harmful pospiviroids has been published recently (Botermans et al. 2013), this technique has the drawback that it requires a series of other real-time RT-PCR experiments for the confirmation of the first analysis and for the identification of the detected viroid with a similar analytical sensitivity. The need for a real-time thermocycler and a renewed stock of TaqMan probes could be too expensive for many small labs. An alternative solution is to use conventional RT-PCR despite its lower analytical sensitivity as compared to the real-time technology. For routine detection, labs often combine two

individual (RT-)PCRs: one with a generic primer pair, Pospi1-FW/Pospi1-RE or Vir 1/Vir 2 (EPPO, 2002), and one with the primer pair VidFW/VidRE to allow the detection of as many pospiviroids as possible. However, in addition to requiring two (RT-)PCRs, some isolates can be missed. Tests indeed showed that at least one isolate of CLVd (GenBank accession no. FM995506) (Steyer et al. 2010) is not recognized by the VidFW/VidRE primer pair. The analytical sensitivity of the test proposed here is up to 10,000 times lower than the one proposed by Botermans et al. (Botermans et al. 2013). However, the relative limit of detection of a 100-fold dilution, which allowed 100 % repeatability and reproducibility of the present test, is sufficient in most routine analyses in which fewer than 100 samples are pooled together. Although the goal of this study was to allow the labs that are willing to use the present method under accreditation after verification rather than full validation, care should be taken to redefine the relative limit of detection under their own lab conditions. This assessment should be carried out at least for CLVd, for which the least sensitive results were obtained, with the relative limit of detection being about a 100-fold dilution. The new combination of adapted primers proposed here potentially provides routine quarantine labs with a simple, cost-effective, and robust detection of a large number of strains of all known pospiviroids as well as their correct identification by direct sequencing. This technique is thus suitable for routine analysis of samples, pospiviroid identification, and the follow-up of pospiviroid outbreaks.

4 Inter-laboratory comparison of four RT-PCR based methods for the generic detection of pospiviroids in tomato leaves and seeds

4.1 Abstract

Four pospiviroid detection methods consisting of a pair of RT-PCR (ANSES 1-2), a pair of real time RT-PCR (Botermans 1-2) and two single RTPCR methods (Luigi and Olivier) were evaluated for their relative accuracy, diagnostic sensitivity, diagnostic specificity, analytical sensitivity and reproducibility through a comparison in eight laboratories. All detection methods were tested on 13 tomato leaf and nine tomato seed samples as well as on 100-fold dilutions of the corresponding RNA extracts. Two different RNA extraction kits and three combinations of RNA extraction and RT-PCR kits were assessed. According to the statistical analysis of the results, ANSES 1-2 method provided the best performance criteria whatever the matrix consisted of, while the relative accuracies of Botermans 1-2 and Olivier methods were not significantly different from ANSES 1-2 for seed and leaf samples respectively. The results also showed that the relative accuracy of the Luigi and Olivier methods significantly increased when primer concentrations were raised to 1 µM, the latter method giving the best relative accuracy of the test in these conditions. No statistical differences were observed between accuracies obtained for the two extraction kits or the three combinations of extraction and RTPCR kits used. ANSES 1-2 method is proposed as international standard for the generic detection of pospiviroids on tomato leaves and seeds.

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Marta Luigi, Helena Lasner, Irena Mavrič-Pleško. 2016. Inter-laboratory comparison of four RT-PCR based methods for the generic detection of pospiviroids in tomato leaves and seeds. Eur J Plant Pathol 144: 645. DOI: 10.1007/s10658-015-0803-8

4.2 Introduction

The genus Pospiviroid comprises nine species which remain latent on numerous ornamental plants. Eight of them can cause damage on citrus trees, tomato, sweet pepper, potato or chrysanthemum. *Potato spindle tuber viroid* (PSTVd) and *Chrysanthemum stunt viroid* (CSVd) are quarantine organisms in many countries (EPPO 2019). Therefore and because of the outbreaks of *Tomato planta macho viroid* (TPMVd), *Tomato apical stunt viroid* (TASVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Columnea latent viroid* (CLVd) and *Citrus exocortis viroid* (CEVd) reported in tomato crops and the outbreaks of *Pepper chat fruit viroid* (PCFVd) reported on sweet pepper and tomato, reliable detection methods are needed to manage these diseases. Although vegetative multiplication and mechanical transmission are the most efficient transmission routes for these viroids, the transmission through seeds has also been demonstrated in tomato (Kryczyński et al. 1988; Antignus et al. 2007; Singh and Dilworth 2009; Candresse et al. 2010). Pospiviroid detection methods thus have to be applicable on tomato seeds as well as on a variety of ornamental and edible plant species leaves. While pospiviroids can be readily detected on a great number and variety of leaf samples (EFSA PLH 2011), the performance of detection methods used in routine laboratories on seed samples is still largely unknown. Different methods are currently available for the generic detection of pospiviroids (Monger et al. 2010; Tiberini and Barba 2012; Torchetti et al. 2012; Botermans et al. 2013; Zhang et al. 2013; Luigi et al.

2014; Olivier et al. 2014), but still no official method has been designated. Therefore, the National Reference Laboratories have to carry out extensive performance comparisons of available methods in order to select the most efficient one on host plant leaves as well as on tomato seeds. This work presents the results of an inter-laboratory comparison of four methods consisting of a pair of RT-PCR (ANSES 1-2; ANSES 2013), a pair of real time RT-PCR (Botermans 1-2; Botermans et al. 2013), and two single RT-PCR methods, Luigi (Luigi et al. 2014) and Olivier (Olivier et al. 2014), which were tested on 22 tomato leaf and seed samples and their respective 100-fold water dilutions. The evaluation of these methods was performed using the performance criteria defined in the EPPO pest management standards PM 7/ 76 (2) and PM 7/98 (2) (EPPO 2010b, 2014). Because logistic regressions are better suited for proportional data than ANOVA performed on arcsine-square-root transformed data and less affected by unbalanced designs (Jaeger 2008; Bolker et al. 2009; Warton and Hui 2011), statistical discriminations of protocols and methods as well as factors like extraction/RT-PCR kits and laboratories were based on logit models in which the performance criteria were considered as the dependent variables. To account for the random effects of laboratories in most of analyses, mixed models were used. According to the results of this inter-laboratory comparison, ANSES 1-2 method is proposed as international standard for the generic detection of pospiviroids on tomato leaves and seeds.

4.3 Materials and methods

4.3.1 Samples

Twenty-two freeze dried and powdered tomato samples (13 consisting of leaves and nine of seeds) from either healthy or mechanically inoculated

plants were prepared in one laboratory according to the same procedure, tested for their homogeneity, coded and randomly distributed to the eight laboratories to ensure a blind test. The pospiviroid species and their isolates used in the test are presented in Table 4. Out of the nine seed samples, two were co-infected with CLVd and CEVd and two were co-infected with CLVd and TASVd. In order to assess the analytical sensitivity of methods, three tomato leaf and four tomato seed samples were diluted 25-fold in their respective matrix. Moreover, each laboratory was asked to dilute 100-fold in water (2 µL of RNA extract in 198 µL of RNase-free water) each of the 22 total RNA extracts. Forty-four samples were thus tested in each laboratory (Table 4).

Table 4: Viroid isolates and matrix/dilution combinations used in the inter-laboratory test

Viroid	GenBank accession no.	Matrix	Dilution			
			Undiluted	1/25 [a]	1/100 [b]	1/2500 [a,b]
CEVd	HG739073	Leaves	v		c	
CEVd and CLVd	HG739076 and FM995507	Seeds	v	v	c	c
CLVd	AY372392	Leaves	v		c	
CLVd	FM995507	Leaves	v		c	
CLVd	FM995507	Seeds	v	v	c	c
PSTVd	FM998542	Leaves	v		c	
PSTVd	AY372400	Leaves	v	v	c	c
PSTVd	X17268	Leaves	v		c	
PSTVd	FM998542	Leaves	v	v	c	c
PSTVd	FM998542	Seeds	v	v	c	c
TASVd	HG739072	Leaves	v		c	
TASVd	HG739071	Leaves	v	v	c	c
TASVd and CLVd	HG739072 and FM995507	Seeds	v	v	c	c
Negative control	-	Leaves	v		c	
Negative control	-	Seeds	v		c	

[a] dilution in the corresponding matrix

v: dilutions available in sample sets

c : dilutions performed in individual laboratories

[b] dilution in water

CEVd: *Citrus exocortis viroid*; CLVd: *Columnea latent viroid*; PSTVd: *Potato spindle tuber viroid*; TASVd: *Tomato apical stunt viroid*

4.3.2 RT-PCR based methods

Each laboratory was invited to apply four different methods on the 22 RNA extracts of viroid isolates and their 100-fold dilutions. Two methods consisted of a pair of either end-point RT-PCR (ANSES 1-2) or real time RT-PCR (Botermans 1-2) protocols (because a separate (real-time) RT-PCR for the detection of CLVd is required for these methods) and the two other methods consisted of two single end-point RT-PCR protocols (Luigi and Olivier). To allow the use of extraction and PCR kits available in participating laboratories, these latter were not considered as being part of the protocols. Therefore in this study, protocols consisted of the following parameters specified in Table 5: primer and probe composition and concentration, PCR reaction volumes, quantity of total RNA extracted per reaction and (real time) RT-PCR cycling programs. However, in order to be able to evaluate potential efficacy differences between extraction and RT-PCR kits, the kit choice was limited to ensure that at least two labs use the same combination of total RNA extraction and RT-PCR kits for the four end-point PCR protocols. The use of real time RT-PCR reagent kits was left to the appraisal of the laboratories. The kits used by each participating laboratory are presented in Table 6.

Table 5: RT-PCR Mix compositions and real time RT-PCR cycles of each protocol of the four tested methods

Primer/Probe name	Reference	Protocol/ Method code	Primer final concentration	Reaction Volume	RNA extract volume	Denaturation	RT step	PCR cycles	Final Elongation
TCR-F 1-1; TCR-F 1-3; TCR-F 1-4; TCR-F PCFVd; TCR-F IrVd; TR-R1; TR-R CEVd; TR-R6	Botermans et al. 2013	Botermans 1	0.3 µM	25 µL	2 µL		48°C for 30 min	40 cycles at 95°C for 15 s, 60°C for 1 min	
pUCCR			0.1 µM						
CLVd-F; CLVd-F2; CLVd-R		Botermans 2	0.3 µM						
CLVd-P			0.1 µM						
Pospi1deg-FW; Pospi1s-RE pCLV4s	Olivier et al. 2014	Olivier	0.24 µM		1 µL		50°C for 30 min	39 cycles at 94°C for 30 s, 60°C for 45 s and 72°C for 45 s	72°C for 2 min
POP1-FW	Luigi et al. 2014	Luigi	0.3 µM	50 µL	2 µL		50°C for 30 min	35 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 1 min	72°C for 7 min
POP3-FW			0.2 µM						
POP-REV			0.4 µM						
Pospi 1-FW Pospi 1-RE	Verhoeven et al. 2004	ANSES 1	1 µM	25 µL	1 µL		50°C for 30 min	15 cycles at 95°C for 30 s, 62°C for 90 s and 72°C for 45 s and 30 cycles at 95°C for 30 s, 59°C for 90 s and 72°C for 45 s	72°C for 7 min
pCLV4 pCLV4R	Spieker, 1996	ANSES 2	1 µM	50 µL	1 µL	95 °C for 3 min, 4°C for 10 min	50°C for 30 min	30 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min	72°C for 5 min

Buffer and enzyme concentrations were used as recommended by the kit manufacturers, dNTPs were added at 200 µM each and RNase inhibitor or DTT was not used. For real time RT-PCRs, the cut-off value was set at 32 Ct. Samples with Ct values between 32 and 37 were considered as undetermined following the recommendations of the corresponding publication (Botermans et al. 2013) and their final status (positive or negative) was left to the appraisal of the laboratories according to their available validation data.

Table 6: Use of total RNA extraction and (real time) RT-PCR kits in the eight participating laboratories

Lab number	Extraction kit	RT-PCR kit	Real Time RT-PCR kit
1	Rneasy Plant Mini Kit (Qiagen)	SuperScript® One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Thermo Fisher Scientific)	Master Mix (Applied Biosystem)
3			AgPath-ID™ One-Step RT-PCR (Thermo Fisher Scientific)
7			SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Thermo Fisher Scientific)
2		OneStep RT-PCR Kit (Qiagen)	AgPath-ID™ One-Step RT-PCR (Thermo Fisher Scientific)
4			QuantiFast Probe RT-PCR Plus Kit (Qiagen)
6			M-MuLV Reverse Transcriptase (Thermo Fisher Scientific) and AmpliTaq Gold® Polymerase (Life Technologies)
5	Spectrum plant total RNA Kit (Sigma-Aldrich)	Titan One Tube RT-PCR System (Roche)	Precision OneStep qRT-PCR MasterMix (Primerdesign)
8			One step qRT-PCR MasterMix No ROX (Eurogentec)

Applied Biosystems Inc (Foster City, USA); Eurogentec SA (Seraing, Belgium); Primerdesign Ltd (Southampton, UK); Qiagen (Hilden, Germany); Roche Diagnostics GmbH (Manheim, Germany); Sigma-Aldrich Chemie GmbH (Steinheim, Germany); Thermo Fisher Scientific (Waltham, USA).

4.3.3 Pospiviroid species identification

To identify the RT-PCR detected species, each laboratory was invited to sequence the amplicons of undiluted samples. Pospiviroid species were then identified using BLAST search against the NCBI viroid reference sequences. Alternatively, the amplicons obtained with Luigi protocol were digested with either Alu I or Sau 96I for 60 min (FastDigest enzymes) or for 12 h (conventional restriction enzymes) at 37 °C and separated on 5 % polyacrylamide gels. Species identifications were performed by comparing the restriction band patterns with those available in the reference publication (Luigi et al. 2014).

4.3.4 Evaluation of performance criteria and statistical analysis

Diagnostic sensitivity, diagnostic specificity and relative accuracy were used to evaluate the performance of the individual protocols and the detection methods. The definitions of these performance criteria were taken from PM 7/76 (2) and PM 7/98 (2) standards (EPPO 2010b, 2014). Along with the three above mentioned criteria, the analytical sensitivity was also assessed by comparing the relative accuracy of each method at the four different dilutions (1, 25, 100 and 2500-fold). Analytical specificity was evaluated on the basis of nondiluted samples for each strain/matrix combination. The repeatability was not assessed because no systematic repetition of a same sample/PCR method combination was performed by the participating laboratories, except for the homogeneity tests. Prior to statistical analyses, several laboratory/ method combinations were withdrawn from the datasets. Along with not performed combinations (lab 5/ANSES 2; lab 6/Botermans 1; lab 6/Botermans 2), two experiments not complying with the common protocols of Table 5 (lab 4/ Luigi's and Olivier's methods) because of higher primer concentrations were not taken into account. For

the comparison of the four methods, the results of double (real time) RT-PCR methods (ANSES 1-2 and Botermans 1-2) and the results of the duplex RT-PCR method (Olivier) were combined to allow comparison with the results of Luigi's method. For a given sample, the combination was carried out as follows: two correct results gave a correct combined result; one or two incorrect (false positive/negative) gave an incorrect combined result. However, for the eight co-infected samples, only the results for pospiviroids other than CLVd were taken into account to minimize the bias between Luigi's method and the three other detection methods. To compare the three above-mentioned performance criteria between protocols, methods, total RNA extraction kits, RT-PCR kits and laboratories, mixed logit models or linear mixed models after an empirical logit transformation of the dependent variables were built on the corresponding dataset. The statistical significance of differences between factor levels (i.e., protocols, methods, laboratory and extraction and/or RT-PCR kits) was computed by Tukey's multiple comparison test performed on the corresponding linear models. The reproducibility was assessed by computing the Fleiss Kappa index (Fleiss et al. 2003) for each detection method. The statistical analyses were made with R version 2.15.3 and the packages lme4, irr and multcomp (Hothorn et al. 2008; Gamer et al. 2012; Bates et al. 2013; R Core Team 2013).

4.4 Results

4.4.1 Performance criteria

Table 7 shows the laboratory relative accuracy obtained for each individual protocol and method as well as the mean for each protocol and method. Based on these means, ANSES 1, Botermans 2 and ANSES 1-2 gave the best results for the detection of pospiviroid species other than CLVd, CLVd and

all pospiviroids respectively. To check if these best protocols and method were significantly different from the others and to better understand the strengths and the weaknesses of each method, several statistical analyses were carried out to compare them according to their performance criteria. When it comes to protocols dedicated to CLVd detection, no significant difference was observed between the accuracies of ANSES 2, Botermans 2, Luigi and Olivier protocols. However the four evaluated protocols differed in terms of diagnostic sensitivity and diagnostic specificity. ANSES 2 and Olivier protocols did not result in false positives (i.e., 100 % diagnostic specificity) while Botermans 2 method gave significantly less false negative (i.e., higher diagnostic sensitivity) than ANSES 2, Luigi and Olivier protocols.

Table 7: Laboratory mean relative accuracy (expressed in percent) obtained for each individual protocol and method.

