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# STUDY OF THE INTERACTIONS BETWEEN *ARABIS MOSAIC VIRUS* AND ITS HOST PLANTS

Dissertation
by
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**Study of the interactions between *Arabis mosaic virus* (ArMV) and its host plants**

**Abstract:** *Arabis mosaic virus* (ArMV) belongs to the plant virus genus *Nepovirus* of the family *Secoviridae*. In the wine producing areas southwest of Germany, including Neustadt an der Weinstrasse (NW), ArMV is, along with the *Grapevine fanleaf virus* (GFLV) and the *Raspberry ringspot* virus (RpRSV), two other nepoviruses, a causative agent of the grapevine fanleaf disease, one of the most widespread and damaging virus diseases affecting grapevine. ArMV is transmitted by the nematode vector *Xiphinema diversicaudatum*, and has a wide natural host range. Nepoviruses have two single-stranded positive sense genomic RNAs, which are linked to a VPg at their 5' ends, and polyadenylated at their 3'ends.

ArMV isolates from different hosts and geographical origins were mechanically inoculated onto *Chenopodium quinoas*. The symptoms obtained with ArMV-NW were very mild, whereas ArMV-Lilac and –Lv produced symptoms of different severity. To characterize the symptom determinant(s) encoded by ArMV, fragments corresponding to genes from both RNAs 1 and 2 of full-length infectious clones of ArMV-NW were exchanged by their counterpart of the ArMV-Lv or -Lilac isolates and tested by mechanical inoculations onto *Chenopodium quinoa* for their infectivity and functionality. The results obtained from the first set of clones showed the N-terminal protein of the protein 2A, the movement protein and the protein 1A are involved in the symptoms development.

In *Nicotiana benthamiana*, the establishment rates of infection between ArMV-NW and -Lv differed, however the recovery phenomenon took place around the same time for both isolates, resulting in a disappearance of symptoms in ArMV-Lv-infected plants and a similarly low accumulation of viral RNAs for both isolates. Moreover, the ArMV-NW-recovered plants were not resistant to a secondary infection with ArMV-Lv.

Post-Transcriptional Gene Silencing (PTGS) is an important antiviral defense system in plants. However, numerous viruses have developed a counter-defense strategy, by coding for a protein acting as a suppressor of gene silencing. So far, no suppressor of gene silencing has been identified for nepoviruses. The use of wild-type and GFP-transgenic *Nicotiana benthamiana* 16C for coinfiltration experiments via *Agrobacterium tumefaciens* of constructs containing the GFP and the different genes encoded by ArMV RNAs 1 or 2 allowed to identify the implication of NTB, VPg-Pro and/or VPg-Pro-Pol in the suppression of RNA silencing.

**Étude des interactions entre le virus de la mosaïque de l'arabette (ArMV) et ses hôtes**

**Résumé:** le virus de la mosaïque de l'arabette (ArMV) appartient au genre *Nepovirus* de la famille des *Secoviridae*. Dans les régions viticoles du sud-ouest de l'Allemagne, y compris Neustadt an der Weinstrasse (NW), l'ArMV, le virus du court-noué de la vigne (GFLV) et le virus des taches annulaires du framboisier (RpRSV) sont des agents causals de la maladie de court-noué de la vigne, l'une des maladies les plus répandues et dévastatrices touchant la vigne. L'ArMV est transmis par le nématode vecteur *Xiphinema diversicaudatum* et possède une large gamme d'hôtes naturels. Les Népovirus ont deux ARNs génomiques simple brin de polarité positive, caractérisés par une VPg à leurs extrémités 5 'et par une queue polyA à leurs extrémités 3'.

Différents isolats d'ArMV provenant de différents hôtes et d'origines géographiques différentes ont été inoculés mécaniquement sur *Chenopodium quinoa*. Les symptômes obtenus avec l'isolat ArMV-NW sont très légers alors que les isolats ArMV-Lv et-Lilac produisent des symptômes de gravité différente. Pour caractériser le ou les facteurs déterminant les symptômes codé par l'ArMV, des fragments de gènes correspondant à l'ARN1 et à l'ARN2 de l'ArMV-NW ont été échangés par leurs homologues des isolats ArMV-Lv et-Lilac, et leur pouvoir infectieux et leur fonctionnalité ont été testés par des inoculations mécaniques sur *Chenopodium quinoa*. Les résultats obtenus suite à la première série de clones ont montré que la région N-terminale de la protéine 2A, la protéine de mouvement et la protéine 1A sont impliquées dans le développement des symptômes.

L'établissement de l'infection est différent entre les isolats ArMV-NW et -Lv sur *Nicotiana benthamiana* alors que le phénomène de « recovery » a lieu en même temps pour ces deux isolats, entraînant une disparition des symptômes chez les plantes infectées par l'isolat ArMV-Lv, et une accumulation faible des ARNs génomiques pour ces deux isolats. En outre, les plantes infectées par l'isolat ArMV-NW ne résistent pas à une seconde infection avec l'isolat ArMV-Lv.

Le « Post-transcriptional Gene Silencing » (PTGS) est un système de défense antiviral chez les plantes. Toutefois, de nombreux virus ont développé une stratégie de défense, en codant pour une protéine agissant comme un suppresseur de PTGS. Jusqu'à présent, aucun suppresseur de PTGS n'a été identifié chez les nepovirus. Des expériences de co-infiltration de souches *d'Agrobacterium tumefaciens* contenant soit la construction GFP ou soit la construction virale sur des plantes *Nicotiana benthamiana* et sur des plantes *Nicotiana benthamiana* transgéniques exprimant la GFP ont permis d'identifier l'implication de la protéine NTB, des constructions VPg-Pro et/ou VPg-Pro-Pol dans la suppression du PTGS.

**Untersuchung der Wechselwirkung zwischen dem *Arabis Mosaik Virus* (ArMV) und seinen Wirtspflanzen**

**Zusammenfassung:** Das Arabis Mosaik Virus (ArMV) gehört zum Planzen-Viren Genus *Nepovirus* innerhalb der Familie der *Secoviridae*. In den Weinanbaugebieten im Südwesten Deutschlands, einschliesslich Neustadt an der Weinstrasse (NW), verursacht ArMV, zusammen mit dem *Grapevine fanleaf virus* (GFLV) und dem *Raspberry ringspot virus* (RpRSV), zwei weiteren Nepoviren, die Blattrollkrankheit, eine der am weitest verbreiteten und große Schäden verursachenden Krankheit bei Reben. ArMV wird durch den Nematoden *Xiphinema diversicaudatum* übertragen und hat einen großen Wirtspflanzenkreis.

ArMV Isolate von verschiedenen Wirten und geographischen Ursprüngen wurden mechanisch auf *Chenopodium quinoa* inokuliert. Die durch ArMV-NW verursachten Symptome waren sehr mild, wogegen die durch ArMV-Lilac und –Lv verursachten Symptome von unterschiedlicher Stärke waren. Um die von ArMV kodierten Syptom-Determinanten zu charakterisieren, wurden Fragmente, homolog zu Genen der beiden RNAs 1 und 2 von infektiösen Volllängenklonen von ArMV-NW mit ihren Gegenstücken der ArMV-Lv oder-Lilac Isolate ausgetauscht und ihre Infektiosität und Funktionalität durch mechanische Inokulation auf *Chenopodium quinoa* getestet. Die Ergebnisse, die mit dem ersten Set von Klonen erhalten wurden, zeigten, dass das N-terminale Protein des 2A Proteins, das Movement Protein und das Protein 1A in die Symptomentwicklung involviert sind.

In *Nicotiana benthamiana* etablierten sich Infektionen mit ArMV-NW und –Lv mit unterschiedlicher Häufigkeit, das Recovery Phänomen fand jedoch bei beiden Isolaten ungefähr zum gleichen Zeitpunkt statt und resultierte in einem Verschwinden der Symptome bei ArMC-Lv-infizierten Pflanzen und in einer ähnlich niedrigen Akkumulation viraler RNA bei beiden Isolaten. Darüber hinaus waren die Pflanzen, die nach einer ArMV-Infektion Recovery zeigten, nicht resistent gegenüber einer Sekundärinfektion mit ArMV-Lv.

Das Post-transkriptionelle Gene Silencing ist eine wichtige Abwehr gegen Virusinfektionen bei Pflanzen. Zahlreiche Viren haben jedoch eine Gegenstrategy entwickelt und kodieren für ein Protein, das als Suppressor des Gene Silencing fungiert. Bisher wurde für Nepoviren kein Suppressor des Gene Silencing identifiziert. Ko-Infiltrationsexperimente von wild-Typ und GFP-transgenen *Nicotiana benthamiana* 16C mit Konstrukten, die GFP sowie verschiedene durch die ArMV RNA 1 oder 2 kodierte Gene trugen, erlaubten NTB, VPg-Pro und/oder VPg-Pro-Pol als in die Suppression von RNA Silencing involviert zu identifizieren.

# PREVIEW

Viruses cause many important plant diseases and are responsible for worldwide huge losses in crop production and quality. In the world, all the economical crops can be infected by diverse parasites such as fungi, bacterial, insects, acaroids, nematodes and viruses.

The grapevine is an important economic crop, particularly in France, where the vineyard represents a surface of 872,000 hectares. The grapevine can be infected by bacteria i.e *Xylella fastidiosa* and fungi such as *Plasmopara viticola* responsible for the mildiou disease, *Botrytis cinerea* and *Guignardia bidwellii*

The viruses that infect the grapevines belong to different genera i.e. *Grapevine fanleaf virus* (GFLV, *Nepovirus*), *Arabis mosaic virus* (ArMV, *Nepovirus*), *Grapevine leafroll associated virus* (GFLRaV, *Closterovirus*), *Grapevine virus A* and *Grapevine virus B* (GVA, GVB, *Vitivirus*), *Rupestris stem pitting associated virus* (RSPaV, *Foveavirus*) and *Grapevine fleck virus* (GFkV, *Tymovirus*). The diseases due to these viruses are characterized by leaf deformation, mosaic, degeneration and wilting and are known to reduce the yield and to affect the qualities of the fruits. It is estimated that approximately 60% of the french vineyard are infected by GFLV.

Chemical products used to eradicate the grapevine pathogens, to preserve and save the agricultural economy of the countries are pointed out because of their toxic effects on the environment and on the sanitary health. Therefore, the research and the development of alternative strategies are investigated. For this, it is absolutely necessary to have a better knowledge of the molecular and biological aspects of these pathogens.

The viruses are intracellular parasites that need the host transcription and translation machineries for their multiplication. The plants have developed diverse pathways of defense to eliminate the viruses such as the hypersensitive response and RNA silencing. In response, the viruses evolved a counter-defense, which leads to a *modus vivendi* between the plant and the virus. Indeed, an equilibrium is established between the plant and the virus to allow the survival of both.

During my thesis, I studied the interactions between *Arabis mosaic virus* (ArMV), in particular the NW, Lv and Lilac isolates and herbaceous hosts. My PhD work aimed at the characterization of the ArMV symptom determinants, the identification of suppressor(s) of the RNA silencing and the recovery phenomenon. The manuscript starts with a description of the current knowledge on nepoviruses. First, I describe the diseases induced by ArMV and the transmission of nepoviruses by the nematodes. Thereafter, I address the molecular biology of nepoviruses (virions, genome, functions of the proteins, expression strategy…) with the emphasis made on ArMV and GFLV.

The first chapter of this manuscript concerns the characterization of the viral protein(s) involved in the development of symptoms. After a brief presentation of the external symptoms displayed by

virus-infected plants as well as some examples of symptom determinants identified for other viruses, I described the study that leads to the characterization of ArMV determinants involved in the expression of disease symptoms. This study was performed using three isolates of ArMV, which induce different symptoms on *Chenopodium quinoa* and chimeric full-length cDNA clones of ArMV-NW isolate in which partial and/or complete genes were exchanged by their counterpart of the ArMV-Lv and -Lilac isolates.

The second chapter started with an introduction on RNA silencing, which plays a crucial role in antiviral defence in plants by inhibiting viral accumulation and preventing systemic infection. The first sub-chapter concerns the study of the establishment of the recovery phenomenon on *Nicotiana benthamiana* plants infected either by ArMV-NW or ArMV-Lv. The goal was to see if it is conceivable to develop a strategy of cross-protection of ArMV host plants, using two isolates, a mild and an aggressive ArMV isolates. The last sub-chapter relates the role of RNA silencing and the strategies developed by ArMV against this antiviral defence. I also reviewed the different strategies used by the viruses to inhibit RNA silencing pathway such as the expression of viral suppressor of RNA silencing (VSRs).In order to identify the VSR(s) encoded by ArMV, we used different approaches on *N. benthamiana* and *Arabidopsis thaliana*, which have been already developed for the identification of VSRs of other viruses.

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## Virus Abbreviations

### Nepovirus

*Apricot latent ringspot virus* (ALRSV)
*Arabis mosaic virus* (ArMV)
*Arracacha virus A* (AVA)
*Artichoke aegean ringspot virus* (AARSV)
*Artichoke Italian latent virus* (AILV)
*Artichoke yellow ringspot virus* (AYRSV)
*Beet ringspot virus* (BRSV)
*Blackcurrant reversion virus* (BRV)
*Blueberry leaf mottle virus* (BBLMV)
*Cassava American latent virus* (CALV)
*Cassava green mottle virus* (CGMV)
*Cherry leaf roll virus* (CLRV)
*Chicory yellow mottle virus* (CYMV)
*Cocoa necrotic virus* (CoNV)
*Crimson clover latent virus* (CCLV)
*Cycas necrotic stunt virus* (CNSV)
*Grapevine Anatolian ringspot virus* (GARSV)
*Grapevine Bulgarian latent virus* (GBLV)
*Grapevine chrome mosaic virus* (GCMV)
*Grapevine fanleaf virus* (GFLV)
*Grapevine Tunisian ringspot virus* (GTRSV)
*Hibiscus latent ringspot virus* (HLRSV)
*Lucerne Australian latent virus* (LALV)
*Mulberry ringspot virus* (MRSV)
*Myrobalan latent ringspot virus* (MLRSV)
*Natsudaidai dwarf virus* (NaDV)
*Navel orange infectious mottling virus* (NOIMoV)
*Olive latent ringspot virus* (OLRSV)
*Peach rosette mosaic virus* (PRMV)
*Potato black rinsgpot virus* (PBRSV)
*Potato virus U* (PVU)
*Raspberry ringspot virus* (RpRSV)
*Tobacco ringspot virus* (TRSV)
*Tomato black ring virus* (TBRV)
*Tomato ringspot virus* (ToRSV)

### Other genus

*Barley stripe mosaic virus* (BSMV, *Hordeivirus*)
*Barley yellow mosaic virus* (BYMV, *Hordeivirus*)
*Bean pod mottle virus* (BPMV, *Comovirus*)
*Beet curly top virus* (BCTV, *Geminivirus*)
*Beet necrotic yellowing mosaic virus* (BNYVV, *Benyvirus*)
*Beet western yellows virus* (BWYV, *Polerovirus*)
*Beet yellow virus* (BYV, *Closterovirus*)
*Bhendi yellow vein mosaic virus* (BYMV, *Begomovirus*)
Broad bean wilt virus 2 (BBWV 2, Fabavirus)
*Brome mosaic virus* (BMV, *Bromovirus*)
*Carnation vein mottle virus* (CVMV, Potyvirus)
*Cauliflower mosaic virus* (CaMV, *Caulimovirus*)

*Citrus tristeza virus* (CTV, *Closterovirus*)
*Cocoa swollen shoot virus* (CSSV, *Badnavirus*)
*Cowpea mosaic virus* (CPMV, Comovirus)
*Cucumber mosaic virus* (CMV, *Cucumovirus*)
*Cymbidium mosaic virus* (CyMV, *Potexvirus*)
*High plains virus* (HPV, unassigned genus)
*Lettuce mosaic virus* (LMV, *Potyvirus*)
*Maize streak virus* (MSV, *Mastrevirus*)
*Pea enation mosaic virus* (PEMV, *Enamovirus*)
*Peanut clump virus* (PCV, *Pecluvirus*)
*Pelargonium flower break virus* (PFBV, *Carmovirus*)
*Plum american line pattern virus* (APLPV, *Ilarvirus*)
*Plum pox virus* (PPV, *Potyvirus*)
*Potato virus X* (PVX, *Potexvirus*)
*Potato virus Y* (PVY, *Potyvirus*)
*Prunus necrotic ringspot virus* (PNRV, *Ilarvirus*)
*Raspberry ringspot virus* (RpRSV, *Nepovirus*)
*Red clover necrotic mosais virus* (RCNMV, *Dianthovirus*)
*Rice hoja blanca virus* (RHBV, *Teniuvirus*)
*Rose mosaic virus* (RMV, *Ilarvirus*)
*Tobacco etch virus* (TEV, *Potyvirus*)
*Tobacco mild green mosaic virus* (TMGMV, *Tobamovirus*)
*Tobacco mosaic virus* (TMV, *Tumbovirus*)
*Tobacco necrotic virus* (TNV, *Necrovirus*)
*Tobacco rattle virus* (TRV, *Tobravirus*)
*Tobacco vein mottling virus* (TVMV, *Potyvirus*)
*Tomato bushy stunt virus* (TBSV, *Tobamovirus*)
*Tomato leaf curl Java Virus* (ToLCJAV, *Begomovirus*)
*Tomato spotted wilt virus* (TSWV, *Tospovirus*)
*Tomato yellow leaf curl China virus* (TYLCCNV, *Begomovirus*)
*Tulip breaking virus* (TBV, *Potyvirus*)
*Turnip crinckle virus* (TCP, *Carmovirus*)
*Turnip mosaic virus* (TuMP, *Potyvirus*)
*Turnip yellow mosaic virus* (TYMV, *Tymovirus*)
*Zucchini mosaic virus* (ZYMV, *Potyvirus*)

# NEPOVIRUSES AND *ARABIS MOSAIC VIRUS*

## I. Nepoviruses: disease and transmission

### 1. Nepovirus host specificity

Nepoviruses have a worldwide large natural host range, including various agriculture crops such as grapevine, small fruits, fruit trees or hortical plants. The diseases due to these viruses have an enormous impact on both the productivity and the quality of the cultivated plants. The grapevine fanleaf disease, discovered in the 1950s, is one of the most widespread and damaging nepovirus disease affecting the grapevine. This disease, which is mainly due to *Grapevine fanleaf virus* (GFLV) and at a lesser extent, to *Arabis mosaic virus* (ArMV) and to *Raspberry ringspot virus* (RpRSV), occurs in most temperate regions where *Vitis vinifera* and hybrid rootstocks are cultivated. It has been reported in Asia, Africa, Europe, New Zealand, South Australia, North and South America (Bovey *et al.*, 1990; Martelli and Savino, 1990). About 60% of the French vineyard is infected by GFLV and the economical lost is evaluated to one billion per year (Fuchs *et al.*, 2006). Grapevine losses resulting from the infection by these nepoviruses depend on the virulence of the virus isolate, the susceptibility of the grapevine variety and environmental factors (Bovey *et al.*, 1990; Martelli and Savino, 1990). Different strains of GFLV have been identified in *Vitis vinifera*, *V. rupestris*, cultivar (cv.) Chardonnay, cv. Huxel and cv. Cabernet. GFLV and ArMV cause progressive degeneration and malformations of berries, leaves and canes (Pearson and Goheen, 1991). Fruit quality is also altered with a substantial decrease in sugar content and titrable acidity (Martelli et Savino, 1990; Andret-Link *et al.*, 2004a). Unlike GFLV, ArMV is not confined to grapevine in nature; it can infect 93 plant species, belonging to 28 different families (Murant, 1981) such as raspberry, strawberry, olive, lilac … (Table 1). The grapevine is also infected by other nepoviruses i.e *Grapevine Bulgarian latent virus* (GBLV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine Tunisian ringspot virus* (GTRSV) and *Tomato ringspot virus* (ToRSV).

The small fruits such as strawberry, raspberry, blueberry, black currant and red currant are also hosts for ArMV and several other nepoviruses i.e *Blueberry leaf mottle virus* (BBLMV), *Blackcurrant reversion virus* (BRV), *Cherry leaf roll virus* (CLRV), RpRSV, *Tomato black ring virus* (TBRV), ToRSV and *Tobacco ringspot virus* (TRSV).

Fruit trees growing both in temperate and tropical regions such as peach, apricot, apple, cherry, plum, walnut and almond, are also subjected to infection by nepoviruses: *Myrobalan latent ringspot virus* (MLRSV), *Peach rosette mosaic virus* (PRMV), CLRV, RpRSV and ToRSV.

Many horticultural crops among which hop, soybean, potato, beet and tobacco are infected by nepoviruses: *Artichoke Italian latent virus* (AILV), ArMV, *Arracacha virus A* (AVA), *Beet ringspot virus* (BRSV), GCMV, *Potato black ringspot virus* (PBRSV) and TRSV. Finally, several nepoviruses also induce diseases in ornamental species i.e *Cycas necrotic stunt virus* (CNSV), which was the first nepovirus reported to infect gymnosperm species in the families of *Aizoaceae*, *Amaranthaceae* and *Chenopodiaceae* (Kusunoki *et al.*, 1986).

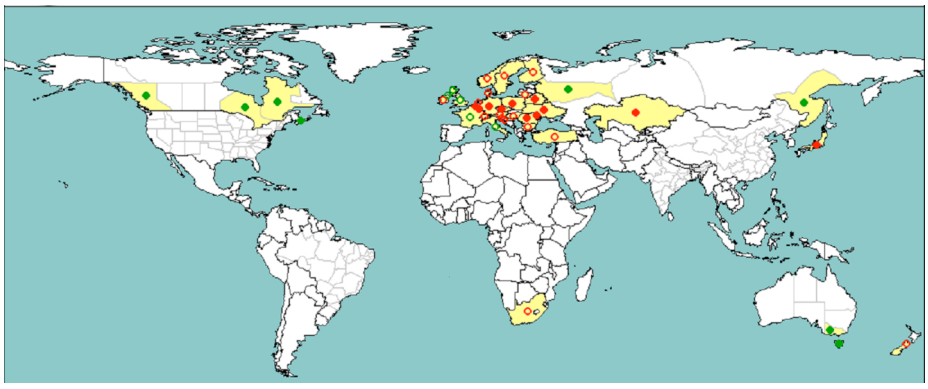
| Isolate | Geographical origin | Exp. Host | Natural host |
|---|---|---|---|
| NW | Hambach, Germany | | Grapevine Pinot gris |
| Bo Ro | Bodenheim, Germany | Grapevine | Grapevine Rondo |
| Bo Ru | Bodenheim, Germany | Grapevine | Grapevine Rülander |
| Dw D | Bad Dürkheim, Germany | Grapevine | Grapevine Dornfelder |
| Ha R | Haärdt, Germany | Grapevine | Grapevine Riesling |
| Lu Ro | Ludwigshöhe, Germany | Grapevine | Grapevine rondo |
| Lu SB | Ludwigshöhe, Germany | Grapevine | Grapevine Pinot noir |
| Se Ru | Seibert, Germany | Grapevine | Grapevine Rülander |
| Sw SR | Schweigen, Germany | Grapevine | Grapevine Schwarzriesling |
| Wa MT | Wachenheim, Germany | Grapevine | Grapevine Müller Türgau |
| We Ch | Weyher, Germany | Grapevine | Grapevine Chardonnay |
| Wl CS | Walheim, Germany | Grapevine | Grapevine Cabernet Sauv. |
| Wl SL | Walheim, Germany | Grapevine | Grapevine St laurent |
| W17 | Eibelstadt, Germany | Grapevine | Grapevine Blaue Sylvaner |
| A2 | Unknown | Grapevine | Grapevine Syrah |
| C1200 | Unknown | Grapevine | Grapevine Chardonnay |
| C1296 | Unknown | Grapevine | Grapevine Chardonnay |
| D8 | Unknown | Grapevine | Grapevine Chardonnay |
| H133 | Unknown | Grapevine | Grapevine Chardonnay |
| H138 | Unknown | Grapevine | Grapevine Chardonnay |
| J86 | Unknown | Grapevine | Grapevine Riesling |
| K43 | Unknown | Grapevine | Grapevine Ugni blanc |
| K77 | Unknown | Grapevine | Grapevine Riesling |
| L3 | Unknown | Grapevine | Grapevine Riesling |
| L7 | Unknown | Grapevine | Grapevine Riesling |
| Muse | Le Grau du roi, France | *C. quinoa* | Grapevine Muscadelle |
| N37 | Unknown | Grapevine | Grapevine Cabernet France |
| N205 | Unknown | Grapevine | Grapevine Ugni blanc |
| P116 | Unknown | Grapevine | Grapevine Ziruck |
| P118 | Unknown | Grapevine | Grapevine Ziruck |
| P119 | Unknown | Grapevine | Grapevine Ziruck |
| P120 | Unknown | Grapevine | Grapevine Ziruck |
| T62 | Unknown | Grapevine | Grapevine Lornet |
| T68 | Unknown | Grapevine | Grapevine Lornet |
| T69 | Unknown | *C. quinoa* | Grapevine |
| T71 | Unknown | Grapevine | Grapevine |
| 6/29 | Unknown | Grapevine | Grapevine |
| 862 | Unknown | *C. quinoa* | Grapevine |
| AB10 | United Kingdom | *C. quinoa* | Sugar beet |
| C | United Kingdom | *C. quinoa* | Raspberry |
| H | United Kingdom | *C. quinoa* | Hop |
| Lilac | United Kingdom | *C. quinoa* | Lilac |
| Olive | Italy | *C. Amar* | Olive |
| PV-46 | Unknown | *C. quinoa* | Ligustrum |
| PV-215 | Unknown | *C. quinoa* | Strawberry |
| PV-216 | Denmark | *C. quinoa* | Phlox |
| PV-217 | Czech republic | *C. quinoa* | Raspberry |

**Table 1: Origin of *Arabis mosaic virus* isolates** (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb)

Many experimental hosts are also used for the propagation of nepoviruses: *Chenopodium quinoa*, in which most nepoviruses induce characteristic symptoms, *C. amaranticolor*, *C. murale*, *Cucumis sativus*, *Nicotiana clevelandii*, *Petunia hybrida* and *Phaseolus vulgaris*.

## 2. Geographic range of nepoviruses

Most nepoviruses are found throughout Europe and North America, as illustrated by the geographical distribution of ArMV (fig. 1) but some virus species that infect grapevines are specifically restricted to individual continents: European species, i.e. *Tomato black ring virus* (TBRV), *Grapevine chrome mosaic virus* (GCMV), *Grapevine Bulgarian latent virus* (GBLV), *Raspberry ringspot virus* (RpRSV) and *Cherry leaf roll virus* (CLRV); America species, i.e. *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV), *Blueberry leaf mottle virus* (BBLMV) and *Peach rosette mosaic virus* (PRMV) (Martelli and Taylor, 1990), North Africa species, i.e. *Grapevine Tunisian ringspot virus* (GTRSV) in Tunisian (Ouertani *et al.*, 1991), Asiatic species, i.e. *Grapevine deformation virus* (GDefV), and a single species found in Turkey, the *Grapevine Anatolian ringspot virus* (GARSV) (Digiaro *et al.*, 2003).



Present and widespread distribution of *Arabis mosaic virus* : Czech republic, Germany, Bulgaria, Luxembourg, Hungary…

Present and restrictive distribution of *Arabis mosaic virus* : Norway, Finland, Sweden, Croatia, Denmark, Ireland, Lithuania, Netherlands, Switzerland, Turkey and South Africa

Present, few occurrences: Canada (British Columbia, Ontario, Quebec), New Zealand, Poland and Australia (Tasmania and Victoria)

Present but no details: United Kingdom, France and Italy

**Figure 1: Map representing the worldwide distribution of *Arabis mosaic virus* (European and Mediterranean Plant protection organization** (EPPO; 2006. 09. 19)).

## 3. Symptoms expressed by host plants upon infection by nepoviruses

### 3.1. External symptoms

Plants infected by nepoviruses display a large range of external symptoms but many of them remain symptomless. The most common symptoms are similar to those observed in plants infected by other viruses: mosaic, necrotic or chlorotic rings (ringspots), leaf flecking and mottling, vein necrosis, enation and plant stunting. The diseases caused by nepoviruses lead eventually to the death of the plant.

Grapevines infected by GFLV isolates express various symptoms, ranging from fanleaf to yellow mosaic, vein banding and ringspots or mottling, depending on the grapevine varieties (Martelli and Savino, 1990; Martelli *et al.*, 1993). Leaves become distorted and asymmetrical with sharply toothed limbs, closer primary veins and an open petiolar sinus. These typical foliar symptoms resemble to a fan, hence the name of fanleaf disease (fig. 2). These symptoms develop early in the spring and persist throughout most of the vegetative season, despite some fading during hot summers (Martelli, 1993). Canes can also be malformed, showing short internodes, double nodes, fasciations and zigzag growth between nodes (Raski *et al.*, 1983). Moreover, the productive life of GFLV-infected vineyards is significantly reduced (15-20 years instead of 30-40 years or longer).

**Figure 2: Comparative production of healthy (left) and *Grapevine fanleaf virus*-infected (right) grapevines cv. *Vitis vinifera Savagnin***
A decrease of the number of berries that reached the maturity is clearly visible in *Grapevine fanleaf virus* -infected plants (Andret-link *et al.*, 2004a).

Concerning the herbaceous hosts, the type and the severity of symptoms depend also on the GFLV strains (Huss *et al.*, 1989; Vuittenez *et al.*, 1964). For example, the inoculation of *Chenopodium quinoa* with GFLV results in leaf distortion, vein banding and chlorotic spots (Izadpanah *et al.*, 2003).

### 3.2. Cytopathology

In addition to external symptoms, nepoviruses also alter the cellular ultrastructure in infected plant; especially they modify the host endomembrane system. GFLV induces the assembly of

endoplasmic reticulum-derived membranous vesicles (Gaire, 1998), named "rosette", which are similar to those described in animal cells infected by poliovirus (Bienz *et al.*, 1994). These cytopathic structures, formed at the early stage of infection, are localized next to the nucleus and serve as sites for the replication of the virus (Ritzenthaler *et al.*, 2002). They are also found in plants infected by *Cowpea mosaic virus* (CPMV, *Comovirus*) (Carette *et al.*, 2000) and *Tobacco etch virus* (TEV, *Potyvirus*) (Schaad *et al.*, 1997). These virus-induced changes of the endoplasmic reticulum (ER) have been also shown for another nepovirus, *Tomato ringspot virus* (ToRSV), using ER marker fused to the green fluorescent protein (GFP) (Han and Sanfaçon, 2003). In epidermal cells of healthy plants, fluorescence was associated with the cortical ER network and with the nuclear envelope (fig. 3A). However, the morphology of the ER was drastically altered in ToRSV-infected cells (fig. 3B), as evidenced by the apparition of large bodies of fluorescence in close proximity to the nucleus (fig. 3C).

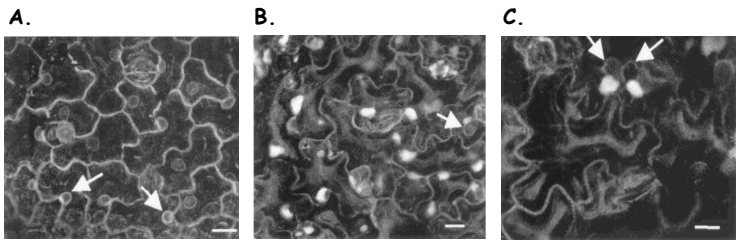



**Figure 3: Confocal fluorescence micrograph of mock-infected or *Tomato ringspot virus*-infected epidermal cells of ER-GFP transgenic *N. benthamiana*** (Han and Sanfaçon, 2003)
A. Epidermal cells of a mock-inoculated leaf; B. Epidermal cells of a *Tomato ringspot virus*-inoculated leaf (4 days post-inoculation), showing aggregates of ER-GFP fluorescence; C. Close-up view of two *Tomato ringspot virus*-infected epidermal cells, showing a perinuclear location of the ER-GFP aggregates. Bars, 20 µm. Arrows point to nuclei. The GFP fluorescence is shown in white over the dark background.

Another cellular modification that occurs upon nepovirus infection is the presence of tubules (~78 nm in diameter), at the level of plasmodesmata (fig. 4). The tubules have an internal diameter compatible with the passage of 28 nm virions in linear arrays. The tubules are unidirectional and are lined with plasma membrane at their outer surface. Their growth is polar and involves the addition of MP subunits at the base of the tubule embedded in the cell wall, thus allowing the protrusion of the tubule into the cytoplasm of the neighbouring cell (Laporte *et al.*, 2003). The tubules permit neoformed virions to invade adjacent cells and so, by cell-to-cell movements to systemically infect the plant through the vasculature networks (see paragraph on virus movement).

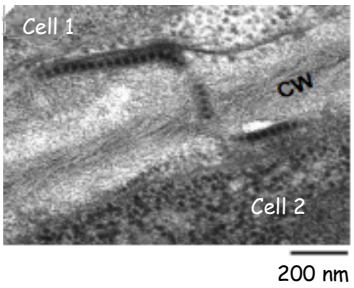



**Figure 4: Tubule across the cell wall (CW) allowing the cell-to-cell movement of *Arabis mosaic virus***
*Arabis mosaic virus* particles in single file can be observed within the tubule (Sanfaçon, 2008).

## 4. Propagation of nepoviruses

The name nepovirus is an acronym of "NEmatode transmitted viruses with POlyhedral particles", proposed by Cadman (1963), although only one-third of these viruses are transmitted by nematodes (fig. 5A left). Currently, it is known that twelve nepovirus species including GFLV, ArMV, TRSV and TBRV, are transmitted by nematodes and that one, *Blackcurrant reversion virus* (BRV) is not transmitted by nematodes but by the eriophyid gall mite (*Cecidophyosis ribis*) and possibly, other Cecidophyopsis species (fig. 5B right). Although, transmission of TRSV predominantly occurs in the soil by nematodes, it may also happen at the aerial part of the plants by the intermediate of arthropods i.e *Thrips tabaci* and *Epitrix hirtipennis* (fig. 5B left). The biological vectors involved in the transmission of several nepoviruses i.e *Grapevine Bulgarian latent virus* and *Myrobalan latent ringspot virus*, could not be identified so far (Harrison and Murant, 1977; Sanfaçon, 2008).

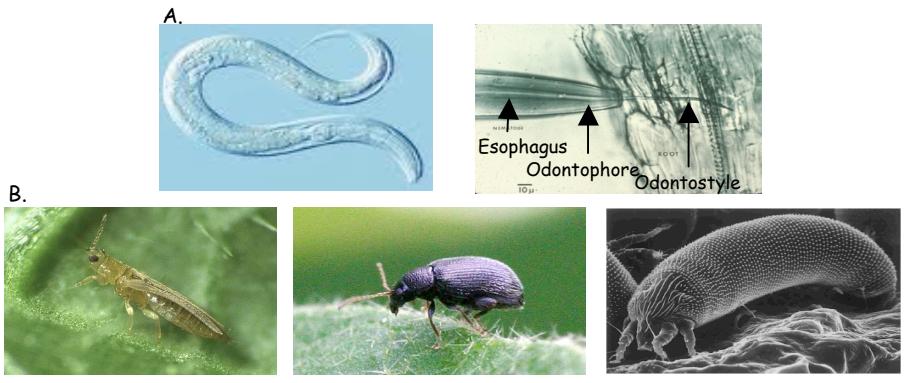



**Figure 5: Vectors involved in the transmission of nepoviruses from plant-to-plant**
A. Soil-inhabiting nematodes are involved in the transmission of nepoviruses: *Xiphinema index* (left) is specifically involved in transmission of Grapevine fanleaf virus. It transmits the virus with the odontostyle when feeding on the roots (right); B. Arthropodes responsible for the transmission of nepoviruses: *Thrips tabaci* (left), *Epitrix hirtipennis* (middle) and *Cecidophyopsis ribis* observed by a scanning electron microscope (right).

At least, nine members of the nepovirus genus such as ArMV, RpRSV, TRSV and CNSV are also seed- and/or pollen transmitted (Card *et al.*, 2007). It has been determined that 50% of the seeds arising from an infected plant lead to infected progeny. Therefore, seeds control following certificate schemes is a prerequisite for international trade to avoid the propagation of these viruses. Many nepoviruses can also be transmitted through grafting and mechanical inoculation. GFLV and ArMV are successfully transmitted to experimental herbaceous hosts (Baldacci *et al.*, 1960; Cadman *et al.*, 1960; Vuittenez, 1960) but hardly to grapevine by mechanical inoculation of a virus-contaminated sap (Nysterakis, 1947; Walter *et al.*, 2000). GFLV can also be transmitted by grafting; it migrates from the infected rootstock to the scion (Lahogue *et al.*, 1995).

### 4.1. Transmission of nepoviruses by nematodes

Three genera of soil-inhabiting nematodes, *Xiphinema*, *Longidorus* or *Paralongidorus,* the two latter belong to the order *Dorylaimida*, family *Londigoridae*, are involved in the transmission of nepoviruses. The interaction between nepoviruses and nematodes is highly specific with two notable exceptions: RpRSV, which is propagated by nematodes belonging to *Longidorus* and *Paralongidorus* genera (Jones *et al.*, 1994) and PRMV, which has also two natural vectors *Xiphinema americanum* and *Longidorus diadecturus* (Klos *et al.*, 1967; Eveleigh and Allen, 1982). The transmission of nepoviruses by nematodes was especially investigated for GFLV, whose natural vector is the ectoparasitic dagger nematode *Xiphinema index* (Hewitt *et al.*, 1958; Das and Raaski, 1968; Andret-Link *et al.*, 2004a). This type of transmission leads in vineyards to a characteristic spherical infected-plant patch distribution due to the concentric propagation of the nematodes in the soil (fig. 6).

**Figure 6: Grapevines infected by *Grapevine fanleaf virus* in a Chardonnay vineyard in the Champagne region in France** (Andret-Link *et al.*, 2004a)
The circular yellow area in the vineyard is a characterisctic of a virus transmission by an ectoparasitic nematode.

### 4.2. Transmission by *Xiphinema* species

#### 4.2.1 Distribution of *Xiphinema* species

Originally, only found in the Mediterranean areas (Walter and Martelli, 1997; Taylor and Brown, 1997), *X. index* is now worldwide distributed. The nematode *X. italiae*, which has also a

Mediterranean distribution (Martelli *et al.*, 1966; Dalmasso, 1970), has been reported to be another vector of GFLV (Cohn *et al.*, 1970) but, this could never being demonstrated experimentally (Martelli and Taylor, 1990).

*X. diversicaudatum* is the common vector of ArMV on grapevine (Harrison and Cadman, 1959; Jha and Posnette, 1959) and *Strawberry latent ringspot virus* on hop (Lister, 1964). This nematode is mainly distributed in western and northern European regions. Several dorylaimid nematodes have been suspected for ArMV transmission but this was never confirmed.

*X. vuittenezi*, which is prevalent in continental European regions, is involved in the transmission of *Grapevine chrome mosaic virus* (Martelli and Sarospataki, 1969).

### 4.2.2 Morphology and reproduction of *Xiphinema* species

*X. index* is characterized by a cylindrical body, about 3.0 mm long for adults, a vulva location approximately at 40% of the body length and a convex-conoid tail with a distinct finger-like protuberance (Loof and Luc, 1989; Vovlas and Larizza, 1994). It possesses a long stylet, named odontostyle, which is a cuticular extension of the odontophore from the esophagus. This stylet migrates from the stoma region and protudes to allow the nematode to feed on the small roots of the plants (fig. 5A right). When the nematode rests, the stylet remains in esophageal position.

The reproduction of *Xiphinema* is parthenogenetic, except for *X. diversicaudatum,* which can reproduce sexually, and thus, males are rather scarce. The duration of *Xiphinema* life cycle is 2 to 14 months or more, depending on biotic and abiotic factors (Taylor and Raski, 1964; Wyss, 2000). The life cycle comprises different stages: eggs lay in the spring, hatch a few days later and mature to adults through four juvenile stages, of which each stage is separated by a molt. Under adverse conditions, including low moisture, low temperature and the absence of host plants, *Xiphinema* undergoes a quiescent phase during which the physiological functions of the nematode are limited and the development is often interrupted (Dalmasso, 1970; Antoniou, 1989). The optimal temperature for reproduction is 24°C and the life cycle is interrupted below 16°C. However, *X. index* can survive in soil at extreme temperatures (Harris, 1979) and retain GFLV for at least 4 years in vineyard soil, stored at 7 to 20°C in the absence of host plants.

### 4.2.3. Acquisition of the virus by *Xiphinema* species

The nematode acquires the virus by feeding on roots of infected plants for a few hours thanks to the odontostyle, which crosses the epidermic barrier of the roots to reach the sieve (fig. 5A right). The feeding of *X. index* induces the formation of galls, which contain enlarged multinucleated cells with dense cytoplasm (Brown *et al.,* 1995; Wyss, 2000). It has been shown that GFLV can be acquired and transmitted by *X. index* within 1 to 10 minutes (Wyss, 2000). The virus particles of ArMV, GFLV and TRSV are mainly localized on the cuticular lining of the esophagus of the *Xiphinema* species (Taylor and Brown, 1997; Mc Farlane, 2003). In the *Longidorus* species, the nepoviruses particles has

been detected in the stylet lumen and attached to the guiding sheath; this was notably shown for RpRSV and TRBV.

*Xiphinema* species can remain viruliferous until 11 months after acquisition of the virus whereas the *Longidorus* species are viruliferous only for 9 weeks. Nematodes are able to acquire and transmit the virus to healthy plants when they are adults and at all juvenile stages (Taylor and Raski, 1964). Nepoviruses are not transmitted from females to eggs and they are lost when the nematodes molt. Indeed, juveniles lose their ability to transmit the virus after molting because of the shedding of the cuticle lining in the odontophore and the esophagus (Taylor and Brown, 1997). Therefore, after molting, the nematode must acquire again the virus by feeding on roots of infected grapevines to become viruliferous.

The specificity of nepoviruses transmission by nematode vector relies on the affinity of virus coat protein for specific carbohydrate moieties in the glycoproteins that are lining the wall of the esophagus (Brown *et al.*, 1995, Wang *et al.*, 2002). To identify the viral molecular determinants involved in the specific transmission of GFLV by *X. index*, chimeric RNA2 constructs were engineered by replacing the 2A, MP, or CP sequences of GFLV with their counterparts in *Arabis mosaic virus* (ArMV) (Belin *et al.*, 1999) because it was known that ArMV is transmitted by *Xiphinema diversicaudatum* but not by *X. index* (Brown *et al.*, 1995). The results obtained with these recombinant viruses led to the conclusion that the coat protein contains the determinant responsible for the specific transmission of GFLV by *X. index* (Belin *et al.*, 2001, Andret-link *et al.*, 2004b). Very recently, Schellenberger *et al.*, (2010) have shown that a stretch of 11 amino acids (positions 188 to 199) of the capsid protein of GFLV, located near to the icosahedral 3 fold axis, is essential for transmission of GFLV by *X. index*.

The release of GFLV is suspected to occur during feeding; a modification of the pH may be important for the release of viral particles from their site of retention by the salivary gland secretions (Wyss, 2000). Apparently, nepoviruses cannot replicate neither in nematodes nor in mites, and consequently, their transmission is considered to be non circulative and semi-persistent (Taylor and Brown, 1997; Brown *et al.*, 1998; Mc Farlane, 2003).

## 5. Prevention of nepovirus transmission

### 5.1. Control of planting material

A basic measure to preserve crops and plant stocks from nepovirus infections is the distribution of virus-free planting material underlying a strict certification scheme (Walter, 1991; Walter and Martelli, 1998). Currently, the control of GFLV infection is based until now on sanitary selection and soil disinfection using nematicides. Although the dissemination of the virus has been reduced by these measures, this control is still inefficient. Moreover, the use of nematicides is largely unsuccessful because the nematode can exist on detached grape roots deep in the soil (Raski and Goheen, 1988;

Demangeat *et al.*, 2005) and it is forbidden in many countries because of environmental toxicity. The rule for infested fields is to respect a long-term quarantine period (4 to 5 years) before replanting, with tests to determine the nematode densities (Esmenjaud *et al.*, 1993; 1994; Belin *et al.*, 2001).

Several procedures to detect GFLV directly in *X. index* populations have been reported. Transmission experiments to bait plants in the greenhouse are an indirect approach to assess the presence of GFLV particles within a nematode population (Taylor and Brown, 1997). Two direct molecular methods are also successfully used to detect GFLV in *X. index*: immunosorbent electron microscopy (ISEM) (Roberts and Brown, 1980; Wang and Gergerich, 1998) and enzyme-linked immunosorbent assay (ELISA) with different combinations of antibodies (Bouquet, 1983; Catalano *et al.*, 1991; Esmenjaud *et al.*, 1992; 1993). Unfortunately, these reliable serological procedures are not very sensitive because they require at least 10 nematodes (Esmenjaud *et al.*, 1993). By contrast, ELISA is routinely and successfully used for GFLV detection with CP-specific antibodies in plant tissues (Esmenjaud *et al.*, 1993; 1994) as well as the reverse transcription-polymerase chain reaction (Rowhani *et al.*, 1993).

### 5.2. Transgenic plants resistant to GFLV and/or ArMV

In the absence or not yet discovered natural resistant grapevine varieties to GFLV and/or to ArMV, several transgenic plants that express engineered resistance genes or pathogen genes (pathogen-derived resistance) have been created over the past 20 years. In grapevine, the most popular strategy has been the expression of the viral coat protein (CP) gene, thus achieving CP-mediated resistance. Transgenic tobacco and grapevine plants expressing the CP of ArMV and GFLV display different levels of virus resistance (Bardonner *et al.*, 1994; Martelli *et al.*, 2000; Gambino *et al.*, 2005). Bardonner *et al.* (1994) reported a significant delay in systemic GFLV infection in transgenic tobacco plants expressing GFLV CP, but no cross-protection against ArMV. High levels of protection against GFLV have been reported in transgenic *Nicotiana benthamiana* expressing non-translatable CP sequences (Monier *et al.*, 2000). However, transgenic grapevines are not resistant to the disease and express different symptoms after inoculation with GFLV (Barbier *et al.*, 1997). Recently, Nolke *et al.* (2009) have developed a new resistance strategy implicating a single-chain antibody fragment (scFvGFLVcp-55) that specifically binds to GFLV CP. The presence of this immune molecule seems to reduce GFLV and ArMV titers in *N. benthamiana*.

Cross-protection is another approach for management of these grapevine viruses. This strategy relies on the use of mild virus strains to protect plants from economic damage caused by closely related, severe virus strains (Fuchs *et al.*, 1997; Lecoq, 1998, Vigne *et al.*, 2009).

## II. The biology of nepoviruses

### 1. Taxonomy of the nepoviruses

#### 1.1. *Picornavirales* order

Nepoviruses belong to the *Picornavirales* order previously referred to as "picorna-like viruses" or "members of the picornavirus-like" superfamily because they ressemble in many aspects to picornaviruses, which infect animals (Le Gall *et al.*, 2008). The members of this order are characterized by: i) a small icosahedral particle(s) of 25-30 nm with a T=3, ii) a positive-stranded RNA genome that may be monopartite or bipartite as in nepoviruses (RNA1 and RNA2), iii) expression of the genome as a polyprotein, which is then cleaved by (a) viral proteinase(s) to generate functional proteins and iv) the same modular arrangement of functional domains within the polyprotein(s). By contrast to picornaviruses, which have only a single RNA molecule, plant viruses have often a bipartite genome, each RNA being enclosed in its own capsid. The domains, which are localized in the polyprotein-encoded RNA1 of bipartite viruses, are a type III helicase (Hel), a cysteine proteinase (Pro) and a RNA-dependent RNA polymerase (Pol). The polyprotein-encoded by the RNA2 contains the capsid domain(s) (CP) at its C-terminus, and upstream of the CP domain, the MP domain involved in the virus movement.

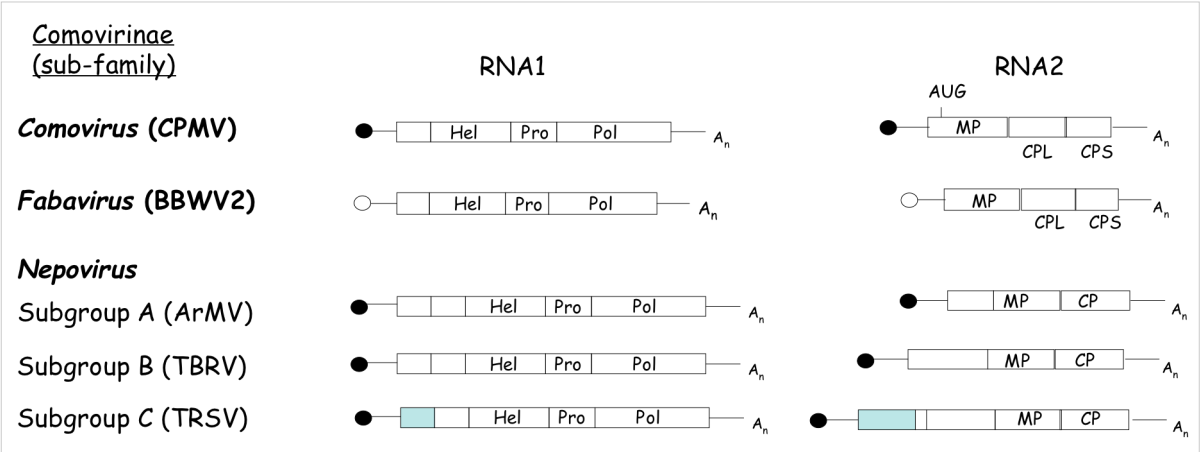



**Figure 7: Genome organization of viruses of the *Comovirinae* sub-family** (Sanfaçon *et al.*, 2009)
The open reading frames are shown by the boxes and the proteinase cleavage sites by the solid vertical lines. The VPg is shown with a sphere (open circle: no experimental evidence for a VPg; a sequence homologous to the VPg of comoviruses is found in the RNA1 of fabaviruses). The RNA2 of comoviruses (and possibly fabaviruses) encodes two polyproteins differing by their translation initiation site as indicated by the AUG above the *Cowpea mosaic virus* (CPMV) polyprotein. The blue boxes represent the areas of sequence identity, at the protein level, with the N-terminus of the subgroup C nepovirus and sadwavirus polyproteins. The different abreviation in this schematic representation are: Hel (Helicase), Pro (3C-like Proteinase), Pol (RNA-dependent RNA polymerase), CP (capsid protein), CPL (capsid protein, large subunit), CPS (capsid protein, small subunit), MP (movement protein), An (polyA), BBWV2 (*Broad bean wilt virus 2*), ArMV (*Arabis mosaic virus*), TBRV (*Tomato black ring virus*) and TRSV (*Tobacco ringspot virus*).

| **Subgroup A** | **Subgroup B** | **Subgroup C** |
|---|---|---|
| *Arabis mosaic virus* (ArMV) | *Artichoke Italian latent virus* (AILV) | *Apricot latent ringspot virus* (ALRSV) |
| *Arracacha virus A* (AVA) | *Beet ringspot virus* (BRSV) | *Artichoke yellow ringspot virus* (AYRSV) |
| *Artichoke aegean ringspot virus* (AARSV) | *Cocoa necrotic virus* (CoNV) | *Blackcurrant reversion virus* (BRV) |
| *Cassava American latent virus* (CALV) | *Crimson clover latent virus* (CCLV) | *Blueberry leaf mottle virus* (BBLMV) |
| *Grapevine deformation virus* (GDefV) | *Cycas necrotic stunt virus* (CNSV) | *Cassava green mottle virus* (CGMV) |
| *Grapevine fanleaf virus* (GFLV) | *Grapevine Anatolian ringspot virus* (GARSV) | *Cherry leaf roll virus* (CLRV) |
| *Potato black rinsgpot virus* (PBRSV) | *Grapevine chrome mosaic virus* (GCMV) | *Chicory yellow mottle virus* (CYMV) |
| *Raspberry ringspot virus* (RpRSV) | *Mulberry ringspot virus* (MRSV) | *Grapevine Bulgarian latent virus* (GBLV) |
| *Tobacco ringspot virus* (TRSV) | *Myrobalan latent ringspot virus* (MLRSV) | *Grapevine Tunisian ringspot virus* (GTRSV) |
| | *Olive latent ringspot virus* (OLRSV) | *Hibiscus latent ringspot virus* (HLRSV) |
| | *Tomato black ring virus* (TBRV) | *Lucerne Australian latent virus* (LALV) |
| | | *Natsudaidai dwarf virus* (NaDV) |
| | | *Navel orange infectious mottling virus* (NOIMoV) |
| | | *Peach rosette mosaic virus* (PRMV) |
| | | *Potato virus U* (PVU) |
| | | *Tomato ringspot virus* (ToRSV) |

**Table 2: List of viruses of the subgroups A, B and C of the nepovirus genus**

### 1.2. *Secoviridae* family

This new family, proposed by Sanfaçon *et al.*, (2009), is an amalgamation of the families *Sequiviridae* and *Comoviridae*.

The viruses from the *Sequiviridae* family are characterized by a monopartite genome and a polyhedral capsid made of three different capsid proteins (CP). This family includes the genera *Sequivirus*, *Waikavirus*, *Cheravirus* and the *Sadwavirus* genus, whose members have only two CPs (Le Gall *et al.*, 2005a; 2005b; 2005c). The previous *Comoviridae* family, now classified as *Comovirinae* sub-family, includes the genera *Nepovirus*, *Comovirus* and *Fabavirus* (fig. 7), which all have a bipartite genome, consisting of RNA1 (~8 kb) and RNA2 (4-7 kb).

### 1.3 *Nepovirus* genus

Nepoviruses differ from comoviruses and fabaviruses by their single large coat protein. Recently, the identification of two distinct domains, upstream of the helicase domain, in the polyprotein P1 encoded by RNA1 led to the proposal that this structural organization is a common feature of nepoviruses (Wetzel *et al.*, 2008); this aspect will be described further in more details (paragraph 3.2).

The nepovirus genus comprises up to now 36 viruses, which have been classified into three subgroups, named A (9 species), B (11 species) and C (16 species) (table 2) according to their serological relationships, the sequence similarities, the length of RNA2 (Mayo and Robinson, 1996; Wellink *et al.*, 2000; Fauquet *et al.*, 2005) and to the identity between the 3' untranslated regions (3'UTR) of RNAs 1 and 2 (Le Gall *et al.*, 1995a).

## 2. General properties of the nepoviruses

### 2.1. Virus particles

Nepoviruses particles consist of non-envelopped icosahedral capsids (26-30 nm in diameter) containing either RNA1 or RNA2 (fig. 8) (Reichmann and Wright, 1965; Diener and Schneider, 1966; Stace-Smith, 1970).

Nepoviruses are generally heat-stable and insensitive to organic solvents and thus, relatively easy to purify from infected plants. Pure virions are obtained by clarification of infected plant extracts with butan-1-ol, alone or mixed with chloroform, followed by differential centrifugation and/or precipitation with polyethylene glycol. Yields of 0.5-5 mg of purified virus particles are usually obtained starting from 100 g of infected leaves.

Nepovirus particles are separated by centrifugation on a sucrose density gradient into three components named T (top), M (middle) and B (bottom), respectively, according to the sedimentation coefficient. The latter depends on the RNA content of the virus particles (table 3):

- ✓ Top component (T) particles sediment at 53S and correspond to empty capsids

- ✓ Middle component (M) particles sediment at 86-128S and contain RNA2 of 3.8-7 kb
- ✓ Bottom component (B) particles sediment at 115-135S and contain either a single RNA1 molecule of 8 kb or two molecules of RNA2 if the latter is less than 4 kb in length.

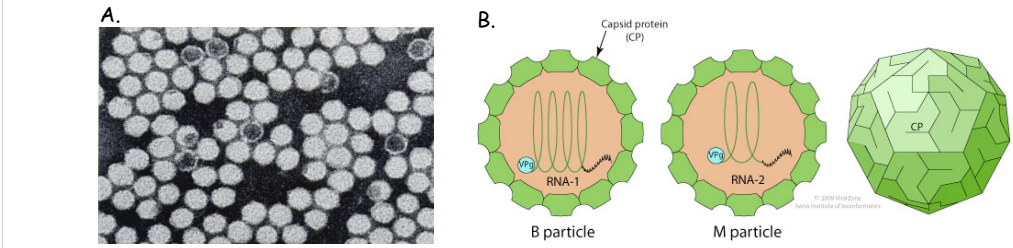



**Figure 8: *Arabis mosaic virus* particles**
A. Electron micrograph of *Arabis mosaic virus* particles; B. Schematic representation of the type B (Bottom) and M (Middle) particles, containing RNA1 and RNA2, respectively (left). The external architecture of the icosahedral capsid is shown at the right. (Source: 2009, viral zone, swiss institut of bioinformatics).

Both M and B components of the subgroup A viruses (GFLV, ArMV) possess an RNA2, whereas only M particles of viruses of subgroups B (TBRV, CNSV) and C (ToRSV, BRV), have an RNA2. However, the RNA2 length differs depending on subgroup B or C, 4.2-4.8 kb and 5.7-6.7 kb, respectively.

| | *Raspberry ringspot virus* | | | *Arabis mosaic virus* | | | *Tobacco ringspot virus* | | |
|---|---|---|---|---|---|---|---|---|---|
| | T | M | B | T | M | B | T | M | B |
| Sedimentation coefficient (S) | 52 | 92 | 130 | 53 | 93 | 126 | 53 | 91 | 126 |
| % RNA (weight) in particles | | 28 | 43 | | 28 | 41 | | 27 | 41 |

**Table 3: Physicochemical properties of the components of three nepoviruses, *Raspberry* ringspot virus, *Arabis mosaic virus*, *Tobacco ringspot virus*** (Stace-Smith, 1970).

### 2.2. Structure of the capsid

Initially, considering the molecular weight of an empty capsid (3.4 $10^6$ Da; Heuss *et al.*, 1981) and that the capsid protein has a molecular weight of 57 kDa, the capsid was predicted to have a T = 1 structure and consequently, to be composed of 60 protein subunits (Buckley *et al.*, 1993). However, the secondary structure and the crystal structure analysis of the ToRSV capsid protein predicted 3 β-barrel domains also kown as jelly-roll structure (Chandrasekar et Johnson, 1998). For this reason, it

was suggested that the capsid of nepoviruses is structurally analogous to the capsid of como- and picornaviruses (Lomonossoff and Johnson, 1991) and that a pseudo T = 3 designation may be more appropriate.

As expected, it has been shown that the GFLV capsid is composed of 60 copies with domains arranged in a pseudo T = 3 icosahedral surface lattice folded into three trapezoid-shaped-β-barrel domains (designated C, B, and A from the N- to the C-terminus, respectively) (Andret-Link *et al.*, 2004b). The B and C domains lie side-by-side around the capsid threefold axes and the prominent protrusion along the fivefold axes is formed by the A domain (Chandrasekar *et al.*, 1997; Chandrasekar et Johnson, 1998). The ArMV particles certainly have the same structural organization as they are composed of 60 coat protein monomers (Takemoto and al., 1985) and serologically related to GFLV (Schellenberger *et al.*, 2010).

### 3. Organization and expression of the genome

In this paragraph, the emphasis is on the organization and expression of GFLV and ArMV, our interest virus.

The genome of nepoviruses consists of two single stranded positive sense RNAs, named RNA1 and RNA2, which are both required for infectivity (Quacquarelli *et al.*, 1976). RNA1 has approximately the same length (7.3 kb) whatever the nepovirus species whereas RNA2 differs in size (3.7 to 7 kb), depending on the nepovirus subgroup. The nucleotide sequence of both RNAs has been determined for several nepoviruses including GFLV strain F13 (Serghini *et al.*, 1990; Ritzenthaler *et al.*, 1991) and the ArMV isolate originating from infected “pinot gris” grapevines in Hambach (Neustadt an der Weinstasse, Germany, hence the name ArMV- NW).

Both RNAs carry a small covalently linked VPg (Viral Protein genome-linked) at their 5’ end and are polyadenylated at their 3’ end (Pinck *et al.*, 1988; Ritzenthaler *et al.*, 1991). The poly(A) tail is encoded by the RNA and not added post-transcriptionally as for the eukaryotic polyadenylated mRNAs. Each genomic RNA encodes a polyprotein, which is proteolytically processed, *in cis* and *in trans* into functional proteins, by the viral proteinase.

The 5’ and 3’ untranslated regions (5’ and 3’ UTRs) of RNA1 and RNA2 contain structural elements that are essential for the initiation of translation and/or replication. Their length varies depending on the nepovirus subgroup. However, all 5’ and 3’ UTRs are A+U rich and have the capacity to form a series of small stem-loops independently of the subgroup.

#### 3.1. Structure of RNA1

The nucleotide sequence of ArMV-NW RNA1 is 7,334 nt-long excluding the poly(A) tail, slightly shorter than the RNA1 of GFLV (7,342 nt). As for GFLV, computer analysis reveals the presence of a single large open reading frame (ORF) encoding a polyprotein of 252 kDa. An amino acid sequence comparison between the polyprotein of ArMV-NW RNA1 and those of other

nepoviruses showed an identity of 75% with the GFLV-F13 polyprotein but only up to 36%, with other nepoviruses.

The 5' UTR of the RNA1 is 70-300 nt-long whereas the 3' UTR varies enormously in length, depending on the nepovirus subgroup, 200-400 nt for subgroup A and B to 1300-1600 nt for subgroup C (Lammers *et al.*, 1999). Concerning the ArMV-NW, the 5' and 3' UTRs of RNA1 have a length of 227 nt and 252 nt, respectively (Wetzel *et al.*, 2004). The 5' UTR of nepoviruses is characterized by the conserved sequence 5'-UG/UGAAAAU/AU/AU/A-3' found close to the translation initiation codon (Fuchs *et al.*, 1989) and therefore this sequence might be involved in the binding of the ribosomal 40S subunit. The 3' UTR contain the consensus sequence 5'-GGACACAAAAAGAUUUU-3' in all subgroups (Fuchs *et al.*, 1989; Serghini *et al.*, 1990; Buckley *et al.*, 1993; Bacher *et al.*, 1994). Another conserved motif, 5'-AAAAGC-3' (Rott *et al.*, 1991; Kreiah *et al.*, 1994) is located upstream of the 3'-poly(A) tail of nepoviruses genomic RNAs.

### 3.2. Polyprotein P1: cleavage and function(s) of the mature proteins

#### 3.2.1 Processing of polyprotein P1

The polyprotein P1, encoded by RNA1, is submitted to multiple cleavages, *in cis* and *in trans*, by the viral proteinase. The cascade of cleavages leads to the release of processing intermediates, which contain two or more protein domains and finally, to the mature proteins. The intermediate polyproteins are also detectable in infected plant cells and thus may have activities different from those of the mature proteins but nevertheless important for the biological cycle of nepoviruses. The accumulation of these products strongly suggests that some cleavage sites are preferentially recognized by the viral proteinase. Slow release of mature proteins by processing of stable intermediates at suboptimal cleavage sites may provide a regulatory mechanism to control the accumulation of specific proteins during the replication cycle.

The ArMV polyprotein P1 encompasses the arrangement of functional domains as in the polyproteins of the other members of the *Picornavirales* order, namely a type III helicase (NTB), a VPg, a proteinase (Pro) and a RNA-dependent RNA polymerase (Pol) (fig. 9A). These four proteins are located in the 2/3 C-terminal part of the P1 polyprotein. Two additional proteins, 1A and 1B, located upstream of the NTB protein, form the N-terminal region of P1. The 1B domain has been identified by *in vitro* assays, which revealed that it is separated from 1A and NTB domain by two cleavage sites, respectively (Wetzel *et al.*, 2008). These authors have shown that 1A and 1B are equivalent to the XI and X2 domains of the polyprotein P1 of ToRSV.

The polyprotein P1 of ArMV is cleaved at five different sites (fig. 9B), which are from the N- to the C-terminus, the dipeptides Cys/Gly (1A-1B), Gly/Val (1B-NTB), Cys/Ser (NTB-VPg), Gly/Glu (VPg-Pro) and Arg/Gly (Pro-Pol). Moreover, Wetzel *et al.* (2008) have suggested that the 1A-Pro precursor of ArMV-NW is predominantly cleaved *in trans* at the 1A-1B and NTB-VPg sites and that

the 1B-NTB is a suboptimal cleavage site. This hypothesis is reinforced by the fact that large amounts of the 1B-NTB intermediate accumulate in infected cells whereas 1B and NTB cleavage products are detected in smaller quantities. Therefore, it will be necessary to determine the efficiency of cleavage at the ArMV 1B-NTB site *in vivo* and to compare the relative accumulation of the 1B-NTB, 1B and NTB proteins in infected plants.

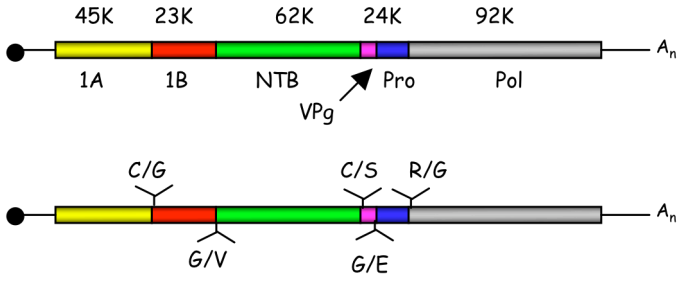



**Figure 9: Genome organization of the RNA1 of ArMV** (Wetzel *et al.*, 2008)
A. Representation of the six maturation products from the N- to the C-terminus of polyprotein P1 referred to as 1A, 1B, NTB, VPg, Pro and Pol, respectively. Their molecular weights are indicated in kDa (K); B. The cleavage sites recognized by the ArMV proteinase are indicated by amino acid single letter code. C: cysteine; G: glycine; V: valine, S: serine; E: acide glutamique; R: arginine. The black sphere represents the VPg at the 5' end and $A_n$, the poly(A) tail at the 3' end. The horizontal lines correspond to the non-coding regions.

Concerning GFLV, five maturation products were identified from the N- to the C-terminus of polyprotein P1, referred to as 1A (46 kDa), $1B^{Hel}$ (88 kDa), $1C^{VPg}$ (3 kDa), $1D^{Pro}$ (24 kDa), and $1E^{Pol}$ (92 kDa). These proteins are generated by processing of the polyprotein at Cys/Ala, Cys/Ser, Gly/Glu, and Arg/Gly cleavage sites, respectively (Pinck *et al.*, 1991; Ritzenthaler *et al.*, 1991; Margis *et al.*, 1994). The processing is predominantly intramolecular (*cis*-cleavage) although an intermolecular cleavage (*trans*-cleavage) of the N-terminal site has been reported for GFLV. Amino acid sequence alignments of the P1 N-terminal region of nepoviruses revealed the presence of the cleavage sites characterized in ToRSV and ArMV, suggesting that the presence of the two protein domains upstream of NTB, referred to as 1A or X1 and 1B or X2, is a common feature of the polyprotein P1 of nepoviruses (Wetzel *et al.*, 2008).

### 3.2.2 Functions of the mature proteins

- **The protein 1A**

The function of protein 1A, which corresponds to the N-terminal part of the polyprotein P1, is unknown up to now. Further studies will be necessary to decipher the biological role of this protein in the replication cycle of nepoviruses.

- **The protease cofactor (1B)**

Rott *et al.* (1995) have identified within the N-terminal region of the polyprotein P1 of nepoviruses, a consensus sequence which presents some similarities with the protease cofactor domain (32 kDa) of *Cowpea mosaic virus* (CPMV), the type member of the *Comovirus* genus (Vos *et al.*, 1988). The CPMV 32 kDa protein modulates the activity of the proteinase (Peters *et al.*, 1992). The conserved amino acid sequence is F-X27-W-X11–L-X21–L-X–E (Xn refers to the number of amino acids between conserved residues). For this reason, a role has been attributed to this part of the nepovirus polyprotein by Ritzenthaler *et al.* (1991) and Rott *et al.* (1995). It has been proposed that it interacts with the NTB-VPg-Pro-Pol precursor and that it induces in this way, a conformation of this precursor, which renders the cleavage sites more accessible to the viral proteinase.

Wetzel *et al.* (2008) have demonstrated by *in vitro* translation assays that the P1 N-terminal region acts as a cofactor of the ArMV proteinase but there is no evidence that 1B (X2) protein is sufficient by itself to play this role, as described for the CPMV 32 kDa. Studies performed on the ToRSV X2 protein showed that this protein is targeted to the endoplasmic reticulum (ER) and that its binds to ER by the intermediate of multiple domains in particular, two C-terminal transmembrane helices (Zhang and Sanfaçon, 2006). Therefore, the protein 1B may participate in the anchorage of the viral replication complex in the ER-derived membranes.

- **The NTB (Nucleoside Triphosphate)-Binding protein**

The NTB protein of nepoviruses contains motifs similar to those found in RNA helicases (Gorbalenya *et al.*, 1989a), in particular the highly conserved 'A' and 'B' sites, G-X4-GKS/T (Xn refers to the number of amino acids between conserved residues) and DD/E, respectively, which are involved in the binding of nucleoside triphosphates (NTP) (Gorbalenya and Koonin, 1990). It also possesses two membrane-binding domains, a C-terminal transmembrane domain and a putative N-terminal amphiphatic helix (Rott *et al.*, 1995). The presence of a transmembrane domain suggests that the NTB protein and/or a larger precursor corresponding to the intermediate cleavage product, may act as a membrane anchor for the replication complex (Wang *et al.*, 2004). Indeed, the mature NTB protein and NTB-VPg intermediate polyprotein are found in association with ER-derived membranes in ToRSV infected plants (Han and Sanfaçon, 2003).

- **The genome-linked viral protein (VPg)**

The VPg is a small protein of 24-28 amino acids, that is covalently linked to the 5' end of both viral RNAs, by a phosphodiester bond involving its N-terminal serine and the first nucleotide of the RNAs (Pinck *et al.*, 1990; Serghini *et al.*, 1990; Fuchs *et al.*, 1989). Its sequence is very variable between nepoviruses of the three subgroups, except the conserved motif $E/D_{1-2}$-$YX_{1-2}$-$RNX_{1-2}$-R

(Mayo *et al.*, 1994). By contrast, the sequence of the ArMV VPg, SEPRLEEGYIPRNKVSRISRT (Wetzel *et al.*, 2004), is very similar to that of GFLV (Pinck *et al.*, 1991).

The VPg of picornaviruses is not involved in translation but plays a central role in the replication of the RNA genome. It is urydylated in the presence of the viral RNA polymerase (3D $^{pol}$) and act as a primer for the synthesis of both RNA strands (Paul *et al.*, 1998; Steil and Barton, 2009). During the replication cycle of nepoviruses, the VPg is translocated into the lumen of the endomembranes (Han and Sanfacon, 2003; Wang *et al.*, 2004; Zhang *et al.*, 2005) while the RNA replication presumably occurs on the cytoplasmic face of the membrane. Since the VPg and the replication complex are physically separated, it is unlikely that the VPg serves as primer for the replication of the nepovirus genome.

By contrast to picornaviruses, degradation of the VPg by a treatment of the nepoviral RNAs with a protease, abolishes (TRSV, TBRV) or diminishes (RpRSV) the infectivity of viral RNAs, indicating that this protein is important for infectivity at the early stage of the replication cycle. The VPg of potyviruses is known to play an important role in their infectivity through interactions with translation initiation factors (elF4E, elF(iso)4E, elF4G and elF(iso)4G). These interactions are crucial for the accumulation of potyviruses in host plants, as disruption confers resistance to infection (Robaglia and Caranta, 2008; German-Retana *et al.*, 2008). Very recently, Gallois *et al.* (2010) showed that *Turnip mosaic virus* (TuMV) variants with mutated VPg overcome this resistance in elF(iso)4E, elF(iso) 4G1/G2 knockout *Arabidopsis* plants.

Huang *et al.*, (2010) have shown that the potyviral VPg interacts with a cellular RNA helicase-like protein (AtRH8) and that this interaction is essential for infection. The colocalization of this cellular protein with the chloroplast bound-virus vesicles suggests a possible role of AtRH8 in viral genome translation and in replication. An interaction between VPg and elF(iso)4E was observed with *Tomato ringspot nepovirus* (Leonard *et al.*, 2002). The VPg is not necessary for infectivity but dispensable for *in vitro* translation of the RNAs of *Tobacco ringspot virus* (Chu *et al.*, 1981), *Tomato black ring virus* (Koenig and Fritsch, 1982) and *Cherry leaf roll virus* (Hellen and Cooper, 1987). By contrast, the VPg is required neither for infectivity nor for *in vitro* translation of *Cowpea mosaic virus*, a member of the subfamily *Comoviridae* (Stanley *et al.*, 1978).

The removal of the covalently linked VPg did not affect the *in vitro* translation of cDNA-derived transcripts of subgroup A nepoviruses (GFLV, ArMV, RpRSV), indicating that the interaction between VPg and elF4E or other cellular translation factors, is not a prerequisite for the expression of the nepoviral polyproteins.

- **The protease**

The large polyproteins encoded by the nepovirus RNA1 and RNA2 are cleaved *in cis* and *in trans* into mature proteins and intermediate precursors by the viral proteinase (Pro). The nepovirus

proteinase is a chymotrypsin-like cysteine proteinase (Margis *et al.*, 1991; Margis and Pinck, 1992), related to the 3C Pro of picornaviruses (Gorbalenya *et al.*, 1989b). The catalytic triad of the 3C Pro of picornaviruses is constituted by histidine (H), glutamic acid (E) or aspartic acid (D), glutamine (Q) and cysteine (C) (Bazan & Fletterick, 1988; Gorbalenya *et al.,* 1989b). The cleavage sites recognized are dipeptides formed by an aspartic acid (E) or glutamine (Q) and glutamic acid (D) or serine (S) or methionine (M) (Bazan and Fletterick, 1990; Dewalt *et al.*, 1989; Gorbalenya *et al.*, 1989b).