Laboratory	Pospiviroid species other than CLVd			CLVd			Pospiviroids			
	ANSES1	Botermans1	Olivier (other than CLVd)	ANSES2	Botermans2	Olivier (CLVd)	ANSES1-2	Botermans1-2	Luigi	Olivier
1	79.5	61.4	72.7	68.2	63.6	70.5	68.2	43.2	50	63.6
2	79.5	84.1	83.3	63.6	50	78.6	70.5	40.9	28.6	81
3	97.7	88.6	63.6	81.8	70.5	63.6	93.2	63.6	38.6	47.7
4	90.9	75	97.7 [a]	84.1	79.5	88.6 [a]	86.4	63.6	77.3 [a]	97.7 [a]
5	63.6	75	79.5	na	86.4	63.6	na	70.5	29.5	61.4
6	86.4	na	77.3	72.7	na	72.7	77.3	na	54.5	68.2
7	93.2	86.4	75	77.3	86.4	72.7	86.4	81.8	29.5	65.9
8	84.1	70.5	75	72.7	90.9	72.7	77.3	68.2	40.9	65.9
mean	84.4	77.3	78	74.3	75.3	72.9	79.9	61.7	43.6	68.9

na: data not available

a : value not taken into account in Tables 5 to 6

The second analysis was performed on samples either healthy or singly infected by a pospiviroid species other than CLVd to compare ANSES 1, Botermans 1, and Luigi and Olivier (real time) RT-PCR protocols. As regards the detection of the pospiviroid species other than CLVd, the analyses showed that the Olivier method was significantly less accurate than ANSES 1 protocol and Luigi's method was significantly less accurate than the other three protocols. The results showed that the difference was due to diagnostic sensitivity. No significant differences in diagnostic specificity between tested protocols/methods were found. The influence of matrix (seeds or leaves) was investigated by performing statistical analyses on three datasets: all samples, seed samples or leaf samples. When combined results of the four methods were compared for their relative accuracy for the 22 total RNA extractions and their 100-fold dilutions, three statistical groups appeared: ANSES 1-2 method gave significantly more accurate results than Botermans 1-2 and Olivier's methods, these two latter methods giving significantly more accurate results than Luigi's method (Table 8 top).

Table 8: Relative accuracy, diagnostic specificity and diagnostic sensitivity obtained for either all samples, seed samples or leaf samples for the four tested methods.

Type of samples		Number of samples/ Lab	ANSES 1-2			Botermans 1-2			Luigi			Olivier		
			Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
All samples	Accuracy	44	79.9	9.1	a	61.7	14.8	b	38.8	10.4	c	64.8	9.8	b
	Specificity		85.7	28.3	ab	60.7	31.8	b	89.3	19.7	a	89.3	19.7	a
	Sensitivity		79.3	8.5	a	61.8	14.3	b	33.7	13.1	c	62.4	11.6	b
			ANSES 1-2			Botermans 1-2			Luigi			Olivier		
			Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
Seed samples	Accuracy	18	68.3	10.5	a	54.8	14.1	ab	35.3	11.9	c	42.2	12.5	bc
	Specificity		92.9	18.9	a	57.1	34.5	b	100	0	a	85.7	24.4	a
	Sensitivity		65.2	11.3	a	54.5	16.8	a	27	13.5	b	36.7	16.5	b
			ANSES 1-2			Botermans 1-2			Luigi			Olivier		
			Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]	Mean	Sd	Group [a]
Leaf samples	Accuracy	26	89.7	9.9	a	66.5	22.2	b	41.2	12.3	c	80.2	13.8	ab
	Specificity		78.6	39.3	a	64.3	47.6	a	78.6	39.3	a	92.8	18.9	a
	Sensitivity		88.7	8.9	a	66.7	20.7	b	38.1	15.9	c	79.2	14.4	ab

[a] Statistical group according to Tukey's test are mentioned for each performance criterion/method combination

Results obtained for seed samples showed a similar pattern as for the whole set of samples. Botermans 1-2 method, however, statistically grouped with both ANSES 1-2 and Olivier's method, the latter being not significantly different from Luigi's method (Table 8 middle). For leaf samples, in terms of accuracy, ANSES 1-2 and Olivier's methods scored best, followed by Botermans 1-2 method (not significantly different from Olivier's method) which itself was significantly better than Luigi's method (Table 8 bottom). In the three latter analyses, the observed accuracy differences were mainly explained, especially for Luigi's and Olivier's methods, by a lack of diagnostic sensitivity (Table 8). Analytical specificity showed that all pospiviroid isolates are detected by all protocols. However, limited amplification was obtained with Luigi's protocol for the three PSTVd isolates on leaf and seed samples and for one of the TASVd isolates provided only as leaf samples. Aweak amplification was also observed for CLVd isolates on seeds both for ANSES 2 and Olivier's protocols (data not shown). Analytical sensitivity was assessed using the relative accuracy of the four tested methods for each of the four dilutions: 1, 25, 100 and 2500-fold (Table 9). Overall, for leaf samples, ANSES 1-2 and Olivier's methods gave the best relative accuracy at each of the four levels of dilution. Botermans 1-2 gave a lower accuracy at 25- fold dilution whereas Luigi's method showed a significantly lower accuracy at all dilution levels. For seed samples, the accuracies of all four detection methods were not significantly different for undiluted samples, Luigi's and Olivier's methods showed a significantly lower accuracy at 25 and 100-fold dilutions. The relative accuracy at 2500-fold was very low for each of the four methods.

Table 9: Analytical sensitivity investigated through relative accuracies obtained for pospiviroid species detection by the four tested methods at the four dilution levels.

	Number of samples/ Lab	ANSES 1-2			Botermans 1-2			Luigi			Olivier		
Seed samples													
		Mean	Sd	Group [c]	Mean	Sd	Group [c]	Mean	Sd	Group [c]	Mean	Sd	Group [c]
1	5	83	21	a	69	30	a	63	18	a	71	11	a
25 [a]	4	82	12	a	61	24	ab	33	28	b	38	34	b
100 [b]	5	77	8	a	63	31	ab	37	14	b	43	29	b
2500 [b]	4	25	32	a	21	37	a	0	0	a	8	14	a
Leaf samples													
1	10	87	17	a	70	32	ab	54	17	b	86	18	a
25 [a]	3	90	16	a	43	37	b	10	16	b	90	16	a
100 [b]	10	89	11	a	77	21	a	49	16	b	79	17	a
2500 [b]	3	86	18	a	43	32	ab	5	13	b	57	32	a

a dilution in the corresponding matrix

b dilution in water

c Statistical group according to Tukey's test are mentioned for each performance criterion/method combinatio

4.4.2 Total RNA extraction and (real time) RT-PCR kit comparison

No significant difference was observed between the two total RNA extraction kits (P=0.9) or between the three total RNA extraction/RT-PCR kit combinations (P≥ 0.39) when the results of the four (real time) RT-PCR tested methods or of the three RT-PCR tested protocols were respectively considered (data not shown).

4.4.3 Pospiviroid species identification

A total of 169 amplicons were sequenced and allowed a correct identification of pospiviroid species in 91.1% of cases, a wrong assignment in 8.3 % and an undetermined result in 0.6 %. Wrong assignments in sequencing were probably due to contamination with positive controls, considering the best hits in blast for these sequences and the fact that these errors came from only one laboratory. Twelve amplicons from Luigi's protocol were digested with endonuclease. The restriction of these amplicons allowed a correct assignment for 83.3 %, led to a wrong assignment in 8.3 % and did not allow any identification in 8.3 % of cases. Fragmentary data did not allow conducting a statistical analysis to compare both identification methods.

4.4.4 Variability between laboratories and reproducibility

Because laboratory number four obtained the best relative accuracy for Luigi and Olivier methods using 1 µM of each primer rather than the lower recommended concentrations (Table 5), reason why these lab/method combinations were not taken into account in all the other analyses, the significance of differences between this laboratory and the seven other

laboratories was evaluated. Tukey's test showed that relative accuracy of the laboratory number 4 for Luigi and Olivier methods was significantly higher compared to each of the seven other laboratories ($P \leq 0.002$) while these latter were not significantly different from each other ($P \geq 0.35$).

The calculation of Fleiss Kappa indexes for the relative accuracy allowed to compare the reproducibility of the four tested methods and to obtain the following ranking: Luigi, ANSES 1-2, Olivier and Botermans 1-2 with the indices of 0.6 (moderate agreement), 0.368 and 0.303 (fair agreement) and 0.132 (slight agreement) respectively.

4.5 Discussion

Although participating laboratories did not use the same total RNA extraction and real time RT-PCR kits and despite the fact that two-thirds of samples were diluted at least 25-fold and more than a quarter of samples were close to the detection limit of the six tested protocols, the values of the performance criteria obtained were satisfactory and allowed a comparison of different pospiviroid detection methods. Method ANSES 1-2, derived from Verhoeven et al. (2004) and Spieker (1996) provides the best results for almost all performance criteria tested, except for reproducibility and diagnostic sensitivity of ANSES 2 for CLVd infected samples. Olivier method also derived from Verhoeven et al. (2004) and Spieker (1996) but consisting of a single RT-PCR, allows an accurate detection of pospiviroid in leaf samples. Nevertheless, a general lack of diagnostic sensitivity in tomato seed samples, especially for CLVd infected samples, decreased its accuracy. However, when the concentration of primers for this method was raised to 1 µM, the best combined relative accuracy of the inter-laboratory comparison (97.7 %) was obtained (Table 7, lab 4). Botermans 1-2 method showed relative accuracy not significantly different from ANSES 1-2 for seed

samples, but significantly lower for leaf samples (Table 8) and the generally accepted advantage of real time RT-PCR over conventional RT-PCR regarding the sensitivity could not be demonstrated in this case. The analysis of raw data showed that the substantial loss of relative accuracy between the two individual protocols and the combined Botermans 1-2 method (Table 7) was mainly due to the non-overlapping conjunction of false negatives of Botermans 1 protocol and false positives of Botermans 2 protocol. Luigi's method was significantly less accurate than the other protocols or methods due to its lack of specificity against PSTVd isolates and one of the two TASVd isolates. This problem was probably linked, at least for PSTVd isolates, to mismatches between the last seven nucleotides of POP1-FW primer 5' end and the targeted region in the genome of the concerned isolates. Although a trend of decreased accuracy for seed samples was observed, it was not possible to draw any conclusions about the matrix effect (leaves or seeds), since different pospiviroid isolates were used for the individual matrices and pospiviroid concentrations in the samples were unknown. The reproducibility of methods was rather low ranging from slight agreement (Botermans 1-2) to fair agreement (ANSES 1-2 and Olivier) and moderate agreement (Luigi). The general lack of reproducibility can be explained by the great proportion of samples which were close to the limit of detection of the different protocols/ methods and which were consequently either positive or negative depending on the laboratory. Considering that sequencing data were too fragmentary and RFLP data were only provided by one lab and only for Luigi's method, no statistical analysis could be performed to compare both identification methods. However, because RFLP identification was linked to Luigi's method, which proved to be less accurate than other tested methods and because sequencing proved to be effective and allowed detecting PCR

contaminations, sequencing should be preferred. The capacity of sequencing to correctly assign species, and thus distinguish between regulated and non-regulated species, as well as genotyping the isolates involved in outbreaks should also be considered as an advantage of RT-PCR over real time RT-PCR. In the light of the above, considering relative accuracy as the most informative performance criterion, ANSES 1-2 method should be considered as the best method of this inter-laboratory comparison, although it cannot be excluded that a modified version of Olivier's method, which is both quicker and less expensive than the former, is also more accurate. ANSES 1-2 should thus be considered as international standard for the generic detection of pospiviroids on tomato leaves and seeds.

5 Innate immunity activation and RNAi interplay in Citrus exocortis viroid—tomato pathosystem

5.1 Abstract

Although viroids are the smallest and simplest plant pathogens known, the molecular mechanisms underlying their pathogenesis remain unclear. To unravel these mechanisms, a dual approach was implemented consisting of *in silico* identification of potential tomato silencing targets of pospiviroids, and the experimental validation of these targets through the sequencing of small RNAs and RNA ends extracted from tomatoes infected with a severe isolate of Citrus exocortis viroid (CEVd). The generated RNA ends were also used to monitor the differentially expressed genes. These analyses showed that when CEVd symptoms are well established: (i) CEVd are degraded by at least three Dicer-like (DCLs) proteins and possibly by RNA-induced silencing complex (RISC), (ii) five different mRNAs are partially degraded through post-transcriptional gene silencing (PTGS), including argonaute 2a, which is further degraded in phasiRNAs, (iii) Dicer-like 2b and 2d are both upregulated and degraded in phasiRNAs, and (iv) CEVd infection induced a significant shift in gene expression allowing to explain the usual symptoms of pospiviroids on tomato and to demonstrate the constant activation of host innate immunity and systemic acquired resistance (SAR) by these pathogenic RNAs. Finally, based on *in silico* analysis, potential immunity receptor candidates of viroid-derived RNAs are suggested.

Olivier Thibaut, Bragard Claude. 2018. Innate Immunity Activation and RNAi Interplay in Citrus Exocortis Viroid—Tomato Pathosystem. Viruses 10*: 587.*

5.2 Introduction

Viroids are the smallest known plant pathogens consisting of small single-stranded and circular RNA molecules (Diener et al. 1993). Although viroids differ from most RNA viruses by the fact that they do not code for any protein, replicate in the nucleus or in the chloroplast, and form imperfect double-stranded RNA (dsRNA) structures, like RNA viruses, viroids are able to activate and resist RNA silencing (Wang et al. 2004), also termed RNA interference (RNAi), activate systemic acquired resistance (SAR) (López-Gresa et al. 2016), and probably innate immunity (Zheng et al. 2017). Recently, the ability of plants to recognize viral dsRNAs as pathogen-associated molecular patterns (PAMPs) and mediate PAMP-triggered immunity (PTI) has been demonstrated (Niehl et al. 2016).

In light of these discoveries, viroids thus offer an opportunity to: (i) better understand how RNA silencing works, (ii) figure out if plant defenses possess pattern recognition receptors (PRRs) or disease resistance proteins which are able to recognize RNAs as PAMPs or effectors and trigger PTI or effector-triggered immunity (ETI) respectively (Moon and Park 2016), (iii) comprehend how viroids can elicit SAR (Fu and Dong 2013), and (iv) unravel the possible interplay between these three major plant defense systems.

Besides these unanswered questions related to defense strategies, the underlying molecular mechanisms leading to viroid symptom induction and modulation are also still largely unknown, despite more than 30 years of research efforts. Currently, three main theories—which are potentially interconnected—are put forward to explain how pospiviroids trigger the first reactions: (i) the first hypothesizes interaction between viroids and unknown plant proteins which would initiate symptom development (Schnölzer et al. 1985), the two others postulate that viroid-derived small interfering RNAs (vd-sRNAs) trigger the modulation of important protein

synthesis through (ii) RNA directed DNA methylation (RdDM) (Pelissier and Wassenegger 2000) or (iii) post transcriptional gene silencing (PTGS) (Itaya et al. 2001).

It is now well established that viroid and virus RNA structures are recognized by host ribonuclease III-like proteins, Dicer-like (DCL), and cleaved into 21-24-nucleotide vd-sRNAs (Wang et al. 2012; Katsarou et al. 2016). Transcriptional gene silencing relying on sequence complementarity between vd-sRNAs and host genome is thus a possible way through which pospiviroids induce symptoms; this was demonstrated for a variant of Peach latent mosaic viroid inducing peach calico symptom (Malfitano et al. 2003). Although several PTGS targets of *Potato spindle tuber viroid* (PSTVd) have been identified, e.g. PlantWD-40 repeat proteins (Aviña-Padilla et al. 2015), two genes involved in gibberellin or jasmonic acid biosynthesis (Wang et al. 2011a), a chloride channel protein CLC-b-like, and a ribosomal protein S3a-like (Adkar-Purushothama et al. 2017), divergences between studies exist, and the mechanisms leading to symptoms remain elusive.

The potential induction of symptoms through RdDM was experimentally tested by Wassenegger et al. (Wassenegger et al. 1994) who demonstrated that PSTVd cDNA integrated into the genome of *Nicotiana tabacum* becomes specifically methylated upon PSTVd replication. However, no methylation of non-transgenic plant DNA due to viroid infection was demonstrated, and the RdDM guide molecules seem to be longer than the 24-nt vd-sRNAs (Dalakouras et al. 2013).

Regarding the protein-viroid interactions, several tomato proteins were found to interact with viroids (Wolff et al. 1985; Werner et al. 1995; Gómez and Pallás et al. 2001; Owens et al. 2001), but although some, like Virp1 (Kalantidis et al. 2007), was found to be essential for their infectivity, the roles they play in symptom modulation are not trivial. Apart from their

interaction with proteins of the RNAi machinery, viroid-derived dsRNAs could directly bind to PRR/disease resistance proteins and trigger PTI/ETI. In mammals, the protein kinase RNA-activated (PKR) is, for instance, known to bind dsRNAs and phosphorylate the translation initiation factor eIF2α, which leads to translation initiation repression. Despite early reports of plant PKR (pPKR) activity in virus-infected plants (Hiddinga et al. 1988; Langland et al. 1995; Chang et al. 1999), no specific kinase could be purified, and the sequence of a putative PKR ortholog is absent from plant genomes (Immanuel et al. 2012). However, the recent discovery of a dsRNA-induced immunity independent of dicer-like proteins (Niehl et al. 2016) offers hope in the search for a plant dsRNAs receptor.

While it is thought that pospiviroids replicate latently in many solanaceous ornamentals, eight species of the genus (Owens et al. 2011) replicate and produce symptoms on tomatoes. This suggests that the induction of symptoms would be independent from the replication and trafficking, and would be rather specific to some susceptible species. However, some mild pospiviroid strains are known to induce very few symptoms on the fast-growing tomato, so that it cannot be excluded that the impact of pospiviroids on slow-growing ornamentals might be difficult to highlight. Conversely, tomato lethal strains of pospiviroids have been identified.

In order to understand how plants activate their defenses against viroids, and how these pathogens escape such defenses while inducing symptoms, a pathosystem consisting of tomato plants cv. Heinz 1706 was infected with a severe isolate of *Citrus exocortis viroid* (CEVd), and was studied through the lens of the transcriptome and the degradome using high-throughput sequencing technologies. Basically, a dual approach was implemented consisting of: (i) prior in silico identification of potential targets of pospiviroids on tomato coding sequences, (ii) the experimental validation of

PTGS and RdDM targets through small RNA and parallel analysis of RNA ends (PARE) sequencing, and (iii) the differential gene expression analysis between infected and mock tomato plants by the quantification of PARE and small RNA reads in all known genes.

These analyses allowed us to (i) highlight new tomato PTGS targets, which unravel both counter defense strategies of pospiviroids and plant defense mechanisms, (ii) further understand the decay pathways of pospviroids, (iii) gain new insights in the transcriptional shift induced by pospiviroids, demonstrating the activation of the innate immunity and SAR by CEVd, and explaining the symptom induction of these pathogens on tomatoes, and (iv) propose potential plant receptors triggering innate immunity and SAR in pospiviroid infected plants.

5.3 Materials and methods

5.3.1 *In silico* analyses of artificial pospiviroid derived vd-sRNAs

Potential tomato PTGS targets of pospiviroids, as well as the pospiviroid genomic regions involved in these interactions, were found using BWA version 0.7.12 (Li and Durbin et al. 2009). 21 nt artificial vd-sRNAs were generated from a set of 891 pospiviroids complete genomes: 313 *Potato spindle tuber viroid* (PSTVd); 128 *Chrysanthemum stunt viroid* (CSVd); 253 CEVd; 83 *Columnea latent viroid* (CLVd); 11 *Mexican papita viroid* (MPVd); 58 *Pepper chat fruit viroid* (PCFVd); 28 *Tomato apical stunt viroid* (TASVd); and 11 *Tomato chlorotic dwarf* (TCDVd). These were circularized by copying the first 21 nucleotides at the end of each sequence. The domains to which the artificial vd-sRNAs belonged were computed according to the position of the vd-sRNA central nucleotide in an annotated alignment of the 891

pospiviroids sequences, and based on the domain boundaries identified by Keese and Symons (Keese and Symons et al. 1985). To allocate the mapping sites to tomato genomic features, intergenic regions, exons and introns, the tomato coding sequences (ITAG3.2), and the feature file "models.gff", provided by the International Tomato Genome Sequencing Project, were used. Minimum free energies of duplexes were computed according to Serra and Turner (Serra and Turner et al. 1989).

5.3.2 CEVd inoculation

A *Solanum laxum* isolate of CEVd (12/0288/VI.4) inducing severe stunting, leaf chlorosis, and deformations, as well as leaf necrosis on tomatoes cv. Minibel, was inoculated to tomato plantlets cv. Heinz 1706-BG (Tomato Genetics Resource Center accession: LA4345) at the first true-leave stage with a mix of 100 mg of infected or healthy tomato leaves in 2 mL of distilled water. Inoculated plants were grown at 26/24 °C day/night temperatures with a 16 h daylight photoperiod, and pospiviroid infections were checked by RT-PCR (Olivier et al. 2014). A pair of both healthy and infected plants were selected for RNA extraction and sequencing.

5.3.3 RNA extractions and sequencing

The top 5 cm of two infected plants and two healthy plants consisting of leaves, stem, petioles, and apex were sampled 47 days after the inoculation and approximately at the middle of the light phase. The samples were immediately crushed in liquid nitrogen and stored at −80 °C until extraction. Small RNAs and total RNAs were extracted using a Spectrum Plant Total RNA kit (Merck, Darmstadt, GermanySigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions, for both types of RNAs. Four small RNAs and 4 PARE Illumina libraries were prepared and sequenced on

Illumina NextSeq500 by GenXPro GmbH (Frankfurt am Main, Germany). Small RNA libraries were prepared by selecting 18-64 bp long RNAs and using "TrueQuant" adapters. PARE libraries were prepared following a protocol derived from German et al. (German et al. 2009), but with "TrueQuant" adapters, and skipping the MmeI digestion step to allow the selection of longer fragments of uncapped RNAs. Small RNA and PARE reads[1] are available at SRA accession: SRP150214 (Table S1).

5.3.4 *In silico* analyses of experimentally generated sequences

Alignments of small RNA and PARE reads on CEVd were performed using BWA on a newly-constructed CEVd sequence allowing 2 mismatches for small RNAs and alignment seeds of 17 nucleotides for PARE reads.

Potential silencing sites were identified by mapping of CEVd-derived small RNAs on tomato genomes (version SL3.0) supplemented with tomato chloroplast and mitochondrion genome sequences (accession numbers: NC_007898.3 and NC_035963.1 respectively). For this analysis, BWA was used, and 3 mismatches per duplex were allowed. To assess the differential expression of genes, PARE reads were mapped on tomato coding sequences (release ITAG3.2) supplemented with publicly-available tomato miRNAs using BWA and a mapping seed of 17 nucleotides. Mapped reads were quantified in each gene for the four samples using Salmon (Patro et al. 2017). Differential gene expression analysis based on read quantification was then carried out using the package Edger (Robinson et al. 2010; McCarthy et al. 2012) in R version 3.2.3. Gene ontology enrichment and analysis was performed using the package TopGO (Alexa and Rahnenfuhrer et al. 2016) in R.

[1] Reads were trimmed using the software Cutadapt (Martin 2011) and provided free of adapters.

To highlight phasi-RNA activation, microRNA genes differential expression and hot spots of vd-sRNA in genes, small RNAs sequences were mapped on tomato coding sequences (release ITAG3.2) supplemented with publicly-available tomato miRNAs using BWA by allowing a maximum of 2 mismatches. Gene and microRNA-derived small RNAs were quantified for each gene and screened for quantification differences using the same procedure as for PARE reads. PhasiRNA detection was performed using the software UNITAS 1.5.1 (Gebert et al. 2017).

Detection of vd-sRNA triggered PTGS cleavages was performed visually by looking at each potential PTGS site identified by mapping, as described above, for differences of PARE 5' end frequencies between infected and mock samples. When consistent frequency differences were found, the statistical significance of these differences was assessed according to Poisson regressions using R. Vd-sRNAs involved in the PTGS sites were then identified by considering the cleavage site between the 10th and 11th nucleotides of the corresponding 21 nt long vd-sRNAs.

To report the differences in read quantification, the log to base 2 of the fold change (logFC), i.e., the normalized number of reads in CEVd-infected samples divided by the normalized number of reads in mock samples was used. The statistical significance of this fold change was expressed using the false discovery rate (FDR).

5.4 Results

5.4.1 Analysis of tomato PTGS targets of *in silico* generated pospiviroid vd-sRNAs

Mapping analysis of in silico-generated vd-sRNAs on tomato CDS showed that the similarities between pospiviroids and tomatoes are distributed in the five genomic domains of pospiviroids in a similar way for the eight

pospiviroid species able to infect tomatoes: TASVd, CLVd, CEVd, PSTVd, PCFVd, CSVd, TCDVd, and MPVd (Figure 11A). Statistical differences were found between each of the five domains in terms of proportions of mapped vd-sRNAs for each of the eight pospiviroid species, and regardless of the strand polarity. The terminal right domain collected the highest number of mapping hits, while the variable domain showed the smallest number (Figure 11A).

Although tomato/pospiviroid similarities are distributed in the five domains in a similar manner for all studied pospiviroid species (Figure 11A), in-depth analysis of these results showed that no potential PTGS target is shared among all eight species, and about three quarters of similarities are only shared between strains of the same species (Figure 11B and S2a). Nevertheless, despite this diversity, several gene ontology terms associated with hypothetical target genes were significantly overrepresented, among which terms linked to cytoskeleton (mainly kinesins), kinases, membrane transporters, and 1,3-beta-D-glucan biosynthesis were the most overrepresented (Table S2b highlighted in gray, purple, yellow and orange respectively).

In total, 2303 potential PTGS targets were found to be shared between at least two pospiviroid species: 639 kinases, including many MAP and receptor like kinases (RLKs), 73 RNA-binding proteins, 89 disease resistance proteins, 55 WD40 repeat-like superfamily proteins, and 47 pentatricopeptide repeat-containing proteins (Table S2a). Remarkably, proteins related to RNA silencing were also present in the list, like argonautes (1, 2 and 5), two suppressors of gene silencing 3 (SGS3) involved in PTGS (Solyc05g005450 and Solyc11g066990), as well as one factor of DNA methylation 1 (Solyc03g117040) and one protein INVOLVED IN DE NOVO 2 (Solyc05g054420) involved in RdDM. Of these 2303 gene targets,

309 were found to be upregulated in the infected samples analyzed in this study.

Strikingly, among the four genes found as potential common targets shared between seven pospiviroid species and whose duplexes minimum free energy were lower than −25 kcal/mol, two TIR-NB-LRR genes (Solyc08g005510 and Solyc11g011060) presenting similarity with TMV resistance protein N, and an endoplasmic reticulum-type calcium-transporting ATPase 3 (Solyc07g022790), were highlighted. Similarly, shared between five pospiviroid species, two disease resistance proteins, i.e., a protein homologous of At1g12280 belonging to the NB-LRR family (Solyc02g037540.2.1) and an uncharacterized protein (Solyc05g007170.3.1), were found as additional potential targets. The analysis of secondary structures of the corresponding vd-sRNA/mRNA duplex confirms some of the many interesting potential PTGS targets, since genes related to plant disease, RNA-binding proteins, or transcription factors showed thermodynamically stable duplexes (Table S2a).

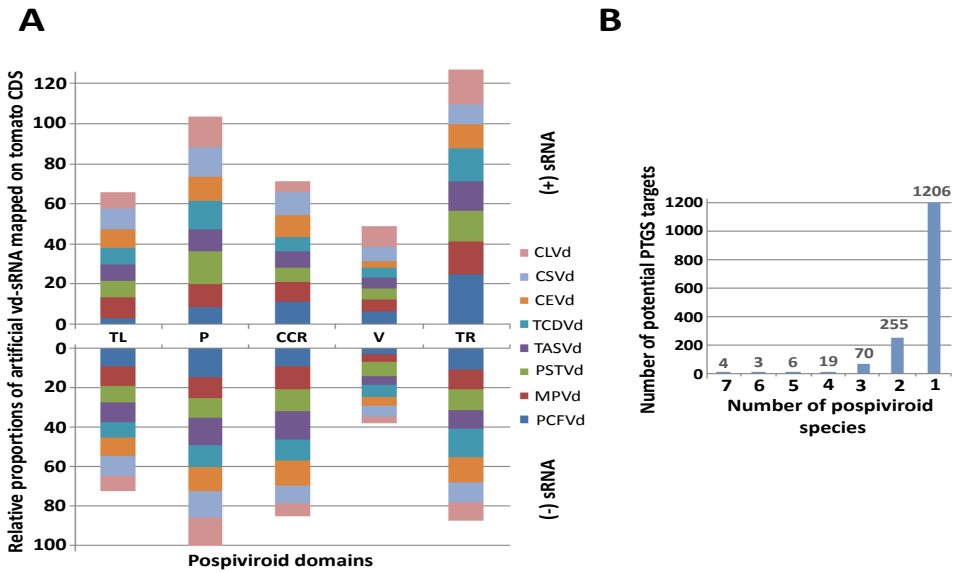

Figure 11. (**A**) Distribution among the five pospiviroid domains of the percentage of artificial 21 nt vd-sRNA mapping to tomatoes CDS for each of the eight pospiviroid species able to infect tomatoes (three mismatches/indels allowed). The upper part of the bar graph shows targets of the positive strand of pospiviroids; the lower part shows targets of the negative strand. Statistical differences between domains are represented as binomial regression groups (P < 1e-5 above the upper bar of each domain. The five pospiviroid domains are mentioned according to the following abbreviations: TL—Terminal left; P—Pathogenicity, C—Conserved central region; V—Variable; TR—Terminal right. (**B**) The number of potential PTGS targets shared among pospiviroid species according to the mapping of *in silico*-generated vd-sRNAs from a set of 891 complete genome sequences of pospiviroids. Here, only the most thermodynamically-stable sites of Table S2a (ΔG ≤ −25 kcal/mol) were reported in this graph.

In order to validate this strategy of PTGS target discovery, a severe strain of CEVd and the fully-sequenced *Solanum lycopersicum* cv. Heinz 1706-BG, were chosen for the analysis of both viroid and tomato degradomes.

5.4.2 CEVd degradome and its tomato silencing targets

The mapping of sequenced-RNAs on the CEVd genome showed that, in agreement with pospiviroid RT-PCR results, CEVd-derived reads were only present in infected samples. The analysis of single nucleotide variants (SNVs) revealed that three CEVd haplotypes, with a maximum of two SNV differences, were present in the infected plants (GenBank accessions: MH476291, MH476292 and MH476293). No other virus or viroid could be detected in the infected and mock-inoculated samples based on BLAST analyses of small RNA reads on a large set of plant viruses and viroids.

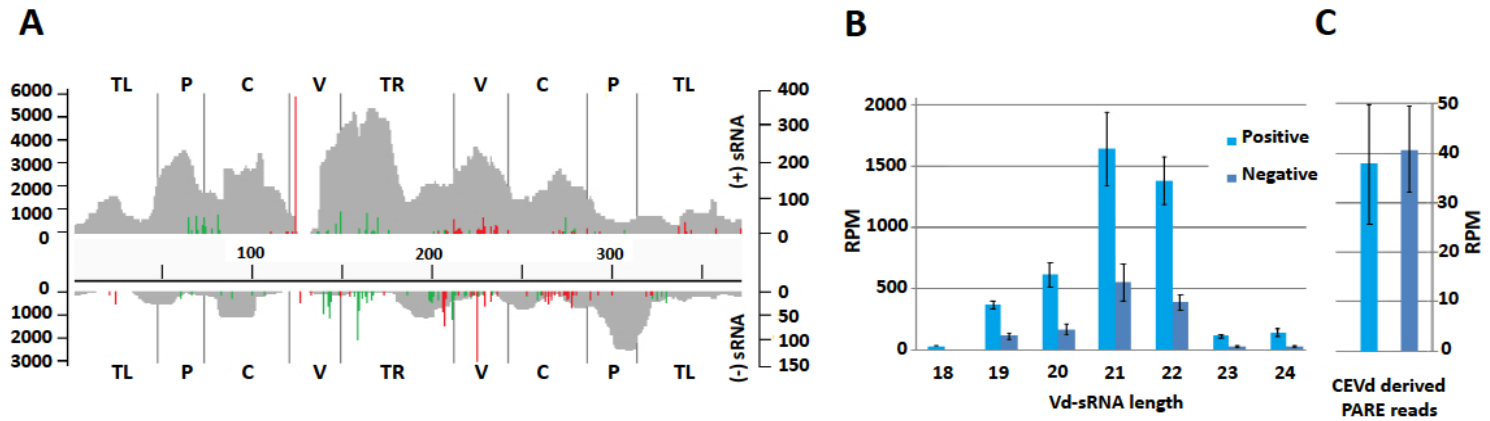

Figure 12. (**A**) Distribution of vd-sRNAs along the CEVd genome (positive strand above and negative strand below) and in the five domains. (18-24 nt). Most frequent 5' and 3'ends of RNA ends are depicted in green and red respectively. The left scale refers to vd-sRNA position count, while the right scale refers to the number of 5' and 3' RNA ends. The five pospiviroid domains are mentioned according to the following abbreviations: TL—Terminal left; P—Pathogenicity, C—Conserved central region; V—Variable; TR—Terminal right. (**B**) Distribution of vd-sRNA according to their length and polarity. Means of read per million (RPM) are depicted with error bars showing the standard deviations observed between biological replicates. (**C**) Mean abundances (in RPM) of CEVd derived PARE reads for both polarities.

The distribution along the CEVd reference genome of the reads from the two replicates of small RNAs and PARE sequences showed very similar patterns indicating a good repeatability (data not shownFigures S1, S2 and S3). The number of reads was higher for the positive strand than for the negative strand, and peaks on both strands were clearly visible (Figure 12A). Size distribution of vd-sRNAs showed that 21-nt and 22-nt sRNAs were the most abundant for both polarities (Figure 12B). The comparison of vd-sRNA maps on CEVd genome for each sRNA length (from 18 to 24-nt) showed very similar patterns between biological replicates for both polarities. These similarities were especially pronounced for 21, 22, and 24-nt vd-sRNAs (Figures S1 and S2). While positive strand vd-sRNAs were three times more represented than their negative counterparts (totals of 32,647 and 9,519 respectively), remarkably, CEVd-derived RNA ends showed a relatively balanced polarity repartition (Figure 12C).