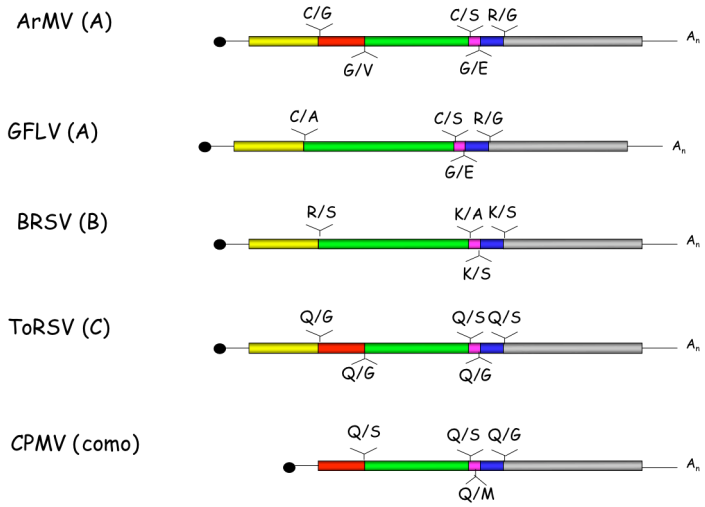



**Figure 10: Cleavage sites in the polyprotein P1 of como- and nepoviruses** (Wetzel *et al.*, 2008)
The polyprotein P1 of *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV) (subgroup A *Nepovirus*), *Beet ringspot virus* (BRSV, subgroup B *Nepovirus*), *Tomato ringspot virus* (ToRSV, subgroup C *Nepovirus*) and *Cowpea mosaic virus* (CPMV, *Comovirus)* are represented by boxes. The yellow box corresponds to the 1A or X1 product, the red box to the 1B or X2 product (Co-Pro for CPMV), the green box to the NTB product, the mauve box to the VPg, the blue box to the protease and the grey box to the RNA polymerase. The vertical lines represent the cleavage sites. The dipeptides recognized by the proteinase, are indicated above each cleavage site: A: alamine; C: cysteine; G: glycine; E: acid glutamic; K: lysine; M: methionine; Q: glutamine; R: arginine; S: serine. The black sphere and $A_n$ represent the VPg and the poly(A) tail, respectively. The 5' and 3' non-coding regions are depicted by horizontal lines.

The protease of nepoviruses from the subgroups A (GFLV, ArMV) and B (BRSV) do not have a histidine in their catalytic site; this residue is replaced by a leucine (Han *et al.*, 2002). The proteinase of the nepovirus subgroup A cleaves after a cysteine (C), arginine (R) or glycine (G) (Serghini *et al.*, 1990; Pinck *et al.*, 1991; Blok *et al.*, 1992; Buckley *et al.*, 1993; Margis *et al.*, 1993) whereas that of subgroup B hydrolyzes the sites where the first residue of the dipeptide is a lysine (K) or an arginine (R) (fig. 10) (Demangeat *et al.*, 1991; Hemmer *et al.*, 1995). The cleavage of the polyprotein by the proteinase of subgroup C nepoviruses was shown to occur after a glutamine (Q), asparagine (N) or aspartate (D) (Bacher *et al.*, 1994; Carrier *et al.*, 1999; Latvala *et al.*, 1998). For example, ToRSV

cleavage sites consist of a glutamine linked to a glycine or a serine (Hans and Sanfaçon, 1995; Wang *et al.*, 1999; Carrier *et al.*, 1999).

- **RNA-dependent RNA polymerase**

The RNA-dependent RNA polymerase (RdRp) is characterized by the conserved tripeptide GDD within D-xxxx-D-xn-G-xxx-T-xxx-N-xn-GDD motif (Argos, 1988), which is found in all polymerases (Poch *et al.*, 1989). This enzyme is involved in the replication of both nepoviral RNAs. It has no proofreading activity and consequently, errors are incorporated into the genome at a rate of $10^{-3}$ to $10^{-4}$ per replication cycle.

### 3.3. Structure of RNA2

The RNA2 of nepoviruses encodes a polyprotein denoted P2. As already mentioned, the size of this genomic RNA differs depending on the virus species; it is one a criteria used to classify nepoviruses into subgroups. RNA2 does not support its own replication but requires the viral replicative functions encoded by RNA1 (Viry *et al.,* 1993). RNA2 codes for the proteins necessary for the propagation of the virus namely the capsid protein and the cell-to-cell movement protein (Ritzenthaler *et al.,* 1995a).

The GFLV RNA2 is 3,774 nt-long with a single ORF, coding for a polyprotein of 122 kDa. Like GFLV, ArMV-NW isolate has only one type of RNA2 whereas the ArMV-S isolate (strain Syrah grapevine), originated from Bulgaria, possesses two distinct species of RNA2, called RNA2-U and RNA2-L, respectively (Loudes *et al.*, 1995), which differ slightly in size. Both RNA2 species of ArMV-S, the RNA2 of ArMV-NW and three Japanese ArMV isolates have been fully sequenced whereas only partial sequences of the RNA2 from other isolates of ArMV, such as ArMV-Lilac, are available (Steinkellner *et al.*, 1989; Bertioli *et al.*, 1991; Imura *et al.*, 2008).

ArMV-NW RNA2 has a length of 3,820 nt excluding the poly(A) tail and contains a single ORF, which encodes an 1110 amino acids long polypeptide (122 kDa) (Wetzel *et al.*, 2001). The comparison of the RNA2 sequences revealed an overall identity of 82-84% between the ArMV-NW and the ArMV-2L and -2U, respectively, and 72% between ArMV-NW and GFLV. The regions coding for the movement protein (MP) and the coat protein (CP) are the most conserved between the ArMV and/or GFLV isolates whereas the sequences specific for the 2A protein display the lowest identity.

The 5’ UTR of ArMV-NW RNA2 (295 nt) is longer than the 5’ UTR of ArMV-NW RNA1 (227 nt). It contains several direct repeats in particular, the sequence GAGUUUAAGAAACUC (motif I, fig. 11) that might be involved in the formation of stem-loop structures. Such secondary structures were already described for the RNA2 5’ UTR of several GFLV isolates (GFLV strain F13, 242 nt of

length) and *Grapevine deformation virus* (GDefV, 236 nt of lenght) (fig. 11) (Wetzel *et al.*, 2001; Ghanen-Sabanadzovic *et al.*, 2005).


**Figure 11: Primary and secondary structures of the RNA2 5' untranslated region of *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV) and *Grapevine deformation virus* (GDef)**
The conserved direct repeats (motif I) are framed and the corresponding secondary structures are depicted below the sequence alignment.

The 3' UTR has a length of 192 nt in ArMV-NW and 212 nt in GFLV. By contrast, the 3' UTRs of RNA2 and RNA1 of *Blackcurrant reversion virus* are very long, about 1360 nt. The 3' UTR contains several conserved sequences among the nepoviruses, which strongly suggests that they are functional importance: GGACACAAAAAGAUUUU (Serghini *et al.*, 1990, Wetzel *et al.*, 2004) and the GUUUGUCCUUU. Some of these conserved motifs are also found in the 3' UTR of RNA1, suggesting that these motifs are specifically recognized by the RNA-dependent RNA polymerase during the replication of the viral genome. For example, 90% identity was found between the last 160 nt of the 3' UTR of the ArMV-NW RNA1 (252 nt) and RNA2 (198 nt) (Wetzel *et al.*, 2004).

### 3.4 Polyprotein P2: cleavage and function(s) of the mature proteins

#### 3.4.1 Processing of polyprotein P2

Polyprotein P2 is cleaved *in trans* by the RNA1-encoded protease into three proteins (fig. 12A): 2A (homing protein, 28 kDa), MP (movement protein, 38 kDa) and CP (capsid protein, 56 kDa). The two cleavage sites recognized by the protease of GFLV are the dipeptides cysteine (C)/alanine (A) and arginine (R)/glycine (G) (fig. 12B) (Serghini *et al.*, 1990; Margis *et al.*, 1993b). Processing of the ArMV P2 polyprotein probably occurs in the same way since the same dipeptides are found at the junctions between 2A/MP and MP/CP. For ToRSV, a new cleavage site (presumably at dipeptide

Q/G) has been predicted in the N-terminal part of P2 to allow the release of two proteins, a 34 kDa protein, assuming translation initiation at the first AUG codon, arbitrarily designed X3, and a 71 kDa protein, X4 (Jafarpour *et al.*, 2009).

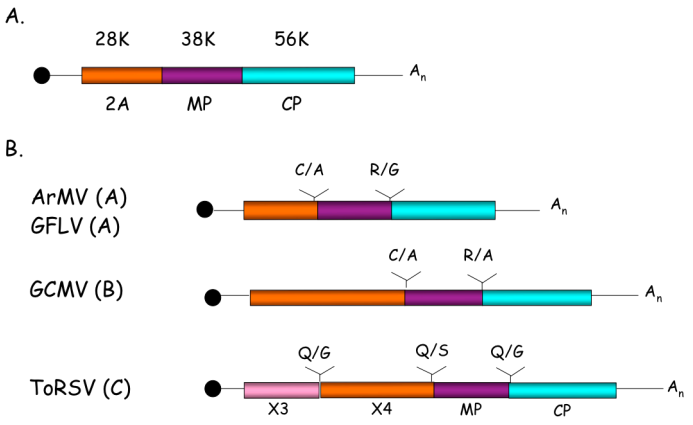



**Figure 12: Gene organization of the RNA2 ArMV**
A. Representation of the three maturation products, from the N- to the C-terminus, of polyprotein P2 referred to as 2A, MP, CP. The black sphere represents the VPg and the $A_n$, the poly(A) tail; B. Cleavage sites in the P2 polyproteins of nepoviruses (Carrier *et al.*, 1999). The polyproteins P2 of *Arabis mosaic virus* (ArMV) and *Grapevine fanleaf virus* (GFLV) (two subgroup A nepoviruses), *Grapevine chrome mosaic virus* (GCMV, subgroup B nepovirus) and *Tomato ringspot virus* (ToRSV, subgroup C nepovirus) are represented by boxes. The pink box represents the X3 product, the orange box the 2A or X4 proteins, the purple box, the MP and the turquoise box, the CP. The vertical lines represent the cleavage sites: A: alamine; C: cysteine; G: glycine; Q: glutamine; R: arginine; S: serine.

### 3.2.2. Functions of the mature proteins

- **The protein 2A**

The N-terminal protein 2A of polyprotein P2 differs in size depending on the nepoviruses (Mayo *et al.*, 1996). The 2A of TBRV and GCMV have a molecular weight of about 40 and 50 kDa, respectively (Demangeat *et al.*, 1991), whereas those of GFLV and ArMV are smaller, about 28 kDa (Margis *et al.*, 1993b). The analysis of the 2A sequences from the different ArMV and GFLV isolates revealed three major domains, the amino-terminus, the central core and the carboxy-terminus domains. The core domain, with the exception of a few additional amino acids in ArMV-2U, and the carboxy-terminus domain show a high level of similarity between the ArMV and GFLV isolates whereas the amino-terminus domain is not conserved among these species (Wetzel *et al.*, 2001; 2002).

The exact role of protein 2A is not totally elucidated but experiments performed on GFLV have shown that this protein is required for RNA2 replication and that it colocalizes to the GFLV replication site, which is located close to the nucleus. Therefore, protein 2A or P2 cleavage

intermediates could act as a homing protein by addressing RNA2 to the virus replication sites in the perinuclear area (Gaire *et al.*, 1999). Based on these results, we assume that the protein 2A of other nepoviruses including ArMV fulfills the same function during the infectious cycle.

- **The movement protein (MP)**

The movement protein of nepoviruses forms tubular structures that protrude from the cell wall and modifies plasmodesmata, as shown in GFLV-infected cells (Ritzenthaler *et al.*, 1995b). These structures allow the transport of the virions from cell-to-cell, according to the so-called CPMV transport model (Lazarowitz and Beachy, 1999, Talianski *et al.*, 2008). The movement protein of GFLV behaves as an intrinsic membrane protein that is probably inserted into the lipid bilayer of membranes *via* a hydrophobic domain (Laporte *et al.*, 2003). MP is transported by the intermediate of Golgi-derived vesicles along microtubules to plamodesmata, where it polymerizes into tubular structures (Laporte *et al.*, 2003). The LL and YXXΦ motifs (where X refers to any amino acid residues and Φ refers to hydrophobic residues with a bulky side chain), located near to the N-terminus of the GFLV MP, might represent an intrinsic MP transport signal, governing MP intracellular movement (Laporte *et al.*, 2003). The LPL motif is a signature for the MP of viruses that moves from cell-to-cell through tubules (Mushegian, 1994; Bertens *et al.*, 2000).

By sequence alignment with the MP of GFLV-F13, a putative movement protein was identified within the polyprotein P2 of ArMV isolates (Serghini *et al.*, 1990; Margis *et al.*, 1993). The ArMV MP has almost the same size as the GFLV counterpart (346 versus 348 aa). At the amino acid level, the identities range from 92-97% between the ArMV isolates, from 95-99% between the GFLV isolates and 84-89% between ArMV and GFLV isolates (Wetzel *et al.*, 2002).

- **The capsid protein (CP)**

The capsid protein is multifunctional. It plays an important role in virus transmission, genome encapsidation, cell-to-cell and long-distance movements of the virions in plants (Andret-Link *et al.*, 2004a; Belin *et al.*, 1999; Callaway *et al.*, 2001; Gaire *et al.*, 1999; Margis *et al.*, 1993; Quacquarelli *et al.*, 1976; Ritzenthaler *et al.*, 1995a, 1995b; Serghini *et al.*, 1990).

Two motifs, located respectively in the N- and C- terminal parts of the capsid protein, are characteristic for nepoviruses (Le Gall *et al.*, 1995b): $FxGx_6FDAYx(R/K)$ and FxFYGR(S/T). The C-terminal motif is involved in the stabilization of the quaternary structure of the capsid. The N-terminal motif on the surface of the CP (Chandrasekar and Johnson, 1998) might be involved in the movement of the virus *in planta*.

As described above, the capsid protein is involved in virus retention within the vector esophageal region and on the stylet (Whitcombe *et al.*, 1999; Belin *et al.*, 2001; Andret-Link *et al.*, 2004b). Moreover, studies performed on *Tobacco rattle virus* (TRV) suggest that the C-terminal of the

CP, which forms a flexible arm protruding from each capsid subunit, may act as a link to the nematode *via* a carbohydrate-containing components (Brown *et al.*, 1995).

Considering the biological importance of the CP, only limited genetic variations can be tolerated in order to ensure the perpetuation of the virus (Andret-Link *et al.*, 2004b; Belin *et al.*, 1999). Therefore, CP appeared to be an ideal antigenic target for antibody-mediated resistance against GFLV. This was confirmed when the CP-specific scFvGFLVcp-55 antibody was constitutively expressed in *N. benthamiana*. Indeed, the expression of this immune molecule conferred resistance to GFLV and resulted in the complete elimination of GFLV. Moreover, its significantly reduced viral titer of ArMV in these *N. benthamiana* plants (Nolke *et al.*, 2008).

## III. Nepovirus replication cycle

Nepoviruses are introduced in the host cell by their vector. After uncoating, the two RNA molecules are translated by a mechanism that is not fully understood. The polyproteins are processed probably co-translationally, as described for other picorna-like viruses, into structural and non-structural proteins. After replication of the genome on membranous vesicles and assembly, the newly formed virions invade the plant by short- and long-distance movements (fig. 13).

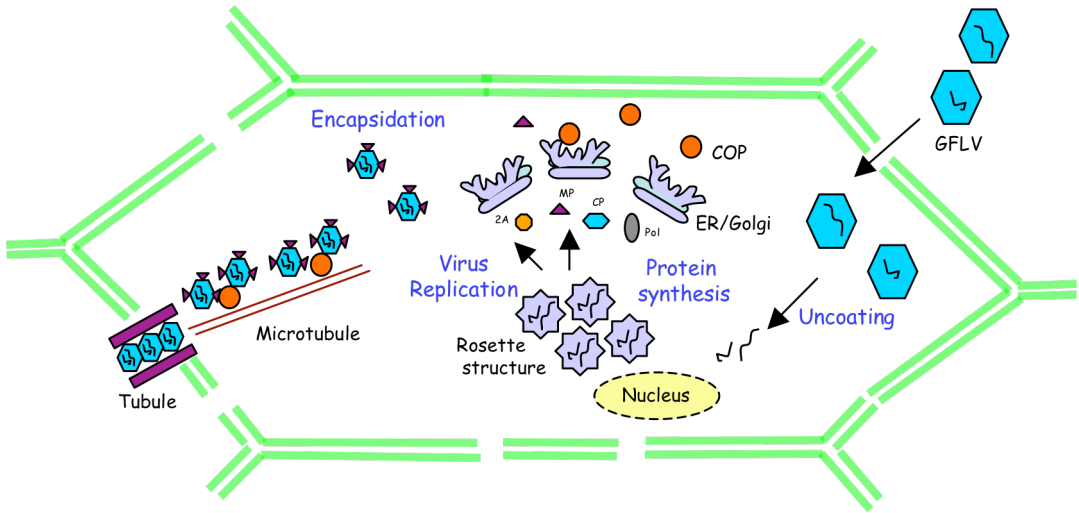



**Figure 13: Schematic representation of the infectious cycle of *Grapevine fanleaf virus*** (Andret-Link *et al.*, 2004b)
After cell entry and decapsidation of GFLV particles, the viral RNAs are translated on endoplasmic reticulum (ER)-derived membranous vesicles. RNA1- and RNA2-encoded polyproteins are proteolytically processed by the viral protease within the viral compartment where mature proteins accumulate. However, the movement protein (MP) seems to be rapidly transported to the cell periphery. The MP might be transported together with the CP or viral particles on Golgi-derived secretory vesicles (COP) along microtubules (MT). At the cell periphery, probably at the level of plasmodesmata, the MP self-assembles into tubular structures through the viral particles move from cell-to-cell.

## 1. Translation

Two major mechanisms are used to translate eukaryotic messenger RNAs (mRNAs), a cap-dependent ribosomal scanning and the internal entry of ribosomes, at the level of a highly structured region called internal ribosomal entry site (IRES). The latter mechanism was first described for picornaviruses and in particular for poliovirus (Pelletier and Sonenberg, 1988; Doudna and Sarnow, 2007). Subsequently, IRES have been discovered in other animal viruses and in plant viruses as well as in a few animal and plant cellular mRNAs. In the cap-dependent scanning strategy, concerning mainly capped and polyadenylated mRNAs, translation initiation is stimulated through mRNA circularization. This circularization involves the cap-bound elF4E factor, the poly(A) binding protein (PABP) interacting with the poly(A) tail and the scaffolding factor elF4G, which simultaneously interacts with elF4E and PABP. It allows the selection of full-length mRNA and favors the initiation of translation by the ribosomes and the released at the stop codon. Apart from the cap and poly(A) tail, the 5' and 3' UTRs of many eukaryotic mRNAs also contribute to the regulation of translation through a variety of mechanisms. The regulatory role of UTRs in translation is particularly important for viral RNAs that lack either a cap structure or a poly(A) tail or both. It has been shown that the 5' and 3' UTRs form RNA closed-loop structure thus mimicking the circularization of cellular mRNAs. In fact, a closed-loop structure formed throught interactions between an IRES in the 5′ UTR and the poly(A) tail was proposed for several uncapped but polyadenylated plant and animal viral RNAs (Bergamini *et al.*, 2000; Svitkin *et al.*, 2001). A direct base pairing interaction between IRES and the 3' UTR was also described in *Picornaviridae* (Dobrikova *et al.*, 2003, 2006; Lopez de Quinto *et al.*, 2002).

Such interactions are also relevant for the translation of the RNA1 and RNA2 of the *Black currant reversion virus* (BRV), a member of the subgroup C of nepovirus. The 5' UTRs of RNA1 and RNA2 are very short (66 and 61 nt, respectively), A+U rich and contain a small stem-loop. At the opposite, the 3' UTRs of both BRV RNA1 and RNA2 are extremely long (1360 and 1363 nt, respectively) and can be folded into several stem-loops, one of which acts as a cap-independent translation enhancer (CITE). This 3' secondary structure is involved in a long-distance base-pairing interaction with the stem-loop present in the 5' UTR (Karetnikov and Lehto, 2008). This interaction is necessary for a maximal stimulation of translation but the poly(A) tail of BRV RNA2 also substantially enhances translation (Karetnikov *et al.*, 2006). Moreover, the 5' UTR contains several sequences complementary to the 3' UTR and the plant 18S rRNA thus allowing an internal entry of the 40S ribosomal subunit (fig. 14) (Karetnikov and Lehto, 2007). To our knowledge, there is no information available concerning the expression strategy used by other nepoviruses. However, it is likely that the translation mechanism involves both long-distance RNA-RNA interactions between the 5' and 3' UTRs and internal ribosomal entry through a direct interaction between the 5' UTR and the plant 18S rRNA. This hypothesis is reinforced by the fact that the 5' and 3' UTRs of nepovirus RNAs,

including ArMV and GFLV, also contains secondary structures that may be potentially implicated in such interactions.

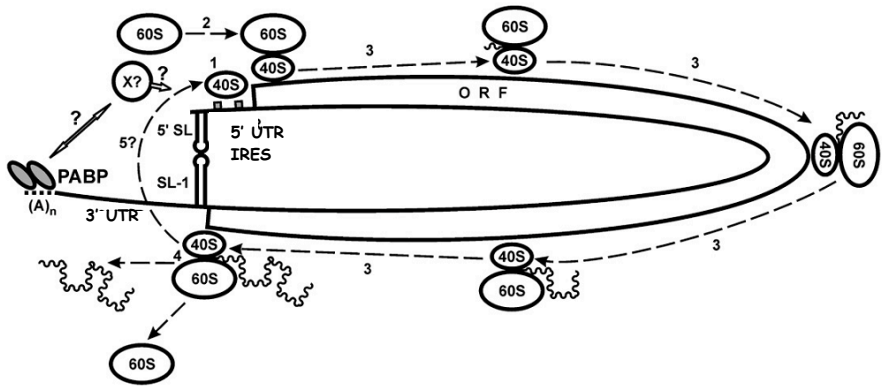



**Figure 14: *A hypothetical model describing the mechanism of the translation of Blackcurrant reversion virus* RNAs** (Karetnikov *et al.*, 2008)
The 40S ribosomal subunit is recruited to the 5′ UTR IRES: base pairing between the plant 18S rRNA and complementary sequences of the BRV 5′ leader (shown as two small gray boxes) may facilitate the recrutement of the 40S. After the start codon recognition, the 60S ribosomal subunit associates with the 40S to form the 80S initiation complex. During translation elongation, the polyprotein (thin curly line) is synthesized. After translation termination, the 80S complex is dissociated and the polypeptide is released. The base pairing between the 5′ stem-loop (SL) of the BRV 5′ leader and the SL-1 of the 3′ UTR leads to the circularization of the viral RNA and might facilitate the recycling of the ribosomal subunits to the 5′ UTR. An alternative way of mRNA circularization is supposed to involve a putative interaction of the poly(A)-binding protein (PABP) with a yet unidentified factor X, which in turn interacts directly or through other factors, with the 5′ leader. The eukaryotic translation initiation, elongation and termination factors are not shown.

The involvement of VPg in translation is under debate since several years and in particular, since the discovery that this small viral protein binds to translation initiation factors. The VPg of *Calicivirus*, an animal virus, interacts with elF3 and elF4E (Daughenbaugh *et al.*, 2003; Goodfellow *et al.*, 2005) and those of potyviruses interacts with elF4E, elF(iso)4E, and PABP (Leonard *et al.*, 2000, Robaglia and Caranta, 2006). More recently, *in vitro* experiments performed by Karetnikov *et al.* (2006) showed that artificial mRNAs flanked by BRV UTRs was efficiently translated without VPg thus leading to the conclusion that VPg is not necessary for the translation of the viral RNA, at least *in vitro*.

### 2. Replication of nepovirus RNAs

The replication complex of many positive-sense RNA viruses is associated with ER membranes. However, membranes of vesicles (endosomes, peroxysomes and lysosomes) or organelles (mitochondria and chloroplasts) can also serve as sites for RNA replication (Salonen *et al.*, 2005). These structures constitute the subcellular compartments that allow a local concentration of replication

proteins and protection of the viral RNA from degradation (Schwartz *et al.*, 2002).

The nonstructural RNA1-encoded proteins are sufficient for GFLV replication (Viry *et al.*, 1993). Aggregates of RNA1-derived viral components accumulate around the nucleus of the host cell, 48 h after transfection with GFLV-RNA1 (Ritzenthaler *et al.*, 2002). In contrast, RNA2 depends on RNA1 translation products for its replication and for the processing of polyprotein P2. RNA2 needs to integrate the replication complex initiated by RNA1. Presumably, the protein 2A targets RNA2 to the replication site or interacts with the same cellular structure as RNA1-derived proteins. Indeed, it was shown that the protein 2A is required for RNA2 but not for the RNA1 replication (Ritzenthaler *et al.*, 2002).

Nepoviruses replicate in association with membranous vesicles that are located at the periphery of the nucleus (Gaire *et al.*, 1999). These vesicles derived from the ER and the Golgi apparatus, assemble into inclusion bodies called “rosette” (Ritzenthaler *et al.*, 2002) by analogy with those observed in animal cells infected by *Poliovirus* (Bienz *et al.*, 193; 1994). RNAs, double-strand RNA replication forms, host and viral proteins are detected in these rosette structures (Boguszewska-Chachulska and Haenni, 2005; Noueiry and Ahlquist, 2003; Sanfacon, 2008; White and Nagy, 2004). Ritzenthaler *et al.* (2002) have shown that GFLV replication is sensitive to brefeldin A, a fungal metabolite known to disrupt the secretory pathway and to induce the formation of an ER-Golgi hybrid compartment in tobacco cells, suggesting that the COP (coat protein-coated vesicles that transport proteins from organelle to organelle) vesicular trafficking mechanism could recruit ER-derived membranes. These authors also found that cerulenin, which inhibits the type II fatty acid synthase, affects also GFLV replication, indicating that *de novo* phospholipid biosynthesis is required for efficient GFLV replication.

Viral poteins are targeted to the replication site in their mature form and/or as intermediate cleavage products. In the case of ToRSV, the mature NTB and the intermediate polyprotein NTB-VPg-Pro-Pol are integral membrane proteins that co-fractionate with the replication complex (Chrisholm *et al.*, 2007), suggesting that the intermediate is brought into the replication complex through the interaction with one or several viral membrane proteins. However, Wang *et al.* (2004) showed that NTB–VPg has the ability to associate with membranes in the absence of other viral proteins (fig. 15). In fact, the NTB protein, which is an integral membrane protein in contrast to VPg, Pro and Pol, is probably essential for the assembly of the replication complex.

Because replication presumably takes place on the cytoplasmic side of the membranes, the VPg domain present on the luminal face of the membranes (detected *in vivo* and *in vitro*) is unlikely to participate actively in the replication of the genome. This finding was somewhat surprising because the VPg plays a key role in the replication of picornaviruses; it serves as primer to the RNA-dependent RNA polymerase for the synthesis of both RNA strands (Steil *et al.*, 2010; Steil and Barton, 2009).

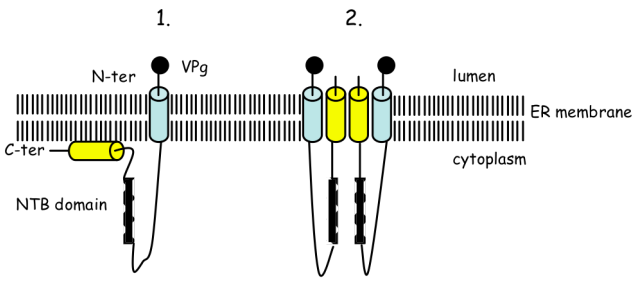



**Figure 15: Model for the insertion of the NTB-VPg protein into ER membranes** (Zhang *et al.*, 2005)
The NTB-VPg product initially associates as a monomer with the membrane (part 1). The N-terminal amphipathic helix (represented by the yellow cylinder) is parallel to the membrane whereas the C-terminal helix (represented by the blue cylinder) crosses the membrane. In a second step (part 2), the protein oligomerizes through the N-terminal amphipathic helix and probably also the C-terminal helix to form an aqueous pore. To simplify the drawing, only two monomers are shown interacting in the structure. However, formation of the pore would imply the interaction of at least four NTB-VPg molecules.The region containing the NTB domain is shown by a black box. The dark sphere represents the VPg.

## 3. Movement of nepoviruses in the host plant

Plant viruses spread from the initially infected cells to the rest of the plant following distinct stages. First, the virus moves intracellularly from the sites of replication to plasmodesmata, an intercellular channel, to infect neighbouring cells. This cell-to-cell movement also designated short-distance movement allows the virus to reach step by step the phloem sieve tubes. Therefrom, the virus invades the whole plant; this long-distance movement causes systemic infection of the host plant. Concerning multipartite viruses, systemic infection achieves only when all virus components i.e RNA1 and RNA2 for nepoviruses or experimentally the corresponding transcripts, are co-inoculated to the host plant (Viry *et al.*, 1993).

### 3.1. Short-distance movement

Cell-to-cell movement of plant viruses occurs in the form of virions or a nucleic acid-protein complex (for review, Taliansky *et al.*, 2008). It is mediated by one or several viral movement proteins (MPs) assisted for some viruses by the capsid protein, which acts as an auxiliary MP either actively or by protecting the genome from degradation (McLean *et al.*, 1997; Ehlers and Kollmann, 2001; Scholthof, 2005).

Nepovirus-infected cells are characterized by the presence of tubular structures containing virus-like particles. The GFLV MP, an intrinsic membrane protein, alone is sufficient to induce the formation of these tubular structures in intact plant cells or protoplasts. The GFLV MP apparently interacts with the plasmodesmata located proteins (PDLP), which are specifically located in the

plasma membrane lining the plasmodesmata (Thomas *et al.*, 2008; Ritzenthaler C., personal communication). As PDLP are present at the bases of tubules formed by MP, they might act as receptor for GFLV MP. The virus movement occurs in two distinct steps: intracellular movement of virus particles from the perinuclear site of RNA synthesis to the cell periphery and then, intercellular movement across the cell wall, through the tubules.

The GFLV MP is probably transported from its site of synthesis in association with the ER–bound replication complex, to specific foci in the cell wall, where it assembles into tubules (Laporte *et al.*, 2003). This intracellular movement depends on the secretory pathway and the cytoskeleton. By analogy with comoviruses, it was suggested that an interaction between GFLV MP and the capsid protein is necessary to facilite the passage of the virions through the tubules and thus to enable nepoviruses to spread from cell-to-cell.

### 3.2. Long-distance movement

In long-distance movement, the viruses use the vascular system to invade the entire plant; they spread along with the flow of photoassimilates (Atabekov & Dorokhov, 1984; Hull, 1989; Matthews, 1991; Maule, 1991). As for cell-to-cell movement, viruses are transported *via* the phloem as either the nucleic acid-protein complexes or virions. No information is available currently concerning the long-distance movement of nepoviruses but it is likely that they circulate as virions given that this is the form used for the cell-to-cell movement.

## IV. Nepoviruses and their satellites

Satellites are sub-viral agents whose genome replication and/or encapsidation depend on the co-infection of the host cell by a helper virus. Satellites were described for animal viruses i.e *Hepatitis D virus* (Taylor and Palchat, 2010), Amoeba virus i.e Spoutnik (La Scola *et al.*, 2008), plant DNA viruses i.e *Geminivirus* DNA β satellite (Briddon and Stanley, 2006; Kumar *et al.*, 2010) and several plant RNA viruses. Satellites reduce or intensify disease symptoms caused by the helper virus.

Satellite RNA molecules are associated with some nepoviruses (Mayo *et al.*, 1995; Murant *et al.*, 1982), such as GFLV-F13, ArMV-NW, TBRV, BRV…(table 4). Nepoviral satRNAs can be classified into two different groups, according to the size of their genome and whether or not, they encode a protein: the small B-type satellites and the circular D-type satellites (Roossinck *et al.,* 1992; Fritsch & Mayo, 1989). *Arabis mosaic virus* (ArMV) and *Chicory yellow mottle virus* (CYMV) are the only known nepoviruses, which can support both types of satellite.

### 1. Type B satellite RNAs (satRNAs)

The type B satRNAs are typically 1100 to 1400 nt in length and encode a non-structural protein (39-48 kDa) of unknown function (Fritsch *et al.*, 1993). These RNA molecules carry a VPg at their 5’

end and are polyadenylated at their 3' end like the nepovirus genomic RNAs. There is no sequence homology in the untranslated regions between the satellites and the helper virus RNA molecules, except for a consensus sequence of 11 nucleotides (UG/UGAAAAAU/AU/AU/A) at the 5' extremity (Fritsch and Mayo, 1989; Fuchs *et al.* 1989).

The satRNAs vary in size depending on the isolate: 1139 nt, 1104 nt, 1092 nt and 1114 nt for the ArMV-NW (Liu *et al.*, 1990), the ArMV-Lilac, the ArMV-Hop and the GFLV-F13 (Fuchs *et al.*, 1989), respectively. Conserved sequences are found at the 5' and 3'-termini of these satellites.

The protein encoded by the satRNAs, named P3, differs in length: 338 amino acids for ArMV-Lilac, 349 for ArMV-NW and 360 for ArMV-Lilac. Concerning the protein P3 of the satellite associated with GFLV-F13, it contains 341 residues (Fuchs *et al.*, 1989). Comparison of the primary structure of the P3 proteins revealed that an identity of 57% between ArMV-NW and ArMV-Lilac and -Hop and 64% between ArMV-NW and GFLV-F13. The protein P3 is highly hydrophilic and basic (Fuchs *et al.*, 1989) and possesses S/TxR/K phosphorylation motifs (Leader *et al.*, 1988; Fritsch *et al.*, 1993). It is detected in subcellular membrane fractions and nucleus-enriched fractions (Moser *et al.*, 1992). P3 seems to be required for the satellite replication (Hans *et al.*, 1992, 1993; Hemmer *et al.*, 1993; Liu *et al.*, 1993; Oncino *et al.*, 1995), but interaction between the satRNA-encoded protein and the helper virus replicase has not yet been demonstrated.

Both the 5' and the 3' UTRs have been shown to be important for nepoviral satRNA replication (Hans *et al.*, 1993, Hemmer *et al.*, 1987). However, replication only occurs with strains, which are related serologically (Hans *et al.*, 1992).

The presence of type B satellites usually does not affect the symptoms induced by the viruses and does not modify the replication level of their helper viruses (Fritsch *et al.*, 1993). In the case of ArMV-Lilac, the large satellite has been shown to exacerbate as well as attenuate disease, depending on the host plants (Liu *et al.*, 1991; Fritsch *et al.*, 1993).

Type B satellite RNA molecules are encapsidated in the helper virus capsid either alone or together with a molecule of RNA2 (Murant and Mayo, 1982).

### 2. Type D satellite RNAs

The type D satRNAs are smaller (0.3-0.46 kb) than the type B satRNAs and they are circular (Mayo *et al.*, 1995, Buzayan and Bruening, 1995). They have neither a VPg nor a poly(A) tail and they have no coding capacity. The first 60 nt at the 5' end of these satellites are strongly homologous and form a "hammerhead" structure, which is involved in the self-cleavage of concatemeric molecules obtained upon replication of the satellite RNA by a "rolling circle" mechanism (Hitsamatsu *et al.*, 1997) and then encapsided (Linthorst and Kaper, 1984; Bruening *et al.*, 1991; 1995).

In general, type D satellites have more effect on the symptoms induced by the helper virus than the type B satellites. This was notably observed in hop plants infected with ArMV, where the

nettlehead symptoms were associated with the presence of this type of satRNA in the infected plants (Davies et Clark, 1983). However, the satRNA of TRSV diminished the viral titer of the helper virus and the severity of symptoms (Passmore *et al.*, 1995).

| Nepovirus | Accession number | Size (nt) | Protein (kDa) | References |
|---|---|---|---|---|
| B type satellites | | | | |
| *Arabis mosaic virus* (ArMV) | NC003523 | 1104 | 39 | Liu *et al.*, 1990 |
| *Grapevine Bulgarian latent virus* (GBLV) | - | ~1800 | ND | Gallitelli *et al.*, 1983 |
| *Grapevine fanleaf virus* (GFLV) | NC003203 | 1114 | 37 | Fuchs *et al.*, 1989 |
| *Tomato black ring virus* (TBRV) | NC003890 | 1375 | 48 | Hemmer *et al.*, 1987 |
| *Chicory yellow mottle virus* (CYMV) | NC006452 | 1145 | 40 | Rubino *et al.*, 1990 |
| *Blackcurrant reversion virus* (BRV) | NC003872 | 1432 | 44 | Latvala-Kilby*et al.*, 2000 |
| D type satellites | | | | |
| *Arabis mosaic virus* (ArMV) | NC001546 | 300 | -[b] | Kaper *et al.*, 1988 |
| *Tobacco ringspot virus* (ToRSV) | NC003889 | 359 | -[b] | Buzayan *et al.*, 1986 |
| *Chicory yellow mottle virus* (CYMV) | NC006453 | 457 | -[b] | |

**Table 4: Some satellites of nepoviruses** (Mayo and Robinson, 1996, Sanfaçon, 2008)
The molecular masses of the proteins encoded by the satellites were calculated from the nucleotide sequence; [b] no translation product; ND: not determined.

# CHARACTERIZATION OF SYMPTOM DETERMINANT(S) ENCODED BY ARABIS MOSAIC VIRUS

# INTRODUCTION

## I. Symptoms of virus-infected plants

Symptoms are defined as external (macroscopic) and/or internal (microscopic) modifications observed in plants upon infection by a pathogen (virus, bacteria or fungi), in adverse environmental conditions, upon nutrient depletion or herbicide treatment. The same symptoms can be induced by distinct viruses or even by other pathogens or particular environmental conditions. Therefore, dependence on symptoms displayed by a host plant for identification of a virus may lead to much confusion. Nevertheless, virus names usually include the most important symptom expressed by the principal host or by the host from which it was described at first.

Most plant viruses induce external symptoms in susceptible plants but some viruses may infect a plant without producing any visible sign of disease, in particular when a very mild strain of the virus or when the virus infects a tolerant host. Moreover, a virus does not necessarily cause a disease in all parts of the infected plant, as described for the recovery phenomenon, where plants recover from infection in newly emerging leaves.

In general, plant viruses induce foliar symptoms (alteration of the pigmentation, necrosis and developmental abnormalities) but viruses may also induce similar symptoms in other parts of the infected plant i.e stems, roots, fruits…Moreover, they generally cause a reduction of the plant size. The symptoms result from physiological alterations, including decreases in photosynthesis and a hormonal imbalance induced by the plant virus. The severity and the type of symptoms depend on the virus species or isolates, on the host plant and on environmental conditions (temperature, light intensity, soil nutrient…).

Concerning the internal symptoms, also termed cytopathological effects, they correspond to ultrastructural changes induced by the virus such as proliferation of endomembranes, disorganization of the cytoskeleton or alteration of the chloroplast and mitochondria envelopes... Cells infected by viruses often contain inclusion bodies in the cytoplasm and/or in the nucleus. The inclusion bodies formed by one or several proteins may be amorphous; these viral bodies called viroplasms usually serve as sites of virus replication but they can also have specialized functions like the electron-lucent viroplasms in cells infected by *Cauliflower mosaic virus* (CaMV, *Caulimovirus*), which are involved in the transmission of the virus by aphids. Some viral proteins can assemble into specific shaped inclusion bodies such as the pinwheels formed by the cylindrical inclusion CI of potyviruses. Crystalline inclusion bodies result from an organized assembly of newly formed virions and are often observed in cells infected by viruses, which are unable to move from cell-to-cell as virus particles i.e. the *Tobacco mosaic virus* (TMV, *Tobamovirus*).

Infection of plants by viruses is responsible for great economical losses worldwide because it is detrimental to crops both quantitatively and qualitatively. Indeed, it results in lower production or

performance i.e. the lower yield of fruits may be due to a reduction of both their size and their number and depreciate the value of the crops (fruits, legumes…) and thus, the plants are no more marketable.

In the following part, I will briefly described some common external symptoms of plants infected by viruses and give a few examples of viral determinants involved in the expression of symptoms.

### 1. Foliar symptoms of virus-infected plants

Phytoviruses essentially induce symptoms in leaves because they replicate in the mesophyll, parachym and companion cells. Usually, the symptoms are not restricted to the virus-inoculated leaves (local symptoms) but also develop on the new emerging leaves (systemic symptoms), as the viruses spread along the vascular system of the plant. Symptoms can be classified into three types of phenotype: abnormal pigmentation (chlorosis, mosaic, ringspot), necrosis and deformations.

#### 1.1. Abnormal pigmentation of virus-infected leaves

Light and dark green areas define leaf mosaic. This phenotype is due to a deficiency in chlorophyll pigments that results from the arrest of the chlorophyll synthesis, the destruction of the chlorophyll and/or alteration of the chloroplast thylakoids. The leaves become yellow when xanthophylls and carotinoid pigments predominate over chlorophyll. Several types of mosaic have been described depending on the pigmentation level and the location of the chlorotic areas. When chlorophyll-deficient cells are grouped in islets and irregularly distributed over the entire surface of the limb, these symptoms correspond to light green or yellow typical mosaics (fig. 1A). The symptom is called vein-mosaic or vein-banding when the loss of pigmentation occurs near and along the veins (fig. 1B), and yellowing veins when the chlorophyll are very depleted in the veins (fig. 1C).

The shape and size of leaf areas deficient in chlorophyll are also taken into account for the symptom terminology: mottle if the edges of the area are diffuse, fleck if the mosaic is small and sharp, speckle for larger and circular mosaic and chlorosis for foliar yellowish spots. In monocotyledons, the virus infection cause stripes or streaks of tissues, whose colour is lighter than the rest of the leaf and which run parallel to the length of the leaf, for example the *Barley stripe mosaic virus* (BSMV, *Hordeivirus*, fig. 1D).

Many viral plant diseases lead to concentric rings and irregular chlorotic lines on leaves and sometimes (fig. 1E), on fruits and vegetative organs (tubercle). The symptom is called ringspot when the virus-infected zone is confined to the rings, for example in plants infected by *Raspberry ringspot virus* (RpRSV, *Nepovirus*, fig. 1F).

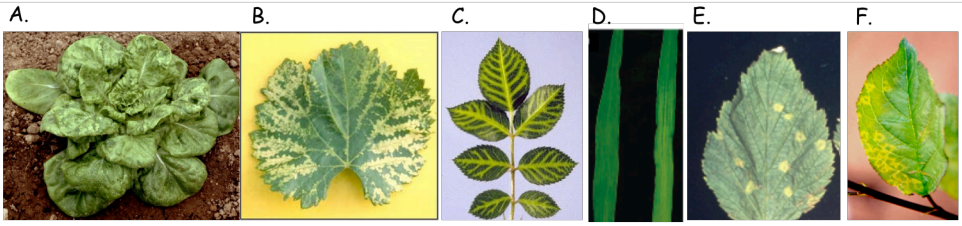



**Figure 1: Mosaic symptoms on leaves induced by viral infection** (EPPO)
A. Mosaic symptoms on lettuce caused by *Lettuce mosaic virus* (LMV, *Potyvirus*); B. Vein-banding on grapevine leaf infected by *Grapevine fanleaf virus* (GFLV, *Nepovirus*); C. Yellow veins on rose infected by *Rose mosaic virus* (RMV, *Ilarvirus*); D. Streak symptoms on stem of barley plant infected by *Barley stripe mosaic virus* (BSMV, *Hordeivirus*) at the left and a healthy stem at the right; E. Chlorotic ring symptoms on *Japanese plum* caused by *Plum American line pattern virus* (APLPV, *Ilarvirus*); F. Ringspot symptoms on raspberry infected by *Raspberry ringspot virus* (RpRSV, *Nepovirus*).

### 1.2. Necrosis

Necrosis is characterized by a brown colour that may appear when the infected cells die (fig. 2A and 2D); the size of the brown areas is variable depending on the spread of the virus. A layer of dead surface cells often characterizes ringspots (fig. 2B); therefore, the latter are named necrotic ringspots. Sometimes, necrosis spreads along the vascular tissues in parallel with the virus movement in the leaf; this is notably visible in virus-infected monocotyledon plants i.e. maize infected by *High plains virus* (HPV, unassigned genus, fig. 2C).

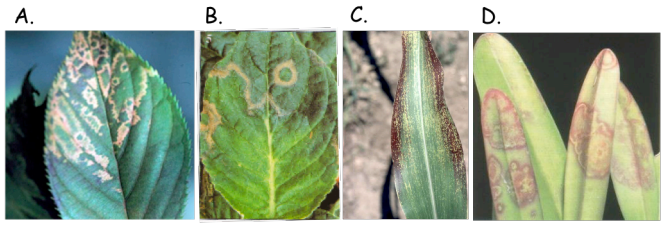



**Figure 2: Necrosis symptoms on leaves induced by viral infections** (EPPO)
A. Necrotic ringspot symptoms on prunus infected by *Prunus necrotic ringspot virus* (PNRV, *Ilarvirus*); B. Necrotic ringspot and irregular lines on a gloxinia leaf caused by *Tomato spotted wilt virus* (TSWV, *Tospovirus*); C. Necrotic streaks on maize infected by *High plains virus* (HPV, unassigned genus); D. Sunken spots and lines of necrotic tissue on orchid leaves infected by *Cymbidium mosaic virus* (CyMV, *Potexvirus*).

### 1.3. Developmental abnormalities

An unequal growth of cells infected by the virus can cause distortions of the leaf. These

distortions appear more or less corrugated, bloated or cramped. The term leaf rolling is characterized by a pronounced epinasty of leaf due to a slow growth in a one side (fig. 3A). The term enation is used when some outgrowths appear at the level of the vein i.e. *Pea enation mosaic virus* (PEMV, *Enamovirus*, fig. 3B). Wilting may also affects the aerial part of the plants and it is frequently followed by the death of the plant.

A viral infection can also cause malformations in any part of the plant by affecting the production of growth regulators. This hormonal imbalance in the infected plant can cause hyperplasia (increase in the number of cells) or hypertrophy (increase in size of organs) and hypoplasia (reduced cell number) or atrophy (reduced size of organs). The deviations of the plant metabolism due to viral replication often result into delayed development of the infected plants, such as stunting or dwarfism. Stunting corresponds to a reduction in size of the leaves (fig. 3C), flowers, fruits and roots and to a shortening of petioles and internodes. Growth abnormalities can also concern roots, as illustrated by the uncontrolled proliferation of root hairs (rhizomania) of sugar beets infected by *Beet necrotic yellowing mosaic virus* (BNYVV, *Benyvirus*) or the stem, whose swelling in cocoa plants is induced by the *Cocoa swollen shoot virus* (CSSV, *Badnavirus*).

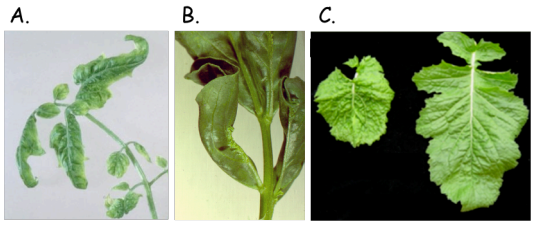



**Figure 3: Developmental abnormalities on leaves caused by viral infection**
A. Leaf rolling/curling symptoms on tomato infected by *Tomato yellow leaf curl virus* (TYLCV, *Begomovirus*); B. Enation symptoms on a pea infected by *Pea enation mosaic virus* (PEMV, *Enamovirus*); C. Stunting symptoms on the leaf infected by *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) at the left, the healthy leaf being at the right.

### 2. Symptoms on flowers of virus-infected plants

Viral infections of ornamental plants have a significant impact on the marketing of these plants because they often led to alterations in the pigmentation, deformations or necrosis of the flowers.

The flower breakings affecting the sepals or the petals arise from the absence of anthocyanin pigments. Bleached or faded areas are observed in the petals, where the virus multiplies. Sometimes, this colour break symptom has been highly wished by merchants and use as a selling point. One of the most famous examples is the so-called “Rembrandt tulip”, which is characterized by white and colour streaks of the tulip flower. This symptom due to the infection of tulip by the *Tulip breaking virus* (TBV, *Potyvirus*), was represented as early as the XVII $^{th}$ century in Rembrandt’s paintings, hence the

name of « Rembrandt tulip » (fig. 4A). Colour break symptoms on the flowers were also described in other plants such as *Nicotiana sanderae*, pelargonium and gerbera infected by *Tobacco mild green mosaic virus* (TMGMV, *Tobamovirus*), *Pelargonium flower break virus* (PFBV, *Carmovirus,* fig. 4B) and CMV, respectively.

Necrosis of flowers is frequently observed on tulips infected by *Tobacco necrotic virus* (TNV, *Necrovirus*) but some orchids also show some necrosis upon infection by *Cymbidium mosaic virus* (CyMV, *Potexvirus*).

Breaking flowers and necrosis are often accompanied by deformations of the floral parts or abortion of petals. One of the most spectacular symptoms on flowers is observed when the carnation is infected by *Carnation vein mottle virus* (CVMV, *Potyvirus*). Indeed, this infection results in crinkled petals giving a huddle up aspect of the flowers (fig. 4C).

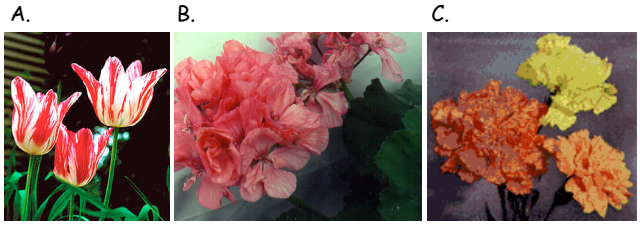



**Figure 4: Flower breakings and deformation of flowers resulting from viral infection**
A. "Rembrandt tulip" due to infection by *Tulip breaking virus* (TBV, *Potyvirus*); B. Pelargonium infected by *Pelargonium flower break virus* (PFBV, *Carmovirus*); C. Crinkled carnation due to infection by *Carnation vein mottle virus* (CVMV, *Potyvirus*).

### 3. Symptoms observed on fruits and tubercles of virus-infected plants

Several viruses alter the quality and the quantity of the fruits in particular those of stones trees thus, leading to important economical losses i.e Sharka disease due to the infection of prunus or peach trees by the *Plum pox virus* (PPV, *Potyvirus*, fig. 5A). The symptoms induced on fruits and tubercles are identical to those observed on leaves: necrosis, mosaic, abnormal growth…For example, *Potato virus Y* (PVY, *Potyvirus*, fig. 5B) provokes necroses in potato tuber. Distorsion of fruits is often observed on eggplant infected by *Tomato bushy stunt virus* (TBSV, *Tombusvirus*, fig. 5C) or on pumpkins infected by *Zucchini mosaic virus* (ZYMV, *Potyvirus*, fig. 5D). Viruses may cause wart-like outgrowth on fruits but conversely, others suppress normal outgrowths i.e *Datura metel* infected by a potyvirus produced fruits that lack normal spines.

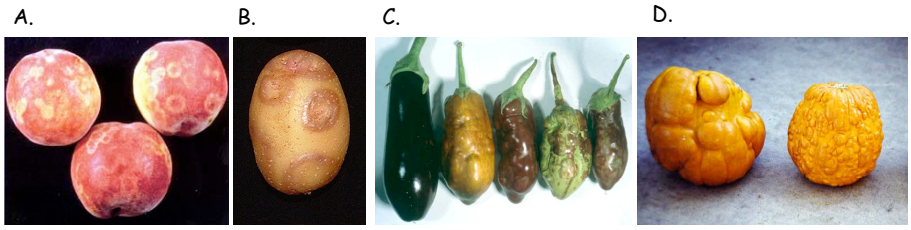



**Figure 5: Illustration of some symptoms on fruits resulting from viral infection** (EPPO and USDA) A. Abnormal pigmentation of peaches infected by *Plum pox virus* (PPV, *Potyvirus*); B. Necrosis on Potato infected by *Potato virus Y* (PVY, *Potyvirus*); C. Deformation of eggplant fruits infected by *Tomato bushy stunt virus* (TBSV, *Tombusvirus*) (healthy fruit at the left and infected fruits at the right); D. Deformation of pumpkins by the *Zucchini mosaic virus* (ZYMV, *Potyvirus*).

## II. Viral determinants involved in the induction of disease symptoms

The development of disease symptoms clearly results from a complex interplay between virus-host interactions that are involved in virus replication, virus movement through the host plant, suppression of RNA silencing, and cellular reprogramming. Virus-host interactions directly affect the host physiology and/or subvert specific cell pathways or processes including hormone regulation, cell cycle control, host transport, degradation of proteins and others. For instance, the Geminivirus Rep protein and the Nanovirus Clink protein are involved in cell reprogramming, the TMV replicase interacts with the host auxin-responsive pathway and naturally, many viral RNAi suppressors interferes with RNAi machinery components and thus, impair the host RNAi surveillance system and the regulation of the expression of nuclear genes by small non-coding RNAs (miRNAs and ta-siRNAs). Nevertheless, many aspects implicated in the establishment of viral disease symptoms are still unknown in part owing to the physiological variability associated with virus infection (Culver and Padmanabhan, 2007).

In the following section, we present three examples of viral determinants involved in the expression of symptoms in virus-infected plants.

### 1. The protein P6 of *Cauliflower mosaic virus* (CaMV)

The *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) is a pararetrovirus characterized by a double-stranded circular DNA genome. It infects essentially Brassicaceous plants (cabbage, *Arabidopsis*, colza…) but some strains can also infect Solanaceous plants (*Datura*, *Nicotiana*). The symptoms induced by the CaMV are chlorosis, mosaic, vein-clearing and stunting depending on the virus strain, the host ecotype and the environmental conditions (Melcher *et al.*, 1986).

Among the six proteins encoded by CaMV genome, the multifunctional protein P6 (62 kDa), which is the most divergent protein among the CaMV strains, is the major symptom determinant. It is involved both in local (chlorotic or necrotic) and in systemic (stunting) symptoms. Notably, this was

demonstrated by using chimeric CaMV DNAs in which ORF VI, coding for P6, was substituted by the homologous sequence from three strains (Cabb-B, D4 and CM184), which induce different symptoms independently of the host plant (Schoelz *et al.*, 1986): the D4 strain causes chlorotic vein-clearing mosaics and is able to induce systemic infection, characterized by stunting in Solanaceous hosts such as *D. stramonium* and *Nicotiana bigelovii* whereas CM184 and Cabb-B are unable to infect *D. stramonium* and *N. bigelovii* systemically and induce rather necrotic than chlorotic lesions at the local level (Lung *et al.*, 1972).

The role of P6 in the expression of symptoms of infected plants was further confirmed by generating transgenic *Arabidopsis* plants expressing this viral protein. The phenotype of these plants is similar to those of CaMV-infected *Arabidopsis* (Cecchini *et al.*, 1997). To identify the sequence or residues of this protein involved in the symptomatology induced by CaMV, Daubert and Routh (1990) realized site-directed mutagenesis within the ORF VI.

Although the P6 protein seems to be the major determinant of the symptom induction, specific symptom are associated to separate regions of the CaMV genome at least in turnip plants; this was demonstrated by studying a series of hybrids of CaMV genomes between a severe virus strain and a mild strain (Stratford and Covey, 1989). A region containing part of gene VII - the corresponding protein was never found in infected plants - and gene I are involved in the rate of spread of systemic vein clearing symptoms. The large intergenic region and part of gene VII influences the degree of leaf chlorosis, the region containing part of gene III, gene IV, and part of gene V control timing of initial systemic symptom appearance and finally, the region containing parts of gene I and II influence plant stunting. In summary, the induction of symptoms depends on the synergy of the several CaMV determinants whose genes segregate along the viral genome. Very recently, it was demonstrated that P6 is a suppressor of RNA silencing (Love *et al.*, 2008) as it was expected because this function is usually associated with viral pathogenicity factors (Ruiz and Voinnet, 2009). These functions may explain why several cellular genes are up or down-regulated in *Arabidopsis* transgenic plants expressing P6 (Geri *et al.*, 1999).

### 2. The P25 encoded by *Beet necrotic yellow vein virus* (BNYVV)

The *Beet necrotic yellow vein virus* (BNYVV), a member of the genus *Benyvirus*, is the causative agent of the sugar beet rhizomania (Tamada, 1975). This disease is characterized by browning of vascular budles in the taproot, leading to its stunting and loss of sugar content and by an abnormal proliferation of secondary roots (fig. 6A). At later stage of infection, the virus can move to the leaves and induce typical symptoms; a foliar chlorosis and veinal yellowing associated with the necrosis of leaf tissue areas (fig. 6B).

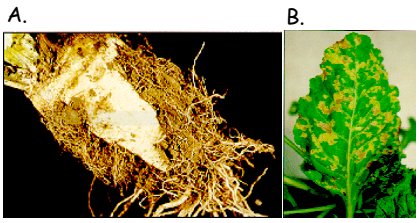



**Figure 6: Symptoms on sugar beet infected by *Beet necrotic vein yellow virus* (BNYVV, *Benyvirus*)**
A. Lateral root proliferation on the tap (rhizomiana);
B. Vein yellowing and necrotic lesions on beet leaves.

The genome of BNYVV consists of five positive ssRNAs (RNA1 to RNA5), which all together encode 9 proteins. Mechanical inoculation of RNA1 and RNA2, which code for the functions involved in virus replication and movement, in association with other viral RNAs showed that RNA3 is involved in the viral disease and in the expression of symptoms. Indeed, inoculation of RNA1 and RNA2 alone caused no visible symptoms while the presence of RNA3 greatly increased the virus titer in taproots and led to severe reduction of the plant yield. These observations suggested that RNA3 is implicated in the multiplication and spread of the virus in the root tissues and thus, in rhizomania symptoms (Tamada, 1975; Tamada and Abe, 1989; Koenig *et al.*, 1991).

The involvement of protein P25 encoded by RNA3 in leaf symptoms was further demonstrated by site-directed mutagenesis (Jupin *et al.*, 1992). Indeed, no yellow lesions were observed on *Tetragonia expansa* leaves when the expression of P25 was impaired by a frameshift.

P25 is also the determinant responsible of the rhizomania disease (Tamada *et al.* 1999). After serially passages on *C. quinoa*, two mutant forms were obtained containing deletion in RNA3. The authors showed that the P25 is directly responsible for the development of rhizomania symptoms, in susceptible and resistant sugar beet cultivars, by mechanical co-inoculation of RNA1 and RNA2 with RNA3 or a RNA3 deletion mutant, which does not code for the C-terminal part of P25. Very recently, Peltier *et al.* (2010) showed that arabidopsis transgenic plants expressing P25 display aroot proliferation phenotype and major hormonal and transcriptomic changes.

Although RNA3-encoded P25 is the major determinant in the symptom expression and rhizomania disease, RNA4 has also an effect on symptoms expression (Rahim *et al.*, 2007). Indeed, virus isolates containing RNA4 but not RNA3 produce strong chlorotic lesions in *T. expansa* leaves and severe symptoms in *N. benthamiana*. Moreover, it was demonstrated by mutagenic analysis that P31, which is encoded by RNA4, can reinforce the symptoms induced by P25 in some Beta species in the presence of P25.

### 3. The protein 2b of the *Cucumber mosaic virus* (CMV)

The *Cucumber mosaic virus* (CMV) is the type member of the genus *Cucumovirus* in the family *Bromoviridae*. The genome of CMV consists of three single-stranded positive sense RNA molecules (RNA1, RNA2 and RNA3), which code for five proteins. The 2b protein is synthetized from a subgenomic RNA of RNA2; its ORF overlaps with the 3'-terminus of ORF2a, which is expressed from the genomic RNA2 (Ding *et al.*, 1994).

CMV has a wide host specificity; it infects a great variety of vegetables and ornamental plants. The symptoms observed upon infection are severe mosaic, stunting and filiformism of the vegetables, chlorosis or necrosis depending on the CMV strains (fig. 7).

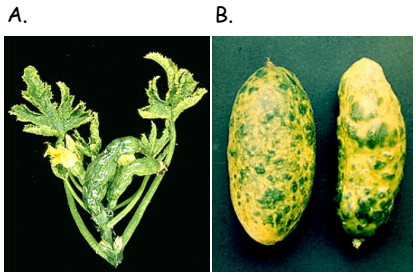



**Figure 7: Symptoms expressed by zucchini and cucumber plants infected by *Cucumber mosaic virus* (CMV, *Cucumovirus*)**
A. Filiformism and stunting symptoms on zucchini leaves and deformation of the fruits; B. Necrosis symptoms on cucumber fruits.

It was demonstrated that the protein 2b is involved in the expression of symptoms using hybrid genomic constructs obtained from mild and virulent CMV strains (Ding *et al.*, 1995; 1996; Shi *et al.*, 2003; Du *et al.*, 2007; Lewsey *et al.*, 2009). This result was further confirmed by the fact that infection of 2b defective CMV strains showed no symptoms or milder symptoms than the wild-type virus (Soards *et al.*, 2002; Wang *et al.*, 2004). Specific domains and/or amino acids of protein 2b involved in the induction of symptoms were characterized (Du *et al.*, 2008). The expression of specific symptoms has also been associated to specific amino acid on the capsid protein and in a few cases to 1a or 2a proteins (for review, Palukaitis and Garcia-Arenal., 2003). The involvement of the protein 2b of CMV in symptom induction is indisputably related to its functions in viral long-distance movement, suppression of RNA silencing (Brignetti *et al.*, 1998; Diaz-Pendon *et al.*, 2007) and to its antagonistic activity against the salicylic acid-mediated defence response.

These three examples clearly show that several proteins and/or nucleic acid sequences are

involved in the causal chain leading to the display of disease symptoms and that the latter result from multiple virus-plant interactions that disturb the host physiology. However, the environmental (experimental) conditions in virus infection also influence the type of symptoms thus, rendering it particularly difficult to link mechanistic causalities with the induction of specific symptoms. Characterization of the viral determinant(s) responsible in the expression of symptoms and the underlying interactions with host components will allow a better knowledge of the viral effects on host physiology and thus of the virus infection cycle. Certainly, it will also allow to understand why a same virus induces different symptoms on different host plants and why different strains of a virus induce little or no symptoms on the same host. A better understanding of the mechanisms involved in the disease symptoms will probably promote new insights into strategies designed to combat viruses and thus, to reduce the economic damage caused by the viruses.

# RESULTS

### I. Host range and symptoms of ArMV infected plants

ArMV is, with GFLV and RpRSV, one of the most important causative agents of the grapevine fanleaf disease. Like other nepoviruses, ArMV has a wide natural and experimental host range that includes woody and herbaceous hosts among which a number of economically important crops such as raspberry, strawberry, olive and grapevine.

Several herbaceous indicator plants can be infected by ArMV and displayed typical symptoms. For example, *Chenopodium quinoa* infected by ArMV develops chlorotic local lesions followed by a systemic mottling (Murant, 1970) and *Petunia hybrida* infected by this virus shows local chlorotic lesions or small necrotic rings. However, not all ArMV strains induce distinct symptoms and some of them do not infect plants systemically.

The symptoms developed by ArMV-infected plants are very common but variable depending on the host plants: mosaic, vein clearing, leaf mottling and flecking, stunting and several forms of deformation including enations (fig. 8). Three Japanese isolates of ArMV were studied for their pathological properties: ArMV-Li (Lily) and ArMV-NA (Narcissus) isolates induce severe necrotic spots on *C. quinoa* whereas the ArMV-BU (Butterbur) isolate causes symptomless infection on these plants (Imura and al., 2008). The severity of the symptoms depends on specific virus-host interactions and on environmental conditions. In many herbaceous hosts and in particular in *Nicotiana* species, symptoms develop on the inoculated leaves and on the first upper systemic leaves whereas the new emerging systemic leaves remain free of symptoms although the virus is present. This phenomenon, where the leaves appeared healthy albeit they contain virus, is termed recovery.

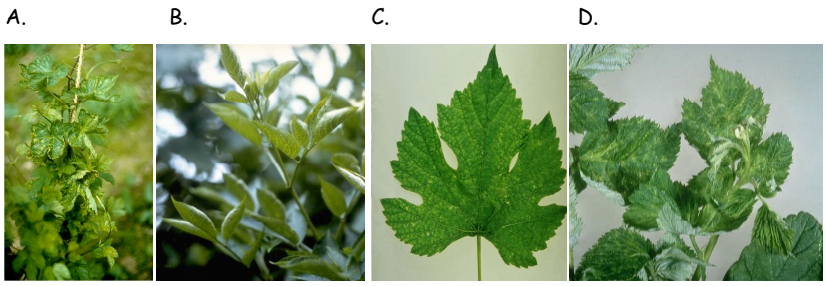



**Figure 8: Symptoms caused by *Arabis mosaic virus* infection on different host plants** (EPPO)
A. Symptoms of "nettlehead" on hop leaves (Eppler, Institut für Phytopathologie und angew. Zoologie, Giessen, Germany); B. *Sambucus nigra* (elder) with vein clearing and leaf rolling (Eppler, Institut für Phytopathologie und angew. Zoologie, Giessen, Germany); C. Mosaic on grapevine (Institut für Planzenschutz im Weinbau, Bernkastel/Kues, Germany); D. Flecking symptoms on raspberry (*Rubus idaeus* cv.) (CSL, York, GB - British Crown).

No information was available concerning the viral determinants responsible for the expression of symptoms of the grapevine fanleaf disease neither for ArMV nor for GFLV and RpRSV.

In order to characterize the symptom determinant(s) of ArMV, we constructed recombinant ArMV-NW genomes where partial and/or complete genes were substituted by their counterpart of two other ArMV isolates, ArMV-Lv and ArMV-Lilac. These chimeric constructs were tested for infectivity and symptoms development on herbaceous plants. This approach needed first to clone and sequence the genome (RNA1 and RNA2) of ArMV-Lv and ArMV-Lilac isolates and to identify the expression of the symptoms induced by the ArMV isolates on several herbaceous plants in order to find the plant species suitable for this study.

### 1. Symptoms induced by the ArMV-NW, -Lv and -Lilac isolates on herbaceous hosts

Four herbaceous plants known to be susceptible to nepovirus infection *Chenopodium quinoa* (family *Chenopodiaceae*), *Nicotiana benthamiana*, *Nicotiana tabacum* and *Nicotiana glutinosa* (family *Solanaceae*) were mechanically inoculated with ArMV-NW, ArMV-Lv and ArMV-Lilac to determine which plant(s) might be appropriate to characterize the determinant(s) of ArMV involved in the expression of disease symptoms. Such a plant should be infected by the three ArMV isolates but express different symptoms upon viral infection.

#### 1.1. *Chenopodium quinoa* (*C. quinoa*)

*C. quinoa* plants infected with ArMV-NW displayed only very mild symptoms or remained symptomless (fig. 9A). By contrast, inoculation of these plants with ArMV-Lv led to severe chlorotic and necrotic symptoms both on inoculated and systemic leaves at 7 days post-infection (dpi). A few

days later, between 10 and 14 dpi, the apical part of these plants showed a very severe necrosis (Fig. 9B), leading eventually to the death of these plants. Yellow mottling or mosaic was observed both on inoculated and systemic leaves of *C. quinoa* infected with the ArMV-Lilac isolate (fig. 9C). The severity of these symptoms was influenced by the environmental conditions i.e they were less severe when the temperature in the greenhouse was higher than 22°C.

The viral titer in systemic leaves of the infected *C. quinoa*, was determined by ELISA, using polyclonal antibodies raised against the capsid protein (anti-CP) of ArMV-NW. Similar absorbance values were obtained for ArMV-NW, ArMV-Lv or ArMV-Lilac isolates, indicating that the type of symptoms on infected *C. quinoa* plants were specifically induced by these isolates independently of their replication rate.

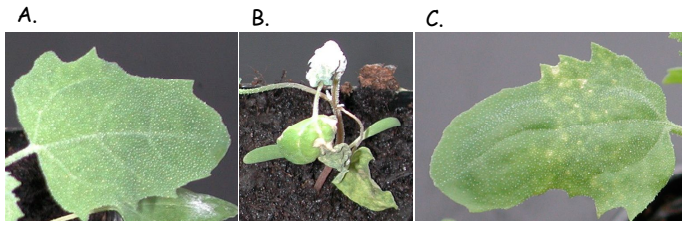



**Figure 9: Symptoms displayed by *C. quinoa* leaves infected by ArMV-NW, ArMV-Lv and ArMV-Lilac isolates**
A. Light mosaic on a systemic leaf caused by ArMV-NW infection; B. Very severe necrosis on the systemic leaves and the apex of plants infected by ArMV-Lv; C. Yellow mottling/mosaic on a systemic leaf infected by ArMV-Lilac. These symptoms were observed at 14 days post-inoculation (dpi).

### 1.2. *Nicotiana benthamiana* (*N. benthamiana*)


**Figure 10: Symptoms expressed on *N. benthamiana* leaves, 7 days after mechanical inoculation with ArMV-NW, ArMV-Lv and ArMV-Lilac isolates**
A. Systemic leaves of plants inoculated with ArMV-NW; B. Mosaic on the first systemic leaves infected by ArMV-Lv; C. Necrosis on a systemic leaf infected by ArMV-Lilac.

Symptoms were not observed on *N. benthamiana* plants inoculated with ArMV-NW, two weeks

after inoculation (fig. 10A) albeit this isolate replicated, as evidenced by the detection of the CP by ELISA. By contrast, infection of *N. benthamiana* with ArMV-Lv induced a severe mosaic on the first systemic leaves at 7 dpi (fig. 10B). However, the emerging new leaves remained symptomless. Concerning the ArMV-Lilac isolate, plants displayed a necrotic spots on the first systemic leaves (fig. 10C) but, as for ArMV-Lv, the new leaves of ArMV-Lilac-infected plants were symptomless.

### 1.3. *Nicotiana tabacum* (*N. tabacum*)

*N. tabacum* plants mechanically inoculated with ArMV-NW, -Lv or -Lilac isolates displayed no symptoms (mosaic, mottling and necrosis) neither on inoculated and nor on systemic leaves even three weeks after inoculation, suggesting that none of these isolates replicated in this herbaceous plant. To verify this hypothesis, we determined the presence of the ArMV capsid protein (CP) from plant extracts by ELISA. The CP could only be detected in the extract from *N. tabacum* inoculated with ArMV-Lv thus, indicating that ArMV-Lv is the sole isolate, among the three we tested, which is able to replicate on *N. tabacum* however, inducing no symptom. To confirm this, we also performed northern blots on total RNAs extracted from systemic leaves, using as probe a cDNA labelled with $^{32}P$ by random priming (fig. 11).

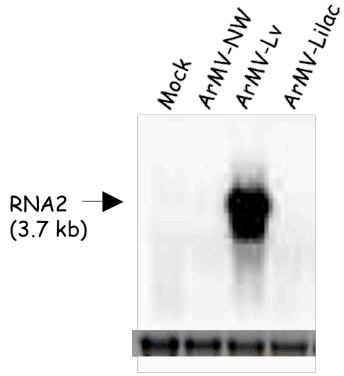



**Figure 11: Detection by northern blot of RNA2 in systemic leaves of *N. tabacum* inoculated with ArMV-NW, ArMV-Lv or ArMV-Lilac isolates**
RNAs were extracted from systemic leaves and electrophoretically fractionated on a 1.5% agarose gel under denaturing conditions. RNA was probed with a $^{32}P$-radiolabelled cDNA obtained by PCR amplification of the MP gene of ArMV-NW. The loading control (LC) showed below the northern blot corresponds to rRNAs stained with ethidium bromide.

We chose a probe complementary to the RNA2-located gene coding for the movement protein (MP) of ArMV-NW for two reasons: i) previous northern assays have shown that this cDNA hybridizes to the RNA2 of ArMV-NW, -Lv and -Lilac, as expected since the MP gene is with the CP

gene, highly conserved between the three ArMV isolates (identity ≥ 94%) and ii) RNA2 (3.7 kb) is usually replicated with a high efficiency compared to RNA1 (7.3 kb). This asymmetrical replication is probably due to the fact that MP and CP encoded by RNA2 are required in large amounts for the systemic infection of plants. Indeed, nepoviruses move from cell-to-cell in the form of particles (180 CP/particles) through long tubules formed by numerous MP molecules (Takemoto and al., 1985; Ritzenthaler *et al.*, 1995b).

The results of the northern blots showed that RNA2 was only detected in *N. tabacum* plants infected by ArMV-Lv. Any radioactive signal could be detected in total RNAs from plants inoculated with ArMV-NW and ArMV-Lilac.

### 1.4. *Nicotiana glutinosa* (*N. glutinosa*)

*N. glutinosa* plants inoculated with ArMV-NW, -Lv or -Lilac remained symptomless. However, the two latter isolates replicated in *Nicotiana* species since their CP could be detected by ELISA. By contrast, the CP of ArMV-NW was not immunodetected, suggesting that this isolate is unable to replicate in *N. glutinosa*.

Taken together, our results indicate that *C. quinoa* is among the four herbaceous plants that we tested, the only one which is appropriate to study the ArMV determinants involved in the expression of symptoms (table 1). *C. quinoa* can be infected by the three ArMV isolates and it develops specific symptoms depending on these isolates. *N. benthamiana* is the sole *Nicotiana* species, which is susceptible to the ArMV-NW, ArMV-Lv and ArMV-Lilac isolates, but symptoms are only expressed on plants inoculated with the ArMV-Lv and -Lilac isolates although the new emerging leaves do not display no symptoms.

| | ArMV-NW | | ArMV-Lv | | ArMV-Lilac | |
|---|---|---|---|---|---|---|
| | Infectivity | Symptoms | Infectivity | Symptoms | Infectivity | Symptoms |
| *C. quinoa* | + | Light mosaic/- | + | Chlorotic/necrosis | + | Mosaic/ mottling |
| *N. benthamiana* | + | - | + | Mosaic | + | Necrotic spots |
| *N. tabacum* | - | - | + | - | - | - |
| *N. glutinosa* | - | - | + | - | + | - |

**Table 1: Infectivity of ArMV-NW, ArMV-Lv and ArMV-Lilac isolates on herbaceous plants and symptoms observed on infected plants**

## II. Cloning and sequencing of the genome of ArMV-NW, ArMV-Lv and ArMV-Lilac isolates

### 1. ArMV-NW full-length infectious clones

The genome of the ArMV-NW isolate, originating from infected « Pinot gris » grapevines in Hambach, in South West of Germany near Neustadt an der Weinstrasse (NW), was cloned and sequenced by Wetzel *et al.* (2001; 2004). The virus was propagated on *C. quinoa*, purified on sucrose gradient and finally, the viral genomic RNAs were extracted, as described in material and methods. Specific and/or degenerate primers were designed based on conserved motifs of the RNA genome of nepoviruses and used, for the cloning and sequencing of the cDNAs corresponding to genomic RNA1 and RNA2 of ArMV-NW (fig.12).