The numbers of 5’ and 3’ ends of RNA ends for each genomic position were assessed, and the most frequent sites (corresponding to outliers according to Poisson distribution) were plotted on plus and minus vd-sRNA maps (Figure 12A). This analysis showed that several positions along the CEVd genome are overrepresented in terms of 5’ and 3’ extremities of RNA ends.

Unexpectedly, in both polarities, the positions with the most abundant RNA end extremities were those of 3’ RNA ends, both flanking the GC-box of the V domain in opposite strands. Groups of 3’ and 5’ RNA ends hot spots were also clearly visible across the genome in both polarities, indicating preferential ends/cleavages at these sites (see also Figure S3).

The potential tomato targets of gene silencing were identified by the mapping of vd-sRNAs on tomato genome rather than CDS in order to check both RdDM and PTGS potential targets. All vd-sRNA mapping sites on tomatoes the tomato genome were then clustered into one of the three

following genomic features: intergenic regions, exons, and introns (Figure 13).

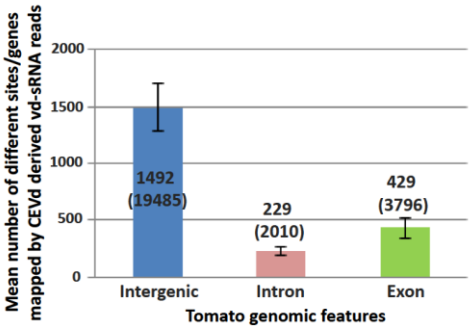

Figure 13. Mean number of different sites/genes mapped by CEVd derived sRNAs for each of the three tomato genomic features: intergenic region, exon (3' and 5'-UTR included), and intron. Standard deviations between samples are depicted as error bars. Mean number of reads for each feature are shown in parentheses.

To assess the possible RdDM induced by CEVd, the differential expression of genes located less than 1 kb from intergenic vd-sRNA mapping sites were checked. The analysis demonstrated that eight genes located less than 1 kb downstream of a vd-sRNA intergenic mapping site were downregulated, as expected in RdDM (Table S3, lines highlighted in green). Impact on expression of gene body methylation is less clear, but many sites in introns and exons could serve as RdDM sites directed by vd-sRNAs.

Likewise, possible PTGS targets were evaluated for genes where vd-sRNAs mapped in exons, by assessing the difference in normalized number of 5' RNA ends in infected samples compared to mocks at expected PTGS sites. Moreover, the differential quantification of small RNAs surrounding the vd-sRNA mapping site was assessed to check the possible production of phased, secondary, small interfering RNAs (phasiRNAs). These analyses showed that five genes, out of the 261 common genes presenting vd-sRNA

similarities in both infected samples (Table S4a), were significantly targeted by vd-sRNAs (Figure 14).

The first PTGS target was a gene annotated as a translational activator gcn1 (Solyc03g025160.3.1), for which an accumulation of vd-sRNAs and a significantly higher number of 5’ RNA ends were observed in infected samples compared to mocks (P = 0.003) (Figure 14A). Although gcn1 was predicted as a potential PTGS target using pospiviroid artificial vd-sRNA, this gene was not found to be differentially expressed in infected samples according to PARE read quantification. These vd-sRNAs derived from a piece of the negative strand overlapping the upper TL and P domain junction, corresponding to the virulence modulating (VM) region (Riesner et al. 1983). Pospiviroids artificial vd-RNAs analysis showed that several tested strains of CEVd (14 out of 253 sequences) and all 28 tested strains of TASVd shared this gene target.

The second gene coding for the argonaute 2A (Solyc02g069260.3.1) showed a higher number of 5’ RNA ends at the vd-sRNA mapping site (P = 0.019). The CEVd domain involved being a part of the positive strand of the lower V domain. Remarkably, the mapping of small RNAs from infected samples presented a two-hit pathway phasiRNAs profile triggered in 5’ by vd-sRNAs and 1361 nt downstream by the microRNA, miR403 (Figure 14B). However, it should be noted that miR403 was not found to be differentially expressed in infected samples compared to mock, and that both infected and mock samples presented a characteristic degradation profile at the miR403 PTGS site. It is also worth noting that argonaute 2A was the gene for which the difference in terms of mapped small RNAs between infected and mock samples was the highest and the most significant (logFC 11.1, FDR 3.50e-24 × 10−24). This gene was also found to be significantly upregulated according to PARE quantification (logFC 2.7, FDR: 0.0002). Pospiviroids

artificial vd-RNAs analysis showed that several tested strains of CEVd (18 out of 253 sequences) and of PCFVd (23 out of 58 sequences) shared this argonaute 2A target site.

A third gene, coding for a putative RNA-binding protein (Solyc08g076660), although not differentially expressed according to RNA ends, was found to present a significantly higher number of PARE read 5' ends than in mocks ($P \leq 0.01$) at four positions within the most vd-sRNA targeted locus (Figure 14C). The analysis of small RNA sequences at this locus showed that they all derived from the negative strand of the 5' part of the VM region covering a region of 26 nt. The gene Solyc08g076660 was also found to collect significantly more small RNAs in infected plants (logFC 6.9, FDR 3.1e-12 × 10−12), primarily due to this vd-sRNAs hot spot. Concerning the function of this CEVd PTGS target, BLASTX analysis of the corresponding mRNA revealed a 100% identity with a protein predicted as a nucleolar protein 12 (GenBank accession: XP_004245924.1), whose exact role is unknown, but which was found to bind to mRNA (Castello et al. 2012). Pospiviroids artificial vd-RNAs analysis also showed that this target is shared within almost all tested strains of CEVd (248 out of 253 sequences) and TASVd (27 out of 28 sequences), but is confined to these species.

The fourth PTGS site was an epoxide hydrolase (Solyc06g009170) targeted by vd-sRNAs from the terminal left of CEVd (Figure 14D). In silico analysis showed that this site is shared between most of the analyzed strains of TASVd and CEVd.

In the fifth PTGS target, vd-sRNAs from the central conserved region form a duplex with mRNAs of a poorly-characterized transmembrane 9 protein (Solyc06g065030) (Figure 14E). In silico analysis showed that this site is shared between many strains of TASVd, CSVd and CEVd.

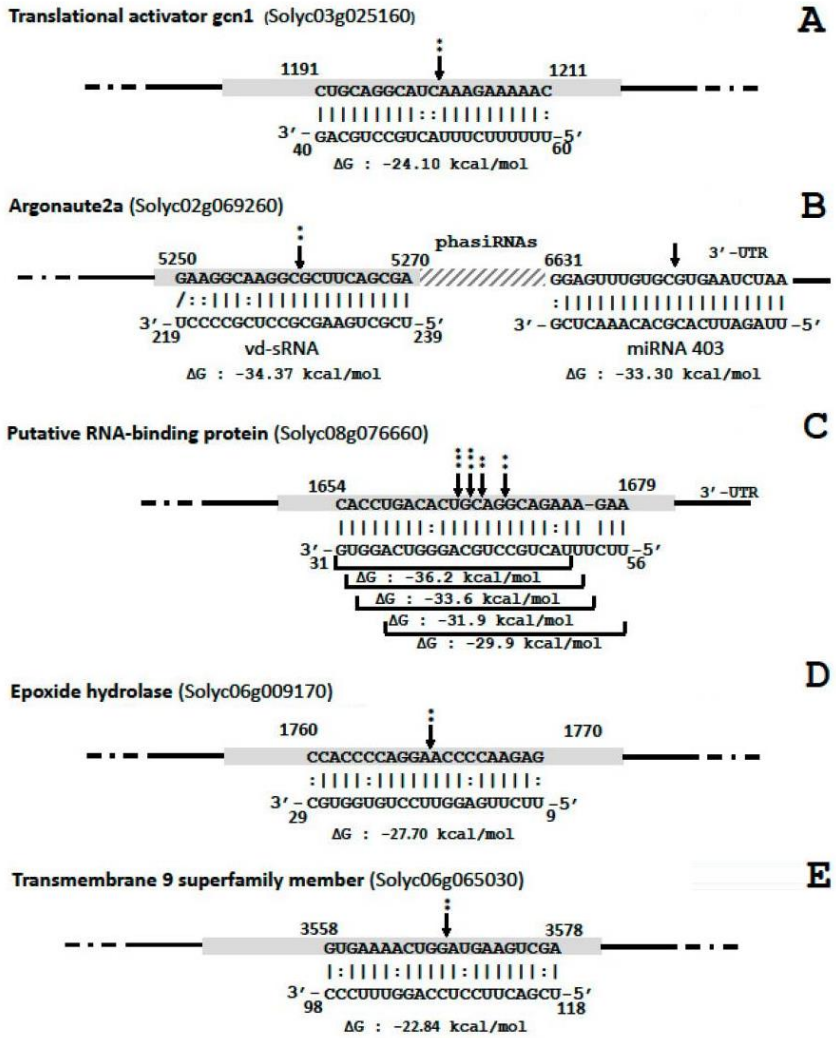

Figure 14. PTGS targets site of CEVd vd-sRNAs of the five genes highlighted in this study: (**A**) translational activator gcn1, (**B**) argonaute 2A, (**C**) putative RNA-binding protein, (**D**) epoxide hydrolase and (**E**) putative RNA-binding protein. The arrows indicate the predicted RISC mediated cleavage sites with their respective probability computed according to Poisson's regression of normalized counts of 5′ RNA ends (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001). Complementarity between nucleotide pairs was depicted as follows: | for Watson-Crick base pairs, / for wobble base pairs and : for mismatches. Coding regions of genes are depicted as gray rectangles; intron and 3′-UTR are represented as black lines. The sequences are shown in the complementary polarity. The PairFold online tool was used to predict the minimum free energy (ΔG) of RNA duplexes.

5.4.3 Tomato transcriptome changes upon CEVd infection

The analysis of the differential expression of RNA ends showed that in CEVd infected plants, 4097 and 3519 genes/ncRNAs were significantly upregulated and downregulated respectively compared to the mock-inoculated plants (|logFC| > 1 and FDR < 0.05) (Table S5). The quantification of small RNAs showed that 67 and 66 genes/ncRNAs collected significantly more and less small RNA respectively in CEVd infected plants (|logFC| > 1 and FDR < 0.05) (Table S6).

According to PARE reads, gene ontology (GO) enrichment analysis revealed that many GO terms were significantly overrepresented in upregulated and downregulated genes identified in infected plants (Tables S7 and S8 respectively).

Because the gene content of the highlighted GO terms greatly overlapped, to avoid redundancy, ontology enriched genes were reported according to the following categories: kinases, pathogenesis-related (PR) genes, transcriptions factors, oxidation-reduction process, protein ubiquitination, DNA methylation and chromatin remodelling, RNAi, and hormone related genes. Also, when it comes to gene annotation, BLASTX analysis of differentially-expressed genes revealed that several discrepancies arose. BLASTX confirmation was thus performed for some specific genes reported hereafter.

5.4.3.1 Kinases

The first category consists of kinases and phosphorylation GO terms which were the most significantly overrepresented in infected sample upregulated genes. In total, 352 upregulated kinases were highlighted in this analysis. Mitogen-activated (MAP) kinases direct cellular responses to a large array

of stimuli. Here, 27 MAP kinases were found to be upregulated, including some known key players in innate immune response: ANP2, MKK2, MPK8 and MPK3. Yet, seven calcium/calmodulin-dependent protein kinases (CDPKs), which may trigger the plant immunity upon cell calcium influx, were found to be upregulated (Table S9).

Besides these MAP kinases, many groups of kinases showing homologies between each other and with known or supposed PRRs were also found to be upregulated in infected plants, including 53 lectin receptor-like kinases (45 G-type and 8 L-type), including 5 LECRK-like kinases involved in innate immunity (Wang and Bouwmeester et al. 2017), 23 LRK10-like homologous to a receptor-like protein kinase involved in wheat rust resistance (Feuillet et al. 1998), 12 RLKs homologous to Arabidopsis gene At3g47570 showing similarity to the gene RXam1 conferring resistance against a strain of *Xanthomonas axonopodis* pv. *manihotis* (Tatis et al. 2018), three homologs of FLS2 known as the PRR that determines the specific perception of the bacterial flagellin flg22 (Gómez-Gómez and Boller et al. 2000), one EFR known as PRR interacting with the peptide elf18 (derived from bacterial EF-Tu) (Kunze et al. 2004), and one homolog of Hcr9-4E providing resistance against *Cladosporium fulvum* (Westerink et al. 2004). Beside immunity-related kinases, 21 kinases showing homology with RLP12-like proteins involved in meristem maintenance were also upregulated in infected plants (Table S9).

5.4.3.2 Disease resistance and PR genes

The majority of disease-resistance genes in plants encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins. In the present study, 63 genes annotated in ITAG3.2 CDS as either disease resistance proteins and/or NBS-LRR, TIR-NBS-LRR, and CC-NBS-LRR were found to be

significantly upregulated in infected plants. Among these genes, 20 putative late blight resistance R1, 15 TMV resistance protein N-like, four disease resistance RPP-like (for resistance to *Peronospora parasitica*), three resistance gene analogs (RGA), two disease resistance protein RPM1 and one disease resistance protein RPS2 conferring resistance to *Pseudomonas syringae*, were found. Beside NBS-LRR, an extracellular leucine-rich repeat (eLRR) gene, Ve2, responsible for verticillium wilt disease resistance, was also found among the upregulated genes (Table S10).

Because the expression of PR proteins is a marker of SAR induction, upregulated genes were checked for the presence of these proteins. This analysis showed that members of 16 families of PRs (Jain and Khurana et al. 2018) were present among significantly upregulated genes. A summary of these genes is presented in Table 10 and mentioned in detail in Table S11.

Table 10. Significantly upregulated PR genes (LogFC > 1 and FDR < 0.05) in infected plants clustered according to their PR family, supposed functions (Liu and Ekramoddoullah et al. 2006; Oide et al. 2013; Breen et al. 2017; Jain and Khurana et al. 2018) and mean overexpression (log FC).

PR Family	Upregulated Genes	Supposed Functions	Mean Overexpression
1	13 genes related to PR-1	Multiple roles ranging from antimicrobial function and defense signal amplification to potential sterol or effector recognition	6.5
2	12 glucanases	Modulators of callose and salicylic acid-dependent defense responses	5.1
3, 4, 8 and 11	18 chitinases	Antifungal properties	5.4
5	5 thaumatins, 3 osmotins and 1 PT-5x	Membrane permeabilizing functions	5.8
6	23 proteinase inhibitors (PIs)	Proteinase inhibitors	4.6
7	16 subtilisin-like proteases and 3 P69 proteins	Proteinases	3.6
9	23 peroxidases	Peroxidases	3.7
10	a S-norcoclaurine synthase 2-like a TSI-1 (tomato stress induced-1)	Antimicrobial activity and in vitro ribonuclease activity, enzymatic activities in plant secondary metabolisms, ligand-binding ability	8.3
12	3 defensin-like proteins	Membrane permeabilizing functions	5.7
13	a thionin-like protein	Membrane permeabilizing functions	2.3
14	9 lipid transfer proteins	Membrane permeabilizing functions	4.8
15	4 germins	Multiple enzymatic, structural and receptor functions	7.3
16	5 germin-like proteins	Multiple enzymatic, structural and receptor functions	2.6

5.4.3.3 Transcriptions factors (TFs)

Ontology enrichment showed that the GO term “DNA binding transcription factor activity” was significantly overrepresented in upregulated genes. In total, 249 genes annotated as TFs were found to be significantly upregulated, including 37 putative WRKY known as elicitor-responsive transcription factors, 22 MYB related, 21 NAC domain-containing proteins, 18 ethylene-responsive, 10 heat shock factors, six MADS-box proteins, six nuclear TFs Y, four LOB domain-containing proteins, four bZIP among which two BZIP17, and a BZIP60 involved in salt and osmotic stress responses and in the activation of unfolded protein response (UPR) respectively, three TGA (TGA2.1, TGA2.3 and TGA4), two pathogenesis-related genes transcriptional activators (PTI5 and PTI6) which activate the defense genes of plants, two ethylene-insensitive 3, 2 MYC (MYC2, MYC3) involved in jasmonic acid (JA) signaling pathways, and a DELLA protein GAI which probably represses the gibberellin (GA) signaling pathway. Remarkably, the key regulator of salicylic acid (SA)-mediated gene expression in systemic acquired resistance, NPR1 protein, was also found among the upregulated genes (Table S12).

Conversely, 153 putative TFs were found to be significantly downregulated. A large diversity was represented among these: six sigma factors (sig A, B, C, D and F) known to regulate the specific transcription of chloroplast related genes, several TFs involved in development and/or floral induction: four auxin response factors, three scarecrow-like proteins, three squamosa promoter-binding-like proteins. Like for upregulated TFs, many uncharacterized TFs were found (Table S12).

5.4.3.4 Oxidation-reduction process

Ontology enrichment revealed many genes involved in oxidation-reduction processes, including 83 cytochromes P450 primarily involved in the formation of terpenoids (Table S13), 23 peroxidases already cited as PR-9 were present and 4 prolyl 4-hydroxylases (P4H4, P4H6, P4H9 and P4H10) which catalyze the post-translational formation of 4-hydroxyproline in -Xaa-Pro-Gly- sequences in proline-rich protein like the plant cell wall glycoproteins: extensins, hydroxyproline-rich glycoproteins, lectins and arabinogalactan proteins, and 13 aminocyclopropane carboxylate oxidases involved in the synthesis of ethylene.

5.4.3.5 Protein ubiquitination

In this study, 36 E3 ubiquitin-protein ligases involved in ubiquitination were found to be significantly overexpressed in infected plants (Table S14). This can reveal the polyubiquitination of proteins and their targeting for destruction by the proteasome. However, it can also be linked to the modification of protein's activity, interactions, or localization. Remarkably, a Avr9/Cf-9 rapidly-elicited protein 1 induced by *Cladosporium fulvum* was found among these ubiquitin-protein ligases.

5.4.3.6 DNA methylation and chromatin remodeling

Highlighted by the nucleosome GO term which was overrepresented in the upregulated genes (P = 0.0001), 27 histone genes (4 H1, 4 H2A, 4 H2B, 10 H3 and 5 H4) were found to be overexpressed in infected samples indicating a significant chromatin remodeling. Likewise, the two core subunits of the condensing complex (SMC2 and 4) were upregulated in infected plants. When it comes to methylation, DRM2, a cytosine methyltransferase involved in de novo methylation in a sequence-

independent manner (Zhang et al. 2018), was found to be strongly overexpressed (LogFC = 4).

Conversely, a chromatin remodeling protein EBS-like supposedly involved in preventing histone acetylation through methylated histone binding and 2 DNA (cytosine-5)-methyltransferases (CMT3 and 1B-like) were found to be downregulated (CMT3 being a methyltransferase that is required for the maintenance of DNA methylation at CHG sites (Lindroth et al. 2001)).

The situation regarding the enzymes involved in histone modifications was difficult to clarify, since several histone acetyltransferases, deacetylases, and histone-lysine N-methyltransferases were found to be both up and downregulated. Moreover, a BLASTX analysis of several of those genes did not allow us to confirm their annotation in ITAG3.2. release (Table S15).

5.4.3.7 RNAi

The RNA silencing machinery was also highlighted in this gene expression analysis, and in order to understand how this machinery is impacted during CEVd infection, all genes and microRNA (miRNA) precursors were isolated from the list of differentially-expressed RNA ends and small RNAs (Table 11).

Table 11: Overexpression of the RNAi machinery components in CEVd infected tomato plants.

Tomato Gene ID	RNAi Machinery Components	PARE		Small RNA		Number of phasiRNA Detected
		Log FC	FDR	Log FC	FDR	
Solyc06g048960.3.1 [a]	Dicer-like 2a	-	-	2.2	0.002	44
Solyc11g008540.2.1 [a]	Dicer-like 2b	3.0	4.80e-5	4.2	5.27e-11	53
Solyc11g008530.2.1 [b]	Dicer-like 2d	6.2	8.92e-6	5.2	3.09e-12	-
Solyc07g005030.3.1 [a]	Dicer-like 4	1.6	0.0008	-	-	-
Solyc02g069260.3.1 [a]	Argonaute 2a	2.7	0.0002	11.1	3.50e-24	102
Solyc01g008960.3.1 [a]	Argonaute 4a	1.3	0.0003	-	-	-
Solyc03g114140.3.1 [a]	RNA-dependent RNA polymerase2	1.18	0.0007	-	-	-
NR_108010	MIR4376	-	-	3.2	1.86e-8	
NR_108011	MIR6022	1.3	0.04	1.80	0.006	
NR_130104	MIR171e	-	-	2.50	0.018	
LM611859	MIR403	-	-	2.3	0.04	
NR_130094	MIR9474	1.5	0.002	-	-	
NR_108004	MIR5300	5.7	0.009	-	-	
LM611867	MIR9472	5.5	0.012	-	-	

[a] PARE reads present in both infected and mock plants; [b] PARE reads only present in infected plants.

The analysis of Dicer-like protein (DCL) in infected plants showed that (i) according to PARE sequencing, DCL 2b was upregulated in infected plants; while the expression of DCL 2d was activated, (ii) DCL 2a, 2b and 2d were also significantly degraded into small RNAs. The mapping pattern of siRNA on the latter three DCL2 mRNAs indicated RNAi degradation through the production of phasiRNAs, which was confirmed for DCL 2a and 2b using UNITAS. Carefully checking of mappings allowed us to rule out the involvement of DCL 2c, to which some PARE and small RNA reads mapped due to sequence similarities shared between the four DCL 2 paralogs. Furthermore, the analysis of RNA end sequences also showed that DCL 4 was significantly upregulated in infected plants, and DCL1 and DCL3 were expressed in both mock and infected plants, but no difference of expression could be found (Table S16).

When it comes to argonautes, only argonaute 2a was found to be both significantly upregulated and degraded through phasiRNA production in infected plants. Argonaute 4a, known to be involved in DNA methylation, was also found to be upregulated. Mapping quality check allowed us to rule out the expression of argonaute 2b and 3 in this study. Argonautes 1, 5, 6, 7, and 10 were found to be present but not differentially expressed between mock and infected plants (Table S17).

RNA-dependent RNA polymerases (RdRP) were also checked for differential expression. Among the five expressed RdRP (2 RdRP2, 1 RdRP5 and 2 RdRP6), only one RdRP2 was found to be upregulated (Table S18).

Several miRNAs/precursors miRNAs were also found to be upregulated according to PARE quantification, and differential quantification of small RNAs highlighted that miR4376, which is known to regulate the expression of an autoinhibited Ca(2+)-ATPase (Wang et al. 2011c), a potential key

player in innate immunity, was significantly overrepresented in infected samples.

It is worth noting that several genes involved in RNAi, SAR, and innate immunity, presented a higher number of both small RNAs and RNA ends in infected samples, indicating a potential control of these genes through RNAi (Table S19). However, except for a leucine-rich-repeat receptor-like protein (Solyc12g009520.2.1), DCL 2b, DCL 2d, and argonaute 2a, the scattered distribution of mapped small RNAs on most of these genes, as well as the size of these small RNAs (often greater than 24-nt), were not compatible with an RNAi origin. Conversely, the four identified genes showed a sRNA distribution consisting of a series of peaks, which is compatible with RNAi degradation in infected plants, and was confirmed for three genes as phasiRNAs using UNITAS.

5.4.3.8 Translation

Many GO terms enriched in the downregulated genes showed that the translation machinery is highly-affected by CEVd infection. Among the genes concerned, 54 ribosomal proteins (35 chloroplastics), 23 tRNA-synthases and methyltransferases, 7 elongation factors, 3 translation initiation factors (2 chloroplastic), and 2 chloroplastic peptide chain release factors, were downregulated (Table S20).

Pentatricopeptide repeat-containing proteins (PPR) bind RNA in a sequence-specific and influence processing, splicing, editing, stability, and translation of RNAs. In this study, 113 PPRs were found to be downregulated in infected plants in which 25 were associated with mitochondrion and 37 with chloroplast. Conversely, 11 putative PPRs were upregulated in infected plants (Table S21).

5.4.3.9 Photosynthesis

Several genes related to photosynthesis and included in different GO terms were significantly downregulated in infected plants. All major protein complexes involved in photosynthesis were impacted: the light-harvesting complexes were affected with the down regulation of 32 chlorophyll a/b-binding proteins as well as 4 proteins involved in chlorophyll synthesis, 38 proteins associated with photosystems I and II were downregulated, 2 proteins involved in the NDH complex as well as 7 subunits of the chloroplastic ATP synthase, and a subunit of the cytochrome b6-f complex, were also downregulated (Table S22).

5.4.3.10 Membrane and excreted proteins

Membrane-related GO terms were significantly overrepresented in both up and downregulated genes, indicating a profound change in cell and organelle membranes. Besides the reported changes occurring in the chloroplastic membranes, a diverse array of membrane proteins was found to be downregulated in infected plants, including 19 NRT1/ PTR FAMILY proteins involved primarily in nitrate and peptide transport, 13 proteins detoxification, 6 PIN and 6 WAT1-related proteins involved in auxin transport and homeostasis, 11 aquaporin-like proteins which facilitate the transport of water across cell membrane, and 11 sugar transporters (Table S23).

When it comes to upregulated membrane proteins, many membrane proteins were also highlighted, including 13 ABC transporters (4 being pumps for glutathione S-conjugates), 6 copper transporters, as well as 7 purine permeases (Table S23).

Besides membrane proteins, excreted proteins were also highlighted in upregulated genes in the ontology enrichment. Along with the

aforementioned PR genes, the aforementioned several genes, also known to be involved in biotic stresses, have been found: six phytosulfokines, peptide hormones which trigger cellular dedifferentiation and redifferentiation upon binding to their membrane receptor, four expansin-like proteins which induce wall stress relaxation, two metalloendoproteinases which may play a role in the degradation and remodeling of the extracellular matrix in response to stresses, and two serpin-like proteins which are potent inhibitors of mammalian chymotrypsin-like serine proteases (Table S24).

5.4.3.11 Hormone related genes

Because for each studied hormone, related genes were found to be both up and downregulated, the relative numbers of up and downregulated genes were compared through binomial regression in order to identify the general trends (Table S25 and S26). This analysis showed that for four hormones related to plant defense, i.e., salicylic acid, abscisic acid, ethylene, and jasmonic acid, the proportions of upregulated genes was highly significantly different from the proportion of downregulated genes ($P < 0.0001$). Conversely, for cytokine, relatively more downregulated genes were found than their upregulated counterparts ($P = 0.05$). No significant differences were found for auxin, brassinosteroid and gibberellin related genes.

5.5 Discussion

This study evidenced CEVd degradation, tomato PTGS targets of CEVd, and tomato transcriptome response upon CEVd infection. In silico analysis of potential pospiviroid PTGS targets allowed us to further confirm and extrapolate the experimental results to other pospiviroids.

In terms of methodology, although PARE read quantification is not generally used to identify differentially-expressed genes, the information provided in this publication was supported by the fact that (i) most of the degradome is not the result of small RNA-mediated cleavage (Addo-Quaye et al. 2009), (ii) the global shape of gene read maps were very similar in infected and mock samples in hundreds of visually inspected genes, (iii) an exhaustive check of CEVd triggered PTGS sites was performed visually by mapping, and (iv) results of this analysis strongly correlated with previous studies and symptoms development (see here after), as well as the already demonstrated SAR activation (López-Gresa et al. 2016).

5.5.1 CEVd degradation

The strand polarities of vd-sRNAs found in this study showed that these latter derived primarily from the positive strand of CEVd. The mappings of these vd-sRNAs on CEVd genome also showed the characteristic patterns consisting of variable peaks and valleys along each strand as observed for different viroids (Bolduc et al. 2010, Diermann et al. 2010, Wang et al. 2011a). The distribution of vd-sRNA lengths, along with the expression of the different DCLs, indicate that DCL1, DCL2s, DCL3, and DCL4 could all be involved in the degradation of viroidal RNAs. However, while the overexpression and activation of DCL2b and 2d respectively can clearly be linked to the high amount of 22-nt vd-sRNAs observed, the overexpression of DCL4 is difficult to assign to 21-nt vd-sRNA production, since it could also be involved in the enhanced phasiRNA degradation observed in the infected samples, as well as in RdDM (Deleris et al. 2016). Since DCL4 is thought to need long, near-perfect dsRNA as substrate (Fukudome and Fukuhara et al. 2017), and as almost no symmetry was found between positive and negative strands in terms of 21-nt vd-sRNAs indicating imperfect dsRNA

origin, the data suggests that these latter sRNAs are mainly produced by DCL1 (Figure 15A). This would also explain the divergent role of DCL4 observed between RNA viruses which are negatively affected by DCL4 compared to PSTVd whose concentration decreases with that of DCL4 (Katsarou et al. 2016). Perfect long dsRNA structures of virus replicative intermediates would be targeted by DCL4, while viroids would remain protected from DCL4 degradation thanks to their imperfect dsRNA structures.

In agreement with this hypothesis, the analysis of RNA ends confirmed the presence of a significant number of predominant subgenomic copies of CEVd, as demonstrated by Minoia et al. (2015). This suggests the involvement of these CEVd genomic fragments, mainly from the positive strand, in the formation of dicer recognizable imperfect dsRNAs. These imperfect dsRNA degradations would also explain the presence of a significant amount of vd-sRNAs of unexpected sizes (18 to 20-nt and 23-nt long), which follow similar mapping patterns of what would be their canonical, 21, 22, or 24-nt duplex counterparts (Figures S1 and S2).

It is also worth noting that the polarity ratio discrepancy observed between vd-sRNAs and RNA ends indicate that positive copies of CEVd are more prone to DCL degradation, while the negative copies are more targeted by RISC. The RISC degradation of negative strand being supported by hot spots of 5’ extremity of RNA ends located where peaks of complementary vd-sRNAs can be observed and the fact that AGO2A is both upregulated in infected samples and targeted by the viroid. This evidence and hypothesis thus supports a complex process of viroid RNAi degradation which is compatible with both the findings of Minoia et al. (2014) that PSTVd titer decreases with the overexpression of AGO1, AGO2, AGO4, and AGO5, and the model developed in Katsarou et al. (2016), where RISC degradation of

viroids allows us to understand why PSTVd titer increases when DCL4 is suppressed. They also allow us to understand why RNAi degradation of CEVd, and more broadly, pospiviroids, leads to asymmetric profiles of vd-sRNAs between positive and negative strands, contrary to what its presumed action on perfect dsRNA would suggest.

5.5.2 Silencing targets of CEVd

Out of the five PTGS sites highlighted in this study, three could directly modulate the fitness of CEVd variants and/or the symptoms caused by the severe isolate used here.

The mRNA of the translational activator gcn1, partially silenced by CEVd, is known to be involved in general protein synthesis repression and increased translation of specific mRNAs through its interaction with eukaryotic initiation factor 2 alpha kinase Gcn2 (eIF2-α kinase Gcn2) in response to stress. Considering that GO term enrichment suggests that protein synthesis was found to be dramatically repressed by CEVd, it could be speculated that this repression is controlled by this translational activator, which stimulates the eIF2-alpha phosphorylation mediated by GCN2 kinase. Interestingly, eIF2-alpha is a central player in the innate response to infection in mammals, and many viruses have evolved strategies to avoid its phosphorylation, which triggers the repression of translation initiation (Walsh et al. 2013). Moreover, eIF2-alpha is also the substrate of the PKR which is activated upon double-stranded viral RNAs binding in mammalian. It should be noted, however, that no pPKR has been found so far (Immanuel et al. 2012), and that GCN2 does not seem to respond to viral infection in plants (Zhang et al. 2008).

When it comes to the nucleolar protein 12, it could be hypothesized that the PTGS inactivation of this RNA-binding protein would either slow down

the detection of the viroid by the plant defense or avoid the hindrance of essential pospiviroid sites by this undesired plant protein.

The potential roles of AGO2a, whose mRNA is degraded through phasiRNA production triggered by vd-sRNAs, is developed above and in the following paragraphs.

Concerning the involvement of vd-sRNAs in RdDM, in silico analysis showed that the promoter region of eight downregulated genes showed similarities with vd-sRNAs. Many genes also presented similarity with vd-sRNAs in their introns and exons, possibly leading to DNA methylation. Furthermore, proteins involved in de novo DNA methylation, i.e., DRM2, DCL2, DCL4, and AGO4, were found to be upregulated in infected samples. However, although 21-22 nt sRNAs could presumably trigger PolV-RdDM, it is worth noting that none of the CEVd-derived sRNAs highlighted in this study spanned the canonical 24 nt length required for PolIV-RdDM (Bolduc et al. 2010). Moreover, further analyses would be needed to confirm whether these DNA targets would be specifically methylated independently of the global methylation changes observed in plants facing biotic stresses (Deleris et al. 2016).

In silico analysis of potential silencing sites of pospiviroid derived sRNAs in tomato showed striking overrepresentation of some gene ontology terms, indicating that the nucleotide sequences of these non-coding RNAs are not only under a selection pressure to maintain their conformation involved in protein interaction, but also to allow the downregulation of specific host proteins through silencing. Remarkably, this analysis showed highly significant overrepresentation of cytoskeleton related terms which highlighted several kinesin genes. Although plant viruses depend on a cytoskeleton for their intracellular transport, little is known about viroid-cytoskeleton interaction. However, it is worth noting that when the 4/1

protein, showing similarity with kinesin but absent in tomato, was downregulated by silencing in tobacco, increased long distance movement of PSTVd was observed (Morozov et al. 2014). Yet, the microtubule network, to which kinesins bind, is known to be involved in immunity response by delivering cell wall components and potentially also other defense agents. Moreover, several effectors alter the function of microtubule-associated proteins during the immune response (Park et al. 2018). Interestingly, β-1, 3-glucan biosynthesis, which is related to callose deposition between the cell wall and the plasma membrane to limit the penetration of pathogens and the cell-to-cell movement of viruses and viroids (Flores 2016), was also highlighted in overrepresented ontology terms. Gene ontology enrichment also revealed potential PTGS sites in many genes related to innate immunity and RNAi. Remarkably, disease resistance proteins able to trigger ETI were found to be among the most stable PTGS duplexes and the most conserved pospiviroid genomic regions.