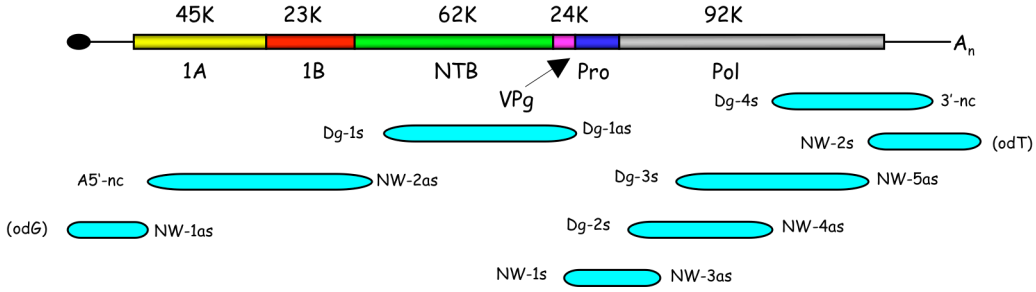



**Figure 12: Cloning strategy of the genomic RNA1 of ArMV-NW**
The different RT/PCR-generated products and the corresponding couple of primers are represented under the genomic map of ArMV-NW RNA1. The polyprotein P1 encoded by RNA1 contains from its N-terminus: the protein 1A (45 kDa), the protein 1B (protease cofactor, 23 kDa), the NTB protein (helicase, 62 kDa), the VPg (3 kDa), the protease (Pro, 24 kDa) and finally the RNA-dependent RNA polymerase (Pol, 92 kDa). The black circle represents the VPg covalently bound to the 5' end and $A_n$ corresponds to the poly(A) tail at the 3' end. The thin lines represent the non-coding sequences.

ArMV-NW cDNAs already available in the laboratory (Wetzel *et al.*, 2001) were used whenever possible to be assembled step by step in the vector pCassII (Shi *et al.*, 1997), based on the presence of unique restriction sites in the ArMV-NW RNA1 or RNA2 sequences.

A one-step overlap extension PCR (Urban *et al.*, 1997) was used to fuse the cDNAs corresponding to the 5' ends of the viral RNAs to the 35S promoter transcription start of the plasmid pCassII such as the first transcribed nucleotide corresponds exactly to the 5' end of the viral RNAs. Indeed, Hans *et al.* (1992) demonstrated that the presence of extra nucleotides at the 5' end of the viral RNAs of nepoviruses had a dramatic effect on the infectivity of the transcripts. In contrast, the presence of additional nucleotides at the 3' end had less inhibitory effect on the virus replication. An inhibitory effect of 5' non-viral extensions have also been demonstrated for synthetic transcripts of TBRV (Greif *et al.*, 1990) and CMV satellite (Masuta *et al.*, 1988) and for RNAs obtained by *in vitro* transcription of genomic cDNAs of animal and plant viruses (Ziegler-Graff *et al.*, 1988). Clones

containing a poly(U) with 30 uridyl residues were used for the 3' ends of RNA1 and RNA2 in order to add a poly(A) tail by transcription of the cDNAs. For regions of the viral genome for which no clones with appropriate restriction sites were available, unique restriction sites, which generated silent mutations in the coding sequence, were integrated in the primer sequences, and RT/PCR were performed with these primers on purified ArMV-NW RNAs. The resulting RT-PCR products were cloned, sequenced and used for further subcloning.

The cDNA clones, FL1 and FL2, corresponding to the full-length RNA1 and RNA2 of ArMV-NW are infectious. Indeed, when they are inoculated *C. quinoa* plants, the latter displayed similar symptoms as those inoculated with ArMV-NW isolate. Therefore, we used these clones to realize chimeric constructs for the characterization of the ArMV symptom determinants.

## 2. ArMV-Lv genomic RNAs

### 2.1. Cloning and sequencing of the ArMV-Lv genomic RNAs

The ArMV isolate from *Ligustrum vulgare*, named ArMV-Lv, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The virus was propagated on *C. quinoa* and purified by differential centrifugation and precipitation with butanol and PEG solution. The viral RNAs were extracted from virions, as described in materiel and methods. The RT-PCR products generated from total viral RNAs, using specific and/or degenerate primers, were sequenced directly or cloned before sequencing. The sequence of the 5' end was obtained upon a 5' RACE experiment whereas the sequence of the 3' end was determined from a cDNA obtained by RT-PCR using an oligo d(T) primer. The cDNAs were sequenced in both directions and the outcoming sequences were compiled and analysed using the DNAsis program package (Hitachi). These sequences have been submitted to the Genbank/EMBL database and have been assigned with the accession numbers EU617326 and EU617327 (Dupuis *et al.*, 2008).

The two RNAs of ArMV-Lv, RNA1 and RNA2, have a length of 7,334 nt and 3,812 nt, respectively, excluding the poly(A) tail. RNA1 contains a single ORF encoding a large polypeptide (P1) of 2,285 amino acids (MW 252 kDa), preceded by a 5' UTR of 229 nt-long and followed by a a 3' UTR of 247 nt-long. Concerning RNA2, the unique ORF codes for a polypeptide (P2) of 1,118 amino acids (MW 122 kDa); the ORF is flancked by 5' and 3' UTRs whose lengths are 261 nt and 194 nt, respectively.

The ArMV-Lv polyproteins display the same modular arrangement of functional domains, as described for the ArMV-NW polyproteins. Therefore, the organization of the polyprotein encoded by RNA1, starting from the N-terminus, is: a protein of unknown function (1A), the cofactor of protease (1B), the helicase (NTB), the VPg, the protease and the RNA-dependent RNA polymerase. The polyprotein encoded by RNA2 consists of three proteins, a protein of 28 kDa (2A), which is involved in the replication of the RNA2, the movement protein (MP) and the coat protein (CP).

### 2.2. Comparison of the sequences of ArMV-NW and the ArMV-Lv genomes

The comparison of the coding sequences of ArMV-Lv and -NW isolates revealed an identity of 81% and 86% for RNA1 and RNA2, respectively. At the amino acid (aa) level, the identity of the polyproteins varies from 73% to 97% (fig. 13). The lowest identity, 78% and 73%, was observed for 1A and 2A, respectively, two proteins whose function(s) are not well defined, and for the VPg (78%). The highest identities were found for the MP (97%) and the CP (96%).

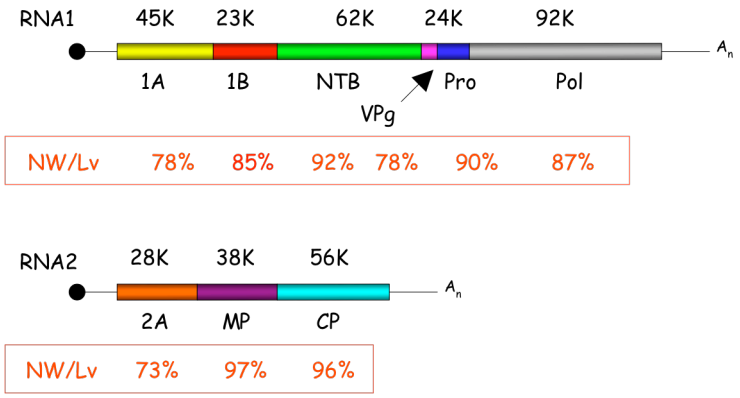



**Figure 13: Representation of the genomic RNA1 and RNA2 of ArMV and comparison of the primary structure of polyproteins P1 and P2 of the ArMV-NW and -Lv isolates**
The polyproteins P1 and P2 encoded by RNA1 and RNA2, respectively, are represented by colored boxes, each colour corresponding to a mature protein. The percentage of amino acid identities between ArMV-NW and -Lv are mentioned under each protein. The black spheres represent the VPg at the 5' end and $A_n$, corresponds to the poly(A) tail at the 3' end. The thin lines represent the non-coding sequences.

Concerning the protein 1A or also named X1, which is the most divergent ArMV proteins, bioinformatics analysis (Blast P) revealed nevertheless a short stretch of amino acid sequence (27 residues) that is conserved in several nepoviral polyproteins (fig. 14). This conserved motif is found in the N-terminal part of both polyproteins, P1 and P2, of nepoviruses belonging to subgroup C i.e *Blackcurrant reversion virus* (BRV) and *Tomato ringspot virus* (ToRSV) and in the N-terminal part of the polyprotein P1 of nepoviruses from subgroup A. The biological significance of this motif has not been elucidated up to now.

The analysis of the non-coding sequences of ArMV-Lv revealed the presence of putative stem-loop structures in the 5' and 3' UTRs of both RNAs (Dupuis *et al.*, 2008). Such structures were found at similar positions in GFLV, *Grapevine chrome mosaic virus* (GCMV) and *Tomato black ring virus* (TBRV) (Wetzel *et al.*, 2001 and reference therein).

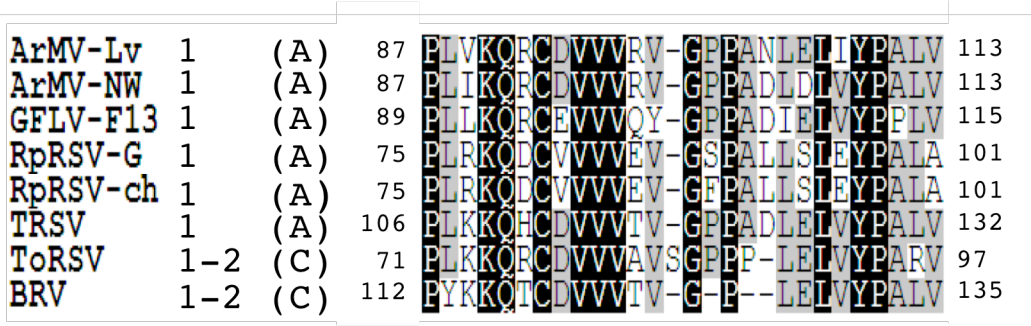



**Figure 14: Comparison of the conserved sequence located in the N-terminal region of the polyproteins P1 (1) and P2 (2) encoded by the genome of ArMV-Lv and -NW isolates and other nepoviruses**

Dashes correspond to gaps introduced to optimise the alignments. Conserved amino acids (aa) are boxed in black whereas the aa found in at least five sequences are boxed in grey. The nepovirus subgroups are shown as (A) and (C). The numbering indicates the location within the polyproteins.

### 3. Sequence of the RNA2 of the ArMV-Lilac isolate

The complete sequence of the genomic RNA2 of the ArMV-Lilac isolate (Wetzel *et al.*, unpublished) was also determined, using the same procedure as for the ArMV-Lv. The sequence of the genomic RNA1 of this isolate is currently being determined.

We compared the primary structure of the polyprotein P2 of ArMV-Lilac and ArMV-NW (fig. 15). As expected, the most divergent protein is the protein 2A, with an identity of only 64% while the MP and CP present 94% of sequence identity.

Preliminary results concerning the sequence of RNA1 of ArMV-Lilac indicate that the proteins 1A, VPg and Pro have an identity of 78%, 78% and 89% with their counterparts of the ArMV-NW polyprotein P1, respectively.

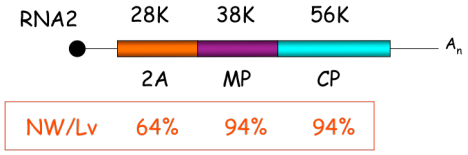



**Figure 15: Comparison of the polyprotein P2 sequence of ArMV-NW and -Lilac isolates**

The polyprotein P2 is represented with the regions coding for the three proteins: 2A, MP and CP. The percentage of amino acid (aa) identities between ArMV-NW and -Lilac is mentioned in the red frame. The black sphere represents the VPg located at the 5' end and $A_n$ to the poly(A) tail. The thin lines correspond the non-coding sequences.

## III. Identification of the ArMV determinants responsible for the expression of symptoms on host plants

Harrisson *et al.* (1972; 1974) have mentioned that both RNA1 and RNA2 of *Raspberry ringspot virus* (RpRSV, *nepovirus*) code for symptom determinants, suggesting that the distribution of such determinants on both genomic RNAs is a common feature of nepoviruses. These authors showed that RNA2 is responsible for yellowing symptom on infected *Petunia hybrida* whereas RNA1 carries determinants for other symptoms including necrosis and stunting. Therefore, both infectious cDNA clones, FL1 and FL2 were used to construct chimeric cDNAs in which complete or partial genes were substituted by their counterpart of ArMV-Lv or ArMV-Lilac isolates.

The chimeric cDNAs FL1 and FL2 were inoculated to *C. quinoa* plants together with the wild-type FL2 or FL1 cDNA, respectively, to constitute a complete nepoviral genome in order to see if the inserted DNA of the chimeric clone induces specific disease symptoms. Indeed, as seen previously *C. quinoa* plants express specific symptoms depending on the ArMV-NW, -Lv and -Lilac isolates. The characterization of the ArMV symptom determinant(s) should be facilitated by the fact that wild-type ArMV-NW induces on *C. quinoa* very mild symptoms or no symptom.

### 1. Characterization of symptom determinants encoded by the RNA2 of ArMV

First, we investigated the role of the 2A, MP and CP proteins encoded by RNA2, in the expression of symptoms on *C. quinoa* plants. The chimeric cDNA clones were constructed using single restriction sites in the sequence of ArMV-NW RNA2.

ArMV-Lv or -Lilac sequences coding for the three proteins were amplified from total RNAs extracted from infected plants, by RT-PCR using specific primers, containing at their 5' ends, the restriction sites allowing the insertion of the cDNAs in the FL2 clone. After amplification, the cDNAs were digested with the restriction endonucleases and swapped with the corresponding fragments on the ArMV-NW FL2. The sequence of the chimeric clones was controlled in order to ensure that we did not interrupt the coding sequence.

The chimeric FL2 construct was mechanically inoculated together with the FL1 cDNA clone onto *C. quinoa* plants at a four leaves stage. In parallel, we also infected this host plant with the wild-type ArMV-NW clones (FL1+FL2), which served as reference. The cDNA, under the control of the 35S promoter in the pCassII vector, were transcribed in the plant by the cellular RNA polymerase II, probably as capped RNAs; the latter were also polyadenylated because the cDNAs contained a poly(U) sequence at their 3' end of the cDNA. Symptoms development was followed up to 21 dpi and the presence of virions and/or their components was determined using different molecular techniques (ELISA, RT-PCR and northern blots). Several independent experiments were performed with 4 plants per assay, under the same environmental conditions (temperature, light…).

### 1.1. Protein 2A

We focused first our investigation on the protein 2A, located at the N-terminus of polyprotein P2, because it is the most divergent ArMV proteins. Therefore, it might be involved in the expression of symptoms.

The corresponding cDNA fragment of the ArMV-NW was replaced in the FL2 cDNA by the 2A gene of the ArMV-Lv or Lilac, respectively (fig. 16). The restriction sites used for the construction of these chimeric clones were *Bal*I, located at the 5' end of the 2A gene, and *EcoN*I, present 80 nucleotides upstream of the sequence coding for the cleavage site 2A/MP of the polyprotein P2.

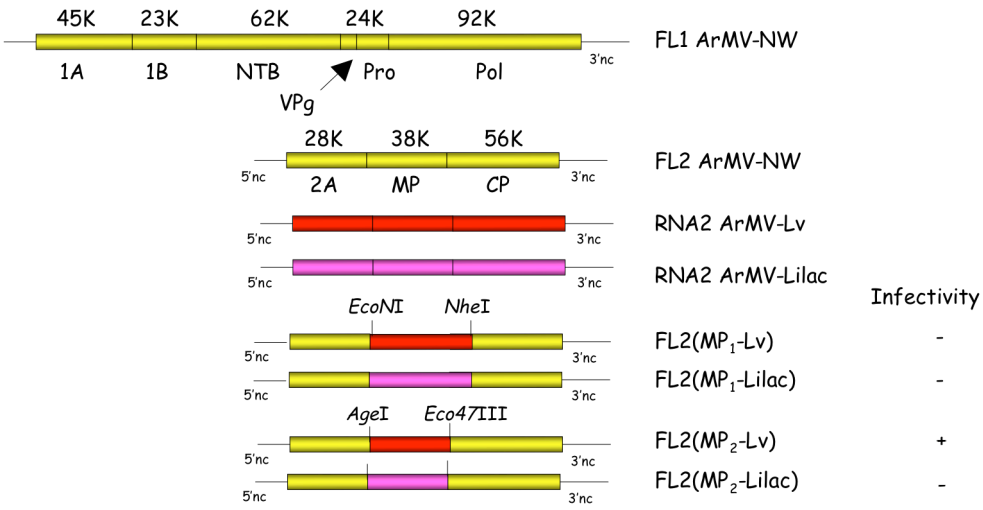



**Figure 16: Representation of the chimeric constructs obtained by substitution of the 2A gene in the FL2 cDNA by the homologous gene of ArMV-Lv or ArMV-Lilac isolates**
The polyproteins P1 and P2 of the ArMV-NW are represented by yellow boxes. The P2 of ArMV-Lv and ArMV-Lilac are represented by red and pink boxes, respectively. This colour code is used in this figure and in the others to depict the chimeric constructs. The restriction sites used for the construction of chimeric FL2 are indicated. The VPg and the poly(A) are not represented. The infectivity on *C. quinoa* of the different cDNA combinations (FL1 + chimeric FL2) determined by ELISA, is given at the right of the figure. The + or - signs indicate if the plants were infected or not.

The infectivity of these different chimeric constructs, FL1(ArMV-NW) + FL2(2A-Lv), FL1(ArMV-NW) + FL2(2A-Lilac) and ArMV-NW (FL1+FL2) was determined by ELISA, using polyclonal antibodies directed against the CP of ArMV-NW, on total proteins extracted from systemic leaves of *C. quinoa*, at 15 dpi. Similar absorbance values were obtained with the plants inoculated with the different chimeric constructs and wild-type ArMV-NW, indicating that the chimeric constructs were infectious and that the protein 2A of Lv and Lilac did not impair the replication of the NW RNA2. This was confirmed by the northern blots that we realized on total RNAs extracted from

systemic leaves with a probe specific for the MP gene (fig.17). Indeed, the levels of RNA2 were similar in plants inoculated with chimeric or wild-type ArMV-NW. Amplification by RT-PCR and sequencing of the 2A gene of chimeric RNA2 constructs from total RNAs extracted from infected plants showed that no mutation occurred in the 2A gene of Lv and Lilac in the course of the infection.

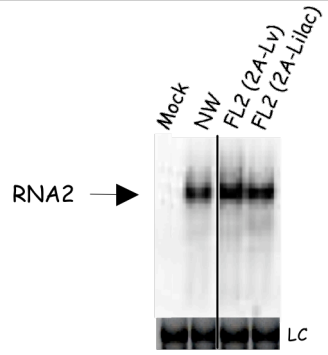



**Figure 17: Analysis by northern blot of the RNA2 level in systemic leaves of *C. quinoa* infected by wild-type and chimeric ArMV**
Total RNAs from mock-inoculated or infected leaves were extracted and after fractionation on a denaturating agarose gel (1.5%), probed with a radiolabelled NW-MP cDNA. The loading control (LC) is presented below the northern blot.

Infection was not delayed when the ArMV-NW 2A gene was replaced by its counterpart from the ArMV-Lv or -Lilac isolates in the FL2, compared to the control infection experiment performed with the wild-type viruses. Indeed, the symptoms were visible on inoculated leaves at 7 dpi and on systemic leaves of *C. quinoa* plants at 15 dpi with all viral combinations. However, they were different from those observed with the wild-type isolate. We observed light ringspots on the inoculated and systemic leaves of *C. quinoa* with chimeric FL2 containing the gene 2A of the Lilac whereas wild-type ArMV-Lilac induced a mosaic (fig. 18). By contrast, inoculated and systemic leaves of *C. quinoa* infected with a chimeric ArMV-NW expressing the protein 2A of the Lv isolate displayed a mild mosaic instead of necrosis, as observed with ArMV-Lv.

At 18 dpi, the symptoms progressively faded away and no symptom could be observed in the new emerging leaves. The retro-inoculation of *C. quinoa* performed with sap prepared from systemic leaves of plants inoculated with the construct FL1 + FL2(2A-Lv), FL1 + FL2(2A-lilac) led to the same phenotypes. The plants contained similar amounts of virions and/or CP as shown by ELISA. These results indicate that the protein 2A and/or its gene can modulate the expression of symptoms independently of the virus titer and that the type of symptom depends on the viral context.

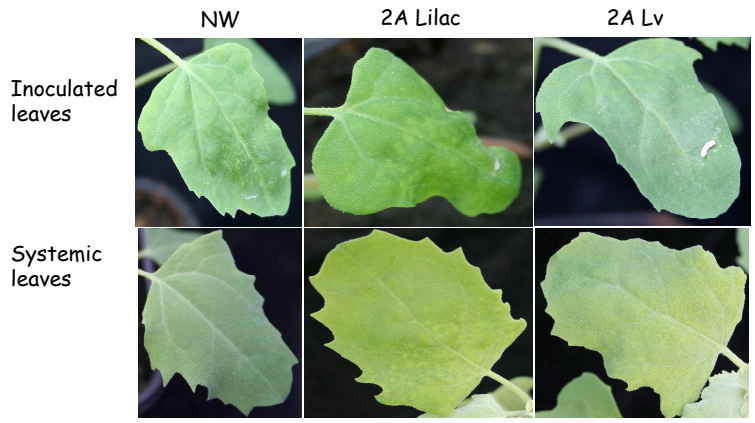



**Figure 18: Symptoms induced on inoculated and systemic leaves of *C. quinoa* by the wild-type ArMV-NW and the chimeric NW constructs containing in its RNA2 the gene 2A of Lv or Lilac**
The plants were mechanically inoculated with FL1 + FL2 cDNAs (NW) or with FL1 associated the FL2 cDNA containing the gene 2A of Lilac (2A Lilac) or Lv (2A Lv). Plants observed at 15 dpi.

Comparison of the amino acid sequence of the protein 2A from the three ArMV isolates clearly showed that the C-terminal part of the protein is highly conserved whereas the N-terminal region is variable, in particular for the Lilac isolate (fig. 19). The N-terminus of the protein Lilac 2A is shorter compared to its counterpart from the Lv and NW isolates, essentially due to the deletion of two sequences of 13 residues (positions 24 to 37) and 15 residues (positions 74 to 90). The protein 2A of ArMV-NW has a deletion of a stretch of 9 aa (positions 88 to 97) compared to Lv.


**Figure 19: Alignment of the amino acid sequence of the protein 2A sequences of the ArMV-NW,-Lv and -Lilac isolates** (Wetzel *et al.*, 2002)
Conserved amino acids are boxed in black; amino acids found in two of three sequences are boxed in darken grey. The amino acid, which belongs to the same class, are boxed in clear grey.

The variability of the N-terminal part of protein 2A strongly suggest that this region might be particularly responsible for the symptoms observed on systemic leaves of *C. quinoa*. In order to verify

this hypothesis, we realized chimeric constructs where the 5' region of the 2A-NW gene was exchanged by the corresponding region of ArMV-Lv or -Lilac, using the restriction sites *Bal*I and *Xho*I (fig. 16). Additionally, we deleted in the FL2 cDNA, the sequence coding for the amino-terminal region of the NW 2A gene, using the same restriction sites. The chimeric constructs, FL2(2A-Nter-Lilac) and FL2(2A-Nter-Lv) and the FL2 deletion mutant, co-inoculated with the FL1, were infectious on *C. quinoa* plants, as evidenced by the immunodetection of the capsid protein in systemic leaves. Symptoms displayed by the systemic leaves of plants inoculated with these constructs were similar to those observed with the FL2 constructs containing the full-length 2A gene of Lilac and Lv, namely ringspots with FL2(2A-Nter-Lilac) and a mosaic with FL2(2A-Nter-Lv). Symptoms were never observed with the ArMV-NW mutant coding for the N-terminal deleted 2A protein. These results indicate that the N-terminal part of the 2A gene is not necessary for viral replication. On the other hand, this protein seems to play a role in the expression of symptoms on *C. quinoa*.

### 1.2. Movement protein (MP)

The *Eco*NI and *Nhe*I restriction sites present in the RNA2 nucleotide sequence were used to construct FL2 chimeric cDNAs, containing the MP gene of the Lilac or Lv isolates. The *Nhe*I site is located 60 nucleotides downstream of the MP/CP cleavage site (fig. 20).

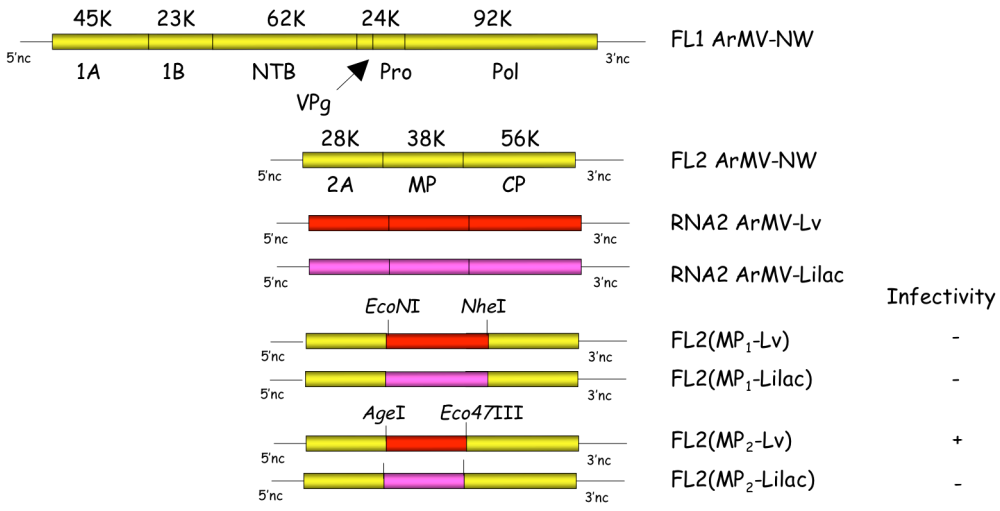



**Figure 20: Representation of the chimeric FL2 constructs containing the MP gene of Lv and Lilac isolates**
The polyproteins P1 and P2 of the ArMV isolates are represented in yellow, red and pink for NW, Lv and Lilac isolates, respectively. The infectivity of the cDNAs on *C. quinoa* was determined by ELISA (+ for infectious and - for non-infectious). $MP_1$ and $MP_2$ correspond to proteins obtained by the hydrolyzis of the wild-type and mutated cleavage sites, respectively.

The CP of ArMV-NW was detected in systemic leaves by ELISA, at 15 dpi, to determine the

infectivity of the different chimeric constructs, FL1(ArMV-NW) + FL2($MP_1$-Lv), FL1(ArMV-NW) + FL2($MP_1$-Lilac) and FL1 + FL2 of ArMV-NW on *C. quinoa*.

The chimeric clones possessing $MP_1$-Lv or -Lilac ($MP_1$ corresponds to wild-type cleavage sites) co-inoculated with FL1 did not produce symptoms on *C. quinoa*, suggesting that these constructs were not infectious; this was confirmed by the negative results of the immunodetection assays (ELISA). The loss of infectivity of the viral cDNAs could be due to the fact that chimeric P2 polyproteins were not processed correctly by the NW protease encoded by RNA1 although the 2A/MP and/or MP/CP cleavage sites are conserved in the three ArMV isolates, C/A and R/G, respectively (fig. 21). We hypothesized that the non-conserved residues upstream of the cleavage sites (4 for 2A/MP and 1 for MP/CP) may be critical for the cleavage of P2 by the NW proteinase. Therefore, we generated new constructs, named $MP_2$-Lv and $MP_2$-Lilac in which we conserved the NW sequences coding for the 2A-MP and MP-CP junction in the chimeric polyprotein P2. The introducing of *Age*I and *Eco47*III restriction sites in the ArMV-NW RNA2 sequence allowed us to swap the fragments corresponding the MP sequence without affecting the cleavage sites.

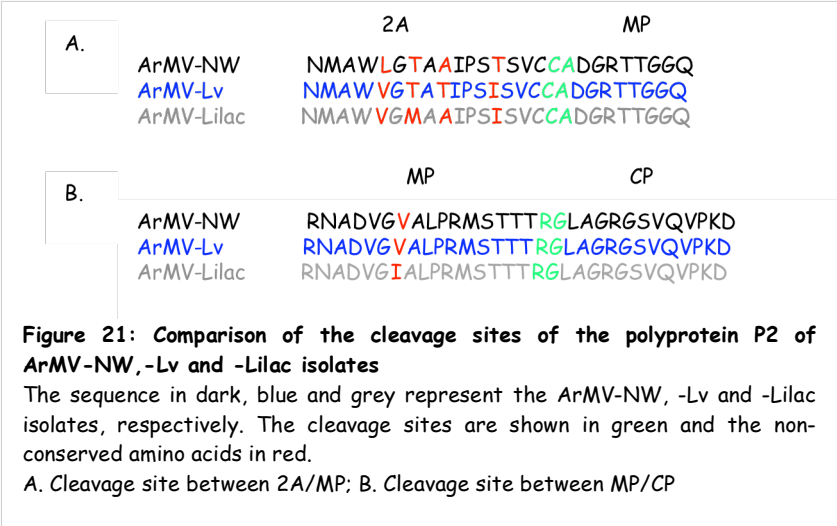



**Figure 21: Comparison of the cleavage sites of the polyprotein P2 of ArMV-NW,-Lv and -Lilac isolates**
The sequence in dark, blue and grey represent the ArMV-NW, -Lv and -Lilac isolates, respectively. The cleavage sites are shown in green and the non-conserved amino acids in red.
A. Cleavage site between 2A/MP; B. Cleavage site between MP/CP

Very light mosaic symptoms were observed on *C. quinoa* inoculated with FL1 and FL2($MP_2$-Lv) at 15 dpi (fig. 22A). By contrast, the $MP_2$-Lilac construct did not induced symptoms similar to the wild-type ArMV-NW, used as control. The results of northern blots performed on total RNAs from systemic leaves at 15 dpi showed that ArMV-NW and FL1 + FL2($MP_2$-Lv) construct were infectious: the RNA2 could be detected in both cases almost at the same level (fig. 22B). The absence of RNA2 in *C. quinoa* plants inoculated with FL1 + FL2($MP_2$-Lilac) strongly suggest that this chimeric NW construct was unable to systemically infect these plants. This was confirmed by ELISA realized on plant extracts with anti-CP antibodies. Indeed, the CP could not be detected in the systemic leaves of

*C. quinoa* plants inoculated with FL1 + FL2($MP_2$-Lilac) whereas it was found in plants inoculated with the wild-type ArMV-NW and the chimeric virus expressing the MP of the Lv isolate.

In conclusion, the MP of the ArMV-Lv isolate might be involved in the expression of symptoms on *C. quinoa*. Our study also indicates that amino acid sequences at proximity of the cleavages sites are important for the processing of polyprotein P2 by the NW proteinase since the chimeric cDNA were infectious only when we mutated the non-conserved residues in order to restore the homologous sequence surrounding the cleavage site of the NW P2. Additional investigations are required to determine why the MP-Lilac construct was not infectious and if it is involved in the symptoms development on *C. quinoa*.

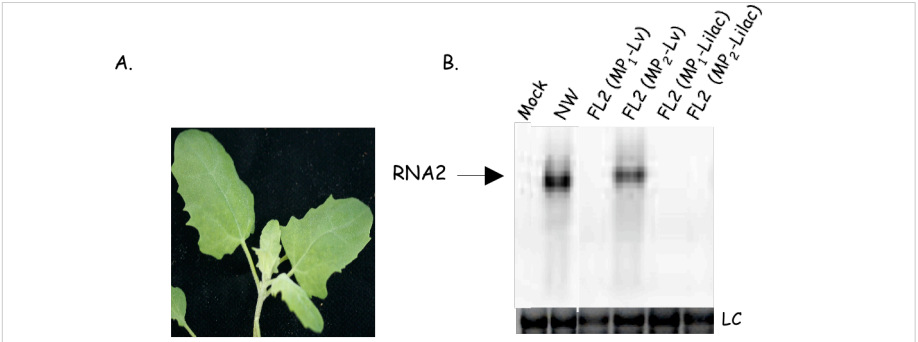



**Figure 22: Symptoms of *C. quinoa* systemic leaves infected by ArMV-NW expressing a chimeric P2 polyprotein containing the Lv MP protein and detection of RNA2 in plants inoculated with different viral combinations**
A. Light mosaic symptoms on systemic leaves infected by FL1+ FL2($MP_2$-Lv) at 15 dpi; B. Total RNAs from *C. quinoa* mock-inoculated or inoculated with various chimeric FL2 associated with FL1 constructs were probed on northern blots with a radioactive cDNA specific for the NW MP. The loading control (LC) is shown below the northern blot.

### 1.3. Capsid protein (CP)

Chimeric FL2 cDNAs containing the sequence coding for the CP of Lv (FL2 (CP-Lv)) or Lilac (FL2(CP-Lilac)) isolates were generated by mutagenesis of the FL2 cDNA clone, using the *Nhe*I restriction site, located downstream of the cleavage site MP/CP and the *Nsi*I restriction site present at the end of the CP coding region (fig. 23).

At 15 dpi, the *C. quinoa* plants inoculated with chimeric constructs did not induce symptoms although, both FL1 + FL2(CP-Lv) and FL1 + FL2(CP-Lilac) were infectious, as evidenced by the presence of RNA2 in systemic leaves.

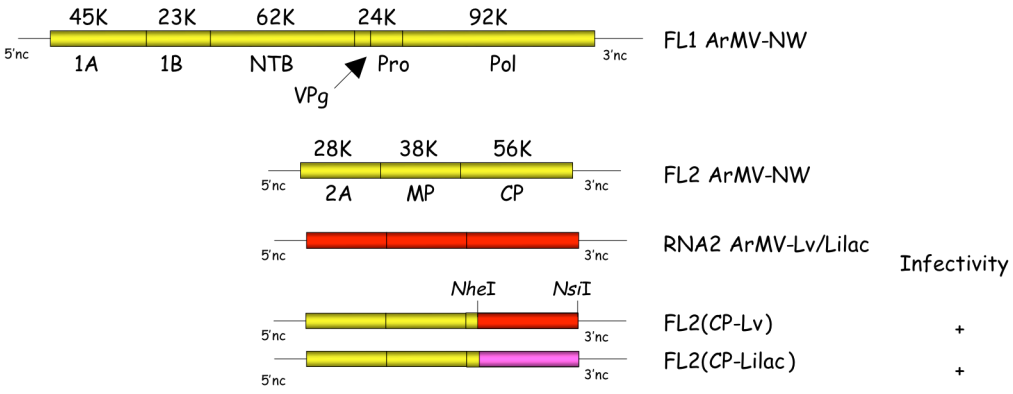



**Figure 23: Representation of the chimeric FL2 constructs containing the CP gene of Lv and Lilac isolates**
The polyproteins P1 and P2 are represented in yellow, red and pink for the ArMV-NW, -Lv and -Lilac isolates, respectively. The infectivity of these viruses analysed by ELISA are indicated at the right of the figure. The + or - correspond to the infectious or non-infectious results, respectively.

However, as shown by the autoradiograph of the northern blots, the level of RNA2 is extremely low for the FL2(CP-Lilac) chimeric construct compared to that of wild-type RNA2 or that obtained wit FL1 + FL2(CP-Lv) (fig. 24). This difference might be due to an inefficient replication of the viral RNA and/or to cell-to-cell movement of the chimeric virus and consequently, these results must be confirmed.

These results suggest that the ArMV coat protein plays no role in the development of symptoms on *C. quinoa*.

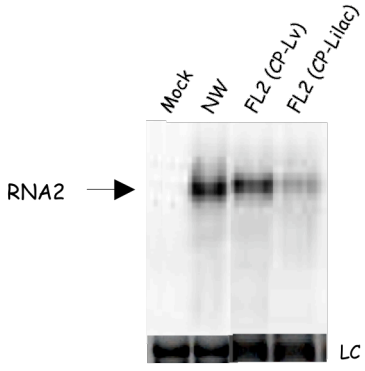



**Figure 24: Northern blot performed on total RNAs of systemic leaves of *C. quinoa* plants inoculated with FL1 associated with FL2, FL2(CP-Lv) or FL2(CP-Lilac)**
Total RNAs were separated on a denaturing gel (1.5%) and hybridized with NW MP cDNA labelled radioactively by random priming. The loading control (LC) is mentioned below the northern blot.

## 2. Characterization of symptom determinants encoded by the RNA1 of ArMV

In order to identify symptom determinants encoded by the RNA1, we used the same strategy as for RNA2. We generated different chimeric NW constructs by replacing completely or partially one or several genes in the ArMV-NW FL1 cDNA by their counterparts of ArMV-Lv RNA1. The chimeric cDNAs were then co-inoculated with the full-length FL2 clone on *C. quinoa* plants. For example, the restriction sites *Sex*A1/*Bsu*36I were used to swap sequences corresponding to the 1A gene between ArMV-NW and -Lv. For the NTB gene, the *Xho*I/*Xho*I restriction sites located within the NTB genes were used. The restriction sites were chosen in order to conserved the 1A/1B, 1B/ NTB and NTB/VPg original cleavages sites of the NW polyprotein P1, respectively. For the others chimeric constructs, we used restriction sites localized upstream or downstream of the cleavage sites (fig. 25).

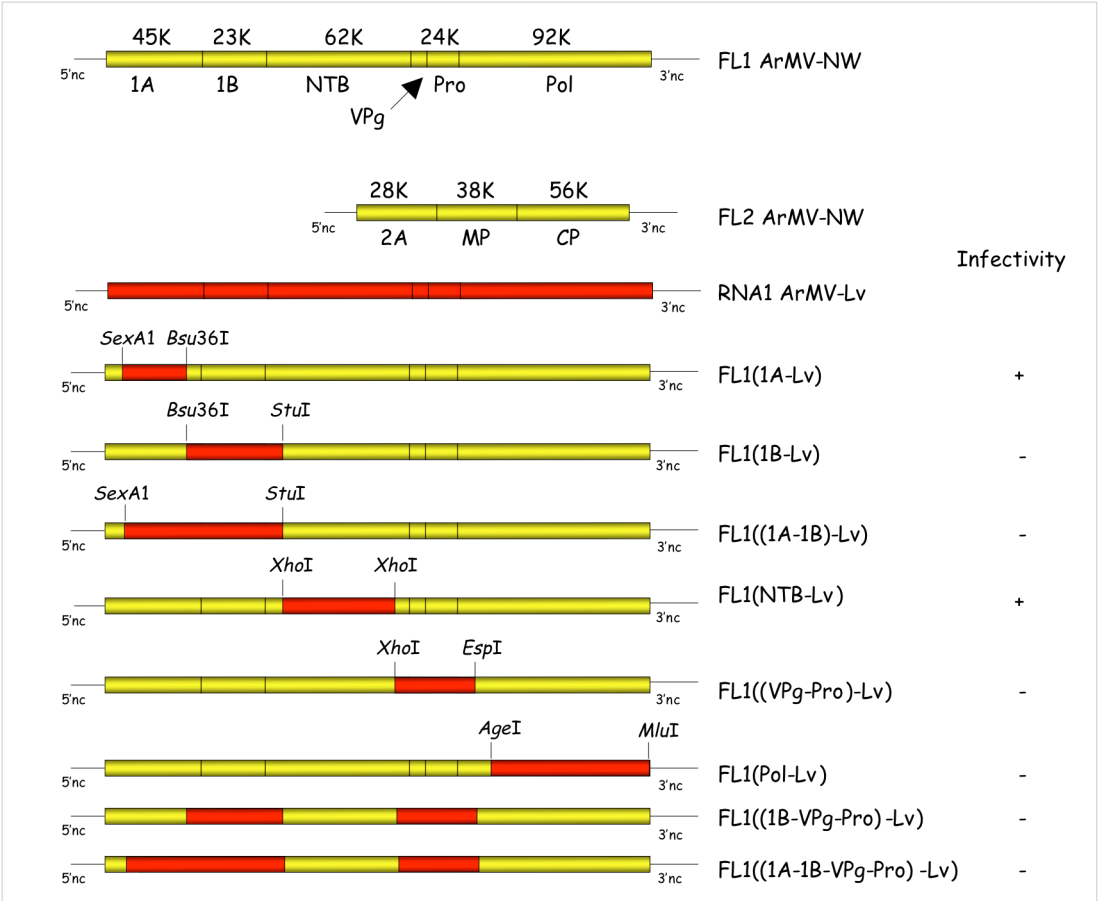



**Figure 25: Schematic representation of the NW-RNA1 chimeric constructs**
The polyproteins P1 and P2 of the ArMV-NW are represented in yellow colour and the Lv polyprotein P1, in red. The complete and partial proteins of the Lv isolate are drawn in red in the chimeric NW polyproteins P1. The restriction sites used for the construction of the chimeric FL1 cDNAs are shown. The infectivity of the different constructs, determined by ELISA, is indicated at the right of the figure with + or - signs.

Only two chimeric constructs were infectious on *C. quinoa*, those coding for the 1A protein and for the NTB protein of Lv, respectively. The CP was immunodetected by ELISA in the systemic leaves, at 15 dpi. By contrast, the other clones were not infectious probably because these constructs encompassed a cleavage site, which was most likely not recognized by the NW protease. The biological importance of the cleavage sites and/or the amino acids context for virus infectivity was already observed when we studied the symptom determinants, harboured by RNA2. Therefore, it will be necessary to restore the initial cleavage sites in all the chimeric constructs.

The plants expressing the protein 1A of ArMV-Lv developed a light mosaic whereas those infected with ArMV-NW producing Lv-NTB protein displayed no symptom, similarly to plants infected with ArMV-NW. These preliminary results suggest that protein 1A might contribute to the expression of symptoms on *C. quinoa* plants infected by the ArMV-Lv isolate.

# DISCUSSION

Infection of host plants with viruses usually leads to symptoms, which may differ greatly depending on the virus isolate, the plant species, the stage of infection and the environmental conditions. The symptoms result from specific interactions between plant factors and the virus that perturb the metabolism of the plant at different levels. The development of symptoms and their severity is controlled by one or several genetic determinants of the host plant (Sicard *et al.*, 2008). For instance, monogenic and semidominant traits control the expression of symptoms in *Arabidopsis thaliana* infected by *Tobacco ringspot virus* (Lee *et al.*, 1996). A single recessive gene operates to control the symptom development in *A. thaliana* plants infected by *Beet curly top virus* (BCTV, *Geminivirus*) (Park *et al.*, 2002). However, the mechanism controlling the expression of symptoms may be more complex, as described for the *A. thaliana* plant infected by *Cauliflower mosaic virus* (CaMV) pathosystem, where three distinct quantitative trait loci contribute to the development of disease symptoms (Callaway *et al.*, 2000). The viral determinants involved in the expression of symptoms were also studied in several pathosystems. Individual or multiple viral determinants may interact with host factors to modulate the specific symptoms of a given virus infection. For example, it has been shown that the capsid protein of *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) are responsible for the production of chlorotic symptoms (Dawson *et al.*, 1988; Shintaku *et al.*, 1992). Additionally, induction of symptoms such as mosaic and stunting upon CMV infection has been attributed to the 2b protein (Ding *et al.* 1996; Shi *et al.*, 2002; Du *et al.*, 2008).

### 1. Infectious chimeric clones

We studied the viral determinants involved in the expression of symptoms of host plants infected with ArMV using the ArMV-NW, -Lv and -lilac isolates through gene-swapping experiments. The mild ArMV-NW isolate induced no symptom or very light mosaic on *Chenopodium quinoa* and *Nicotiana benthamiana*. By contrast, ArMV-Lv is a virulent isolate, as it induces a necrosis and a severe mosaic on *C. quinoa* and *N. benthamiana* plants, respectively, and it able to infect *N. tabacum* and *N. glutinosa*. ArMV-Lilac can be considered as a moderate isolate since virus-infected *C. quinoa* and *N. benthamiana* plants only displayed a mosaic.

*C. quinoa* and *N. benthanamiana* mechanically inoculated with the infectious cDNA clones of the ArMV-NW isolate, corresponding to RNA1 and RNA2, respectively, manifested no or very light symptoms, as observed when the plants were infected with the ArMV-NW native isolate. Therefore, the research of viral determinants involved in the symptoms development could be conducted undertaken with chimeric cDNAs obtained by swapping complete and/or partial genes in the ArMV-NW full-length cDNAs with their counterpart of ArMV-Lv and ArMV-Lilac isolates. This experimental strategy has been successfully used to characterize several symptoms determinants (Carrère *et al.*, 1990; Burgyan *et al.*, 2000; Krause-Sakate *et al.*, 2004; Yang *et al.*, 2007). *C. quinoa*

was the most suitable herbaceous host for our study because it expresses different symptoms when infected with the ArMV-NW, -Lv and -Lilac isolates.

In contrast to ArMV infection, the infection of *C. quinoa* plants with the viral cDNAs starts with the production of capped and polyadenylated transcripts as the cDNAs are under the control of the 35S promoter. This indicates that the attachment of the VPg at the 5' end of the viral RNAs is not a prerequisite for the infectivity of ArMV, at least for translation.

**2. Symptom determinants of ArMV encoded by RNA2**

Analysis of the symptoms expressed by *C. quinoa* plants mechanically inoculated with different chimera cDNAs showed that 2A plays a role in the development of symptoms. Indeed, the infected plants displayed ringspots and a light mosaic when the chimeric NW isolate expressed the 2A protein originating from the ArMV-Lilac and -Lv isolates, respectively. At the opposite, *C. quinoa* plants infected in the same conditions with the wild-type ArMV-NW, were symptomless. The development of the symptoms on this herbaceous plant in not attributable only to the protein 2A because the phenotype of *C. quinoa* plants infected with the ArMV-Lv isolate led to a severe phenotype, necrosis, whereas the Lv-protein 2A expressed in a ArMV-NW context induced a mosaic. Alternatively, if necrosis is attributable to the protein 2A, ArMV-NW proteins might modulate the virulence of the ArMV-Lv isolate.

We also showed that the variability in symptoms is not due to an altered transcription level of the genomic RNAs since similar levels of RNA2 could be detected in systemic leaves and consequently, the development of symptoms is unrelated with the virus titer in the infected plants. Indirectly, this result also indicates that the hybrid ArMV particles spread from cell-to-cell with the same efficiency as the wild-type isolate. However, we were unable to determine if the expression of the mosaic and the ringspots is correlated with the levels of protein 2A in the infected plants because of the lack of antibodies raised against protein 2A. Burgyan *et al.*, (2000) showed that the lack of systemic necrosis is not due to an altered transcription level of viral RNAs in the case of *Tomato bushy stunt virus*.

The protein 2A can be divided into two parts, the C-terminal part which is highly conserved among the different ArMV isolates and the N-terminal part, which is variable in sequence and in length. Only a few amino acids are conserved in this region and the 2A protein of ArMV-NW and ArMV-Lilac are shorter compared to the ArMV-Lv homologue. The N-terminal region of Lilac 2A protein is notably characterized by several deletions.

Therefore, we assumed that the variable N-terminus of 2A carries the symptom determinant whereas the C-terminal region is specifically involved in the replication of RNA2. It was shown that the polymorphism of the pathogenic factor p23 of *Citrus tristeza virus* (CTV) is also responsible for the discrimination between mild and severe isolates (Sambade *et al.*, 2003). The involvement of the N-

terminal part of 2A protein in the symptomatology was confirmed when we inoculated *C. quinoa* plants with ArMV-NW viruses coding for a chimera protein 2A, whose the N-terminal region was substituted by its counterpart of Lv and Lilac isolates. In both cases, the symptoms induced were similar to those obtained when the full-length protein 2A of these isolates were expressed in an ArMV-NW context. It would be interesting to determine if the additional sequences in the 2A protein of the Lv isolate carry the determinant for the mosaic symptom. Kagiwada *et al.*, (2005) have shown that the nature of a single amino acid residue in the region of the C-terminus of RNA-dependent RNA polymerase of *Potato virus X* is responsible for the type of symptoms (necrosis, ringspots and mild mosaic) induced in infected *Nicotiana* plants. Similarly, studies performed on virulent potyvirus Y isolates showed that two residues in the C-terminal part of the Hc-Pro protein are specifically involved in tobacco vein necrosis on *N. tabacum* (Tribodet *et al.*, 2005). However, it was recently suggested that other element(s) are involved in the expression of this symptom, in addition to the C-terminal fragment of Hc-Pro (Hu *et al.*, 2009).

We also observed that the deletion of the N-terminal region of the protein 2A did not impair the replication of ArMV-NW and systemic infection on *C. quinoa*. This result strongly suggests that the C-terminal region of this protein is responsible for the addressing of RNA2 to the virus replication site and that protein 2A exerts this "homing" function independently of the function associated with its N-terminus.

The movement protein (MP) and/or the capsid protein (CP) of some viruses are also involved in the development of symptoms (Bol, 2005; Yang *et al.*, 2007). The movement protein of *Tomato spotted wilt virus* (TSWV, *Tospovirus*) expressed in transgenic plants, induces viral disease-like symptoms probably by inducing the deposition of callose at the plasmodesmata thereby blocking the transport of sucrose (Rinne *et al.*, 2005). The capsid protein of TMV and CMV are responsible for the formation of chlorotic symptoms (Dawson *et al.*, 1988; Shintaku *et al.*, 1992). We observed a moderate role of the MP of ArMV-Lv in the induction of symptoms since *C. quinoa* plants infected with the recombinant ArMV-NW only displayed a mild mosaic. This could not be confirmed with the MP of Lilac isolate because the chimeric NW-MP-Lilac virus was not infectious in contrast to the NW-MP-Lv recombinant, although we modified the amino acid context of the cleavage site, as for the NW-$MP_2$-Lv construct, to permit the processing of polyprotein P2 by the NW RNA1-encoded protease. We assume that the NW protease was unable to cleave P2 between the MP and CP sequences since the homology between the P2 polyproteins of NW and Lv isolates is higher than between the NW and Lilac P2 polyproteins.

The CP of ArMV is apparently not involved in the development of symptoms. *C. quinoa* plants infected with the chimeric NW expressing the Lv capsid protein were symptomless albeit this virus replicated and spread in the plant with the same efficiency as the wild-type ArMV-NW isolate, as evidenced by almost the same levels of RNA2 in the systemic leaves. At the opposite, the presence of the Lilac CP sequence in the NW P2 polyprotein reduced considerably the efficiency of the systemic

infection of *C. quinoa*; only low amounts of RNA2 could be detected in the non-inoculated upper leaves. This might be due to inefficient cleavage of P2 and/or to altered stability of the NW RNAs in the presence of the Lilac capsid protein.

To confirm the involvement of protein 2A in the development of symptoms in plants infected by ArMV, we envisaged to produce *Arabidopsis* transgenic plants expressing this viral protein. This strategy was successfully used for studying several symptom determinants of plant virus i.e. the p23 of CTV (Ghorbel *et al.*, 2001; Fagoaga *et al.*, 2005) and the P6 of CaMV (Zijlstra and Hohn, 1992; Cecchini *et al.*, 1997; Yu *et al.*, 2002). Cecchini *et al.* (1997) have shown that the P6 protein of CaMV, which is a key symptom determinant during virus infection, is sufficient to induce a symptom-like phenotype including stunting, chlorotic and vein-banding, when it is expressed as transgene in *A. thaliana*. These authors have also shown that P6 forms perinuclear electron-dense viroplasms in these transgenic plants, as in a viral infection context. Unfortunately, this strategy seems not to be adapted for the ArMV 2A protein if we refer to studies performed on *Grapevine fanleaf virus* (GFLV). The behaviour of protein 2A, when transiently expressed in uninfected tobacco BY2 protoplasts, is different from that observed in the viral context. When fused to GFP, the 2A protein appeared as punctuate structures evenly distributed in the cytoplasm whereas in cells co-transfected with GFLV RNAs, it was predominantly found in the proximity of the nucleus along with the VPg and the viral protease (Gaire *et al.*, 1999). Therefore, it would preferable and even much easier to substitute the 2A coding sequence in the RNA2 of the Lv and the Lilac isolates by the homologous sequence of ArMV-NW to see if this exchange modifies the virulence of the two former isolates. This should also reveal the type of symptoms induced by the other viral determinant(s) as ArMV-NW does not induce any symptom on *C. quinoa*.

### 3. Symptom determinants of ArMV encoded by RNA1

Harrison *et al.*, (1974) suggested that both genomic RNAs of *Raspberry ringspot virus* (RpRSV) carries symptom determinants, RNA2 being responsible for the yellowing of *Petunia hybrida* leaves while RNA1 induces other types of symptom.

Exchanging several coding sequences in the RNA1 of ArMV-NW by their counterparts of Lv isolate led only to two infectious chimeric viruses, namely those expressing the Lv-1A and -NTB proteins, respectively. The Lv-1A protein might play a role in the expression of symptoms in *C. quinoa* plants because the leaves displayed a light mosaic while the plants infected with the ArMV-NW isolate and the chimeric NTB-Lv construct were symptomless. The implication of NTB in the symptom development has been demonstrated for *Bean pod mottle virus* (BPMV, *Comovirus*) (Gu and Gabrial, 2005).

The involvement of several viral components in the symptoms development has been described for RNA and DNA viruses. For instance, the systemic necrosis induced in *N. glutinosa* by the CMV

subgroup I strains is due to the co-action of the 2b protein and the overlapping C-terminal region of the 2a protein (Du *et al.*, 2008). At least, two viral determinants encoded by two separate RNAs are also implicated in the pathogenesis of *Beet necrotic yellow vein virus* (Rahim *et al.*, 2007). The RNA3-encoded P25 protein is involved in the induction of rhizomiana symptoms in sugar beet (Koenig *et al.*, 1991; Tamada *et al.*, 1999; Peltier *et al.*, 2010) and in severe local lesions on leaves ((Tamada *et al.*, 1989; Jupin *et al.*, 1992) while the p31 protein expressed from RNA4 enhances slightly the expression of symptoms in some Beta species and is involved in the induction of severe symptoms in *N. benthamiana* plants. We can also quote the case of *Maize streak virus* (MSV, *Mastrevirus*) movement and capsid proteins, whose co-expression elicits severe symptoms whereas individual expression of these proteins results in reduced symptoms severity (Van der Walt *et al.*, 2008).

Presumably, several ArMV determinants are also implicated in the symptoms development. We do not exclude that cleavage intermediate products of the polyprotein(s) might also contribute to the expression of disease symptoms in ArMV-infected plants in addition to mature proteins. Indeed, cleavage intermediates of picorna-like viruses are known to be involved in the viral pathogenesis i.e. the proliferation of endomembraneous vesicles. Wetzel *et al.*, (2008) have shown that some cleavages of the nepoviral polyproteins are sub-optimal leading to an unequal accumulation of viral mature proteins during the course of the infection. Therefore, the production of the viral mature proteins and their precursors may vary from one isolate to another and/or depend on the host plant if cellular factors are required for the processing of the polyproteins. An involvement of the protein precursor(s) in disease symptoms would render it particularly difficult to decipher the contribution of each viral determinant in the type and/or severity of the symptoms.

The role of the non-coding regions of the viral RNAs in the expression of symptoms should also be investigated since some reports indicate that viral RNA sequence directly control the disease symptoms in plants. For example, the 5' non-coding region of *Grapevine chrome mosaic virus* cloned in a viral vector derived *Potato virus X* (PVX) induces in several *Nicotiana* species, necrotic symptom instead of the vein clearing and mosaic symptoms, which are normally observed in these plants infected with PVX (Fernandez *et al.*, 1999). The 3' end of *Tobacco vein mottling virus* is also involved in the symptoms development (Rodriguez-Cerezo *et al.*, 1991). More recently, Krause-Sakate *et al.*, (2005) came to the conclusion that systemic wilting of some lettuce cultivars infected by the *Lettuce mosaic virus* (LMV, *Potyvirus*) AF199 isolate is attributable to the viral RNA rather than to the corresponding proteins.

In conclusion, our results indicate that the ArMV protein 2A is involved in the development of symptoms but it is probably not the unique viral determinant since the symptoms induced by this protein differ in *C. quinoa*, depending on the viral context. Therefore, other unidentified determinants must contribute to the expression of the disease symptoms. One of these candidates could be the

protein 1A albeit its contribution seems to be moderate. Moreover, the 2A and 1A proteins act not in synergy to induce symptoms although, it was shown that these proteins interacts themselves in GFLV (Amari et al., 2010).

How this protein elicits the symptoms is still an open question as no information is available concerning its exact function during the ArMV infectious cycle. Characterization of the host partners of protein 2A and a comparative analysis of the transcriptome of ArMV-infected and mock-inoculated plants would certainly give an insight in the perturbation of physiological mechanisms leading to the disease symptoms.

It has been demonstrated that many viral determinants involved in the expression of the disease symptoms and/or controlling their severity are frequently suppressors of the plant RNA silencing antiviral defense system. For instance, the potyvirus Hc-Pro, the tombusvirus p19 and the *Cucumber mosaic virus* 2b are characterized as well as suppressors and symptom determinants in virus-infected plants (for review, Burgyan, 2008). Indeed, transgenic expression of silencing suppressors in herbaceous plants often produces phenotypes that resemble to viral symptoms (Van der Wilk *et al.*, 1997; Dunoyer *et al.*, 2004; Silvahy and Burgyan, 2004). The viral silencing suppressor can exert its function by interfering with the miRNA pathway, which is implicated through the regulation of the expression of transcription factors, in the control of plant development leading to developmental abnormalities (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004). Therefore, the characterization of the ArMV suppressor of RNA silencing became a priority in our project that aimed at unravelling the mechanisms(s) of the ArMV pathogenesis.

# RNA SILENCING

## I. RNA silencing mechanism

### A. Description of RNA silencing

RNA silencing is a generic term for sequence-specific suppression of gene expression. RNA silencing was discovered in transgenic petunia plants in which supplementary copies of the chalcon synthetase (CHS) gene, introduced in the nuclear genome, were suppressed together with the endogenous gene copy (Napoli *et al.*, 1990). The introduction of a chimeric petunia CHS gene in petunia plants was realized in order to reinforce anthocyanin pigment in the petals but surprisingly resulted in the complete arrest of anthocyanin synthesis. This mechanism triggered by a foreign sequence and resulting in the co-suppression of both the endogene(s) and the transgene, is called post-transcriptional gene silencing (PTGS) in plants, quelling in fungi (Cogoni and Macino, 1999) and RNAi in nematodes (Fire *et al.*, 1998), Drosophila (Kennerdell and Carthew, 1998), and mice (Bahramian and Zarbl, 1999).

RNA silencing can also be induced by a sequence or a transgene that present homology with an endogenous gene (Finnegan and McElroy, 1994; Matzke and Matzke, 1996; Meyer, 1995; Crispin *et al.*, 1997). This was notably demonstrated with plant viruses, whose genome share some homologies with transgenes or endogenous genes present in host nuclear DNA (English *et al.*, 1990; Lindbo *et al.*, 1993; Guo *et al.*, 1997; Ruiz *et al.*, 1998). However, it was also reported that viruses could trigger RNA silencing without homologies with the host genome (Ratcliff *et al.*, 1997; Covey *et al.*, 1997; Al-Kaff *et al.*, 1998).

RNA silencing may act both at the RNA and the DNA levels. Mechanisms of silencing at the RNA level include mRNA cleavage or translational repression whereas RNA silencing at the DNA level involves DNA and/or histone methylation and subsequent transcriptional gene silencing (TGS) through heterochromatin formation and maintenance (Bartel, 2004; Jones-Rhoades *et al.*, 2006). RNA silencing is involved in various fundamental processes as an adaptative protection against viruses (Voinnet, 2001), in genome defense against mobile DNA elements (Ketting *et al.*, 1999) and in developmental regulation of gene expression (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). However, all these manifestations of RNA silencing rely on the action of small RNA (sRNA) molecules of 21 to 24 nucleotides (nt) in length, which originate from the processing of the dsRNA trigger (Hamilton and Baulcombe, 1999; Elbashir *et al.*, 2001). During PTGS, these sRNA molecules control stability or regulate translation of their mRNA targets by guiding endogenous effector complexes (Hammond *et al.*, 2000; Bartel, 2004; Jones-Rhoades *et al.*, 2006).

### B. Different steps in RNA silencing mechanism

#### 1. Initiation

In a broad range of eukaryotic organisms, RNA silencing is triggered by a double-stranded RNA (dsRNA), which is the key initiator molecule (Fire *et al.*, 1998). The dsRNA can be delivered

exogenously or produced *in vivo*.

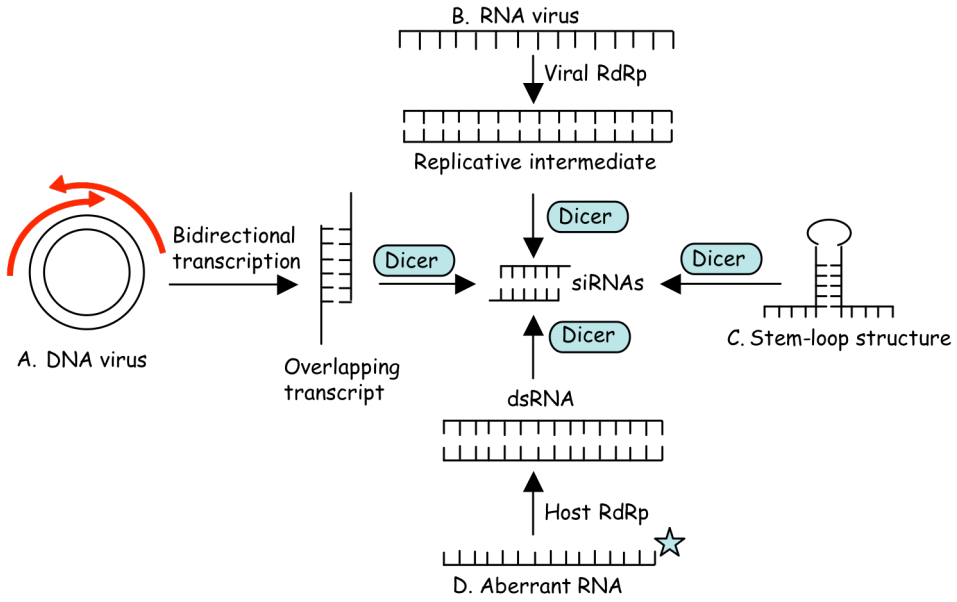



**Figure 1: Four different sources of dsRNA serve as substrates for plant Dicer-like enzymes to produce short-interfering RNAs (siRNAs)**
A. Circular dsDNA viruses produce dsRNA by symmetrical transcription of overlapping reading frames; B. SsRNA viruses produce dsRNA replication intermediates; C. Some viral transcripts or ssRNA genomes have extensive secondary structures; D. DsRNA synthesized by plant host RNA-dependent RNA polymerase (RdRP or RDR) enzyme, using viral ssRNA as a template.

The source of dsRNAs (fig. 1) can be provided by RNA viruses during the replication of their genome or by the presence of fold back structures (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Sijen *et al.*, 2001). It was also shown that the host RNA-dependent RNA polymerase (RpRd or RDR) converted single-stranded RNA (ssRNA) into dsRNA that becomes at its turn an initiator molecule (Wassenegger *et al.*, 2006). DsRNA can also derive from overlapping transcripts or from the transcription of inverted repeat constructs (Waterhouse *et al.*, 1998). Once RNA silencing has been triggered, dsRNA is cleaved by Dicer-like protein (DCLs) in plants into double-stranded short-interfering RNAs (siRNAs) of 21-25 bp (Hamilton and Baulcombe, 1999; Elbashir *et al.*, 2001).

### 2. Processing step

In *Arabidopsis thaliana*, four Dicer-like proteins, named DCL1 to DCL4, are involved in the processing of dsRNAs. The size of the siRNAs duplexes, resulting from the cleavage of dsRNAs, is dependent on the Dicer-like protein. The siRNAs of 21 bp-long are produced by DCL1 or by DCL4

whereas siRNAs of 22 bp, 24 bp are generated by DCL2 and DCL3, respectively. DsRNA processing, also called dicing, is facilitated by the assistance of dsRNA-binding proteins (DRB). After dicing, both strand of the siRNAs have a 5'-phosphate and a 3'-hydroxyl group at their extremities and the duplexes are characterized by 2 nt overhangs at the 3' ends (Hammond, 2005). Later, the 3' ends are methylated at the 2'OH of the ribose by the methyltransferase HUA ENHANCER 1 (HEN1) to protect the siRNAs from degradation by cellular nucleases and from the oligouridylation; the methylation enhances the stability of the siRNAs.

The siRNAs that act at the level of the chromatin are retained in the nucleus whereas if they are involved in PTGS, they are exported in the cytoplasm thanks to the exportin-5 homologue HASTY (HST).

### 3. Effector step

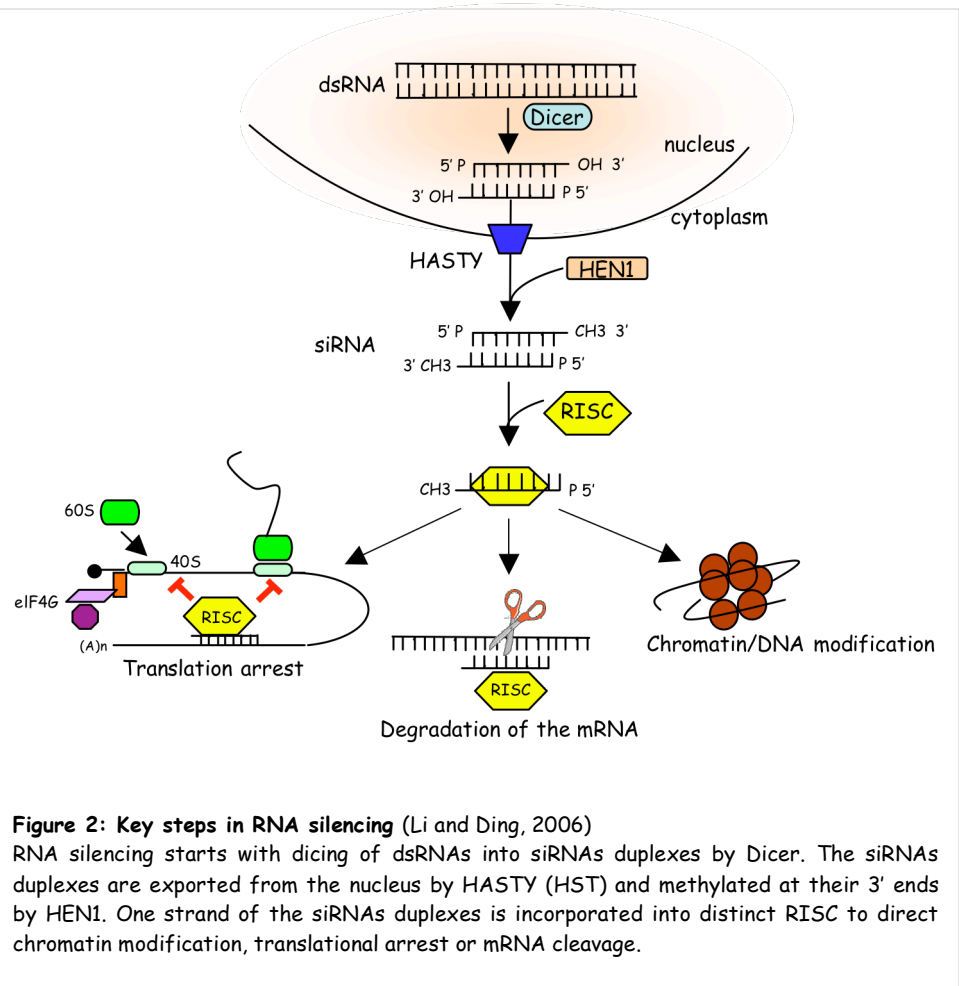



**Figure 2: Key steps in RNA silencing** (Li and Ding, 2006)
RNA silencing starts with dicing of dsRNAs into siRNAs duplexes by Dicer. The siRNAs duplexes are exported from the nucleus by HASTY (HST) and methylated at their 3' ends by HEN1. One strand of the siRNAs duplexes is incorporated into distinct RISC to direct chromatin modification, translational arrest or mRNA cleavage.

During the effector step of RNA silencing (fig. 2), one strand of the siRNAs is recruited into a macromolecular complex, the RNA-Induced Silencing Complex (RISC) or the RNA-induced

Initiation of Transcriptional Silencing complex (RITS), which direct sequence-specific PTGS or TGS, respectively (Hammond *et al*., 2000; Ekwall, 2004), indicating that the siRNA must unwind prior to its incorporation into RISC. The incorporated strand, named the selected guide strand, adresses RISC to the target whereas the siRNA strand which is not incorporated, named passenger strand, is degraded (Lau *et al.*, 2001; Schwartz *et al.*, 2004). RISC has different activities: (i) endonucleolytic cleavage of the target mRNA by a perfect or near perfect base-pairing between siRNAs and the targeted sequences (Llave *et al.*, 2002); (ii) translational repression when there is partial complementarity (Aukerman and Sakai, 2003; Chen, 2004) and (iii) DNA cytosine and/or histone methylation. The translational repression mechanism is not fully understood but there is evidence that RISC mediates sequestration of target transcripts away from the translational machinery into cytoplasmic foci termed Processing-bodies (Rossi, 2005). Another possibility is the binding of RISC to multiple target sequences within a mRNA and thus interfere with the ribosome movement along the transcript (Tolia and Joshua-Tor, 2007). The effector molecule of RISC is the protein Argonaute (AGO). Ten AGO proteins were identified in Arabidopsis as well as their roles (Vaucheret *et al.*, 2008).

**4. Maintenance**

The siRNA/RISC complex can prime against any cytoplasmic RNA species sharing sequence homology with the triggering molecules. RNA silencing is maintained by the re-initiation of the mechanism by the action of cellular RDRs, which can generate many dsRNAs from a primary siRNA or the resulting cleavage product perceived as aberrant RNA. The generation of these dsRNAs provides a new source of substrates for DCLs and consequently, leads to the accumulation of many secondary siRNAs raised against the target molecule. Therefore, this amplification process permits to RNA silencing to keep pace with the replication of the viral RNA (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Xie *et al.*, 2004). In the case of the heterochromatin-silencing pathway, this step ensures that a few molecules of transposon RNA suffice to suppress all copies of a transposable element (Martienssen *et al.*, 2004, Baulcombe, 2004; 2007). The action of RDRs occurs either by a primer-dependent mechanism or a primer-independent mechanism (fig. 3).

The primer-dependent process implies the presence of the primary siRNAs from viruses, transgenes or transposons to produce dsRNAs by the RDR. The resulting dsRNA is then cleaved by DCL4 to gives rise to 21 nt secondary siRNAs (Sijen *et al.*, 2001; Vaistij *et al.*, 2002). These secondary siRNAs are derived not only from the initiator region but also from adjacent regions of the initial target sequence (Voinnet *et al.*, 1998; Vaistij *et al.*, 2002). In *Arabidopsis*, this primer-dependent process also called transitivity requires RDR6 and RNA helicase-like proteins, SDE3 and SDE5 (Dalmay *et al.*, 2001, Himber *et al.*, 2003).

In the second mechanism, dsRNA is produced from a viral or transposon ssRNAs. These ssRNAs have some structural particularities such as the absence of a cap or of a poly(A) tail and thus, it might be considered as aberrant RNAs. The conversion of ssRNA into dsRNA is performed by

RDR6 in plant, in the presence of SGS3, which binds dsRNA probably to stabilize the RDR6-produced dsRNA (Bateman, 2002; Fukunaga and Doudna, 2009). The resulting dsRNA is then used by DCL4 to produce secondary siRNAs (Yoshikawa *et al.*, 2005; Vaucheret *et al.*, 2006).

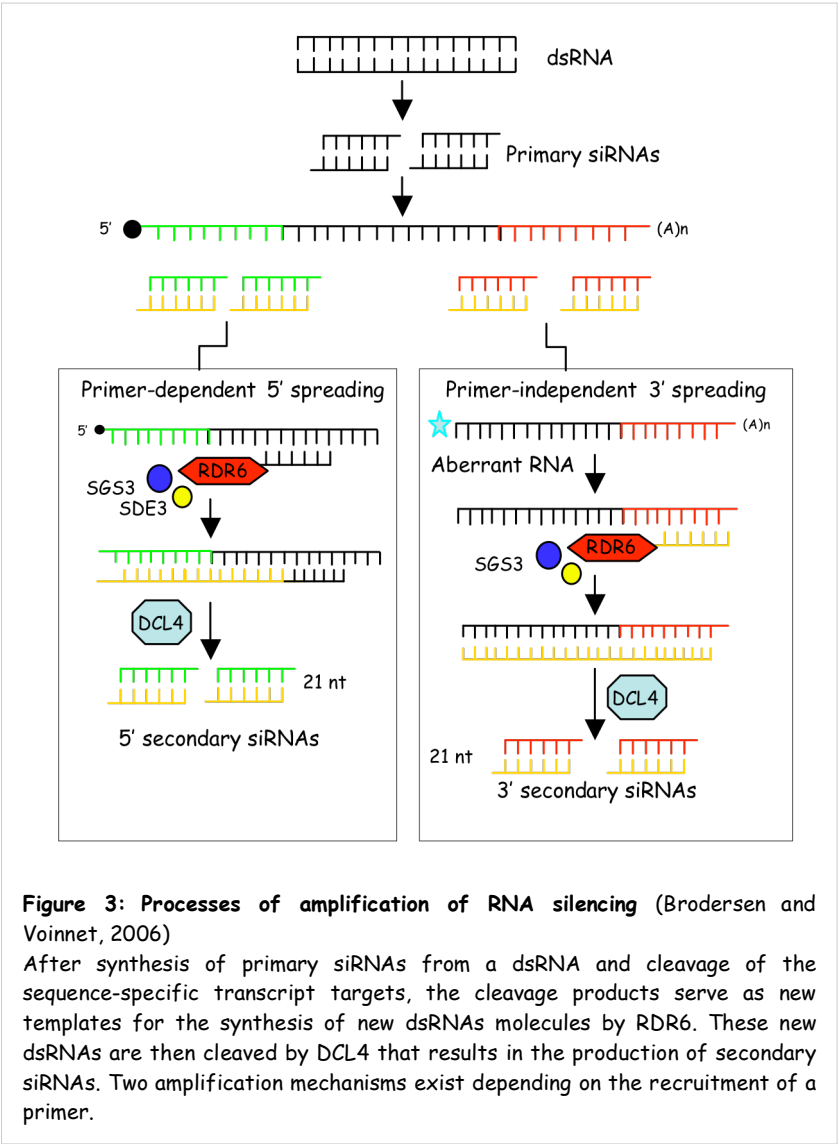



**Figure 3: Processes of amplification of RNA silencing** (Brodersen and Voinnet, 2006)
After synthesis of primary siRNAs from a dsRNA and cleavage of the sequence-specific transcript targets, the cleavage products serve as new templates for the synthesis of new dsRNAs molecules by RDR6. These new dsRNAs are then cleaved by DCL4 that results in the production of secondary siRNAs. Two amplification mechanisms exist depending on the recruitment of a primer.