5.5.3 Innate immunity activation

The transcriptome analysis showed that tomato plants react to CEVd by a major gene expression shift supposedly governed by kinases, transcription factors, chromatin remodeling, and DNA methylation (Figure 15). The consequences of this shift are the downregulation of genes involved in photosynthesis and in protein synthesis and the massive overexpression of a large array of genes involved in innate immunity. The CEVd activation of genes like MAP kinases, CDPKs, defense related TFs, and members of 16 PR families, is thus fully compatible with the induction of the innate immunity proposed by Zheng et al. (Zheng et al. 2017) and with the results obtained in several other studies looking for PSTVd impacts on the tomato

transcriptome (Owens et al. 2012; Więsyk et al. 2018) and metabolism (Bagherian et al. 2016).

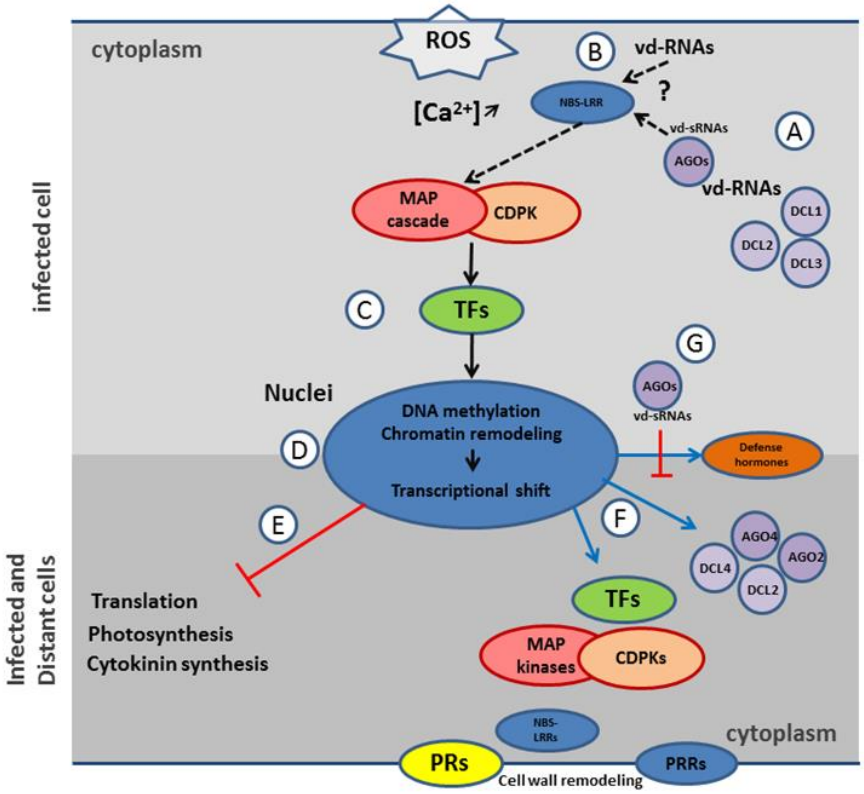

Figure 15. Hypothetical and simplified model of innate immunity and SAR activation by pospiviroids: **(A)** In infected cells, the viroid derived RNAs (vd-RNAs) are degraded by Dicer-like (DCL) 1, 2, and 3 into viroid-derived small RNAs (vd-sRNAs) which are loaded in argonautes (AGOs) to further degrade vd-RNAs. **(B)** vd-RNAs or AGOs loaded with vd-sRNAs are recognized directly or indirectly by nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins which trigger effector-triggered immunity (ETI). **(C)** After reactive oxygen species (ROS) production and Ca2+ cytoplasmic influx, MAP kinase cascade (MAP cascade) and calcium-dependent protein kinases (CDPK) activate transcription factors (TFs), which transduce the innate immune signal. **(D)** Host DNA undergoes methylation and chromatin remodeling which lead to a massive transcriptional shift. **(E)** This shift induces a repression of translation, photosynthesis, and cytokinin biosynthesis, which leads to symptoms. **(F)** This shift also induces the systemic activation of immunity defenses through the overexpression of innate immunity related proteins ranging from pathogen sensors, e.g., pattern recognition receptors (PRRs) and NBS-LRRs, to signal transductors, e.g., MAP kinases, CDPKs, TFs, and defense hormones, and eventually, to defense proteins, e.g., RNAi machinery, PR genes, and cell wall remodeling proteins. **(G)** As counter-defense, pospiviroids contain specific sequences in their genome which, when present in vd-sRNAs and loaded in AGOs, trigger the PTGS degradation of mRNAs involved in dedicated defense.

This work also confirmed the activation of SAR observed by López-Gresa et al. (2016), which is probably linked to the systemic overexpression of the complete arsenal of innate immunity ranging from pathogen sensors (PRR and NBS-LRR), to signal transduction (MAP kinases, CDPKs, TFs, defense hormones), and eventually, to defense proteins (RNAi machinery, PR genes, cell wall remodeling proteins) (Figure 15F). Indeed, it could be speculated that this general reinforcement of cell defenses makes the plants more prompt to react to further infection, whatever the pathogen and PRs are well known as SAR markers. These results, together with previous results (Owens et al. 2012; Zheng et al. 2017; Więsyk et al. 2018), thus showed that pospiviroids are able to consistently activate the innate immunity and SAR of tomato, even in the late stage of infection (47 dpi here), but that this arsenal fails to curb these pathogens.

How tomato activates its innate immunity in response to CEVd is not known, but the proteins highlighted through the mapping of artificial pospiviroid vd-sRNAs and RNAi components are good candidates for both the recognition of vd-RNAs and the activation of innate immunity and SAR. The overexpression of DCL2s and DCL4 would suggest a predominant role of RNA silencing machinery, and it could be hypothesized that a protein-protein interaction with DCL2 and/or the silencing of essential regulating genes by DCL2 derived phasiRNAs are the initial triggers upon pathogenic dsRNA infection. Remarkably, BLASTX analyses of mammal dsRNA receptors RIG-1 and MDA5, using tomato genome as a reference, revealed similarities with DCL3 and DCL4 respectively. When it comes to the possible activation of the innate immunity by DCL2 phasiRNAs, it should be noted that a stringent mapping of these phasiRNAs on tomato CDS conducted in this study did not reveal any good candidates for immunity activators. Interestingly, although the mechanism is still unknown, AGO2 has been

found to play an essential role in antiviral defense (Harvey et al. 2011; Wang et al. 2011b). Remarkably, *ago2* mutants showed increased symptoms but no impact on the viral accumulation. In the present study, the fact that this severe isolate of CEVd triggers both the upregulation and the degradation of AGO2A suggests that AGO2 also plays a pivotal role in antiviroidal defense and modulation of symptoms. The recognition of vd-sRNA loaded AGO2A by innate immunity receptors could also be an interesting avenue of future research (Figure 15B).

Recently, dsRNAs-triggered immunity operating independently of RNA silencing in plant was also demonstrated (Niehl et al. 2016). However, the specific dsRNA receptor was not identified. Considering these findings, together with the absence of pPKR, the TIR-NBS-LRR and the NB-LRR proteins identified by in silico analysis as common potential PTGS target of pospiviroids would constitute good RNA receptor candidates for the direct or indirect recognition of vd-RNAs and the induction of ETI and SAR (Figure 15B). Unfortunately, too few PARE reads were available at the corresponding PTGS sites to confirm the importance of these proteins. Another explanation for these striking common potential immunity sensor targets would be the inactivation of plant immunity for viruses able to allow the transmission of pospiviroids in their natural hosts by trans-encapsidation.

When it comes to symptom induction, the long-lasting impediment of photosynthesis and protein synthesis, along with the potential protein degradation activity, certainly impacts the growth and the phenotype of CEVd infected plants. Yet, the overall downregulation of genes involved in cytokinin production/signaling could also explain the reduction of the apical meristem dominance in pospiviroid infected plants. Since cytokinin is an important plant growth regulator involved in cell division and

differentiation, the typical dwarfing symptom and epinastic leaves induced by pospiviroids could be linked to the phenotypes observed in tobacco showing reduced concentrations of cytokinin (Werner et al. 2001) (Figure 15E).

Moreover, the drastic impact of pospiviroid on all complexes of the photosynthesis machinery, especially chlorophyll biosynthesis and chlorophyll binding proteins, should have a link with the typical chlorosis symptoms observed in the top leaves of pospiviroid infected tomatoes, and was particularly visible with the CEVd strain used in the two tested tomato varieties (cv. Minibel and cv. Heinz 1706). As innate immunity activation is associated with the production of reactive oxygen species (ROS), it could also be hypothesized that the appearance of necrosis observed in leaf vascular tissue is due to a continuous oxidative stress (Baker and Orlandi et al. 1995).

To explain how pospiviroid strains modulate symptoms, several variables probably come into play, among which the most important are probably the efficiency of viroid replication and trafficking, viroid degradation, and the silencing of plant targets.

So far, only the RNAi is known to constitute an effective defense against viroids. However, RNAi can also have a deleterious effect on the plant, since its action can lead to undesired silencing of essential genes. In the present study, the degradation of AGO2A possibly leads to a better viroid replication, and thus, to increased symptoms. The gene silencing through PTGS highlighted in this study would also explain, at least in part, the additional alteration of the host phenotype induced by the severe strain of CEVd used.

5.6 Conclusions

This work confirmed, at the transcriptome level, the activation of innate immunity and SAR by pospiviroids. The downregulation of photosynthesis, translation, and cytokinin-related genes in infected plants provided a good explanation of the symptoms caused by pospiviroids in tomato. The data generated suggests a joint degradation of CEVd by DCLs and RISC from subgenomic copies. The results also confirmed the key role of AGO2 which is both upregulated and degraded in samples infected with the severe strain of CEVd used. In silico analysis of potential PTGS sites indicated that evolution shaped pospiviroid sequences, not only for conformation maintenance, but also to target the silencing of specific host genes. Based on this computer analysis, and considering the experimental results, potential innate immunity receptors recognizing viroid-derived RNAs were suggested, allowing us to propose a first hypothetical model of ETI and SAR activation by pospiviroids.

5.7 Supplementary Materials:

Available online at https://www.mdpi.com/1999-4915/10/11/587/s1

Figure S1: vd-sRNA maps on the positive strand of CEVd genome for each sRNA length, Figure S2: vd-sRNA maps on the negative strand of CEVd genome for each sRNA length, Figure S3: PARE reads maps on positive and negative strands of CEVd genome, Table S1: Summary of sequencing libraries, Table S2a: Potential targets of artificial vd-sRNAs of pospiviroids, Table S2b: Gene ontology enrichment of potential PTGS targets of artificial vd-sRNAs, Table S3: Potential RdDM targets of CEVd derived sRNAs in promoter regions of tomato, Table S4a: PTGS site of CEVd derived sRNAs, Table S4b: Normalized count of PARE 5' ends for the five PTGS targets of vd-sRNAs, Table S5: up and downregulated genes according to PARE reads

quantification, Table S6: Genes for which different amounts of mapped small RNA reads were found in infected compared to mock samples, Table S7: Gene ontology enrichment in genes overexpressed in infected samples, Table S8: Gene ontology enrichment in genes downregulated in infected samples, Table S9: Differentially expressed kinase related genes, Table S10: Differentially expressed disease resistance genes, Table S11: Differentially expressed PR encoding genes, Table S12: Differentially expressed transcription factor encoding genes, Table S13: Differentially expressed cytochrome P450 genes, Table S14: Differentially expressed E3 ubiquitin-protein ligases genes, Table S15: Differentially expressed chromatin related genes, Table S16: Expression of Dicer-like related genes, Table S17: Expression of argonaute related genes, Table S18: Expression of RNA-dependent RNA polymerase related genes, Table S19: Disease related genes upregulated in infected plants for both PARE and small RNA reads, Table S20: Downregulated genes related to translation, Table S21: Differentially expressed pentatricopeptide genes, Table S22: Downregulated genes related to photosynthesis, Table S23: Differentially expressed genes related to membrane proteins, Table S24: Upregulated excreted protein genes, Table S25: Expression of hormone related genes, Table S26: Expression of hormone related genes statistical analysis.

6 General conclusions and future prospects

Viroids are fascinating biological entities which, despite their simple nature, still hide many secrets when it comes to their origin, their routes of natural transmission, the way they induce symptoms and, more generally, how they interact with their hosts.

As viroids can be major harmful pathogens, the understanding of these fundamental aspects may also have very practical consequences on how to manage the diseases they cause. The present thesis focused both on practical and fundamental aspects of these diseases by the setup and the assessment of a new generic detection method of pospiviroids, and the dissection of the molecular mechanisms underlying the interaction between the pospiviroids and their hosts, mostly using the model CEVd and tomato (Olivier and Bragard 2018).

On the other hand, in conjunction with this thesis, a European research project (DEP2) was initiated through the network Euphresco, now hosted at the EPPO, to broaden the scope of the thesis and coordinate the efforts of the European researchers on topics related to pospiviroids. This project allowed testing the newly developed detection method across eight different official labs (Olivier et al. 2016) and brought new information to assess the risk of interspecific host transmission (Van Bogaert et al. 2016) as well as seed transmission (Faggioli et al. 2015). Also, the lack of efficacy of the only approved pospiviroid disinfectant in EU was highlighted (Olivier et al. 2014).

6.1 Detection methods: toward an EU and global consensus.

In the absence of European Reference Laboratories (EURLs) for plant diseases, the National Reference Laboratories (NRLs) themselves had to assess the performance of the detection methods they choose/develop and to compare them with other existing methods. On the other hand, because most of NRLs try to comply with the international standard ISO/IEC 17025, they also have to evaluate their ability to perform these methods on blind samples.

In order to meet these needs, NRLs try to take part in inter-laboratory comparisons (third-line controls) in which they can test their own methods or methods recommended by EPPO in its diagnostic protocols (Phytosanitary Measures series no. 7 - PM7). However, the lack of available ring tests, the operational and analytical constraints imposed by the ring test organizers as well as the paucity of results and the difficulty to interpret qualitative results often prevent the NRLs to fully assess their methods and clearly identify the strengths and weaknesses of each tested method.

The test performance study organized in the framework of DEP2 was developed and the results were analyzed to respond, as much as possible, to the needs of NRLs concerned by the detection of pospiviroids. The results of this comparison allowed the participating laboratories to assess four different methods on tomato leaves and seeds with a minimum of constraints while still permitting them to get a detailed overview of performance criteria of each tested method.

The designation process of EURLs is currently underway and the assessment and the choice of official methods to apply in the EU as well as the

organization of ring tests should be in their core business in the near future. However, the Euphresco network will certainly continue to play an important support and a prospective role, beside and upstream of these EURLs. Also, from a legal point of view, quarantine viroid species are currently transferred to regulated non quarantine pests (RNQPs) to allow the set into force of the Regulation (EU) 2016/2031 repealing the former quarantine directive 2000/29/EC.

Improving detection methods is an ever-evolving process for which the highlighting of new related pathogens or the unravelling of new transmission routes may require further development. Furthermore, the implementation of new regulations, as stated in the previous paragraph, can change the trajectory or stop/freeze this development. Also, the emergence of new technologies such as HTS fosters new diagnostic strategies which favor broad spectrum screenings at the expense of specific detection methods and poses new challenges as stated in the introduction of this work (see Chapter 1.5). Recently, a comparison between the two main strategies of HTS enabling the detection of viroids: sRNA and ribosomal RNA depleted total RNA, showed that the former provides a higher recovery of viroids reads while the latter gave better results in terms of *de novo* assembly with a higher proportion of genomes covered (Pecman et al. 2017; Visser et al. 2016). In this work, a better breadth of CEVd genome coverage was also observed with RNA ends sequencings compare to small RNAs ones. It should be noted however that 3.5 times more nucleotides were sequenced in RNA ends than with sRNAs and that no ribosomal RNA depletion was performed, making the comparison with previous studies difficult. Similarly to the study of Kutnjak et al (2015), no striking variant differences were observed between assembled CEVd

genomes from sRNA and PARE reads indicating that, like for PVY, both sequencing strategies provide a reliable diversity assessment of viroid populations.

Despite the upcoming designation of EURLs and the drafting of an EPPO standard dedicated to pospiviroid detection, a new Euphresco project aiming at ring-testing pospiviroid detection methods: PospiTest, has been launched. This demonstrates the continual need of official laboratories to test their newly developed and routine methods. Meanwhile, the **International Plant Protection Convention** (**IPPC**) is also publishing global diagnostic standards, essentially based on EPPO standard, like the **International Standard for Phytosanitary Measures** (**ISPM**) 27-annex 7 dedicated to the detection of PSTVd. The predominant role of EPPO standard in the drafting of ISPM reflects the leading role of European countries in providing a phytosanitary framework for the world trade of plant material.