### 5. Propagation of the systemic signal of RNA silencing

RNA silencing can extend beyond its site of initiation because of the movement of signalling molecules (Lakatos *et al.*, 2004). Indeed, the signal can induce RNA silencing in neighboring or distant tissues that do not contain the initial trigger such as a transgene or a replicating virus, from shoot to root and vice-versa, (Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997; Voinnet *et al.*, 2000). The DCL4-dependent 21 bp and DCL3-dependent 24 bp siRNAs duplexes, derived from an inverted-repeat transgene, to mediate long-distance silencing through the phloem in RDR6-

independent background in *Arabidopsis* and through graft junction (Himber *et al.*, 2003; Dunoyer *et al.*, 2007; Dunoyer *et al.*, 2010a, 2010b; Molnar *et al.*, 2010).

This systemic RNA silencing in plants provides cell-to-cell communication that affects gene expression during development, immunizes cells ahead of the viral infection and introduces epigenetic effects (Voinnet and Baulcombe, 1997; Voinnet *et al.*, 2000). Two types of propagation of systemic signal have been identified in *N. benthamiana*: cell-to-cell and long-range movements.

The cell-to-cell movement occurs through plasmodesmata, which connect plant cells with the notable exception of mature guard cells (fig. 4.2 and fig. 4.3). It was demonstrated that after introduction of 35S-GFP construct in transgenic 35S-GFP plants, the signal moved to the growing point of the plant (Voinnet *et al.*, 1998).

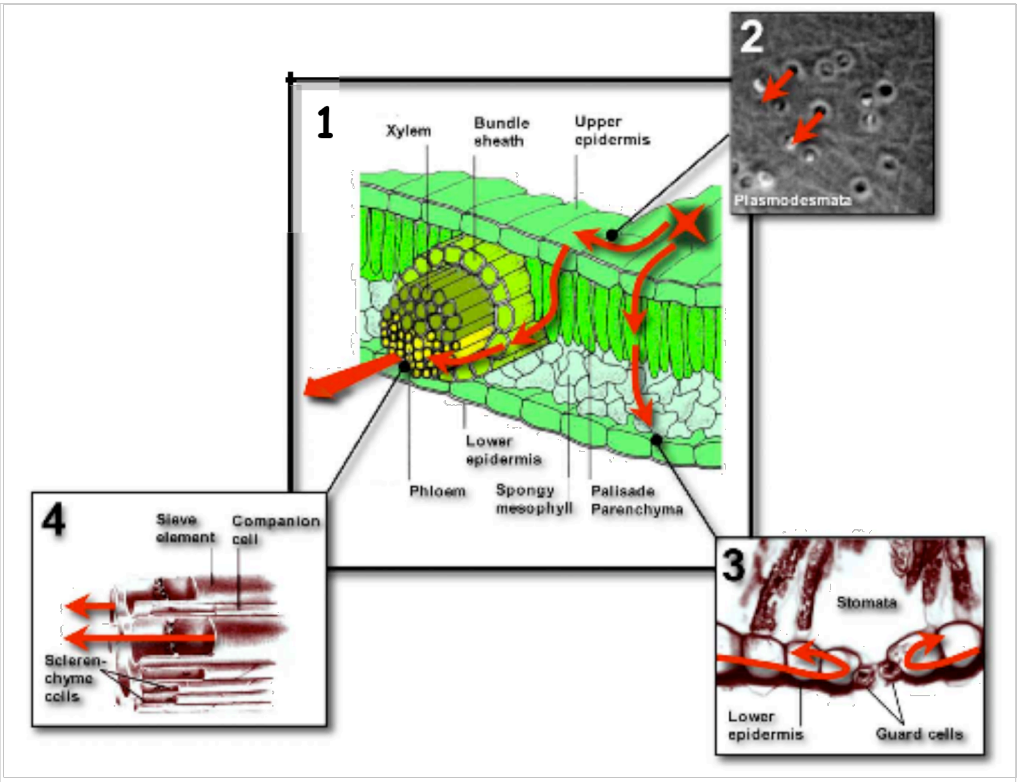



**Figure 4: The cell-to-cell and the long-range movements of the systemic silencing in plants** (Voinnet, 1998)
1. Schematic representation of the propagation of the signal (red) in a cross section leaf. The red star corresponds to the initiation site; 2. Electron micrograph of plasmodesmata connecting two adjacent cell, which allow the movement of the signal from cell-to-cell in the upper epidermis after initiation of silencing; 3. The silencing moves towards the lower epidermis but is excluded from stomata guard cells; 4. Long distance spread of the signal in the phloem sieve elements after it has crossed the bundle sheath.

RNA silencing that spreads through plasmodesmata, does not exceed a nearly constant number of 10-15 cells (Voinnet *et al.*, 1998; Himber *et al.*, 2003; Dunoyer and Voinnet, 2005).

The long-distance movement via the vascular system allows the propagation of siRNAs in the entire lamina of leaves (Voinnet and Baulcombe, 1997; Voinnet *et al*., 1997; 1998; 2005; Palauqui *et al.*, 1997; Guo *et al.*, 2002). It occurs when the signal has crossed the bundle sheath through the phloem sieve elements, which is also involved in the transport of organic nutrients, RNA and proteins throughout plants (fig.4.4).

## C. Different effectors involved in RNA silencing

### 1. DICER- like proteins

#### 1.1. Characteristics

Class I RNaseIII enzymes, found in bacteria and yeasts, contain a single RNase III domain linked to a dsRNA-binding domain (dsRBD) whereas class II and class III enzymes have two RNaseIII catalytic domains. Dicer family proteins, originally identified in *Drosophila melanogaster* are type III endoribonuclease (Bernstein *et al.*, 2001). They recognize and slice specifically dsRNAs to generate either small-interfering (si)RNAs or micro (mi)RNAs.

Dicer-like proteins (DCLs) have a molecular weight of approximately 200 kDa and contain from the N-terminal domain, a DEXH-helicase, a RNA helicase-C, a DUF283 domain, a PAZ (for "Piwi/Argonaute/Zwille") protein-protein interaction domain, two tandem RNase III domains and one of two dsRNA-binding domains (dsRBD) (fig. 5A).

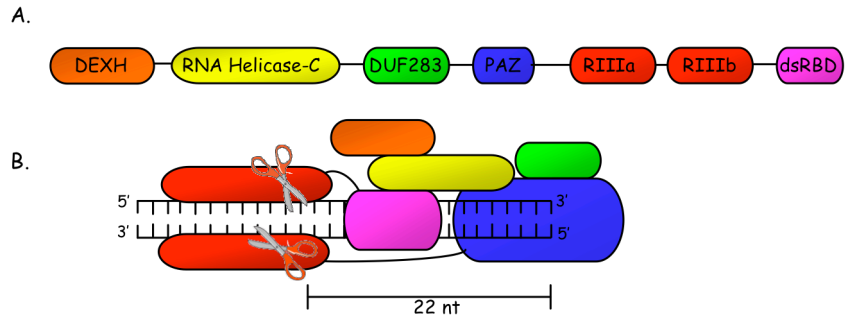



**Figure 5: Representation of RNase III endoribonuclease and model for Dicer catalysis**
A. Schematic representation of functional domains that characterized type III endoribonuclease; B. Model of the catalytic action of DCLs as described by Zhang *et al.*, (2004). The PAZ domain binds the 2-nt 3' overhangs of a dsRNA. The RNase III domains form a pseudo-dimer. Each domain hydrolyzes one strand of the substrate. The binding site of the dsRBD is not defined and the function of the helicase domain is not known.

The DUF283 domain, which consists of three β-strands and two α-helices, is involved in the protein-protein interaction. It mediates the heterodimerization with specific dsRNA binding proteins

(DRBs) (Qin *et al.*, 2010). PAZ domain is highly conserved among DCL proteins and mediates both homo- and heterodimerization (Cerutti *et al.* 2000). This domain of 130 amino acids (aa) reveals a high degree of similarity with an oligonucleotide-oligosaccharide binding (OB) fold motif, known to bind nucleic acids (Berstein *et al.*, 2001). The RNaseIII domain seems to regulate the endonucleolytic cleavage of dsRNAs into siRNAs. The slicing activity requires the interaction of the two catalytic residues of each RNase III domain because the active site is formed across the interface of this dimer.

Many models have been proposed to explain the action of DCLs on dsRNAs (Carmell and Hannon, 2004; Zhang *et al.*, 2004; Hammond *et al.*, 2005). It is likely that the 2-nucleotides of the 3′-end overhangs, typical of Dicer products, are a consequence of the alignment of the two RNase III domains on the dsRNAs (Hammond *et al.*, 2005). The production of siRNAs of 22 bp in this model depends on the close positionning of adjacent Dicer RNase III domains (fig. 5B). It was also suggested that the distance between the PAZ domain and the two RNase III domains determines the length of the siRNAs (MacRae *et al.*, 2006).

### 1.2. Diversities of DCLs in plants

*A. thaliana* possesses four Dicer-like (DCL) proteins. They share 40% to 47% amino acids similarity in the RNA helicase domain and the two RNase III domains but the other functional domains are not conserved. Each DCL produces a well-defined class of siRNAs. DCLs are associated with double-stranded RNA-binding (DRB) proteins that serve as essential cofactors, leading to the hypothesis that the different types and sizes of siRNAs are presumably due to the specific association of each DCL with a different DRB.

DCL1 prefers substrates with hairpin-like structures and processes fold-back dsRNA precursors to release miRNAs (Xie *et al.*, 2004; Bouché *et al.*, 2006). It is the only DCL protein in plants that is able to generate both miRNAs and siRNAs. Indeed, DCL1 can also produce RDR6-dependent endogenous siRNAs in the absence of the three others DCL proteins (DCL3, DCL4 and DCL2) (Bouché *et al.*, 2006). DCL1 is involved in plant development and its suppression is embryo-lethal (Mc Elver *et al.*, 2001; Schauer *et al.*, 2002). In fact, the loss of function in *dcl1* mutant induces morphological defects including reduced size, abnormal leaf form, loss of axillary buds and altered floral morphology (Jabcosen *et al.* 1999; Park *et al.* 2002; Reinhart *et al.* 2002).

DCL3 produces siRNAs of 24 bp, which are involved in heterochromatin formation and are also associated in RNA silencing of retroelements and transposons (Hamilton *et al.*, 2002; Tang *et al.*, 2003).

DCL4 and DCL2 are implicated in viral defense. The substrates diced by DCL4 are replicative intermediates of viral RNA, dsRNAs generated by RDR6, hairpin-like RNA and inverted-repeat transgenes (Dunoyer *et al.*, 2005). The products are 21 nt-long siRNAs and mediate PTGS. DCL4 is

also implicated in the production of trans-acting (ta)-siRNAs involved in the proper leaf development (Gasciolli *et al.*, 2005; Adenot *et al.*, 2006).

DCL2 are also implicated in the production of viral siRNAs (vsRNAs) (Xie *et al.*, 2004). It was demonstrated that DCL2 rescues antiviral silencing if DCL4 is genetically inactivated or suppressed. DCL2 produces vsRNAs with a length of 22 nt (Bouché *et al.*, 2006; Deleris *et al.*, 2006; Fusaro *et al.*, 2006). It also synthesizes stress-related natural-antisense-transcript (nat)-siRNAs in the context *dcl1-dcl4* plants by interacting with DRBs that normally bind to DCL1 or DCL4. This observation indicates a possible redeployment of DRBs towards the available DCLs (Bouché *et al.*, 2006).

Redundancies in the different DCLs and an interconnection between the diverse endogenous pathways of RNA silencing have been observed. Indeed, DCL2 and DCL4 can produce RDR2-dependent heterochromatic siRNAs in the absence of DCL3 (Mlotshwa *et al.*, 2008), as DCL3 can substitute DCL4 to produce ta-siRNAs (Gasciolli *et al.*, 2005).

### 2. DsRNA-binding proteins

In *Arabidopsis*, five dsRNA binding proteins (DRB) were identified, named HYPONASTIC 1 (HYL1) and DRB2 to DRB5 (Qi and Hannon, 2005; Vaucheret *et al.*, 2006). These proteins are characterized by the presence of two dsRNA-binding domains (dsRBD1 and dsRBD2) in their N-terminal part. DRB proteins interact physically and specifically with DCLs and optimize the processing of DCL substrates into specifically sized siRNAs (Hiraguri *et al.*, 2005, Yu *et al.*, 2005).

Arabidospis DRB1, known as HYL1, is required for the efficient and precise processing of pri-miRNA during plant miRNA biogenesis in Dicing bodies in the nucleus (Han *et al.*, 2004; Vazquez *et al.*, 2004a; Hiraguri *et al.*, 2005; Kurihara *et al.*, 2006; Fang and Spector, 2007). HYL1, which preferentially binds dsRNAs *in vitro* (Lu and Fedoroff, 2000), possesses additionally to the dsRBDS, a nuclear localization sequence (NLS) and a putative protein-protein interaction domain, which could be involved in the interaction of HYL1 with DCL1 and AGO1 (Fang and Spector, 2007). It seems that DRB proteins facilitate the transfert of siRNAs into RISC.

It was also demonstrated that DRB4 interacts with DCL4 and enhances the production of endogenous ta-siRNAs (Hiraguri *et al.*, 2005; Nakazawa *et al.*, 2007). Consistently, *hyl1* and *drb4* mutants exhibit phenotypes similar to *dcl1* and *dcl4* mutants and are affected in the production of miRNAs and ta-siRNAs, respectively (Han *et al.*, 2004; Vasquez *et al.*, 2004; Adenot *et al.*, 2006).

### 3. HUA ENHANCER1 (HEN1)

HEN1 is an S-adenosyl methionine (SAM)-binding methyl-transferase. In plants, HEN1 adds a methyl group on the ribose 2' hydroxy moiety of the 3' terminal nucleotide of the miRNAs and siRNAs duplexes (Yang *et al.*, 2006; Yu *et al.*, 2005). HEN1 is localized in both the nucleus and the cytoplasm, suggesting that methylation of miRNAs and siRNAs could occur in both compartments (Fang and Spector, 2007). *Hen1* mutants exhibit pleiotropic phenotypes and are defective in siRNA

and tasiRNA silencing, in addition to miRNA biogenesis (Chen *et al.*, 2002; Park *et al.*, 2002; Boutet *et al.*, 2003; Vazquez *et al.*, 2004a). Methylation protects the siRNAs and miRNAs from uridylation and degradation as evidenced by the presence of additional nucleotides, primarily uridines, on their 3' ends, when hen1 gene is inactivated (Li *et al.*, 2005).

## 4. Argonaute proteins

### 4.1. Characteristics

The RNA-induced silencing complex (RISC) is a multicomponent complex that regulates gene expression mediated by the sequence complementarity between siRNA and its target. The RISC is characterized by the presence of at least one Argonaute (AGO) protein. AGO proteins are mainly implicated in the specific cleavage of complementary mRNAs to a central position of a siRNA/mRNA duplex (Hammond *et al.*, 2000, 2001; Bernstein *et al.*, 2001; Elbashir *et al.*, 2001). The phosphate group at the 5' end of the siRNA strand has been shown to be required for efficient RNA silencing (Nykanen *et al.*, 2001; Schwarz *et al.*, 2002) The cleavage of the substrate occurs at the phosphodiester bond at a distance of 10-11 nucleotides from the 5'end of the guide siRNA (Elbashir *et al.*, 2001a, 2001b; Haley and Zamore, 2004; Martinez and Tuschl, 2004).

AGO proteins (~100 kDa) are highly basic proteins and consits of four domains: an N-terminal, PAZ, Mid and a C-terminal PIWI domains (fig. 6A), among which PAZ and PIWI domains are particularly conserved (Cerutti *et al.*, 2000).

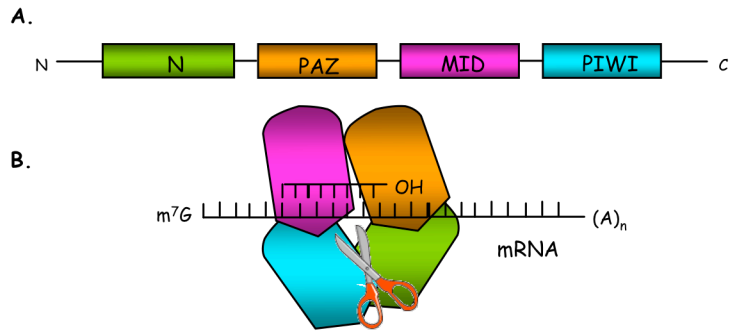



**Figure 6: Representation of Argonaute protein** (Yang *et al.*, 2009)
A. Schematic representation of functional domains ; B. Cartoon model for AGO-mediated mRNA cleavage

The PAZ domain of 130 aa has been identified both in Argonaute proteins and in DCL-proteins (Bernstein *et al.*, 2001a). In AGOs, PAZ is composed of two subdomains linked by a cleft constituted of aromatic residues, which binds the two-nucleotides overhang at the 3' end of the guide strand

siRNA (Lingel *et al.*, 2003; Song *et al.*, 2003; Tolia *et al.*, 2007; Hutvagner *et al.*, 2008) (fig. 6B), while the 5' phosphate of the siRNA is located at the interface between the Mid and PIWI domains through an interaction with a divalent metal ion.

The PIWI domain of 300 aa, at the C-terminus of Argonaute, defines with PAZ domain a groove for substrate binding. It is structurally similar to ribonuclease H (RNase H) domain, a class of RNA endonucleases that normally cleave the RNA strand of a DNA–RNA duplex, whereas Piwi proteins are active on RNA–RNA hybrids. In fact, PIWI domain confers the endonucleolytic (or 'slicer') activity on RISC (Liu *et al.*, 2004; Song *et al.*, 2004; Ma *et al.*, 2005). This domain contains three conserved catalytic residues composed of the two aspartates and a histidine, called the 'DDH' motif, which is analogous to the 'DDE' catalytic motif of RNase H (Yang *et al.*, 1995). The endonucleolytic cleavage of RNA by the PIWI domain generates a 5′-phosphate and a 3′-hydroxy group (Hammond *et al.*, 2005; Farazi *et al.*, 2008).

### 4.2. Diversities of AGO proteins

Currently, ten AGO proteins were identified in *Arabidopsis*, named AGO1 to AGO10 (for review, Vaucheret *et al.*, 2008). AGO1 is involved in plant development in *Arabidopsis* (Vaucheret *et al.*, 2006). Indeed, *ago1* mutants exhibit numerous phenotypic abnormalities such as radialized leaves, abnormal infertile flowers and in some cases, shoot apical meristem defects (Bohmert *et al.* 1998; Lynn *et al.* 1999). AGO1, which has a nuclear and cytoplasmic localization (Fang and Spector, 2007), preferentially associates with siRNAs that have a 5'-terminal uridine (Mi *et al.*, 2008, Montgomery *et al.*, 2008; Tadeka *et al.*, 2008). AGO1 has been identified as a slicer that uses both miRNAs and siRNAs as guides (Baumberger *et al.*, 2005; Qi *et al.*, 2005) and to inhibit mRNA translation (Brodersen *et al.*, 2008). It was shown that *ago1* mutants were hypersensitive to viral infection (Morel *et al.*, 2002; Zhang et al, 2006), suggesting clearly the implication of AGO1 in the antiviral pathway. This was notably confirmed by Zhang *et al.* (2006), who detected siRNAs, derived from *Cucumber mosaic virus* and *Turnip yellow mosaic virus* (TYMV, *Tymovirus*), in Flag-AGO1 immunoprecipitates recovered from virus-infected Flag-AGO1 plants. AGO10, which is the closest paralogue of AGO1, is involved in translation inhibition using miRNAs as guide (Brodersen *et al.*, 2008; Lanet *et al.*, 2009).

AGO2 and AGO3 have a degenerate "DDH" motif (Nowotny *et al.*, 2005). AGO2 preferentially associates with siRNAs that have a 5'-terminal adenosine (Mi *et al.*, 2008, Takeda *et al.*, 2008) whereas AGO5 seems to preferentially associate with siRNAs that have a 5' terminal cytosine, however their biological relevances have yet not to be determined (Takeda *et al.*, 2008).

AGO4 is involved in transcriptional gene silencing (TGS). Indeed, it was shown that AGO4 cleaves siRNA targets and directs cytosine methylation and chromatin modification (Zilberman *et al.*, 2003; 2004; Qi *et al.*, 2006). AGO4 interacts with the C-terminal domain of NRPD1b and localizes to distinct bodies in the nucleolus (Li *et al.*, 2006; Pontes *et al.*, 2006). It was proposed that AGO4

functions at target loci through two distinct and separable mechanisms. Firstly, AGO4 can recruit DNA methylation components in a manner independent of its catalytic activity. Secondly, secondary siRNAs are generated through the catalytic activity of AGO4 to reinforce silencing by methylation (Qi *et al.*, 2006). AGO6 and AGO9 have a redundant function with AGO4 and mediate cytosine methylation of DNA targets (Zheng *et al.*, 2007).

AGO7 seems to participate in antiviral pathway and in the transition between young and adult leaves and it is needed for the TAS3 tasiRNAs pathway (Falhgren *et al.*, 2006). Therefore, it was suggested that AGO7 might be part of an RNA silencing effector complex which is specific to a particular stage of plant development (Hunter *et al.*, 2003, Morris *et al.*, 2008).

**5. RNA-dependent RNA polymerases**

The RNA-dependent RNA polymerases (RDRs) catalyze the extension of a RNA primer, from the 3' end, annealed to a RNA template. The catalytic domain of RDRs contains the motif DxDGD, which is a characteric of a metal-chelating active site (Cheestham *et al.*, 2000; Cramer *et al.*, 2002).

In Arabidopsis, there are six RDRs named AtRDR1, AtRDR2, AtRDR3a, AtRDR3b, AtRDR3c, AtRDR6 (SDE1/SGS-2).

Transitive silencing in plants requires the production of secondary siRNAs, which is dependent of RDR6. As aformentioned, RDR6 can act both in a primer-independent and in a primer-dependent process and thus, RNA silencing in plants spreads from 5' to 3' and 3' to 5' ends (Voinnet *et al.*, 1998; Mourrain *et al.*, 2000; Vaistij *et al.*, 2002; Petersen *et al.*, 2005). RDR6 participates also in the biogenesis of ta-siRNAs (Peragine *et al.*, 2004; Vazquez *et al.*, 2004).

RDR1, which is induced by salicylic acid treatment or pathogen infection, is also required for the amplification of RNA silencing pathway that silences transgenes and viruses (Wang *et al.*, 2010) whereas RDR2 takes part in the chromatin-silencing pathway (Xie *et al.*, 2004).

**II. Endogenous RNA silencing pathways**

The endogenous RNA silencing pathways include the miRNA, ta-siRNA and natural cis-antisense pathways.

**A. Micro-RNAs (miRNAs) pathway**

The miRNAs negatively regulate gene expression in plants and animals and this regulation is crucial for development. The accumulation of the miRNAs is regulated spatio-temporally in response to environmental stimuli. The miRNAs are also involved in the regulation of genes during the plant development and the biotic and abiotic stresses. The genes targeted by miRNAs are involved in meristem identity and maintenance, patterning, cell division, hormone signaling and developmental timing (Rhoades *et al.*, 2002; Xie *et al.*, 2003).

**1. Biogenesis of miRNAs**

The miRNA biogenesis includes many steps in the nucleus and in the cytoplasm (Papp *et al.*, 2003; Bartel *et al.*, 2004). The MIR genes, usually located in the intergenic or intronic regions of the nuclear genome, originated from random mutations in initially perfect inverted repeat (IR) genes to progress in shortened hairpins with mismatches and bulges. In a first step, the MIR gene is transcribed by RNA polymerase II into a pri-miRNA (fig.7). It expression is tissue or cell-type specific according the role of the miRNA(s) in patterning and maintenance of differentiated cell states (Bartel and Bartel, 2003; Parizotto *et al.*, 2004; Voinnet, 2010).

The pri-miRNA is characterized by a cap, a poly(A) tail and an imperfect fold-back structure that contains mismatches and GU wobbles. It might be stabilized by the RNA-binding protein DAWDLE (DDL) (Lee *et al.*, 2004; Yu *et al.*, 2008; Voinnet, 2009). The pri-miRNA is cleaved in a stem-loop precursor of approximately 70-300 nt, named pre-miRNA, in nuclear dicing bodies by DCL1 (Grishok *et al.*, 2001; Park *et al.*, 2002; Reinhart *et al.*, 2002; Xie *et al.*, 2005). This processing requires the HYL-1, the zinc finger protein SERRATE (SE) and the cap-binding proteins (CBP): CBP20 and CBP80 (Han *et al.*, 2003; Vazquez *et al.*, 2004a; Lobbes *et al.*, 2006; Kurihara *et al.*, 2006; Yu *et al.*, 2008, Dong *et al.*, 2008; Kim *et al.*, 2008). Then, the pre-miRNA is processed by DCL1 (Schauer *et al.*, 2002) into a miRNA/miRNA* duplex (Dunoyer *et al.*, 2004). One strand of the duplex corresponding to the mature miRNA is incorporated into RISC whereas the miRNA* defined as the passenger strand, is normally degraded (Tomari *et al.*, 2004; Baulcombe *et al.*, 2005). However, a recent study suggests a potential biological role for miRNA* in Drosophila (Okamura *et al.*, 2008), as it was previously suggested for the miRNAs encoded by the *Simian virus 40* genome (Sullivan *et al.*, 2005). The miRNA duplexes of 21-24 bp in lenght have biochemical features similar to siRNAs with 2 nt-long 3' overhangs and 5' phosphate/3' hydroxyl ends (Vazquez *et al.*, 2006). The miRNA is methylated by HEN1 and probably exported to the cytoplasm by HASTY, which is the plant homologue of exportin-5 (Park *et al.*, 2005).

MiRNAs regulate gene expression by directing the cleavage of target mRNAs by AGO1 (Llave *et al.*, 2002; Hutvagner *et al.*, 2002; Ambros *et al.*, 2004, Qi and Hannon, 2005). After cleavage, the 5' fragment of the mRNA is degraded by the exosome whereas the 3' fragment is digested by the exonuclease XRN4, which degrades uncapped mRNAs (Souret *et al.*, 2004). The miRNA can also act *in trans* to inhibit the translation of targeted mRNAs by AGO1 or AGO10, depending of the degree of complementary between the miRNAs and their target and the presence of the enzyme KATANIN (KTN1) and the decapping enzyme VARICOSE (VCS) (Brodersen *et al.*, 2006; Lanet *et al.*, 2008; Brodersen *et al.*, 2008; Voinnet, 2009). Some miRNAs might be retained in the nucleus to act at the level of chromatin silencing. Recently, it was shown that the 22 nt miRNAs trigger the RDR6-dependent secondary siRNAs pathway (Cuperus *et al.*, 2010).

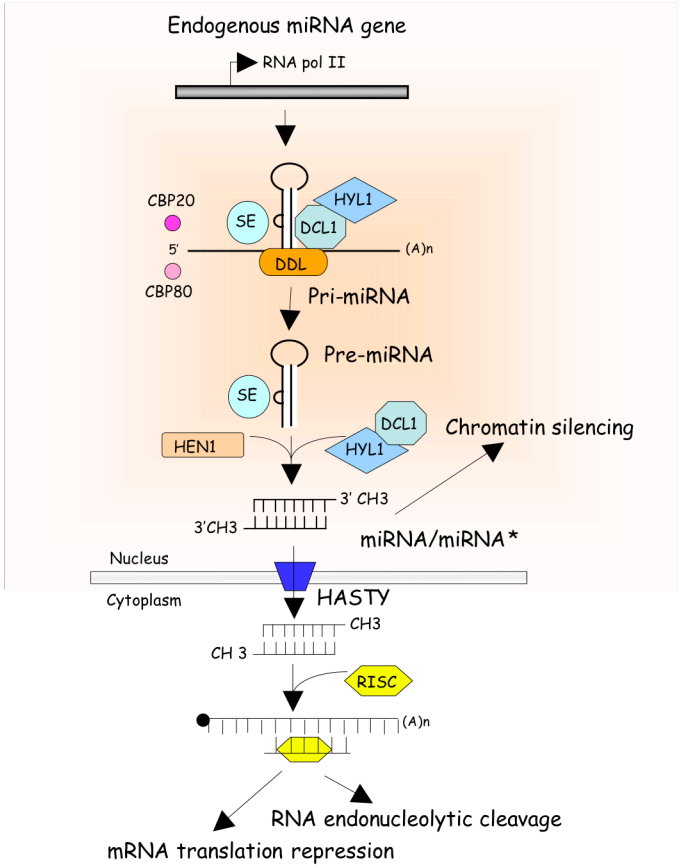



**Figure 7: Biogenesis and activity of miRNAs in *Arabidopsis*** (Brodersen *et al.*, 2006; Voinnet, 2009) The primary miRNA transcript (pri-miRNA) that exhibits a stem loop structure(s), are synthesized by RNA polymerase II. Then, the pri-miRNA is cleaved into pre-miRNA by DCL1 in the presence of HYL1, SE, DDL, CPB20 and CPB80. In the nucleus, methylation of miRNAs is performed by the combined action of DCL1, HYL1 and HEN1. After nuclear export by HASTY, one strand of duplex miRNA is incorporated into RISC and lead to either degradation or inhibition of translation of RNA targets. It is also possible that some miRNAs induce DNA methylation or chromatin silencing.

## 2. Targets of miRNAs

The plant miRNAs target a series of genes that are important for normal plant development, hormonal control and a variety of biotic and abiotic stresses (Ruiz-Ferrer and Voinnet, 2008). They can act at the transcriptional or posttranscriptional levels.

MiR159 targets the MYB transcription factors involved in a plant hormone response (Palatanik *et al.*, 2003). A gibberellic acid (GA) stimulus could lead to an increase in MYB33, MYB65 and MYB101 that would initiate flowering and, directly or indirectly, to an increase in miR159 (Millar and Gubler, 2005). Two other miRNAs, miR165 and miR166, have been identified to target the five Class III HD-Zip gene family members present in the *Arabidopsis* genome: PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV), ATHB8, and ATHB15 (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002). These HD-ZIP transcription factors influence the abaxial and the adaxial polarity in leaves

and stems (Emery *et al.*, 2003). Some miRNAs are involved in the regulation of floral development. Indeed, miR156 is involved in the vegetative phase as well as in floral transition (Wang *et al.*, 2007). Other miRNAs act at many stages of development by targeting NAC transcription factor or by modulating the auxin signalling (Mallory *et al.*, 2004). For example, miR160 and miR167 seem to be implicated into shoots and roots development by targeting auxin response factor (ARF) genes (Mallory *et al.*, 2005; Liu *et al.*, 2008) whereas miR164 regulates NAC1 that mediates auxin signalling in lateral root (Guo *et al.*, 2005). In addition, the effectors involved in the miRNA pathway can be triggered themselves by the miRNAs to regulate the process, as miR162 which targets the mRNA encoding DCL1 (Xie *et al.*, 2003) and thus, negatively regulates DCL1 synthesis. Concerning AGO1 component of RISC, it is regulated by miR168 (Vaucheret *et al.*, 2004).

### B. Trans-acting siRNAs pathway

The trans-acting siRNAs (ta-siRNAs) represent an endogenous class of small interfering RNAs (siRNAs) that act *in trans*, hence the name ta-siRNAs (Vaucheret *et al.*, 2006, for review Allen *et al.*, 2010). The endogenous transcripts, whose cleavage is mediated by ta-siRNAs, are implicated in the transition from a juvenile to an adult phase of vegetative development before flowering, such as the morphology and the polarity of the leaves (Xie *et al.*, 2005; Gasciolli *et al.*, 2005; Adenot *et al.*, 2006).

#### 1. Biogenesis of ta-siRNAs

For their biogenesis, the ta-siRNAs require the components of both miRNA and siRNA pathways (Allen *et al.*, 2010). The ta-siRNA pathway is initiated by Pol II transcription to yield TAS transcripts that contain miRNA target site(s) (fig. 8).

In Arabidopsis, six TAS genes (TAS1a, b, c TAS2, TAS3 and TAS4), that encode non-protein coding transcripts, have been identified. After transcription, the TAS precursors (pri-ta-siRNA) are exported to the cytoplasm where they are targeted by miRNAs-guided AGOs (figure 8). TAS1a, TAS1b, TAS1c and TAS2 transcripts are targeted by miR173, TAS3 transcript is targeted at two conserved sites by miR390 (Allen *et al.*, 2005; Xie *et al.*, 2005) and TAS4 trnascript by miR828 (Rajagopalan *et al.*, 2006). The annealing of specific miRNAs on the TAS transcript allows the recruitement of the miRNA pathway-effectors AGO1-DCL1-HEN1-HYL1. So, the TAS transcripts are cleaved at specific positions by the corresponding miRNA/RISC to generate products, which will serve as template for the ta-siRNAs synthesis. (Rajagopalan *et al.*, 2006; Vazquez *et al.*, 2004b; Perargine *et al.*, 2004; Gasciolli *et al.*, 2005).

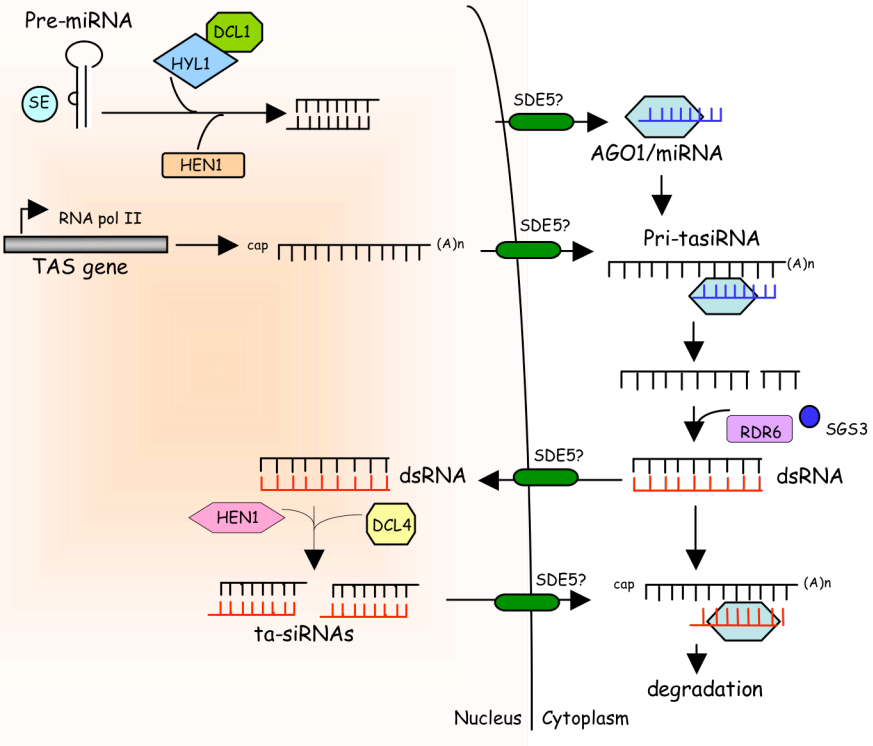



**Figure 8: Trans-acting pathway in *Arabidopsis*** (Brodersen and Voinnet, 2006; Vasquez *et al.*, 2010)
Firstly, a precursor, called pri-tasiRNA, is cleaved via a specific miRNA. Following this cleavage, the generated products are converted into dsRNAs by RDR6 and SGS3 in the cytoplasm. The synthesis of tasiRNAs from dsRNAs is realized by DCL4 in nucleus. Then, the tasiRNAs target RNAs in the cytoplasm for their degradation. The traffic of ta-siRNAs between the nucleus and the cytoplasm could be due to SDE5.

After cleavage by miRNAs, the 3' end products for TAS1a, TAS1b, TAS1c and TAS2 and the 5' end products for TS3 of the TAS transcripts are converted into dsRNAs by RDR6. SGS3 participates also in this conversion stabilizing the dsRNAs (Yoshikawa *et al.*, 2005). Then, the dsRNAs are imported in the nucleus, probably by the SDE5 protein and cleaved by DCL4 in the presence of DRB4 to generate the 21 nt ta-siRNAs (Peragine *et al.*, 2004; Vasquez *et al.*, 2004; Dunoyer *et al.*, 2005; Hernandez-Pinzon *et al.*, 2007). Finally, the ta-siRNAs are incorporated into RISC and guide cleavage of complementary mRNAs. AGO1 (TAS1 and presumably TAS2) or AGO7 (TAS3) are implicated in the regulation of the target genes at the post-transcriptional level (Hunter *et al.*, 2003; Baumberger *et al.*, 2005; Adenot *et al.*, 2006; Howell *et al.*, 2007). Contrary to miRNAs, the ta-siRNAs can move from cell-to-cell and thus generate a gene-expression gradient allowing organ polarization (Chitwood *et al.*, 2009).

### 2. Targets of ta-siRNAs

Many genes in plants are involved in the proximodistal, adaxial-abaxial and mediolateral leaf development. The most famous are auxin response factor/Ettin genes, (ETT)/ARF3 and ARF4 (Chen *et al.*, 1999; Sawa *et al.*, 1999; Siegfried *et al.*, 1999; Kumaran *et al.*, 2002) and the KANADI genes (Eshed *et al.*, 2001; Pekker *et al.*, 2005), which are the determinants of the abaxial fate of the leaf. The PPR (PHABULOSA/PHAVOLUTA/REVOLUTA) genes family controls the leaf shape by specifying adaxial identity (McConnell *et al.*, 1998; 2001; Emery *et al.*, 2003; Zhong and Ye, 2004; Prigge *et al.*, 2005). These factors and some MYB transcription factors such as the ASYMMETRIC LEAVES1 (AS1) in *Arabidopsis*, which also determines the adaxial fate (Xu *et al.*, 2003), are the targets of ta-siRNAs (Adenot *et al.*, 2006; Fahlgren *et al.*, 2006; Garcia *et al.*, 2006; Backman *et al.*, 2008).

A good illustration of the implication of ta-siRNAs on the leaf development is the ta-siRNAs of TAS3. Garcia *et al.*, (2006) showed that TAS3-ta-siRNAs target ETT/ARF3 mRNA and ARF4 mRNA that induces the downregulation of the FIL genes in the abaxial domain of the leaf.

## C. Natural cis-antisense transcripts-associated siRNAs (nat-siRNAs)

Like the ta-siRNAs pathway, the biogenesis of nat-siRNAs involves the miRNA and the siRNA pathways.

The dsRNA is formed by overlapping of two transcripts (Borsani *et al.*, 2005), one of the transcripts being constitutively expressed while the second transcript is induced by specific conditions. Such an overlapping was notably described for SRO5 and P5CDH transcripts, which are produced in antisense orientation (Wang *et al.*, 2005).

These cis-antisense transcrips are processed by DCL2 to generate nat-siRNAs species of 24 nt in length. These primary nat-siRNAs anneal to the transcript that is then converted into dsRNA by RDR6 and SGS3. Thereafter, the dsRNA is cleaved by DCL1, but it is likely that NRPD1a, which is a subunit of the DNA-dependent RNA polymerase IVa complex (Pol IVa), contributes also in the accumulation of the secondary nat-siRNAs of 21 nt by amplifying the transcript templates of RDR6 (fig. 9). These secondary nat-siRNAs are named *cis*-acting siRNAs (casiRNAs) because they promote the silencing of the constitutive transcripts that generate them.

The induction of the natural cis-antisense transcripts-associated siRNAs occurs in response to a high salinity conditions and thus, it is assumed that the nat-siRNAs contribute to the stress adaptation (Borsani *et al.*, 2005). Indeed, the down-regulation of P5CDH results in an increase of proline synthesis, which is a physiological response to confer salt tolerance.

Other numerous cis-antisense genes have been identified in plant, suggesting that these nat-siRNAs might be very important in the regulation of endogenous gene expression (Yamada *et al.*, 2003; Chen, 2005).

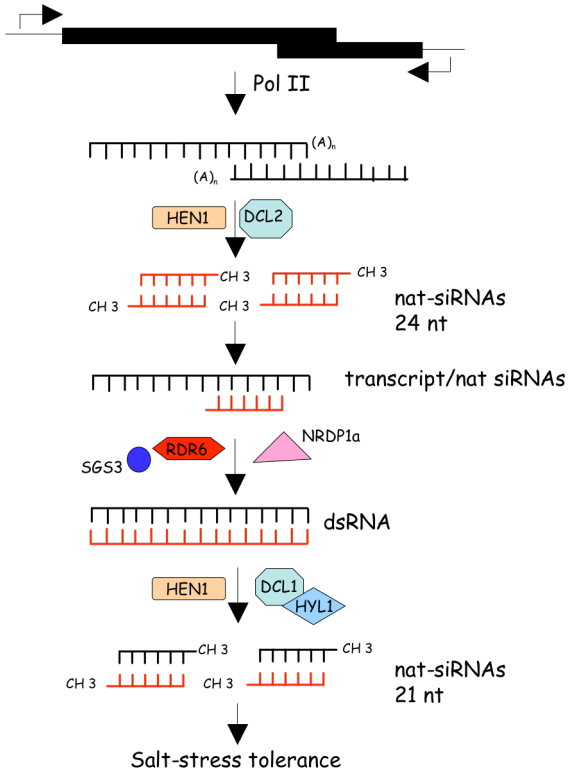



**Figure 9: Natural-antisense transcript pathway in *Arabidopsis*** (Vasquez *et al.*, 2006)
Double-stranded region resulting from pairing of cis-antisense transcripts is processed by DCL2 to generate 24 nt nat-siRNAs that guide cleavage of one transcript through an as yet unidentified AGO protein. RDR6 and SGS3 synthesize a strand complementary to cleave fragments of mRNA target leading to a dsRNA that is processed by DCL1. Resulting 21 nt nat-siRNAs reinforce cleavage of mRNA targets. NRPD1a could be involved in an amplification loop using the dsRNA generated by RDR6 and SGS3 as a template.

## III. Transgenes and RNA silencing

The introduction of transgenes such as an inverted-repeat construct as well as a sense transgene can also induce RNA silencing (Brodersen and Voinnet, 2006).

### A. Inverted-repeat PTGS

After transcription of an inverted-repeat construct (IR-construct), the transcript is characterized by a fold-back structure that activates PTGS (Beclin *et al.*, 2002; Giordano *et al.*, 2002; fig. 10). This pathway is called inverted-repeat PTGS (IR-PTGS).

Two dicers, DCL4 and DCL3, are involved in the processing of the dsRNAs generated from an IR-construct. It was demonstrated that DCL4 is an essential component in the SUC-SUL system, which is an IR transgene of the SULPHUR gene expressed under the phloem-companion cell-specific promotor, SUC2 (Dunoyer *et al.*, 2007). This SUC-SUL construct triggers SUL silencing and results in

vein-centered chlorosis (Deleris, 2006).

The siRNAs of 24 nt direct the chromatin methylation and consequently transcriptional silencing (Llave *et al.*, 2002) whereas those of 21 nt guide the cleavage of the transcripts corresponding of the transgene by AGO1/RISC. The 21 nt/AGO1 complex are also involved in the silencing movement (Morel *et al.*, 2002; Dunoyer *et al.*, 2010a; 2010b). DCL2 might also be involved in IR-PTGS because it processes some endogenous DCL4 substrates into 22 nt siRNAs in the absence of DCL4 (Gasciolli *et al.*, 2005; Xie *et al.*, 2005; Dunoyer *et al.*, 2007).

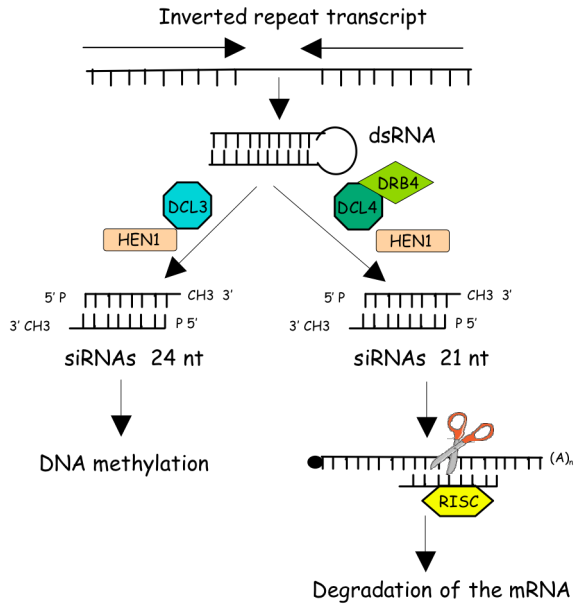



**Figure 10: Inverted-repeat post-transcriptional gene silencing (IR-PTGS)** (Brodersen and Voinnet, 2006)
The transcription of an inverted-repeat construct (IR) produces a transcript with a stem-loop structure. This structure induces the IR-PTGS and is cleaved into siRNAs of 21 nt or 24 nt by DCL4 and DCL3, respectively. Both siRNA species are methylated at their 3' termini by HEN1. The siRNAs of 24 nt can direct DNA or histone modification at homologous loci whereas the one strand of 21 nt siRNAs is incorporated into AGO1-loaded RISC to guide endonucleolytic cleavage of homologous RNA, leading to its degradation.

### B. Sense-PTGS

There are several examples showing that single-copy transgenes producing sense transcripts can also trigger PTGS. It seems that the high expression level of a transgene can lead to the accumulation of aberrant RNAs (abRNAs), which are characterized by a lack of 5' capping and/or a 3' poly(A) tailing (fig. 11). RDR6 converts abRNAs into dsRNAs and subsequently, induces the degradation of all transgene transcripts by the intermediate of the S-PTGS pathway (Gazzani *et al.*, 2004). Other factors are also required in this sense-PTGS (S-PTGS), such as SGS3, HEN1 and the putative RNA

helicase SDE3 (Mourrain *et al.*, 2000; Dalmay *et al.*, 2001; Glazov *et al.*, 2003; Boutet *et al.*, 2003). The dsRNAs generated by RDR6 are sliced by DCL4 to produce siRNAs of 21 nt. These siRNAs can be used as primers by RDR6 to increase the production of dsRNAs from single-stranded templates (transitivity phenomenon). The 21 nt siRNAs can also be associated with AGO1/RISC to provok the degradation of the new synthetized transcripts, as in the IR-PTGS (Morel *et al.*, 2002). The resulting cleavage products could be perceived as aberrant RNAs and thus, could promote further production of dsRNAs, resulting in an amplification of the reaction.

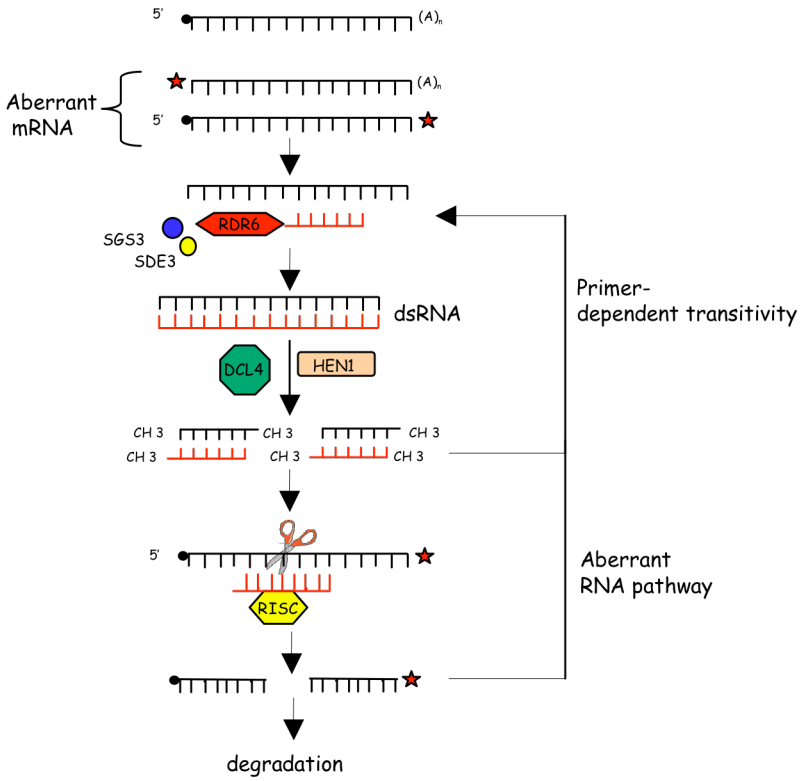



**Figure 11: Sense-post-transcriptional gene silencing pawthay (S-PTGS)** (Brodersen, 2006) The high level of transgene expression leads to the synthesis of aberrant transcripts, which induce S-PTGS. These aberrant features are a lack of a poly(A) tail or lack of 5' capping. The aberrant RNAs are converted into dsRNAs by the combined action of RDR6, SGS3 and SDE3. The resulting dsRNA is then processed by DCL4, producing siRNAs of 21 nt.

## IV. DNA methylation pathway and heterochromatic siRNAs

In eukaryotes, the DNA is wrapped around histone proteins, forming chromatin fibres The chromatin can be in two forms: the euchromatin, which is less condensed and accessible for transcription and replication and the heterochromatin form, which is the highly condensed and inaccessible for these processes.

Most heterochromatin, composed of repeated DNA sequences, is found near centromeres and telomeres. Heterochromatin is characterized by two sets of modifications: methylation of cytosine residues and methylation of the histone H3 at lysine K9 and K27 residues in *Arabidopsis* (Jackson *et al.*, 2004). In *Arabidopsis*, the proteins involved in these modifications are the cytosines methyl-transferases DRM1 and DRM2, the chromatin remodelling protein DRD1, the methyltransferase1 (MET1) for maintenance of methylation at CG sites and Chromo methyltransferase (CMT3) for maintenance at CNG (N represent any nucleotides) and asymmetrical CNN sites (Chan *et al.*, 2005; Matzke *et al.*, 2005). These modifications are important for chromatin remodeling, condensation and control of numerous genetic processes in the cell, including replication, transcription, DNA repair, recombination and gene transposition (Lippman and Martienssen, 2004; Grant-Downton and Dickinson, 2005; Grewal and Jia, 2007). In addition to methyltransferases, the DDM1 (Decrease in DNA Methylation, a chromatin remodeling helicase) is the major factor for maintaining methylation in both CG and non-CG contexts (reviewed by Bender, 2004; Chan *et al.*, 2005; Gehring and Henikoff, 2007).

**A. DNA methylation pathway**

Transcriptional-gene silencing (TGS) was firstly demonstrated in transgenic plants, where *de novo* methylation of the transgene promoter sequence correlated directly with its transcriptional inactivation (Matzke *et al.*, 1994; Park *et al.*, 1996). This silencing depends on the presence of dsRNA like other pathways. Therefore, TGS pathway can be triggered by the introduction of inverted repeat sequences or a sense-transgene, which are processed into siRNAs and cause DNA methylation of homologous promoters resulting in epigenetic silencing of the downstream gene (Mette *et al.*, 2000; Jones *et al.*, 2001). Transposons, retroelements, repetitive DNA and some regions surrounding centromeres also trigger TGS (Chan *et al.*, 2004; Matzke and Birchler, 2005; Huettel *et al.*, 2006; Wierzbicli *et al.*, 2008). In plants, RNA viruses were also reported to direct DNA methylation of homologous genes (Wassenegger *et al.*, 1994, Baulcombe, 2004). The first evidence for this type of silencing, called RNA-directed DNA methylation (RdDM) came from experiments with viroid-infected tobacco plants. When the RNA of a viroid was integrated as a transgene into the tobacco genome, cDNA copies of the viroid became methylated only during replication of the homologous inoculated viroid (Wassenegger *et al.*, 1994). These results indicated that the presence of the replicating viroid RNA could induce methylation of homologous DNA sequences.

RdDM typically involves methylation of both CG and non-CG sequences. The siRNA-directed DNA methylation in plants is also linked to histone methylation (Soppe *et al*., 2002; Zilberman *et al*., 2003; reviewed by Grant-Downton and Dickinson, 2005; Kanazawa *et al*., 2007).

### B. Heterochromatic siRNAs

The siRNAs corresponding to several endogenous silent loci, including retrotransposons, 5S rDNA and centromeric repeats are referred to as repeat-associated siRNAs (ra-siRNAs), cis-acting siRNAs (ca-siRNAs) or heterochromatin siRNAs (hc-siRNAs) (Hamilton *et al.*, 2002; Lippman *et al.*, 2004; Chan *et al.*, 2005; Kasschau *et al.*, 2007). In plants, ra-siRNAs of 23-25-nt are the most abundant siRNA class. Their biogenesis occurs in nuclear Cajal body-like structures and requires the activity of Pol IVa, RDR2, DCL3 and HEN1 (Xie *et al.*, 2004; Lu *et al.*, 2005; Herr *et al.*, 2005; Onodera *et al.*, 2005; Matzke *et al.*, 2009).

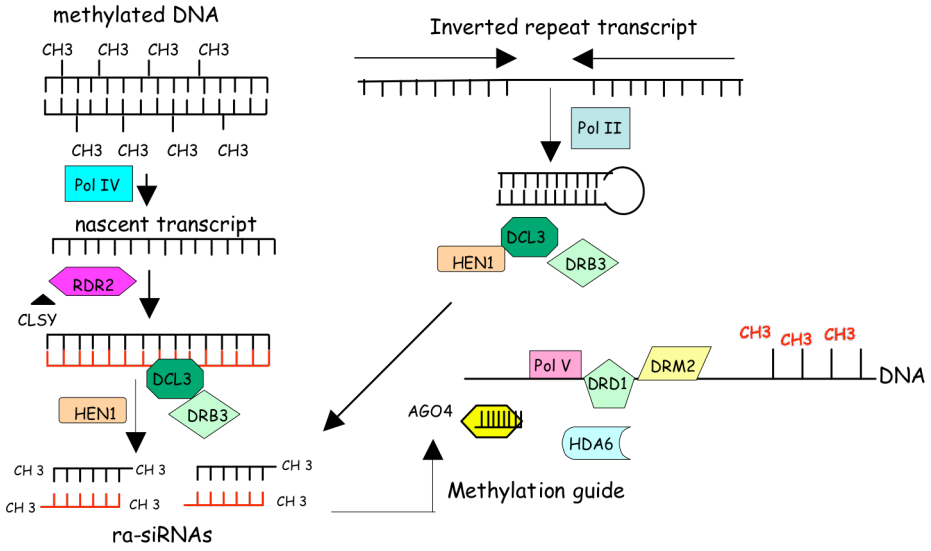



**Figure 12: Schema illustrating chromatin-targeted RNA silencing** (Brodersen *et al.*, 2006)
A nascent Pol II or Pol IV transcript from methylated DNA or an aberrant transcript, respectively, form a dsRNA. The dsRNA is then processed by DCL3 with CLSY into 24 nt siRNAs. These siRNAs-loaded AGO4 are recruited to guide chromatin modifications rather than RNA cleavage. They can also guide sequential activities of histone deacetylases (HDA6), chromatin remodeling factors (DRD1) and/or DNA methyl-transferases (DRM).

Non-protein-coding RNAs are produced from methylated loci by Pol IVa (DNA-dependent RNA polymerase containing the NRPD1a and NRPD2 subunits), which preferentially transcribes methylated DNA (Onodera *et al.*, 2005) or aberrant RNAs generated from methylated loci (Vaucheret *et al.*, 2005). These transcripts are converted into dsRNAs by RDR2 (Li *et al.*, 2005; Kasschau *et al.*, 2007; Pikaard *et al.*, 2008; Mosher *et al.*, 2008) in the presence of CLASSY (CLSY), which facilitates the processing of these transcripts by DCL3 (Xie *et al.*, 2004; Smith *et al.*, 2007) although partially redundant functions of DCL2, DCL3 and DCL4 in this pathway have been reported (Henderson *et al.*, 2006)(fig. 12). After methylation by HEN1 (Li *et al.* 2005; Xie *et al.*, 2004), the ra-siRNAs loaded

into RNA-induced transcriptional silencing complexe (RITS). RITS, which contains at least AGO4 (Zilberman *et al.*, 2003) or AGO6 or AGO9 in some cases (Zheng *et al.*, 2007; Havecker *et al.*, 2010), mediate *de novo* methylation of cytosines within DNA sequences complementary to the siRNAs by DRM1 and DRM2 (Cao *et al.*, 2003; Zilberman *et al.*, 2003; 2004; Matzke *et al.*, 2006). Moreover, AGO4 has been shown to physically interact with the C-terminal domain of the largest subunit of Pol V, which interacts with intergenic loci through the chromatin-remodelling factor DRD1 (Kanno *et al.* 2004; 2008). Pol V generates transcripts, which bind the siRNAs that guide heterochromatin modification usually associated with cytosine methylation and/or histone deacetylation (Li *et al.*, 2006; El-shami *et al.*, 2007; Wierzbicki *et al.*, 2008; 2009; Matzke *et al.*, 2009). Other factors, such as DMS3 and the putative histone deacetylase HDA6 (Aufsatz *et al.*, 2002), are also involved in the promoter methylation.

The ra-siRNAs of 24 nt maintain methylation of DNA and repress histone modifications on certain retroelements and repetitive DNA to ensure genome stability (Hamilton *et al.*, 2002; Xie *et al.*, 2004; Matzke *et al.*, 2005) or direct *de novo* DNA methylation (Stam *et al.*, 1998; Pelissier *et al.*, 1999; Luff *et al.*, 1999). This *de novo* DNA methylation results in the change of the chromatin configuration/methylation of the silenced locus DNA and thereby the silencing of genes at the transcriptional level (TGS) (Matzke *et al.*, 2005).

## V. RNA silencing and Phytoviruses

Plants have against various pathogens a natural passive defense based on the presence of the rigid cell wall. They have also developed some active defense mechanisms upon recognition of pathogens such as viruses.

The first common active defense identified is the so-called hypersensitive response (HR). This mechanism programs the death of the cells surrounding the primary infection site. This response is induced when the host plant, carrying a resistance gene (*R*), is challenged by a pathogen, carrying a matching avirulence gene (*Avr*) (Keen, 1990). The HR is characterized by the formation of necrotic lesions, an oxidative burst, alterations in cell wall structure, increase in endogenous salicylic acid levels and activation of defense-related genes, including genes encoding the pathogenesis-related (PR) proteins (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997; Yang *et al.*, 1997). In addition to these local responses, the uninfected part of the plant develops the systemic acquired resistance (SAR), which leads to enhanced resistance against the initial or unrelated pathogens (Ryals *et al.*, 1996; Sticher *et al.*, 1997). In conclusion, HR mechanism allows a local containment of the invading pathogen at entry sites.

Among host defense responses, RNA silencing has emerged as an important natural antiviral mechanism in plants (Ratcliff *et al.*, 1997; Vance and Vaucheret, 2001; Voinnet, 2001, 2005; Baulcombe, 2004; Ding *et al.*, 2004; Wang and Metzlaff, 2005). The implication of the RNA silencing

against the viruses comes from different observations. Several studies have demonstrated the capacity of a virus to induce RNA silencing if it shares sequence similarities with a transgene or an endogenous gene. Mutant plants affected in the silencing pathways are hypersusceptible to viral infection. The identification of the recovery phenomenon allowed to demonstrate that RNA silencing can be triggered a virus. Moreover, the detection of siRNAs of 21-24nt of length derived from the *Potato virus X* (PVX, *Potexvirus*), confirmed the antiviral role of RNA silencing (Hamilton *et al.*, 1999). Another irrefutable demonstration is the discovery of virus-encoded proteins, which suppress RNA silencing (Brigneti *et al.*, 1998).

### A. Manifestation of RNA silencing in plants infected by viruses

Plants respond to pathogen invasion by regulating the expression of their genes, as shown by two phenomena, the Virus-Induced Gene Silencing pathway (VIGS) and recovery.

#### 1. Virus-Induced Gene Silencing (VIGS)

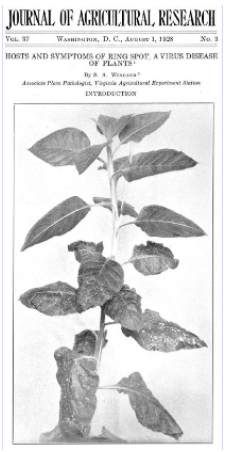
Observations in transgenic plants lead to the discovery that viruses can trigger RNA silencing. It was shown that transgenes derived from viruses are able to induce RNA silencing; this process is called Virus-Induced Gene Silencing (VIGS). In this system, the viruses may also be the targets if they share some similarities in the nucleotide sequence with these transgenes (Lindbo *et al.*, 1993; Kamagai *et al.*, 1995). It was demonstrated that PTGS is implicated in the degradation of both the transgene mRNA and the RNA genome of the virus (Lindbo *et al.*, 1993; Smith *et al.*, 1994; Goodwin *et al.*, 1996; Guo and Garcia, 1997). An illustration is the experiment conducted in transgenic *Nicotiana benthamiana* plants expressing high levels of GFP, in combination with a PVX vector that expresses the GFP (Anandalaskshmi *et al.*, 1998). In this system, the GFP transgene becomes completely and systemically silenced as well as the virus, which is completely eliminated from the plant.

In conclusion, the virus induces and is triggered by the PTGS. This finding led to the proposal that PTGS may be a natural virus resistance mechanism in plants (Baulcombe, 1996; Pruss *et al.*, 1997). Later, it was shown that the induction of PTGS could occur in the absence of any known homology between the inducing virus and the plant genome (Ratcliff *et al.*, 1997; Covey *et al.*, 1997; Al-Kaff *et al.*, 1998). According to this idea, PTGS would be activated naturally in virus-infected plants and artificially in transgenic plants, when the transgene or its RNA is perceived as part of a virus (Ratcliff *et al.*, 1997).

#### 2. Recovery phenomenon

The recovery phenomenon is observed as a form of host resistance in response to plant virus infection (Mathews, 1992). The name recovery comes from the disappearance of the symptoms at the time of the infection. In fact, a plant shows symptoms at the early phase of the infection, which

disappear or become milder in the new emerging tissues later in the infection. Wingard *et al.*, (1928) did the first description of the recovery phenomenon in tobacco plants infected by *Tobacco ringspot virus* (TRSV, *nepovirus*; fig.13).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HOSTS AND SYMPTOMS OF RING SPOT, A VIRUS DISEASE OF PLANTS[1]

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INTRODUCTION

**Figure 13: Recovery in tobacco plants infected with *Tobacco ringspot virus***
The original legend (Wingard *et al.*, 1928) to the figure is "Turkish tobacco plant 23 days after inoculation with ringspot". In this study, it was described that only the inoculated leaves show a necrosis while the upper leaves are asymptomatic and immune to the virus.

At this time, these observations did not permit to explain the resistance to secondary infection. It was observed that tobacco plants expressing a mild strain of *Tobacco mosaic virus* (TMV, *Tobamovirus*) are resistant to a related severe strain of TMV (Yamaya *et al.*, 1988a, 1988b). Therefore, the recovered plants are subsequently "cross-protected" against further infection with the same or closely related strains of the initially infecting virus. Moreover, low levels of viral RNA characterize these plants besides to be symptom-free (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al Kaff *et al.*, 1998; Ratcliff *et al.*, 1999). In fact, the recovery is accompanied by a decreasing virus accumulation in the infected plants and accumulation of siRNAs derived from virus by PTGS (Ratcliff *et al.*, 1997; Covey *et al.*, 1997; Ratcliff *et al.*, 1999). So, the siRNAs accumulated during the primary virus infection can destroy the viral RNAs from a secondary infection that share sequence homologies. Thus, the host recovery seems to be a consequence of the activation of the RNA silencing as antiviral defense. Moreover, the discovery of a systemic signal of RNA, which can move systemically in plants (Palauqui *et al.*, 1997; Voinnet *et al.*, 1997; 1998; Palauqui *et al.*, 1998, Dunoyer *et al.*, 2010a), confirm the possibility of a plant to be activated and to respond more rapidly to the viral infection.

## B. Molecular basis of the viral RNA silencing

### 1. Origin of the dsRNAs

Viruses characterized by a single-strand positive RNA genome represent the vast majority of plant viruses. During their replication, the viral-encoded RDR synthetizes a dsRNA intermediate product (fig. 14A), which is the principal source of dsRNA from plant RNA viruses (Ratcliff *et al.*, 1999; for review, Ruiz-Ferrer and Voinnet, 2008).

DNA viruses can also trigger RNA silencing through the presence of fold-back structures in their transcripts (Szittya *et al.*, 2002; Molnar *et al.*, 2005). *Cauliflower mosaic virus* (CaMV, *Caulimovirus*) activates RNA silencing through a stem-loop structure present in the 5'end of the 35S RNA (Moissard *et al.*, 2004; fig. 14B).

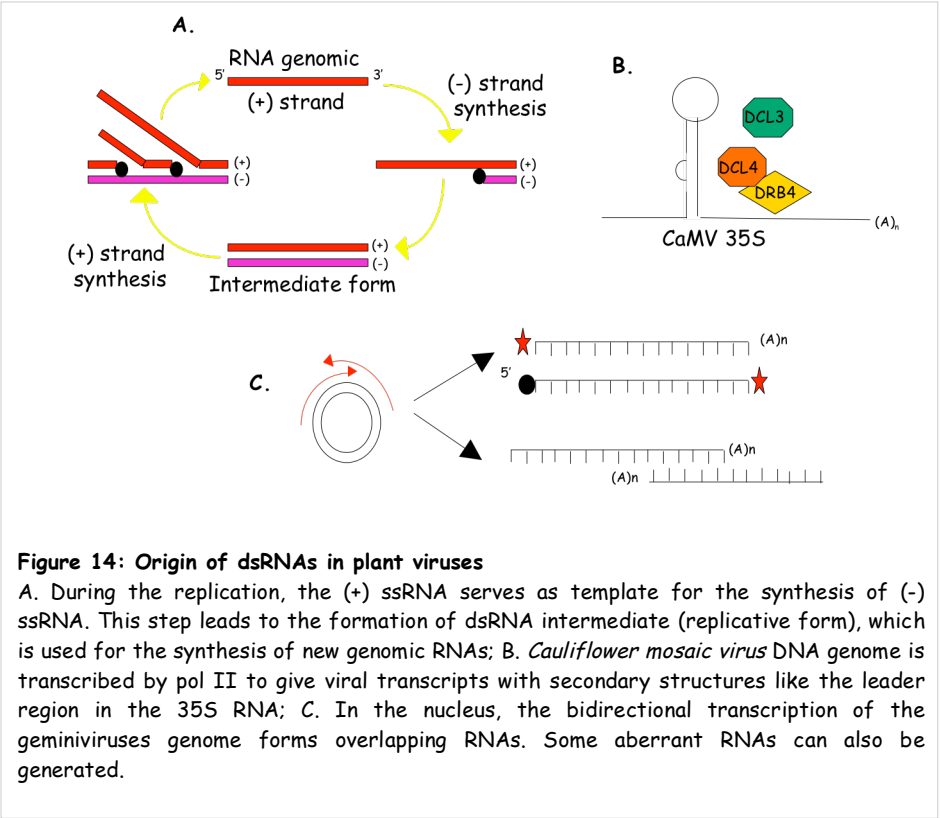


**Figure 14: Origin of dsRNAs in plant viruses**
A. During the replication, the (+) ssRNA serves as template for the synthesis of (-) ssRNA. This step leads to the formation of dsRNA intermediate (replicative form), which is used for the synthesis of new genomic RNAs; B. *Cauliflower mosaic virus* DNA genome is transcribed by pol II to give viral transcripts with secondary structures like the leader region in the 35S RNA; C. In the nucleus, the bidirectional transcription of the geminiviruses genome forms overlapping RNAs. Some aberrant RNAs can also be generated.

Another source of dsRNAs can derive from converging promoters, as described in geminiviruses, which are plant ssDNA viruses. The symetrical transcription of the geminiviral genome leads to two transcripts that overlap at their 3'end. Therefore, dsRNA is formed by annealing of the 3'end of these mRNAs (fig. 14C). DsRNAs can also be generated during the geminivirus replication cycle by the abundance of the mRNA that serves as template for the host-RDRs or by the presence of

folded structures (Vanitharani *et al.*, 2005).

**2. Effectors involved in the viral-RNA silencing**

DCL4 is the main dicer implicated in the slicing of the viral RNA into viral-derived short-interfering RNAs (vsRNAs) in *Arabidopsis*. Therefore, the degradation of the viral RNA is accompanied with the accumulation vsRNAs of 21 nt. It was also shown that DCL2 substitutes DCL4, when the latter is absent or inactivated and slices the RNA into vsRNAs of 22 nt (Blevins *et al.*, 2006; Bouché *et al.*, 2006; Deleris *et al.*, 2006; Fusaro *et al.*, 2006; Diaz-Pendon *et al.*, 2007). The involvement of these dicers was notably demonstrated by the fact that viruses are able to accumulate in the *dcl2-dcl4* mutant background. In the case of infection with *Tobacco rattle virus* (TRV, *Tobravirus*) and CMV, vsRNAs of 24 nt are also detected, which apparently enhance antiviral silencing (Deleris *et al.*, 2006, Diaz-Pendon *et al.*, 2007). DCL1 seems to play a minor role in antiviral response because a low accumulation of vsRNAs of TRV was detected in *dcl2-dcl3-dcl4* mutant background (Deleris *et al.*, 2006).

Concerning the DNA viruses, the four DCLs are implicated in the antiviral defense in *Arabidopsis*. It was shown that CaMV-derived vsRNAs of 24 nt and 21 nt were produced in abundance by DCL3 and DCL4, respectively, and DCL2 can replace DCL4, as for RNA viruses (Blevins *et al.*, 2006; Moissard and Voinnet, 2006). The implication of DCL3 in the antiviral defense against DNA viruses has been demonstrated by the increase of the CaMV accumulation only in the *dcl2-dcl3-dcl4* triple mutants background (Moissard and Voinnet, 2006). A hypothesis to explain the role of DCL3 is its capacity to induce the methylation of the viral minichromosome, probably in order to abolish the transcription of the genome. In *dcl2-dcl3-dcl4* mutants, vsRNAs of 21 nt were also detected in low quantity, suggesting a possible action of DCL1 on the DNA virus accumulation. It was proposed that DCL1 acts early in the dicing pathway by excising the 35S leader region (the major source of CaMV-derived vsRNAs) from the primary transcript to facilitate its subsequent processing by DCL4 and DCL3 (Moissiard and Voinnet, 2006). At the level of RISC, 21 nt and 22 nt siRNAs are incorporated in AGO1, the major effector of PTGS and/or AGO7, which are involved in the degradation of the viral RNA (Zhang *et al.*, 2006, Morris *et al.*, 2008, Qu *et al.*, 2008) whereas the 24 nt siRNAs are incorporated into RISC containing AG04. In fact, the slicing activity of the DCLs is not alone sufficient to disturb the viral multiplication (Deleris *et al.*, 2006), since *ago1* and *ago7* mutant plants are highly susceptible to the viruses (Morel *et al.*, 2002).

**3. Amplification and propagation of RNA silencing**

As already mentioned, the siRNAs of 21 nt and 24 nt move over long-range distances in RDR6-independent background in *Arabidopsis* (Dunoyer *et al.*, 2007; Molnar *et al.*, 2010; Dunoyer *et al.*, 2010a). An extensive silencing movement, called transitivity, can also occur through reiterated short-

distance cell-to-cell signalling events, involving secondary siRNAs (Himber *et al.*, 2003; fig.15).

The siRNAs implicated in the cell-to-cell movement can be converted into dsRNA by RDR6 and SDE3 in the presence of homologous transcripts as templates. The new synthesis of dsRNAs leads to the production of secondary siRNAs of 21 nt by DCL4 (Vaistij *et al.*, 2002). The biogenesis of secondary siRNAs of *Tobacco rattle virus* (TRV) involved the combined action of RDR1, RDR2 and RDR6 (Donaire *et al.*, 2008).

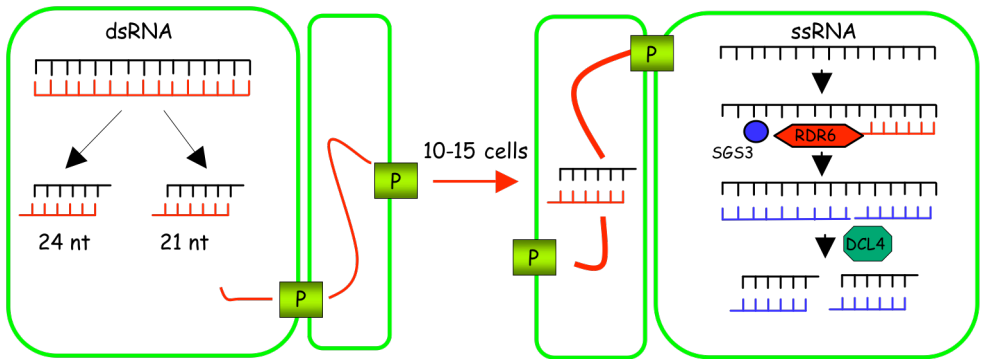


**Figure 15: A re-initiative model for cell-to-cell transgene silencing movement**
The short-range cell-to-cell movement can be initiated from one single cell and occurs over 10-15 cells by propagation of the siRNAs through plasmodesmata. The long-range movement is dependent upon RDR6 and SDE3, which use homologous transcripts as templates to produce new dsRNA. Then, this dsRNA molecule is processed by DCL4 into secondary siRNAs of 21 nt.

# INFECTION OF *NICOTIANA BENTHAMIANA* BY TWO ARMV ISOLATES AND RECOVEY PHENOMENON

# INTRODUCTION

Nepoviruses have been shown to induce in infected plants a phenomenon called “recovery”, i.e *Tomato black ring virus* (TBRV) and *Tomato ringspot virus* (ToRSV) (Ratcliff *et al.*, 1997; Jovel *et al.*, 2007; Siddiqui *et al.*, 2008). Recovery, which was first described by Wingard (1928), is characterized by an initial symptomatic infection followed by symptoms attenuation or elimination in the newly emerging leaves. Another characteristic is that the upper leaves of the recovered plants are resistant to a secondary infection with the same virus. However, the plant sap extracted from recovered leaves, when used as inoculum, is able to induce typical symptoms on young healthy plants (Wingard, 1928). Later, it was shown that the recovery phenomenon was accompanied by a reduction of the viral RNA level (Covey *et al.*, 1997; Ratcliff *et al.*, 1997, 1999). Additionally, the resistance to a secondary infection with the same virus was shown to be sequence-specific, suggesting that the RNA silencing, which is an antiviral defense mechanism activated by double stranded RNAs (Baulcombe, 2004, 2005; Voinnet, 2005), might be involved and responsible for the reduced virus titer.

Recently, several reports involving viruses from and within different virus families showed different behaviours in relation to recovery, revealing an unexpected degree of complexity for this phenomenon. For example, the *Malva vein clearing virus* (MVCV, *Potyvirus*) was absent from recovered tissues (Lunello *et al.*, 2009), whereas for geminiviruses, recovery was accompanied by a reduction of the viral DNA titers (Chellappan *et al.*, 2004; Rodriguez-Negrete *et al.*, 2009; Hagen *et al.*, 2008). At the level of the geminiviruses viral-derived small RNAs (vsRNAs), it seems to be no correlation between their accumulation and the symptomatology. Indeed, vsRNAs of the *African cassava mosaic virus* (ACMV, *Begomovirus*) was found at higher level in recovered leaves than in symptomatic leaves (Chellappan *et al.*, 2004), whereas the opposite situation was observed with the *Pepper golden mosaic virus* (PepGMV, *Begomovirus*) (Rodriguez-Negrete *et al.*, 2009) or the *Cucurbit leaf crumple virus* (CuLCrV, *Begomovirus*) (Hagen *et al.*, 2008). For nepoviruses, a reduction of viral RNA accumulation was observed in recovered leaves for TBRV (Ratcliff *et al.*, 1997) and *Tobacco ringspot virus* (TRSV, *Nepovirus*)(Siddiqui *et al.*, 2008). However, no commensurate viral RNA reduction was observed in recovered leaves from plants infected by ToRSV (Jovel *et al.*, 2007). Moreover, the lack of vsRNAs in recovered tissues of TRSV-infected plants correlated with the small amounts of genomic viral RNAs in these tissues (Siddiqui *et al.*, 2008).

In this part, we describe the establishment of infection and the establishment of the recovery phenomenon of the ArMV-NW and ArMV-Lv isolates on *Nicotiana benthamiana* and the role played by the recovery phenomenon in relation to a secondary infection with the homologous or a heterologous virus isolate. These results are also appended as an article entitled “Different establishment rates of infection and recovery in *N. benthamiana* between a mild and a virulent isolate of Arabis mosaic nepovirus” (Dupuis *et al.*, 2010) that will be submitted for publication.

# RESULTS

## I. ArMV-Lv isolate induces recovery in infected *Nicotiana benthamiana* plants

The recovery phenomenon for an ArMV infection was investigated in the host plant *Nicotiana benthamiana*, using in a first approach, the ArMV-Lv isolate, which induces severe symptoms on various plants. Plants, at the 3-4 leaves stage, were rub-inoculated with a sap prepared from *Chenopodium quinoa* leaves infected by ArMV-Lv.

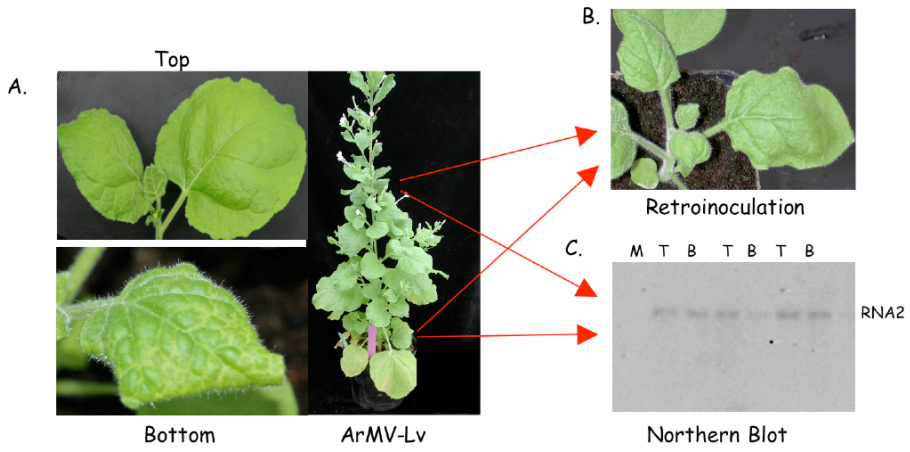


**Figure 1: Study of recovery phenomenon on *Nicotiana benthamiana* infected by ArMV-Lv**
A. Symptoms of *N. benthamiana* infected by ArMV-Lv after 4 weeks. Top corresponds to the new emerging leaves, which are symptomless and bottom, to the first systemic leaves that appeared after inoculation of the plant with ArMV and displayed a mosaic; B. Symptoms after retroinoculation of *N. benthamiana* with a sap of recovered or non-recovered leaves; C. Northern blot performed on total RNAs extracted from symptomatic (bottom, B), asymptomatic leaves (Top, T) and mock-inoculated plants (M) using as probe the radiolabelled MP cDNA. This experiment has been realized on three plants.

*N. benthamiana* plants infected with the ArMV-Lv isolate showed a severe mosaic on the first systemic leaves (bottom leaves) whereas the upper leaves (top leaves) did not show any symptoms (fig. 1A). These observations suggested that the recovery phenomenon took place.

Northern blots were performed with total RNAs extracted from these plants to determine the accumulation level of ArMV RNA2 in both symptomatic and recovered leaves, 4 weeks after inoculation (fig. 1C). This experiment was performed using a radioactive probe specific for the MP coding sequence, which is the most conserved sequence (97% homology) of the RNA genome of the different ArMV isolates. The results showed that the viral RNA could be detected in the symptomatic and the asymptomatic leaves and these leaves contained the same amounts of RNA2. Therefore, the

virus was present and it replicated at the same level in the symptomatic as well as in the asymptomatic leaves. The retro-inoculation of *N. benthamiana* plants with a sap prepared from these leaves (with or without symptoms) produced a similar phenotype; the first leaves displayed a mosaic whereas the upper leaves were symptomless (fig. 1B). In conclusion, the recovery was activated on *N. benthamiana* plants following the mechanical inoculation of the ArMV-Lv isolate and the virus present in the recovered leaves was still replicating and infectious upon retro-inoculation.

## II. Investigation of the recovery phenomenon

The recovery phenomenon was investigated in more details, at different times after inoculation (5, 7, 9, 11, 13, 16, 20 and 25 dpi) of *N. benthamiana* plants with ArMV-Lv, the symptoms displayed by the leaves and the viral titer were conducted. In parallel, we also studied the recovery phenomenon in *N. benthamiana* plants infected by the mild isolate ArMV-NW.

### 1. Symptoms development in *N. benthamiana* inoculated with ArMV-NW or ArMV-Lv

*N. benthamiana* plants were inoculated with extracts containing approximately the same amount of ArMV-Lv or ArMV-NW. During the course of the infection, symptoms could never be visualized neither in the inoculated leaves nor in systemic leaves in the plants inoculated with ArMV-NW. By contrast, a light mosaic was observed at 9 dpi in the systemic leaves of *N. benthamiana* plants infected by ArMV-Lv (fig. 2).

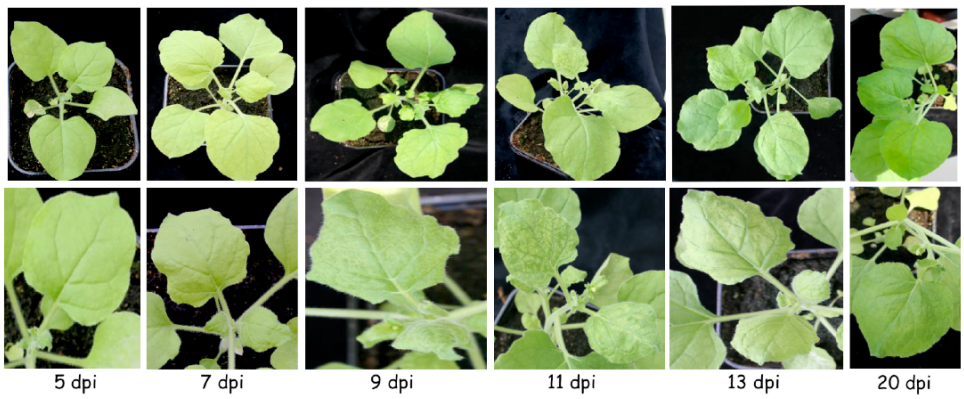


**Figure 2: Symptoms development on *N. benthamiana* plants infected by ArMV-Lv**

At 11 dpi, this mosaic became more pronounced on the systemic leaves but the new emerging leaves were symptom-free at 13 dpi. The latters were still symptomless at 16 and 20 dpi. A severe mosaic did also develop on ArMV-Lv inoculated leaves but strikingly these could not be seen before 16-20 dpi. In conclusion, the *N. benthamiana* plants are able to recover after infection by the aggressive Lv isolate of ArMV and apparently, the recovery phenomenon takes place, approximately

at 13 dpi. As the ArMV-NW isolate did not induce external foliar symptoms, we could not determine the time course of recovery and if it really occurred.

### 2. Titer of ArMV-NW and ArMV-Lv isolates in recovered *N. benthamiana* plants

The accumulation of ArMV RNA2 in systemic and inoculated leaves of *N. benthamiana* plants infected either by ArMV-NW and -Lv isolates was determined at different times after inoculation. Northern blots were performed with the probes corresponding to the 2A cDNA of ArMV-NW or Lv, respectively, labelled radioactively by random priming (fig. 3). The 2A coding sequence is the most divergent genomic sequence, 73% of identity between ArMV-NW and ArMV-Lv isolates.

In the inoculated leaves of *N. benthamiana*, the viral RNA2 of the NW and Lv isolates were detected at 5 dpi and at 7 dpi, respectively. The RNA2 of the NW and Lv isolates reached its maximal accumulation level at 16 dpi but the Lv isolate replicates faster in *N. benthamiana* plants than the NW isolate, as shown by the results of the northern blot (fig. 3). We can notice that the amount of RNA2 of the ArMV-NW isolate increased considerably within 3 days from 13 dpi. After 16 dpi, the quantity of RNA2 remained more or less stable for both isolates, however the RNA2 of ArMV-Lv accumulated at higher levels than its counterpart of ArMV-NW. In conclusion, our results suggest that the ArMV-Lv isolate accumulates in higher concentration in *N. benthamiana* than the NW isolate.

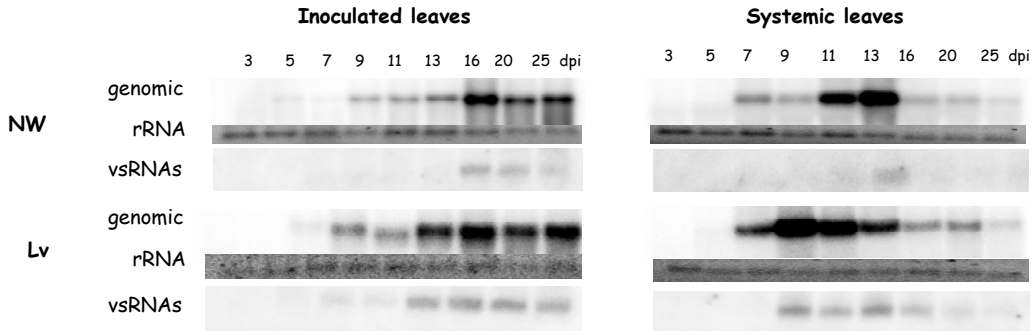


**Figure 3: Northern blots performed on RNA from inoculated and systemic leaves of *N. benthamiana* infected with ArMV-NW or -Lv isolates**

Total RNAs from inoculated and systemic leaves of *N. benthamiana* plants infected by ArMV-NW or -Lv were extracted and 5 μg were fractionated on denaturing gel. The probes used were the radiolabelled 2A cDNA of NW and Lv, respectively. The loading controls corresponding to the gels are shown below each northern blots.

Genomic: viral genomic RNA2, rRNA: ribosomal RNAs, vsRNA: viral-derived small interfering RNAs, dpi: days post-inoculation

Concerning the systemic leaves, RNA2 is detected at 7 dpi for NW and Lv, but the maximal level was obtained at 13 dpi for NW and at 9 dpi for Lv. For both isolates, the viral RNA2 content decreased, 2-3 days after it reached its maximum, at 16 dpi for NW and at 13 dpi for Lv but was still detectable at 25 dpi. Interestingly, even if the level of RNA accumulation was similar between these two isolates at the plateau of infection, plants infected by ArMV-NW did not display symptoms. This difference can be potentially explained by the drastic diminution of the quantity of RNA2 observed in systemic leaves of plants infected with NW whereas for Lv, this decrease was slower. This can reflect a difference in the efficiency of the suppressor of RNA silencing encoded by these two isolates of ArMV and/or in their silencing suppression strategy.

Taken together, these results strongly suggested that the recovery took place but both ArMV isolates were still present in the recovered leaves. The establishment of the infection in *N. benthamiana* is faster for the virulent isolate ArMV-Lv than for the milder isolate ArMV-NW. However, both are equally affected by the host antiviral RNA silencing once the recovery phenomenon has taken place in the plant. One can notice the correlation at 9, 11, 13 dpi between the symptoms severity and the quantity of RNA2 in the case of ArMV-Lv.

In order to determine if the decrease of the viral RNAs was due to the RNA silencing (see part RNA silencing and ArMV), we analyzed the accumulation of the viral-derived small RNAs (vsRNAs) for both isolates (fig. 3). Low molecular weight RNAs were fractionated on a polyacrylamide gel under denaturing conditions, transferred onto a membrane and hybridized with the 2A radiolabelled probes. The analysis of the vsRNAs showed that the accumulation levels of these RNAs correlated with those of the corresponding viral RNAs, suggesting that the vsRNAs derived from the degradation of the viral genomic RNAs, as expected due to the activation of the RNA silencing.

**3. Characterization of ArMV-derived small RNAs**

In order to map the ArMV-derived vsRNAs, which were detected in both the inoculated and systemic leaves of ArMV-infected *N. benthamiana* plants, approximatively 1 kbp-long fragments covering the entire genome of ArMV-NW or -Lv were amplified by RT-PCR, electrophoretically separated, and blotted. Small RNAs from ArMV-NW or -Lv infected *N. benthamiana* were purified, end-labelled with [$\gamma$-$^{32}$P] and used as probes.

The mapping of the ArMV-NW-derived vsRNAs gave the strongest signals of hybridization with the fragments corresponding to the RNA2, mainly the 5' non-coding region, 2A, MP and the CP genes, the signal being slightly weaker for the fragment corresponding to the C-terminus of the CP gene and the 3' non-coding region (fig. 4). The signals observed with the fragments corresponding to the RNA1 were generally weaker, in particular, those corresponding to the 5' and 3' terminal regions of this RNA. The difference between RNA1 and RNA2 could be explained by a difference of the ratio of these viral RNAs. Indeed, nepoviral RNA2 is replicated at a higher efficiency than RNA1. However, some regions on the RNA1 hybridized more strongly to the siRNAs than others, mainly

those coding for the 1B and the protease-polymerase for ArMV-NW, indicating that these regions are preferentially targets of RNA silencing machinery.

The mapping of the ArMV-Lv-derived vsRNAs gave also the strongest signals for the fragments corresponding to the 5' non-coding region, 2A and MP genes whereas the hybridization corresponding to the CP gene and 3' non-coding region fragments were weaker, in the range of those obtained for the fragments corresponding to the RNA1. The sequences coding for the 1B and the amino-terminal half of the polymerase genes for ArMV-Lv seem to be preferentially targeted for degradation.

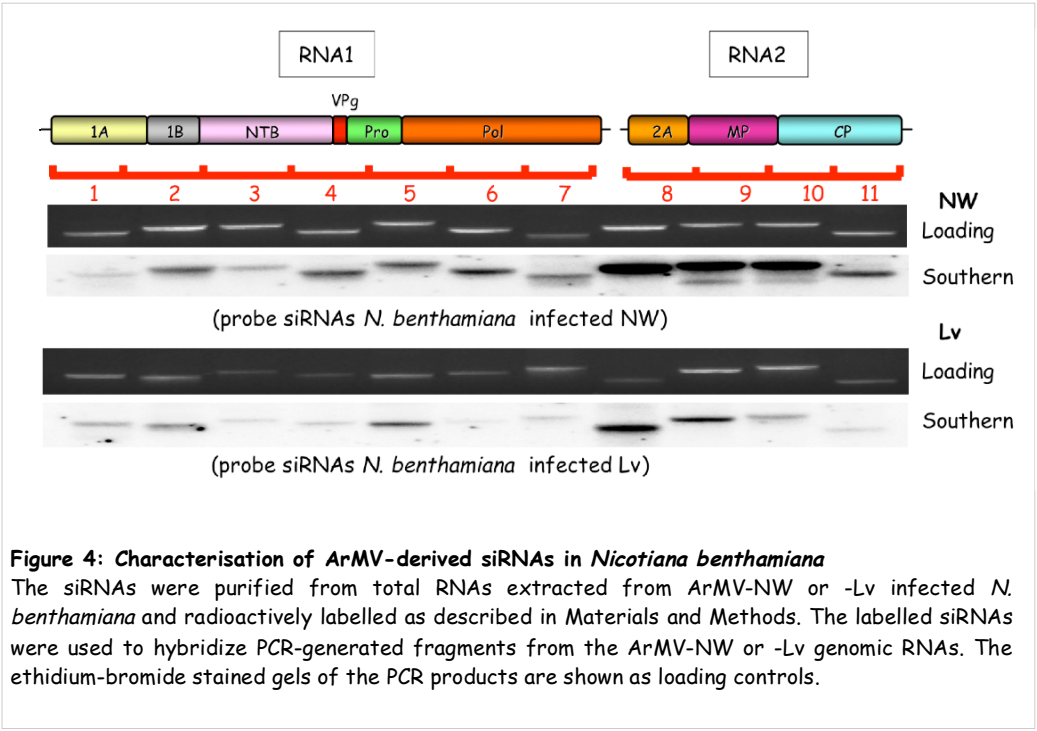


**Figure 4: Characterisation of ArMV-derived siRNAs in *Nicotiana benthamiana***
The siRNAs were purified from total RNAs extracted from ArMV-NW or -Lv infected *N. benthamiana* and radioactively labelled as described in Materials and Methods. The labelled siRNAs were used to hybridize PCR-generated fragments from the ArMV-NW or -Lv genomic RNAs. The ethidium-bromide stained gels of the PCR products are shown as loading controls.

## III. Immunity tests in *N. benthamiana* infected by ArMV-NW or -Lv isolates

It has been described concerning the recovery phenomenon that the plants preliminary infected with a virus are resistant to a second infection with the same virus. To determine if it is also the case for ArMV, we tested the ArMV-NW and ArMV-Lv isolates.

### 1. Homologous immunity assays

To test the homologous immunity, *N. benthamiana* plants were inoculated with ArMV-NW and ArMV-Lv, respectively. At 20 dpi, time required for recovery, the top leaves were inoculated with the same virus isolate.

We did not observe any symptom due to a secondary infection by ArMV-NW or ArMV-Lv, at 7 dpi and 16 dpi, neither on the inoculated leaves nor on the new emerging leaves of the recovered *N. benthamiana* plants inoculated either by ArMV-NW or -Lv, respectively (fig. 5A).

Northern blot analysis on total RNAs extracted from these leaves showed that the RNA2-NW was only detected at 13 dpi in the inoculated leaves whereas it was already present at 7 dpi in the systemic leaves, suggesting that at this time, the accumulation level of RNA2 in the inoculated leaves is more important that its degradation whereas in the systemic leaves, the RNA2-NW is targeted by RNA silencing. Indeed, the amount of RNA2 of ArMV-NW decreased at 9 dpi in the systemic leaves whereas this of ArMV-Lv was constant at the time of the infection in the inoculated and systemic leaves. Although, we reproduce a similar profile observed from a primary infection, the amounts of viral RNAs in inoculated and systemic leaves after the establishment of the recovery were lower to those observed for the primary infection, indicating that the plants were immune to a secondary infection by the same virus (Fig. 5B).

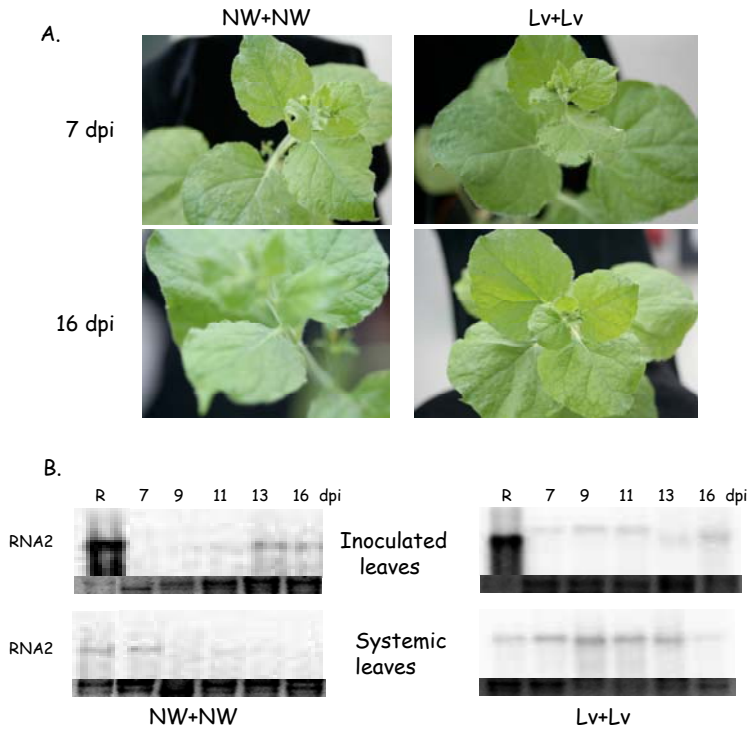


**Figure 5: Effect of a homologous secondary infection on ArMV-NW or –Lv recovered *Nicotiana benthamiana***

A. Phenotype of recovered *N. benthamiana* plants from ArMV-NW and ArMV-lv infection after a homologous secondary infection with the same ArMV isolates, at 7 and 16 days post inoculation (dpi); B. Total RNAs were extracted, blotted, and probed with the radiolabelled 2A cDNA of ArMV-NW or –Lv, respectively, as described in material and methods. The loading controls are shown below the northern blots. R: recovered state control, collected before the second inoculation.

### 2. Heterologous immunity assays

The isolates ArMV-NW and -Lv have 81% and 85% identity at the nucleotide level between their RNAs 1 and RNAs 2, respectively. To evaluate if the recovered *N. benthamiana* plants were also immune to a secondary infection with a heterologous isolate, ArMV-NW or Lv-recovered plants were inoculated with ArMV-Lv or -NW, respectively. Plants were monitored for symptoms development after the second inoculation, at 7 dpi and 16 dpi, and viral RNA accumulations were assessed by northern blots. For this, membranes were hybridized with probes corresponding to the highly variable 5' end region of the 2A gene of ArMV-NW or -Lv, respectively.

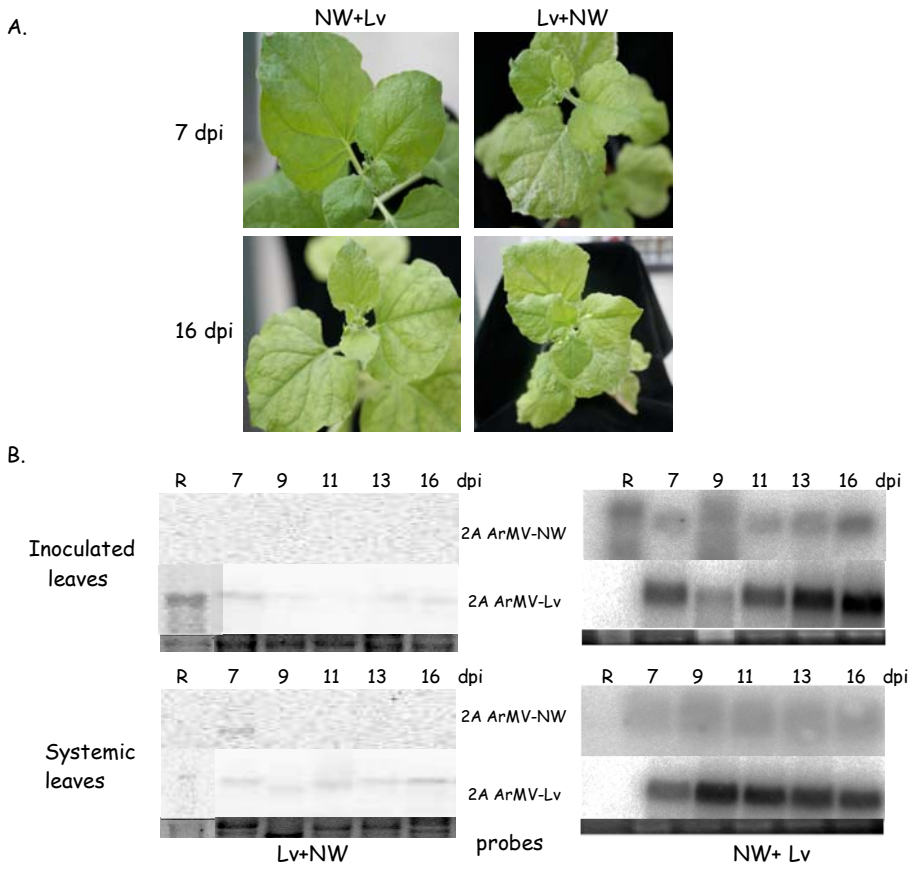


**Figure 6: Effect of a heterologous secondary infection on ArMV-NW or –Lv recovered *Nicotiana benthamiana***

A. Phenotype of recovered *N. benthamiana* plants from ArMV-NW and ArMV-Lv infection after a heterologous secondary infection with ArMV-Lv and -NW isolates, respectively, at 7 and 16 days post inoculation (dpi); B. Total RNAs were extracted, blotted, and probed with the radiolabelled 2A cDNA of ArMV-NW or –Lv, respectively. The loading controls are shown below the northern blots. R: recovered state control, collected before the second inoculation.

The plants were all symptomless whatever the combination of viruses used in this study (fig. 6A). The analysis by Northern blots showed that ArMV-NW was unable to accumulate in ArMV-Lv recovered leaves, suggesting that the ArMV-Lv recovered plants were immune to ArMV-NW. The ArMV-Lv RNAs accumulation was not affected by the secondary infection, remaining at the level of the recovered status (fig. 6 left). However, ArMV-Lv was able to replicate in ArMV-NW recovered leaves. In inoculated leaves, the ArMV-Lv RNAs were detected from 7 dpi, reaching a plateau around 13dpi. At the same time the accumulation of the ArMV-NW RNAs remained unchanged at the recovery level. In systemic leaves, ArMV-Lv RNAs were detected from 7 dpi, followed by an increase of the accumulation at 9 dpi, and slightly decreasing from 13 dpi. At the same time, low accumulations of ArMV-NW RNAs were detected from 7 dpi, which remained at the recovery level (fig. 6 right). These results suggest that the ArMV-NW induced recovery did not confer any immunity towards ArMV-Lv. Overall, the secondary infection of ArMV-Lv onto ArMV-NW recovered plants followed a pattern similar to a primary infection, except that no symptom could be seen in the secondary infection.

# DISCUSSION

### 1. Comparison of the establishment of infection and recovery between mild and virulent isolates of ArMV

The observation of different rates of infection, but similar rates of establishment of the recovery phenomenon between two isolates of ArMV confirms the complexity of the plant host-virus interactions. The differences observed in the infection rate are unlikely due to different virus titers of inoculum prepared from infected *C. quinoa* plants because both isolates are able to systemically infect with high titers, as confirmed by ELISA. We rather hypothesized that the mature proteins and the intermediate products of these isolates are expressed at different levels in the plants, due to the cleavage efficiency of the polyproteins of ArMV-NW and -Lv isolates by the viral-encoded protease. Wetzel *et al.*, (2008) have shown that the cleavage sites along the polyprotein P1 of ArMV-NW were hydrolyzed by the viral protease with different efficiencies and thus, a slower release of the mature proteins and/or their precursors could be responsible for the delayed infection, when compared to ArMV-Lv. However, we do not totally exclude that the interactions between the viral proteins and the putative host factors are more or less effective between depending on the ArMV isolates. The ArMV-Lv protein(s) could interfere earlier and/or more efficiently with the plant antiviral defense response, hence allowing that the infection starts earlier. Alternatively, the ArMV-Lv could encode a protein with a stronger suppressor of gene silencing activity than the homologue of ArMV-NW isolate, however, this suppressive activity is not very effective because the plants recovered. No suppressor of gene silencing has yet been identified for ArMV or for another nepoviruses. In both cases, recovered plants contained low amounts of virus, suggesting that the virus managed to a certain extent to evade the plant defense mechanisms. Similar results were obtained with TRSV (Siddiqui *et al.*, 2008) and TBRV (Ratcliff *et al.*, 1997) but they differed from those reported for ToRSV (Jovel *et al.*, 2007), where no commensurate reduction of viral RNAs was observed in recovered leaves. However, similar differences were also reported for viruses from the family *Geminiviridae* and their respective recovery patterns (Chellappan *et al.*, 2004; Carrillo-Tripp *et al.*, 2007; Hagen *et al.*, 2008; Rodriguez-Negrete *et al.*, 2009), revealing an even higher degree of complexity. Each virus or isolate of a virus seems to have its own particular pattern in its relationship to his host.

### 2. Implication of RNA silencing in recovery phenomenon

The accumulation of viral-derived siRNAs detected in infected plants correlated with the accumulation of viral genomic RNAs. This result was consistent with that observed with TRSV (Siddiqui *et al.*, 2008), for which siRNAs could not be detected in recovered tissues, which contain only small amounts of viral genomic RNAs.

The mapping of the ArMV-NW or -Lv -derived siRNAs showed a generally similar pattern to

the one described for ToRSV (Jovel *et al.*, 2007), the vast majority of these small RNAs generated by slicing from the RNAs 2. This could be explained by the fact that the RNA2 of ArMV are replicated at a higher level than RNA1 and consequently, it is present in larger amount than RNA1 in infected plants. No other particular stable secondary structures (Mfold) seem to exist neither in RNAs 1 nor in RNAs 2 that would preferentially promote the generation of siRNAs. Therefore, their distribution differed along RNAs 1 and the discrepancy observed between the RNAs 2-derived siRNAs of ArMV-NW and -Lv remains unclear. The 5' region of RNA2 seems to be a preferential target of RNA silencing. It would be interesting to sequence the siRNAs to identify more precisely which part of the RNA2 is targeted by RNA silencing. Indeed, it is interesting to see if it is the replication intermediate or the secondary motifs which trigger the establishment of the antiviral defense (Molnar *et al.*, 2005; Ho *et al.*, 2006).

### 3. Immunity and cross-protection approach

Cross-protection is an alternative approach of the use of nematicides to protect grapevine against the viruses. This strategy relies on the use of mild strains to protect plants from economic damage caused by closely related, severe virus strain (Yamaya *et al.*, 1988; 1989; Fuchs *et al.*, 1997; Lecoq *et al.*, 1998; Vigne *et al.*, 2009). In our case, the first inoculation with a mild strain seems to no protect the plants against a strain more, which is more virulent whereas the virulent strain does not permit the replication of the mild strain, as already suggested in the case of two strains of GFLV (Vuittenez *et al.*, 1976). Moreover, ArMV and GFLV are related serologically (Diaz and Harrison, 1963) and present some identical motifs in their sequences.

When secondary infections were performed with the homologous or heterologous isolates of ArMV, the expected immunity was observed, except when ArMV-Lv was inoculated onto ArMV-NW recovered plants. In this case, the kinetic of the infection, including the accumulation of the RNA2, was similar to that of a primary infection. However, ArMV-Lv did not produce any symptoms during the secondary infection. The identity levels between ArMV-NW RNAs 1 and 2 and the corresponding sequences of ArMV-Lv are 81% and 85% respectively, at the nucleotidic level. Stretches of 25-40 nucleotides with 100% identity were found throughout both genomic RNAs between the two isolates, indicating that the siRNA generated from a isolate should able to recognize and degrade the viral RNAs of the other isolate, as observed for ArMV-NW when inoculated onto ArMV-Lv recovered plants. It is unclear which viral or plant factor allowed ArMV-Lv to overcome or evade the ArMV-NW induced recovery, while however being unable to overcome or evade its own induced recovery. This question remains to be answered. It would also be interesting to perform experiments of immunity between the mild strain of ArMV-NW and GFLV and RpSRVS and the virulent strain of ArMV-Lv and GFLV and RpRSV. Indeed, GFLV and RpRSV are other viruses involved in the grapevine fanleaf disease.

Cross-protection has been reported for other nepoviruses i.e *Tomato black ring virus* strains in tobacco (Harrison, 1958), *Tomato ringspot virus* (Wingard, 1928), which protect also tobacco against *Cherry leaf roll virus* (Fulton, 1975). Huss *et al.* (1989) studied the cross-protection between ArMV and GFLV on *C. quinoa*. Plants infected with the mild isolates, GFLV-GH or ArMV-S, were partially protected against a second infection with the virulent strain GFLV-F13. The symptoms were less severe and the amount of capsid protein in the infected plants was reduced compared to *C. quinoa* plants primary inoculated with this virulent GFLV strain. By contrast, a primary inoculation with either GFLV-GH or ArMV-S had no effect on the virulent ArMV-862 strain. The effectiveness of cross-protection in naturally infected vineyards was recently tested by Komar *et al.* (2008). The incidence of challenge GFLV infection was significantly reduced in grapevines cross-protected with mild strains GFLV-GH and ArMV-Ta compared to control grapevines. Cross-protection is a complex mechanism implicated various virus-host interactions, which may differ depending on the viral strains, the hosts and possible the environmental conditions (temperature, light, moisture).

# RESEARCH OF A PUTATIVE SUPPRESSOR OF RNA SILENCING ENCODED BY ARMV

# INTRODUCTION

As RNA silencing in plants prevents virus accumulation, plant viruses have evolved various strategies to counteract this defense mechanism. These strategies are based on the expression of silencing suppressor proteins, called Viral Suppressors of RNA silencing (VSRs) (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). Moreover, there is a relation between RNA silencing suppression and the phenotypes observed during systemic infection by plant viruses. In fact, the majority of the silencing suppressors identified are pathogenicity factors encoded by the viruses that enhance the viral accumulation and pathogenicity (Pruss *et al.*, 1997; Voinnet *et al.*, 1999). It is hypothetized that VSRs contribute to the severity of viral symptoms, indirectly by facilitating virus accumulation and spreading and directly by modifying endogenous siRNA-regulated pathways.

These VSRs probably evolved independently in different virus groups because they are structurally diverse and there are no common sequence motifs. Moreover, these proteins appear to act against different stages of the RNA silencing mechanism (Voinnet *et al.*, 1999; Anandalakshmi *et al.*, 2000; Llave *et al.*, 2000; Mallory *et al.*, 2001; Diaz-Pendon *et al.*, 2007).

## I. Discovery of viral suppressors of RNA silencing

### 1. Synergistic disease

The synergistic viral disease occurs when two heterologous viruses coinfect the same host. This coinfection results in the accentuation of symptoms and accumulation of virus (Damirdagh and Ross, 1967).


**Figure 1: Synergistic disease in tobacco plants infected with *Potato virus X* (PVX) and *Potato virus Y* (PVY)** (Mlotshwa *et al.*, 2008)
Infection by PVX or PVY shows mild symptoms whereas plants infected by both viruses develop a severe necrosis in systemic infected leaves.

Many studies on the synergism involve a member of the potyvirus group of plant viruses (fig. 1) and led to the discovery that potyviral helper-component protease (Hc-Pro) encoded by *Potato virus Y* (PVY, *Potyvirus*) is a suppressor of RNA silencing (Anandalakshmi *et al.*, 1998; Ruiz *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). It was found that transgenic plants expressing the 5' proximal region of the *Tobacco etch virus* (TEV, *Potyvirus*) genome (termed the P1/Hc-Pro sequence) develop synergistic disease when infected with a broad range of plant viruses (Pruss *et al.*, 1997).

### 2. Pathogenicity determinants

As described above, the discovery that viruses encode silencing suppressors came from experiments performed to understand the phenomenon of synergism. The 2b protein of CMV was also identified as a silencing suppressor (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998). This viral protein also enhances the accumulation of PVX and accentuates the disease symptoms and when it is expressed from PVX vector, it suppresses PTGS, as Hc-Pro (Pruss *et al.*, 1997). A key to the identification of new silencing suppressors came from the observations that Hc-Pro and 2b had been previously characterized as pathogenicity determinants that means as factors that are not strictly required for viral replication but needed for efficient accumulation at the cellular and/or whole plant level. By extrapolation, many viral pathogenicity determinants would be identified as suppressors of gene silencing and more generally, many viruses would have the ability to suppress PTGS.

## II. Experimental strategies for the identification of silencing suppressors

Several methods have been used to identify plant viral suppressors of silencing (reviewed by Moissiard and Voinnet, 2004; Li and Ding, 2006). These methods are reported below.

### 1. *Agrobacterium*-mediated transient suppression assay

When a 35S-GFP transgene is agro-infiltrated into *N. benthamiana* plants that are already transformed with a GFP transgene, named 16C (Baulcombe *et al.*, 1998), the infiltrated patch appears bright green due to the transient GFP expression superimposed on fainter green fluorescence from the transgene (Voinnet *et al.*, 1998).

The GFP mRNA is detected at 2 days post-infiltration (dpi), but after a peack at 3 dpi, its level declines dramatically through 6 dpi (Johansen *et al.*, 2001). The decrease of the GFP mRNA is accompanied by the accumulation of GFP-specific short (21 nt) and long siRNAs (24 nt). Moreover, the infiltrated patch becomes uniformly red fluorescent (Voinnet *et al.*, 1998), indicating that the newly infiltrated transgene and the resident GFP transgene are locally silenced. This local silencing precedes "systemic silencing", in which GFP expression is suppressed in newly emerging, non-infiltrated leaves of the GFP transgenic plants (Voinnet *et al.*, 1998; 2005). Short-distance systemic

silencing leads to the development of a red ring, around the infiltration zone whereas long-distance systemic silencing results in the spreading of red areas in upper leaves (fig. 2).


**Figure 2: Initiation, propagation and completion of GFP systemic silencing in transgenic *N. benthamiana* 16C** (Voinnet *et al.*, 2005)
1. Following syringe-infiltration of *Agrobacterium tumefaciens* expressing GFP, the local silencing is characterized by the apparition of a red ring surrounding the infiltrated zone; 2. The silencing signal is propagated in the vein and in the lamina; 3. Silenced plant in which the GFP silencing invaded all tissues.

In the patch-test assay, the candidate suppressor protein is co-delivered to plants with the GFP reporter construct that triggers RNA silencing of the GFP transgene via *Agrobacterium tumefaciens*. The delivery by infiltration of this mix of recombinant *A. tumefaciens* allows the expression of both the silencing inducer and the putative suppressor in the same infiltration zone. The transgenic GFP mRNA is rapidly degraded in the absence of a silencing suppressor whereas its usually accumulates in the presence of a suppressor (Voinnet *et al.*, 2000; Llave *et al.*, 2000; Hamilton *et al.*, 2002; Tadeka *et al.*, 2002; Bucher *et al.*, 2003).

### 2. Heterologous expression assay and symptoms severity

Another procedure for the identification of a viral-encoded suppressor is the PVX-based heterologous expression (fig. 3A). In this experiment, the candidate protein is expressed from a viral vector containing the PVX. The identification of a suppressor protein is based on the synergism phenomenon that results in a dramatic increase of the symptoms.

### 3. Reversal of transgene-induced gene silencing

This experiment involves the recovery of the GFP expression in 16C *N. benthamiana* plants. In a first step, 16C plants are silenced by agroinfiltration of the GFP transgene (fig. 3B); the appearance

of red area in the upper leaves visualizes the establishment of the silencing. In a second step, a silenced plant is crossed with a transgenic plant that expresses the putative viral suppressor (Anandalakshmi *et al.*, 1998; Kasschau and Carrington, 1998). If the latter can revert the RNA silencing induced by the GFP transgene, green fluorescence spots will appear on the whole plant.

Another assay to identify a possible effect of the putative suppressor on the reversion of RNA silencing is to express the candidate protein from a heterologous viral vector, such as PVX. Then, the recombinant viral vector is inoculated onto the silenced plants (Pruss *et al.*, 1997). If the protein is a suppressor, GFP will be produced leading to green fluorescence when the plants are observed under UV light.

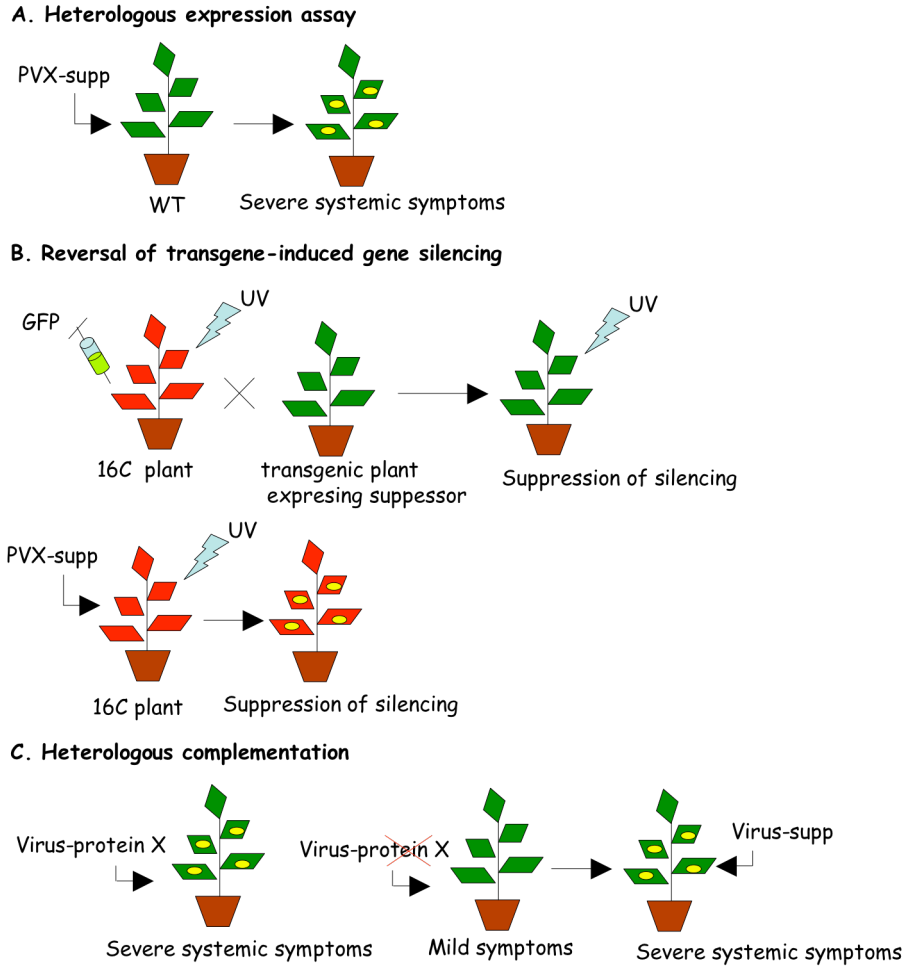


**Figure 3: Experimental strategies for preliminary characterization of silencing suppressors** (Moissard *et al.*, 2004)
PVX: *Potato virus X* given here as an example of heterologous viral vector; Supp: silencing suppressor; UV: ultraviolet illumination; GFP: *A. tumefaciens* strain in which the T-DNA has been modified to contain a transgene expressing GFP; Protein X: a protein characterized as pathogenicity determinant. The red colour corresponds to the establishment of GFP RNA silencing and the yellow spots to the GFP green fluorescence.

### 4. Heterologous complementation

In this system, the gene for the candidate silencing suppressor protein replaces a known suppressor of silencing in its viral context. If the candidate protein complements functionally the suppressor, it is considered as a silencing suppressor protein (Moissard *et al.*, 2004, fig. 3C).

### 5. Experiments to test the suppression activity on the systemic signal

One commonly assay is based on the co-infiltration of separate *A. tumefaciens* cultures harboring the putative suppressor and the GFP onto *N. benthamiana* (Guo and Ding, 2002; fig. 4).

In the absence of a functional suppressor, the silencing of the GFP expression occurs within 3 dpi and the signal is propagated in the plant. Therefore, the plant appears completely red under UV light. If the suppressor inactivates or blocks the spread of the signal of the RNA silencing, the expression level of GFP is stabilized beyond 7 dpi and the plant remains green fluorescent.

Another possibility to identify a suppressor affecting the systemic signal is the grafting assay. In this assay, transgenic rootstocks carry an inverted-repeat (IR) constructs, corresponding to the 5' portion (referred to as 'GF') of the GFP transgene while the recipient shoot tissues (scions), express the full-length GFP transgene. The establishment of the RNA silencing in the scion, characterized by the loss of the GFP expression, confirm the graft-transmission of systemic signal (Brosnan, 2007). The spread of the systemic signal is estimated by the accumulation of siRNAs corresponding to the GF part. It is also possible to study the transitivity process by monitoring the accumulation of the 3′ secondary siRNAs, using a probe corresponding to the P region of the GFP gene whereas the 5′ secondary siRNAs accumulation is determined, using a probe corresponding to the GF region (Zhang *et al.*, 2008).

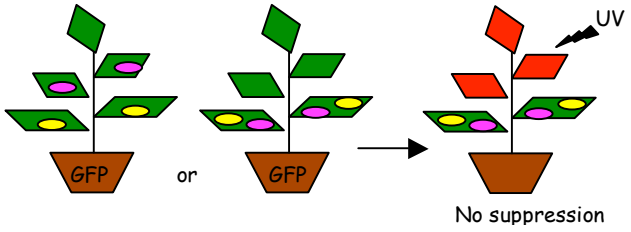


**Figure 4: Schematic representation of the effect of the suppressor on the GFP systemic signal**
The yellow ring corresponds to the agro-infiltrated GFP and the purple ring, to the agro-infiltrated suppressor. In the absence of suppression, the upper leaves appear red under UV lamp whereas in the presence of the suppressor, the expression of the GFP is maintained in the upper leaves.

## III. Properties and mode of action of some silencing suppressors

Viral suppressors of RNA silencing (VSRs) from different viruses do not block the same stage in the silencing process (Pantaleo *et al.*, 2007; Voinnet, 2005). Many of the VSRs are RNA binding proteins and thus, they can bind long dsRNAs as well as siRNAs while other suppressors block more downstream stages in silencing by targeting Argonaute proteins. VSRs may also interfere with the silencing signal that moves between cells and through the phloem of the plant (Palauqui *et al.*, 1997; Voinnet *et al.*, 1997) or directly either with the effector proteins involved in the RNA silencing pathway or with the products generated by these effectors. Several studies have shown that transgenic expression of silencing suppressors can alter the accumulation and/or the function of miRNAs, leading to developmental abnormalities related to the loss of function of miRNAs. Indeed, some of the symptoms resulting from virus infection are probably the consequence of perturbation of the miRNA pathway.

In this following part, I present the mode of action of some VSRs to illustrate the diversity and therefore, the difficulty to define a putative suppressor.

### 1. Suppression by dsRNA binding activity

#### 1.1. Hc-Pro protein

The RNA genome of potyviruses is translated into a polyprotein, which is processed into eleven mature proteins by three virus-encoded proteinases: P1-Pro, Hc-Pro and NIa (fig. 5).

The helper component-proteinase (Hc-Pro), encoded by *Potato virus Y* (PVY, *Potyvirus*), is a multifunctional protein required both for the maintenance of genome replication, aphid-mediated transmission, cell-to-cell and long-distance movement through the plant and polyprotein processing (Maia *et al.*, 1996). It was the first identified suppressor of RNA silencing. P1/Hc-Pro has been shown to enhance the replication and the pathogenicity of a broad range of heterologous plant viruses (Pruss *et al.*, 1997; Kasschau *et al.*, 1997; 1998).

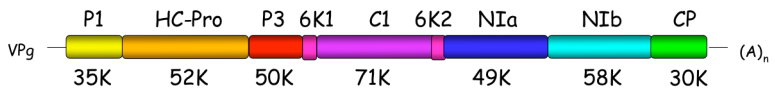


**Figure 20: Genomic organization of the potyvirus genome** (9.7 kb)
Coloured boxes depict the mature proteins obtained upon cis and trans cleavages of the polyprotein.

Hc-Pro suppresses both transgene- and virus-induced silencing (Anandalaskshmi *et al.*, 1998; Kasschau *et al.*, 1998; Brigneti *et al.*, 1998); it interferes with the accumulation of primary and secondary siRNAs by preventing the degradation of the mRNA and dsRNA (Mallory *et al.*, 2002;

Kasschau *et al.*, 2003; Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Mlotshwa *et al.*, 2005; Moissiard *et al.*, 2007). Indeed, Hc-Pro binds siRNAs and dsRNAs probably by forming head-to-tail homodimers that sequester siRNA duplexes and prevent them from entering the RISC (Lakatos *et al.*, 2006; Merai *et al.*, 2006). Some studies showed that it affects also the miRNA accumulation by its RNA binding capacity, resulting in the inhibition of the miRNA-mediated cleavage of target mRNAs and induction of developmental defects in *Arabidopsis* (Kasschau *et al.*, 2003; Chapman *et al.*, 2004, Dunoyer *et al.*, 2004).

Moreover, Hc-Pro interacts with a calmodulin-related-protein, called rgsCaM (regulator of gene-silencing-calmodulin-like protein), and activates its expression. As, rgsCaM may act as an endogenous suppressor of silencing *via* a calcium-dependent regulatory pathway, it was suggested that the suppression of the PTGS by Hc-Pro could be due to the amplification of this suppressor (Anandalakshmi *et al.*, 2008).

### 1.2. P19 protein

The P19 protein (fig. 6A), encoded by the *Tomato bushy stunt virus* (TBSV, *Tombusvirus*), is a symptom determinant (Dalmay *et al.*, 1993), an elicitor of the hypersensitive response on *N. tabacum* and it induces a systemic necrosis in *N. benthamiana* (Scholthof *et al.*, 1995a). Later, it was demonstrated that it suppresses RNA silencing in patch-test assays (Voinnet *et al.*, 1999) and prevents the accumulation of GFP specific short and long siRNAs (Hamilton *et al.*, 2002; Silhavy *et al.*, 2002; Takeda *et al.*, 2002; Voinnet *et al.*, 2003) in transgenic plants.

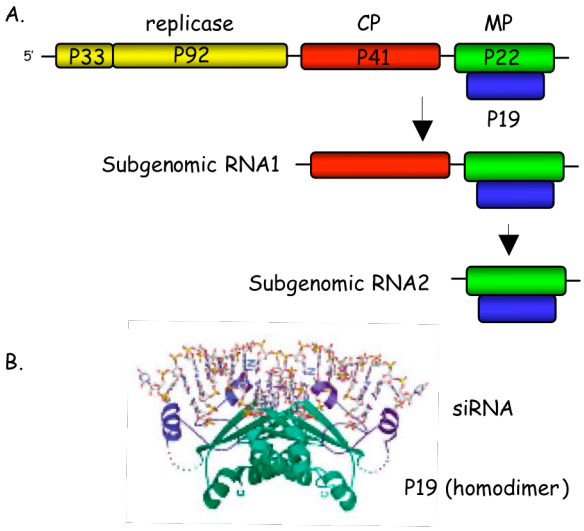


**Figure 6: P19, VSR of *Tomato bushy stunt virus* (TBSV)**
A. Organization of the genomic and subgenomic RNAs of TBSV (4.7 kb). The boxes represent the mature proteins; B. Representation of P19/siRNA complex (Vargason *et al.*, 2003).

P19 forms as Hc-Pro a head to tail homodimers by interaction of tryptophan residues from each monomer and to the formation of α-helix brackets that surround the ends of the siRNA base-paired region (Vargason *et al.*, 2003; Ye *et al.*, 2003, for review, Scholthof *et al.*, 2006; fig. 6B). Moreover, both miRNA and its complementary sequence (miRNA*) accumulate in transgenic arabidopsis expressing P19 (Chapman *et al.*, 2004"), whereas in wild type plants miRNA* is undetectable (Dunoyer *et al.*, 2004), leading to the conclusion that P19 prevents the incorporation of miRNAs and siRNAs into RISC (Chapman *et al.*, 2004; Li *et al.*, 2004).

### 1.3. P21 protein

*Beet yellows virus* (BYV, *Closterovirus*) encodes P21 (fig. 7A) that suppresses RNA silencing in an IR-PTGS assay (Reed *et al.*, 2003).

It was demonstrated that P21 binds siRNA and miRNA duplexes *in vitro* and in transgenic plants (Chapman *et al.*, 2004; Merai *et al.*, 2006; Lakatos *et al.*, 2006; Yu *et al.*, 2006). The crystal structure showed that P21 forms an octameric ring whose inner surface might be involved in RNA binding (Ye and Patel, 2005; fig. 7B). It inhibits initiation of RISC assembly by siRNA sequestration but cannot impair preassembled RISC activity (Lakatos *et al.*, 2006). It was also suggested that P21 may compete with HEN1 for miRNA/miRNA* duplex substrates.

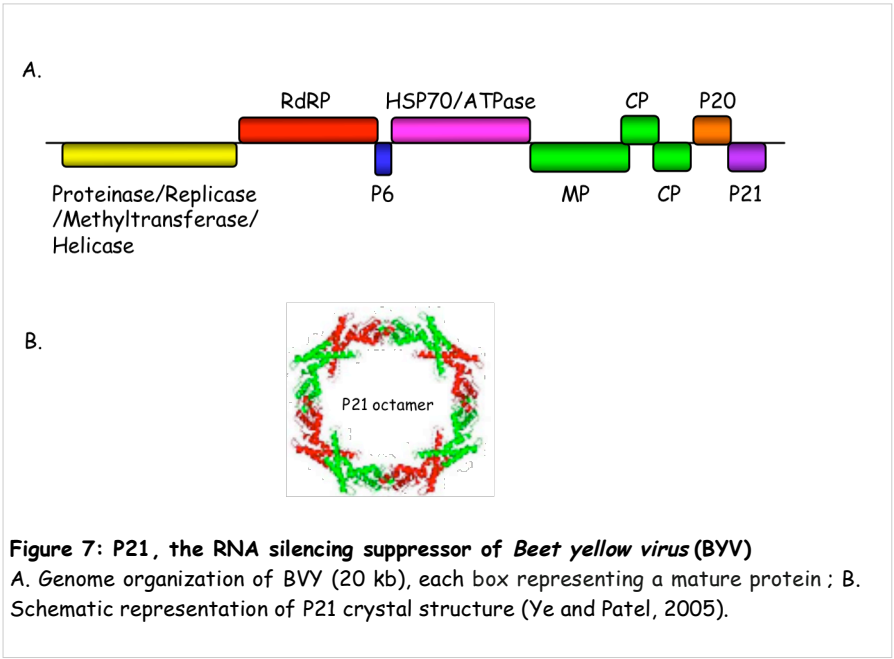


**Figure 7: P21, the RNA silencing suppressor of *Beet yellow virus* (BYV)**
A. Genome organization of BVY (20 kb), each box representing a mature protein ; B. Schematic representation of P21 crystal structure (Ye and Patel, 2005).

### 1.4. P15 protein

The P15 protein of *Peanut clump virus* (PCV, *Pecluvirus*) is a small cysteine-rich protein. The depletion of this protein results in a significant decrease in accumulation of progeny PCV RNAs. Because the protein does not co-localize with the sites of viral replication, it was postulated that its

indirect effect on PCV accumulation could result from suppression of a host defense system (Dunoyer *et al.*, 2001). This suppression activity needs the coiled-coil-mediated dimerization of P15 (Dunoyer et *al.*, 2002). Recently, it was shown that P15 sequester siRNAs and miRNAs, as described for P19 (Merai *et al.*, 2006; Wadsworth; oral communication).

## 2. Suppression of RNA silencing by inhibiting DCL function and/or Agornaute activity

### 2.1. P38 protein

The suppressor P38 (capsid protein) of *Turnip crinckle virus* (TCV, *Carmovirus*) has several important roles in virus infection (fig. 8). It is needed for systemic movement and cell-to-cell movement of the virus in *N. benthamiana* (Hacker *et al.*, 1992; Li *et al.*, 1998). It also interacts with a transcription factor of the NAC family that results in resistance response in Arabidopsis (Ren *et al.*, 2000; Kachroo *et al.*, 2000).

P38 is a strong silencing suppressor of PTGS; it blocks both local and systemic silencing preventing the accumulation of detectable levels of siRNAs in infiltrated leaves (Qu *et al.*, 2003; Thomas *et al.*, 2003). P38 was shown to inhibit DCL4 function, using plant *dcl*-mutants (Deleris *et al.*, 2006). P38 mimics host-encoded glycine/tryptophane (GW)-containing proteins that normally are required for RISC assembly and/or function (El-shami *et al.*, 2007), preventing by this study, the incorporation of the siRNAs and miRNAs into RISC. It has also the capacity to interfere with RNA silencing by binding AGO1 (Azevedo *et al.*, 2010).

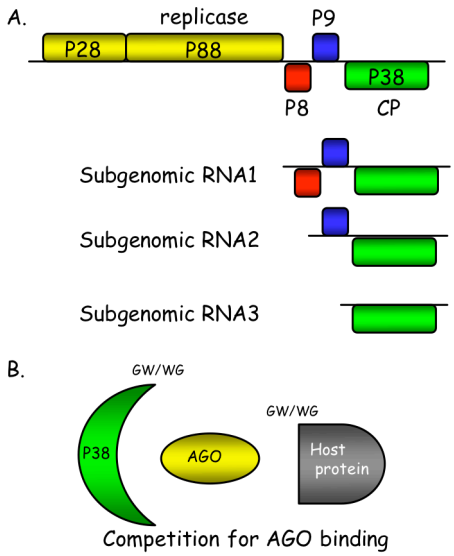


**Figure 8: P38 of *Turnip crinckle virus* (TCV)**
A. Genomic organization of TCV, each box representing a mature protein; B. The motif GW/WG of P38 mimics cellular GW/WG repeats contained in host proteins, required for RISC assembly and/or function and thus, blocks AGO (Jin *et al.*, 2010).

### 2.2. CMV 2b protein

The 2b protein of *Cucumber mosaic virus* (CMV, *Cucumovirus*) suppresses RNA silencing at different levels. It could prevent intercellular spread of RNA silencing signals by reducing the production RDR-dependent siRNAs (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Waterhouse *et al.*, 2001; Guo *et al.*, 2002). Indeed, it was demonstrated that 2b protein could directly bind to sRNAs, in particular, siRNAs (Goto *et al.*, 2007). It also interferes with the slicer activity of AGO1 and AGO4 by binding at the interface between the PAZ-containing module and a part of the PIWI domain of the AGO1 (Zhang *et al.*, 2006; Ruiz *et al.*, 2007; Gonzalez *et al.*, 2010). CMV 2b protein, which has a nuclear localization, is also able to prevent methylation of transgene DNA (Lucy *et al.*, 2000; Dalmay *et al.*, 2000).

### 2.3. P0 protein

The P0 protein of *Beet western yellows virus* (BWYV, *Polerovirus*, fig. 9A), strongly inhibits intracellular RNA silencing but has no effect on the propagation of the silenced state outside the zone of its initiation (Pfeffer *et al.*, 2002). P0 suppresses RNA silencing by preventing RISC assembly and by triggering the degradation of the AGO1 protein (Baumberger *et al.*, 2007; Csorba *et al.*, 2010).

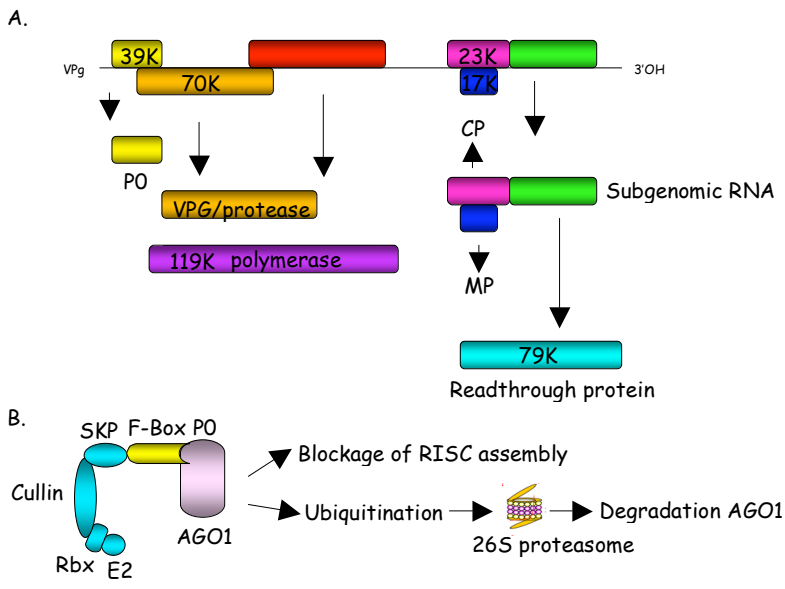


**Figure 9: P0 of poleroviruses**
A. Organization of the poleroviruses genomic and sug-genomic RNAs; B. RNA silencing suppressor activity of P0 leading to the degradation of AGO1 by the proteasome (Bartolamiol *et al.*, 2008).

Indeed, P0 has a F-box-like domain, which permits the interaction with *Arabidopsis* kinase-related protein 1 (SKP1) orthologs, ASK1 and ASK2 (Pazhouhandeh *et al.*, 2006), which are the components of the SKP1-Cullin-F box (SCF, also know as SCF ubiquitin-ligase complex) family of E3 ubiquitin ligases. P0 acts as an F-box protein in an SCF complex, interacts with AGO1 and triggers its degradation by the 26S proteosome (fig. 9B) (Bortolamiol *et al.*, 2007; 2008; Csorba *et al.*, 2010).

## 3. Other suppressor activities

### 3.1. P25 protein

The movement protein P25 encoded by the first gene of the "triple gene block" of *Potato virus* X (PVX, *Potexvirus*) is an RNA helicase (Kalinima *et al.*, 2002). It suppresses the systemic silencing signal but not the local silencing triggered by virus replication (Voinnet *et al.*, 2000, Hamilton *et al.*, 2002).

By contrast to HC-Pro, it appears that P25 does not reverse an established RNA silencing (Brigneti *et al.*, 1998). Furthermore, it does not prevent DNA methylation of the 35S-GUS transgene in the tobacco host (Mallory *et al.*, 2003).

### 3.2. Combination of viral proteins

The *Citrus tristeza virus* (CTV, *Closterovirus*) genomes encodes three suppressors, P23, P20 and the capsid protein (CP), which exhibit distinct features in silencing suppression (Lu *et al.*, 2004). P23 suppresses the intracellular silencing but does not prevents neither intercellular silencing nor DNA methylation of the target transgene whereas P20 and CP proteins interfere with the intercellular silencing (Guo *et al.*, 2002).

### 3.3. RNA as a silencing suppressor

The suppression activity is usually associated with viral-encoded proteins. However, it was demonstrated that the RNA of *Red clover necrotic mosais virus* (RCNMV, *Dianthovirus*) can also have RNA silencing suppressor activity.

In a transient assay, RCNMV interferes with the accumulation of siRNAs generated from a hairpin and with miRNA biogenesis. However, it was demonstrated that the viral proteins encoded by RNA1 of the RCNMV, P27, RNA polymerase and CP, were not sufficient to suppress sense-transgene mediated RNAi (S-RNAi) (Takeda *et al.*, 2005). RNA1 was identified as the essential component for suppressing the S-RNAi suggesting a strong link between the viral replication machinery and the RNA silencing machinery. A possible mechanism for RNAi suppression through RNA replication is the recruitement and the sequestration of the host proteins involved both in the replication of positive-strand RNA viruses and RNA silencing machinery. The suppression activity of RNA1 is enhanced by the presence of the movement protein encoded by RNA2 (Powers *et al.*, 2008).

# RESULTS

The ArMV-NW and ArMV-Lv isolates infect *N. benthamiana* and *C. quinoa* plants. The former most often induces no symptom whereas the Lv isolate causes a mosaic or a necrosis on these host plants, respectively (see part symptom determinants and ArMV). When we investigated the recovery phenomenon, we observed that the amounts of viral RNAs drastically decreased in the course of the infection but the virus was never completely eliminated (see part recovery phenomenon and ArMV), suggesting that these ArMV isolates have developed a counter-defensive strategy, which is more or less adapted against the antiviral RNA silencing response.

Viruses are both the inducers and the targets of RNA silencing. Therefore, they evolved to produce one or several proteins, which are involved in the suppression of RNA silencing. It is generally accepted that all plant viruses have at least one suppressor of RNA silencing, named VSR for Viral Suppressor of RNA silencing, which can interfere at different levels in RNA silencing (for review, Wadsworth and Dunoyer, 2009). They totally differ by their structures and their functions (movement protein, replicase...). This diversity renders particularly difficult to identify the VSR of a defined virus species. Nonetheless, the VSR are almost pathogenic factors responsible for the induction of symptoms in infected plants (Brignetti *et al.*, 1998) suggesting that the movement and 2A proteins of ArMV might be VSRs as they seem to be involved in the expression of symptoms in *C. quinoa*.

Two main strategies are frequently used to identify VSR(s) (Moissard and Voinnet, 2004), the agrobacterium-mediated transient suppression assay and the expression of the putative suppressor from a recombinant viral vector (PVX vector). We applied these techniques to characterize the VSR(s) encoded by ArMV using wild-type *N. benthamiana* and the GFP transgenic *N. benthamiana* plants, called 16C (provided by D. Baulcombe, Plant breeding Institut, Cambridge, UK).

### 1. Replication of ArMV-NW on DCL-deficient Arabidopsis plants

The Dicer-like proteins process dsRNAs into specifically sized siRNAs. DCL4 catalyzes the formation of 21 nucleotides (nt) long siRNAs from viral double-stranded RNA (dsRNA) upon infection with RNA and DNA viruses (Xie *et al.*, 2004). In absence of functional DCL4, 22 nt and 24 nt-long siRNAs are produced from viral dsRNA molecules by DCL2 and DCL3, respectively (Bouché *et al.*, 2006, Deleris *et al.*, 2006; Fusaro *et al.*, 2006, Diaz-Pendon *et al.*, 2007). To identify if one or several DCL(s) are involved in antiviral defense against ArMV, we studied the replication of ArMV-NW in *A. thaliana* (ecotype Col 0*)* mutants deficient in one, two or three DCLs: *dcl2*, *dcl3*, *dcl4*, *dcl2-3*, *dcl3-4*, *dcl2-4*, *dcl2-3-4* mutants. These mutants were available in the laboratory of Olivier Voinnet (IBMP, Strasbourg).

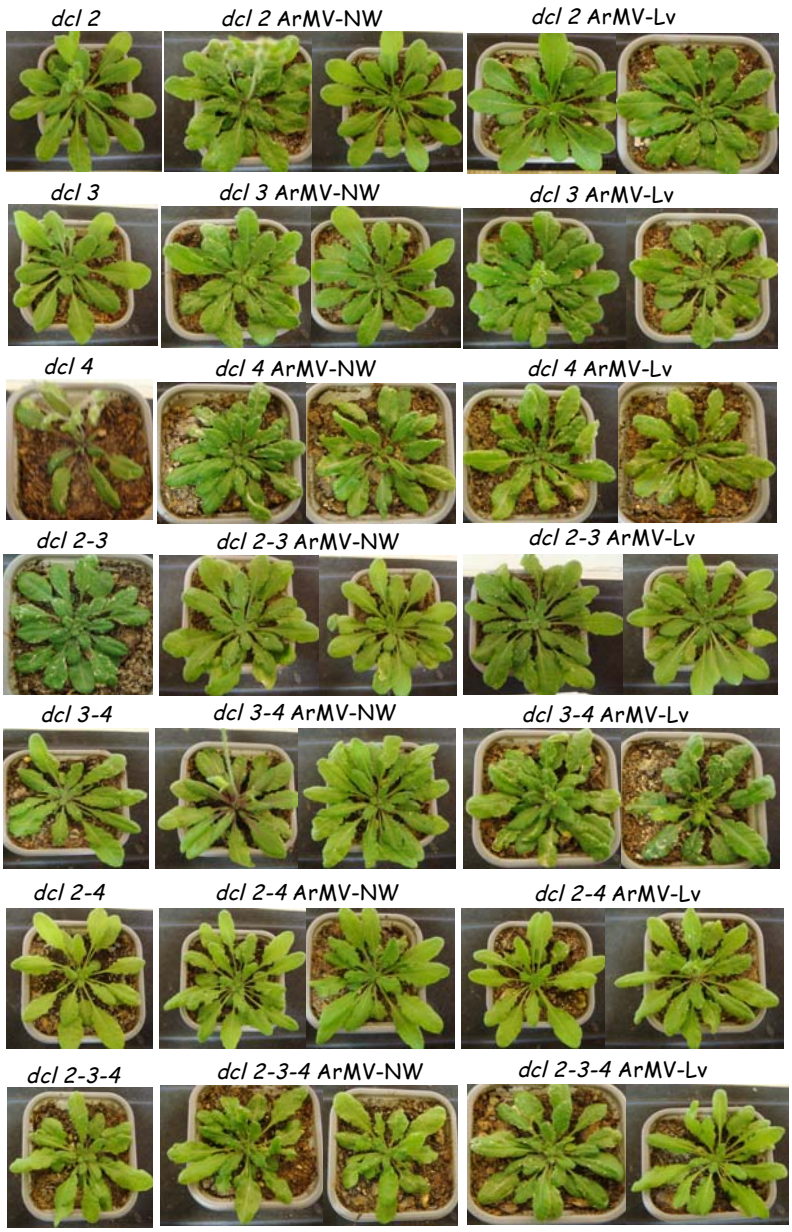


**Figure 10: Dicer-deficient plants mock-inoculated and infected with ArMV-NW or -Lv isolates**
The different mutants of Dicer (dcl) in *Arabidopsis thaliana* plants *dcl 2*, *dcl 3*, *dcl 4*, *dcl 2-3*, *dcl 3-4*, *dcl 2-4*, *dcl 2-3-4* were inoculated with the ArMV-NW and -Lv isolates and the symptoms severity were observed at 15 dpi.

The *Arabidopsis* mutants and wild-type plants were inoculated with a sap obtained from *C. quinoa* plants infected with ArMV-NW or -Lv isolates, at 7 dpi. The inoculated leaves of wild-type *Arabidopsis* plants showed curling and mosaic but the systemic leaves were free of symptom, at 15 dpi. The same symptoms were observed on DCL-deficient plants infected either by ArMV-NW or -Lv (fig. 10).

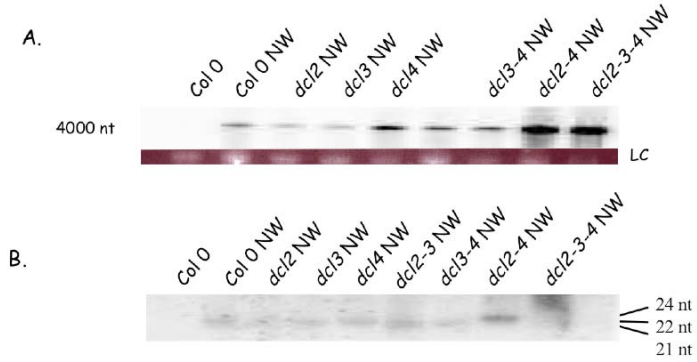


**Figure 11: Analysis by northern blots of the amount of RNA2 and viral-derived siRNAs (vsRNAs) in systemic leaves of DCL-deficient *Arabidopsis* plants infected by ArMV-NW, at 15 days post-inoculation**

A. Total RNAs from mock-inoculated and infected systemic leaves were extracted, fractionated (5 μg) on a denaturing agarose gel (1.5%) and probed with a radiolabelled NW-2A cDNA. The loading control (LC) is shown below the autoradiograph; B. Class of vsRNAs generated in mock-inoculated or ArMV-NW infected Dicer-deficient Arabidopsis plants. 20 μg of total RNAs was fractionated on polyacrylamide-urea gel and probed with the radiolabelled NW-2A cDNA. The analysis of the hybridization was made by phosphoimager after overnight exposure.

Analysis of the RNA2 content of ArMV by northern blots, using a 2A specific radiolabelled cDNA as probe, showed that wild-type plants infected with ArMV-NW contained reduced levels of RNA2. Almost, the same amount of RNA2 was detected in *dcl2* and *dcl3 Arabidopsis* mutants whereas about two fold higher levels of RNA2 were found in *dcl4*, *dcl2-3* and *dcl3-4* mutants. RNA2 accumulated at high levels in *dcl2-4* and *dcl2-3-4 Arabidopsis* plants compared to the single or other double mutants and to wild-type *Arabidopsis* (fig. 11A). The same results were obtained with plants infected with the ArMV-Lv isolate. These results indicate, as described for other plant RNA viruses (Wadsworth and Dunoyer, 2009), that DCL4 is the main effector protein implicated in the defense against ArMV infection. However, DCL2 also cleaves ArMV RNAs since RNA2 accumulated in higher amount in *dcl2-4 Arabidopsis* plants than in *dcl4 Arabidopsis* plants.

The involvement of DCL4 and DCL2 in the cleavage of the genomic RNAs of ArMV-NW, was confirmed by the detection of 21 nt and 22 nt-long viral-derived siRNAs (vsRNAs) in the mutant

plants deficient in DCL2 and DCL4, respectively (fig. 11B). In *dcl2*, *dcl3* and *dcl2-3* mutants and in wild-type infected plants, we found vsRNAs of 21 nt, as expected whereas in *dcl4* and *dcl3-4* mutants, we detected 22 nt vsRNAs, demonstrating that DCL2 can rescue compromised DCL4 activity. Analysis of small RNAs in the infected *dcl2-4* mutant revealed an accumulation of 24 nt vsRNAs, which are DCL3-dependent. However, the cleavage of the ArMV RNAs by DCL3 is not very efficient as shown by the presence of similar amounts of RNA2 in *dcl2-4* mutant and in the triple-mutant *dcl2-3-4*. No vsRNAs could be detected in the *dcl2-3-4* triple mutant, suggesting that DCL1 is not involved in the antiviral defense against ArMV.

In conclusion, DCL4 and DCL2 are involved in the antiviral defense against ArMV-NW in *A. thaliana* and DCL4 is the mainly dicer. DCL3 may also contribute to the immunity against ArMV because vsRNAs of 24 nt were detected in plants containing inactivated DCL4 and DCL2 proteins.

**2. Effect of ArMV-NW and -Lv on the accumulation of RNA silencing effectors**

Besides DCLs, argonaute proteins (AGOs), dsRNA binding proteins (DRBs) and cellular RNA-dependent RNA polymerases (RDRs) are also core components of the plant RNA silencing machinery involved in antiviral defense (Ruiz-Ferrer and Voinnet, 2009). In *A. thaliana*, there are 4 DCLs, 5 DRBs, 10 AGOs and 6 RDRs. We determined by western blots if the infection of *Arabidopsis* plants with ArMV-NW or ArMV-Lv isolates modifies the level of DCL1 (~190 kDa), DCL3 (~150 kDa), DCL4 (~170 kDa), DRB4 (~50 kDa) and AGO1 (~130 kDa); specific antibodies raised against these proteins were available at IBMP. We compared the amounts of these components in mock-inoculated and ArMV-infected *Arabidopsis* plants, at 15 dpi. For this study, we used extracts from buds because these organs are enriched in RNA silencing components (Dunoyer *et al.*, 2007). Proteins were separated on 6% or 12% polyacrylamide gels, under denaturing conditions.