In the framework of this thesis, a generic detection method was developed to allow a simple, cost-effective, and robust detection of a large number of strains of all pospiviroid species recognized by the ICTV as well as their correct identification by direct sequencing of amplicons. This result was confirmed through an inter-laboratory comparison which highlighted the fact that the lack of sensitivity of the test regarding CLVd could be fixed by increasing the concentration of the primers. This detection method is particularly adapted to labs handling small number of samples with a relatively high rate of positive which request sequencing. Considering the persistent questions regarding the seed and insect transmission rate of pospiviroids and the spatial distribution of these pathogens in the shoot-

apical meristem, the newly developed detection method is also indicated as a sensitive tool to study these fundamental and major aspects.

Regarding the inter-laboratory comparison, it should be pointed out that many difficulties were encountered for it organization. Production of infected tomato seeds was particularly challenging and the quality checks performed during this production indicated that seeds are more often contaminated externally with infected flesh than truly positive in agreement with the low rate of seed transmission obtained by Faggioli et al. (2015). Secondly, an innovative statistical approach had to be applied for interpreting the results since a variety of conditions had to be compared. Administrative issues related to the need of import permit required for infected material transportation throughout Europe also had to be addressed. Moreover, the absence of dedicated budget also made the organization of this inter-laboratory comparison difficult.

Like for the validation of detection methods, the fact that pospiviroids are genetically diverse and have a broad host range makes the establishment of comprehensive inter-laboratory comparisons impossible. Throughout the existing network of labs analyzing plants for the presence of pospiviroids, a variety of methods have been validated with different reagents. Moreover, the changing regulations, which will probably limit the obligation to carry out testing to a few host crops, also currently prevent the organizers of inter-laboratory comparisons to make the best choice in terms of matrices to test. In this context, it is essential to clearly define the goal of the inter-laboratory comparison through preliminary discussions between labs, and in particular NLRs, together with the recently appointed EURLs coordinators.

However, it should be pointed out that when laboratories comply with ISO/IEC 17025 with a given detection method, whatever their type of scope (fixed or flexible), expensive validations/verifications have already been performed and any changes to their methods result in additional validation/verification and administrative expenses. So, unless detection method are imposed by law, without any real advantage to change their accredited methods, those laboratories will probably keep on using their validated methods, ISO accreditation being legally above EURLs/EPPO recommendations.

6.2 Activation of innate immunity by pospiviroids

So far, no effective measures to cure a crop infected with viroids have been found and a massive infection by these pathogens can lead to high economic losses. Understanding the molecular interactions between viroids and their hosts is thus of primary importance to figure out the determinants of the host plant susceptibility to these pathogens and how the resistance/tolerance of plants could be improved. Beyond such direct practical application, viroids, as the simplest pathosystem known, offer a unique opportunity to further understand how the innate immunity works. Considering the results obtained in previous studies which showed the accumulation of PR proteins during pospiviroid infection (Camacho Henriquez and Sänger 1984; Lucas et al. 1985; Granell et al. 1987; Vera et al. 1989; Domingo et al. 1994; Gadea et al. 1996; Tornero et al. 1996; Itaya et al. 2002; Owens et al. 2012), the upregulation of cell wall and stress hormones related genes (Owens et al. 2012; Bagherian et al. 2016; Więsyk et al. 2018), the activation of SAR (López-Gresa et al. 2016) as well as the upregulation of innate immunity related genes (Owens et al. 2012; Zheng et al. 2017), the present work has shown that innate immunity is activated

upon viroid infection (Olivier and Bragard 2018). Furthermore, beyond the scope of viroids, similar questions about the innate immunity activation by viral nucleic acids also are of major importance for the sustainable control of viral diseases in crops (Nicaise 2017). Currently, viral nucleic acid features are considered as PAMP and thus thought to activate PAMP-triggered immunity (PTI) upon their recognition by pattern recognition receptors (PRRs). However, these hypothetical PRRs recognizing these viral RNA/DNA are unknown and this PTI hypothesis suffers from a major conceptual flaw since PRRs are known to harbor their recognition capabilities in the apoplast while the viral RNA/DNA are found within cells.

In the present work, although confirmations with other techniques would be useful, the analysis of differentially expressed genes (DEGs) between infected and mock tomato plants allowed to demonstrate that the significant transcriptome shift observed in this study and in previous works is primarily linked to the activation of the innate immunity of the host. In-depth analysis of DEGs allowed a comprehensive look at the molecular mechanisms of SAR which indicates that beside the production of PR proteins, plants also overexpress a large array of genes involved in ETI, PTI and SAR allowing the broad-spectrum and rapid detection of further infections. This work also demonstrated that the two branches of PTI: i.e. MAPK cascades and CDPKs are activated since genes from both branches were found upregulated.

Concerning the technical approach used, considering the large number of random degradation sites observed in the sequence datasets and the limited budget available in addition to other considerations mentioned in chapter 5.5., PARE reads were used for DEG analysis. The biological origin of mRNA random degradations is currently unknown, however, the presence

of RNA endonucleases with random cleavage activity is suspected in plant (Zhang and Guo 2017) and, of course, degradations, due to the presence of RNAses in the environment, during extraction, RNA transportation and library preparation cannot be ruled out. Although the results obtained in this thesis using PARE reads for DEG analysis are supported by previous studies, confirmation with other HTS technologies such as RNA-seq or MACE-seq would be useful to ascertain them.

If the downstream signaling cascade of PTI is relatively well known, the ETI counterpart remains largely obscure. In particular, since PTI and ETI are thought to share many downstream pathways, the way ETI is able to trigger subcellular Ca^{2+} influxes, one of the very first and critical steps in PTI to activate CDPKs, is still unknown. Also, since PTI MAPK cascades are seemingly activated by heterotrimeric G proteins (Su et al. 2015; Miller et al. 2016), the link between these latter proteins and ETI remain to be established. Several recent studies show that PRR co-receptors of PTI are also involved both in ETI (Yang et al. 2010; Kørner et al. 2013; Nicaise and Candresse 2017) and dsRNA-triggered innate immunity (Niehl et al. 2016). These co-receptors could then constitute the pivotal link between ETI and PTI since some of them are thought to interact with heterotrimeric G proteins (Aranda-Sicilia et al. 2015; Liang et al. 2016).

In this study, *in silico* analysis performed using artificial pospiviroid derived siRNA allow formulating hypotheses about RNA-triggered innate immunity and possible links between ETI and PTI:
Remarkably, among the most conserved and thermodynamically plausible PTGS sites predicted *in silico*, several proteins potentially able to trigger ETI were highlighted. Although this finding suggesting that pospiviroids are

recognized by this immunity triggers requires further experimental validations, this hypothesis would allow understanding both the recent findings which showed that RNAs alone can trigger innate immunity independently of DCLs (Niehl et al. 2016) and the innate immunity activation observed in this work. These observations open a valuable path of research which would hopefully allow integrating viroids in this general concept of ETI rather than in PTI as it is currently thought for viral RNAs. If this stands true, parallels could be drawn with mammalian NLRs which are able to detect non-self RNAs through the direct or indirect interactions. In mammalians this innate immunity activation leads to the production and excretion of interleukin-1β which triggers inflammatory response through binding to specific transmembrane receptor, called IL-1R, showing structural homologies with the plant PRRs involved in PTI (i.e. external LRR and internal TIR domains) (Imler and Hoffmann 2001). According to this parallel between mammalian and plant innate immunity directed against non-self RNAs, it could be hypothesized that, in plants, viroid/virus RNAs are first detected by NRLs and trigger ETI which, in turn, leads to the production of unknown excreted proteins able to trigger directly or indirectly the PTI/DTI pathway. On the basis of these considerations and on the evidence that PRR co-receptors are involved in immunity response enhancement (Yang et al. 2010; Kørner et al. 2013; Nicaise and Candresse 2017), it would then be the second working model developed in the introduction section – i.e. direct interaction between virus/viroid RNAs with NLRs- possibly combined with the fourth model - indirect activation of PTI through DAMPs- which are the most plausible models (Figure 16).

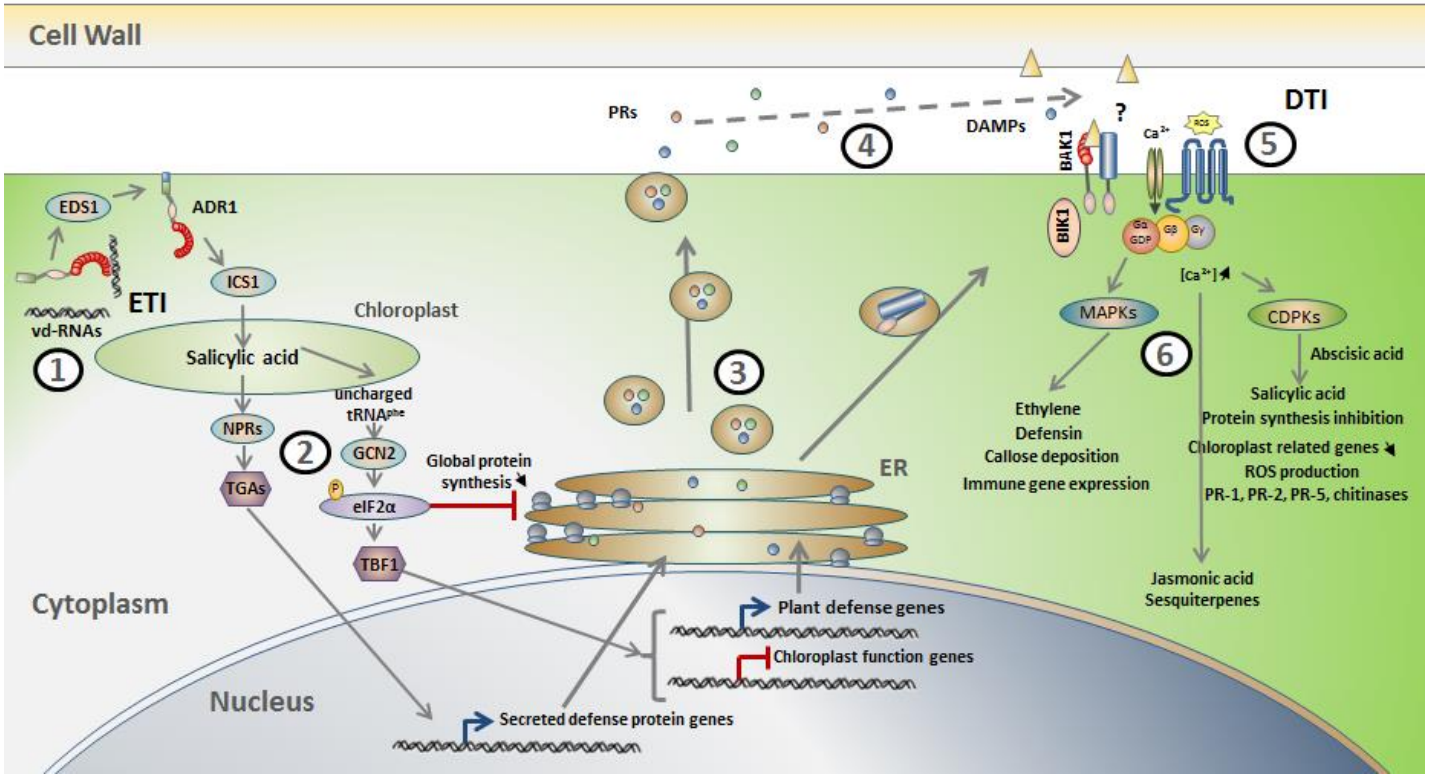

Figure 16: Proposed working model of pospiviroid-triggered innate immunity: 1) viroid-derived RNAs (vd-RNAs) are recognized directly by TIR-NBS-LRRs (TNLs). Activated TNLs interact with the nucleocytoplasmic lipase-like protein Enhanced Disease Susceptibility 1 (EDS1). EDS1 needs ADR1 to activate isochorismate synthase 1 (ICS1) and thus the

production of salicylic acid (SA) in the chloroplast. 2) SA production induces the activation of NPRs regulatory proteins which interact with TGAs transcription factor controlling the expression of PR genes that are associated with systemic acquired resistance (SAR). SA also enhances the presence of uncharged $tRNA^{phe}$ which activate the GCN2 kinase. GCN2 then activates eIF2α which induces a global reduction of protein synthesis and activates the transcription factor TBF1. TBF1 represses chloroplast related genes and induces defense related genes. 3) Pathogenesis-related (PR) proteins and defense related genes mRNAs are translated and processed in the endoplasmic reticulum (ER). 4) Molecules among which PRs are secreted in the apoplast and trigger the release of damage-associated molecular patterns (DAMPs) or mount directly a DTI through their recognition by unknown receptors and co-receptors like BAK1 and the associated kinase BIK1. 5) DTI activation leads to Ca^{2+} influx from the apoplast to the cytoplasm and the production of reactive oxygen species (ROS). 6) These activate CDPKs and MAPKs which, in turn, activate the production of innate immunity molecules whose presence is supported in this work.

The fact that viroids, and thus non-self RNAs, might mount ETI is an important discovery which would allow integrating viroids in the general models of plant innate immunity from which these pathogens have been so far excluded. To test this important hypothesis, the protein-RNA interactions suggested in this work could be tested using different experimental methods like: electrophoretic mobility shift assay (EMSA), systematic evolution of ligands by exponential enrichment (SELEX), RNA pull-down assay, RNA footprinting, RNA immunoprecipitation (RIP) or UV-induced cross-linking immunoprecipitation (CLIP).

Furthermore, the way calcium influxes are triggered in ETI is a key issue to understand: (i) how ETI and PTI are connected, (ii) how ETI can trigger HR through, among others, the activation of CDPK1 and CDPK2 which require calcium influx. The calcium-transporting ATPase (Solyc07g022790) found as a potential PTGS target of six pospiviroid species is certainly an interesting protein to investigate in this respect. Interestingly, BLASTX analysis of Solyc07g022790 mRNA indicated that this gene codes for an endoplasmic reticulum-type calcium-transporting ATPase 3. It is worth recalling here that ER stress, which is associated with HR and SAR, is probably triggered by a disruption of Ca^{2+} subcellular homeostasis (Krebs et al. 2015; Bahar et al. 2016). Interestingly, the transcription factor BZIP60 involved in the unfolded protein response (UPR) was found to be upregulated in CEVd infected tomatoes. Along with the many upregulated genes associated with protein secretion highlighted in this work, these considerations suggest that ER stress and UPR are also activated in pospiviroid infection.

Regarding the transducin/WD40 repeat-like superfamily protein (Solyc07g066140.1.1) found as another potential PTGS target of six

pospiviroid species, it is worth recalling here that: (i) a WD-40 repeat proteins has been proposed as pospiviroid PTGS target (Aviña-Padilla et al. 2015), (ii) in addition to Solyc07g066140, 54 other WD40 containing proteins were found as potential PTGS target and (iii) Gβ, the central component of heterotrimeric G protein allowing the activation of MAPK cascade and interacting with PRR co-receptors (see above), is a WD40 repeat-containing protein. However, BLASTX of Solyc07g066140 mRNA found maximum homology with an uncharacterized *WD repeat-containing protein C2A9.03*. whose role, like the WD-40 repeat protein found by Aviña-Padilla et al. (2015), remains to be determined.

6.3 Symptoms induction by pospiviroids

As a biological indicator of pospiviroids and recently sequenced plant species, *Solanum lycopersicum* (tomato) is a valuable plant model to understand how viroids induce their typical growth reduction and chlorosis symptoms in other hosts.

The impact of innate immunity activation demonstrated in the present work together with the evidence available in the literature allows pinpointing the pivotal role of chloroplasts on pospiviroid symptom expression. Indeed, it has been demonstrated that viroid symptoms are enhanced by the amount and the quality of light available (Hollings and Stone 1973; Harris and Browning 1980; Schlemmer et al. 1985; Grasmick and Slack 1985). On the other hand, the chloroplast is considered as a key regulator balancing immunity response and plant growth according to the available light energy (Stael et al. 2014). The chloroplasts are also thought to trigger hypersensitive response by allowing maximum ROS and SA production when maximum light is available (Roden and Ingle 2009; Zurbriggen et al. 2009; Mur et al. 2010). Although chloroplasts are a very

important source of ROS during immunity response, the way chloroplastic ROS production is regulated is not well understood. Interestingly, Xu et al. (2012) showed that ROS levels increased in all light-harvesting chlorophyll a/b-binding protein (LHCB) mutants they have tested suggesting that ROS accumulation is due to imbalanced photosynthetic antenna complex. It is also worth noting that LHCB genes are downregulated by intensive light and the phytohormone abscisic acid (ABA) (Staneloni et al. 2008) and are considered to have a pivotal role in modulating chloroplast functions and thus plant growth (De Montaigu et al. 2010; Thines and Harmon 2010). The present work showed that 32 LHCBs were downregulated and that several genes involved in ABA synthesis were upregulated. Altogether, these data suggest that ABA induces and/or amplifies the growth reduction observed in viroid infected plants by reducing the expression of LHCBs which, in turn, lead to chloroplastic ROS production and contribute to chlorosis symptom development.