As shown in figure 12, plants infected either with ArMV-NW or ArMV-Lv and mock-inoculated plants contained the same amounts of DCL1 and DCL3. It was necessary to load high amounts of plant extracts, at least 300 μg, to immunodetect DCL1 suggesting that the latter is present at a low level in buds whereas 100 μg were sufficient to detect the other components.

The level of AGO1, the component responsible for the slicer activity in RISC, was slightly higher in ArMV-infected *Arabidopsis* plants, compared to healthy plants. By contrast, ArMV-infected plants contained large amounts of DRB4, the co-factor of DCL4, in particular in plants infected with the NW isolate. Unfortunately, we were unable to properly visualize DCL4 because the antibodies cross-reacted with many other cellular proteins. Nevertheless, we can conclude from these results that the replication of ArMV-NW and -Lv in *Arabidopsis* plants had not influence on the expression of DCL1 and DCL3. By contrast, the expression of DRB4 is enhanced in the infected plants, in agreement with the fact that DCL4 is the main effector protein in the defense against ArMV and viruses, in general.

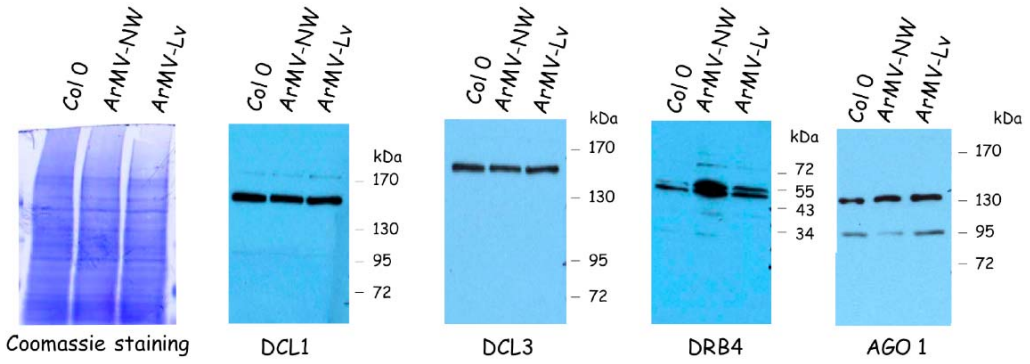


**Figure 12: Detection by westen blots of effectors involved in RNA silencing, in buds of mock-inoculated and ArMV-infected of *A. thaliana* Col 0 plants**
Protein extracts from buds (100 µg or 300 µg) were fractionated on a polyacrylamide gel (6% or 12 %) in the presence of 0.1% SDS and after transfer onto a membrane, detected with antibodies against DCL1, DCL3, DRB4 and AG01, respectively. The gel stained with Coomassie blue is shown at the left.

## 3. Study of the effect of ArMV-NW and -Lv isolates on GFP silencing in the 16C transgenic *N. benthamiana* plants

### 3.1. Effect of ArMV replication on the establishment of the GFP silencing

Transgenic *N. benthamiana* plants, which constitutively express the green fluorescent protein (GFP) under the control of the 35S promoter, called 16C, are frequently used to characterize the RNA silencing suppressor activity of viral proteins (Brignetti *et al.*, 1998). In this system, RNA silencing is initiated by the introduction in this transgenic plant of a replicon containing the homologous GFP gene. This leads to the post-transcriptional gene silencing (PTGS) of the GFP genes and the plant becomes red under ultraviolet (UV) light due to the fluorescence of the chlorophyll. If the 16C plant is co-infiltrated with the GFP replicon and a vector coding for a suppressor of RNA silencing, the plant remains green fluorescent due to the inhibition of the RNA silencing pathway.

We have seen that ArMV-NW induces no symptom on *N. benthamiana* plants although it replicates with the same efficiency as other ArMV isolates whereas the ArMV-Lv isolate causes a mosaic on the first systemic leaves, at 7 dpi but the emerging new leaves remain symptomless; this is the recovery phenomenon.

To determine if ArMV suppresses RNA silencing, 16C transgenic *N. benthamiana* plants were inoculated with a sap obtained from *C. quinoa* leaves infected with ArMV-NW or -Lv isolates. At 7 dpi, the GFP silencing was induced in the ArMV-infected *Nicotiana* plants with the hypervirulent strain GV3101 of *A. tumefaciens* containing the binary-Ti plasmid (pBIN) coding for GFP (provided by O. Voinnet). The effect of ArMV isolates on the PTGS of the GFP transgene and the exogenous GFP gene was analyzed by monitoring the fluorescence of GFP under UV light, at 12, 19 and 33 dpi.

PTGS occurred in mock-inoculated and ArMV-NW-infected transgenic plants through the plants at 12 dpi and the systemic GFP silencing signal spread in the new emerging leaves of the plants

at 19 dpi, as evidenced by the red veins and limbs (fig. 13). The plants appeared completely red under UV light at 33 dpi. The GFP was also completely silenced in 80% of the 16C plants infected with ArMV-Lv but 20% of them were still green fluorescent at 33 dpi, indicating that the silencing of GFP was suppressed in these plants by the Lv isolate. The ability of ArMV-Lv to suppress RNA silencing in some plants but not in others, was surprising since all of them contained the same viral titer as evidenced by ELISA, using antibodies directed against the capsid protein.

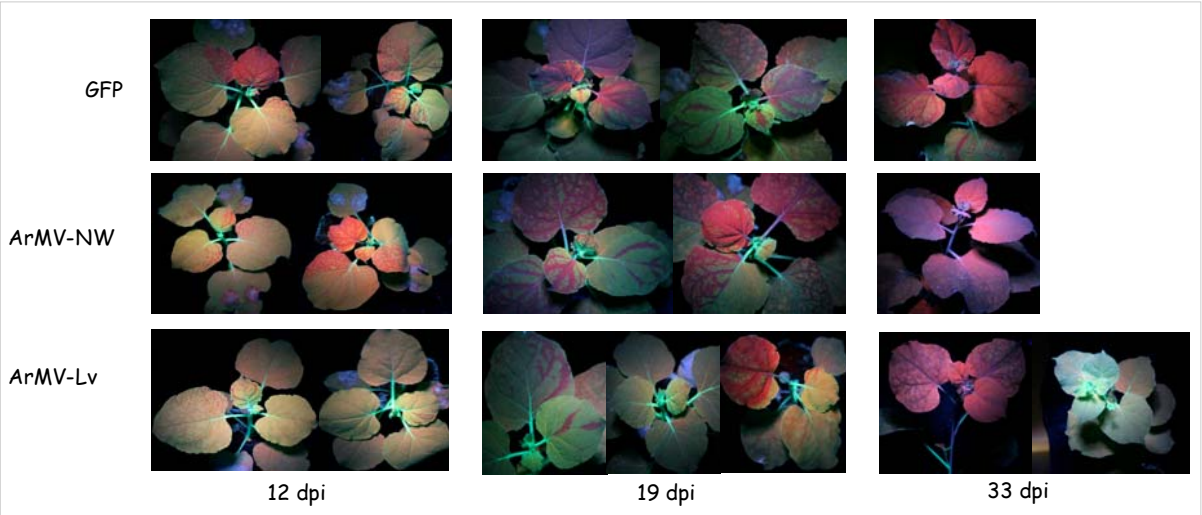


**Figure 13: Establishment of the GFP silencing in mock-inoculated or ArMV-infected 16C transgenic *N. benthamiana* plants**
The plants were observed under UV light at 12, 19 and 33 days post-inoculation (dpi) of the plants infected with the ArMV-NW or ArMV-Lv isolates.

### 3.2. Effect of ArMV replication on the initiation step of GFP silencing

Three steps characterize RNA silencing: initiation, systemic propagation and maintenance of the silencing state.

To determine if ArMV-Lv or NW isolates have an effect on the intracellular RNA silencing, we followed the green fluorescence in 16C transgenic plants on 2 or 3 separate patches per leaf, agro-infiltrated with the GFP replicon, 7 days after inoculation of these plants with each ArMV isolate. The fluorescence of the GFP was analyzed under UV light at 2, 4 and 6 days after agro-infiltration of the GFP replicon (fig. 14).

At 2 dpi, GFP fluorescence could be observed at the level of the infiltrated zones in infected and GFP agro-inoculated 16C plants. Its intensity was due to the transient GFP expression superimposed on fainter green fluorescence from the transgene. The green fluorescence disappeared on the infiltrated patches of all plants, infected or not, at 4 dpi, indicating that RNA silencing of the GFP occurred. At 6 dpi, the silenced patches were surrounded by a intense red line which superimposed the red background of the leaves due to the expression of the chlorophyll, meaning that the non-cell

autonomous movement of the systemic signal of RNA silencing occurred through a zone of 10-15 cells, as described by Himber *et al.*, (2003).

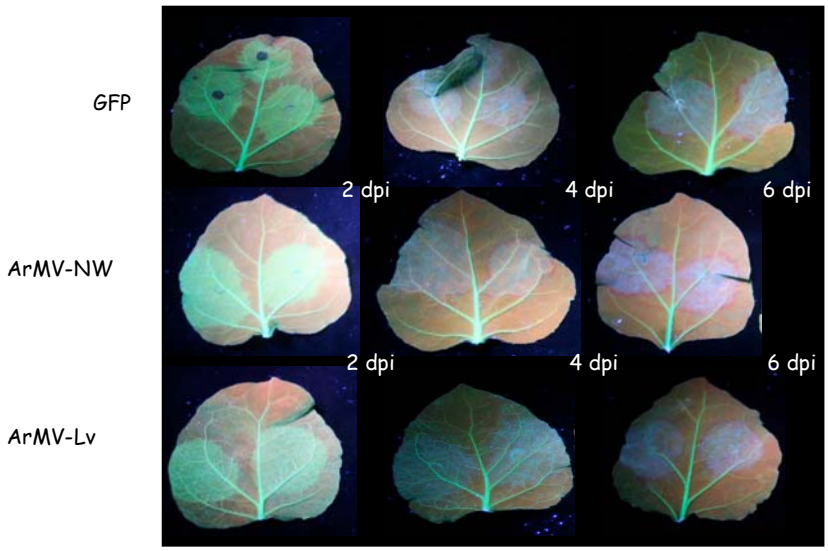


**Figure 14: Patches of infected or non-infected 16C transgenic *N. benthamiana* plants agro-inoculated with the GFP replicon**
The plants were observed under UV light at 2, 4 and 6 dpi. The green fluorescence corresponds to the expression of the GFP genes. The red line around the silenced patches (bluish area) results from the cell-to-cell propagation of the RNA silencing of the GFP.

Northern blot assays were performed on total RNAs from cut-off agro-infiltrated patches to detect the GFP mRNA (~ 1 kb). The levels of GFP mRNA coincided with the intensity of the GFP fluorescence observed in the patches of infected and non-infected plants, at different times after agro-infiltration (fig. 15).

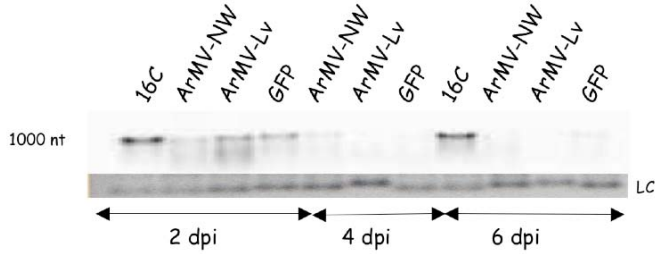


**Figure 15: Northern blot of the GFP mRNA present in the agro-infiltrated patches of infected and non-infected 16C transgenic *N. benthamiana* plants**
Total RNAs (5 μg) were extracted from the agro-infiltrated patches, fractionated on a denaturing agarose gel (1.5%) and probed with a radiolabelled GFP cDNA. The hybrids were visualized by phosphoimager after overnight exposure. The loading control (LC) is shown.

As expected, the amounts of GFP mRNA were higher in the non-agro-infiltrated 16C plants at 2 dpi compared to the GFP agro-infiltrated plants because the silencing of GFP was not triggered in these plants; this level remained constant at 6 dpi. At 2 dpi, the GFP mRNA could be visualized at reduced levels in the agro-infiltrated plants, infected or not with ArMV. At 4 and 6 dpi, the mRNA was hardly detectable in all infiltrated plants with GFP replicon owing to RNA silencing. Therefore, these results indicate that ArMV-NW and -Lv isolates did not interfere with the initiation of GFP silencing.

### 3.3. Effect of ArMV replication on the maintenance step of GFP silencing

To determine if the replication of ArMV has an effect on the maintenance of RNA silencing, 16C transgenic plants silenced upon agro-infiltration of the GFP replicon, were inoculated with ArMV-NW or -Lv isolates after propagation of RNA silencing in these plants, when they were totally red under UV light. The plants inoculated either with NW or Lv isolates, remained red 14 days after inoculation although the virus replicated, as evidenced by the appearance of disease symptoms in plant infected by ArMV-Lv (we remind that no symptom is induced by ArMV-NW infection), indicating that these isolates was unable to revert the established silencing of GFP.

## 4. Identification of suppressor(s) of RNA silencing encoded by ArMV-NW or -Lv

### 4.1. Study performed with the ArMV-NW infectious cDNA clones

The involvement of ArMV genomic RNAs in the PTGS pathway was investigated by agrobacterium-mediated transient suppression assays also called patch-tests. In this test, the plasmid coding for the putative RNA silencing suppressor (VSR) is co-delivered with the GFP replicon, which triggers the GFP silencing, in the same agro-infiltration zone on wild type or 16C *N. benthamiana* plants (fig. 16). In the absence of RNA silencing suppression activity, the GFP mRNA is rapidly degraded and the infiltrated patches become red under UV light whereas in the presence of a VSR, the GFP mRNA is stabilized and consequently, the infiltrated patches remain green fluorescent.

This experiment was performed with the genomic RNAs of ArMV-NW isolate for which we had the full-length FL1 and FL2 cDNA clones, corresponding to RNA1 and RNA2, respectively. These cDNAs were cloned into the binary vector pZP200 and used to transform the strain ATHV of *A. tumefaciens*. The ArMV B satellite (1104 nt) was also tested because several studies showed that satellites are involved in the suppression of RNA silencing (Cui *et al.*, 2005; Chellappan *et al.*, 2005; Saeed *et al.*, 2007).

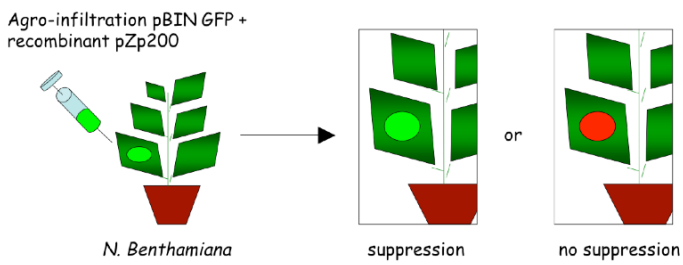


**Figure 16: Agrobacterium-mediated transient suppression assay**
A mix of two recombinant agrobacteria, containing the putative suppressor of RNA silencing and the GFP replicon, respectively, was co-delivered with a syringe at the lower face of the leaf. The fluorescence of the GFP is perpetuated at the infiltration zone in presence of a suppressor. By contrast, infiltrated patch appears red in absence of a suppressor.

Each cDNA (for simplification, cDNAs will be designated RNA1, RNA2 and satellite) was tested separately and in combination with other cDNAs, including RNA1+RNA2 and RNA1+ RNA2+satellite combinations that mimic authentic ArMV infection in nature. Plants agro-infiltrated with the GFP replicon alone served as control for the establishment of PTGS and those infiltrated with the recombinant plasmid coding for the P19 suppressor of *Tomato bushy stunt virus* (Hamilton *et al.*, 2002), as positive control for suppression of RNA silencing.

### 4.1.1. Patch-tests in 16C transgenic *N. benthamiana* plants

At 5 dpi, the patches of all agro-infiltrated plants appeared green fluorescent however with variable intensities i.e. plants infiltrated with the satellite construct and satellite with RNA1 and/or RNA2 combinations were less fluorescent than those infiltrated with RNA1, RNA2 or RNA1+RNA2 constructs (fig. 17A). As expected, the fluorescence was less intense in plants only agro-inoculated with the GFP replicon and strong in plants agro-infiltrated with P19, this viral protein being considered as a very efficient RNA silencing suppressor.

At 7 dpi, all infiltrated patches were less fluorescent and surrounded by a red line due to the propagation of the RNA silencing signal, except plants in which GFP cDNA was co-delivered with RNA1, RNA2 and RNA1+ RNA2 and P19 constructs thus, suggesting that both RNA1 and RNA2 of ArMV-NW might have an effect on the local RNA silencing. However, we can notice that this local silencing occurred with the ArMV RNAs co-expressed with the satellite in the plants.

To confirm that ArMV RNAs have an effect on the stabilization of GFP mRNA, northern blots were performed on total RNAs extracted from the agro-infiltrated patches at 5 dpi and 7 dpi to determine the level of GFP mRNA. As shown in figure 17B, the amounts of GFP mRNA coincided more or less with the fluorescence intensity of the infiltrated zones. At 5 dpi, plants infiltrated with the RNA1, RNA2 or RNA1+RNA2 constructs contained slightly more GFP mRNA than the plants that

received the satellite alone or in combination with RNA1 and/or RNA2. The level of GFP mRNA in the plants that expressed RNA1, RNA2 or RNA1+RNA2 was similar to that found in non-infiltrated 16C transgenic plants, suggesting that viral RNAs are involved in the suppression of RNA silencing. As expected, the GFP mRNA accumulated at very high levels in the plants that expressed P19. At 7 dpi, the differences in the levels of GFP mRNA present in the infiltrated patches were less pronounced, in particular, in the plants that received RNA1, RNA2 or RNA1+RNA2. The quantity of mRNA was similar to that found in plants agro-inoculated only with the GFP replicon and more important than the level of GFP mRNA of the infiltrated zones containing the satellite alone or associated with ArMV RNAs. However, we have to stress that the profiles of green fluorescence and GFP mRNA were heterogeneous between plants agro-infiltrated with the same construct, depending on the plant development stage and in particular, on the size of the leaves.

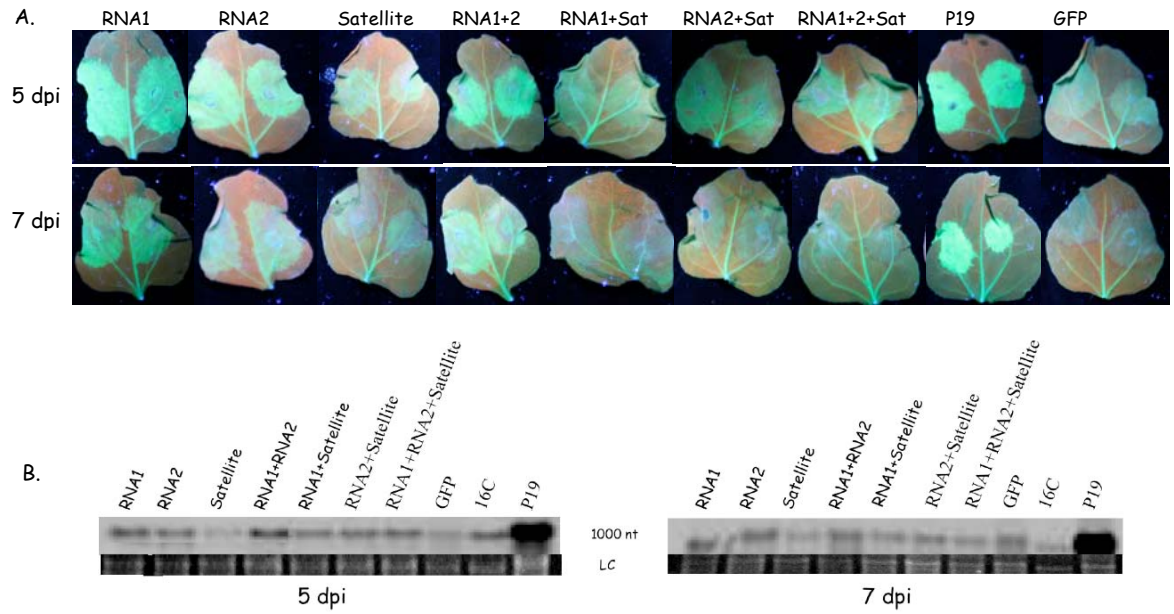


**Figure 17: Agrobacterium-mediated transient suppression assays with the ArMV RNA constructs**
A. Analysis of the patches agro-infiltrated with the RNAs constructs in the 16C transgenic *N. benthamiana* plants at 5 and 7 dpi, under UV light. The viral cDNA constructs were mixed with the GFP at OD 1:1 and agro-infiltrated in the same patch except for the RNA1+RNA2 combination at 1:1:2 OD and the RNA1+RNA2+satellite combination at OD 1:1:1:3; B. The northern blots were realized with RNAs extracted from the infiltration zones at 5 and 7 dpi. For the analysis, 5 μg of each sample were fractionated on denaturing 1.5% agarose gel and probed with the radiolabelled GFP cDNA. After hybridization, the membrane was washed and the hybrids visualized by phosphoimager after overnight exposure. The loading controls (ethidium bromide staining) are shown below the northern blots.

These discrepancies led us to agro-infiltrate both sides of a same leaf, one side with the GFP replicon and the other side with the GFP replicon and the viral cDNA constructs (fig. 18).

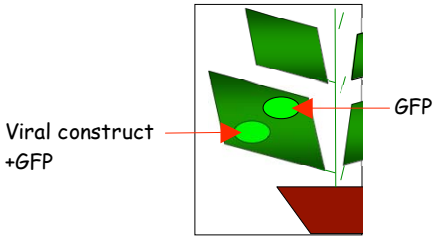


**Figure 18: Experimental procedure to study the effect of the RNA constructs on GFP silencing in 16C transgenic *Nicotiana benthamiana* plants**
The recombinant plasmid containing the putative suppressor and the GFP replicon were infiltrated with a syringe at the left of the leaf whereas the GFP replicon alone, as negative control, was delivered at the right of the same leaf.

These experiments were performed with RNA1, RNA2, the ArMV satellite and the RNA1+RNA2. The 5' and 3' non-coding sequences (UTRs) of the ArMV RNA2 were also tested to determine if their secondary structures interfere with RNA silencing. The 16C plants were also infiltrated with the empty vector to ensure that the latter has no effect on PTGS (negative control) and with the TBSV P19 construct (positive control). The RNA silencing suppression activity was determined by analysing the GFP fluorescence under UV light but also with the phosphoimager, which is more sensitive and specifically detects the fluorescence due to GFP by comparising between the fluorescence emitted under FITC laser (GFP-Long Pass, 525-540 nm) and under the cy3 laser (GFP-Band Pass; 515-560 nm), respectively.

At 3 dpi, the green fluorescence intensity, observed under UV light and with phosphoimager, was similar in the opposite patches of the same leaf whatever the constructs (fig. 19), confirming previous observations that GFP fluorescence is retained in *N. benthamiana* 2 to 3 days after agro-infiltration of the GFP replicon (Voinnet and Baulcombe, 1997). At 5 dpi, no fluorescence was visible in the patches of plants agro-infiltrated with the 5' and 3' UTRs constructs, the ArMV satellite and the empty vector while astonishingly, the patches agro-inoculated only with the GFP replicon remained green fluorescent. By contrast, the fluorescence was unaffected in plants infiltrated with the RNA1 and RNA2 constructs, P19 and to a lesser extent, with the RNA1+RNA2 combination. At 7 dpi, the patch that expressed P19 was intensively fluorescent. The expression of the GFP was maintained in plants infiltrated with the RNA1, RNA2 and the co-expression of the both constructs, at 7 dpi whereas it was totally abolished on the other side of the leaf in which we delivered only the GFP replicon, suggesting that both ArMV RNAs are involved in RNA silencing suppression functions.

Northern blots were realized on high and low molecular weight RNAs extracted from the infiltrated zones, using as probe a GFP cDNA radioactively labelled by random priming, to assess the levels of GFP mRNA and the corresponding siRNAs.

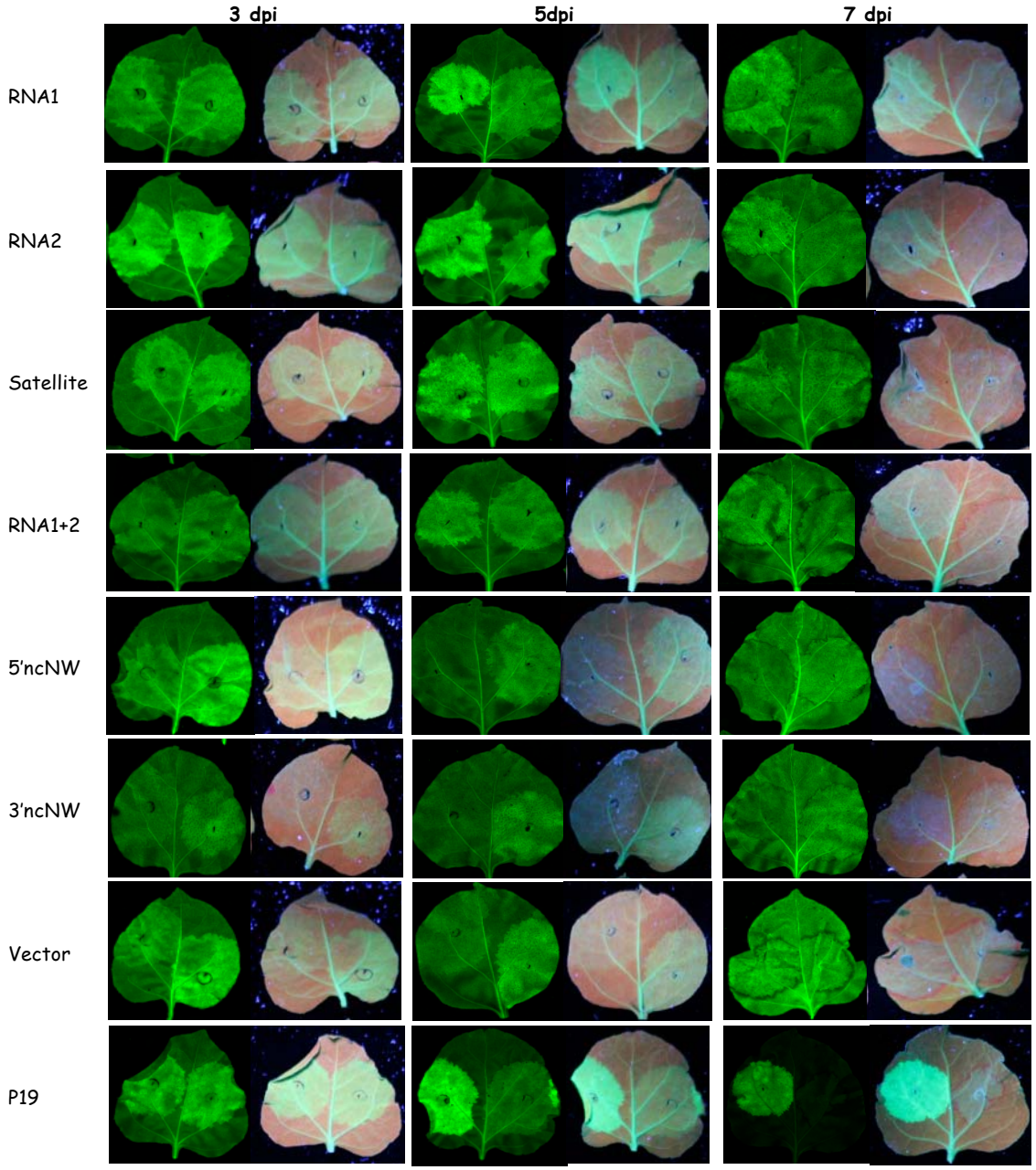


**Figure 19: Observation of the infiltration zones of the viral RNAs constructs on 16C transgenic *N. benthamiana* plants under UV light and phosphoimager**
The patch corresponding to the viral RNAs constructs and the GFP replicon was infiltrated with a syringe at the left and the patch for the GFP alone at the right of the leaf. The analyses by phosphoimager (FITC laser) are represented at the left whereas those obtained under UV light are shown at the right.The times of analysis selected were the 3, 5 and 7 dpi.

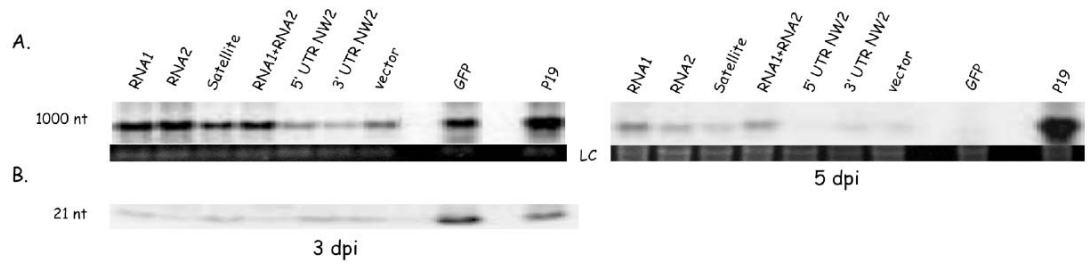


**Figure 20: Detection by northern blots of GFP mRNA and the corresponding siRNAs in 16C transgenic *N. benthamiana* plants agro-infiltrated with *A. tumefaciens* containing different ArMV cDNAs**
High (5 μg) and low (10 μg) molecular weight RNAs were analyzed by northern blots (A and B), respectively, using total RNAs extracted from the different infiltrated zones at 3 and 5 dpi. High molecular weight RNAs were fractionated on a 1.5% agarose gel in the presence of formaldehyde whereas low molecular weight RNAs were separated on a 20% polyacrylamide gel containing 8M urea. The RNAs were probed with the radiolabelled GFP cDNA. The hybridization signal were visualized by phosphoimager after overnight exposure. The loading controls (LC) are shown.

At 3 dpi, similar amounts of GFP mRNA were detected, in the patches co-infiltrated with the GFP replicon and RNA1, RNA2, RNA1+RNA2 and the satellite constructs or P19 and in the patches infiltrated only with the GFP replicon (control) (fig. 20A left). The leaves that received the 5' UTR, 3' UTR or the empty vector contained reduced levels of GFP mRNA. At 5 dpi, low levels of GFP mRNA were found in the patches that expressed the ArMV RNAs (RNA1, RNA2, and RNA1+RNA2) in presence of GFP replicon whereas the mRNA was undetectable in the control patches agro-infiltrated with GFP replicon (fig. 20A right). At 7 dpi, the foliar zones infiltrated with the 5' UTR, 3' UTR or the empty vector were depleted of GFP transcripts, as expected since no fluorescence could be observed in these patches, at 5 dpi. A similar profile was observed with RNAs- agro-infiltrated patches, at 7 dpi. These results confirmed that both ArMV RNAs are involved in the suppression of local RNA silencing and that their VSRs are barely efficient, at least in *N. benthamiana* compared to the TBSV P19.

GFP-derived siRNAs were present at a very low level in the patches that received all viral constructs, except for the GFP control and P19, for which we found larger amounts of siRNA (fig. 20B). Concerning the 5' and 3' UTRs of ArMV RNA2, they had apparently no effect on the GFP silencing since the northern patterns displayed for GFP mRNA and siRNA were similar to that obtained with the empty vector.

The accumulation of GFP in the infiltrated patches was also determined by western blots, using polyclonal anti-GFP antibody since its expression can be impaired at the translation level through the annealing of non-coding small RNAs (siRNA and/or miRNA) to the mRNA. Analysis of total proteins extracted from the infiltrated patches revealed that there was no difference in the amounts of GFP

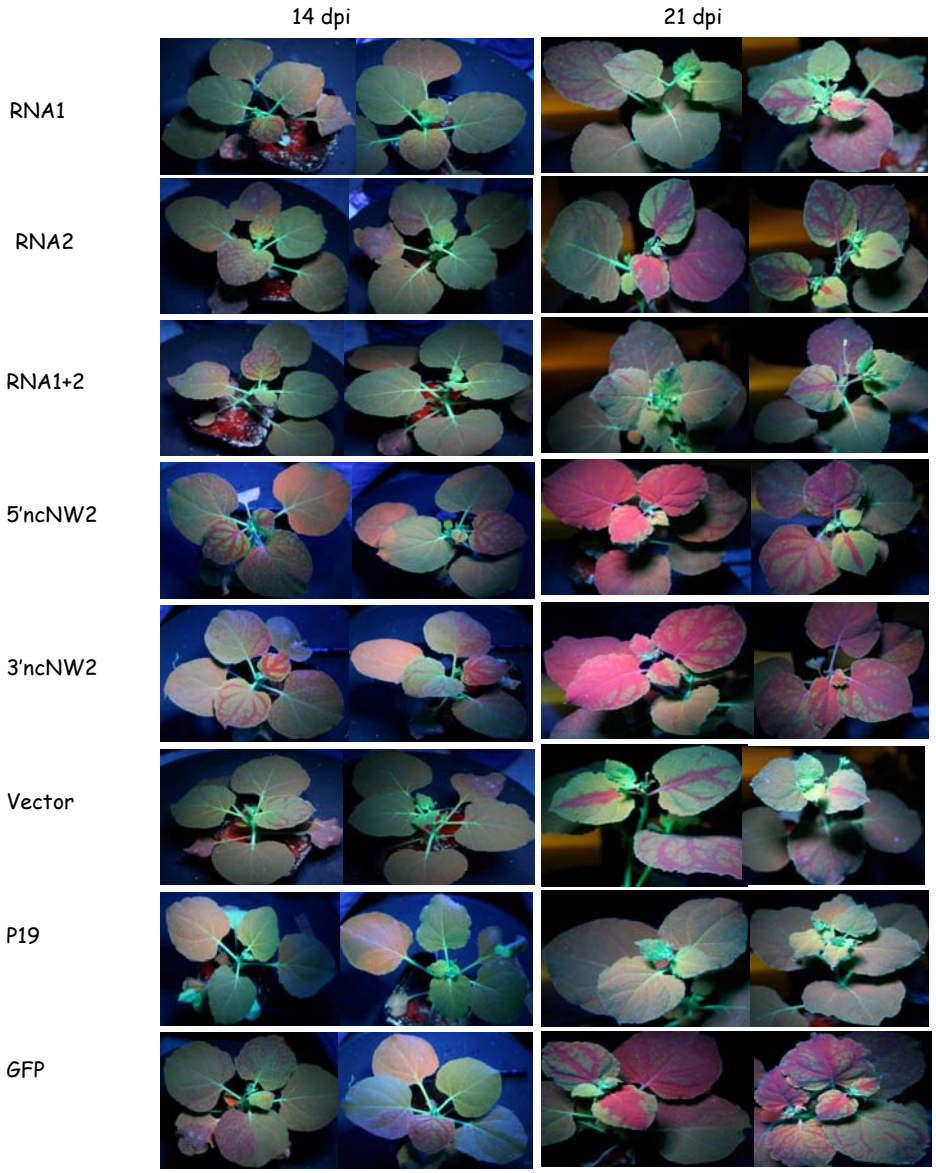


**Figure 22: Observation of the propagation of the systemic signal of RNA silencing in 16C transgenic *N. benthamiana* plants agro-infiltrated with viral constructs**
Photos correspond of the silenced plants agro-infiltrated with the different RNAs constructs, at14 and 21dpi. The apparition of the red limb or nervures means the establishment of the systemic silencing of the GFP in 16C plants.

between the patches containing viral RNAs and those containing the untranslated regions and GFP, at 5 dpi, suggesting that the suppression of GFP silencing did not operate at the translational level.

### 4.1.2. Study of the systemic signal in 16C transgenic *N. benthamiana* plants

We also studied the effect of the RNA constructs on the propagation of the systemic signal of GFP silencing. For this, 16C plants were agro-infiltrated as already described and then, the systemic silencing was followed by observation of the plants under UV light (fig. 21). The red fluorescence on the limb and the veins of the new emerging leaves appears upon systemic silencing of the GFP.

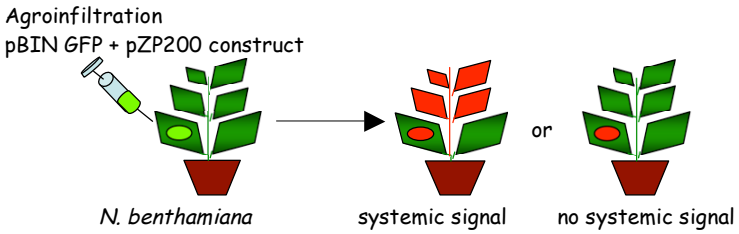


**Figure 21: Experimental procedure to study the systemic signal of GFP silencing**
A mixture of agrobacteria containing the putative VSR and GFP replicon was co-delivered by infiltration with a syringe at the lower face of the leaf. At 14 dpi, the fluorescence was observed under UV light in the upper leaves and new emerging leaves. The plant appears red when the propagation of the silencing occurred.

At 14 dpi, red fluorescence appeared in the main veins and the limb with all viral constructs (RNA1, RNA2, RNA1+RNA2) but the red fluorescence was particularly intense in plants agro-infiltrated with the plasmids coding for the 5' and 3' UTRs of ArMV RNA2 (fig. 22). These plants became red at different degrees at 21 dpi whereas plants transiently expressing P19 were still green fluorescent at 21 dpi.

Together, these results suggest that ArMV RNA1 and RNA2 contribute to the suppression of GFP silencing in 16C transgenic *N. benthamiana* plants but neither RNA1 nor RNA2 inhibit the intercellular movement of GFP silencing in contrast to P19 of TBSV.

### 4.1.3. Patch-tests in wild-type *N. benthamiana* plants

The introduction of a single copy transgene can produce aberrant sens transcripts, which are recognized by the RNA silencing pathway: this silencing is called sense PTGS (S-PTGS). In order to determine if the ArMV constructs have an effect on S-PTGS, we performed the same experiments, as described above on wild-type *N. benthamiana* plants. The infiltrated zones of the leaves are green

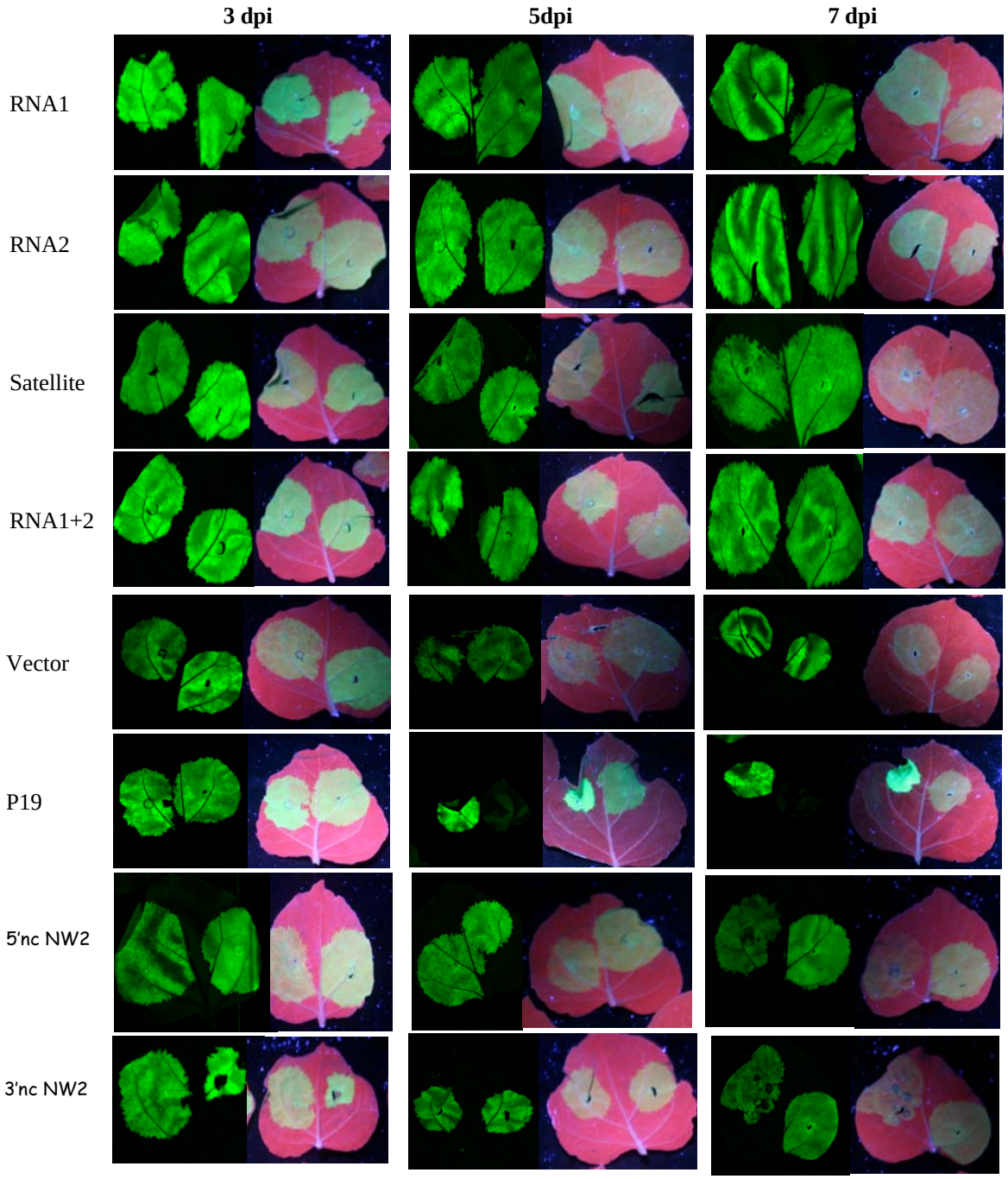


**Figure 23: Study of the effect of the viral constructs on the S-PTGS in *N. benthamiana***
The patch corresponding to the viral constructs and the GFP replicon was infiltrated with a syringe at the left and the patch for the GFP alone at the right of the leaf. The analyses of the infiltration zone at, 5 and 7 dpi, by phosphoimager (FITC laser) are represented at the left whereas these obtained under UV light are shown at the right.

fluorescent and the surrounding limb black when they were observed with the phosphoimager, in contrast to 16C transgenic *N. benthamiana* plants, for which they were totally green fluorescent due to the constitutive expression of GFP in these plants.

At 3 dpi, no significant difference was observed between the right (GFP alone) and the left (GFP + viral constructs) patches for all assays but two days later (5 dpi), the green fluorescence of the patches infiltrated with GFP replicon and RNA1, RNA2 and RNA1 + RNA2 constructs was slightly stronger than the fluorescence in the opposite patches agro-infiltrated with the GFP replicon (fig. 23). The fluorescence persisted at 7 dpi in the leaves that received the ArMV cDNAs but no so intensively as in the P19 control, suggesting that RNA1 and/or RNA2 suppress S-PTGS but less efficiently than P19. At the opposite, the fluorescence totally disappeared in the infiltrated zones that received the ArMV satellite and the vector, at 7 dpi, suggesting that the satellite did not impair the S-PGTS.

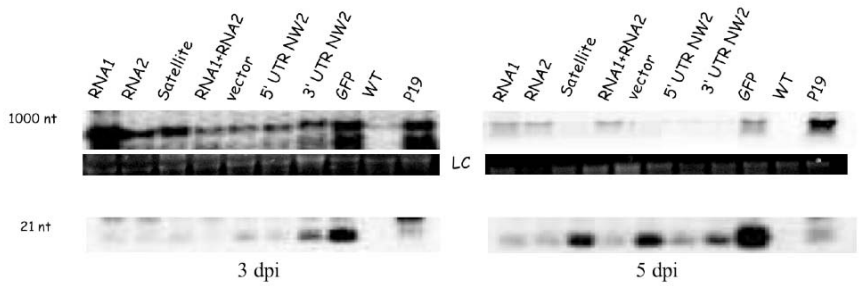


**Figure 24: Analysis by northern blots of GFP mRNA and the corresponding siRNAs in *N. benthamiana* plants agro-infiltrated with *A. tumefaciens* containing different ArMV cDNAs**
Northern blot were performed with high (A) and low (B) RNAs (5 μg and 10 μg, respectively) isolated from total RNAs extracted from the infiltrated zones at 3 and 5 dpi. High molecular weight RNAs were fractionated on a 1.5% agarose gel in the presence of formaldehyde whereas low molecular weight RNAs were separated on a 20% polyacrylamide gel containing 8M urea. After transfer, the membranes were incubated with radiolabelled GFP cDNA and after stringent washes, the hybrids were detected with the phosphoimager. The loading controls (LC) are shown.

The GFP mRNA was detected by northern assays in the plant patches, transiently transformed with the viral constructs but not in the mock-infiltrated wild-type (WT) *N. benthamiana* plants, at 3 dpi. The levels of GFP mRNA in the infiltrated zones were variable depending on the viral construct, see for example the GFP mRNA content in RNA1, 5' UTR, WT and P19 lanes (fig. 24A left). Surprisingly, the mRNA level was higher in the zone in which we co-delivered GFP cDNA with ArMV RNA1 than with P19, but this result was rather due to the rate of transformation than to the efficiency of viral RNA1 to suppress the S-PTGS. At 5 dpi, the same amounts of GFP mRNA were detected in the control patches (infiltrated only with the GFP) and in those agro-infiltrated with RNA1,

RNA2 and RNA1+RNA2. As expected, a high level of this mRNA was present in the plants agro-infiltrated with P19 (fig. 24A right). The GFP transcript was barely or not detected in the plants infiltrated with the others constructs i.e. the 5' and 3' UTRs, the satellite and the vector.

The GFP-derived siRNAs (21 nt) were abundant in the patches infiltrated with the GFP cDNA and with the 3' UTR at 3 dpi (fig. 24B left). Traces of these siRNAs were also found in the plants agro-infiltrated with the other constructs, except in WT *N. benthamiana*. The same profile of GFP-derived siRNAs was observed at 5 dpi but for *N. benthamiana* only transformed with the GFP cDNA and in plants infiltrated with the satellite, the 5' UTR and the vector, the level of siRNAs increased considerably within two days (fig. 24B right). At the opposite, the patches still fluorescent at 5 dpi (RNA1, RNA2, RNA1+RNA2 and P19) contained siRNAs at reduced levels. These molecular analyses confirmed that the expression of ArMV RNA1 and/or RNA2 interfered with the S-PTGS pathway.

The levels of GFP in plants were also determined by western blotting. We focused our study only on the RNA1, RNA2 and RNA1+RNA2 constructs because the satellite, the vector and the non-coding regions did not show any effect on the intensity of the GFP fluorescence and on the GFP mRNA accumulation.

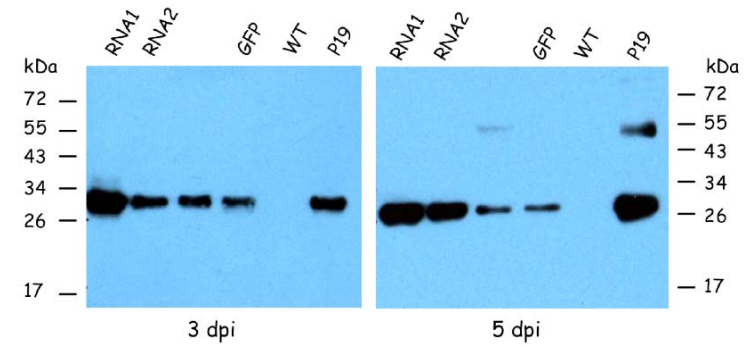


**Figure 25: Immunodetection of GFP in patches agro-infiltrated with viral cDNA constructs on *N. benthamiana* plants**
Proteins were fractionated on a denaturing polyacrylamide gel and detected by chemiluminescence (ECL) after incubation of the membrane with a specific antibody raised against GFP.

At 3 dpi, the amounts of GFP perfectly correlated with the GFP mRNA levels in the agro-infiltrated plants (fig. 25). At 5 dpi, GFP accumulated at a high level in plants infiltrated with RNA1, RNA2 and P19 cDNAs however, the plants expressing P19 contained ten fold more GFP mRNA (the ratio was determined with the phosphoimager). The band at 54 kDa in the P19 lane probably corresponds to a GFP dimer. As the level of GFP mRNA was reduced in the zone infiltrated with the viral constructs at 5 dpi, we assume that the higher amounts of GFP results from the accumulation of

GFP although, it is likely that RNA silencing suppression activity of ArMV RNAs also contributed to this steady state level of GFP.

### 4.2. Characterization of the RNA silencing suppressor(s) of ArMV-NW

To identify the ArMV protein(s) involved in the suppression of (S)-PTGS, transient suppression assays were performed in 16C *N. benthamiana* plants, as described previously. The sequences coding for the ArMV mature proteins or precursors were amplified by RT-PCR and cloned in the pGJpRT vector. The recombinant plasmids were introduced in the ATHV strain of *A. tumefaciens*.

#### 4.2.1. Patch tests in 16C *N. benthamiana* plants

The leaves of 16C plants were co-infiltrated with recombinant plasmids coding for the viral proteins and the GFP, respectively.

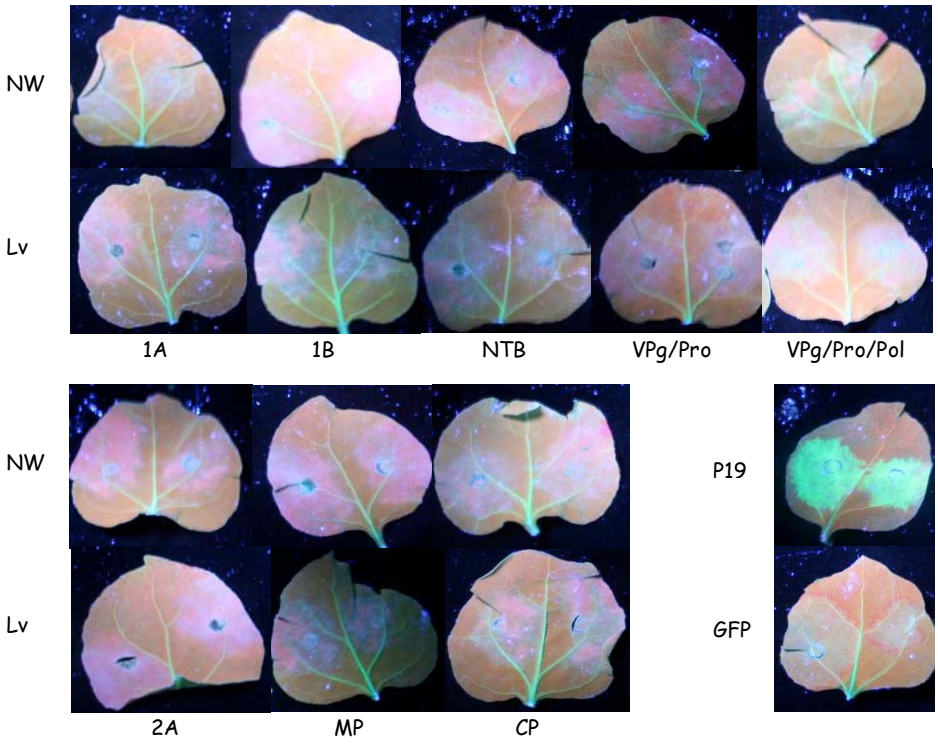


**Figure 26: Observation under UV light of the patches infiltrated with the different ArMV cDNAs in 16C *N. benthamiana*, at 5 dpi**
Culture of recombinant *A. tumefaciens* containing the GFP replicon and ArMV cDNAs, respectively, were mixed (OD 1:1) and infiltrated in 16C transgenic *N. benthamiana* plants.

We focused our investigation on the mature proteins 1A, 1B, NTB and on the intermediate cleavage products, VPg-pro and Vpg-Pro-Pol encoded by RNA1 and on the three proteins expressed

from RNA2, namely the 2A protein, the MP and the CP. The cDNA coding for the viral RNA-dependent RNA polymerase (Pol) could not be cloned in *E. coli* because the expression of the viral polymerase was toxic for the bacteria.

The patch tests did not permit to characterize RNA silencing suppression activity for none of the ArMV proteins or precursors thereof. The fluorescence of the patches infiltrated with both the GFP and the viral cDNAs disappeared, at 5 dpi (fig. 26) similarly to those agro-infiltrated only with the GFP cDNA. By contrast, the green fluorescence was maintained with P19.

To determine, if the absence of suppression was due to an inefficient expression of the viral proteins, the sequences coding for the viral proteins were fused either at the 5' or 3' ends to the HA (hemagglutinin from Influenza virus) sequence in order to detect the viral proteins in the transformed *N. benthamiana* plants by using commercial anti-HA antibodies. We used this strategy to detect the ArMV proteins because antibodies against the viral proteins were not available. Western blot performed with anti-HA antibodies on the proteins extracted from the infiltrated patches led only to the detection of the MP (38K) and CP (56K) proteins, suggesting that the other viral proteins were not produced at least, at a detectable level (fig. 27).

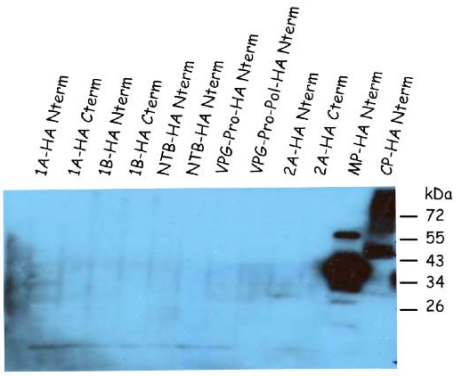


**Figure 27: Detection of HA-tagged ArMV proteins in the agro-infiltration patches of *N. benthamiana* plants**
Proteins were fractionated on a denaturing gel, probed with a specific antibody raised against HA-tag and detected by chemiluminescence (ECL).

We repeated this experiment with the GV3101 strain of *Agrobacterium* to deliver the viral constructs into *N. benthamiana* plants, this strain being already used to transform the plants with the GFP cDNA. One side of the leaf was agro-infiltrated with the GFP replicon and the opposite side, with the GFP replicon and the viral cDNA. No or dimmed fluorescence, compared to the fluorescence emitted by the leaves agro-inoculated only with the GFP cDNA, could be observed in the patches that were co-infiltrated with the 1A, 1B, 2A, CP and MP constructs, at 3 dpi, suggesting that these viral

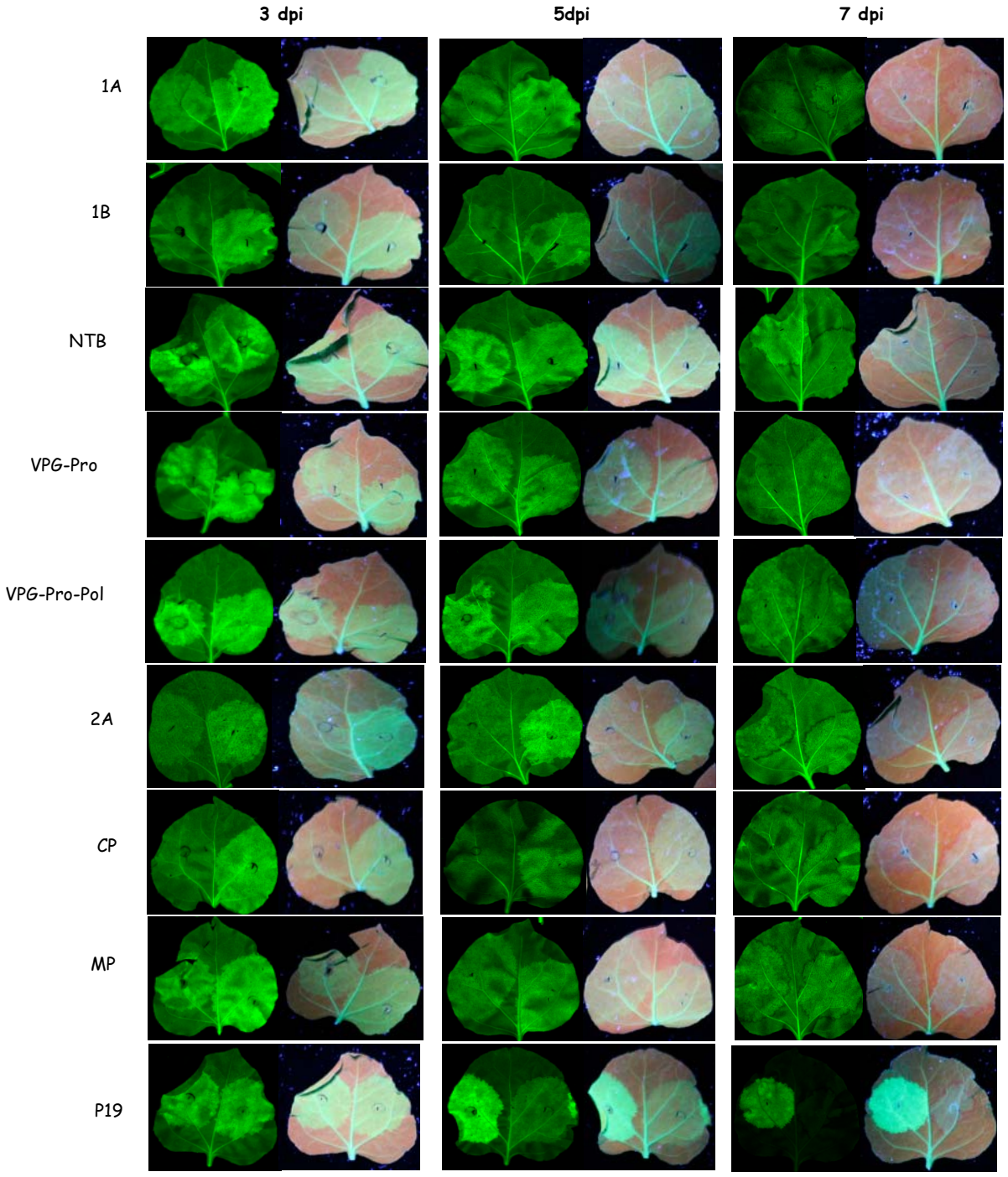


**Figure 28: Observation under UV light and phosphoimager of the infiltration zone of the viral constructs in 16 transgenic *N. benthamiana* plants**
The patch corresponding to the viral constructs and the GFP replicon was infiltrated with a syringe at the left and the patch for the GFP alone at the right of the leaf. The analyses by phosphoimager (FITC laser), at 3, 5 and 7 dpi are represented at the left whereas those obtained under UV light are shown at the right.

proteins were unable to suppress the silencing of GFP (fig. 28). We hypothetize that the viral proteins were expressed, at least the MP and CP, as shown in the previous experiment. Fluorescence was detectable with the NTB, VPg-Pro and VPg-Pro-Pol constructs but no conclusion could be drawn at this stage and even at 5 dpi, concerning the eventual capacity of these proteins to suppress RNA silencing because green fluorescence was still detected in the control patches. However, at 7 dpi, the fluorescence was suppressed on the control patches and the latter were bordered by a red line while fluorescence was clearly visible in the patches infiltrated with cDNAs coding for the NTB, VPg-Pro and VPg-pro-Pol constructs. These observations led to the hypothesis that these proteins of ArMV might be involved in the suppression of the PTGS.

To confirm this hypothesis, we performed northern blots on RNAs isolated from agro-infiltrated patches in order to analyse the GFP mRNA and GFP-derived siRNAs. The patches agro-infiltrated with the NTB, VPg-Pro, VPg-Pro-Pol, CP and P19 constructs contained levels of GFP mRNA similar to that found in patches agro-inoculated only with the GFP cDNA, at 3 dpi. Lower amounts of GFP transcripts were detected in patches infiltrated with the 1A, 2A, 1B and MP constructs (fig. 29A left). At 5 dpi, the quantity of GFP mRNA decreased significantly in all patches except those expressing P19 and the mRNA was totally absent in the patches infiltrated only with the GFP cDNA (fig. 29A right).

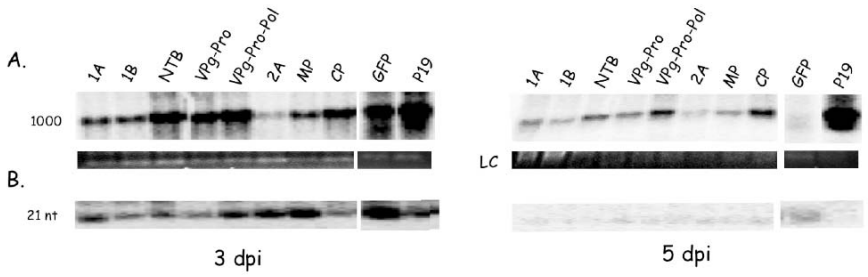


**Figure 29: Detection by northern blots of GFP mRNA and the corresponding siRNAs in 16C transgenic *N. benthamiana* plants agro-infiltrated with *A. tumefaciens* transformed with ArMV cDNAs**
High (5 μg) and low (10 μg) molecular weight RNAs were analyzed by northern blots (A and B), respectively, using total RNAs extracted from the different infiltrated zones at 3 and 5 dpi. They were fractionated as described in figure 24. The RNAs were probed with the radiolabelled GFP cDNA. The hybridization signals were visualized by phosphoimager after overnight exposure. The loading controls (LC) are shown.

Analysis of 21 nt-long GFP-specific siRNAs by northern blot showed that they accumulated at high levels in the patches infiltrated with GFP, 1A, 2A and MP cDNAs at 3 dpi (fig. 29B left), respectively, suggesting that suppression activity was not associated with these viral proteins. At the

opposite, siRNAs were less abundant in the patches agro-infiltrated with 1B, NTB, VPg-Pro and CP, which contained significant amounts of GFP mRNA. At 5 dpi, the siRNAs were almost at the detection threshold except for those present in plants infiltrated with GFP replicon (fig. 29B right).

We also realized western blots to determine the quantity of GFP in 16C *N. benthamiana* plants. The GFP protein was detected in higher amounts in the patches infiltrated with the VPg-Pro-Pol and P19 constructs than in the 1A, NTB, VPg-Pro, 2A, MP and CP infiltrated patches, at 3 dpi (fig. 30). At the opposite, the GFP was not detected in the plants infiltrated with the 1B cDNAs. The levels of GFP were unchanged at 5 dpi, except for patches expressing P19, which contained more GFP than at 3 dpi.

Taken together, our results suggest that the NTB protein and two intermediate cleavage products of the polyprotein P1, VPg-Pro and VPg-Pro-Pol, might be involved in PTGS suppression.

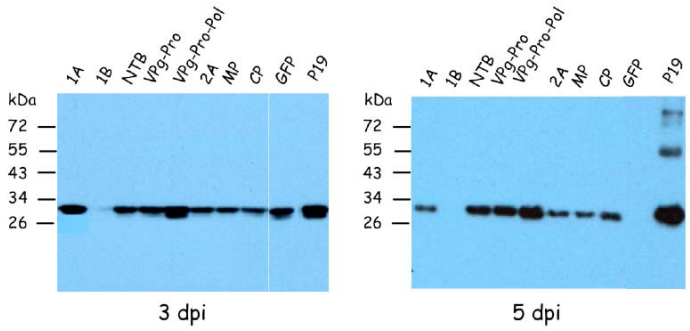


**Figure 30: Accumulation of GFP in patches agro-infiltrated with viral constructs on 16C transgenic *N. benthamiana* plants**
Proteins extracted (10 µl) were fractionated on denaturing polyacrylamide gel, transferred onto a membrane and probed with antibodies against GFP; the immuno-complexes were detected by chemiluminescence (ECL).

### 4.2.2. Influence of ArMV proteins on the systemic signal in 16C plants

The effect of the ArMV proteins on the GFP systemic silencing was also investigated on 16C transgenic plants (fig. 31). At 14 dpi, plants observed under UV light displayed red veins and limbs for all constructs except for the NTB and P19 clones. At 21dpi, only some plants agro-infiltrated with the NTB cDNA remained green thus, indicating that GFP was synthetized in these plants as those that expressed P19. Therefore, the NTB protein of ArMV presumably intervenes at the initiation step of PTGS and interferes with the short distance propagation of the silencing signal.

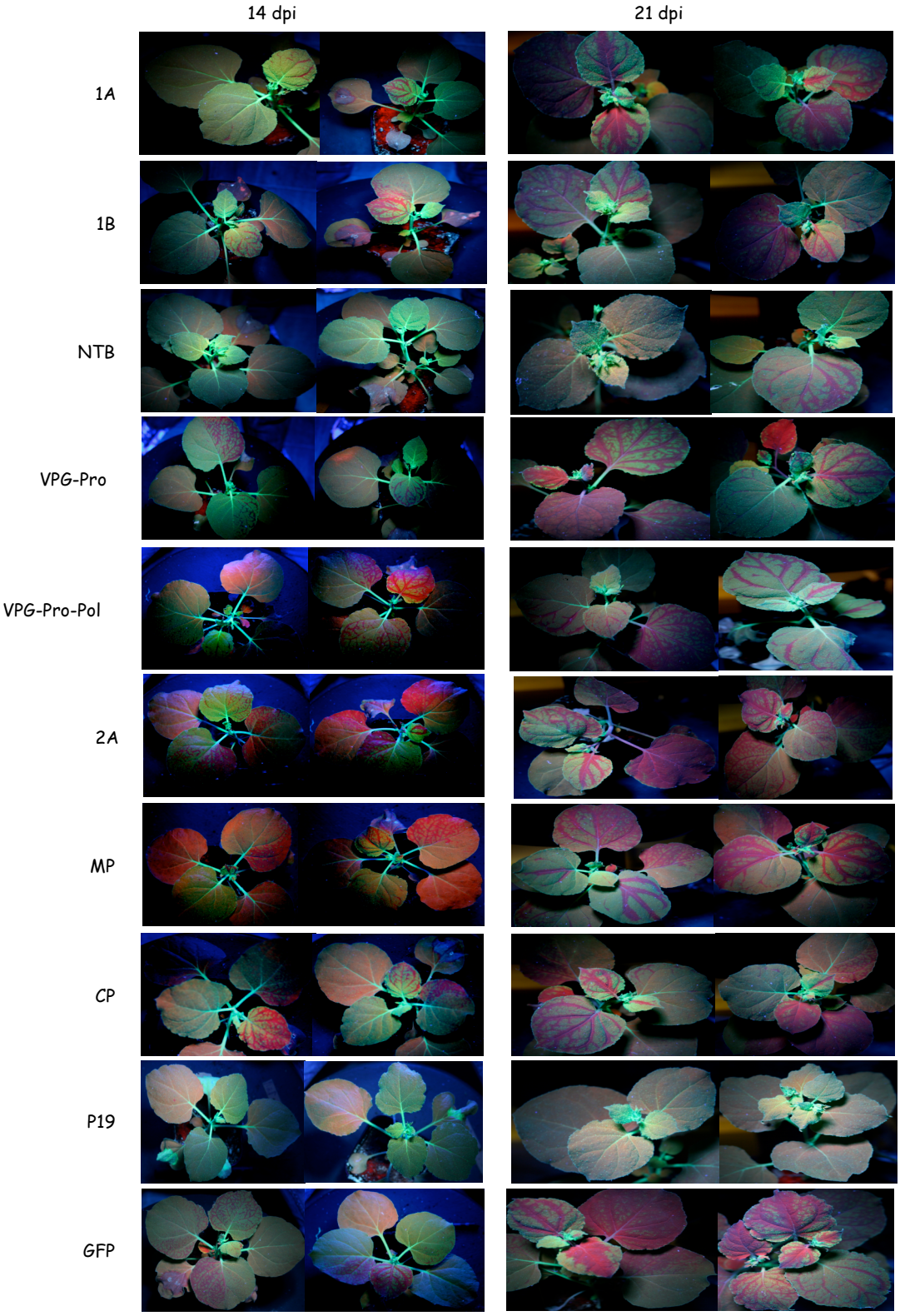


**Figure 31: Observation of the propagation of the systemic signal of the RNA silencing in 16C transgenic *N. benthamiana* plants**
Photos corresponding of the silenced plants agro-infiltrated with the viral constructs, at 14 and 21 dpi. The apparition of the red limb or nervures means that the establishment of the systemic silencing of the GFP in 16C.

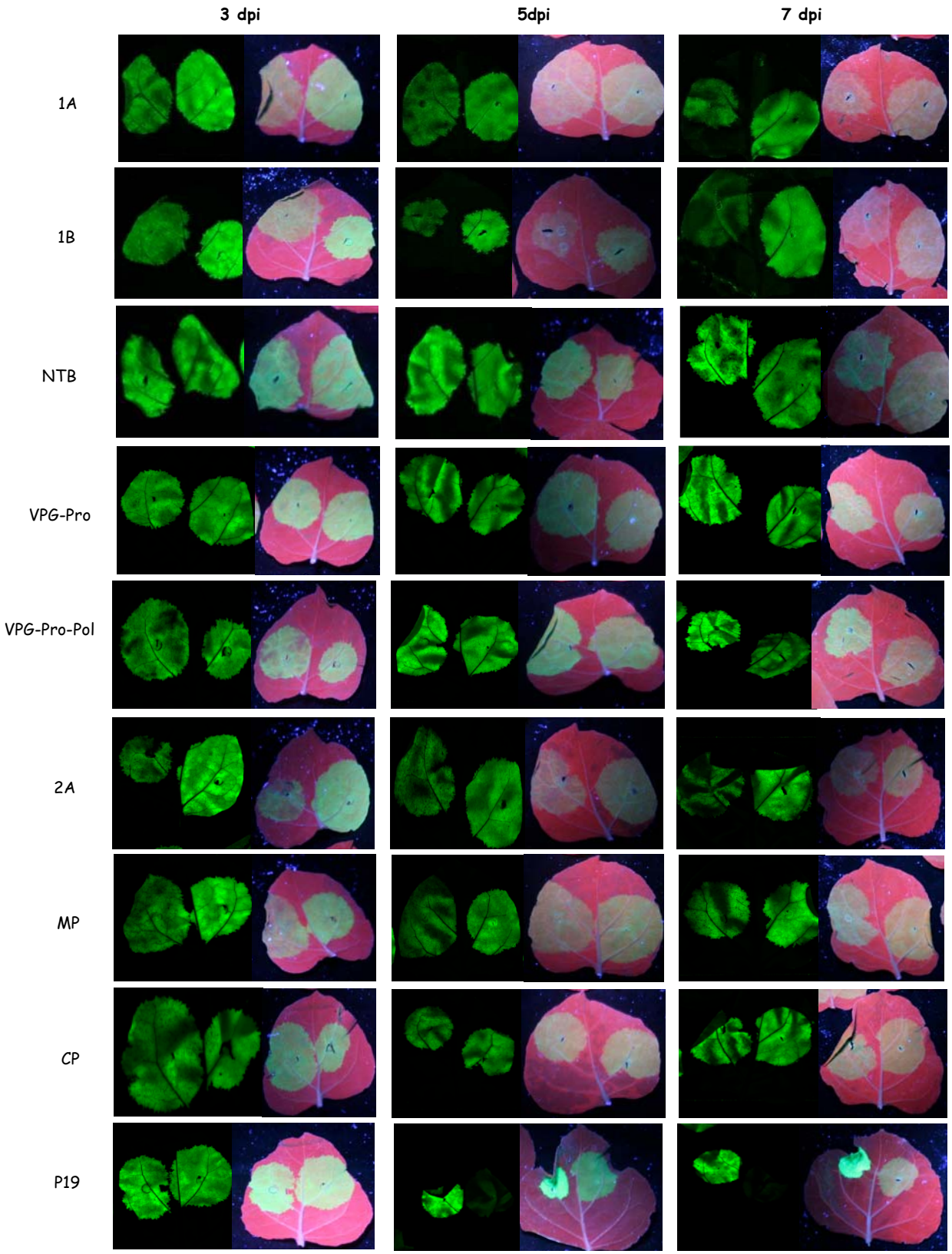


**Figure 32: Study of the effect of the viral constructs on the S-PTGS in *N. benthamiana***
The patch corresponding to viral constructs and the replicon GFP was infiltrated with a syringe at the left and the patch for the GFP alone at the right of the leave. The analysis of the infiltration zone, at 5 and 7 dpi. by phosphoimager (FITC laser) are represented at the left whereas these obtained under UV light are shown at the right.

### 4.2.3. Patch-test in wild type *N. benthamiana* plants

To see if ArMV proteins have an effect on the S-PTGS pathway, patch-tests were realized on wild-type *N. benthamiana* plants to set free from a rapid initiation of RNA silencing of the GFP gene. Moreover, experiments using 16C plants might not permit to detect the suppression of the RNA silencing in the presence of protein having a weak suppressor activity. Indeed, GFP-derived siRNAs accumulate very rapidly in the 16C transgenic plants, after introduction of a GFP replicon, leading to a prompt degradation of the GFP mRNA and consequently, to the extinction of the green fluorescence. The rapid degradation of the mRNA is notably due to the production of secondary siRNAs by the intermediate of the RDR6 activity.

At 3 dpi, the foliar patches co-infiltrated with the GFP gene and with the 1A, 1B, 2A or MP constructs were less fluorescent than those expressing only the GFP (fig. 32). No significant difference was observed between NTB, CP, VPg-Pro and VPg-Pro-Pol and the control patches. However, at 5 dpi and at 7 dpi, the fluorescence of the zones infiltrated with the NTB, VPg-Pro and VPg-Pro-Pol cDNAs was stronger than that of the GFP patches suggesting these (poly)proteins have an effect on the S-PTGS. At 7 dpi, the fluorescence was no more detectable with the other viral constructs, 1A, 1B, 2A, MP and CP.

The patches agro-infiltrated with the NTB, VPg-Pro, VPg-Pro-Pol, GFP and P19 constructs contained higher levels of GFP mRNA than those which received other ArMV sequences (fig. 33).

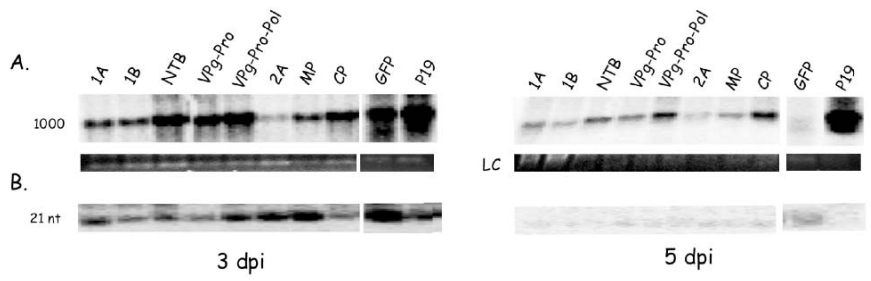


**Figure 33: Detection by northern blots of GFP mRNA and the corresponding siRNAs in wild-type *N. benthamiana* plants agro-infiltrated with *A. tumefaciens* containing different ArMV cDNAs**
High (5 μg) and low (10 μg) molecular weight RNAs were analyzed by northern blots (A and B, respectively), as described previously using total RNAs extracted from the infiltrated zones at 3 and 5 dpi. The RNAs were probed with the radiolabelled GFP cDNA. The hybridization signals were visualized by phosphoimager after overnight exposure. The loading controls (LC) are shown.

The steady state level of the GFP mRNA correlated with the GFP-derived siRNA pattern. The latter accumulated in plants that contained low amounts of GFP and vice-versa, except for GFP and 1B. In fact, the GFP siRNAs, corresponding to the low detection of the GFP mRNA, accumulated at a

higher level in the 1A, VPg-Pro-Pol, 2A and MP infiltration patches than in the NTB, VPg-Pro and CP infiltrated patches. Low levels of GFP mRNA were detected in all plants at 5 dpi, except in those infiltrated with the VPg-Pro-Pol and CP constructs, which contained significant amounts of GFP mRNA. As expected, huge amounts of GFP mRNA were found in plants agro-infiltrated with the P19 construct. Concerning the accumulation of the siRNAs at 5 dpi, they were hardly detectable in the plants transformed with the ArMV cDNAs but still detected in the GFP infiltrated zone. At 7 dpi, the GFP mRNA was only revealed in plants that contained P19.

The production of GFP in the infiltrated plants was assessed by western blots. At 3 dpi, it was not easy to distinct an effect of the viral cDNAs on the production of GFP (fig. 34). However, we observed an accumulation of GFP with the NTB, VPg-Pro, VPg-Pro-Pol constructs at 5 dpi compared to the other viral cDNAs but the GFP level was similar to that observed in the agro-infiltrated patches containing GFP alone. At 7dpi, the amount of GFP was significantly higher in plants that received the NTB construc compared to the other patch-tests, except for P19 in agreement with the fluorescence intensity of the plants at this stage. This suggests that NTB could play a role in the accumulation of the GFP.

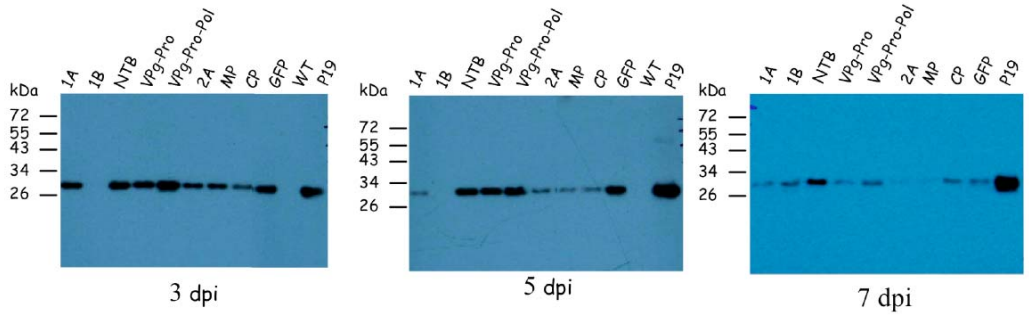


**Figure 34: Western blots of GFP in leaves of *N. benthamiana* plants agro-infiltrated with viral constructs**
Proteins were fractionated by SDS-PAGE and probed with a specific antibody raised against GFP. The immune complexes were detected by chemiluminescence (ECL).

### 5. Identification of suppressor(s) of RNA silencing by a heterologous expression approach

Synergistic viral diseases of higher plants are caused by the interaction of two distinct viruses or occasionally by two strains of the same virus. They are characterized by dramatic increase in symptoms and in accumulation of one of the co-infecting viruses (Damirdagh and Ross, 1967). For example, it has been demonstrated that the synergistic disease of tobacco plants resulting from a co-infection with *Potato virus X* (PVX, *Potexvirus*) and potyviruses such as *Potato virus Y* (PVY, *Potyvirus*), is characterized by an increase in symptom severity, a change in the regulation of PVX RNA replication, and an increase in the accumulation of PVX (Pruss *et al.*, 1997). This synergism is

due to suppression of PTGS by the potyvirus because these syndroms do not occur in plants infected by PVX alone. Brigneti *et al.*, (1998) have demonstrated by expressing PVY-encoded proteins in a PVX vector that the viral suppressor of RNA silencing is the Hc-Pro protein. This strategy also allowed identifying P19 of *Tomato bushy stunt virus* and protein 2b of *Cucumber mosaic virus* as silencing suppressors, upon their expression from the PVX vector (Pruss *et al.*, 1997; Brigneti *et al.*, 1998; Voinnet *et al*., 1999).

Therefore, we studied the synergistic effect between PVX and ArMV-NW, -Lv or -Lilac to see if these ArMV isolates accentuate the symptoms induced by PVX infection. For this, *N. benthamiana*, which is host for both PVX and ArMV, was inoculated with a mixture of saps obtained from host plants infected by ArMV and PVX, respectively.

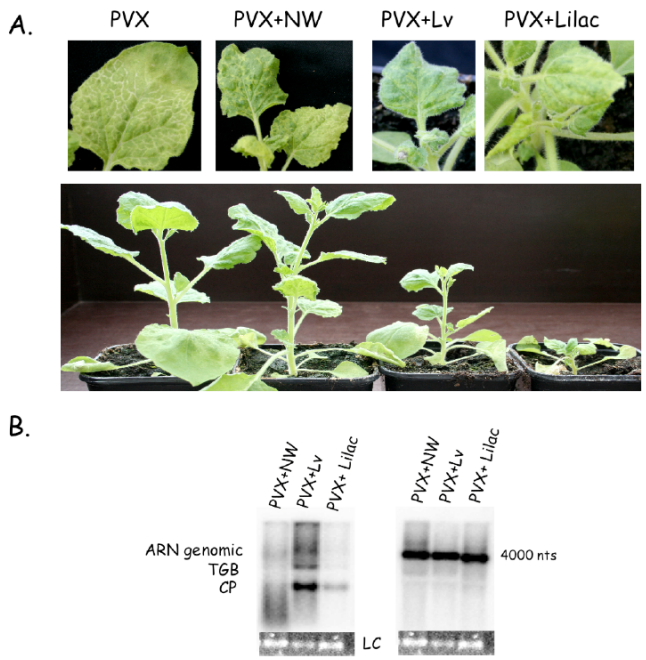


**Figure 35: Study of the synergy of PVX and ArMV co-infection in *N. benthamiana***
A. Symptoms of plants infected by PVX and PVX+ArMV at 21 dpi; B. Northern blots peformed on total RNAs extracted from the systemic leaves of ArMV infected plants probed with the 3' non-coding region of PVX cDNA (left) and the ArMV- NW MP cDNA (right).

At 21 dpi, the time required for the expression of the PVX symptoms, we compared the severity of symptoms between PVX-infected plants and the PVX + ArMV-infected plants (fig. 35A). The mosaic was more severe on systemic leaves of plants infected with PVX+ArMV than in plants infected with PVX alone. Moreover, the plants co-infected with PVX and ArMV-Lv or -Lilac isolates were smaller than those co-infected with PVX and ArMV-NW or infected only with PVX.

Northern blots were performed on total RNAs from infected plants using radiolabelled cDNAs corresponding to the MP gene of ArMV and the 3' non-coding region of PVX, respectively. The replication efficiency of the ArMV isolates was not affected by the presence of PVX, as shown by the presence of same quantities of RNA2 in plants co-infected with PVX and the NW, Lv and Lilac isolates, respectively (fig. 35B right). At the opposite, the genomic and subgenomic RNAs of PVX could only be detected in plants co-infected with the Lv and at a reduced level with the Lilac isolate; we only found the subgenomic RNA coding for the CP. In the presence of ArMV-NW, the PVX RNAs were partially degraded (fig. 35B left). Therefore, the severity of the symptoms in plants co-infected with PVX and ArMV isolates correlated with the replication and/or stability of PVX genome and thus, we hypothetized that the virulence of PVX was enhanced thanks to a suppressor activity of RNA silencing encoded by ArMV.

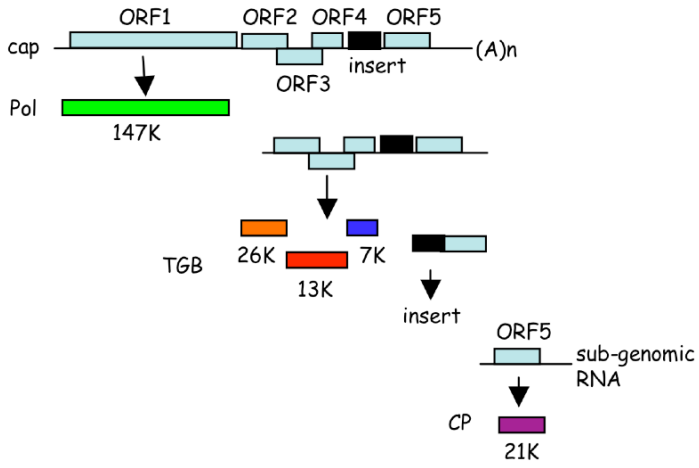


**Figure 36: Schematic representation of the PVX genome**
The PVX ORFs are represented by blue boxes and the ArMV coding sequence by a black box. The first ORF codes for the RNA polymerase (147K). The expression of the other proteins involves the synthesis of sub-genomic RNAs. The first sub-genomic RNA allows the translation of the triple gene block (TGB) corresponding to the 26 kDa, 13 kDa and 7 kDa proteins and of the ArMV-insert. The second sub-genomic RNA encodes the CP.

In order to characterize the putative ArMV-encoded suppressor, in the context of a PVX infection, the ArMV coding sequences were cloned in the PVX-vector, called pP2C2S (Baulcombe *et al.*, 1995). This vector contains the full-length cDNA of PVX under the control of bacteriophage T7 RNA polymerase promoter. Infectious PVX RNAs were produced by *in vitro* transcription of the PVX vector by the T7 RNA polymerase, after its linearization by digestion with endonuclease *Spe*I (Baulcombe *et al.*, 1995). The sequences coding for the proteins of the ArMV-NW, -Lv or -Lilac isolates were inserted in the *EcoR*V site, between the ORFs 4 and 5 of the PVX cDNA (fig. 36).

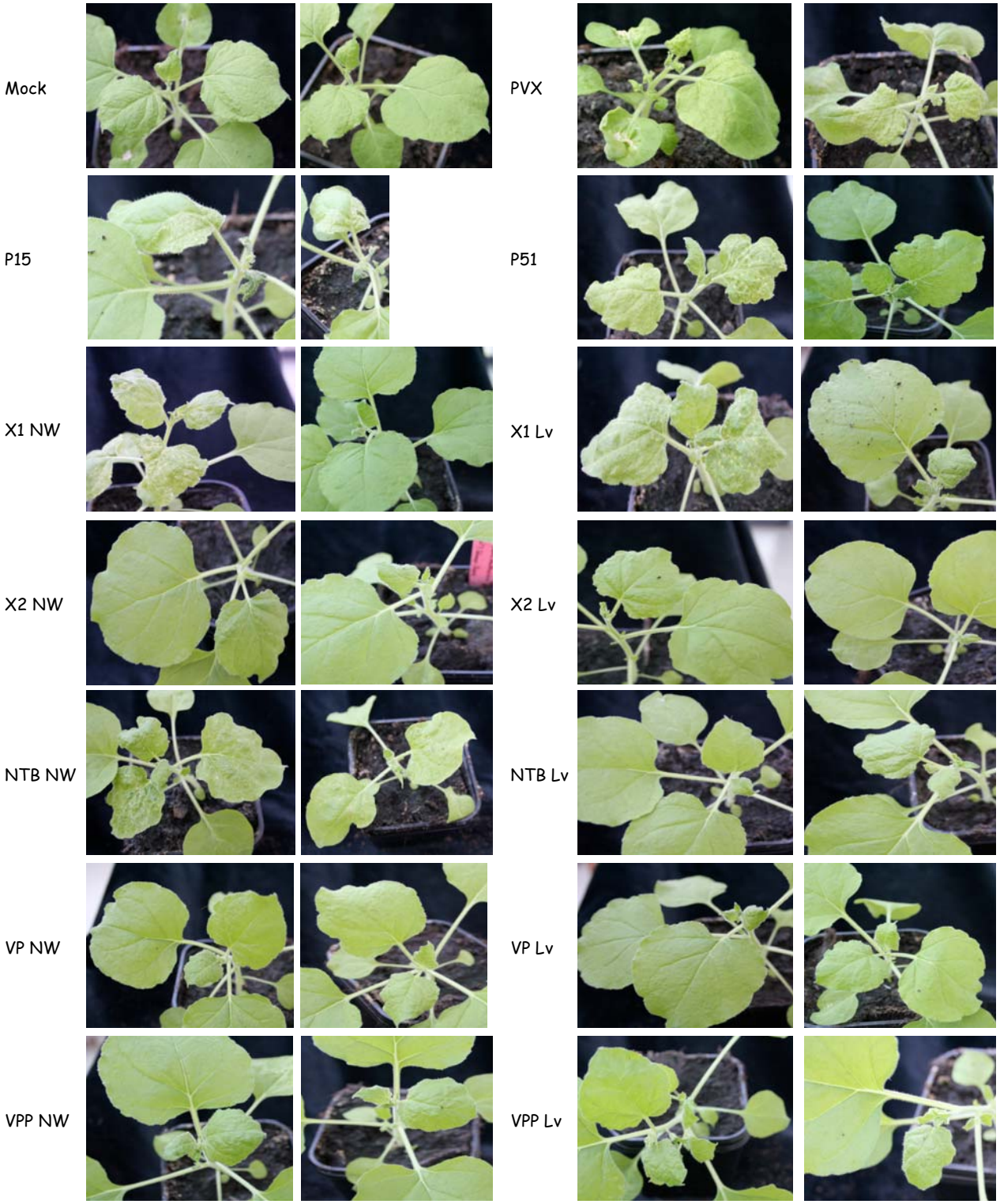


**Figure 37: Symptoms development in *N. benthamiana* plants infected by PVX or infected by RNA1 constructs-PC2S2 of ArMV-NW, and -Lv isolates**

1A (X1); 1B (X2); NTB, VP (VPG-Pro) and VPP (VPG-Pro-Pol) are the genes tested of the RNA1 of ArMV-NW and -LV inserted in pC2S2. P15 and P51 are proteins encoded by the *Peanut clump mosaic virus*

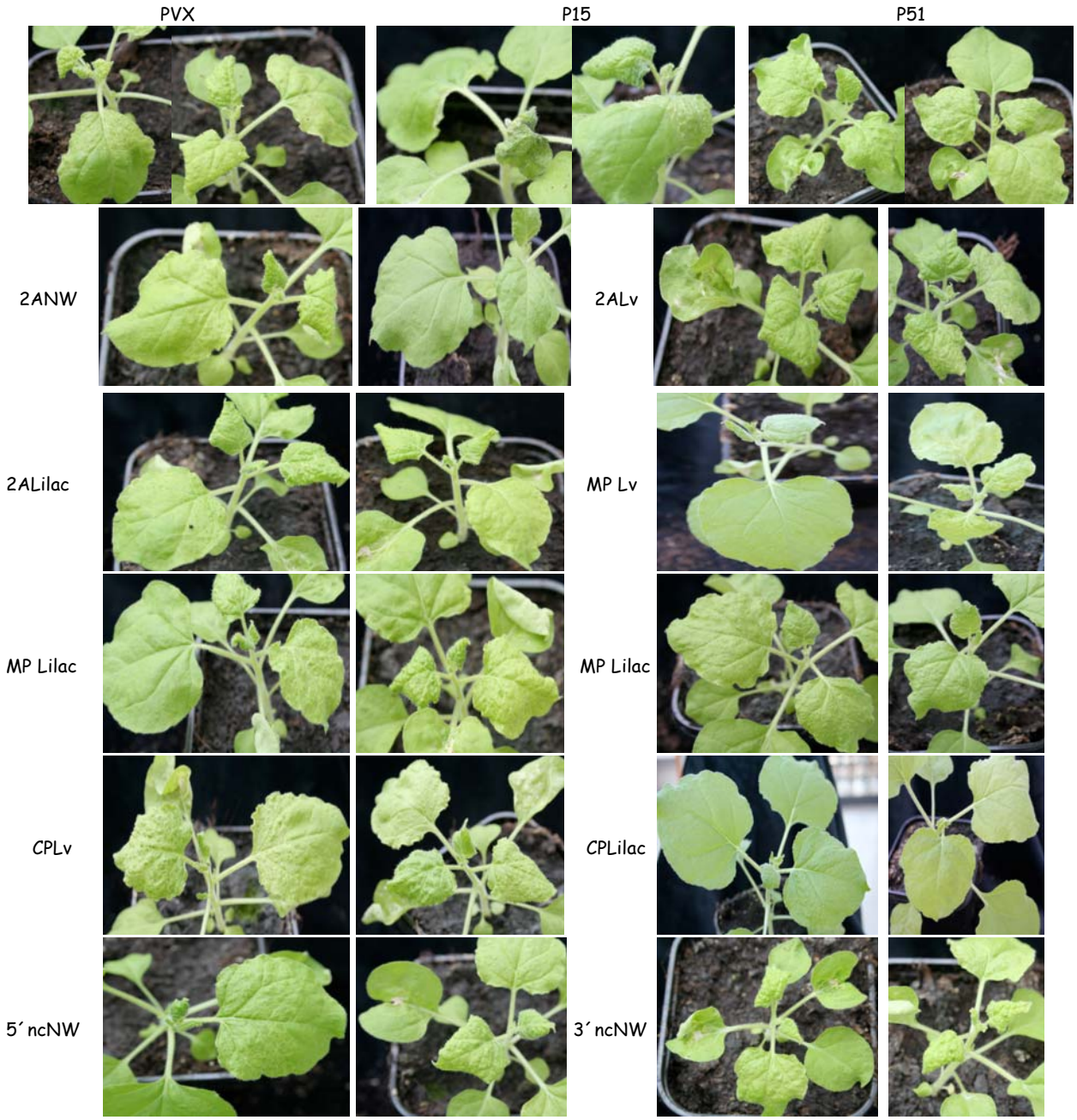


**Figure 37: Symptoms developement in *N. benthamiana* plants infected by PVX or infected by RNA2 constructs-PC2S2 of ArMV-NW, -Lv and -Lilac isolates**

Nc for non-coding region of the RNA2 of ArMV-NW

In this way, the ArMV proteins are expressed from a subgenomic RNA following duplication of the promotor sequence of the CP (Baulcombe *et al.*, 2005). *N. benthamiana* plants were mechanically inoculated with the chimeric transcripts and their symptoms were compared to those developed by plants infected with PVX. In parallel, we inoculated *N. benthamiana* with two recombinant pP2C2S clones, containing the protein P51 of *Peanut clump virus* (PCV, *Pecluvirus*) used as negative as it has no suppression activity and the protein P15 of the same virus, a known suppressor of RNA silencing, respectively (Dunoyer *et al*., 2002).

The same quantities of PVX and chimeric PVX transcripts were inoculated onto plants. The plants infected with the P15 pP2C2S construct showed necrosis on the new emerging leaves, at 21 dpi in contrast to the P51-expressing transcript, which did not modify the symptoms that are normally induced upon a natural PVX infection. When the VPg-Pro-Pol coding sequence of ArMV was inserted in the PVX vector, the plants were asymptomatic (fig. 37). Concerning the other chimeric constructs, no significant difference in the severity of the mosaic was observed between the plants infected with the wild-type PVX and with the chimeric viruses but a delay in the expression of the mosaic was noticed with the 1B, NTB and VPg-Pro constructs.

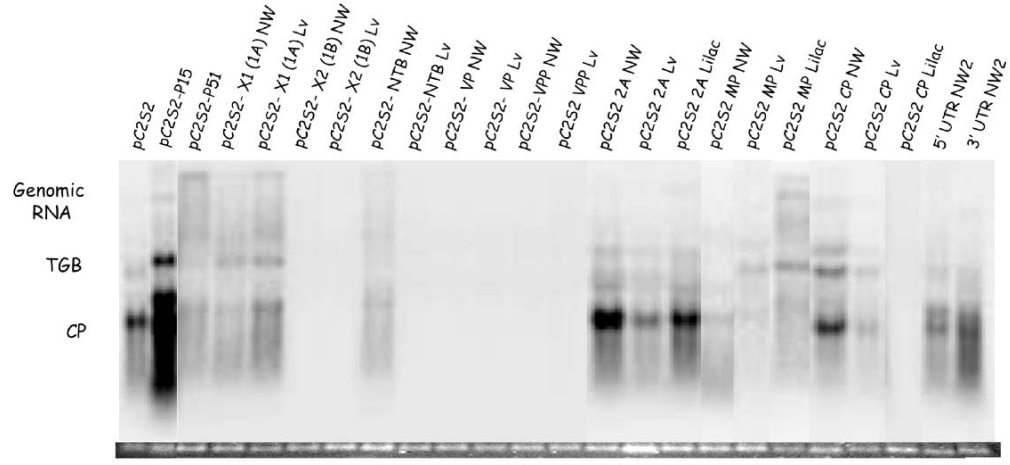


**Figure 38: Accumulation of the genomic and sub-genomic RNAs of PVX in *N. benthamiana* plants inoculated with different pC2S2 constructs**
5 μg of total RNAs were fractionated and probed with the radiolabelled 3' non-coding region of the PVX genome. Hybrids were analysed by phosphoimager after overnight exposure. The position of the genomic and sub-genomic RNAs, coding for the TGB and CP, are indicated at the left.

The levels of PVX genomic and subgenomic RNAs were determined by northern blots on total RNAs extracted from *N. benthamiana* plants, at 21 dpi with a labelled probe able to hybridize to the 3' non-coding region (fig. 38). As expected, the genomic and the subgenomic RNAs of PVX (TGB and

CP) could be easily detected in plants inoculated with the P15-pC2S2 construct since P15 suppresses RNA silencing. By contrast, plants infected with PVX contained low amounts of subgenomic RNAs; the genomic RNA could not be detected. Concerning the PVX constructs containing the ArMV sequences coding for 1B, VPg-Pro and VPg-Pro-Pol of the NW and Lv isolates and the NTB-Lv proteins, they were unable to replicate in *Nicotiana* plants or they were rapidly degraded after their inoculation onto plants. Indeed, no radioactive signal could be detected with these chimeric transcripts. By contrast, the PVX subgenomic RNA coding for the CP was present in plants inoculated with the chimeric PVX transcripts harbouring the 2A-ArMV and CP-NW sequences. Except, the PVX-2A-Lv construct, we found significantly higher amounts of PVX subgenomic RNA than in PVX-infected *N. benthamiana* plants.

These preliminary results suggest that the 2A protein of the ArMV-NW and -Lilac isolates could be involved in the suppression of RNA silencing, in our experimental conditions.

### 6. Effect of the replication of ArMV on the endogenous pathways of the RNA silencing

Since several years, it is known that RNA silencing is also implicated in the regulation of the cellular pathways, including differential development and metabolism (Baulcombe *et al.*, 2004; Brodersen and Voinnet, 2006; Dykshoom *et al.*, 2007). In addition to their role in defense, silencing pathways have important roles in gene regulation at the transcriptional and post-transcriptional levels through the action of small endogenous RNAs, micro-RNAs and trans-acting siRNAs, which are generated by a common core of effector proteins. As viral suppressors can impair the function of these proteins, we analyzed the accumulation of the micro-RNAs and trans-acting siRNAs in *A. thaliana* (ecotype Col 0) plants infected either by ArMV-NW or -Lv.

#### 6.1. Effect of ArMV replication on the micro-RNAs pathway

The micro-RNAs (miRNAs) pathway involves the combined action of DCL1 and DCL4, HYL1, HEN1 and AGO1 or AGO7, leading to the inhibition of the translation or the degradation of the target mRNA (Brodersen and Voinnet, 2006; Brodersen *et al.*, 2008). To determine if the replication of the ArMV-NW and -Lv isolates affects this pathway, we studied three miRNAs: miRNA168, miRNA162 and miRNA159, which target the mRNA encoding the AGO1, DCL1 and the MYB proteins, respectively (Martin and Paz-Ares, 1997; Jin and Martin, 1999). MYB proteins are transcription factors that regulate genes involved in the identity/or number of floral organs, leaf shape, abaxial-adaxial leaf asymmetric and lateral root formation (Rhoades *et al.*, 2002, Emery *et al.*, 2003).

*Arabidopsis* plants inoculated with ArMV-NW or -Lv showed a mosaic, at 15 dpi on the inoculated leaves as well as curling of leaves with the ArMV-Lv isolate. Other morphological changes were not observed at the level of the stem, leaves and floral organs (fig. 39).


**Figure 39: Symptoms expressed by *A. thaliana* (Col 0) plants infected by ArMV-NW or -Lv isolates.**

Infection of *Arabidopsis* plants by ArMV was confirmed by northern blot assays performed on total RNAs from plants at 7 dpi and 14 dpi, using a probe that hybridizes to the 2A coding sequence. ArMV RNA2 was already detected at 7 dpi and at 14 dpi in plants inoculated with NW and Lv isolates but the Lv RNA2 was at least twice as abundant as the NW RNA2, indicating that the Lv isolate replicated more efficiently in *Arabidopsis* than the NW isolate (fig. 40A).

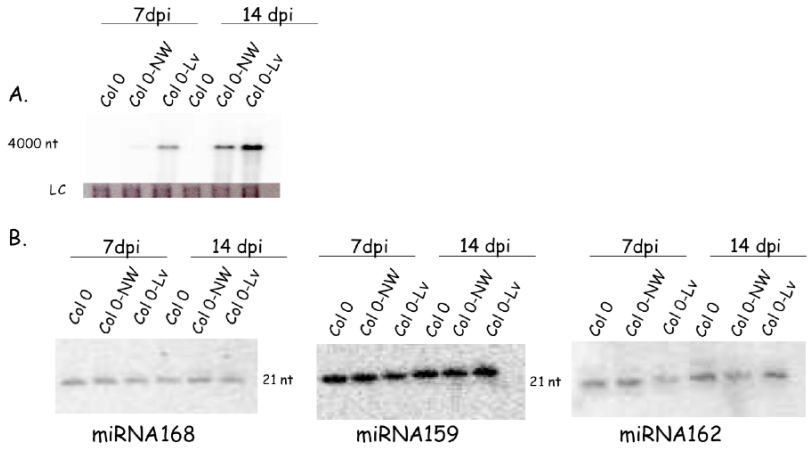


**Figure 40: Accumulation of viral ArMV and miRNAs in *Arabidopsis* Col 0 plants inoculated with ArMV-NW or Lv**

A. 5 μg of RNA were fractionated on a denaturing agarose gel (1.5%) and probed with the cDNAs labelled radioactively by random priming that correspond to the sequence encoding the 2A protein of ArMV-NW and Lv, respectively. The loading control is shown below the gel; B. The results of the northern blot experiments obtained with the low molecular weight RNAs (10 μg of the RNA loaded) are presented at the right. The hybridizations were performed with miRNA168, miRNA162 and miRNA159 labelled by the polynucleotide kinase in the presence of $^{32}$P-γ-ATP. The same membrane of the northern experiments was used for the three probes, after stripping

Northern assays performed on small RNAs showed that miR159 and miR168 accumulated at the same level in *Arabidopsis* plants infected either by ArMV-NW or -Lv and in mock-inoculated plants, at 7 dpi and 14 dpi. Concerning miR162, we observed a non-significant increase of the quantity

at 14 dpi compared to 7 dpi in plants infected with ArMV-Lv (fig. 40B). Taken together, these data indicate that replication of ArMV has no effect on the accumulation of these particular miRNAs and consequently, confirm that ArMV species does not impair the expression of AGO1 and DCL1 involved the processing of these miRNAs (see paragraph 2).

### 6.2. Effect of ArMV infection on the trans-acting siRNAs pathway

In the endogenous pathway of the RNA silencing leading to the production of trans-acting RNAs (ta-siRNAs), the pre-tasiRNA are converted into dsRNA by the action of RDR6 and SGS3 giving rise to the mature ta-siRNAs (21 nts). The RDR6-SGS3 involvement is reminiscent of siRNA biogenesis in S-PTGS. We compared the accumulation of ta-siRNAs in the leaves of healthy and ArMV-infected Arabidopsis Col 0 plants (fig. 41). For this, membranes that served for the study of miRNA, were stripped and hybridized with a specific probe to detect ta-siRNA255. No significant difference in the ta-siRNA255 accumulation was observed in leaves at 7 dpi and at 14 dpi, indicating that the replication of ArMV has no effect on the accumulation of the trans-acting siRNA255 and thus, ArMV does not impair the activity of the effectors involved in this pathway.

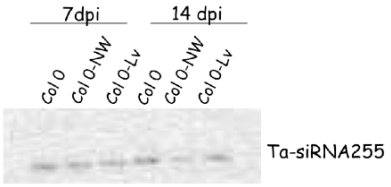


**Figure 56: Analysis of the effect of ArMV-NW or -Lv infection on the accumulation of ta-siRNA255 in Arabidopsis plants**

# DISCUSSION

Replication of ArMV RNAs probably occurs in membranous vesicles derived form endoplasmic reticulum like for other nepoviruses (Gaire *et al.,* 1999; Ritzenthaler *et al.,* 2002; Han and Sanfaçon, 2003), suggesting that the ArMV genome and its replicative forms are not exposed to the RNA silencing machinery. It was already described for *Brome mosaic virus* (BMV, *Bromovirus*), which also replicates in membrane-bound vesicles, that the viral RNAs are protected from degradation by the host ribonucleases, including ribonucleases of the RNA silencing machinery (Schwartz *et al*., 2002). Moreover, the RNA genome of ArMV should also be protected from silencing during its intra- and intercellular movements since it moves as virions (Ritzenthaler *et al.,* 1995b; Laporte *et al.,* 2005). However, ArMV does not completely escape the RNA silencing machinery, as evidenced by the reduced levels of viral RNA observed in recovered leaves.

### 1. ArMV isolates and RNA silencing

Experiments performed on RNA silencing of GFP in 16C transgenic *Nicotiana benthamiana* plants infected with ArMV, showed that ArMV-NW and ArMV-Lv isolates were unable to prevent the initiation of RNA silencing. However, ArMV-Lv seems to delay and/or to hamper the systemic movement of the GFP silencing signal in contrast to the ArMV-NW isolate. We also observed that neither ArMV-Lv nor ArMV-NW were able to fully revert established silencing against the GFP transgene as described for the *Tomato ringspot nepovirus* (Jovel *et al.*, 2007).

The class of 21 nt-long RNAs represents the majority of virus-derived small RNAs (vsRNA) found in wild-type *Arabidopsis thaliana* infected with ArMV, indicating that DCL4 is the primary antiviral Dicer implicated in the silencing of the ArMV RNA. This was confirmed upon infection of *dcl2* and *dcl3* mutant plants with ArMV and by the fact that the level of DRB4, the co-factor of DCL4, increased in ArMV-infected *Arabidopsis* plants. When DCL4 was inactivated, DCL2 rescued antiviral silencing by generating 22 nt-long vsRNAs. The latter were not detected in ArMV-infected wild-type plants, confirming the surrogate role of DCL2 in antiviral defense. DCL3-dependent 24 nt-long vsRNAs were only produced when both, DCL4 and DCL2, were inactivated, confirming that viral RNAs can be substrates for DCL3 in the absence DCL4 and DCL2 (Deleris et al., 2006). The presence of the 24 nt-long RNA suggests a functional redundancy among DCLs rather than an antiviral activity of DCL3 (Brodersen and Voinnet, 2006). At the opposite, no significant contribution of DCL1 was found against ArMV because DCL1-dependent 21 nt-long RNAs did not accumulate in a triple *dcl2-dcl3-dcl4* mutant background. In summary, DCL4 and DCL2 are the main slicers of the ArMV RNAs in *Arabidopsis* as described for many other plant viruses, in particular for RNA viruses (Xie *et al.,* 2004; Bouché *et al.*, 2006, Deleris *et al.*, 2006; Fusaro *et al.,* 2006, Diaz-Pendon *et al.*, 2007).

The detection of viral RNAs in ArMV-infected *Arabidopsis* plants suggested that ArMV is able

to counteract the host RNA silencing machinery. The hypothesis that ArMV codes for a RNA silencing suppressor is reinforced by the fact that the symptoms induced by *Potato virus X* (PVX) are accentuated when plants are co-inoculated with PVX and ArMV. However, the ArMV silencing suppressor(s) are barely efficient, compared for example to P19 of the *Tomato bushy stunt virus* (TBSV), since only low levels of viral RNAs are found in ArMV-infected plants (see part II), The ArMV-Lv isolate is more virulent than ArMV-NW as evidenced by the accumulation of large amounts of viral RNA, a high rate of systemic infection and the induction of severe symptoms. This suggested that the ArMV-Lv isolate is more adapted to its hosts and in particular, that its RNA silencing suppressor is more efficient that the NW homologue. However, the presence of similar amounts of viral RNAs in recovered leaves (see part IIA) upon infection with ArMV-NW and -Lv isolates does not corroborate this hypothesis.

Disease symptoms induced upon viral infection result from interactions between viral and cellular components, that perturb the plant physiology. In particular, they are due to the fact that viral RNA suppressors interfere with the regulation of the expression of cellular proteins, as the host gene-silencing and antiviral defense pathways operate through common effectors and mediator molecules. Therefore, we investigated the effect of ArMV infection on the endogenous RNA silencing pathway. Our preliminary results showed that ArMV infection did not modify the accumulation levels of miRNA159, miRNA162, miRNA168 and of ta-siRNA255 in *Arabidopsis*. However, we cannot exclude that ArMV infection affects others miRNAs or ta-siRNAs or miRNAs* rather than miRNAs, as it was observed for the P19 suppressor of TBSV, Hc-Pro of potyviruses and p21 of *Beet yellow virus* (Chapman *et al.,* 2004; Dunoyer *et al.,* 2004). Indeed, some suppressors have the capacity to bind single-stranded RNAs and thus, can stabilize miRNA*, the passager strand of miRNA duplexes which is normally degraded (Chapman *et al.,* 2004; Dunoyer *et al.,* 2004; Csorba *et al.,* 2007).

**2. Suppressive effect of viral RNAs of ArMV**

To characterize the ArMV suppressor(s) of RNA silencing (VSR), we used two experimental approaches: agrobacterium-mediated transient suppression assay and expression of ArMV sequences cloned in a PVX vector. The first approach consisted to induce PTGS or S-PTGS of GFP in 16C transgenic and wild-type *N. benthamiana* plants, respectively, by agro-infiltration of a GFP replicon together with a viral construct to see if the latter suppresses RNA silencing. In the second approach, the presence of a RNA silencing suppressor results in the modification of the symptoms and in higher levels of sub-genomic and genomic PVX RNAs, in *N. benthamiana*.

ArMV-NW RNA1 and RNA2 seem to suppress RNA silencing when they are expressed separately, in PTGS (16C transgenic plants) and S-PTGS (wild-type plants) assays but they were unable to impair the systemic spread of the GFP silencing signal. Moreover, no synergetic effect in the suppression of RNA silencing could be observed when both genomic RNAs were co-expressed in *N.*

*benthamiana* plants in order to mimic an authentic ArMV infection. The suppression efficiency of the ArMV RNAs is relatively weak compared to that of the suppressor P19 of TBSV. Indeed, the GFP mRNA accumulated only at low levels in *N. benthamiana* plants in the presence of the ArMV RNAs whereas huge amounts of GFP mRNAs were found in the same experimental conditions in plants that expressed P19. Whether the suppression activity is due to the ArMV RNAs themselves instead of the encoded polyproteins and/or the mature proteins cannot be excluded. Indeed, it has been demonstrated that the replication and accumulation of the RNA of *Red clover necrotic mosaic virus* is involved i*n* the suppression of RNA silencing Takeda *et al.,* (2005).

The expression of ArMV RNA1 was more efficient in the suppression of RNA silencing than RNA2, suggesting that RNA1 codes for a VSR which has a stronger activity than the VRS encoded by RNA2. However, this might also be due to a dose effect in our experimental conditions, RNA1 having the capacity to replicate in the cytoplasm in contrast to RNA2. Indeed, once RNA1 has been produced by the cellular RNA polymerase II, it can be expressed into proteins (precursors and mature proteins) among which the RNA-dependent RNA polymerase and the proteinase (Viry *et al.,* 1993; Ritzenthaler *et al.,* 1999) and consequently, it can replicate at a high level in *N. benhamiana* plants. At the opposite, the production of RNA2 is totally dependent on the cellular transcription machinery. Finally, as the viral RNAs are synthesized in the nucleus, we can also hypothetize that RNA2 is more susceptible than RNA1 to degradation by nuclear-located DCLs because of the presence of specific fold-back structures. For instance, it has been demonstrated that the secondary structure in the leader region of pregenomic RNA of the *Cauliflower mosaic virus* is targeted by the four DCLs in Arabidopsis (Moissiard and Voinnet, 2006; Blevins *et al.,* 2006).

Silencing suppression can be mediated by RNA rather than by proteins, as demonstrated by the resistance of viroids to RNA silencing however they do not code for any proteins (Wang *et al*., 2004; Ameres *et al.,* 2007). It was shown that double-stranded regions of viroids are resistant to RISC and that viroid-derived RNAs with 5'-U termini might saturate AGO1, the effector involved in antiviral defense. Several animal viruses also use RNAs to inhibit the defense system of their hosts, the best example being the double-stranded virus-associated RNAs (VA1).The latter binds to the RNA-dependent protein kinase (PKR) and thus, prevents its activation that normally leads to inhibition of translation initiation.

Fernandez *et al.,* (1998) showed that the 5' non-coding region of the RNA2 of *Grapevine chrome mosaic nepovirus* (GCMV) dramatically exacerbates the symptoms of PVX in *N. benthamiana* (Fernandez *et al.,* 1998). These authors hypothetized that the stem-loop structures localized in the 5' non-coding region of GCMV may influence the development of necrosis, either by interfering with host factors, or by stabilizing some essential structure. However, they did not demonstrate that the 5' non-coding region of GCMV is able to suppress RNA silencing. The presence of conserved sequences containing notably U stretches and stable secondary structures in the 5' and 3' non-coding regions of ArMV RNAs, led to the hypothesis that these untranslated regions might play a role in the suppression

of RNA silencing. However, preliminary results obtained with the untranslated regions of the ArMV-NW isolate, indicate that they have no effect on RNA silencing neither in wild-type nor in 16C transgenic *N. benthamiana* plants. Moreover, the expression of these regions from a PVX vector did not modify the symptoms induced by PVX.

How the viral transcripts, expressed in the nucleus from viral cDNAs, operate to suppress RNA silencing, remains unanswered. We cannot exclude that the suppression activity primarily occurs in the nucleus.

Like RNA1 and RNA2, ArMV-NW did not suppress systemic RNA silencing when PTGS was triggered in ArMV-infected 16C transgenic *Nicotiana* plants, by agro-infiltration of the GFP replicon. By contrast, ArMV-Lv had an effect on the systemic spread of RNA silencing of the GFP thus indicating that ArMV isolates evolved different strategies to counteract the RNA silencing pathway. It would be useful to obtain full-length infectious Lv cDNA clones to investigate the counterdefense strategy of this virulent ArMV isolate.

### 3. NTB and VPg and/or protease and/or polymerase suppressors of RNA silencing?

Both ArMV RNAs are apparently able to suppress RNA silencing, suggesting that at least, two viral components (RNAs and/or proteins) are implicated in this function. It was shown that *Citrus tristeza virus* (CTV, a *Closterovirus*), encode several distinct VSRs, which each exhibits distinct features in silencing suppression (Lu *et al.*, 2004). Indeed, P23 suppresses intracellular silencing whereas P20 and the capsid protein (CP) acts at the intercellular level.

For this study, we had to face a major difficulty, the inability to determine if the ArMV proteins were properly expressed in the plants infiltrated with agrobacteria harbouring the corresponding cDNAs. Indeed, upon expression of hemagglutinin (HA)-tagged ArMV proteins (antibodies raised against the ArMV proteins are not available), we could only detect by western blot assays, using anti-HA antibodies, the movement and the capsid proteins (MP and CP, respectively) in total protein extracts. The failure to detect the other ArMV proteins might be due to the fact that they are probably anchored in the membrane derived from the endoplasmic reticulum as described for the proteins of *Tomato ringspot nepovirus* (ToRSV) and that, the yield of extraction was below the threshold of detection (Han *et al.*, 2003; Wang *et al.*, 2004; Zhang *et al.*, 2005; Chrisholm *et al.*, 2007). Fusion of the viral proteins to a fluorescent protein should be a prerequisite for the forthcoming investigations to ensure that these proteins are really expressed upon agro-infiltration of host plants.

Despite the difficulties encountered in this study, it seems that the VPg-Pro and VPg-Pro-Pol constructs of ArMV interfere with the establishment of RNA silencing of the GFP transgene while in the S-PTGS context, only VPg-Pro-Pol has an effect on the GFP mRNA accumulation. As the VPg-Pro-Pol polyprotein contains the protease activity, we do not know if the interference with RNA silencing is due to the polyprotein and/or to the mature proteins as they can be released from this

precursor by *cis*-cleavages. The biological relevance of cleavage intermediates during the replication cycle of many viruses, which use the polyprotein-cleavage strategy, has been described (Sawicki, 1994; Shirako and Strauss, 1994). Therefore, it is not excluded that ArMV cleavage intermediates are directly involved in the suppression of RNA silencing or contribute to this function by reinforcing the activity of VSR(s). To answer this question, it would be necessary to test constructions coding for the mature proteins (VPg, protease and pol) and, at the opposite, to test precursors whose cleavage sites are inactivated by mutagenesis.

The ArMV NTB protein might also be a good candidate for the suppression of RNA silencing since the GFP was still detectable at 7 dpi in patches agro-infiltrated with NTB and GFP replicon whereas with other viral constructions, GFP was no more detectable. As the NTB protein was unable to preserve the GFP mRNA from degradation in these assays, we hypothetize that it presumably intervenes at the silencing signalling step. The interference of NTB with the systemic spread of the GFP silencing could be explain by its capacity to bind nucleic acids and to its helicase activity. Indeed, many VSRs suppress RNA silencing through the binding of sRNAs i.e. P19 (Silhavy *et al.,* 2002; Chapman *et al.,* 2004; Dunoyer *et al*., 2004), P21 of *Beet yellow virus* (BYV, *Closterovirus*, Lu *et al*., 2004), P15 of PCV (Merai *et al.*, 2006), Hc-Pro (Lakatos *et al*., 2006; Merai *et al.,* 2006), NS3 of *Rice hoja blanca virus* (RHBV, *Teniuvirus*, Hemmes et al., 2009), P38 of TCV (Thomas et al., 2003) and γb of *Barley yellow mosaic virus* (BYMV, *Hordeivirus*, Yelina *et al.,* 2002) and P126 of *Tobacco mosaic virus* (TMV, *Tobamovirus*, Csorba *et al.,* 2007). For instance, the sequestration of the 21 nt siRNA duplexes by P19 precludes the cell-to-cell movement of the systemic signal ahead of the infection front. Immunoprecipitation of NTB expressed in transgenic plants coupled with the detection of siRNA* and miRNA* should determine if NTB sequesters vsRNAs as it was shown for P19, Hc-Pro and P21 (Chapman *et al.,* 2004; Dunoyer *et al.* 2004). Naturally, such a strategy should also be envisaged for other putative VSRs of ArMV. Characterization of the cellular component(s) which are co-immunoprecipitated, might allow to decipher the steps of the RNA silencing pathway which are targeted by the viral suppressors.

The type B-satellite of ArMV-NW had no effect on the RNA silencing contrary to the satellites of genimiviruses which are involved in the suppression of RNA silencing as demonstrated for the BC1 protein of satellite DNA B of *Tomato leaf curl Java Virus* (ToLCJAV, *Begomovirus*), *Bhendi yellow vein mosaic virus* (BYMV, *Begomovirus*) and *Tomato yellow leaf curl China virus* (TYLCCNV, *Begomovirus*). This protein impairs the propagation of the systemic signal of RNA silencing (Cui *et al.,* 2004; Gopal *et al.*, 2007; Kon *et al*., 2007).

The heterologous expression of the ArMV proteins from the PVX vector, as an alternative approach, did not confirm our previous results mainly because many pC2S2 recombinants vectors were unable to replicate as evidenced by the absence of genomic and subgenomic RNAs of PVX. We suppose that the insertion of sequences with a length that exceeds 1,000 nt could be limiting for the replication of the PVX or for the synthesis of subgenomic RNAs. However, preliminary results

indicate that protein 2A might be involved in the suppression of RNA silencing. The genomic and subgenomic RNAs of PVX accumulated at higher levels in comparison to PVX infection, when the protein 2A of ArMV-NW, -Lv and -Lilac isolates was expressed from the PVX vector. However, surprisingly, protein 2A did not modify the symptoms albeit we identified this protein as a symptom determinant in ArMV infection.

Two other strategies for the assessment of VRS were described and could be used to identify the ArMV VSR(s); i) agrobacterium-mediated delivery of the GFP mRNA by using a GFP-tagged minireplicon of BYV (miniR-GFPΔp21) and ii) the development of a new VSR assay based on the biological properties of *Turnip crinkle virus* (TCV) deleted of the capsid protein. In the first experiment, the identification of the VSR is based on the increase of replicon infectivity and the number of fluorescent cells is 25 fold higher than that of miniR-GFPΔp21 (Dolja et al., 2006). Concerning the second assay, the increase in foci size of TCV-sGFP in *N.benthamiana* preinfiltrated with a viral candidate is directly correlated with suppressor activity and not a cell-to-cell movement function. Moreover, this assay permits to utilize two delivery methods (cytoplasmic through infectious transcripts and nuclear through agroinfection).

The use of other susceptible herbaceous hosts should also be considered due to the difference in symptoms severity and in the accumulation of viral RNAs depending on the ArMV isolate.

# CONCLUSION

The grapevine fanleaf disease is one of the most widespread and damaging nepovirus disease of grapevines. This disease is mainly due to *Grapevine fanleaf virus* (GFLV) and to a lesser extent, to *Arabis mosaic virus* (ArMV) and *Raspberry ringspot virus*. Unlike GFLV, ArMV has a large wild host range. It infects not only grapevines but also several other cultivated plants worldwide distributed such as strawberries, raspberries and hop.

A basic measure to preserve crops and plant stocks from ArMV infection is the supply of plant material declared free of virus contamination following a strict certification scheme. Soil fumigation and/or fallow at least during one year, are also strategies to limit the spread of the ArMV-induced diseases. However, a better comprehension of the host-virus interactions could be a good challenge to develop new alternatives of protection and to combat ArMV infection.

The aim of my Ph.D work was to identify the viral determinant(s) involved in the expression of symptoms and to study the strategy and factor established by the virus to induce disease, in particular to identify the ArMV suppressor(s) of RNA silencing.

**1. Symptoms expressed by ArMV**

The most common symptoms induced by ArMV are mosaic, ringspots, mottling, necrosis and several growth abnormalities including enations and stunting. The type of symptoms mainly depends on the host plant, the virus isolate and growth conditions. In order to identify the viral determinant(s) involved in the ArMV-induced symptoms, we studied three isolates of ArMV from different origins: ArMV-NW isolated from grapevine, ArMV-Lv from *Ligustrum* (privet*)* and ArMV-Lilac from lilac. These viral isolates induce different symptoms on herbaceous hosts (*Chenopodium quinoa, Nicotiana benthamiana, N. tabacum* and *N. glutinosa*): ArMV-NW does not induce any symptom or eventually, a mild mosaic whereas ArMV-Lilac and -Lv are moderate and virulent isolates, respectively, on herbaceous plants. The latter displayed upon infection with these ArMV isolates, mosaic or necrosis depending on the plant species. ArMV-Lv is the sole isolate able to infect the four herbaceous plants whereas the two others are restricted to *C. quinoa* and *N. benthamiana*.

The viral determinant(s) involved in the expression of symptoms were investigated using chimeric cDNA constructs obtained by exchanging sequences of the full-length cDNAs of ArMV-NW with their counterparts of ArMV-Lv and -Lilac isolates. This study permitted to determine that the N-terminal region of the protein 2A encoded by RNA2 is involved in the development of symptoms. This region is very divergent among ArMV isolates compared to the other viral proteins. However, as this protein induces different symptoms on the same host plant, depending on the viral context (native or chimera viruses), other viral proteins should participate to the expression of symptoms. The protein 1A and the movement protein (MP) encoded by RNA1 and RNA2, respectively, are presumably also involved in the symptoms development but their contribution seems to be of minor importance. As described for several plant viruses, the disease symptoms probably result from a synergistic effect of

these different proteins and their precursors; the latter are intermediate cleavage products of the polyproteins encoded by the both ArMV RNAs.

Transgenic plants expressing the proteins 1A and 2A and the MP, respectively, should permit to demonstrate unambiguously that these ArMV proteins are symptom determinants. The characterization of their host partners should give insights in the cellular pathways perturbed by these viral proteins and in the underlying mechanism leading to the expression of symptoms.

### 2. The recovery phenomenon in *Nicotiana benthamiana* upon infection with ArMV

ArMV-NW and ArMV-Lv are both able to systemically infect *N. benthamiana* plants but the replication of ArMV-Lv is more efficient as demonstrated by the presence of large amounts of viral RNA compared to the those produced by the ArMV-NW isolate, at the same time of infection. However, the levels of ArMV-Lv and -NW RNAs decrease simultaneously during the course of the infection and finally, plants infected with ArMV-Lv or ArMV-NW, contain very low and identical amounts of RNA. The decrease of the viral RNAs was accompanied by an accumulation of the viral-derived small interfering RNAs (vRNAs). The mapping of ArMV-NW and -Lv-derived vsRNAs showed that the vast majority of these small RNAs, were generated by the slicing of RNA2. The difference between the levels RNA1- and RNA2-derived siRNAs is probably due to the fact that RNA2 replicates better than RNA1 however, we cannot totally exclude that RNA2 is a favourite target of the RNA silencing machinery.

The phenomenon corresponding an initial symptomatic infection followed by symptoms attenuation or elimination accompanied by a decrease of the viral RNA, is called recovery (Wingard, 1928; Covey et al., 1997; Ratcliff et al., 1997; 1999). It was already described for another nepoviruses (Jovel et al., 2007; Siddiqui et al., 2008) and for several other plant viruses. Recovery results from the activation of RNA silencing machinery involved in antiviral defense (Baulcombe et al. 2004; 2005; Voinnet et al., 2005). The difference in the behaviour of ArMV-Lv and ArMV-NW in *N. benthamiana* suggested that the former isolate developed a counterdefense strategy that is more effective at least at the beginning of the systemic infection, than that of the ArMV-NW.

The establishment of recovery is often associated with the capacity of the plant resist to a second infection. This immune property has been exploited since many years to develop of a cross-protection strategy, in which a first inoculation of the plant with a mild viral strain protects against a closely related virulent strain (Fulton, 1975; Yamaya *et al.*, 1988; 1989; Huss *et al.*, 1989; Fuchs *et al.*, 1997; Lecoq *et al*. 1998). The effectiveness of this approach was recently tested in natural vineyards, using mild strain of GFLV (Komar *et al.*, 2008). However, this cross-protection strategy is not applicable to all virus strains. Indeed, our study reveals that a protection against ArMV mild (NW) and virulent (Lv) isolates is only induced upon a primary infection with the virulent strain and not the contrary, suggesting that cross-protection might involve various virus-host interactions that may be

different depending on the viral isolates and their hosts. Moreover, the defense and the counterdefense strategies deployed by the plant and virus, respectively, may be responsible for this discrepancy. Therefore, the knowledge of the viral counterdefense mechanism is a prerequisite to better understand the host-virus interactions.

### 3. RNA silencing and ArMV

RNA silencing is an evolutionarily conserved system that functions as an antiviral mechanism in plants and animals. To counteract RNA silencing, viruses express silencing suppressor proteins (VSRs), which are diverse in sequence and structure depending on the virus (For a review, Wadsworth and Dunoyer, 2010).

When I started my thesis, many studies have been carried out to identify the effectors involved in this antiviral pathway in *Arabidopsis.* In parallel, different experimental approaches have been developed to identify new VSRs and to determine how they suppress RNA silencing (Moissard and Voinnet, 2004; Li and Ding, 2006). It appears that several VSRs are defined as RNA binding proteins that interfere with RNA silencing pathway by sequestering siRNAs while others, target argonaute proteins or interfere with the silencing signal (for review, Wadsworth and Dunoyer, 2009). These VSRs were also defined as pathogenicity factors and their transgenic expressing result to developmental abnormalities (Pruss *et al.,* 1997; Voinnet *et al.,* 1998; Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998). Therefore, the characterization of VSR(s) encoded by ArMV has been investigated, following the identification of symptom determinants which could be good candidates for VSR(s) and the study of the recovery phenomenon in plants infected with ArMV-NW and -Lv.

We infected *Arabidopsis* plants deficient in one, two or three dicer-like proteins (DCL) with ArMV-NW and -Lv isolates in order to identify the DCL involved in the degradation of the ArMV genomic RNAs. As described for other viruses, DCL4 and DCL2 are the main dicers involved in the slicing of the viral RNAs of ArMV (Voinnet, 2005; Deleris *et al*., 2006; Fusaro *et al*., 2006; Diaz-Pendon *et al*., 2007). VsRNAs of 21 nt, resulting from slicing of RNA by DCL4, were detected in majority in ArMV-infected plants whereas DCL2-specific 22 nt-long vsRNAs, were generated upon DCL4 inactivation.

ArMV-Lv isolate is able to interfere with the spread of the systemic signal of RNA silencing. In order to characterize the viral factor(s) involved in this counterdefense, two experimental approaches have been used: agrobacterium-mediated transient assay and expression of suppressor by recombinant PVX, resulting in enhanced symptoms. The involvement of the ArMV RNAs was also addressed, out of a viral context, to determine if they have the capacity to suppress RNA silencing, Our results led to the conclusion that RNA1 and RNA2 of the ArMV-NW isolate, and at a lesser extent the combination thereof, have the capacity to interfere with the establishment of the GFP silencing but not with the propagation of the systemic signal. A similar conclusion was drawn with *Red clover necrotic mosaic*

*virus:* RNA1 was identified as the main component of the suppression of S-RNAi. However, ArMV-NW was unable to suppress the systemic silencing of GFP whereas ArMV-Lv impaired it when the GFP silencing was triggered in ArMV-infected plants thus, suggesting that the suppression activity of the ArMV-encoded VSRs may depend on the stage of viral infection.

Preliminary results obtained when we tried to identify ArMV VSRs, indicate that VPg-Pro and/or VPg-Pro-Pol cleavage intermediates and/or the mature proteins interfere with the initiation step of RNA silencing and NTB, on the propagation of the systemic silencing. We could not conclude concerning the eventual role of protein 2A in the suppression of RNA silencing.

The relationships between viruses and their hosts are very complex and involve several factors, as suggested by the observations and results obtained during my thesis. The various analysis discussed show a strong overlapping between these different mechanisms but also the involvement of other pathways not study in this thesis. Therefore, the study of the impact of the viral infection at the level of the plant should be considered as a whole, by studying the perturbations induced by the virus and the cellular factors involved in the establishment of viral infection according the stage of infection. The identification of the viral factors implicated in the host range must be undertaken to determine if the severity and variability of the symptoms may be explained at this level. As already suggested in previous analysis, the role of the processing intermediates must also be investigated and does not confine to the mature protein. The levels of accumulation of these cleavage products must also pursued more precisely according the ArMV isolates.

# MATERIAL AND METHODS

# I. Material

### 1. Different host plants

Two indicator plant species are preferentially used for the characterization of *Arabis mosaic virus* (ArMV) determinants involved in the expression of symptoms, *Chenopodium quinoa* and *Nicotiana benthamiana*. However, others herbaceous hosts susceptible to nepoviruses such as *Nicotiana tabacum* and *Nicotiana glutinosa*, are also tested with the ArMV isolates.

*Nicotiana benthamiana*, wild type and 16C transgenic plants (provided by Dr. Baulcombe, Cambridge University, UK) are used to identify the ArMV RNA silencing suppressor(s). The 16C transgenic plants contain a single copy of the gene coding for the green fluorescent protein (GFP) and consequently, appear green fluorescent under UV light whereas the wild type plant are red due to the absorption of UV by the chlorophyll.

Wild type Arabidopsis thaliana (ecotype Columbia, col 0) and several single, double and triple *dcl* mutants in which the Dicer-like gene(s) are knockout, are also used for the study of RNA silencing suppression: *dcl2*, *dcl3*, *dcl4*, *dcl2-3*, *dcl3-4*, *dcl2-4* and *dcl2-3-4* mutants (provided by P. Dunoyer, IBMP, France).

### 2. Viruses and infectious clones

Several *Arabis mosaic virus* isolates are studied: ArMV-NW (NW for Neustadt and der Weinstrasse, isolate of grapevine), ArMV-Lv (Lv for Ligustrum vulgare isolate) and ArMV-Lilac. The infectious cDNA clones corresponding to RNA1 and RNA2 of ArMV-NW, designed FL1 and FL2 (FL for Full-length), respectively, are available in the laboratory. These cDNAs are cloned into the vector pCass II (see paragraph 4.2).

The cDNA of the PVX RNA genome is cloned in pC2S2 vector (obtained from P. Dunoyer, IBMP, France) to synthesize the corresponding transcripts by *in vitro* transcription assays.

### 3. Bacteria strains

#### 3.1. *Escherichia coli* strains

Two E. coli strains are used for cloning: *E. coli* Invα F´ and *E. coli* JM110 (Invitrogen). Their *lac*ZΔ M15 genotype permits the selection of recombinant plasmid by β galactosidaseę α complementation.

#### 3.2. *Agrobacterium tumefaciens* strains

*Agrobacterium tumefaciens* is a plant pathogen responsible of the crown gall disease. The infection of the plants occurs by the intermediate of the *A. tumefaciens* Ti plasmid which contains the opine genes, the virulence genes and the oncogenes. The virulence (vir) genes products are responsible for the transfer of T-DNA from the bacteria to the host nuclear DNA and thus, for the

transformation of the plant whereas the proteins encoded by the oncogenes (onc), induce tumors. The different strains of *Agrobacterium* used to transfer heterologous genes to the plants, contain a disarmed Ti plasmid, this means that the plasmid does not have any onc genes, but still harbour the vir genes. Two A. tumefaciens strains are used: GV3101 *Agrobacterium* C58C51 (Rifampicin and Kanamycin resistant) (Koncz and Schell 1986) and ATHV *Agrobacterium* C58C1 (Rifampicin resistant, Kanamycin sensible) (Hood *et al.* 1986).

## 4. Plasmids

### 4.1. pTPCR vector

The cloning vector pTPCR (Wassenegger *et al.*, 1994, fig. 2) is obtained by insertion of a *Kpn*I/*Bam*HI fragment (26 bp) into the vector pT3T7-lac (Boehringer Mannheim) in order to insert additional restriction sites in the cloning cassette. The latter is located within the LacZ gene, which codes for the β-galactosidase. Expression of this enzyme is visualized by the appearance of a blue coloration due to the degradation of the X-Gal substrate, previously added to the culture media. When a DNA fragment is inserted in the cassette, the expression of β-galactosidase is impaired and the colonies remain colourless.

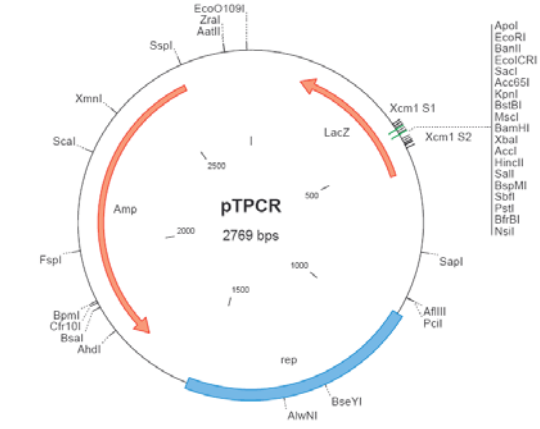


**Figure 1: Genetic map of the pTPCR vector**

### 4.2. pCass II vector

The pUC19-based cloning vector pCassII (Ding *et al.*, 1995) contains the 35S promoter in which the enhancer sequence is duplicated for high transcription rates of the cloned DNA and a 35S terminator (fig. 2). Therefore, pCassII suits especially for the production of infectious viral full-length clones. It contains also a resistance gene against ampicillin and a LacZ gene expressing β-galactosidase.

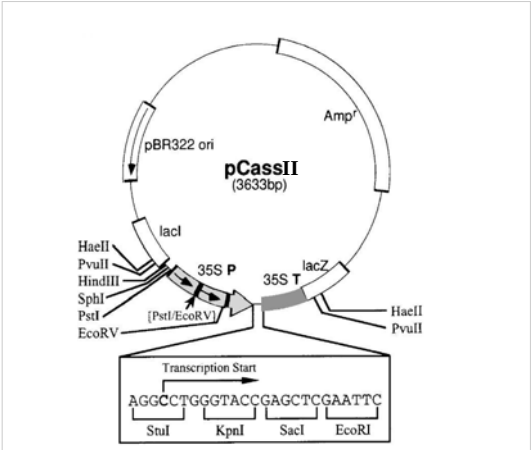


**Figure 2: Representation of the pCassII vector**

### 4.3. pPZP200 vector

The binary vector pPZP200 (Hajdukiewicz *et al.*, fig. 3) contains a bom site, which permits the mobilisation of the plasmid DNA from *E. coli* to *Agrobacterium*, an origin of replication functional in both *E. coli* (PBR322) and *Agrobacterium* (pVS1) and a resistance gene against spectinomycin. This vector is used in the case of agroinfiltration of the full-length cDNA of the ArMV-NW isolate.

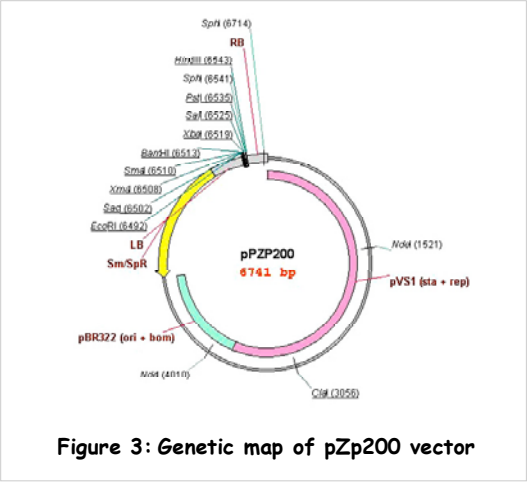


**Figure 3: Genetic map of pZp200 vector**

### 4.4. pGJpRT vector

The pGJpRT vector (Galetzka *et al.*, fig. 4) is used for the cloning in Agrobacterium of genes of ArMV-NW and ArMV-Lv isolates. It confers resistance to spectinomycin and streptomycin.

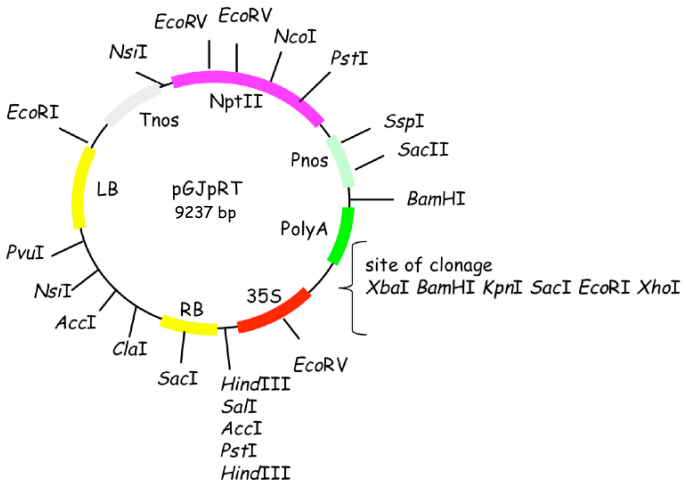


**Figure 4: Genetic map of pGJpRT vector**

### 4.5. pBin vector

This vector is used to insert in the plant nuclear genome, the GFP gene under the control of 35S promoter and the nos (nopaline synthase) terminator. This pBin-GFP, derived from pBin19 (Bevan *et al.*, 1984), contains an origin of replication both for *E. coli* and *A. tumefaciens*. pBin19 carries the *lacZ'* gene, the kanamycin-resistance gene ($kan^R$) and the two boundary sequences from the T-DNA region of the Ti plasmid. These boundary sequences recombine with the plant chromosomal DNA, thus allowing the insertion of DNA interest. This strain is selected under rifampinicin and kanamycin. The pBin-GFP is used to transform the 16C transgenic *N. benthamiana* plants to induce RNA silencing of the GFP transgene.

## II. Methods in molecular biology

### A. DNA

#### 1. Preparation of plasmid DNA

One to 5 ml of LB (10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0), containing the appropriate antibiotics, are inoculated with a single colony of recombinant bacteria, using a toothpick. After incubation overnight at 37°C under shacking, the culture is centrifuged at 5000 g during 5 minutes at room temperature. Then, the supernatant is discarded and the pellet, containing the bacteria, is resuspended in 100 µl of P1 solution (50 mM glucose, 25 mM tris-HCl, 10 mM EDTA, pH 8). Bacteria are lysed by addition of 200 µl of P2 solution (0.4 mM NaOH, 2% SDS) and incubation at room temperature for 5 minutes. Proteins, chromosomal DNA and RNAs of high molecular weight (HMW) are precipitated by addition of 150 µl of the P3 solution (3 M sodium acetate, pH 5.2). After

homogenisation, the tube is placed on ice during 5 minutes. The precipitate is removed by centrifugation, 15 minutes at 18,000 g, at 4°C. The supernatant is mixed with 800 µl isopropanol and incubated for 2 minutes at room temperature. The DNA is recovered by a centrifugation, 5 minutes at 20,000 g, washed with 200 µl of ethanol 70% and finally, after drying, dissolved in 20 µl of water.

Highly purified plasmid DNA for cloning or sequencing is obtained using the same procedure but, after elimination of the precipitate by centrifugation, the supernatant is loaded on the QIAprep spin column. The latter is then centrifuged 1 minute at 11,000 g and washed with 750 µl PE buffer. The DNA eluted with 50 µl 10 mM Tris-HCl, pH 8.5 by centrifuging the column at 11,000 g for 1 minute.

The protocol is almost the same when large amounts of DNA are required i.e for mechanical inoculation to plants. Usually, plasmid DNA is extracted from a 25 ml or 100 ml bacteria culture, using the JESTAR Plasmid Midiprep Kit" (Genomed). The recombinant bacteria are treated with P1, P2 and P3 solutions, as described above. After centrifugation at 16,000 g for 10 minutes at room temperature, the supernatant is applied on a JETSTAR 2.0 column equilibrated with solution E5. After loading, the column is washed with solution E5 (20 ml or 60 ml depending on the volume of bacteria culture) and the DNA is eluted with solution E6 (5 ml or 15 ml). The DNA is precipitated with 0.7 volume of isopropanol (3.5 ml or 10.5 ml) and centrifuged at 20,000 g at 4°C for 30 minutes. To finish, the plasmid DNA is washed with 70% ethanol and centrifuged again. After air-drying for 10 minutes, the pellet DNA is dissolved in a suitable volume of water.

## 2. Amplification of DNA by PCR (polymerase Chain Reaction) or by RT-PCR (Reverse Transcriptase-PCR)

### 2.1. PCR

This technique allows *in vitro* exponential amplification of a DNA template by a series of polymerization reactions. The reaction requires two oligodesoxyribonucleic primers (forward- and reverse primers), which frame in two orientations the sequence to amplify.

Two thermostable DNA polymerases are used depending on the aim of our experiments: *Taq* (*Thermus aquaticus*) polymerase (5 PRIME) for miniprep analysis and Pfu (*Pyrococcus furiosus*) polymerase (Finnzymes), for the cloning experiment, because the latter has a high processivity. The DNA sequence is amplified in the following reaction mix:

Miniprep analysis (Taq polymerase)

| | |
|---|---|
| DNA template (1-150 ng) | x µl |
| 10x Taq buffer | 2.5 µl |
| 10 mM dNTP mix | 1 µl |
| Forward Primer 10 pmole | 1 µl |
| Reverse Primer 10 pmole | 1 µl |
| *Taq* DNA polymerase | 0.25 µl |
| Water to | 25 µl |

Cloning (Pfu polymerase)

| | |
|---|---|
| DNA template (1pg-10 ng) | x µl |
| 5x Phusion HF buffer | 4 µl |
| 10 mM dNTP mix | 0.4 µl |
| Forward Primer 10 pmole | 1 µl |
| Reverse Primer 10 pmole | 1 µl |
| Phusion DNA polymerase | 0.6 µl |
| Water to | 20 µl |

The PCR cycles are performed as following:

| | | |
|---|---|---|
| Initial denaturation | 94 °C for 5 min | 98 °C for 30 s |
| Denaturation | 94 °C for 30s | 98 °C for 10 s |
| Annealing | x°C for x s | x°C for x s |
| Primer extension | 72 °C for x s | 68 °C for x 1min/kb |
| Final extention | 72 °C for 7 min | 68 °C for 7 min |

The temperature for the annealing depends on the Tm of the primers and the time required for the annealing and the extension steps depends on the length of the sequence to amplify. The denaturation, the annealing and the primer extension steps are repeated 30 times in the presence of either Taq DNA polymerase or Pfu DNA polymerase.

### 2.2. RT-PCR

When DNA is amplified starting from RNA template, the first step is performed with a reverse transcriptase (RT), a RNA-dependent DNA polymerase in order to synthesize a cDNA.

The superscript (SSII) one-step RT-PCR with Platinum Taq system (Invitrogen) is used for this reaction. This system contains a SuperScript II Reverse Transcriptase, which has been engineered to reduce RNAse H activity and to increase its thermal stability and, a Platinum *Taq* DNA polymerase. It is used for combined cDNA synthesis and PCR, in a single tube, using specific primers. The composition of the reaction mix and the conditions used are as following:

| | |
|---|---|
| RNA template (1pg-10 ng) | x µl |
| 2x reaction mix | 10 µl |
| Forward Primer 10 pmole | 1 µl |
| Reverse Primer 10 pmole | 1 µl |
| RT/ Platinum *Taq* mix | 0.4 µl |
| DEPC water to | 20 µl |

| | |
|---|---|
| cDNA synthesis | 45 °C for 30 min |
| Initial denaturation | 94 °C for 2 min |
| Denaturation | 94 °C for 15 s |
| Annealing | x°C for x s |
| Primer extension | 72 °C for 1min/kb |
| Final extention | 72 °C for 7 min |

After the reverse transcription step, the cDNA is amplified by 35 to 40 PCR cycles.

### 2.3. Rapid amplification of cDNA ends (RACE)

This technique allows a rapid amplification of cDNA corresponding to the 3’ and/or 5’ ends of transcripts (3’ and 5’ RACE- PCR, Invitrogen).

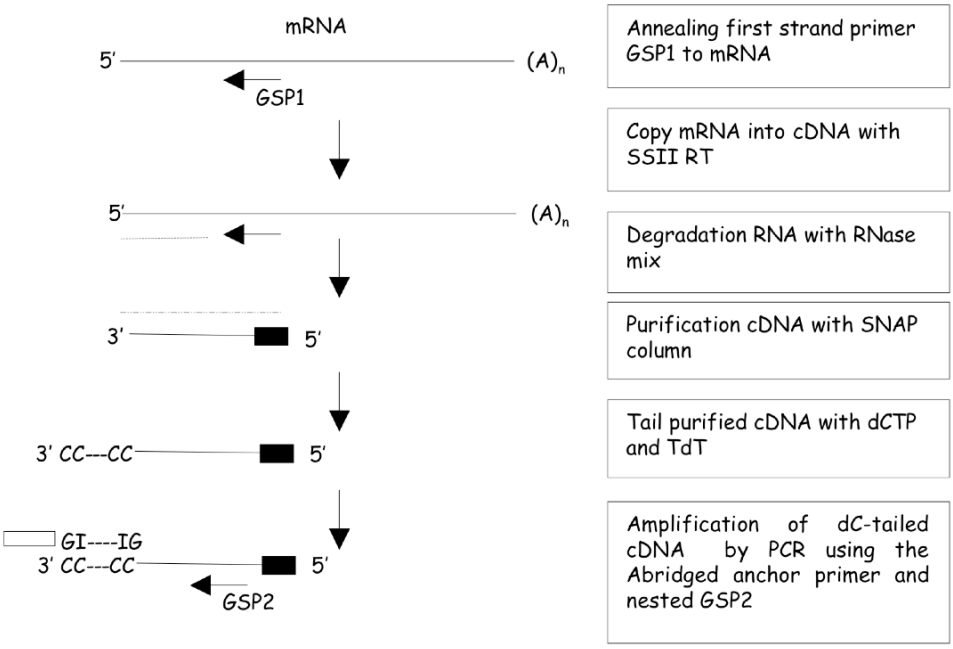


**Figure 5: Schematic representation of the 5' RACE process**

5' RACE-PCR begins using mRNA as a template, for a first round of cDNA synthesis, by reverse transcription using an anti-sense (reverse) oligonucleotide primer that recognizes a known sequence in the gene of interest; the primer is called a *gene specific primer* (GSP1). The RNA template is then removed by RNase treatment and the unincorporated dNTPs and GSP1 are eliminated using a SNAP column. After, a homopolymeric tail of dCTP is added to the 3' end of the cDNA by the terminal deoxynucleotidyl transferase (TdT), creating a primer-binding site at this end of the cDNA. To finish, PCR amplification is accomplished using Taq polymerase, a nested gene specific primer (GSP2) that anneals to a site located within the cDNA molecule and a novel deoxyinosine-containing primer (abridged anchor primer), which permits amplification from the homopolymeric tail. This strategy allows the amplification of unknown sequences between the GSP2 and the 5' end of the mRNA (fig. 5).

### 2.3.1. First strand cDNA synthesis

One to 5 µg RNA are mixed with 2.5 pmoles of GSP1 primer (5'-AGT GCA GCA GCA CTG GGA AC-3') in a final volume of 15.5 µl. The mixture is incubated for 10 minutes at 70°C to denature RNA and chilled on ice for 1 minute. Then, the following components are added in the order given below.

| | |
|---|---|
| 10x PCR buffer | 2.5 µl |
| 25 mM $MgCL_2$ | 2.5 µl |
| 10 mM dNTP mix | 1 µl |
| 0.1 M DTT | 2.5 µl |

The reaction mix, warmed up at 42°C for 1 minute, is added with 1 µl of Superscript II reverse transcriptase (2U/µl) and first incubated at 42°C for 50 minutes followed by an incubation at 70°C for 15 minutes to inactivate the enzyme. The residual RNA is eliminated in presence of 5 units of DNase free RNase A at 37°C for 30 minutes.