When it comes to photosynthesis, it is worth adding that SA synthesis which has been shown to be likely induced in this work is also known to downregulate - through a cascade involving: GCN2, eIF2α and TBF1 - several regulatory and structural proteins of chloroplasts: PsbR subunit of photosystem II, chloroplast o-succinylbenzoyl-CoA ligase, plastid ribosomal protein, a subunit of the chloroplast NAD(P)H dehydrogenase complex and components involved in thylakoid membrane biogenesis (Pajerowska-Mukhtar et al. 2012). This is in agreement with the downregulation of chloroplast related proteins observed in this work. Remarkably, Pajerowska-Mukhtar et al. (2012) showed that loss-of-function mutants for the downregulated chloroplast genes they found display several developmental defects like: pale pigmentation, dwarfism and lethality.

Due to high transcriptional shift it can produce, the transcription factor TBF1 is thought to be a key player to rapidly reprogram cellular transcription which diverts energy resources to cope with pathogens at the expense of growth and development (Pajerowska-Mukhtar et al. 2012). It is also interesting to recall here that GCN2 is both activated by the mammalian dsRNA sensor PKR and by GCN1 that has been found to be targeted by CEVd through PTGS in this study. The role of GCN1 is likely to mediate the activation of the kinase GCN2 by facilitating transfer of uncharged tRNAs from the ribosomal A-site to the tRNA-binding domain in GCN2 (Sattlegger and Hinnebusch 2000; Castilho et al. 2014).

As stated before, the long-lasting impediment of photosynthesis and protein synthesis, along with the potential protein degradation activity observed in this study, certainly impacts the growth and the phenotype of CEVd infected plants. The overall downregulation of genes involved in cytokinin production/signaling could also explain the reduction of the apical meristem dominance in pospiviroid infected plants. Moreover ABA synthesis, which is likely enhanced in CEVd infected plants, contributes to repress the vegetative growth. Eventually, ROS production that is often associated with innate immunity activation especially under high light intensity and LHCB gene repression is probably associated with the observed necrosis observed in tomato infected with pospiviroids.

Altogether, these data suggest that viroid symptoms are mainly due to the long-lasting activation of innate immunity rather than an undesired silencing effect of vd-siRNAs. In fact, symptoms could actually be more pronounced for viroid isolates unable to sufficiently repress the innate immunity response of the host. However, it cannot be excluded that vd-siRNAs also have deleterious effects on the plant, since their action can lead

to undesired silencing of essential genes. In the present study, the degradation of AGO2, which is obviously involved in several essential but still elusive pathways (see section 1.5.1.2.) as well as the PTGS targeting of the non-characterized nucleolar protein, could have an adverse effect. Also, it could be suggested that the degradation of AGO2A possibly leads to a better viroid replication, and thus, to increased symptoms.

6.4 RNAi interplay

If RNA silencing degradation of viroids is now well established, the exact mechanisms by which this happens remain elusive. In this work, the mapping of viroid derived RNA sequences along the CEVd genome further demonstrated that subgenomic copies of pospiviroids are involved in the degradation of viroids and that not only Dicer-like proteins but also RISC come into play in this process.

Because DCLs show functional redundancies and multiple roles in development, defense and epigenetics, it is particularly difficult to dissect their respective role in pospiviroid degradation. Moreover, the presence of several DCL isoforms further complicates the situation. If imperfect dsRNA structure of viroids can theoretically be first targeted by DCL1, a significant number of pospiviroid subgenomic copies observed in this study allows hypothesizing alternative degradation where other DCLs can come into play. Indeed, the presence of stretches of viroid derived ssRNAs lacking 5'cap or 3'poly(A)-tail allow the action of RDR6. RDR6 can thus produce perfect dsRNAs which allows the degradation by DCL2, DCL3 and DCL4. Since these perfect dsRNAs are supposedly rather short, less than one half of rod-like structures i.e. <90 bp if it happens at random, the role of DCL4, which shows a higher efficiency for dsRNA longer than 100bp, should be

mitigated. DCL2 and DCL3 , whose pivotal role in pospiviroid degradation has been recently demonstrated (Katsarou et al. 2016), should then respectively produce the 22-nt and 24-nt vd-sRNAs observed in pospiviroid infected plants.

When it comes to the degradation through secondary sRNA production of DCL2a, DCL2b and DCL2d observed in the present study, it is worth noting that a recent work also conducted in tomato but with two viruses: TMV and PVX showed the same phenomenon (Wang et al. 2018). The scientists involved in this latter study showed that this phased sRNA degradation was triggered through a feed-back loop involving the 22 nt-long miR6026. The same study showed that, similarly to the present work, DCL2b was upregulated but not DCL2a. This finding indicated a striking similarity between virus and viroid infection in terms of DCL2 impacts. Interestingly, Wang et al. (2018) also showed that DCL2a/b dependent sRNA were primarily 22-nt long from transposable element. They thus suggest that DCL2a and DCL2b play a role in genome stability. The striking similarity of DCL2s regulation expression and defensive role in both viroid and virus infections is thus an interesting topic to explore and is further commented in chapter 6.5. Moreover, the phasiRNA production from DCL2 mRNA certainly involves DCL4 and this would thus explain the epistatic effect of DCL4 on DCL2 observed by Qin et al. (2017) who suggested that DCL2 impedes cell-to cell spread of **virus-induced RNA silencing** (**VIGS**).

It is worth noting that the preferential cleavage sites observed in this study might also give information about the way pospiviroids are cleaved during their replications. Indeed, little is known about the way (+)-stranded oligomers are cleaved into monomers. It is currently assumed that class III RNases are involved in this process (Gas et al. 2008).

As stated before, the polarity ratio discrepancy between vd-sRNAs and RNA ends observed in this study indicates that positive copies of CEVd are more prone to DCL degradation, while the negative copies are more targeted by RISC. The RISC degradation of negative strand being supported by hot spots of 5' extremity of RNA ends located where peaks of complementary vd-sRNAs can be observed and the fact that AGO2A is both upregulated in infected samples and targeted by the viroid. This evidence and hypothesis thus support a complex process of viroid RNAi degradation, compatible with both the findings of (Minoia et al. 2014) that PSTVd titer decreases with the overexpression of AGO1, AGO2, AGO4, and AGO5, and the model developed in (Katsarou et al. 2016), where RISC degradation of viroids allows to understand why PSTVd titer increases when DCL4 is suppressed. This also explains why RNAi degradation of CEVd, and more broadly, pospiviroids, leads to asymmetric profiles of vd-sRNAs between positive and negative strands, contrary to what its presumed action on perfect dsRNA would suggest.

Remarkably, AGO2, highlighted in this study, is thought to be a multifunctional argonaute. Besides being a major player in antiviral defense, AGO2 has also been found to mediate both *de novo* DNA methylation by recruiting 21-22-nt siRNAs (Pontier et al. 2012) and promoting the exocytosis of PR1 (Zhang et al. 2011). Considering that a major DNA methylation and chromatin remodeling are likely activated upon CEVd infection and that global methylation changes are observed in plants facing biotic stresses (Deleris et al. 2016), it could be hypothesized that AGO2 plays a pivotal role in the initiation of *de novo* methylation required in this process. The specific role of AGO2 in DNA methylation during innate immunity activation is thus an interesting avenue to explore.

6.5 Pospiviroid host tissue invasion and transmission

As mentioned in chapter 1.3.2.4, pospiviroids are thought to readily invade all parts of their hosts with the notable exception of SAM and floral organs that are differentially protected according to viroid-host combinations. Experimental evidence demonstrated that these observed differences rely at least on the host ability to protect these vital tissues: (i) by callose deposition (Zhang et al. 2014b), (ii) by specific trafficking factors (Zhu et al. 2002) and (iii) by a mechanism which requires RDR6 (Di Serio et al. 2010). Conversely, the ability of viroids to invade the whole plant and in particular SAMs and floral organs depends on specific sequence motifs contained in their genome (Zhu et al. 2002).

The results obtained in this work suggest that pospiviroid variants have been selected to avoid, or at least modulate, the activation of the innate immunity and especially the immune pathways which lead to callose deposition (chapter 5.4.1.). These results thus support previous observations of Zhang et al. (2014b) that the differential invasion of SAMs observed between two varieties of Argyranthemum could be explained by callose deposition in the plasmodesmata of cells located below SAMs. Obvious links can also be made with the work of Adkar-Purushothama et al. (2015) who showed that vd-siRNA deriving from the VM region of PSTVd target at least two callose synthase genes. This latter work also showed that the ability of PSTVd to target *callose synthase* mRNAs through PTGS greatly affected the accumulation of viroids and the severity of disease symptoms. Although no study of PSTVd distribution in SAM and floral organs was provided in the study conducted by Adkar-Purushothama et al. (2015), based on the data mentioned in this section, it could be hypothesized that callose deposition and viroid dedicated counter-defenses play a pivotal role

in terms of SAM and flower invasion. This tempting hypothesis, surely require further experimental evidence, as pinpointed by Flores (2016), but it provides a strikingly simple explanation to many pending questions about symptomatology, viroid aggressiveness, seed transmission rate, success rate of sanitation in meristem culture.

However, the protective role of RDR6 against SAM invasion by PSTVd observed in *N. benthamiana* (Di Serio et al. 2010) seems to steer against this hypothesis since impaired RNAi would lead to reduced silencing of callose synthase genes and thus better protection of SAM. To explain this apparent discrepancy, it could be suggested that: (i) RDR6 has little or no role in the production of vd-sRNAs involved in the silencing of callose deposition coming from the VM region, (ii) RDR6 is required by the plant to stimulate its general lines of defenses and/or, (iii) to reduce the viroid titer in the plant by targeting other regions of the pospiviroid genome. It is worth noting that similar influence of RDR6 on *Potato virus X* has been demonstrated (Schwach et al. 2005). Since RDR6 is involved in the production of phasiRNAs, parallels could be drawn with DCL2 degradation which was observed both in the present work and for viruses (Wang et al. 2018). Indeed, DCL2 is thought to degrade both viroids (Katsarou et al 2016) and viruses (Garcia-Ruiz et al. 2010). Additionally, DCL4 which is thought to play opposite roles in viral and viroidal infections (Katsarou et al 2016), is also likely involved in DCL2 degradation together with RDR6. Wang et al. (2018) suggested that the strong change in endogeneous siRNAs observed upon virus-induced DCL2 degradation, especially in terms of 24-nt siRNAs, could lead to epigenetic modifications. It could also be hypothesized that the epigenetic modifications that are suggested by the analysis of DEGs in this work and observed in biotic stresses (Deleris et al. 2016) and SAR (Fu

and Dong, X. 2013) could be the direct/indirect consequences of DCL2 degradation which constitute a major change in RNAi.

It is also tempting to hypothesize that the trafficking of 24-nt siRNAs activated upon viroid/virus infection and the subsequent DCL2 degradation (Wang et al. 2018) constitutes the main systemic SAR signaling pathway which would also allow the priming phenomenon and the transgenerational immune memory of SAR (see chapter 1.5.1.1.2.2). This hypothesis would be supported by the fact that phloem is known as the conduit for both systemic RNA silencing and systemic acquired resistance (SAR) (Molnar et al. 2010; Shah et al. 2014).

On the other hand, the striking overrepresentation of GO terms related to cytoskeleton in the list of potential PTGS targets of pospiviroid derived sRNAs (Chapter 5.4.1) is also an interesting path for future research when it comes to viroid trafficking. As mentioned before, cytoskeleton has been associated with PD SEL regulation (Zambryski and Crawford 2000; Su et al. 2010). According to the new data generated in this work, two different mechanisms regulating PD SELs through cytoskeleton could be involved in the restriction of pospiviroid cell-to-cell movements: (i) the PD aperture regulation by actin filaments and (ii) the callose delivery at PD mediated by microtubules. Also, cytoskeleton could be involved in the intracellular degradation of viroids. The in-depth analysis of cytoskeleton related genes highlighted in this work could shed light on this still poorly understood but essential topic for both viroids and viruses.

6.6 Host-pospiviroid co-evolution and characterization of their relationship

The present work also indicates that the genome of pospiviroids is under a selection pressure which retains sequences allowing to degrade, through PTGS, mRNAs coding for proteins involved in viroid dedicated plant defense or hindering essential viroid sites. The study of these PTGS targets is an opportunity to decipher the evolutionary arms race which took place both in pospiviroids sequence and in the genome of their host.

Although viroids are primarily regarded as pathogens because of their most visible impact and their status of quarantine agents, their beneficial effects are also considered in agriculture. In the citrus industry for instance, trials and commercial use of *Citrus dwarfing viroid* as dwarfing agent have been successfully tested to increase yields thanks to earlier and more abundant fruit settings. Moreover, beside their dwarfing ability, viroids can provide some protective effects against fungi as it was observed in citrus trees coinfected with CEVd and *Phoma tracheiphila* (Solel et al. 1995). Although the mechanism underlying this protection is not known, it is presumably attributed to the production of PR proteins (Hadidi 1988). The present work, which demonstrates the upregulation of a large array of immunity and SAR related genes upon CEVd infection, together with many supporting publications showing the accumulation of PR proteins during pospiviroid infection (Camacho Henriquez and Sänger 1984; Lucas et al. 1985; Granell et al. 1987; Vera et al. 1989; Domingo et al. 1994; Gadea et al. 1996; Tornero et al. 1996; Itaya et al. 2002; Owens et al. 2012), upregulation of cell wall and stress hormones related genes (Owens et al. 2012; Bagherian et al. 2016; Więsyk et al. 2018), activation of SAR (López-Gresa et al. 2016) as well as upregulation of innate immunity related genes (Owens et al.

2012; Zheng et al. 2017), are in agreement with this hypothesis which indicates that mild strains of viroids could be used to prime the SAR and thus reinforce the broad spectrum immunity of the host. This immunization capability of viroids was also observed and used to control viral infections like those caused by *Cocoa swollen shoot virus*, *Papaya ringspot virus*, *Tobacco mosaic virus* and *Citrus tristeza virus* (CTV). However, except for CTV in citrus, the protections were not long-lasting (Hadidi et al. 2003). It could be speculated from these observations that viroids activate the broad spectrum immunity of the host but if no specific defense is present in the genotype of the host against the co-infecting agent this latter cannot be defeated but only delayed. Cross-protection effects observed between viroid strains and species (Niblett et al. 1978; Khoury et al. 1988; Duran-Vila and Semancik, J.S. 1990; Singh et al. 1990) are also consistent with this hypothesis although, when it comes to closely related pathogen species, cross-protection is thought to be related to RNAi (Flores et al. 2005).

Although viroid infections present some advantages in intensive citrus orchards, the benefits of using viroids are sometimes considered as too low to compensate for the losses incurred when drought or stress conditions are experienced and the higher risk of damages due to uncontrolled transmission to susceptible varieties.

Considering this evidence, it cannot be excluded that, in their natural environment, host plants of viroids can benefit from the infection of a mild strain. According to the observations reported here before, it could be for instance hypothesized that, in the natural habitat of Citrus, deep tropical and subtropical forests undisturbed by abiotic factors, Citrus individuals able to withstand the presence of viroids could have a double selective advantage over their susceptible counterparts: (i) they tolerate more

efficiently subsequent fungal and viral diseases and, (ii) they benefit from earlier and more abundant fruit settings which would speed up the reproduction of viroid tolerant trees.

On the other hand, viroids would be selected for their ability to be multiplied and to invade their hosts as well as to withstand plant defenses. Unable to produce protein effectors to defeat host defenses, the small size of viroids would be the result of a conflicting selection towards the reduction of the probability to silence essential host genes while retaining the ability to silence dedicated defenses (RNAi, immunity RNA sensors and signaling, and gene involved in the control of PD SEL). The small size of viroid would also allow them to improve their ability to avoid the PD SEL which likely restrains their trafficking from cell to cell. Considering the result of this present work, it cannot be ruled out that the co-evolution between viroids and their natural hosts, although driven by an evolutionary arms race, could have come to mutualistic relationships where both plant and viroid benefit from each other. These beneficial relationships would, however, be fragile equilibriums turning into parasitism on the occasion of mutations either in the viroid or in the host (upon transmission to susceptible hosts). Viroid could foster unsusceptible hosts not only by selecting them for their ability to withstand their presence but also by making them more prone to resist diseases and by accelerating their reproduction. Through the study of molecular interactions taking place between viroids and their hosts, it is thus the history of their co-evolution which unfolds. Although several protein-viroid interactions have been identified, so far, this approach did not allow having a comprehensive overview on how viroid-host pathosystems work and what are the main drivers of their co-evolution. The original approach adopted in this work which relies on RNA similarities between viroid and tomato genome

brought a new and global perspective on the co-evolution which appears to be primarily driven by an evolutionary arms race. The application of this approach based on RNA similarities to other viroids and viruses and other host genomes would certainly allow unraveling new molecular interactions and maybe other evolution drivers while confirming those highlighted in this study. The simple nature of viroids thus makes them a perfect tool to study plant-pathogen coevolution. Considering that at least one viroid can replicate in yeast (Delan-Forino et al. 2011), similar *in silico* analysis with non-plant genomes could also be trialed to help tracing the phylogenetic origin of viroids. The present work suggests that pospiviroids are particularly adapted to their plant hosts and have evolved under a strong selection pressure exerted by plant defenses to restrict both their replication and trafficking.

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