### 2.3.2. SNAP column purification of cDNA

The cDNA is mixed with 120 µl of binding solution 6 M NaI and then transferred to a SNAP column. The column is centrifuged at 16,000 g for 20 seconds to bind the DNA on the column. Thereafter, the column is submitted to a first wash with 0.4 ml of cold 1x wash buffer and two additional washes with 0.4 ml of cold 70% ethanol. To recover the purify cDNA, the spin cartridge insert is transferred into a new tube and 50 µl of sterilized, distilled water (preheated at 65°C) is added to the spin cartridge. The cDNA is eluted by centrifugation at 16,000 g for 20 seconds.

### 2.3.3. Tailing of cDNA with terminal deoxynucleotidyl transferase (TdT)

A homopolymeric dCTP tail is added to the 3' end of the cDNA in the following reaction mix:

| | |
|---|---|
| 5x tailing buffer | 5 µl |
| DCTP 2 mM | 2.5 µl |
| Purified cDNA sample | 10 µl |
| DEPC water | 6.5 µl |

After addition of the components, the reaction mixture is incubated for 2-3 minutes at 94°C, chilled for 1 minute on ice and incubated for 10 minutes at 37°C in the presence of 1 µl TdT (1U/µl). The reaction is stopped by inactivation of TdT for 10 minutes at 65°C.

### 2.3.4. Amplification by PCR of dCTP-tailed cDNA

The reaction is performed in the following mixture:

| | |
|---|---|
| 10x Taq buffer | 2.5 µl |
| $Mg(Oac)_2$ 2 mM | 0.5 µl |
| GSP2 5'-ATG AAA ATT CTC GTG GGG TT-3' | 1 µl |
| Abridged anchor primer | 1 µl |
| dNTP 10 mM | 0. 65 µl |
| dC-tailed cDNA | 5 µl |
| 5 PRIME *Taq* polymerase (5U/µl) | 0.25 µl |
| Water | 18.10 µl |

The conditions for the amplification of the dCTP-tailed cDNA by PCR are:

| | | |
|---|---|---|
| Denaturation | 94 °C for 5 min | One time |
| Denaturation | 94 °C for 20 s | 40 times |
| Annealing | 42 °C for 10 s | 40 times |
| Primer extension | 72 °C for 10 s | 40 times |
| Final extention | 72 °C for 5 min | One time |

The amplification yield is determined by the analysis of 5 µl of the 5' RACE products on agarose gel.

**3. Analyse of DNA by electrophoresis on agarose gel**

DNA is fractionated on 0.7 to 2% agarose gels depending on its size. The gel is prepared either in 1x TAE buffer (4 mM tris-HCl, 0.8 mM acetic acid, 0.1 mM EDTA, pH 8) or in 1xTBE buffer (90 mM tris-HCl, 90 mM boric acid, 0.1 mM EDTA, pH 8). The gel also contained ethidium bromide (0.5 µg/ml) to directly visualize DNA under UV light (Multi-purpose Image System). The DNA samples are mixed with 0.2 volume of gel tracking dye (0.15% bromophenol blue, 15% ficoll 400, 10mM EDTA, pH8) before loading on the gel. The DNA is separated on the agarose gel at 100-130 V in electrophoresis buffer (1x TAE or 1xTBE) in parallel with DNA size marker (Fermentas).

**4. Purification of DNA fragments**

DNA fragments are purified from agarose gel, after electrophoresis, using the "QIAquick Gel Extraction Kit" protocol (QIAgen). The band containing the DNA fragment is excised from the agarose gel with a scalpel, and added after weighing, with 300 µl of QG buffer per 100 mg of gel. The gel slice is melted at 50$^{o}$C for 10 to 15 minutes with occasional mixing during the incubation to obtain a homogenous solution. The yield of DNA fragments <500 bp and >4 kbp could be improved by mixing the solution with 100 µl isopropanol per 100 mg of gel. Then, the solution is transferred into the QIAquick column in order to bind the DNA onto its silica membrane. The column is centrifuged for 1 minute at 11,000 g and washed with 750 µl of PE buffer and a quick centrifugation is realized. Finally, the DNA is eluted by addition of 50 µl of buffer EB (10 mM tris-HCl, pH 8.5) or $H_2O$ and centrifugation for 1 minute at 11,000 g.

**5. Digestion of DNA with restriction enzymes**

DNA molecules are incubated with an appropriate amount of endonuclease(s), usually 1U per 0.5 µg of DNA in a final volume of 20 µl, in the buffer recommended by the suppliers (1x final concentration). The digestion is performed for 1h30 to 2 hours at 37°C. The digestion products are analyzed using an aliquot of the reaction mix (2.5 µl) and purified by a phenol/chloroform extraction to eliminate the proteins, followed by an ethanol precipitation to concentrate the DNA.

The volume of the reaction mix is adjusted to 200 µl with TE buffer (1 mM EDTA, 10 mM tris-HCl pH 8) and then, added with one volume of phenol/chloroform. After vigorous vortexing for a few seconds, the emulsion is centrifuged at 20,000 g for 5 minutes at room temperature. The upper aqueous phase, which contained the nucleic acids, is taken and treated with a volume of isoamyl alcohol/chloroform (vol 24:1). After centrifugation, the nucleic acids are precipitated by addition of 2.5 volumes of ethanol and 0.1 volume of sodium acetate 3M, pH 5 at -20°C for 30 minutes. The DNA is pelleted by centrifugation at 20,000 g for 25 minutes, at 4°C, washed with 1 ml of ethanol 70%, followed by a centrifugation at 20,000 g for 15 minutes. At the end, the pellet is dried and the DNA dissolved in 10 µl water.

**6. Dephosporylation**

The plasmid linearized with the restriction enzyme(s) is dephosphorylated at the 5' ends with the shrimp alkaline phosphatase (SAP, Roche) to prevent its self-ligation during cloning experiments. The reaction is placed 1 hour at 37°C in a mix (10 µl) containing digested plasmid (~ 500 ng), 1x SAP buffer and 2 units of SAP. The enzyme is inactivated upon incubation at 65°C for 15 minutes.

**7. Ligation**

The insertion of DNA in the linearized vector is done in the presence of T4 DNA ligase (Fermentas) with an insert /plasmid ratio of 3/1 to optimize the ligature and a total amount of DNA which should not be more than 300 ng. The reaction mix (10 µl) contains in addition to the linerarized vector and the DNA fragment(s) of interest, T4 DNA ligase buffer and T4 DNA ligase (5U/µl; 0.5 µl for cohesive ends and 1 µl for blunt ends). The reaction mix is incubated overnight at 16°C and then, the DNA is purified by phenol/chloroform extraction, as described above and dissolved in 10 µl water.

**8. Transformation of bacteria**

**8.1. Preparation of bacteria for use by the heat-shock process**

Cells are made competent by a process that uses calcium chloride and heat shock. The standard procedure is the following.

One hundred µl of bacteria cells frozen at -80°C or one fresh colony picked are grown with 250 ml sterile SOB medium (20 g/l trypton, 5 g/l yeast extract, 200 mg/l NaCl, 200 mg/l KCl, 2 g/l $MgSO_4 \cdot 7\ H_2O$, pH 7) on a shaker at 37 °C until the culture reached an $OD_{600\ nm}$ of 0.6. The bacteria are placed on ice for 10 minutes and then, harvested by centrifugation at 5,000 g for 10 minutes, at 4 °C. The bacteria pellet is resuspended gently on ice in 1/4 volume of ice cold $MgCl_2$ for 3 to 5 minutes. Thereafter, the cell suspension is centrifuged at 4,000 g for 10 minutes. The bacteria pellet is

resuspended on ice in 9/20 volume of ice cold $CaCl_2$ and then placed on ice at least 20 minutes. After centrifugation at 4,000 g for 10 minutes, the bacteria are resuspended in 1/50 volume of ice cold, sterile 85 mM $CaCl_2$ in 15% glycerol w/v.

### 8.2. Transformation of competent bacteria cells

Transformation is performed using *E. coli* Invα F´ or JM110 strain. The standard procedure is described below (retransformation conditions are in the brackets). Fifty µl (Invα: 10 µl, JM110: 50 µl) of competent cells are gently thawed on ice and added with 5 µl (1 µl) of the ligation reaction. The bacteria suspension is incubated on ice for 30 minutes (10 minutes) and then, incubated at 42°C for 45 seconds and is placed immediately on ice for at least 2 minutes. This heat-shock allowed DNA to enter in the bacteria cells. The tube is added with 200 µl (90 µl) of SOC-medium and incubated for 1 hour (30 minutes) at 37°C, under shaking to allow the bacteria to recover cell wall.

After incubation, 150 µl (total volume) of the suspension is plated on a LB agar media (1% trypton, 0.5% yeast extract, 1% NaCl, 1,2% agar, pH 7.0) in Petri dishes, containing the appropriate antibiotic, 100 µg/ml of ampicillin or 50 µg/ml of spectinomycin. The plate is incubated for at least 12 hours at 37°C. When recombinant plasmids are selected by β-galactosidase enzymatic activity, 20 µl of X-Gal (40 µg/ml) is added on a LB agar plate.

## B. RNA

### 1. Plant total RNAs Extraction

Two approaches are used to purify RNAs depending on its further utilization. RNAs used for cDNA synthesis by RT-PCR, are extracted and purified on columns using the D-Genos kit whereas those analyzed by northern blots are obtained following the classical extraction procedure with the TRI-Reagent solution from Sigma

### 1.1 Kit from D-Genos

This method enables the simultaneous RNA extraction of 24 samples without any centrifugation. The RNA obtained with the D-genos kit is very pure but the yield is extremely low. The EasyPrep System contains 3 plates, two of them can be loaded with columns (first step: yellow, second step: blue) and one with 1.5 ml microcentrifuge tubes (fig. 6). In addition, there is a carrier for the plates with a waste tray at the bottom and the sealed pressure well on the top. The EasyPrep Pump Unit allows two different pressure settings in a certain time frame: 0.25 bars or 0.5 bars are used to pass the homogenised sample through the anion-exchange-resin column.

**Figure 6: D-Genos EasyPrep System** (Pharmacia Biotech.) At the Right, we can see EasyPrep Processing; this Unit is ready for RNA extraction with 3 columns. At the Left, we find the EasyPrep Pump Unit.

The procedure indicated by D-Genos manufacturer is the following, separated in distinct steps:

-Preparation of the column: 1 ml E1 buffer is loaded on the yellow column. The fixation of the resin into the column is realized by exerting an overpressure of 0.5 bars.

-Preparation of plant extract: leaves (~10 cm²) from infected plant are ground in 1ml of extraction buffer (Bioreba). Two hundred µl of the crude extract of each sample are mixed with 120 µl E2 buffer (117 µl $E2_1R$ buffer and + 3 µl $E2_2$ buffer/ 5 reactions) and incubated at 37°C for 30 minutes and then, after addition 1.5 ml E3R buffer, at room temperature for 5 minutes. During this incubation, the plate containing the yellow column is placed in the process unit.

- Fixation of the RNA on the resin: the totality of the solution is loaded on the yellow column and incubated at room temperature for 5 minutes. The fixation of the RNA on the resin is realized by exerting an overpressure of 0.25 bars.

-Washing of the resin: to eliminate the unbound RNA, 1 ml of buffer E4 is loaded on the yellow column. The washing is realized by exerting an overpressure of 0.25 bars and this step is repeated three times.

-Elution of RNA: 800 µl E5 buffer is added on the plate 1, containing the yellow columns, and the all is incubated at room temperature for 1 minute. During the incubation, the plate 2 containing the blue column is placed in the elevator. The elution of the RNA into the blue column is realized by exerting an overpressure of 0.25 bars for 4 minutes.

-Precipitation of RNA: 640 µl E6 buffer is added to the solution containing the RNA in the blue column (plate 2). Then, the reaction is incubated at room temperature for 10 minutes. After incubation, the plate 2 is placed in the process unit by eliminating the yellow column on the pate 1. The fixation of the RNA on the blue column is exerted by an overpressure of 0.5 bars.

-Washing of the RNA: To wash, 1 ml E7 buffer is added and an overpressure of 0.5 bars is exerted. Then, 100 µl E8 buffer is added and incubate at room temperature for 5 minutes.
- Elution of the RNA: the plate containing the microcentrifuge tubes is placed in the elevator. The elution of the RNA is realized by exerting an overpressure of 0.5 bars. This purified RNA could be used directly for the RT-PCR amplification (5-10 µl).

### 1.2. RNA Extraction using TRI-Reagent

The TRI-Reagent is a mixture of guanidine thiocyanate and phenol in a monophosphate solution. The protocol, using a TRI-Reagent solution (Sigma), is an effective method for isolating total RNA from fresh or frozen tissues or cells.

One hundred mg tissue sample is ground in liquid nitrogen and 1 ml of Tri-Reagent is added to the sample. The crude extract is then transferred in an Eppendorf tube and incubated at room temperature for 5 minutes. After incubation, 0.2 to 0.3 ml of chloroform is added to the extract and the tube is vortexed thoroughly for 30 seconds. The tubes are incubated for 10 minutes at room temperature with 1 or 2 additional vortex steps meanwhile before centrifugation at 20,000 g for 15 minutes, at 4°C. The upper aqueous phase containing the RNA is transferred into a new Eppendorf tube and the RNA is precipitated by addition of 1 volume of isopropanol at room temperature for 20 to 30 minutes. The RNA is recovered by centrifugation at 20,000 g for 20 minutes at 4°C, washed with 0.4 ml of 70-80% ethanol, dried for 5 minutes and dissolved in 30 to 50 µl water or 50% formamide. The RNA is quantified at OD 260 nm with the Nanodrop system before its storage at -20°C.

## 2. Analyse of RNA by Northern blot

### 2.1. Fractionation of RNA on agarose gel

High molecular weight RNAs are fractionated on an agarose gel 1.2% in the MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7) containing 1% deionised formaldehyde.

One volume of denaturing solution (500 µl formamide, 120 µl formaldehyde, 200 µl MOPS 10x, 120 µl bidistilled $H_2O$, 1 µl ethidium bromide) is added to one volume of the RNA sample (3-10 µg). The mixture is incubated for 15 minutes, at 65 °C and then immediately placed on ice to denature the RNAs. After addition of 0.5 volume of loading dye (30 % ficoll, 10 mM EDTA, 0.25 % bromophenol blue, 0.25 % cyanol of xylen), the RNAs are separated on the agarose gel covered with 1x MOPS, at 5 V/cm$^2$ for 4 to 5 hours and at room temperature.

After electrophoresis, the agarose gel is shaked for 10 minutes in 100 ml of 0.05 M NaOH to introduce breaks in RNA in order to improve the transfer of the high molecular weight RNAs onto the membrane. The gel is neutralized by immersion in 100 ml of 10x SSC (1.5 M NaCl, 0.15 M Na-citrate, pH 7.2) for 20 minutes. This step is repeated twice. The transfer of the RNAs is realized as usual. The gel is layed on the Whatman 3MM paper sinking in 10x SSC and overlayed with the

membrane (Millipore), three sheets of Whatman 3MM paper, paper towels and finally, a weight of about 400 g. The transfer of the RNA is realized by capilarity of 10x SSC through the agarose gel, for at least 12 hours. After transfer, and then, the membrane is washed in 2x SSC for 10 minutes and the RNA molecules are cross-linked to the membrane by UV light with a Stratalinker apparatus (1,200 Joules×100).

### 2.2. Preparation of the radioactive probes

#### 2.2.1. Random-priming labelling

Random decamers are annealed to a denatured DNA template and labelled with radioactive dNTPs which are incorporated into the new DNA strands by Klenow fragment. For this labelling, we used the decalabel DNA labelling kit (Fermentas). In a first step, the DNA template is denatured in boiling water for 5 to 10 minutes in the presence of the random primers and then cooled at room temperature to allow the primers to hybridize to the DNA strands. This step is realized in the following mix:

| | |
|---|---|
| DNA template (100 ng) | 10 µl |
| Decanucleotide in 5X reaction buffer | 10 µl |
| Water, nuclease free to | 40 |

Then, the following components are added to the mix:

| | |
|---|---|
| Mix C (0.33 mM dGTP, 0.33 mM dATP, 0.33 mM dTTP) | 3 µl |
| $\alpha$ $^{32}$P-dCTP (or $\alpha$ $^{32}$P-dATP) 50 mCi/1.85 Mbq | 6 µl |
| Klenow fragment (5 U/µl) | 1 µl |

After incubation for 5 minutes at 37°C, 4 µl of dNTP (0.25mM dATP, 0.25mM dTTP, 0.25mM dCTP, 0.25mM dGTP) are added to the reaction mix and incubated for additional 5 minutes at 37°C. The reaction is stopped by addition of 1 µl 0.5 M EDTA, pH 8 and 100 µl hybridization buffer to the mix and heating at 94°C for 5 minutes. Non-incorporated radioactive dNTPs are removed by gel filtration on a Sephadex G50 column.

#### 2.2.2. 5’ end labelling with the T4 Polynucleotide Kinase (PNK)

PNK catalyzes the transfer of the γ-phosphate from ATP to the 5'-OH group of single - and double-stranded DNAs and RNAs, oligonucleotides or nucleoside 3'-monophosphates (forward reaction). The reaction is done, as recommended by Fermentas manufacture, in the following mix:

| | |
|---|---|
| Oligo (10μM) | 2 μl |
| PNK Buffer 10X | 2 μl |
| Water | 10.5 μl |
| $^{32}$P γATP (10mCi/ml) | 5 μl |
| T4 PNK (10 U/μl) | 1 μl |

This reaction mix is incubated at 37°C for 30 to 45 minutes and then filtrated on a Sephadex G25 column to eliminate free dNTPs. The probe is heated at 95°C for 5 minutes then cooled at 4°C for 10 minutes.

### 2.3. Hybridization

The membrane is incubated with the hybridization buffer (Perfect Hybrid Plus buffer, Sigma) for 1 hour. After addition of the labelled radioactive probe, the membrane is placed for 12 hours, at 42°C for an oligonucleotide probe or at 65°C for a DNA or RNA probe. After hybridization, the membrane is washed at 50°C or at 65°C three times with 2x SSC, 2% SDS for 20 minutes each and eventually, under more stringent conditions, with 1x SSC, 1% SDS, to remove probes unspecifically bound to the membrane. Finally, the membrane is dried and exposed with a Fuji-screen and revealed by using a phosphoimager (Phareos FX Plus, molecular imager).

To re-hybridise other labelled probe, the membrane is stripped with 0.5x SSC, 0.5% SDS for 30 minutes at 100°C.

## C. Proteins

### 1. Extraction of proteins from plants

Two patches (~ 1cm in diameter) from leaves are ground in mortar with a little pestle, in presence of 100 μl of 8 M urea. Ten μl of the crude extract are mixed with one volume of Laemmli buffer (4% SDS, 4% β-mercaptoethanol, 125 mM tris pH 6.8, 20% glycerol, 0.03% bromophenol blue) and heated at 100°C for 3 minutes to denature the proteins before analysis by SDS-PAGE.

The so-called method of Tanaka (Hurkman and Tanaka, 1986) is used to extract proteins from buds. Buds frozen in liquid nitrogen are ground in a cold mortar and then, introduced in a microcentrifuge tube. Six hundred μl of Tanaka buffer (0.7 M sucrose, 5 mM EDTA, 0.1 M NaCl, 2% β mercaptoethanol, 2 mM PMSF, 0.5 M tris, pH 8) and 600 μl of phenol are added to the sample. After vigorous and continuous mixing during 5 minutes and centrifugation at 20,000 g for 5 minutes, at room temperature, the upper phase is transferred into a new microcentrifuge. The proteins are precipitated with 1 ml of ammonium acetate /methanol for 20 minutes. The precipitate is centrifuged at 20,000 g for 10 minutes, at room temperature to pellet the proteins. The latter are washed twice with 1 ml of ammonium acetate /methanol. The proteins are dried on ice and resuspended in 80 to 100 μl water and mixing during 10 minutes. Before analysis of the proteins by SDS-PAGE, the samples are

heated for 2 to 3 minutes at 95°C, stirred and centrifuged at 20,000 g for 1 to 2 minutes to separate the insoluble and soluble proteins.

**2. Fractionation of proteins by polyacrylamide gel under denaturing conditions (SDS-PAGE)**

Proteins are separated in polyacrylamide gel electrophoresis in denaturing conditions by the presence of SDS. The polyacrylamide gel consisted of two parts, the upper stacking gel to concentrate the proteins and the resolving gel, where proteins are separated following their molecular weight.

The composition of the resolving gel is the following (for a Acryl/Bis gel of 12%), however, the concentration of Acrylamide/Bisacrylamide depends of the molecular weight of the researched proteins.

| | |
|---|---|
| Tris pH 8.8 | 0.375 M |
| Acryl/Bis- 37.5:1 | 0.29 M |
| SDS | 0.01 % |
| Temed | 0.08 % |
| Persulfate | 0.01 % |

The stacking polyacrylamide gel has a concentration of 5% polyacrylamide and is prepared in 0.15 M tris, pH 6.8. After loading, the proteins are fractionated in the electrophoresis buffer (2.5 mM tris, 19.2 mM glycine, 0.1% SDS), at 100 V, for 2 hours.

**3. Western blot**

After separation by SDS-PAGE, the proteins are blotted on Immobilon-P membrane, previously wetted in ethanol. The gel and the membrane are sandwiched between two Whatmann 3MM paper and two “scotch brite”. The transfer is realized in a transfer buffer (25 mM tris, 25 mM glycine, 25% ethanol) for 1hour, at 300 mA, in the cold room.

After transfer, the membrane is washed with phosphate buffer saline buffer (137 mM NaCl, 2.7 mM KCl, 1.47 mM $KH_2PO_4$, 4.3 mM $Na_2HPO_4$, pH 7.4) containing 1% Tween (PBS-Tween solution) for 15 minutes to eliminate all trace of SDS. The membrane is then saturated in PBS-Tween containing 5% milk for 30 minutes, at 4 °C, to avoid non-specific interactions and incubated overnight with the primary antibody specific for the protein of interest, at 4 °C. Thereafter, the membrane is washed twice with PBS-Tween for 10 minutes, at room temperature to remove the excess of primary antibody and incubated with the anti IgG secondary antibody coupled to peroxydase, in PBS-Tween-5% milk for 2 hours, at 4 °C. The secondary antibody is removed by washing several times with PBS-Tween for 10 minutes, at room temperature. The immune complex is revealed by chemiluminescence reaction using the ECL kit (Amersham).

The proteins can be visualized by staining of the membrane in 0.2% coomassie blue R250, 45% ethanol, 17% acetic acid solution, followed by discoloration in 25% ethanol and 7.5% acetic acid.

## III. Methods in virology

### 1. Production of viral RNAs by *in vitro* transcription

Viral cDNAs (PVX and virus recombinants) cloned in the pC2S2 vector are transcribed *in vitro* by the T7 RNA polymerase, using the RiboMAx Large Scale System (Promega). The viral RNAs are capped at their 5' end during the transcription assays to increase the translation efficiently.

The recombinant plasmid are linearized downstream of the cDNA, at unique restriction sites with restriction endonucleases, purified and then incubated in the following reaction mix:

| | |
|---|---|
| T7 Transcription 5x buffer | 5 µl |
| 25 mM of rATP, RCTP rUTP and 2mM of rGTP | 7.5 µl |
| Linear template (2.5 µg) in nuclease free water | 8 µl |
| Ribo $m^7G$ cap analog, 40 mM | 2 µl |
| Enzyme mix | 2.5 µl |

After homogenization by pipeting, the reaction mix is incubated for 2 to 4 hours, at 37°C. The transcripts are purified by phenol/chloroform extraction and precipitated by addition of 0.1 volume 3M sodium acetate (pH 5.2). The solution is placed on ice for 2 to 5 minutes and then, centrifuged at 20,000 g for 10 minutes in a refrigerated centrifuge. The RNA pellet is washed with 1 ml 70% ethanol to solubilize the salts, dried and dissolved in 20 µl RNAse-free water. The amount of RNA obtained upon transcription is determined by spectrophotometry at wavelength of 260 nm. One optical density at 260 nm corresponds to 40 µg RNA/ml.

### 2. Inoculation of plants with ArMV or infectious clones

Leaves from ArMV infected plants are ground in a frozen mortar in the presence of inoculation buffer (0.03 M $K_2HPO_4$, 0.05 M glycine, 1 M KOH, pH 9.2, 1% bentonite) containing 1% celite, an abrasive that favours the entry of the virus in the leaf epidermis, through micro-lesions. The virus is inoculated to healthy plants at four-six leaves stage by rubbing tow leaves with the virus-contained sap. Then, the leaves are washed with water to eliminate the excess of buffer.

When viral RNA or cDNA are used, two leaves per plants are inoculated with 10 µl of inoculation buffer containing 5 µg of RNA or cDNA. To optimize the infectivity on *Chenopodium quinoa*, the plants are placed in the dark a day before inoculation.

In the case of Arabidopsis plants, the mixture is distributed on five leaves per plants (20 µl/leaves) with the aid of cotton swab.

### 3. Detection of ArMV in infected plants by Enzyme-linked immunoabsorbant assay (ELISA)

ELISA is a biochemical technique, which allows a rapid detection of an antigen and/or antibodies in a sample. ELISA is used as a diagnostic tool in animal and plant pathology, for example to detect the presence of a virus. In the "sandwich ELISA" procedure, the sample with an unknown amount of antigen is immobilized on a polystyrene microtiter plate via capture by an antibody specific for the antigen. This technique includes several steps:

#### 3.1. Preparation of the samples

A leaf of an infected plant is ground in a mortar, in the presence of 1 ml extraction buffer (BIOREBA) until to obtain a homogeneous solution that is then transferred into a tube and chilled on ice.

#### 3.2. Coating of the plate

One µl of anti-ArMV CP IgG (BIOREBA) is diluted in 1 ml of coating buffer (0.015 mM $Na_2CO_3$, 3.4 mM $NaHCO_3$, pH 9.6). The antibody is distributed 100 µl/well on the microtiter plate. Then, the plate is incubated for 2 hours, at 37°C to allow the binding of the antibody. Three washes with PBS 1x (14 mM NaCl, 2,7 mM KCl, 1.76 mM $KH_2PO_4$, 10 mM $Na_2HPO_4$, pH 7.4) are realized after incubation with a mild detergent solution to remove any proteins or antibodies that are not bound.

#### 3.3. Loading of the samples

One fifty hundred µl of plant extracts are loaded into the well. Then, the microtiter plate is incubated overnight, at 4°C to allow the specific binding of the antigen with the antibody. Three washes permit to eliminate the unbound antigens with PBS 1x.

#### 3.4. Detection of the antigen-antibody complexes

To detect antigen-antibody complexes, 1 µl of anti-ArMV CP IgG conjugated to the alkaline phosphatase (BIOREBA) per 1 ml of conjugate buffer (1.98 mM tris, 13.68 mM NaCl, 0.4 µM tween 20, 0.083 µM PVP (Polyvenylpyrrolidon), 0,2 mM BSA (bovine serum albumin), 0.98 µM $MgCl_2$-$6H_2O$, 0.0027 mM KCl, pH 7.4) is prepared and 100 µl are distributed per wells. Then, the microtiter plate is incubated overnight for 5 hours, at 4°C. Three washes to eliminate the excess of anti-ArMV CP IgG-AP are realized. The activity of the alkaline phosphatase is revealed in the

presence of 1 mg of substrate (4-nitro-phenyl-phosphate) (Loewe Biochem) per 1 ml of substrate buffer (1M diethanolamine (DEA), pH 9.6) by incubation for 2 hours in a dark room, at room temperature. The visible signal produced upon cleavage of the substrate, is quantified by Multiskan Ascent (Thermo, electron corporation), at 405 nm, indicating the quantity of antigens in the samples.

#### 4. Virus purification

Virus particles are extracted from infected plants (*Chenopodium quinoa*) and further used to purify the viral RNAs.

One hundred g of infected plants (fresh or frozen) are ground with 100 ml extraction buffer (0.1 M Na phosphate, 0.1 M Ascorbic acid, 0.01 M EDTA, pH 7), in a commercial mixer to obtain a homogeneous extract. The latter is filtrated through cheesecloth to remove plant fibers. The filtrate is added with 8.5% (v/v) butanol and permanently stirred for 15 minutes, at room temperature. After a centrifugation for 20 minutes at 1,000 g, at 10°C to eliminate organelles and insoluble material, the supernatant is mixed for 1 hour, at room temperature with a 5 fold concentrated PEG solution (50% PEG 2000, 0.5 M NaCl, 5% Triton 100) and centrifuged again for 30 minutes at 7,000 g, at 10°C. The pellet enriched in virions is resuspended in 2 ml of 0.1 M phosphate buffer (1M $NaH_2PO_4$, 1M $Na_2HPO_4$, pH 7) by low stirring overnight, at 4°C. Thereafter, the virus preparation is centrifuged for 10 minutes at 7,000 g, at 10°C and the supernatant transferred into a Corex tube and added with 0.1 M Na phosphate buffer pH 7. The solution, which becomes milky, is submitted to an ultracentrifugation for 2h30 at 150,000 g, at 4°C. The resulting pellet is resuspended in 200 µl 0.1 M Na phosphate buffer pH 7 and transferred into an Eppendorf tubes. After a last centrifugation at 20,000 g for 5 minutes, the supernatant which contained the purified virus particles, is stored at 4°C. RNAs are extracted from purified virions with phenol/chloroform as previously described and analyzed by electrophoresis on an agarose gel prepared in TBE buffer (paragraph II. A.3).

## IV. Methods specifically used to study RNA silencing

#### 1. Transformation of plants with *Agrobacterium tumefaciens*

##### 1.1. Electroporation of agrobacteria

Fourthy µl of thawed cells are added with 1 µl of the ligation product and transferred to an electroporation cuvette. The latter is placed on ice for 1 minute and then, in the electroporator (Biorad). The conditions of electroporation are 25 µF for the capacity, 2.5 kV/cm for the voltage and 200 Ω for the resistance. After electroporation, 1 ml of LB is added to the bacteria suspension and transferred into an Eppendorf tube. The tube is incubated for 1hour, at 28 °C, the optimal growth temperature for agrobacteria and finally, 150 µl of the suspension is plated out on a LB agar plate, containing the appropriate antibiotic.

### 1.2. Agroinfiltration

#### 1.2.1. Establishment of RNA silencing in plants

RNA silencing in 16C transgenic *Nicotiana benthamiana* plants is established by infiltration of the agrobacteria containing the GFP replicon. This infiltration is realized with plants at 4 leaves stage and two or three leaves are infiltrated per plants, except the cotyledons, using a syringe of 2 ml without needle. We applied the tip of the syringe on the upper face on the leaf.

The establishment of silencing is visualized by the decrease of the fluorescence at 4 days post-infiltration (dpi) on the leaves and 8 dpi in the systemic leaves. The fluorescence disappearance is observed under UV light and using the FITC (GFP-BP) filter of the phosphoimager.

#### 1.2.2. Patch test

Co-agroinfiltration or patch test is realized to identify a RNA silencing suppressor. For this, agrobacteria (OD at 600 nm) containing the pBin-GFP and the putative silencing suppressor gene are mixed (1:1) in agroinfiltration buffer (10 mM MES, 10 mM $MgCl_2$, 100 μM acetosyringone). Acetosyringone activates the opine genes which are required for plant transformation.

### 2. Isolation and detection of siRNAs

#### 2.1. Purification of siRNAs

Small RNAs, less than 40 nucleotides long, are purified from total RNAs extracted from plants, using the flashPAGE fractionator (Ambion), a miniaturized electrophoresis instrument as recommended by Ambion manufacturer. This system allows large-scale purification of RNAs no longer than 40 nucleotides in about 12 minutes (fig. 7).

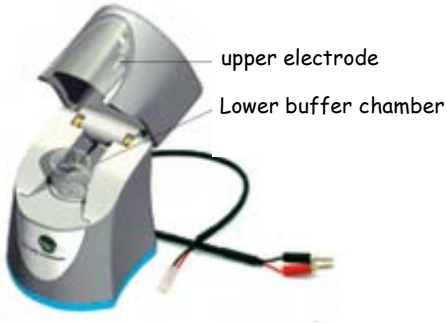


**Figure 7: FlashPAGE fractionator**

Two fifty hundred µl of flashPAGE lower running buffer is introduced in the lower buffer chamber. A flashPAGE Pre-cast gel is inserted into the lower buffer chamber and 250 µl of flashPage upper running buffer is added into this flashPAGE Pre-cast gel cartridge. Equal volumes of RNA or DNA (1-100 µg) and flashPAGE gel loading buffer are mixed with a maximum of 100 µl and then, the samples are heated for 2 minutes, at 95°C and placed on ice before loading onto the gel. Electrophoresis is done at 75-80 V constant voltage until the blue dye begins to exit the gel. The gel is removed and the lower running buffer, which contains the 40nt RNAs, is transferred to a fresh microcentrifuge tube. The RNA is concentrated by ethanol precipitation overnight at -20°C and centrifugation. The RNA pellet is washed with 70% ethanol, dried and resuspended in water.

### 2.2. Detection of siRNAs by Northern blot

Northern blot is also done to visualize the RNAs of 19-25 nucleotides produced during PTGS. Low molecular weight RNAs are separated on a 17.5% polyacrylamide gel (acrylamide/bis-acrylamide 19:1) prepared in 1x TBE in presence of urea 6M, under denaturing conditions. A pre-run is done for 20 to 30 minutes, at 80 V before loading the samples with 0.5x TBE buffer. 10-15 µg of RNA in 100% formamide are denatured by heating at 95°C for 5 minutes and chilled on ice for to 10 minutes. The samples are loaded on the gel after mixing with 0.25 volume of 4x loading buffer (50% glycerol, 50 mM tris pH 7.7, 5 mM EDTA, 0.03% bromophenol blue) and separated by electrophoresis at 80 V, at room temperature.

After fractionation, a Hybond-NX membrane (Amersham) is placed onto the polyacrylamide gel and both are put in between Whatmann 3MM paper and two “scotch brite”. The small RNAs are electro-transferred onto the membrane in 0.5x TBE for 1 hour, at 80 V/300 mA and at 4°C. After transfer, the membrane is equilibrated in 2x SSC for 10 minutes and the RNA molecules are covalently bound to the membrane by UV cross-linking (1,200 Joules×100).

Detection of siRNAs are performed using radiolabelled probes, as described in paragraph II. B. 2.2.2.

### 3. Mapping of siRNAs by southern blot

The siRNAs were mapped along the ArMV-NW and -Lv genome by southern blots. The fragments of 1000 bp were amplified by PCR or RT-PCR using specific primers.

Equal amount of each DNA fragment (160 ng) are separated on 1% agarose gel. After running, the gel is equilibrated in 1x transfer solution (120 mM NaCl, 80 mM NaOH) for 30 minutes and the transfer of the DNA onto a Hybond-N+ membrane is realized by capillarity overnight. After transfer, the membrane is incubated in neutralizing buffer (1.5 M NaCl, 0.5 M tris-HCl, pH 7.5) for 15 minutes and then, is prehybridized for at least 1 hour at 65°C in the hybridization buffer (Sigma

buffer). After addition of the $^{32}$P γATP-siRNA, the membrane is hybridized for 12 hours. The next day, the membrane is washed at 65°C in SSC/SDS buffers (paragraph II. B. 2.3).

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# ANNEX

**INFECTION AND RECOVERY RATES IN NICOTIANA BENTHAMIANA BETWEEN A MILD AND A VIRULENT ISOLATE OF ARABIS MOSAIC NEPOVIRUS**

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**ABSTRACT**

The establishment rates of infection and recovery of two distinct isolates of the Arabis mosaic nepovirus were investigated in *Nicotiana benthamiana*. The infection of the virulent isolate ArMV-Lv did occur more rapidly than the one of the mild isolate ArMV-NW, and the highest viral RNAs accumulation of ArMV-Lv coincided with the presence of symptoms on the plants. The recovery phenomenon took place around the same time for both isolates, resulting in similarly low accumulation of viral RNAs for both isolates. The viral-derived small interfering RNAs (vsRNAs) accumulation was in correlation with the viral genomic RNAs accumulation for both isolates. Recovered plants were immune to a secondary infection with the same virus for both isolates. However, when the secondary infection was performed with the heterologous isolate of ArMV, while ArMV-NW was unable to replicate in ArMV-Lv recovered plants, ArMV-Lv on the other hand was able to overcome the ArMV-NW-induced recovery, and replicated in a similar way to a primary infection.

**Keywords**: ArMV, recovery, immunity

## INTRODUCTION

Nepoviruses, a plant virus genus of the family *Secoviridae* (Sanfacon et al., 2009), have been shown to induce in infected plants a phenomenon called "recovery", which was first described by Wingard (1928). Recovery is characterised by an initial symptomatic infection followed by symptom attenuation or elimination in the newly emerging leaves. Another characteristic is that the upper leaves of the recovered plants are resistant to a secondary infection with the same virus. However, the plant sap extracted from recovered leaves, when used as inoculum, is able to induce typical symptoms on healthy plants (Wingard, 1928). Later, it was shown that the recovery phenomenon was accompanied by a reduction of the viral RNA accumulation (Covey et al., 1997; Ratcliff et al., 1997, 1999). Additionally, the resistance to a secondary infection with the same virus was shown to be sequence-specific, suggesting that the RNA silencing, which is an antiviral defense mechanism activated by double stranded RNAs (Baulcombe, 2004, 2005; Voinnet, 2005), might be involved and responsible for the reduced virus titer.

Recently, several reports involving different viruses from and within different virus families showed different behaviours in relation to recovery, revealing an unexpected degree of complexity for this phenomenon. For the Malva vein clearing potyvirus for example, the virus was absent from recovered tissues (Lunello et al., 2009), whereas for geminiviruses, recovery was accompagnied by a reduction of the viral DNA titers (Chellappan et al., 2004; Rodriguez-Negrete et al., 2009; Hagen et al., 2008). At the level of the geminiviruses viral-derived small interfering RNAs (vsRNAs), there seems to be no correlation between their accumulation and the symptomatology. For the *African cassava mosaic* virus, a higher accumulation of vsRNAs was observed in recovered leaves than in symptomatic leaves (Chellappan et al., 2004), whereas the opposite situation was observed with the *Pepper golden mosaic virus* (Rodriguez-Negrete et al., 2009) or the *Cucurbit leaf crumple virus* (Hagen et al., 2008). For nepoviruses, a reduction of viral RNA accumulation was observed in recovered leaves for the *Tomato black ring virus* (Ratcliff et al., 1997) and *Tobacco ringspot virus* (TRSV)(Siddiqui et al., 2008). However, no commensurate viral RNA reduction was observed in recovered leaves from plants infected by *Tomato ringspot virus* (Jovel et al., 2007). Moreover, the lack of vsRNAs in recovered tissues of TRSV-infected plants correlated with the small amounts of genomic viral RNAs in these tissues (Siddiqui et al., 2008).

Nepoviruses have two positive sense, single stranded genomic RNAs, called RNA1 and RNA2. These RNAs are polyadenylated at their 3' end and have a covalently attached small genome-linked viral protein (VPg) at their 5' end (for a review, see Mayo and Robinson, 1996). The complete nucleotide sequences of the two genomic RNAs of the grapevine isolate NW of *Arabis mosaic virus* (ArMV) (Wetzel et al., 2001; 2004), as well as those corresponding to the *Ligustrum vulgare* (Lv) isolate of ArMV have been reported (Dupuis et al., 2008). The RNA1 is translated into a polyprotein, which is

proteolytically matured by the RNA1-encoded proteinase into 6 final products referred to as X1 (of unknown function), X2 (protease cofactor), NTB (helicase), VPg, the proteinase and the polymerase (Wetzel et al., 2008 and references therein). The RNA2 is translated into a polyprotein, which is proteolytically matured by the RNA1-encoded proteinase into 3 final products referred to as 2A (homing protein, implicated in RNA 2 replication), the movement protein and the coat protein (Wetzel et al., 2001 and references therein). These two isolates differ in their virulence. ArMV-NW produces on *Chenopodium quinoa* very mild or no symptoms while ArMV-Lv induces extremely severe apical necrosis, eventually leading to the death of the plant.

In this manuscript, we describe the establishment rates of infection and recovery between these two ArMV isolates and the role played by the recovery phenomenon in relation to a secondary infection with the homologous or a heterologous virus isolate.

## MATERIALS AND METHODS

### Viruses and plants

ArMV-NW infectious clones corresponding to the genomic RNAs 1 and 2 (Dupuis et al., manuscript in preparation) were used to inoculate *Chenopodium quinoa* plants. The ArMV-Lv isolate (Dupuis et al., 2008) was obtained from the Deutsche Sammlung Mikroorganismen und Zellkulturen GmbH, Messerweg 11/12, 38104 Braunschweig, Germany (DSMZ, code name PV046). The viruses were propagated on *Chenopodium quinoa*. For virus inoculations, *Chenopodium quinoa*-infected material was ground in 0,03M $K_2HPO_4$ buffer pH 9.2 containing 1% bentonite and 1% celite, and rub-inoculated onto *Chenopodium quinoa* or *Nicotiana benthamiana* plants.

### Northern blot experiments

Total RNAs were extracted from plants using the TRIreagent (Sigma), following the recommendations of the manufacturer. For the detection of the viral genomic RNAs, 5 µg of total RNAs were separated in a 1% formaldehyde-agarose gel, blotted by capillarity on a nylon membrane (Hybond N+, Amersham) in 20x SSC buffer (1.5M NaCl, 0.15 M trisodium citrate, pH 7.0), and cross-linked under UV light (310 nm). For the detection of the siRNAs, 10 µg of total RNAs were separated by denaturing polyacrylamide gel electrophoresis, electroblotted on a Hybond N+ membrane, and cross-linked under UV light. For the preparation of the probe, 25 ng of purified PCR-amplified fragments corresponding to the ArMV-NW or –Lv 2A gene or 5' non-coding region, were labelled with [α-$^{32}$P]dCTP (3,000 Ci/mmol, Perkin-Elmer) using the Decalabel DNA labelling kit (Fermentas), following the recommendations of the manufacturer. The membranes were hybridised at 42°C for the low molecular weight RNAs (LMW) and at 65°C for the high molecular weight RNAs (HMW) for 16 hours in 1x PerfectHyb Plus buffer (Sigma). Post-hybridisation washings were performed at 50°C for

LMW and at 65°C for HMW, in 2% SDS, 2x SSC (3 times) and 1% SDS, 1x SSC (1 time). In cases of hybridisations with different probes, membranes were stripped at 100°C in 0.5% SDS and 0.5% SSC, and rehybridised with the next probe as described above. Fuji screens and a scan phosphoimager Pharaos FxPlus molecular imager (Biorad) were used for the revelation of the hybridisation signals.

**Mapping of siRNAs**

Fragments of approximately 1,000 nt were amplified from both ArMV-NW and –Lv genomes (the primer sequences are available upon request), purified, and equal amounts (160 ng) separated on 1% agarose gels. The gels were equilibrated in transfert solution (120 mM NaCl, 80 mM NaOH) for 30 minutes, and the fragments transferred by capillarity onto Hybond N+ membranes for 12 hours. The membranes were then incubated in a neutralising solution (1.5M NaCl, 0.5 M Tris-HCl pH 7.5) for 15 minutes. To purify and label the siRNAs, total RNAs were extracted from ArMV-NW or –Lv infected *Nicotiana benthamiana* as described above, and the siRNAs purified from there using the FlashPAGE fractionator System (Ambion), following the recommendations of the manufacturer. The purified siRNAs were dephosphorylated using the calf intestinal phosphatase (Roche) and end-labeled with [γ-$^{32}$P]dATP (3,000 Ci/mmol, Perkin-Elmer) using the T4 polynucleotide kinase (Fermentas). The hybridisations were performed as described above.

**RESULTS**

**Establishment of infection and recovery rates of ArMV-NW and –Lv on *Nicotiana benthamiana***

*Nicotiana benthamiana* plants were mechanically inoculated with ArMV-NW or -Lv and the symptoms development monitored. Both isolates were susceptible to systemically infect *N. benthamiana*, as they were detected in both inoculated and systemic leaves by ELISA (data not shown). ArMV-NW did not produce any symptoms, neither on the inoculated nor the systemic leaves. On the other hand, ArMV-Lv induced a yellow mottling or mosaic at 7-9 days post inoculation (dpi) on the first new emerging leaves. However, the following leaves had fully recovered, as they were symptomless. Symptoms did also develop on ArMV-Lv-inoculated leaves, but these could not be seen before 16-20 dpi.

A time course analysis of viral RNAs accumulation was realised from ArMV-NW or -Lv infected plants. For this, the inoculated leaves and the systemic top leaves were collected from two plants for each sampling time at 3, 5, 7, 9, 11, 13, 16, 20 and 25 dpi, as described in materials and methods. In the inoculated leaves, the establishment of the infection was similar between ArMV-NW and -Lv. In fact, the viral RNAs from both isolates became detectable by northern blots at 9 dpi and increased until 16 dpi, where they seemed to reach a plateau (**Figure 1**). For ArMV-Lv, the accumulation of the

viral RNAs at the plateau level coincided with the apparition of the symptoms on the inoculated leaves.

In systemic leaves, the viral RNAs were detectable from 7 dpi for both isolates. The accumulation of viral RNAs for ArMV-NW increased steadily to reach a maximum accumulation at 13 dpi, from when it drastically droped (16 dpi). For ArMV-Lv, the maximum accumulation of viral RNAs was reached at 9 dpi, which coincided with the time of the apparition of symptoms on the first new emerging leaves. After this, the viral RNAs started to decrease (**Figure 1**), to reach a low accumulation similar to ArMV-NW (16 dpi). However, despite the important decrease of the viral RNAs accumulation for both isolates, the virus was not completely eliminated and remained detectable even at 25 dpi. The recovered leaves, when used as inoculum for inoculations onto *N. benthamiana* or *Chenopodium quinoa*, were able to produce a normal virus infection, indicating that the virus was still infectious in the recovered leaves. Identical patterns were observed for both ArMV-NW or -Lv in three independent experiments.

In order to determine if the decrease of the viral RNAs was due to antiviral RNA silencing, we analysed the accumulation of the vsRNAs for both isolates. The analysis of the vsRNAs showed that their accumulation were in correlation with those of their corresponding viral RNAs (**Figure 1**). These results suggest that the vsRNAs derived from the degradation of the viral genomic RNAs, and that the decrease of the viral genomic RNAs is a consequence of the activation of the RNA silencing.

Taken together, these results show that the establishment of the infection in *N. benthamiana* is faster for the virulent isolate ArMV-Lv than for the milder isolate ArMV-NW. However, both are equally affected once the recovery phenomenon has taken place in the plant. For ArMV-Lv, we observed a correlation between the establishment of the symptoms and the accumulation of the viral RNAs in both the inoculated and systemic leaves. Interestingly, similar levels of accumulation of viral RNAs were observed in systemic leaves between ArMV-NW (at 13 dpi) and ArMV-Lv (at 9 dpi). However, no symptoms could be seen for ArMV-NW. This difference could be due to the drastic diminution of viral RNAs observed for ArMV-NW at 16 dpi, while the decrease observed for ArMV-Lv was more progressive. This could reflect a difference in the efficiency of a putative viral-encoded suppressor of RNA silencing between these two isolates, and/or a different efficiency in their silencing suppression strategy.

**Characterisation of ArMV-derived vsRNAs**

In order to characterise the ArMV-derived vsRNAs, which were detected in both the inoculated and systemic leaves of ArMV-infected *N. benthamiana*, approximatively 1 kb fragments covering the entire genome of ArMV-NW or -Lv were amplified, electrophoretically separated, and blotted. Small RNAs from ArMV-NW or -Lv infected *N. benthamiana* were purified, end-labeled with [γ-32P] and used as probes.

The mapping of the ArMV-NW-derived vsRNAs gave the strongest signals of hybridization with the fragments corresponding to the RNA2, mainly the 5' non-coding regions, 2A, MP and the CP genes, the signal being slightly weaker for the fragment corresponding to the C-terminus of the CP gene and the 3' non-coding region. The signals observed with the fragments corresponding to the RNA1 were generally weaker (**Figure 2**). The mapping of the ArMV-Lv-derived vsRNAs gave also the strongest signals for the fragments corresponding to the RNA2, essentially the 5' non-coding region, 2A and MP genes. The signals corresponding to the CP gene and 3' non-coding region fragments were weaker in the range of those obtained for the fragments corresponding to the RNA1. However, some regions on the RNA1 were hybridised more strongly to the siRNAs than others, mainly those corresponding to the X2 and the protease-polymerase genes for ArMV-NW, and the X2 and amino-terminal half of the polymerase genes for ArMV-Lv (**Figure 2**). These patterns were reproducible for both ArMV-NW or –Lv in three independent experiments.

In conclusion, the vsRNAs essentially derived from the RNA2. This is most likely linked to the fact that the viral RNA2 is present more abundantly in the infected cells than the viral RNA1.

**Immunity experiments**

In the recovery phenomenon, it was described that the plants were immune to a second infection by the same virus. To investigate to which extend the ArMV-NW or -Lv recovered plants were immune to a secondary infection, secondary infections were performed with the homologous virus or with the heterologous isolate (ArMV-Lv or -NW).

At a stage of 20 dpi, when the recovery is fully established for both isolates, the top leaves of ArMV-NW or -Lv infected plants were inoculated with ArMV-NW or ArMV-Lv. A time course analysis was realised. For this aim, the inoculated leaves and the systemic top leaves were collected for each sampling time, at 7, 9, 11, 13, and 16 dpi. Total RNAs were extracted, separated electrophoretically, blotted and probed as described in materials and methods. As expected, for both ArMV-NW or -Lv, a secondary infection with the homologous virus did not produce any symptoms, neither on the inoculated leaves nor on the systemic ones. Northern blots analysis showed that the levels of viral RNAs accumulation detected in these leaves were similar to those observed for the primary infection after the establishment of the recovery, indicating that the plants were immune to a secondary infection by the same virus (**Figure 3**).

**Hetero-immunity experiments**

The isolates ArMV-NW and -Lv have 81% and 85% identity at the nucleotidic level between their RNAs 1 and RNAs 2, respectively, with 100% identity stretches of 25-40 nucleotides found throughout both genomic RNAs. To evaluate if the recovered plants were also immune to a secondary infection with the heterologous isolate, ArMV-NW or Lv-recovered plants were inoculated with ArMV-Lv or NW, respectively. Plants were monitored for symptoms development, and viral RNAs

accumulation assessed by northern blots. Probes corresponding to the highly variable N-terminal region of the 2A gene of ArMV-NW or -Lv were used to be able to differentiate both isolates. In neither of these two cross-protection experiments, symptoms could be visualized neither on inoculated nor systemic leaves. The analysis by northern blots showed that ArMV-NW was unable to accumulate in ArMV-Lv recovered leaves, suggesting that the ArMV-Lv recovered plants were immune to ArMV-NW. The ArMV-Lv RNAs accumulation was not affected by the secondary infection, remaining at the level of the recovered status (**Figure 4**). However, ArMV-Lv was able to replicate in ArMV-NW recovered leaves. In inoculated leaves, the ArMV-Lv RNAs were detected from 7 dpi, reaching a plateau around 13 dpi. At the same time the accumulation of the ArMV-NW RNAs remained unchanged at the recovery level. In systemic leaves, ArMV-Lv RNAs were detected from 7 dpi, followed by an increase of the accumulation at 9 dpi, and slightly decreasing from 13 dpi. At the same time, low accumulation of ArMV-NW RNAs was detected from 7 dpi, which however remained at the recovery level (**Figure 4**). These results suggest that the ArMV-NW induced recovery did not confer any immunity towards ArMV-Lv. Overall, the secondary infection of ArMV-Lv onto ArMV-NW recovered plants followed a pattern similar to a primary infection, except that no symptoms could be seen in the secondary infection.

## DISCUSSION

The observations that different rates of infection, but similar rates of establishment of the recovery phenomenon take place between a mild and a virulent isolate of ArMV is an indication of the degree of complexity of the plant host-virus relationship. The differences observed in the infection rate are unlikely to be due to a difference in virus accumulation in the inoculum, as both isolates are able to systemically infect *Chenopodium quinoa* with high titers, as confirmed by ELISA. One hypothesis could be that the different viral-encoded proteins are expressed differently in these isolates. The viral-encoded proteins of ArMV-Lv could be cleaved from its polyproteins more efficiently by the viral-encoded protease than the corresponding proteins of ArMV-NW, as it was shown that the different proteins of the polyprotein 1 of ArMV-NW were cleaved by the viral-encoded protease with different efficiencies (Wetzel et al., 2008). In the case of ArMV-NW, a slower release could be responsible for the delayed infection, when compared to ArMV-Lv. Another hypothesis could be that the interaction between the different viral-encoded proteins and putative host factors are more or less effective between these isolates. In this case, the ArMV-Lv protein(s) could interfere earlier and/or more efficiently with the plant defense response, hence allowing the infection to take earlier. Alternatively, the ArMV-Lv could encode a protein with a stronger suppressor of gene silencing activity than the corresponding protein of ArMV-NW. However, no suppressor of gene silencing has yet been identified for ArMV-Lv or -NW and for another nepoviruses. Both isolates were then equally affected once the recovery phenomenon had taking place, suggesting that the effect of the putative suppressor encoded by the virus had been countered by the plant defense mechanisms. In both cases, low amounts

of virus remained, suggesting that the virus managed to a certain extent to evade the plant defense mechanisms. These results were similar to those obtained with TRSV (Siddiqui et al., 2008) and TBRV (Ratcliff et al., 1997). However, they differed from those reported for ToRSV (Jovel et al., 2007) where no commensurate reduction of viral RNAs was observed in recovered leaves. Similar differences were reported for different viruses from the family *Geminiviridae* and their respective recovery patterns (Chellappan et al., 2004; Carrillo-Tripp et al., 2007: Hagen et al., 2008; Rodriguez-Negrete et al., 2009), suggesting that each virus or isolate of a virus has its own particular pattern in its relationship to his host.

The accumulation of viral-derived siRNAs detected in the plants correlated with the accumulation of viral genomic RNAs. This result was consistent with those observed with TRSV (Siddiqui et al., 2008), for which siRNAs could not be detected in recovered tissues, where there were only small amounts of viral genomic RNAs. On the other hand, ToRSV-derived siRNAs were only present in infected plants at low accumulation (Jovel et al., 2007).
The mapping of the ArMV-NW or -Lv -derived siRNAs on their respective genomic RNAs showed a generally similar pattern to the one described for ToRSV (Jovel et al., 2007), the vast majority of them deriving from the RNAs 2. This could be explained by the fact that the RNAs 2 of ArMV are present in larger amounts in infected plants than their corresponding RNAs 1. No other particular features were detected in the sequences of neither RNAs 1 nor 2 that would preferentially promote the generation of siRNAs. It is therefore unclear why their distribution differed within different areas of RNAs 1, or between the RNAs 2 of ArMV-NW and -Lv.

When secondary infections were performed with the homologous isolates of ArMV on recovered plants, the expected immunity was observed, for both the mild and virulent isolate of ArMV. However, while the expected immunity was observed when ArMV-NW was used for the secondary infection on ArMV-Lv recovered plants, ArMV-Lv inoculated onto ArMV-NW recovered plants was able to replicate following a pattern similar to a primary infection, with the exception that no symptoms could be seen in the secondary infection. The identity levels between the two isolates of ArMV RNAs (81% and 85% between RNAs 1 and 2 respectively at the nucleotidic level, with stretches of 25-40 nucleotides with 100% identity found throughout both genomic RNAs) should have been sufficient for the gene silencing machinery to recognise and degrade the ArMV-Lv RNAs, as observed for ArMV-NW when inoculated onto ArMV-Lv recovered plants. It is unclear which viral or plant factor(s) or which interaction(s) between viral and/or plant proteins allowed ArMV-Lv to overcome or evade the ArMV-NW induced recovery, while however being unable to overcome or evade its own induced recovery. These questions remain to be answered.

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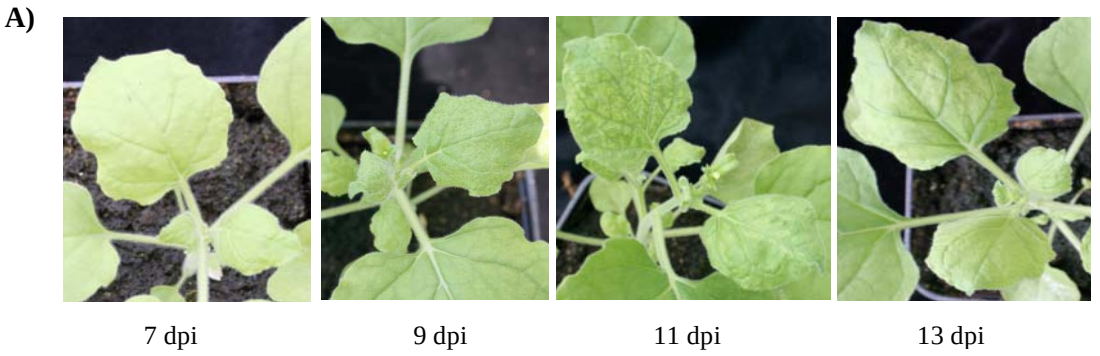
**Figure 1**: Characterisation of the recovery phenomenon in ArMV-infected *Nicotiana benthamiana*.

**A)** Establishment of the symptoms on the systemic leaves in ArMV-Lv -infected plants.

**B)** Northern blot analysis of ArMV-NW or –Lv inoculated *Nicotiana benthamiana* plants. PCR-amplified fragments corresponding to the 2A genes of ArMV-NW or –Lv were radioactively labeled and used as probes for hybridization of the ArMV-NW or –Lv genomic RNAs and siRNAs respectively in inoculated and systemic *Nicotiana benthamiana* leaves. Genomic: viral genomic RNA 2, rRNA: ribosomal RNAs, vsRNA: viral-derived small interfering RNAs, dpi: days post inoculation.



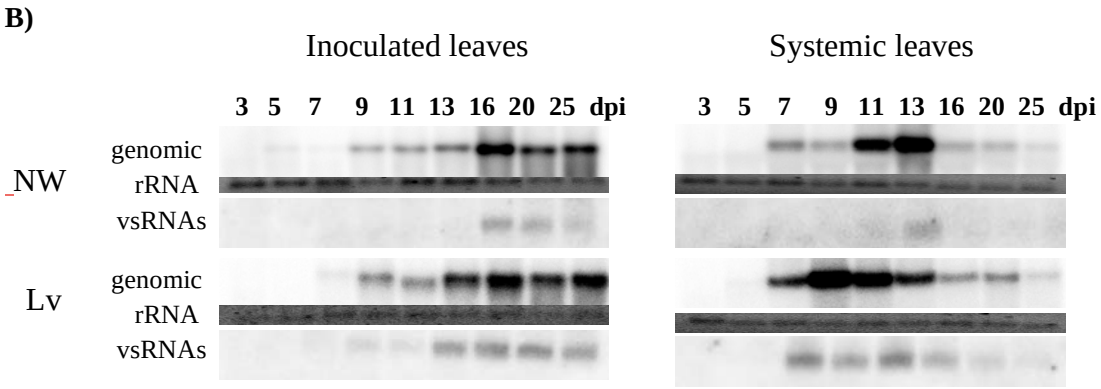



**Figure 2**: Characterisation of ArMV-derived siRNAs in *Nicotiana benthamiana*. The siRNAs were purified from total RNAs extracted from ArMV-NW or –Lv infected *Nicotiana benthamiana* and radioactively labelled as described in Materials and Methods. The labelled siRNAs were used to hybridise PCR-generated fragments from the ArMV-NW or –Lv genomic RNAs. The ethidium-bromide stained gels of the PCR products are shown as loading controls.

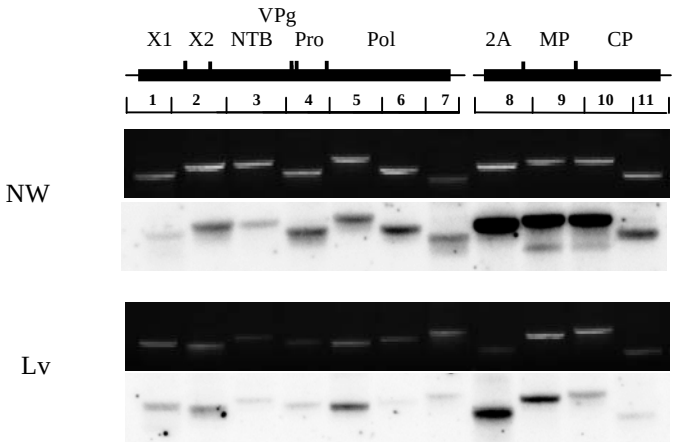


**Figure 3**: Effect of a homologous secondary infection on ArMV-NW or -Lv recovered *Nicotiana benthamiana*. Systemic and inoculated leaves were collected between 7 and 16 days post inoculation (dpi). R: recovered state control, collected before the second inoculation. Total RNAs were extracted, blotted, and probed, as described in Materials and methods. The probe corresponded to the 2A gene of ArMV-NW or -Lv, respectively. The loading controls are shown under the Northern blots.

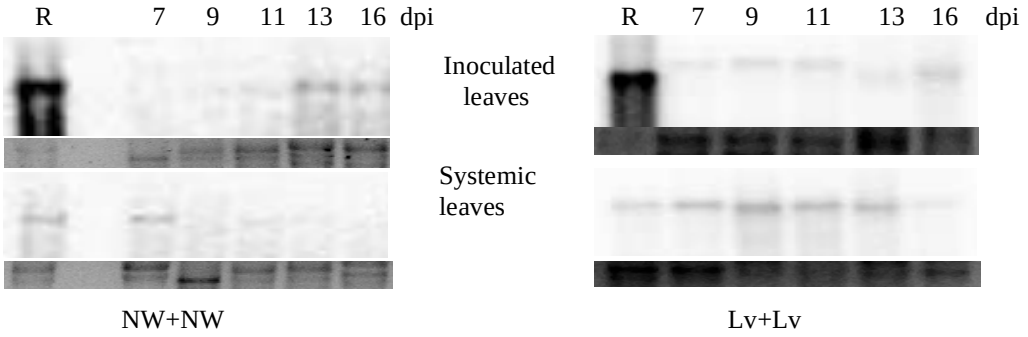
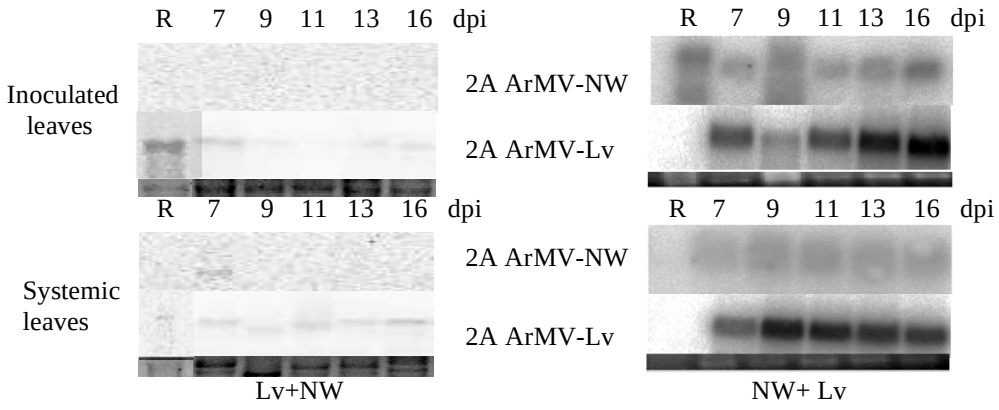


**Figure 4**: Effect of a heterologous secondary infection on ArMV-NW or -Lv recovered *Nicotiana benthamiana*. Systemic and inoculated leaves were collected between 7 and 16 days post inoculation (dpi). R: recovered state control, collected before the second inoculation. Total RNAs were extracted, blotted, and probed as described in Materials and methods. Probes corresponding to the N-terminal region of the 2A gene of ArMV-NW or -Lv, which corresponds to the most variable region between the two isolates, were used. The loading controls are shown under the Northern blots.



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ANNOTATED SEQUENCE RECORD

# Complete genome sequence of a virulent isolate of *Arabis mosaic virus* from privet (*Ligustrum vulgare*)

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Arabis mosaic virus (ArMV), a member of the genus *Nepovirus* of the family *Comoviridae*, has polyhedral virions and is transmitted by the nematode vector *Xiphinema diversicatum*. The serological, biological and particle properties of ArMV have been described previously [6, and references therein]. Nepoviruses have two positive-sense, single-stranded genomic RNAs, which have a covalently attached small genome-linked viral protein (VPg) at their 5′ end, and whose 3′ ends are polyadenylated [for a review, see 3]. Nepoviruses have been divided into three subgroups (A–C) based on the length of RNA 2, serological properties of the virions and cleavage site specificity of the viral proteinase [3]. The complete genome of the grapevine isolate NW of ArMV (subgroup A) has been determined [7, 8]. The genomic organisation of ArMV-NW was similar to that of the fully characterised grapevine fanleaf virus strain F13 [7, 8, and references therein]. Recently, an additional cleavage site between two protein domains upstream of the nucleotide binding protein (NTB) on the RNA-1-encoded polyprotein of ArMV was identified [10]. It was suggested that these two protein domains, corresponding to the X1 and X2 domains in tomato ringspot virus (ToRSV, subgroup C nepovirus) [5], is a common feature among nepoviruses [10]. In this paper, we report the complete RNA 1 and RNA 2 sequences of a virulent isolate of ArMV from privet (*Ligustrum vulgare*, Lv). Sequence comparisons between this isolate and the corresponding sequences of other ArMV isolates and other nepoviruses are also reported.

The ArMV-Lv isolate (PV46) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Messeweg 11/12, 38104 Braunschweig, Germany. ArMV-Lv was propagated on *Chenopodium quinoa*, purified, and the RNAs extracted as described previously [7]. Total RNA extracted from infected leaves of *C. quinoa* using the Omniprep RNA extraction kit (Ivagene, France) was used as template for RT/PCR using the One-Step RT/PCR System (Invitrogen) with specific and/or degenerate primers designed from available ArMV sequences. An oligo(dT) primer was used in combination with an ArMV RNA 1- or RNA 2-specific primer to amplify the 3′ end of the two viral RNAs. Purified viral RNAs were used for 5′RACE experiments, following the instructions of the supplier of the 5′RACE kit (Invitrogen). RT/PCR products were cloned and sequenced, or sequenced directly, and each nucleotide was sequenced at least three times and in both directions. Sequences were compiled and analysed using the DNAsis program package (Hitachi). The genomic databases were searched online for similarities at the National Centre for Biotechnology Information Blast network server [1].

ArMV-Lv, when mechanically inoculated onto *C. quinoa*, produced very severe chlorotic and necrotic symptoms on inoculated and systemic leaves. Ten to 14 days after inoculation, the apical part of the plant started necrosing, leading eventually to the death of the plant. In contrast, ArMV-NW produced only very mild or no symptoms on *C. quinoa*. When ArMV-Lv was inoculated onto *Nicotiana benthamiana*, a severe mosaic was observed on the first systemic leaves. The following leaves, however, were symptomless. ArMV-NW, on the other hand, did not

The sequences presented in this paper have been submitted to the Genbank/EMBL database and have been assigned the accession numbers EU617326 and EU617327.

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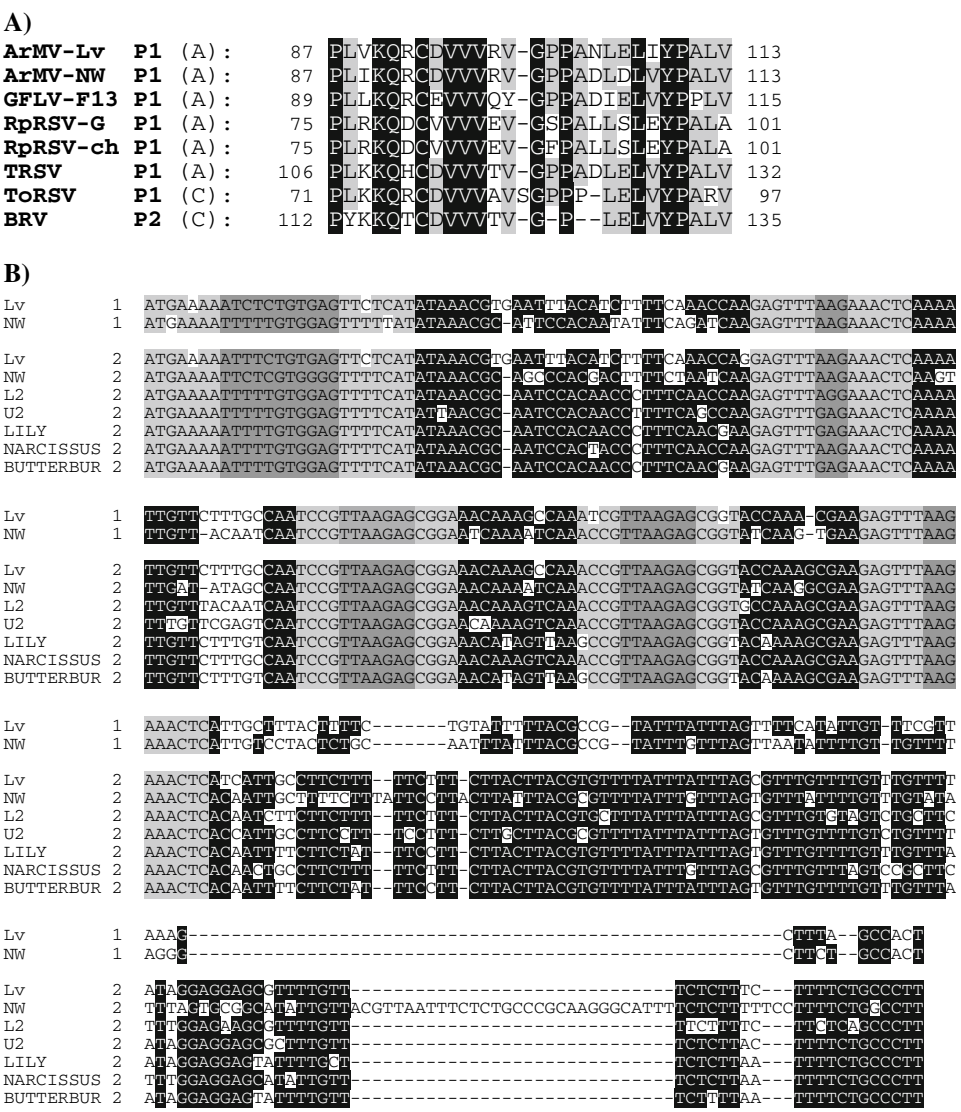


**Fig. 1** Sequence comparisons between ArMV-Lv and other isolates of ArMV or nepoviruses. *Dashes* correspond to gaps introduced to optimise the alignments. **a** Comparison of a sequence motif in the polyproteins 1 or 2 of different nepoviruses. Conserved amino acids are boxed in *black*; amino acids found in at least five of eight sequences are boxed in *grey*. The nepovirus subgroups are shown as (A) and (C); P1 and P2 refer to polyprotein 1 or 2, respectively. The *numbering* indicates the location within the polyproteins. **b** Comparison of the nucleotide sequences of the 5′ non-coding regions of RNA 1 and RNA 2 of ArMV-Lv with those of other ArMV isolates. Conserved nucleotides are boxed in *black*. Conserved putative stem and loop structures are boxed in *light grey* (stem) and *dark grey* (loop). 1 and 2 refer to RNA 1 and 2, respectively

produce symptoms on *N. benthamiana*. In ELISA, comparable absorbance values were obtained for ArMV-Lv- and ArMV-NW-infected plants, suggesting that the difference in symptomatology was not due to a lower virus titre in plants (not shown).

The complete sequence of the ArMV-Lv RNA 1 was 7,334 nt long excluding the poly(A) tail. A single large open reading frame (ORF) was found, encoding a polypeptide of 2,285 amino acids (MW 252,856 = 252K). This putative ORF was preceded by a 229-nt 5′ non-coding region and followed by a 247-nt 3′ non-coding region. The overall levels of RNA 1 nucleotide and amino acid sequence identities between ArMV-Lv and ArMV-NW were 81 and 86%, respectively. A gene-by-gene comparison between ArMV-Lv and ArMV-NW RNA 1 revealed the highest identity (92%) in the NTB proteins and the largest differences (78% identity) both in the N-terminal X1 proteins [10] of unknown function and in VPg. Interestingly, a BlastP search with the X1 protein of ArMV-Lv revealed a short stretch of amino acids that are conserved in the X1 protein of several nepoviruses of subgroup A and ToRSV [4] of subgroup C. For blackcurrant reversion virus

(BRV, previously referred to as blackcurrant reversion associated virus) [2], which also belongs to subgroup C, this motif was found at the N-terminus of the polyprotein encoded by RNA 2 (Fig. 1a). So far, this motif was found in all the nepoviruses of subgroups A and C for which complete genomic sequences are available. On the other hand, this motif, whose significance is unknown, was not found in any of the fully sequenced nepoviruses from subgroup B.

The complete sequence of ArMV-Lv RNA 2 was 3,812 nt long excluding the poly(A) tail. A single large ORF was found encoding a polypeptide of 1,118 amino acids (MW 122,994 = 122K). This putative ORF was preceded by a 261-nt 5′ non-coding region and followed by a 194-nt 3′ non-coding region. The overall levels of RNA 2 nucleotide and amino acid sequence identities between ArMV-Lv and ArMV-NW were 85 and 91%, respectively. A gene-by-gene comparison between ArMV-Lv and ArMV-NW revealed the highest level of identity between the movement proteins (97%), whereas the N-terminal 2A proteins were most diverse (73% identity).

Analysis of the non-coding sequences of ArMV revealed conserved putative stem-loop structures in the 5′ non-coding regions of both RNA 1 and RNA 2 (Fig. 1b). Such putative structures were also found in similar positions in GFLV, grapevine chrome mosaic virus (GCMV) and tomato black ring virus (TBRV) [7, and references therein]. Interestingly, for raspberry ringspot virus (RpRSV), also belonging to subgroup A, such conserved structures were found in the 3′ non-coding regions of RNA 1 and RNA 2, but not in their 5′ non-coding regions [9].

The availability of complete genome sequences of two ArMV isolates differing strikingly in symptomatology on *C. quinoa* and pathogenicity in *N. benthamiana* provides a good basis for studying symptom and pathogenicity determinants in the ArMV genome. Establishing full-length infectious clones of ArMV-NW and -Lv for exchanging certain genome fragments would now be the next step towards the identification of ArMV genes involved in symptomatology and pathogenicity. This would permit deeper insights into the molecular biology of ArMV and nepoviruses.

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